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Investigation of Dosage Distributions of Polyvinyl Siloxane Dental Impression Shields for Head and Neck Radiotherapy with Thermoluminances Dosimeters

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ABSTRACT

Objective: This study aimed to assess the effects of denture materials on dose distribution on a head and neck radiotherapy-appropriate model and calculate the thickness of a stent by polyvinyl siloxane dental impression material for shielding scattered radiation from dental restorations.

Methods: In the first step of the study, 5mm diameter and 5mm height of cylindrical dental material of titanium, zirconia lithium disilicate were irradiated with 6-Megavoltage photons from a clinical linear accelerator. In the second step, dental materials at the center of polyvinyl siloxane thicknesses of 5, 10, and 20mm were irradiated with 2 Gray and 10 Gray fractional doses. Measurements were made using three thermoluminescent dosimeters positioned laterally. The percentage backscattered dose and percentage dose decrease values were calculated to interpret the results.

Results: According to the result, dosages scattered from dental materials increased for samples irradiated with 2Gy; a decreased dose was reported for samples irradiated with a 10Gy. 5mm PVS samples provided higher dose attenuation than others. Regardless of dental material, it is seen that the attenuation intensities calculated from TLD-100 dosimeters ranged from 22.7 to 38,62 for 2Gy, and 10.01 to 38,87 for 10Gy.

Conclusion: Dental material alters the scattered radiation. In irradiated head and neck cancer patients, a 5mm thick guard is sufficient to prevent radiation diffused from dental materials in clinical usage.

Keywords: Radiotherapy, polyvinylsiloxane, TLD, dose distribution.

1. INTRODUCTION

Radiotherapy is a fundamental part of cancer treatment protocols. It is very competent and helps to take control of the tumor. However, its toxic effects on healthy tissues within the irradiation area are negative aspects of treatment also. If the targeted area is around the oral cavity, oral functions like eating and chewing of cancer patients become painful, negatively affecting life quality (1). Radiotherapy in head and neck cancers (HNRT) is challenging both clinicians and patients due to dental materials such as amalgam, dental ceramic, and titanium. The tooth with restoration made from different dental materials acts as a heterogeneous substrate inducing secondary electron scattering in different ways, primarily backward. Besides, an increase in out-of-field exposure may raise the incidence of radiotherapy-related secondary malignancies. Thereby they convert contamination of neighbor healthy tissues with extra doses of radiation (2). Enhanced radiation dosage is a crucial determinant of dental complications as far as the dimension and position of the irradiating areas (3).

In the studies that concentrated on dental restorative materials, researchers have investigated the interaction of radiotherapy with teeth, gold, amalgam, composite, ceramic, zirconia, and titanium (2,4–6). While Reitemeier *et al.*(7) said that dental materials might produce up to a 2-fold increase in excess radiation, Chin *et al*. (5). declared increased scattering radiation dosage approximately four times more than other materials studied. All studies above recommended that if there are restored teeth with dental materials in the targeted area during HNRT, surrounding tissue adjacent to these teeth should be protected from backscattering radiation stem from the presence of restoration itself. A protective intraoral stent could eliminate the adverse effects that developed early stage of treatment and lasted three-four weeks even after treatment completion related to HNRT (8–10). The use of a spacer with shielding capacity can avoid dose augmentation induced by backscatter radiation from dental materials (7).

Since their excellent chemical and mechanical durability, polysiloxane polymers have been employed for radiation

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protection studies in nuclear applications (11). Nevertheless, there are a few studies about the usefulness and effectiveness of polyvinyl siloxane (PVS) impression material with different metal additives as a shielding material (12,13). According to these studies, PVS-metal composites are equally effective as traditional shielding alloys. Authors preferred PVS over conventional materials owing to advantages of PVS like the ease and fast of fabricating, not necessarily precise equipment, and techniques, and more comfortable for patients. Although a PVS-metal composite stent may be effective in the case of intraoral radiotherapy adjacent teeth with dental restorations, the metal in the composite structure also creates considerable backscattered radiation. In another research, Kawamura used regular PVS impression material as a proton beam stopper to protect the tongue during proton treatment of a 75-year-old patient with gingival squamous cell carcinoma (14). He compared dose-volume histograms of the tongue with and without PVS and the relative linear stopping power of PVS using converted CT data and a model simulation. At the end of the study, the authors declared that PVS might be a promising proton beam stopper option.

As a result of the literature review, no study has been found on whether PVS dental impression material efficiently blocks the radiation emitted by dental materials. Thus, the current study was designed to (1) determine the effects of contemporary denture materials on dose distribution on an appropriate model for HNRT and (2) identify the thickness of a stent made from PVS dental impression material for shielding backscattered radiation from dental restorations.

2.METHODS

This study researched whether high and medium viscosity PVS impression materials could attenuate or block the scattering radiation from dental materials. The experimental setup which was followed during the investigation is shown in Fig 1.

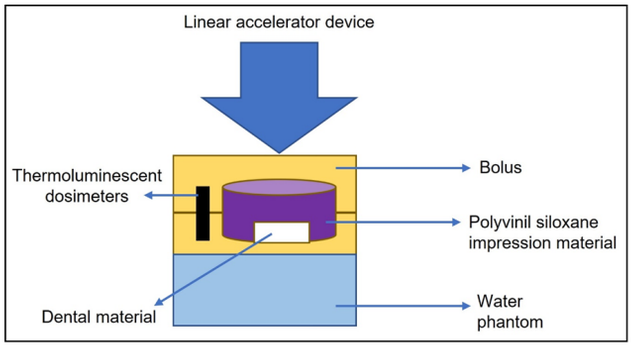

Figure 1. *An illustration of the experimental design*

To imitate the dental restorations, cylindrical specimens with 5mm diameter and 5mm height were produced in equal amounts for each group of dental material. Highly translucent zirconia (3Y-TZP) blocks (Nacera Pearl 1, Doceram Medical Ceramics, Germany) were cut by CAD/CAM (Ceramill Motion; Amanngirbach, Germany) and sintered according to the manufacturer's recommendations for monolithic zirconium specimens (n=5). Titanium specimens (n=5) were produced from Grade 4 pure titanium (Astra Tech Implant System; Dentsply Sirona, Charlotte, NC, USA). The lithium disilicate specimens (n=5) were fabricated by milling out of blocks with CAD/CAM (IPS e.max CAD; Ivoclar Vivadent, Schaan, Liechtenstein).

For this study, it was decided to prepare the samples for both high-viscosity (Kulzer, Variotime Dynamix Heavy Tray) and medium-viscosity (Kulzer, Monophase/Dynamix Monophase) groups with 5mm, 10mm, and 20mm thicknesses around the dental materials PVS-based elastomeric impression materials were obtained using a device (Pentamix®; ESPE) that mixes the base and catalyst homogeneously following the manufacturer's instructions and put into the mold (Fig 2). In the experimental setup, the sample of the study group containing dental material at the center of PVS was placed in soft tissue-equivalent bolus material (Superflab Bolus Material; CNMC Co, Nashville, TN, USA) (Fig 3).

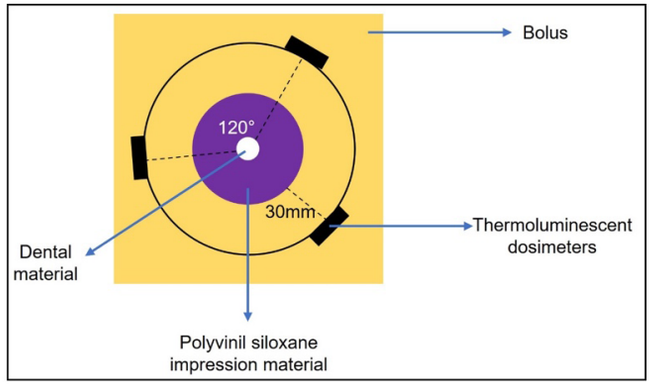

Figure 2. *An illustration of TLD dosimeter's locations*

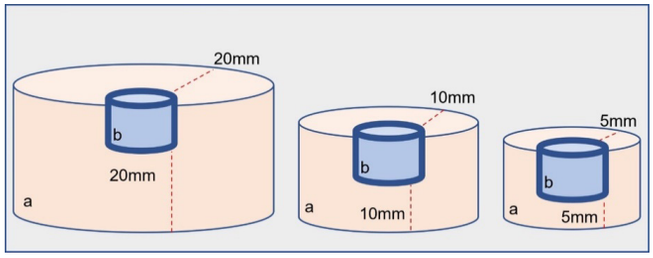

Figure 3. *An illustration of dental material specimens within polyvinyl siloxane*

Under the bolus, the RW3 solid water phantom was put to stop backscattering. Then, the setup prepared separately for each sample was positioned on the radiotherapy device where the central beam was right in the center of the dental material during irradiation. A 6-MV photon beam was applied with a clinical linear accelerator (Clinac Ix; RapidARC, Varian Medical Systems, USA) (5). The radiation source was

positioned to be away 100 cm from the impression material. While irradiation, the source-skin distance (SSD) technique was performed at the field of 15x15mm doses of 2Gy and 10Gy, compatible with The National Comprehensive Cancer Network (NCCN) Guidelines which recommend applying daily and weekly fractional therapeutic radiation in the clinical design of HNRT. The dimension of a pre-calibrated thermoluminescent dosimeter (TLD-100; Harshaw Chemical Company, Solon, OH) was 3.2x3.2x0.89mm, and its spatial resolution was 2mm. TLD was positioned in the lateral direction of the dental material's outer line around a 30mm radius circle with an angle of 120 apart from each other (Fig 4).

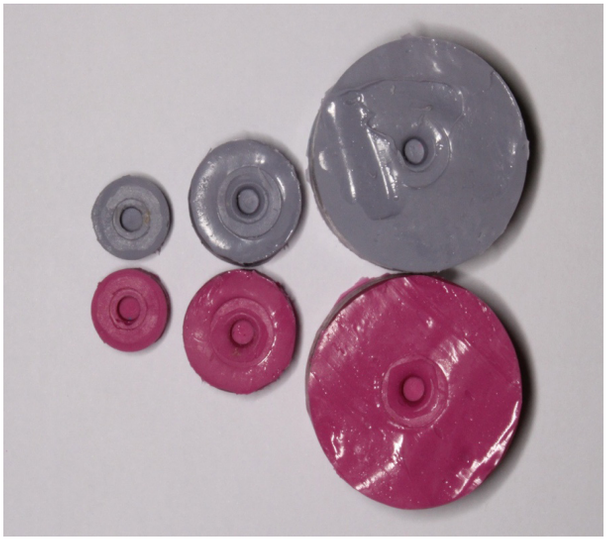

Figure 4. *Polyvinyl siloxane samples with a space at the center for dental material: High-viscosity PVS (Grey) and Medium-viscosity PVS (Pink)*

There were three TLD-100 on a ring with a 30mm radius. Therefore, the distance effect for scattering, which is inversely proportional to the square, was eliminated. In this way, the comparison was made more accurately. The Harshaw TLD reader read TLD-100 dosimeters. This process was repeated three times for each sample. The setup in the control group was produced by titanium, zirconia, and lithium disilicate without PVS impression material.

In this study, interpreted the below situations were interpreted:

(1) Percentage dose difference (PDD) was calculated in the presence of a dental restoration material by the following formula:

PDD = (D2-D1 / D1) x 100

(2) Percentage dose difference (PDD) was calculated in the presence of a PVS around the dental restoration material by the following formula:

PDD = ((D3-D2) / D2) x 100 (15).

D1: the photon dosage in only soft tissue-equivalent bolus material, **D2:** the photon dosage in the presence of a dental restoration material, and **D3:** the photon dosage in the presence of a PVS around the dental restoration material at the exact precise location, respectively.

3. RESULTS

For each dose energy, the precision of the TLD measurements was quantified by calculating the mean response, standard deviation, and percent dose differences for the dental material's group and PVS height.

In the first part of our study, we evaluated the impact of some widely used denture materials on dose distribution in HNRT by medical linac and a TLD dosimetry system. The values of dose distribution in the case of different dental restoration, titanium, zirconia, and lithium disilicate are presented in Table 1.

According to the result, scattered doses increased for samples irradiated with 2Gy; a decreased dose was reported for samples irradiated with a 10Gy. Dose increase was found at 22.57%, 12.32%, and 7.83% for titanium, zirconia, and lithium disilicate, respectively. In contrast, dose decrease was recorded as 20.27%, 12%, and 10.22% for titanium, lithium disilicate, and zirconia, respectively (Table 1).

In the second part of our research, dosimetric distribution after simulated HNRT in the presence of dental material and PVS impression material was evaluated. The dependency on the thickness of PVS specimens concerning dose differences for 2Gy and 10Gy fractional radiation doses is presented in Table 2 and Table 3, respectively.

Table 1. *Absolute backscatter dose and percent dose differences in the presence of different dental restoration materials for 2Gy and 10 Gy fractional radiation*

	Absolute Backscatter Dose (mSV) ±SD					Percent Dose Difference-PDD (%)		
	No restoration$^{\alpha}$	Zirconia$^{\beta}$	Titanium$^{\beta}$	Lithium Disilicate$^{\beta}$		Zirconia	Titanium	Lithium Disilicate
2Gy	8.076±1.7	9.071±3.1	9.899±4.2	8.709±2.9		+12.32	+22.57	+7.83
10Gy	45.336±1.5	40.703±11.8	36.146±6.5	39.895±14.7		-10.22	-20.27	-12

$^{\alpha}$ refers to D1 (the photon dosage in only soft tissue-equivalent bolus material), and $^{\beta}$ refers to D2 (the photon dosage in the presence of a dental restoration material).

The presence of 5mm PVS in both high and medium viscosity reduced the scattered radiation from all dental materials. 5mm PVS samples provided higher dose attenuation than others. Without considering dental material, it is seen that the attenuation intensities calculated from TLD-100s ranged from 22.7 to 38,62 for 2Gy, and 10.01 to 38,87 for 10Gy fractional dosage (Table 2 and Table 3). In addition, according to the results of the study, increases in scattered doses were also observed in the presence of PVS samples.

Table 2. *Absolute scattered dose and percent dose differences in PVS presence with different dental restoration materials for 2Gy fractional radiation.*

	Absolute Backscatter Dose (mSV) ± SD				Percent Dose Differences (%)		
		Zirconia $^{\mu}$	Titanium $^{\mu}$	Lithium Disilicate $^{\mu}$	Zirconia	Titanium	Lithium Disilicate
High viscosity PVS							
	5 mm	6.118±0.7	6.076±1.4	6.464±1.4	-32.55	-38.62	-34.69
	10 mm	8.130±1.6	7.124±1.2	6.744±3.1	-10.37	-28.03	-31.87
	20 mm	6.272±1.7	8.103±4.5	7.249±2.1	-30.86	-18.14	-26.77
Medium viscosity PVS							
	5 mm	7.012±1.9	5.775±0.5	6.333±0.8	-22.70	-36.33	-27.28
	10 mm	6.668±2.9	7.168±3.5	14.758±6.6	-26.49	-20.98	+69.46
	20 mm	8.167±2.3	7.276±3.9	7.924±4.4	-9.97	-19.78	-9.01

$^{\mu}$ *refers to D3 (the photon dosage in the presence of a PVS around the dental restoration material at the exact precise location)*

Table 3. *Absolute scattered dose and percent dose differences in the presence of PVS with different dental restoration materials for 10Gy fractional radiation*

	Absolute Backscatter Dose (mSV) ± SD				Percent Dose Differences (%)		
		Zirconia $^{\mu}$	Titanium $^{\mu}$	Lithium Disilicate $^{\mu}$	Zirconia	Titanium	Lithium Disilicate
High viscosity PVS							
	5 mm	24.880±5.6	32.529±7.3	28.549±7.3	-38.87	-10.01	W-28.44
	10 mm	38.890±15.6	36.233±8.4	38.288±.5	-4.46	+0.24	-4.03
	20 mm	48.850±20.9	33.480±12.6	32.677±14.2	+20.01	-7.38	-18.09
Medium viscosity PVS							
	5 mm	29.530±6.5	32.469±2.7	32.199±8.6	-27.45	-20.23	-20.89
	10 mm	36.431±15.4	40.558±19.7	34.320±5.7	-10.50	-0.36	-15.68
	20 mm	34.229±11	32.276±10.9	34.294±15	-15.91	-20.70	-15.75

$^{\mu}$ *refers to D3 (the photon dosage in the presence of a PVS around the dental restoration material at the exact precise location)*

4. DISCUSSION

This study observed that contemporary prosthodontic dental materials affected dose distribution on an appropriate model for HNRT due to the backscattering effect of the materials themselves. It is demonstrated that dental material may increase the backscattered radiation for 2Gy but reduce for 10Gy fractional dose adjacent area. Authors concluded that these results were the limitation of TLD-100 dosimetry. Namely, the luminescence dosimetry related to high-sensitive LiF:Mg,Cu,P, or Al_2O_3:C has a poor response to the growing ionization density of the radiation field, such as 10 Gy, which may result in an underestimation of the dose values (16). In this study, it is decided to utilize an in-phantom TLD-100 dosimeter based on Lithium Fluoride doped with Magnesium and Titanium (LiF: Mg, Ti) to measure the secondary radiation outside the treatment area. The reason behind using the TLD-100 dosimeter was that it demonstrates close tissue equivalency, compact size, high accuracy, repeatability, and low signal fading (22). Also, it is very convenient for them to use it as an out-of-field dose detector for treatment energies up to 10 MV (23). Three TLD-100 dosimeters were used in an experimental setup to ensure stable dosimetry readings around dental materials. TLD-100 dosimeters were placed at 120 angles away from each other and in a different direction from the central beam to avoid the misleading effect of the irradiation dose for treatment over the scattered radiation measurement.

The increase varied based on the dental material: titanium (22.57%), zirconia (12.32%), and lithium disilicate (7.8%). The present study results have supported the studies conducted by Beyzadeoğlu and Akyol using a TLD dosimeter. Compared with 18% and 12.32% dose enhancement in front of Ti implant, a higher dose increase, 22.57% (17,18) was measured in this study. However, Akyol observed a maximum dose increase for (zirconia) Y-TZP implant material, contrary to our results. The differences in the results might stem from

an experimental design in which they used a human mandible with a root form Ti implant. A few studies are related to the effect of lithium disilicate restoration during HNRT (2,15,19). Tso compared backscattered dose variation from different dental materials at a different distance (0,1,2,3,4, and 5mm) measured using TLD after irradiating with a 6MV photon beam (6). Their results showed that lithium disilicate had the lowest dose enhancement in all distances. The dose increase of zirconia was higher than lithium disilicate at the exact distances because of the higher physical density of zirconia. Accordingly, our results concerning dental materials are consistent with the studies mentioned above. The doses for 10Gy fractional radiation in the current study were 20.27%, 12%, and 10.22%, which attenuated after going through the dental materials for Ti, lithium disilicate, and zirconia (Y-TZP), respectively. In phantom, the electron dosage distribution is affected by the type of restoration materials used and the energy of the electron beam. As expected, the backscattering amplitude decreases when the photon beam's energy increases (20,21).

Protecting healthy tissues in irradiated head and neck cancer patients is essential. Backscattering-induced radiation enhancement has been linked to mucositis in several investigations. During radiation therapy, an adequate thickness of low-Z material (water equivalent) inserted before the tooth can protect the neighboring healthy tissues (7). In this way, the risk of acquiring oral disorders in these people can be minimized by lowering the backscatter radiation caused by dental restorations. A stent with appropriate thickness has been placed in which the tooth is put in the treatment field. It also ensures that the beam does not travel through the dental restorations, but it is not feasible. just PVS impression materials were studied in this research because it is practical and takes a few minutes to shape into a PVS stent whose consistency is excellent and fits into any place (14).

Furthermore, a few research have been conducted on PVS impression material, but in these studies, PVS used various metal additions as a shielding material. Although the PVS-metal composites, according to these investigations, are just as effective as standard shielding alloys, the metal in the composite construction caused significant backscattered radiation (12,13). Also, Feng investigated the impact of a bite block made of polyester film and putty PVS on the dosimetric variables of patients with head and neck cancer (22). Consequently, they indicated that it did not alter the dosage distribution typical of the targeted region.

This study evaluated whether PVS impression material influenced attenuating scattered radiation during HNRT, which was planned with clinical practice in mind. In addition, the effectiveness of PVS sample thickness was investigated, and their dosimetric measures were evaluated for backscattering reduction. The results show us that almost all PVS impression material attenuated the backscattered dose resulting from the dental material. Especially 5mm thickness of PVS was adequate for attenuation at both high and medium-viscosity impression materials. The conclusions of the study by Tso support our results (6). Namely, he said that a dental guard with a 5 mm spacing around all teeth might be built before radiation simulation to mitigate amplified radiation to adjacent tissues, or a stent of any thickness should at least be used to reduce the radiation exposure. Some studies have recommended that utilizing 3mm of water-density material can protect the oral mucosa against excessive doses (5,7,12,15). In this study the effect of 5, 10, and 20mm thickness of PVS was inestigated, taking into account the work of Wang declared that 4mm of a layer of PVS effectively attenuated the radiation scattered from the on metal-polysiloxane composite (13). To the best of our knowledge, no other research with the same object as this one, a study about the appropriate thickness of a stent made from only PVS impression material for HNRT, exists. Other results of our findings were that 10 and 20mm of PVS samples showed significantly less dose attenuation of both 2Gy and 10Gy irradiation except for high viscosity PVS for the 2Gy group. Increased dose attenuation might be originated from the mass density effect of PVS in 10 and 20mm groups because physical density and electron density per cm^3 is known to play roles in perturbation in dose distribution, particularly for higher energy photons (23). Consequently, the scattering of secondary electrons in PVS material itself might cause dose enhancement. Contrary to our expectation that PVS samples would decrease scattered radiation from dental materials, three measurement values of 10mm and 20mm PVS samples with different dental materials showed increases. No defined conclusion could be made about these exceptions with the experimental results. To investigate how these parameters may impact dose distribution, future studies could made. Also, there were a few limitations in the study. This study only evaluated the dose distribution along with dental material and PVS samples. Also, the experimental setup employed a simple shape to conform to the factual dose enhancement affected by the curve of a tooth, dental implant, or dental restoration. Future research might investigate alteration in tooth form, tooth density, soft tissue density, and restorative material to provide more information about dose variation.

5. CONCLUSION

This research effectively analyzed the dosage distribution for a simulated human oral cavity using real-world contemporary denture materials in this research. According to the study's findings, the presence of dental restoration material alters the scattered radiation in phantom, depending on the kind of restoration material and the intensity of the electron beam. Using polyvinyl siloxane dental impression material as a dental guard around restored teeth with contemporary dental material may help minimize irradiation of nearby normal tissues at higher doses. The guard's thickness of 5mm is adequate to provide a shielding effect against backscattered

radiation regardless of the dental restorative materials and is more practical in clinical usage in terms of a stent size.

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Author Contributions:

Research idea: Y.D., Ç.A.

Design of the study: Y.D., Ç.A.

Acquisition of data for the study: H.Ö.U., Y.D

Analysis of data for the study: H.Ö.U., Y.D.

Interpretation of data for the study: H.Ö.U.

Drafting the manuscript: H.Ö.U, Y.D.

Revising it critically for important intellectual content: H.Ö.U, Y.D., E.B.G.A.

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Clinical and Experimental Health Sciences

Surface Plasmon Resonance Binding Study on the Interaction of Acetazolamide and Bovine Serum Albumin

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ABSTRACT

Objective: Serum albumins are major plasma proteins in systemic blood circulation and act as transport proteins for endogenous and exogenous compounds such as drugs. In pharmaceutical applications, it is essential to characterize how drugs bind to serum albumin in the evaluation of drug candidates. Surface plasmon resonance (SPR) is fast, real-time, label-free optical based detection technique that offers the monitoring of molecular interactions, analyzing binding reactions and determining the affinity constants with real-time and high sensitivity. Acetazolamide (AZA) is used in the treatment of epilepsy and glaucoma.

Methods: To determine the binding kinetics of AZA-Bovine serum albumin (BSA) interaction, (i) SPR gold sensor surface was functionalized, (ii) amine coupling procedure was applied to activate the surface group and BSA was immobilized on functionalized sensor surface, (iii) the concentration series of AZA (10, 25, 50, 75, 100, 150, 200 and 250 µM) was injected to SPR system and (iv) kinetic values were measured using the software of SPR system.

Results: 5 mM MUA was coated for surface functionalization. 250 µg/mL BSA as ligand, 30 µL/min flow rate, 1X PBS buffer (pH 7.4) and 10 mM acetate buffer (pH 5.2) as running and coupling buffers, respectively, were performed for SPR binding study. According to result, equilibrium constant (K_D) of AZA-BSA was determined as 67.72 µM.

Conclusion: In this study, we investigated the AZA-BSA binding interaction using SPR system based on Kretchmann configuration. The study designed with fast, label-free and real-time approach will provide valuable knowledge for pharmaceutical and clinical applications.

Keywords: Acetazolamide, bovine serum albumin, protein-drug interaction, surface plasmon resonance.

1. INTRODUCTION

Acetazolamide with molecular weight of 222.24 g/mol possesses physicochemical properties such as (i) white–yellowish crystalline powder, (ii) poorly soluble and (iii) soluble in alkaline conditions (1,2). AZA is mainly used in the treatment of epilepsy (3,4) and glaucoma (5,6) and some studies have focused on its antibacterial (7), antitumor (8), teratogenic (9) features, esterase (10-12) and intraocular pressure-lowering activities (13). After oral use, absorption of AZA is entirely actualized in gastrointestinal tract (14). AZA is nearly 95% bound to plasma proteins (15). The drug does not undergo metabolic changes in the body and is excreted by kidney in the urine within 24 hours (16).

Blood consists of great number of blood cells and plasma. The plasma also contains many plasma proteins including human serum albumin (HSA), α1-acid glycoprotein, lipoproteins (17) and globulins (18). HSA is well characterized plasma protein in intravascular compartment. Due to extraordinary ligand-binding capacity, the transport of many endogenous and exogenous compounds such as fatty acids, steroids, thyroid hormones and drugs is one of the main functions of albumin (19). It also has essential functions such as free radical scavenging, regulation of capillary membrane permeability and colloid osmotic pressure. It constitutes approximately 55% of the total protein content in the plasma of a healthy individual, which makes it the most abundant protein of the plasma (20). HSA is an important biomarker of various diseases including post-menopausal obesity, ischemia, rheumatoid arthritis, acute graft-versus-host disease and cancer. In addition to being used as a biomarker, HSA is also used in the treatment of various diseases such as hypoalbuminemia, burns, shock, hypovolemia, trauma, hemorrhage, acute respiratory distress syndrome, chronic liver disease, acute liver failure and hemodialysis. In addition to that, biotechnological applications of HSA, including surgical sealants and adhesives, biochromatography, fusion

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proteins, ligand trapping and implantable biomaterials, have been reported (21).

Serum albumins can be obtained from different mammalian species and these proteins show various similarities in physico-chemical characteristics (22). HSA and BSA are often used in biochemical and biophysical studies due to their similar folding, well known primary structure and possible binding interactions with various small molecules (23). BSA and HSA display around 76% sequence homology and a repeating pattern of disulfides. Although sequence homology does not seem too high, physico-chemical properties do not vary significantly. Due to binding properties, medical care availability and low cost, BSA is usually used in binding affinity experiments over HSA (24).

Plasma protein-drug interactions have been investigated for many years since plasma proteins establish an important part of the human proteome (25). Drugs are transported in the circulation either free or bound to plasma proteins. There are many drugs that bind reversibly to plasma proteins, making plasma proteins a crucial factor in determining the pharmacokinetics and pharmacological effects of drugs (26). Only free drugs have the capacity to interact effectively with the target. Therefore, it is important for there to be a balance between the drug and the carrier so that it is strong enough to aid in the transport but also weak enough to release the drug to the target (27). Serum albumins include several binding regions which have different affinities for analytes and bindings of these analytes with albumin occur at the side I and II on the protein (28). Several established techniques can be used to evaluate BSA-compound interactions including isothermal titration calorimetry, quartz crystal microbalance, spectroscopic (UV/VIS, fluorescence) techniques, FT-IR and SPR (29,30).

SPR is an optical based detection and/or sensing tool which offers real-time and label-free immunoassays having high specificity and sensitivity with short response time (31). Due to these unique characteristics, SPR has emerged a powerful technology in diagnosis (32), environmental monitoring (33), cellular imaging (34), genotyping (35), analysis of biomolecular bindings/interactions (36) between ligands and analytes generating kinetic and affinity data (37). SPR is classified as (i) prism coupling, (ii) integrated optical waveguide coupling, (iii) fiber coupling and (iv) grating coupling according to sensing structure (38) and prism-based or Kretchmann's configuration types are the most widely used SPR sensors (39). Aluminum, copper, gold and silver have been used as metal surface layer to generate surface plasmons for SPR sensing platforms (40). However, gold is the most commonly used surface layer for SPR instruments because of its outstanding durability, chemical stability and low oxidizing power (Figure 1) (41). Several functional chip surface modifications have been designed to enhance ligand and/or receptor immobilization in protein-drug binding investigations such as carboxymethylated dextran (42), 11-mercaptoundecanoic acid (MUA) (43) and 3-mercaptopropionic acid (44) on gold surface.

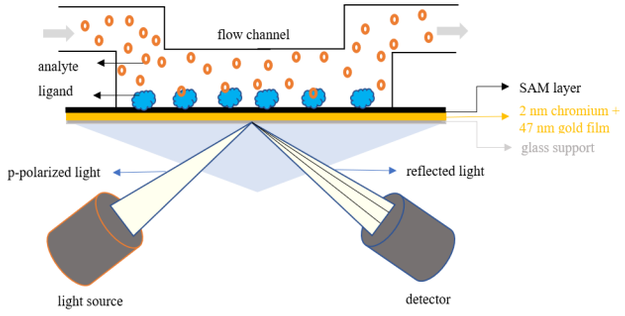

Figure 1. *Schematic representation of SPR sensor based on Kretchsmann configuration and sensor chip configuration.*

Understanding the binding affinity and pharmacological activity of drugs in both normal and disease states is useful in terms of drug design and the use of effective medication and treatment for patients (45,46). To the best of our knowledge, the interaction of AZA and BSA has not been evaluated using the SPR technique. Due to the broad use of AZA, its interaction with albumin needs to be addressed. Therefore, we investigated the binding properties of AZA with BSA using SPR method in this study.

2. METHODS

2.1. Chemical and Materials

Acetazolamide, bovine serum albumin, 11-mercaptoundecanoic acid, *N*-ethyl-*N*'-(3-(dimethylamino)propyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), ethanolamine hydrochloride (EA-HCl), KH_2PO_4, Na_2HPO_4, sodium acetate, NaOH and hydrogen peroxide (H_2O_2) were purchased Sigma-Aldrich (Saint Louis, MO, USA). KCl and NaCl were commercially obtained from Carlo Erba. Sulfuric acid (H_2SO_4), acetic acid and ethanol were purchased from Isolab (Turkey). The bare gold SPR sensor chip and matching fluid were obtained from Biosensing Instrument Inc. (Tempe, AZ, USA). BSA, stock solutions of chemicals and H_2O_2 were stored at 4°C in refrigerator, EDC was kept at – 20°C in a freezer.

2.2. Instrumentation

All water used in the preparation of samples and buffers was obtained from a water purification system (Direct Q®3 UV, Millipore Corp., France). Single channel pipettes were used for liquid transfer (Eppendorf Research Plus, Eppendorf AG, Germany). Routine weighing measurements in the study were performed by an analytical balance (Ohaus PA224C, Ohaus Corp., USA). The pH values of the solutions were determined with a pH meter (Seven Compact, Mettler Toledo, Switzerland). A vortex mixer (ZX3 Advanced Vortex Mixer, Velp Scientifica, Italy) was used to mix small volume of liquids and an ultrasonic bath (Elmasonic S 60 H, Elma Schmidbauer GmbH, Germany) was used to degas of the solutions.

Binding study of AZA-BSA was analyzed using BI-4500A SPR instrument (Biosensing Instrument Inc., Tempe, AZ, USA) with p-polarized laser light (λ= 670 nm). SPR instrument was combined with autosampler, syringe pump as a flow injection system, computer and software as control system.

2.3. Preparation of Self-Assemble Monolayer (SAM) on Gold Chip

All chip surfaces were cleaned with piranha solution [H_2SO_4/H_2O_2 (7:1 v/v)] before the functionalization of gold surfaces. Then, the clean gold chip was immersed in the 5 mM MUA solution for 24 hours at 22°C for functionalization of the chip surface. After 24 hours, the chip was rinsed 3 times with ethanol to remove unbound MUA.

2.4. BSA Immobilization on Gold Chip

Before the experiment, the device was rinsed with phosphate-buffered saline (PBS) (pH 7.4) buffer and the functionalized chip was mounted onto prism of the SPR device. Sensor temperature was set to 25°C. Syringes were filled with degassed PBS buffer. A stable baseline was achieved before the experiment. During the initial phase, only the PBS buffer was run on the functionalized gold chip. For the activation of MUA modified gold surface, EDC-NHS mixture 1:1 (v/v) (NHS 0.05 M + EDC 0.2 M) was injected for 600 sec with the flow rate of 30 µL/min. BSA (250 µg/mL, prepared in 10 mM acetate buffer, pH 5.2) was injected with the flow rate of 30 µL/min for 200 sec immediately after activation. BSA was not injected to the reference channel. After BSA injection, 1.0 M EA-HCl solution was injected onto the surface to block non-specific binding. Before kinetic analysis, the sensor surface was cleaned with 50 mM NaOH for 5 minutes. PBS was used as running buffer during the entire procedure.

2.5. Kinetic Analysis of AZA Interaction with BSA

All binding interactions between AZA-BSA were performed using double referencing and DMSO calibration protocols. Different concentrations of AZA solution (10, 25, 50, 75, 100, 150, 200 and 250 µM) were injected to the channels for 60 sec with the flow rate of 30 µL/min to calculate the kinetic parameters. After each injection 50 mM NaOH solution was used for regeneration process for 60 sec. Since BSA was not immobilized to second channel, this channel was used as reference. PBS (pH 7.4) was used as running buffer during the entire procedure. All kinetics of AZA-BSA binding were used to calculate the association rate constant (k_a), the dissociation rate constant (k_d) as well as the equilibrium constant (K_D) by SPR Data Analysis Software of Biosensing Instrument (version 3.10.5).

3. RESULTS

3.1. Construction of SAM and BSA Immobilization

MUA is one of the commonly used long-chain alkanethiol in the preparation of SAMs for SPR binding studies. MUA is negatively charged chemical at neutral pH (47). Surface functionalization should be designed to avoid insufficient analyte binding signal and/or some secondary effects like including steric hindrance and mass limited transport. 1 mM and 5 mM concentration of MUA were preferred in the immobilization of albumin onto the SAMs (36,48). In the study, 5 mM MUA demonstrated better surface coverage density for surface demand of BSA than 1 mM MUA. This functionalized sensor surface is one of the widely used sensor surface for amine coupling applications (43,49). As seen in Figure 2, freshly prepared EDC-NHS solution was applied to MUA functionalized SAM on the gold sensor surface for activating channels. After activation with EDC-NHS, BSA was immobilized on MUA based SAM. In this process, firstly the signal increased sharply as soon as the activation mixture contacted on SAM surface. The signal continued high level during the exposure of activation mixture. This mixture activates the MUA which increases surface quality. After exposure of activation mixture, signal turned to baseline. Then BSA solution was injected to SPR system. Signal increased relatively high levels which showed successful BSA immobilization on activated sensor surface (50). And EA-HCl solution was injected to block remaining active binding sites. This step is also crucial for removing loosely bound proteins from activated sensor surface (51). All steps including activation, ligand immobilization and blocking have been illustrated in Figure 3. In this study, the ligand has been injected into a channel for binding analysis and another channel was applied as reference channel. In binding experiments using SPR, the bulk index of refraction shifts and non-specific signals and also temperature drift are compensated by the reference channel (52).

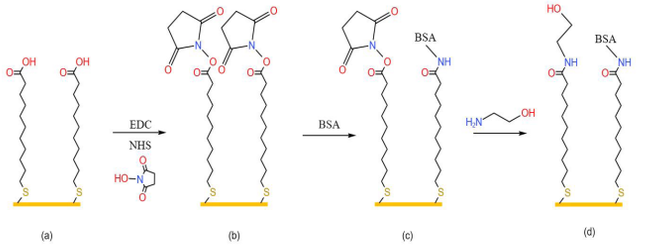

Figure 2. *BSA as a ligand immobilization process on MUA based SAM: (a) MUA coated gold chip surface, (b) activation with NHS-EDC, (c) immobilization of BSA, (d) blocking of remaining activating groups (50).*

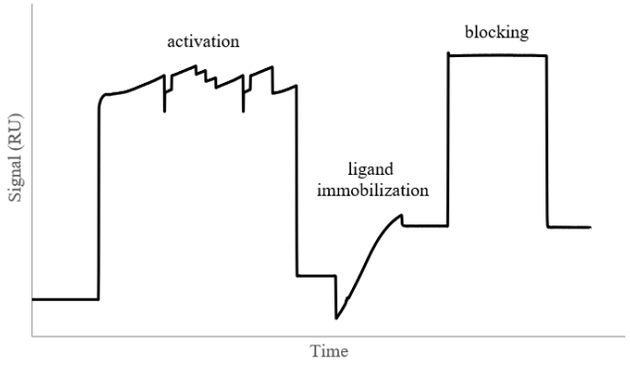

Figure 3. *SPR sensorgram illustrations of BSA immobilization step on 11-MUA based SAM.*

3.2. SPR Data Analysis

Association rate constant (k_a) demonstrates the number of complex formations formed per second. Dissociation rate constant (k_d) shows fraction of complexes decayed per second. k_a and k_d were carried out by SPR studies. Finally, equilibrium constant (K_D) which illustrates the ligand affinity to any molecule was calculated (53). k_a, k_d and K_D values were calculated by BI-Data Analysis Program. As seen in Figure 4, dose response sensorgrams of AZA and BSA were investigated after reference subtraction. According to result, association rate constant (k_a), dissociation rate constant (k_d), and equilibrium constants (K_D) values of AZA interaction with BSA are 12.84×10^3 (1/M × s), 8.69×10^{-1}(1/s) and 67.72 µM, respectively.

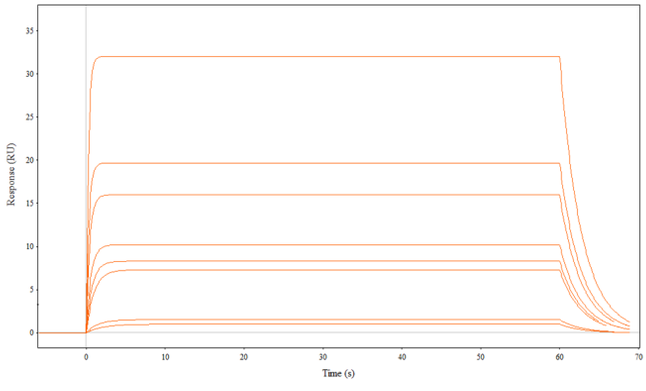

Figure 4. *Simulated SPR binding curves of BSA with AZA at different concentrations (10-250 µM).*

4. DISCUSSION

Most drugs have the ability of binding albumins in systemic blood circulation (54,55). Moderate binding to proteins is ideal in most cases. In therapeutic applications, when affinity binding of receptor and analyte is too high, effective drug action cannot occur. If the molecular binding is too weak, the drug can undergo some possible fates, it can be metabolized and eliminated by kidney filtrations (56). HSA typically exhibits an ability to bind endogenous and exogenous compound/ligands with affinity constants in the range of 10^{-4} to 10^{-6} M (57). AZA possesses moderate affinity for albumin under *in vitro* conditions (58). The decrease in the binding of AZA with albumin will cause changes in the distribution and elimination of the drug. The increased concentration of free drug will cause both active tubular secretion from the kidneys and increased uptake by red blood cells (59).

There have been many investigations in the drug-serum albumin binding in recent years (60). The interactions of levothyroxine (61), resveratrol (62), diazepam, warfarin, phenylbutazone, iodipamide, azapropazone, tolbutamide, iophenoxic acid, indomethacin, furosemide, bucolome, sulfisoxazole, diclofenac and more drugs with albumin have been analyzed by several methods (45). Kurkov et al. (58) showed the *in vitro* binding behavior of AZA on HSA. According to result, AZA showed medium binding affinity to HSA. Another useful study conducted by Rich et al. (63) contains equilibrium constants values obtained by SPR. In this study, ten drug compounds including naproxen, digitoxin, sulfadimethoxine, ketanserin, pyrimethamine, rifampicin, salicylic acid, coumarin, salbutamol and warfarin which used as control group were injected to SPR system to analyze the binding interactions with HSA.

SPR based binding studies of HSA with cyclolinopeptides (64), rosuvastatin (36) and BSA with piperacillin (65), sunitinib malate (53), rifampicin (24) and neomycin (66) were performed to determine the drug affinities to serum albumins at temperatures of about 298 K. Table 1 illustrates that neomycin, piperacillin, rifampicin and sunitinib malate bind to the BSA molecule with equilibrium constants of 1.50-2.22×10^{-5} M. Rosuvastatin shows high affinity to HSA with a low K_D value (1.55×10^{-8} M). And other peptide-based biomolecules have the binding affinity for HSA in the range of 8.27×10^{-5}-1.43×10^{-2} M.

Table 1. *Equilibrium constant values of some drug interactions with albumins at nearly 298 K.*

Drug	Ligand	Equilibrium constant (K_D)	T (K)	Ref
Cyclolinopeptides	HSA	8.27×10^{-5} – 1.43×10^{-2} M	~298	64
Rosuvastatin	HSA	1.55×10^{-8} M	298	36
Piperacillin	BSA	2.22×10^{-5} M	298	65
Sunitinib malate	BSA	1.91×10^{-5} M	296	53
Rifampicin	BSA	1.50×10^{-5} M	298	24
Neomycin	BSA	2.21×10^{-5} M	298	66
Acetazolamide	BSA	6.77×10^{-5} M	298	This study

5. CONCLUSION

SPR system based on Kretchmann configuration was performed to monitor binding of AZA to immobilized BSA on MUA based SAM. SPR is a fast, sensitive, label-free and non-invasive optical based detection techniques and

also allows real-time monitoring in drug-protein binding studies requiring small sample volumes. There is growing interest in pharmaceutical industry about drug-serum albumin interactions to determine accurate and reliable pharmacokinetics of the therapeutic agents. The study designed with fast, label-free and real-time approach will provide valuable knowledge about the affinity between AZA and BSA for pharmaceutical and clinical applications.

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Research idea: EAT
Design of the study: EAT, FGA
Acquisition of data for the study: EAT, FGA
Analysis of data for the study: EAT
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Clinical and Experimental Health Sciences

Effect of Reboxetine Treatment on BDNF, Synaptophysin, and PSD-95 Levels in the Spinal Dorsal Horn of Rats with Diabetic Neuropathy

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ABSTRACT

Objective: It is known that neuropathic pain is accompanied by alterations in the levels of neurotrophic factors and synaptic proteins in the microenvironment of the spinal dorsal horn. Such changes contribute to hyperalgesia and allodynia processes; thus, analgesic drugs can exert their pharmacological effects by affecting the expressions, levels, or functions of these endogenous substances. In this study, based on the knowledge that reboxetine (a selective noradrenaline reuptake inhibitor) has the potential for antihyperalgesic efficacy in diabetic neuropathy, we aimed to examine the probable effects of this drug on diabetes-induced changes in brain-derived neurotrophic factor (BDNF), synaptophysin (the pre-synaptic marker of synaptic integration), and postsynaptic density-95 (PSD-95) (the postsynaptic marker of synaptic integration) levels in the superficial laminae of the dorsal horn.

Methods: Experimental diabetes was induced by a single-dose injection of streptozotocin (STZ) (50 mg/kg) in rats. After four week-long induction period of painful diabetic neuropathy, rats were treated orally with 8 mg/kg reboxetine for two weeks. Hyperalgesia responses were evaluated by using the Randall–Selitto and Hargreave's tests. Following the pain tests, immunohistochemical studies were performed.

Results: Two weeks of reboxetine administration increased the reduced paw withdrawal thresholds and shortened the paw withdrawal latencies of diabetic rats in neuropathic pain tests, indicating the antihyperalgesic efficacy of this drug. Moreover, augmented BDNF and synaptophysin levels in diabetic rats reversed by reboxetine treatment. However, there was no alteration in the densities of PSD-95, in both STZ-diabetic and reboxetine-treated STZ-diabetic rats.

Conclusion: The obtained results suggested that inhibition of central sensitization and modulation of spinal plasticity seem to be pharmacological mechanisms underlying reboxetine's antihyperalgesic effects on diabetic rats. However, further studies are still needed to clarify the exact mechanism of action.

Keywords: Brain-derived neurotrophic factor, diabetes mellitus, neuropathic pain, postsynaptic density-95, synaptophysin.

1. INTRODUCTION

Neuropathic pain is a highly complex pain condition that usually results from nerve injury or dysfunction of the nervous system. Amputations, disc herniation, traumas, tumors compressing nerves, some chemotherapeutic agents, some viral infections, and some diseases are among the main reasons causing neuropathic pain (1). *Diabetes mellitus* is one of the major causes of neuropathy. Chronic exposure to hyperglycemia leads to detrimental changes in the nervous system's sensory and motor (somatic and autonomic) components (2,3,4). This metabolic disease gives rise to abnormal sensory symptoms such as hyperalgesia (augmented pain sensitivity), and allodynia (painful sensation against innocuous stimuli) (5). Patients with diabetic neuropathy may experience burning, tingling, shooting, sharp, lancinating, or sometimes electric shock-like sensations (6).

Polyol pathway hyperactivity, oxidative and nitrosative stress, microvascular changes, channels sprouting, microglial activation, central sensitization, and alterations in synaptic plasticity are the main mechanisms underlying the physiopathology of neuropathic pain in diabetes (6).

Neurotrophins, which regulate the growth, maintenance, and apoptosis of neurons in the developing nervous system as well

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as injured neurons, have been reported for their substantial roles in the development and transmission of neuropathic pain (1,7). These endogenous molecules have been shown to contribute to the pathogenesis of neuropathic pain as they have critical roles in the complex mechanisms that underpin peripheral and central sensitization (8,9,10). Moreover, results of numerous studies demonstrated that the changes in synaptic morphology and synaptic protein levels in spinal dorsal horn neurons are also associated with the development of chronic pain (11,12,13). The enhanced synaptic plasticity of nociceptive interneurons in this region has been suggested as the basis of central sensitization in neuropathic pain (14). Especially synaptophysin (a presynaptic vesicle glycoprotein that is an indicator of synaptic connections' efficiency) and PSD-95 (a scaffold protein on the postsynaptic membrane playing a critical role in the modulation of the size and shape of dendritic spines) are important synaptic proteins mediating enhancement of synaptic plasticity and playing critical roles in the development of neuropathic pain (13). In this context, reducing neuropathy-induced alterations in neurotrophic factors and synaptic protein levels in the dorsal horn may be an important strategy to alleviate chronic pain.

Reboxetine is a potent and selective noradrenaline reuptake inhibitory antidepressant (15). It has been reported that this drug has analgesic activity in acute and chronic pain (16,17,18). The catecholaminergic and opioidergic systems mediated beneficial effects of reboxetine against diabetes-induced neuropathic pain have also been shown previously (19). However, the potential efficacy of this drug on diabetes-induced changes in spinal neurotrophic factors or synaptic proteins has not been clarified, yet. Therefore, in this study, we aimed to investigate the effect of reboxetine on the brain-derived neurotrophic factor (BDNF), synaptophysin, and postsynaptic density-95 (PSD-95) densities in the spinal dorsal horn of rats with diabetic neuropathy, in order to clarify molecular mechanisms underlying the antihyperalgesic effects of this drug.

2. METHODS

2.1. Animals

Inbred male Sprague Dawley rats weighing 300–350 g at the same age were obtained from the Anadolu University Research Unit for Experimental Animals, Eskişehir, Turkey. Rats were housed under controlled temperature at 24 °C, 12/12-h light/dark cycle, and 50% humidity in well-ventilated rooms. The rats had access to food and water provided ad-lib. The Local Ethics Committee on Animal Experimentation confirmed the experimental protocol (Protocol code 2022-15 and date of approval 11.05.2022).

2.2. Establishment of Streptozotocin-Induced Experimental Diabetes

Streptozotocin (STZ) was used for the experimental diabetes induction (20). The rats were denied access to food overnight before the STZ injection. STZ was dissolved in citrate buffer (pH=4.5; 0.1 M). A single dose of STZ (50 mg/kg) through the tail vein was administered to the rats of the diabetic groups. An equal volume of citrate buffer was injected into the tail veins of the healthy controls. Plasma glucose levels were quantified after 72 hours of the STZ administration. The rats were considered diabetic with over 300 mg/dL plasma glucose levels.

2.3. Drug Administrations

4 weeks after inducing the experimental diabetes model, reboxetine was administered to the diabetic rats at doses of 8 mg/kg (*p.o.*) for 2 weeks. This is the effective antihyperalgesic dose of reboxetine in rats with diabetic neuropathy (19). Rats in the healthy and diabetic control groups were administered a physiological saline solution (0.9% sodium chloride), that was used to dissolve reboxetine.

2.4. Assessment of Neuropathic Pain

Mechanical hyperalgesia was evaluated by using the Randall–Selitto analgesiometer (Ugo-basile, Varese, Italy), as described previously (20). The device is used to apply an increasing pressure stimulus to the dorsal parts of the hind paws of rats and the force (grams) with which the rat withdraw its paw was accepted as the mechanical nociceptive threshold. The maximum force to be applied was determined as 250 grams in order to prevent tissue damage (19,20).

Thermal hyperalgesia was evaluated with the Hargreave's test device (Ugo-basile, 37370, Verase, Italy). This test is based on measuring the duration of the "paw withdrawal" reaction of rats against radiant heat focused on their hind paws. At the beginning of the test, the rats were placed in the plexiglass compartments of the device and waited for 30 minutes to acclimate to the environment. Then, paw withdrawal latency (time between activating the heat source and withdrawal of the back paw), was recorded with an accuracy of 0.1 seconds by the automatic timer of the device. Response times were calculated by taking the average of three measurements made with a 5-minute interval. Heat was not applied for more than 20 seconds in order to avoid a possible paw damage (19,20).

2.5. Spinal Cord Dissection

After completion of neuropathic pain experiments, rats were anesthetized with halothane and perfused transcardially with 0.1 M pH 7.4 phosphate-buffered saline (PBS) and then 4% paraformaldehyde in PBS (20). Midlumbar L4-L5 segments of the spinal cord were dissected and stored at 4 °C overnight in the fixative solution.

2.6. Immunohistochemistry of Spinal Dorsal Horn

After dehydration through an ascending concentration series of ethanol, spinal tissues were cleared in xylene and embedded in paraffin blocks (20). Transverse sections of the L5-level spinal cord (21) were collected by a microtome and sections (3 µm thick) were mounted on positively charged slides. To prevent antigen masking, the sections were treated with 1:10 EDTA Buffer (AP-9004-999 Thermo Scientific) under high pressure and cooled down to room temperature. Endogenous peroxidase activity was blocked to prevent non-specific background staining with 3% hydrogen peroxide solution (TA-125-HP ThermoScientific). Thermo Scientific UltraVision protein block solution (TA-125-PBQ Thermo Scientific, USA) was applied to slides for 10 minutes after washing with PBS.

Mouse monoclonal anti-synaptophysin antibody [SY38] (ab8049) 1:10 was used to investigate presynaptic protein expression; rabbit polyclonal anti-PSD-95 antibody-synaptic marker (ab18258) 1:250 was used to investigate postsynaptic protein expression and recombinant anti-BDNF antibody [EPR1292] (ab108319) 1:250 was used to label BDNF immunopositive somas and terminals in the spinal sections, respectively. After incubation with primary antibodies (Abcam PLC, Cambridge, UK) for 2 hours at room temperature, the sections were incubated with Primary Antibody Amplifier Quanto (TL-125-QPB), HRP Polymer Quanto, and Ultra V Block (TA-125-UB) for 30 minutes at room temperature, respectively. Each preparation was washed with PBS for all steps of UltraVision™ Quanto Detection System HRP (Thermo Scientific, USA). Sections were washed for 10 minutes three times in PBS. After that, sections were exposed to 2 µg/ml 3,3' – diaminobenzidine for 2-3 minutes. After the reaction product was revealed, sections were washed, dehydrated, placed in xylene for 5 minutes and coverslipped.

2.7. Light Microscopy, Immunohistochemical Analysis

Photomicrographs were obtained through the light microscope with Olympus CX31RTSF (Olympus GmbH, Hamburg, Germany) with an integrated camera with a 4x objective lens and LCmicro (Olympus GmbH, Hamburg, Germany) imaging software. Digital photomicrographs were taken under a 40x lens from all prepared sections and then evaluated by using the ImageJ 1.50i (U.S. NIH, Bethesda, MD, USA) image analysis program.

For calculating immunoreactivity, the rectangular fields (200 x 100 µm), including the superficial layer (laminae I and II), were taken from four consecutive sections at 200 µm intervals per L4-L5 spinal segments as presented in Figure 1. The auto thresholding tool of the ImageJ program was used to evaluate the percentage of immunoreactive area in the superficial laminae of the dorsal horn. All individual values were averaged to procure a mean value for each animal (21).

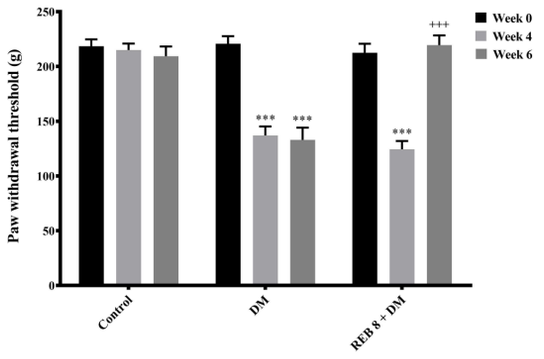

Figure 1. *Change in the paw-withdrawal thresholds of control (normoglycemic) and diabetic rats and the effects of reboxetine (8 mg/kg) treatment on these alterations, in the Randall–Selitto test. Significant difference compared to week 0 group $^{***}p < 0.001$; significant difference compared to week 4 group $^{+++}p < 0.001$. Two-way repeated-measures analysis of variance, followed by the Bonferroni test, n =8 (REB: Reboxetine, DM: Diabetes mellitus).*

2.8. Statistical Analysis

Statistical analysis of the data was carried out with GraphPad Prism (ver. 8.3.4.). The data obtained from Randall-Selitto and Hargreaves tests were evaluated using two-way repeated measures ANOVA followed by Bonferroni multiple comparison test. The data obtained from immunohistochemical studies were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD multiple comparison test. $p<0.05$ was considered significant.

3. RESULTS

3.1. Results of Neuropathic Pain Tests

Figure 2 demonstrates the changes of paw-withdrawal thresholds in the control and diabetic rats and the effect of reboxetine treatments on these alterations, in the Randall–Selitto test.

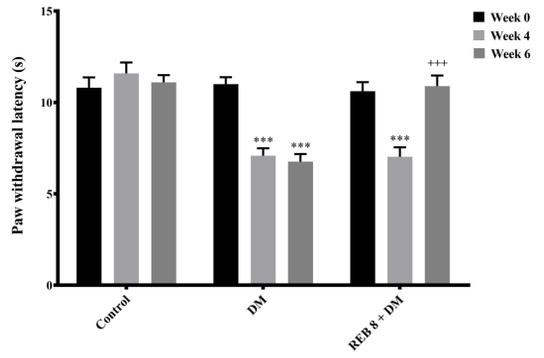

Figure 2. *Change in the paw-withdrawal latencies of control (normoglycemic) and diabetic rats and the effects of reboxetine (8 mg/kg) treatment on these alterations, in the Hargreave's test. Significant difference compared to week 0 group $^{***}p < 0.001$; significant difference compared to week 4 group $^{+++}p < 0.001$. Two-way repeated-measures analysis of variance, followed by the Bonferroni test, n =8 (REB: Reboxetine, DM: Diabetes mellitus).*

Two-way repeated-measures ANOVA results indicated that animals' withdrawal threshold in this test were affected not only by the treatment factor [F (2, 21) = 28.77, P < 0.001], but also by the time factor [F (2, 42) = 39.12, P < 0.001]. In addition, a significant interaction was found between treatment and time [F (4, 42) = 20.78, P < 0.001].

The Bonferroni test showed that no changes were observed in the paw-withdrawal thresholds of normoglycemic rats, throughout the experiments. However, paw-withdrawal thresholds of diabetic rats, measured at fourth (p<0.001) and sixth (p<0.001) weeks, were significantly lower than the values measured at the beginning of the experiments. Moreover, subacute administration of reboxetine at 8 mg/kg daily dose for two weeks produced marked increases (p<0.001) in the paw-withdrawal thresholds of diabetic rats (Figure 2).

Figure 3 demonstrates the changes of paw-withdrawal latency in the control and diabetic rats and the effect of reboxetine treatments on these alterations, in the Hargreave's test. Two-way repeated measures ANOVA results indicated that animals' paw withdrawal latency in this test were affected by both the treatment factor [F (2, 21) = 22.94, P < 0.001] and the time factor [F (2, 42) = 16.71, P < 0.001]. In addition, a significant interaction was found between treatment and time [F (4, 42) = 14.57, P < 0.001].

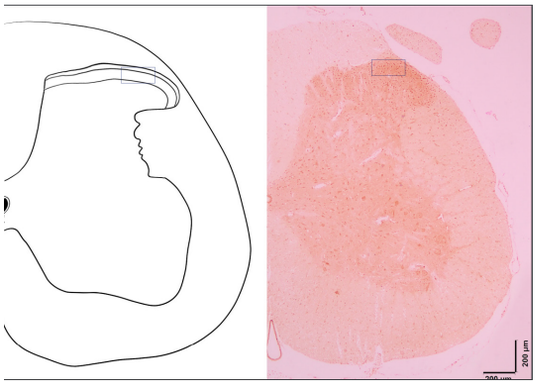

Figure 3. *Representative drawing and image of the spinal cord for the region of interest for immunohistochemical analysis.*

The Bonferroni test revealed that, similar to Randall–Selitto test, no changes were detected in the paw-withdrawal latencies of normoglycemic rats, during the experiments. On the other hand, paw-withdrawal latencies of diabetic rats, measured at fourth (p<0.001) and sixth (p<0.001) weeks, were significantly lower than the values measured at the beginning of the experiments. Furthermore, subacute administration of reboxetine at 8 mg/kg daily dose for two weeks induced notable increases (p<0.001) in the paw-withdrawal latencies of diabetic rats (Figure 3).

3.2. BDNF Immunoreactivity in the Spinal Dorsal Horn

Representative images of BDNF immunoreactivities in the superficial layers of the spinal dorsal horn were presented in Figure 4. In these photomicrographs, the brown-stained regions represent BDNF-IR neurons and synaptic terminals of the first-order sensory neurons in the spinal sections. BDNF immunostainings revealed that 6-week STZ-induced diabetes increases the immunoreactive areas compared to non-diabetic healthy rats (Figures 4A and 4B) and 2-week reboxetine treatment has ameliorated the BDNF overexpression (Figure 4C).

Alterations of the BDNF-IR % area in the spinal dorsal horn [F (2, 15) = 11.35; *p<0.01*] were presented in Figure 4D. Tukey's multiple comparison tests showed that rats with experimental diabetes significantly elevated BDNF-IR % area in the marginal layer and *substantia gelatinosa* of the spinal dorsal horn compared to healthy rats. On the other hand, 2-week reboxetine treatment administered to diabetic rats significantly reduced the percentage of BDNF-IR areas compared to non-treated diabetic rats.

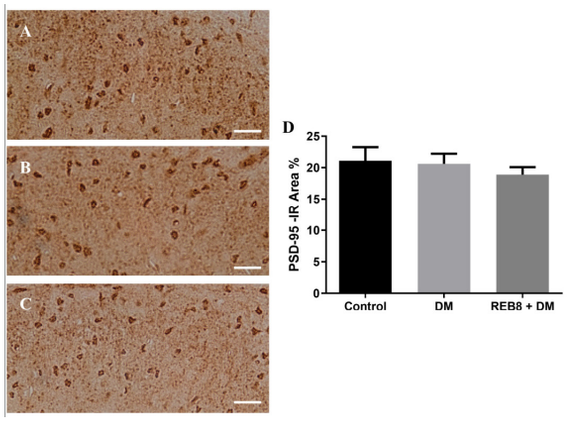

Figure 4. *Representative images of BDNF immunopositive transverse sections of L5 level spinal dorsal horn* ***(A)*** *physiological saline-administered healthy rats (Control) and* ***(B)*** *physiological saline-administered diabetic rats (DM),* ***(C)*** *8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM), Scale bar: 20µm.* ***(D)*** *Percentage of BDNF-IR area in the superficial dorsal horn of healthy rats (Control), physiological saline-administered diabetic rats (DM), or 8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM). The significant difference compared to the control group* ****p<0.001; Significant difference compared to the DM group* $^{+}$*p<0.05; One-way analysis of variance, followed by Tukey's HSD multiple comparison test, n=6 (REB: Reboxetine, DM: Diabetes mellitus).*

3.3. Immunoreactivity of Synaptic Proteins in the Spinal Dorsal Horn

Representative images of synaptophysin immunoreactivities in the superficial layers of the spinal dorsal horn were presented in Figure 5. Brown-stained regions represent synaptophysin-IR synaptic terminals of somatosensory neurons. It can be observed that the synaptophysin immunoreactivities were increased in diabetic rats compared

to normoglycemic animals (Figures 5A and 5B) and reboxetine treatment for 14 days (Figure 5C) significantly reversed this augmentation in the superficial layers of the spinal dorsal horn.

Alterations of the synaptophysin-IR % area in the superficial dorsal horn [$F(2, 15) = 6.129$; $p<0.05$] were presented in Figure 5D. Obtained results from the posthoc test indicated that the percentage of synaptophysin-IR areas was significantly elevated in rats with experimental diabetes than in healthy rats. On the other hand, 2-week reboxetine treatment in diabetic rats normalizes the increased synaptophysin levels.

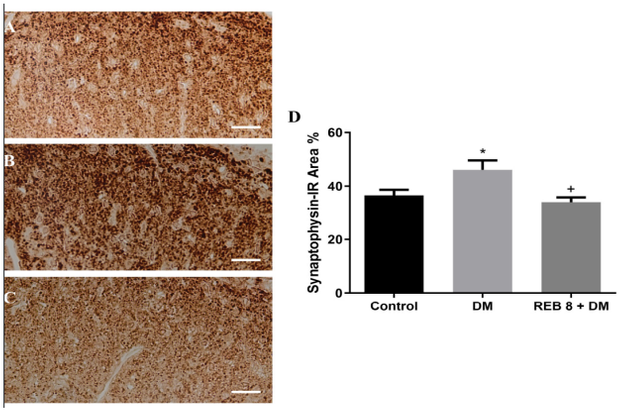

Figure 5. *Representative images of synaptophysin immunopositive transverse sections of L5 level spinal dorsal horn* ***(A)*** *physiological saline-administered healthy rats (Control) and* ***(B)*** *physiological saline-administered diabetic rats (DM),* ***(C)*** *8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM), Scale bar: 20µm.* ***(D)*** *Percentage of the synaptophysin-IR area % in the superficial dorsal horn of healthy rats (Control), physiological saline-administered diabetic rats (DM), or 8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM). The significant difference compared to the control group* $^{*}p<0.05$*; Significant difference compared to the DM group* $^{+}p<0.05$*; One-way analysis of variance, followed by Tukey's HSD multiple comparison test, n=6 (REB: Reboxetine, DM: Diabetes mellitus).*

Representative images of PSD-95 immunoreactivities in the superficial layers of the spinal dorsal horn were presented in Figure 6. Brown-stained regions represent PSD-95 immunoreactive postsynaptic terminals and cell bodies of dorsal horn neurons. It can be seen that the PSD-95 immunoreactivities did not alter between the experimental groups (Figures 6A-6C).

Alterations of the PSD-95 immunoreactive area % in the superficial dorsal horn [$F(2, 15) = 0.459$; $p>0.05$] were presented in Figure 6D. Tukey's HSD multiple comparisons test for PSD-95 immunoreactivities has revealed that the healthy and diabetic groups did not show any differences. Although reboxetine treatments slightly tend to reduce PSD-95 immunoreactive area % compared to untreated diabetics, the difference is not significant.

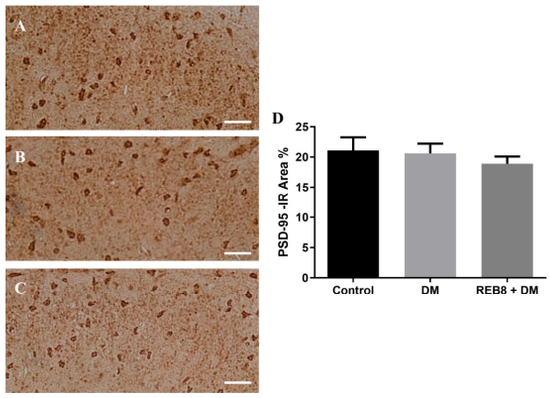

Figure 6. *Representative images of PSD-95 immunopositive transverse sections of L5 level spinal dorsal horn* ***(A)*** *physiological saline-administered healthy rats (Control) and* ***(B)*** *physiological saline-administered diabetic rats (DM),* ***(C)*** *8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM), Scale bar: 20µm.* ***(D)*** *Percentage of PSD-95 – IR area in the superficial dorsal horn of healthy rats (Control), physiological saline-administered diabetic rats (DM), or 8 mg/kg reboxetine-treated diabetic rats (REB 8 + DM). One-way analysis of variance, followed by Tukey's HSD multiple comparison test, n=6 (REB: Reboxetine, DM: Diabetes mellitus).*

4. DISCUSSION

In the first step of the study, diabetes was induced with STZ. After four weeks, Randall–Selitto and Hargreave's tests were performed to evaluate whether hyperalgesia developed in rats (19,20). The results of these tests revealed that four-week diabetic rats had reduced paw withdrawal thresholds and shortened paw withdrawal latencies indicating that diabetic animals developed hyperalgesia against both mechanical and thermal nociceptive stimuli. On the other hand, two weeks of reboxetine treatment significantly increased the reduced paw withdrawal thresholds and shortened paw withdrawal latencies of diabetic rats. These findings confirm our previous results reporting the antihyperalgesic efficacy of reboxetine against diabetes-induced hyperalgesia (19).

In the second step of this study, based on the antihyperalgesic activity of reboxetine on diabetes-induced neuropathic pain, we aimed to investigate the potential effect of this drug on diabetes-induced alterations in spinal neurotrophin and synaptic protein levels.

BDNF is a member of neurotrophins, essential for neuronal differentiation, maturation, survival, and synaptic plasticity (22,23). It is also a critical protein in the microglia-neuron signaling pathway. In the spinal dorsal horn, an increment of BDNF release by neurons and microglia would lead to the disinhibition of nociceptive neurons and enhancement of excitatory synaptic transmission (24,25). The primary sensory neurons are known to synthesize BDNF, which can be transported anterogradely to the axon terminals in the spinal dorsal horn (26,27). BDNF binds to tyrosine kinase receptor B (TrkB) on the second-order sensory neurons. This triggers intracellular signaling cascades through phosphorylation

and alters synaptic transmission (28,29). Increased BDNF expression caused by a peripheral tissue or nerve injury diminishes the presynaptic and postsynaptic GABAergic inhibition, which leads to thermal and static mechanical hypersensitivity, respectively (30). In addition, BDNF-induced activation of the dorsal horn NR2B-containing N-methyl-D-aspartate (NMDA) receptors (NMDA-2B) has been associated with developing and maintaining neuropathic pain (31). Another mechanism suggested for the role of BDNF is related to its regulatory role on the expression and activity of the TRPV1, which plays roles in the transmission of mechanical, chemical, and thermal nociceptive stimuli (32).

As a modulator of central and peripheral nociception, BDNF has been studied widely in experimental animal models, and several studies point out the importance of this protein in the pathophysiology of neuropathic pain (10,24,33,34). It has been reported that targeting this neurotrophin might be a beneficial therapeutic strategy for chronic and persistent pain treatment (35). Therefore, we investigated the potential efficacy of reboxetine on the BDNF immunoreactivity in the dorsal horn, in painful diabetic neuropathy (PDN).

Obtained results of this study demonstrated that six-week STZ-induced diabetes increased the BDNF immunoreactivity in the superficial laminae of the dorsal horn. This data is aligned with the previous papers reporting the overexpression of BDNF in several neuropathic pain models (1,31,36,37). Moreover, our experimental data supported some recent findings about the alterations of BDNF levels in diabetes-induced neuropathic pain conditions. For example, in a recent study, twenty-six-day-long STZ-induced diabetes was reported to increase BDNF expression significantly in the ipsilateral spinal cord in Western-Blot and immunohistochemical measurements compared to the control group (28). Similarly, in a study evaluating BDNF / TrkB expressions in comorbid depression with chronic unpredictable mild stress model in STZ-induced painful diabetic neuropathic rats, BDNF / TrkB expressions were found to be significantly increased in both the spinal dorsal horn and DRG (38). Based on the role of BDNF in diabetes-induced neuropathic pain, we investigated whether an antihyperalgesic drug reboxetine alters the expression of this neurotrophin in dorsal horns. Our results indicated that a two-week treatment of diabetic rats with reboxetine significantly decreased the BDNF overexpression in the superficial laminae of the dorsal horn. This finding suggested that the beneficial effect of this drug on diabetic neuropathic pain might be related to the inhibition of central sensitization induced by enhanced BDNF levels.

In addition to neurotrophic factors, changes in synaptic plasticity induced by synapse-associated proteins also play a critical role in the development of neuropathic pain (13,14,39,40). Increased synaptic plasticity of nociceptive interneurons in the spinal dorsal horn has been reported as the source of central sensitization in neuropathic pain (14). Long-term potentiation of synaptic plasticity of nociceptive neurons in the spinal dorsal horn requires the participation of both pre-and postsynaptic structures (41). Active zones are the sites specialized for the exocytosis of synaptic vesicles at the pre-synaptic axon terminals (42). The size of the active zone is an important morphological parameter of synapses that effectively reflects the area of neurotransmitter release. Increased synaptic vesicle proteins in the pre-synaptic active zones may lead to synaptogenesis, thus resulting in neuropathic pain (43).

Synaptophysin is the first cloned marker of synaptic vesicles and the second most abundant synaptic vesicle glycoprotein (14,44). This protein is the main component of the small vesicles of neuroendocrine cells and neurons and modulates the synaptic vesicle cycle (45). The level of synaptophysin is an agreeable indicator of the synapse number and synaptic connections' efficiency. In addition, synaptophysin expressions in DRG and the spinal dorsal horn have been associated with the severity of neuropathic pain (14). Therefore, we examined the alterations of synaptophysin levels in the superficial dorsal horn of diabetic rats. Our findings indicated that six-week diabetes increased the levels of synaptophysin in the axon terminals of the primary sensory neurons forming synapses in the spinal dorsal horn. These results correlate with some previous findings indicating the diabetes-induced increase in synaptophysin immunoreactivity and numerical enhancement of synapses in the dorsal horn (13,14,40). It can be suggested that this augmentation in the presynaptic region increases the release of neurotransmitters and neuromodulators, provides faster and more intense transmission of the nociceptive signal, and reduces the pain threshold. Our study examining the potential effect of reboxetine on this increased synaptophysin immunoreactivity in the spinal dorsal horn demonstrated that administration of this drug for 2 weeks significantly reversed the diabetes-induced alterations. These findings suggest that the analgesic effect of reboxetine against diabetic neuropathic pain may be related to its inhibitory effect on synaptophysin overexpression in the dorsal horn, as well as BDNF.

Forming and developing the postsynaptic terminals in excitatory synapses is as crucial as pre-synaptic partners for synaptic plasticity in neuropathic pain (46,47). PSD-95 also known as SAP-90 encoded by the DLG4 gene is a main postsynaptic scaffolding protein in excitatory glutamatergic neurons. This protein is a member of the membrane-associated guanylate kinase (MAGUK) superfamily. Structurally, it has three PDZ domains in the membrane that anchor receptor proteins to cytoskeletal components and an SH3-GUK (Src homology 3-guanylate kinase) domain (48,49). PSD-95, located at the postsynaptic compartment of excitatory synapses called "postsynaptic density", is a regulator of synaptic plasticity and synaptogenesis (49). This postsynaptic marker has been reported to lie behind central sensitization in neuropathic pain via interacting with glutamatergic NMDA and AMPA receptors (14). In this study, in order to clarify diabetes-induced alterations in the postsynaptic terminals, we investigated the immunoreactivity of PSD-95 in the superficial dorsal horn of the spinal cord. Obtained findings showed that levels of

PSD-95, the postsynaptic marker of synaptic integration, have not changed for diabetic rats. Considering the findings of this study pointing to synaptophysin overexpression in diabetic conditions, it can be suggested that the unchanged expression of PSD 95 may be a compensatory mechanism following excessive neuromediator release from presynaptic neurons. Indeed, in a recent study, Calabrese et al. also reported that one-month-long diabetes in rats induced a significant increase in presynaptic proteins (synapsin-1 and syntaxin-1) but they did not detect an alteration in diabetes on the expressions of PSD proteins (PSD-95, GluN1, GluN2A, and GluN2B) in the spinal dorsal horn (50). The findings of this study are consistent with our data. On the other hand, some other studies suggested that diabetic animals showed an enhancement in the levels of both synaptophysin and PSD-95 (13,14). The contradictory findings may be related to the differences between the experimental conditions. Actually, more detailed and time-dependent experiments are needed to clarify changes in synaptic protein expressions during diabetes progression.

In this study, our further experiments about the effect of 14-day-reboxetine-treatment on PSD-95 immunoreactivity in the superficial dorsal horn of diabetic rats revealed that PSD-95 has not been changed following the drug administrations, which suggests that alteration in PSD-95 expression did not mediate the efficacy of reboxetine on diabetic neuropathic pain.

A limitation of this study is the lack of data on the effects of reboxetine on normoglycemic rats, as reboxetine treatment may also cause significant changes independent of diabetic conditions. Indeed, previous studies reporting a direct effect of reboxetine on brain BDNF levels in healthy (51,52) and depressed (53,54,55) subjects may point to the potential of this drug to alter spinal neurotrophin levels. Similarly, reboxetine may also have direct effects on synaptophysin levels. Therefore, it is clear that new studies investigating the effects of reboxetine treatment on neurotrophin and other synaptic protein levels are needed.

5. CONCLUSION

In conclusion, the results of the present study revealed that subacute reboxetine administration in diabetic rats suppresses the over-expressions of both BDNF and synaptophysin in the spinal dorsal horn. Therefore, it can be suggested that reboxetine may reveal its antihyperalgesic effect on PDN by preventing the establishment of central sensitization and weakening the presynaptic neurotransmitter release.

To our knowledge, this is the first study associated with the antihyperalgesic effect of reboxetine with some molecular mechanisms in the superficial dorsal horn of diabetic rats. On the other hand, assessing the effects of reboxetine on diabetes-induced changes in the structural synaptic plasticity parameters such as, the number of dendritic branches, the width of the synaptic cleft, and the thickness of postsynaptic matter, etc. may provide further understanding of the exact activity mechanism of this drug against neuropathic pain.

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Acquisition of data for the study: NTY, UIU
Analysis of data for the study: NTY, UIU
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Clinical and Experimental Health Sciences

Relationship Between Staging and Grading of Periodontitis and Periimplantitis: A Retrospective Study

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ABSTRACT

Objective: The purpose of this retrospective study was to determine the relationship between peri-implantitis and stage/grade of periodontitis.

Methods: Records of 171 periodontitis patients with 318 dental implants were screened. Classification of diagnosed patients with periodontitis were done by both stage (1, 2, 3 and 4) and grade (A, B and C). The conditions of the peri-implant tissues were assessed as no peri-implantitis or peri-implantitis. Marginal bone loss severity of implants diagnosed with peri-implantitis, prosthesis type (single, bridge), location of dental implants (maxilla, mandibula, anterior and posterior), smoking (yes/no) and diabetes history (yes/no) of the patients were also evaluated. Analysis was done at implant level.

Results: A total of 203 (63.8%) dental implants were diagnosed with peri-implantitis. There were statistical differences in the stage and grade of periodontitis between implants diagnosed with no peri-implantitis and peri-implantitis ($p<.05$). All of the dental implants in stage 4 periodontitis patients were diagnosed with peri-implantitis. Staging (1/2 versus 3/4) and grading (A/B versus C) of periodontitis had significant effects on the marginal bone loss of implants (radiographically ≥25% or <25% of the implant length) diagnosed with peri-implantitis. The marginal bone loss risk increased 3.86 times in stage 3/4 compared to stage 1/2 and 3.16 times in patients with grade C periodontitis compared to grade A/B.

Conclusion: The outcome of this study indicates that peri-implantitis was quite prevalent in dental implant patients with periodontitis, depending on the stage/grade. The severity of peri-implant marginal bone loss of implants was related to higher-level staging and grading of periodontitis.
Keywords: Alveolar bone loss, peri-implantitis, periodontitis

1. INTRODUCTION

Periodontitis is a widespread disorder; the severe form of periodontitis ranks 6th as one of the most common diseases (1). A questionnaire published in 2015 revealed that almost 50% of the adult (aged ≥30 years) population present periodontitis. Additionally, the incidence of periodontitis is 68% in individuals aged ≥65 years (2). It has a multifactorial mechanism in which host response, environmental and acquired factors, local factors, drugs used, genetic predispositions play an important role, usually pathogenic bacteria.

Peri-implantitis is a pathogenic condition that causes the progressive bone loss around dental implants (3). In the literature, there are similarities in the pathogenesis and etiology of periodontitis and peri-implantitis. The etiology of both diseases is thought to be due to the presence of a microbial biofilm (4). Peri-implantitis and periodontitis are both chronic inflammatory diseases due to a biologically destructive interplay between subgingival microbial biofilm and the host immunoinflammatory response, which can cause destruction of tooth/implant supporting tissue and result in tooth/implant loss. In the 2017 World Workshop, there is significant evidence that patients with a history of periodontitis, a lack of routine maintenance treatment, and poor biofilm control are more likely to acquire peri-implantitis (3). Potential theories for the relation between periodontitis and peri-implantitis include that periodontitis patients may have a defective host immune response, more pathogenic bacterial species, or a greater bacterial load (5). A number of studies have examined the peri-implantitis prevalence and its correlations with either current periodontitis or a history of periodontitis. In a 10-year cohort clinical trial (6), 45 patients who had no previous periodontitis history and 8 patients underwent implant treatment after their periodontal treatments were completed, and the 10-year peri-implantitis incidence was 29% in patients with periodontitis compared to 6% in the non-periodontitis subjects. In a systematic review (7), patients with periodontitis had a higher overall percentage of biologic complications, such as implant loss, compared to non-periodontitis patients. Daubert et al.(8) revealed that severe form of periodontitis was significant risk indicator with an unadjusted risk ratio of 7 of all examined variables for peri-implantitis. According to a review (3),

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there are studies reporting that the risk of developing peri-implantitis is not associated with patients with a history of periodontitis (aggressive or chronic) and associated with an increase of 2.2-19 times. Derks et al. (9) reported a risk ratio of 4 for moderate/severe peri-implantitis in patients with current periodontitis in a 9-year follow-up of 588 patients. There are also studies stating that periodontitis is not related to peri-implantitis (10, 11). A cross sectional study (11) involving 134 patients failed to show an increased risk of peri-implantitis patients who had a history of periodontitis. There are limited studies examining the association between peri-implantitis and periodontitis based on the latest classification. Ravida et al. (12) reported that there was an association between the grade of periodontitis and the occurrence of implant failure. Yamazaki et al. (13) found that the peri-implant disease prevalence was higher in patients who had Stage 4 periodontitis. Considering the World Workshop in 2017 periodontitis and peri-implantitis case definitions could lead to more accurate comparisons and analyzes to explore in revealing potential associations. Thus, in this retrospective study, it was aimed to determine the association between the stage and grade of periodontitis and the presence and severity of peri-implantitis.

2. METHODS

The ethical approval of the present retrospective study was granted by the Clinical Research Ethics Committee of Faculty of Dentistry Marmara University (Protocol number: 2022.092). The protocol of this study was in accordance with the principle stated in the Helsinki Declaration of 1975, as revised in 2013. The data was collected from patient charts, both physical and electronic, received between January 2018 and September 2022 at the Faculty of Dentistry, Marmara University, Turkey. For inclusion in the present study, subjects had to fulfill the following criteria: patient with one or more implants in functions for at least 1 year, patients with fixed prosthesis placed on dental implants, patients diagnosed with periodontitis, patient with reliable and available demographic, medical, radiographical and periodontal data. The exclusion criteria were as follows: non-periodontitis patients, patients who use overdentures or all-on-four/six, patients with unclear or incomplete data.

Periodontitis was identified according to Word Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions in the 2017 (14). The current classification is based on the stages and grades of periodontitis. In staging, the severity and extent of periodontitis were assessed in this study. The severity of periodontitis was based on the periodontal breakdown of the worst-affected tooth and classified as Stages 1, 2, 3, and 4. Generalized, localized (<30% of teeth affected) and molar/incisor pattern are three categories used to describe the extent of periodontitis. Grade of periodontitis is determined using indirect evidence of progression rate. Bone loss at the worst-affected tooth (calculated as radiographic bone loss which is a percentage of root length divided by the patient's age) in the dentition as a function of age. Grade A is characterized as a slow rate of progression, Grade B as having a moderate rate of progression and Grade C as having a rapid rate of progression.

Case definition of peri-implantitis was made according to current classification system of peri-implant diseases and conditions guidelines (15). In this study, implants were evaluated for the peri-implantitis or no peri-implantitis. Moreover, the most coronal implant-bone contact point was determined radiographically to represent the interproximal marginal bone level and was quantified as a percentage of implant length to categorize the degree of bone loss (<25%, 25%-50% or >50% of the implant length) (12). The number of implants, their location (maxilla, mandibula, anterior and posterior) and prosthesis type (single, bridge), smoking (yes/no) and diabetes history (yes/no) of the patients were also assessed.

2.1. Statistical Analysis

Chi-squared test was applied for analysis of data. Binary logistic regression analysis was performed to determine associations between marginal bone loss of implants diagnosed with peri-implantitis and periodontal status (stage/grade). A statistical software package (SPSS v20.0for Windows, IBM, Chicago, IL) was used for statistical analysis. Data analysis was done at the implant level. When $p<.05$, the differences were regarded as significant.

3. RESULTS

A total of 171 periodontitis patients with 318 implants composed of 105 (61.4%) females and 66 (38.6%) males, with a mean age of 49.3±11.6 years (range 21 to 74 years) were included in the present study. Most of the patients were non-smokers (84.0%) and only 10.2% had a history of diabetes. At implant level, 203 (63.8%) implants were diagnosed with peri-implantitis (Table 1). No statistically significant difference was detected between smoking status, presence of diabetes, and peri-implant health status ($p>.05$). There was no significant difference between the locations and archs of the implants and the presence of peri-implantitis ($p>.05$). There were statistical differences in the stage and grade of periodontitis between implants diagnosed with peri-implantitis and no peri-implantitis ($p<.05$). Of the patients with peri-implantitis, 6.9% had stage 1 periodontitis, 34.5% stage 2, 50.2% stage 3 and 8.4% stage 4. All of the dental implants in stage 4 periodontitis patients were diagnosed with peri-implantitis. In terms of grading, patients diagnosed with peri-implantitis were 5.9% in grade A, 67.5% in grade B, and 26.6% in grade C. The extent of periodontitis was generalized in the majority of both the study population (79.6%) and patients diagnosed with peri-implantitis (82.7%). According to the severity of marginal bone loss, the distribution of implants with peri-implantitis in the stage and grade of periodontitis are presented in Figure 1 and Table 2 ($p<.05$). Binary logistic regression model outcomes showed that grading (A/B versus C) and staging (1/2 versus 3/4) significantly affected the

marginal bone loss (>25%) of implants diagnosed with peri-implantitis (Table 3). The marginal bone loss risk increased 3.86 times in stage 3/4 compared to Stage 1/2 and 3.16 times in patients with grade C periodontitis compared to grade A/B.

Table 1. *Comparison of demographic characteristics, periodontal status and features of the implant according to peri-implantitis status*

Variables	Total N (%)	Peri-implantitis status		p
		Peri-implantitis N (%)	No Peri-implantitis N (%)	
Number of implants	318 (100.0)	203 (63.8)	115 (36.2)	
Smoking				.203
Yes	51 (16.0)	37 (18.2)	14 (12.2)	
No	267 (84.0)	166 (81.8)	101 (87.8)	
Diabetes				.341
Yes	32 (10.2)	18 (9.1)	14 (12.2)	
No	286 (89.9)	185 (91.1)	101 (87.8)	
Stage				**.000**
1	44 (13.8)	14 (6.9)	30 (26.1)	
2	109 (34.3)	70 (34.5)	39 (33.9)	
3	148 (46.5)	102 (50.2)	47 (40.0)	
4	17 (5.3)	17 (8.4)	0 (0.0)	
Grade				**.000**
A	39 (12.3)	12 (5.9)	27 (23.5)	
B	206 (64.8)	137 (67.5)	69 (60.0)	
C	73 (23.0)	54 (26.6)	19 (16.5)	
Extent				.058
Localized	64 (20.1)	34 (16.7)	30 (26.1)	
Generalized	254 (79.9)	169 (83.3)	86 (73.9)	
Arch				.908
Maxilla	162 (50.9)	104 (51.2)	58 (50.4)	
Mandible	156 (49.1)	99 (48.8)	57 (49.6)	
Position				.873
Anterior	50 (15.7)	33 (16.3)	17 (14.8)	
Posterior	268 (84.3)	170 (83.7)	98 (85.2)	
Prosthesis type				.482
Single-unit	145 (45.6)	96 (47.3)	49 (42.6)	
Multi-unit	173 (54.4)	107 (52.7)	66 (57.4)	

Chi-square test, p<.05.

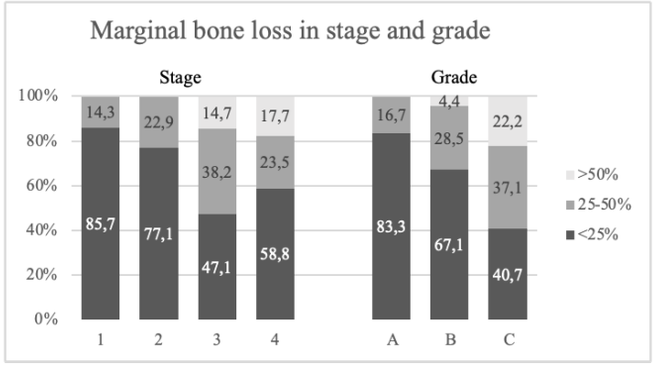

Figure 1. *Distribution of implants diagnosed with periimplantitis according to marginal bone loss severity in stage and grade periodontitis*

Table 2. *Comparison of marginal bone loss severity of implants diagnosed with periimplantitis with periodontal status*

	Marginal Bone Loss N (%)				p
	Total N=203	<25 N =124	25-50 N=61	>50 N=18	
Stage					.000
1	14 (6.9)	12 (9.7)	2 (3.3)	0 (0.0)	
2	70 (34.5)	54 (43.5)	16 (26.2)	0 (0.0)	
3	102 (50.2)	48 (38.7)	39 (63.9)	15 (83.3)	
4	17 (8.4)	10 (8.1)	4 (6.6)	3 (16.7)	
Grade					.000
A	12 (5.9)	10 (8.1)	2 (3.3)	0 (0.0)	
B	137 (67.5)	92 (74.2)	39 (63.9)	6 (33.3)	
C	54 (26.6)	22 (17.7)	20 (32.8)	12 (66.7)	

Chi-square test, p<.05.

Table 3. *Binary logistic regression analysis of ≥25% marginal bone loss of implants diagnosed with periimplantitis in association with periodontal status (stage/grade)*

	OR	95% CI	p
Stage			
1-2	*Ref*	2.047-7.263	.000
3-4	3.856		
Grade			
A-B	*Ref*	1.659-6.007	.000
C	3.157		

OR:odds ratio; CI:confidence interval; p<.05.

4. DISCUSSION

Modern dentistry is very interested in the biological issues that can arise with osseointegrated dental implants. Peri-implantitis is a pathological disorder that affects the tissues around dental implants and is characterized by a progressive loss of implant supporting bone and inflammation of the peri-implant connective tissue. There are conflicting findings in the data on prevalence and risk factors/indicators of peri-implantitis. In this retrospective study, the relationship between peri-implantitis and stage/grade of periodontitis was evaluated.

A history of periodontitis is a risk indicator or factor for peri-implantitis in the literature (15). According to a meta-analysis, patients with periodontitis had a 2.3 times higher risk of developing peri-implantitis than periodontally healthy individuals (16). Roccuzzo et al. (17) showed a peri-implantitis prevalence of 47.2% in severe periodontitis patients and 27% in moderate periodontitis patients. According to Pjetursson et al. (18), the periodontitis patients with residual periodontal probing depths ≥5 mm had significantly more risk for implant loss and peri-implantitis. In patients with severe periodontitis, residual probing depths ≥6mm including >10% of sites after treatment were found to be a significant risk factor for development of peri-implantitis. Additionally, implants applied to patients who had previously tooth loss due to periodontitis were significantly susceptible to develop peri-implantitis and showed 0.5 mm more marginal bone loss after 5 years (16). Most of the studies examining the relationship between periodontitis and peri-implantitis in

the literature are based on the classifications before 2017. Romandini et al. (19) used the AAP/CDC case definitions for periodontal status assessment and the 2017 World Workshop for the peri-implantitis definition. According to authors, peri-implantitis prevalence was 12.4% in healthy subjects and 27.9% in periodontitis patients. To best of our knowledge, there are only two publications evaluating the association between the new classification of periodontitis and peri-implantitis. Ravida et al. (12) found that the prevalence a peri-implantitis prevalence of 33.3% in stage 1 and 2, 17.2% in stage 3, and 35.5% in stage 4 periodontitis patients. Although there was an increasing trend for stage 4, they did not find a significant relationship between the peri-implantitis prevalence and severity of periodontitis. In a recent study, Yamazaki et al. (13) found that in stage 4 patients, peri-implantitis prevalence was significantly higher. In two previous studies, although the number of patients diagnosed with peri-implantitis in grade B and C periodontitis patients were higher than in grade A periodontitis patients, none of the differences were statistically significant. In the present study, peri-implantitis were higher in stage 3 and 4, and grade B and C periodontitis.

Since the case definitions in 2017 World Workshop for peri-implantitis did not support categorization between severity levels of peri-implantitis based on the extent of marginal bone loss. Ravida et al. (12) evaluated the severity of peri-implantitis in terms of the degree of marginal bone loss. The severity of marginal bone loss was radiographically categorized <25%, 25-50% or >50% of the implant length. The increased severity of marginal bone loss (>25%) was significantly affected by grade of periodontitis (C versus A/B), whereas not by staging. Patients having a previous history of Grade C periodontitis compared to Grades A/B experienced a 7.6-fold greater risk of severe marginal bone loss. In a recent research (19), marginal bone loss was calculated from radiographs by calculating the distance between the fixture/abutment joint and the marginal bone level with a digital caliper, and averaging the mesial and distal bone resorption. Marginal bone loss was assessed by classifying it as <3 mm and ≥3 mm, Stage 4 was found to be significantly higher in the marginal bone loss ≥3mm group. Similar to previous studies, the severity of peri-implant marginal bone loss was also linked in this study to higher-level staging and grading of periodontitis.

Smoking has been linked to periodontal disease through a number of processes, including disruptions in the inflammatory and responses of the host to possible periodontal pathogens, changes to the subgingival microbial populations, and impaired tissue healing capacity that causes an unbalanced state of tissue homeostasis (20). According to Karoussis et al., (6) only 6% of implants in non-smokers had peri-implantitis, compared to 18% of all implants in smokers. While 3 cross-sectional researches confirm these outcomes, with odds ratios of 32 (21), 3 (22) and 5 (23), there are also studies reporting no higher risk in smokers (8, 24). Smoking does not currently appear to be a risk factor or signal for peri-implantitis, according to conclusive evidence (15). No significant association was detected between peri-implantitis and smoking in the present study. This could be as a result of the limited number of smokers, the self-reporting of smoking histories utilized in this study, and the definition of smoking status.

Hyperglycemia-induced release of advanced glycation end products and a variety of common risk variables of a genetic, microbiological, and lifestyle character are among the mechanisms underlying correlations between diabetes mellitus and periodontal disease (25). According to several studies, an increased risk of peri-implantitis exists in diabetic patients. Ferreira et al. (16) showed that individuals with diabetes (24.13%) had a higher risk of developing peri-implantitis than non-diabetic patients (6.56%) and an OR of 1.9 was recorded. Daubert et al. (8) demonstrated a 3-fold risk for peri-implantitis in diabetes patients at the time of implant placement. Ravida et al. (12) recorded that 19.6% of individuals with peri-implantitis did not have a diagnosis of diabetes, while 29.6% had a diagnosis of diabetes, but this difference was not statistically significant. Recently, Romandini et al. (19) have failed to show an association between diabetes and peri-implantitis. Similarly, in this study, no association was showed between the diagnosis of diabetes and peri-implantitis. It is thought that there is a lack of power due to the low percentage of patients with a history of diabetes among patients included in the study. Additionally, it was stated in the 2017 World Workshop that there is not available evidence to determine whether diabetes is a peri-implantitis risk indicator or factor (15).

The risk factors of periimplantitis associated with the implant-supported prosthesis have been established in the literature. Ill-fitting/ill-designed fixed and cement retained restorations are considered as risk factors for periimplantitis (26). Previous studies (19, 27) have also noted a correlation between the presence of peri-implantitis and the type of restoration (single crowns versus bridges), which may be clarified by the more challenging accessibility to oral hygiene practices. Contrary to previous studies, in the present study, there was no difference between single and bridge prostheses in terms of the occurrence of peri-implantitis.

The main limitation of the present study is the use of existing data in the system due to its retrospective nature. Limitations include the absence of implant brand names in the records and the fact that each dental implant was placed and began to function at a different time. Moreover, there is strong evidence in the literature that the risk of developing peri-implantitis is increased in patients with poor plaque control skills and no regular maintenance care after implant therapy (15). Only the association was evaluated in this retrospective study, further prospective clinical researches are needed to investigate the cause-effect relationship.

5. CONCLUSION

The outcome of this study indicates that peri-implantitis was quite prevalent in dental implant patients with periodontitis,

depending on the stage/grade. The severity of peri-implant marginal bone loss of implants was related to higher-level staging and grading of periodontitis. Prior to receiving implant treatment, patients' periodontitis stage and grade may prove to be a useful risk indicator of developing periimplantitis. To support this conclusion, additional clinical research is necessary.

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Clinical and Experimental Health Sciences

Obesity Awareness Among Elementary School Students: A Controlled Before – After Study

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ABSTRACT

Objective: Obesity is a metabolic disorder that occurs due to excessive body fat accumulation and can lead to physical and emotional problems. Preventing and treating obesity in childhood and adolescence is crucial. The aim of this study was to evaluate the effectiveness of obesity awareness education provided to fourth-grade elementary school students.

Methods: An experimental study with pretest-posttest control group design was conducted. The study sample consisted of a total of 663 students, 344 students in the experimental group and 319 students in the control group, who attended the fourth grade of two elementary schools in Istanbul and met the inclusion criteria.

Results: The sample included 326 girls (49.2%) and 337 boys (50.8%). The mean (SD) BMI was 18.45 (3.49) in the experimental group and 18.04 (3.00) in the control group. 73% (n = 251) of the students in the experimental group and 77.7% (n = 248) of the students in the control group stated that obesity only made walking/running difficult. There was no difference in obesity awareness scores between the experimental and control groups before the education (p=0.92). However, at 1 week and 1 month after the education, the experimental group had significantly higher scores compared to the control group (p<0.001 for both).

Conclusion: Our results showed that fourth-grade elementary school students were aware of obesity, but their awareness increased significantly compared to the control group after receiving obesity awareness education. This study showed that raising children's awareness would help avoid factors that could lead to obesity.

Keywords: Obesity, health education, school-age children, awareness

1. INTRODUCTION

The World Health Organization (WHO) defines obesity as "excessive fat accumulation that presents a risk to health" (1). Obesity is a public health problem that negatively affects both the duration and quality of life (2,3). Numerous international organizations have declared obesity an epidemic that poses a growing threat to children and adolescents worldwide, especially in developed countries (4,5). The significant increase in childhood obesity also predisposes to type 2 diabetes, asthma, sleep apnea, hypertension, cardiovascular disease, musculoskeletal system disorders, increased mortality and morbidity rates in adulthood, low self-esteem, and emotional and social problems (2-4).

The WHO reported that 39 million children under the age of 5 were overweight or obese in 2020 and over 340 million children and adolescents aged 5-19 were overweight or obese in 2016 (6). In the United States, the prevalence of obesity in children aged 2-19 years is 18.5% (7). In Türkiye, the prevalence of obesity is reported to be 8.5% in children aged 0-5 years and 8.2% among children and adolescents aged 6-18 years (8).

The literature indicates that a child's diet is shaped by their eating habits during infancy, that the dietary habits of parents can cause obesity, and that changes in the gut microbiota can also be a precipitating factor in the development of obesity (9). Moreover, obesity can also be triggered by environmental factors in children with genetic predisposition (10). With rapid urbanization, the increased consumption of ready-to-eat foods, irregular eating habits, as well as low levels of physical activity and more sedentary lifestyles increase the risk of obesity (4). For these reasons, obesity prevention and treatment in childhood and adolescence is becoming increasingly important (4,11).

Children spend the majority of their waking hours in school (12). Therefore, the school setting provides access to large numbers of children and is a critical environment for introducing health services and strategies and promoting health behaviors (12,13). The earlier in life health-promoting behaviors are learned, the more permanent and effective they will be (14). In addition, within the team that provides school health services, school nurses play a vital role in protecting student

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health (15). School nurses have important responsibilities in the fight against obesity at health promotion (15,16). The aim at health promotion is to maintain a healthy weight or reduce weight to within normal limits (3). School nurses can take precautions to lower the risk of obesity or overweight, and can act as educators and role models for school children in developing healthy dietary and physical activity habits (12,16). In the management of obesity, nursing interventions such as education, care, and support are important for devising, implementing, and evaluating strategies to solve the problem of obesity in children (17).

Obesity is a serious global health problem, and its prevention, early diagnosis, and treatment is becoming increasingly urgent (3,9). We believe that this study will raise awareness about childhood obesity among school children and contribute to similar studies.

The aim of this study was to evaluate the effectiveness of obesity awareness education provided to fourth-grade elementary school students. The hypothesis was **"**obesity awareness education program is effective to achieve obesity awareness for elementary school children"

2. METHODS

2.1. Study Design

An experimental study with pretest-posttest control group design was conducted between February and April 2017 at two different primary state schools in Istanbul.

2.2. Participants

The study included fourth-grade students enrolled in two elementary schools in Istanbul who met the selection criteria. Sample size was determined by performing power analysis using the G*Power (v3.0.10) program. The power of the study is expressed as 1-β (β = Type II error probability) and in general, studies should have at least 80% power (20). The standard deviation of the total score of the Obesity Awareness Scale (OAS) used in this study was reported as 58.28± 8.66 in the literature (19). Using this value, the total sample size for 90% power at an alpha error level of 0.05 and effect size 0.30 was 644 subjects. The sample comprised a total of 663 students, 344 in the experimental group and 319 in the control group. 25 students in the control group did not participate in the follow-up tests because they were not at school on the day of data collection. Because of that study completed 319 students in control group. Which school will be in the experimental group and which will be in the control group was determined by drawing lots. Because the OAS is designed to measure obesity awareness in children aged 10-14 years, the study was conducted with students in the fourth grade (10 year age group). Inclusion criteria for the study were being a fourth-grade student in the participating schools and not having autism or any mental disability. Exclusion criteria were being absent from school during data collection and not consenting to participate in the study. All the students who met the inclusion criteria participated the study.

2.3. Instruments

Data were collected using a sociodemographic information form and the OAS.

Sociodemographic information form: The form consisted of 16 questions (11 closed-ended, 5 open-ended) prepared by the researchers in line with the literature (20) to evaluate the sociodemographic characteristics of the students participating in the study.

Obesity Awareness Scale (OAS): This scale was developed by Allen (21) and adapted to Turkish by Kafkas and Özen (20). The scale measures obesity awareness in children aged 10-14 years. The Turkish version of the instrument contains a total of 20 items in 3 domains. The Obesity Awareness subscale contains 9 items, the Diet subscale contains 6 items, and the Physical Activity subscale contains 5 items. Each item is rated on a 4-point Likert scale from "strongly disagree" (1 point) to "strongly agree" (4 points). The total score ranges from 20 to 80, with higher total score indicating a higher level of obesity awareness.

2.4. Data Collection

The study was conducted in two different schools to prevent group interactions. Both schools in the study operated using a double-shift schedule (morning and afternoon). Therefore, data collection was carried out during both the morning and afternoon sessions. Data were collected from the experimental and control groups in the students' classrooms under the supervision of the researchers. First, the sociodemographic information form and OSA were administered to both groups as the pretest. Data collection lasted approximately 20 to 30 minutes. After completing the forms, the students in the experimental group received a 30-minute education on obesity awareness on the same day by researchers in their classrooms.

2.5. Intervention

The education covered topics such as the definition of obesity, the prevalence of obesity in children, problems caused by obesity, the causes of obesity, and how to recognize obesity. In the posttests, the entire experimental group completed the OSA again 1 week and 1 month after the education. The students in the control group also completed the OSA at 1 week and 1 month after the pretest, without receiving the education.

2.6. Data Analysis

Statistical analyses were performed using NCSS (Number Cruncher Statistical System) 2007 software (Kaysville, Utah, USA; License No: 167.594.8377483; Serial No: N7H5-J8E5-D4G2-H5L6-W2R7). Kolmogorov-Smirnov test was used to determine whether continuous variables were normally distributed. Data were not normally distributed.

Mann-Whitney U test was used to evaluate differences between the experimental and control groups. Friedman test was used to compare the means of multiple dependent groups, and pairwise comparisons were performed using Wilcoxon paired rank test to determine the source of significant differences. The results were evaluated within a 95% confidence interval. Statistical significance was accepted at p values less than 0.05. For categorical variables, frequency and percentage values were presented as descriptive statistics.

2.7. Ethical Considerations

Approval to conduct the study was obtained from the Marmara University Institute of Health Sciences Ethics Committee (approvel date 07.11.2016 and number 88) and written consent obtained from T. C. Istanbul Governorship Provincial Directorate of National Education (13.01.2017-493278). However, a written informed consent was obtained from all children participating in the study and their families. After completing data collection from the experimental and control groups, the students in the control group were also given the obesity awareness training.

3. RESULTS

3.1. Characteristics of the Participants About Obesity

The characteristics of the participants are shown in Table 1. The sample included 326 girls (49.2%) and 337 boys (50.8%). The mean (SD) BMI was 18.45 (3.49) in the experimental group and 18.04 (3.00) in the control group. There was no difference between experimental and control group according to pre-test results of OSA (U=54639.50; p=0.92). 73% (n = 251) of the students in the experimental group and 77.7% (n = 248) of the students in the control group stated that obesity only made walking/running difficult. However, it was found that the same group did not have sufficient information about other health problems caused by obesity (diabetes, kidney disease, circulatory disorder).

3.2. The Effect of Obesity Awareness Education

There was no difference in obesity awareness scores between the experimental and control groups before the education (p=0.92). However, at 1 week and 1 month after the education, the experimental group had significantly higher scores compared to the control group (p<0.001 for both). In within-group comparisons, the experimental group showed significant increases in OSA score at 1 week and 1 month after the education when compared to before the education (p<0.001). Pairwise comparisons showed that OSA scores at both 1 week and 1 month were higher than before the education, and that the score at 1 month was higher than at 1 week (Table 2). Comparison of the students' OSA scores based on their sociodemographic characteristics is shown in Table 3.

Table 1. *Characteristics of the participants about obesity*

Characteristic	Experimental group (n = 344)		Control group (n = 319)	
	n	%	n	%
What is obesity?				
Extremely overweight	265	77.0	267	85.9
Diabetes	46	13.4	27	8.7
Kidney disease	18	5.2	7	2.2
Circulatory disorder	15	4.4	10	3.1
How many hours a day do you watch TV or play with the computer?				
Less than 2 hours	209	60.8	219	68.7
2 hours or more	135	39.2	100	31.3
Do you eat or drink while watching television or using computers?				
Yes	147	42.7	100	31.3
No	197	57.3	219	68.7
Do you have breakfast regularly every morning?				
Yes	289	84	275	86.2
No	55	16	44	13.8
How often do you consume food/drinks like soda, chips, hamburgers, candy, etc.?				
Everyday	13	3.8	26	8.2
A few times a week	132	38.4	147	46.1
Several times a month	109	31.7	89	27.9
Never	90	26.2	56	17.6
How many hours a night do you sleep?				
Less than 8 hours	110	32.0	120	37.6
8 hours or more	234	68.0	199	62.4
Do you do sports?				
Yes	276	80.2	267	83.7
No	68	19.8	52	16.3
Do you eat before going to bed at night?				
Yes	132	38.4	107	33.5
No	212	61.6	211	66.1
Do you get junk food/drinks (soda, candy, etc.) from your friends?				
Yes	86	25.0	49	15.4
No	258	75.0	270	84.6

Table 2. *Comparison of obesity awareness scale scores of the children before and after the education*

Obesity Awareness Scale scores		Experimental group (n=344)		Control group (n=319)		Test value
		Median (Q1-Q3)	Mean rank	Median (Q1-Q3)	Mean rank	U; p
OAS total score	Pretest[a]	56 (50-63)	1.65	56 (49-62)	1.89	54639; 0.92
	Posttest (1 week)[b]	61 (55-66)	2.12	57 (49-65)	2.05	**42773; <0.001**
	Posttest (1 month)[c]	62 (57-66)	2.22	57 (50-65)	2.06	**93813; <0.001**
	χ^2; p	**66.967; <0.001** b>a c>a c>b		**5.931; 0.06**		

χ^2: Friedman test U: Mann-Whitney U test Significant results shown in bold (p<0.05)

Tablo 3. *Comparison of obesity awareness scale scores according to sociodemographic characteristics about obesity*

Characteristic		Experimental group(n=344)		Control group(n=319)		Test value
		Median (Q1-Q3)	Mean rank	Median (Q1-Q3)	Mean rank	U; p
How many hours a day do you watch TV or play with the computer?						
< 2 hours	Pretest	57 (50-63)	1.62[a]	56 (49-63)	1.87	22725.50; 0.90
	Posttest (1 week)	61 (55-66)	2.17[b]	57 (50-65)	2.06	**17701; <0.001**
	Posttest (1 month)	62 (57-66)	2.20[c]	58 (50-65)	2.07	**18006; <0.001**
	Test value (χ^2; p)	**46.06; <0.001 a<b; a<c; b<c**		5.88; 0.05		
≥ 2 hours	Pretest	56 (51-63)	1.70[a]	56 (51-62)	1.94	6746.50; 0.995
	Posttest (1 week)	60 (55-65)	2.04[b]	56 (49-62.75)	2.02	**5214; 0.003**
	Posttest (1 month)	62 (56-66)	2.26[c]	56 (49.25-64.75)	2.04	**4908.50; <0.001**
	Test value (χ^2; p)	**23.02; <0.001 a<b; a<c;b<c**		0.58; 0.74		
Do you have breakfast regularly every morning?						
Yes	Pretest	57 (51-63)	1.67[a]	56 (49-62)	1.90	38327; 0.466
	Posttest (1 week)	61 (55-66)	2.12[b]	56 (49-65)	2.06	**30278.50; <0.001**
	Posttest (1 month)	62 (57-66)	2.21[c]	57 (49-65)	2.04	**29374; <0.001**
	Test value (χ^2; p)	**50.80; <0.001 a<b; a<c; b<c**		4.42; 0.10		
No	Pretest	54 (46-61)	1.57[a]	56 (52-62)	1.86	1008.50; 0.213
	Posttest (1 week)	59 (54-64)	2.13[b]	58 (49-64)	1.95	1002.50; 0.197
	Posttest (1 month)	61 (55-64)	2.30[c]	60 (52-64)	2.19	1090; 0.507
	Test value (χ^2; p)	**17.29; <0.001 a<b; a<c; b<c**		2.55; 0.27		
How many hours a night do you sleep?						
< 8 hours	Pretest	56 (49-63.25)	1.71[a]	56 (49.25-64)	1.96	6380; 0.662
	Posttest (1 week)	60 (54-66)	2.05[b]	56 (47-63.75)	1.99	**4892; 0.001**
	Posttest (1 month)	62.50 (55.75-66)	2.24[c]	55.50 (48-64)	2.05	**4797.50; <0.001**
	Test value χ^2; p)	**16.41; <0.001 a<b; a<c; b<c**		0.44; 0.79		
≥ 8 hours	Pretest	56 (51-62)	1.63[a]	56 (49-62)	1.85[a]	22755.50; 0.684
	Posttest (1 week)	61 (55-66)	2.16[b]	57 (50-65)	2.08[b]	**18778; 0.001**
	Posttest (1 month)	61 (57-66)	2.22[c]	58 (50-66)	2.07[c]	**18420; <0.001**
	Test value (χ^2; p)	**51.97; <0.001 a<b; a<c; b<c**		**7.20; 0.02 a<b; a<c; c<b**		
Do you do sports?						
Yes	Pretest	58 (51-63)	1.67[a]	56 (50-63)	1.90	35823.50; 0.576
	Posttest (1 week)	61 (55.25-6)	2.13[b]	57 (49-65)	2.03	**28263.50; <0.001**
	Posttest (1 month)	62 (56.25-66)	2.20[c]	58 (50-65)	2.07	**28770.50; <0.001**
	Test value (χ^2; p)	**48.68; <0.001 a<b; a<c; b<c**		4.31; 0.11		
No	Pretest	52.50 (47-59.75)	1.60[a]	55 (47.50-60)	1.85	1614; 0.414
	Posttest (1 week)	60 (52.25-65)	2.09[b]	57 (49-62)	2.12	1455; 0.097
	Posttest (1 month)	61.50 (57-65)	2.32[c]	54.50 (50-64)	2.04	**1238.50; 0.005**
	Test value (χ^2; p)	**19.52; <0.001 a<b; a<c; b<c**		2.08; 0.35		

χ^2: Friedman test U: Mann-Whitney U test Significant results shown in bold (p<0.05)

4. DISCUSSION

In this study, obesity awareness scores at 1 week and 1 month after the education were significantly increased compared to before the education (Table 2). The students included in the study were 10 years old. Raising obesity awareness may be easier in elementary school children due to their enthusiasm for and openness to learning and their high capacity for acquiring knowledge and skills. Making the education provided to children interesting and engaging may result in the correct behaviors being more memorable and easier to apply to their lives. Furthermore, the high retest results at 1 month after the education show that the education was effective and remembered by the children. We also believe that the interactions between the participating students made the education memorable.

In this study, obesity awareness scores increased significantly in all children who received the education, regardless of their daily screen time (Table 3). In a study on obesity, it was observed that students were active most of the time at school, whereas in the time they spent at home, they were mostly inactive (in front of a television or computer) and their diet changed. Healthy eating and physical activity habits that start in childhood set the stage for adulthood (11). Studies have demonstrated an increase in food intake and BMI in children who watch television for more than 2-3 hours a day (22,23). In addition, while watching television, children may be influenced by unhealthy foods seen in advertisements or programs and their consumption of these foods may increase, and peers may also influence each other (24,25). These issues were covered during the education provided in this study and it was seen that the education was effective in raising awareness of these topics.

In present study, children who ate breakfast regularly and those who did not had increased obesity awareness scores after the education (Table 3). These findings show that the education was effective. Skipping breakfast in children prevents a healthy eating schedule, which can lead to obesity. Efforts to promote a healthy eating schedule in schools should aim to establish favorable dietary behaviors throughout life (26,27). Breakfast is one of the main meals of the day and has an impact on academic success as well as physical growth and development (28).

Another factor that can contribute to obesity is sleep duration. There are many studies demonstrating the relationship between obesity and sleep (29,30). For children, sleep is as important as nutrition and growing up in a safe environment. Children with sleep problems have increased secretion of hunger hormones and decreased secretion of satiety hormones; in other words, there are also irregularities in the hormones that regulate energy balance. Children who do not sleep enough have a greater desire to eat and feel tired during the day. Therefore, they tend to eat more in order to feel more energetic. This results in the intake of more energy than the body needs, which it then stores as fat (31). All of these factors were also discussed in the obesity awareness education and it was found that the education increased awareness on this subject (Table 3).

While the median score of the students who reported not doing sport was 52.50 before the education, it increased significantly to 60 at 1 week after the education and 61 at 1 month after the education (Table 3). Chang and Kim (32) reported that children's physical activities were low due to reasons such as watching television, playing video games, and using computers, and that a low proportion of the students exercised. The results of our study showed that the education provided was effective both in students who did and did not engage in sports. In addition, the fact that the median scores of the students who did not do sport were very close to those of students who did sport suggests that these students may be ready to adopt healthy lifestyle behaviors. It is crucial that students are encouraged to do sport regularly and make it a lifestyle in order to prevent obesity.

4.1. Limitations

The terms such as body mass index (BMI), diabetes, kidney disease, and circulatory disorder in the OSA were difficult for the students to understand. The researchers explained these terms to the students during data collection.

5. CONCLUSION

Our results showed that fourth-grade elementary school students were aware of obesity, but their awareness increased after receiving obesity awareness education. The hypothesis was proved. Considering that healthy lifestyle behaviors gained during childhood will directly affect adult life, providing obesity awareness education to students in schools is imperative.

5.1. Implications of This Paper

Raising children's awareness may help avoid factors that can lead to obesity. School curricula should incorporate content related to obesity in the subject of health education. In terms of protecting and improving the health of school children, it is also important to include pediatric and school/pediatric nurses who will organize health education for family, teachers, students, and school employees. School/pediatric nurses can assume duties in the fight against obesity such as developing healthy cafeteria interventions and encouraging healthy eating habits, educating classroom teachers to be good role models for their students, and promoting physical activity. National action plans can be organized to reach communities and children collectively, such as giving obesity awareness messages in the press and media, increasing appropriate health programs on television and broadcasting attention-getting slogans during programs with large audiences, planning activities where children and families can participate together, and providing practical healthy lifestyle education.

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Clinical and Experimental Health Sciences

Examination of Factors Affecting the Tendency of Intensive Care Nurses Towards Medical Errors: A Multicenter Study in Turkey

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ABSTRACT

Objective: This study aimed to examine the factors affecting the tendency of intensive care nurses towards medical errors.

Methods: The data of this descriptive and cross-sectional were collected using a Personal Information Form and the Medical Error Trend Scale in Nursing. The population of the study consisted of a total of 647 intensive care unit nurses who worked at two research and training hospitals located in the province of Istanbul and one university hospital located in the province of Edirne. The inclusion criterion was being a registered nurse working in intensive care units for at least two months, and it was aimed to reach the entire population. The sample included 349 nurses (participation rate: 53.12%) who completed the questionnaire.

Results: While76.5% of the participants were female, their mean age was 28.96±5.70 years, and 73.4% had graduated from universities. The order of significance of the four independent variables, which were determined to have significant effects on the Medical Error Trend Scale in Nursing scores of the participants, was satisfaction with working in the intensive care unit, the number of patients per nurse, having an intensive care nurse certificate, and weekly working hours.

Conclusion: The tendencies of the nurses who worked for 40 hours per week, those who were satisfied with working in the intensive care unit, those who provided care for 1 or 2 patients per day, and those who had an intensive care nurse certificate towards medical errors were lower in comparison to the others.

Keywords: Intensive care units, medical errors, nurses

1. INTRODUCTION

The Joint Commission on Accreditation of Healthcare Organizations (JCAHO) defines medical errors as the patient's being damaged due to inappropriate and unethical behavior, and inadequate and negligent actions of healthcare professionals (1). Medical errors are a serious public health problem and pose a threat to patient safety. Healthcare organizations need to establish a culture of safety that focuses on health system improvement by viewing medical errors as challenges that must be overcome (2).

In recent years, medical errors have become some of the most significant healthcare problems in countries experiencing transformations in their healthcare systems (3,4). Posing a threat to patient safety, medical errors are stated to be a major cause of morbidity and mortality and lead to economic losses (5). It is reported that medical errors, the third leading cause of death, lead to more than 250,000 deaths per year in the United States (6). It is seen that 6.7-21% of medical error cases are related to nurses in Turkey (7, 8).

Medical errors lower morale and reduce the motivation of healthcare professionals while causing patients and society to distrust healthcare professionals and the system (9). The current literature focuses on studies in this field and the care environment to find problems with medical errors and develop a solution to these problems (3).

Since high-risk practices are performed more in intensive care units, medical errors may be seen more frequently, as well. It is reported that almost all intensive care patients experience a life-threatening medical error, and patients on average are exposed to 1.7 errors per day. A 10-bed ICU could be anticipated to produce more than 6,200 error reports per year (10).

It is stated that medical errors in intensive care units arise from the high-risk conditions of patients (e.g., age, sex, clinical status), the complex work environment, stress induced by alarm and other medical equipment sounds, workload, and excessive working hours (11,12). Factors such as the lack of experience of nurses, lack of communication skills, physical fatigue, increasing numbers of patients per nurse (above the recommended standards), lack of motivation, environmental factors, lack of attention, and educational factors also

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contribute to medical errors and affect patient safety (13). Among medical errors that commonly occur during the provision of healthcare are adverse drug reactions, catheter-associated urinary tract infections, central line-associated bloodstream infections, injury from falls and immobility, obstetric adverse events, pressure ulcers, surgical site infections, venous thrombosis, ventilator-associated pneumonia, and the wrong site/wrong procedure in surgery (2,14,15). Nurses are responsible for preventing unintended consequences related to procedures and treatments to be performed on the patient and providing care for the patient in a safe environment (16). To prevent medical errors in intensive care, it is important to find the factors affecting tendencies towards medical errors (3).

This study aimed to examine the factors affecting the tendency of intensive care nurses towards medical errors.

The research question:

- What are the (professional and individual) factors affecting the tendency of intensive care nurses towards medical errors?

2. METHODS

2.1. Study Design and Participants

This descriptive and cross-sectional study was carried out in five hospitals with the most intensive care unit beds, located in the Istanbul and Edirne provinces of Turkey between May and December 2018.

The population of the study consisted of a total of 647 intensive care nurses who worked at two university hospitals (n:119 and n:110) and two research and training hospitals (affiliated with the Ministry of Health) (n:170 and n:145) located in the province of Istanbul and one university hospital located in the province of Edirne (n:103). The inclusion criterion was being a registered nurse working in intensive care units for at least two months, and it was aimed to reach the entire population. The sample included 349 nurses (participation rate: 53.12%) who completed the questionnaire.

According to the R^2 Value of 0.15, considered as the primary result of this study, which was obtained in the regression analysis, it was determined that five independent variables were effective on the tendency of nurses towards medical errors. The effect size was found as f^2: 0.18 (moderate effect size), and the power of the sample to represent the population was found as 1.00 (100%) in the post hoc power analysis conducted with the G*Power (3.1.9.2) program. Thus, it was determined that the sample size in the study was sufficient.

2.2. Data Collection Tools

The data were collected using a Personal Information Form and the Medical Error Trend Scale in Nursing.

The Personal Information Form was prepared by the researchers in line with the literature, and it consisted of items questioning the descriptive characteristics (e.g., gender, age, educational background) and professional characteristics (e.g., weekly working hours, number of patients per nurse, willingness to work in intensive care units) of the participants.

Medical Error Trend Scale in Nursing (METSN) was developed by Özata and Altunkan. It consists of 49 items and five subscales, including drug and transfusion applications (18 items), hospital infections (12 items), patient monitoring and material safety (9 items), falls (5 items), and communication (5 items). The scale has a 5-point Likert-type scoring system, and each item has the response options of 1 evaluated as "never", 2 evaluated as "rarely", 3 evaluated as "sometimes", 4 evaluated as "usually", and 5 evaluated as "always". The lowest and highest scores that can be obtained from the scale are 49 and 245. The total score is used in the evaluation of the scale. A higher total score is interpreted as a lower tendency of nurses to make medical errors. The Cronbach's alpha coefficient of the scale was reported as 0.95 by Özata and Altunkan. In this study, we found the Cronbach's alpha coefficient of the Medical Error Trend Scale in Nursing as 0.94 (0.89 for drug and transfusion applications, 0.83 for hospital infections, 0.80 for patient monitoring and material safety, 0.67 for falls, and 0.68 for communication).

2.3. Ethical Considerations

The study was approved by the ethics committee of Trakya University (No:2018/156). At the beginning of the study, the aim and method of the study were explained to the nurses who were reached, and their written informed consent was obtained. Ethical principles of the Declaration of Helsinki were taken into account in the study.

2.4. Data Analysis

The analysis of the data was performed using the "SPSS for Windows 21.0" software package, through parametric and nonparametric descriptive statistical analyses. In data analysis, in terms of descriptive statistics, frequencies and percentages were used for the categorical data, and means and standard deviations were used for the numeric data. Whether the numeric variable, METSN scores, had a normal distribution was evaluated based on skewness (-1.12) and kurtosis (0.99) values, and the data were found to have a normal distribution. In the comparison of the mean METSN scores of the participants based on the independent variables, the independent-samples t-test method was used for the variables that had two groups. For the variables that had three or more groups, one-way analysis of variance (ANOVA) (post hoc analysis: Tukey's HSD test) and Kruskal-Wallis test (post hoc analysis: Bonferroni-corrected Mann-Whitney U Test and Tukey's test) were used in independent groups. The independent variables that affected the scale scores of the participants in the primary analyses were evaluated by multiple regression (backward method) analysis. A post hoc

power analysis was conducted to test the adequacy of the sample size. The significance level was accepted as p<0.05.

3. RESULTS

It was found that 76.5% of the participants were female, their mean age was 28.96±5.70 years, and 73.4% had graduated from universities (Table 1). There was no statistically significant difference between the mean METSN scores of the participants based on their age group, gender, or level of education (p> .05, Table 1).

METSN mean scores were found to be significantly higher among the married participants in comparison to the participants (p< .02) and among the participants who had children in comparison to those without children (p< .001) (Table 1).

Table 1. *Comparison of the Medical Error Trend Scale in Nursing mean scores of the participants based on their descriptive characteristics (N= 349)*

Characteristics	n	%	METSN score X̄ ±SD	Test	p
Age					
<30 years	225	64.5	227.81±14.95ıt	t: 1.852	0.065
≥30 years	124	35.5	230.81±13.69		
Gender					
Female	267	76.5	229.78±14.03	t: 1.962	0.052
Male	82	23.5	225.94±15.93		
Level of education					
High school	28	8.0	227.54±15.24		
Undergraduate	27	7.7	235.41±11.09	KW: 7.644	0.054
Graduate	256	73.4	228.34±14.39		
Postgraduate	38	10.9	227.84±16.67		
Marital status					
Single	200	57.3	226.86±15.85	t: 3.149	**0.002**
Married	149	42.7	231.38±12.18		
Having children					
Yes	253	72.5	227.41±15.02	t: 3.347	**0.001**
No	96	27.5	232.74±12.56		

t: independent-samples t-test, df: 347
KW: Kruskal-Wallis test, df: 3
METSN: Medical Error Trend Scale in Nursing

The mean total experience of the participants in nursing was 6.32±5.82 years, their mean experience in the ICU was 4.87±5.00 years, their mean weekly working hours were 11.33±4.30 hours, and their mean value of the average number of patients per nurse was 3.02±1.14.

METSN mean scores were found to be significantly higher in the participants who worked in the profession of nursing (p< .001) or in the ICU (p< .05) for more than five years in comparison to those who worked in the profession or the ICU for five years or shorter (Table 2).

Table 2. *Comparison of the Medical Error Trend Scale in Nursing mean scores of the participants based on their work-related characteristics (N: 349)*

Work-Related Characteristics of Nurses	n	%	METSN score X̄ ±SD	Test	p
Type of hospital					
University Hospital	120	34.4	228.90±15.55	t: .022	0.983
Hospital affiliated to MoH	229	65.6	228.86±14.05		
Type of intensive care unit					
Reanimation	159	45.6	227.84±14.09	F: 1.942	0.122
CVS/Coronary ICU	38	10.9	233.66±11.66	(SD: 3/345/348)	
Internal/Surgical ICU	59	16.9	227.39±14.96		
Pediatric/Neonatal ICU	93	26.6	229.65±15.89		
Total number of years in nursing					
≤ 5 years[a]	199	57.0	226.38±15.89	F: 7.061	**0.001**
6-10 years[b]	84	24.1	232.11±10.49	(df: 2/346/348)	a < b, c
> 10 years[c]	66	18.9	232.30±13.48		
Number of years in ICU					
≤ 5 years[a]	241	69.1	227.20±15.29	F: 5.574	**0.004**
6-10 years[b]	74	21.2	231.97±12.71	(df: 2/346/348)	a < b, c
> 10 years[c]	34	9.7	234.06±10.44		
Hours worked per day					
≤ 8 hours	173	49.6	230.46±13.29	t: 1.760	0.079
> 8 hours	176	50.4	227.32±15.60		
Hours worked per week					
40 hours[a]	103	29.5	233.78±11.04	F: 10.466	**0.000**
41-50 hours[b]	159	45.6	228.08±14.84	(df: 2/346/348)	a > b, c
51-60 hours[c]	87	24.9	224.54±16.12		
Average number of patients per nurse					
1-2 patients[a]	116	33.2	231.43±12.17	F: 5.825	**0.003**
3 patients[b]	171	49.0	226.20±15.83	(df: 2/346/348)	a, c > b
≥ 4 patients[c]	62	17.8	231.48±13.89		
ICU Nurse Certification					
Yes	121	34.7	232.74±12.07	t: 3.947	**0.000**
No	228	65.3	226.83±15.36		
Satisfied with working in ICU?					
Yes	301	86.2	230.00±13.85	t: 3.683	**0.000**
No	48	13.8	221.81±16.93		
Thinking of leaving ICU					
Yes	89	25.5	224.63±16.36	t: 3.231	**0.001**
No	260	74.5	230.33±13.63		

F: Analysis of variance for independent groups, df: intergroup/intragroup/total degrees of freedom
t: Independent-samples t-test, df: 347
METSN: Medical Error Trend Scale in Nursing

METSN mean scores were found to be significantly higher in the participants who worked 40 hours per week in comparison to those who worked more than 40 hours per week (p< .05, Table 2). METSN mean scores were also found significantly higher in the participants with ICU nurse certification in comparison to those without ICU nurse certification (p< .001, Table 2). The mean METSN score of the participants who were satisfied with working in the ICU was significantly higher in comparison to those who were not satisfied (p< .000), and the mean score of those who did not think of leaving the ICU was significantly higher in comparison to those who thought of leaving the ICU (p< .001) (Table 2).

The METSN scores of the participants are given in Table 3.

Table 3. *Medical Error Trend Scale in Nursing scores of the participants, N: 349*

Scale and Subscales		Min-Max	$\bar{x}$ ±SD
Medical Error Trend Scale Overall Score		179-245	228.88±14.56
METSN Subscales	Drug and transfusion applications	63-90	85.27±5.40
	Hospital infections	38-60	56.32±4.10
	Patient monitoring and material safety	27-45	40.44±4.21
	Falls	11-25	23.11±2.23
	Communication	14-25	23.74±1.91

The effects of the 11 independent variables determined to be effective on the METSN scores of the participants in the primary analyses were evaluated together with multiple regression analysis (backward method). According to the correlation analysis and multicollinearity statistics, there was no high-level autocorrelation between the independent variables included in the regression model (Table 4). Five independent variables (number of years in the ICU, willingness to work in the ICU, having children, intention to leave the ICU, and total number of years in nursing) among the variables included in the regression model were sequentially excluded from the regression model since they did not have a sufficient effect on the METSN scores of the participants.

The order of significance of the five independent variables, which were determined to have significant effects on the METSN scores of the participants according to the b coefficient, was as follows (from the most significant to the least significant): satisfaction with working in the ICU, the number of patients per nurse, having an ICU nurse certificate, weekly working hours, and marital status. These five independent variables explained 15% of the total variance in the Medical Error Trend Scale in Nursing scores of the participants (Table 4).

The METSN score of the participants who were satisfied with the ICU they worked at was 8.21 points higher than the score of those who were not satisfied. The METSN score of the participants who provided care for 1-2 patients or more than 4 patients per day increased by 6.33 points in comparison to the score of those who provided care for 3 patients per day. The METSN score of the participants with an ICU nurse certificate was 4.81 points higher than the score of those without a certificate. The METSN score of the participants who worked for 40 hours per week was 4.34 points higher than the score of those who worked for more than 40 hours per week. The mean Medical Error Trend Scale in Nursing score of the married participants was 4.04 points higher than the score of the single participants.

Table 4. *Effects of the independent variables on the Medical Error Trend Scale in Nursing scores of the participants: Multiple regression analysis results, N: 349*

Independent Variables	B	Std. Error	b	T	p	95% Confidence Interval for B		Collinearity statistics	
								Tolerance	VIF
(Constant)	213.89	2.29		93.386	0.000	209.39	218.40		
Number of patients cared for	6.33	1.47	0.22	4.299	0.000	3.44	9.23	0.956	1.046
Satisfaction with the unit	8.21	2.11	0.19	3.897	0.000	4.06	12.35	0.986	1.014
ICU nurse certification	4.81	1.59	0.16	3.029	0.003	1.69	7.94	0.907	1.103
Hours worked per week	4.34	1.64	0.14	2.647	0.008	1.11	7.56	0.929	1.077
Marital status	4.04	1.55	0.14	2.613	0.009	1.00	7.09	0.885	1.130
R: 0.40 *Adjusted R2: 0.15* *F: 12.99* *p: 0.000* *Durbin Watson: 2.05*									

4. DISCUSSION

According to the primary results of the study, the medical error trend of the nurses who were satisfied with working in the ICU, those who provided care for one or two patients, those who had ICU nurse certification, and those who worked for 40 hours per week decreased. It has been determined that the risk of medical errors significantly increases in nurses who work for more than 40 hours per week (17,18), their medication errors, the fall-induced injuries of patients, and the nosocomial infections of patients increased (19), and there is a positive correlation between this variable and pressure ulcers, lack of communication skills, and patient complaints (20). Akın Korhan et al. did not find a significant correlation between METSN mean scores and weekly working hours (21). Zarea et al. did not find a significant correlation between medication errors and overtime (9). In their study examining the effects of shift duration (8-h/12-h shifts) on the quality of patient care, Estabrooks et al. (22) concluded that the evidence to determine the negative effect of shift duration was insufficient. However, evidence shows that the attention, alertness, and decision-making skills of nurses with longer shift durations are affected since their fatigue levels increase, and their sleep quality deteriorates (18,20,23,24). In our sample, we determined that daily working hours did not have a significant effect on the tendency of the participants to make medical errors. Our findings supported the results of previous studies regarding the fact that excessive weekly working hours increase the tendency of nurses to make medical errors. Conflicting results in the literature make it difficult for us to make clear inferences. Further studies are recommended for precise results.

Studies in the literature have pointed out that increasing numbers of years in nursing would enable nurses to make fewer medical errors as their professional knowledge and skills increase accordingly (25). In this study, it was found that the mean Medical Error Trend Scale in Nursing scores of the participants who had worked in nursing or in the ICU for more than five years were significantly higher than those who had worked for five years or shorter. Among the variables included in the regression model, it was determined that the independent variables including the number of years in nursing and the number of years in the ICU did not have a sufficient effect on the Medical Error Trend Scale in Nursing scores of the participants. Similarly, there are many studies revealing that the professional experience of nurses does not affect their medical error rates (21,26,27). On the other hand, there are also results showing that, as the professional experience of nurses decreases, their tendency towards medical errors and medication errors increases, as well (28-31). Further studies are required to reveal the effect of the number of years spent by nurses in the profession of nursing and in the ICU on medical errors and reach more precise judgments.

In the sample of our study, it was determined that the tendency of the participants who provided care for 1-2 patients to make medical errors was lower (higher METSN score by 6.33 points) than those who provided care for 3 patients. Inadequate nurse staffing leads unintended consequences to occur more frequently (32,33). It was reported that, thanks to reducing the number of patients per nurse, medical errors could decrease, patient outcomes could be improved, and the quality of nursing care could increase (34,35). Yüksel Koçak and Yaman (30) underlined that the higher the number of patients per nurse, the higher the rate of medical errors**,** while Uğurlu and Vural (27) stated that there was a significant correlation between the number of patients per nurse and medical errors. Zarea et al. (9) and Cheragi et al. (36) found significant correlations between the number of patients per nurse and medication errors. For the quality of patient care and patient safety in the ICU, the number of patients per nurse and the patient-to-nurse ratio should be determined by considering the level of care requirements. The patient-to-nurse ratio in a level 3 ICU is recommended to be 2:1. Moreover, for patients who receive supportive care like extracorporeal membrane oxygenation, as the number of organ support treatments increases, this ratio is recommended to be 1:1 and even 1:2 (37,38). In accordance with the literature, our results confirmed that an increase in the number of patients per nurse is associated with unintended consequences.

In our sample, we found that the tendency the participants with ICU nurse certification to make medical errors was lower than the tendency of those without certification. In the literature, it has been stated that nurses with inadequate professional knowledge make more medical errors (25,27,39). Hajibabaee et al. (39) pointed out that nurses who attended training programs on drug applications made fewer medication errors. The finding that the tendency of the participants with certification to make medical errors was lower was an expected result in this study, which was consistent with the literature.

The tendency of the participants of this study who served at the ICU at their own request towards medical errors was found to be lower than those who worked unwillingly. Hajibabaee et al. (39) and Bolandianbafghi et al. (40) revealed that as the job satisfaction levels of nurses increased, the rates of their medication errors decreased**.** The finding in our study indicated that voluntarily serving at the ICU improves motivation and might reduce the tendency nurses to make medical errors.

In our study, no significant relationship was found between the METSN scores of the participants and their age, gender, or level of education. Akın Korhan et al. (21) similarly did not find any significant relationship between the METSN scores of nurses and their age, gender, or level of education. Yiğitbaş et al. (5) determined no significant relationship between METSN scores and age or level of education, while tendency towards medical errors was higher in female nurses. Zarea et al. (9) stated that the gender of nurses and their level of education did not affect their medication errors, while Shahrokhi et al. (26) pointed out that age and

gender did not affect these error rates, and Cheragi et al. (36) and Uğurlu and Vural (27) determined that age did not affect these rates. Hajibabaee et al. (39) underlined that as age and level of education increase, the rate of medication errors decreases. Bolandianbafghi et al. (40) reported that younger and inexperienced nurses make more medication errors. It is seen that the results of different studies on the topic in the relevant literature have been different.

Limitations of the study

The tendencies of the nurses towards medical errors were measured based on the self-reports of the participants. Therefore, the results may not always reflect their tendency towards medical errors. Further studies are recommended to reveal the correlation between demographic variables and tendencies towards medical errors and reach more precise judgments.

5. CONCLUSION

The tendencies of the nurses who worked for 40 hours per week, those who were satisfied with working in the intensive care units, those who provided care for 1 or 2 patients, and those who had intensive care nurse certificates towards medical errors were lower in comparison to the others.

To reduce the tendency of nurses towards medical errors in ICUs, reducing the number of patients per nurse (1 or 2 depending on the patient's care requirements), setting weekly working hours as 40 hours and below, and offering more intensive care certificate training programs may be recommended.

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Clinical and Experimental Health Sciences

Neuropsychological and clinical correlations of optical coherence tomography findings in patients with schizophrenia

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ABSTRACT

Objective: There are increasing studies examining retinal fiber layer (RNFL) and ganglion cell layer (GCL) thinning in patients with schizophrenia. However, the results are controversial, and clinical and cognitive reflections of these findings remain unclear. With this study, we aim to examine retinal abnormalities and establish correlations with cognitive and clinical parameters.

Methods: In this cross-sectional study, we examined 29 patients with schizophrenia and 13 age and gender-matched healthy controls. All participants underwent psychometric assessment, neuropsychological tests, and optical coherence tomography (OCT) measurements. The retinal fiber layer and ganglion cell layer thickness were used as retinal parameters.

Results: Five patients dropped out during the OCT measurement process, 24 patients with schizophrenia and nine healthy controls were included in the analysis. There was no statistically significant difference between groups in measuring retinal nerve fiber layer or ganglion cell layer thicknesses. The verbal fluency test score negatively correlated with left RNFL superior ($\rho = -.422$, $p < .05$). STROOP response duration positively correlated with right RNFL on average ($\rho = .551$, $p < .05$), left RNFL on average ($\rho = .498$, $p < .05$), right RNFL superior ($\rho = .507$, $p < .05$), left RNFL superior ($\rho = .461$, $p < .05$) and right RNFL temporal values ($\rho = .434$, $p < .05$). STROOP response error was also positively correlated with right RNFL temporal thickness ($\rho = .430$, $p < .05$). STROOP response duration was positively correlated with right GCL total ($\rho = .646$, $p < .01$), right GCL superior ($\rho = .658$, $p < .01$) and right GCL inferior ($\rho = .596$, $p < .01$) thickness.

Conclusion: We did not find a significant relationship between reduced RNFL or GCL thickness and cognitive impairment. However, we had several positive correlations between cognitive task scores and RNFL and GCL thicknesses. Additionally, our study did not correlate symptom severity and clinical severity parameters with reduced RNFL or GCL thickness.

Keywords: schizophrenia, OCT, retina, cognitive impairment, clinical severity

1. INTRODUCTION

Schizophrenia is a life-long mental disorder, the pathophysiology of which has not yet been clarified, causing significant deterioration in the individual's functionality and quality of life. In addition to the positive and negative symptoms observed in schizophrenia patients, cognitive deficiency constitutes one of the main symptom clusters of the disease. Studies have revealed that patients with schizophrenia have widespread impairments in many cognitive domains, such as executive functions, attention, verbal learning, memory, and verbal fluency, and have shown that cognitive dysfunction seen in schizophrenia predicts poor functionality and inadequate treatment response (1-3).

Recent meta-analyses have shown that the initial level of functionality and early diagnosis and treatment are the most important predictors of a patient's functionality and emphasized the importance of biomarkers that can be detected early in schizophrenia (4, 5). In addition to numerous structural and functional neuroimaging studies conducted for this purpose, studies examining retinal structure changes in patients with schizophrenia have increased. Retinal evaluation is suggested as a candidate biomarker for schizophrenia (6-8). Optical coherence tomography (OCT) is an easy-to-apply, non-invasive retinal imaging method that has come to the forefront and gives information about retinal nerve fiber layer thicknesses, macular volume, and macular thickness, which provides an idea about many neurodegenerative processes so far (9-12). Although thinning of the peripapillary retinal nerve fiber layers (pRNFL) has been shown in several OCT examinations performed in patients with schizophrenia to date (13, 14), studies examining the relationship of retinal

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changes with clinical symptoms or cognitive functions are limited and revealed inconsistent results (15-17). With this study, firstly, we aim to compare the RNFL and ganglion cell layer (GCL) thickness between patients and healthy controls. Secondly, we aim to elucidate the relationship between clinical and cognitive impairments and retinal alterations that may occur in patients with schizophrenia.

2. METHODS

2.1. Sample and Study Procedure

In this cross-sectional study, researchers evaluated a consecutive series of 50 patients with schizophrenia who were under treatment at the Marmara University Hospital's psychiatry outpatient clinic in Istanbul, Turkey. Diagnosis of schizophrenia was made through several sessions with two psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders 5th edition criteria (DSM 5, APA 2013). Patients aged 18-50 who had been stable clinically for at least three months were referred to the research psychiatrist after a routine psychiatric evaluation. Subjects with mental retardation, alcohol, and substance use disorder, history of head trauma leading to unconsciousness, and degenerative neurological, immunological, or systemic disease that may affect the visual pathways were excluded from the study. Also, 20 healthy controls matched with patients regarding age, gender, and education were recruited for the study. After the first evaluation, individuals who met the inclusion criteria were subjected to ophthalmological examination. Participants who have a primary ophthalmologic disease (glaucoma, retinal disease, age-related macular degeneration, diabetic retinopathy, degenerative myopia); myopia, hyperopia, or astigmatism ≥1 diopter, and a pathology such as cataract, corneal leukoma or vitreous hemorrhage that may affect the ocular examination and optical coherence tomography measurement were not included to the study. Following the initial step, the sample consisted of 29 patients, and 13 healthy controls were invited within five days for psychometric and neurocognitive assessment and OCT measurement. The whole of the measurements was conducted on the same day of evaluation. Five patients dropped out during the OCT process, 24 patients with schizophrenia and nine healthy controls were included in the final analysis. The study followed the Declaration of Helsinki and the International Conference on Harmonization/Good Clinical Practice Guidelines. All participants provided written informed consent, and the local ethics committee approved the study (approval number: 09.2017.432).

2.2. Data acquisition

2.2.1. Psychometric Measures

The participants' sociodemographic features, patients' clinical information about the history of schizophrenia, and current clinical characteristics were obtained through a semi-structured data form developed by the research team. Psychopathological assessment was performed using The Positive and Negative Syndrome Scale for Schizophrenia (PANSS). The scale examines the severity of the disease under three subheadings: positive symptoms, negative symptoms, and general psychopathology. It evaluates 30 symptom-oriented items on a 7-point scale; 1 means 'absent' and 7 means 'excessive'. The range for the Positive and Negative Scales is 7-49, and the range for the General Psychopathology Scale is 16-112. The total PANSS score is simply the sum of the sub scales (18).

2.2.2. Assessment of Cognitive Functions

A battery of neuropsychological tests was designed to assess the impairments in various cognitive domains. Wechsler Memory Scale-Revised (WMS-R) and WMS-R visual reproduction subscale was utilized for assessing visual memory (19), Digit Span test was used to evaluate verbal attention and working memory (19), the Stroop Color–Word Interference Test (SCWIT) was utilized for selective visual attention and executive functions (20), the Trail Making Test-A (TMT-A) and Trail Making Test-B (TMT-B) was used for assessing mental flexibility, eye tracking and motor speed (21) and finally Turkish version of the Phonemic Verbal Fluency Test was used to measure the verbal fluency (22, 23).

2.2.3. Measurement of Retinal Nerve Fiber Layer (RNFL) and Ganglion Cell Layer Thickness

OCT was measured by a qualified ophthalmologist who was blind to the participants on the same day of cognitive evaluation. For RNFL analysis, the optic nerve head (ONH) protocol of RTVue (Optovue Inc., Foremont, CA, USA) was used. The software automatically defines the optic cup as the intersection of the inner nerve head boundary and a parallel line 150µm above the joining line of the retinal pigment epithelium tips. The RNFL thickness map was generated from RNFL thicknesses measured around an area of 3.45 mm in diameter around the disc center. Mean, superior, inferior, temporal, and nasal hemisphere RNFL thicknesses were provided. Macular parameters were obtained using the GCL protocol of RTVue (Optovue Inc., Foremont, CA, USA). The GCL thickness was measured from the internal limiting membrane to the inner plexiform layer boundary. The following GCL parameters are provided: average, superior, and inferior thickness. The global loss volume (GLV), representing the average GCL loss over the entire GCL map, and the focal loss volume (FLV), representing the local GCL loss using a pattern deviation map to correct for overall absolute changes, were also computed.

2.3. Statistical Analysis

All statistical analyses were conducted using SPSS Version 22.0. We utilized the Chi-square test to compare groups of categorical variables. Comparisons of patients and healthy controls for the continuous variables were undertaken using

Mann-Whitney U tests. Spearman-Brown correlation analysis was used to examine interrelations among continuous variables. For all analyses, the significance was defined as p<.05.

3. RESULTS

We presented the sociodemographic characteristics of patients and healthy controls in Table 1. Group comparisons revealed no difference between groups regarding age, gender, education level, monthly income, and health habits such as packs of cigarettes smoked per day. The only group differences were in marital status (χ^2= 15.25, *p*<.001), current working (χ^2=27.69, *p*<.001), and occupation status (χ^2=14.63, *p*<.001). The patients were mostly single (89.7%), unemployed (86.2%), and did not have any occupation (55.2%).

We presented the clinical characteristics (i.e., PANSS scores, age at the onset of the illness, duration of the disease, the number of hospitalizations, years past the latest hospitalization, the use of clozapine treatment and the presence of psychosis in the family) of the patients with schizophrenia diagnosis at Table 2.

Table 1. *Sociodemographic characteristics of groups*

	Patient Group (n = 29)		Healthy Controls (n= 13)		Group Comparisons	
	Mean	Std. Deviation	Mean	Std. Deviation	Mann-Whitney U Test	*p*
Age	33.83	9.13	32.23	8.04	171.50	.64
Monthly income (Turkish Liras)	2904.14	456.46	3076.92	202.91	119.50	.06
Packs of cigarette smoked	.62	.66	.96	1.01	156.00	.34
	f	%	f	%	Chi-Square Test	
Gender						
Females	6	20.7%	4	30.8%	.50	.70
Males	23	79.3%	9	69.2%		
Marital status						
Single	26	89.7%	4	30.8%	**15.25**	**<.001**
Married	3	10.3%	9	69.2%		
Education						
Primary school	3	10.4%	4	30.8%		
Secondary school	5	17.2%	4	30.8%	4.69	.20
High school	13	44.8%	3	23.0%		
University/college	8	27.6%	2	15.4%		
Current working status						
Working	4	13.8%	13	100%	**27.69**	**<.001**
Unemployed	25	86.2%	0	0%		
Occupation						
None	16	55.2%	0	0%	**14.63**	**<.001**
Blue collar	6	20.7%	10	76.9%		
White collar	7	24.1%	3	23.1%		

***p<.01. ***p<.001.*

Table 2. *Clinical characteristics of the patient group (n= 29)*

	Mean	Std. Deviation
PANSS Positive Symptoms	16.90	6.78
PANSS Negative Symptoms	22.38	7.64
PANSS General Psychopathology	33.48	11.60
PANSS Total	72.76	23.19
Age at the onset of the illness (years)	21.97	6.59
Duration of the illness (years)	11.63	7.57
Number of hospitalizations	2.55	2.50
Latest hospitalization (years past)	4.9130	2.83
	f	%
Use of clozapine treatment	24	57.1%
Presence of psychosis in family	12	28.6%

PANSS: Positive and Negative Syndrome Scale for Schizophrenia

Table 3. *Comparison of healthy controls and the patient group regarding neuropsychological test scores*

Neuropsychological tests	Healthy controls (n=13)		Patient group (n=29)		Mann-Whitney U	*p*
	Mean Rank	Sum of Ranks	Mean Rank	Sum of Ranks		
TMT-total score	26.19	340.50	19.40	562.50	127.50	.10
TMT-A duration	16.04	208.50	23.30	652.50	117.50	.07
TMT-A error	19.50	253.50	21.70	607.50	162.50	.23
TMT-B duration	16.19	210.50	19.07	419.50	119.50	.42
TMT-B error	16.12	209.50	17.58	351.50	118.50	.63
Digit span – forward	19.00	247.00	22.62	656.00	156.00	.36
Digit span – backward	22.69	295.00	20.97	608.00	173.00	.66
Verbal Fluency Test score	27.92	363.00	18.62	540.00	**105.00**	**.02**
Verbal Fluency Test-perseveration	22.77	296.00	20.93	607.00	172.00	.67
Verbal Fluency Test – out of category	19.00	247.00	22.62	656.00	156.00	.39
Verbal Fluency Test – special names	22.81	296.50	20.91	606.50	171.50	.65
STROOP – reading duration	13.58	176.50	25.05	726.50	**85.50**	**.004**
STROOP – reading correction	17.85	232.00	23.14	671.00	141.00	.20
STROOP – response duration	17.46	227.00	21.27	553.00	136.00	.34
STROOP – response error	19.69	256.00	20.15	524.00	165.00	.92
STROOP-response correction	24.38	317.00	17.81	463.00	112.00	.09
STROOP – duration difference	22.04	286.50	18.98	493.50	142.50	.44

**p<.05. **p<.01. TMT: Trail Making Test*

Table 4. *Comparison of patients and healthy controls in terms of retinal nerve fiber layers (RNFL) and ganglion cell layers (GCL)*

	Healthy control (n=9)		Patient group (n=24)		
Retinal Nerve Fiber Layers	Mean Rank	Sum of Ranks	Mean Rank	Sum of Ranks	Mann-Whitney U
Right RNFL mean	19.00	171.00	16.25	390.00	90.00
Left RNFL mean	16.50	148.50	17.19	412.50	103.50
Right RNFL superior	16.72	150.50	17.10	410.50	105.50
Left RNFL superior	17.78	160.00	16.71	401.00	101.00
Right RNFL nasal	18.44	166.00	16.46	395.00	95.00
Left RNFL nasal	14.83	133.50	17.81	427.50	88.50
Right RNFL inferior	20.06	180.50	15.85	380.50	80.50
Left RNFL inferior	15.78	142.00	17.46	419.00	97.00
Right RNFL temporal	19.72	177.50	15.98	383.50	83.50
Left RNFL temporal	16.83	151.50	17.06	409.50	106.50
Ganglion Cell Layers	Mean Rank	Sum of Ranks	Mean Rank	Sum of Ranks	Mann-Whitney U
Right GCL total	19.00	171.00	15.52	357.00	81.00
Left GCL total	21.33	192.00	14.61	336.00	60.00
Right GCL superior	18.11	163.00	15.87	365.00	89.00
Left GCL superior	21.67	195.00	14.48	333.00	57.00
Right GCL inferior	20.56	185.00	14.91	343.00	67.00
Left GCL inferior	20.22	182.00	15.04	346.00	70.00

Note. All Mann Whitney U tests are insignificant at an alpha level of .05.
RNFL: retinal nerve fiber layer GCL: ganglion cell layer

Table 5. *Results of Spearman-Brown correlations among the scores of neuropsychological tests and cell layers for patients (n=24)*

	right_ RNFL_mean	left_ RNFL_mean	right_ RNFL_ superior	left_ RNFL_ superior	right_ RNFL_ nasal	left_ RNFL_ nasal	right_ RNFL_ inferior	left_ RNFL_ inferior	right_ RNFL_ temporal	left_ RNFL_ temporal	right_ GCL_ total	left_ GCL_ total	right_ GCL_ superior	left_ GCL_ superior	right_ GCL_ inferior	left_ GCL_ inferior
TMT-total score	.06	-.08	.06	-.033	.347	.087	.201	.217	-.344	-.176	-.182	-.168	-.268	-.206	-.227	-.133
TMT-A duration	-.12	.12	-.17	.128	-.279	.186	-.164	-.044	.100	.130	-.015	.128	.051	.150	.039	.093
TMT-A error	**.41***	.27	.24	.269	-.052	-.229	.298	.121	**.459***	**.431***	.099	.048	.123	-.018	.026	.022
TMT-B duration	-.09	.03	.06	.100	-.243	.068	-.127	-.123	-.010	-.114	.120	.106	.233	.139	.133	.044
TMT-B error	-.03	-.35	.01	-.156	-.103	-.105	-.053	-.241	-.095	-.265	-.061	-.310	.122	-.229	-.168	-.417
Digit span-forward	-.01	-.23	-.04	-.252	.063	-.229	.111	.060	-.134	-.004	-.127	-.101	-.228	-.147	-.141	-.035
Digit span – backward	-.21	-.25	-.16	-.383	.249	-.082	.078	.032	-.343	-.159	.050	-.096	-.047	-.103	.037	-.050
Verbal Fluency Test score	-.13	-.39	-.08	**-.422***	.251	-.277	.048	-.001	-.351	-.141	-.156	-.299	-.230	-.288	-.144	-.264
Verbal Fluency Test – perseveration	.21	.28	.17	.086	.104	-.030	.294	.309	.022	.139	-.099	-.010	-.085	-.071	-.041	-.021
Verbal Fluency Test – out of category	.11	.19	.10	.222	-.123	.168	-.053	-.120	.318	.258	.242	.241	.318	.261	.229	.173
Verbal Fluency Test – special names	-.10	-.11	.01	-.101	.395	.304	-.066	-.081	**-.427***	**-.459***	-.174	-.124	-.141	-.132	-.085	-.090
STROOP – reading duration	.14	.32	.32	.277	-.216	.231	-.074	.135	.154	.066	.278	.306	.351	.296	.273	.238
STROOP – reading correction	.19	.29	.11	.375	-.143	.404	-.001	.087	.304	-.011	-.052	-.079	.069	-.052	-.072	-.121
STROOP – response duration	**.55****	**.50***	**.51***	**.461***	.035	.350	.310	.204	**.434***	.150	**.646****	.404	**.658****	.397	**.596****	.354
STROOP – response error	.29	.16	.15	.268	.065	.284	.052	-.146	**.430***	-.010	.257	.128	.390	.166	.177	.050
STROOP-response correction	.25	.25	.12	.35	.091	.307	-.068	-.089	.284	.247	.213	.329	.323	.358	.138	.243
STROOP – duration difference	.28	.11	.04	.14	-.073	.137	.413	-.001	.370	.079	.276	.007	.237	-.022	.249	.003

**p<.05. **p<.01. RNFL: retinal nerve fiber layer GCL: ganglion cell layer TMT: Trail Making Test*

Table 3 compared healthy controls and the patient group on neuropsychological test scores. Patients (Mean Rank = 18.62) had significantly lower verbal fluency indicated by verbal fluency total score than the healthy controls (Mean Rank = 27.92), Mann-Whitney U = 105.00, p=.02. Patients' reading duration in STROOP test (Mean Rank = 25.05) was significantly higher than controls (Mean Rank = 13.58), Mann-Whitney U = 85.50, p =.004. Patients did not significantly differ from healthy controls regarding other scores on neuropsychological tests.

We compared the patient group's and healthy controls' retinal nerve fiber layers (RNFL) and ganglion cell layers (GCL) thickness. As seen in Table 4, groups did not significantly differ regarding RNFL variables or the values on GCL. We further assessed the relationship between cognitive task scores and RNFL and GCL for all participants. We presented the correlations among nerve layers and scores of the neuropsychological tests for the patient group in Table 5.

3.1. Correlations Between the Scores of Neuropsychological Tests and OCT Measures for All Respondents

Spearman Brown correlational analyses on all participants revealed positive relations between TMT total scores and right RNFL nasal values (ρ = .387, p <.05); errors from TMT-A and right RNFL temporal (ρ = .373, p <.05) as well as left RNFL temporal values (ρ = .362, p <.05). For all participants, verbal fluency test perseveration score was positively correlated with left RNFL on average (ρ = .370, p <.05) and left RNFL inferior (ρ = .401, p <.05). In contrast, verbal fluency test special names score was negatively correlated with left RNFL temporal (ρ = – .355, p <.05). STROOP response duration positively correlated with right RNFL on average (ρ = .361, p <.05), left RNFL on average (ρ = .421, p <.05), right RNFL superior (ρ = .410, p <.05) and left RNFL superior values (ρ = .424, p <.05). STROOP reading correction was positively related to left RNFL nasal thickness (ρ = .409, p <.05) for all participants. In terms of the correlation between cognitive task scores and GCLs, STROOP response duration positively correlated with right GCL total (ρ = .420, p <.05), right GCL superior (ρ = .381, p <.05) and right GCL inferior (ρ = .377, p <.05). Finally, STROOP reading correction positively correlated with left GCL superior (ρ = .363, p <.05).

3.2. Correlations Between the Scores of Neuropsychological Tests and OCT Measures for Patients

For the patient group, TMT-A error score positively correlated with right RNFL on average (ρ = .414, p <.05), right RNFL temporal (ρ = .459, p <.05), and left RNFL temporal (ρ = .431, p <.05) thickness. The verbal fluency test score negatively correlated with left RNFL superior (ρ = – .422, p <.05), while the verbal fluency test special names score negatively correlated with right RNFL temporal (ρ = – .427, p <.05) and left RNFL temporal (ρ = – .459, p <.05) values. STROOP response duration positively correlated with right RNFL on average (ρ = .551, p <.05), left RNFL on average (ρ = .498, p <.05), right RNFL superior (ρ = .507, p <.05), left RNFL superior (ρ = .461, p <.05) and right RNFL temporal values (ρ = .434, p <.05). STROOP response error was also positively correlated with right RNFL temporal thickness (ρ = .430, p <.05). STROOP response duration was positively correlated with right GCL total (ρ = .646, p <.01), right GCL superior (ρ = .658, p <.01) and right GCL inferior (ρ = .596, p <.01) thickness.

Table 6. *Correlations between clinical indicators of schizophrenia and retinal and ganglion cell layers*

	PANSS-p	PANSS-n	PANSS-g	Age of onset	Duration of illness	Numbers of hospitalizations	International Consensus Study of Antipsychotic Dosing	Chlorpromazine equivalence (mg)
right RNFL mean	**.501***	**.442***	**.485***	-0.173	-0.115	0	.033	-.031
left RNFL mean	**.460***	0.379	**.483***	-0.236	0.123	0.052	.082	.086
right RNFL superior	0.288	0.268	0.217	-0.041	0.081	0.13	-.217	-.153
left RNFL superior	**.555****	0.332	**.537****	-0.305	0.232	-0.004	-.171	-.137
right RNFL nasal	0.051	-0.007	0.071	-0.051	-0.389	-0.139	-.128	-.223
left RNFL nasal	0.115	-0.023	0.091	-0.276	0.119	-0.026	.084	.115
right RNFL inferior	0.188	0.337	0.139	-0.025	-0.244	0.011	.195	.128
left RNFL inferior	0.182	0.116	0.118	0.016	-0.138	0.044	.352	.375
right RNFL temporal	**.529****	**.532****	**.451***	-0.074	0.051	-0.008	.185	.093
left RNFL temporal	**.429***	0.324	**.515***	-0.187	0.074	-0.052	.055	-.043
right GCL total	0.202	0.282	0.271	-0.068	0.178	-0.157	-.264	-.312
left GCL total	0.278	0.201	0.409	-0.251	0.197	-0.215	-.322	-.337
right GCL superior	0.224	0.401	0.296	-0.055	0.103	-0.094	-.268	-.306
left GCL superior	0.3	0.195	**.461***	-0.314	0.191	-0.239	-.335	-.365
right GCL inferior	0.128	0.233	0.138	0.088	0.154	-0.105	-.122	-.197
left GCL inferior	0.223	0.143	0.307	-0.139	0.132	-0.251	-.242	-.274

**p<.05. **p<.01. PANSS: positive and negative symptom scale PANSS-p: PANSS positive symptom score PANSS-n: PANSS negative symptom score PANSS-g: PANSS general psychopathology symptom score RNFL: retinal nerve fiber layer GCL: ganglion cell layer*

3.3. Correlations Between the Clinical Indicators of Disease Severity and OCT Measures

Finally, we assessed the relationship between clinical features with RNFL and GCL thickness. We did not find any significant correlation between OCT measures and age of onset, duration of schizophrenia, number of hospitalizations, or the dosage of current antipsychotic use. PANSS positive (PANSS-p) scores were correlated with right and left average RNFL thickness, left RNFL superior, and right and left RNFL temporal regions. PANSS negative symptom scores (PANSS-n) were significantly correlated with the right RNFL average and temporal areas. General psychopathology scores (PANSS-g) positively correlated with right RNFL mean, left RNFL average, left RNFL superior, and right RNFL temporal values. Finally, only a significant correlation was detected between PANSS-g and left GCL superior thickness. The details of the correlations are presented in Table 6.

4. DISCUSSION

This study examined the differences in OCT findings between the patients with schizophrenia and healthy participants and explored the relationships between various cognitive functions and retinal structural alterations in patients. Our initial hypothesis in this study was patients would have thinner RNFLs and GCLs than healthy controls. Second, thinning of retinal nerve and ganglion cell layers due to possible neurodegeneration would correlate with the loss of cognitive functions and more clinical severity in patients with schizophrenia. Contrary to our hypothesis, OCT measures did not differ between patients and healthy controls. Regarding cognitive correlations, only verbal fluency task scores were correlated with thinner left RNFL superior, right RNFL temporal, and left RNFL temporal values. Moreover, we detected several positive correlations between poorer cognitive performance and layer thicknesses in several retinal regions. In terms of disease-related clinical parameters, interestingly, there were positive correlations between PANSS scores and mean, superior, and temporal RNFL thicknesses.

The demonstration of thinning of the RNFLs and GCLs in early OCT studies prompted researchers to pursue new research to find a new biomarker in schizophrenia (24, 25). In addition to various studies showing thinning of the peripapillary retinal nerve layers, (13) there is a considerable number of studies that did not detect significant atrophy in RNFLs in patients with schizophrenia (16, 26, 27). Studies suggest that longer duration of the illness (28), comorbid medical conditions such as hypertension or diabetes (27), and the presence of recent illness episodes (29) affect the thinning of RNFL and GCL or macular thicknesses. Age, body mass index, and metabolic syndrome were also reported as confounding factors on the relationship between schizophrenia and RNFL thickness (26). Our analysis also did not determine any significant difference in RNFL or GCLs between patients and healthy controls. This negative finding may be related with non-psychiatric medical conditions of the participants which we did not assess in our study.

The association of cognitive functioning with RNFL thickness in healthy individuals was reported in a previous study. The authors noted that better cognitive performance was related to a thicker RNFL in only young individuals, but this association diminished in older age groups (30). In schizophrenia, a few studies assessed the cognitive correlations of retinal findings (17, 31, 32). One study found that lower scores of immediate memory and visuospatial functions of the RBANS (Repeatable Battery for the Assessment of Neuropsychological Status) were correlated with a decline in RNFL thickness. In contrast, not found any correlation between language, attention, delayed memory, and RNFL (31). The other study of the same group reported a significant correlation between RNFL thickness thinning and lower scores on the Stroop Color Word reading test (32). Finally, a recent study examined the correlations between executive functioning, attention, memory/learning and OCT results. Thinning of the right inner plexiform layer and left macula were associated with worse executive functioning and attention. However, they did not find a significant correlation between cognitive task scores and the thinning of RNFL, GCL, and choroid (17). Our study found no significant correlation between deterioration in executive functions, attention, working memory, and thinning of the RNFL or GCL layers. Interestingly, we even found a relationship between the poorer scores in verbal fluency, STROOP response duration, response error, and thicker RNFL and GCL. When all the results are put together, the results seem inconsistent and complex.

Finally, concerning the correlation between clinical severity and OCT measures, we did not find any significant relationship between RNFL, GCL thinning, symptom severity, or other disease-related severity indicators. This finding is consistent with many studies in the literature that did not find a relationship between disease severity and OCT findings (17, 25, 28, 33, 34).

Several limitations should be acknowledged. First, our sample size is relatively small; given the limited power, non-significant results should be approached with caution. Second, according to the total PANSS scores, our sample consisting of patients with moderate illness severity, may restrict the generalizability of our results. Third, we did not assess the confounding factors such as age, smoking, other medical comorbidities, and cardiometabolic factors. Finally, however, we evaluated the current dosage of antipsychotic drugs cross-sectionally; we cannot exclude the possible retinal effects of cumulative antipsychotic use.

5. CONCLUSION

RNFL and GCL thicknesses did not differ between schizophrenia patients and healthy controls, and we did not detect any significant correlation between retinal thinning and cognitive impairment as a result of our cognitive evaluation. Although our results support negative studies

of OCT in schizophrenia, prospective studies conducted in larger samples are required to show whether OCT can be a prognostic marker. In future studies, besides cognitive tasks showing instant performance, evaluating functionality and investigating whether the deterioration in these areas is correlated with the thinning in repetitive OCT measurements will provide more accurate information.

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Design of the study: ASK, MY, AET
Acquisition of data for the study: CŞÇ, MD, EBÇ, SAT
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Clinical and Experimental Health Sciences

Evaluation of the Clinical Effect of Hyaluronic Acid Mouthwash on Palatal Secondary Wound Healing in Diabetic Rats

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ABSTRACT

Objective: The objective of this study was to investigate clinical effect of topically administered hyaluronic acid (HA) mouthwash on healing of secondary palatal wound in diabetic (D) rats.

Methods: 60 Wistar albino male rats were divided into D and non-diabetic (ND) groups. Diabetes was induced to 30 randomly selected rats by initially administering 110 mg/kg of nicotinamide intraperitoneally, followed by 15 min of intraperitoneal injection of 65 mg/kg of streptozotocin solution. 5 mm excisional wounds were made in the centre of the palate. After that, 6 animals from each group were sacrificed. Then, both groups were subdivided into two groups: 0.12% HA mouthwash and saline (S) (n=12 per group), depending on the agent to be administered to the wound area (WA). On days 7 and 14, six rats from each group were sacrificed, and the WAs were measured through photographic measurements utilizing Image J software.

Results: The WA decreased with time in each group ($p<0.05$). A significant difference was detected in the intergroup comparison of the WA on days 7 and 14 ($p<0.05$). On days 7 and 14, the smallest WA was observed in the ND-HA group, while the largest was in the D-S group ($p<0.05$). On day 14, the WA of both HA groups was similar ($p>0.05$) and smaller than that of both S groups ($p<0.05$).

Conclusion: Topical application of HA mouthwash effectively improved secondary wound healing and reduced WA in D and ND rats. Thus, topical application of HA can be used in diabetic palatal secondary wound healing.

Keywords: Wound healing, diabetes mellitus, hyaluronic acid, rat, palate

1. INTRODUCTION

The loss of normal anatomical structure and functional continuity is referred to as a wound (1). A complex series of biochemical and cellular processes known as wound healing are required in order to repair tissue damage and preserve tissue integrity. It can be divided into four overlapping phases: hemostasis, inflammation, proliferation, and remodeling (2,3). Clot formation, inflammation, re-epithelialization, angiogenesis, formation of granulation tissue, wound contraction, formation of scar tissue, and tissue remodeling are all normal stages of the wound healing process (4). Many local and systemic factors can affect one or more phases of wound healing and lead to inappropriate or impaired tissue repair (5).

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, impaired insulin action, or both (6). DM is one of the well-known systemic diseases that impairs wound healing by disrupting one or more biological mechanisms involved in the healing process (7). One of the well-known complications of DM is impaired wound healing, which leads to chronic wounds. Chronic wounds have disruption of wound healing phases and therefore wound does not heal within a normal time frame (5). In addition, as a result of hyperglycemia, there is a decrease in endothelial cell proliferation, angiogenesis, the formation of granulation tissue, and collagen synthesis (8). Due to all of these factors, DM increases the risk of infection in wounds and delays wound healing. In the case of oral wounds, DM patients have increased susceptibility to bacterial invasion and wound infection; as a result, bacteraemia can cause systemic inflammation and sepsis. Moreover, oral infections and poor wound healing in DM patients might impair the body's natural ability to heal (9).

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Hyaluronic acid (HA) is a non-sulfated polysaccharide that is a member of the glycosaminoglycan family and is an essential part of the extracellular matrix of skin, connective tissue, and synovial joints (10). Bacteriostatic, anti-inflammatory, anti-oedematous, osteoinductive, and pro-angiogenic characteristics of HA's highly biocompatible and non-immunogenic nature promote wound healing (11). The effect of HA is related to its molecular weight. In comparison to lower molecular weight HA, higher molecular weight HA could be more effective for increasing tissue regeneration, the anti-inflammatory response, and accelerating wound healing (12). HA promotes wound healing by reducing the negative effects of inflammation during the stages of granulation tissue formation and remodeling and increasing cell proliferation, reepithelialization, angiogenesis, regeneration, and inflammatory response (13,14,15,16). Additionally, higher molecular weight HA accelerated the healing of diabetic (D) wounds and protected against infection by enhancing the antioxidant defence system (17).

The success of surgical procedures depends on the healing of the wound. In recent years, it has been demonstrated that topically applied HA promotes clinically beneficial wound healing outcomes in both animal studies and clinical trials on secondary wound healing (11,18,19). To the best of our knowledge, based on findings of previous research, topical application of higher molecular weight HA demonstrated a positive effect and enhanced D dorsal secondary wound healing (12,17). However, there is no study on the efficacy of higher molecular weight HA on the healing of secondary palatal wound in diabetic (D) rats. Thus, the objective of this study was to investigate clinically the effectiveness of topically administered HA mouthwash in palatal wound healing in D and non-diabetic (ND) rats by assessing the wound closure.

2. METHODS

The protocol of present study was approved by the Istanbul University Animal Experiments Local Ethics Committee on March 26, 2021 (Protocol no: 2021/08). Our protocol and the manuscript were created according to the ARRIVE Guidelines, Animal Research: Reporting of in Vivo Experiments.

2.1. Animals

60 male Wistar albino rats that were 3 months old and weighed 250–300 g were obtained from the Istanbul University Aziz Sancar Institute of Experimental Medicine, where the experiment was conducted. Female rats were not included in the study as hormonal changes may affect wound healing. Rats were placed in standard experimental cages with a maximum of 3 rats in a cage and were kept under standard conditions with a 12-h light and dark cycle, a temperature of 22 ± 1°C, and a relative humidity of 40–60%. Animals were fed a standard diet of pellets and water ad libitum. Sixty animals were randomly divided into two main groups: an ND group (n=30) and a D group (n=30) via a computer-generated randomization table.

2.2. Induction of Diabetes

After 30 rats were fasted overnight, a single dose of 65 mg/kg streptozotocin (STZ) (ChemCruz, Santa Cruz Biotechnology, Dallas, TX) dissolved in distilled water was injected intraperitoneally, 15 min after 110 mg/kg nicotinamide (Acros Organics BV, Geel, Belgium) was administered intraperitoneally to induce diabetes. In order to prevent hypoglycemia due to massive pancreatic insulin release, 6 h after STZ administration, a 5% glucose solution was given to rats in the first 24 h. Following 72 h of STZ injection, blood was withdrawn from the animals' tail veins, and fasting blood glucose was measured using glucose reagent strips and a glucose meter (eBsensor Blood Glucose Monitoring System, Visgeneer Inc., Taiwan*)*. The rats were classified as D when their fasting blood glucose levels were above 200 mg/dl and were utilized for this study (20).

2.3. Wound Creation

The animals were anesthetized intraperitoneally with 100 mg/kg ketamine hydrochloride (Ketalar, Eczacıbaşı, Türkiye) and 10 mg/kg xylazine hydrochloride (Rompun, Bayer, Germany). All surgical procedures were performed by an experienced researcher (EA). After general anesthesia, each rat was stabilized, and the mouth was opened using a retractor. A circular excisional wound with a diameter of 5 mm was made in the centre of the palatal mucosa using a disposable punch biopsy tool (Kai Medical, Kai Industries Co., Ltd., Seki, Japan). Following bleeding control, the wounds were left for secondary healing. On day 0, after wound induction, six animals from the ND and D groups were sacrificed immediately. Then, the ND and D groups were subdivided into two groups according to the agents with a computer-generated randomization table: ND with the HA (ND-HA) group (n=12), ND with the saline (ND-S) group (n=12), D with the HA (D-HA) group (n=12), and D with the saline (D-S) group (n=12). Six animals from each group were sacrificed with decapitation on days 7 and 14 after surgery.

2.4. Hyaluronic Acid and Saline Application

Without touching the wound, according to the treatment group, 1 ml of saline or higher molecular weight (1000-1800 kDa) 0.12% HA (Gengigel® First-aid, Ricerfarma SRL, Milano, Italy) was applied directly to the wound for 1 minute using a syringe (Beybi, Istanbul, Türkiye) with a blunt cannula. This process was repeated twice a day, in the morning and evening, for one week. After 2 hours of HA or saline application, animals were fed a standard diet of pellets and water ad libitum.

2.5. Clinical Evaluation

All animals were sacrificed on days 0, 7, and 14 after surgery. Maxillae were separated, and the palate specimens were photographed at a constant distance and magnification using a Nikon F-3 camera (Nikon Corp., Tokyo, Japan). Using

ImageJ software (National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij), the photographs of the wound area (WA) were analyzed, and calibrated with a 10-mm-long periodontal probe (Williams probe, Hu-Friedy Manufacturing Inc., Chicago, IL, USA). All measurements were carried out by the same blinded researcher (HSY).

2.6. Statistical Analysis

The primary outcome of this study was WA measurements. The sample size was determined using data from research with a comparable design. (21). A sample of 5 rats per group would have 80% power to detect a difference of 1 mm^2 WA between the HA and control groups, assuming that the standard deviation is 0.56. 6 rats were enrolled in each group, considering possible dropouts. Statistical analyses were performed using statistical software (IBM SPSS version 24, Chicago, IL, USA). Mean and standard deviation are used to present the results. To examine the distribution of variables, the Shapiro-Wilk test was used. For intragroup multiple comparisons, the Friedman test was applied since the data were not normally distributed. If significance was found, pairwise comparisons using the Wilcoxon Signed Rank test were carried out. Intergroup comparisons among groups were performed by the Kruskal-Wallis test. If significance was found, the Mann-Whitney U test was used for pairwise comparisons between groups. $p<0.05$ was considered statistically significant.

3. RESULTS

Clinical images of the WA of all groups on days 0, 7, and 14 are presented in Figure 1. Clinical examination of the wounds revealed that all groups' wounds gradually healed over time. Slow wound healing was observed at 7th day postoperatively in the ND-S, D-HA, and D-S groups.

Table 1 shows the mean WA measurements of all groups on days 0, 7, and 14 post-surgery. The mean WA between the ND and D groups at baseline (day 0) was not found to be statistically different (19.40±1.34 mm^2 and 21.65±2.89 mm^2, respectively) ($p>0.05$). There was a significant decrease in the mean WA of all groups on day 14 compared to the baseline ($p<0.05$).

Table 1. *Inter and intra group comparison of wound area (mm^2)*

	Groups													
	ND (a) (n=6) Mean±SD	D (b) (n=6) Mean±SD	ND-HA (c) (n=12) Mean±SD	ND-S (d) (n=12) Mean±SD	D-HA (e) (n=12) Mean±SD	D-S (f) (n=12) Mean±SD	$p^{*(a-b)}$	$p^{\#(c-d-e-f)}$	$p^{*(c-d)}$	$p^{*(c-e)}$	$p^{*(c-f)}$	$p^{*(d-e)}$	$p^{*(d-f)}$	$p^{*(e-f)}$
Day 0	19.40±1.34	21.65±2.89					0.578							
Day 7			14.00±0.68	17.95±0.36	16.95±0.40	20.25±0.61		0.000	0.004	0.004	0.004	0.004	0.004	0.004
Day 14			4.64±2.16	12.62±0.95	6.18±1.68	15.20±1.58		0.000	0.004	0.332	0.004	0.004	0.023	0.004
p^{**}			0.002	0.002	0.002	0.009								
$p^{\#\#(0-7)}$			0.026	0.026	0.026	0.459								
$p^{\#\#(0-14)}$			0.026	0.026	0.026	0.026								
$p^{\#\#(7-14)}$			0.026	0.026	0.026	0.026								

*SD; Standart deviation, ND; Non-diabetic, D; Diabetic, HA; Hyaluronic acid, S; Saline, * Mann Whitney U test, # Kruskal Wallis test, ** Friedman test, ## Wilcoxon test, p<0.05.*

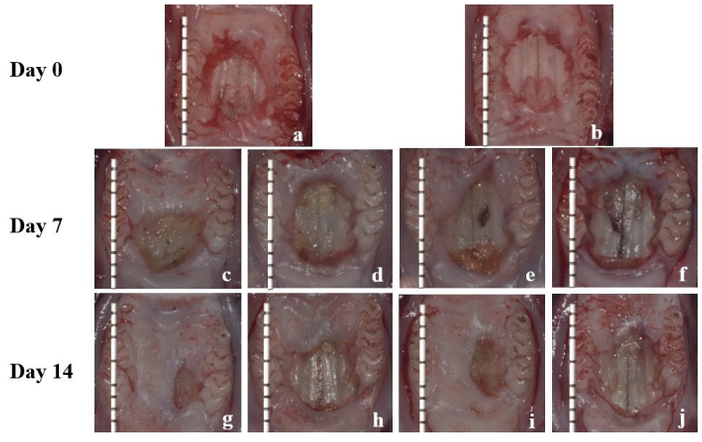

Figure 1. *Representative clinical images of the palatal wound area in (a) non-diabetic group and (b) diabetic group at day 0, (c) non-diabetic HA group, (d) non-diabetic saline group, (e) diabetic HA group and (f) diabetic saline group at day 7, (g) non-diabetic HA group, (h) non-diabetic saline group, (i) diabetic HA group and (j) diabetic saline group at day 14 after surgery.*

Significant differences were found in the intergroup comparison of the WA on days 7 and 14 ($p<0.05$). On days 7 and 14, the smallest WA was observed in the ND-HA group (14.00±0.68 mm^2 and 4.64±2.16 mm^2, respectively), while the largest WA was observed in the D-S group (20.25±0.61 mm^2 and 15.20±1.58 mm^2, respectively) ($p<0.05$). On day 7, the WA in the ND-HA group was found to be significantly smaller than the D-HA group ($p<0.05$). However, there was no statistically significant difference in WA between the ND-HA and D-HA groups on day 14 ($p>0.05$). Additionally, on days 7 and 14, the WA in the D-S groups was detected to be larger than the ND-S group ($p<0.05$). Furthermore, the WAs of the ND-HA and D-HA groups were significantly smaller than the ND-S and D-S groups on day 14 ($p<0.05$).

4. DISCUSSION

HA promotes wound healing by reducing inflammation, increasing collagen formation, and accelerating angiogenesis (12,14,16). However, the benefits of HA are still controversial, and there is no data about using HA mouthwash in D palatal secondary wound healing. Therefore, the goal of this study was to investigate the clinical effects of HA mouthwash application on WA in D rats. Based on the findings of this study, evaluating HA mouthwash clinically and the decrease in WA, no significant difference was observed between the D and ND HA groups on day 14 after surgery, suggesting that applying HA mouthwash to palatal secondary wound healing was efficient in both the D and ND groups.

In terms of the healing process, wound healing consists four distinct but overlapping phases (2). During the first week following surgery, the inflammatory phase of oral wound healing occurs. The first fibroblasts appear at the wound site between the end of the inflammatory phase and the beginning of the proliferative phase (24–48 hours post-injury). After reaching a peak on day 7, they gradually return to normal levels by day 14 (22). Accordingly, fibroblast migration along the fibrin network causes reepithelialization to begin at the edges of the wound (23). Hence, it is possible to see that the wound clinically heals. Therefore, in our study, the WA assessment was done on days 7 and 14.

According to our study, the ND-HA and D-HA groups showed greater clinical wound healing compared to the ND-S and D-S groups on days 7 and 14. The WA in the HA groups was smaller than the saline groups on day 14. The wound closure in ND and D rats was similar on the 14th day following the application of HA. In this study, HA significantly accelerated the clinical healing of wounds in ND and D rats. There are several studies on humans that evaluated the topical HA applied to the donor area following a free gingival graft promotes palatal wound healing (11,24) and on animals that examined the efficiency of HA in secondary wound healing of oral mucosa or skin (12,19,21,25). Chen et al (12), in a study evaluating cutaneous wound healing in D rats, reported that the higher molecular weight of HA significantly accelerated wound healing and reduced the time required for healing compared to the untreated D control group. Lee et al (25) compared HA gel and film in secondary wound healing by creating wounds on the tongue surfaces of systemically healthy rats. Although no significant difference was found between the groups on day 3, they observed that HA gel and film accelerated clinical wound healing on day 7 than the untreated control group. Hammad et al (21) reported that HA gel facilitated clinical palatal wound closure and reduced palatal WA on days 7 and 14 compared to chlorhexidine digluconate gel, allantoin gel, and placebo gel in systemically healthy rats. Taşkan et al (19) discovered that in the HA gel-applied group, the palatal WA was smaller on days 3, 7, 14, and 21 as than the placebo group. This is the first study that was conducted to investigate efficacy of the 0.12% HA mouthwash in the secondary healing process of palatal wounds on the D and ND rats by assessing the wound closure clinically. The results of our study on days 7 and 14 were consistent with the results of the abovementioned studies reporting favouring the ND-HA group. Moreover, our study demonstrated that HA mouthwash could increase palatal wound healing in D rats. According to the findings of previous studies and our study, HA application leads to improved secondary wound healing in ND and D rats by enabling polymorphonuclear leukocytes and macrophages to migrate and adhere to the inflamed area, inducing the production of pro-inflammatory cytokines by fibroblasts and keratinocytes that support the inflammatory response, promoting angiogenesis, stabilizing the granulation tissue, and re-establishing the epithelium (26).

One of the limitations of the present study was the short follow-up period (14 days) in palatal secondary wound healing in ND and D rats. Another limitation was that this study only used one HA concentration. Therefore, further investigations with a longer follow-up period in different concentrations of HA and various wound types on D rats are needed before such firm conclusions can be obtained.

5. CONCLUSION

Within the limitations of this study, topical application of HA mouthwash demonstrated a positive effect that improved secondary wound healing and reduced WA in D and ND rats. Thus, topical application of HA can be used in diabetic palatal secondary wound healing.

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Research idea: HSY
Design of the study: HSY
Acquisition of data for the study: EA
Analysis of data for the study: EA, SDD, HSY
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Clinical and Experimental Health Sciences

Otoprotective Mechanisms of Carvone As An Antioxidant Agent Against Ototoxic Damage Caused By Paclitaxel

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ABSTRACT

Objective: Ototoxicity is cellular damage caused by the use of solid treatments as chemotherapeutics in critical illnesses like cancer. The generation of free radicals is linked to fluctuating hearing loss caused by chemotherapeutics. Antioxidants can help to prevent ototoxicity-related oxidative damage. Carvone (CVN) is a monoterpene with excellent antioxidant properties that protect against oxidative damage. This study investigates the biochemical and functional aspects of CVN's putative otoprotective mechanisms against paclitaxel (PCX)-induced ototoxicity.

Methods: 24 Wistar albino rats were assigned into four different groups: Control, CVN, PCX, and PCX+CVN. Once a week, the control group received saline. The PCX group received 5 mg/kg PCX intraperitoneally once a week (4 times). Once a week, the CVN group received 50 mg/kg intraperitoneally. The PCX+ CVN group received 5 mg/kg PCX followed by 5 mg/kg CVN once a week. All animals were subjected to deterioration product otoacoustic emission testing before (day 0) and after drug administration (day 23).

Results: PCX showed an ototoxic effect by weakening otoacoustic emission values. PCX leads to significant otoacoustic emission value shifts ameliorated by CVN co-treatment (for 2000Hz $p< .001$, for 4000 levels $p< .01$, for 6000Hz $p< .001$, and for 8000 Hz $p< .01$ in PCX+CVN group). Furthermore, the PCX group had significantly greater malondialdehyde levels and significantly lower glutathione levels in the cochlear tissues, compared to the other groups. Co-administered CVN with PCX reversed these effects, making oxidative stress parameters close to those of the control group (for GSH levels $p< .001$, for MDA levels $p< .01$ in the PCX+CVN group).

Conclusion: According to the findings, CVN appears to preserve cochlear function in rats against the disruptive effects of PCX.

Keywords: Antioxidant, carvone, ototoxicity, oxidative stress, paclitaxel.

1. INTRODUCTION

Damage to the inner ear by noise, ototoxic pharmaceuticals, aging, and several disorders leads to hearing loss. Some anticancer drugs, such as cisplatin and paclitaxel (PCX), have been linked to ototoxicity (1). Today, the increase in the usage of chemotherapeutic drugs as the prevalence of cancer rises is increasing the incidence of ototoxic hearing loss. The persistence of hearing loss caused by chemotherapeutic drugs leads to multiple decreases in the quality of life of cancer survivors, and this situation appears as a severe health problem. As a result, there is still a clinical need for treatments to avoid chemotherapeutic-induced ototoxicity.

PCX, a microtubule-stabilizing drug, is among the most effective broad-spectrum chemotherapy (2, 3). PCX alters tubulin polymer balance by increasing microtubule stability, preventing depolarization of the microtubule network, and inhibiting the G2/M phase, demonstrating its anticancer effect (4). It is among the most extensively used anticancer drugs, showing activity in various cancers, including breast, endometrial, lung, and cervical carcinoma (5). PCX has a variety of side effects, the most serious of which is peripheral neuropathy, which has significant dose-limiting toxicity. The impacts of PCX on microtubule polymerase impede axonal transport, causing sensory neurons in the dorsal root ganglia to be damaged and sensory nerve conduction velocity to be reduced, resulting in peripheral neuropathy (6). Even though many chemotherapeutic agents have been demonstrated to have ototoxic effects in considerable detail, there needs to be more research on the ototoxic effects of PCX in the literature (2). However, given the harm that PCX does to sensory neurons, it is assumed that it has a similar effect on neurons in the cochlear, as evidenced by recent investigations (3, 7). In one study, researchers found that 71% of patients developed neuropathic symptoms after paclitaxel administration and that paclitaxel produced early sensory dysfunction and led to permanent neuropathy (8). In another study, the incidence

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of audiogram-confirmed hearing loss in patient groups given platinum only, taxane only, and platinum and taxane ranged from 52.3% to 71.4%. There was no difference between the three chemotherapy groups regarding hearing loss incidence or effects (9).

The accumulation of reactive oxygen species (ROS), which cause damage in cochlear cells and can lead to cell death via apoptosis, is presently thought to be the mechanism of cochlear toxicity caused by chemotherapeutic drugs. Minimizing ROS generation or boosting the antioxidant system is essential to prevent ototoxic damage from cochlear oxidative stress induced by the excessive free radical formation in the inner ear (1). Exogenous antioxidants were shown in studies to protect against ototoxic damage by increasing the ROS scavenger system and reinforcing the deficient endogenous antioxidant synthesis (3, 10-13).

Carvone (CVN) is derived from a variety of pharmaceutical and aromatic plants (cumin, dill, and mint). CVN is a monoterpene with unique pharmacological effects, including anti-inflammatory, anti-tumor, anti-diabetic, antibacterial, fungicidal, and antioxidant capabilities (12). CVN had protective effects against free radical-induced tissue damage reducing consumption of glutathione (GSH) and to decreased elevated malondialdehyde (MDA) levels in a range of experimental models (15-17). However, no studies were conducted to determine whether CVN protects against ototoxicity. Based on the background, this study aimed to assess the preventive efficacy of CVN against ototoxicity induced by PCX in a rat model.

2. METHODS

2.1. Ethics and Animal Handling

The experiments in this study, authorized by the Animal Experiments Ethics Committee at Ataturk University (ATADEM) (Number: 42190979-000-E.160.011.96726), were undertaken by the principles of the Guide for the Care and Use of Laboratory Animals. Twenty-four male Wistar albino rats (250-300 g) were purchased from the ATADEM at the University of Ataturk. Animals were kept under conventional room and environmental settings (12 hours light/12 hours dark, 22°± 3°C temperature, 55%-10% moisture, background noise level less than 50 dB). Throughout the trial, rats were fed ad libitum.

2.2. Drugs and Anesthesia

The dosages of CVN (Sigma-Aldrich Chemical Company, Darmstadt, Germany) and PCX (Sindaxel; Actavis Drug Co., Istanbul, Turkey) utilized were 50 and 5 mg/kg for the rat per weight, respectively, based on previous research (3, 7, 18, 19), and both were given intraperitoneally. Before each recording for otoscopic examination, 50 mg/kg ketamine HCL (Ketalar; Pfizer, Istanbul, Turkey) and 10 mg/kg xylazine (Xylazinbio; Bioveta, Ankara, Turkey) anesthetic mixture was given intraperitoneally.

2.3. Experimental Procedures

To perform an otoscopic examination and evaluate distortion product otoacoustic emissions (DPOAE, The MADSEN Capella device, Natus Medical Denmark), all rats were anesthetized intraperitoneally (50 mg/kg ketamine hydrochloride-10 mg/kg xylazine) before the start of the study. The rats were assigned into four groups of six each: Control, CVN, PCX, and PCX+CVN. The size of the experimental groups' samples was established based on prior similar studies (11, 20). Animals in the control group received intraperitoneal 1ml/kg normal saline once a week (1., 8., 15., 22. days). Animals in the PCX group received 5 mg/kg of PCX intraperitoneally once a week (1., 8., 15., 22. days). Animals in the CVN group were given 50 mg/kg intraperitoneally once a week (1., 8., 15., 22. days). Animals in the PCX+ CVN group received 5 mg/kg PCX, followed by 5 mg/kg CVN 30 minutes later once a week (4 consecutive weeks). The process for giving the drug is outlined in Table 1 and Figure 1. The second DPOAE measurements were taken one day following the last drug delivery (23. Day), and the experiment was ended by giving the rats high-dose anesthesia. The rats' cochleas were extracted and preserved in appropriate conditions for biochemical experiments.

Table 1. *Experimental groups, dosing schedules and procedures*

Groups	Dose	Procedures
CONTROL	1ml/kg	Saline for 1., 8., 15., 22. days (i.p.)
PCX	5 mg/kg	PCX for 1., 8., 15., 22. days (i.p.)
CVN	50 mg/kg	CVN for 1., 8., 15., 22. days (i.p.)
PCX+ CVN	5 mg/kg-	First PCX and 30 min later CVN for 1., 8., 15., 22. days (i.p.)

PCX:Paclitaxel, CVN:Carvone, i.p.:Intraperitoneal

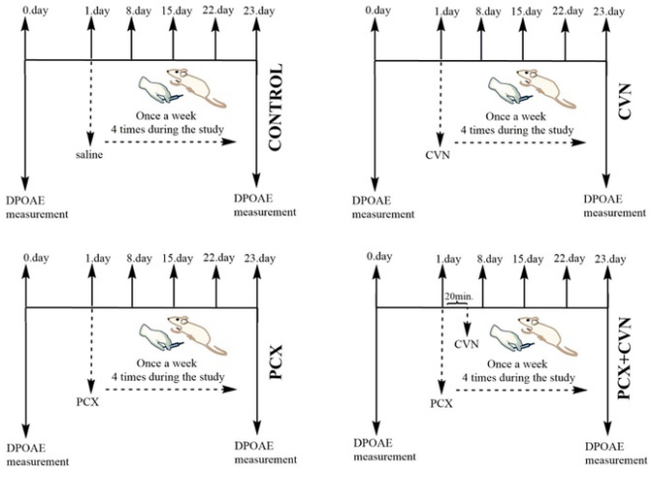

Figure 1. *Drug application protocol. Procedure for administering carvone and paclitaxel. PCX: Paclitaxel, CVN: Carvone.*

2.4. Test of Audiological Function

DPOAE dimensions were determined by inserting a fitting probe into the external ear canal under general anesthesia in a quiet setting, following the rats who had already received an otoscopic examination. The difference between the levels of L1 and L2 was kept at 10 dB SPL (sound pressure level) (L1 = 65 dB SPL, L2 = 55 dB SPL). The test was set to two separate frequencies, ratio f1/f2 = 1.22, to obtain the most powerful response. Measurements were recorded in the distortion product gram (DPgram) form (3). DPgram measurements were carried out at 4 frequencies between 2000 and 8000 Hz. Signal Noise Ratio (SNR) values of 3 dB or higher were assumed to indicate positive.

2.5. Test of Biochemical Parameters

The harvested cochlea tissues were pulverized using liquid nitrogen in a tissue grinder (The Tissue Lyser II – Qiagen, Hilden, Germany) and then homogenized with the appropriate buffer (PBS) as previously stated (21). An ELISA reader was used to quantify GSH (Sigma-Aldrich Chemical Company, Darmstadt, Germany, CS0260-1KT)(22) and MDA (Sigma-Aldrich Chemical Company, Darmstadt, Germany, MAK085-1KT) (23) levels in the supernatant from each sample. The mean ± standard deviation (SD) was used to express the data.

2.6. Statistics

IBM Corp.'s SPSS 21.0 (Armonk, NY, USA) application was used for data analysis. The mean and standard deviation were used to present all the data. Shapiro-Wilk test, skewness, kurtosis, QQ plot, and histograms were used to determine the data's normality. Parametric tests were utilized since the values were normally distributed. For homogeneity of variances, the Levene test was performed. Tukey's Significant Difference test was used for homogenous variances, and the Games Howell test was utilized for non-homogeneous variances. The variations in DPOAE amplitudes within the group were compared using a paired T-test before and after medication delivery (days 0 and 23). In all tests, a p-value less than .05 was considered statistically.

3. RESULTS

3.1. Results of Audiological Function Test

Table 2 summarizes the DPOAE measurements of the groups on day 0, which is shortly before any drug administration, and on day 23, which is one day after the last administering drugs. For all frequencies tested, DPOAE values at day 0 were not meaningfully different ($p > .05$). DPOAE thresholds alter statistically significantly before and after PCX treatment. Following PCX treatment, DPOAE values (at day 23) declined at all frequencies. At all frequencies of DPOAE measurements on days 0 and 23, no significant change in DPgram values was detected in all groups, excluding the PCX group. On the 23rd day, DPOAE values were significantly lower in the PCX group than in the Control group. On the 23rd day, no significant difference was found between the DPOAE measurements of the CVN and PCX+CVN groups compared to the control group. When the DPOAE measurements of the PCX and PCX+CVN groups were compared on the 23rd day, it was observed that the DPOAE values that had dropped with PCX had dramatically increased with the CVN application (Fig 2).

Table 2. *Intra-group comparison of pre – and post-treatment degradation product otoacoustic emission (DPOAE) thresholds.*

GROUPS	PRE/ POST	2000	4000	6000	8000
CONTROL	PRE	3.00±0.35	8.70±0.63	21.7±0.64	23.6±1.1
	POST	2.85±0.16	8.20±0.68	22.06±0.75	23.49±1.02
PCX	PRE	3.00±0.35	8.71±0.53	21.77±0.59	21.56±1.64
	POST	-2.6±0.16 [a]	6.14±0.30 [a]	11.11±0.34 [a]	12.23±0.85 [a]
CVN	PRE	3.18±0.20	9.48±0.45	22.56±1.18	22.31±1.33
	POST	3.31±0.50	10.24±0.68	19.74±1.79	22.56±0.91
PCX+CVN	PRE	3.00±0.35	8.71±0.53	22.14±1.20	22.31±1.33
	POST	2.70±0.20	9.15±0.69	16.88±0.20	20.75±1.76

[a] *(p-values < .05); Within the PCX group was compared PRE and POST medications statistically significant. Paired T-test was utilized for intra-group comparison. PCX:Paclitaxel, CVN:Carvone, i.p.:Intraperitoneal. The values are represented as mean ± SD.*

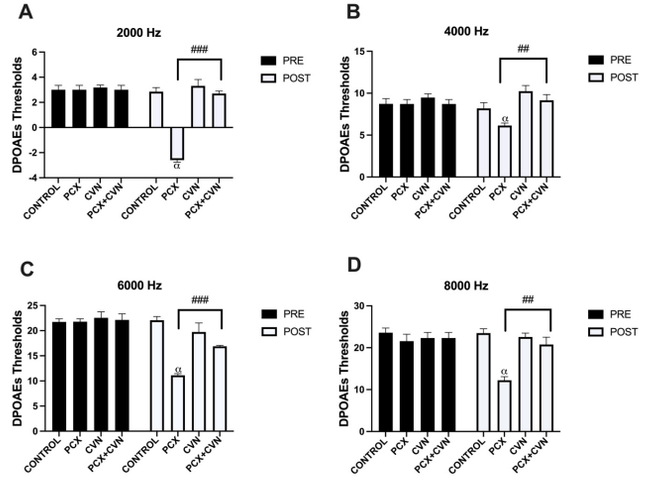

Figure 2. *The intergroup comparison of pre and post-treatment hearing threshold values (A:2000Hz; B:4000Hz; C:6000Hz; D:8000Hz). Degradation product otoacoustic emission (DPOAE) thresholds on day 0 (pretreatment) and day 23 (post-treatment). The DPOAE thresholds differed among the three groups ($^{\alpha}p < .05$ for the control vs. PCX by repeated-measures ANOVA with Games Howell posthoc test). The DPOAE thresholds in the PCX + CVN group on day 23 were attenuated compared with those in the PCX group ($^{\#\#}p < .01$, $^{\#\#\#}p < .001$ for the PCX vs. PCX + CVN groups by repeated-measures ANOVA with Games Howell posthoc test). The values are represented as mean ± SD.*

3.2. Results of Biochemical Parameters Test

Figures 3A and B present the results of the GSH and MDA levels, respectively. There was a substantial increase in MDA levels compared to the control group, while significant reductions in GSH levels were noted in rats given just PCX. When the CVN group was compared to the control group, there was no significant difference in MDA and GSH levels. However, when comparing the PCX+CVN group to the PCX group, there was a substantial rise in MDA levels and a significant decline in GSH levels. Additionally, the PCX+CVN group's MDA and GSH levels were identical to the control group.

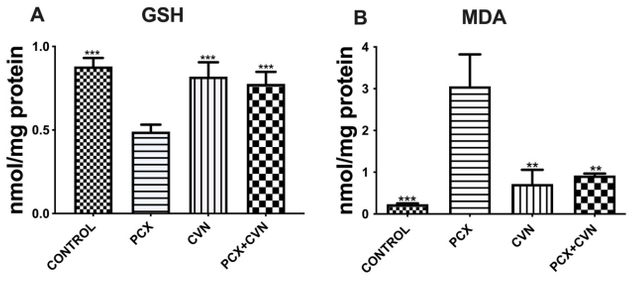

Figure 3. *The effect of carvone on oxidative stress parameters (A: GSH; B: MDA) in groups that underwent paclitaxel-induced ototoxicity. Statistical comparisons were run using one-way ANOVA followed by Tukey's test. The PCX group was compared with the other groups; *p < .05, **p < .01, and ***p < .001 marks were used. The values are represented as mean ± SD.*

4. DISCUSSION

This study investigated whether CVN may protect against ototoxicity after exposure to PCX. CVN's preventive effects on oxidative damage in the cochlea following PCX treatment were evaluated biochemically. DPOAE results confirmed PCX-induced ototoxicity. DPOAE results also demonstrated that CVN had a protective effect against PCX ototoxicity, which aligned with our biochemical findings.

In the related literature, various exogenous otoprotector substances, such as thymoquinone (24), carvacrol (3), curcumin (25), and resveratrol (26), have been utilized to reduce ototoxicity caused by various chemotherapeutics by "scavenging" free radicals at a preliminary phase and mitigating oxidative injury via their antioxidant properties.

The pathophysiological processes of generation of ROS, reduction in the antioxidant system, DNA damage, oxidative alterations in proteins, and enhanced lipid peroxidation all contribute to the development of ototoxic consequences associated with chemotherapeutics. Considering these physiological processes, the reason for focusing on antioxidant therapy in ROS-related hearing loss is that administering exogenous antioxidants can prevent inner ear damage via up-regulation of endogenous antioxidant production and the ROS scavenger system (1, 27).

CVN has been demonstrated to be a biologically active molecule in numerous in vitro and in vivo experiments, suggesting that it could be a promising therapeutic candidate (14). Indeed, CVN's potent antioxidant properties make it an attractive candidate for therapeutic development in various oxidative stress-related disorders. Many working groups have examined and documented the antioxidant effects of CVN (14, 16). Compared to α-tocopherol (28), employed as a reference antioxidant in a study, the total antioxidant activity test revealed that CVN exhibited a robust antioxidant activity. Since alpha-tocopherol has been shown to protect against cisplatin-induced ototoxicity in previous research, CVN, which has shown to have more potent antioxidant activities, may have a more significant protective impact than alpha-tocopherol in ototoxicity. Various in vitro techniques, such as lipid peroxidation, 2,2-dipenyl-1-picrylhydrazil (DPPH), and the phosphomolybdenum assay, have been used to examine CVN's antioxidant capacity. As a result, CVN was discovered to have inhibitory activity against thiobarbituric acid reactive species (TBARS), causing the DPPH radical to be scavenged and the reduction of molybdenum, Mo(VI), to Mo(III) (V)(29). So, the potential protective effects of CVN, which is renowned for its powerful antioxidant and anticarcinogenic capabilities, against PCX-induced ototoxicity were explored in this work. To our knowledge, this is the first report to demonstrate a protective impact of CVN against ototoxic damage in the literature.

Some exogenous antioxidant agents used to prevent chemotherapeutic-induced ototoxicity must provide reliable protection without reducing the anticancerous impact potential of chemotherapeutics to be considered an optimal otoprotector. According to various in vitro studies based on cell culture assays, CVN shows antiproliferative effects against a range of cancer cell lines. In an in vivo study of 7,12-dimethylbenz(a)anthracene-induced skin carcinogenesis, CVN had a chemo-preventive effect (14). In another study, CVN showed anticancer activity by inhibiting the proliferation of myeloma KMS-5 cells (28). CVN reduced the migration of breast cancer cell lines and triggered apoptosis in a study testing its antiproliferative and apoptotic activities on breast cancer cells (31). CVN has also been found to protect the retina and optic nerve against PCX-induced cytotoxicity (32). Given this background, it is reasonable to believe that CVN will contribute to the efficacy of the chemotherapeutic treatment in combination and protect against the chemotherapeutic drug's harmful aspects.

Although the cellular mechanisms by which chemotherapeutics such as cisplatin cause the loss of outer hair cells and subsequent degeneration of the organ of corti have been described, the cellular mechanisms by which PCX causes the loss of outer hair cells and subsequent degeneration of the organ of corti are not well understood (2, 7). Even though several anticancer drugs have been demonstrated to be ototoxic, there is very little information on the effects of PCX on the inner ear (7, 33). Because all the adverse effects found with other anti-neoplastic medications like cisplatin in combination use have been traced to drugs

other than PCX, this is the case (34-37). However, some studies demonstrate that PCX can cause sensorineural hearing loss in mice and some histological alterations (38). The pathways that cause cell death in non-proliferating hair cells and inner ear neurons may differ dramatically from the anticancer therapies' traditional modes of action (2). PCX inhibits cell growth and other cellular processes by stabilizing microtubules and preventing microtubule depolymerization from delivering its anti-neoplastic impact (4). Although PCX, like other anti-neoplastic agents, has various adverse effects, peripheral neuropathy is the most prevalent. PCX-induced tubulin polymerization in neurons limits axonal transport, which may cause peripheral neuropathy (39). Sensory abnormalities and decreased sensory nerve conduction velocity are caused by PCX's neurotoxic effects on the dorsal root ganglia. The neurotoxic effects of PCX on peripheral glial cells are assumed to be the cause of its adverse effects on peripheral axons (40, 41). Because β-tubulin isoforms are found in both hair cells and neurons, it is not surprising that considerable damage occurs in both auditory nerve fibers and spiral ganglion neurons, given the recognized destructive mechanism of PCX (42, 43). Due to PCX's possible ototoxic mechanism, it is expected to exacerbate ototoxicity when used with other anti-neoplastic drugs known to induce hearing loss (2). Even though the molecular and cellular causes of PCX ototoxicity are unknown, it is thought that the drug's recognized neurotoxic effects on peripheral glial cells are also responsible for its adverse effects on auditory nerve fibers and spiral ganglion neurons. In this situation, PCX stimulates the generation of free oxygen radicals in both auditory nerve fibers and spiral ganglion neurons, causing oxidative damage to the cochlea and eventually death, according to the recognized damaging mechanism of PCX. PCX was found to have an ototoxic effect in the cochlea by triggering caspase 3 activation, a hallmark of intrinsic apoptosis (7). The triggering of the generation of free oxygen radicals that cause oxidative damage is required for the induction of apoptosis by activating the caspase pathway (7). Excessive ROS generation depletes GSH, causing antioxidant enzymes in the cochlea to be inhibited. The antioxidant defense mechanism is then depleted, resulting in an increase in lipid peroxidation and also cellular damage. High MDA levels are a hallmark of this condition. MDA, a biochemical marker reflected the level of oxygen-free radical and lipid degradation in tissues, is generated as a result of oxidative damage caused by the impacts of ROS. MDA causes changes in ion transport, enzyme activities, structural damage to biological macromolecules such as lipids, proteins, and DNA and disruption of cell membranes. Since it has oxidative stress and inflammatory properties, not only one of the metabolites of cellular damage but also one of the substances that cause cell damage, it plays a role in determining the level of cellular damage (44, 45).

Chemotherapeutic agents that cause ototoxicity have been shown in research to raise MDA levels while decreasing GSH, an essential non-enzymatic endogenous antioxidant (10, 11). PCX raised MDA levels while lowering GSH levels in our study, consistent with previous findings (3, 8, 9). These data indicate that oxidative stress plays a role in the cochlear damage caused by PCX-induced ototoxicity. At the same time, MDA levels were reduced, whereas GSH levels increased due to CVN therapy in this study. According to Mengyuan et al. (15), CVN reversed PCX-induced ototoxicity, reducing oxidative stress and exhibiting otoprotective action due to these findings. CVN dose-dependently decreases MDA levels in rats induced neuronal injury by cerebral I/R. Also, Asle-Rousta et al. (16) showed that CVN reduced MDA levels and enhanced GSH levels in the livers of immobilized rats. Zhao and Du (46) confirmed that CVN increases GSH content and decreases MDA levels on lipopolysaccharide (LPS)-induced acute lung injury in mice. In light of these studies, based on the data in our study, CVN functions as an antioxidant by activating the PCX-mediated reduced antioxidant enzyme system and reducing ROS production.

In the early stages of chemotherapeutic-induced ototoxicity, clinical consequences can be seen. Ototoxicity manifests clinically as gradual, permanent, and dose-dependent sensorineural hearing loss. DPOAE is a simple and inexpensive test frequently used in research to demonstrate hearing loss in the cochlea (47). The DPOAE test was utilized in our research to demonstrate functional impairment in the cochlea. The test was given to all rats twice: once before the first drug was given and again after the last drug was given. According to our DPOAE responses, the DPOAE values of rats receiving PCX were considerably lower than both day 0 and day 23 of all the other groups' DPOAE values at all frequencies. This outcome also demonstrated the ototoxic impact of PCX. Furthermore, compared to the PCX group, rats given CVN with PCX showed considerably greater DPOAE readings. Based on this finding, CVN appears to have a functional protective effect against cisplatin-induced ototoxicity.

It needs to be noted that this experimental research is limited by the need for histopathological examinations. Therefore, further in-depth investigations are necessary to elucidate the protective properties of CVN against PCX-induced ototoxicity in the cochlear tissue, specifically from a histopathological perspective.

5. CONCLUSION

PCX successfully induced ototoxicity, evident from the reductions in DPOAE results and biochemical findings. CVN shows distinct signals of protection against PCX ototoxicity after administration. In conclusion, the findings of this study indicate that CVN effectively mitigated oxidative stress parameters by elevating MDA levels ($p < .01$) and significantly reducing GSH levels ($p < .001$). Additionally, CVN demonstrated a beneficial effect on the levels of DPOAE ($p < .01$ at 8000Hz), thereby providing protection against PCX-induced damage. However, more detailed research is needed to determine the optimal dose of CVN before it can be used in clinical practice. In addition, CVN can be used with chemotherapeutic drugs due to its significant anticancer

effect and may be a suitable drug option against ototoxicity, a side effect of chemotherapeutic drugs.

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Acquisition of data for the study: BD, FA
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Clinical and Experimental Health Sciences

Frequency of Sphenoid Sinus Pneumatization in Panoramic Radiographs

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ABSTRACT

Objective: Sphenoid sinus is an irregular cavity in the skull and sphenoid sinus pneumatization (SSP) is a variation of the sphenoid sinus that can observe on the radiographs when it reaches large sizes. It is aimed to evaluate the prevalence of SSP in panoramic radiographs in this study.

Methods: Panoramic radiographs of 500 patients were performed in this study. The patients who applied to our department with miscellaneous dental complaints were over the age of 14. The gender and age of the patients were recorded and the right, left, or bilateral visibility of SSP was examined. The study subjects were classified into four age categories and SSP prevalence was evaluated with gender, age, and visibility by statistical analyses.

Results: Female patients were more than males (78%, and 22% respectively). SSP was found in approximately one-third of the patients (33%) in the panoramic radiographs. Of these, 69 (41.8%) were on the right only, 40 (24.2%) on the left only, and 56 (33.9%) were bilateral. SSP was more common in women (24.2%) and was mostly detected in group 2 (14.4%). Any significant difference was not found in terms of right, left, and bilateral SSP, gender, and age groups. (p ›.05).

Conclusion: SSP can be superposed in the zygomatic arch as a radiolucency that can be misdiagnosed as a cyst. SSP was observed in approximately one-third of patients in this study so dentists should be aware of superimposed surrounding structures of the maxillofacial region for accurate diagnosis.
Keywords: Sphenoid sinus, pneumatization, panoramic radiography, dentistry

1. INTRODUCTION

The sphenoid sinus is seated centrally and posteriorly within the sphenoid bone. It is an irregular cavity that is adjacent to many important anatomic structures, such as the cavernous sinuses, internal carotid artery, and optic nerve (1,2). The sphenoid sinus is a potential cavity at birth and develops postnatally, reaching its original size usually during adolescence (3,4).

The sphenoid sinus pneumatization (SSP) is variable and its degree varies from absent to wide (2,5). The pneumatization extends into the pterygoid process, lesser and greater wings of the sphenoid bone, anterior and posterior clinoid processes, vomer, ethmoid bones, occipital bone, and the clivus (5).

In some cases, if the sphenoid sinus is extensive, the dentists can incidentally observe the SSP on the zygomatic arch in the panoramic radiography. Although SSP can be observed in some cases, diagnosing SSP can be difficult with conventional radiographic images because of its adjacent relationship with intracranial structures and deep location. The SSP can appear on the panoramic radiograph in three ways: a radiolucency superimposed on part of the zygomatic arch; an image with an aspect similar to the pterygomaxillary fissure or when the image is loculated, similar to a cystic lesion (5).

Panoramic radiography is a useful two-dimensional imaging modality in dentistry for providing a general impression of the jaws and surrounding structures in a single projection. Moreover, it is a simple, low-dose, and cheap method to examine the jaws, maxillary sinus, zygomatic arch, and adjacent anatomic structures. Misinterpretation in the dental radiographic examination may occur due to distortion and superimposition of anatomic structures and a limited two-dimensional view in the panoramic images. When SSP appears in the panoramic radiograph, it may be misdiagnosed as a cyst by dentists (6). SSP can be evaluated by various imaging methods e.g. computed tomography, and cone-beam computed tomography (CBCT) in the previous studies (2,4,7). Only a few cases of SSP had reported in the panoramic radiograph. Therefore, in this study, the frequency of SSP was investigated on panoramic images and it was aimed to raise awareness about SSP in the dental radiographic examination.

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2. METHODS

This retrospective study was performed on panoramic radiographs of 500 patients. The patients who applied to our department with miscellaneous dental complaints were over the age of 14. Necessary permissions were obtained from the non-invasive ethics department of the university (decision number: 2020/12-16). All images were obtained from the same panoramic machine J. Morita Veraviewepocs 2D (J.Morita Mfg. Corp Kyoto/Japan, 14.8 seconds at 65 kV 5-7.5 mA). The patients who had craniofacial anomalies and maxillofacial trauma and the panoramic images that were not evaluated clearly were excluded from the study. A dentomaxillofacial radiologist (M.K.) who had 4 years experienced evaluated the images. On the panoramic radiography, well-defined, unilocular, or multilocular radiolucencies superimposed on the zygomatic arch were accepted as SSP (Figures 1 and 2). The gender and age of the patients were recorded and the right, left, or bilateral visibility of SSP was examined. The age ranges of the patients were divided into 4 different groups: Group 1; 14-30 age, group 2; 31-50 age, group 3; 51-65 age, group 4; 65 years and older. The power test analysis determined that the study's power value was 96% with an effect size 0.17, a Z alpha of 0.05, and 500 sample sizes. The collected data were evaluated in the Statistical Package for the Social Sciences (ver.22; IBM Corporation, Armonk, NY, USA) program. Data analysis was performed with the Pearson chi-square test and results with a p-value less than .05 were considered statistically significant.

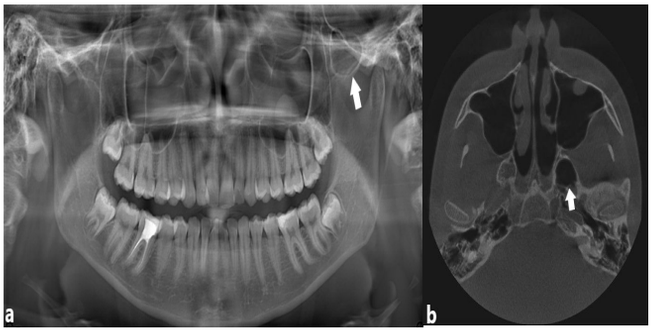

Figure 1. *Sphenoid sinus pneumatization (SSP) on the left zygomatic arch in the panoramic image (a) and axial cone-beam computed tomography image (b).*

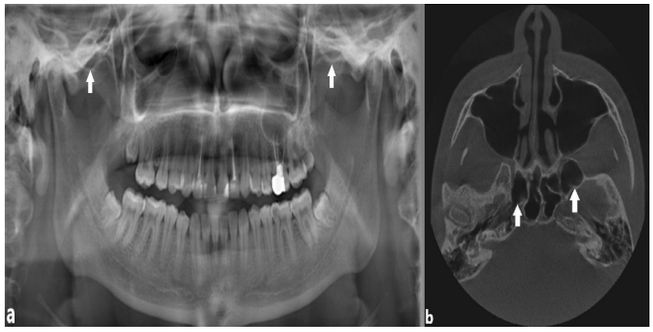

Figure 2. *Bilateral sphenoid sinus pneumatization (SSP) on the right and left zygomatic arch in the panoramic image (a) and axial cone-beam computed tomography image (b).*

3. RESULTS

A total of 500 patients' panoramic radiographs were evaluated, whose ages were ranging from 14 to 88 years with a mean of 39.95±15.34. Female patients (78%) were more than male patients (22%) (Table 1). In terms of age groups, most patients were in group 2 followed by group 1 (Table 1). SSP was found in 33% of all patients, of these 41.8% were on the right, 24.2% on the left, and 33.9% were bilateral. While 24.2% of female patients (n=121) had SSP, 8.8% of male patients (n=44) had SSP on the panoramic images. SSP was most common female patients, right side and group 2 (Table 2). The presence of SSP in female and male patients is as in table 3 (p›.05). Any significant difference was not found in gender, age groups, right, left, and bilateral SSP.

Table 1. *The frequency of the patient's age groups and gender.*

	Frequency (n)	Percentage (%)
Age groups		
Group 1 (14-30 aged)	166	33.2
Group 2 (31-50 aged)	214	42.8
Group 3 (51-65 aged)	83	16.6
Group 4 (65 aged and above)	37	7.4
Sex		
Female	390	78
Male	110	22

Table 2. *Distribution of sphenoid sinus pneumatization by gender and age groups.*

	Gender			Age Groups				
	Female (n %)	Male (n %)	Total (n %)	Group 1 (n %)	Group 2 (n %)	Group 3 (n %)	Group 4 (n %)	Total (n %)
Right	47 (68.1)	22 (31.9)	69 (100)	25 (36.2)	31 (44.9)	11 (15.9)	2 (2.9)	69 (100)
p-value	.033			.472				
Left	31 (77.5)	9 (22.5)	40 (100)	15 (37.5)	16 (40)	7 (17.5)	2 (5)	40 (100)
p-value	.937			.878				
Bilateral	43 (76.8)	13 (23.2)	56 (100)	20 (35.7)	25 (44.6)	5 (8.9)	6 (10.7)	56 (100)
p-value	.816			.342				

Chi-square test p ›.05

Table 3. *Distribution of sphenoid sinus pneumatization prevalence according to gender and age groups.*

	Gender				Age Groups					
	Female (n %)	Male (n %)	Total (n %)	p value	Group 1 (n %)	Group 2 (n %)	Group 3 (n %)	Group 4 (n %)	Total (n %)	p value
Present	121 (24.2)	44 (8.8)	165 (33)	.85	60 (12)	72 (14.4)	23 (4.6)	10 (2)	165 (33)	.48
Absent	269 (53.8)	66 (13.2)	335 (67)		106 (21.2)	142 (28.4)	60 (12)	27 (5.4)	335 (67)	
Total	390 (78)	110 (22)	500 (100)		166 (33.2)	214 (42.8)	83 (16.6)	37 (7.4)	500 (100)	

Chi-square test p ›.05

4. DISCUSSION

The anatomical variations of the sphenoid sinus are highly variable, it was found that be remarkably common in the various population. SSP is of variations of sphenoid sinus and large sizes of SSP including surrounding structures optic nerve, internal carotid artery, cavernous sinus, maxillary and the cranial nerves III, IV, and VI, and some of these structures may protrude or also be involved by the sphenoid sinus (2,5). Due to its proximity to anatomical structures, the degree and size of SSP have an important role in surgical planning for endoscopic skull base cases.

SSP occurs toward various adjacent anatomical structures but the pterygoid process and anterior clinoid process pneumatization of sphenoid sinus was determined frequently in the previous studies (2,8,9). Pterygoid process pneumatization (PP) is defined if it extends beyond a horizontal plane crossing the vidian canal (2). Considering the anatomical regions of PP in line with the literature and confirmed with CBCT images' the patients, detected SSPs of the study were thought to be suitable for PP.

Development of the sphenoid sinus begins in the intrauterine period and continues until after puberty. For this reason, patients who were aged over 14 were included in this study, and no significant relationship was found between age and the incidence of SSP.

A radiographic assessment is a part of the dental examination routine which is a key role for diagnose, decisive differential and final diagnosis, and treatment plan of several pathological conditions of the maxillofacial region. Panoramic radiography is used commonly by dentists for this purpose. It is a valuable, easy-to-implement, cheap diagnostic tool used in dentistry. However, the panoramic image is a two-dimensional projection of the maxillofacial region with multiple distortions and superimpositions (6). Due to this may lead to misinterpretation of certain pathological conditions like SSP, dentists should have knowledge of the jaws, maxillary sinus, and zygomatic processes and their adjacent structures for correct diagnosis. In case reports with SSP on the panoramic radiographs, SSP was observed as a well-defined, unilocular, or multilocular radiolucent image with a corticated border in the region of the pterygoid process (5,10,11). In this study, SSP was accepted as a well-defined radiolucency with a sclerotic margin superposed on the region of the pterygoid process. Also, for the patients with SSP who had both panoramic and CBCT images, radiolucency in the region of the pterygoid process on the panoramic radiograph was confirmed by CBCT images (Figures 1 and 2).

SSP has been evaluated in computed tomography, CBCT, or magnetic resonance images (4,12,13). The prevalance of pterygoid plate pneumatization of sphenoid sinus varies between 15% and 37% in the CT studies (12,13), and 38.9% in CBCT studies (8). SSP was found in approximately one-third of the (33%) dental patients' panoramic radiographs, which is consistent with the literature.

Generally, the SSP is symptomless and no treatment is required. Although swelling and eye proptosis due to excessive pneumatization were reported in a few studies that were not new, any symptoms were not stated in recent case reports of SSP (5,10,11). On the one hand, excessive sphenoid sinus is an advantage in accessing the lesions involving the middle cranial fossa, foramen magnum, and retroclival region, on the other hand, protrusion and dehiscence of vital surrounding structures (e.g. vidian canal, optic nerve, internal carotid artery) may occur due to the existing large pneumatization of the sphenoid sinus (2,7).

The male patients' size was the limitation of this study. The predominance of female patients in the sample may have influenced the statistical results. Besides, this study was carried out with the participation of Turkish dental patients. Further studies with much larger sample sizes and different nationalities can contribute to improving the literature.

5. CONCLUSION

SSP that one of the variations of the sphenoid sinus is relatively common in the population. In dentistry, SSP can be superposed on the panoramic radiographs as a radiolucent lesion that can be misdiagnosed as a cyst or other pathologies. Dentists should be aware of superimposed anatomic surrounding structures of the maxillofacial region and an existing suspected lesion in the panoramic images' diagnosis should be affirmed by the advanced imaging modalities (e.g. cone-beam computed tomography).

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Design of the study: MY, MK.
Acquisition of data for the study: MK.
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Drafting the manuscript: MY, MK.
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Clinical and Experimental Health Sciences

A New Perspective on the Evaluation of Comorbidity Indices on Survival in Non-Small Cell Lung Cancer

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ABSTRACT

Objective: Mortality studies are interpreted by considering comorbid diseases related to the main disease. Existence, number, and type of comorbid diseases can have an important effect on prognosis. There are various comorbidity indices to include the effects of comorbid diseases in the model. With a new perspective, we aimed to emphasize the importance of evaluating the combination of comorbid diseases in cancer survival.

Methods: Retrospective cohort, data were collected from cases with Non-Small Cell Lung Cancer treated in Department of Chest Diseases. Initially, the effects of their comorbid diseases on the duration of survival were calculated with univariate analysis, then examined according to number of comorbidities, lastly their specific combinations' Hazar Ratio were calculated with Cox multivariate analysis. The most used comorbid indices in the literature were also included.

Results: Out of 247 non-small cell lung cancer (NSCLC) cases analysis, 220 (89%) were men. Median duration of follow-up was 277 days, at the end of the follow-up 197 cases had died. HR of two comorbid diseases in cases was 1.80, but 59.52 for the combination of "diabetes and interstitial lung disease" and 3.76 for "diabetes and previously cancer". Existing comorbid indices had no significant effect on survival time (p:0.684; 0.101; 0.273; 0.567, respectively).

Conclusion: We have offered a new perspective which takes into comorbid diseases related to main disease and specially their combinations when the risk is estimated in survival research. Accurate assessments of the list of comorbid diseases related to main disease hold significant importance in advancing this field.

Keywords: Comorbidity index, comorbid disease, survival analysis, non-small cell lung cancer

1. INTRODUCTION

Many types of research consider comorbid diseases significant, for instance survival not only dependent on pathologic stage, prognosis, age, and sex, but also on other factors such as comorbid diseases (1,2). Additionally, comorbid diseases can affect the diagnosis, treatment, prognosis, and outcome (3). In the literature, the effects of comorbid diseases are listed in various forms, such as scoring, severity of the comorbid diseases etc. (3). Alvan Feinstein noted that "the failure to classify and analyze comorbid diseases has led to many difficulties in medical statistics" in the 1970s (4). Previous comorbid indices approached more general to comorbid disease types, followed by age-adjusted or specific-disease comorbid indices (2,3,5). Comorbid indices have been used frequently in studies on cancer, although there is no specific type of measurement or gold standard for cancer patients and comorbidity can wield an important role in various types of research, and in some oncology studies it has a greater impact than age (6).

The ''Cumulative Illness Rating Scale (CIRS-1968)'', the ''Kaplan-Feinstein Classification (KFC-1974)'', the ''Charlson Comorbidity Index (CCI-1987)'' and the ''Index of Co-Existent Disease (ICED-1987)'' are valid and reliable and commonly used approaches to measure comorbidity that can be used in clinical research (4). Also, the most used is the CCI, the most detailed is the CIRS with scoring sheet, and the most complicated is ICED with scoring and also physical condition, The KFC is a useful and realistic comorbidity index for clinical diabetes research because of specifically designed for diabetes (6,7,8). In addition to these, more current and specific indices such as Modified Charlson Comorbidity Index, Elixhauser Comorbidity Measures, Ovarian Cancer Comorbidity Index (OCCI) are also available (6,9,10,11). These kinds of comorbid indices have been used regardless

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of the main disease however effect of comorbid diseases is changeable depending on the type of the main disease (10). Comorbidity indices are used in the studies or is tried to select the most suitable index for the study by comparing them, but the interaction of comorbid diseases was unobserved.

The purpose of our study is to comorbid diseases' effects on the survival time according to the specific combinations of their, by regarding the most used comorbid indices in existing literature. The most suitable dataset that motivated our study was the survival parameters of non-small cell lung cancer cases along with their comorbid diseases. Through this approach, we aim to underscore that different evaluation methods can yield different outcomes, impacting both result interpretation and the ability to predict prognosis within the area of comorbid studies.

2. METHODS

This study has been approved by the Ethics Committee of Istanbul University, Cerrahpasa Medical Faculty.

A retrospective cohort study was performed patient records from the Department of Chest Diseases 1998 to 2012. A homogeneous group was created from 455 cases by selecting 247 cases with non-small cell lung cancer (NSCLC) with no surgical operation and just taken chemotherapy, curative radiotherapy, chemotherapy-radiotherapy, and chemotherapy-palliative radiotherapy. Data collected by file review included type of treatment, survival status, survival time, comorbid diseases, age, diseases stages, smoking status, and gender. Comorbid diseases that were projected by senior consultant when selected are chronic obstructive pulmonary disease (COPD), diabetes, coronary heart disease, renal failure, asthma, interstitial lung disease, previously cancer. Sample size being insufficient by nature for reliable multivariate analysis, data was folded by four for more clearly statistical results when multivariate analysis.

Summary statistics of continuous data were presented as mean, standard deviation (SD), and median to describe the cases' characteristics. Categorical data were presented as frequencies and proportions. The normality of data distribution was assessed through the Shapiro-Wilk Test. The examination of inter-group differences in the context of two independent samples relied on either the Mann-Whitney U Test or the Independent Student t Test, depending on normal distribution of data. Survival Analysis was conducted utilizing the Kaplan-Meier method and evaluating survival difference between groups was tested using the Log-Rank Test. (12, 13). Risk factors on survival were determined by univariate and multivariate Cox proportional hazard analysis (14,15,16). All data analysis was conducted utilizing the Statistical Package for the Social Sciences (SPSS) v.28. Reported outcomes were accompanied by 95% confidence intervals, and statistical significance was considered at $p<0.05$.

3. RESULTS

Of 247 non-small cell lung cancer (NSCLC) cases analysis, 89% (n:220) were men and 11% (n:27) were women. The mean age at time of diagnosis was 62.15±9.95 years (median:62), ranged from 34 to 87 years. Median duration of follow-up was 277 days, at the end of the follow-up 197 cases had died, 50 cases have still lived. Some cases have some comorbid diseases like diabetes, COPD, coronary heart disease, renal failure, asthma, interstitial lung disease, previously cancer. The highest rate of these comorbid diseases was coronary heart disease with 13% (n:32) and the least rate was renal failure with 1% (n:2). 172 cases did not have any comorbid disease therefore 25.5% of cases had just 1 comorbid disease, 3% of cases had 2 comorbid diseases and 2% of cases had 3 or more comorbid diseases. The most frequent diseases stage was 3b (43.7%) and 7.6% of cases never smoked, 34.2% of cases quit smoking, and 58.2 of cases still smoke.

No statistical difference was found in ages between male-female or died-alive (p:0.096; t=-1.704, and p:0.070; Z=-1.813, respectively). The difference between female and male for survival time was not statistically significant (514.04±637.59 median 247, and 461.20±554.47 median 287.50, respectively. p:0.809; Z=-0.241). The median survival time for NSCLC survival was 332 days (95% Confidence Interval (CI) 305.931-358.069) with Kaplan-Meier Analysis (Figure 1). Log-Rank Analysis didn't indicate a statistically significant difference in two genders' survival (p:0.529; x^2=0.396). When evaluating risk factors for survival time with Cox proportional hazard analysis confirmed a statistically significant effect for age, diseases stages, and smoking status but didn't confirm for gender (p:0.002, <0.001, <0.001, 0.209, respectively).

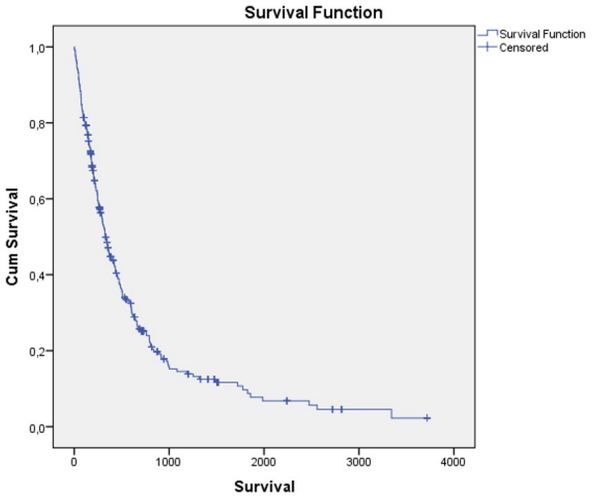

Figure 1. *Kaplan-Meier survival graph for NSCLC*

In univariate analysis, diabetes, COPD, renal failure and asthma comorbid diseases were not a statistically significant effect (p:0.255; 0.317; 0.404; 0.337, respectively) but the following three comorbid diseases significantly affected

survival time of cases: coronary heart disease (HR: 1.27; 95% CI:1.038-1.564) interstitial lung disease (HR: 12.29; 95% CI:7.308-20.652), previously cancer (HR: 1.62; 95% CI:1.131-2.306) (Table 1). In multivariate analysis, same comorbid diseases were still significantly effect on survival time and also they become more significant than univariate analysis, coronary heart disease (HR: 1.32; 95% CI:1.067-1.632), interstitial lung disease (HR: 13.17; 95% CI:7.816-22.187), previously cancer (HR: 1.68; 95% CI:1.174-2.402) (Table 2).

Table 1. *Univariate analysis results of comorbid diseases*

					95% CI for HR	
	β	S.E.	p	HR	Lower	Upper
Diabetes	0,141	0,124	0,255	1,151	0,903	1,468
COPD	0,144	0,144	0,317	1,155	0,871	1,531
Coronary Heart Disease	0,242	0,105	0,020*	1,274	1,038	1,564
Renal Failure	-0,297	0,356	0,404	0,743	0,370	1,492
Asthma	-0,243	0,253	0,337	0,784	0,477	1,288
Interstitial Lung Disease	2,508	0,265	<0,001*	12,285	7,308	20,652
Previously Cancer	0,480	0,182	0,008*	1,615	1,131	2,306

** p<0.05 significant, S.E.: Standard Error, HR: Hazard Ratio; CI: Confidence Interval*

Table 2. *Multivariate analysis results of comorbid diseases*

					95% CI for HR	
	β	S.E.	p	HR	Lower	Upper
Diabetes	0.117	0.128	0.362	1.124	0.875	1.444
COPD	0.099	0.145	0.494	1.105	0.831	1.468
Coronary Heart Disease	0.277	0.108	0.010*	1.320	1.067	1.632
Renal Failure	-0.229	0.357	0.522	0.796	0.396	1.601
Asthma	-0.333	0.260	0.201	0.717	0.430	1.194
Interstitial Lung Disease	2.578	0.266	<0.001*	13.168	7.816	22.187
Previously Cancer	0.518	0.183	0.005*	1.679	1.174	2.402

** p<0.05 significant, S.E.: Standard Error, HR: Hazard Ratio; CI: Confidence Interval*

Table 3 shows that analysis of total combinations in numbers; when cases who have two comorbid diseases whatever they are, compared with cases who haven't any comorbid disease (reference category), two comorbid diseases were significantly effect on survival time (HR: 1.80; 95% CI:1.225-2.640) and also founded same thing for cases who have four comorbid diseases whatever they are (HR: 9.94; 95% CI: 3.653-27.032).

If two comorbid diseases at random instead of content of combinations that have two diseases such as "diabetes+interstitial lung disease" or "diabetes+previously cancer", it might be inconvenient to show for comorbid diseases' effect on survival time; HR of 2 comorbid diseases in cases was 1.80 but the HR of "diabetes+interstitial lung disease" combination comorbid disease was 59.52 as the HR of "diabetes+previously cancer" combination was 3.76 (Table 4). Although there was not a statistically significant difference for cases who have three comorbid diseases (Table 3), the HR of "diabetes+COPD+coronary heart disease" was 2.31 HR. Cox regression survival graph for comorbid disease and their combinations at Figure 2. When evaluating age, diseases stages, and smoking status (were significantly in univariate analysis) as risk factors for NCSLC survival time in all combined comorbid diseases, and combinations that were significant already are still significant and also some combinations' HR were increase.

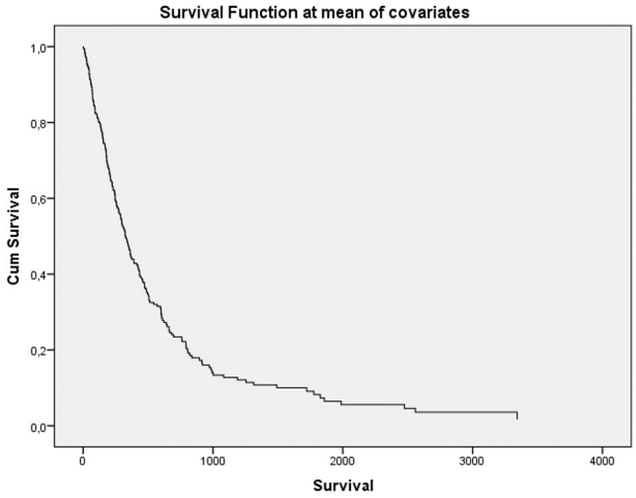

Figure 2. *Cox regression survival graph for comorbid diseases* combinations.

''Charlson Comorbidity Index'', ''Kaplan-Feinstein Classification'', ''Index of Co-Existent Disease'' and ''Cumulative Illness Rating Scale for Geriatrics'', had no significant effect for NSCLC on survival time (p: 0.684; 0.101; 0.273; 0.567, respectively).

Table 3. *Number of total comorbid diseases*

						95% CI for HR	
	n	β	S.E.	p	HR	Lower	Upper
0 Comorbid Disease	688			<0.001*			
1 Comorbid Disease	252	0.142	0.084	0.091	1.152	0.978	1.357
2 Comorbid Diseases	32	0.587	0.196	0.003*	1.798	1.225	2.640
3 Comorbid Diseases	12	0.122	0.292	0.675	1.130	0.637	2.005
4 Comorbid Diseases	4	2.296	0.511	<0.001*	9.937	3.653	27.032

** p<0.05 significant, S.E.: Standard Error, HR: Hazard Ratio; CI: Confidence Interval*

Table 4. *Multivariate analysis results of comorbid diseases with their combinations*

	β	S.E.	p	HR	95% CI for HR	
					Lower	Upper
Comorbid Combination			<0.001			
Diabetes	-0.012	0.164	0.942	0.988	0.716	1.363
COPD	-0.125	0.194	0.519	0.882	0.603	1.291
Coronary Heart Disease	0.264	0.123	0.033*	1.302	1.022	1.658
Renal Failure	-0.252	0.357	0.480	0.777	0.386	1.564
Asthma	-0.009	0.292	0.976	0.991	0.559	1.758
Interstitial Lung Disease	2.380	0.303	<0.001*	10.803	5.970	19.546
Previously Cancer	0.241	0.229	0.292	1.273	0.813	1.994
Diabetes+Coronary Heart Dis.	0.138	0.357	0.698	1.149	0.570	2.314
COPD+ Coronary Heart Dis.	0.377	0.357	0.291	1.458	0.724	2.938
Diabetes+Interstitial Lung Dis.	4.086	0.550	<0.001*	59.517	20.262	174.823
Diabetes+ Previously Cancer	1.323	0.505	0.009*	3.755	1.396	10.103
COPD+ Previously Cancer	0.843	0.504	0.094	2.323	0.866	6.233
Diabetes + COPD + Coronary Heart Dis.	0.838	0.357	0.019*	2.312	1.148	4.658
Diabetes + Coronary Heart Dis. + Asthma	-0.594	0.503	0.237	0.552	0.206	1.479
Diabetes + COPD + Coronary Heart Dis. + Interstitial Lung Dis.	2.412	0.512	<0.001*	11.158	4.093	30.420

** p<0.05 significant, S.E.: Standard Error, HR: Hazard Ratio; CI: Confidence Interval*

4. DISCUSSION

Comorbid diseases, which were significant in the univariate analysis, were still significantly effect on survival time in the multivariate analysis and also they become more significant than univariate analysis. When we regard all types of comorbid combinations in analysis as it is expected much more significant results and HR increase much more. Researchers require appropriate methods to adjusted results for underlying differences in cases' survival time (3,4,17). It is crucial to understand the impact of comorbidity on cases' survival to develop the accurate estimate of survival (8,18). Comorbid diseases in NSCLC have been examined with different approaches such as singular, total number of comorbid diseases, Charlson Comorbidity Index (1,19,20,21). Hovewer, as it is understood in our study, it is necessary to particularize real effects of comorbid diseases by doing together univariate analysis and multivariate analysis. Also, comorbid diseases should be taken together into consideration with their combinations not just single. Comorbidities that existing shouldn't be taken with number of total comorbid combination because different combination of comorbid diseases that have same number of diseases can have different power and significance. Interstitial lung disease was found to be the most influential comorbid disease, both in univariate, multivariate analyses and in combination with other comorbid disease. The combinations of diabetes and previous cancer, as well as COPD and previous cancer, were found to be effective.

In our study, an interesting point, none of most used comorbid indices had significant effect for NSCLC on survival time. In the light of these findings, it can be said that some comorbid diseases' power was hide or increased by other diseases, although there are some valid and reliable indices to measure effect of comorbidity that can be used in the literature (22,23,24). There aren't any indices that involve a comorbid disease list that is enough for all main diseases, different indices are necessary due to the presence of diverse comorbid diseases that can potentially impact the prognosis of various main diseases. Some comorbid indices may not include some comorbid diseases that have important effect for a main disease therefore using such indices isn't capable of an appropriate and confidential prediction, untrustworthy and can't specify required details as a result of this estimations will be fallacious. Indices that assumed same power for every disease can be misleading.

Our study is a pioneering work in providing a statistical perspective to the clinic, emphasizing the importance of extensive data collection, and paving the way for prospective research.

5. CONCLUSION

In conclusion, the inclusion of comorbid diseases is an essential aspect of survival research; however, accurate assessment is crucial for generating trustworthy results. Considering the importance of this aspect, research led by clinicians to compile comprehensive lists of comorbid diseases related to main disease, while also considering the severity of these comorbidities for indexing purposes, holds significant importance in advancing this field.

Limitations

The number of samples is small for the validity of the model in our study. Studies with larger data are needed. There are some potential limitations such as unrecorded severity of comorbid diseases in retrospective data.

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Clinical and Experimental Health Sciences

Radiographic Evaluation of the Effect of Vitamin D3 Supplementation on Regeneration of Calvarial Bone Defects in Rats

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ABSTRACT

Objective: The present study was aimed to evaluate radiographically the effect of orally administered vitamin D3 on guided bone regeneration in calvarial critical size defects (CSD) in rats.

Methods: Two calvarial CSD were created in 12 male Sprague-Dawley rats. One of the defects was left empty (E defect), while the other one was treated with deproteinized bovine bone graft and collagen-based resorbable membrane (GM-filled defect). Following surgical intervention, rats were randomly assigned into two groups; the control group was administered distilled water, and the test group was treated with 2 µg /kg vitamin D3 by gavage once a day for 8 weeks. Radiological images were obtained from rats on 4th and 8th weeks. The area fraction of newly formed osteoid was determined using Image Fiji Analysis Software.

Results: The percentages of area fraction in the GM-filled defects were statistically higher than the E defects in both study groups at 4th and 8th weeks (p<.0001). In both E and GM defects, the percentage of area fraction was higher at weeks 4 and 8 in the test group compared to the control groups (p<.0001). In comparison to the other groups, the GM-filled defect in the test group had the highest mean percentage of area fraction (p<.0001).

Conclusion: This study demonstrated that healing of CSD could be evaluated by radiography and Vitamin D3 improves bone healing, particularly when guided bone regeneration is used in rats with CSD at the calvaria.

Keywords: Bone regeneration, radiography, vitamin D3.

1. INTRODUCTION

Alveolar bone loss continues to be a concern in the field of oral rehabilitation since congenital illnesses, tumors, and trauma can all cause major face bone abnormalities that are difficult to correct both functionally and aesthetically (1, 2). As a well-known fact, if the optimal method is not used for bone formation, the natural structure of the bone cannot be accomplished, and as a result, unfavorable fibrous tissue forms during the healing process (3). Guided bone regeneration (GBR), is a well-established method for treating bone defects (4). This procedure allows for the filling of a space maintained by either resorbable or non-resorbable barrier membranes with bone, allowing for the regeneration of bone tissue (5, 6). An essential component of the procedure is the membrane inhibits apically downgrowth of epithelium. Biocompatibility, clinical management, integration by the host tissues, the capacity to create space, and acceptable mechanical and physical properties are positive attributes of the membrane used for GBR (7). The first generation of barrier membranes consisted of non-resorbable membranes. These membranes typically exhibit biocompatibility and the ability to create space (8). However, non-resorbable membranes require a second surgical procedure to be removed. A second generation of membranes made of resorbable materials as collagen-based membranes were established and widely used in a variety of clinical situations (7). Osteogenesis, osteoinduction, and osteoconduction are three different processes that bone regeneration can be achieved (9). Allografts, xenografts, alloplasts, and autogenous bone are the major types of bone graft materials (8). Xenografts are made by deproteinizing cow, horse, and pig bone tissue with the removal of organic material. Its benefits include having a porous structure that is similar to that of human cancellous bone, being high in osteoconduction because it acts as a support structure for the new bone formation and being reasonably priced when compared to other bone graft materials (8, 10). Deproteinized bovine bone material

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is the most widely used clinical product among xenograft materials due to its stable and excellent bone formation ability (11). According to research, bone graft materials covered by barrier membranes were well preserved and exhibited osteoconductive properties; additionally, the bone grafts could maintain stability enough to be successfully incorporated into the healthy bone as the membranes were employed in combination (12). This contributed to a positive regenerative outcome by providing sufficient space (13). According to the results of the prior studies, using collagen membrane in conjunction with xenografts may improve bone regeneration (14).

Studies on vitamins have expanded as it has been clear how beneficial nutrition is to human health. A fat-soluble hormone, vitamin D refers to two compounds: vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol); it is converted by the liver and kidneys into an active form of Vitamin D3 (1,25(OH)2D3). The anabolic effect of 1,25(OH)2D3 on bone metabolism is well known (15). It is essential both for calcium and phosphorus homeostasis, which affects bone remodeling (16, 17). The discovery that vitamin D3 has receptors with a high affinity for osteoblastic cells provides more validity to the idea that vitamin D3 regulates bone production and mineralization. Several studies have demonstrated that vitamin D3 administration increases fracture healing (18), implant osseointegration (19), bone density and regeneration (20, 21). Hong et al. (21) concluded that vitamin D has a positive effect on bone regeneration in the study in which they examined the effects of topical and systemic vitamin D3 applications on bone density and regeneration. The findings demonstrated that topical treatment of D3 expedited the formation of new bone and increased bone density, but this method had a lower effect than systemic vitamin D3 administration. There has been also substantial research on the essential functions of vitamin D in the control of calcium homeostasis and bone metabolism. However, there is still a lack of comprehensive information on the effects of cholecalciferol on bone healing and regeneration in dentistry (20).

Radiographic methods, histologic and histomorphometric analyses can be used to evaluate the healing of bone regeneration. Radiographic assesments have been used to examine the effect of various treatment concepts on bone formation. They offer the potential benefit of being less expensive and time-consuming than histologic examination; however, the validity of these assessments has not yet been thoroughly investigated (22). A small number of studies have investigated the accuracy of the evaluation of bone regeneration using standardized conventional radiographs.

In the present study, we hypothesized that vitamin D3 and GBR can improve bone healing in a rat model. Thus, the purpose of this study was to evaluate radiographically the effect of orally administered vitamin D3 on GBR in a critical-size defect (CSD) model at the calvaria of rats.

2. METHODS

2.1. Animals

Our study was approved by the Acıbadem University Animal Experimentation Ethics Committee (protocol no. 2020/32). The authors followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines 2.0 from 2020. Twelve male, 4-month-old (mean weight 300–350 g), healthy Sprague Dawley rats were used in the study. The rats were placed in pairs in purpose-built cages at the Acıbadem University Experimental Animal Research Laboratory, with a 12-h light/dark cycle at 21°C ± 2°C and with ad libitum access to rat food and water during the entire experiment. Every cage had a limit of 2 rats to contain in the shelter. Surgical procedures were performed under general anesthesia, and all efforts were made to minimize animal suffering.

According to the power analysis performed by using the values obtained from an animal study (10) having similar defect size with the present study and comparing new bone formation between the groups, at a 80% power and 5% significance level, a minimum of 6 rats per group and a total of 12 rats were found necessary.

2.2. Surgical Procedures

To minimize suffering, animals were anesthetized with a combination of ketamine (10% Ketasol; Richter Pharma AG, Wels, Austria), and xylazine (Rompun, Bayer, Leverkusen, Germany) with 35 and 3 mg/kg respectively. The scalp was shaved and cleaned with povidone-iodine after general anesthesia. The skin, subcutaneous tissue, and periosteum were reflected, exposing the parietal bones, after a 2 cm-long midline incision was made along the sagittal suture (Figure 1a). For the 5 mm defects, two full-thickness, non-suture associated bone defects were trephined in the left and right sides of the parietal bone under constant normal saline irrigation. Surgery was performed carefully to prevent injury to the cranial dura mater. The one side defects were left empty (E defect) while a collagen-based resorbable membrane (circular membrane with a diameter of 6 mm on the midline) (BioGide®) and a bovine bone graft (BioOss®) were applied in the other side defects (GM-filled defect) of all animals (Figure 1 b,c,d,e). An absorbent suture (Vicryl 3-0, 4-0; Ethicon Inc., NJ, USA) was used to seal the subcutaneous tissue, and the skin was left to recover. Following surgery, animals were given intramuscular injections of the antibiotic Ceftriaxone (Rocephin, Roche, Nutley, New Jersey, USA), 25 mg/kg, for 3 days, and the analgesic Carprofen (Rimadyl, Pfizer, New York, USA), 4 mg/kg, 24 hours a day, for 3 days.

2.3. Experimental Groups

The animals were randomly divided into two groups by a researcher (HOO) after the rats awoke from anesthesia following the procedure; the control group (n=6) was given distilled water, and the test group (n=6) was given 2 µg /kg vitamin D3 by gavage once a day for 8 weeks. Twenty four

hours following the final vitamin dose, all animals were euthanized by anesthetic overdose and sacrificed at week 8.

2.4. Radiographic Analysis

An X-ray machine (Siemens Arcadis Avantic C-Arm, Berlin, Germany) was used to take radiographs of the samples collected in week 4 and 8 (Figures 1f and 1g). The radiographic images were taken under exposure parameters of 7 mA, 0.03 s, and 70 kV with the X-ray beam perpendicular to the bone defect areas parallel to the floor. A standard threshold was used to include all areas of high density in order to quantitatively calculate the entire area of the newly produced osteoid. Each defect's osteoid region and the bone defects (n=12) were detected. Using Image Fiji Analysis Software (Olympus Image Analysis Software 5.0, Tokyo, Japan), the newly formed osteoid area fraction was determined. The radiographic evaluation is performed by the same researcher (GNV).

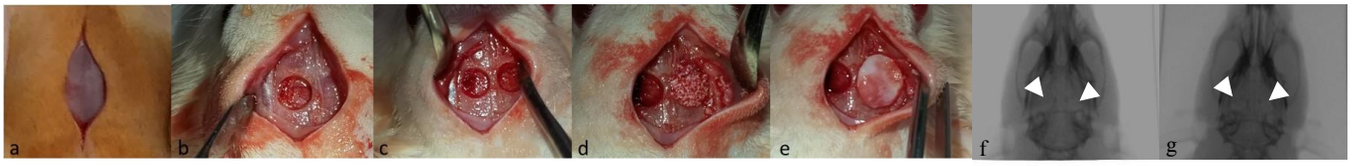

Figure 1: *Creation of the defects.* ***a)*** *Midline incision design from the frontal to the occipital region,* ***b)*** *Critical size defect on the left side,* ***c)*** *Creation of two critical size defects of 5 mm,* ***d)*** *Empty defect (Left) and defect filled with deproteinized bovine bone graft (Right),* ***e)*** *Empty defect (Left) and defect covered by collagen-based resorbable membrane after filling with deproteinized bovine bone graft (Right),* ***f)*** *Radiographic image of the Control group and* ***g)*** *of the Test group at week 8.*

2.5. Statistical Analysis

GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, USA) was used for data analysis. Each quantitative result was presented as the mean ± standard deviation (SD). The intragroup comparisons were performed with the two-way ANOVA with post-hoc test, the differences between study groups were determined with two-way ANOVA supplemented by Tukey's multiple comparison test. P values of <.05 were as statistically significant.

3. RESULTS

No postoperative complications, infections, changes in animal behavior, body weight, or general health issues were seen in any of the rats following surgery or over the course of the study (up to 8 weeks). In all rats, healing of the tissue at the surgical sites was uneventful. Radiographic images of the rat calvarias from both groups were obtained at weeks 4 and 8. The percentage of the area fraction according to newly formed osteoid values at weeks 4 and 8 are presented in Figure 2. At week 4, the area fraction in the control group for the GM-filled defect was 13.11±0.87%, but it was only 11.0±0.94% for the E defect. At week 8, the percent area fraction in the control group was 14.15±0.69% for GM-filled defects and 12.19±0.39% for E defects. In the test group, at both 4th and 8th weeks, GM-filled defect (18.62 ± 0.49 and 19.81 ± 0.70%, respectively) showed higher area fraction than E defect (15.83 ± 0.41 and 17.10 ± 0.66%, respectively). It was determined that the values at 8th week were statistically higher than 4th week in both E defect and GM-filled defect in study groups (p<.05). In addition, the values in the GM-filled defect were statistically higher than the E defect in the study groups both at week 4 and 8. The percentage of area fraction, in both E and GM defects was higher at weeks 4 and 8 in the test group compared to the control groups (p<.0001). GM-filled defect in test group exhibited the highest mean percentage of area fraction between all groups (p<.0001).

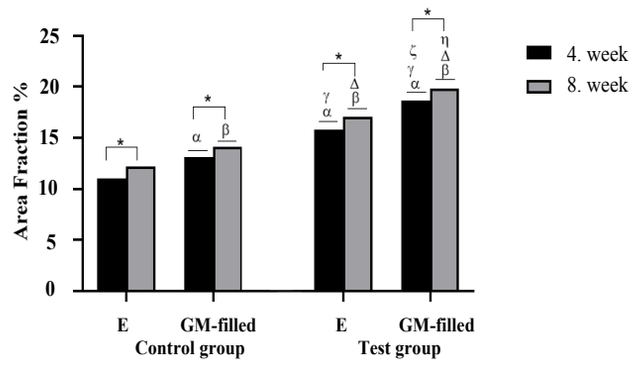

Figure 2. *The percentage of area fraction for all groups. [α]Compared to the Control group E defect at week 4 with p<.0001, [β]Compared to the Control group E defect at week 8 with p<.0001, [γ]Compared to the Control group GM-filled defect at week 4 with p<.0001, [Δ]Compared to the Control group GM-filled defect at week 8 with p<.0001, [ζ]Compared to the Test group E defect at week 4 with p<.0001, [η]Compared to the Test group E defect at week 8 with p<.0001; *p<.05, 8th week versus 4th week in all groups, Two-Way ANOVA test.*

4. DISCUSSION

The present study was designed to observe the effect of vitamin D3 on bone regeneration using a rat calvarial critical size defect model during instead of for 4 and 8 weeks and

evaluate new bone formation through radiological analysis. The outcomes showed that the administration of 2 µg/kg vitamin D from the first postoperative day could actually accelerate new bone formation in calvarial defect areas. Radiographic images taken at weeks 4 and 8 following surgery showed higher new osteoid formation.

Since it provides crucial data on physiological and pathological circumstances that could be used to develop better clinical interventions, the use of animal models for in vivo research has been favored (23). When using bone substitutes in an animal model to evaluate bone regeneration, it is essential to verify that the substituted material conformed to the concept of CSD. It was identified by Schmitz and Hollinger in 1986 as the smallest intraosseous diameter that will never spontaneously heal over the course of the animal's life or the research (24). According to the species of the animal and the site of the defect, this concept has several thresholds. Critical size defect has been utilized extensively in the improvement and establishment of a wide variety of regenerative materials and procedures, and is one of the most reliable and popular in vivo models in the field of bone regeneration. A CSD is one that does not repair during the period of the investigation (25). The diameter of the rat calvarial CSD has been the point of contention in the literature, with reports ranging from 4 mm to 8 mm, emphasizing the requirement of a control group in every investigation (23). The 5 mm diameter, however, has been widely accepted as a critical-size calvarial defect in healthy rats (26, 27). The use of a 5 mm CSD has the advantage of allowing for the establishment of two defects per animal, therefore allowing fewer animals to be included in the experiment; avoiding the inclusion of the sagittal suture, hence reducing the potential of midsagittal sinus lesions (28). The use of standard calvarial defects with a diameter of 5 mm in rats allows evaluation of the effects of bone substitutes used in GBR.

The follow-up periods in this experiment were only 4 and 8 weeks. In the study of Gosain et al. (29) on critical size calvarial defects in rats, the recovery in the 8th week after surgery was 30.1% greater than in the 4th week; they stated that it was only 7.7% greater at the 12th week compared to the 8th week. Accordingly, in a rat model, the critical period between the 4th and 8th week after injury was found to be sufficient for evaluating total recovery (30). Besides, 8 weeks was the right amount of time to evaluate late repair, including bone remodeling, bone regeneration, and graft material absorption by new bone tissue (31). Consistent with the literature, in this study, as the results after 4 weeks demonstrated less newly formed osteoid at the defect sites in both the control and test groups, suggesting that 4 weeks was insufficient time to complete the bone healing process.

The gold standard in regeneration is autologous bone grafting, but this method has limitations, such as longer recovery periods for graft harvesting, volume restrictions for the bone, restrictions in supply and donor site morbidity (32). Furthermore, using autogenous bone frequently necessarily requires a second surgical site and prolonged perioperative time (33). Advances in the use of bone substitutes to replace autogenous grafts improve both the patient's and the surgeon's operating conditions. Allograft also regularly has supply limitations (34). It can cause an immunogenic reaction, has a less consistent clinical outcome, and is only available in limited quantities. A xenograft is derived from a nonhuman species. As a result, antigenicity is significantly higher than that of allografts; it requires more sterile processing, which may result in decreased osteoinductive properties. These grafts may be less expensive and more readily available due to the abundance of donors. Additionally the shelf life is also generally long because of the extensive sterilization processes (35). Bio-Oss® is a deproteinized bovine bone mineral that is biocompatible and has low resorption and excellent bone conduction. Therefore, the slow degradation process of this product may help to maintain the stability of regenerated bone (36). Additionally, it has 60–70% porosity per unit volume and no organic components. No immunological responses have been reported to its clinical use (37). Bio-Oss® has been extensively researched in several studies over the last few decades, with several authors confirming its osteoconductive potential in animal or clinical studies (10, 38). Bio-Gide® is a natural bilayer collagen membrane that has a fibrous surface in addition to a cell-occlusive surface to protect the wound site during healing and enable protein deposition (33). It promotes consistent bone regeneration and perfect tissue connection (39). Comparing collagen membranes to non-resorbable membranes, several studies have found that collagen membranes may support even greater bone regeneration and wound healing (40). In order to protect the initial coagulum, the Bio-Gide® membrane combines with the surrounding tissues. After that, it optimally dissolves to enable the series of biological events that lead to regeneration (14). These membranes are among the most studied in the literature because they play important structural support roles, and collagen is the primary component of connective tissues. (41). The combination of Bio-Oss® and Bio-Gide® significantly lowers graft resorption, enables uncomplicated recovery, decreases morbidity, and minimizes patient discomfort (42). Considering the properties of these materials, in the present study in which these materials were used in GBR, there was no immune reaction in the GM-filled defects in both the vitamin-administered and non-vitamin-treated groups. Our study also demonstrated that new bone formation was higher in GM-filled defects compared to E defects at 4th and 8th weeks. In addition, new bone formation at week 8 was higher in both groups compared to week 4. Similar to our study, Fadel et al. (33) found that new bone formation was significantly higher in defects treated with the combination of Bio-Oss® and Bio-Gide® at both 4 and 8 weeks compared to empty defects, in bone regeneration in rats. Moreover, new bone formation was higher at week 8 than week 4 in both groups.

Studies on the administration of vitamins for bone healing and formation have become more popular in recent years. A limited number of studies on the effects of vitamin

administration on guided bone regeneration are available. However, there is currently insufficient evidence to support the idea that administering of vitamin D3 may have a positive effect on osteoblasts and stimulate bone regeneration. Animal studies revealed that administering vitamin D3, either systemically or locally, had a positive effect (21, 43, 44). Hong et al. (21) demonstrated that orally administering vitamin D3/Ca in addition to alloplastic grafts enhanced new bone formation and bone volume in dogs. Cignachi et al. (45) discovered that vitamin D3 helps to improve bone regeneration including in rats with induced diabetes. In one single study by Han et al. (46) the effect of eldecalcitol (ELD), an active vitamin D3 analog, on bone regeneration in 64 rats was investigated; the results showed that the systemic administration of ELD could improve new bone formation as evidenced by an increased bone volume and speeded mineralization. In another study on rats, it was observed that systemic administration of vitamin D3 increased the osseointegration of implants (47). The results of our study, which showed that orally administered vitamin D3 increases new bone formation radiographically in rats, is consistent with previous studies showing that dietary vitamin D3 consumption increases bone formation. In this study, it was also found that vitamin D3 promoted increased bone production, particularly in GM-filled defects.

The effect of various treatment concepts on bone formation has been evaluated using radiographic evaluations. Many animal studies have used different radiography techniques to assess bone regeneration (48-50). All of these studies used radiographic analysis without any histologic components. In this study, we were able to evaluate new bone formation radiographically in all defects at 4 and 8 weeks. However, to the best of our knowledge, no study that radiographically examines the effect of orally vitamin D3 administration on bone regeneration is present in the literature. One limitation of this study is that only radiographic analysis was performed to assess the effect of vitamin D3 supplementation on bone regeneration in rats.

6. CONCLUSION

Based on the results of this study, we can conclude that at week 4 and 8, radiographic evaluation can be utilized to identify new bone formation in CSD in rats and orally administered vitamin D3 enhances bone formation in CSD at the calvaria of rats.

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Clinical and Experimental Health Sciences

N-Acetylcysteine Ameliorates 5-Fluorouracil-Induced Ovarian Injury in Rats

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ABSTRACT

Objective: Although 5-fluorouracil (5-FU) is one of the most commonly used chemotherapeutics worldwide, it has been shown that 5-FU administration can cause reproductive toxicity in recent years. N-acetylcysteine (NAC) is the precursor of glutathione, the most important endogenous antioxidant molecule and is known for its effective antioxidant and anti-inflammatory properties. Although NAC is one of the most studied antioxidant molecules, its curative effect against ovarian damage caused by 5-FU has not been demonstrated to date. It was therefore aimed to investigate whether NAC is therapeutic against 5-FU-induced ovotoxicity in this study for the first time.

Methods: Rats were first exposed to a single dose of 5-FU (100 mg/kg) and then treated with NAC (10 and 20 mg/kg) for three days. The oxidative stress, inflammation and apoptosis markers in ovarian tissues were also determined using spectrophotometric methods. Ovarian tissues were also evaluated histologically.

Results: It was revealed that the levels of oxidative stress, inflammation and apoptosis biomarkers in ovarian tissue increased by 5-FU administration ($p<.005$). Treatments with NAC significantly restored these damages dose-dependently ($p<.005$). Moreover, these biochemical findings were confirmed by histological examination.

Conclusion: NAC can be considered as a potential therapeutic molecule against 5-FU-induced reproductive toxicity, as it can abolish the ovarian toxicity caused by 5-FU by reducing oxidative stress, inflammation and apoptosis.

Keywords: 5-Fluorouracil, apoptosis, ınflammation, n-acetylcysteine, oxidative stress, ovotoxicity

1. INTRODUCTION

The incidence of cancer is increasing all over the world and chemotherapeutic drugs are frequently used in the treatment of cancer (1,2). Since chemotherapeutics have a low therapeutic index, they affect not only target malignant cells but also healthy cells (3). In young women, side effects of chemotherapeutics are seen on the reproductive system and gonadal damage, permanent ovarian failure and menopause may develop after chemotherapy (4). 5-fluorouracil (5-FU) is one of the most used chemotherapeutics (2). It is a drug that causes decreased DNA synthesis mainly by inhibiting thymidylate synthase. It also interferes with RNA processing and protein synthesis (5). It is frequently used against colorectal, breast, stomach, pancreatic, head and neck cancers (6). In addition to leukopenia, hemolytic anemia and thrombocytopenia, 5-FU administration may cause some side effects such as stomatitis, mucositis, diarrhea and various organ toxicity (2). Recent studies have shown that 5-FU, one of the most frequently used chemotherapeutics in the world, causes ovarian damage (5,7). Since there is no approved treatment protocol or a specific antidote to be used against the toxic effects of chemotherapeutic use, different toxicity prevention strategies are currently being studied meticulously (1,8).

N-acetylcysteine (NAC) is a water-soluble molecule and a precursor of glutathione (GSH) (9,10). It has been shown to has antioxidant, anti-inflammatory and anticancer activities (11,12). Although NAC has been shown to abolish the damage caused by various chemotherapeutics in liver, heart and kidney tissues (4,10,11), there is no research on its effect on 5-FU-induced ovotoxicity. Thus, we hypothesized that NAC may have therapeutic effects against 5-FU-induced

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ovarian damage, and in this study, we aimed to determine the effects of NAC against 5-FU-related ovarian damage in an experimental model for the first time.

2. METHODS

2.1. Animals

A total of 30 healthy female Sprague-Dawley rats (weighing 200±20 g) were used to conduct the current study. All rats were kept in clean plastic cages at an ambient temperature of 22±2°C and subjected to a 12 h photo period of light-dark cycle. All experimental procedures were approved by Local Animal Research Ethics Committee of Karadeniz Technical University (Protocol No: 2021/30). The estrus stages of the rats were evaluated by vaginal smear and only the rats determined to be in estrus period were included in experiments (13).

2.2. Experimental Design

The rats were divided into five groups. Control group was given physiological saline for four day. 5-FU group was given 5-FU (100 mg/kg) in 1st day and saline followed 3 days. 5-FU+NAC groups were given 5-FU in 1st day and NAC (10 mg/kg and 20 mg/kg, respectively) followed 3 days. *Per se* NAC group (only 20 mg/kg) was given physiological saline in 1st day and NAC (20 mg/kg) for 3 days. All the treatments were given via intraperitoneally (IP). All rats were sacrificed by cervical dislocation on day 5 (8,14) and their ovarian tissues were removed. Half of the tissues were stored at – 80 ℃ for biochemical examinations, while the other half were subjected to 10% formaldehyde fixation for histological analysis. Doses of 5-FU (15,16) and NAC (17,18) were determined according previous studies.

2.3. Biochemical Analysis

Ovarian tissue samples (approximately 20 mg) were homogenized in PBS using a homogeniser. The protein levels were determined using a commercial colorimetric kit (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL). Total oxidant status (TOS) and total antioxidant status (TAS) levels were determined using commercial kits (Rel Assay Diagnostics, Gaziantep, Turkey). The ratio of TOS to TAS was accepted as oxidative stress index (OSI) and calculated the following formula (14):

$$\text{OSI (arbitrary unit)} = \frac{\text{TOS (μmoL hydrogen peroxide equivalent/L)}}{\text{TAS (mmoL trolox equivalent/L)}} \text{ x } 100$$

Malondialdehyde (MDA) levels were determined using previously described method (19). The tissue levels of oxidative stress [superoxide dismutase (SOD), glutathione (GSH), 8-hydroxy-2'-deoxyguanosine (8-OHdG)], inflammation [interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α)] and apoptosis (caspase-3) biomarkers were determined using ready-to-use ELISA kits (Fine Biotech Co. Ltd, Wuhan, China).

2.4. Histological Analysis

Tissue samples were fixed in in 10% formaldehyde for two days and then placed in paraffin blocks after routine tissue follow-up. Sections of 5 μm were stained with H&E and examined under a light microscope. The prepared slides were evaluated using previously described scale (20,21). Analyzes were performed by a pathologist unfamiliar with the groups using the coding system.

2.5. Statistical Analysis

A sample size of 6 animals per group has been provided the appropriate power (1–β=0.8) to identify significant differences in MDA (adjusted α=0.016 for two comparisons), taking into account an effect size d=2.0, a two-sided t-test, and a sample size ratio=1 (G*Power 3.1.9.2, Kiel University, Kiel, Germany). Statistical analyses were made by using the SPSS version 23.0 (Chicago, IL, USA) statistical package software. The Shapiro-Wilk test was used to check whether the data were normally distributed. Data were expressed as the median [interquartile range, 25-75% (IQR)] for non-normal distribution. As all of the MDA data fitted non-normal distribution, the Kruskal Wallis test was used for overall comparison of the groups. Comparisons between groups were performed using the Mann-Whitney U test with the Bonferroni correction. Regarding the Bonferroni correction, α=0.05/10 = 0.005 was established to have statistical significance.

3. RESULTS

All biochemical findings were presented in Table 1. Results indicated that 5-FU administration elevated MDA, TOS, OSI and 8-OHdG levels compared to control group (all p=.004). However, the levels of oxidative stress parameters in 5-FU+NAC (20 mg/kg) group were lower compared to 5-FU group (all p=.004).

The TAS, SOD and GSH levels of 5-FU group were lower than control group (all p=.004). In 5-FU+NAC (10 mg/kg) and 5-FU+NAC (20 mg/kg) groups, SOD and GSH levels were significantly higher than 5-FU group (all p=.004).

The IL-6, TNF-α and caspase-3 levels of 5-FU group were higher than control group (all p=.004). In 5-FU+NAC (10 mg/kg) group, only TNF-α levels was lower than 5-FU group (p=.004). The levels of all these parameters in 5-FU+NAC (20 mg/kg) group were lower compared to 5-FU group significantly (p=.004). Also, there was no difference between control group and *per se* (only 20 mg/kg NAC) group in terms of ovarian biochemical parameters (p>.005).

Histopathological features of ovarian tissues obtained from all groups were shown in Figure 1 and quantified in Table 2. Administration of 5-FU significantly increased vascular congestion and edema in the ovarian tissue compared with control group (p=.003 and p=.004, respectively). However, NAC (20 mg/kg) treatment alleviated the levels of vascular congestion significantly compared with 5-FU group (p=.003).

Also, there was no difference between control group and *per se* (only 20 mg/kg NAC) group in terms of ovarian histological findings (p>.005).

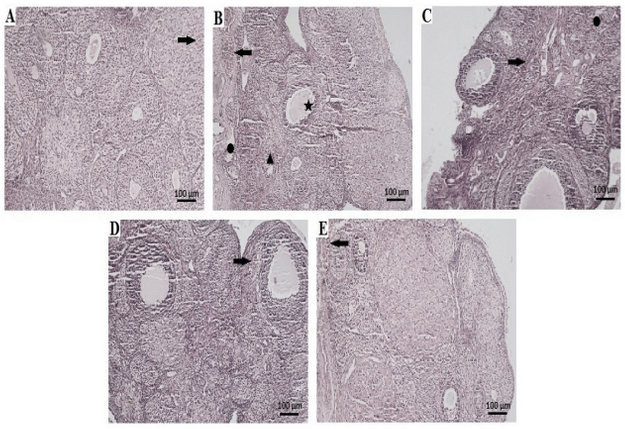

Figure 1. *Histopathological examination of rat ovarian tissues stained with H&E (×100).* ***(A) Control group;*** *black arrow: vascular congestion.* ***(B) 5-FU group;*** *black arrow: vascular congestion, filled circle: edema, filled triangle: follicular degeneration, black star: leukocyte infiltration.* ***(C) 5-FU+10 mg/kg NAC group;*** *black arrow: vascular congestion, filled circle: edema.* ***(D) 5-FU+20 mg/kg NAC group;*** *black arrow: vascular congestion.* ***(E) 20 mg/kg NAC (per se) group;*** *black arrow: vascular congestion.*

4. DISCUSSION

Although chemotherapeutics are indispensable drugs in cancer treatment, their low selectivity is a problem waiting to be solved (14). It has been suggested that increased ROS, inflammation and apoptosis, and suppression of antioxidant system are main mechanisms in 5-FU-induced tissue damage (6,15). ROS formation induced by 5-FU increases lipid peroxidation (15). It is known that increased ROS causes protein and DNA damage, especially lipids in cells (22-24). MDA is considered the most important indicator of lipid peroxidation level (25). The higher MDA levels obtained in 5-FU group indicate that 5-FU induces lipid peroxidation in this study. NAC applications alleviated MDA formation dose-dependent manner. Consistently, it was previously reported that NAC could exert tissue protective activity by inhibiting holoxan, cisplatin, doxorubicin and cyclophosphamide-induced lipid peroxidation (4,10,26,27).

TOS and TAS measurement is a useful, fast and simple method to evaluate the complex oxidative mechanism of a pathology (28,29). Reduced GSH, a low molecular weight tripeptide, is an antioxidant molecule. It is known that increased GSH oxidation increases susceptibility to lipid peroxidation (27). The fact that 5-FU administration caused higher OSI levels

Table 1. *Effects of NAC on biochemical parameters of ovarian tissues rats*

	Control	5-FU	5-FU+NAC (10 mg/kg)	5-FU+NAC (20 mg/kg)	NAC (20 mg/kg)
TOS (µM H_2O_2 equivalent/L)	11.9 (10.8-12.6)	34.8 (24.0-47.6)[a]	18.1 (15.9-23.0)	12.2 (11.5-12.7)[b]	12.5 (12.2-13.3)
TAS (mM trolox equivalent/L)	0.55 (0.51-0.57)	0.30 (0.22-0.32)[a]	0.33 (0.19-0.52)	0.59 (0.35-0.66)	0.51 (0.39-0.54)
OSI (arbitrary unit)	2.24 (2.14-2.31)	12.1 (10.3-14.2)[a]	5.99 (4.28-8.61)[a]	2.18 (1.87-3.36)[b]	2.44 (2.35-3.34)
MDA (nmol/mg protein)	16.9 (15.7-18.1)	46.3 (44.8-48.5)[a]	28.8 (19.9-48.7)	16.2 (12.5-22.5)[b]	18.4 (11.3-20.5)
8-OHdG (ng/mg protein)	3.92 (3.63-4.88)	8.47 (7.49-9.64)[a]	6.77 (3.52-9.02)	5.17 (4.27-5.87)[b]	4.57 (3.04-5.36)
SOD (ng/mg protein)	3.57 (2.44-3.76)	0.99 (0.94-1.33)[a]	2.17 (1.90-2.76)[b]	3.10 (2.30-4.43)[b]	2.87 (1.69-4.61)
GSH (µg/mg protein)	12.5 (9.55-15.18)	5.45 (5.1-6.33)[a]	8.3 (7.55-9.28)[b]	11.3 (9.95-14.55]b	12.1 (9.23-16.4)
IL-6 (pg/mg protein)	1166.3 (1029.7-1418.6)	3192.7 (2750.3-3515.9)[a]	2076.0 (1568.9-2646.4)	1343.4 (708.2-1821.1)[b]	1052.9 (748.2-1431.0)
TNF-α (pg/mg protein)	142.9 (129.1-161.5)	515.3 (476.4-569.4)[a]	257.2 (163.8-355.8)[b]	170.8 (143.8-182.5)[b]	141.6 (100.1-164.3)
Caspase 3 (ng/mg protein)	2.84 (2.43-3.41)	7.45 (6.34-9.82)[a]	5.37 (4.34-7.02)	3.77 (2.84-4.90)[b]	2.44 (1.68-3.29)

5-FU: 5-fluorouracil, NAC: N-acetylcysteine, TOS: total oxidant status, TAS: total antioxidant status, OSI: oxidative stress index, MDA: malondialdehyde, SOD: superoxide dismutase, GSH: glutathione, 8-OHdG: 8-hydroxy-2'-deoxyguanosine, IL-6: interleukin-6, TNF-α: tumour necrosis factor-alpha.

P-values according to Kruskal-Wallis variance analysis and followed the Mann-Whitney U test with the Bonferroni correction.

Data were expressed as medians with a 25th and 75th percentile interquartile range (IQR).

[a]p<.005 compared with control group, [b]p<.005 compared with 5-FU group.

Table 2. *Histopathological findings of experimental groups*

	Control	5-FU	5-FU+NAC (10 mg/kg)	5-FU+NAC (20 mg/kg)	NAC (20 mg/kg)
Vascular congestion	1 (0-1)	2.5 (2-3)[a]	1 (1-2)	1 (0-1)[b]	1 (0-1.25)
Edema	0 (0-0.25)	2 (1-2)[a]	1 (0.75-1)	0 (0-1)	0 (0-1)
Hemorrhage	0 (0-0)	1 (0-1)	0 (0-0.25)	0 (0-0)	0 (0-0.25)
Follicular degeneration	0 (0-0)	1 (0-1)	0 (0-1)	0 (0-0.25)	0 (0-0.25)
Leukocyte infiltration	0 (0-0)	1 (0-1)	0 (0-1)	0 (0-0.25)	0 (0-0.25)

5-FU: 5-Fluorouracil, NAC: N-acetylcysteine.

P-values according to Kruskal-Wallis variance analysis and followed the Mann-Whitney U test with the Bonferroni correction.

Data were expressed as medians with a 25th and 75th percentile interquartile range (IQR).

[a]p<.005 compared with control group, [b]p<.005 compared with 5-FU group.

and lower GSH levels showed that oxidative stress mediated ovotoxicity. NAC treatment attenuated ovarian injury by modulating oxidative stress. Consistently, it was previously reported that NAC could exert tissue protective activity by modulating methotrexate, cisplatin and doxorubicin-induced oxidative stress (11,30-34).

DNA is one of the most important targets of ROS and shows increased 8-OHdG DNA damage. 8-OHdG levels in ovarian tissues were therefore measured to determine the level of free radical-mediated DNA damage in this study (20). Elevated 8-OHdG levels in the 5-FU group indicated that DNA damage mediates ovotoxicity. Especially, NAC (20 mg/kg) application to 5-FU administered rats restored these changes. Similar with our results, it has been reported that NAC can exert a renoprotective effect against cisplatin-induced damage by inhibiting DNA damage (35).

SOD is one of the most important enzymes protecting the cell against ROS attacks (25), and 5-FU is known to increase tissue damage by suppressing SOD levels (6). Our results showed that systematic 5-FU administration suppressed SOD expression, while NAC treatments abolished this inhibition in dose-dependently. Consistently, it was previously reported that NAC could exert tissue protective effect against chemotherapeutic-induced tissue injury via modulating antioxidant enzymes (26,32,34).

Inflammation has been suggested as a second mechanism in 5-FU-induced toxicity (7). TNF-α and IL-6 are considered two of the main inflammatory cytokines (31). The elevated levels of these markers in 5-FU-treated rats indicates that 5-FU toxicity is mediated by inflammation. NAC applications to 5-FU administered rats restored these changes with its previously demonstrated anti-inflammatory activity (8,11,20). Consistently, it was previously reported that NAC could exert tissue protective effect against chemotherapeutic-induced injury via modulating inflammation (31,36).

Apoptosis is proceed by the activation of caspases, which are cysteine-dependent aspartate specific protease (21). Increased oxidative stress and inflammation levels can trigger apoptosis through caspase-3 activation (14). Therefore, it has been reported that compounds with anti-apoptotic properties may be useful agents in the prevention of chemotherapeutic-induced toxicity (21). The elevated caspase-3 levels in 5-FU-treated rats indicated that 5-FU toxicity is mediated by apoptosis. NAC (20 mg/kg) applications to 5-FU administered rats significantly restored these changes. These results were consistent with previous reports (35,37,38).

Histological evaluation results showed signs of increased vascular congestion and edema in the ovarian tissue of rats treated with 5-FU. Treatment with NAC (20 mg/kg) reversed 5-FU-induced ovarian injury. Similar with our findings, it is reported that NAC exhibits ovoprotective effect against ischemia/reperfusion (I/R) injury. In the same study, histological examination showed that NAC treatment improves edema, vascular congestion, hemorrhage and follicular degeneration findings caused by I/R damage (20).

Our study has a few limitations. First, the therepeutic effect of two concentrations of NAC were evaluated in this study. It was determined that 20 mg/kg dose of NAC could restore biochemical and histological changes better than 10 mg/kg dose of NAC against 5-FU induced ovarian damage. Therefore, future studies should evaluate the ovoprotective effect of NAC at doses above 20 mg/kg. Second, the molecular mechanisms involved in 5-FU-induced ovotoxicity or the ovoprotective effects of NAC in rats have not been investigated in detail. Therefore, further studies are needed to explain in detail how NAC improves 5-FU-related ovotoxicity. Third, the outcome of 5-FU and NAC administration in rats may not reflect the overall effects of such therapy in patients.

5. CONCLUSION

This study showed that NAC (especially the dose of 20 mg/kg) may have therapeutic effects against 5-FU-induced ovarian damage. This effect is thought to be mediated, at least in part, by antioxidant and anti-inflammatory activities of NAC. However, the use of NAC against 5-FU-induced ovotoxicity needs to be supported by more extensive *in vivo* and clinical studies.

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Clinical and Experimental Health Sciences

Percutaneous Cholecystostomy is a Feasible and Safe Option for High-Risk Acute Cholecystitis Patients.

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ABSTRACT

Objective: The best option for treating high-risk patients with acute cholecystitis is still being determined. We evaluated our high-risk acute cholecystitis patients in whom we preferred percutaneous cholecystostomy and aimed to determine whether this approach was safe and feasible.

Methods: We retrospectively reviewed our 71 patients in whom we performed percutaneous cholecystostomy between May 2019 and July 2023. All procedures were performed with local anaesthesia under ultrasonographic guidance via the transhepatic route. The catheters were removed when the signs of acute cholecystitis were significantly regressed or when the catheters were found to be ineffective. The catheters of those eligible for surgery were removed during the operations.

Results: All procedures were successful without failure, and no significant complications developed in the post-intervention period. Pain at the catheter insertion site (20 patients) was the leading minor complication, and in one patient, bilioma was detected and percutaneously drained. The procedure was ineffective in 7 patients (9.85%), and the catheters were removed. In 22 patients (31%), interval cholecystectomy surgery was performed. In the subgroup of patients whose catheters were adequate and were not operated (5 patients), the catheters were removed after an average of 27.2 days. The mean length of stay was 9.6 days, and four patients died (5.6%) during the index hospitalization.

Conclusion: Our findings suggest that percutaneous cholecystostomy is a feasible, safe and highly effective treatment option for acute cholecystitis in high-risk patients.

Keywords: Acute cholecystitis; percutaneous cholecystostomy; patient outcomes.

1. INTRODUCTION

In the population, the estimated prevalence of gallstones is 10–15%, and at least one-fourth of patients with gallstones will develop complications. Acute calculus cholecystitis is the first clinical presentation in 10–15% of the cases (1). Cholecystectomy is the most common therapeutic approach, and the 2020 World Society of Emergency Surgery (WSES) updated its guideline and recommends laparoscopic cholecystectomy “as soon as possible, within seven days from hospital admission and ten days from the onset of symptoms” (2). However, considering the wide range of clinical scenarios, laparoscopic cholecystectomy can not be considered a ‘one fit all’ option. In high-risk patients, deciding the best option for the treatment is still complicated due to advanced-stage malignancies, severe comorbidities, and poor general conditions. For this subgroup of patients, Tokyo 2018 guidelines recommend considering percutaneous cholecystostomy as an alternative to emergent cholecystectomy (3).

We evaluated the electronic medical records of 71 high-risk patients diagnosed with acute cholecystitis between May 2019 and July 2023 in whom we preferred percutaneous cholecystostomy. We aimed to determine whether percutaneous cholecystostomy is a safe and feasible option to be preferred in this patient group by evaluating the procedure’s success rate, complications, efficacy and impact of this intervention on patient outcomes.

2. METHODS

The study was conducted following the Declaration of Helsinki (as revised in 2013) and was approved by Kartal Dr. Lütfi Kırdar City Hospital Cinical Trials Review Board and Ethics Committee (2022/5l4/238/8 – 29.11.2022).

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Percutaneous cholecystostomy was performed in 71 patients diagnosed with acute cholecystitis with clinical, laboratory or radiological findings between May 2019 and July 2023. The main reason for preferring percutaneous cholecystostomy in this patient group was the high risk of surgery due to comorbidities and age. Besides comorbidities and age, percutaneous cholecystostomy was preferred in patients whose primary or metastatic malignant diseases were localized in the periampullary region, causing obstruction and cholecystitis. However, surgery was not possible because of the extent of the disease.

The same interventional radiologist performed all percutaneous cholecystostomy procedures. The procedures were performed under local anaesthesia and ultrasonography guidance using the transhepatic and Seldinger techniques. The same interventional radiologist performed post-procedure follow-ups of the patients.

The catheters were removed when the signs of acute cholecystitis were significantly regressed or when the catheters were found to be ineffective. The catheters of those who became eligible for surgery were also removed during the operation.

3. RESULTS

In our percutaneous cholecystostomy cohort, 31 patients were male, 40 were female, and the mean age was 70.8 (40–99). Pre-procedure mean blood pressure of the patients was 115/75 mmHg, heart rate was 85/min, and body temperature was 36.9 ^{0}C. Fourty-four patients had significant abdominal pain and had Murphy positivity in their clinical examinations. Laboratory and ultrasonographic findings diagnosed 27 patients without significant abdominal pain. Forty patients (60.6%) had obstructive jaundice. The mean total bilirubin level was 9.58 mg/dl (0.3-22 mg/dl), direct bilirubin was 6.57 mg/dl (0.1-20.6 mg/dl), and white blood cell count was 11.159 /microliter (3-36 /microliter). Gallbladder wall thickness was normal in 19 patients (26.8%).

In thirty-four patients (48%), cholecystitis developed due to gallbladder stones. Mechanical compression of a primary periampullary tumour or metastases of a different primary in this localization was the aetiology of acute cholecystitis in 34 patients (48%). Gallbladder stones were not detected in 30 of these 34 patients. Three patients (4%) were diagnosed with acalculous cholecystitis, and all those patients were significantly symptomatic and had hydropic gallbladders (transverse diameter > 5 cm) that necessitated palliation.

Problems related to the cardiovascular system were the leading comorbidity in our cohort, 29 patients had coronary artery disease, and 26 patients had high blood pressure. Chronic renal failure was present in 6 patients.

All percutaneous cholecystostomy procedures were uneventful, and no significant complications developed in the early post-procedure period. Among the minor complications, the most common was pain at the catheter insertion site, defined by 20 patients (28.2%). Bilioma was detected in one patient and drained. Five patients were followed up in the intensive care unit, and others were in the regular ward after the procedure. Bile samples were sent from all patients after the procedure, but only 18 patients had bile culture positivity. *Escherichia coli* was the predominant microorganism reported in 9 patients, and *Enterococcus faecium* was reported in four patients.

Despite revisions throughout the follow-ups, the procedure was ineffective in 7 patients (9.85%), and the catheters were removed.

In 22 patients (31%), interval cholecystectomy was performed alone or as a part of a more complicated operation like radical pancreaticoduodenectomy. The timing of those operations varied a lot from one day to seven months after the cholecystostomy procedures.

There was a subgroup of patients whose catheters were effective and were not operated. The number of patients in that subgroup was 5 (7%), and catheters of those five patients were removed after an average of 27.2 days (20-32 days).

The mean length of stay was 9.6 days, and four patients died (5.6%) during that index hospitalization. One patient died due to COVID-19 infection.

4. DISCUSSION

Emergency laparoscopic cholecystectomy and percutaneous cholecystostomy are the leading options for treating high-risk acute cholecystitis patients. Unfortunately, available data is limited; only a few studies compare the two treatment modalities and only one randomized clinical trial (4). A recent systematic review and meta-analysis stated that most studies have low-quality evidence (5). Meta-analysis notified that percutaneous treatment is inferior in treating acute cholecystitis in high-risk patients due to the higher incidence of complications than cholecystectomy. However, this inferiority is most likely related to an association between various patient-side factors and conditions and the severity of acute cholecystitis. We still need high-quality data and more concrete evidence to decide the preferred option for high-risk patients.

We retrospectively analyzed our 71 high-risk patients in whom we performed percutaneous cholecystostomy to palliate the emergent issue and improve the general condition. Our primary motivation for preferring percutaneous cholecystostomy for those critically ill patients with severe comorbidities was their increased postoperative mortality risk when undergoing a surgical procedure. This risk might reach up to 20%, according to our previous experiences and published series (6). In our study, we found that four patients died (5.6%) during that index hospitalization period, and one of those patients died not due to the underlying acute cholecystitis or our intervention but due to COVID-19 infection. Thus we can claim that percutaneous

cholecystostomy has a lower mortality risk than emergency surgery for this fragile group.

All our procedures were successful (100%) and only had minor procedural complications like pain at the catheter insertion site (28.2%) and bilioma. Indeed this high success rate with low procedural complications is familiar to us, and many authors published relatively same results (6).

One of the technique's most critical problems is drainage's ineffectiveness. In this subgroup of patients, adequate drainage cannot be achieved despite revision attempts, the procedure is considered ineffective, and the catheter is removed. In our study, we failed in 7 patients (9.85%), and despite the revision attempts, the procedure was considered ineffective, and the catheters were removed. Similar results were noted in different centres, and the authors reported that tube-related problems and tube dysfunction were more troubling than the complications of the procedure itself (7). Catheter dislodgement has also been frequently documented following placement by others (8.9), but we did not find any dislodgement in our cohort. All in all, our findings also support the idea that rates of procedural complications were low compared to dysfunction.

A recently published retrospective cohort study evaluated 132 patients who underwent percutaneous cholecystostomy for acute cholecystitis. No significant variations were detected in the intraoperative and perioperative outcomes between patients undergoing interval cholecystectomy within versus after eight weeks from percutaneous cholecystostomy placement. They found that patients may benefit from undergoing interval cholecystectomy after the 8-week cut-off after percutaneous cholecystostomy. However, they also showed that very long periods between percutaneous cholecystostomy and interval cholecystectomy procedures may increase the risk of more extended ICU stays (10). In our study, 22 patients (31%) had interval cholecystectomy. Cholecystectomy was performed either as a part of a more complex operation, like radical pancreaticoduodenectomy or alone. The timing of those operations also varied a lot, compatible with the published series. We found that surgeries had been scheduled the next day of the procedure or seven months after. On the flip side, we must be aware of the fact that an essential part of our study includes patients treated during the COVID-19 pandemic era and of course, it is tough, if not impossible, to express numerically the effect of the pandemic on the timing and delay of cholecystectomy operations. However, when we evaluated the details of the patient's electronic records, the impact of the pandemic was felt in patients who had late surgery. Nevertheless, when we consider the outcome data of our patients, we should be reluctant to early surgery.

We have certain limitations. Notably, it is a retrospective and non-randomized study evaluating a heterogenous and critically ill patient population. Since a part of the study was carried out during the pandemic, the different dynamics of this period may have impacted both the durations, like the timing of the surgeries and the results.

5. CONCLUSION

Our findings suggest that percutaneous cholecystostomy is a feasible, safe, highly effective and cost effective treatment option for acute cholecystitis in high-risk patients. We need prospective randomized studies to determine its superiority to emergency cholecystectomy in this fragile population. We also need high-quality data to decide the best timing of interval cholecystectomy after percutaneous cholecystostomy and have room for improvement for the tube dysfunction problems.

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Clinical and Experimental Health Sciences

Comparison of the Healthy Lifestyle Behaviors of Resident Russian Women and Their Children in Antalya with Their Turkish Peers: A Descriptive Study

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ABSTRACT

Objective: The migration movement from Russia to Antalya is predominantly women and children. The aim of this research was to compare the healthy lifestyle behaviors of Russian women residing in Antalya and their primary school children with their Turkish peers.

Methods: This is descriptive comparative research. The study with a purposive sample method included students in grades 1-8 of one private Russian primary school and their mothers, as well as students from three private Turkish schools in the same region and their mothers to ensure similarity. 122 Turkish and 76 Russian mothers and child couples participated in the study. Russian women and Turkish peers completed Healthy Lifestyle Behaviors Scale and the International Physical Activity Questionnaire. The Nutritional Behavior Scale and the Child Physical Activity Questionnaire (CPAQ) were fulfilled by children.

Results: Russian women were better healthy lifestyle behaviors than Turkish peers in health responsibility (z=-3,91; p<.05), physical activity (z=-3.13; p<.05), nutrition (z=-6.36, p<.05), and interpersonal relations (z=-2.98, p<.05). Russian children consume more healthy food than their Turkish peers (z=-3.53, p<.05). There was no statistically significant difference between the mean CPAQ total score of Turkish and Russian children (z=-1.32, p>.05).

Conclusion: Russian women had higher indicators of a healthy lifestyle and physical activity level than Turkish women. Russian children were consuming more healthy foods than Turkish peers. Also, there was a positive relationship between the health behavior of children and their mothers. The effect of cultural background on the lifestyle behaviors of women and children should be examined in more detail.
Keywords: Women, child, healthy lifestyle, cross-cultural comparison, immigrant

1. INTRODUCTION

Due to its migration mobility, Turkey is the country where immigrants and refugees come both for settlement purposes and for transit to Europe (1-3). Among the foreigners residing in Antalya, Russians rank first with 16,724 people (4). It has also been pointed out that the transnational movement from Russia to Antalya is the migration of women and young people (4-7). Due to the high level of education, Russian women living in Antalya belong to the middle and high income groups. This differentiates them from the immigrant profile known to be disadvantaged.

Healthy lifestyles are collective patterns of health-related behaviour based on choices from options available to people (8). The four most common healthy lifestyle choices are not using alcohol use, not smoking, healthy nutrition, and exercise. Rest and relaxation, preventive health check-ups, and similar health-related activities also constitute healthy lifestyle choices. The higher the social class, the greater the range of lifestyle choices and the probability of realizing them; conversely, the lower the class, the more limited the choices and the lower the probabilities for realization. Age, gender, race/ethnicity, culture, families, friends, and communities also affect lifestyle choices (9). Children's activities of daily living, eating habits and obesity were found to be associated with parents' behaviors (10-12). It is supported by research that there are similarities and differences between Turkish and Russian cultures, although there are relative similarities between the two cultures, it has been determined that there are significant differences in daily life and eating habits (5, 6). According to, a social structure that nurtures and reinforces healthy lifestyles for the individual has not been established in Russia (13). Therefore, negative health lifestyles have become the norm for many people. Some reports documented this lifestyle as heavy smoking, low physical activity, and obesity. Among the causes of premature death in Russia, inactivity is the seventh factor of premature death causes. It is calculated

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that the level of physical activity in Russia decreased by 18%, and this data is estimated to be 32% in 2030 (14). In 2012, the Russian Ministry of Health published the recommendations of the World Health Organization (WHO) "to provide medical assistance to adults to optimize physical activity" (15). Despite this, the physical activity level of the population in Russia remains low. Other indicators of unhealthy lifestyle are obesity and smoking. According to WHO data, 59.8% of Russia's adult population (over 20 years old) are overweight and 26.5% are obese, and 11% of women in Russia smoke (16).

When the lifestyle behaviours of Turkish women were examined in various studies, it was determined that the lack of physical activity was the weakest lifestyle behaviour. Obesity and smoking are other unhealthy lifestyle problems in Turkish women. According to the Turkey Health Survey, in 2019 was reported 24.8% of women were obese and 30.4% were pre obese. In Turkey, the percentage of women who smoke every day increased from 13.3% in 2016 to 14.9 % in 2019 (17). According to these data, the rate of obesity and smoking is higher among Turkish women than adult Russian women.

Parents' lifestyle, behaviour, culture, nutrition, activity, problem-solving approach and similar activities affect their children (18). Studies have revealed that children's lifestyle behaviours are greatly influenced by their mothers; for example, mothers of obese children have been found to have higher body mass index (11, 12, 19). Parents play a vital role in preventing childhood obesity as they have a great influence on many of their children's behaviours such as diet, physical activity and sleep. Since children's behaviour is shaped through observation and adaptation, it is important for parents to lead and reinforce a healthy lifestyle (20).

Nurses in countries like Turkey that constantly accept immigrants are increasingly providing care to incoming individuals with different cultural backgrounds. For this reason, it is important to know the healthy lifestyle behaviours of immigrants from different cultures (21). In order to provide culturally adequate nursing care to individuals with different cultural backgrounds, nurses should be able to identify and understand the cultural similarities and differences of the individuals they care for, and then integrate the values and preferences of care recipients into their nursing care (22). The aim of this research is to compare the healthy lifestyle behaviour of Russian women residing in Antalya and their primary school aged children with their Turkish peers. The questions we seek answers to in this research;

1. Is there a difference between the healthy lifestyle behaviours of Russian and Turkish women living in Antalya?

2. Is there a difference between Russian and Turkish women in terms of physical activity level?

3. Is there a difference between the nutritional behaviours, physical activity levels, and obesity frequency of Russian and Turkish children?

4. Is there a relationship between the health behaviours of children and their mothers?

2. METHODS

2.1. Study Design and Sample

This descriptive comparative study was conducted in Antalya, Turkey in the spring semester of 2020-2021 academic year in four primary schools, one of which is a Russian school. The population of the research consists of children and their mothers enrolled in grades 1-8 in a Russian Primary School affiliated to the Directorate of National Education and three Turkish Primary Schools in Antalya. A purposive sample was used in this study. Those who accepted to participate in the study in a Russian and three Turkish schools were included. In the definition of Russian and Turkish women's nationality, it was taken into account that she had spent half of her life in the country where she was born. Forty out of the 116 Russian students who were eligible for the study, were not reached during the data collection phase due to the COVID-19 pandemic. So, in the Russian study group 65.5% of the population was reached. For comparison, it was aimed to take at least one time the number of Turkish students, and 1.6 times the number was reached with 122 women and child pair participants. The study was completed with 122 Turkish and 76 Russian mother-and-child couples.

2.2. Data Collection

At the time of data collection of the study, the data of both women and children were collected through an online survey, since distance education was started due to the COVID-19 Pandemic. Children completed the questionnaires under the supervision of their mothers. Questionnaires in Russian for Russian women and questionnaires in Turkish for Turkish women were created using the Google Drive program. Since the children knew Turkish, questionnaires in Turkish were used in both groups.

2.2.1. Data Collection Tools for Women

Women's age, education, employment status, height, weight and harmful habits were measured by self-report. Body Mass Index (BMI) calculated by researcher.

2.2.2. Healthy Lifestyle Behaviours Scale (HLBS) was developed in 1987 by Walker et al. Turkish **(HLBS-TR)** validity and reliability study of HLBS was performed (23). Its Russian adaptation **(HLBS-RU)** was made in 2018 by Petrash et al. The content, scoring and sub-dimensions of HLBS-TR and HLBS-RU are the same as the original scale. The scale consists of 52 items in total and has 6 sub-items, these are; health responsibility, physical activity, nutrition, spiritual development, interpersonal relationships and stress management. All items of the scale are positive. The rating is a four-point Likert scale; never = 1, sometimes = 2, often = 3, regularly =4. The lowest score for the whole scale is 52, and the highest score is 208. The increase in the score indicates healthy lifestyle behavior. Alpha coefficient reliability value of HLBS-TR is .94 and it varies between .79-.87 in sub-dimensions (23). Alpha coefficient reliability of HLBS-RU is

.94 and it varies between.79-.87 in sub-dimensions (24). In this study, the Cronbach's Alpha value of HLBS was found to be .97 for Turkish women and .96 for Russian women.

2.2.3. International Physical Activity Questionnaire (IPAQ) was developed in 2003 by Craig et al. IPAQ was subjected to a reliability and validity study carried out in 14 centres in 12 countries during the year 2000, and it was demonstrated that IPAQ have acceptable measurement properties for monitoring population levels of physical activity among 18 – to 65-year-old adults in diverse settings (25). The Turkish validity and reliability study was carried out in 2010 (26), and the Russian validity and reliability study was carried out in 2014 (27). The IPAQ short form consists of 7 questions and includes all kinds of physical activity (at work, travel time (walking or cycling), time spent for homework or in the garden, spare time activities / sports). In the evaluation, it is checked that all activities are performed for at least 10 minutes each. Physical activity levels; are classified as physically inactive (<600 MET-min/week), low physical activity level (600-3000 MET-min/week), and adequate physical activity level (>3000MET-min/week). A MET is 1 MET of body energy, which is equal to about 3.5 ml of oxygen consumption. The more the body works during the activity, the higher the MET value. When making calculations for IPAQ Short-Form, minutes, days and MET values (multiples of resting oxygen consumption) are multiplied and a score is obtained as MET-minute / week IP. The criterion validity for the Turkish short form r =.69 was reported (26).

2.2.4. Data Collection Tools for Children

Children's age, gender, height, weight and harmful habits were measured by self-report. BMI calculated by researcher.

Table 1. *Distribution of descriptive characteristics of Turkish and Russian women and their children*

Descriptive characteristics			Turkish (n=122)	Russian (n=76)	p
WOMEN'S descriptive features	Country of birth (n, %)		122 (%61.6)	76 (%38.4)	
	Age (Mean±SD)		41.25 ± 5.7	39.61 ± 5.6	.04[a*]
	Education (n, %) **	Primary school	1 (%50.0)	1 (%50.0)	.86[b]
		Secondary school	6 (%42.9)	8 (%57.1)	
		High school	19 (%70.3)	8 (%29.7)	
		University and above	96 (%61.9)	59 (%38.1)	
	Employment (n, %)	Working	90 (%72.0)	35 (%28.0)	.001[b*]
		Not working	32 (%43.9)	41 (%56.1)	
	Smoking (n, %)	Yes	41 (%70.6)	17 (%29.4)	.09[b]
		Now	81 (%57.8)	59 (%42.2)	
	BMI (n, %) ***	Underweight		3 (%100.0)	.006[b*]
		Normal	82 (%56.9)	62 (%43.1)	
		Overweight	37 (%88)	5 (%12)	
		Obese	3 (%33.3)	6 (%66.7)	
CHILDREN'S descriptive features	Country of birth (n, %)		137 (%69.2)	61 (%30.8)	
	Age (Mean±SD)		10.46 ± 2.86	10,99 ± 2.56	.34[a]
	Gender (n, %)	Male	68 (%65.3)	36 (%34.7)	.25[b]
		Female	54 (%57.4)	40 (%42.6)	
	Family type (n, %)	Nuclear family	107 (%66.4)	54 (%33.6)	.01[b*]
		Extended family	4 (%36.4)	7 (%63.6)	
		Broken family	11 (%42.4)	15 (%57.6)	
	Bad habits (n, %)	Yes	2 (%33.4)	4 (%66.6)	.14[b]
		No	120 (%62.5)	72 (%37.5)	
	BMI (n, %)***	Underweight	9 (%64.2)	5 (%35.8)	.08[b]
		Normal	54 (%54.5)	45 (%45.5)	
		Overweight	24 (%80.0)	6 (%20.0)	
		Obese	35 (%63.6)	20 (%36.4)	

*p <.05, **The analysis was made by making high school and below and University groups, ***Analysis was performed by excluding the underweight group. SD: standart deviation, BMI: Body Mass Index, [a]Mann Whitney U test, [b]Chi-square test*

Table 2. *Evaluation of the Healthy Lifestyle Behavior Scale and Physical Activity Questionnaire of Women*

Women's HLBS	Turkish (n=122) (Mean±SD)	Russian (n=76) (Mean±SD)	p, 95% confidence interval Cohen's d		
Health responsibility	23.50±5.43	26.28±4.53	**.000**[a]*	.82 – .82	.58
Physical activity	18.84±4.87	21.22±5.28	**.002**[a]*	.86– .86	.46
Nutrition	23.30±4.83	28.22±4.88	**.000**[a]*	.85 –.86	1.0
Spiritual development	25.86±6.05	25.93±5.21	.923[a]	.89 –. 89	
Interpersonal relations	25.42±5.36	27.72±4.48	**.003**[a]*	.86 – .86	.43
Stress management	20.554±4.39	20.54±5.56	.926[a]	.82 – .82	
HLBS Total Score	137.47±26.73	149.92±25.34	**.005**[a]*	.96 –.96	.40
Women's IPAQ	10.55±3.01	11.69±2.91	**.008**[a]*	.79 –.80	.39

**p<0.05, [a]Mann Whitney U test, HLBS: Healthy Lifestyle Behaviours Scale, SD: standart deviation, IPAQ: International Physical Activity Questionnaire*

2.2.5. Nutritional Behavior Scale (NBS) measures the heart health-promoting (low-fat and low-salt) food consumption habits of children and was developed within the scope of the CATCH project, which aims to improve heart health and reduce the risks of cardiovascular disease (28). The Turkish validity and reliability of the scale was performed (29). The NBS consists of a total of 14 items to be filled in by marking which of the two comparable foods the student ate the most. It gets – 1 for unhealthy food and +1 for healthy food. The total score ranges from – 14 to +14 points. A high total score indicates healthy eating habits. NBS's Kuder-Richardson 20 internal consistency was .68 and test-retest reliability was.74. The content validity index of the Turkish version of the scale was.96, the internal consistency reliability coefficient (Kuder-Richardson 20) .68, and the Russian version was .95 (30). In our study, it was found to be .95.

2.2.6. Child Physical Activity Questionnaire (CPAQ) was developed in 1997 by Kowalski et al (31) in order to determine the physical activity level of children. The Turkish adaptation of the CPAQ was made by Erdim and Ergün (32), and the Cronbach's Alpha value was found to be .86. CPAQ contains 10 items. It examines the physical activities of the child in the last seven days and the frequency of these activities. The CPAQ separately measures physical activity in spare time, school physical education classes, breaks, lunch break, after school, evenings, weekends and spare time. In addition, the days of the week that physical activity is performed and the situation that prevents physical activity are also evaluated. Each item of the CPAQ is evaluated on a 5-point scale. Low physical activity is indicated by "1" and high physical activity is indicated by "5". In the sample of this study, the reliability value was calculated as .92 for Turkish children and .90 for Russian children.

2.3. Data Analysis

Licensed SPSS (Statistical Package for Social Science) 23.0 program of Akdeniz University was used for statistical analysis of the data. In categorical data, comparison was made with Chi-Square, and in continuous data, since the data did not show normal distribution, analysis was made with the Man Whitney U test. A value of p<.05 was accepted as the level of significance. Cohen's d was calculated to evaluate the effect size of the difference between groups (https://www.psychometrica.de/effect_size.html). The relationship between the health behavior of women and children was made with Spearman Correlation.

Table 3. *Comparison of nutritional behaviors, physical activity and BMI averages of Turkish and Russian children*

Variables	Turkish (n= 122) (Mean±SD)	Russian (n=76) (Mean±SD)	p	Confidence interval	Cohen's d
NBS	1.45±0.15	1.53±0.11	**.000**[a]*	.95 –.95	.52
CPAQ					
PA in spare time	1.67±0.63	1.67±0.44	.25[a]	.88 –.88	
PA in physical education classes at school	3.20±1.26	3.26±1.33	.67[a]	.86 –.86	
PA at school breaks	2.95±1.39	3.12±1.49	.40[a]	.81 –.81	
PA at lunch breaks	3.02±1.28	2.64±1.51	**.05**[a]*	.82 –.82	.29
PA after school)	2.72±1.11	3.03±1.18	.08[a]	.80 –.81	
PA in the evenings	2.63±0.98	2.93±1.16	.06[a]	.80 –.85	
PA on the weekend	2.60±0.94	2.89±0.92	**.05**[a]*	.80 –.82	.29
PA done in spare time	2.29±0.99	2.63±1.07	**.02**[a]*	.80 –.82	.32
PA by days of the week	2.41±0.88	2.66±1.04	.19[a]	.89 –.89	
Situation that prevents PA	1.93±2.49	1.91±0.29	.49[a]	.83 –.83	
CPAQ Total	2.09±0.57	2.19±0.54	.19[a]	.91 –.91	
BMI Values	19.7±3.9	19.6±3.5	.96[a]	.80–.82	

**p<0.05, [a]Mann Whitney U test, SD: standart deviation, NBS: Nutritional Behavior Scale, CPAQ: Child Physical Activity Questionnaire, PA: Physical Activity, BMI: Body Mass Index,*

2.4. Ethical Aspects of the Study

The study was approved by both Akdeniz University's Clinical Research Ethical Board (22.07.2020 IRB number KAEK-571) and Directorate of Antalya National Education (01.09.2020-E.11784220). Informed consent was prepared in Russian for Russian participants and in Turkish for Turkish participants. If the participant gave his consent on the first page of the electronic questionnaires, other parts of the questionnaire were opened.

Table 4. *Relationship between Mothers' and Children's Nutritional Behavior Scale and Physical Activity Questionnaire Values®*

Variables		Mothers' Physical Activity Questionnaire, Nutrition and BMI values (N=198)			
		Physical Activity	Nutrition	IPAQ	BMI
Children's NBS, CPAQ and BMI values (N= 198)	NBS	.18*	.35**	.18*	-.02
	CAPQ total score	.20**	.29**	.20**	.10
	PA in spare time	.10	.17*	.21**	.10
	PA in physical education classes at school	.10	.20**	.14	.04
	PA at school breaks	.10	.18*	.14*	-.06
	PA at lunch breaks	.12	.07	.13	.01
	PA after school	.29**	.23**	.13	-.09
	PA in the evenings	.22**	.20**	.05	-.04
	PA on the weekend	.19**	.15*	.15*	-.06
	PA done in spare time	.13	.16	.15*	.03
	PA by days of the week	.14	.30**	.82**	.06
	Situation that prevents PA	-.04	-.06	.05	.03
	BMI	.01	-.03	-.07	.19**

*®Spearman correlation analysis *p<0.05; **p<0.01, NBS: Nutritional Behavior Scale, CPAQ: Child Physical Activity Questionnaire, PA: Physical Activity, BMI: Body Mass Index,*

3. RESULTS

It was found that Turkish and Russian women have the same level of education, the unemployment rate of Russian women is higher than that of their Turkish peers, and the level of smoking and overweight among Russian women is lower than that of their Turkish peers. Russian women were younger than Turkish peers. Among Russian children, the rate of those with broken families and harmful habits is higher than their Turkish peers. The rate of overweight and obese children is lower for Russian children than their Turkish peers (Table 1).

The difference between the HLBS mean scores of Turkish and Russian women was found to be statistically significant (z=-2.79; p<.05). It was seen that Turkish women got the highest score in the spiritual development sub-dimension (25.86±6.05) and the lowest score in the physical activity sub-dimension (18.84±4.83). It was seen that Russian women got the highest score from the nutrition sub-dimension (28.22±4.88) and the lowest score from the stress management sub-dimension (20.54±5.56). When the HLBS sub-dimensions of Turkish and Russian women were compared; the mean scores of Russian women in the health responsibility (z=-3.91; p<.05), physical activity (z=-3.13; p<.05), nutrition (z=-6.36, p<.05) and interpersonal relations (z=-2.98, p<.05) sub-dimensions were found to be higher than their Turkish peers, and the difference was statistically significant (p<.05). When the IPAQ mean scores of Turkish and Russian women were compared, it was found that the mean score of Russian women (11.69±2.91) was higher than their Turkish peers (10.55±3.01) and it was statistically significant (z=-2.66), p<.05) (Table 2).

The difference between the mean NBS scores of Turkish and Russian children was statistically significant (z=-3.53, p<.05). When compared in terms of healthy and unhealthy food consumption; it was found that Russian children (1.53±0.11) consumed more healthy food than their Turkish peers (1.45±0.15) and the difference was statistically significant. Turkish children's physical activity scores during lunch break are higher than their Russian peers. Russian children's physical activity scores at weekends and during spare time were higher than their Turkish peers. There was no statistically significant difference between the mean CPAQ total score of Turkish and Russian children (z=-1.32, p>.05) (Table 3).

Directly proportional weak correlation (r = – .35 was found between the feeding behaviors of the mothers and the feeding behaviors of the children, and directly proportional very weak correlation was found between the physical activity of the mothers (IPAQ) and the physical activity of the children (CPAQ). Directly proportional and strong relationship (r =.82) was found between the physical activity of the children according to the days of the week and the physical activity of their mothers. Directly proportional very weak correlation (r =.19) was determined between the BMI value of the children and the BMI value of the mothers (Table 4).

4. DISCUSSION

This study is the first to compare the healthy lifestyle of Russian women residing in Antalya and their primary school-aged children with their Turkish peers living in the same region. In our study population, all demographics except education level are different (Table 1). About three-quarters of both Turkish and Russian women had a university degree or higher. Russian women were slightly younger than their Turkish peers, and the rate of unemployed was high. The fact that the rate of unemployed Russian women is higher than their Turkish peers may be related to the fact that Russian women who work seasonally in the tourism sector define themselves as not working. Smoking and being overweight

are more common in Turkish women than in their Russian peers. The fact that the rate of smoking and being overweight among Russian women is lower than their Turkish peers differs from the profile of immigrant women. There are more studies reporting that smoking, being overweight, and obesity are more common among immigrant women (33-35). The good level of physical activity of the Russian women in our study group may explain their lower rate of being overweight. In addition, Russian women being a little younger may be associated with being less overweight. In our study, the smoking rate of Turkish women was found to be higher than their Russian peers. In Turkey, as the education level of women increases, the rate of smoking also increases (36). The education of the majority of Turkish women in our study group is university level. This profile explains the high rate of smoking among women in our study group.

The fact that geographical boundaries are not prohibitive and people move to other countries voluntarily or through forced migration can affect lifestyle and health behavior. In studies examining the profile of those who migrated to Antalya from Russia to work and live it was reported that the rate of women with a high education level working in the tourism sector is high (6,7). The voluntary emigration of Russian immigrants in Antalya due to better climate, living, and working conditions may make their profile different from the forced immigrant profile. As a matter of fact, the findings of this study show that the healthy lifestyle behaviors of settled Russian immigrant women and children are similar to or better than their Turkish peers (Table 2). Although there are many studies showing that immigrants' health behaviors are generally more negative in studies comparing immigrants to local people (37, 38) studies showing better or similar health behaviors are limited (39). This situation can be explained by the fact that the majority of the Russian women interviewed for this study, similar to their Turkish peers, are at a university or higher education level and they are in a high socio-economic class. The healthy lifestyle behavior of Russian women was better of their Turkish peers. This situation may be related that they voluntarily migrated to Antalya, had a well economic situation, and had been in Turkey for a longer period of time might be related. In this study, it was determined that Russian women with higher education levels had better health and lifestyle behaviors in general. In a study conducted in Russia (40), high adherence to a healthy lifestyle was associated with female gender, older age, urban residence, high educational status, and absence of family. Since the socio-demographic characteristics of the Russian women in our study also showed such a profile, they may have shown better healthy lifestyle behaviors. However, in our sample poor stress management in Russian women may have resulted from the difficulty in coping with the stressors brought on by immigration. This situation may have resulted from both the cultural differences between Turkish and Russian women as well as their immigration and settled life situation. The physical activity level of Russian women was found to be higher than their Turkish peers. It has been determined that the level of healthy lifestyle behavior of internal immigrant women in Turkey is lower (41). Otherwise, immigrant women with social security have healthier behavior. In the same studies, it was determined that migrant women got the highest score from the spiritual development sub-dimension and the lowest score from the physical activity dimension. The fact that the behaviors of women in studies (41) examining the profile of internally immigrated women in Turkey are similar to those of Turkish women in this study indicates that cultural behavior patterns are strong. In studies conducted in Russia (33-35) it has been observed that the healthy lifestyle behavior of Russian women is similar to the healthy lifestyle behavior of Russian women in this study. Similarly, in previous studies examining the healthy lifestyle behavior of Turkish women (42,43) the lowest behavior was physical activity, and the higher sub dimension was spiritual development which is consistent with the health behavior profiles of Turkish women in this study. The fact that Russian women show healthier behavior patterns than their Turkish peers within the scope of the study can be associated with socio-cultural behavior patterns. For example, professional participation in sports activities from childhood is common in Russian culture. This may be reflected in behavior in adulthood.

Among Russian children, the rate of those with broken families and harmful habits is higher than their Turkish peers (Table 1). This may be related to the separation in Russian-Turkish marriages due to differences in thought in Russian and Turkish cultures as it is stated in previously reported studies (6,7). Previous studies have reported that harmful habits are common among immigrant children (39, 44, 45). In this study, unlike the literature, there was no difference in the ratio of those with harmful habits between Russian and Turkish children. The rate of overweight and obese children is lower in Russian children than their Turkish peers. This situation is thought to be related to the fact that the Russian children interviewed for this study both show healthier eating behavior and that the level of their physical activity at the weekend and during spare time is higher than their Turkish peers (Table 3). In our study population, weekend and free time physical activity scores of Russian children were found the higher than the Turkish peers. In our study population, weekend and free time physical activity scores of Russian children were found the higher than the Turkish peers. In the study, it is thought that both Russian and Turkish children are at a similar socioeconomic level as they are taken from private schools in the same region. This difference may be due to cultural behavior patterns. It has been found that Russian children consume more healthy food than their Turkish peers. Although there was no difference in the total physical activity level of Russian and Turkish children, there was a difference in terms of the time period in which the activity was performed. Russian children's weekend and spare time physical activity scores were higher than their Turkish peers, and Turkish children's lunch break physical activity scores were higher than their Russian peers. This shows that Russian children make extra effort for physical activity during non-school time. Unlike this study, in studies investigating

the nutritional behavior of immigrant children and physical activity status, it has been observed that the nutritional and physical activity behavior of immigrant children are generally low (46, 47). This situation can be associated with the fact that the immigrant Russian children interviewed for this study belong to the upper socioeconomic class. Yet another reason may be the effect of starting professional sports activities at an early age in Russian life style. In addition, the fact that there is a positive relationship between mothers and children's physical activity in our study group shows the family's influence on daily life activities.

Directly proportional weak correlation was found between the nutritional behaviors, body mass index and physical activity of women and their children, and directly proportional strong correlation was found between the physical activity of women and the physical activity of their children according to the days of the week (Table 4). In the studies conducted, it was stated that the physical activity status of the mothers, the level of their knowledge about nutrition levels, their attitude and eating habits at home especially affect the general health status, nutrition and activity habits of school-aged children (48, 49). Within the scope of this study, the strong relationship between the physical activity of women and their children on certain days of the week may be related to the fact that mothers and children do physical activity together. According to another view, this can be explained by the fact that women who do physical activity plan activities for their children.

5. CONCLUSION

It was found that Russian women have higher indicators of a healthy lifestyle and physical activity level than Turkish women. It has been determined that Russian children consume more healthy food than Turkish children. Physical activity levels of Turkish and Russian children were similar. However, there was a difference in terms of time and place of physical activity. Turkish children were doing their total activity at school. Russian children, on the other hand, were performed their physical activities on the weekend and in their free time. It has been determined that there is a directly positive relationship between the health behavior of children and their mothers. In future, the lifestyle behaviors of Turkish and Russian immigrants, who are at a lower socioeconomic level, can also be examined. Also, the factors that motivate the healthy lifestyle behaviors of Russian immigrants living in Antalya might be examined. It is recommended to focus on the areas that need improvement in the nutritional behaviors of Turkish children, to carry out studies to gain healthy eating habits, and to re-evaluate the health behaviors of immigrants in the long term.

5.1. Limitation and Strength of the Study

Our data were based on participants' self-report. Therefore, it carries the risks of research using subjective data. Since face-to-face education was suspended in schools during the COVID-19 pandemic, the data of both women and children were collected with electronic questionnaires. On the other hand, the high reliability of measurement tools and the collection of women's data in their mother tongue through questionnaires eliminate this limitation. The education level of the majority of the Russian mothers included in this study is university level and their economic level is better than the general population because their children attend a paid private school. Therefore, it should be taken into account that the study represents a Russian immigrant group with only a high socioeconomic level.

Recommendations for practitioners

It is known that the number of temporary and permanent immigrants from Russia to Turkey has increased in recent years. The factors that push and attract people to migrate can change over time. For this reason, evaluating the healthy lifestyle behaviors of immigrants from every socioeconomic level who migrated for repulsive reasons is recommended. In order to provide culturally sensitive health care, health professionals should recognize the immigrant profile in society.

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Design of the study: SG, SE
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Analysis of data for the study: SE,SG
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Clinical and Experimental Health Sciences

The Effect of Layer Thickness and Light Intensity on the Degree of Conversion, Microhardness and Cytotoxicity of Bulk Fill Composite Resins

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ABSTRACT

Objectives: The aim of this study was to evaluate the effects of polymerizing bulk fill composite resins at different thicknesses and different light intensities on the degree of conversion, microhardness and cytotoxicity of the composites.

Methods: Two different bulk fill composite resins were used in this in vitro study: Sonic Fill 2, Filtek Bulk Fill. Samples prepared from both composites with a thickness of 2, 4 and 6 mm were polymerized in 2 different power modes. The degree of conversion, microhardness and cytotoxicity of these samples were measured.

Results: As the layer thickness of the bulk fill composite resins increased, the degree of conversion of the lower surfaces of the composites decreased significantly (p <.05). When the lower and upper surface microhardness ratios of bulk fill composite resins were examined, 2 mm thick samples of both composites polymerized by both polymerization methods and 4 mm thick samples polymerized in standard power mode exceeded the acceptable threshold value of 0.80. According to the results of the WST-1 experiment; cell viability decreased as the layer thickness of bulk fill composites increased.

Conclusions: The upper surface degree of conversion and microhardness values of the bulk fill composite resins examined were higher than the lower surface values. According to the results of the WST-1 experiment; as the layer thickness of the bulk fill composites increased, the cell viability decreased, the cytotoxic properties increased.

Keywords: Cytotoxicity, filtek bulk fill, hardness, polymerization, sonic fill

1. INTRODUCTION

When developing composite resin materials, which are frequently used in restorative dentistry, the most emphasized issue is the polymerization mechanism, which significantly affects the physical and mechanical properties of the composite. The effectiveness of polymerization affects many properties of the composite such as its mechanical properties, biocompatibility, volumetric shrinkage and the tensile forces formed during this process, degree of conversion (polymerization degree) and polymerization depth (1). When polymerization is not provided sufficiently, the physical and mechanical properties of the composite resin may weaken over time. Manufacturers have produced bulk fill composites that are polymerized in a single layer of up to 4 mm, saving time for the physician and patient, to simplify and speed up the placement of resin-based composites in large layers in the posterior region (2). Bulk fill composites can be applied as a single layer of 4-6 mm thickness, especially with their increased translucency and the presence of polymerization modulators; and have low shrinkage stress and a high degree of conversion (DC) at this depth (3). The percentage of carbon-carbon double bonds (-C = C-) converted into single bonds (-C-C-) to form a polymeric resin is defined as the degree of conversion, and the degree of conversion values reported for conventional composite resins range from 52 to 75% (4). The degree of conversion is an important parameter for evaluating the optimum clinical performance of resin-based composite materials (5). Polymerization depth, degree of conversion, polymerization shrinkage, linear thermal expansion coefficient, elastic modulus, abrasion resistance, the C factor, etc. parameters such as these affect the clinical success of composite restorations.

The clinical success of the composite restoration depends on various parameters such as polymerization depth, polymerization degree, polymerization shrinkage, linear thermal expansion coefficient, elastic modulus, abrasion resistance, C factor, etc. (4) Among these parameters, the degree of conversion is directly related to physical and mechanical properties such as strength, hardness, solubility,

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color changes and biocompatibility (5). Hardness determines the mechanical properties of polymerized restorative materials. Increasing the hardness increases the resistance against scratching and abrasion and increases clinical success by preventing the material from deforming against the incoming forces. In many studies; the polymerization depth of composite resins was defined based on microhardness measurements on the upper and lower surfaces of the composite resin. It has been reported in these studies that 0.80 can be used as a critical acceptable minimum threshold (6). When composite resins are not sufficiently polymerized, their physical and mechanical properties weaken and residual monomer is released into the environment. These residual monomers can cause estrogenic, genotoxic and cytotoxic effects (7). Possible toxicological reactions are evaluated in vitro using cell cultures in cytotoxicity tests. Provides detailed information on cytotoxicity tests, cell membrane and organelles, protein and DNA synthesis, cell division, cell viability and death (8).

The degree of conversion of bulk fill composites has been shown to be comparable to conventional composites (9). However, there are no data on the extent to which a layer thickness of up to 6 mm compared to a layer thickness of 2 and 4 mm affects the degree of conversion, microhardness and cytotoxicity of bulk fill composites. The aim of this study is to examine the degree of conversion, microhardness and cytotoxicity of two different bulk fill composite resins polymerized at different layer thicknesses and different light intensities. The null hypotheses tested are:

(I) The irradiance applied does not affect the degree of conversion, microhardness and cytotoxicity of the composites.

(II) Thickness of composites does not affect their degree of conversion, microhardness and cytotoxicity.

(III) The applied irradiance and composite thickness do not cause an increase in the cytotoxicity of the composite over time.

2. METHODS

Ethical approval is not required for this study. The effect sizes were determined using the results obtained from previously published studies (9-11). G*Power 3.1 (University of Heinrich Heine, Dusseldorf, Germany) indicated that the sample size used in each test exhibited a power of 0.85 (α = .05).

2.1. Preparation of Samples

Sonic Fill 2 (Kerr Corp., Orange, CA, USA) and Filtek Bulk Fill (3M ESPE, Seefeld, Germany) bulk fill composite resins were used in this study. Detailed information on the selected materials is listed in Table 1. For the preparation of the samples, three different teflon molds of 5 mm diameter, 2, 4 and 6 mm thickness were used. Mylar strip was placed on the bottom surface of the teflon mold. Bulk fill composite resins (A2) were placed in the teflon mold. The samples were polymerized from the upper surface of the teflon mold using a light unit (Valo Ultradent, South Jordan, UT). For polymerization, the light device was used in the standard power mode (1000 mW/cm^2-20 sec) in one of the groups and in the extra power mode (3200 mW/cm^2-3 sec) in the other. A total of 60 samples were prepared from two different bulk fill resin composites, with two different irradiances and three different layer thicknesses, 5 samples for each group. While the same samples were used for microhardness and degree of conversion, new samples were prepared for cytotoxicity tests. The samples were kept dry in amber colored bottles at room temperature (25°C) for 24 hours.

Table 1. *Manufacturers and compositions of bulk fill composites used in the study*

Materials	Composition	Filler Ratio (w/v)	Manufacturer
Filtek Bulk Fill (FBF)	Bis-GMA, UDMA, Bis-EMA, ytterbium trifluoride, zircon silica	%64.5 / %42.5	3M Espe, St.Paul, USA Lot number: N899704
Sonic Fill 2 (SF)	Bis-GMA, TEGDMA, EBPADMA, glass oxide, silicon dioxide	%83.5 / %66	Kerr, Orange, CA, USA Lot number: 6599433

Bis-GMA: Bisphenol A-glycidyl methacrylate, UDMA: Urethane Dimethacrylate, Bis-EMA: Bisphenol A Ethoxylate Dimethacrylate, TEGDMA: Triethylene Glycol Dimethacrylate, EBPADMA: Ethoxylated bisphenol a dimethacrylate.

2.2. Measuring the Degree of Conversion

An ATR-FTIR spectrometer (Perkin-Elmer, Waltham, USA) was used to measure the degree of conversion of bulk fill composite resin samples kept in amber bottles. FTIR spectra ranging from 400 to 4000 cm^{-1} were documented by 32 scans at a resolution of 4 cm^{-1}. Measurements were made on the upper and lower surfaces of cured composite resin samples. The degree of conversion (Equation 1) was determined according to the following equation using changes in the absorbance density ratios of aliphatic C = C to aromatic C-C in the cured and uncured states.

$$\text{Degree of conversion}(\%) = \left(1 - \frac{\left(\frac{A_{aliphatic}}{A_{aromatic}}\right)_{cured}}{\left(\frac{A_{aliphatic}}{A_{aromatic}}\right)_{uncured}}\right) x100 \quad (1)$$

The top and bottom DC ratio (Equation 2), which shows the change in the degree of polymerization with depth, was calculated according to the formula:

$$\text{Degree of conversion ratio}\ (\%) = \frac{\text{Degree of conversion}_{(\text{bottom})}}{\text{Degree of conversion}_{(\text{top})}} \text{x}100 \quad (2)$$

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2.3. Microhardness Measurement

The hardness measurement of the samples was made after 24 hours. The notching tip of the Vickers (Micromet, Buehler, USA) device was positioned perpendicular to the surface to be measured, a 200 g load was applied for 15 s and microhardness was measured from the lower and upper surfaces of the samples. The average of three measurements made on each surface was determined as the hardness value of that surface.

2.4. Cytotoxicity Test

In the cytotoxicity test, a total of 180 samples were prepared, 5 samples in each group and different samples for the 1st, 7th and 21st days. WST-1 (water-soluble tetrazolium) analysis was applied to determine the cytotoxicity of bulk fill composite resins. The L929 mouse fibroblast cell line to be used in the study (Şap Institute, Turkey) was first stained with blue fluorescent DAPI (4′,6-diamidino-2-phenylindole) dye in terms of Mycoplasma transmission, and the result was negative. Cells were inoculated into 24-well culture dishes at $1x10^4$ cells /cm^2 and kept at 37°C and in an incubator containing 5% CO_2 to adhere to the surface overnight. The discs prepared from bulk fill composite resin materials in a sterile environment were sterilized under ultraviolet light (laminar flow sterile cabinet, Class II, Heraus, Hanau, Germany) for 20 minutes. Bulk fill composite discs were placed in the prepared experimental environment and incubated in low glucose DMEM (Dulbecco's Modified Eagle's Medium) medium containing 10% fetal bovine serum (FBS), 1% penicillin / streptomycin. L929 cells that were not treated with bulk fill composite resin materials were used as the control group. WST-1 test was performed to determine cell viability and cytotoxicity at the end of the 1st, 7th and 21st days after placing the bulk fill composite discs in the experimental environment. After the bulk fill composite resins were removed from the experimental environment, WST-1 Cell Proliferation Assay Reagent (Roche) was added at a ratio of 1:10 (30 µl WST-1 reagent to 270 µl medium). After 2 hours of incubation under appropriate conditions (37°C, in an incubator containing 5% CO_2), absorbances were read using a 450 nm wavelength monochromatic microplate reader (Microplate Reader, VersaMax, Molecular Devices, USA).

2.5. Statistical Analysis

The obtained data were statistically analyzed using the SPSS 22 (IBM Corp., Armonk, NY, USA) package program. Whether the samples were normally distributed or not was examined with the Kolmogorov-Smirnov test. According to the results obtained, Kruskal-Wallis and Mann-Whitney U tests were used for the analysis of microhardness and degree of conversion. According to the results of cell viability measurements, one-way analysis of variance and Tukey multiple comparison test were used to determine the statistical differences between groups. In addition, the Anova test was used for repeated measurements in order to examine the changes in cell viability on the 1st, 7th and 21st days. The significance level for all results was set at $p = .05$.

3. RESULTS

3.1. Results of Degree of Conversion Measurements of Bulk Fill Composites

When the layer thicknesses of Sonic Fill 2 (SF) and Filtek Bulk Fill (FBF) groups polymerized in the standard power mode were compared separately, there was no statistically significant difference among the average degree of conversion ($p>.05$), while there was a statistically significant difference among the groups polymerized in the extra power mode ($p <.05$) (Table 2).

When the effect of irradiance on each composite group and each layer thickness was examined separately, there was no statistically significant difference between the groups of FBF composite prepared only in 2 and 6 mm thickness (Table 2).

As the layer thickness increases in SF and FBF groups polymerized in standard and extra power mode, the average degree of conversion ratio decreased statistically significantly *(p<.05)* (Table 2).

Table 2. *The ratio of degree of conversion (%) of SF and FBF groups from the lower surface to the upper surface after polymerization in different light irradiance and different increment thicknesses.*

	Layer thickness	1000 mW/cm² – 20 s (Standart power)	3200 mW/cm² – 3 s (Extra power)
SF	2 mm	78.70 ± 9.31 ^{a,C}	94.40 ± 5.30 ^{a,C}
	4 mm	57.03 ± 7.84 ^{x,B}	24.49 ± 4.54 ^{x,AB}
	6 mm	27.41 ± 6.01 $^{+,A}$	16.28 ± 6.46 $^{+,A}$
FBF	2 mm	83,24 ± 7.65 ^{a,C}	88.36 ± 7.41 ^{a,C}
	4 mm	49.23 ± 8.25 ^{x,B}	33.42 ± 3.12 ^{y,B}
	6 mm	31.13 ± 9.88 $^{+,A}$	34.38 ± 6.37 $^{-,B}$

SF: Sonic Fill, FBF: Filtek Bulk Fill. Different lower case letters and symbols show the statistical difference between SF and FBF samples polymerized at the same thickness and same light irradiance according to the Paired Sample T test. Different capital letters indicate the difference between the groups in the column according to the One Way ANOVA test (p< .05).

3.2. Results of Microhardness Measurements of Bulk Fill Composites

3.2.1. Lower-Upper Surface Microhardness Ratio

When the lower-upper surface microhardness ratios were compared, the clinically acceptable threshold value of 0.80 was exceeded by the 2 and 4 mm thick samples polymerized in the standard power mode of both composites and the 2 mm thick samples polymerized in the extra power mode (Figure 1) (Table 3).

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When the lower-upper surface microhardness ratios of the 2 and 4 mm thick samples of both composites were compared, 2 mm thickness samples prepared in the extra power mode showed statistically higher microhardness ratios than the samples prepared in 4 mm thickness. There was no statistically significant difference between the samples prepared in the standard power mode (p>.05) (Table 3).

Table 3. *Microhardness ratio (%) of SF and FBF groups to the upper-lower surface after polymerization in different light irradiance and different increment thicknesses.*

	Layer thickness	1000 mW/cm^2 – 20 s (Standart power)	3200 mW/cm^2 – 3 s (Extra power)
SF	2 mm	0.90 (0.05) ^{BC}	0.84 (0.05) ^{D}
	4 mm	0.85 (0.05) ^{B}	0.60 (0.05) ^{C}
	6 mm	0.64 (0.18) ^{A}	0.14 (0.05) ^{A}
FBF	2 mm	0.90 (0.06) ^{BC}	0.85 (0.04) ^{D}
	4 mm	0.91 (0.05) ^{B}	0.53 (0.07) ^{C}
	6 mm	0.73 (0.05) ^{A}	0.49 (0.07) ^{B}

SF:Sonic Fill, FBF: Filtek Bulk Fill. Different capital letters indicate the difference between the groups in the column according to the One Way ANOVA test, (p< .05).

When the light intensities were compared, samples polymerized at 4 and 6 mm thicknesses and standard power mode from SF and FBF composite resins showed a higher microhardness ratio than samples polymerized in the extra power mode (p <.05) (Figure 1).

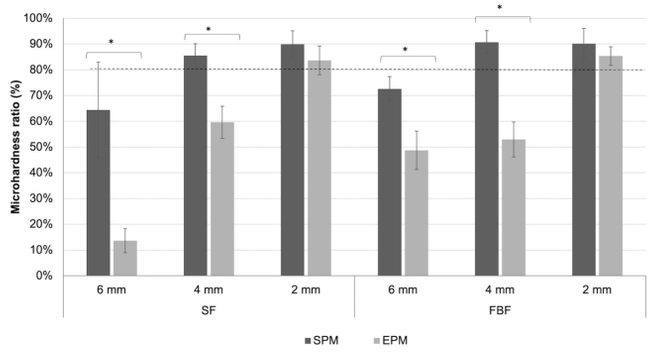

Figure 1. *Upper-lower surface average microhardness ratios (%) of SF and FBF groups. The * sign indicates that there is a statistical difference between columns belonging to the same thickness within the composite groups. The green line indicates the 80% hardness rate, which is considered to be the clinical threshold for microhardness in the literature.*

3.3. Findings of Cytotoxicity Tests of Bulk Fill Composites

The average cell numbers of the groups (composite resins tested and the control group) are shown in Figure 2. The control group in the WST-1 test referred to the group in which only cells were used instead of bulk fill composite samples. There was a statistically significant difference between the WST-1 test results on the 1st, 7th and 21st days among the samples belonging to the control group (p <.05) (Figure 2).

In the 6 mm thick samples of the SF composite polymerized in the standard power mode, the number of cells decreased statistically significantly from day 1 to day 21 (p <.05) (Figure 2). Although there were changes in cell numbers between the 1st, 7th and 21st days of the FBF composite groups, there was no statistically significant difference (p >.05) (Figure 2).

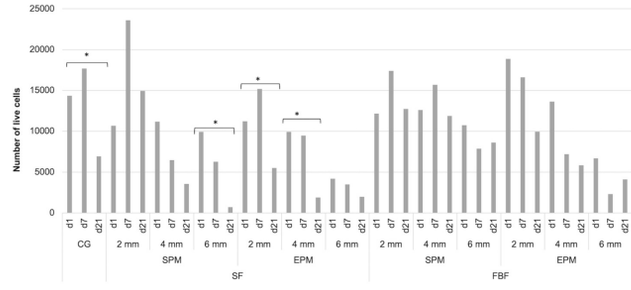

Figure 2. *Comparison of WST-1 test results on days 1, 7 and 21 of the same samples of Sonic Fill 2 and Filtek Bulk Fill composites. (* sign indicates that there is a statistical difference between the columns belonging to the same sample within the composite groups. d1: 1st day, d7: 7th day, d21: 21st day, SPM: standart power mode, EPM: extra power mode, CG: control group)*

There was no statistically significant difference in terms of cell viability at the end of the 1st day between FBF and SF samples prepared in standard power mode at all layer thicknesses (p >.05) (Figure 3). Cell viability from 2 mm to 4 mm did not decrease in samples polymerized in the extra power mode, while cell viability was reduced in samples with 6 mm thickness (Figure 3).

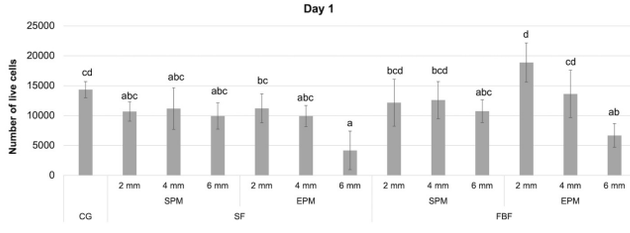

Figure 3. *Comparison of the results of the WST-1 test on day 1 of Sonic Fill 2 and Filtek Bulk Fill composite samples prepared at different light irradiances and 2,4,6 mm thickness. (Different lower case letters indicate statistical difference between columns.)*

At the end of the seventh day, cell viability decreased as the layer thickness increased in the SF and FBF groups polymerized in the extra power mode and in the FBF groups polymerized in the standard power mode (Figure 4).

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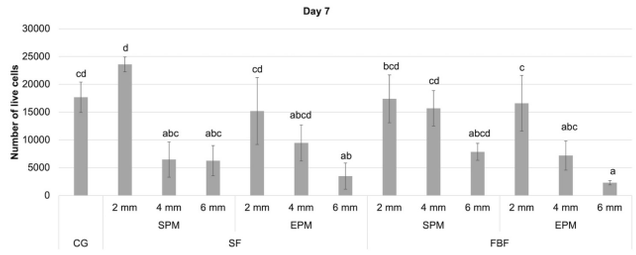

Figure 4. *Comparison of the results of the WST-1 test on day 7 of Sonic Fill 2 and Filtek Bulk Fill composite samples prepared at different light irradiance and 2,4,6 mm thickness. (Different lower case letters indicate statistical difference between columns).*

When compared with the control group at the end of the 21st day, the cell numbers in all groups were found to be similar to the control group (p>.05) (Figure 5).

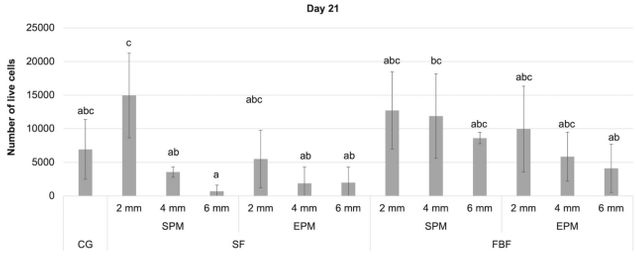

Figure 5. *Comparison of the results of the WST-1 test on the 21st day of the Sonic Fill 2 and Filtek Bulk Fill composite samples prepared at different light irradiance and 2,4,6 mm thickness. Different lowercase letters show that there is a statistical difference between the columns. (CG:Control grup, SPM: Standart power mode, EPM:Extra power mode)*

4. DISCUSSION

The improvements in the mechanical and aesthetic properties of the materials used in the posterior area have brought the expectation of ease of use. The bulk fill technique developed for this purpose and the materials applied with this technique have become very popular today (12). Although bulk fill composites have many advantages, they also have disadvantages. An increase in water absorption and water solubility and a decrease in microhardness may cause weakening of physical and mechanical properties. This may affect the clinical behavior of the restoration due to reasons such as color stability, surface roughness, restoration gloss and hydrolytic degradation (13, 14).The chemical stability, and physical and mechanical properties of composite resins that are not polymerized enough are reduced, and they also have a potentially toxic effect on pulpal tissues (15).

Few studies in the literature have evaluated the cytotoxicity of bulk fill composites. However, different parameters such as cytotoxicity test method, cell type, cell-material contact method and exposure time used in these studies make it difficult to compare the results of these studies (10, 15, 16).

There are studies in the literature showing that monomer release from composites continues in the long term (9, 17-19). The decrease in the number of cells in some groups compared to the control group on the 7th and 21st days in our study suggests that the release of cytotoxic substances from the composite resins may continue after the first 24 hours. These findings support the results in the literature.

Similar to the results of this study, the study of Şişman et al. found a decrease in the number of cells on the 7th, 14th and 21st days compared to the control group. As in our current study, they used FBF and SF bulk fill composites with a diameter of 5 mm and a thickness of 4 mm, differently, they used dental pulp stem cells (10). In the study of Şişman et al., the number of viable cells in the FBF group increased from day 1 to day 7, and decreased on day 21, but this decrease was not statistically significant. This result supports the result of our study since it yields similar results with the 4 mm thick FBF group polymerized in the standard power mode with the same diameter and thickness used in our study. In the same study, while the number of live cells increased from day 1 to day 21 in the SF group, in our study, the number of live cells decreased from day 1 to day 21 in the SF group polymerized in standard power mode, but this decrease was not statistically significant. This difference may be related to the fact that the light intensities (1200 mW / cm^2-1000 mW / cm^2) of the light devices used in both studies is different and the cells used in the WST-1 test are different.

The amount of monomer may increase if the applied light time is insufficient and the wavelength of the light used is not sufficient for the polymerization of the material or the composite is prepared in excessive thicknesses. In our study, it was determined that the number of viable cells in samples prepared with a thickness greater than the manufacturer's instructions and polymerized at high irradiance in a short time compared to the other groups. Based on these results, the parts related to cytotoxicity of the first, second and third null hypotheses were rejected.

The amount of light available to stimulate the photoinitiator is significantly reduced from the upper surface to the lower surface as a result of the absorption and scattering of light by the composite itself or the surrounding tissue (20, 21). The data we obtained in our study support this information. As the thickness of both composites increased, the degree of conversion decreased as the thickness increased, since sufficient light could not reach the lower regions to excite the photoinitiators. For this reason, the part of our second null hypotheses that the composite thickness does not affect the degree of conversion was rejected.

Jain et al. investigated the effects of polymerizing four different bulk fill composite resins at two different layer thicknesses and two different light intensities (1000 mW/cm^2-1400 mW/cm^2) on the degree of conversion immediately after polymerization and 24 hours after polymerization. When the study findings were examined, it was observed that when the irradiance increased, the degree of conversion was higher on the lower and upper surfaces in both thicknesses of

the two bulk fill composite resins (5). When compared with the data we obtained in our study, when the irradiance was increased, the rate of degree of conversion of SF composite samples prepared with 2 mm thickness increased statistically significantly. Except for the samples of FBF composite prepared in 2 and 6 mm thickness, the rate of the degree of conversion decreased significantly in all groups. For this reason, the part of the first null hypothesis related to the degree of conversion was rejected.

In addition, samples of SF composite prepared at 4 and 6 mm thickness and polymerized in extra power mode showed a lower degree of conversion rate compared to FBF samples of the same thickness. This can be explained by the lower translucency confirmed by previous studies compared to Sonic Fill's other bulk fill composites (22, 23). Low translucency affects light transmission and adversely affects the degree of conversion (24).

Comparing the microhardness values of the top and bottom surfaces of composite specimens is another way of assessing the degree of conversion and depth of cure of the material (25, 26).

Previous studies have reported that an increase in the degree of conversion increases the surface microhardness (27, 28).

In examining the polymerization values of composite resins, it is not sufficient to evaluate only the surface microhardness where light is applied. In many studies, it has been reported that the microhardness values of the upper surface closest to the light device and the lower layers should be proportioned, and when this ratio falls below 80%, the polymerization should be considered insufficient (29, 30). According to different researchers, acceptable curing depth is obtained when the bottom-top microhardness ratio of the composite resin is at least 80% (11, 29, 31). According to the data obtained, when the microhardness ratios are examined, it is seen that the bulk fill composite resins used outside the manufacturer's instructions (6 mm layer thickness and polymerization in extra power mode) are below 0.80. The degree of conversion ratios are in parallel with microhardness ratios and groups with microhardness ratio of 0.80 and above have degree of conversion ratios. Clinicians may be advised to pay attention to cavity depth when using these composites. When these results were evaluated, the microhardness parts of the first and second null hypothesis were rejected.

In some cases, clinicians may lose the concept of depth in deep cavities and fall outside the manufacturer's instructions. In this study, a layer thickness of 6 mm was investigated as the worst case for clinicians. Limitations of this study; traditional composite resin was not used as a control group and cytotoxicity was not investigated on dental pulp cells. In this sense, the results obtained in our study give preliminary information about the cytotoxic effects of the tested bulk fill composite resins. Further in vitro tests related to the results obtained from this study should be carried out and the results should be supported by animal experiments.

5. CONCLUSIONS

In this study, the effects of polymerization of Sonic Fill 2 and Filtek Bulk Fill composites at 2, 4 and 6 mm thickness at two different light intensities (standard power mode-20s and extra power mode-3s) on the degree of conversion, microhardness and cytotoxicity of the composite was investigated.

The degree of conversion and microhardness of both bulk fill composites were below the clinically accepted threshold when used at irradiance and thickness not conforming to the manufacturer's instructions. WST-1 test results show parallelism with these results. In addition, it can be said that monomer release continues after the first 24 hours in both bulk fill composites and causes a cytotoxic effect. In line with these results, although bulk fill composite resins provide many advantages to clinicians, they may have insufficient physical and mechanical properties and show cytotoxic effects when the manufacturer's instructions are exceeded.

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Research idea: AD, CB
Design of the study: AD, CB
Acquisition of data for the study: SGB
Analysis of data for the study: CB
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Clinical and Experimental Health Sciences

The Effect of The Musical Snow Globe Used in Infancy Vaccination Applications on The Level of Pain: A Randomized Controlled Study

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ABSTRACT

Objective: This randomized controlled trial was conducted to investigate the effect of a musical snow globe on reducing pain associated with vaccine administration in infants 2-6 months of age.

Methods: The sample of the study consisted of 78 infants (experimental group (EG):39, control group (CG): 39 babies) who applied to the family health center between the specified dates and met the case selection criteria. The Face, Legs, Activity, Crying, and Comfort (FLACC) Scale was used to assess infants' pain before, during, and after immunization. Heart rate and oxygen saturation in the infants' blood were measured as part of the study. During vaccine administration, infants in the experimental group were shown a snow globe with music, while infants in control group were vaccinated with a routine health application.

Results: There was no statistically significant difference (p>.05) between the SpO2 values before the interventions (EG: 99.46 CG: 99.56) and the SpO2 values after the interventions (EG: 96.30 CG: 96.05) of the babies in the experimental and control groups; In heart rates, there was no statistically significant difference (p>.05) between befoere the interventions (EG:127.31 CG:127.71) and after the interventions (EG:140.10 CG:147.66) values. However, a significant difference was found between SpO2 (EG: 95.20 CG: 93.23) and heart rate values (EG: 145.76 CG: 157.33) during the intervention (p<.05). There was no statistically significant difference between the FLACC scores (EG: 0.23 CG: 0.28) of the babies in both groups before the intervention (p>.05); however, the FLACC scores of the infants in the experimental group were lower than the infants in the control group at the time of the intervention (EG: 3.89 CG: 6.92) (p<.05) and afterwards (EG: 2.82 CG: 5.18) (p<.05). A significant difference was found between the babies in the experimental and control groups in terms of crying time. Babies in the experimental group cried for a shorter time (EG: 140.09 CG: 193.44) (p<.05).

Conclusion: The study showed that musical snow globe is an effective tool for reducing pain during vaccinations in infants.

Keywords: Infants, vaccine application, pain, musical snow globe

1. INTRODUCTION

Vaccine administration is one of the most common invasive and painful procedures children face in the neonatal period Infants are exposed to a total of 24 injections through vaccination in the first two years of life in United States (1). In Turkey, the total number of injections for routine vaccinations is twenty (2). The high number of vaccinations in healthy infants and children and the psychological trauma caused by vaccination pain necessitate intervention against the pain that occurs during vaccination (3).

Pain was considered as a fifth life finding and was stated that all infants, including preterm infants, experience the negative effects of pain (4). Therefore, the management of acute pain in infants and children is of great importance due to invasive procedures. The pain that occurs during immunization can affect the child physically, emotionally, and behaviorally if not properly managed. Deterioration in breathing patterns, inadequate oxygenation of tissues, changes in blood pressure, increases in pulse rates and oxygen consumption, and enlargement of pupils are among the negative physiological conditions caused by pain (5,6). Children may experience emotional and behavioral problems, such as difficulty socializing, anxiety, stress disorders, and rapid distraction (7).

Pharmacologic and nonpharmacologic measures are used to control infant pain during vaccine administration. However, due to some adverse reactions to pharmacologic measures, the use of nonpharmacologic measures has greatly increased (8). Some studies have been conducted to alleviate neonatal painful initiatives using one or more sensory stimuli based on the neonates' senses of sight, hearing, touch, taste, and smell (8,9).

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When selecting these methods, it is important to consider the child's age and developmental stage and that the method is appropriate for the child. It is necessary that attention distraction methods address the child both visually and auditorily, especially in infancy (10-13). The use of attention redirection methods in infants and children can prevent the child from focusing on pain, and the pain they feel can be reduced by redirecting attention (13,14). Studies have shown that skin-to-skin contact (13,15), the use of sucrose solution (16), foot reflexology (17), breastfeeding and maternal breast milk odor (15,18), the Exor Baby Music Mobile (19), and heat and cold applications are effective in treating infant pain (20).

2. METHODS

2.1. Design

This randomized, controlled trial was conducted in newborns 2 to 6 months of age admitted to a family health center for vaccination from June to December 2020.

The study was submitted to Clinical Trials, Protocol ID 121.020.20100140, Clinical Trials ID No: NCT04772430.

2.2. Sample

The inclusion criteria of the study were: Age between 2 and 6 months, term birth or greater than 38 weeks of gestation at birth, no congenital or chronic health problems, enrollment in the family health center for vaccination, no use of an analgesic medication in the past 4 hours, accompaniment by a parent, and no crying before vaccination. The study was conducted in two family health centers. For randomization, the experimental and control groups were drawn by lot among the family health centers.

In order to determine the intervention and control groups in the study, one family health center intervention and the other family health center control group were determined by drawing lots. The drawing of lots was independently made by a nurse other than the researcher.

Power analysis performed with the G-power 3.1.9.2 program showed that the minimum sample size required was 64, with a confidence level of 95% (margin of type 1 error =.05) and a power level of 90% (margin of type 2 error =.10). Forty newborns were included in each group in case there were situations that might disturb the homogeneity of the groups in terms of case losses and variables affecting pain.

In the first phase of the study, 166 newborns who came to both family health centers for vaccination were included. However, 67 infants were excluded from the study because they did not meet the inclusion criteria, 10 infants because their parents did not want to participate in the study, and 9 infants because they cried before use. The parents of two infants declined to participate in the study during enrollment. Seventy-eight infants completed all phases of the study. Figure 1 describes the study procedure. The design, conduct, and reporting of this study conformed to the guidelines of the Consolidated Standards of Reporting Trials

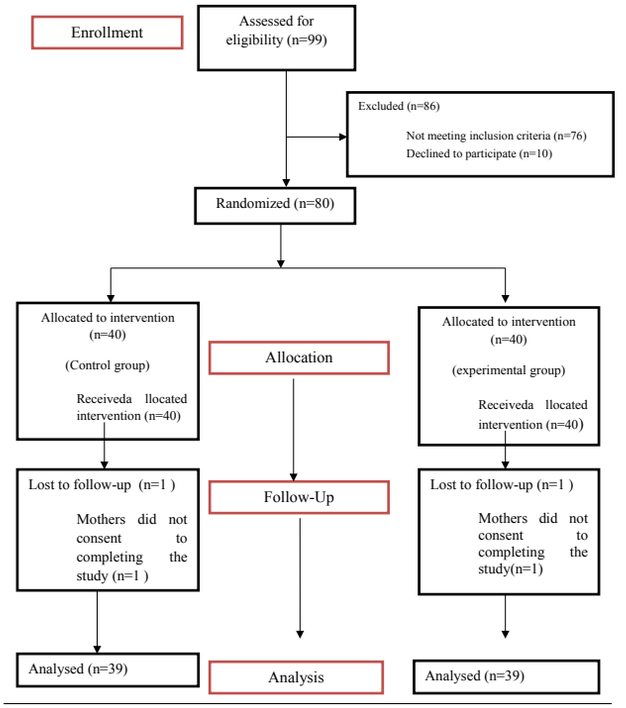

Figure 1. *CONSORT Diagram of this study.*

2.3. Instruments

Baby introductory information form

The form created by the researchers consists of two parts. The first part contains 10 questions about the baby's sex, age, weight, height, number of previous vaccinations, type of diet, nutritional status before vaccination, and duration of feeding.

Registration form

This is the form that records the infant's oxygen saturation level (SpO_2) before, during, and after use, crying duration, pulse, and respiratory values.

Face, Legs, Activity, Cry, Consolability (FLACC) Scale

Developed in 1997 by Merkel et al, this is a behavioural observation scale used in the assessment of procedural and postoperative pain in children aged two months to seven years. The FLACC Pain Scale score is determined by evaluating five categories such as facial expression, leg movements, activity, crying, and comfort (21). The adaptation of the scale to Turkish language was done by Şenaylı et al. (22).

In the current study, the intraclass correlation coefficient (ICC), which indicates the correlation between FLACC scores obtained during and after vaccination, was calculated by two independent observers under the same conditions to measure the inter-rater reliability of the FLACC scale. The ICC was .99, .97, and .98 before the procedure, during the procedure, and after the procedure, respectively.

Pulse oximeter device

In the study, a Mindray brand device was used to determine infants' oxygen saturation and heart rate before, during, and after paediatric use. Calibration of the device is checked annually. The PM-60 is a small and lightweight device for immediate and uninterrupted monitoring of SpO_2 and heart rate. The 2.4-inch colour screen LCD continuously displays SpO_2 and heart rate values.

Delta chronometer SW 305

It is simple and practical to use and operates on a 1.5 V battery. The chronometer was put into operation when sampling began, and each step of the process was performed in sequence.

The snow globe

The snow globe used in the study was in soft shades of pink, cream, and yellow. Inside the snow globe was a baby figure sleeping over the moon. The snow globe was 18x15 cm, had the shape of a glass dome and the snow machine inside constantly created the impression of snow falling on the scene. The snow globe was accompanied by classical music and worked continuously when the button was turned on.

2.4. Conducting the Study

Parents who agreed to participate in the study were asked to fill out the Baby Introduction Form. The temperature of the intervention room where the vaccination was performed was set at 24°C. The humidity was 30%, the lighting was 1000 lux, and the noise level was 35 dB(A) during the day, which met occupational health and safety requirements. At the facility where the study was conducted, these measurements are taken regularly for occupational safety reasons. No other procedures were applied in the intervention room during vaccination to avoid external factors. No one was allowed to enter the room except the baby and its parents, the nurse who administered the vaccine, and the researcher.

The infants were placed supine on a couch for vaccination. All infants were awake and wearing clean diapers during the injection, and their parents were in the same room. The pulse oximeter was attached to the baby's right wrist on the stretcher, and the baby's heart rate and SpO_2 were recorded prior to vaccination. This device was left on the baby's wrist throughout the procedure. Then, the researcher and a nurse separately measured the FLACC scale values. Before vaccination, the timer was kept ready, which was activated as soon as the baby began to cry. The baby's injection site was opened by the nurse and cleaned with 70% ethyl alcohol according to aseptic principles. The vaccines were injected into the vastus lateralis muscle at a 90° angle using a 23-mm needle. The injection lasted approximately 20 seconds. After the procedure, light pressure with dry cotton was applied to the inoculated area. The timer was turned on until the baby stopped crying. The infants' heart rate and SpO_2 were recorded during sampling, and the degree of pain was assessed using the FLACC scale. Two minutes after administration of the vaccination, the heart rate and SpO_2 were recorded again, and the level of pain was assessed using the FLACC scale. The administration of the vaccination took an average of one minute in each of the babies.

After the pain scores were assessed with the FLACC scale, in contrast to the infants in the control group, the snow globe was placed at a distance of 20-30 cm so that the infants in the experimental group could see it before inoculation. The snow globe was kept in operation throughout the vaccination. All infants in the experimental and control groups were injected with the same vaccine by the same nurse.

2.5. Ethical Aspects of the Research

Parental informed consent, facility approval, and ethics committee approval (date: 24.06. 2020 and number: 2020-13) were obtained.

2.6. Data Analysis

The SPSS 22.0 program was used for statistical analysis. Mean-standard deviation (minimum-maximum), number, percentages, Kolmogrov-Smirnov test, chi-square, Mann-Whitney U test, Friedman test, and T test for paired samples were used to analyze the data. Results were analyzed with a 95% confidence interval, and p <.05 was considered statistically significant (23,24).

3. RESULTS

The study found that the infants in both groups were similar in terms of sex, age, weight, length, number of previous vaccinations, type of diet, nutritional status before vaccination, and duration of feeding, and no statistically significant difference was found between them (p>.05) (Table 1).

Table 1. *Descriptive characteristics of babies*

Characteristics	EG (n=39) Mean±SS	CG (n=39) Mean ±SS	Total (n=78) Mean ±SS	Test Value Z	p
Age (month)	3.79±1.50	3.74±1.60	3.79±1.54	-0.146	.88[a]
Weight (g)	6523±984.55	6608±117.85	6565±1047.33	0.357	-.77[a]
Length (cm)	60.66±4.32	60.05±5.31	60.35±4.82	-0.561	.57[a]
Number of previous	4.46±1.93	4.35±2.04	4.41±1.97	-0.228	.82[a]
Gender	n (%)	n (%)	n (%)		
Girl	21 (53.8)	22(56.4)	43(55.1)	0.523	.82[b]
Boy	18(46.2)	17(43.6)	35(44.9)		
Nutrition					
Yes	25 (64.1)	33 (84.6)	58(74.4)	4.303	.38[b]
No	14 35.9)	6(15.4)	20(25.6)		
Diet					
Breast milk	39 (100.0)	33(84.6)	72(92.3)	6.500	.39[b]
Breast milk+ formula	0 (0.0)	6 (15.4)	6 (7.7)		

[a]*Mann Whitney U Testi* [b]*Ki-Kare Testi p<.05*

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Table 2. *Comparison of the physiological parameters and pain scores of the groups according to the procedure time*

Groups		Before transaction[a]	During transaction[b]	After Transaction[c]	p	p[c-d]	p[c-e]	p[d-e]
EG	FLACC scores	0.23± 0.62	3.89± 2.95	2.82± 2.15	.001[f]	.001[g]	.001[g]	.003[g]
CG		0.28±0.82	6.92 ± 2.14	5.18 ± 2.23	.001[f]	.001[g]	.001[g]	.001[g]
	p	.768[a]	.001[a]	0.001[a]				
EG	Mean heart rates	127.31±15.47	145.76±20.08	140.10±19.42	.001[f]	.001[g]	.001[g]	.002[g]
CG		127.71±14.72	157.33±18.95	147.66±20.78	.001[f]	.001[g]	.001[g]	.002[g]
	p	.905[a]	.011[a]	.101[a]				
EG	Mean SpO_2 level	99.46±1.33	95.20±3.42	96.30±2.62	.001[f]	.001[g]	.001[g]	.004[g]
CG		99.56±1.27	93.23±2.92	96.05±2.08	.001[f]	.001[g]	.001[g]	.001[g]
	p	.347[a]	.008[a]	.635[a]				

[c]Before transaction, [d]During transaction, [e]After Transaction, [f]Friedman test, [g]Wilcoxon signe dranks test, [a]Mann Whitney U Test

There were no statistically significant differences between the SpO_2 values of the infants in the experimental and control groups before (EG: 99.46 CG: 99.56) (p>.05) and after the interventions (EG: 96.30 CG: 96.05) (p>.05); however, a significant difference (p<.01) was found between their SpO2 values during the intervention (EG: 95.20 CG: 93.23). The infants' SpO_2 values were lower during the intervention than before and after the intervention. There were statistically significant differences between infants in both groups in terms of SpO_2 values before, during, and after the intervention (p<.001) (Table 2, Figure 2).

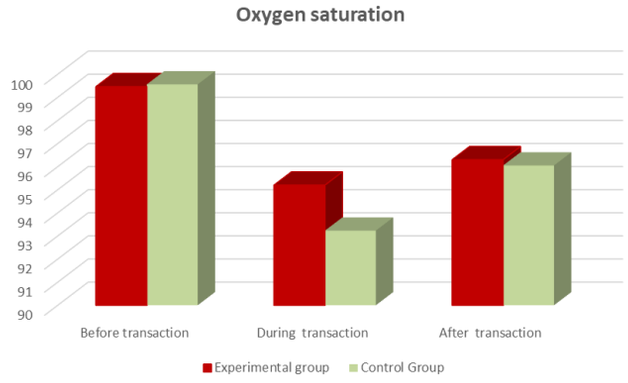

Figure 2. *Spo2 mean value distributions of experimental and control group infants according to the time of procedure*

Although no statistically significant differences were found between the heart rates of the infants in the experimental and control groups before (EG:127.31 CG:127.71) (p>.05) and after the interventions (EG:140.10 CG:147.66) (p>.05), a significant difference (EG: 145.76 CG: 157.33) (p<.01) was found during the intervention. Infants' heart rate values were higher during the procedure than before and after the procedure. There were statistically significant differences between the infants of both groups in terms of heart rates before, during and after the procedures (p<.001) (Table 2, Figure 3).

No statistically significant differences were found between the FLACC scores of the infants in both groups before the intervention (EG: 0.23 CG: 0.28) (p>.05); however, the FLACC scores of the infants in the experimental group were lower than those of the infants in the control group during (EG: 3.89 CG: 6.92) (p<.001) and after (EG: 2.82 CG: 5.18) (p<.001) the intervention. There was a statistically significant difference between FLACC scores of infants in both groups before, during, and after interventions (experimental group, p<.001; control group, p<.001) (Table 2, Figure 4). A significant difference was found between the infants in the experimental and control groups in terms of crying duration. It was observed that the infants in the experimental group cried for a shorter time (EG: 140.09 CG: 193.44) (p<.001) (Table 3).

Table 3. *Comparison of the mean total crying time of groups*

Groups		Mean ±SD	Median	Min	Max
EG	Crying times	140.09±1.09	40	0.00	540
CG		193.44±2.50	1.85	0.00	720
p		.001[a]			

[a]Mann Whitney U Test, SD: Standart deviation

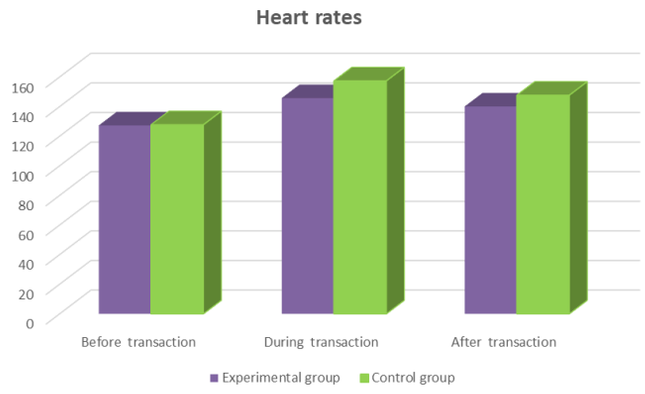

Figure 3. *Heart rates mean value distributions of experimental and control group infant saccording to the time of procedure*

4. DISCUSSION

The assessment of pain in infants is difficult because they cannot express themselves verbally. Therefore, physiological variables are the parameters that allow pain assessment (18,25,26). In this study, the infants' heart rates and oxygen saturation were measured before, during, and after vaccination to allow physiological assessment.

The increase in heart rate of infants in the experimental group during vaccination was significantly lower than that

of infants in the control group (Figure 3). The decrease in oxygen saturation was significantly higher in infants in the control group than in those in the experimental group during vaccination (Table 2, Figure 2).

In the study conducted by Jain, Kumar, and McMillan (25) to determine the effect of massage on pain levels during heel blood sampling in infants, no significant differences were found between infant groups in oxygen saturation levels before, during, and after the procedures, and the heart rate of infants in the massage group was lower (25). In the study conducted by Ozkan et al. (26), it it was found that oxygen saturation decreased less during the procedure in the group that received foot massage during blood sampling at the heel of newborns (26). Maternal breast milk odour (18), breastfeeding (10,27), and ShotBlocker application (28) were found to have positive effects on infants' heart rates during procedures with needles.

The verbal utterance of the individual is the most accurate and precise way to assess pain; however, it is not possible to assess the pain of infants with verbal utterance. Therefore, pain assessment in infants can be done using behavioural responses. The FLACC scale is one of the scales commonly used to assess pain in infants. In this study, the FLACC mean scores of infants in the control and experimental groups did not show statistically significant differences before the interventions. However, it was found that the FLACC values of infants in the experimental group were statistically significantly lower than those of infants in the control group during and after vaccination (Table 2, Figure 4). The results of this study suggest that the audible snow globe is effective in reducing infants' pain. Similar results have been obtained in previous studies. In the study conducted by Susilawati et al. (29), it was found that electrical nerve stimulation through the skin can reduce injection-related pain during vaccination in infants. In the study by Hogan et al. (30), tactile stimulation was reported to be effective in reducing pain in infants. Raouth Kostandy et al. (13) found in their study that skin-to-skin contact had a positive effect on reducing pain during immunizations in infants. In the study by Efe and Erkul (31), breastfeeding during vaccination was reported to be effective in reducing pain. The effectiveness of music and pressure application in reducing pain during vaccination was found in the study conducted by Kant (14). Göl and Altuğ Ozsoy (32) found in their study that rapid vaccine injection without aspiration and manual pressure application before vaccination effectively affected pain intensity and duration of crying in infants.

Crying is a form of communication in infants and children. It is one of the most important responses of infants during invasive and painful procedures. Crying is an observable and assessable behaviour in pain assessment (32). In the study by Ipp et al. (33), it was found that the longer the infants' crying lasted during immunisation, the higher their pain scores were. In this study, it was observed that infants in the control group cried longer during vaccination than infants in the experimental group (Table 3). The previous studies also indicated that various distraction methods were effective in reducing the duration of crying during vaccination. In the study by Dilli et al. (10), breastfeeding during vaccination was found to reduce the duration of crying in infants younger than six months. In the study by Ozdemir and Güdücü Tüfekçi (19), it was observed that infants who were vaccinated in a room where music was played from a cell phone cried for a shorter time than those not distracted by a device.

No study was found in the literature that evaluated the effectiveness of the snow globe in reducing pain during vaccination in infants. Therefore, this study is considered the first research to evaluate the effectiveness of snow globe in reducing pain during vaccination in infants.

5. CONCLUSIONS

In the study, it was found that the infants who were made to watch a musical snow globe during vaccination had less pain, less increase in heart rate, and less decrease in oxygen saturation during and after vaccination. This study suggests that the musical snow globe is effective in reducing pain during vaccinations in infants.

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Design of the study: BA, TKA
Acquisition of data for the study: BA, TKA
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Clinical and Experimental Health Sciences

Accuracy of Dental and Chronological Age Estimation in A Sample Turkish Caucasion Children: Comparison of Demirjian's and Willems Methods

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ABSTRACT

Objective: The purpose of this study is to apply Demirjian's and Willems' methodologies and to define whether there are any discrepancies in predicting dental age versus chronological age in a sample Turkish Caucasian children.

Methods: A total of 150 Turkish Caucasian children with known chronological age and gender were chosen. The chronological age was determined by subtracting the date of birth from the date of the radiograph, and it was expressed as a number with two decimal places. Each age group was determined to have a minimum sample size of 12 and a maximum sample size of 27. All panoramic radiographs were scored according to the criteria of Demirjian's and Willems methodologies with Onyx Ceph 3.1.54 software.

Results: The dental ages of the cases ranged from 4.82 to 15.66 years calculated by the Demirjian's method, with an average of 9.47±2.27 years, while the Willems method of the cases ranged from 4.13 to 14.34 years calculated by the Demirjian's method, with an average of 8.87±2.24 years. According to Demirjian's method, in the developmental evaluation of dental age, 45.3% of boys were found to have a statistically higher chronological age than girls ($p<.05$), while no statistically significant difference was found between dental age and chronological age in developmental evaluation according to Willems method ($p>.05$).

Conclusion: The Willems method was shown to be more accurate in determining dental age in Turkish children. Further studies on large population groups and diverse ethnicities are required to increase the reliability and repeatability of the results.

Keywords: Dental age, chronological age, demirjian's method, willems method

1.INTRODUCTION

Estimating chronological age is critical in many domains, including forensic medicine, pediatric endocrinology, archaeology, and clinical dentistry. The chronological age is determined by the phases of maturity of various tissues (1). Skeletal age, morphological or somatic age, secondary sex character age, and dental age are some of them (1,2). Somatic maturity refers to yearly increases in height or weight (3). Secondary sexual traits such as voice changes in boys and menarche in girls show sexual maturity (3). These maturity indicators have limited value because they can be applied only after serial recordings of height or the inception of puberty (2). Skeletal maturation is an complementary part of individual patterns of growth and development (4). Skeletal maturity evaluation approaches include visual observation of the growing bones' form and size changes (2). The foot, the ankle, the hip, the elbow, the hand, and the wrist, as well as the cervical vertebrae, have been used to calculate skeletal age using a variety of approaches (2). On the basis of hand wrist radiography procedures, these approaches were used to determine skeletal age (4). The basic premise is that osseous abnormalities in the hand and wrist are markers of broader skeletal changes (5). In orthodontics, radiographs of the cervical vertebrae, as well as hand and wrist radiographs, are routinely used to determine skeletal maturation stage (6).

Dental maturity is the last physiologic measure. Estimation of dental age comprises two principles that have been used to determine tooth eruption and mineralization stage of dental tissues (7). The stage of tooth production has been presented as a more valid criterion for determining dental maturity than the stage of tooth eruption, and it is commonly used to assess and forecast age. This information is helpful for diagnosis and treatment management in clinical dentistry (8). Because intraoral or panoramic radiographs may be used to identify dental developmental phases, physiologic maturity can be easily assessed in most orthodontic or pediatric dentistry clinics without the need of hand wrist radiography (2). Dental age is of great importance to the orthodontist when planning therapy for various forms of malocclusions in maxillofacial development. In archaeology and forensic odontology, age estimations can assist in the identification process, particularly when there is no information on the deceased, as well as provide skeletal and dental information about historical populations (9). Also in pediatric endocrinopathies,

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recognising of dental age may help practitioner to diagnose and evaluate the development of child (1).

Methods for estimating the age of children based on their dental development, such as the atlas method and scoring systems. In addition, radiological and morphological approaches are employed on adults (10). Among several methods of age assessment, the Demirjian's method which was described in 1973 and 2928 French-Canadian parentage of panoramic radiographs is commonly used (1,11).

A meta-analysis of the results from several research on different populations using Demirjian's method revealed that the Demirjian data set overstated the age of men and females by 6 months on average (12). Willems used Demirjian's approach in a Belgian research with the intention of minimizing the error rates of the method (13). Although just a few studies evaluating it have been published, this approach was more accurate than the Demirjian's method for determining dental age (14-18).

Demirjian's approach in Turkish parentage has been published in a number of studies (19–26), however few studies have compared Demirjian's and Willems methods (27,28). Applying Demirjian's and Willems' methods to a sample of Turkish Caucasian children will enable this study to determine whether there are any differences between dental age and chronological age estimates.

2.METHODS

2.1. Subjects

A total of 150 Turkish Caucasian children of known chronological age and gender were chosen. There were 75 boys and 75 girls, ranging in age from 4 to 14 years. Panoramic radiographs were selected from the patient record database of Marmara University's Faculty of Dentistry's Oral Diagnosis and Radiology Department between 2013 and 2015. The panoramic radiographs were obtained using Planmeca Promax (Planmeca Oy, Helsinki, Finland). The inclusion criteria for the sample were the availability of a high-quality orthopantomogram in their clinical records and the absence of a medical or surgical history that might impact the existence and development of permanent teeth. This research eliminated children with congenital or systemic illnesses, unclear panoramic radiographs, and aplasia of permanent mandibular teeth. The study protocol numbered as 09.2015.171 was approved by Clinical Research Ethics Committee, Marmara University Faculty of Medicine on 03.07.2015. Prior to analysis, all patient data and information were rendered anonymous and de-identified.

The chronological age was calculated by subtracting the date of birth from the date of the radiograph and was represented with two decimal places. According to chronological age, the sample was separated into nine groups of one year each. For each age group, a minimum sample size of 12 and a maximum sample size of 27 were determined for finite population.

2.2. Dental age estimation method

Demirjian et al. (1) and Willems et al. (13) scoring standards were used to all panoramic radiographs. For the digital technique, direct digital panoramic radiographs were recorded in the Joint Photographic Experts Group format and transferred to the OnyxCeph3™ 3.1.54 (Image Instruments, Chemnitz, Germany) dental analysis software for analysis. Digital measurements were evaluated using a 23-inch Acer 1920×1080-pixel HP Reconstruction PC monitor. The seven mandibular teeth on the left were scored. First, the calcification stage of each tooth was used to categorize its stage from 'A' to 'H'. Stage 0 indicated the absence of the case, but Stage 1 indicated the radiolucent bud prior to calcification (Fig. 1). Using the Willems technique, the dental maturity of the left mandibular seven permanent teeth was scaled similarly to the Demirjian method, but the maturity score for each tooth was determined using the Willems method's tables. As mentioned by Demirjian et al. (1) and Willems et al., each score was transformed into a dental age by gender (13).

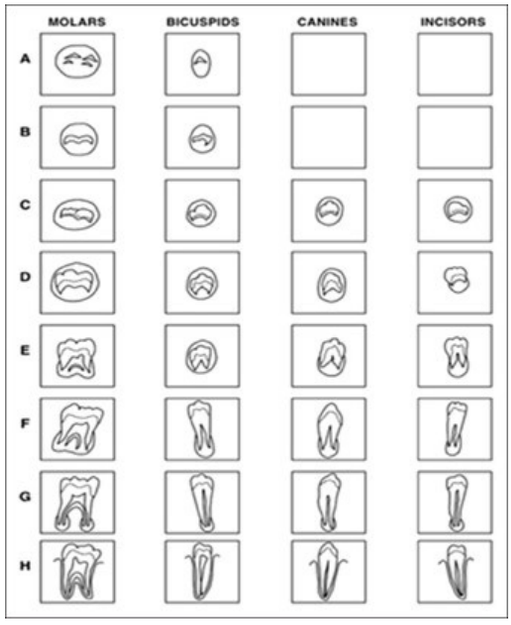

Figure 1. *Schematic drawing of the developmental stages of teeth.*

All panoramic radiographs was examined by a single oral and maxillofacial radiologist (EI). To measure dependability, all photos were reexamined by the same examiner three months later. Cohen's kappa coefficient was used to determine the reproducibility of the statistics.

2.3. Statistical Analysis

The data were analysed with the IBM SPSS (Statistical Package for Social Sciences) Statistics 22 (IBM SPSS, Turkey) was used for Windows 15.0 software. Descriptive statistical methods (mean, standart deviation) were used for the evaluation of the data. For the comparison of quantitative data paired sample t test was used and also comparison of

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qualitative data Chi-square test was used. P values < .05 were considered statistically significant.

3.RESULTS

The patients ranged in age chronologically from 4.45 to 13.47, with an average age of 9.07 ±2.12 years. Demirjian's dental age estimation technique revealed that the patients' dental ages ranged from 4.82 to 15.66 years, with an average of 9.47±2.27 years. With an average age of 8.87 ± 2.24 years, the method-determined Willems dental ages ranged from 4.13 to 14.34 years. Girls' chronological ages ranged from 5.22 to 13.47 years, with an average of 9.05± 2.2 years, while boys' chronological ages ranged from 4.45 to 12.79 years, with an average of 9.09±2.06 years. According to Demirjian's dental age estimation method, girls' dental ages ranged from 4.82 to 15.66 years, with an average of 9.64 ± 2.29 years. With an average age of 9.29 ± 2.26 years, boys with dental issues ranged in age from 4.82 to 14.58 years. Girls' dental ages, as determined by Willems method, ranged from 4.18 to 13.84 years, while boys' dental ages ranged from 4.13 to 14.34 years.

In the developmental evaluation of dental age conducted using Demirjian's method, it was discovered that 45.3% of boys had a statistically higher chronological age than girls (p= .043), whereas in the developmental evaluation conducted using Willems method, there was no statistically significant difference between dental age and chronological age (p>.05) (Table 1).

Table 1. *Developmental evaluation of gender, chronological age, dental age with Demirjian's and Willems methods*

	Gender		p
	Girls	Boys	
	n (%)	n (%)	
Demirjian's method dental age			
Higher than chronological age	22 (29.3%)	34 (45.3%)	**.043***
Lower than chronological age	53 (70.7%)	41 (54.7%)	
Willems method dental age			
Higher than chronological age	49 (65.3%)	46 (61.3%)	**.611**
Lower than chronological age	26 (34.7%)	29 (38.7%)	

*Chi-square test; * p< .05*

Demirjian's method revealed that for girls, the average dental development age was substantially higher than chronological age (p= .001). Additionally, chronological age was statistically considerably higher than usual for years of dental development age (p= .006) when calculated using Willems method. There is no statistically significant difference between the two methods for boys' average age of dental development (p> .05) (Table 2).

According to Demirjian's method for dental development, the average dental age of the patients was statistically substantially higher than the average chronological age for the age ranges of 5.00–5.99 and 6.00–6.99 years (p= .001). Demirjian's method for dental development also revealed that the average dental age of the patients for the age range of 7.00–7.99 years was statistically substantially higher than the average chronological age (p= .011). There was no statistically significant difference between the Demirjian's method-calculated dental development ages and the patients' chronological ages for the age ranges of 8.00–8.99, 9.00–9.99, 10.00–10.99, 11.00–11.99, and 12.00 –12.99 years (p> .05) (Table 3) (Fig 2).

The average chronological age of the patients was statistically substantially higher than the average dental age determined using the Willems method for the age ranges of 5.00–5.99 and 12.00–12.99 years (p< .05). There was no statistically significant difference between the chronological ages of the patients and the dental development ages calculated using the Willems method for the age ranges of 6.00–6.99, 7.00-7.99, 8.00 –8.99, 9.00 –9.99, 10.00 –10.99 and 11.00 –11.99 years (p> .05) (Table 4) (Fig. 2).

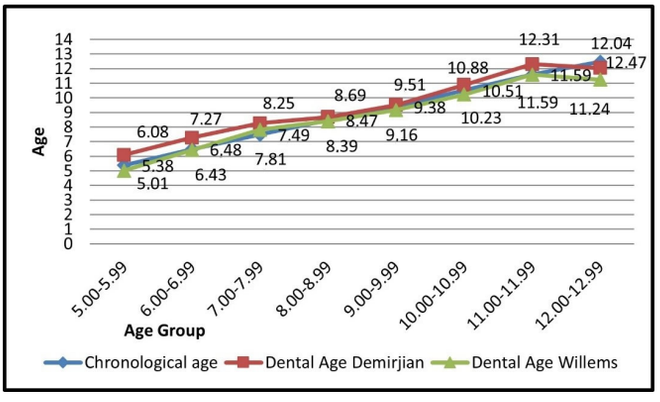

Figure 2. *Age groups according to chronological age, dental age calculated by the methods of Demirjian's and Willems*

For patients adopting the Demirjian's method for dental development, the average dental age was statistically significantly higher than the average chronological age for girls between the ages of 5.00-5.99, 6.00-6.99, 7.00-7.99 and 8.00-8.99 (p< .05). The average dental age of the patients using the Demirjian's method for dental development was statistically substantially higher than the average chronological age for boys aged 5.00–5.99 and 6.00–6.99 (p< .05) (Table 5).

According to the Willems method for dental development, the average chronological age of girls between the ages of 12.00 – 12.99 was statistically substantially higher than the average dental age of the patients (p< .05). No statistically significant differences were observed in the age ranges of 5.00–5.99, 6.00–6.99, 7.00–7.99, 8.00–8.99, 9.00–9.99, 10.00-10.99, and 11.00–11.99 years between the patients' chronological ages and the dental development ages determined using the Willems method (p> .05) (Table 6) (Fig 3).

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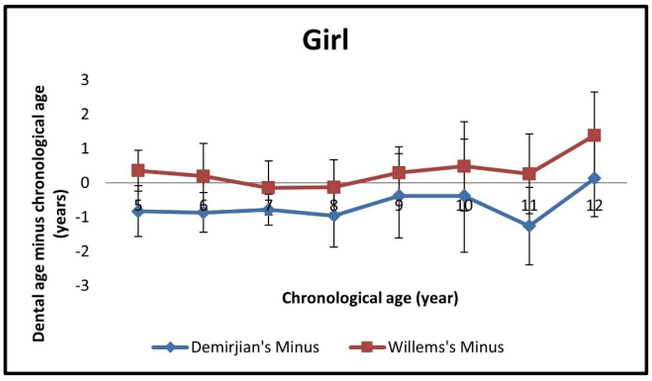

Figure 3. *Dental age minus chronological age for girls*

According to the Willems method for dental development, the average chronological age of boys aged 8.00–8.99 was statistically substantially higher than the average dental age of the patients (p= .044). There were no statistically significant variations between the patients' chronological ages and the dental development ages determined using the Willems method for the age ranges of 5.00–5.99, 6.00–6.99, 7.00–7.99, 9.00–9.99, 10.00–10.99, 11.00–11.99, and 12.00–12.99 years (p> .05) (Table 6) (Fig 4).

The intra-observer reproducibility tests resulted in almost perfect agreement according to Landis and Koch guidelines (29) K = 0.839 for intra-observer agreement.

Table 2. *The amount of deviation from the chronological evaluation of dental development years regarding gender and age by Demirjian's and Willems methods*

Gender	Chronological Age	Demirjian's method dental age	Age Difference	p	Chronological Age	Willems method dental age	Age Difference	p
	Mean±SD	Mean±SD	Mean±SD		Mean±SD	Mean±SD	Mean±SD	
Girls	9.05±2.2	9.64±2.29	-0.59±1.14	.001*	9.05±2.2	8.71±2.17	0.34±1.04	.006*
Boys	9.09±2.06	9.29±2.26	-0.2±1.28	.174	9.09±2.06	9.02±2.31	0.07±1.14	.621

*SD: Standart Deviation, Paired sample t testtest; * p< .05*

Table 3. *Evaluation of the deviation amount of dental age and chronological age calculations according to age groups with Demirjian's method*

Age range	Number of Child	Chronological Age	Demirjian's method dental age	Age difference	p
	n	Mean±SD	Mean±SD	Mean±SD	
5.00-5.99	15	5.38±0.38	6.08±0.82	-0.69±0.64	**.001****
6.00-6.99	12	6.48±0.32	7.27±0.58	-0.79±0.49	**.001****
7.00-7.99	21	7.49±0.3	8.25±1.17	-0.76±1.25	**.011***
8.00-8.99	20	8.47±0.29	8.69±0.81	-0.22±0.82	**.241**
9.00-9.99	26	9.38±0.27	9.51±1.29	-0.13±1.19	**.589**
10.00-10.99	27	10.51±0.26	10.88±1.53	-0.37±1.52	**.216**
11.00-11.99	16	11.59±0.39	12.31±1.84	-0.72±1.57	**.087**
12.00-12.99	13	12.47±0.42	12.04±1.33	0.43±1.23	**.233**

*Paired sample t test, * p< .05, **p< .01*

Table 4. *Age groups according to chronological age, dental age calculated by the method of Willems*

Age range	Number of Child	Chronological Age	Willems method dental age	Age Difference	p
	n	Mean±SD	Mean±SD	Mean±SD	
5.00-5.99	15	5.38±0.38	5.01±0.66	0.37±0.52	**.014***
6.00-6.99	12	6.48±0.32	6.43±0.93	0.06±0.86	**.818**
7.00-7.99	21	7.49±0.3	7.81±1.21	-0.33±1.24	**.242**
8.00-8.99	20	8.47±0.29	8.39±0.51	0.08±0.54	**.495**
9.00-9.99	26	9.38±0.27	9.16±0.9	0.22±0.79	**.166**
10.00-10.99	27	10.51±0.26	10.23±1.29	0.28±1.28	**.267**
11.00-11.99	16	11.59±0.39	11.59±1.66	0.01±1.47	**.996**
12.00-12.99	13	12.47±0.42	11.24±1.38	1.23±1.28	**.005****

*Paired sample t test ; *p< .05, **p< .01*

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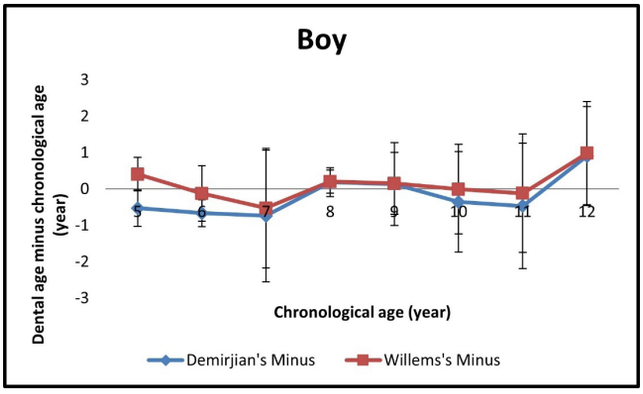

Figure 4. *Dental age minus chronological age for boys*

Table 5. *The amount of deviation of boys and girls between the chronological age and developmental dental age by Demirjian's method*

	Age Range	Number of Child	Chronological Age	Demirjian's method dental age	Age Difference	p
		n	Mean±SD	Mean±SD	Mean±SD	
Girls	**5.00-5.99**	8	5.5±0.29	6.34±0.91	-0.83±0.74	**.015***
	6.00-6.99	7	6.52±0.29	7.39±0.48	-0.87±0.58	**.007****
	7.00-7.99	11	7.36±0.29	8.14±0.4	-0.79±0.45	**.001****
	8.00-8.99	7	8.39±0.34	9.35±0.94	-0.96±0.92	**.032***
	9.00-9.99	13	9.39±0.23	9.78±1.36	-0.38±1.23	**.282**
	10.00-10.99	16	10.51±0.27	10.89±1.73	-0.38±1.65	**.375**
	11.00-11.99	5	11.88±0.09	13.14±1.04	-1.26±1.13	**.067**
	12.00-12.99	8	12.49±0.5	12.36±1.17	0.13±1.12	**.748**
Boys	**5.00-5.99**	7	5.24±0.44	5.78±0.65	-0.53±0.5	**.031***
	6.00-6.99	5	6.44±0.38	7.11±0.71	-0.67±0.37	**.015***
	7.00-7.99	10	7.63±0.26	8.36±1.69	-0.74±1.81	**.230**
	8.00-8.99	13	8.51±0.27	8.34±0.43	0.18±0.4	**.138**
	9.00-9.99	13	9.37±0.33	9.24±1.21	0.13±1.14	**.693**
	10.00-10.99	11	10.52±0.27	10.88±1.27	-0.36±1.38	**.408**
	11.00-11.99	11	11.45±0.39	11.93±2.04	-0.47±1.73	**.385**
	12.00-12.99	5	12.44±0.31	11.54±1.54	0.9±1.36	**.214**

Paired sample t test ; p< .05, **p< .01*

Tablo 6. *The amount of deviation of boys and girls between the chronological age and developmental dental age by Willems method*

	Age Range	Number of child	Chronological age	Willems method dental age	Age Difference	p
		n	Mean±SD	Mean±SD	Mean±SD	
Girls	**5.00-5.99**	8	5.5±0.29	5.15±0.71	0.35±059	**.138**
	6.00-6.99	7	6.52±0.29	6.33±0.85	0.19±0.96	**.618**
	7.00-7.99	11	7.36±0.29	7.51±0.74	-0.15±0.78	**.547**
	8.00-8.99	7	8.39±0.34	8.52±0.77	-0.13±0.8	**.678**
	9.00-9.99	13	9.39±0.23	9.1±0.88	0.29±0.76	**.196**
	10.00-10.99	16	10.51±0.27	10.03±1.38	0.48±1.3	**.164**
	11.00-11.99	5	11.88±0.09	11.62±1.07	0.26±1.16	**.638**
	12.00-12.99	8	12.49±0.5	11.11±1.31	1.38±1.27	**.018***
Boys	**5.00-5.99**	7	5.24±0.44	4.85±0.6	0.4±0.46	**.062**
	6.00-6.99	5	6.44±0.38	6.57±1.12	-0.13±0.76	**.730**
	7.00-7.99	10	7.63±0.26	8.15±1.55	-0.53±1.64	**.336**
	8.00-8.99	13	8.51±0.27	8.31±0.31	0.2±0.32	**.044***
	9.00-9.99	13	9.37±0.33	9.22±0.95	0.15±0.85	**.523**
	10.00-10.99	11	10.52±0.27	10.53±1.14	-0.01±1.23	**.975**
	11.00-11.99	11	11.45±0.39	11.58±1.92	-0.12±1.63	**.808**
	12.00-12.99	5	12.44±0.31	11.46±1.6	0.98±1.42	**.198**

Paired sample t test ; p< .05*

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4. DISCUSSION

Tooth development is commonly used to determine dental maturity and age. This information aids in diagnostic and treatment planning in clinical dentistry. In archaeology and forensic odontology, age estimate techniques can help in determining the age of death of a deceased child and provide vital information about former populations. Age estimation is also important in immigration administration when birth documents are lacking or contested in order to determine physiological age (8,14).

Panoramic radiographs were employed to evaluate dental maturity because they are widely available in dental clinics, the mandibular region is clearly visible, and intraoral radiography without image distortion is difficult to create (2,16-19).

There are many standard scales available for rating the tooth calcification stage. In the present study, the approach provided by Demirjian et al was chosen because its criteria are focused on form and proportion of root length, utilizing the perceived importance to crown height rather than absolute length. In radiography, shortened or lengthened projections of growing teeth have no effect on the validity of evaluation (2,7,11).

Willem's dental age estimate method, a variant of Demirjian's method, was also adopted in the current investigation since it gave more accurate age estimation than Demirjian's methods (11, 27,28). This is supported by our findings.

Enlarging the sample size might ensure more appropriate information about distrubution of the dental developmental stages (2). The present study consisted of 150 subjects; 75 males and 75 females with similar sample size of many studies (7, 11, 16). Some studies provide us with a total of tooth scores for each year of age, and we utilized this to predict age in 1-year intervals (8).

It is suggested that tooth mineralization relative to stages of skeletal maturation be considered individually for genders (2). The tooth development rate was higher in females than in males and have shown differences by gender for 10 years of age and above because of hormonal factors and puberty (8, 14, 15). This is in agreement with our findings where girls were dentally more advanced than boys for the dental maturation (1, 7, 15, 18, 21). Similarly, the chronological age of the patients between 12 and 13 years is statistically significantly higher than the average of the dental development age assessed by the Willems method in this study.

In this study the average age of dental development is statistically significantly higher than the chronological age average calculated by Demirjian's and Willems method for cases up to 6 years. The Demirjian's method revealed the smallest differences between chronological and estimated dental ages in boys aged 8 to 9 years and females aged 9 to 11 years. Willems method revealed the smallest differences between chronological and estimated dental ages in boys aged 10 to 11 years and girls aged 8 to 9 years. The current study contradicted a prior result that age may be predicted more correctly in younger children than in older children (8).

The most differences between the chronological and estimated dental ages were observed in the 12 to 13 year age groups in boys and 11 to 12 year age groups in girls in Demirjian's method and 12 to 13 year age groups in boys and 12 to 13 year age groups in girls in Willems method. This higher overestimation of the dental age observed in the older children in this study was probably due to the pubertal growth changes related during this age period (19).

No universal consensus has been adopted to classify the identifiable human groups based on ethnicity. A country which is defined by a geographical boundary could contain different ethnic groups of varying genetic predispositions. Individual evaluations of these ethnic groups need to be performed to obtain a detailed understanding of dental growth among these groups (13, 15, 17). In Maber et al.'s (8) study there were no significant differences across ethnic groups for any technique for either gender, and data from both ethnic groups were merged. While many ethnicities are prevalent in the Turkish population, we did not take this into account in our study.

5. CONCLUSION

The current study's findings confirm prior research that indicate the dependability of the Demirjian's method as it may be used to determine tooth production phases in the Turkish population. The Willems method, on the other hand, found to be more accurate in assessing dental age in Turkish children. Further research is needed on large population groups and different ethnicity in order to improve the reliability and reproducibility of the results.

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Clinical and Experimental Health Sciences

Effects of Online Nutrition Training Program About Mediterranean Diet on Anthropometric Measurements and Diet Quality in Overweight and Obese Adolescent Girls

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ABSTRACT

Objective: This study was conducted to evaluate the effect of an online nutrition training program about the Mediterranean diet for 8 weeks in overweight and obese female high school students on anthropometric measurements and diet quality.

Methods: A total of 86 students between the ages of 14-18 years were included in the study, and they were divided into two groups as nutrition training (n=44) and control groups (n=42). The Mediterranean Diet Quality Index (KIDMED) scores were calculated and anthropometric measurements of groups were taken by the dietician at the beginning and at the end of the study.

Results: The KIDMED score of the nutrition training group was 4.59±2.40 at the beginning, later on it increased to 7.43±2.57 after the program (p= .001). After the program, it was determined that the difference between the KIDMED scores of the nutrition training and control groups were statistically significant (p= .034), however the decrease in body weight (p= .09), BMI (p= .64), and waist circumference (p= .06) were similar between groups.

Conclusion: As a result of the study, it can be said that online nutrition training program about the Mediterranean diet for 8 weeks may affect the diet quality positively of overweight and obese female adolescents, but long-term programs should be planned to determine the effects on anthropometric measurements.

Keywords: Adolescent, anthropometry, diet, mediterranean diet, obesity

1. INTRODUCTION

Adolescent obesity is a major public health problem that is becoming more frequent in both emerging and industrialized countries, and it is linked to genetic, environmental, psychological, socioeconomic, and cultural variables. It has been stated that the prevalence of obesity in school age and adolescence has increased approximately 4.5 times in the last 40 years (1). In the 2016 report of the World Health Organization (WHO), 18% of children and adolescents aged 5-19 years were reported to be overweight and obese (2). According to the results of Turkey Nutrition and Health Survey-2017 (TBSA-2017), 34.0% of adolescents between the ages of 15-18 years in Turkey are overweight, 27.8% are obese and 3.7% are morbidly obese (3). While 18.6 percent of children and adolescents aged 9-18 living in the Turkish Republic of Northern Cyprus (TRNC) are overweight, 16.2% are obese (4).

Overweight and obese adolescents are at the risk of various health problems, including insulin resistance, hyperinsulinemia, type 2 diabetes, hypertension, hyperlipidemia, asthma, sleep apnea, chronic hypoxemia, orthopedic problems, depression and anxiety (1). Adolescent obesity is also associated with the child school absenteeism and reduced quality of life (5).

In order to minimize and control adolescent obesity, international and local policies and programs have been created and implemented all around the world. On the other hand, school-based intervention programs are frequently preferred to control obesity in adolescents (6-9).

Schools are ideal places to provide nutrition training for children and adolescents and also evaluate their nutritional status (10-12). In the period from pre-school to high school, comprehensive and regular nutrition training given by dietitians in schools is very important to improve nutritional status, academic performance and public health (8). In addition, nutrition training programs can help adolescents gain awareness and become more conscious individuals by providing the skills, social support and environmental reinforcement they need to adopt healthy eating behaviors (13).

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The goal of school-based nutrition training programs is to promote intake of vegetables, fruits, legumes, and whole grain foods while decreasing consumption of items rich in energy, saturated fat, and salt (8,14). These diet related suggestions are largely based on the Mediterranean diet (MD). The MD is widely considered as one of the healthiest diet models. With increased adherence to the MD, it is possible to protect and improve health against obesity and other chronic diseases (15,16).

In a cross-sectional study, 1643 adolescents aged 11-16 years were included and it was determined that the risk of being overweight and obese decreased by 30% with increasing adherence to the MD (17). In a recent study, it was emphasized that 8-week nutrition training about the MD given to adolescents aged 11-16 years, increases the level of nutrition knowledge and therefore that may be effective in preventing future health problems (18).

Today, in order to provide behavioral change, nutrition training using digital technologies comes to the forefront (19). In order to create a permanent behavioural change in adolescents, it is recommended that nutritional intervention programs to be made using innovative multimedia technology tools (20). Due to the intense use of computers and the internet both at school and outside of school (21), it is suggested that online nutrition training can be beneficial in increasing the level of nutrition knowledge, especially in overweight and obese adolescents (22).

This study was conducted to evaluate the effect of an online nutrition training program about the MD adaptation on anthropometric measurements and diet quality for 8 weeks in overweight and obese female high school students.

2. METHODS

2.1. Study Population and Desing

This case-control study was conducted between December 2019 and May 2020. The study included a total of 86 adolescent females aged 14-18 years who were overweight or obese (≥ 85th percentile) according to the WHO's Body Mass Index (BMI) percentile for age and gender (23), and who did not have any chronic health concerns other than obesity. Because of the higher frequency of food addiction, stress, and depression among obese adolescent girls, it was projected that this group would have a greater need for nutrition training (24).

Initially, the girls were evaluated with a questionnaire on age, nutritional habits, and adherence to the MD. Overweight and obese adolescent girls were analyzed in terms of age, anthropometric measures, energy, macronutrients, fiber intake, and mean KIDMED score. They were randomly separated into two groups, with no statistically significant difference between the groups (Table I). A simple randomization method was used in this study.

It was calculated that 86 cases, 43 in each group, would be needed for the study, assuming that the pretest-posttest data would be tested using the Wilcoxon signed-rank test, with an error probability of 0.05 and a statistical power of 80%. In the study, 44 participants were included in both groups but 2 people in the control group were excluded from the study because they could not be reached at the end of the study. The study was completed with 44 adolescents in the nutrition training group and 42 adolescents in the control group.

The purpose, plan, and reasons for the research were explained to the individuals and they were invited to participate in the study voluntarily. Written informed consent was obtained from the parents of all students included in the study. This study was deemed ethically appropriate by the Eastern Mediterranean University (EMU) Scientific Research and Publication Ethics Committee with the decision dated 8.11.2019 and numbered 2019/25-05. Since the study was carried out in a state high school in Famagusta District, official permission was also obtained from the General Secondary Training Department of the Ministry of National Training and Culture of the Turkish Republic of Northern Cyprus.

The dietitian provided nutrition instruction for the MD compliance to the nutrition training group for 45 minutes once a week for a total of 8 weeks. The control group was given general nutrition training for 1 hour, only once face to face, by the dietitian.

The nutrition training group received a nutritional via online nutritional information training for 8 weeks. Training content is divided by weeks and that is the MD and its importance (week 1), grains (week 2), vegetables and fruits (week 3), olive oil (week 4), fish (week 5), chicken, red meat, egg (6th week), milk and dairy products (7th week), legumes and nuts (8th week) respectively. A WhatsApp group has been created and the dietitian narrated a video and question-and-answer training were supplied to the nutrition training group via this group. Additionally, varied brief messages about the MD were distributed routinely over the WhatsApp group to reinforce the information, three days a week. In this eight-week study, the anthropometric measurements and adherence to the MD of both groups before and after the study were taken and evaluated by the dietitian. In addition, the amount of variation between the body weight loss and other anthropometric measurements of the individuals at the end of 8 weeks was also calculated.

2.2. Anthropometric Measurements

The body weight of the individuals was taken with a standard digital scale while they wear thin clothes and without shoes. The height measurement of the individuals was taken with a non – flexible tape measure on the fronkfort plane with paying attention to the fact that their feet were united. Waist circumference was measured from the midpoint between the lower rib bone and the cristailiac with a non-flexible tape measure parallel to the ground. The measurement of hip circumference was also measured by standing on the side of the person and measuring from the highest point of the hip

(25). In addition, individuals' BMI value, waist/hip ratio and waist/height ratio were calculated.

2.3. Energy and Nutrient Intake

Foods consumed by individuals for 3 consecutive days (2 weekdays, 1 weekend) were questioned by 24-hour food consumption recording method. Energy, macronutrient and fiber intake were calculated with the Nutrition Information System (BEBIS 8.2) program.

2.4. Mediterranean Diet Quality Index (KIDMED)

The Mediterranean Diet Quality Index (KIDMED) was used to evaluate adherence to the MD and diet quality. This index is a simple tool developed and widely used to evaluate the nutritional habits and nutritional status of children and adolescents (26,27). Also, diet quality in children and adolescents can be measured using the KIDMED index (27). KIDMED consists of 16 yes-no questions; For the 6th, 12th, 14th and 16th questions, yes answers are scored as – 1 point, while yes answers for other questions are scored as +1 point. As a result, the total score ranges from – 4 to 12. In the present study, adherence to the MD was classified into three levels based on KIDMED scores: score 8-12, optimal adherence to the MD; score 4-7, average adherence to the MD; and score ≤3, very poor adherence to the MD (26).

2.5. Statistical Analyses

The data of the research were analysed using the Statistical Package for the Social Sciences (SPSS) 20.0 program. In descriptive statistics, frequency and percentagewere used for categorical variables, and mean, standard deviation (SD) lower and upper values were used for numerical variables. The normality of numerical variables to normal distribution was evaluated using Kolmogorov-Smirnov and Shapiro-Wilk tests. Pearson Chi-Square test was used to compare categorical variables between groups. To compare the numerical variables, the control group and the nutrition training group were compared before and after the training, the dependent samples t-test (Paired samples t-test) and the independent samples t-test (Student t-test) was used for the comparisons between the groups. Hypotheses were tested at 5% significance level.

3. RESULTS

This research was conducted with a total of 86 overweight and obese female high school students aged 14-18 years, 44 in the nutrition training group and 42 in the control group. Age, energy, macronutrient and fibre intake, anthropometric measurements and KIDMED scores of the adolescents included in the nutrition training group and the control group were similar (Table 1).

Table 1. *Comparison of age, energy, macronutrient intakes, anthropometric measurements and KIDMED scores of adolescents*

Energy and Nutrients	Control Group (n=42)		Nutrition Training Group (n=44)		p
	$\bar{X}$ ± ss	min-max	$\bar{X}$ ± ss	min-max	
Age +	16.28 ± 0.21	15-18	16.37 ± 0.32	14-18	0.88
Macronutrient intake +					
Energy (Kcal)	1560.09 ± 452.33	1028.43-2255.50	1748.48 ± 302.73	1171.75-2170.32	0.08
Protein (%)	20.33 ± 2.57	16.03-21.22	19.13 ± 3.10	14.01-27.05	0.07
Protein (g)	79.28 ± 12.90	41.20-119.65	83.59 ± 14.07	41.21-124.06	0.09
Fat (%)	35.47 ± 5.61	20.03-47.88	38.87 ± 6.02	21.02-46.32	0.06
Fat (g)	60.66 ± 16.11	23.87-117.99	75.39 ± 18.4	27.59-111.19	0.06
CHO (%)	44.13 ± 6.58	37.11-63.01	41.98 ± 6.58	35.69-60.88	0.06
CHO (g)	172.10 ± 16.16	95.09-330.72	178.18 ± 20.96	101.29-325. 20	0.08
Fiber (g)	16.15 ± 3.39	8.89-25.76	16.41 ± 2.52	10.20-23.21	0.16
Anthropometric measurements +					
Height (cm)	161.81 ± 7.04	150.0-178.0	161.48 ± 6.69	150.0-184.0	0.84
Body Weight (kg)	74.96 ± 9.91	58.0-103.5	74.69 ± 9.60	60.0-109.5	0.83
BMI (kg/m²)	28.53 ± 2.71	24.4-35.4	28.56 ± 2.90	23.0-37.7	0.82
Waist circumference (cm)	82.06 ± 8.86	64.0-98.0	82.54 ± 9.10	67.0-106.0	0.99
Hip circumference (cm)	99.25 ± 10.10	81.0-122.5	101.68 ± 10.38	83.0-126.0	0.32
Waist/Hip ratio	0.82 ± 0.07	0.65-0.94	0.80 ± 0.07	0.64-0.98	0.22
Waist/Height ratio	0.50 ± 0.05	0.41-0.62	0.51 ± 0.06	0.43-0.70	0.59
KIDMED +					
Overall score	5.15 ± 2.45	0-9	4.59 ± 2.40	0-10	0.22
BMI percentile classification by age, n (%) *					
Overweight (85-95th percentile)	15 (35.7)		25 (55.5)		0.06
Obese (>95th percentile)	27 (64.3)		20 (44.5)		

$\bar{x}$: Mean, sd: Standard deviation

+ Independent sample t-test was used for comparison between groups.

**Pearson Chi-square test was used for comparisons between groups*

When the adherence to the MD was evaluated, 26.2% and 19.0% of the adolescents included in the control group showed poor and optimal adherence to the MD before the training. While 11.9% and 28.6% of them achieved poor and optimal adherence to the MD after the training, respectively. In the nutrition training group, 31.8% and 11.4% of the adolescents showed poor and optimal adherence to the MD before nutrition training; 20.4% and 43.2% of them achieved poor and optimal adherence to the MD after training respectively (Table 2).

There was no statistically significant difference in the anthropometric measurements of the adolescents included in both groups after the training compared to the values before the training (p> .05). In addition, the anthropometric measurements of the adolescents included in the nutrition training group and the control group after the training were similar (p> .05). In addition, the KIDMED score of the nutrition training group was 4.59±2.40 before the training and 7.43±2.57 after the training. The KIDMED score of the control group was 5.15 ± 2.45 before the training and 6.26±2.15 after the training. There was a statistically significant difference in the KIDMED scores of both groups obtained before and after the training (p= .001). In addition, after the training, the difference between the pre – and post-training KIDMED scores of the adolescents in both groups was found to be statistically significant (p= .026) (Table 3).

The decrease in body weight (p= .09), BMI (p= .064) and waist circumference (p= .06) of the adolescents included in both groups after the training was found to be statistically similar. In addition, the increase in the KIDMED scores of the adolescents included in the nutrition training group after the training was found to be higher than the control group (p= .034) (Table 4).

Table 2. *Comparison of KIDMED score distributions of both groups before and after training*

		Control Group				Nutrition Training Group			
		BT (n=42)		AT (n=42)		BT (n=44)		AT (n=44)	
		n	%	n	%	n	%	n	%
KIDMED score	Low adherence	11	26.2	5	11.9	14	31.8	9	20.4
	Medium adherence	23	54.8	25	59.5	25	56.8	16	36.4
	Optimal diet quality	8	19.0	12	28.6	5	11.4	19	43.2
	p_1	0.06							
	p_2	**0.039***							
	p_3	**0.011***							
	p_4	**0.024***							

BT:before training, AT:after training
p1: difference between nutrition training and control groups BT
p2:control group BT-AT difference
p3: nutrition training group BT-AT difference
p4: difference between nutrition training and control groups AT
Pearson Chi-square test was used for comparisons between all groups.
** p < 0.05*

Table 3. *Evaluation of anthropometric measurements and KIDMED scores of adolescents included in both groups*

	Control Group n=42			Nutrition Training Group n=44			
	BT	AT	p_1	BT	AT	p_2	p_3
	± ss	$\bar{x}$ ± ss		$\bar{x}$ ± ss	$\bar{x}$ ± ss		
Anthropometric measurements							
Height (cm)	161.81 ± 7.04	161.94 ± 7.05	0.99	161.48 ± 6.69	161.49 ± 6.70	0,99	0.84
Body Weight (kg)	74.96 ± 9.91	73.59 ± 10.57	0.47	74.69 ± 9.60	73.19 ± 9.87	0.59	0.90
BMI (kg/m²)	28.53 ± 2.71	28.02 ± 2.91	0.39	28.56 ± 2.90	28.04 ± 2.85	0.53	0.75
Waist circumference (cm)	82.06 ± 8.86	81.52 ± 8.98	0.69	82.54 ± 9.10	81.63 ± 9.37	0.63	0.95
Hip circumference (cm)	99.25 ± 10.10	98.83 ± 10.45	0.80	101.68 ± 10.38	100.91 ± 10.50	0.74	0.38
Waist/Hip ratio	0.82 ± 0.07	0.82 ± 0.07	0.68	0.80 ± 0.07	0.80 ± 0.07	0.82	0.30
Waist/Height ratio	0.50 ± 0.05	0.51 ± 0.07	0.73	0.51 ± 0.06	0.50 ± 0.05	0.41	0.98
KIDMED							
Overall score	5.15 ± 2.45	6.26 ± 2.15	**0,001***	4.59 ± 2.40	7.43 ± 2.57	**0,001***	**0,026***

BT:before training, AT:after training, x̄: Mean, sd: Standard deviation
p1:control group BT-AT difference
p2:nutrition training group BT-AT difference
p3: difference between nutrition training and control group AT
p1-p2: Independent sample t-test was used for comparison between groups.
p3: Independent sample t-test was used for comparison between groups.
** p < 0.05*

Table 4. *Comparison of KIDMED, body weight, BMI and waist circumference differences of adolescents included in both groups after training*

	Control Group (n=42)		Nutrition Training Group (n=44)		p
	$\bar{x}$ ± ss	min-max	$\bar{x}$ ± ss	min-max	
KIDMED	1.11 ± 1.52	-1 – 6	2.84 ± 1.63	-2 – 7	**0.034***
Body Weight (kg)	-1.36 ± 1.24	-8 – 0	-1.50 ± 1.36	-6 – 0	0.09
BMI (kg/m²)	-0.51 ± 1.29	-3.5 – 0	-0.52 ± 1.19	-3.3 – 0	0.64
Waist circumference (cm)	-0.54 ± 1.03	-5 – 0	-0.91 ± 1.45	-6 – 0	0.06

Independent sample t-test was used for comparison between groups.
$\bar{x}$: Mean, sd: Standard deviation
** $p < 0.05$*

4. DISCUSSION

Nutrition training about the MD, which is given to adolescents regularly, increases the level of nutrition knowledge, thus it can be effective in reducing the risk of future health problems (15-18). In this study, it was determined that the nutrition training about the MD, which was given online regularly for 8 weeks to overweight and obese adolescent girls, improved diet quality compared to the general nutrition training given face-to-fact. However it had no effect on anthropometric measurements.

In another study, it was shown that in adolescents aged 10-14 years, the quality of diet measured with KIDMED was low, and the level of nutritional knowledge was associated with eating habits (28). The diet quality of children and adolescents aged 6-16 years in Italy was evaluated by KIDMED, showing that 16.7% had low, 63.7% moderate and 19.6% optimal diet quality (29). In a study that conducted with 1231 adolescents aged 12-17 years, it was determined that 30% of the adolescents had high and 15.7% of them had low MD quality. It was determined that 32.0% of the boys and 25.2% of the girls had a high MD quality (30). In a study conducted in Turkey, it was determined that 17.9% of adolescents aged 10-14 years had adhered to low, 59.2% had moderate, and 22.9% optimal diet quality. On the other hand, 18.4% of the girls found to have low, 56.8% moderate, 24.9% optimal diet quality (28). Recently conducted studies in Turkey with adolescents have been found that adolescents show Mediterranean diet at a low-moderate level (31,32). Consistent with these studies 26.2% of the adolescents included in the control group had low, 19.0% optimal diet quality, and 31.8% adolescents included in the nutrition training group had low, 11.4% optimal diet quality in this study (Table 2).

In a cross-sectional study conducted with 520 children aged 8-17 years, it was shown that children with normal body weight were more likely to adapt to the MD than those who were overweight and sedentary (33). Accordingly, while high body weight and BMI may negatively affect the quality of the MD (33), the diet quality measured by KIDMED in adolescents may also vary according to regional and cultural eating habits (34).

It is known that diet quality and the level of knowledge about healthy and proper nutrition in adolescence are quite low. The importance of implementing intervention programs to improve this situation is emphasized (35-38). Improving the level of nutrition knowledge and awareness in obese adolescents positively affects their nutritional behaviors and diet quality (36). Adolescence is an important period in which behavior and attitude development in health is ensured, and nutrition training programs carried out in schools contribute to the acquisition of correct eating habits (8). In the study conducted by Şahingöz et al. (18), it was found that 25.0% of the 11-16-year-old adolescents had low, 56.6% of them moderate and 18.4% of them had optimal KIDMED scores before the training. The training given for 18 hours for 8 weeks increased the KIDMED scores. It was determined that the score decreased from 6.06 ± 2.16 to 5.26±2.65 in the control group, increased from 5.20±2.45 to 11.84±0.52 in the experimental group, and finally, 100% of the experimental group reached the optimal diet quality score (18). In this study, the KIDMED score of the nutrition training group before the training was (4.59±2.40) and (7.43±2.57) after the training ($p< .05$); the KIDMED score of the control group was (5.15±2.45) before the training and (6.26±2.15) after the training ($p< .05$). In addition, the difference between the KIDMED scores of the adolescents included in the nutrition training group and the control group after the training was statistically significant ($p< .05$) (Table 3) besides the increase in the KIDMED scores of the adolescents included in the nutrition training group after the training was higher than the control group ($p< .05$) (Table 4). Similarly, it has been determined that the training given for 18 hours for 8 weeks in 11-16 years old adolescents increased KIDMED scores. For this reason, it is recommended to provide nutrition training from an early age and to increase the level of nutrition knowledge in order to minimize adult health problems (18).

Nutrition training programs given regularly in schools are of great importance in reducing the prevalence of obesity in adolescents (6-9). It is stated that the risk of being overweight and obese at school age decreases with the

increase in adherence to the MD (33). It is emphasized that it is important to improve the quality of diet in order for a significant decrease in BMI value and changes in body composition to occur in adolescents (39). In a recent study found that increasing adolescents MD like food choices led to a significant reduction in glucose and lipid profile. In relation to this, it has been emphasied to maintain a MD diet to increase adolescent health status (40).

As the prevalence of excessive weight gain and related diseases among young people increases, the frequency of developing and applying computer-based programs for nutrition training is also increases. Computer interventions have the potential to provide standardized and effective programs alongside rapid technology (22). It has been shown that internet-mediated obesity prevention programs in young people can provide significant improvement in nutritional behaviors (41,42). There was no statistically significant difference in the anthropometric measurements of the adolescents included in both groups after the training compared to the values before the training ($p > .05$). In addition, the anthropometric measurements of the adolescents included in the nutrition training group and the control group after the training were similar ($p > .05$) (Table 3). Likewise, the decrease in body weight, BMI and waist circumference of the adolescents included in both groups after the training was found to be statistically similar ($p > .05$) (Table 4).

It was determined that after the internet-mediated nutrition training program given to adolescents to prevent obesity, there was an improvement in healthy eating habits. However it had no effect on the decreases of obesity prevelance with an average age of 15.2 years (43). According to the results of different studies, it is stated that there is an improvement in food intake as a result of nutrition training but the effect on BMI is controversial (44,45). As a result of a systematic review of nutrition training programs conducted in schools, it was determined that nutrition training programs had a positive effect on obesity in 8 out of 24 studies including 25896 children, while 16 were ineffective (46). When the meta-analysis of the 11 studies conducted was examined, it was determined that there is no significant difference in BMI values when the nutrition training group and the control group compared as a result of the intervention programs carried out at schools (44).

Supporting components of nutrition training programs applied to adolescents such as curricula, method of application, length, and inclusion of the family in the program differ depending on the programs (44-46). It is emphasized that a significant decrease in BMI can be achieved, especially in programs lasting 1-2 years, however there is no significant difference can be observed between groups in intervention programs lasting less than 6 months (44). In a different study, it is suggested that at least 1 year of follow-up is required for a school-based online obesity prevention program to achieve a significant reduction in BMI (43).

Considering a study with different results from these findings, it is emphasized that the importance of including families in these programs during the implementation of behavior change programs for diet and physical activity in overweight and obese adolescents aged 13-16 years (39). According to a systematic review of randomized controlled trials, when nutrition training for 10-18 year-olds is theoretically based as part of training in schools and is incorporated into training by school staff, in concordance with parents and families as well as it includes changes in school canteens and cafeterias, it may have significant effects on adolescent nutrition (47). Multicomponent lifestyle interventions to adolescents in the school environment may provide a first step in behavior changes and provide grounds for future prevention programs in adolescent (48). In addition, it is also emphasized that it is important to maintain individual nutrition programs in order for a significant change in BMI in obese adolescents (29,49). Also individual programs in adolescents can be effective in reducing BMI and resolving nutrition related issues (49).

5. CONCLUSION

As a result of this study, it can be specified that nutrition training about the MD, which have been given online for 8 weeks to overweight and obese adolescent girls, can positively affect diet quality. However more comprehensive and long-term programs should be planned in order to be effective on anthropometric measurements. Among the limitations of the study may be indicated as this study covers a short-term nutrition training of 8 weeks and that it is not repeated after a certain period of time in order to determine whether the effect of the training continues. For this reason, it is recommended that the online nutrition training given to obese adolescent girls be repeated at regular intervals in order to have a positive effect on body weight. It is also recommended that similar extended studies have to be planned for boys and girls adolescents/children.

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Clinical and Experimental Health Sciences

Development and the Validity and Reliability Study of the Birth Health Belief Scale

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ABSTRACT

Objective: This study aims to develop a measurement tool based on the Health Belief Model to assess pregnant women's attitudes and beliefs about the mode of delivery.

Methods: A 65-item draft scale consisting of five sub-scales was used for the development of the Birth Health Belief Scale (BHBS). The draft scale was administered to 336 pregnant women. Data were analyzed using SPSS 18.0. Analyses included Kendall's W test, Cronbach's alpha, Kaiser Meyer Olkin (KMO) Test, Bartlett's Test, and Exploratory Factor analysis (Principal component analysis).

Results: Analysis results showed that the 5-point Likert scale consisted of 34 items and five factors. Cronbach's alpha coefficient was calculated as 0.974. Item analysis results revealed that the item-total and item-remainder correlations were significant ($p<0.001$).

Conclusion: The Birth Health Belief Scale was determined to be a valid and reliable measurement tool.

Keywords: Attitude, birth, belief, health belief model, scale.

1. INTRODUCTION

Preferred mode of delivery is one of the factors that play a role in the early diagnosis of risks for the woman and her baby and ensure a healthy outcome by performing appropriate interventions during pregnancy. Considering the mother's and baby's health, vaginal delivery should be preferred primarily (1). Cesarean delivery is preferred when the mortality and morbidity risk for the mother and/or baby is high in spontaneous vaginal delivery, when certain complications arise, or when spontaneous vaginal delivery is impossible (1-3). Since 1985, the World Health Organization (WHO) has stated that the optimum cesarean rate in all deliveries is 10-15% and reported that maternal and neonatal mortality and morbidity do not decrease if the cesarean rate is higher than this value. On the other hand, the frequency of cesarean delivery is rapidly increasing worldwide making it the most commonly performed major abdominal operation (1-6).

Although it is difficult to indicate a certain cause for the increase in cesarean section rates, medical, institutional, legal, psychological, and sociodemographic factors are known to contribute (7-9). On the other hand, the mother's desire is one of the main factors that contribute to the increase in cesarean section rates (8). For this reason, it is important to determine the common underlying reasons behind women's wanting or preferring cesarean section without the presence of medical reasons (10). The Health Beliefs Model is one of the most frequently used concepts for determining health-related individual factors, leading to positive health behaviors, and planning health trainings (11). The Health Belief Model, which is a motivation theory, has focused on understanding what motivates individuals for doing or not doing health-related actions (12,13). According to this model, behavioral changes require changing individuals' perceptions (14). Perceptions that have effects on health behaviors in the model include susceptibility perception, seriousness perception, benefits perception, barriers perception, and health motivation and self-efficacy perception. Susceptibility perception is the threat or risk perceived by the individual in her health condition; seriousness perception refers to how seriousness is perceived by the individual according to the outcomes of a disease; benefit perception refers to the perceived benefit for decreasing catching the diseases; barrier perception refers to perceived individual barriers for realizing the recommended health behaviors, and

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self-efficacy perception refers to the individual belief, efficacy, determinism, and self-confidence for realizing the health behavior to reach expected outcomes (13-16).

The Health Belief Model is frequently used in obstetrics and gynecology to help individuals acquire behaviors to protect and improve health. Today, the model has started to be used to determine women's mode of delivery preferences and the factors affecting these preferences (16-18). The literature includes no measurement tools based on the Health Belief Model on this issue. The purpose of this study is to develop a measurement tool based on the Health Belief Model for evaluating beliefs and attitudes of pregnant women about mode of delivery and to conduct a validity and reliability study of the measurement tool.

2. METHODS

2.1. Study Design

This methodological study was designed to develop a measurement tool based on the Health Belief Model in order to assess the attitudes and beliefs of pregnant women about mode of delivery. The study was carried out in two phases. While the first phase included the development of the draft form of the Birth Health Belief Scale (BHBS), the second phase included the evaluation of the psychometric properties.

2.2. Development of the Birth Health Belief Scale

The steps for developing a Likert-type attitude scale, which are listed below, were followed to prepare the scale.

2.2.1. Creating an Item Pool

After reviewing the pertinent literature, the researchers wrote positively keyed, negatively keyed, and neutral items to evaluate the attitudes and beliefs of pregnant women about mode of delivery considering the cognitive, affective and behavioral dimensions (2,8,12,15-19). Special attention was paid to ensure that the scale had the features representing the sub-scales of the Health Belief Model, that the statements were clear and understandable, and that they did not mean differently. Then a pool consisting of 65 items was created.

2.2.2. Receiving Expert Opinions

The items created by the researchers were presented to 10 professors in Obstetrics and Gynecology Nursing. Experts were requested to evaluate the statements presented to them and then rate them as 1=not relevant, 2=somewhat relevant (needs major changes), 3= quite relevant (needs minor changes), and 4=highly relevant. The content validity index (CVI) was found high as $\geq .932$ $p < .05$ and items with this feature were added in the pre-trial form. The responses given to the items in the perceived benefit, perceived barriers, perceived caring/severity, perceived self-efficacy and motivation, and perceived sensitivity sub-scales are rated from 1 to 5. The perceived disability and perceived sensitivity sub-scales of the 65-item draft scale are reverse scored.

2.2.3. Pre-Trial of the Scale

The questionnaire was pilot tested with 50 nulliparous pregnant women, independent of the study, by using the face-to-face interview technique to find out whether the questions were comprehensible and how long it takes to complete it. No changes were made in the questionnaires after the pre-trial.

2.2.4. Item Analysis

The item-total score correlations of each sub-scale of the draft scale were examined. Eight items (3,13,27,28,35,52,55,63) with item-total score correlations below 0.30 were removed from the scale, and the number of items was reduced to 57.

2.2.5. Target Population and the Sample

All pregnant women who applied to the Pregnancy Outpatient Clinic of Sivas Numune Hospital between August 2019 and January 2020 formed the target population of the study. In methodological studies, the sample size should be between five and ten times more than the number of items in the scale (19). The sample size was determined as 325 in the present study. The sample included 336 primigravida pregnant women who were literate and agreed to participate in the study.

2.3. Psychometric Evaluation of the Birth Health Belief Scale

The data were analyzed using the SPSS 18.0. The Kendall's W test was used for the content validity of the scale. The reliability of the scale was assessed using Cronbach's alpha, and item total score correlations were assessed for item reliability. Kaiser Meyer Olkin Test, Bartlett's Test and Exploratory Factor analysis were used to determine the construct validity of the scale.

2.4. Ethical Issues

Ethical approval was obtained from Sivas Cumhuriyet University Non-Invasive Clinical Research Ethics Committee (04.07.2019; 2019-07/35), and written permission was obtained from Sivas Provincial Health Directorate.

3. RESULTS

The mean age of pregnant women was 25.6 years. Of all the participating women, 40.8% had a bachelor's degree, 92% had a nuclear family, 52.1% did not work and 65.8% perceived their income level as moderate, 73.2% had planned pregnancy, 86.6% had no miscarriages previously, and 72% preferred vaginal delivery (Table 1).

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Table 1. *Sociodemographic characteristics of the participating pregnant women*

N=336	
	n (%)
Education	
Primary school	3 (0.9)
Junior High School	20 (6.0)
Senior High school	100 (29.8)
Associate's Degree	55 (16.4)
Bachelor's Degree	137 (40.8)
Master's Degree / Doctorate	21 (6.2)
Family type	
Nuclear family	309 (92.0)
Extended family	27 (8.0)
Employment status	
Employed	161 (47.9)
Not employed	175 (52.1)
Economic status	
Income less than expenses	40 (11.9)
Income equal to expenses	221 (65.8)
Income more than expenses	75 (22.3)
Is the pregnancy a planned one?	
Unplanned and unwanted	10 (3.0)
Unplanned but wanted	80 (23.8)
Planned and wanted	246 (73.2)
History of miscarriage	
Yes	45 (13.4)
No	291 (86.6)
Preferred mode of delivery	
Normal (Natural / Vaginal)	242 (72.0)
Cesarean section	94 (28.0)
Mean age/year	25.6±2.35

3.1. Construct Validity

The adequacy of the sampling and the suitability of the correlation matrix were tested before the factor analysis was performed. The result of Kaiser-Meyer-Olkin sampling adequacy test was .915. Bartlett's sphericity test result was X^2=31966.840, which is considered highly significant (p<.001). Exploratory factor analysis was performed to determine the construct validity of the scale. The varimax rotation method was conducted for the factor analysis.

Exploratory factor analysis of the scale items showed that the factor loads ranged between.63 and .85. The factors presented in the table are as follows: factor 1: perceived self-efficacy and motivation, factor 2: perceived benefits, factor 3: perceived sensitivity, factor 4: perceived caring / severity, and factor 5: perceived barriers.

According to the exploratory factor analysis results, confirmatory factor analysis (CFA) was performed to determine the compatibility of the five-factor structure with the predicted theoretical structure. Path diagram and goodness of fit criteria were examined for the evaluation of the CFA. Multiple fit indices were used for CFA. Evaluations included the goodness of fit index (GFI), the adjusted goodness of fit index (AGFI), comparative fit index (CFI), the root mean square error of approximation (RMSEA), and the root mean square residual (RMR). Analysis results indicated that the fit statistics calculated using the confirmatory factor analysis were at an acceptable level (Table 2).

Table 2. *Fit indices and acceptable index values of the final scale*

Index	Normal Value	Acceptable value	The scale's value
χ^2/SD	<2	<5	3.54
GFI	>0.95	>0.90	.90
AGFI	>0.95	>0.90	.91
CFI	>0.95	>0.90	.90
RMSEA	<0.05	<0.08	.59
RMR	<0.05	<0.08	.71

χ^2/SD: Chi Square/Standart Deviation; GFI: Goodness of Fit Index; AGFI: Adjusted Goodness of Fit Index; CFI: Comparative Fit Index; RMSEA: Root Mean Square Error of Approximation; RMR: Root Mean Square Residual

Figure 1 shows the path diagram of the Birth Health Belief Scale. Fit indices of the scale were found as χ^2/SD value= 3.54, GFI= .90, AGFI= .91, CFI= .90, RMSEA= .59 and RMR= .71. CFA indicated that all the fit indices of the Birth Health Belief Scale were adequate.

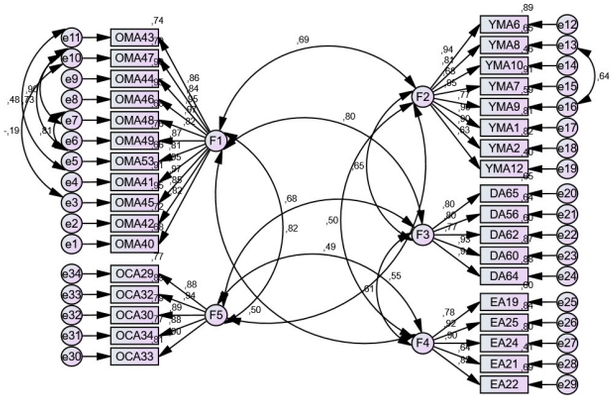

Figure 1. *Path diagram of the birth health belief scale*

OMA: Perceived Self-Efficacy and Motivation; OCA: Caring / Severity; YMA: Perceived Benefits; DA: Perceived Sensitivity; EA: Perceived Barriers

Regression coefficients among the sub-scale items created as a result of factor analysis were calculated by examining the item total score reliability coefficients. The analysis of the standardized coefficients revealed that the factor loads were high, standard error values were low, t values were significant (p <.001), and R^2 values were high.

3.2. Reliability Analysis

3.2.1. Cronbach's alpha internal consistency coefficient

Cronbach's alpha coefficient of the scale was calculated as .974. Cronbach's alpha value for each sub-scale was >.50 and thus reliability was sufficient. The internal consistency coefficient analysis results showed that the Birth Health Belief Scale was a highly reliable scale (Table 3).

Table 3. *Cronbach's alpha internal consistency values*

Sub-scales of the Birth Health Belief Scale	The number of the items	Cronbach's Alfa Value
Perceived self-efficacy and motivation	11	.977
Perceived benefits	8	.946
Perceived sensitivity	5	.932
Perceived caring / severity	5	.953
Perceived barriers	5	.909
Birth Health Belief Scale total	34	.974

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Table 4. *Test-retest results*

Groups	Lower %27	Upper %27	p	Test	Re-Test	p
	Mean±SD	Mean±SD		Mean±SD	Mean±SD	
Perceived Self Efficacy and Motivation	1.72±0.95	4.78±0.16	**.000**	3.77±1.47	3.81±1.46	.420
Perceived Benefits	3.41±1.22	4.87±0.16	**.000**	4.41±0.96	4.46±1.05	.350
Perceived Sensitivity	1.65±0.74	4.56±0.69	**.000**	3.63±1.57	3.68±1.56	.180
Perceived Caring / Severity	1.43±0.75	4.97±0.12	**.000**	3.39±1.65	3.42±1.70	.501
Perceived Barriers	1.80±1.30	4.75±0.34	**.000**	2.81±1.45	2.89±1.54	.102
Birth Health Belief Scale	2.08±0.68	4.79±0.12	**.000**	3.70±1.20	3.69±1.24	.820

SD: Standart Deviation

T-test conducted within the scope of the item analysis showed that the differences were statistically significant for all the groups (p<.001). These results demonstrated that the measurements done with this scale were sensitive enough to distinguish the differences. The test-retest method was used to measure time invariance of the scale and its sub-scales. This method is carried out by administering the scale to the same group twice at a 2-week interval. Test-retest was carried out with 40 participants. Test-retest analysis results indicated that the scale expressions were consistent (p>.05) (Table 4).

4. DISCUSSION

The basic step of the scale development is the conceptual and theoretical definition of the feature to be measured (20). The first phase of the scale development included reviewing the pertinent literature, which states that preparing 3 or 4 times more than the number of items required or if possible, even a higher number of items is useful for item analysis (20,21). Taking this into consideration, a 65-item draft of the scale was developed. In the second phase, experts were consulted to test the language and content validity of the 65-item draft scale. According to Özdamar (2016), the language and content validity is the feature of a scale to inspect the objectives determined concerning the subject. At least three experts are recommended to be consulted to confirm the content validity of the scale (22). In this study, the opinions of 10 experts were obtained considering the recommendations reported in the literature. Content validity evaluations performed using the statistical techniques consist of the stages of content validity ratio (CVR) and content validity index (CVI) (20). For the 10 experts, the CVR was.80 (22). Hence, as no items were below the minimum value of.80, no items were removed from the scale. The CVI obtained by calculating the average of the calculated CVR values of the 65 items was .932. This finding shows that the Content Validity of the remaining 65 items of the scale was statistically significant as CVI was greater than CVR. Statistically significant chi-square value of the Bartlett's test (p<.001) shows that data were suitable for factor analysis.

Load values of the items in the factor should be high. If the factor load of each item is less than.30 or the difference of the factor loads of the item in two different factors is less than.10, the item is removed from the scale and the analysis process is continued (23). Therefore, in order to ensure that the scale is more reliable, the minimum value of the factor load was determined as .45, and the items with a factor load below .45 were excluded from the analysis. Consequently, 23 items were excluded from the scale. Exploratory factor analysis of the scale items showed that factor loads ranged between.63 and.85 and were collected under 5 factors with a variance of 83.056%. These factors were determined as the sub-scales of the Health Belief Model: perceived self-efficacy and motivation, perceived benefits, perceived sensitivity, perceived caring/severity, and perceived barriers. The higher the variance ratio is, the stronger the factor structure of the scale is. The perceived self-efficacy and motivation sub-scale which consists of eleven items (1-11) assesses the pregnant woman's belief in spontaneous vaginal delivery. The perceived benefits sub-scale consisting of eight items (12-19) assesses to what extent the pregnant woman is aware of the benefits of the spontaneous vaginal delivery in terms of her health. The perceived sensitivity sub-scale consisting of five items (20-24) assesses to what extent the pregnant woman is at risk in terms of not having vaginal delivery and wanting to have cesarean delivery. The perceived caring/severity sub-scale consisting of five items (25-29) assesses the individual threat causing the person not to have spontaneous vaginal delivery. The perceived barriers sub-scale consisting of five items (30-34) assesses the perceived barriers to a healthy and successful spontaneous vaginal delivery. There are a limited number of studies in the literature evaluating the effect of women's mode of delivery preferences using the Health Belief Model. Loke et al. (2015) reported that action cues, utility, and perception of seriousness from Health Belief Model components affect women's decision about the mode of delivery (17). Hassani et al. (2016) determined that the Health Belief Model based the education program positively affected women's awareness and perception of choosing the safest mode of delivery (24).

If a scale is accepted as valid, then it should be tested for its reliability (23). The scale is considered not reliable if Cronbach's alpha value is 0.00<0.40, has low reliability if it is 0.40<0.60, reliable if it is 0.60<0.80, and highly reliable if it is 0.80<1.00 (21). The Cronbach's Alpha value of this scale we developed is 0.974, which indicates that the scale has high reliability.

After the Pearson moments correlation coefficients conducted to test the item validity were calculated for the item residual and item total analyses, all the items in the scale were considered to have a significant relationship at the level of 0.00 with the total score. In the process of item distinctiveness, the difference between the item average scores of the lower group (lower 27%) and the upper group (upper 27%) determined according to the total scores of the test was compared using the independent samples t-test, and the item discrimination indices of each item yielded statistically significant results at the level of 0.005. Item total analysis showed that the correlations of all the items were positively significant.

The second approach in determining the consistency of the measurements is the calculation of the correlation between two measurements performed by giving a test to the same individuals under the same conditions at a certain time interval. This method is called the test – retest method (23). The Pearson correlation coefficient between the scores obtained from these two tests is calculated. The correlation coefficient should not be below.70 (21). Pearson correlation analysis performed to calculate the test – retest (external consistency) values indicated significant relationships between the two administrations of all the items. This finding shows that the scale is reliable.

5. CONCLUSION

In conclusion, it was determined that the Birth Health Belief Scale was a valid and reliable measurement tool. The BHBS was developed for nulliparous pregnant women who had never given birth. In line with these results, it is recommended that the scale be used as a reliable tool in determining the level of belief and tendency towards vaginal birth in all nulliparous pregnant women.

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Clinical and Experimental Health Sciences

Postgraduate Dental Students' Knowledge Levels Toward Medication-Related Osteonecrosis of the Jaws

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ABSTRACT

Objective: Medication-related osteonecrosis of the jaws (MRONJ), is often described as a side-effect of bisphosphonates within the dental school curriculum. However, as highlighted in the current literature, some antiresorptive and antiangiogenic drugs may also cause MRONJ. This study aimed to investigate the awareness and knowledge of post-graduate dental students (PDSs) from different specialty/doctoral programs towards MRONJ.

Methods: An electronic questionnaire containing 28 questions in 3 different sections focusing on demographic characteristics, general information, and clinical attitude, was prepared. Two-thousand PDSs from 27 universities were invited to participate in the survey in December 2021. The obtained data were evaluated statistically using descriptive statistics and the Chi-Square test (p=.05).

Results: The response rate of the survey was 10%. The number of PDSs showed a homogeneous distribution for each specialty, whereas the number of women participants was higher than that of men (p<.05). In the general information section, the highest correct answer rates belonged to students from Oral and Maxillofacial Surgery and Oral and Maxillofacial Radiology departments. However, there was no statistically significant difference between different dental specialties regarding correct answer rates (p>.05). PDSs had higher rates of correct answers to general information questions about antiresorptive drugs than for antiangiogenic drugs. 92% of participants stated that they obtained their knowledge about MRONJ from their undergraduate education.

Conclusion: The findings of this study reveal the necessity of updating the dental school curriculum in line with the current literature on MRONJ, as well as including more postgraduate courses on MRONJ during the specialty/doctoral education period.

Keywords: Bisphosphonate-associated osteonecrosis of the jaw, dental education, dental students, medication-related osteonecrosis of the jaws, postgraduate dental students

1. INTRODUCTION

Medication-related osteonecrosis of the jaws (MRONJ) is an adverse effect characterized by the progressive destruction of the jawbones caused by drugs used in the treatment of metabolic bone diseases, rheumatologic diseases, and some cancer types (1). After it had first been reported as a bisphosphonate-induced side-effect in 2003, the American Association of Oral and Maxillofacial Surgeons (AAOMS) named this adverse effect as bisphosphonate-related osteonecrosis of jaws (BRONJ) in 2009 (2,3). However, the term BRONJ was later updated as MRONJ in consequence of the recent case reports presenting that the other antiresorptive drugs such as denosumab and some antiangiogenic drugs may have similar side-effects and cause destruction of the jawbones (4).

Even though the side-effect of antiresorptive and antiangiogenic drugs on bone tissues concerns the entire skeletal system, MRONJ tends to be observed more frequently in jawbones because of their higher vascularization rate, remodeling capacity, and turn-over speed (5). Moreover, since the infectious agents in the oral cavity can easily spread to the jawbone through teeth, gingival grooves, and extraction cavities, the jawbones are more prone to the development of MRONJ (6). Therefore, eliminating the focal infections and gaining the optimum health of oral tissues before antiresorptive and antiangiogenic therapy is of great benefit in preventing the development of MRONJ (5). In agreement with this statement, the AAOMS emphasizes the importance of consulting the patients with dentists and maintaining oral hygiene before the antiresorptive or antiangiogenic therapy (4). Hence, it is critical to increase the

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awareness and knowledge of dentists about antiresorptive, antiangiogenic drugs and the development of MRONJ, as well as evaluate their attitudes and behaviors toward these patients. Considering that a multidisciplinary approach has the utmost importance in the prevention and treatment of MRONJ, it is also essential for dentists from different specialties to have equal knowledge levels about MRONJ.

In the literature, there are too many studies investigating the awareness levels of dentists and physicians toward MRONJ (7-12). However, only a few involve or focus on undergraduate dental students (UDSs) (13-15), and there is no study specifically for post-graduate dental students (PDSs). It is noteworthy that most studies are directed toward antiresorptive drugs (7,10,11,14,16,17), whereas a limited number of them address antiangiogenic drugs (8,9,12,15). To our knowledge, there are only three studies including PDSs as a part of questionnaire participants (18-20). However, in those studies, PDSs did not have a homogeneous distribution according to their fields of specialty, and the questions within the scope of the survey were only about bisphosphonates and antiresorptive drugs.

Therefore, this study aimed to investigate the awareness and knowledge of PDSs, who are currently studying in different specialty/doctoral programs, toward MRONJ with a questionnaire containing questions oriented to both antiresorptive and antiangiogenic drugs.

2. METHODS

This study was approved by the Institutional Scientific Research and Publication Ethics Board (No: 2022 – 1159) and was carried out by the Declaration of Helsinki. An online questionnaire was designed by three oral and maxillofacial radiologists with different years of experience, according to the position paper of AAOMS (4) and a review of the related studies (8,16). The questionnaire consisted of twenty-eight questions and three sections as followed: Demographic characteristics, general information about MRONJ, and clinical attitude.

In the demographic characteristics section, six questions were asked to the participants to record their gender, age, year of graduation, specialty/doctoral program and the university where they have been receiving their postgraduate education. In the general knowledge section, participants' knowledge of MRONJ was investigated by twelve questions focusing on the definition of MRONJ, risk factors related to MRONJ, antiresorptive and antiangiogenic drug indications as well as their mechanism of action and route of administration. In the last section, the clinical attitude of PDSs toward MRONJ patients and the source of their knowledge about MRONJ were questioned.

The questionnaire was first administered to a pilot group of 20 PDSs in order to verify the validity, reliability and comprehensibility of the questions. Later then, 2000 PDSs studying in different specialty/doctoral programs at 27 universities in Turkey were invited to participate in the online questionnaire.

The data obtained were evaluated statistically using IBM SPSS Statistics 20.0 (SPSS Inc., Chicago, IL). Descriptive statistics were used to analyze the answers given to the demographic characteristics section. In order to compare the data between PDSs from different fields of dental specialties, Pearson's chi-square test was used. For all data, a p value <.05 was considered significant.

3. RESULTS

3.1. Demographic Characteristics

Two hundred PDSs (147 women and 53 men) studying in 27 different universities with a mean age of 26.7 ± 2.7, were included in the questionnaire. The demographic information of the participants obtained from the first section is summarized in Table 1. Even though PDSs showed a homogeneous distribution for each specialty/doctoral program, the number of women participants was significantly higher than that of men (p<.05).

Table 1. *Demographic information of PDSs.*

Demographic characteristics		N (%)
Gender	Women	147 (73.5%)
	Men	53 (26.5%)
Age	23-24	23 (11.5%)
	25-30	167 (83.5%)
	31-35	5 (2.5%)
	35+	5 (2.5%)
Year of graduation	Before 2015	9 (4.5%)
	2015	9 (4.5%)
	2016	15 (7.5%)
	2017	34 (17%)
	2018	45 (22.5%)
	2019	55 (27.5%)
	2020	29 (14.5%)
	2021	4 (2%)
Specialty/doctoral program	Oral and Max. Surgery	27 (13.5%)
	Oral and Max. Radiology	30 (15%)
	Prosthodontics	23 (11.5%)
	Restorative Dentistry	29 (14.5%)
	Endodontics	27 (13.5%)
	Periodontology	21 (10.5%)
	Orthodontics	20 (10%)
	Pediatric dentistry	23 (11.5%)

PDSs: Post-graduate dental students

3.2. General Knowledge About MRONJ

PDSs studying in the Departments of Oral and Maxillofacial Surgery and Oral and Maxillofacial Radiology had the highest correct answer rates to the general knowledge questions. However, when the total correct answer rates of different specialty/doctoral program were compared, the differences were found to be statistically insignificant (p>.05).

Table 2a. *PDSs' general knowledge about MRONJ.*

General Knowledge Questions		N (%)
Do you have knowledge about MRONJ?	Yes No	190 (95%) 10 (5%)
Which of the following criteria is required for the diagnosis of MRONJ according to the AAOMS?† (more than one answer can be marked)	Current or previous treatment with antiresorptive or antiangiogenic agents No history of radiation therapy to the jaws or obvious metastatic disease to the jaws Exposed bone or bone that can be probed through an intraoral or extraoral fistula in the maxillofacial region that has persisted for longer than 8 weeks	152 (76%) 109 (54%) 196 (98%)
Which of the following procedures and situations are among the risk factors associated with MRONJ? (more than one answer can be marked)	Dentoalveolar surgery Anatomical region Prosthesis-related trauma Dental infection Tobacco/alcohol use Type of cancer Duration/dose of the antiresorptive drug Concomitant corticosteroid use with antiresorptive and/or antiangiogenic therapy	187 (93.5%) 150 (75%) 153 (76.5%) 148 (74%) 123 (61.5%) 104 (52%) 183 (91.5%) 138 (69%)
Which of the following drugs/treatments can cause MRONJ? (more than one answer can be marked)	Bisphosphonate Antiangiogenic drugs Denosumab Radiotherapy Bisphosphonate, antiangiogenic drugs and denosumab	197 (98.5%) 143 (71.5%) 117 (58.5%) 54 (27%) 83 (41.5%)
Which of the following disease(s) requires antiresorptive therapy? (more than one answer can be marked)	Osteoporosis Osteogenesis imperfecta Paget's disease Multiple myeloma Malignant tumor metastasis I don't know	185 (92.5%) 61 (30.5%) 97 (48.5%) 125 (62.5%) 135 (67.5%) 3 (1.5%)
Which of the following disease(s) requires antiangiogenic therapy? (more than one answer can be marked)	Osteoporosis Gastrointestinal tumors Renal carcinoma Neuroendocrine tumors Malignant tumor metastasis I don't know	39 (19.5%) 61 (30.5%) 89 (44.5%) 78 (39.5%) 132 (66%) 44 (22%)
What is the route of administration of antiresorptive/antiangiogenic drugs?	Only oral Only intravenous Only subcutaneous Oral and intravenous Oral, intravenous and subcutaneous	1 (0.5%) 0 0 160 (80%) 39 (19.5%)

†According to the 2014 position paper of AAOMS (4)

PDSs: Post-graduate dental students, MRONJ: Medication-related osteonecrosis of the jaws, AAOMS: American Association of Oral and Maxillofacial Surgeons

According to the results presented in Table 2a, 95% of PDSs had knowledge about MRONJ. On the other hand, when the PDSs were asked about the definition of MRONJ suggested by the 2014 position paper of the AAOMS, only 52.5% answered this question correctly by selecting all three diagnostic criteria of MRONJ. The most stated risk factors related to MRONJ were dentoalveolar surgery and duration/dose of the antiresorptive drug, whereas the least were smoking/alcohol use and type of cancer (Table 2a).

When the answers according to the drug groups causing MRONJ were evaluated, eighty-three (41.5%) PDSs who chose bisphosphonate, denosumab, and antiangiogenic drugs together were aware that these three drug groups may have this side-effect. However, 54 (27%) participants answered this question incorrectly by identifying radiotherapy as a possible cause of MRONJ. Furthermore, when the answers given to the question about the route of administration of antiresorptive/antiangiogenic drugs were examined, only 19.5% of the participants knew about subcutaneous administration as well as oral and intravenous administration of antiresorptive and antiangiogenic drugs (Table 2a).

Most PDSs (92.5%) knew the drug mechanism of antiresorptive drugs. The relation between the route of administration and the risk of developing MRONJ was correctly identified by 83% of the participants. On the other hand, only 29% of PDSs were aware of the association between the different diseases treated with varying doses of antiresorptive drugs and the risk of MRONJ (Table 2b).

Among the antiresorptive drug groups, zoledronate (92%) and alendronate (85.5%) were the most frequently known ones, whereas denosumab was chosen by 55.5% of PDSs. Bevacizumab (31.5%) was the most commonly known antiangiogenic drug (Table 3). While 57% of the PDSs stated that they did not know any antiangiogenic drug, the rate of this option for the antiresorptive drugs was only 5.5%. Moreover, the participants had more knowledge about indications of antiresorptive drugs (66.5%) than that of antiangiogenic drugs (32.5%). Thirty-nine (19.5%) PDSs incorrectly selected osteoporosis as an indication for antiangiogenic drug use (Table2a).

3.3. Clinical Attitude and Information Sources About MRONJ

Table 4 summarizes the clinical attitudes of PDSs towards MRONJ patients, as well as the information sources about MRONJ. 100% and 96% of PDSs agreed that a comprehensive oral and dental examination is necessary prior to antiresorptive and antiangiogenic therapy, respectively. However, only 38.5% of PDSs stated that a patient was referred to them for dental consultation before antiresorptive therapy; and the rate was even lower (14%) for antiangiogenic therapy. 92% of the participants obtained their knowledge about MRONJ from undergraduate dental education. While internet and books/journals were selected respectively by 38.5% and 38% of PDSs, the rate for postgraduate dental education was only 21.5%.

Table 2b. *PDSs' general knowledge about MRONJ.*

General Knowledge True/False Questions		N (%)
Antiresorptive drug use reduces osteoclast function and bone remodeling (True)	True False I don't know	185 (92.5%) 10 (5%) 5 (2.5%)
The risk of developing MRONJ is higher for orally administered antiresorptive drugs than for intravenously administered drugs (False)	True False I don't know	12 (6%) 166 (83%) 22 (11%)
Compared with cancer patients treated with antiresorptive drugs, osteoporosis patients have a lower risk of developing MRONJ (True)	True False I don't know	58 (29%) 60 (30%) 82 (41%)

PDSs: Post-graduate dental students, MRONJ: Medication-related osteonecrosis of the jaws

Table 3. *PDSs' familiarity with antiresorptive and antiangiogenic drugs.*

Antiresorptive drugs	N (%)	Antiangiogenic drugs	N (%)
I dont't know any	11 (5.5%)	**I dont't know any**	114 (57%)
Alendronate (Fosamax)	171 (85.5%)	**Bevacizumab (Avastin)**	63 (31.5%)
Ibandronate (Bonviva)	104 (52%)	**Aflibercept (Zaltrap)**	12 (6%)
Zoledronate (Zometa)	184 (92%)	**Sunitinib (Sutent)**	21 (10.5%)
Risedronate (Actone)	55 (27.5%)	**Sorafenib (Nexavar)**	15 (7.5%)
Pamidronate (Aredia)	68 (34%)	**Temsirolimus (Torisel)**	33 (16.5%)
Clodronate (Bonefos)	50 (25%)	**Everolimus (Certican)**	22 (11%)
Denosumab (Prolia)	111 (55.5%)		

PDSs: Post-graduate dental students

Table 4. *PDSs' clinical attitude and information resources regarding MRONJ.*

Clinical attitude questions		N (%)
Is a comprehensive oral and dental examination necessary prior to antiresorptive therapy?	Yes No I don't know	200 (100%) 0 0
Is a comprehensive oral and dental examination necessary prior to antiangiogenic therapy?	Yes No I don't know	192 (96%) 2 (1%) 6 (3%)
Should patients who are planned to use antiresorptive drugs be informed about the importance of oral and dental health?	Yes No I don't know	199 (99.5%) 0 1 (0.5%)
Should patients who are planned to use antiangiogenic drugs be informed about the importance of oral and dental health?	Yes No I don't know	195 (97.5%) 1 (0.5%) 4 (2%)
Is it necessary to consult a medical doctor before dental treatment of the patients using antiresorptive drugs?	Yes No I don't know	198 (99%) 0 2 (1%)
Is it necessary to consult a medical doctor before dental treatment of the patients using antiangiogenic drugs?	Yes No I don't know	197 (98.5%) 0 3 (1.5%)
Has a patient been referred to you for a dental examination prior to antiresorptive therapy by a medical doctor?	Yes No	77 (38.5%) 123 (61.5%)
Has a patient been referred to you for a dental examination prior to antiangiogenic therapy by a medical doctor?	Yes No	28 (14%) 172 (86%)
Are you familiar with international guidelines on risk factors and management of MRONJ?	Yes No I don't know	74 (37%) 96 (48%) 30 (15%)
Information resources	Undergraduate dental education Postgraduate dental education Congress/Seminar Internet Books/Journals National guidelines Other	184 (92%) 43 (21.5%) 57 (28.5%) 77 (38.5%) 76 (38%) 5 (2.5%) 10 (5%)

PDSs: Post-graduate dental students, MRONJ: Medication-related osteonecrosis of the jaws

4. DISCUSSION

The term osteonecrosis of jaws (ONJ) was first included in the undergraduate dental curriculum as a side-effect of bisphosphonate drugs in the 2006-2007 academic year and gained more importance with the AAOMS 2009 position paper on BRONJ (3,18). After the 2014 update of the AAOMS (4), which highlighted denosumab and antiangiogenic therapies as possible causes of osteonecrosis of the jaws, these drug groups also began to be included in the dental curriculum. According to Deveci and Uğar Çankal (18), the revised undergraduate curriculum in line with the AAOMS reports has a direct relationship with the knowledge levels of dentists about MRONJ. In agreement with this statement, previous studies suggest that younger dentists tend to be more knowledgeable about MRONJ, since MRONJ-related dental education is a recent concept in the undergraduate dentistry curriculum (7,16,21).

Ekmekcioglu et al (9) conducted a questionnaire among general dentists and specialists and showed that the latter were significantly more aware of MRONJ. With respect to their result, they concluded that postgraduate dental training is an effective factor for the dentists' knowledge levels about MRONJ. Similar to the mentioned study, Alhussain et al (21) reported that specialist dentists were more knowledgeable about MRONJ than general dentists. Concerning the aforementioned investigations, the present study only included recently graduated dentists who were currently under specialty/doctoral training in order to evaluate and compare the knowledge levels of different dental specialties using a homogeneously distributed group of participants.

To our knowledge, there are only three investigations involving PDSs among participants (18-20). Sahin (19) and Patil et al

(20) divided respondents into two groups and evaluated correct answers based on dentists' experience in surgical procedures and different years of experience, respectively. However, a comparison between different specialty/doctoral programs was not performed in both investigations (19,20). Although Deveci and Uğar Çankal (18) compared the correct answer rates between different dental specialties in their study, the participants did not show a homogeneous distribution according to their specialty/doctoral programs. Moreover, all three studies focused on antiresorptive drugs, thus awareness and knowledge level toward antiangiogenic drugs was not questioned (18-20). Therefore, to the authors knowledge, this is the first study evaluating the awareness and knowledge of PDSs from different specialty/doctoral programs towards MRONJ with a questionnaire covered both antiresorptive and antiangiogenic drugs.

The results of the present study revealed that oral and maxillofacial radiologists and surgeons were able to achieve higher correct answer rates than other dental specialties. Even though our result was statistically insignificant ($p>.05$), many studies agreed that oral and maxillofacial surgeons are more knowledgeable about MRONJ (19,21-23). This can be explained by the fact that MRONJ patients are encountered more frequently in university dental hospitals (22), and the mentioned specialties particularly have more experience with such patients. The reason for mentioning only oral surgeons regarding the high level of MRONJ knowledge in the previous questionnaires may be the absence (21-23) or the small number (18) of oral and maxillofacial radiologists in such studies. More investigations involving an equal number of participants from all dental specialties may be helpful to determine the actual knowledge levels of different specialties.

As observed in the current research, almost all PDSs knew the term MRONJ, but only half of them were able to identify the MRONJ definition described by the AAOMS. Even though this indicates a lack of knowledge among recently graduated dentists, the percentage obtained in the present study was still higher than that of the previous studies conducted with general and specialist dentists. Al-Eid et al (8) showed 35.1% of Saudi Arabian dentists knew the correct definition of MRONJ, while Almousa et al (12) reported a rate of 28.1%. Considering studies involving PDSs, Patil et al (20) stated 35% of the respondents were able to recognize the MRONJ definition. Despite the higher level of knowledge in the current study, it is alarming that only 54% of PDSs were confident that "no history of radiation therapy to the jaws or obvious metastatic disease to the jaws" (4) was a necessary criterion for MRONJ diagnosis. Consistent with this result, it is also worrying that 27% of respondents in the present research accepted radiotherapy as an MRONJ-causing treatment, and this rate was even higher (59.5%) in the study of Al-Eid et al (8).

Besides the diagnostic criteria of MRONJ, awareness of MRONJ-related risk factors is also critical for the management and treatment strategies for these patients. According to a Saudi Arabian research, the most cited risk factor regarding MRONJ was dentoalveolar surgery, consistent with the results of the present study, while corticosteroid therapy was identified only by 13.5% (8). Similarly, in a French study, less than a third (29.7%) of the participants were aware that concomitant corticosteroid therapy may increase the risk of developing MRONJ (16). In contrast to these investigations, the awareness level in the present study was found much higher (69%), indicating that corticosteroids have adequate importance within the MRONJ-related dental curriculum of Turkey. Almousa et al (12) reported that tobacco use was the most stated risk factor with a percentage of 52.5% in their study, which included both dentists and dental students. Additionally, they also observed that dentists were significantly more aware of smoking as a risk factor compared to students (12). Arnaud et al (16) conducted a questionnaire among general and specialist dentists, most of whom were over 30, and obtained a remarkably higher rate (92.1%) about this topic. According to the results of the present study, in which participants mainly were under the age of 30, 61.5% of PDSs identified tobacco use as a risk factor. Even though more than half of PDSs were aware of this risk factor, the percentage was still lower than that of Arnaud et al (16), which points to a possible lack of knowledge among younger dentists about this topic. A similar conclusion can also be reached for another risk factor, cancer type, considering only 52% of PDSs accepted it as an MRONJ-related risk factor.

In the questions where the indications of antiresorptive and antiangiogenic drugs were asked, the most cited indication for antiresorptive therapy was found as osteoporosis, whereas for antiangiogenic therapy as malignant tumor metastasis. Previous studies reporting osteoporosis as the most recognized therapeutic indication for bisphosphonates confirm this conclusion (15,18). Since the therapeutic indication questions in the current study had more than one correct answer, participants who chose three or more correct indications were accepted to have given the correct answer. Accordingly, two-thirds (66.5%) of respondents were able to answer this question correctly. On the other hand, only 14.5% of PDSs could identify all the diseases treated with antiresorptive therapy.

The most commonly known antiresorptive drugs in the current study were alendronate and zoledronate, consistent with the previous studies (9,12,13). It was a reasonable result, since physicians who prescribe antiresorptive medication usually prefer the aforementioned drug groups (24). Considering dentists' familiarity level of denosumab, two similar studies held in Spain and Turkey, including both general dentists and specialists, reported different knowledge rates, 61.7% and 18.4%, respectively (7,9). In the present questionnaire, in which all the participants were under specialty/doctoral training, 58.5% of PDSs were able to identify denosumab. It is noteworthy that this rate is closer to the results of the research from Spain, rather than the research from Turkey, where more than half (74.5%) of the respondents had no specialty/doctoral education (9). Even though this implies the effectiveness of postgraduate dental education, according

to the results of the present study, the primary source of information on MRONJ was found as undergraduate dental education with a rate of 92%. This finding was in agreement with the previous investigations (16,18,19). It is important that only 21.5% of PDSs identified postgraduate education as an information resource, which emphasizes the major role of undergraduate training in MRONJ knowledge.

Many studies in the literature acknowledge that recently graduated younger dentists are more knowledgeable about MRONJ, regardless of their field of specialty (7,16,21). However, the results of the present study revealed that even after eight years of the AAOMS updating the term BRONJ as MRONJ, PDSs are still more confident in their knowledge of antiresorptive drugs. This also highlights that although younger dentists have become more aware of MRONJ over the years (18), they still have insufficient knowledge about antiangiogenic drugs (12,16). On the other hand, the fact that only a few of the respondents (14.5%) in the current study identified all indications of antiresorptive therapy is thought-provoking about the adequacy of dental education regarding antiresorptive drugs.

The questionnaire in the present study was conducted based on the 2014 position paper of AAOMS (4), as there was no new update available at the time of the study design. Recently, the AAOMS released a 2022 update that includes changes in MRONJ definition criteria, medications, and management strategies (25). In this study, PDSs were asked about the 2014 definition of MRONJ, and the survey did not include a novel monoclonal antibody, Romosozumab, among drug familiarity questions. Therefore, this creates a limitation for the present study. Further investigations based on the AAOMS 2022 position paper, questioning the revisions about diagnosis and management strategies may be helpful to enlighten how undergraduate and specialist/doctoral education curricula overlap with the current literature.

5. CONCLUSION

The present investigation is of great importance in the following respects: It is the first study in which the participant group consisted only of PDSs as well as the questionnaire covered both antiresorptive and antiangiogenic drugs. Additionally, PDSs had a homogenous distribution according to their specialty/doctoral programs, which allowed us to compare different fields of dental specialties in terms of knowledge level about MRONJ. Although oral and maxillofacial radiologists and surgeons were found to be more knowledgeable, there was a remarkable lack of knowledge across all specialties, particularly with regard to antiangiogenic drugs. Therefore, the authors of this study suggest that both undergraduate and postgraduate dental education should be revised in line with the updated literature on MRONJ.

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Acquisition of data for the study: EA
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Clinical and Experimental Health Sciences

Research on the Determination of the Physicochemical and Sensory Characteristics of the Wine Produced by Malolactic Fermentation from *Sauvignon Blanc, Merlot* and *Kalecik Karası* Grapes

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ABSTRACT

Objective: The aim of this study is to examine how malolactic fermentation and classical fermentation affect the physicochemical and sensory properties of wines made from red and white grapes.

Methods: In our research, we opted for Sauvignon blanc variety in white grapes and locally produced Kalecik karası and French Merlot variety in red grapes all of which are recognised as important grape varieties both in our country and around the world. To preserve the natural aroma of the grape, start the fermentation rapidly and ensure formation of a balanced amount of glycerol, the use of Saccharomyces cerevisiae strain among vine yeasts deemed suitable. Oenoccus oeni MBR®UVAFERM®BETA (2x1011CFU/g) (Lallemand Inc., France) strain was used for malolactic fermentation. Sensory analyzes of the produced wines, chromatographic analysis of organic acids, and pysico-chemical analyses of products which are formed as a result of the processing of the fruits, were also made. For this purpose, the panelists evaluated the wines according to seven different criteria and the evaluation was made on a nine-point hedonic scale, and the most liked sample was given 9 points and the least liked one was given 1 point. Chemical and sensory properties of the produced wines were evaluated statistically.

Results: As a result of our study, it has been determined that the amount of ash in red wines is higher which indicates higher amount of grape extracts obtained from Kalecik karası and Merlot grapes.When compared according to fermentation types, the amount of lactic acid increased in wines produced by malolactic fermentation whereas the amount of phenolic compounds was higher in ethyl alcohol concentrations, and these values decreased with malolactic fermentation. Additionally, the accommodation of two foreign origin grapes one of which is red and the other one white, country's geography has been revealed in this study with the characteristics of the wine produced.

Conclusion: The harmony of two foreign grapes, one red and one white grape, to the geography of our country was revealed with the characteristics of the wine produced. As a result of the thesis study, it was found that malolactic fermentation improved the quality of the wine, making it more pleasurable. In this context, the results of the research has the quality and attributes that will shed light on winemakers.
Keywords: Malolactic fermentation, phenolic compounds, wine, maceration.

1. INTRODUCTION

Vine is the resource material of wine and belongs to the *Vitis* genus of the *Vitaceae* Family. *Vitis* genus includes two species namely *V. vinifera* and *V. muscadinia* (1). Archeological excavations show that Anatolian peninsula is the homeland of the first vine tree, and it is known that wine has been produced in different ways since 4000 BC, spreading to the Hittites, Lydians and other civilizations (2). Fresh grapes can be divided into three groups according to their consumption patterns; table grapes, dried grapes, wine grapes (3). *Papazkarası, Öküzgözü, Boğazkere, Kalecik Karası, Pinot Noir, Gamay* and *Merlot* can be named among the grape varieties that give quality red wine. *Kalecik Karası* initially produced in Ankara later started to be produced in Thrace region as well. In our country *Kalecik Karası* and *Merlot* varieties attract increasing interest and *Kalecik Karası* and *Merlot* wines are sold at the highest prices in the markets (4). The main factors affecting the ripening of grapes and the composition of the wine are; grape variety, maturity state, ecological factors, nurturing, diseases and presence of damage. Too high or too low air temperature during ripening, excessive or insufficient rainfall, or excessive irrigation reduce the synthesis of phenol compounds. In addition, the amount of phenol components in red wines is 20-25 times more than white wines. The reason why red wines are rich in phenol components is the pulp fermentation applied during the production process (5). Generally, alcohol fermentation, which is a biological process like all other fermentations, is used in winemaking. During fermentation, besides ethyl alcohol and carbon dioxide, low amounts of glycerin, acetic acid, acetaldehyde, high alcohols and many substances that affect the aroma of the wine produced are formed (6,7). Another fermentation applied to wines is malolactic fermentation, which is generally preferred for red wines; however it can also be applied in white wines with high acidity. During malolactic fermentation, malic acid (2-10 g/L) is reduced entirely and this causes the pH of the wine to rise and

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the taste to change, giving way to increased sensory properties and permanence. In addition, acetaldehyde, diacetyl and high alcohol contents also change, contributing to the aroma of the wine (8,9). The aim of this study is to investigate the effects of wine production methods by classical and malolactic fermentation on the sensory and physicochemical properties of the samples obtained, and to determine the physico-chemical composition and sensory properties of the samples obtained by both methods. In addition, the study is aimed to reveal the accommodation of two foreign origin grapes, one of which is red and the other white, to the geography of our country, by the characteristics of the wine produced.

2. METHODS

2.1. Collection of research material and processing into wine

Sauvignon blanc from white grape varieties, *Kalecik karası* of native origin and *Merlot* of France origin from red grape varieties were used as research material. The research materials were harvested on 10.10.2012 from vineyards of Tekirdağ Viticulture Research Institute (40° 58' 25' 'N – 27° 28' 53'' E, Altitude: 575m). Brix, acid, pH and density analyzes of the grapes were made prior to harvesting. At the end of the analysis, maturity indices were calculated and to proceed the harvest was decided. The harvest of the grapes, whose maturity was determined by performing raw material analyzes, was carried out in a controlled manner, and the samples were carefully collected from beginning, middle and end of the vineyard and each furrow. Raw material analyzes were carried out in the laboratory of Tekirdağ Viticulture Research Institute. Following the harvest of approximately 60 kg of each grape variety, the grapes were brought to a private wine establishment where the selection and removal process of bruised and dented grapes was initially carried out. Later, the stems were separated from the grain using a grape mill and the mash was prepared by cracking the grain. The following processes were applied for red and white grapes as shown in the wine production flow charts. White wine production flow chart is shown in Supplementary Figure 1. and the red wine production flow chart is shown in Supplementary Figure 2. After the grain cracking process, 25 mg/L potassium metabisulfite was added to the mash as a preservative before pressing and the mash was mixed again. To preserve the natural aroma of the grape, start the fermentation rapidly and ensure formation of a balanced amount of glycerol, the use of *Saccharomyces cerevisiae* strain among vine yeasts deemed suitable (10). In alcohol fermentation, *S.cerevisiae* N JB3 (Selection CIVAM cor 8) was used for white grapes, *S.cerevisiae* N 7303 (INRA NARBONNE) was used for red grapes, and *Oenoccus oeni* MBR®UVAFERM®BETA ($2x10^{11}$CFU / g)(Lallemand Inc., France) was used for yeast and malolactic fermentation. 5L, 10L and 15L glass and pet demijohns were used for the must fermentation. Viusal inspections of the fermentation process were monitored from fermentation heads attached to demijohns, and demijohns were preserved in temperature controlled rooms. 75 cL brown wine bottles were used as packaging material for the final product.

2.1.1. Fermentation of white grape

Hydraulic basket presses were used in pressing. In fermentation, the "first wort" flowing spontaneously without any applied pressure was combined with the "second must" obtained after the first pressing and the amount of must obtained was measured as approximately 35L. YEPD broth was used for activation of the culture, the culture was transferred to the broth under aseptic conditions and incubated for 48 hours under 28°C after being mixed. At the end of this period, the culture was grown up to the volume that will be used in fermentation in the same medium, then separated from the liquid part using centrifugation, re-suspended in 0.85% saline before inoculation and added to the vessels, at the rate of 0.25% (V/V), where the fermentation will be carried out. The number of microorganisms in the added culture was determined to be $1.1x10^9$, in YEPD agar after 48 hours in 28°C. The obtained must was transferred to a demijohn and fermentation caps were attached, after the mouths were sealed with paraffin, it was left to fermentation at cellar temperature (15±2°C). Density analyzes were performed between the 0th and 21st days of fermentation, taking care to make measurements at the same time every day or every other day and the decision to end the fermentation process was taken according to the densitometry measurements.

2.1.2. Fermentation of red grape

Dry yeast *S.cerevisiae* N 7303 INRA NARBONNE which will be used for alcohol fermentation was activated as made with white grapes, the number of microorganisms in the culture at the end of activation was determinedas $1.2 x10^9$. The active culture was added to the must 0.25% (V / V). Red grapes were left to the grain fermentation at 20±2°C for a week (11). At the end of the grain fermentation, the red grapes were pressed using hydraulic basket presses. In fermentation, the "first must" flowing spontaneously without applying pressure was combined with the "second must" obtained after the first pressing, and 34 and 35L must obtained from *Merlot* and *Kalecik karası* grapes respectively, were transferred to demijohns. Demijohns were then left to fermentation at cellar temperature (15±2°C). Density analyzes were performed between the 0th and 21st days of fermentation, taking care to make measurements at the same time every day or every other day and the decision to end the fermentation process was taken accordingly.

2.1.3. Transfer and resting in red and white wine

The first transfer process was applied to the white wines left to fermentation at the end of December 2012, the inactive microorganisms settled at the bottom of the container were separated from the must. While transferring helps the wine to mature, over-transfer and contact with air will cause excessive oxidation of the wine and flattening of the taste and aroma, so the transfer was done carefully. The transfer process was repeated at the beginning of February and density measurements were made. Since the density measurement results for white wine of *S.blanc* variety the were detected as 990 fermentation was deemd as complete. The amount of must at the end of the fermentation was determined as 33 liters. The measurement results for red wine of *Kalecik karası* variety were 990, and *Merlot* were determined as 995 therefore it was decided that the fermentation was completed. The amount of must at the end of the fermentation has been measured as approximately 32L for both *Kalecik karası* and *Merlot* varieties.

2.1.4. Malolactic fermentation

MRS Broth was used in the activation of the lyophilized culture, the culture was transferred to the liquid medium under aseptic conditions, after stirring, it was left to incubation at 37°C under microaerophilic conditions for 48 hours. At the end of the period, the culture was grown up to the volume that will be used in fermentation in the same medium, then separated from the liquid part using centrifugation, re-suspended in 0.85% saline before inoculation and added at 5% (V / V) to the containers where the fermentation will be carried out. The number of microorganisms in the added culture was determined to be $1.1x10^9$, in MRS agar medium after 48 hours in 37°C. Sulfurization was avoided during malolactic fermentation, as excess sulfur would harm the bacteria. Demijohns with fermentation caps sealed were kept at 18–20°C. After approximately 2 weeks, completion of malolactic fermentation was observed and the wines were left to rest.

2.1.5. Bottling and aging

After the end of malolactic fermentation, the samples obtained were filtered with plate filter using 8 plates (Pall SeitzSchenk Filtersystems GmbH, Germany) .Samples were filled into 70 cL glass bottles after filtration, and the bottles were closed with corks and labeled. No collage (clarification) process has been applied, as the phenol component of wines will be determined later.

2.2. Physico-chemical analyzes on wines

The physico-chemical analyzes of the product obtained from the processing of fruits were carried out in the Food Analysis Laboratory of Tekirdağ Viticulture Research Institute, and the chromatographic analysis of organic acids were carried out in the Liquid Chromatography (HPLC) Laboratory of Bursa Food and Feed Control Center Research Institute. Citric acid, oxalic acid, malic acid, tartaric acid and lactic acid standards used in the determination of organic acids by HPLC were obtained from SigmaAldrich Co. LLC. HPLC (Agilent 1100, Agilent Technologies, USA), C8 HPLC column (ACE 150X4.6 mm), 0.45 µm membrane filters (Agilent, screw tap 5182-0716), shaking water bath (julabo SW22, JULABO Labortechnik GmbH, Seelbach, Germany), ultrasonic water bath (VWR Ultrasonic cleaner, VWR International GmbH, Darmstadt, Germany), centrifuge (Heraeus Megafuge 40R Centrifuge, Thermo Fisher Scientific Inc. USA), spectrophotometer (Optizen 322OUV, Optizen Labs LLC, Warsaw, Poland) has been used for Organic acid determination.

2.3. Sensory Analyzes of the wines

The sensory analyzes of the wines in the thesis were carried out by 35 panelists aged between 25-40 who had no experience in wine tasting. During the analysis, attention was paid to the basic rules such as a bright environment, the absence of foreign odor, the selection of the appropriate glass and the tasting time. The form used in sensory analysis is shown in Appendix 1.The panelists evaluated the wines according to seven different criteria and the evaluation was made on a nine-point hedonic scale, where the most liked sample was given 9 points and the least liked one was given 1 point. The analysis form for the sensory evaluation is given in the Appendix. Panelists scored in a bright and closed environment without being affected by each other. Evaluation was made by taking the average of the points given by each panelist for each criteria group (12,13).

2.4. Statistical Analyses

The chemical properties of the wines produced were evaluated statistically. The data obtained as a result of chemical analysis of 6 groups of wines, two varieties from each of the three grape types used, were evaluated using the variance analysis with the Version 8 (SAS Institute Inc. 2008) statistical program.

3. RESULTS

Results of the raw materials chemical analyses and physicochemical and sensory analyses of the wines produced in our study are given together with the variance analyses.

3.1. Raw material analysis results.

Table 1. *Analysis results of raw materials before fermentation.*

Grape Varieties	pH	Brix% (Dry matter)	Acid (g / L)	Maturity Index	Density (density)
Sauvignonblanc	3.20	24.6	9.0	25.4	1108
Kalecik karası	3.41	22.6	11.06	17.6	1095
Merlot	3.50	23	7.2	29.2	1100

The density values were 1.108 for *S.blanc*, 1.095 for *Kalecik karası*, and 1100 for *Merlot*. According to the raw material analysis obtained, pH values were; 3.20 for *S.blanc*, 3.41 in *Kalecik karası*, 3.50 in *Merlot*, and brix intervals and acid values are in parallel with the literature studies. Brix values were found as 24.6% in *S.blanc* variety, 22.6% in *Kalecik karası* and 23% in *Merlot* variety. The acid content of the raw materials used in the study was determined as 9.0 g/L in *S.blanc* variety, 11.06 g/L in *Kalecik karası*, and 7.2 g/L in *Merlot* variety. Raw material maturity indices were determined as 25.4 in *S.blanc* variety, 17.6 in *Kalecik karası* and 29.2 in *Merlot* variety.

3.2. Chemical analysis results of wine samples

The chemical analysis results obtained in the study are given in Table 2. At the end of our study, the lowest density values of the wines produced were determined as 0.9901 for *S.blanc* malolactic wine, and the highest as 0.9952 for *Merlot* malolactic wine. In a study conducted in our country, the density of wines was determined between 0.9934-0.9970 (14). In a study using different yeast species, it was stated in the experiments that the specific gravity of wines varied between 0.9260 and 0.9940 (15). In a study conducted with wine density, the density of black grape wine was found to be 0.9864 and the density of white wine to be 0.9867 (16). According to a study conducted with red wines, the density in red wines is between 0.9917-0.9927 (17).

The dry matter value of *S.blanc* classic wine was 20.90 g/L, the dry matter value of malolactic *S.blanc* wine was 21.1g/L. The dry matter value of the *Kalecik karası* classic wine was 26.0 g/L. The dry matter value of the malolactic wine of *Kalecik karası* was 27.6 g/L. The dry matter value of *Merlot* classic wine was 29.5 g/L and the dry matter value of *Merlot*'s malolactic wine was found as 25.5 g/L.

Table 2. *Chemical analysis results of wine samples.*

Wine Types	Density 20/20°C	Alcohol%, V/V	Dry matter g/L	Ash g/L	Ash thickness	Total Acid g/L	Acid g/L	pH	Total SO_2 mg/L	Free SO_2 mg/L	Sugar g/L
S. blanc Classic	0.9902 ± 0.1^{a}	12.5 ± 0.17^{a}	20.9 ± 0.26^{d}	1.61 ± 0.35^{c}	23.6 ±0.94^{c}	6.38 ± 0.11^{c}	0.38 ± 0.06^{a}	3.33 ± 0.0^{c}	25 ± 0.69^{d}	8 ± 0.64^{at}	0.97 ± 0.10
S. blanc ML	0.9901 ± 0.05^{a}	12.5 ± 0.0^{a}	21.1 ± 0.05^{d}	1.52 ± 0.15^{c}	20.8 ± 0.25^{d}	6.38 ± 0.16^{c}	0.42 ± 0.04^{a}	3.29 ± 0.05^{c}	25 ± 1.52^{d}	7 ± 1.15^{e}	1.27 ± 0.18^{e}
Kalecik karası Classic	0.9943 ± 0.50^{a}	12.3 ± 0.28^{a}	26.0 ± 1.38^{c}	2.51 ± 0.04^{ab}	35.6 ± 0.81^{a}	6.68 ±0.11^{bc}	0.40 ± 0.05^{a}	3.71 ± 0.02^{a}	37 ± 0.83^{b}	15 ± 0.66^{b}	2.07 ± 0.03^{d}
Kalecik karası ML	0.9929 ± 0.06^{a}	12.3 ± 0.35^{a}	27.6 ± 1.11^{b}	2.39 ± 0.07^{ab}	32.8 ± 0.20^{b}	7.13 ± 0.06^{a}	0.34 ± 0.07^{a}	3.71 ± 0.01^{a}	45 ± 3.52^{a}	19 ± 1.05^{a}	3.42 ± 0.01^{a}
Merlot Classic	0.9952 ± 0.51^{a}	12.1 ± 0.35^{a}	29.5 ± 0.65^{a}	2.87 ± 0.16^{a}	32.8 ± 1.38^{b}	6.90 ±0.15^{ab}	0.35 ± 0.03^{a}	3.65 ± 0.21^{b}	33 ± 1.05^{bc}	12 ± 0.62^{c}	2.52 ± 0.36^{b}
Merlot ML	0.9941 ± 0.58	12.5 ± 0.32	25.5 ± 1.45^{c}	2.86 ± 0.05^{a}	32.8 ± 0.62^{b}	7.05 ±0.07^{ab}	0.36 ± 0.03^{a}	3.65 ± 0.08^{b}	30 ± 1.73^{cd}	9 ± 0. 79^{d}	2.21 ± 0.23^{c}

P≤0.05; There is a statistically significant difference between the averages shown in different letters in the LSD test.
Classic: Control groups produced by classical fermentation.
ML: Experimental groups produced by malolactic fermentation

In a study conducted on red and white wines, the amount of dry matter was determined as 22.2-24.0 g/L in white wines and 25.8-31.5 g/L in red wines (14). When we look at the dry matter results of the wines obtained in our study, it is compatible with the dry matter amounts given in the previously mentioned studies. When we look at the studies on the alcohol content of wines, it has been seen that the values vary between 8.8% and 14% (17-20). Ash contents were determined as 1.61 g/L for *S.blanc* classic wine, 1.52 g/L for *S.blanc* malolactic wine, 2.51g/L for *Kalecik karası* classic wine, and 2.39g/L. for its malolactic wine. The ash content of *Merlot* classic wine is 2.87g/L and that of malolactic wine is 2.86g/L. Ash thickness values were measured as lowest in malolactic wine of *S.blanc* variety with 20.8 g/L and highest in classical wine of *Kalecik karası* variety with a value of 35.6 g/L. Ash is the sum of non-flammable substances in wine. Although its amount in wine is less than that of must, it varies according to the processes applied to the wine (21). Kubilay (1996) states that there is 1.4-1.6 g/L ash in white wine and 1.9-2.3 g/L ash in red wine. In another study, ash amounts in red wines were found between 2.5 and 2.9 g/L. Ash thickness on the other hand is between 26.4 and 29.2 (17).

PH values of wines were found as; 3.33 in S.blanc classical wine, 3.29 in malolactic wine, 3.71 in both types of Kalecik karası wines, and 3.65 in both types of Merlot wines. During fermentation, organic acids, together with their salts, remain stable in the wine and the pH of the wine is kept constant in the range of 2.90-4.00, ensuring a healthy fermentation (22). Total SO_2 amounts were determined as 25 mg/L in both wine types of *S.blanc* variety; 37 mg/L in *Kalecik karası* classical wine and 45 mg/L in its malolactic wine; 33 mg/L in *Merlot* classical wine and 30 mg/L in malolactic wine. Free SO_2 amounts were found as; 8 mg/L in *S.blanc* classic wine, 7 mg/L in its malolactic wine; 15mg/L in *Kalecik karası* classical wine and 19 mg/L in malolactic wine; 12 mg/L in *Merlot* classical wine and 9 mg/L in malolactic wine. Total acid contents were the lowest in two ypes of *S.blanc* wines with 6.38 g/L and the highest in *Kalecik karası* malolactic wine with 7.13 g/L. The amount of volatile acid was found to be the least amount in *Kalecik karası* malolactic wine with 0.34g/L and the most in *S.blanc* malolactic wine with 0.42g/L.

The amount of volatile acid in Turkish wines is between 0.2-0.8 g/L. According to Turkish wine regulations, acid should not exceed 1.8 g/L. According to the EC wine regulations, the maximum amount of volatile acid is 1.1 g/L for white wines and 1.2 g/L for red wines (23). According to this information, the volatile acid amounts in of our study are in accordance with the Turkish wine charter.

Sugar content of wines were detected as in *S.blanc* malolactic wine, 1.27 g/L in *Kalecik karası* classic wine, 3.42 g/L in *Kalecik karası* malolactic wine, 2.52 g/L in *Merlot* classic wine and 2.21 g/L in its malolactic type.

3.3. Phenolic contents of wine samples.

The contents of phenolic substances found and detected in the wines produced in our study are given in Table 3.

Table 3. *Phenolic contents of the obtained wines*

Grape variety	Phenolic Compounds (Catechins) mg/L	Tannin (tannic acid) g/L	Anthocyanin mg/L
S.blanc Classic	311 ± 2.96^{d}	0:32 ± 0.15^{d}	ND
S.blanc ML	291 ± 1.45^{d}	0.30 ± 0.03^{d}	ND
K. karası Classic	2743 ± 33.42^{a}	2.83 ± 0.20^{a}	54.2 ± 2.36^{c}
K. karası ML	2288 ± 25.05^{b}	2.21 ± 0.05^{c}	48.5 ± 3.10^{d}
Merlot Classic	2093 ± 17.15^{c}	2.29 ± 0.12^{b}	136.9 ± 6.17^{a}
Merlot ML	2053 ± 11.20^{c}	2.14 ± 0.04^{c}	98.5 ± 4.01^{b}

P≤0.05; In the LSD test, there is a statistically significant difference between the averages shown with different letters
ND: Not Detected

Phenolic compounds detected in wines; catechin amount were measured as 311 mg/L in *S.blanc* classic, 291 mg/L in *S.blanc* malolactic; 2743 mg/L for *Kalecik karası* classic, 2288 mg/L for *Kalecik karası* malolactic; 2093 mg/L for *Merlot* classic and 2053 mg/L for *Merlot* malolactic wines. Tannin amounts were detected as 0.32 g/L in *S.blanc* classic wine, 0.30 g/L in *S.blanc* malolactic wine; 2.83 g/L in *Kalecik karası* classic wine, 2.21 g/L in *Kalecik karası* malolactic wine; 2.29 g/L in *Merlot* classical wine and 2.14 g/L in *Merlot* malolactic wine. The amount of anthocyanin was determined as 54.2 mg/L in *Kalecik karası* classic wine, 48.5 mg /L in *Kalecik karası* malolactic wine; 136.9 mg/L in *Merlot* classic wine and 98.5 mg/L in *Merlot* malolactic wine.

3.4. Analysis results of organic acids in wines

The amount of organic acids detected in the study is given in Table 4. In addition, the HPLC chromatogram and calibration curves of the analyzed organic acids are given in Figures 1-5.

Table 4. *The amount of organic acids in the obtained wines.*

	Lactic Acid ng/µL	Oxalic Acid mg/L	Tartaric Acid mg/L	Malic Acid mg/L	Citric Acid mg/L	T/M	M/L
***S.blanc* Classic**	1.71±0.08^{ab}	0.085±0.01^{a}	4.02±0.05^{a}	2.25±0.02^{d}	Trace	1.78±0.04^{b}	1.31±0.07^{c}
***S.blanc* ML**	2.06±0.10^{a}	0.065±0.01^{a}	2.96±0.08^{b}	1.24±0.02^{e}	Trace	2.38±0.03^{a}	0.60±0.04^{c}
***Kalecik karası* Classic**	1.28±0.04^{b}	0.075±0.01^{a}	3.06±0.06^{b}	3.03±0.04^{a}	Trace	1.01±0.00^{c}	2.36±0.05^{a}
***Kalecik karası* ML**	1.53±0.53^{ab}	0.060±0.02^{a}	1.79±0.14^{d}	2.76±0.08^{b}	Trace	0.65±0.04^{e}	1.92±0.71^{ab}
***Merlot* Classic**	1.59±0.06^{ab}	0.075±0.01^{a}	2.62±0.02^{c}	3.10±0.01^{a}	Trace	0.84±0.01^{d}	1.95±0.07^{ab}
***Merlot* ML**	1.69±0.07^{ab}	0.055±0.00^{a}	1.59±0.10^{d}	2.36±0.02^{c}	Trace	0.67±0.04^{e}	1.40±0.07^{b}

P≤0.05; In the LSD test, there is a statistically significant difference between the averages shown with different letters.
Trace: Below the working range.

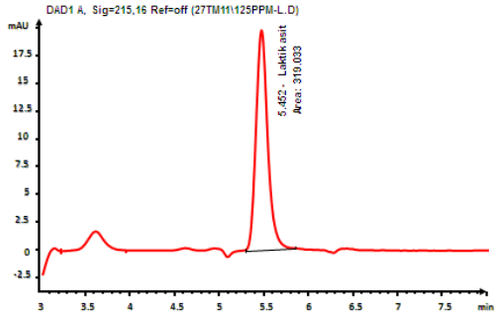

Figure 1.A-HPLC *chromatogram of Lactic acid standard.*

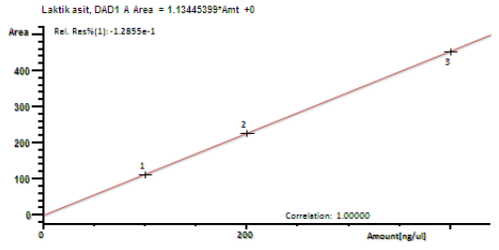

Figure 1.B *– Calibration curve for lactic acid.*

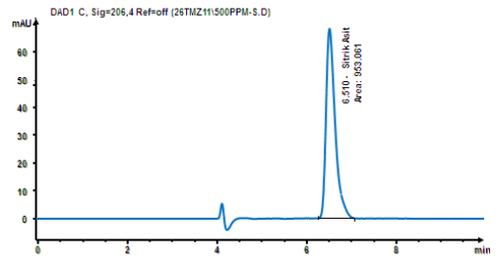

Figure 2.A *– HPLC chromatogram of citric acid standard.*

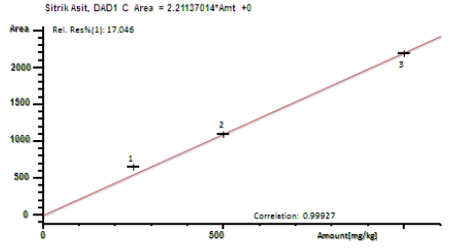

Figure 2.B *– Calibration curve for citric acid.*

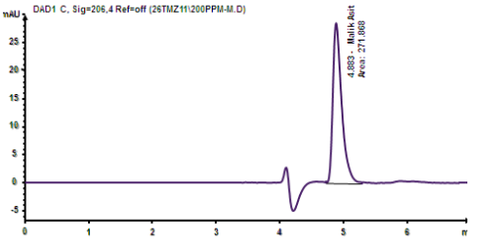

Figure 3. *A – HPLC chromatogram of malic acid standard.*

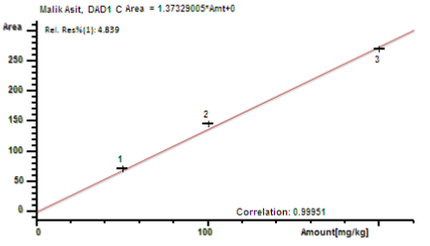

Figure 3. *B – Calibration curve for malic acid.*

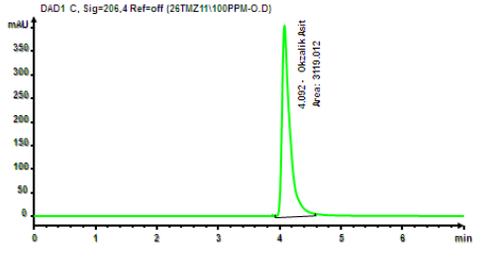

Figure 4. *A – HPLC chromatogram of oxalic acid standard.*

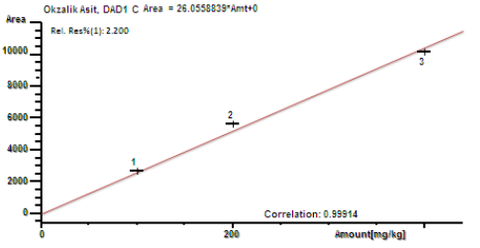

Figure 4.B *– Calibration curve for oxalic acid*

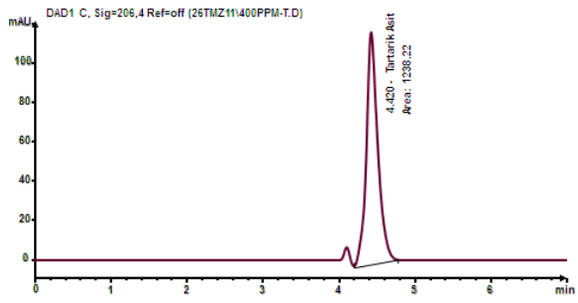

Figure 5. *A – HPLC chromatogram of tartaric acid standard.*

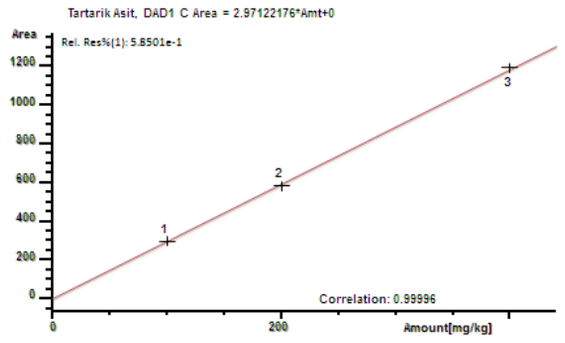

Figure 5. *B – Calibration curve for tartaric acid.*

Table 5. *Wavelengths and accuracies of researched organic acids.*

Compound	Wavelength (nm)	RT (d)	Accuracy R^2
Lactic Acid	360	5:45	1.00000
Malic Acid	280	4.88	0.99951
Oxalic Acid	360	4:09	0.99914
Tartaric Acid	320	4:42	0.99996
Citric Acid	320	6.510	0.99927

RT: *Retention time*

The wavelengths, retention times and accuracies of the samples analyzed at three wavelengths, 280, 320 and 360, are given in Table 5. The accuracy of the compounds range from 0.99914-1.00000. As seen in table 4, Lactic acid contents, among the organic acids studied in the produced wines, for wines produced with classical fermentation was determined between 1.28–1.71 ng/mL and for wines produced by malolactic fermentation, it was found between 1.53–2.06 ng/mL. The amount of oxalic acid for wines produced by classical fermentation was between 0.075-0.085 mg/L, while for wines produced by malolactic fermentation, the value is between 0.055-0.065 mg/L. The determined amount of tartaric acid for wines produced by classical fermentation was 2.62–4.02 mg/L whereas it was found between 1.59-2.96 mg/L for wines produced by malolactic fermentation. The amount of malic acid is between 2.25-3.10 mg/L for wines produced by classical fermentation, and between 1.24-2.76 mg/L for wines produced by malolactic fermentation.

3.5. Sensory analysis results

Sensory analysis results of the produced wines are given in Table 6. Wines were scored out of 9 while performing sensory analysis.

It was observed that the color scores of the samples were between 7.85-8.42, clarity scores 7.28-8.42, bouquets points 6.42-7.57, fullness scores 6.48-7.54, fragrance scores 6.62-7.54, taste points 6.57-8.01 and general taste points 7.00-8.14. When we look at the sensory analysis results in terms of taste; *S.blanc*'s wine produced by traditional fermentation scored 6.57 points, and wine produced by malolactic fermentation scored 7.57 points. While traditional wine of *Kalecik karası* scored 7.57 points, malolactic type was appreciated more and got 8.01 points. The traditional wine of *Merlot* scored 7.01 points, and the malolactic wine 7.57 points.

Table 6.The *scores of the wines obtained as a result of the sensory analysis.*

Varieties	Color	Clarity	Bouquets	Fullness	Fragrance	Taste	General Likes	Group Average
SC	7.85±0.69	7.28±1.38	6.42 ± 1.51	6.48±1.45	6.62 ± 1.32	6.57± 1.81	7.00±1.63	6.88±1.39
SM	8.28 ± 0.95	7.85± 1.21	7.57 ± 0.97	7.53± 0.95	7.58 ± 0.81	7.57± 0.97	7.85± 1.06	7.74± 0.98
Mean	8.06 ± 0.82	7.56± 1.29	6.99 ± 1.24	7.0 ± 1.2	7.1 ± 1.06	7.07± 1.39	7.42± 1.34	7.31± 1.19
KC	8.42 ± 0.53	7.85± 0.69	7.02 ± 1.21	7.42± 1.20	7.12 ± 1.20	7.57± 1.25	7.71± 1.27	7.58± 1.05
KM	8.42 ± 0.53	8.42± 0.53	7.28 ± 1.25	7.54± 1.18	7.50 ± 0.82	8.01± 0.81	8.14± 0.69	7.90± 0.83
Mean	8.42 ± 0.53	8.13± 0.61	7.15 ± 1.23	7.48± 1.19	7.31 ± 1.01	7.79± 1.03	7.92± 0.98	7.74± 0.94
MC	8.01 ± 0.53	8.03± 0.81	7.28 ± 0.95	7.25± 0.93	7.22 ± 1.31	7.01± 1.41	7.71± 1.11	7.50± 1.00
MM	8.14 ± 0.48	8.14± 0.69	7.57 ± 0.97	7.52± 0.90	7.54 ± 1.22	7.57± 0.97	8.01± 0.81	7.78± 0.86
Mean	8.07 ± 0.50	8.08± 0.75	7.42 ± 0.96	7.38± 0.91	7.38 ± 1.26	7.29±1.19	7.86± 0.96	7.64± 0.93
Minimum	7.85	7.28	6.42	6.48	6.62	6.57	7.00	6.88
Maximum	8.42	8.42	7.57	7.54	7.58	8.01	8.14	7.90
Average	8.19	7.93	7.20	7.30	7.26	7.38	7.74	7.56

Abbreviations: SC: S. blanc Classic, SM: S.blanc, Malolactic KC: Kalecik karası Classic, KM: Kalecik karası Malolactic MC: Merlot Classic MM: Merlot Malolactic

3.6. Statistical Analysis

Results of the variance analysis of the wines obtained at the end of our study are given in Appendix Table 2. According to the results of variance analysis, while the yeast used did not have a statistically significant effect on the density, alcohol and pH values of the wines, it had a significant effect on the dry matter, ash, ash thickness, free and total sulfur, total acid and volatile acid values (*P <0.01*).

Lactic acid culture does not seem to have a statistically significant effect on density, alcohol, total sulfur, volatile acid and pH values of wines, however it has a significant effect on dry matter, ash, ash thickness, total acid, free sulfur and sugar values (*P <0.01*).

When the culture and yeast interaction is examined; It has no significant effect on density, alcohol, ash, volatile acid and pH values, whereas a significant effect was determined on dry matter, total and free sulfur, sugar, ash content and total acid values (*P <0.01*). In addition, the groups have a significant effect on anthocyanin, catechin and tannin values (*P*<0.01).

According to the results of variance analysis, there is no statistically significant effect of the yeast used on the organic acids of the wines (*P*<0.01). Variance analysis also revealed that the yeast used had a statistically significant effect on the groups in terms of clarity, bouquets and fullness in the sensory analysis test of the wines (*P <0.01*).

While no significant effect was observed in classical varieties in terms of fragrance parameter, a significant effect was found in malolactic varieties (*P* <0.01). It was determined that the yeast and lactic acid bacteria used had a significant effect on the groups in terms of taste values (*P*<0.01).

Culture and yeast interaction had a significant effect on fullness, odor and bouquets values, although no significant effect was observed on taste, color, clarity and general taste parameters (*P <0.01)*.

4. DISCUSSION

The density results of the wine samples obtained in our study were in parallel with the studies carried out and it was determined that they were within normal limits. Dry matter amount in wines fluctuates within a wide range however it is desired to be between 13-45 gram per liter. According to the Turkish food codex wine regulations, dry matter should be at least 18g in white wines (23). When we look at the dry matter results of the wines obtained in our study, it is compatible with the dry matter amounts given in the previously mentioned studies. The density is low in wines rich in alcohol, and the density increases as the amount of dry matter increases (11). A parallel result was found in our study. When we look at the wine samples, *Merlot,* which has a higher density than the others has a high amount of dry matter. A striking point in the study is that the density of *Kalecik karası* wine made with malolactic fermentation decreased more than the others.

As a result of our study, it was determined that the values of the wines obtained did not change much. Although in the *Merlot* variety it is observed that the alcohol value of the wine produced by malolactic fermentation increases compared to other wines, according to the results in the literature and Turkish standards, the alcohol amounts at the end of our study are within the normal values. Although the ash and ash thickness of the wines obtained as a result of our study is parallel to other studies, it has been determined that the amount of ash in red wines is higher. This result shows us that the extract amouts *of Kalecik karası* and *Merlot* grapes that we studied are higher. It is not preferred that the pH of the wine be higher than 3.50. This is because; As the pH of the wine increases; it becomes susceptible to oxidation reactions, unwanted color changes, protein instability and bacterial fermentation. Moreover the effectiveness of SO_2 on the wine also decreases (5). The pH values obtained in our study are in accordance with the specified values. Sulfur dioxide inactivates the oxidase enzyme in the structure of the product to be processed into wine, prevents oxidation by binding oxygen, and has an antiseptic effect on microorganisms (24). The highest and lowest amounts of sulfur added to prevent oxidation in our research were found in *Kalecik karası* and *Sauvignon blanc* varieties respectively.

Total acidity is determined by titration and gives the amount of free mineral and organic acids in the wine It is stated that the organic acid content of the wine and the changes in the acid content give information about the ripening or contamination of the wine. For example, an increase in the ratio of acetic acid and lactic acid gives information about the ripening of the wine (25). According to the Turkish Food Codex Alcoholic Beverages Regulation, wines must contain at least 4.5 g/L total acid (23). In red wines, higher diacetyl content occurs depending on the wine production technique and malolactic fermentation time (9). In another study, the total acidity of red wines ranges between 6-7 g/L (17). As a result of our study, when we look at the total acid amounts, the acid values of malolactic fermentation were higher in red wines, but remained the same in white wines.

Less volatile acid formation is observed in high acid wines, and therefore it can be thought that the presence of high acid reduces volatile acid formation (16).

In a study conducted, it was reported that the reducing sugar ratios in the samples obtained ranged from 0.35% to 0.86% and the average sugar content was stated as 0.58% (26). According to the results of another study, the amount of sugar in red wines was determined between 2.4-4.9 g/L. (17). According to the results of our research, the amount of sugar in wines is compatible with the studies.

As the shell contact time increases, the total nitrogen content increases (27). It has been shown in studies that total phenolic compound extraction increases at high fermentation temperatures (28,29). In a different study, it has been shown that the effect of temperature on total phenolic compounds was in the first few days of mash fermentation (3-4 days). And its effect decreases in the following days (30). In addition, the

extraction of phenolic compounds is faster in the presence of ethyl alcohol (31). As a result of our study, the amount of phenolic compounds was higher in ethyl alcohol concentrations, while these values decreased with malolactic fermentation.

Phenolic compound content values showed great differences in many studies. In a study using 18 different wine samples, it was stated that the total phenolic compound content of wines was between 2.5 and 3.6 g/L (32). There are studies that draw attention to the fact that the total phenolic compound content in red wine in terms of gallic acid is between 1000-4000 mg/L and this value is found as 6500 mg/L in some samples (33,34). According to a study, it was stated that the content of phenolic compounds in wines obtained from the *S.blanc* variety ranged from 50-2000 mg/L in gallic acid (34). However, as it is known, the amount of phenolic substance can vary according to factors such as grape variety, soil and climate. In another study, it was reported that the phenolic content in red wines was between 1882 mg/L (17). The values we found in our study are consistent with the results of the mentioned study.

According to some researchers, "cold" and "enzyme-applied cold maceration" processes generally yield high total anthocyanin amounts in wines (35), while according to some, the total amount of anthocyanins is higher in wines produced by classical maceration (36).

In the study conducted with the wines obtained from Sangiovese grapes of Tuscany region, the total anthocyanin amount was determined as 98 mg/L and 154 mg/L in wines obtained by cold maceration application (under nitrogen gas at 5ºC) (37). These results are consistent with the values obtained in our study.

In another study, the catechin and epicatechin amounts in enriched red wines were determined as 41.34 mg/L and 14.89 mg/L, respectively. These values were determined as 29.41 mg/L and 12.14 mg/L in enriched white wines. The total phenol and antioxidant capacity averages were determined to be 2155.26 mg/L, 414.36 mg/L respectively for red and white wines. Researchers explain the differences in phenol level and antioxidant capacity between red and white wines with the differences in grape variety, climatic conditions and the fermentation methods applied, especially emphasizing the beneficial effect of red wine on health (38).

Tannin amounts should be 1,5 g/L in normal red wines and between 2-2,5 g/L in dark and heavy red wines. The amount of tannins is not desired to be high, as excess tannins will give the wine a bitter taste (18). Although the values obtained from our study are in accordance with the previous studies, amount of tannins in the *Kalecik karası* wine was found higher, however this did not affect the results obtained in the sensory analysis. When the organic acid concentrations determined by HPLC-DAD technique were examined in all varieties, it was seen that the amount of tartaric acid was the highest in general. According to a study, it was found that the highest amount of acid in grapes is tartaric acid (39).

While monitoring the amount of tartaric acid and malic acid, it was determined that the amount of oxalic acid was the least. When compared according to fermentation types, the amount of lactic acid increased in wines produced by malolactic fermentation, while the amounts of tartaric acid, malic acid and oxalic acid were found to be higher in wines produced by classical fermentation. When we look at the sensory evaluation results; In terms of color, two types of *Kalecik Karası* wine take the first place, followed by *Merlot*. In terms of clarity, the malolactic type of *Kalecik Karası* takes the first place. Clarity average was determined as 7.92 in all wines. Although the shades of the colors varies according to the type of grapes, white wines that appear brown and brownish are generally oxidized. In red wines with higher amout of acids, the color becomes bright red (11).

In terms of Bouquets, the malolactic variety of *Merlot* grape got the highest score. Bouquets average was determined as 7.19 in all wines. This is the aroma found in grapes and transferred to wine which is formed during fermentation and resting. These aromatic substances in structure of esther, aldehyde, alcohol and ketones, which are numerious, affect the overall quality of the wine (11). Looking at the fullness results, the malolactic varieties of red wines got the highest score. These results were found in parallel with the amount of acid and other properties we found in the chemical results. Wines that are not full are thin, juicy and without bitterness. Full wines on the other hand, leave a greasy character in the mouth (40). In terms of taste scores, malolactic type *Kalecik karası* wine ranked first. The taste average of all wines was determined to be 7.38. Low acidity creates a, flat and insipid wine. A bitter taste is sometimes seen in red wines. Sweetness is an important criterion for sweet table wines (11). When we evaluate the sensory analysis results in total, the total point average of all wines was found to be 7.73. According to the results of the total evaluation, the most admired variety is the wine produced by malolactic fermentation of *Kalecik karası,* followed by the malolactic wine of the *Merlot* variety.

5. CONCLUSION

In this research, malolactic fermentation involving lactic acid bacteria was carried out following the yeast fermentation in the production of classical wine with two red and one white grape varieties. Afterwards, the effects of malolactic fermentation carried out under controlled conditions on the physico-chemical and sensory qualities of the young wines produced were evaluated. The wine samples obtained were examined in terms of organic acid changes, phenolic compositions and sensory properties.

The obtained results showed higher amounts of phenolic compounds in ethyl alcohol concentrations, however these values decreased with malolactic fermentation. While the amount of lactic acid increased in wines produced by malolactic fermentation, the levels of tartaric acid, malic acid and oxalic acid were found to be elevated in wines produced by classical fermentation. Tartaric acid amounts were found to be the highest among all organic acids in general. Wines produced by malolactic fermentation were more appreciated than the control group consisting of traditional wines. This difference was not considered to be significant in terms of color, however it was regarded important in terms of other

sensory criteria (P<0.01). Subsequently, it was determined that red grape varieties responded more positively to MLF in terms of wine production technology than white grape varieties and these differences were found to be significant (P<0.01). As a result of sensory analysis, the most popular wine type is *Kalecik karası* wine produced by malolactic fermentation. This was followed by the malolactic wine of the *Merlot* variety. It has been observed that malolactic fermentation also reduces the amount of tannins, nevertheless affects the flavors of the produced malolactic wines favorably. In addition, the harmony of two foreign grapes, one red and one white grape, to the geography of our country was revealed with the characteristics of the wine produced. As a result of the thesis study, it was found that malolactic fermentation improved the quality of the wine, making it more pleasurable. In this context, the results of the research has the quality and attributes that will shed light on winemakers.

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Acquisition of data for the study: AGA, AY, ZS
Analysis of data for the study: AGA, GK, AY,
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APPENDIX 1

Sensory Evaluation Analysis Form

Please give your score between 1-9 in the relevant box.. Age :..... Gender : M □ W □ Do you smoke? M □ W □	1awful	4 not bad	7 good
	2 very bad	5 I neither liked nor disliked	8 very good
	3 bad	6 tolerable	9 excellent

varieties	Color	Clarity	Bouquets	Saturity	Fragrance	Taste	General Appreciation	Group Average
Savignon blanc								
Savignon blanc+ML								
average value								
Kalecik Karası								
Kalecik Karası+ML								
average value								
Merlot								
Merlot+ML								
average value								

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Clinical and Experimental Health Sciences

Awareness of the Protective Measures Against Covid-19 Among Turkish Dentists: A Questionnaire Survey

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ABSTRACT

Objective: The COVID-19 pandemic has deeply affected the entire world. This study aims to evaluate Turkish dentists' awareness of and protective measures against COVID-19 infection.

Methods: A web-based questionnaire consisting of 35 closed-ended and multiple-choice questions was prepared according to Turkish Dental Association guidelines. The questionnaire was divided into three domains: demographic data, precautionary measures, and attitude. Ethical clearance was obtained from the Istanbul University institutional review board. questionnaire was sent to the dentists registered with the Turkish Dental Association. The minimum sample size was calculated as 756 individuals. The Chi-Square test was used to evaluate the difference for categorical variables. Statistical significance was accepted as $p < .05$.

Results: A total of nine hundred and ninety-seven questionnaires was analyzed. Five hundred and sixty-three respondents (56.5%) were general dentists, and 434 (43.5%) were specialists. Seven hundred and thirty-five (73.7%) dentists were found to wear disposable gowns, N95/FFP2 masks, and face shields. About half (48.1%) were confident of avoiding infection. Twenty-one (2.1%) subjects were SARS-Cov-2 positive. A majority of both dentists who had COVID-19 (71.4%) and dentists who did not have it (92.7%) agreed that COVID-19 is an occupational risk ($p < .001$).

Conclusion: Although nearly two-thirds of the respondents reported using personal protective equipment, greater precautionary measures should be taken by dental staff to protect the patients from COVID-19 and other contagious diseases.

Keywords: Awareness, covid-19, dentistry, pandemic, protective measures

1. INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2) was officially announced to the world on January 8, 2020, under the name COVID-19. This infectious disease is transmitted from person to person by airborne droplets or by direct contact with affected cases or contaminated surfaces (1). The first case of COVID-19 detected in Turkey was announced by the Ministry of Health on March 11, 2020 and the country's first death due to the virus occurred on March 15, 2020. On April 1, 2020, the Minister of Health announced that COVID-19 cases had spread all over Turkey.

Significant progress has been made in vaccinating against the disease. However, because no definitive method has been presented thus far for its control and treatment, the recommended standard has been to control the source of infection (2).

COVID-19 is transmitted between individuals through close contact and droplets, and it is known that health care service providers or others who have close contact with infected patients are at high risk of contracting the disease. Dentists and dental staff are considered high-risk due to both their working conditions and the nature of their contact with patients (3).

At the beginning of the pandemic, it was decided to postpone emergency and non-essential dental treatments until a second statement, and lists of these emergency and mandatory services were published. Clinical guidelines prepared by the Turkish Dental Association (TDA)'s scientific committee on April 17, 2020, were delivered to dentists via the TDA website and by SMS (4). Later, elective procedures could be resumed given the necessary infection control measures were taken, although the provision of emergency services was to be prioritized (5).

Personal protective equipment (PPE) refers to special coverings designed to protect dental health care personnel

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from exposure to or contact with infectious agents. These include gloves, face masks, protective eyewear, face shields, and protective clothing (e.g., reusable or disposable gowns, jackets, and lab coats). Dental health care personnel should wear N95/FFP2 masks that cover their noses and mouths during procedures that are likely to generate splashes or sprays of blood or bodily fluids during the pandemic (6, 7).

The literature reveals dentists' knowledge, attitudes, and perceptions regarding measures for viral infection control; however, survey results show that the preventive measures Turkish dentists take are not satisfactory (8-12). Our null hypothesis is that dentists do not adequately adhere to COVID-19 prevention measures. This cross-sectional study aimed to evaluate Turkish dentists' awareness of and protective measures against COVID-19 from the nation's first pandemic-related lockdown onward.

2. METHODS

Approval for this cross-sectional online survey was obtained from Istanbul University Faculty of Dentistry's ethics committee (decision date and number: 22.07.2020 – 2020/47). The study protocol was registered on ClinicalTrials (NCT05175820). Informed consent was obtained from the participants prior to their contribution.

2.1. Subjects

The sample consisted of graduated dentists working in Turkish state-affiliated oral and dental health institutions, private clinics, hospitals, or universities who were registered with the TDA until June 2020, and who accepted voluntary participation in the study.

The sample size was calculated with the OpenEpi program (a web-based epidemiologic and statistical calculator) (13). The population (N) consisted of 22,275 dentists who were registered with the TDA. Since there is no information about dentists' working conditions and precautions during the pandemic period in Turkey, the prevalence (p) was determined as 50%. The minimum sample was calculated using the $n = [DEFF*Np(1-p)]/ [(d^2/Z^2_{1-\alpha/2}*(N-1)+p*(1-p)]$ formula. The sample size was found to be 756 by taking the prevalence of 50% (P), 95% confidence interval, 5% margin of error (d), and taking the design effect (DEFF) as two. This study was carried out in September 2020.

2.2. Questionnaires

The questionnaire consisted of 35 questions compiled from both previous studies (14, 15) and local guidelines (4). In the pilot study, questionnaires were sent to 20 dentists and feedback was received in case of problems with the clarity of the questions and their online answering. The questions were grouped as follows: demographic information (6 items), physical conditions of the clinic (5 items), protective equipment for employees (2 items), regulation of appointments and patient admission (4 items), treatment procedures and infection protocols (10 items), attitudes towards COVID-19 (8 items).

To measure the participating dentists's knowledge about COVID-19-related procedures, the survey asked multipl-choice questions regarding May 21, 2020 TDA clinical guidelines for protective equipment, sterilization and disinfection, and considerations in arranging appointments (4).

The questionnaires were shared via e-mail. The dentists were informed of the privacy policy regarding data collection and were allowed to participate after having provided voluntary consent.

The Checklist for Reporting Results of Internet E-Surveys (CHERRIS) guidelines were used for data interpretation (16). Questions regarding professional experience, gender, age, region of residence, workplace, and the decision to practice or not to pratice during the pandemic were also coded to test whether the COVID-19 measures differed according to various demographic groups.

2.3. Data Collection

Data was collected via Google Forms® (Google LLC, Mountain View, CA, USA). The survey questions were prepared based on TDA guidelines and a compilation of related questions from the literature, and the survey was accessible via a web link delivered to participants by email.

2.4. Statistical Analysis

Data analysis was conducted by means of statistical software (IBM® SPSS® Statistics version 28, Armonk, NY, USA). The Chi-Square test was used to evaluate the difference for categorical variables. Statistical significance was accepted as $p<0.05$.

Multiple linear regression analysis was performed to estimate the fulfillment of 13 requirements in the clinical setting. The backward method was used while performing multiple linear regression analysis. Durbin Watson statistic was used in the evaluation of autocorrelation. The collinearity limit value was 2.5 (17).

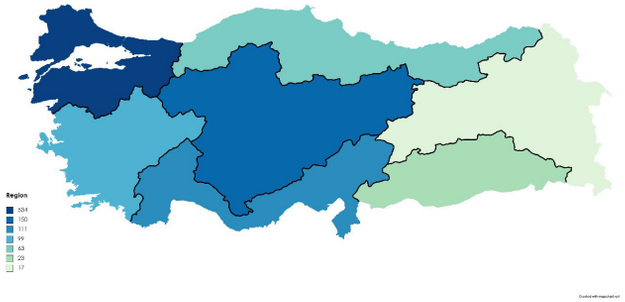

Figure 1. *Distribution of participants. The majority of dentists participated from regions including the Marmara, Central Anatolia, Mediterranean and Aegean.*

The participants' mean age was 39.9±11.7 (median 38.0). More than half (60.6%) dentists were female, and 43.5% of them were specialists. Most of them work in private clinics (Table 1).

Table 1. *Characteristics of the participants.*

Characteristic		n	%
Gender			
	Male	393	39.4
	Female	604	60.6
	Total	997	100
Specialty			
	DDS	542	54.4
	PhD	312	31.3
	MSc	143	14.3
	Total	997	100
Type of postgradute education			
	No postgradute education	563	56.5
	Pedodontics	183	18.4
	Ortodontics	70	7
	Prosthetics	53	5.3
	Oral & maxillofacial surgery	46	4.6
	Periodontology	39	3.9
	Endodontics	21	2.1
	Conservative dentistry	19	1.9
	Oral & maxillofacial radiology	3	0.3
	Total	997	100
Working type			
	Private	741	74.3
	Public	256	25,7
	University hospital	0	0
	Total	997	100
Experience			
	<15 years	501	50.3
	≥15 years	496	49.7
	Total	997	100
Regions			
	Aegean Region	99	9.9
	Black Sea Region	63	6.3
	Central Anatolia Region	150	15.0
	Eastern Anatolia Region	17	1.7
	Marmara Region	534	53.6
	Mediterrranean Region	111	11.1
	Southeastern Anatolia Region	23	2.2
	Total	997	100

Table 2 shows the participants' responses to the questions regarding COVID-19 prevention in the dental setting, adherence to guidelines, and awareness and attitudes. The use of rubber dams was significantly higher among specialists than among general dental practitioners (p< .05). They were also significantly fewer COVID-19 infections among dentists who used rubber dams than those who did not (p< .05). Dental specialists were confident in infection control procedures, and they used surgical caps and face shields together with N95/FFP2 masks (p< .05). Dentists who were confident in infection prevention measures were significantly less likely to have COVID-19 infection than were dentists in other groups (p< .05). Most participants (92.3%) agreed that COVID-19 poses a risk to dentists. The use of PPE was found to be significantly higher among dentists working in private clinics than those working in public clinics (p= .005, p< .001, p> .05 for N95/FFP2 mask, surgical cap, and face shield, respectively) (Table 3).

Table 2. *Response of the participants to infection prevention and COVID-19 awareness-related questions.*

Questions	n	%
Do you have an isolated examination room?		
Yes	874	87.7
No	123	12.3
Total	997	100
Does examination room contain only one dental unit?		
Yes	850	85.3
No	147	14.7
Total	997	100
Is there a window opening to the outside in the examination room?		
Yes	863	86.6
No	134	13.4
Total	997	100
Is there any objects such as magazines, toys that may affect cross-infection in the waiting room?		
Yes	241	24.2
No	756	75.8
Total	997	100
Do you operate the air conditioner while the patients are in the clinic?		
Yes	394	39.5
No	603	60.5
Total	997	100
Do you have sufficient stock of d disposable gowns, N95/FFP2 masks and googles/face shields for all staff in the clinic?		
Yes	735	73.7
No	262	26.3
Total	997	100
Do you practice with four-hand technique to control cross-infection?		
Yes	675	67.7
No	322	32.3
Total	997	100
Do you evaluate the first dental history of the patients by telephone or video talk before they come to the clinic?		
Yes	342	34.3
No	655	65.7
Total	997	100
Do you inform your patients to come alone or with a maximum of 1 companion, if possible?		
Yes	881	88.4
No	116	11.6
Total	997	100
Do you measure patients' temperature before they enter your clinic?		
Yes	808	81
No	189	19
Total	997	100
Do you give information to your patients to be present at the clinic no more than 5 minutes before the appointment?		
Yes	717	71.9
No	280	28.1
Total	997	100
Do you ensure disinfection and ventilation of the environment for at least 15 minutes after each treatment?		

Yes	795	79.7
No	202	20.3
Total	997	100
Do you keep the door of the clinic room closed during the treatment?		
Yes	657	65.9
No	340	34.1
Total	997	100
Do dentists wear N95/FFP2 mask in your clinic?		
Yes	902	90.5
No	95	9.5
Total	997	100
Do dentists wear surgical cap in your clinic?		
Yes	819	82.1
No	178	17.9
Total	997	100
Do dentists wear face shield in your clinic?		
Yes	947	95
No	50	5
Total	997	100
Do staff wear N95/FFP2 masks in your clinic?		
Yes	646	64.8
No	351	35.2
Total	997	100
Do staff wear surgical cap in your clinic?		
Yes	681	68.3
No	316	31.7
Total	997	100
Do staff wear face shield in your clinic?		
Yes	748	75
No	249	25
Total	997	100
Do you use HEPA filter?		
Yes	265	26.6
No	732	73.4
Total	997	100
Do you use 1.5% hydrogen peroxide, 0.2% povidone iodine, or 0.05-0.25% sodium hypochlorite rinsing before treatment?		
Yes	584	58.6
No	413	41.4
Total	997	100
Do you disinfectate dental impressions/prosthetics/appliances before sending them to the laboratory?		
Yes	760	76.2
No	110	11
Don't work with laboratory	127	12.7
Total	997	100
Do you disinfectate prosthetics/appliances before applying them to the patient?		
Yes	794	79.6
No	84	8.4
Dont' apply prosthetics/appliances	119	11.9
Total	997	100
Do you use rubber dam?		
Yes	114	11.4
No	749	75.1
Don't practice with restorative/ endodontics	134	13.4
Total	997	100
Do you run the compressor-operated instruments empty for 10-15 seconds after the patient leaves to prevent reabsorption?		
Yes	448	44.9
No	549	55.1
Total	997	100
Do you autoclave the handpieces used for each patient?		
Yes	372	37.3
No	625	62.7
Total	997	100
Did you get COVID-19 infection?		
No	976	97.9
Yes, hospitalized due to COVID-19	6	0.6
Yes, quarantined at home due to COVID-19	15	1.5
Total	997	100
Did you suspend to go working during the COVID-19?		
Yes	826	82.8
No	171	17.2
Total	997	100
How is your current pace of work compared to the pre-COVID-19 period?		
Didn't change	271	27.2
Practiced less	592	59.4
Only intervened dental emergencies	104	10.4
Stopped practicing	30	3
Total	997	100
Do you feel confident about the COVID-19 precautions?		
Definitely not confident	48	4.8
Not confident	111	11.1
Undecided	358	35.9
Confident	421	42.2
Definitely confident	59	5.9
Total	997	100
Did you receive any education about COVID-19?		
Yes	417	41.8
No	580	58.2
Total	997	100
Do you consider yourself to have sufficient knowledge about COVID-19?		
Yes	438	43.9
No	74	7.4
Partially	485	48.6
Total	997	100
Do you agree with the following statement?: "I believe the COVID-19 infection poses a risk to dentists."		
Definitely not agree	42	4.2
Neither or nor agree	35	3.5
Agree	269	27
Definitely agree	651	65.3
Total	997	100

Table 3. *Comparison of dentists working in private and public dental services in terms of using PPE.*

PPE n (%)	Private	Public	p
N95/FFP2	659(73.1)	243(26.9)	.005
Surgical cap	585(71.4)	234(28.6)	< .001
Face shield	698(73.7)	249(26.4)	.052

Chi-squared test, PPE: personal protective equipment.

Compared to before the pandemic significant differences were observed between the working status of the dentists who grouped according to their professional experience (p< .001). The majority of dentists with less than 15 years of experience (68.3%) have not changed their working situation after pandemic" (Table 4).

Table 4. *Comparison of dentists' experience and the change of working status before the pandemic.*

	Professional experience		p
Working types, n(%)	< 15 years	≥ 15 years	
No change	185(68.3)	86(31.7)	
Less than before the pandemic	258(43.6)	334(56.4)	< .001
Only emergency	46(44.2)	58(55.8)	
Not working	12(40.0)	18(60.0)	

Chi-squared test

According to the linear regression model, the factors affecting the 13 items were found to be positively related to age, extent of COVID-19 education, and full protection of the dentist and dental staff (p< .001) (Table 5).

Table 5. *Linear regression model of factors affecting the application of 13 items.*

	ß (95% CI)	p
Age (ref:<38 years old)	0.84 (0.64-1.04)	< .001
Having education on COVID-19	0.36 (0.15-0.56)	.001
Dentists using full personal protective equipment	1.16 (0.89-1.44)	< .001
Dental staff using full personal protective equipment	2.12 (1.88-2.36)	< .001
(Constant)	5.23 (5.00 – 5.46)	< .001

R2= .462

4. DISCUSSION

COVID-19 appeared at the end of 2019 and spread rapidly around the world, attaining pandemic status and becoming a serious threat to public health. Infected individuals may be asymptomatic or require mild or intensive care, the latter involving airway support and having serious consequences, and, potentially, leading to death. While this and similar central approaches are fundamental to managing the pandemic, it is also important for individuals to comply with the measures. It is necessary to determine the factors affecting social harmony, to ensure society's full participation, to increase awareness by eliminating the lack of information, and to facilitate attitude and behavioral change. In this context, it has been necessary to gather social data in order to make regulations for disease prevention. This study evaluated Turkish dentists' knowledge about COVID-19, their adherence to guidelines, and their attitudes.

The World Health Organization publishes regularly updated guidelines for infection prevention, control, and management. In Turkey, the TDA created clinical guidelines in line with the recommendations of international and national health institutions (4).

According to these guidelines, the number of patients who visit clinics only to receive information should be reduced by teledentistry. In Menhadji et al.(18), both dentists and patients had positive perceptions of teledentistry. In the present, dentists' use of teledentistry was quite low. It is expected that teledentistry will become a widespread practice, especially during lockdown periods.

If patients' body temperature is >37.5°C, they should be isolated at home or hospitalized (19). In a study conducted among Lebanese dentists, 91% of dentists stated that patients' temperatures had been measured (20). The present study found that temperature measurement was performed at a lower rate.

To reduce the risk of contamination, adequate ventilation of the waiting area and examination room is very important. Ideally, patients should be admitted in negative pressure rooms with reduced occupancy, or isolated rooms with good ventilation (60 L/s per patient for natural ventilation) (21, 22). Most of the participants in the present study had isolated examination room and single-unit rooms. Most also had a window opening to the outside. However, the use of HEPA filters during treatment was low.

In addition to the standard precautions (hand hygiene, wearing gloves and surgical masks), dental staff should use PPE, including FFP2/FFP3 filtered masks, disposable caps/gowns, face/eye protectors. Since rotary instruments create atmospheric aerosols, dentists and dental staff should use appropriate goggles or face shields and cover their heads with suitable protective clothing (23) —even if they are using rubber dams—to reduce the risk of infection (24). Hleyhel et al. (20) surveyed 323 Lebanese dentists and reported that 93.2% of the participants and 84.8% of the dental staff members wore PPE. Kamran et al. (25) stated that 69% of Pakistani dentists wear N95 masks, 80% use eye protection, 82% use face shield and 73% use protective clothing. A study conducted in Brazil in May 2020 reported that the use of N95 masks, face shields, and disposable gowns was 71%, 84%, and 66%, respectively (26). The use of PPE was found to be higher in our study than in a study conducted among 1,958 people during Turkey's first lockdown (March 2020). Duruk et al. (27) found that nearly half of dentists (46.37%) had difficulties obtaining PPE. The equipment shortage may have been resolved in the months following the first lockdown. However, dental staff's use of PPE was lower than dentists'. In this regard, dentists should guide their staff and educate them about infection prevention.

Due to the oral spread of aerosols, it is recommended that dental patients use 1.5% hydrogen peroxide, 1% povidone-iodine, or 0.05% hypochlorous acid as a mouthwash before undergoing dental procedures (28). Similar to the results of a previous study (25), nearly half the dentists participating in the present study had their patients rinse their mouths before the procedure.

The use of a rubber dam not only increases the success of dental treatments but also a significantly reduces the risk of bacterial atmospheric contamination (29). During the pandemic, the use of rubber dams was reported to be 37.8% (20) among Lebanese dentists and 28% among Pakistani dentists (25). In a study conducted in Turkey (27), it was reported to be 13.84%, and in the present study only 11.4% for restorative procedures.

At the beginning of the pandemic, there was a period when elective dental procedures were postponed. Many dental institutions have been economically affected due to possible COVID-19 infections among dentists or dental staff members, unstable patient flows, and the inevitable cost of PPE. The British Dental Association reported that only 8% of its members were confident of maintaining their financial stability (30). Wolf et al. (31) reported that 1.4% of a total of 1,324 dentists had closed their clinics by the end of 2020 due to the economic situation. Most of the dentists participating in the present study reported that they had to reduce their practice by a minimum of 0-10%. In a study of 875 dentists in Poland, 71.2% of the participants were reported to have postponed or suspended their practice (22). Cheng et al. (32) found that dentists' desire to open a new dental clinic or work in a hospital between had decreased with COVID-19. Some dentists kept their clinics open on average 1.1 days a week during lockdown (20), while some of them (80%) closed their offices until the number of COVID-19 cases had decreased (25). Compared with the results of a study conducted in Turkey (27), dentists' working rates increased slightly, and there was no change in the rate of patient admissions for dentists with less than 15 years of experience. Mutluay et al. (33) found that dentists' working rates were higher in their study conducted in November 2020.

The TDA has provided information and support via clinical guidelines and online courses to help dentists adapt to the conditions of the pandemic. Duruk et al. (27) reported that 26.65% of Turkish dentists received COVID-19 training in the first month of the pandemic. Since the present study covered the first six months of the pandemic, the rate of dentists receiving training about COVID-19 was found to be higher.

One of the present study's limitations is that it was impossible to check for duplicate participation of the same person. Since IP addressed could not be obtained via Google forms, duplication could not be controlled. Additionally, dentists with low computer literacy did not respond to the online questionnaire or consider academic surveys important due to people's concerns in the early days of the pandemic. Another limitation was that 45% of the participants were specialists, which displays a volunteer bias. The fact that the researchers who conducted the study are pediatric dentists may also have contributed to the higher response rate of dentists with the same specialization.

The study's strengths lie in its inclusion of dentists living in seven regions of Turkey, the ability to reach many dentists by sharing the questionnaires online, and the fact that the participants could express their opinions more easily without an interviewer present. Furthermore, thish study's predate the availability of vaccines, when dental health care workers' occupational risks were very high.

For the safety of the public, the reasons for dentists do not comply with infection prevention practices should be examined, and strategies targeting the obstacles they encounter should be developed.

5. CONCLUSION

The COVID-19 is expected to be on our agenda for years to come, and society's dental and oral treatment needs must be met. This study's null hypothesis is accepted: dentists do not take adequate COVID-19 or infectious disease-prevention measures. Awareness of both COVID-19 and future infectious threats must be raised in dentistry practices, and dental health care workers must take the necessary precautions against infection.

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Clinical and Experimental Health Sciences

The Effect of Education, Telephone Monitoring on Self-Efficacy and Shock Anxiety of Implantable Cardioverter Defibrillator Patients

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ABSTRACT

Objective: Implantable cardioverter defibrillators (ICDs), which are used in the treatment of fatal ventricular arrhythmias, have many life-saving benefits. On the other hand, it has negative effects in terms of physical, social and psychological aspects by commonly causing anxiety and depression. The aim of this study is to examine the effects of education and telephone monitoring on self-efficacy and shock anxiety in patients with implantable cardioverter defibrillator implantation.

Methods: 65 patients hospitalized for ICD insertion were randomly divided into intervention (n=33) and control (n=32) groups. The intervention group was given a training booklet prepared by one-on-one training about ICD. Telephone monitoring was performed once every two weeks for three months and then once a month. No intervention was applied to the control group. Self-Efficacy and Outcome Expectation Scales, Florida Shock Anxiety Scale were applied in the third and sixth months after ICD implantation in both groups.

Results: The self-efficacy scores at the sixth month were higher in the intervention group than in the control group (p=.03). There was no difference between the shock anxiety scores at 3 months (p=.58) and 6 months (p=.64) between the groups. Shock anxiety mean scores of both groups in the 6th month are lower than the mean scores in the 3rd month (p<.01). It was found that self-efficacy and outcome expectation scores decreased as shock anxiety scores increased.

Conclusion: It was determined that the intervention increased the level of self-efficacy and did not make a difference in terms of and shock anxiety. It should be aimed to reduce shock anxiety and accompanying physical problems by supporting patients with various nursing interventions that will increase self-efficacy during adjustment periods.

Keywords: Implantable cardioverter defibrillator, self-efficacy, anxiety, phone call, training.

1. INTRODUCTION

Implantable cardioverter defibrillators (ICDs) are technical devices that automatically detect fatal ventricular arrhythmias such as ventricular fibrillation and ventricular tachycardia and apply the necessary treatment. ICDs have been frequently used to provide out-of-hospital early defibrillation since the 1980s (1-5) There has been a noticeable reduction in cardiovascular death rates after the initiation of treatment of arrhythmias with the ICD (5,6)

It is known that living with an implanted device and experiencing ICD shock causes various harms to the patient and the family (7). Patients with ICD are at risk for symptoms related to anxiety disorders and depressive episodes due to sudden exposure to shock, and concern that the device is not working properly or that any activity in daily life could cause shock (8). In different studies, the levels of clinical anxiety and depression have been shown to be 24-87% and 24-33%, respectively (9-12)

Shock anxiety is defined as fear of possible shocks and avoidance of activities that may trigger shock (13,14). Even in individuals who have not experienced shock, fear of shock can increase anxiety and avoid some behaviors, create a feeling of limitation in activities in daily life, and can be an important cause of morbidity (15-19). Diagnosis and treatment of anxiety is important for the prevention of morbidity (18).

Self-efficacy may be a key factor in post-implantation compliance of ICD patients. Those with low post-implantation belief in self-efficacy may overestimate the difficulties in the adaptation period (1,20). Studies have emphasized that training on living with ICD is important during the compliance period (21,22). Telephone monitoring method has been used in many studies due to its convenience and cost-effectiveness in reaching patients with ICD and other chronic diseases (1,23). In this study,

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telephone monitoring was preferred in the follow-up of patients with ICD who usually live far from the hospital and have disability in terms of driving.

2. METHODS

In our study, it was aimed to examine the effects of education and telephone monitoring on self-efficacy and outcome expectations, which are prominent in the adjustment periods of individuals with ICD, and on shock anxiety, which is a common problem.

The research was conducted between March 2016 and March 2017 in the Cardiology Department of a Training and Research Hospital in Istanbul. The research was planned as quasi-experimental and prospective.

2.1. Sample

Power analysis was performed using the G*Power (v3.1.7) program to determine the number of samples. According to Cohen's effect size coefficients; it was decided that the number of cases in the groups should be taken as a minimum of 30, considering that there should be at least 26 people in the groups and there might be losses during the study process after the calculation made by assuming that the evaluations to be made between two independent groups would have a large effect size (d=0.80). The study inclusion criteria were patients over 18 years of age, about to have/newly inserted ICDs, being reachable by phone, who usually live far from the hospital and have disability in terms of driving and agreeing to participate in the study. Exclusion criteria were hearing and cognitive problems (such as dementia), presence of uncontrolled co-morbidities (such as cancer), use of medication for anxiety/depression, and refusing to participate in the study.

Among the 69 patients who were found to be eligible for inclusion criteria in a training and research hospital in Istanbul between March 2016 and 2017; two patients were excluded from the study because they were not discharged because of mental retardation and one patient was not discharged due to co-morbidities. In order to ensure randomization, the patients were divided into intervention group and control group, including 34 and 32 patients respectively, according to the odd or double admission date. A patient included in the intervention group was excluded from the study because of reporting that the follow-up will continue in another hospital. The study was terminated with 65 patients, 33 in the intervention group and 32 in the control group (Figure 1).

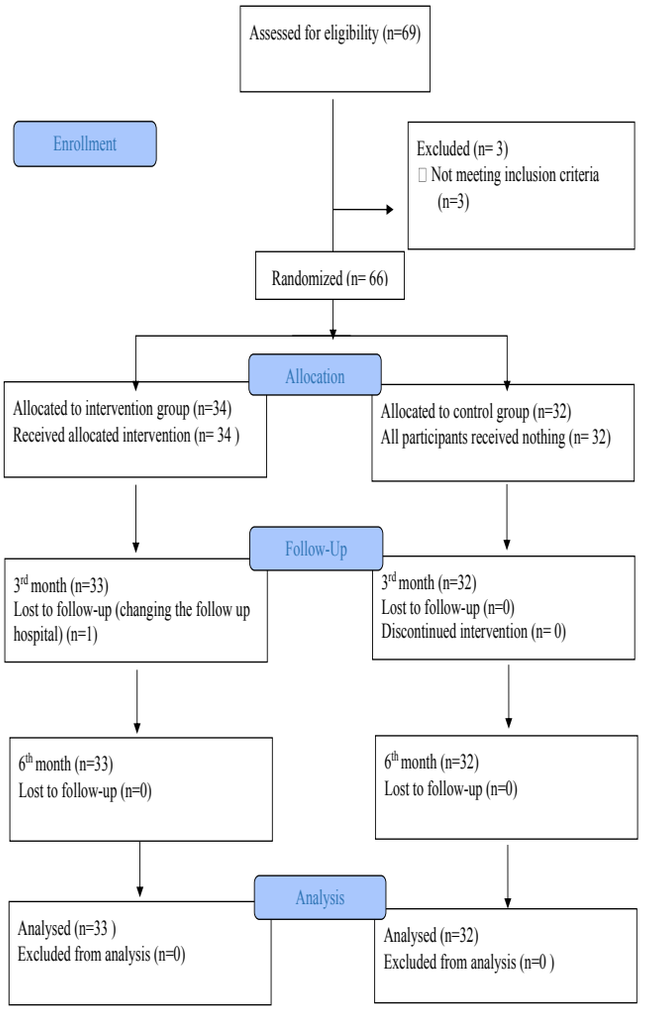

Figure 1. *Flow chart of the inclusion and exclusion of participants.*

2.2. Data Collection Tools

Self-efficacy and outcome expectations scales after ICD implantation were developed by Dougherty, Johnston, and Thompson in 2007 to measure self-efficacy and outcome expectations of ICD patients. The scale has two sub-dimensions: self-efficacy and outcome expectations after ICD implantation. Items are scored in the range of 0 (not at all sure) to 10 (very sure). Higher scores obtained from the scale mean that self-efficacy increases. The outcome expectations scale, on the other hand, consists of seven items and focuses on perceived self-management behaviors after ICD implantation. Items are scored between 1 (definitely true) – 5 (definitely not true). Higher scores obtained from the scale mean that outcome expectations increase (20). The validity and reliability of the scales for the Turkish population was made by Alkan and Enç in 2014. After the validity and reliability study, the scale consisted of a total of 15 items. Cronbach's alpha coefficients were found to be .87 and .75 (24). Cronbach's Alpha coefficients for self-efficacy at 3rd and 6th months in our study was found to be .96 and .94, respectively. On the other hand, Cronbach's Alpha coefficient

for outcome expectations at 3rd and 6th months was found to be .94 and .98.

Florida shock anxiety scale: It was developed by Kuhl et al. in 2006 to determine the shock anxiety levels of ICD patients. In the evaluation of the scale, the items are scored between 1-5. The total scores obtained from the scale vary between 5 and 50, and higher scores mean that the patient's level of shock anxiety is high (15). The validity and reliability of the scale for Turkish population was made by Alkan and Enç in 2014. Cronbach's alpha coefficient was found to be .87 (25). The Cronbach's Alpha coefficients for the shock anxiety scale in our study were found to be .96 at 3rd month and .95 at 6th month.

Training Booklet: The booklet, which consists of the content of the training given to the intervention group patients, was prepared by the researcher after examining the sample booklets and consulting the expert opinion (three nursing faculty members and two cardiology physicians). It was given to patients after one-on-one training. The training booklet include information about the normal heart rhythm, abnormal heart rhythms (ventricular tachycardia and fibrillation), individuals who need the ICD, description of ICD and its placement, the practices to be performed in the clinic after the procedure, the follow-up process, the possible risks and the daily life with the ICD (driving, physical activity, when to start sexual life, etc.), points to be considered in electromagnetic interaction and frequently asked questions. Answers to the following questions: 'Answers to the questions 'Will I realize I have an ICD in my body, what will I feel when ICD shocks, will there be a warning before shocking, what should I do after shock, can I travel abroad, will I continue to take my medications, what is the difference between a pacemaker and ICD', are explained in the frequently asked questions section. It is a 25-page booklet in A4 format.

Checklist for phone calls: The form prepared by the researchers in the light of the literature (15,21,26,27) for use in telephone interviews with intervention group patients include; several questions adapted from the scales for self-efficacy, outcome expectations and shock anxiety in order to make the telephone interviews in a semi-structured order.

The checklist for phone calls includes questions such as the name of the patient who was interviewed, the number of interviews, the duration of the interview, whether the ICD shocked, how it felt if it shocked, whether there was a situation of concern or distress (walking, running, exercising, sleeping, pain, drugs, sexual intercourse, returning to work, cardiac arrest/rhythm disturbance, ICD is given shock, ICD is not given shock) .

2.3. Data Collection Method

The intervention and control group patients received the routine care applied in the hospital. In the routine care of the hospital, there is no training on ICD, only the procedure is explained in order to obtain an informed consent.

Data collection method from intervention and control groups is shown in Figure 2.

In the first interview with the intervention group patients, their consent was obtained after providing informative information about the study. The phone number of the researcher was given to the patients after filling out the patient identification form and they were told that they could call whenever they wanted. The patients were trained after completing the introduction form. One or two relatives of the patient were also included in the training. The patient was in a comfortable sitting position, the door of the room was closed, and the training was conducted in such a way that face-to-face interaction would occur. The patients and their relatives were given the opportunity to ask questions during the training. The training was carried out for durations varying between 35-60 minutes from patient to patient. At the end of the training, the training booklet prepared for the patients was delivered. In the first three months after discharge, the patients were called every two weeks, and the questions included in the checklist were asked to the patients and the questions of the patients were answered. Phone calls lasted an average of 10-15 minutes. On the control days of the third month, after the control was over, SE-ICD, OE-ICD and FSAS were filled in with a face-to-face interview. The average time to fill the scales took 20-30 minutes. During the remaining months of the follow-up, the patients were called once a month to ask the questions included in the checklist for phone interviews, and the questions of the patients were answered. SE-ICD, OE-ICD and FSAS were filled in by face-to-face interview after the control was completed on the sixth month control days.

The patients in the control group were told that they would be called by the researcher to learn the control days by filling out the patient information form face to face. In the third and sixth months, the patients were called by phone and the control days were learned. These interviews lasted an average of 2-3 minutes. SE-ICD, OE-ICD and FSAS were filled in face-to-face interviews after the control was completed on the control days in the third month and sixth month. The average time to fill the scales took 20-30 minutes. After the research was completed in the sixth month, the patients in the control group were educated and given a training booklet.

2.4. Data Analysis

While evaluating the study data, Student's t-Test for two-group comparisons of normally-distributed parameters, and Mann-Whitney U test for two-group comparisons of non-normally-distributed parameters were used when comparing quantitative data in addition to descriptive statistical methods (Mean, Standard Deviation, Median, Frequency, Ratio, Minimum, Maximum). Kruskal Wallis test was used in comparisons of three or more groups that do not show normal distribution. Wilcoxon Signed Ranks test was used for in-group comparisons of non-normally distributed parameters. Pearson Chi-Square test was used to compare qualitative data. Spearman's Correlation Analysis was used

to evaluate the relationships between variables. Significance level was determined as p< .05.

2.5. Ethical Considerations

The research was conducted in accordance with the Declaration of Helsinki. Written permission, in order to conduct the research, was obtained from the Health Sciences Institute Ethics Committee of a university, with protocol number 115 dated 26/10/2015. Written permission was obtained from the Training and Research Hospital where the study was conducted. Written consent was obtained from the patients included in the study, after they were informed about the study with an information form and necessary explanations were given. At the end of the research, the control group was trained and a training booklet was given.

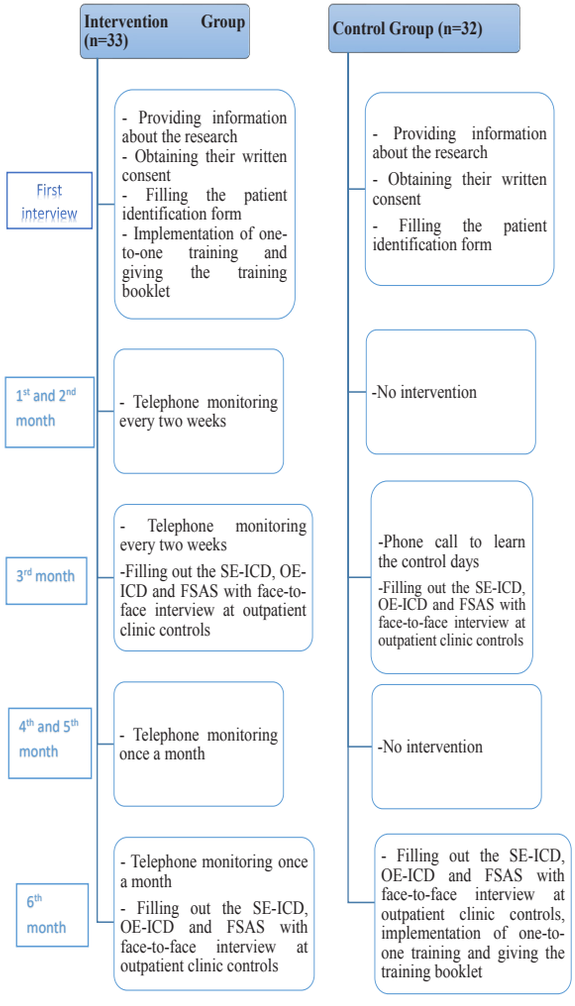

Figure 2. *Study application plan*

3. RESULTS

Information on demographic and some medical characteristics of the groups is given in Table 1. It was found that there was no difference between the groups in terms of these characteristics (Table 1). When the mean age of the participants was examined, it was seen that the intervention group was 55.88±15.57, the control group was 55.72±15.79, and the majority of the patients in both groups were male (75.8%, 78.1%) and married (75.8%, 68.8%). When ICD shocks are examined; It was determined that 48.5% of the intervention group and 40.6% of the control group experienced shock at the 3rd month. On the other hand, it was determined that 27.3% of the intervention group and 37.5% of the control group experienced shock at the 6th month (Tablo 1).

Table 1. *Comparison of sociodemographic and medical characteristics of intervention and control group patients*

Characteristics		Intervention group (n=33)	Control group (n=32)	Homogeneity	
				X^2/t	*p*
Age (year)	*Min-Max (Median)*	18-79 (58)	20-76 (58)	***0,041***	[c]***0,97***
	Meant±SD	55,88±15,57	55,72±15,79		
Gender; *n (%)*	**Female**	8 (24,2)	7 (21,9)	***0,051***	[d]***0,82***
	Male	25 (75,8)	25 (78,1)		
Marital status; *n (%)*	**Married**	25 (75,8)	22 (68,8)	***0,398***	[d]***0,53***
	Single	8 (24,2)	10 (31,2)		
Educational status; *n (%)*	**Literate**	3 (9,1)	3 (9,4)		
	Primary school	18 (54,5)	12 (37,5)		
	Secondary school	5 (15,2)	3 (9,4)	***1,847***	[d]***0,17***
	High school	6 (18,2)	10 (31,2)		
	University	1 (3,0)	4 (12,5)		
	Primary school and below	21 (63,6)	15 (46,9)	***8,741***	[d]***0,07***
	Secondary school and above	12 (36,4)	17 (53,1)		
Working status; *n (%)*	**Working**	9 (27,3)	14 (43,7)	***2,226***	[d]***0,33***
	Not working	10 (30,3)	6 (18,8)		
	Retired	14 (42,4)	12 (37,5)		
Economic status; *n (%)*	**Income less than expenses**	4 (12,1)	4 (12,5)	***0,002***	***0,96***
	Income equal to/ more than expenses	29 (87,9)	28 (87,5)		
Living with; *n (%)*	**With spouse**	13 (39,4)	11 (34,4)	***0,807***	[d]***0,67***
	Spouse and child	12 (36,4)	10 (31,2)		
	Other	8 (24,2)	11 (34,4)		
Chronic disease; *n (%)*	**Not present**	15 (45,5)	12 (37,5)	***0,423***	[d]***0,52***
	Present	18 (54,5)	20 (62,5)		
3rd month shock status; *n (%)*	**Not present**	17 (51,5)	19 (59,4)	***0,406***	[d]***0,52***
	Present	16 (48,5)	13 (40,6)		
6th month shock status; *n (%)*	**Not present**	24 (72,7)	20 (62,5)	***0,777***	[d]***0,38***
	Present	9 (27,3)	12 (37,5)		

[c]*Student-t test;* [d]*Pearson Chi-Square Test*

While the 3rd month self-efficacy scores did not differ according to the groups (p=.32 p> .05); It was seen that the 6th month self-efficacy scores were higher in the intervention group than in the control group (p= .03). It was found that the 6th month self-efficacy scores were higher than the 3rd month scores in both groups (p< .01) (Table 2).

Table 2. *Comparison of self-efficacy, outcome expectation, and shock anxiety of intervention and control group patients*

Scales	Follow-up time		Intervention group (n=33)	Control group (n=32)	*Z*	[a]*p*
Self-efficacy	**3rd month**	*Min-Max (Median)*	26-90 (60)	25-90 (54)	***-0,992***	***0,32***
		Mean±SD	59,09±19,26	54,25±18,42		
	6th month	*Min-Max (Median)*	44-100 (75)	36-100 (61,5)	***-2,123***	***0,03****
		Mean±SD	72,67±15,21	63,69±16,41		
		Z	***-4,886***	***-4,700***		
		[b]***p***	***<.01***	***<.01***		
Outcome Expectation	**3rd month**	*Min-Max (Median)*	15-25 (21)	5-25 (20,5)	***-0,930***	***0,93***
		Mean±SD	21,00±3,23	20,63±4,51		
	6th month	*Min-Max (Median)*	5-25 (23)	18-25 (25)	***-0,624***	***0,53***
		Mean±SD	22,24±4,05	23,06±2,56		
		Z	***-2,889***	***-3,476***		
		[b]***p***	***<.01***	***<.01***		
Shock Anxiety	**3rd month**	*Min-Max (Median)*	10-44 (23)	10-45 (21,5)	***-0,560***	***0,58***
		Mean±SD	23,30±10,32	22,13±10,18		
	6th month	*Min-Max (Median)*	10-37 (20)	10-39 (16,5)	***-0,466***	***0,64***
		Mean±SD	19,33±8,75	19,81±8,47		
		Z	***-4,347***	***-3,451***		
		[b]***p***	***<.01***	***<.01***		

[a]*Mann Whitney U Test;* [b]*Wilcoxon Signed Ranks Test; *p<,05*

The 3rd month (p= .93) and 6th month (p= .53) outcome expectation scores did not differ between the groups. It was found that the 6th month outcome expectation scores were higher than the 3rd month scores in both groups (p< .01) (Table 2).

Shock anxiety scores at 3 months (p= .58) and 6 months (p= .64) did not differ between the groups (p> ,05). In both groups, 6th month shock anxiety scores were lower than 3rd month scores (p< .01) (Table 2).

Table 3. *The Relationship between self-efficacy, outcome expectation, and shock anxiety*

Scales		Total (n=65)		Intervention group (n=33)		Control group (n=32)	
		3rd month	6th month	3rd month	6th month	3rd month	6th month
Self-efficacy – Outcome expectation	*r*	0,476	0,334	0,443	0,368	0,518	0,418
	p	***<.01***	***<.01***	***0,01****	***0,03****	***<.01***	***0,02****
Self-efficacy – Shock anxiety	*r*	-0,756	-0,690	-0,792	-0,680	-0,750	-0,692
	p	***<.01***	***<.01***	***<.01***	***<.01***	***<.01***	***<.01***
Outcome expectation – Shock anxiety	*r*	-0,548	-0,466	-0,534	-0,362	-0,550	-0,596
	p	***<.01***	***<.01***	***<.01***	***0,04****	***<.01***	***<.01***

*r: Spearman's Correlation Coefficient; *p<,05; **p<,01*

4. DISCUSSION

In this study, it was found that the self-efficacy of individuals with ICD who underwent training and telephone follow-up was increased compared to the control group, and there was no difference in outcome expectations between the groups. In another study in which telephone support was applied to individuals with ICD, it was found that self-efficacy was increased (27). After ICD implantation, patients need to develop behavioral changes in order to adapt to their new situation. Self-efficacy and outcome expectations are important concepts in developing adaptive behavior (20,28). Studies have shown that it is possible to achieve improvements in many parameters during the adjustment period of patients with ICD with various interventions. Cowan et al. (2001), applied a psychosocial education program to the intervention group patients and found that the intervention reduced the deaths from cardiovascular causes in the patients and did not affect the heart rate variability, anxiety, depression and anger in their study where they followed up every six months for two years (29). In the study of Smeulders et al. (2007), in which they applied a patient-centered self-management program to patients with ICD in groups, they found that the patients' self-efficacy levels increased and their satisfaction with the program was higher (30). Yardımcı & Mert (2019) stated that there was improvement in many sub-dimensions of the quality of life of patients with ICD, to whom they applied web-based training (31). In our study, it is seen that training and telephone counseling similarly improve self-efficacy.

As a result of this study, it was determined that there was no significant difference between the groups in terms of shock anxiety, and the shock anxiety of both groups was lower at 6 months. In another study conducted with the Turkish population, it was found that there was a significant decrease in state anxiety in patients with ICD who underwent a planned education and follow-up program, and there was no significant difference between the intervention and control

groups (17). It is thought that these two similar results are due to the fact that patients generally display a fatalistic attitude. Flemme et al. (2012) also found that one of the most common methods used by patients to cope with depression and anxiety symptoms and deterioration in quality of life is 'fatalism' (32). There is only a weak relationship between the experience of shock and shock anxiety; therefore, even in the absence of actual device firing, shock anxiety persists (33). Not knowing where and when shocking will occur, and lack of knowledge about what to do during shocking are reasons for anxiety in individuals with ICD. It has been accepted that education and post-discharge follow-up conducted with different methods in various qualitative and quantitative studies, increase the quality of life by reducing uncertainty and anxiety in patients (17,34-37).

It is a known fact that anxiety contributes to poor outcomes in morbidity and mortality in cardiology (33). Shock anxiety is an important problem that has the potential to cause different problems in individuals with ICD. Nursing interventions are needed for the patients in order to cope with problems about returning to work, sexual life and other physical restrictions in the post-ICD period (27,32,38). In the study of Mlynarska et al. (2020); having concerns about ICD has been reported to have a major impact on physical, psychological, and social vulnerability (39). In a study, it was determined that the sexual functions of those with high shock anxiety were poor (10).

It is necessary to correct the false beliefs of patients that lead them to avoid activities that may cause an increase in heart rate. In addition to training on ICDs, interventions such as simple stress management techniques such as deep relaxation or breathing exercises that provide a degree of control over heart rates and reduce distress can be planned in line with this purpose (40).

It is seen in the studies in the literature that different attempts are made to reduce the shock anxiety of patients with ICD. Sears et al. (2007) found that the stress and shock management program in six-week sessions and in a one-day psycho-educational workshop reduced the anxiety and cortisol level in saliva in patients with ICD, while the decrease in anxiety level was faster in the six-week program (41). The patients with ICD in the study of Salmoirago-Blotcher et al. (2013), received mindfulness meditation training over the phone and it was seen at the end of the study that the awareness levels and anxiety levels of the intervention group patients were more positive than the control group (36). In a study that applied a yoga program to patients with ICD (42) and in another study that applied training based on web-based social cognitive learning theory (31), it was found that the intervention group had a significant reduction in shock anxiety.

Similar results were observed for both groups at 3 and 6 months in this study when the relationship between self-efficacy, outcome expectations and shock anxiety was examined. A high level of negative correlation was found between self-efficacy and shock anxiety scores for both groups. It was observed that there was a positive weak-moderate relationship between self-efficacy and outcome expectation scores, and a moderate negative relationship between shock anxiety and outcome expectations. Morken et al. (2014) found that shock anxiety increased in patients with ICD as the support received from healthcare professionals was decreased (14). The relationship between self-efficacy and shock anxiety in our study also supports this study. Education and telephone follow-up intervention increased the self-efficacy of the patients and caused a decrease in shock anxiety.

4.1. Study Limitations

The limitations of the study are that the study was conducted in a single center, the number of samples was small, and the intervention was not blinded for the researchers and the participants.

5. CONCLUSION

This study showed that self-efficacy increased in the intervention group, which received training and telephone follow-up six months after ICD insertion, compared to the control group, while outcome expectations and shock anxiety were not different between the groups. It is noteworthy that shock anxiety decreased in both groups at the sixth month. The high negative correlation between self-efficacy and shock anxiety shows us how important nursing interventions are to increase the self-efficacy of patients. It should be aimed to reduce shock anxiety and accompanying physical problems by supporting patients with various nursing interventions that will increase self-efficacy during adjustment periods.

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Peer-review: *Externally peer-reviewed.*
Author Contribution:
Research idea: BA, SO
Design of the study: BA, SO
Acquisition of data for the study: BA
Analysis of data for the study: BA, SO
Interpretation of data for the study: BA, SO
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Clinical and Experimental Health Sciences

Proteomic Analysis of Gingival Crevicular Fluid During Tooth Eruption

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ABSTRACT

Objective: Gingival crevicular fluid (GCF) is a biological fluid that has the unique capacity to reflect changes in periodontium to its protein composition, making it ideal for potential biomarkers. There is limited information about the mechanism of tooth eruption, for which GCF might provide valuable knowledge. This study aimed to provide a proteomic approach to investigate the composition of GCF obtained from two different supraosseous tooth eruption stages of permanent molars changes.

Methods: GCF samples were taken from a total of 26 healthy children, whose permanent molar just emerged from the gingiva (5-8 years old) and the occlusal equilibrium stage (9-13 years old). Proteins were extracted with Bio-Rad Rehydration Buffer followed by Zeba™ Spin Desalting Column. GCF samples were separated with two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by mass spectrometry-based protein identification.

Results: A new optimized protocol with enriched protein extraction from GCF samples was developed. Several proteins related to tooth eruption were detected. It was determined that keratin type II cytoskeletal 4 and keratin type I cytoskeletal 9 decreased and albumin increased in GCF protein content in erupting teeth compared to those in the occlusal equilibrium phase.

Conclusion: This methodology, which we have applied for 2D-PAGE of GCF, can also be a source for other studies. There is huge diagnostic potential in mass spectrometry technologies, this study can be carried forward by using other approaches.

Keywords: Tooth eruption, gingival crevicular fluid, proteomic analysis, two-dimensional gel electrophoresis, mass spectrometry.

1. INTRODUCTION

Tooth eruption is defined as all the movements that occur from the area where the tooth develops within the dentoalveolar structure until it reaches its functional position in the oral cavity (1). The process begins with the formation of primary teeth in the sixth week of intrauterine life and after the eruption and occlusal balancing of the third molars, it continues passively for a lifetime (1). Steedle and Proffit (2) divided eruptive movements into 6 phases, three pre-functional stages and three post-functional stages. They are classified as follicular growth, pre-emergent eruptive spurt, post-emergent eruptive spurt, juvenile occlusal equilibrium, circumpubertal occlusal eruptive spurt, and adult occlusal equilibrium (2). Most of our knowledge of the tooth eruption mechanism is based on animal studies, and although they provide very valuable information, they cannot directly reflect the process in humans (3).

The gingival crevicular fluid (GCF) is a biological fluid in the gingival sulcus surrounding the teeth, with a tooth on one side and epithelium on the other side, and is present as a transudate or inflammatory exudate originating from blood plasma (4). GCF has received major attention due to its unique capacity to reflect changes in the gingival area to its protein composition (4). It locally generates materials such as inflammatory mediators, host inflammatory cells, locally produced extracellular proteins, microbial plaque, and antibodies directed against dental plaque bacteria (5). Although various methods have been described for the GCF collection, such as capillary tube and gingival washing, the paper strip method is the most frequently employed one due to its easy and fast usage and is considered a non-invasive technique (6).

Although the development of mass spectrometry (MS) technology has led to extensive proteome documentation of

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body fluids such as plasma, whole saliva, parotid secretion, or minor gland secretion saliva in the dental field, the number of large-scale proteome analysis studies for GCF components is still very limited (4). One of the main reasons that biochemical or MS studies of GCF protein content are limited is because GCF is present in very small quantities (0.2 – 0.5 µL per site) in the healthy periodontium of adults (4). Other limitations are dynamic protein range and highly abundant protein content (4). Abundant proteins such as albumin or immunoglobins restrict the identification of low-level proteins (4).

With the progression of MS technologies, large-scale data on the protein content of GCF have been shown but most studies have focused on finding markers for periodontal diseases among adults (4,7,8). Few proteomic studies have been performed with GCF of pediatric subjects. To the best of our knowledge, proteomic analysis of GCF about tooth eruption has not been studied before. Tooth eruption is a complex and multifactorial process that is still not fully understood. Multiple tissue changes occur during this process, such as bone apposition and resorption, and the development of root and periodontium. One of the main hypotheses states that periodontal ligaments promote eruption by generating tension and compression of collagen fibers and fibroblasts (9), which makes us consider that some information about eruption may be concealed in the gingival sulcus pocket. We conducted two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of GCF obtained from apparently partially erupted permanent molars as compared with fully erupted permanent molars. This study aimed to investigate how the protein level in GCF changes during two different tooth eruption stages of permanent mandibular molars. And an additional aim is to contribute to the development of the 2D-PAGE protocol for GCF.

2. METHODS

2.1. Subjects

This study was approved by the Marmara University Faculty of Dentistry Clinical Research Ethics Committee (2019-271). Informed consent was obtained from each parent of pediatric subjects (26 children, 14 girls, 12 boys) who applied to Marmara University Faculty of Dentistry Department of Pediatric Dentistry. They were selected to participate in this study based on the following inclusion criteria; having general systemic and mental health, as well as healthy oral soft tissues and periodontal health (Silness-Löe Plaque Index (PI) score 0 or 1), not taking any antibiotics in the last 30 days, and absence of any orthodontic appliances, crowns or any tooth with pulpal pathology. Only right and left permanent mandibular first molars without any caries, sealants, fillings, or enamel hypoplasia were sampled (#36 and #46). Subjects abstained from brushing their teeth, chewing gum, eating, or drinking for at least 1.5 h before the visit. Subjects were examined in two groups according to the eruption stage of the tooth classified by Steedle and Proffit (2):

1 – Eruption Group (ER): Children aged between 5-8, whose permanent mandibular first molar tooth is just emergence the gingiva, clinically at least half of the molar tooth's occlusal surface is covered with gums and a radiologically wide-open-apex. It corresponds to the beginning of the post-emergent eruptive spurt phase (2) (Fig.1).

2 – Occlusal Equilibrium Group (OE): Children aged between 9-13 whose permanent mandibular first molar tooth fully erupted, clinically reach occlusion and contact with its antagonist tooth, and radiologically root length developed and narrowed apex. It corresponds to the beginning of the juvenile occlusal equilibrium phase (2) (Fig. 1).

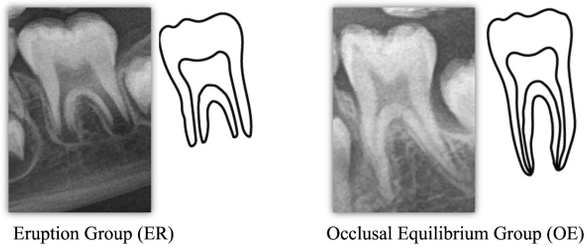


Figure 1. *Radiographic images and illustrations of subject groups: Eruption Group (ER) and Occlusal Equilibrium Group (OE).*

Before sample collection, an intra-oral examination was performed by a single investigator (SYA) under the reflector light. For periodontal evaluation, PI was evaluated by dryness of the teeth before the examination. After sample collection with paper strips, probing pocket depth (PPD) of molars was measured from mesiobuccal point of the sampling tooth with a Williams-type periodontal probe (0103.A0.01, Dentag, Maniago, Italy).

2.2. GCF Study Groups and Sample Collection

This study was carried out with eruption group subjects (n=3) and occlusal equilibrium group subjects (n=3), care was taken to ensure that factors such as age, gender, and tooth number were similar. GCF sampling site was first irrigated with 20 mL of isotonic saline (NaCl 0.9%, Polifarma, Tekirdag, Turkiye). GCF sample was collected with a sterile Periopaper strip (Oraflow Inc., New York, U.S.A.) by using the intrasulcular method after the site was protected from saliva contamination by cotton rolls (10). The Periopaper strip was gently inserted into the buccal sulcus until minimum resistance was sensed (1 mm) and left in place for 40 seconds (Fig. 2). Samples observed to be contaminated with blood or saliva were discarded. Clean Periopaper strips were placed in Eppendorf tubes which were stored at – 80 °C for further processing.

2.3. GCF Protein Extraction

All studies after sampling were carried out at Yeditepe University Proteomics and Mass Spectrometry Laboratory (YediPROT). Apical 2-3 mm of each collected Periopaper strip was cut with sterile scissors and placed in Bio-Rad Rehydration Buffer at 20 °C for 10 minutes. Zeba™ Spin Desalting Column (7K MWCO, Thermo Fisher, U.S.A.) was employed to remove any impurities from the protein sample that might affect further analysis following Zeba™ Spin instructions (Fig. 2).

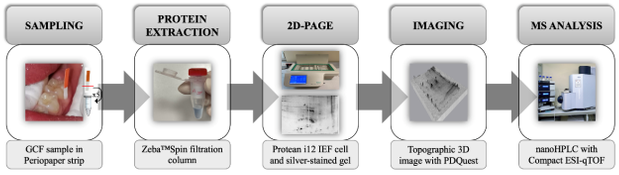


Figure 2. *Study Workflow*

2.4. Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Approximately 130 µL of GCF protein sample was rehydrated onto ReadyStrip™ immobilized gradient strips (IPG Strips, 11cm pH 4-7, Bio-Rad, U.S.A.) under mineral oil at 20 °C for 16 h. Next, isoelectric focusing (IEF) was performed with Protean i12 IEF Cell (Bio-Rad, U.S.A.) (Fig. 2) following the protocol given in Table 1. Precast 8-16% polyacrylamide gels (Bio-Rad Criterion TGX Stain-Free Protein Gels, U.S.A.) were chosen for the second dimension with 1:10 Precision Plus Protein™ WesternC™ Standards (Bio-Rad, U.S.A.). GCF proteins were separated with SDS-PAGE and visualized with ProteoSilver™ Silver Stain Kit (Sigma-Aldrich Inc., U.SA.).

Table 1. *Isoelectric focusing running protocol*

Step	Voltage [V]	Gradient	Current [µA]	Time [HH:MM]	Volt Hours
1	250	Rapid	50	0:20	HH:MM
2	8000	Gradual	50	1:00	HH:MM
3	8000	Rapid	50	26000	Volt Hr
4	750	Hold	50		

µA: Microampere, H:Hour, M:Minute

2.5. Gel Image Processing

Silver-stained gels were scanned using ChemiDoc XRS+ (Bio-Rad, U.S.A.) and analyzed by the PDQuest software (Ver.8.0.1.55 Bio-Rad, U.S.A.) (Fig. 2). Eruption group was called as ER (n=3) and occlusal equilibrium group as OE (n=3). Background subtraction and protein spot detection were processed automatically.

2.6. MS Analysis

The protein spots selected by the PDQuest were diced into smaller pieces to be destained completely by using 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. Next, protein samples were reduced with 10 mM dithiothreitol and alkylated with 100 mM iodoacetamide under dark for 30 min. Following, protein samples were digested with trypsin (MS Grade – Gold Promega, Madison, WI, U.S.A.) at 37 °C for 16 h. The peptide samples were extracted and concentrated by using vacuum centrifugation. They were introduced into Thermo Dionex UltiMate™ 3000 RSLCnano using a pre-concentration setup with Acclaim PepMap RSLC C18, 2 µm, 100Å column (Mobile phase A: 100% H2O + 0.1% formic acid, Mobile phase B: 100% acetonitrile + 0.1% formic acid). This separation was followed by the analysis with NanoBooster Captivespray™ UHR Quadrupole Time-of-Flight (Bruker Compact, Germany). HyStar program for the control of the whole process, otofControl for MS, Compass Data Analysis for the generation of .mgf files and mascot-based SwissProt database search, and Biotools to list the proteins identified were employed to finalize the analysis.

3. RESULTS

3.1. GCF Study Groups

The mean age, gender, sampled tooth number, PI and PPD values of the two groups are given in Table 2. A total of 6 gels were run by repeating two groups in triplicate, and 5 Periopaper strip samples were used for each gel. The mean age was 6.84 years in the eruption groups, and 10.19 years in the occlusal equilibrium groups.

Table 2. *The mean age, gender, sampled tooth number, mean PI scores of two groups*

Eruption Group	n	Gender (F/M)	Mean Age (Week)	Tooth No (36/46)	PI Mean	PPD Mean
ER-1	5	2/3	356.0	2/3	0	0.9
ER-2	5	3/2	355.6	3/2	0	0.8
ER-3	5	3/2	358.4	3/2	0	0.7
Total	15	53% F 47% M	356.67 (age*:6.84)	53% #36 47% #46	0	0.8
Occlusal Equilibrium Group	n	Gender (F/M)	Mean Age (Week)	Tooth No (36/46)	PI Mean	PPD Mean
OE-1	5	2/3	528.4	3/2	0.6	0.8
OE-2	5	2/3	530.2	2/3	0.4	0.7
OE-3	5	3/2	535.8	2/3	0	0.6
Total	15	47% F 53% M	531.47 (age*:10.19)	47% #36 53% #46	0.33	0.7

*ER:Eruption Group, OE:Occlusal Equilibrium Group, F:Female, M:Male, PI: Silness-Löe Plaque Index, PPD: Probing Pocket Depth *1 year is calculated as 52.17 weeks.*

3.2. Optimization for Protein Extraction from GCF Samples

To develop an optimized protein extraction protocol, urea buffer and rehydration buffer (Bio-Rad ReadyPrep 2-D Rehydration Buffer, U.S.A.) were selected for the first step of this study. We evaluated the ability of extraction of these two methods by one-dimensional SDS-PAGE, and we observed

more diversified protein bands with rehydration buffer as a protein extraction solution (Fig. 3a).

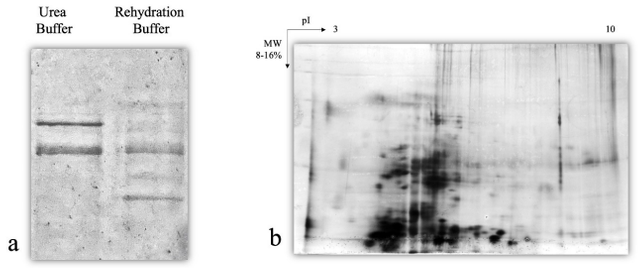


Figure 3. *a. One-dimensional SDS-PAGE gel image of two different extraction buffers; Urea buffer and Bio-Rad Rehydration Buffer b. Silver stained two-dimensional SDS-PAGE gel image of GCF sample, using wide range IPG strips (pH 3-10).*

3.3. Two-Dimensional Polyacrylamide Gel Electrophoresis

For the first dimension, the IEF procedure, a total of 4 hours and 40 minutes was followed. After the second dimension was performed, gels were stained with silver, and these 2D-PAGE gels were scanned, and their visual gel images are displayed in Fig. 4. These images demonstrate that the majority of the proteins detected have a low molecular weight below 50 kDa, and the majority of these proteins aggregated in the pH range of 5 to 6.5.

3.4. Gel Image Processing

There were two groups with 3 replicates, Gel ER-3 was chosen as the master gel and all other gel-to-gel spot matchings were performed against this gel in PDQuest software. A greater number of spots were detected in the OE group (avg. 89) than ER group (avg. 67.6). It was found 86 protein spots were in the master gel, and 62% and 69% of all spots matched in group ER while group OE this rate resulted as 36%, 28% and 25% (Table 3).

Table 3. *Detected spots on each gel and their matched spot number, rate and correlation coefficient to master gel.*

Gel Name	Group	Spots	Matched	Match Rate-1**	Match Rate-2***	Correlation Coefficient
ER-1	ER	55	38	69%	43%	0.292
ER-2	ER	62	39	62%	44%	0.389
ER-3*	ER	86	86	100%	97%	1.000
OE-1	OE	95	27	28%	30%	0.314
OE-2	OE	101	26	25%	29%	N/A
OE-3	OE	71	26	36%	29%	0.250

** Master Gel; **Match Rate-1: The percentage of matched spots relative to the total number of spots on the gel;***Match Rate-2: The percentage of matched spots on the gel relative to the total number of spots on the master.*

Spot quantity can be calculated by PDQuest, which is the total intensity of a defined spot in the gel image. This corresponds to the amount of protein in the actual spot in the gel and it is calculated automatically during spot detection (11). The spot quantitation graphs are shown in Table 4 with protein spots number. The protein spots numbered 4302, 5401, and 8201 were detected in all gels of both groups and protein spots numbered 6301 and 6302 were observed in all gels of ER group while none of the gels of the OE group. The protein spots numbered 1401 and 4201 were observed in all gels of the OE group while only one gel of ER group. The locations of these protein spots on the gels were reported in Fig. 4.

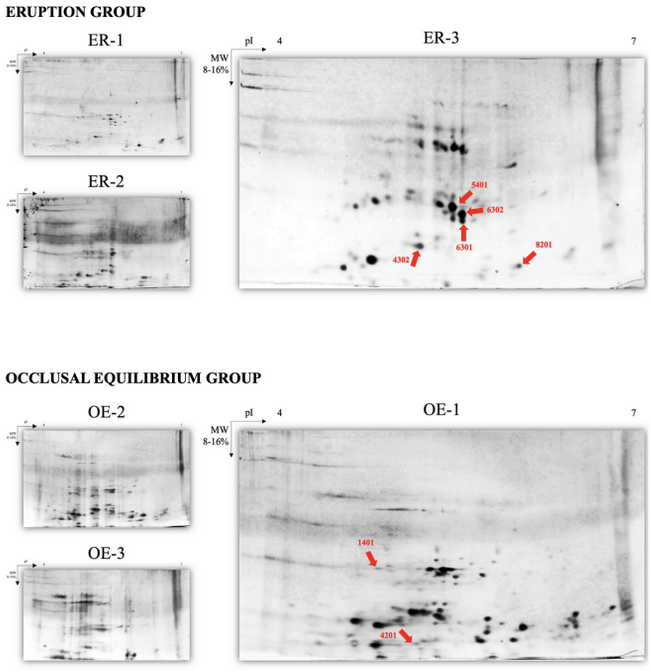
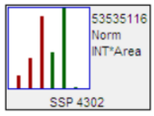
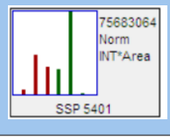
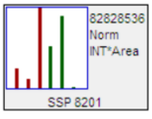
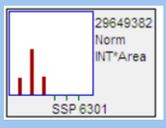
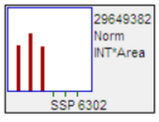
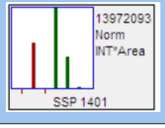
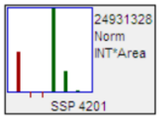


Figure 4. *Visual images of silver-stained gels. Protein spots that were analyzed with MS were marked.*

3.5. Identification of Proteins with Mass Spectrometry

Seven spots, four of which were differentially expressed between two groups and three of which were similarly expressed on both six gels, were selected for mass spectrometry analysis. After the in-gel digestion protocol, peptides were analyzed by mass spectrometry. A total of 7 proteins were identified from the spot samples, particularly keratins, albumin, and apolipoprotein A1 (Table 4).

Table 4. *Commonly and differentially expressed proteins between ER and OE groups and their quantitation graphs. Trypsin hits were omitted.*

Num. of spot and quantitation graph	Accession	Description	Mass	Score	Num. of significant matches
#4302 	K1C10_HUMAN	Keratin, type I cytoskeletal 10 OS=Homo sapiens OX=9606 GN=KRT10 PE=1 SV=6	58792	74	1
	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens OX=9606 GN=KRT1 PE=1 SV=6	65999	63	1
#5401 	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens OX=9606 GN=KRT1 PE=1 SV=6	65999	621	20
	K1C10_HUMAN	Keratin, type I cytoskeletal 10 OS=Homo sapiens OX=9606 GN=KRT10 PE=1 SV=6	58792	275	9
	APOA1_HUMAN	Apolipoprotein A-I OS=Homo sapiens OX=9606 GN=APOA1 PE=1 SV=1	30759	248	6
#8201 	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens OX=9606 GN=KRT1 PE=1 SV=6	65999	101	3
#6301 	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens OX=9606 GN=KRT1 PE=1 SV=6	65999	580	18
	K22E_HUMAN	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens OX=9606 GN=KRT2 PE=1 SV=2	65393	305	9
	K1C10_HUMAN	Keratin, type I cytoskeletal 10 OS=Homo sapiens OX=9606 GN=KRT10 PE=1 SV=6	58792	507	12
	ALBU_HUMAN	Albumin OS=Homo sapiens OX=9606 GN=ALB PE=1 SV=2	69321	52	1
#6302 	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens OX=9606 GN=KRT1 PE=1 SV=6	65999	341	13
	ALBU_HUMAN	Albumin OS=Homo sapiens OX=9606 GN=ALB PE=1 SV=2	69321	55	1
#1401 	K2C4_HUMAN	Keratin, type II cytoskeletal 4 OS=Homo sapiens OX=9606 GN=KRT4 PE=1 SV=5	56109	84	2
	K1C9_HUMAN	Keratin, type I cytoskeletal 9 OS=Homo sapiens OX=9606 GN=KRT9 PE=1 SV=3	62027	65	1
#4201 	K2C1_HUMAN	Keratin, type II cytoskeletal 1 OS=Homo sapiens OX=9606 GN=KRT1 PE=1 SV=6	65999	165	5

4. DISCUSSION

Over the last century, various theories have been presented to understand the mechanisms of tooth eruption, but still, the mechanism behind this process is not yet fully unraveled (12). It has been shown that the tooth follicle regulates osteogenesis and osteoclastogenesis by secreting many different chemical mediators and plays a major role, particularly in the intraosseous phase of tooth eruption (13-18). While the dental follicle is necessary for eruption, it transforms into the periodontal ligament, once the tooth emerges from the gingiva (17). It is thought that extracellular matrix components such as collagen and fibronectin in the periodontal ligaments ensure tooth movement by creating tension and compression during the eruption (19). Several studies suggest that biochemical changes in GCF seem to reflect changes in the periodontal ligament (20, 21) and collection of this fluid is easy and non-invasive. Based on that

information, we designed this study considering that the biomarkers related to the supraosseous phase of the tooth eruption can be observed in GCF.

Since GCF is mostly in the field of periodontology and the gingiva gives serious reactions to more inflammatory conditions, comparative proteomic studies in GCF have been carried out mostly among adult patients of periodontal health and disease states (5, 7, 8, 22-26). Very few proteomic studies on GCF were conducted in pediatric samples (9, 27-29). In these studies, GCF samples were collected and analyzed with mass spectrometry-based tools to be able to demonstrate the differences in GCF composition in permanent and primary teeth, root resorption status, and pubertal growth states (9, 27-29).

When the tooth emerges into the oral cavity, the tooth movement rate is accelerated until the tooth reaches its occlusal contact. The post-emergent eruptive spurt phase, in which the fastest tooth movement occurs in the oral cavity, is thought to be worth investigating. While planning the study groups, mandibular permanent first molars were preferred for sampling. The most important reason for choosing it is that permanent molars are not succedaneous which means they do not replace any primary teeth. The presence of a shedding primary tooth while the permanent tooth is erupting may cause damage to gingival tissues, the gingiva may bleed, and the GCF content would be altered because of the exfoliated primary tooth. Therefore, the change between the post-emergent eruptive spurt phase and the occlusal equilibrium phase was examined in permanent first molars (2).

Due to the limited amount of GCF samples and their protein harvest, our first approach was to enrich the protein extraction output from GCF samples. Therefore, we compared two separate extraction solutions, urea buffer and rehydration buffer for their protein yield. Rehydration buffer provided more versatile protein bands, while urea buffer demonstrated only a few but dense protein bands when samples were separated on one-dimensional SDS polyacrylamide gel (Fig. 3a). For the next steps, we employed the rehydration buffer to extract proteins from GCF samples to examine the possibility of a wide range of proteins involved in the tooth eruption. We tested our protein extraction buffer efficiency with an additional clean-up step including Zeba™ Spin column to avoid any interference that might affect the resolution of two-dimensional polyacrylamide gels (Fig. 3b). When compared to the method in the study of Tsuchida et al (25), conventional urea buffer combined with ultrafiltration as the extraction method, our optimized protocol demonstrated higher resolution images of more protein spots which were not occluded due to the background noise or vertical streaks. However, the selection of the wide pI range for the IPG strip (3 to 10) caused spot overlapping due to similar molecular weight and a pI range of 5 to 6 for the GCF proteins extracted. For this reason, IPG strips of pH 4 to 7 were employed for the rest of the study. In the second dimension, corresponding GCF protein samples were separated with 8-16% gels followed by visualization with ProteoSilver™ silver stain kit.

When triplicates of 2D gel images of sample groups were compared, more protein spots were visualized in the OE group (OE, avg. 89; ER, avg. 67.6) according to PDQuest, suggesting that protein diversity and/or the number of protein modifications were higher in the OE group (Table 3). Later, we selected the spots displaying different intensities for in-gel trypsin digestion and mass spectrometry analyses to identify their protein contents (Fig. 4). We received hits for keratin type I cytoskeletal 10 (K1C10), keratin type II cytoskeletal 1 (K2C1), apolipoprotein A-1 proteins in spots number 4302, 5401 and 8201, and the proteins seen in these spots were detected in all gels of both groups (Table 4). The proteins we identified in 6301 and 6302 numbered spots were keratin type II cytoskeletal 1 (K2C1), keratin type II cytoskeletal 2 epidermal (K22E), keratin type I cytoskeletal 10 (K1C10) and albumin (Table 4). These spots were displayed only in ER group gels. We identified keratin type II cytoskeletal 4 (K2C4), keratin type I cytoskeletal 9 (K1C9), and keratin type II cytoskeletal 1 (K2C1) in spots numbered 1401 and 4201, and the intensity of these spots were lower in the ER group (Table 4). We encountered K1C9, K1C10, K2C1, K2C4, and K22E in our GCF samples due to the known expression of different types of cytokeratins in gingival pockets (7). Cytokeratins, which are cytoskeleton structural proteins, form intermediate filaments and are considered reliable markers of development and differentiation in epithelial cells (28).

The gingival sulcus is composed of oral sulcular epithelium and junctional epithelium. These components are a barrier against bacterial penetration and have a high turnover rate that allows rapid replacement of damaged cells and tissues (30). The normal turnover rate in healthy periodontium is known to be one of the most rapid of all epithelial tissues, and the keratin content in the crevice would be expected to be significantly greater than in other physiological fluids (30). In this study, among the keratins, K1C10 and K2C1 types were seen in similar amounts in both groups, while K2C4 and K1C9 type keratins were detected very rarely in the ER group but were found in the OE group. Elevation of the K2C4 and K1C9 proteins in GCF may be associated with the maturation of gingival tissue and permanent molar or with increasing age.

Bostanci et al (7) identified 25 different keratins and showed K1C9, K1C10, K2C1, and K2C4 were less regulated in aggressive periodontitis compared to healthy adult subjects. We also mention that K2C4 and K1C9 proteins are suppressed in tooth eruption, which might suggest a similarity in these two specific keratin types between tooth eruption and periodontal disease. When the tooth penetrates the oral mucosa, periodontal destruction occurs until the tooth crown fully emerges, and the gingival tissues give a physiological inflammatory response (9). On the other hand, Huynh et al (31) compared K1C9, K1C10, K2C1, and K22E levels in healthy, gingivitis, and chronic periodontitis adult subjects, and reported that the level of these proteins

was similar in healthy and gingivitis groups while elevated during periodontitis.

One of the most abundant proteins in GCF is albumin, which takes part in transport by binding to hormones, cytokines, and lipoproteins (7). Albumin was detected in pellicle and saliva, as well as in GCF samples (32). Bickel et al (33) and Carneiro et al (5) evaluated the albumin levels of GCF from healthy and periodontal disease adult subjects and reported that albumin levels were higher in periodontal disease groups. In this study, we observed that albumin levels increased in the eruption group as well. If the eruption of the tooth is considered a physiological inflammation status, albumin levels in GCF may indicate similar reactions just as in periodontal disease. When the protein profiles of pubertal subjects were compared to the post-pubertal, serum albumin was found to be higher in the pubertal group (29). The albumin levels in our GCF samples from the younger group demonstrate advanced intensities while it gets lower through ageing, which may imply the age-association effect on the albumin content of GCF (Table 4).

Apolipoprotein A-1 (ApoA-1) is the major protein component of high-density lipoprotein (HDL) (34). It was detected in both groups demonstrating no significant difference, similar to the studies identify ApoA-1 in their analysis (Table 4) (8, 23, 25, 29, 35). Only Moriya et al (28) reported that the ApoA-1 level was upregulated in the GCF of the permanent teeth compared to the primary teeth.

The periodontium is very vascular and highly permeable, therefore a variety of biomolecules in periodontal tissues might infiltrate the gingival sulcus making GCF an important biomarker. In this study, we tried to identify the GCF protein expression changes in two different phases of supraosseous tooth eruption. Proteins showing similarities and differences between these groups were determined. Overall, we report that the ER group displays slight similarities to the protein characterization in periodontal disease states (5, 7, 33). When the tooth first emerges from the gingiva, there might be a temporary physiological inflammatory response similar to that in periodontal disease, but not pathological. This study, which is the first GCF proteome study on tooth eruption, can be seen as a new way to look for the unknown components of this process. Although there are some limitations to conducting a proteomic study on GCF samples, such a relatively small sample volume with limited protein output and masking of low-concentration proteins by high-abundance proteins (31), it would still be manageable by optimizing protein extraction along with the application of new combinations of proteases to enrich peptide composition for mass spectrometry to identify more of the proteins. Our findings from this study support that GCF holds great potential to unravel the biochemical events relating to physiological and pathological conditions.

5. CONCLUSION

We present a mass spectrometry-based proteomics approach for the analyses of GCF, and we report several proteins related to tooth eruption. The novel protein extraction method for GCF resulted in better resolution for 2D-PAGE and image quantitation, applicable for future studies.

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Acquisition of data for the study: SYA
Analysis of data for the study: SYA, HC
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Clinical and Experimental Health Sciences

Assessment of Micro-Gap in Hybrid Abutment-Crowns Fabricated with Different Materials

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ABSTRACT

Objective: The aim of this in vitro study is to evaluate the micro-gap changes in three dimensions after thermodynamic loading between hybrid abutment crowns made of different materials and implants with internal conical connection.

Methods: A total of 10 morse cone connection implants (Straumann Bone Level Implant, Institut Straumann AG, Basel, Switzerland) were used. In this study, two study groups were formed using lithium disilicate glass-ceramic (LD) and polymethyl methacrylate (PMMA) in hybrid abutment-crown production (n=5). Hybrid abutment-crowns were fabricated by CAD/CAM system. Hybrid abutment crowns were designed and manufactured digitally. A 4-month of clinical cycle was applied to the samples in the chewing simulator. The micro-gap at the implant-abutment interface was visualized with micro-CT before and after thermodynamic loading. Micro-gap change was determined using these obtained images. For comparisons, independent t-test was used.

Results: When comparing the micro-gap volumes before and after aging, no significant difference was observed between the LD and PMMA groups. The micro-gap increase after loading was 0.68 ±0.209 in the LD group and 0.45 ±0.373 in the PMMA group. Although the increase was higher in the LD group, there is no statistically significant difference between two groups.

Conclusion: he micro-gap in the interface of implants and hybrid abutment crowns increased after aging. Hybrid abutment-crown material affected the micro-gap increase, but it was not statistically significant.

Keywords: Micro-gap, hybrid abutment-crown, implant-abutment connection.

1. INTRODUCTION

The implant-abutment connection (IAC) is the transition point from surgery to the prosthetic stage and it is the primary determinant of the success and stability of the implant-supported prosthesis (1). It has been stated that the implant-abutment connection is an important factor that determines the long-term prognosis of the treatment (2). Mismatch at the implant-abutment attachment interface can cause increased stress at the connection part, leading to screw loosening, screw breakage, and implant overloading (3,4). This situation can also lead to peri-implant pathology by causing microleakage and bacterial colonization (5,6,7). Various connection types have been developed to eliminate micro-gap caused by incompatibility between the implant and abutment. Currently, the use of conical connections is most recommended to avoid micro-gaps (8,9).

In addition to the implant abutment connection, the choice of abutment and material used in restoration production is important for the long-term success of the treatment (10,11). With the development of CAD/CAM systems, hybrid restorations prepared by fixing the restoration on the original titanium abutment of the implant system have become popular (12). Hybrid restorations produced as a combination of titanium abutments and various materials are very advantageous in terms of low cost and easy application (13) (14). In addition, soft tissue modeling can be performed during healing using hybrid restorations in immediate loading applications (15,16). Hybrid restorations used in the immediate loading protocol can be fabricated from ceramics such as lithium disilicate (LD) or from different materials such as hybrid ceramics, composites and polymethylmethacrylate (PMMA) (17,18,19). Many studies have been conducted to evaluate the effect of abutment production techniques and restoration materials on the implant-abutment connection (11,20). However, more studies are needed to evaluate the effects of all-ceramic and polymer materials, which are

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increasingly used in fabricating hybrid restorations, on the implant-abutment connection. The aim of this study is to evaluate the micro-gap volume between internal morse cone implants and hybrid abutment-crowns manufactured from two different materials before and after dynamic loading.

2. METHODS

2.1. Design of Study

After thermodynamic loading, the effect of different restoration materials on the micro-gap change between the implant and abutment was evaluated. In this present study, these steps were followed to evaluate the micro-gap changes: production of monolithic hybrid abutment-crowns from LD blocks and PMMA blocks with a digital system for implants, cementation of the produced restorations on titanium bases, loading of the prepared hybrid abutment crowns on the implants, three dimensionally (3D) evaluation before aging, aging equal to 4-month oral use with a chewing simulator, after aging 3D evaluation, 3D superimposition of the obtained images, determination of micro gap change and statistical analysis of the results were performed respectively.

Hybrid abutment crowns with LD blocks (IPS E-max CAD, Ivoclar Vivadent, Schaan, Liechtenstein) and PMMA blocks (Telio CAD, Ivoclar Vivadent, Schaan, Liechtenstein) were prepared for the Straumann Bone Level Implants (Institut Straumann AG, Basel, Switzerland). The Straumann bone level implant-abutment connection is morse-conical. This connection has a 15º tapered structure and four slots. Ten implants with a diameter of 4.1mm and a length of 10mm were included in this study. Two study groups were formed for LD and PMMA materials. Five hybrid abutment crowns were fabricated from each material (n=5). Study groups and sample numbers are given in Table 1.

2.2 Preparation of Samples

For the fabrication of the crowns, a ti-base abutment (TiBase S BL 4.1 L, Sirona, Bensheim, Germany) was placed on the implant. Scan post (ScanPost, Sirona, Bensheim, Germany) were placed on the ti-base abutment, and digital impressions were taken with a Cerec Omnicam intraoral camera (Sirona, Bensheim, Germany). After the optical impression process, STL data obtained with CEREC SW 4.5.1 software (Sirona, Bensheim, Germany) on a portable computer were transferred to CEREC inLab 4.5.1 program (Sirona, Bensheim, Germany). A first premolar crown compatible with the ti-base was designed by paying attention to anatomical details. The designed crown data were saved as STL data and transferred to CEREC SW 4.5.1 (Sirona, Bensheim, Germany) software. Production of the crown was completed with the CAM unit CEREC MCX (Dentsply-Sirona Dental Systems, Bensheim, Germany). All processes to complete the crystallization and polishing of the restoration were performed with a Programat P 310 porcelain furnace (Ivoclar Vivadent, Schaan, Liechtenstein). After checking the compatibility of the restoration with the ti-base abutment, the other LD crowns were produced with the same steps. The same design and milling processes were applied for PMMA (Telio CAD, Ivoclar Vivadent, Schaan, Liechtenstein) crowns. Polishing of PMMA crowns was finished with brush bur.

According to company instructions, all restorations were cemented onto ti-base abutments with Multilink Hybrid Abutment Cement (Ivoclar Vivadent, Schaan, Liechtenstein).

The implants were embedded in acrylic using a silicone index. The prepared hybrid abutment crowns were loaded onto the implants with a torque wrench. A load of 35 N was applied with a torque wrench.

Table 1. *Sample distribution of the study groups according to tested materials. (LD= Lithium disilicate ceramic; PMMA=Polymethyl methacrylate)*

Group	Materials (Product name, manufacturing company)	N
LD	Lithiumdisilicate ceramic (IPS e.max CAD, Ivoclar Vivadent, Schaan, Liechtenstein)	5
PMMA	Polymethyl methacrylate (Telio CAD, Ivoclar Vivadent, Schaan, Liechtenstein)	5

2.3. Determination of the Initial Micro-gap

Before aging, all samples were scanned with the micro-tomography device (Skyscan 1174, Skyscan, Kontich Belgium) to determine the initial micro-gap volume. After finishing the scanning process, 3D images were obtained by rendering the radiographic image sequences taken during 180° rotation. CTan (Bruker, Kontich, Belgium) software was used to determine the micro-gap volume, and CTVol (Bruker, Kontich, Belgium) software was used for the 3D analysis of the images.

Figure 1. *All samples prepared to place in the chewing simulator*

2.4. Aging of the Samples

Thermodynamic aging of the samples was performed on a dual-axle chewing simulator (SD Mechatronic Chewing Simulator CS-4.2, Willytech, Munich, Germany). Hybrid

restoration-implant complexes were fixed in the sample holders of the device with acrylic (Figure 1). Metal parts of the device are fixed in the upper compartment for dynamic loading application. Samples were simulated 80,000 cycles of chewing, equivalent to approximately four months of clinical use. Dynamic loading was performed with 50 N at 5-55ºC.

2.5. Determination of Micro Gap Change After Loading

After loading, samples were scanned a second time with microtomography, and 3D radiographic images were obtained. 3D images of each sample before and after loading were superimposed on three axes (x, y, z). The superimposing process was performed with Skyscan Data Viewer (Bruker, Kontich, Belgium) software. The area to measure of micro-gap in the implant-abutment interface was determined on these superimposed images. In the determined areas, the change was determined by calculating the micro-gap volume before and after dynamic loading.

Table 2. *Comparison of micro-gap before and after aging*

Groups	LD	PMMA	P value
Micro-gap Before Aging (%) (Mean+SD)	2,65 ± 0,338	2,71 ± 0,59	0,863
Micro-gap After Aging (%) (Mean+SD)	3,33 ± 0,444	3,16 ± 0,719	0,663

Independent t test; Mean±Standart Deviation
LD= lithium disilicate ceramic; PMMA=polymethyl methacrylate

2.6. Statistical Analysis

The IBM SPSS (Statistical Package for Social Sciences) for Windows V22 (SPSS Inc, Chicago, USA) program was used to evaluate the findings obtained in this present study. Evaluations were done at 95% confidence interval and $p<0.05$ significance level. The assumption of normal distribution was checked with the Shapiro-Wilk test. Before and after aging micro-gap values and micro-gap changes after aging of the two groups using different restoration materials were evaluated. An Independent t-test was used as parametric test assumptions were provided in comparisons.

Table 3. *Comparison of micro-gap changes*

Groups	Mean+SD (%)	P value
1.LD	0.68 ±0.209	0,273
2.PMMA	0.45 ±0.373	

Independent t test; Mean±Standart Deviation
LD= lithium disilicate ceramic; PMMA=polymethyl methacrylate

3. RESULTS

Representative µCT images of implant-abutment junction surface before and after aging from both study groups are shown in Figure 2-3. In this study, comparisons between groups were achieved with percentage (%) values. Comparisons of the micro-gap before and after aging are given in Table 2. Micro-gap was observed in all samples regardless of condition. No significant difference was observed when the two groups' micro-gap volumes before and after aging were compared. (p= 0,663 and p>0,541). After loading in both groups, an increase in the micro-gap volume was determined. The mean micro-gap increase (%) was %0,68 ± 0.209 in the LD Group and %0,45 ± 0,37 in the PMMA Group. Although the increase was higher in the LD Group, no statistically significant difference was found (p= 0,273) (Table 3).

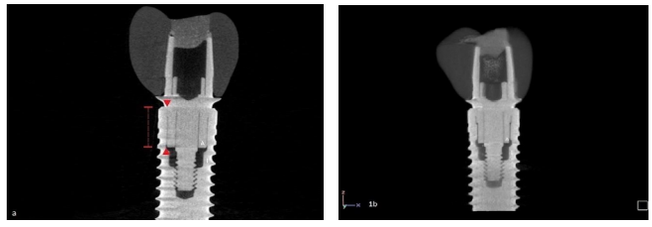

Figure 2. *Representative micro-CT images for LD group before (a) and after (b) aging. The dashed lines show the measured area (3.0x magnification). (I = Implant body, A = Abutment).*

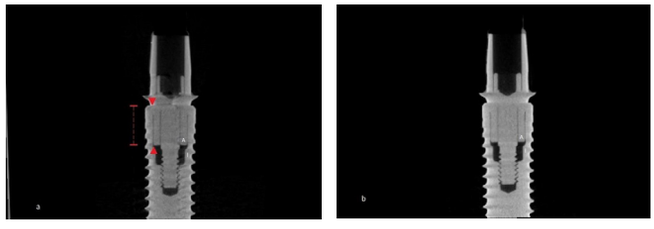

Figure 3. *Representative micro-CT images for PMMA group before (a) and after (b) aging. The dashed lines show the measured area (3.0x magnification). (I = Implant body, A = Abutment).*

4. DISCUSSION

Incompatibility, which creates micro-gaps and loss of stabilization between the implant and abutment, causes mechanical and biological problems. It has been reported that the increased mechanical stress on the connection components, the implant and the bone tissue surrounding the implant neck may cause preload loss or mechanical problems such as screw loosening/breakage (21,22,23,24). The micro-gap increase can cause bacterial leakage, micro-movements and wear between two components that will affect osseointegration (6,11,25). Rack et al. reported that the micro-gap increase occurred under cyclic loading in different internal conical joint systems, causing the micro-motion range to expand. They concluded that with an increasing mismatch between the two components in the implant abutment joint, the amount of microleakage increases and the mechanical properties of the joint weaken (26). In this present study, it was observed that the micro-gap between implant and abutment increased in short-term loading in both groups.

Many studies have been conducted to evaluate the abutment production technique and the effect of materials used in restoration production on mechanical stability in

implant-supported restorations (11,20). However, there are still not enough studies to understand the mechanical behaviour of all materials and abutment types (27,28) Zordk et al. compared the torque loss of hybrid abutment crowns fabricated with zirconia, lithium disilicate and PEEK materials after thermal aging. And no statistically significant difference was found between these three groups (20). In this present study, micro-gap changes between the hybrid abutment crowns, which were produced by using two different materials, and implants after aging were investigated. However, this increase was similar in hybrid restorations prepared with the same production technique and different materials and fixed on identical titanium bases.

The elastic modulus of the materials that used implant-supported restorations affects the stress distribution from the occlusal face to the implant (29,30). Tribst et al. reported that hybrid restorations with low elastic modulus show better stress distribution (31). In a different study, it has been reported that materials with low elastic modulus have little effect on the micro-gap change (32). In this present study, although the elastic modulus of the restoration materials was different, there was no statistically significant difference between the changes. The micro-gap change was less in the Telio CAD (3.2 GPa) group with a low elastic modulus than in the lithium disilicate glass-ceramic (95 GPa) group with a higher elastic modulus.

5. CONCLUSION

Micro-gaps between implant-abutment existed in all conditions. The micro-gap volume at the interface of implants and hybrid abutment crowns increased after aging. Hybrid abutment-crown material affected the micro-gap increase, but it was not statistically significant.

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Clinical and Experimental Health Sciences

In Vitro Investigation of Shear Bond Strength of Titanium Alloy Bonded to Monolithic Zirconia Prepared Via Different Surface Roughening Methods Using Different Cements

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ABSTRACT

Objective: To evaluate the shear bond strength (SBS) of yttrium-stabilized tetragonal zirconia polycrystals bonded to titanium alloys via different surface treatment methods using four different cements.

Methods: Eighty titanium and monolithic zirconia discs were prepared with computer-aided design/manufacturing (CAD/CAM) technology. All titanium discs and 40 of monolithic zirconia discs were polished by using silicon carbide paper and sandblasted with 50 µm aluminum oxide (Al_2O_3). Tribochemical silica coating was applied to remaining 40 monolithic zirconia discs. The monolithic zirconia discs were divided into eight groups after surface treatment (n=10). Titanium discs were cemented using conventional glass ionomer cement (GIC), resin-modified GIC, self-adhesive resin cement, and dual-cure resin cement. The SBS test was performed using a universal testing machine. The failure patterns were examined by using a scanning electron microscope (SEM). Data were statistically analyzed with one-way analysis of variance (ANOVA), two-way ANOVA and Tukey's test (α<.05).

Results: The SBS values differed according to the surface treatment methods and cements used (p<.001). The highest and lowest SBS values were measured in the tribochemical-silica-coated G-CEM ONE (34.77±5.53 MPa) and Al_2O_3 sandblasted GC Fuji I (3.30±0.77 MPa) cement groups, respectively. Failure analysis revealed that 41.25%, 31.75% and 25% of the failures were cohesive, adhesive, and combined failures, respectively.

Conclusion: The SBS values between the monolithic zirconia and titanium alloy were significantly higher in the resin cement groups containing 10-methacryloyloxydecyl dihydrogen thiophosphate and 10-methacryloyloxydecyl dihydrogen phosphate (p<.05). While adhesive and combined failures were observed at high SBS values, cohesive failures were detected as the bonding values decreased.

Keywords: Cements, shear bond strength, surface roughness, monolithic zirconia, tribochemical silica coating

1. INTRODUCTION

Titanium, which is frequently used in the fabrication of dental implant abutments, has several advantages such as biocompatibility, resistance to abrasion, and sufficient mechanical durability (1). However, the metallic-gray color of the titanium alloys creates an aesthetic problem, especially in submucosal peri-implant tissues (2). Although zirconia abutments can overcome these aesthetic disadvantages, drawbacks such as failure of the implant-abutment junction area and wear at the implant connection limit their clinical use. To eliminate these issues and achieve more aesthetically pleasing results, hybrid abutments have been developed. A hybrid abutment consists of two components: a prefabricated titanium-based substructure, and a zirconia or lithium disilicate ceramic superstructure. The ceramic superstructure is bonded to the titanium base abutment using cement (1,2,3,4,5,6,7). Thus, the advantages of the two materials include a combination of the durability of titanium and the aesthetic properties of ceramic materials (8). According to previous studies, the clinical success of hybrid abutments depends on the cementation technique (4,7,8).

It has been reported that conventional and resin cements have been used in the cementation of hybrid abutments. (9,10). Resin cements are the material of choice for the cementation of fixed implant restorations due to their high bonding ability with metals and ceramics, wide aesthetic color options, favorable mechanical properties, high strength, superior retention, and low solubility in the oral cavity (9,10). Moreover, in the presence of multifunctional monomers such as 10-methacryloyloxydecyl dihydrogen thiophosphate (10-MDTP) and 10-methacryloyloxydecyl dihydrogen phosphate (10-MDP), the surface wettability of the material increases and crosslinking occurs with the methacrylate groups of the resin cement (11).

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Various surface modification methods such as sandblasting, tribochemical silica coating, hydrophilic acid etching, and laser-based methods are recommended to form a strong mechanical and chemical retention between the resin cement and the ceramic (11,12).

Sandblasting creates a rough surface for the mechanical retention of the cement. Simultaneously, it increases the strength of monolithic zirconia and prevents the spreading of cracks by acting on the compressive stress layer (12). The tribochemical silica coating process not only produces roughness but also chemically activates the ceramic surfaces. As a result of the blasting pressure, the embedded silica and aluminum oxide (Al_2O_3) particles and the binding silane agent react chemically.

The aim of this study was to evaluate the shear bond strength (SBS) between monolithic zirconia and titanium materials that were treated using different surface-roughening methods and to which traditional glass ionomer cement (GIC), resin-modified GIC, dual-cure resin cement and self-adhesive resin cement were applied in vitro. The null hypothesis in this study was that there would be no significant difference in SBS values between the tested cements. The second null hypothesis was that different ways of surface treatment would have no effect on adhesion.

2. METHODS

According to the results of an analysis based on G*Power version 3.1.9.2, (Heinrich – Heine-Universität Düsseldorf, Germany, power = 0.95, a = 0.05, b = 0.05) using on the data of a study (13), the number of speciments to be included in the study in each group was determined to be 10 (Fig. 1). Eighty monolithic zirconia discs with a height of 10 mm and a diameter of 7 mm (GC Initial Zirconia UHT, Tokyo Japan) were fabricated using the with CEREC inLab program (CERECMCX5 Software 18.1 DentsplySirona, Bensheim, Germany), and 80 titanium discs (CopraTi-5 Whitepeaks, Essen, Germany) with a height of 8 mm and a diameter of 12 mm were fabricated by using Dentifa PRO2 (Professional Dental CNC, Istanbul, Türkiye).

The titanium discs were polished using silicon carbide papers of different grit sizes, of P600, P1200 and P2400 (Minitech 233 Presi, Eybens, France), while the monolithic zirconia discs were polished by using P600, P800 and P1200 grit silicon carbide papers (Minitech 233 Presi, Eybens, France) with water cooling. Then, they were cleaned in an ultrasonic cleaner for 5 minutes (14,15).

All the titanium discs and 40 zirconia discs were sandblasted with 50 µm Al_2O_3 particles (Cobra Aluminum Oxide White, Renfert, Germany) under a pressure of 2 bar from a distance of 10 mm for 15 seconds and air dried for 10 seconds. A tribochemical silica coating (CoJet Sand, 3M ESPE, Seefeld, Germany) was applied to the remaining 40 zirconia specimen surfaces from a distance of 10 mm under a 2.8 bar air pressure for 15 seconds. Subsequently, silane (CoJet 3M-ESPE Sil, Seefeld, Germany) was applied for 15 seconds and allowed to dry for 5 minutes (16).

After the surface treatments, the specimens were divided into eight subgroups according to the cement type (n=10).

Conventional GIC (Fuji I, GC, Tokyo, Japan) was applied to the specimen surface (G4, G8) according to the instructions specified by the manufacturer. Resin-modified GIC (FujiCEM Evolve, GC, Tokyo, Japan) was applied to the center of the prepared surface using an automatic tip (G1, G5).

A dual-cure adhesive resin cement (G-CEM LinkForce, GC, Tokyo, Japan) was applied in combination with a universal primer (G-Multi Primer, GC, Tokyo, Japan). The primer was applied to both the titanium and the zirconia surfaces for 15 seconds and air dried for 10 seconds. Then, cement was applied using an automatic tip. (G2, G6).

Self-adhesive resin cement (G-CEM ONE, GC, Tokyo, Japan) was used in combination with an adhesive-enhancing primer (GC AEP, Tokyo Japan). Primers were applied to both surfaces for 10 s and air dried for 5 s. (G3, G7). The cement was applied by using an automatic tip. All resin cements were polymerized for 40 seconds using a 1200 mW/cm² light-emitting diode device (LED Rainbow Curing Light, MDD, Voco, Germany) (Fig.1). During the polymerization, all specimens were maintained under a constant force of 5 Newton (Table 1).

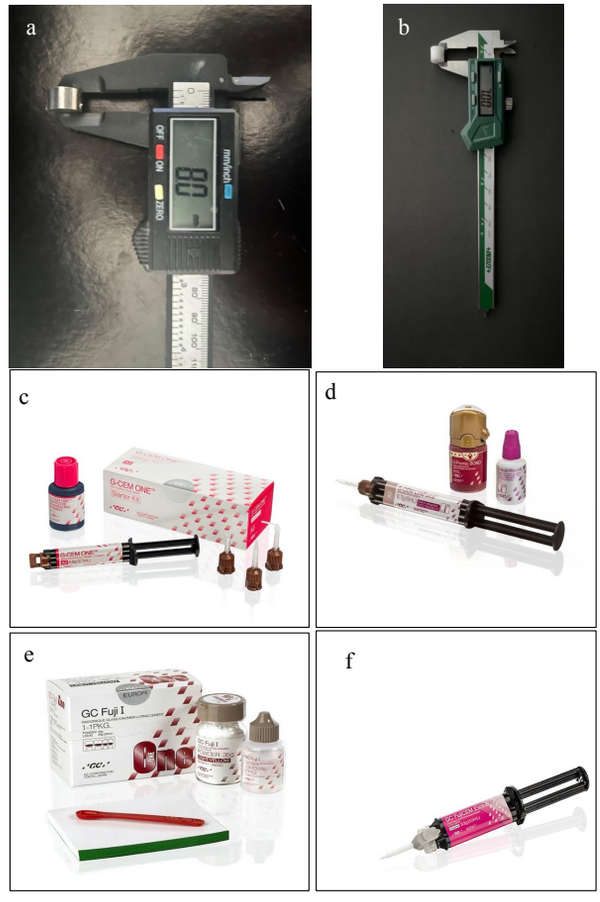

Figure 1. *Titanium disc (a), Zirconia disc (b), G-CEM ONE cement (d), G-CEM LinkForce cement (d), GC Fuji I cement (e), FujiCEM Evolve Cement (f)*

After cementation, all the specimens were placed in a dry-air incubator (Nüve EN 055, Akyurt, Ankara) with distilled water at 37 °C for 24 hours.

***Table 1**. Cements used in this study.*

Material	Main Components	Manufacturer
G-Cem LinkForce	Paste A: Bis-GMA, UDMA, DMA, initiator, pigments Paste B: Bis-MEPP, UDMA, DMA, initiator, Bis-EMA, dibenzoyl peroxide, BHT	GC Corp., Tokyo, Japan
G-Multi Primer	Vinyl silane, phosphoric methacrylate monomer, thiophosphoric ester monomer, methacrylic acid ester, ethyl alchol	GC Corp., Tokyo, Japan
G-CEM Adhesive-Enhancing Primer	Ethanol, 10-MDP, 10-MDTP 4-META, 2-hydroxy-1,3 dimethoxypropane, vanadyl acetylacetonate, 2,6-di-tert-butyl-*p*-cresol	GC Corp., Tokyo, Japan
G-CEM ONE	Paste A: Fluoroaluminosilicate glass, methacrylic acid ester, initiator Paste B: Silica filler, methacrylic acid ester, phosphoric methacrylate monomer, initiator	GC Corp., Tokyo, Japan
Fuji 1	Powder: fluoroalumino silicate glass Liquid: polyacrylic acid, distilled water, silica powder, polycarboxylic acid	GC Corp., Tokyo, Japan
FujiCEM Evolve	Paste A: HEMA, UDMA, Butyl hydroxytoluene, Stabilizer Paste B: Ytterbium trifluoride, Polyacrylic acid, Polybasic carboxylic acid, Quartz	GC Corp., Tokyo, Japan

Bis-GMA: bisphenol-A-glycidyldimethacrylate; Bis-EMA: ethoxylated bisphenol-A-dimethacrylate; MDP: 10-methacryloyloxydecyl dihydrogen phosphate; MDTP: 10-methacryloyloxydecyl dihydrogen phosphate HEMA: 2-hydroxyethyl methacrylate; 4-MET: 4-methacryloyloxyethyl trimellitate; MEPS: methacryloyloxyalkyl thiophosphate methylmethacrylate; UDMA: urethane dimethacrylate; DMA: N, N-dimethylacrylamide.

2.1. Shear Bond Strength Test

To fix the specimens during the SBS measurement, a plate of pure iron (30 mm ´ 10 mm ´ 10 mm), on which the titanium discs could be placed, was prepared (Fig. 2).

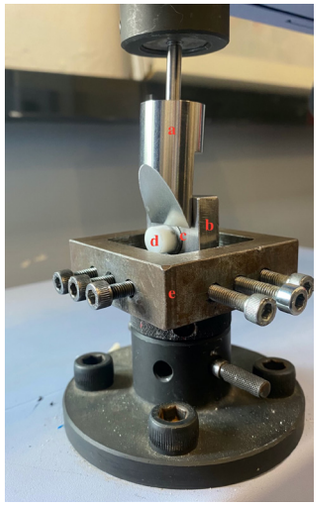

***Figure 2.** Shearing rod (a), The plate of pure iron (b), Titanium disc (c), Zirconia disc (d), Shear bond testing (e)*

The specimens were sequentially placed in a universal testing machine (Shimadzu Corporation, Tokyo, Japan). A blunt-tipped spacer was placed between the titanium and zirconia interfaces, and the specimens were loaded with a speed of 1 mm/min until the zirconia discs were separated from the titanium. The resulting values were obtained by dividing the applied force (N) by the bonded area (mm^2) and were recorded in megapascals.

2.2. Failure Analysis

After the specimens were failured and removed from the test apparatus, the failure sites were observed under a stereomicroscope at 30´ magnification to identify the type of bond failure. Failure types were grouped as adhesive failure (a) at the resin cement-titanium interface, cohesive failure (b) within the resin cement, and combined failure (c), which is a combination of adhesive and cohesive failure. To observe the topographic changes, the specimens were coated with gold (Quorum SC 7620 Sputter Coater, East Sussex, England) and examined using a scanning electron microscope (SEM; Zeiss Evo LS10, Germany) magnifications at 1000´, 2000´, and 5000´.

2.3. Statistical Analysis

Data were analyzed using the IBM Statistical Package for Social Sciences V23 software. Two-way analysis of variance (ANOVA) was used to compare the SBS according to the different etching methods and applied cements. One-way ANOVA was used for analysis of the failure type. Multiple comparisons were performed by using posthocTukey's significant difference test. The statistical significance was set at $\alpha < .05$.

3. RESULTS

The results of two-way ANOVA indicated that both surface treatment methods had a significant effect on the SBS values in the G2, G6 ($p = .013$) and G3, G7 ($p = .006$) cement groups ($p<.001$). A statistically significant difference was also observed between the mean SBS values of the different cement types ($p<.05$) (Table 2).

No significant differences were observed in the SBS values of the G1, G5 ($p = .821$) and G4, G8 ($p=1.00$) cement types for the different surface methods ($p>.05$). Among all groups, G7 exhibited the highest SBS value (Table 2).

According to the failure-type analysis, 41.25% cohesive, 33.75% adhesive and 25% combined failures were observed (Fig. 3) (Table 3). While the cohesive failures were predominantly observed in G1, G4, G5 and G8 groups, adhesive and combined failures were observed in G2, G3, G6 and G7 groups. Cohesive failures were observed in GC Fuji I (G4, G8) cement when compared to G-CEM ONE (G3, G7) and G-CEM LinkForce (G2, G6) cements at a statistically significant level ($p< .001$) (Fig. 4 and Fig. 5).

Table 2. *Shear bond strength values (MPa)*

	Methods			
Cement	**Tribochemical Silane Coating**	**Aluminum oxide Sandblasting**	**p**	**Total Avarage**
GC FujiCEM Evolve	8.19 ± 1.96^{D,E} (G1)	5.85 ± 2.71^{E} (G5)	.821	7.02 ± 2.59^{d}
G-CEM LinkForce	18.55 ± 4.38^{C} (G2)	12.83 ± 2.29^{D} (G6)	.013	15.69 ± 4.49^{a}
G-CEM ONE	34.77 ± 5.53^{A} (G3)	28.64 ± 5.77^{B} (G7)	.006	31.70 ± 6.34^{b}
GC Fuji I	3.34 ± 1.14^{E} (G4)	3.30 ± 0.77^{E} (G8)	1.00	3.32 ± 0.95^{c}
Total Avarage	16.21 ± 12.7	12.65 ± 10.51	.025	14.43 ± 11.72

$^{A\_D}$ There is no difference between cements with the same letter; $^{A\text{-}E}$ No difference between cement and surface roughening method interactions with the same letter (p< .05)

Table 3*. Groups according to the failure type (n=10)*

	Failure Type		
Groups	Cohesive Failure	Adhesive Failure	Combined Failure
G1	7	0	3
G2	0	6	4
G3	0	7	3
G4	10	0	0
G5	7	0	3
G6	0	8	2
G7	0	6	4
G8	9	0	1
Total Average	41.25%	33.75%	25%

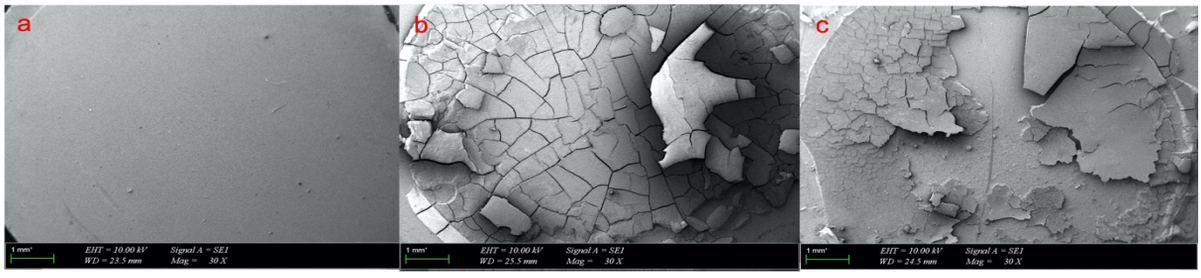

Figure 3. *Scanning electron microscope (SEM) images of failure types at magnification of 30´. (a) Adhesive failure; (b) Cohesive failure; (c) Mixed failure.*

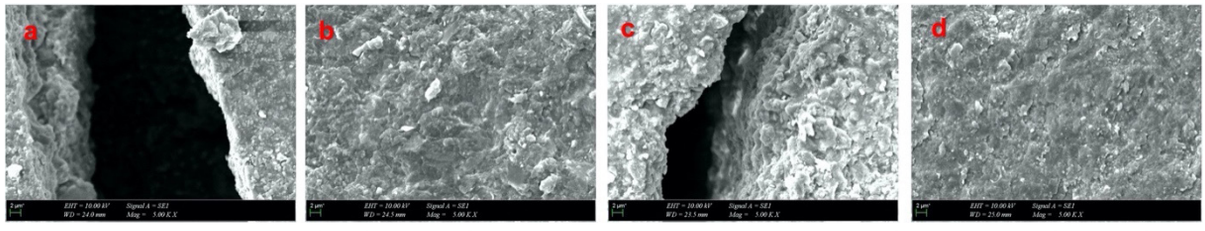

Figure 4. *Scanning electron microscope (SEM) images of Al2O3 sandblasted roughened titanium surfaces at a magnification of 5000´. (a) GC FujiCEM Evolve; (b) G-CEM LinkForce; (c) GC Fuji I; (d) G-CEM ONE.*

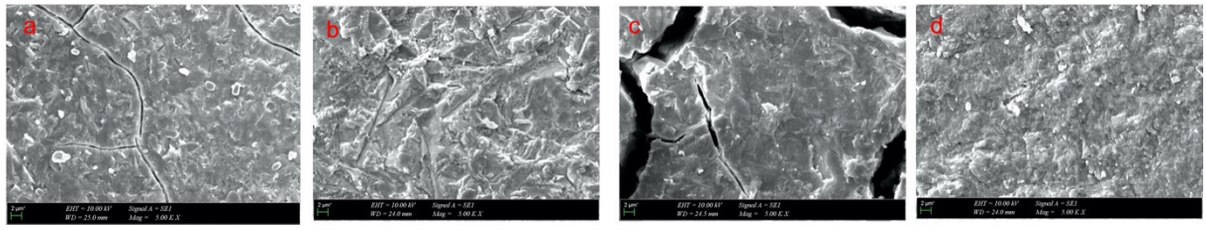

Figure 5. *Scanning electron microscope (SEM) images of titanium surfaces treated with CoJet at 5000´ magnification. (a) GC FujiCEM Evolve; (b) G-CEM LinkForce; (c) GC Fuji I; (d) G-CEM ONE.*

4. DISCUSSION

The study focused on the SBS of Y-TZP bonded to titanium alloys via various surface treatment techniques and cements. Considering the results obtained in this study, the first null hypothesis, that different types of cements do not affect the bonding to the titanium surface, was rejected. Tha statistical analysis revealed a significant difference between the study groups ($p< .001$). The second null hypothesis was rejected because different surface treatment methods affected the bond strength. The CoJet system was significantly more effective in the 10-MDTP and 10-MDP adhesive resin cement groups ($p< .001$).

Al_2O_3 particles (50 µm) have been used in many studies to increase surface roughness and increase the retention of titanium abutments with cements (6,12,17,18,19). Based on these studies, 50 µm Al_2O_3 particles were used for the surface treatment of all titanium surfaces in this study.

The strong and long-term bonding between cement and titanium depends on the constituents, properties, and bonding ability, as well as on the surface properties of the titanium. Therefore, four different categories of luting cements were used in our study.

The results of this study revealed that the SBS values of both Fuji I (G4, G8) and FujiCEM Evolve (G1, G5) groups were significantly lower than those of the adhesive resin groups ($p< .001$). Fuji I, a brand of GIC, provides advantages such as a low cost, relatively improved biocompatibility, fluoride release, and ease of manipulation (20). The results of our study are similar to the results of the study by Fawzy et al (6), where the Fuji I cement primarily exhibited cohesive failure and low SBS values. The FujiCem Evolve cement also showed similar results as the Fuji I cement in our study.

Sandblasting increases the bond strength by enhancing the surface area and roughness. Zhang et al. suggested that sandblasting reduces the strength of zirconia by causing microcracks. However, research has demonstrated that the resin cement infiltrates into the microcracks, thereby significantly increases the strength of the ceramic (12). Moreover, in the CoJet system, the silica particles not only roughened the surface but also promoted chemical retention through the bonding between the silane and the silica-coated zirconia surface (21). Previous studies have reported that finer micro-retentive grooves are observed in the SEM images of CoJet groups than in the groups with Al_2O_3 sandblasting (22,23,24,25). Thus, the CoJet group exhibits higher SBS values than the group with Al_2O_3 sandblasting. In this study, similar to the literature, the SBS values of the CoJet system were observed to be statistically high in the G-CEM ONE (G3, G7) and G-CEM LinkForce (G2, G6) cement groups.

Specimens containing 10-MDP-containing systems are recommended for the long-term adhesive durability of adhesion between monolithic zirconia and resin cement (26). Researchers have speculated that 10-MDP does not hydrolyze because it reacts with the hydroxyl groups on the ceramic surface, provides chemical bonding with zirconia, and contains a long carbonyl chain (11). Specimens treated with self-adhesive resin cement containing 10-MDTP and 10-MDP (G2, G3, G6, G7) showed higher SBS values than those treated with other cement types (27,28). In this study, the SBS was found to be significantly higher in the adhesive cement systems containing 10-MDTP and 10-MDP.

Because shear strength are the most dominant forces during chewing and other jaw movements (29,30,31), the SBS test, which is the most commonly used test method, was used in our study to evaluate the metal-resin bonding efficiency in vitro.

The type of failure also supports the SBS values (32,33). Altan et al. evaluated the SBS values between a monolithic zirconia material obtained by computer-aided design/manufacturing and resin cement obtained after different surface treatments and reported that cohesive failure were observed in groups with low SBS values, whereas combined and adhesive failures were observed in groups with high SBS values (33). In accordance with the findings of Altan et al., cohesive failure occurred in GIC and resin-modified GIC in this study.

The zirconia and titanium-zirconia bonding ability. The bonding ability of titanium and 3Y-TZP is still under investigation, and several bonding protocols consisting of different surface treatments, primers, and luting agents have been reported to have a significant influence on bonding to the zirconia surface. A number of factors (temperature, pH, saliva chemistry, food or drink interaction, presence of microorganism) may interfere bond strength. Śmielak et al. (34) reported greater retentive shear bond strength for polycarboxylate cement and zinc-oxide-eugenol cement compared with Panavia F.2, when the monolithic zirconia disc cemented onto the Ti disc. Shear bond strength values were found to be greater in the groups that were sandblasted with aluminum oxide. Adhesive failures were observed in the Panavia F.2 cement groups.

The simulation of intraoral conditions is significantly challenging in a laboratory setting. Moreover, the negative C-factor effect of the cements and the shrinkage of the resin cement after polymerization, which occurs in clinical conditions, could not be imitated. The thermal cycle, pH changes, and dynamic fatigue load, which were not evaluated in this study, may affect the durability of the resin bond. To confirm the data obtained from this in vitro study, clinical follow-up studies are required in the presence of chewing forces and in the oral environment that can affect the long-term stability of the resin bond.

Considering the results obtained in this study, the first null hypothesis, that different types of cements do not affect the bonding to the titanium surface, was rejected. Tha statistical analysis revealed a significant difference between the study groups ($p<.001$). The second hypothesis was rejected because different surface treatment methods affected the bond strength. The CoJet system was found to be significantly more successful in the 10-MDTP – and 10-MDP-containing adhesive resin cement groups ($p<.001$).

In this study, the post-thermocycling bond strength was not evaluated, this might be considered as a limitation. This matter should be investigated further by comparing differences in initial and postthermocycling bond strength values. Another limitation was that the titanium discs used to provide fundamental information on cement adhesion did not accurately represent the clinical situation of cement flow and distribution between titanium and zirconia surfaces. In addition, only monolithic zirconia material was used, different results might have been provided with different types of materials.

5. CONCLUSION

Within the limitations of this in vitro study, the following conclusions were drawn:

1. Conventional GIC (GC Fuji I) and resin-modified GIC (GC FujiCEM Evolve) exhibited significantly lower SBS values with the titanium surface, whereas the use of self-adhesive resin cements such as G-CEM ONE and G-CEM LinkForce, following the application of 10-MDP and 10-MDTP primer, provided effective bonding to the titanium surface.

2. While cohesive failures occur in conventional and resin-modified GICs with low SBS values, mostly adhesive and combined failures are predominantly observed in groups with high bond strengths.

3. Both sandblasting and tribochemical silica coating methods, which are applied for the surface treatment of monolithic zirconia, gave satisfactory SBS values in the adhesive resin cement groups. As a result of this, G-CEM ONE and G-CEM LinkForce cements can be clinically preferred for cementation of titanium and monolithic zirconia surfaces.

4. Long-term clinical studies are required to prove the validity of the obtained findings, which is in line with the limitations of any in vitro study.

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Acquisition of data for the study: GC, ZŞA
Analysis of data for the study: GC, YUA, ZŞA
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Drafting the manuscript: GC, ZŞA, YUA
Revising it critically for important intellectual content: GC, ZŞA, YUA
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Clinical and Experimental Health Sciences

A Deep Learning Approach to Automatic Tooth Detection and Numbering in Panoramic Radiographs: An Artificial Intelligence Study

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ABSTRACT

Objective: n this study, in order to test the usability of artificial intelligence technologies in dentistry, which are becoming widespread and expanding day by day, and to investigate ways to benefit more from artificial intelligence technologies; a tooth detection and numbering study was performed on panoramic radiographs using a deep learning software.

Methods: A radiographic dataset containing 200 anonymous panoramic radiographs collected from individuals over the age of 18 was assessed in this retrospective investigation. The images were separated into three groups: training (80%), validation (10%), and test (10%), and tooth numbering was performed with the DCNN artificial intelligence software.

Results: The D-CNN system has been successful in detecting and numbering teeth. of teeth. The predicted precision, sensitivity, and F1 score were 0.996 (98.0%), 0.980 (98.0%), and 0.988 (98.8%), respectively.

Conclusion: The precision, sensitivity and F1 scores obtained in our study were found to be high, as 0.996 (98.0%), 0.980 (98.0%) and 0.988 (98.8%), respectively. Although the current algorithm based on Faster R-CNN shows promising results, future studies should be done by increasing the number of data for better tooth detection and numbering results.

Keywords: Artificial intelligence, deep learning, panoramic radiography, tooth numbering.

1. INTRODUCTION

Since its inception in the 1950s, panoramic imaging has grown in popularity and importance as a diagnostic tool. It is a specific radiographic method used to provide a flat image of the jaws' curving surfaces. Curved surface tomography is the fundamental imaging concept. On a single film, entire maxilla, mandible, temporo-mandibular joints, and associated structures are visible. It is used as a pre-scan radiography to evaluate tooth and bone support, locate impacted teeth, and determine the site of dental implants, among other things. It also provides a basic evaluation of the bone state of the jaw and jaw joints, as well as a diagnosis of maxillary and mandibular fractures (1). On the other hand, panoramic radiographs can show significant geometric distortions and have relatively low spatial resolution compared to intraoral radiographs. Significant changes in image projection in the anterior region might arise depending on the patient's posture and the curvature of the jaws. It also lacks the delicate anatomical characteristics revealed in intraoral periapical radiography. However, it has a dosage advantage over several intraoral radiography (2-5).

Artificial intelligence (AI) is described as a machine's ability to replicate intelligent human behavior in order to execute complicated tasks such as problem solving, object and word recognition, and decision making. AI technologies have already achieved significant success in the modern world, and are integrated in our daily lives through search engines, online assistants, and video games. However, it is fast evolving in a variety of sectors, including medicine. In clinical medicine, a wide variety of AI models are being created for automatic disease risk prediction, detection of abnormalities/pathologies, disease diagnosis, and prognosis evaluation. Because of its capacity to provide digitally coded pictures that can be more readily translated into computer

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language, radiology serves as a form of entry point for the application of artificial intelligence in medicine (1, 3, 6-10).

The term machine learning (and its subcategories) refers to a situation where an agent learns whether it has improved its performance on future tasks after making observations about the data given to it. Machine learning is a term coined by Arthur Samuel in 1959 to describe a field of AI where computers learn automatically from a collection of data. Machine learning algorithms change as they are exposed to more data; they do not rely just on rules; they grow with experience, learning to provide precise responses by analyzing enormous volumes of data (11).

Machine learning is frequently separated into two types: supervised and unsupervised learning. The algorithm is provided annotated data ("basic truth" data) to use in the construction of the algorithm in supervised learning. Unsupervised learning requires the system to categorize itself using unlabeled input. Deep learning, and particularly deep convolutional neural networks (also known as DCNN (Deep Convolutional Neural Network) or CNN (Convolutional Neural Network)), are a subset of supervised machine learning that has gotten the most attention in recent years. DCNNs are a sort of supervised learning that use an algorithmic framework based on deep neural networks with several layers. The power of this method resides in its scalability and the neural network architecture's capacity to extract its own meaningful characteristics from data with no additional direction than the labeled input data (11). Neural networks must be "trained" with training datasets before they can "learn." In radiology, they are often hand-labeled picture datasets that are utilized by the algorithm to enhance its fit to the underlying reality. After a network has been trained using a training dataset, it will be evaluated using a different dataset (validation datasets) to assess the model's fit to the new data (3,10-12).

With the emergence of a digital picture archiving and communication network that produced vast volumes of imaging data, AI became a cornerstone of radiology, presenting significant potential for AI training (2,3,13,14). In a prior work, DCNN was used to learn by following a similarly graded component of the brain's visual cortex. Many research in the literature have proved the potential of DCNN approaches to aid practitioners in dentistry (11,12,16-18). Thus, the study aimed to evaluate the function of the diagnostic computer software designed for the evaluation of tooth detection and numbering on panoramic radiographs.

2. METHODS

In this retrospective study, a radiographic dataset containing 200 anonymous panoramic radiographs taken from patients over the age of 18 between January 2021 and January 2022 from the archive of Marmara University Faculty of Dentistry Department of Oral and Maxillofacial Radiology was evaluated. Panoramic radiographs with metal superposition, position errors, motion artifacts etc. were excluded from the dataset. Panoramic radiographs showing teeth such as dental caries, restorative fillings, crowns and bridges, implants etc. were included in the study. The study was conducted in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Invasive Clinical Research Ethics Committee, Marmara University Faculty of Medicine on 04.11.2022 with protocol number 09.2022.1417.

2.1. Radiographic Data Set

All panoramic radiographs were obtained using the Planmeca Promax 2D (Planmeca, Helsinki, Finland) panoramic dental imaging unit with the following parameters:68 kVp, 16 mA,13 s. The radiographic dataset comprised of optimizing panoramic radiographs with the exposure parameters as low as reasonably achievable and as low as diagnostically acceptable.

2.2. Image Evaluation

Each tooth was labeled and numbered on the panoramic x-ray with the "area detection" option in the artificial intelligence assisted diagnosis software program Cranio-Catch (Cranio-Catch, Eskisehir, Turkey) according to the FDI tooth numbering system by the dentist (D.M.) (Figure 1,2).

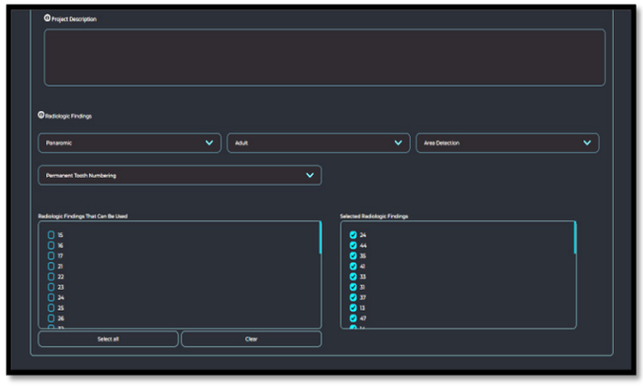

Figure 1. *Cranio-Catch software program, project creation and labeling information screen.*

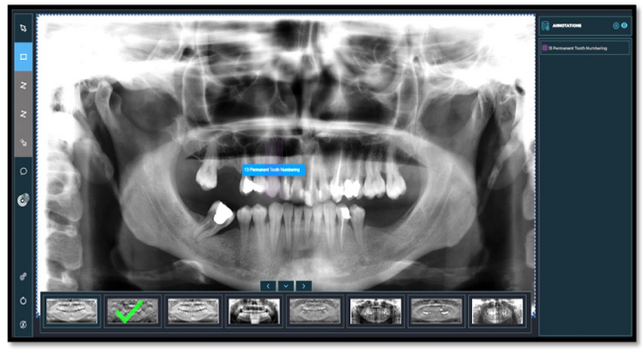

Figure 2. *Example of "Area detection" data labeling for tooth detection and numbering in panoramic radiography.*

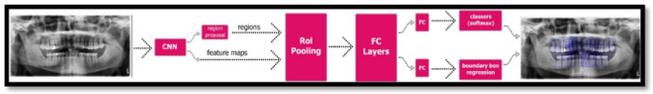

Figure 3. *System architecture and thread detection-numbering chain of operations.*

2.3. Deep Convolutional Neural Network (D-CNN)

A random sequence was generated using the open source Python programming language (Python 3.6.1, Python Software Foundation, Wilmington, DE, USA; retrieved on 01 August 2019 from https://www.python.org). The Inception v2 Faster R-CNN) network implemented with the TensorFlow library was used to build a model for thread detection and numbering. This method consists of 22 deep layers, which can obtain different scale features by applying convolutional filters of various sizes within the same layer.

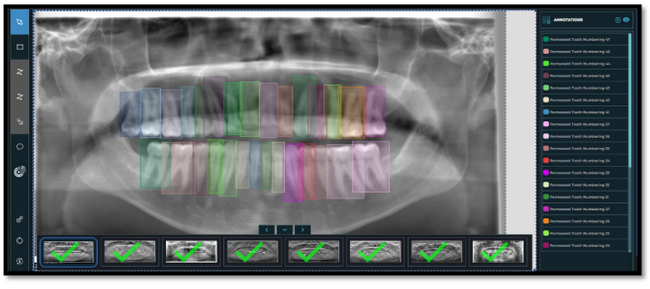

Figure 4. *Automatic tooth detection and numbering model of Craniocatch artificial intelligence software.*

2.4. Model Pipeline

In this study, an AI algorithm (CranioCatch, Eskişehir, Turkey) was developed to automatically detect and number teeth using deep learning techniques, including Faster R-CNN Inception v2 models, in panoramic radiographs. Using Inception v2 architecture as transfer learning, first the transfer values in the cache were recorded and then a fully connected layer and softmax classifier were used to create the final model layers (Figure 5). The training was conducted using 7000 steps on a computer with 16 GB RAM and NVIDIA GeForce GTX 1050 graphics card. The training and validation datasets were used to predict and generate optimal CNN algorithm weighting factors.

Before training, each radiograph was resized from its original dimensions of 2943×1435 pixels to 1024×512 pixels. The training dataset, in which 32 different teeth are labeled at the same time, consists of 160 images.

Numbering on 160 panoramic radiographs in the training group: 11-12-13-14-15-16-17-18-21-22-23-24-25-26-27-28-31-32-33-34-35-36-37-38-41-42-43-44-45-46-47-48 (tooth numbers).

2.5. Training Phase

Images were divided into training (80%), validation (10%) and testing (10%) groups. For each quadrant (regions 1, 2, 3, and 4), 160, 20, and 20 images were randomly allocated to the training, validation, and test groups, respectively.

The CranioCatch approach to detecting teeth is based on a deep CNN using 200,000 epochs trained with faster R-CNN initial v2 with a learning rate of 0.0002. After the model was trained, it was used to identify the presence of teeth (Figure 6).

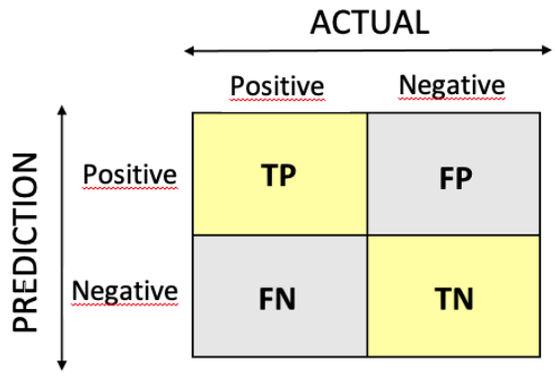

Figure 5. *Confusion matrix.*

2.6. Statistical Analysis

The confusion matrix, a useful table summarizing predicted and actual situations, was used as a metric to calculate the success of the model (Figure 5).

The following procedures and metrics were used to assess the success of the AI model:

• Initially true positive (TP), false positive (FP) and false negative (FN) rates were calculated.

TP: Result where the model accurately predicted the positive class (teeth were correctly detected and numbered on panoramic radiographs).

FP: Result where the model incorrectly predicted the positive class (teeth detected correctly but incorrectly numbered on panoramic radiographs).

FN: The result where the model incorrectly predicted the negative class (teeth were incorrectly detected and numbered on panoramic radiographs).

The following metrics were then calculated using the TP, FP and FN values:

Sensitivity (Recall): TP/ (TP + FN)

Precision: TP/ (TP + FP)

F1 Score: 2TP/ (2TP + FP + FN)

3. RESULTS

The D-CNN system has been successful in detecting and numbering teeth. TP, FP and FN results in all quadrants were determined as 4956, 18 and 100 tooth, respectively. Sensitivity and precision ratios are promising for the detection and numbering of teeth. The estimated precision, sensitivity, and F1 score were 0.996 (99.6%), 0.980 (98.0%), and 0.988 (98.8%), respectively (Table 1).

Table 1. *AI model performance measure value using the confusion matrix*

Measure	Value	Derivations
Sensitivity	0.980	TPR = TP/(TP + FN)
Precision	0.996	PPV = TP/(TP + FP)
F1 score	0.988	F1 = 2TP/(2TP + FP + FN)

TPR:True Positive Rate, Tp: True Positive, FN: False Negative, FP: False Positive, PPV: Positive Predictive Value

4. DISCUSSION

Artificial intelligence (AI) is the realization of tasks such as decision making, recognizing words and objects, and problem-solving using computer software and machines. Deep learning systems, which are a subset of artificial intelligence applications, have been gaining popularity recently and are stated to be promising (14,17). Artificial intelligence and deep learning applications are being used in many areas such as detection of caries in dentistry, detection of orofacial pathologies, orthodontic treatment planning, robotic surgery, dental implant construction and its usage area is expanding. Artificial intelligence applications in dentistry are promising in terms of saving time and effort for physicians, providing support in case of lack of experience, and accelerating archiving and reporting works. It has gained importance both clinically and academically for researchers to follow the developments related to artificial intelligence applications, which are becoming more common in the field of health and expanding their place in the literature and gaining experience in this field (4,18-20).

With the advent of modern imaging modalities and the development of archiving systems, radiology has experienced two significant digital revolutions. These developments were followed by the use of artificial intelligence, especially in radiographic analysis. Especially its compatibility with image processing methods has highlighted dental radiology studies (3,6,10).

Radiologists are using AI diagnostic models not only to assess and report numerous medical images, but also to improve job productivity and obtain more exact outcomes in the precise screening and diagnosis (4,6,20). The emergence of deep learning techniques has increased the performance of automated image analysis methods. The shape, number and position of the teeth are items that a dentist evaluates in the first step in a panoramic x-ray. Modeling tools have been proposed to assist experts as decision supporters for better diagnoses. The primary intent of image segmentation and detection is to aid other automated systems in later processing phases.

Although tooth segmentation and detection research is not new, the use of deep learning methods in the subject is. There are few research on tooth detection, segmentation, and numbering in panoramic radiography in the literature. To fill some of the gaps in the field of dental image analysis, Silva et al. (21) analyzed the performance of four network architectures, Mask R-CNN, PANet, HTC, and ResNeSt, on a dataset of 753 training, 452 validation, and 295 test datasets. The selection of these networks has been made based on their high performance over other datasets, for example for segmentation and detection. As a result of the study, they concluded that the Mask R-CNN solution with an F1 score of 0.902 (90.2%) is much better than the classical methods. This is the first study on sample segmentation, detection and numbering of teeth in panoramic dental x-rays. It has been found that detecting, segmenting, and numbering threads is entirely possible through any of the analyzed architectures, and performance can be significantly improved by choosing the appropriate neural network architecture.

Koch et al. (22) trained a U-Net model on the UFBA-UESC Dental Images dataset and found an F1 score of 0.936 (93.6%) in tooth segmentation in 1200 training and 300 test datasets in 1500 dental panoramic radiographs.

Jader et al. (23) were the first researchers to investigate the detection and segmentation of teeth in panoramic x-ray. They changed the UFBA-UESC Dental Images dataset to include the information of dental samples and created this new dataset as UFBA-UESC Dental Images Deep. A Mask R-CNN powered by ResNet-101 was trained and validated with 193 and 83 images, respectively. The remaining 1224 images of the data set were used for testing and the sensitivity, precision and F1 score were determined as 0.840 (84.0%), 0.940 (94.0)% and 0.880 (88%), respectively.

Tuzoff et al. (24) were the first researchers to apply deep learning to identify and number teeth on panoramic x-rays. On a dataset containing 1352 images for training and 222 images for testing, the sensitivity and precision were 0.994 (99.4%) and 0.994 (99.4%) for tooth detection, respectively, while these values were 0.980 (98.0%) and 0.994 (99.4%) for tooth numbering, respectively. They reported that the result of tooth detection and numbering in panoramic radiographs using a trained CNN-based deep learning model to generate automatic tooth detection according to FDI two-digit notation is promising and AI deep learning algorithms have the potential for practical application in clinical dentistry.

Celik et al. (25) tested the functionality of diagnostic computer program developed to assess missing teeth on panoramic radiography. For the identification of missing teeth, the dataset contains 153 images, 99 intact teeth and 54 missing teeth. The open-source Python programming language and the libraries OpenCV, NumPy, Pandas, and Matplotlib were used to generate a random sequence. For

preprocessing, a pre-trained Google Net Inception v3 CNN network was employed, then dataset transfer learning was taught using 76 images. The model estimate of the images used in training has a success percentage of 94.7%. Estimating 32 images reserved for the exam but not utilized in training yields a 75% success rate. Bilgir et al. (3) found the predicted sensitivity, precision and F1 score measurement on a test data set consisting of 249 panoramic radiographs as 95.5%, 96.5%, and 96.5%, respectively. The deep convolutional neural network algorithm has been successful in detecting and numbering teeth.

Impacted supernumerary teeth are frequently observed in the maxillary incisor region, where they are known as mesiodens. Kuwada et al. (26) used different DL-based AI architectures to identify and classify the presence of impacted supernumerary teeth in the maxillary anterior region on panoramic radiography. Using various testing data, Detect Net obtained the greatest rate of diagnostic efficiency with 0.93 and 0.96 accuracy values for diagnosing the incisal area in terms of the presence or absence of an impacted supernumerary tooth. Detect Net also performed flawlessly, with recall, accuracy, and F-score values of 1.0. Similarly, Mine et al. (27) aimed to apply convolutional neural network (CNN)–based deep learning to detect the presence of supernumerary teeth in children during the early mixed dentition stage. The VGG16 model maintained a high performance in the detection of supernumerary teeth, with accuracy of 82.3%, sensitivity of 85.0%, and specificity of 79.0%. Although further improvements are needed for clinical applications, the CNN-based deep learning is a promising approach for detecting supernumerary teeth.

The artificial intelligence model applied by Prados-Privado et al. (28) obtained 0.992 (99.2%) accuracy in tooth detection and 0.938 (93.8%) accuracy in tooth numbering. In accordance with previous literature, the precision, sensitivity, and F1 scores achieved in our investigation were all high, at 0.996 (99.6%), 0.980 (98.0%), and 0.988 (98.8%). The comparatively low results achieved in investigations on periapical and bitewing radiography can be attributed to the fact that the system first assesses whether the image corresponds to the upper or lower jaw, while CNN-based deep learning in panoramic radiographs is accomplished in fewer steps. Despite the fact that the present approach, which is based on the Faster R-CNN, produced promising results, this study has certain limitations. Further study is needed to analyze different types of CNN architectures and algorithms, as well as to determine the estimation effectiveness of each tooth type (incisors, canines, premolars, and molars) using larger data sets. It is conceivable to develop a system that provides improved tooth detection and numbering results. Future study should also look at the advantages of applying AI to create radiographic images with less radiation exposure. Eventually, it should be also stated that AI is unlikely to replace the dentist-patient interaction in the near future, as humanistic qualities are equally critical in decision-making to manage dental treatment. The AI technologies are meant to assist dental professionals in decreasing misdiagnosis and to operate in harmony with the unique talents of dentists to deliver better, accessible treatment by automating routine elements of dentists' work.

5. CONCLUSION

A deep CNN was suggested in the current study for tooth detection and numbering. The model's high levels of accuracy and sensitivity demonstrated its value for teeth recognition and numbering. AI technology can help clinicians detecting and numbering teeth on panoramic radiographs. Artificial intelligence applications in dentistry are promising in terms of saving time and effort for physicians, providing support in case of lack of experience, and accelerating archiving and reporting works.

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Peer-review: *Externally peer-reviewed.*

Author Contributions:

Research idea: FNP, DM.
Design of the study: FNP, DM.
Acquisition of data for the study: DM, GK.
Analysis of data for the study: DM, GK, ISB.
Interpretation of data for the study: ÖÇ.
Drafting the manuscript: GK, DM, FNP.
Revising it critically for important intellectual content: DM, GK, FNP.
Final approval of the version to be published: FNP.

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Clinical and Experimental Health Sciences

Effect of Zuclopenthixol Acetate on Neural Tube Development in Early Chick Embryos

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ABSTRACT

Objective: Neural tube defects are one of the congenital malformations of the central nervous system. Although the factors that cause the development of neural tube defects and their mechanisms of action are still not clearly explained, genetic predisposition, drug use and some environmental factors are thought to play a role. In this study, it was aimed to investigate the effects of zuclopenthixol acetate (ZA) on neural tube development in a chick embryo model.

Methods: Fourty specific pathogen-free (SPF) eggs were used in the study. The eggs were incubated for 28 hours and divided into four groups of 10 eggs each. At the end of the 28th hours, saline was injected to the control group, while ZA was administered subblastodermically to the experimental groups in 3 different doses (0.7, 1.4, 2.1 mg/kg). At the end of the 48th hours, all the eggs were opened and the embryos were dissected from the embryonic membranes and evaluated morphologically and histopathologically.

Results: When the study groups were evaluated according to the neural tube positions (open or closed), it was found that the neural tube patency increased depending on the ZA dose, which was statistically significant ($p < 0.05$). In addition, morphological developments of embryos were evaluated. Compared to the control group, a statistically significant decrease was observed in the mean somite numbers in all ZA-treated groups, while a significant decrease was found in the mean cranio-caudal length only in the high-dose group.

Conclusion: In this study, it was observed that neural tube and morphological development were adversely affected in the groups treated with ZA in the chick embryo model. It was shown that neural tube closure defects in embryos increased in direct proportion with ZA doses. However, we believe that it will not be possible to fully adapt the results of this study, which was carried out in the chick embryo model, to humans and that more comprehensive research should be conducted.

Keywords: Chick embryo, congenital malformations, neural tube defect, zuclopenthixol acetate

1. INTRODUCTION

The neural tube (NT) is the embryonic structure that forms to the central nervous system, which is made up of the brain and spinal cord. This structure begins to develop around the 17th day of fertilization (early week 3) and completes its development by the middle of the 4th week of embryonic development. Closure of NT begins in the cervical region. It continues uninterruptedly in the form of a zipper, in the cranial and caudal directions. Closure occurs in the midline at 23 days, in the anterior neuropore on 24-25 days and in the posterior neuropore on 25-26 days (1) Neurulation is the bending, elevation, fusion, and remodeling of the neural plate to form NT (2). Disruption of this process can result in neural tube defects (NTD) (3).

Anomalies that occur when the NT does not close are expressed as NTD (4). NTD is one of the congenital anomalies of the central nervous system that occurs during embryogenesis and is caused by the incompleteness of the morphogenetic process of NT closure (5). NTD occurs after NT does not close in the normal period (3rd and 4th weeks of intrauterine life) and as a result, permanent problems often occur (6). Incomplete closure of the cranial end of the NT causes anencephaly or exencephaly, while incomplete closure of the caudal end causes spina bifida of varying degrees (7).

Factors such as race, ethnicity, geographical location, and socioeconomic status are thought to be effective in the development of NTD. However, the etiology of NTD has not been clarified. It has been reported that many factors may lead to the development of NTD, including maternal exposure to hyperthermia and various chemicals during pregnancy, drug use, malnutrition or being obese, low folic acid levels, the presence of diabetes in the mother, and genetic factors (8).

The ideal situation to be achieved during drug therapy while pregnancy is to treat the mother's disease while protecting the fetus from the possible toxic effects of drugs.

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Therefore, it is important to determine the safety of drugs during pregnancy. However, the study of drug safety during pregnancy is fraught with ethical challenges, as it is not possible to study human embryos (9). It is not possible to access and examine the human embryo while it is in the neurulation stage. Therefore, different experimental models, including other mammals, amphibians, and birds, are used to study NT development (5). These models have advantages and disadvantages compared to each other. The fact that the developmental stages of the chick embryo resemble the neuronal and spinal development stages of the human embryo in the first trimester provides advantages for research (10). The ability of chick embryos to grow outside the uterus, the easy manipulation of embryos, and the ability to incubate many eggs and obtain many embryo samples are other advantages of working with a chick embryo model (11).

Some teratogens and drugs, which are thought to be harmful for the development of NT during pregnancy, have been investigated using various experimental methods (12,13). One of these teratogenic agents is zuclopenthixol (14). Zuclopenthixol is an antipsychotic drug with three different formulations (zuclopenthixol dihydrochloride, zuclopenthixol acetate, zuclopenthixol decanoate). It is used in agitation, acute and chronic schizophrenia, and similar psychoses, thought disorders, hallucinations, restlessness, mania, and other psychoses accompanied by aggression (15,16). It is licensed for use in doses of 50-150 mg in acute exacerbations. The chemical formula of ZA is $C_{24}H_{27}ClN_2O_2S$, its molecular weight is 443.04 g/mol (17).

Different experimental models are used in the literature to determine the teratogenic and toxic effects of various natural or artificial chemical agents and to determine safe dose ranges. The chick embryo model is one of these models. The neuronal and spinal developmental stages of the human embryo in the first trimester are like the early (first 7-day period) nervous system development stages in chick embryos (18). Therefore, chick embryos are one of the most suitable models that can be used to investigate neural developmental stages.

Antipsychotic drugs are among the drug groups that are risky to use during pregnancy. As far as we have investigated the results regarding the teratogenic and toxic effects of ZA, which is in this drug group, in the embryo as a result of its use in pregnant women, it is scientifically limited. Therefore, in our study, we aimed to examine the effect of different doses of ZA on NT development in the early chick embryo model.

2. METHODS

Permission was obtained from Afyon Kocatepe University Animal Experiments Local Ethics Committee for this study (Number: 49533702/104; Date: 24.08.2021).

2.1. Laboratory and Incubation Conditions

This study was conducted in Afyonkarahisar Health Sciences University, Faculty of Medicine, Department of Anatomy. Standardization in the incubator was determined as 37.5±0.5°C constant ambient temperature and 60±5% relative humidity. The incubator was run empty for one hour until the desired ideal ambient temperature and relative humidity were reached. Afterwards, the eggs were placed in the incubator with sharp ends pointing down in order to ensure the continuity of the embryos and to have them available at the times desired.

2.2. Experimental Animals

In this study, the eggs were procured from the Veterinarian Control and Research Institute, Bornova, Izmir, Turkey. 65±5 g in weight, specific pathogen-free (SPF) and day 0 fertilized eggs of white chickens.

2.3. Experimental Groups

In the study, 40 fertilized SPF eggs were randomly divided into 4 groups, with 10 in each group. The day they were incubated was considered day 0. Eggs were removed from the incubator at 28 hours after being placed in the incubator. Eggshell was sterilized with 70% ethanol. Under the light, the air sac of the egg was found and the middle point corresponding to this cavity was marked with a pencil. In the area of this sign, holes of approximately 2 cm were opened and injection was made with a Hamilton injector.

ZA was administered via the subblastodermic route in a volume of 30 µL in groups A, B, C and D by Hamilton microinjector (0.7 mg/kg, 1.4 mg/kg and 2.1 mg/kg in groups B, C and D, respectively). Group A served as the control group and was administered 30 µL 0.9% NaCl via the subblastodermic route. After the injections, small windows were sealed with cellophane tape. Then the eggs were hand-turned 180° and placed in the incubator.

2.4. Removal of Chick Embryos

Eggs were removed from the incubator at 48 hours of incubation. The eggshell was broken and only the yolk was placed in a glass container containing sterile ringer lactate or saline. The watch glass was placed in the cup to receive the blastoderm. Then, using fine forceps and fine-tipped scissors, the vitelline membrane was cut over the yolk. The vitelline membrane was separated from the yolk by carefully holding both ends, and the blastoderm adhering to the membrane was advanced in the liquid and placed in the watch glass. Embryos were examined under a light microscope.

2.5. Histological Tissue Follow-Up

Embryo samples obtained were taken into 10% formaldehyde for fixation. Tissues, which were kept in fixation solution for 72 hours, were washed in running tap water and passed through graded alcohol series. It was then cleared with xylol and embedded in paraffin. 5 µm sections were taken from embryos. Embryo sections were placed on the slide. Paraffin was cleaned with xylol. Then the slides were passed through

graded series of alcohol (100%, 96%, 80%, 70%, 50%) and washed in water. Sections were stained with Hematoxylin-Eosin (H&E) to determine the general histological structure. It was then passed through the increasing series of alcohols then xylol. Finally, it was closed with a coverslip using entellan.

2.6. Statistical Analysis

Analysis of all findings was performed using the Statistical Package for the Social Sciences (SPSS) 22.0 program. The data related to NT (open or closed) were analyzed by using c2 test. The somite number and crown–rump length were analyzed by using non-parametric Kruskal-Wallis tests. Dunn test were employed as *post-hoc* tests and $p < .001$ were considered significant.

3. RESULTS

Findings were evaluated according to Hamburger Hamilton staging. Embryos were removed from the eggs at 48th hour according to the normal developmental period. In a 12th stage embryo, the somite count was 16. Head continues to rotate to the left. The closure of the anterior neuropore completes the closure of the neural tube. Telencephalon begins to appear. Primary optic vesicles and optic sac are clearly visible. The heart takes a slight S shape. The head fold of the amnion occupies the entrance to the forebrain. In our study, we investigated the effect of different doses of ZA on the development of NT in chick embryos assumed to have reached the 48th hour and 12th stage.

Group A: Only saline injection was applied to the eggs in the control group. In this group, 10 embryos were evaluated. Embryos were examined morphologically and histopathologically. NT was found to be closed in all embryos and no developmental delay was observed. The head of the embryos had begun to turn to the left. Enlargement of the telencephalon was evident and Rathke's sac could be observed in some of the embryos. All these findings were compatible with stages 13 and 14 according to the Hamburger-Hamilton classification (19), and their developmental stages were normal. The mean cranio-caudal length of the embryos was 772.40±111.47 µm, and the mean somite number was 21.30±2.26.

Group B: Embryos in this group were injected with 0.7 mg/kg ZA. 3 out of 10 embryos evaluated morphologically had open NT. The NT of all the remaining embryos was closed and there was no developmental delay. The head of the embryos began to turn to the left. The anterior neuropore of embryos was closed. The telencephalon was beginning to appear. Primary optic vesicles were prominent. The heart took the shape of the letter S. When all these findings were observed, it was seen that the embryos were compatible with stage 12 according to the Hamburger-Hamilton classification (19). The mean cranio-caudal length was 736.20±87.56 µm, and the mean somite number was 15.9±0.74. It was observed that there was a decrease in both compared to the control group.

Group C: Embryos in this group were injected with 1.4 mg/kg of ZA. 4 out of 10 embryos evaluated morphologically had open NT. A slight cranial bending was observed in the macroscopic examination. The hindbrain was divided into 5 neuromeres. The anterior neuropore had begun to close. Optic vesicles were prominent. Development stage of all embryo, was stage 11 according to Hamburger–Hamilton embryonic classification (19) and were behind the normal developmental stage. The mean somite number of the embryos was 15.20±0.79, and the mean cranio-caudal length was 719.10±114.37 µm.

Group D: Embryos in this group were injected with 2.1 mg/kg of ZA. NT was open in 6 of the embryos. In the macroscopic examination, the brain vesicle, which is the first sign of cranial fold, was seen in the embryos. Optic vesicles were not clear. As a result of the study, these findings, which were seen in all the embryos that received high-dose injection, showed that the embryos were at stage 10-11 according to the Hamburger-Hamilton classification (19) and were behind the normal developmental stage. As a result of the macroscopic and morphological evaluation, the mean cranio-caudal length was 578.10±11.75 µm, and the somite number was 15.0±0.94. It was determined that there was a significant decrease in these parameters compared to the control group.

When the study groups were evaluated according to their NT positions (open or closed), it was found that NT patency increased depending on the ZA dose, which was statistically significant ($p<0.05$), (Figure 1). In addition, as a result of the morphological evaluation, it was determined that there was a decrease in the mean cranio-caudal lengths and somite counts of the embryos depending on the dose. Compared to the control group, a significant decrease was observed in the mean somite counts in all dose groups, while a significant decrease was observed in the cranio-caudal length mean values only in the high dose group ($p<0.001$, Table 1).

Table 1. *Statistical analysis between groups.*

Parameters	Group A	Group B	Group C	Group D	p
Open NT/Closed NT	0/10	3/7[a]	4/6[a]	6/4[a]	p = .036
Rate of Open NT/Total	0%	30%	40%	60%	
Somite Count	21.30±2.26	15.9±0.74[a]	15.20±0.79[a]	15.0±0.94[a]	p = .004* p < .001* p < .002*
Cranio-Caudal Length (µm)	772.40±111.47	736.20±87.56	719.10±114.37	578.10±11.75[a]	p = .004

**Kruskal-Wallis test*

a; A statistically significant difference was found when compared with the control group.

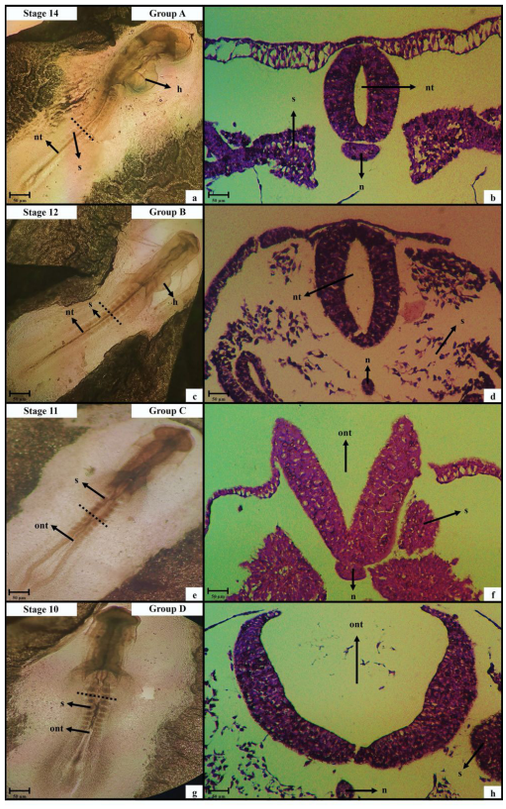

Figure 1. *Evaluation of embryos under the light microscope: (a) image of group A embryo under the light microscope; (b) cross section of an embryo (H&E, X20) after histological staining of group A embryos; (c)) image of group B embryo under the light microscope; (d) cross section of an embryo (H&E, X20) after histological staining of group B embryos; (e) image of group C embryo under the light microscope; (f) cross section of an embryo (H&E, X20) after histological staining of group C embryos; (g)) image of group D embryo under the light microscope; (h) cross section of an embryo (H&E, X20) after histological staining of group D embryos. nt: neural tube; ont: open neural tube; n: notochord; s: somites; h: heart; Group A: Control; Group B: ZA 0.7 mg/kg; Group C: ZA 1.4 mg/kg; Group D: ZA 2.1 mg/kg.*

4. DISCUSSION

In humans, between the 18th and 60th days of pregnancy, defects in nervous system development are more likely to occur. These defects occur as a result of problems occurring during the development of the NT or the reopening of the NT after completing its development (20). Different experimental models such as amphibian, mammalian, poultry, and computer modeling are used in the investigation of NT development.

The highest incidence of many psychiatric disorders in women, occurs during the reproductive years (21). In the literature, the status of being a parent of individuals with a diagnosis of psychiatric illness and the social and psychological status of individuals who have children have been examined. As a result of the studies, although there was a decrease in the fertility rate in psychiatric cases, it was determined that 36% of all individuals and 59% of women with a diagnosis of psychosis included in the study had children (21,22). It is very important to determine the drug safety of women with psychosis in terms of their desire to become a mother and to understand whether the drugs they should use (haloperidol, chlorpromazine, trifluoperazine, zuclopenthixol) have side effects in terms of mother and baby.

Antipsychotic medications may need to be prescribed for pregnant women in some cases. In these cases, the general health of the mother and the baby should not be adversely affected. However, there are no clear data on the potential effects of antipsychotic drugs on the infant. The available data on drug safety are limited, particularly for atypical antipsychotics (23,24). ZA is also in the antipsychotic drug group. Zuclopenthixol is a thioxanthene group neuroleptic drug. Its effect is fast and the duration of activity is 2-3 days. The fact that the side effects are low and mild, and that it can be administered with 48–72 hours intervals, has increased the use of the drug in recent years (25). Although there are various studies on the results of ZA use, no study on the effects of ZA on the fetus, especially on NT developing in the first trimester, was found as a result of the literature search conducted by us. However, animal reproduction studies of drugs in category “C” according to FDA have shown adverse effects on the fetus, and it has been reported that there are no adequate and well-controlled studies in humans. The study was planned in this direction and the effect of ZA on neural tube development in the chick embryo model was investigated.

In a study conducted in rats, the physiological effects of different doses of 3 long-acting neuropsychiatric drugs, including ZA (ZA doses: 0.5, 1 and 5 mg/kg), such as changes in body temperature, spontaneous cage activity and food intake were investigated. As a result of the study (5th-17th hours), food intake was significantly reduced in the medium and high dose ZA groups. In the high-dose ZA group, there was a significant decrease in body temperature at night. They explained that neuroleptics affect body temperature, spontaneous cage activity, and food intake, but that the effects are short-lived and do not have negative consequences for animals, and concluded that further studies are required (26). However, when the results of this study was evaluated in general, it was thought that the short-term negative effects that occurred in rats could also occur in humans after ZA use, and its use during pregnancy could harm the fetus. In our study, the doses to be applied to chick embryos were determined by considering the ZA doses used in rat studies (26,27).

Due to their small molecular size and lipophilic/lyophobic properties, antipsychotic drugs easily pass through the placenta and enter the fetal circulation. Maternal exposure to these drugs affects the fetal exposure level according to the placental crossing rate (28). Sadowski et al. observed in their study that fetuses exposed to antipsychotics were more likely to be premature (10.6%) compared to controls (4.3%) (29). In a study examining the potential relationship between the use of antipsychotic drugs during pregnancy

and gestational diabetes mellitus (GDM) and investigating the existing literature, it was stated that the use of first – and second-generation antipsychotic drugs did not pose a risk of GDM. As a result of the study, the use of ZA during pregnancy is not risky for GDM, but the effect of ZA on the fetus could not be examined (30).

In a study in which the neurodevelopment of 203 children whose mothers were exposed to typical antipsychotics during pregnancy, it was reported that there was no significant difference compared to the population in terms of IQ scores evaluated at the age of four. Major malformations were detected in babies whose mothers used haloperidol (2/78 (2.6%), flupentixol (5/101 (5%)) and zuclopenthixol (8/75 (10.7%)) (31,32).Use of both typical and atypical antipsychotics during late pregnancy may result in delayed development of the nervous system with an increased risk of perinatal complications, including extrapyramidal findings, respiratory distress, and seizures, which may inevitably persist up to 1 year of age (33). However, there is no general information in the literature on the increase of other malformations, although there is a potential concern about the occurrence of neural tube defects due to the use of antipsychotics during pregnancy (34). In addition to the use of antipsychotics, low diet, deficiency in vitamin intake, high maternal obesity rates and low serum folate levels in mothers with psychiatric disorders are thought to be effective in increasing the risk of NTD malformation (35). In a systematic study by Gentile et al., in which they investigated the safety of antipsychotic use in pregnant women, 419 pregnant women using antipsychotic drugs were examined. As a result of the examination, it was reported that a total of 26 congenital malformations with 4 neural tube defects were identified (36). When the results of our study are evaluated in line with this literature information, we think that ZA exposure negatively affects the development of the nervous system and therefore causes neural tube closure defects. It is seen that the concerns about fetal neural tube defects related to the use of antipsychotic drugs in the literature are supported by our study. Although this study was conducted in chick embryos, the results obtained are a reference study showing possible teratogenic effects that may occur with antipsychotic drug exposure in humans.

In a case report reporting the use of zuclopenthixol decanoate (ZD), a different form of ZA, during two consecutive pregnancies of a woman diagnosed with schizophrenia, it was found that a significant improvement was achieved in the clinical condition of the mother after the treatment. ZD was prescribed to the patient who stopped taking the drug on her own due to amenorrhea 6 months before the pregnancy due to compliance problems. Intramuscular (i.m.) depot ZD injection was planned to the patient as 400 mg every two weeks. The first unwanted pregnancy was diagnosed at the 13th week of pregnancy, approximately four and a half months after the start of ZD therapy. The patient was explained about the risks of using ZD during pregnancy. The patient decided to continue both pregnancy and drug therapy freely. The next drug dose was reduced to 200 mg and administered at monthly intervals. It was stated that both babies showed normal development. They reported that ZD is an option that can be used for pregnant women with psychosis, but large and controlled studies are needed to obtain a definitive result. In addition; it is understood that the mother has babies with normal development after ZD use, and that the use of the drug in controlled and certain doses does not have a toxic effect on both the mother and the fetus (37). As a result of our study, it was determined that the nervous system development of embryo was negatively affected depending on the ZA doses, and exposure to high doses of ZA delays fetal development. Therefore, we think that in cases where the use of ZA in pregnant women is necessary, it should be used by determining the dose that is controlled and appropriate for the clinical picture.

In the literature, it is thought that antipsychotic drugs block central dopamine (DA) receptors, leading to an accelerated cycle of DA and accumulation of acid metabolites in the brain (38,39). Although the pathophysiological mechanism of Neuroleptic Malignant Syndrome (NMS) is not known exactly, it is suggested that the blockade of dopamine receptors due to the use of antipsychotics causes this syndrome. NMS has been reported to be associated with both typical and atypical antipsychotics. However, a guideline was published by the American College of Obstetrics and Gynecology, which states that typical antipsychotics are safer than atypical antipsychotics in pregnant women (40). However, it has been stated that changes in the dose and route of administration of antipsychotic drugs increase the risk of NMS (41). We think that ZA, which we applied at different doses in our study, had a negative effect on the development of the nervous system regarding its effect on dopamine receptors.

In a study conducted in rats, it was determined that different doses of ZA (0.7 and 1.4 mg/kg intraperitoneal (i.p.)) increased the malondialdehyde level, decreased the glutathione level and produced a pro-oxidant effect (27). Many factors such as normal oxygen metabolism in living cells, environmental pollutants, radiation, various medical treatment methods and drug use trigger the formation of oxygen-derived free radicals. Antioxidants, which prevent oxidation caused by free radicals in the body and can stabilize this situation, come into play (42,43). In addition to this literature information, it has been determined that antioxidant defense mechanisms are impaired with the increase of oxidative damage due to the deterioration of the oxidant-antioxidant balance. As a result of this situation, it has been reported that nervous system development, membrane transport, mitochondrial energy production, gene expression and receptor-mediated phospholipid-dependent signal transduction may be affected (44,45). We think that neural tube closure defects and negative effects on embryo development observed as a result of our study may overlap with the results of these studies.

5. CONCLUSION

In our study, the possible negative effect of ZA, an antipsychotic drug, on the development of NT was investigated in the

chick embryo model. The effects of ZA on the development of embryos were associated with the injected doses, and it was determined that increasing doses of ZA administration caused midline closure defects during NT formation in early chick embryos during development. In addition, it was observed that the cranio-caudal length and somite counts decreased significantly in the C and D experimental groups compared to the control group, depending on the dose.

Consequently, our study has demonstrated that ZA exerts direct teratogenic effect on the process of NT formation of chick embryo in a dose-dependent manner. However, since we think that it will not be possible to fully adapt the results of this study, which was carried out in the chick embryo model, to humans, we believe that a more comprehensive study should be conducted.

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Author Contributions:

Research idea: GAK, EA, TE
Design of the study: EA, TE
Acquisition of data for the study: GAK, AS, YEK
Analysis of data for the study: EA, TE
Interpretation of data for the study: GAK, EA, TE
Drafting the manuscript: GAK, YEK, AE
Revising it critically for important intellectual content: AE, GAK, AS
Final approval of the version to be published: TE, EA, GAK

Data Availability Statement: *The datasets used and analyzed during the current study are available from the corresponding author upon request.*

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Clinical and Experimental Health Sciences

Cytotoxicity of *Sambucus nigra* L. on Cancer Cell Line and In Vitro Antioxidant Properties

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ABSTRACT

Objective: Essential oils, free fatty acids, flavonoids, glycosides, phenolic acids, carotenoids, vitamins, and minerals are found in significant quantities in the characteristic chemical composition of Sambucus nigra L. This study aimed to evaluate the antioxidant potential of Sambucus nigra L. Fructus and evaluate the cytotoxicity on the cancer cell line.

Methods: The Sambucus nigra L. fruits were collected from Yalova Atatürk Horticultural Central Research Institute in September 2021. The ethanol extract was prepared. Antioxidant property of Sambucus nigra L. fruit extracts was evaluated with 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity, Cupric Ion Reducing Antioxidant Capacity (CUPRAC). Also, total phenolic content, total flavonoid content, and total anthocyanin content were calculated. Liver hepatocellular carcinoma cell line (HepG2) was used for cytotoxicity assay and an 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was applied.

Results: The total phenolic, total flavonoid contents and total monomeric anthocyanins were 9.75±0.92 mg GAE/ mg fruit extract, 0.07437±0.004 mg quercetin/ mg fruit extract, respectively. 0.45±0.0014 mg catechin/gram of fruit extract and 2.08±0.025 mg Cyanidin-3 glucosides/g fruit extract. CUPRAC and DPPH results showed that Sambucus nigra L. extract has strong antioxidant activity. The results of the cytotoxicity assay indicated that while concentrations of the extract increased, the viability of HepG2 decreased.

Conclusion: Our findings suggest that the *Sambucus* fruit extract is particularly rich in antioxidant components that are possibly modulating their beneficial use for hepatocellular malignancies, significantly reducing the number of viable cancer cells and inducing cell death.
Keywords: *Sambucus nigra,* elderberry, cytotoxicity, antioxidants

1. INTRODUCTION

Many herbs have been extensively studied for their antioxidant activity in recent years. It is believed that increased intake of food rich in natural antioxidants is associated with lower risks of degenerative diseases. Sambucus nigra L. is one of them and belonging to the Caprifoliaceae family, is an extremely accessible plant native to the northern hemisphere. Its seeds are rapidly spread by birds and other animals along with forests and roadsides, leading to its spread to different habitats today such as the subtropical regions of Asia, North Africa, and North America (1).

Essential oils, free fatty acids, flavonoids, glycosides, phenolic acids, carotenoids, vitamins, and minerals are found in significant quantities in the characteristic chemical composition of Sambucus nigra. Together with anthocyanins, phenolic acids and flavonols form the main secondary metabolites of Sambucus nigra L. (2). These phenolic compounds have strong antioxidant activities both in vitro and in vivo due to their reducing properties. Phenolic compounds exhibit a variety of biological activities such as anti-inflammatory, antiviral, antiallergic, vasoprotective, and anti-carcinogenic activities (3). In recent years, Sambucus nigra L.'s antioxidant activity and effects have attracted considerable attention. Under normal physiological conditions, the endogenous defence system can scavenge reactive oxygen species (ROS). On the other hand, the endogenous system may be insufficient in an excessive increase in the amount of ROS. Reactive oxygen species emerging with uncontrolled free radical production can cause oxidative damage to biomacromolecules, amino acids, DNA, lipids, and proteins. For this reason, it is a matter of curiosity whether the place of Sambucus nigra L. in human life can be expanded or not (4, 5).

This study aimed to evaluate the antioxidant potential of Sambucus nigra L. fructus and evaluate the cytotoxicity on

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the liver cancer cell line. It is expected that this study will reveal the potential natural antioxidant sources of Sambucus nigra L. fruits and their anti-tumour activity.

2. METHODS

2.1. Materials

The Sambucus nigra L. fruits were collected from the Yalova Atatürk Horticultural Central Research Institute in September 2022.

2.2. Sample preparation and extraction

The fruit sample of Sambucus nigra L. plant was dried in an oven (37°C) and then 100 g of dried samples were weighed, and ethanol extracts were prepared by maceration method (48 hours). The collected ethanol filtrates were then filtered through filter paper. The solvents of the obtained filtrates were evaporated at low pressure and temperature using a rotary evaporator device, and crude extracts were obtained. The obtained crude extracts were kept in the refrigerator at +4 °C.

2.3. Total phenolic content (TPC)

4.5 mL of distilled water was added to the 0.1 mL extracts prepared at concentrations of 5 mg/mL. The mixture was then added with 0.1 mL Folin-Ciocalteu reagent (FCR) (diluted with 1:3 distilled water) and 0.3 mL 2 percent sodium carbonate solution, and the absorbance of mixed was measured at 760 nm 2 hours later against the reference. The total phenolic content was expressed as mg of gallic acid equivalents (GAE) per mg of extract (9).

2.4. Total flavonoid content (TFC)

For total flavonoid analysis, 1 mL of the extracted samples was taken into each tube, and 4 mL of distilled water and 0.3 mL of 5% sodium nitrite ($NaNO_2$) solution were added to it. After 5 minutes, 0.3 mL of 10% aluminium chloride ($AlCl_3$) solution was added, mixed and left for 6 minutes. Then, 2 mL of 1 M NaOH was added and the volume was made up to 10 mL with distilled water. The absorbance values of the samples were measured with a UV-VIS spectrophotometer at a wavelength of 510 nm. Results are given as catechin equivalents/mL.

2.5. Total anthocyanin content

Anthocyanin level was detected by using the pH differential method according to the procedure of Majki´c et al. (10). Total anthocyanin content (TAC) expressed as mg equivalent cyanidin-3-O-glucoside per g fruit extract.

2.6. DPPH radical scavenging activity

10 µL of extracts prepared at 0.5-5 mg/mL concentrations were taken, 240 µL of 0.1 mM DPPH solution was added, DPPH solution was added, and standard solutions were prepared for 1 min. After vortexing, they were left in room conditions and dark for 30 min. Their absorbance was measured against reference using a microplate reader at 517 nm. The control was prepared under the same conditions by using 10 µL methanol instead of the sample and standard material and the absorbance of the control was measured. The experiment was repeated three times and the average was calculated.

2.7. Cupric Reducing Antioxidant Capacity assay (CUPRAC)

In the Cupric Reducing Antioxidant Capacity (CUPRAC) assay, 60 µL each of copper (II) solution, neocuproin solution and ammonium acetate buffer (1 M) were mixed. 10 µL ethanol and 60 µL of extract were added and shake the solution. The solutions were kept in room conditions with their mouth closed for 60 minutes. At the end of this period, absorbance values at 450 nm were measured against the reference solution that does not contain a sample. CUPRAC values were given as mM TroloxE/mg extract (8)

2.8. Cell culture

The human liver hepatocellular carcinoma cell line (HepG2) was from American Type Culture Collection (ATCC), Manassas, VA, USA). All medium and solutions were heated to 37 °C before the process of cell cultivation. The cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with heat-inactivated fetal bovine serum (FBS) (a final concentration of 10%), L-alanyl-L-glutamine and 1% penicillin-streptomycin at 37 °C. It was maintained in 75 cm2 cell culture flasks in the incubator with the condition of 37 °C, 95% air and 5% CO2 for 24 h to confluence. After the incubation of 24 h, all waste medium was discarded and the monolayer cells were detached with trypsin-EDTA in the incubator for 3 min. The suspension was centrifuged in a refrigerated centrifuge at 125 x g for 5 min. The supernatant was discarded and the pellet was suspended in a fresh medium. Then, viable and dead cells were counted by the method of trypan blue (0.4%) staining with a haemocytometer. For the preparation of U-bottom 96-well microplates, 100 µl of the stock viable cell suspension (3 x 105 cell/ml) was seeded in each well (3 x 104 cell/well) and kept in the incubator for 24 h to confluence at least 90%.

2.9. Cytotoxicity assay

The extract was 2-fold serially diluted with maintaining medium at the concentrations of 10, 5, 2.5, 1.25, 0.625 and 0.312 mg/ml. Then, 100 µl of each dilution was added to six-replicated wells of the 96-well microplate confluent with cells. 100 µl of maintaining medium was only added to cell control wells (medium + cell) and blank wells (only medium).

The microplate was incubated at 37 °C and 5% CO2 for 24 h. Inert microscopy (Olympus ix71, Tokyo, Japan) was used to observe the morphological changes of the cells. MTT assay (tetrazolium-based colorimetric assay) was used to determine the cytotoxic effects of the extract by spectrophotometry. MTT solution in PBS (5mg/ml) was prepared and filtered with 0.45 µm sterile syringe filters. MTT solution (10µl) was added to wells. The microplate was incubated for 4 hours at 37 °C in 5% CO2. After incubation, the supernatant was discarded and DMSO (100 µl) was added to the wells. The microplate was gently shaken to solubilize the formazan crystals. A microplate reader (Absorbance 96, Byonoy, Germany) was used to measure the absorbance at a wavelength of 570 nm. The percentage of cell viability and inhibition was calculated in the formula as follows,

Cell viability (%) = (Asample – Ablank)/(Acontrol – Ablank) x 100

Cell inhibition (%) = 100 – [(Asample – Ablank)/(Acontrol – Ablank) x 100]

Asample = absorbance value of test compound, Acontrol = absorbance value of control (cell), Ablank = absorbance value of blank (medium)

CC_{50} was expressed in mg/ml of the extract and calculated by the formula (y=mx+b) of linear regression analysis using the extract concentration-response curve (Fig 1).

2.10. Vitamin C equivalence

The assay was slightly adapted from the methods of Muraina et al. (6) and Liu and Nair (7). L-ascorbic acid (600 µmol) and the extract (1 mg/ml) were prepared in DMSO at two-fold concentrations. 380 µL of MTT solution (1 mg/ml in distilled water) and 20 µL of each dilution were vortexed in a 1.5 ml-eppendorf tube. Then, 400 µL of DMSO was added to all tubes to solve the formazan crystals and the reaction mixture was incubated at 37 °C for 4 h. 150 µL of each reaction mixture was pipetted to a 96-well microplate in quadruplicate. The microplate (Absorbance 96, Byonoy, Germany) was read to measure the absorbance at a wavelength of 570 nm. A standard curve was generated with the dilutions of L-ascorbic acid. Vitamin C equivalents of the extract were calculated with the curve (Fig 2).

3. RESULTS

In the study, the content of bioactive compounds in the extract of Sambucus nigra L. fruit was examined (Table 1). The total phenolic component content of the ethanol extracts of Sambucus nigra L. fruits was 9.75±0.92 mg GAE/mg dried extract, while the total flavonoid component content was 0.07437±0.004 mg quercetin/mg dried extract and 0.45±0.0014 mg catechin/gram dried extract. As for the content of total monomeric anthocyanins in Sambucus nigra L. fruit extracts, it was 2.08±0.025 mg Cyanidin 3 glucosides/g dried extract.

Table 1. *The content of total phenolics, flavonoids and anthocyanins in Sambucus nigra L. fruit extract*

Measure	Mean ± SD
TPCa (mg GAE per g extract)	9.75±0.92
TFC b (mg quercitin per mg extract)	0.07437±0.004
TFCc (mg catechin per g extract	0.45±0.0014
TACd (mg cyanidin 3-glucoside per g extract)	2.08±0.025

TPC: Total phenolic content; TFC: total flavonoid content; TAC: Total anthocyanins content

[a] mg gallic acid equivalent per mg of dry extract

[b] mg kersetin equivalent per mg of dry extract

[c] mg catechin equivalent per g of dry extract

[d] mg cyanidin 3-glucoside equivalent per g of dry extract

In this study, radical scavenging activity (DPPH) and reducing power (CUPRAC) of Sambucus nigra L. fruit extract were investigated (Table 2). As a result of all analyses applied to Sambucus nigra L. fruit extract, it was determined that it has strong antioxidant activity.

Table 2. *Biological activities of Sambucus nigra L. fruit extract*

	Value
DPPH (IC_{50} mg/mL)	0.13±0.057
DPPH (Ascorbic acid)	0.004± 0.007
CUPRAC Value[a] (mM troloks equivalent/mg extract)	45.20±1.530

[a] mM equivalent Trolox per mg of dry extract

DPPH: 2,2-diphenyl-1-picryl-hydrazyl-hydrate; CUPRAC: Cupric Ion Reducing Antioxidant Capacity

The results of the cytotoxicity assay indicated that while concentrations of the extract increased, the viability of HepG2 decreased. The highest percentage of cell viability was determined with the concentrations of 2.5, 1.25, 0.625 and 0.312 mg/ml as 58.8%, 71.2%, 84.2% and 100.9% respectively. The lowest was determined with the concentrations of 1 and 0.5 mg/ml as 39.7% and 30.4% respectively. The linear regression between the concentration of the extract and the cell viability was determined as R2=0.794 (Fig 1). CC50 of the extract was calculated as 5.49 mg/ml by the formula of y = –6.43x + 85.293 in Fig 1.

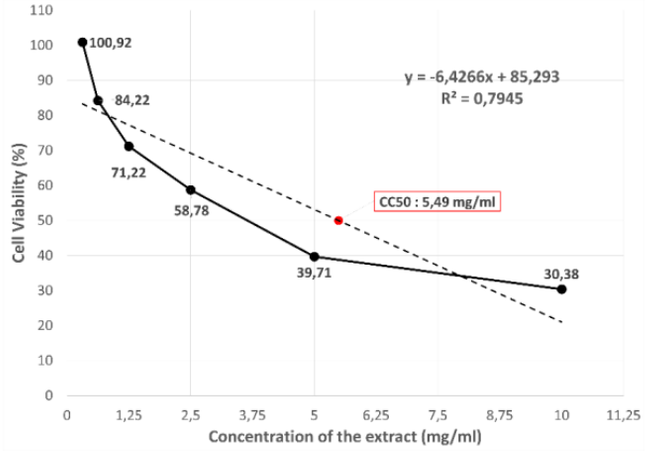

Figure 1. *Effect of elderberry fruit extract on cancer cell viability*

Vitamin C equivalents linearly increased by increasing the concentrations of the extract in dilutions (R2=0.978, Fig 2). Vitamin C equivalents were determined as 671.43, 420, 214.29, 130.95, 34.29 and 11.43 µmol of vitamin C for 1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 respectively.

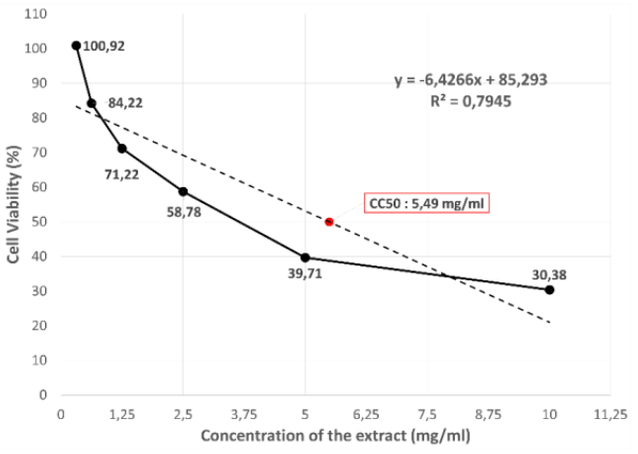

Figure 2. *Vitamin C equivilant of Sambucus nigra L. fruit extract*

4. DISCUSSION

There are many pharmacologically active compounds in polyphenols and flavonoids are among the most prominent. Flavonoids are mostly found in high amounts in plants and are involved in processes such as plant pigmentation, antioxidant capacity, protection from biotic and abiotic stresses, and plant-environment interactions. Anthocyanin gives the plant orange, red and blue colors. Berries are rich in antioxidant components, Sambucus nigra L. is one of these fruits (11).

The total phenolic component content of Sambucus nigra L. has been studied in many studies. TPC values have been determined in different ranges in the literature. It is thought that climatic and topographic differences may also affect berry quality and TPC values in Sambucus nigra L. which is determined by Atkinson and Atkinson (12). In a study examining the effectiveness of different solutions (ethanol and water), the TPC of Sambucus nigra L. extract powder, in which ethanol was used as a solvent, was measured as 339.68±1.47 mg GAE/g dried weight (13). Akbulut et al. (2009) studied the ethanol extract of Sambucus nigra L. and found that it had a high phenolic content (371-432 mg GAE/100 g fresh matter) (14). In the study of Özgen et al., the TPC was found between 2.898 and 5.006 µg GAE/g (15). Another study determined the TPC of different Sambucus nigra L. fruits as 17.115 mg GAE/g (16). Goud and Prasad measured the TPC value of 43±0.98 mg GAE/g dried weight in their study (17). In a review examining the antioxidant properties of elderberries, it was determined that the TPC value varied between 1.91-17.90 mg GAE/g fresh matter (18). In this study, the TPC value was calculated as 9.75±0.92 mg GAE/g extract. The findings of our study correspond to similar ranges with the data in the literature.

Total flavonoid component content (TFC) can be obtained using different standards. In a study the TFC of ethanol extract of Sambucus nigra L. was measured as 840.54±13.46 mg RE/g dried weight (13). In another study, the TFC of methanol extract of Sambucus nigra L. was measured as 15.00±1.12 mg rutin/g dry weight (17). In this study, the TFC value was calculated as 0,07±0,004 mg quercetin/mg extract and 0,45±0,0014 mg catechin/g extract similar to the literature. The flavonoid is one of the phenolic components, so it was an expected result that the phenolic content was higher than the flavonoid content. However, less TFC content was found in our study than the TFC estimates in the literature. Assays used for the estimation of TFC are only equivalent to relative quantities of selected standards or have limitations in analytical methods for quantification. Differences in results may be linked with differences between selected standards.

Berries are generally rich in anthocyanins, Sambucus nigra L. is one of these fruits (19). Özgen et al. (15) examined the antioxidant capacity of different Sambucus nigra L. accessions and found that they contained anthocyanins between 1.308 and 4.004 mg Cy3GE/g. In the study of Domínguez et al., the amount of TAC obtained from Sambucus nigra L. extracts varies between 287.8–645.7 mg/100 g (20). Csorba et al. in their study determined the total anthocyanin content as 854.57 mg GAE/100g (16). In another study, anthocyanin's content in Sambucus nigra L. fruits was found to vary between 660 and 800 mg/100 g (21). Parallel to the literature, TAC was determined at 2.08±0.025 mg Cy3GE/g in the present study.

Studies have established a strong relationship between the phenolic content of a fruit and its ability to scavenge free radicals (22). On the other hand, DPPH radical capture test and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical capture test is characterized by color change in the presence of antioxidants. Özgen et al.(15) evaluated the antioxidant capacity of Sambucus nigra L. by DPPH analysis and it was found to be between 5.4-16.9 µmol troloks equivalent/g(TE/g). In the study conducted by Jakobek et al. (2007), it was determined that Sambucus nigra L. fruits have strong antiradical activity (100.16 mmol TE 1 g-1) in the DPPH method (23). In another study evaluating the antioxidant properties of the methanol extract of Sambucus nigra L. fruits, the DPPH value was calculated as 104.35 ± 0.22 µmol TE/g (24). In the present study, it was determined as 0.004± 0.007 µmol ascorbic acid equivalent/g.

The concentration required to scavenge 50% of the free radicals present is defined as the IC50 value. In a study, 50% scavenging capacity of Sambucus nigra L. was determined as 0.081 (mg/µl dry matter) (25). In a study carried out by preparing an acidified extract of Sambucus nigra L. fruits, lyophilization process was applied to the extracts and it was observed that DPPH free radicals were scavenged from 88.17% to 88.50% (26). In this study, the IC50 value was determined as 0.1311±0.0057 mg/mL.

The CUPRAC method is often preferred to measure the antioxidant capacity of plant materials. Haş et al. evaluated the antioxidant properties of Sambucus nigra L. fruit extract

and found it to be 52.3 ± 0.11 µmol TE/g dry weight (24). In a study, CUPRAC analysis of methanol extract of Sambucus nigra L. flower was performed and it was calculated as 0.42 mmol TR/g (27). In this study, it was calculated as 45.20±1.530 mM trolox equivalent/mg dry extract. In parallel with the literature, it has been shown that Sambucus nigra L. has a strong Copper (II) ion reduction potential.

The tumour is characterized by the establishment of the vessels feeding it as a result of the stimulation of pro-angiogenic factors and the provision of oxygenation. IC50 values are obtained as a result of measuring the cytotoxic effect. A lower IC50 value represents the higher ability of a cytotoxic compound to cause cell death or inhibit cell growth. Saeedi Saravi et. al (28) evaluated the cytotoxic effect of ethyl acetate extract of Sambucus ebulus on cancer (HepG2 and human colon carcinoma) and non-cancer (rat fibroblast and hamster ovary) cell lines. It was shown that the ethyl acetate extract of Sambucus ebulus had higher inhibition effects on cancer cell lines (0.097±0.152 mg/ml) compared to normal cell lines (0.312±0.346 mg/ml), which can act as an anticancer compound. In another study. In a study, HepG2 cells were exposed to different doses of Sambucus nigra L. fruit extracts and it was noted that the cell viability was still 100% at 500 µg/ml (29). However, the present study provides evidence that the ethanol extract of Sambucus nigra fruit acted as a cytotoxic agent on hepatic cancer cells (HepG2) with a CC50 of 5.49 mg/ml. These findings highlight that Sambucus nigra L. significantly reduces viable cancer cells and induces cell death.

The main findings of this study revealed that Sambucus nigra L. fruit extract is rich in antioxidant components. In addition, as expected as a result of the relationship between phenolic components and biological activities, it was observed that both the copper (II) ion reduction potential and the scavenging effect of DPPH radicals of Sambucus nigra L. fruit extract were high. On the other hand, the fact that the efficacy of the bioactive components in the structure of Sambucus fruit extract was not examined separately is a limitation of this study.

5. CONCLUSION

In light of our results and the literature's information on antioxidant and anticancer effects of bioactive components, we can conclude that Sambucus nigra L. with its high antioxidant capacity is a plausible candidate for the prevention of hepatic cancer. However, more research is needed to fully evaluate the safety and effectiveness of Sambucus nigra L.

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Clinical and Experimental Health Sciences

Capillary Hemangioma Oral Cavity: Report of Two Cases

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ABSTRACT

Capillary Haemangioma is a benign vascular tumor that is characterized by blood vessel growth and is usually known to be a developing hamartomatous disease of infancy and childhood. The current case report describes an exceptionally unusual atypical appearance of capillary haemangioma. These lesions confront the doctor with a diagnostic quandary and, if not appropriately controlled, can lead to significant problems.

Keywords: oral hemangioma, oral mucosa, gingiva

1. INTRODUCTION

The term hemangioma has been used to describe vascular abnormalities and proliferations (1). Hemangioma is a condition characterized by abnormal endothelial cell growth and an increase in the number of capillaries. The phrase vascular malformation, on the other hand, refers to a structural abnormality that is not caused by endothelial growth. As a result, vascular abnormalities are classified as either vascular tumors or vascular malformations. Hemangiomas of infancy, congenital hemangiomas, and pyogenic granuloma are examples of vascular tumors, whereas vascular malformations include capillary, venous, lymphatic, and arteriovenous malformations (2).

Although hemangioma is a common benign soft tissue tumor of head and neck, oral cavity lesions are relatively rare. Most common sites of hemangioma in oral cavity are lips, tongue, buccal mucosa and palate in oral cavity. Incidence is higher in females than males (3:1). Hemangioma can be observed congenitally or be seen in older individuals as well (3). Clinically, lesions are soft and generally painless. Also, swelling is not a rare finding. Mass can be described smooth or lobulated sessile or pedunculated with variable size (5).

Hemangiomas are classified on their histological appearance: capillary, cavernous or mixed hemangiomas, Capillary hemangioma is a type which is a unit of small thin-walled vessels of capillary size. Vessels are lined by a single layer of flattened or plump endothelial cells and surrounded by a discontinuous layer of pericytes and reticular fibres. Cavernous hemangiomas are deep, irregular, dermal blood-filled channels which is a unit of thin-walled cavernous vessels or sinusoids that separated by a scanty connective tissue stroma (1-3).

In addition capillary hemangiomas are caused by exuberant neovascular response to infection, local irritation or hormonal irregularity. Swelling might occurs in skin or mucosa to the accompaniment of ulceration and bleeding (5). Therefore, a careful imaging plan is necessary to evaluate the lesion, its neighbour tissues, possible complications. Vascular lesions are hard to operate due to the control of bleeding (6).

Various plans of treatment for hemangioma are popular. Excision, laser therapy, sclerotherapy, cryosurgery, electrodessiciation, embolization, curettage or ligation are individually or combine of these are treatment options (5,6). These reports aimed to represent two capillary hemangiomas, one of which has a unique location.

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2. CASE PRESENTATIONS

2.1. Case 1

In October 2021, a 19-years old Turkish female patient referred to the Department of Oral and Maxillofacial Radiology, Faculty of Dentistry, Marmara University with a swelling in edentulous alveolar ridge of left mandibular first molar. The patient has no systemic condition or regular usage of medicine. Also, the patient claimed that lesion begun to swell after the tooth extraction (left mandibular first molar, Figure 1). The patient declared that first small lesion spontaneously dropped. Then, according to the patient a new lesion started to grow up during one year. Panoramic radiograph did not reveal any bone involment (Figure 2).

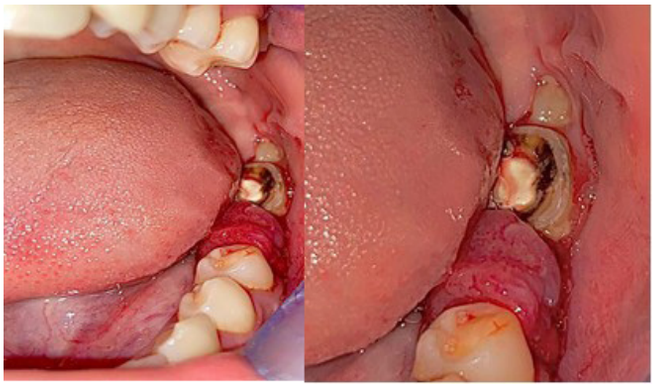

Figure 1. *Intraoral examination of spontaneously bleeding lesion which localized on the edentulous left mandibular fist molar's alveolar ridge. Ulceration and vascular lesion can be well observed.*

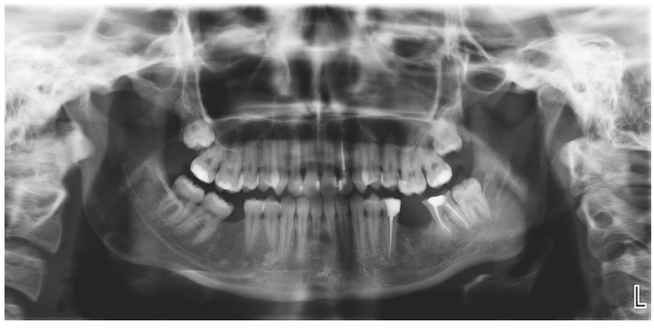

Figure 2. *Panoramic radiograph of the patient in case report 1.*

Extraoral examination including the lymph nodes was insignificant. Intraoral examination revealed a localized lesion which was pedicellated, spontaneously bleeding, hypertrophic and attached gingiva of deciduous mandibular first molar area, at the extraction zone. Lesion was a red mass, covering entire alveolar ridge buccolingually. Due to the clinical appearance and examination, provisional diagnose of pyogenic granuloma was establised.

An excisional biopsy was performed under local anesthesia. The biopsy tissue was sent for the histopathological examination at the Department of Oncologic Cytology and Tumor Pathology, Institute of Oncology, Istanbul University. Bleeding control was granted with pressure, coterization and suturation. The histopathologic features revealed nonceratinize stratified squamous epithelium of composed of many capillary vessels and red blood cells into some of well-shaped vessels (Figure 3a and 3b). Many small capillary vessels were evident. Vascularity was increased with numerous and various size of capillaries. Also, blood vessels and vasoformative tissue under the stratified squamous epithelium was revealed. Few areas of epithelium showed ulceration. The histopathological diagnosis was ulcerous capillary hemangioma. The operation site healed without any complication within 1 week (Figure 4). Five months of follow-up revealed no recurrence.

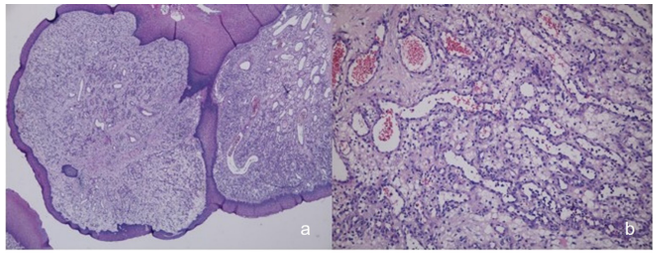

Figure 3a. *Various size of lumens which floored by endothelium under the stratified squamous epithelium (stain: H and E; magnification x40)* ***b.*** *Well shaped, multiple vascular sections. Red blood cells can be seen into some of the vessels (stain: H and E; magnification x200)*

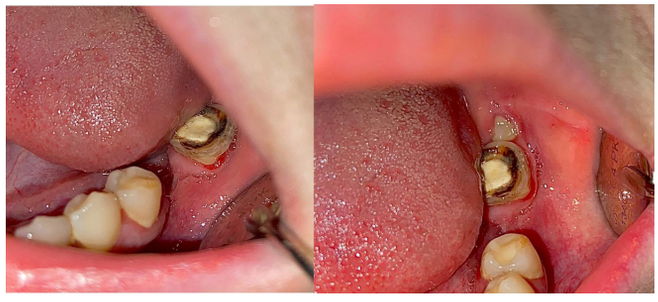

Figure 4. *Healing 1 week after the surgery.*

2.2. Case 2

In December 2021, a 48-years old Turkish male patient referred to the Department of Oral and Maxillofacial Radiology, Faculty of Dentistry, Marmara University with a swelling on anterior palatine mucosa (Figure 5). The patient declared that swelling got larger in a short period of time (15 days). Panoramic and periapical radiography did not reveal any bone involvement (Figure 6). The patient was also suffering from spontaneous bleeding around the lesion. Extraoral examination including the lymph nodes was insignificant. Intraoral examination revealed a localized,

erythematous and hypertrophic lesion of palatinal mucosa, also partially ulcerations and irregular surface was observed. Poor oral hygiene was seen during intraoral examination. Periodontal pocket of 5 mm was measured during periodontal examination of tooth 22. Patient has been chewing with anterior dentition due to the bilaterally edentulous posterior maxilla and has not been using a partial prosthetics.

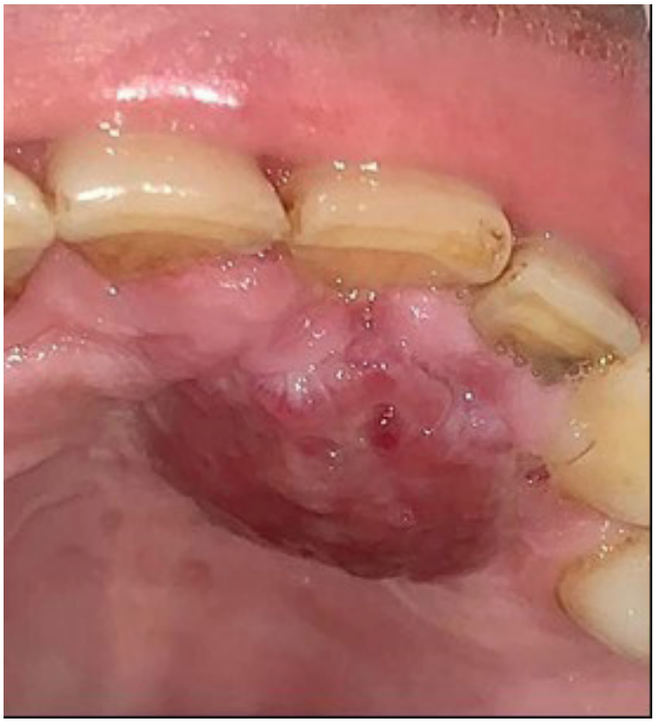

Figure 5. *Intraoral examination of the lesion which positioned in anterior of palatinal keratinized gingiva.*

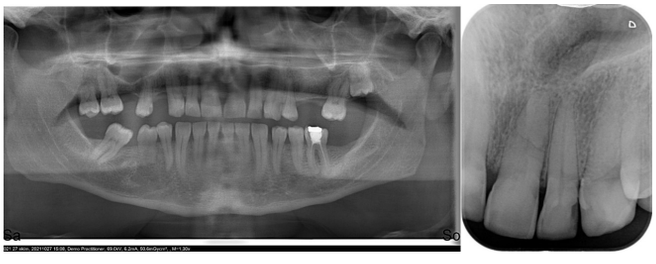

Figure 6. *Panoramic and periapical radiographies of the patient in case 2.*

An excisional biopsy was performed and sent for the histopathological examination. The histopathologic features revealed stratified squamous epithelium. Many lumens with different diameters are observed in most areas. Serum or erythrocytes are observed in the lumens. The histopathological diagnosis for this present case was ulcerous capillary hemangioma. The operation site healed without any complication within 2 weeks. Four months of follow-up revealed no recurrence (Figure 7).

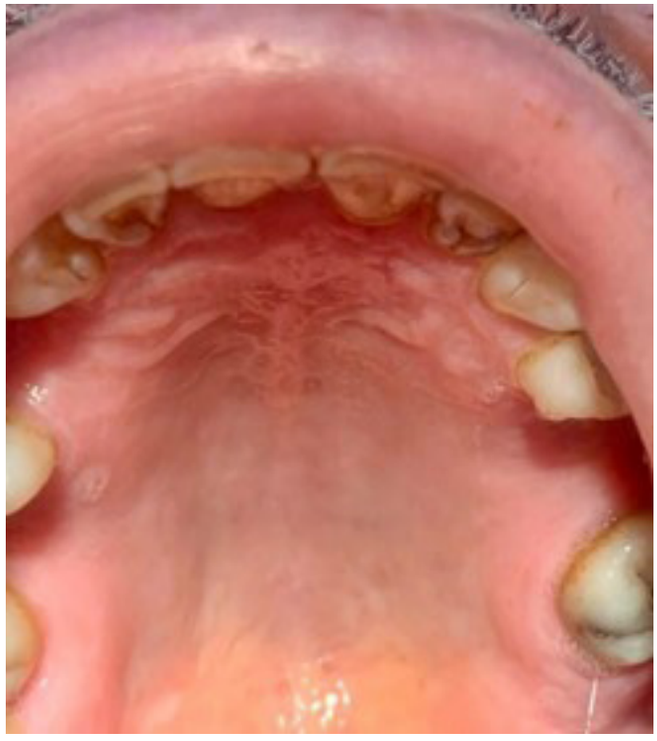

Figure 7. *Postoperative healing, 2 weeks after excisional biopsy.*

3. DISCUSSION

Hemangioma is a benign head and neck lesion that can develop during the neonatal period or occur congenitally (6). Oral hemangiomas account for 14.3% of all mouth benign vascular lesions, according to Correa et al. (7)'s prevalence study. Mumcu et al. (8), on the other hand, observed that the prevalence of oral hemangioma in Turkey is lower than in Germany. Previously, no race-related variations in the incidence of oral capillary hemangioma have been observed. (7-9).

Lobular capillary hemangiomas are rare benign lesions of the oral cavity (1,2). Oral capillary hemangioma lesions might involve the gingiva as though our cases, although the occurrence in the gingival mucosa is rare. The differential diagnosis of hemangiomas are pyogenic granuloma, inflammatory gingival hyperplasia, epulis granulomatosa and squamous cell carcinoma (6,7).

Clinical appearance and size can differ in individuals which brings differential diagnoses (8,9). One of the cases we represent here was reported at 19 years of age and the pre-diagnosis was pyogenic granuloma. The ulcerous view of our case represented was a distractive appearance on the path of diagnose and we considered pyogenic granuloma. Therefore, the biopsy was needed for definitive diagnosis. Similarly, a case report in literature described an exceptionally unusual aberrant appearance of capillary haemangioma on gingiva. In this case, the lesion was identified clinically as pyogenic granuloma but histopathologically as capillary haemangioma (10).

Vascular lesions should be pre-diagnosed with radiographic methods and clinical examination before a proper excision due to the high risk of complications. Ultrasonography is a popular method to examine the vascularity of the lesion. Understanding the vascularity of the lesion is important before the surgery to decrease the risk of complication. Therefore, the pre-diagnose and clinical examination matters for a proper pre-operative imaging. Neighbour tissues are highly important. Size of the lesion can be monstrous. Superficial growth of this benign lesion can breach into soft tissues in various degrees including lymph nodes. Large expansion or penetration of deep soft tissues is also a characteristic entity for cavernous hemangioma (3,9).

Management of bleeding and healing process is a challenge with vascular lesions. Postoperative ulceration is also a common complication (9,11-13). A clinical treatment protocol of da Silva et al. presented that sclerotherapy is a method which degrades the risk of superficial ulceration or scar (11-15). Spontaneous resolution of small lesions can also be seen in small hemangiomas of pediatric population, also Case 1 patient declared that the first small lesion resolved spontaneously (13-15).

4. CONCLUSION

Capillary haemangioma (CH) is a clinically similar condition to pyogenic granuloma that is distinguished by histological findings. Despite the fact that it is asymptomatic, its location and size may necessitate prompt and meticulous management. It frequently provides the clinician with a diagnostic challenge. This demands biopsy of such lesions in order to establish a definitive diagnosis and to ensure correct care and avoidance of consequences. Above all, the surgical excision of CH should be done with care, taking into account intraoperative and postoperative hemorrhage. CH is linked to a higher incidence of postoperative recurrence, necessitating a longer site follow-up.

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Clinical and Experimental Health Sciences

Giant Pulmonary Herniation: A Late and Rare Complication of Minimally Invasive Lung Biopsy for Interstitial Lung Disease

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ABSTRACT

Pulmonary herniation is the protrusion of the lung parenchyma beyond the normal limits of the thoracic cavity. It is a rare entity. In general, the defect in the chest wall is accompanied by increased intrathoracic pressures in the formation mechanism. Usually the cause is blunt-penetrating thoracic trauma, violent cough or previous thoracic surgery with insufficient closure of the chest wall. We report a case with giant pulmonary herniation that developed four years after biopsy in a patient diagnosed with usual interstitial pneumonia by VATS. Although this is a very rare condition in the literature, one of the late and rare complications of diagnostic pulmonary resections with awake VATS may be caused giant pulmonary herniation

Keywords: Pulmonary, diagnostic resection, pulmonary herniation

1. INTRODUCTION

Pulmonary Herniation (PH) is the protrusion of the lung parenchyma beyond the normal limits of the thoracic cavity. It is a rare entity. In general, the defect in the chest wall is accompanied by increased intrathoracic pressures in the formation mechanism. Usually the cause is blunt-penetrating thoracic trauma, violent cough or previous thoracic surgery with insufficient closure of the chest wall. Asymptomatic and minimal PHs can be approached conservatively. Parenchyma incarceration, which is the most feared complication in followed cases, should be considered. Symptomatic and major ones require surgical repair (1-3).

Here, we wanted to present a case with a PH developing four years after biopsy in an Idiopathic Pulmonary Fibrosis (IPF) patient diagnosed with awake VATS.

2. CASE PRESENTATION

A 60-year-old male patient evaluated to our clinic 4 years ago with complaints of shortness of breath and cough. In his history, he had hypertension for 15 years and schizo-affective disorder for 1 year. He was exsmoker for 15 years. When his exposures were questioned, it was learned that he worked in the glass factory for 23 years and retired for 12 years ago.

Chest CT image before the diagnostic surgery, showed peripheral ground glass areas with a peribronchovascular distribution, subpleural reticulation, traction bronchiectasis on the left lingular segment with a lower lobe predominance of the lesions, which was consistent with a probable UIP pattern and surgical lung biopsy was recommended with the decision of the multidisciplinary council (Figure 1A).

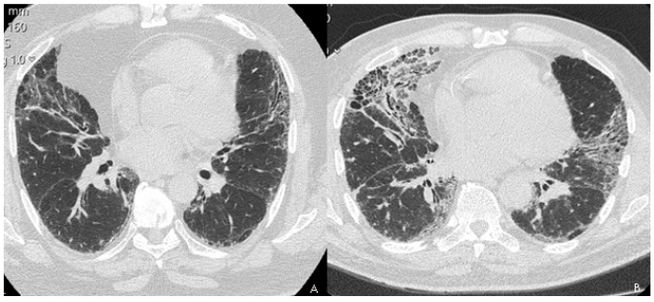

Figure 1. A: *Chest CT image before the diagnostic surgery, showed peripheral ground glass areas with a peribronchovascular distribution, subpleural reticulation, traction bronchiectasis on the left lingular segment with a lower lobe predominance of the lesions, which was consistent with a probable UIP pattern* ***B:*** *Chest CT image after four years from diagnostic surgery, shows remarkable progression in findings consistent with IPF.*

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The patient underwent awake non-intubated uniportal VATS with the help of TEA (Thoracic Epidural Analgesia) and sedation (propofol 1mgr/kg iv, remifentanil 1 mgr/kg iv). A total of two wedge resections were performed with the help of endoscopic GIA from the infiltrated and intact lung parenchyma areas detected in the posterior segment of the right upper lobe of the lung during VATS. The total operation time was 35 minutes. Chest drain was removed at the postoperative 24th hour. The day after he was discharged uneventfully. Awake VATS procedure was preferred to reduce the risks of general anesthesia and intubation and to provide rapid recovery. Pathology result was reported as diffuse focal interstitial pneumonia, lung parenchymal tissue showing heterogeneous fibrosis. No specific findings were demonstrated to suggest silicosis.

The patient was evaluated at the multidisciplinary council again and radiological and morphological findings were consistent with a probable UIP pattern. He was not eligible for lung transplantation due to his psychiatric condition and nintedanib treatment was started. During his treatment, the patient was followed up regularly every six months and remained stable for three years. At the end of the fourth year, although the clinical response of the patient was stable, progression was detected in his radiological findings (Figure 1B). Since his psychiatric disease was under control, the patient was asked again for the transplantation, but he refused again. Beside the progression of the disease, there was also pulmonary herniation at the same time. The patient presented with a complaint of pain and bulging mass on the right side of his chest in the old biopsy area. Bulging was observed on inspection, also bulging increased with breathing and coughing (Figure 2A). PH was detected on chest CT images, in the bulging area which increased when coughing (Figure 2B). Violent couhing period history may be the cause of pulmonary herniation

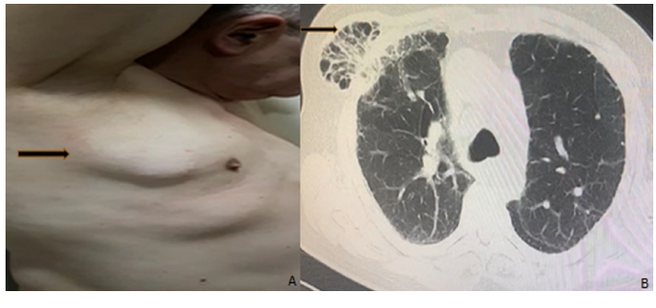

Figure 2. A: *Bulging on the antero-lateral chest wall localized in the old incision site is clearly seen in the 4th year after the operation, marked with arrow.* ***B:*** *Chest CT image of PH at the same period, marked with arrow.*

The patient was operated to repair PH. The operation was performed on the old incision scar with the help of sedo-analgesia (i.v. propofol 0,5 mgr/kg + dexmedetomidine 1 mikrogram/kg). Also, lidocaine 2 mgr/kg was used for local anesthesia. Under the subcutaneous tissue, the PH from the defect in the intercostal region was detected (Figure 3). The herniated lung tissue was pushed into the thoracic cavity and the defect in the intercostal region was primary separate repaired using the no 1 polyglactin. We used the intercostal and serratus anterior muscles as autologous tissue over the repaired defect. The patient was discharged uneventfully on the first postoperative day. After two years of the hernia operation, he passed away due to acute exacerbation of IPF with no recurrence for PH.

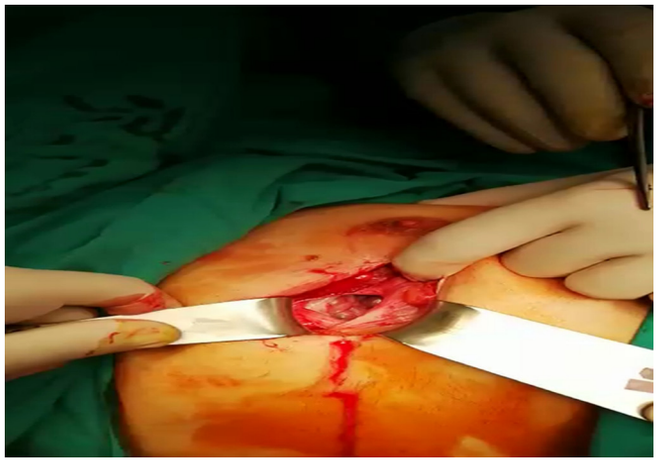

Figure 3. *Operation view of the intercostal defect which causes PH*

3. DISCUSSION

PH is a rare entity and is defined as the protrusion of the pulmonary tissue and pleural membranes beyond the chest cavity through an abnormal opening in the chest wall, diaphragm or mediastinum. PH first described in the 16th century by Morel-Lavallée (5). Although the exact frequency is not known, less than 400 cases of lung herniation have been reported in literature. PH can be congenital or acquired. Acquired hernias are etiologically classified as traumatic, spontaneous, and pathologic. PHs's anatomical locations are cervical, thoracic, diaphragmatic and mediastinal. The mechanism of acquired PHs includes intercostal muscle or thoracic wall weakness (after rib or cartilage fracture) with conditions that increase intrathoracic pressure, such as coughing, sneezing, musical instrument or glass blowing or heavy lifting. (5,6). Our case corresponds to an acquired lung herniation of an operative traumatic origin and violent coughing. This may have been caused by inadequate closure of the intercostal space used during VATS. Particularly in cases where a wound retractor is used, meticulous closure of the intercostal space may prevent the development of the such complication.

The most common clinical presentation of a PH is a soft, tender, subcutaneous mass that may protrude on deep breathing or coughing. Although chest X-ray and thorax USG can be used for diagnosis, chest CT to be taken after Valsalva maneuver is often needed for definitive diagnosis. In the differential diagnosis, subcutaneous emphysema, bronchopleural fistula, chest wall lipoma, chest wall or breast abscess, cutaneous metastasis, seroma, hematoma, pectoralis major tendon rupture should be considered (3-6).

Four years after diagnostic operation our case presented with a complaint of pain and bulging mass on the right side of his chest in the former biopsy area, In our case, the diagnosis was clearly established by chest CT.

The most common complications of VATS are pneumothorax, prolonged air leakage, pain and infections (4-6). PH is an uncommon complication after awake VATS .

PH was described in a few cases in the literature. PH was reported after minimally invasive cardiac surgery and VATS lobectomy (2-7). However, there is no case of PH due to awake lung biopsy for the diagnosis of ILD in the literature.

While conservative treatment can be tried in spontaneous and traumatic minor PH, surgical repair is required in major and symptomatic PH. Bed rest, analgesic, antitussives, antibiotics, chest orthosis and other basic treatment can be used in conservative treatment unless serious findings such as incarceration occur. The purpose of surgical treatment is returns the lung to its proper position and then stitches up the opening or reinforces the weakened area of the chest. Surgery, generally, is not the preferred treatment for lung hernias in the cervical region, no matter how severe. But surgery is routinely performed for lung hernias involving the thoracic wall and diaphragm. Usually, posterior chest wall defects under the scapula do not require surgical repair. Wire sutures, absorbable and non-absorbable materials (such as polytetrafluoroethylene patch, polyglactin, PTFE (PolyTetraFluoroEthylene), dacron, marlex and goretex mesh), autologous tissues, approximation of ribs with monofilament sutures can be used in surgical repair. Which surgical technique will be used depends on the location and size of the hernia and the experience of the surgeon (1,5-7). We successfully used the intercostal and serratus anterior muscles as autologous tissue and performed the approximation of ribs with monofilament separate sutures technique.

4. CONCLUSION

As in our case, we aimed to emphasize that PH, which is a rare complication of diagnostic VATS biopsy, can also be detected in the late period. Although there are other repair methods of the PH, primary closure with autologous tissue reinforcement technique which we also used can be used safely.

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Clinical and Experimental Health Sciences

A Case Report of Metal Induced MRI Accident and Diagnosis by Ultrasonography

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ABSTRACT

Dental radiology has shown great developments in recent years. In addition to conventional radiography techniques, advanced imaging techniques are frequently used in dental radiology when necessary. Magnetic Resonance Imaging (MRI) is an advanced imaging technique that is frequently used because of its high soft tissue resolution and no risk of ionizing radiation. However, it should be noted that MRI also has some risks. Ultrasonography (USG) is a very useful technique for imaging foreign bodies especially superficial ones. The purpose of this case report was to present the USG diagnosis and subsequent surgical removal of a metallic foreign body that interferes with MRI in a cheek of 40 years old female. At the same time, it was aimed to draw attention to the disadvantages of metals for MRI and the role of USG in detecting superficial foreign bodies.

Keywords: Magnetic resonance ımaging, ultrasonography; foreign bodies.

1.INTRODUCTION

Magnetic Resonance Imaging (MRI) was used first by Damadian in 1971 and by Lauterbur in 1973 (1). The most important advantages of MRI are that it does not contain ionizing radiation and it is the technique with the highest soft tissue contrast. Since patients are exposed to a strong magnetic field during MRI, metallic objects in the environment or in the patient's body may be adversely affected by this magnetism. MRI has some contraindications such as; cardiac pacemakers, cerebral aneurysm clips, metallic foreign bodies, vena cava filters, IV stents, middle ear prostheses and orthopedic prostheses.

Ultrasonography (USG) is a technique that uses high-frequency ultrasound waves, does not contain ionizing radiation, and is generally used to examine muscles, tendons, joints, vessels and internal organs that are not behind the bone. USG has many advantages such as allowing simultaneous imaging, being portable, inexpensive, free of radiation, being non-invasive and not affected by metal artifacts. Although the use of USG in dentistry is thought to be limited to the evaluation of salivary glands, cervical lymph node evaluation, facial muscles, face and neck soft tissues; with the development of high-resolution devices in recent years, USG has begun to be used in different areas in our field as well as in the examination of foreign bodies in the head and neck region (2).

The necessity of radiological examination in detecting foreign objects is indisputable. Conventional radiography, Computed Tomography (CT), MRI and USG are generally used to identify foreign bodies (3). The purpose of this case report was to present the MRI accident caused by a metallic foreign body in cheek and its diagnosis by USG followed by surgical removal. At the same time, it was aimed to increase the awareness of the readers about the disadvantages of metals for MRI and the role of USG in detecting superficial foreign bodies.

2.CASE REPORT

A 40-year-old female patient applied to our clinic with an interesting medical history. She stated that MRI was requested for orthopedic discomfort in her arm. For this purpose, when she entered the MRI device, she stated that there was an excessive swelling and pain in the left cheek with the operation of the device and the operator then immediately stopped shooting. The patient was told

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that there was a metallic object in her cheek and MRI could only be performed after this object was removed. When the patient came to us for this purpose, we saw the metallic object superimposed on the left mandibular molar teeth in the Panoramic and periapical films available in the telemedicine system. However, the patient stated that she did not remember any event in her history that could have caused this.

In the clinical bi-digital examination of the patient's cheek, we felt a hardness in that area (Fig. 1). On panoramic radiography, the object was not noticed at first because it was superimposed on the left mandibular first molar with metallic filling (Fig. 2). A metallic object was observed in the periapical film taken in the superior direction, but the location of the object was not fully discernible (Fig. 3). It was decided to take extra-oral and intra-oral USG from the patient for further examination. USG was applied using an Aplio-300 device (Toshiba Corporation, Tokyo, Japan) and 12 MHz linear probe used for extra-oral USG and 18 MHz Hockey stick probe used for intraoral USG. Metallic foreign body was clearly observed in both techniques.

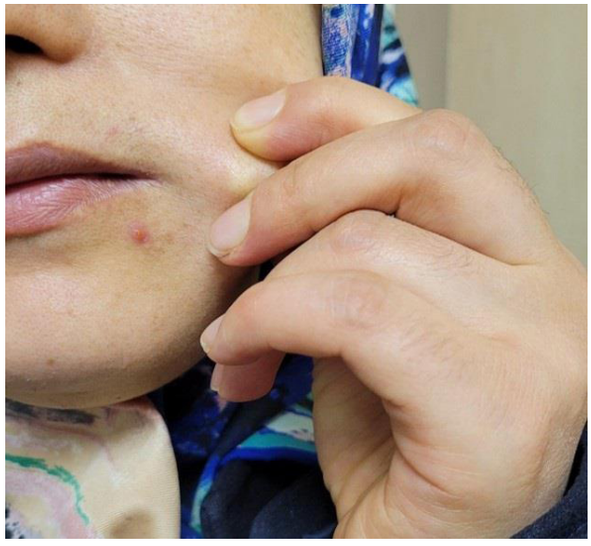

Figure 1. *Clinical examination of the foreign body in the cheek.*

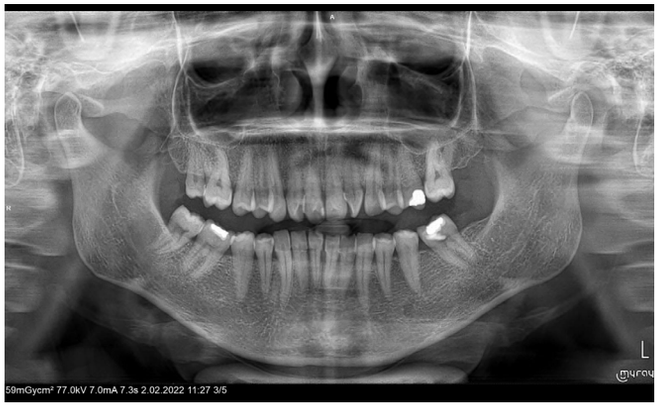

Figure 2. *The metallic object superimposed on the left mandibular first molar with metallic filling is observed on the panoramic radiography.*

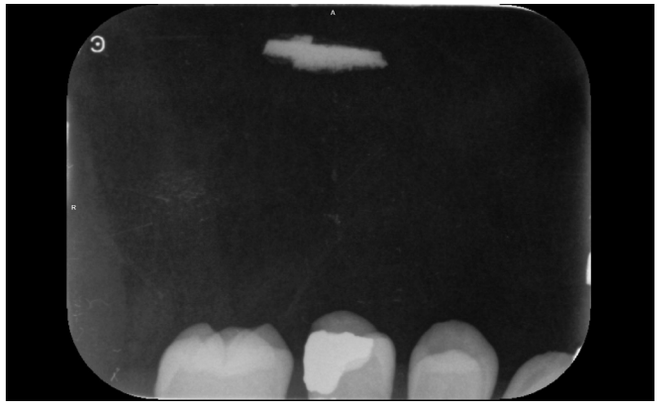

Figure 3. *The periapical radiographic image of the metallic object.*

Extra-oral USG examination revealed an 8.4 mm foreign body with posterior acoustic shadowing in the subcutaneous area (Fig 4). In the intra-oral USG examination, a foreign body with posterior acoustic shadowing of 4.1 mm was observed in the submucosal area (Fig 4). Later, the patient was referred to the surgical service for the removal of this object. The body was removed by intra-oral intervention under local anesthesia (Fig 5). Afterwards, the patient was called for control post-operatively. When there was no problem, the patient was referred for MRI again after the post-operative edema and pain subsided. Afterwards, the patient was able to have an MRI without any problems.

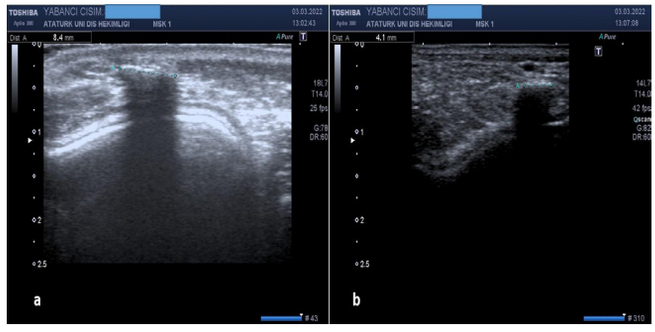

Figure 4. *The ultrasonography of the metallic object revealed a hyper echogenic foreign body with posterior acoustic shadowing. a: Trans cutaneous-approach, b: trans-oral approach.*

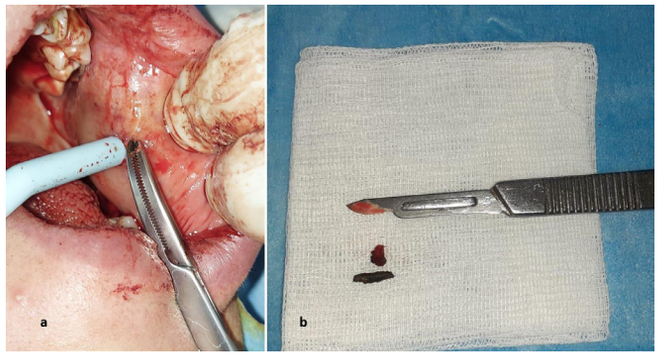

Figure 5. *Removal of the metallic object by intra-oral intervention under local anesthesia. a: The operation, b: The extracted metallic object.*

3. DISCUSSION

MRI is an advanced imaging technique that is frequently used because of its high soft tissue resolution and no risk of ionizing radiation. However, it should be noted that MRI also has some risks for both patients and healthcare personnel (4). Although radiologists are educated about the feasibility and indication of MRI, other doctors who request MRI should also be aware of benefits and risks of MRI. Referring physicians, especially those who know the patient's medical history better, can better know whether their patients have any risky conditions for MRI. This is even more important for patients carrying metallic medical devices.

Ferromagnetic objects in the MRI room or on the patient can be a hazard to both the patient and the staff, as they can be moved by the strong magnetic field. In this respect, necessary precautions should be taken, these objects should be removed from the environment, and a detailed story should be taken from the patients as to whether they have any metallic objects in their bodies. In this case, our patient was unaware that she had such a metallic object on her cheek until the MRI scanning. According to, guidelines to Prevent Excessive Heating and Burns Associated with Magnetic Resonance Procedures, metallic objects that come into contact with the patient's skin should be removed, use insulation material of 1 cm or thicker to prevent skin-to-skin contact and the formation of closed-loops from touching body parts and only materials proven to be MRI safe should be allowed (5).

As it is known, static and time-varying electromagnetic fields are used in MRI. However, although there is no risk of ionizing radiation, these magnetic fields can pose a danger to humans from time to time. Namely, the radiofrequency used in MRI may cause heating of the tissues (6). Fatal MRI accidents have been reported in patients with metallic implants in the literature (7). The most important cause of serious injuries in MRI scans is heating caused by radiofrequency. Namely; in phantom experiments, metal heating was found to reach 75°C following RF exposure (8).

The hazards caused by metallic implants in MRI due to their physical structure can be examined under four headings; torques and slips, movements in metal objects, malfunction in active devices such as pacemakers, and tissue damage due to local heating (9, 10). MRI may cause undesirable conditions in patients with metallic implants such as orthopedic devices (11), cardiovascular devices (12), and cochlear implants (13). With the developments in biomedical engineering, safe MRI devices are now available (12). In addition to all these, metals also cause image artifacts in MRI more severe than in CT (14). In recent years, new protocols have been developed to eliminate these negative interactions of MRI with metals. The most basic factors affecting metal interactions in MRI are the type of metal, MR field strength used, MR protocol and sequence. The location, size, orientation, and configuration of the metal play a role also. Titanium, which is frequently used in many treatment materials today, definitely causes smaller interference and artifact in MRI compared to Cobaltchrome and stainless steel (15-19). Recently developed aluminum-free titanium composites, biodegradable magnesium alloys or radiolucent carbon-fiber-reinforced polymers have reduced unwanted metallic interactions in MRI.

USG is a safe diagnostic method in which internal organs are imaged using ultra sound in medicine. It does not carry the risk of ionizing radiation and is not affected by metal artifacts. USG is used in dentistry generally for several purposes such as; salivary gland diseases, cervical lymphadenopathy, various soft tissue mass, masticatory and neck muscles and as well as novel usage areas such as; maxillofacial fractures, periapical lesions, Temporomandibular Joint, tongue tumors, dental tissues' decay, cracks and fractures, mucosal lesions, periodontal tissues, implant dentistry, evaluation of rapid palatal expansion in orthodontia (20).

Foreign bodies can enter the body unintentionally in different ways due to aspiration, ingestion or insertion and they can be imaged with various techniques such as conventional radiography, USG, fluoroscopy, or CT depending on the type and location of the object (21). In the literature, there are many cases of foreign body in the cheek diagnosed by various radiological methods (22). However, we did not encounter a similar case that caused an MRI accident and was diagnosed with ultrasonography in dentistry. In fact, the use of USG in dental radiology has been increasing in recent years. Different bodies can give different images in different imaging techniques and in different environment. USG with high frequency probes should be preferred to examine superficial foreign bodies even non-opaque ones that cannot be seen on radiography. In this case, USG gave us a clear benefit in imaging the metallic foreign body on the patient's cheek. The metal fragment was clearly observed with its hyperechoic structure and acoustic shadow in both intraoral and extra oral USG examination. In the present case, the size of the object was measured smaller in the intra-oral USG. But it should be said that; size of the object measured by USG can of course also vary depending on the localization of the object and holding angle of the probe. Of course, it may not always be possible to approach the desired area from the inside of the mouth at the desired angle with the USG probe.

Although plain radiographs are sufficient for imaging radio-opaque foreign bodies, they are insufficient for full localization of the object because they do not allow three-dimensional imaging. As a matter of fact, the same inadequacy was encountered in this case as well. As in this case, if the metallic foreign body on the patient's cheek had been detected on time in the previous dental panoramic radiograph, the patient might not have encountered an undesirable situation during MRI. In this respect, every radiographic examination should be examined in detail. Dentists may be the first to notice foreign bodies in the maxillofacial region.

4. CONCLUSION

This case report concluded that, it should be questioned whether patients have metal implants or objects in their

bodies before they were admitted to magnetic field devices such as MRI. Because metallic objects on the patient or inside his body will be strongly attracted by the machine. In addition, this case report reminded us the importance of USG in imaging foreign bodies that may be found in superficial soft tissues.

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Design of the study: F. Ç.
Acquisition of data for the study: Z. B.
Analysis of data for the study: G. A.
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Drafting the manuscript: E.A.G.
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