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Research Article

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Evaluation of morphology and viability of spheroid derived from Insulin-GLase cell line: A model system to understand Type 2 Diabetes Mellitus

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Abstract

Type 2 Diabetes Mellitus (T2DM) is one of the major health issues in the world. The cellular mechanism of T2DM is still not fully understood. It could be studied by using spheroid three-dimensional (3D) culture which is considered representative of the in vivo conditions. Several types of pancreatic β cell lines have been used, one of which is the insulin-GLase (iGL) cell line. This study aims to evaluate the effect of cell density and incubation time on spheroid morphology and cell viability in order to understand which one can be considered as the best option in studying T2DM using iGL cell. Spheroid was made by using the Hanging drop method. The variations of initial seeding cells were 50, 100, 200, and 400 cells/µL then incubated for 1, 2, 3, and 4 days. The evaluated parameters in this study are spheroid morphology and cell viability. Spheroid morphology was observed by using inverted phase contrast microscope integrated with camera (Nikon) and NIS-Elements Analysis D software. Cell viability was determined by using LUNA-II™ Automated Cell Counter (Logos Biosystem). The result of this study showed that spheroid in all of the group cell concentration have formed since the first day and its diameter was significantly increased on the following days ($p<0,05$). The spheroid size was positively correlated with the cell density in group 50-200 cells/µL. A single and stable spheroid morphology was observed in 50-100 cells/µL group. Cell viability in 3D culture system was lower and significantly decreased since day 3 compared to 2D culture ($p <0.05; 0.01$). In conclusion, spheroid derived from iGL cell line with a stable morphology and good viability could be obtained from a cell concentration of 50-100 cells / µL with two days of incubation.

Keywords: iGL, cell density, spheroid, viability

1. Introduction

Diabetes is a major health problem around the world. Corresponding to the International Diabetes Federation (IDF), the incidence of diabetes reached 9.3% or 460 million people of the world's total population in 2019. This number is expected to continuously increase in the next decade. Indonesia is the 7th rank as the country with the highest number of diabetics patient in the world with a range of 10.7 million people. Type-2 diabetes (T2DM) is the type of diabetes with the largest number of patients (IDF Diabetes Atlas 9th edition, 2019). T2DM is caused by inadequate insulin secretion and insulin resistance. Currently cellular and molecular mechanism of T2DM is not fully understood. However, nowadays it can be studied with by using in vitro model of pancreatic β cell culture.

iGL cell line is a cell derived from the rat pancreatic β cell sub-clone. These cells can express insulin-GLase in response to high environmental glucose levels. Human insulin protein were fused with *Gaussia* luciferase protein as a marker of insulin secretion (Suzuki et al., 2017). Another advantage of this cell is its ability to be cultured in 3D system. These cells will form an aggregate that gather and condense which is known as a spheroid. 3D culture as spheroid form is more identical to actual conditions of the in vivo microenvironment than monolayer culture by facilitating the interaction cell to cell and cell to extracellular matrix interaction (Ryu et al., 2019). Moreover, this culture system allows cells to grow from all directions in vitro conditions (Lee et al., 2019). This 3D spheroid culture model can be potentially used to study the cellular conditions and microenvironment of pancreatic β cells and their correlation with the pathomechanism of T2DM. Furthermore, it could be used as an appropriate model for studying the activity and effectiveness of antidiabetic drugs. The spheroid model is a good model for testing the efficacy and toxicity of drugs, as proven in anti-tumor drugs research (Gong et al., 2015).

In this study, the hanging drop method was preferred because it is a relatively easy and simple method to generate the spheroids. It tends to be simple and does not require complicated tools and materials. This technique allows spheroids to form on the edge of the drop. The size of

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spheroid could be controlled by adjusting the volume of drop or the density of cell suspension (Bartosh and Ylostalo, 2014). In comparison to previous research, spheroids from iGL cells were obtained by 3D culture system that using specific plate, this technique is considered as easier and reproducible (Suzuki et al., 2017).

The limitation of the spheroid culture system is the greater the cell aggregate the more likely the cells in the spheroid center are deficient in nutrients and oxygen that causing cell death (Ryu et al., 2019). Therefore, this study aims to understand the effect of the initial cell density and incubation time on the morphology and cell viability of iGL cell spheroids. Furthermore, this is an original study to determine the most appropriate number of initial cell density and incubation time to generate spheroid derived from iGL cells with good shape and viability for advance further research.

2. Materials and methods

2.1. iGL cell preparation and propagation

iGL cells are derived from the rat pancreatic β cells (Cosmo Bio, IGL01C, Japan). The cells thawed in the water bath for two minutes. Then administration of 1 mL of iGL medium culture (Cosmo Bio, IGLM, Japan) into the cryotube. Further, the cell was transferred directly to the 15 mL tube (Corning, 430791, USA) and add the medium until 10mL volume. Next, it was centrifuged at a speed of 300g for five minutes then discards supernatant. The cell pellet then washed with a 5mL medium and centrifuged at a speed of 300g for five minutes. The supernatant was discarded and resuspended with 1mL medium and count the cells with an automatic cell counter (LUNA Automated Cell Counter, Logos Biosystem, South Korea). Seeding the 8×10^5 cells in 100x20 mm dish (Corning, 430167, USA). Medium was replaced on the fourth day. After reached 70-90% of confluency, the cells were passaged.

Passage of the cell was performed by removing the remaining medium followed by washing with PBS (Gibco, 18912014, USA) once. Then 1mL of Trypsin-EDTA 0.05% (Gibco, 25300054, USA) were added and incubate at 37°C for two minutes, subsequently. Next, 10 mL medium were added to deactivate the trypsin. The detached cells were transferred into the new tube, counted, and seeded to a new 100×20 mm dish with $8\text{-}9\times10^5$ cells each dish afterward.

2.2. 3D cell culture with hanging drop method

After cell propagation, cells were collected and divided into four cell density variants which are 50, 100, 200 and 400 cell/µL cell suspension. The cell suspension drops were formed by placing 25 µL cell suspension on the lid of a 100×20 mm bacterial petri dish (SPL,10101, South Korea). The outer side of the dish lid were previously outlined with waterproof marker to make small squares approximately 5×5 mm in size before used, so the cell suspension drop could be placed in a relatively identical distance. 80 drops were prepared for each cell concentration which performed in two petri dishes. 20-25 mL if sterile PBS were added to the bottom part of the dish. Once the all the drop has completed, the lid was flipped quickly and carefully to maintain the drops. Then the lid was used to cover the bottom part of the dish that already contained PBS. The petri dishes were placed in the incubator with 37°C and 5% of CO_2 for 1, 2, 3, and 4 days.

2.3. Spheroid morphology evaluation

The study evaluated spheroid morphology by the size and shape of the spheroid from each variation of cell concentration during different time of incubation. The morphology was observed \using inverted contrast-phase microscope (Nikon, Ts2 Ph Fl, Japan) integrated with camera (Nikon, DS-Fi3, Japan). The spheroid diameter was measured by using NIS-Elements Analysis D 5.20.00 64-bit software. Measurements were performed at 30 drops per treatment (15 drops per dish). When there was an excessive number of spheroids in one drop, the average size would be the final size. Measurements are performed on two orthogonal spheroid diameters (d1 and d2) and then the estimation of diameter (D) is calculated by calculating the root of the multiplication of the two diameters ($D=\sqrt{d1d2}$) (Shi et al., 2018).

2.4. Evaluation of cell viability

Cell viability was analyzed by using trypan blue and LUNA-II™ Automated Cell Counter (Logos Biosystem, South Korea). Cells from 2D and 3D cultures were harvested to produce cell suspense containing single cells. The spheroid was dispersed by using Trypsin-EDTA 0.05% (Gibco, 25300054, USA) and gentle pipetting. Afterwards, each cell suspension was mixed with 10µL Trypan Blue 0.4% with a ratio of 1:1. Then it was approximately homogenized for five seconds by using a 10µL micropipette (Appendorf, USA). next, 10µL mixed cells and trypan blue were removed into the counting slide. Finally, the slide was inserted into the device and the monitor displayed the result immediately after few minutes.

2.5. Data analysis

The data were analyzed using the STATA.12 software. Student's t-tests or two-way analysis of variance and Tukey's post hoc tests are used to determine significant differences between the groups.

3. Results

3.1. Spheroid size and morphology

The formation of cell spheroids began in all variation groups of cell density since the first day after cell seeding. Fig. 1 showed that the spheroids formed most closely resemble round or oval balls. This shape is found in spheroids derived from all variations except 200 cells/µL. In 200 cells/µL variant, the spheroids were found in irregular shape.

The concentration of cells has positive correlation with the diameter of the spheroid. However, the positive correlation only occurred in the group of 50-200 cells/µL. At a higher

cell concentration, 400 cells/µL, the diameter of the spheroid is smaller but found in greater quantities.

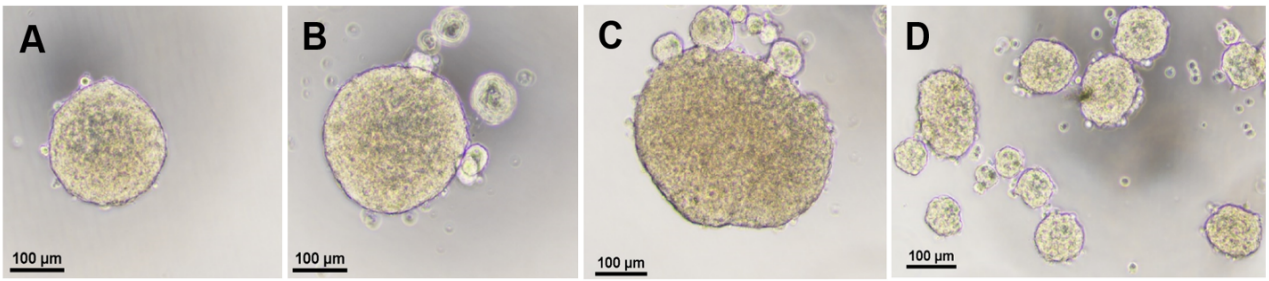

Fig. 1. Spheroid from 50 cells/µL (A), 100 cells/µL (B), 200 cells/µL (C), and 400 cells/µL (D) with two days of incubation (Phase contrast microscope, mag. 100x)

Discrepancies in spheroid size are observed from the first to the fourth day after are illustrated in Fig. 2. Spheroids become larger and more solid as the day went. Concentration and duration of incubation have a significant effect on the size of the spheroid. Moreover, there is a positive correlation between the diameter of the spheroid and the day of incubation.

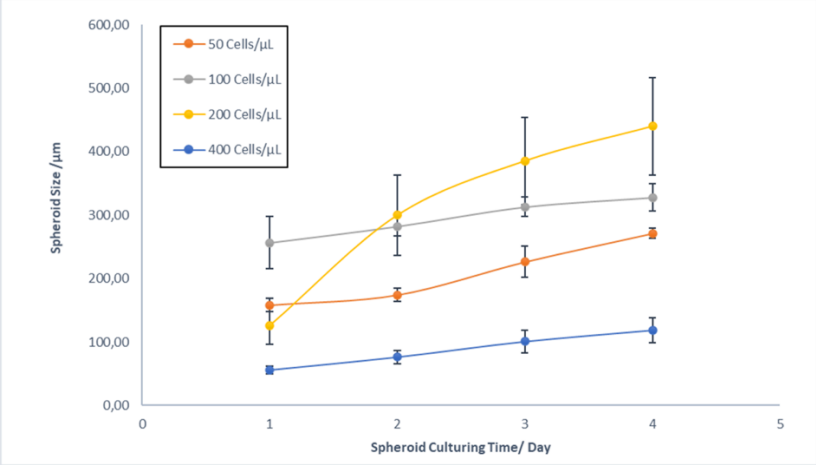

Fig. 2. Size comparison of spheroid from different initial cell seeding densities from day 1 to 4. Data represent the mean ± standard deviation (SD) of each experiment (n=30). Data included was ±2SD. Statistical analysis was performed by to way ANOVA (p=<0.01). There is strong positive correlation between spheroid and day of incubation (Pearson, r=0,4443)

3.2. Spheroid cell viability

The viability of iGL cells cultured in 3D culture system is relatively unstable than 2D monolayer culture. 2D cultures mostly maintain their viability in up to 80% for all groups of cell concentration until the fourth day. During two days of incubation, the 2D and 3D culture systems showed no significant differences for the cell viability in groups of 50,100, and 200 cells/µL. However, the cell viability begins to fall significantly from the third to the fourth day in 3D cultures. Result is shown in Fig. 3.

4. Discussion

An understanding of the cellular mechanism of insulin secretion and its correlation to mechanism of T2DM can be studied with an in-vitro model of pancreatic β cell culture. Several variants of widely used pancreatic β cell line are RIN-m5F (Lai et al., 2014), EndoC-bH1 (Esguerra et al., 2020), INS-1 cells (Baidwan et al., 2017), and iGL (Suzuki et al., 2017). Most of the cells are potentially form 3D structure like spheroid. Spheroid has been counted as appropriate in vitro model for last decade due to it closely mimic the in vivo condition (Bartosh and Ylostalo, 2014). Several methods have been used for generating spheroids i.e., hanging drop, gel embedding, magnetic levitation, and spinner culture (Ryu et al., 2019). Some of these methods need scaffolds (Bresciani et al., 2019). Each of these methods has its own advantages and disadvantages respectively. Appropriate method of the study is selected by considering the research objectives and availability of tools and materials. This study employed hanging drop method due to it is an easy, simple and reproducible method.

This study proved that spheroids from iGL cells can be generated by using 3D culture system with Hanging Drop method as shown in Fig. 1. The size of the spheroid was affected by cell density and time of incubation. Fig. 2 showed that the size of the spheroid was positively correlated with the initial cell seeding number at concentrations of 50-200 cells /µL. This result relatively similar with the previous study by using a variety of cell lines with different cell number such as HIEC-6 (Flampouri et al., 2019), MCF-7 (Gong et al., 2015), HCT-116, UM-UC-3, and HeLa cells (Pereira et al., 2017).

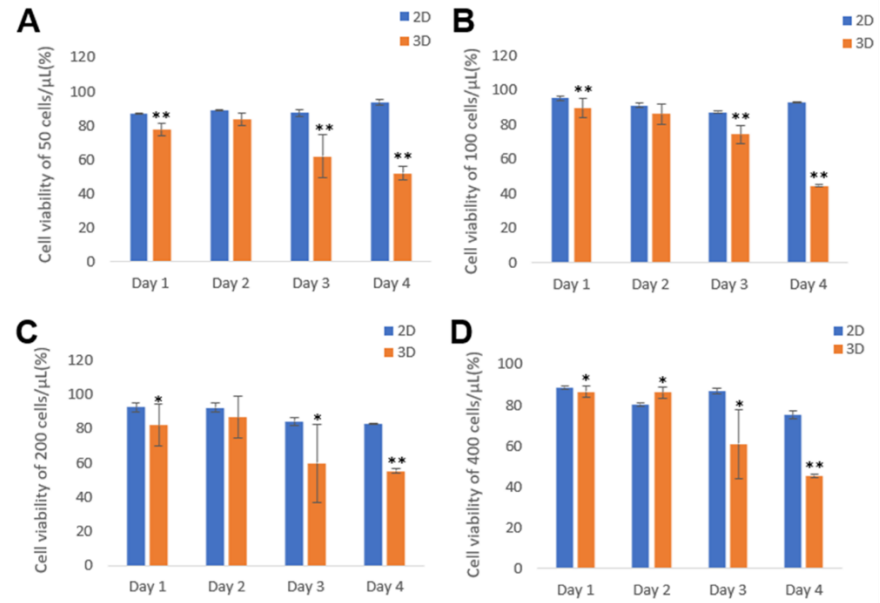

Fig. 3. Cell viability of iGL cells in 3D and 2D culture from 1-4 days of incubation with various cell densities (A-D 50,100,200, and 400 cells/µL respectively). The differences between 2D and 3D were analyzed using student t-test (**p<0.01, *p<0.05)

Fig. 1 illustrated that spheroid derived from iGL cell tends to be stable and consistent morphology in groups of 50 and 100 cells/µL with a round or oval shape and mostly a single spheroid. On contrary, the group of 200 cells/µL had an irregular shape and surrounded by numerous spheroids in smaller size. Different results were found in the cell group of 400 cells/µL. The size of the spheroids of this group tends to be smaller, even smaller than the group of 50 cells/µL. However, greater quantity of the spheroids was found in this groups, rather than other groups that had a single spheroid or multiple spheroids with one large dominant spheroid. It is apparently due to the differences in the microenvironment between groups. The formation of spheroids by cell aggregation is a complex process influenced by the surrounding micro-environment such as extracellular matrix, cell to cell junctions, and dissolved proteins in the medium. Additionally, the formation of spheroids is also determined by the phenotype of the cell type used (Repin et al., 2014). Each cell has a different fusion speed and model. Spheroids can be formed by the fusion of a single cell or cell aggregate thus forming a larger and denser cell aggregate to become a spheroid (Kosheleva et al., 2020).

Fig. 3 showed that the cell viability of spheroid from all groups of cell concentrations showed similar results to 2D monolayer culture in two-day incubation, except for the 400/µL group that tends to have higher viability. However, in general, viability of spheroid cells is most likely lower than 2D monolayer culture due to inequality in gaining oxygen and nutrition. Cells in 2D culture have better access to nutrients and oxygen than a spheroid. The spheroid consists of a multilayered mass of cells that form two zones, the middle and peripheral zones. Cells of the middle zone were lack of nutrients and oxygen so they tend to be in the quiescent phase or cell death either by apoptosis or necrosis. This zone looks darker on observations with a microscope inverted contrast phase (Zanoni et al., 2016). In this study, the dark zone was found in spheroids since the 2nd day of incubation. However, it is still seemed as a small spot. The dark zones expanded from day 3 and almost cover all spheroids on day 5 (data not shown). From this result, we can conclude that iGL cell 3D culture with Hanging Drop method (25µL per drop) allows conducive microenvironment conditions until the 2nd day of incubation. The result relatively consistent with the data of cell viability. The viability of spheroid cells decreased intensely in 3D culture from day 3 of incubation compared to 2D monolayer culture. This result is consistent with a common fact about the increase of incubation time results in decreased nutrients and increased toxic metabolites for cells (Ryu et al., 2019). That was one of the disadvantages of Hanging Drop culture method is the difficulty of the medium replacement process (Bresciani et al., 2019).

Despites of the good results, there are some limitations of this study. First, iGL cells need a special medium and reagents that are relatively more expensive than widely used basic medium. Researchers still unable to maximize the potential of iGL cells which insulin secretion can be detected directly from the fluorescence under the fluorescent microscope. Therefore, further research is required to explore the potential of iGL cells as an in-vitro model to study DMT2 especially in terms of cellular mechanisms of insulin secretion.

In conclusion, we found that the initial cell density and time of incubation have a significant effect on spheroid morphology and cell viability. Spheroid derived from iGL cell line with a stable morphology and good viability could be obtained from a cell concentration of 50-100 cells/µL with two days incubation.

Conflict of interest

There is no conflict of interests in this research.

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Reverse shoulder arthroplasty-early results

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Abstract

Although reverse shoulder arthroplasty is used in massive rotator cuff tears, it is also used in proximal humerus fractures and shoulder arthroplasty revision. In this study, we aimed to examine the early radiological and clinical results of patients undergoing reverse shoulder arthroplasty with different diagnoses. Between 2010 and 2013, reverse shoulder arthroplasty was applied to 10 patients (4 men, 6 women) with the diagnosis of arthropathy due to rotator cuff tear (n: 7), multi-fragmentary proximal humerus fracture (n: 2) and hemiarthroplasty revision (n: 1). The median age of the patients was 74 (64-85) years and the median follow-up was 15.5 (3-35) months. Patients were assessed in terms of joint range of motion, Constant score and Visual Pain Scale (VAS) and radiological examinations before and after surgery. Preoperative active shoulder flexion, abduction, internal and external rotation degrees of the patients were 65, 30, 40 and 50 degrees, and 105, 95, 30, 57.5 degrees respectively in their final controls. Constant score was 20.5 (14-63) preoperatively, and 54.5 (38-64) in the final controls ($p<0.05$). While the preoperative VAS was 7 (3-9), it was found to be 1.5 (1-3) in the final controls ($p<0.05$). As a complication, scapular notching was observed in one patient. Internal external rotation values were better in the rotator cuff arthropathy group than the fracture group. Reverse shoulder arthroplasty, especially in the treatment of patients with rotator cuff arthropathy, has good early results and a low complication rate. We recommend paying attention to the glenoid component location to avoid scapular notching.

Keywords: proximal humerus fracture, rotator cuff arthropathy, scapular notching, reverse shoulder arthroplasty

1. Introduction

Reverse shoulder arthroplasty (RSA), first designed by Paul Grammont in France in the 1980s, extends the moment arm of the deltoid muscle by shifting the shoulder rotation center medially and inferiorly. Thus, active forward flexion and abduction movement of the shoulder is provided with deltoid muscle strength (Grammont and Baulot, 1993). It has a more stable prosthesis design than total and partial shoulder prostheses (Matsen et al., 2007). Although RSA are used in massive rotator cuff tear arthropathy, it has also been found to be used in failed shoulder arthroplasty or internal fixation revisions, shoulder involvement of rheumatological diseases, tumor reconstruction, multi-fragmentary proximal humerus fractures (Boileau et al., 2006; Guery et al., 2006; Matsen et al., 2007; Levy and Badman, 2011; Melis et al., 2012; Shafritz and Flieger, 2012; Schwarz et al., 2021). In this study, we aimed to report the clinical and radiological results of RSA that have been performed with different diagnoses.

2. Materials and Methods

Between 2010 and 2013, a total of 10 patients, four males and six females, underwent shoulder replacement surgeries with RSA. The diagnoses were arthropathy due to massive rotator cuff tear in seven patients, multi-fragmentary proximal humerus fracture in two patients, and hemiarthroplasty revision in one patient. The indications for surgical treatment in patients diagnosed with arthropathy due to massive rotator cuff tear were despite conservative treatment for at least six months to continue the complaint, determining irreparable massive rotator cuff tears in magnetic resonance imaging (MRI) and accompanying pseudo-paralytic shoulder to tears. The pseudo-paralytic shoulder was defined as anterior flexion less than 60 degrees accompanying anterior and superior instability (Shafritz and Flieger, 2012). Preoperative shoulder range of motion was measured with the help of a goniometer. In the preoperative evaluation, each patient underwent standard anterior-posterior and axillary shoulder radiography, MRI in addition to patients with rotator cuff arthropathy, and computed tomography (CT) examinations in addition to those with a proximal humeral fracture. The right shoulder of five patients and the left shoulder of five patients were operated.

In the surgical technique, the deltopectoral approach was used in all patients and the tenotomy was applied to the subscapularis tendon. The midpoint of the glenoid was found,

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and a Kirshner wire was sent from the inferior of this point with 15 degrees inferior, parallel to the scapular body. The glenoid was prepared by removing only superficial subchondral bone using a reamer. The baseplate was fixed to the scapula with at least two screws without using cement. Humerus diaphysis was reamed with suitable reamers and metaphysis was prepared with rasps. The humeral stem was placed with cement in an appropriate degree at 10 degrees retroversion. The glenoid sphere was fixed to the baseplate with a screw. A polyethylene insert of appropriate size was placed after the trials in the humeral system. The joint was reduced, and stability control was checked. In patients operated with the diagnosis of proximal humerus fracture, tuberculum majus, and tuberculum minus sutured anatomically with the help of the sutures (Fig. 1).

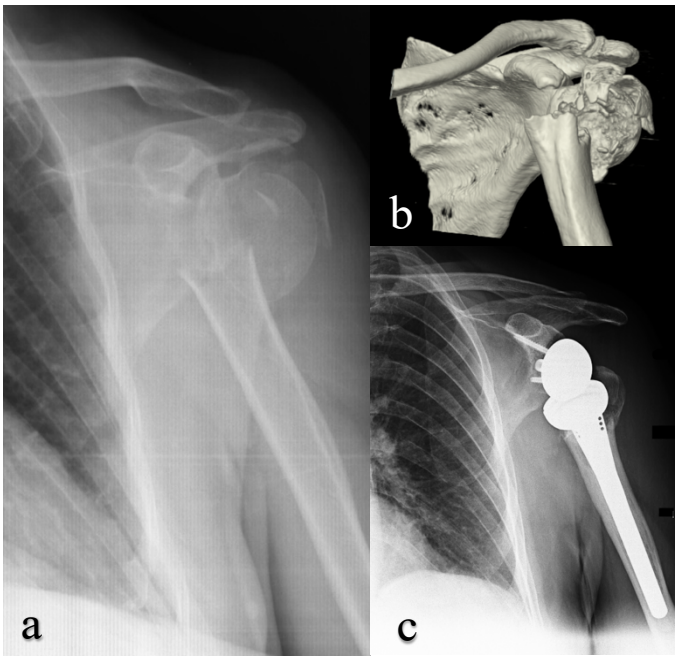

Fig. 1. Pre-operative anterior posterior shoulder radiography (a) three-dimensional reconstruction computerized tomography image (b) and postoperative 4th month control anterior posterior radiography (c) with the diagnosis of multi-fragmentary proximal humeral fracture

Shoulder arm sling was applied in the first six weeks after the operation and passive joint range of motion exercises were started in the early period. Active exercises were started after the sixth week, and muscle-strengthening exercises were performed after 12 weeks.

Patients were evaluated with joint range of motion, Constant and visual pain scale (VAS) scores and direct radiography, in their last controls. Constant score includes pain, degree of activity, and shoulder movements. The median age of the patients was 74 (64-85) years and the median follow-up was 15.5 (3-35) months.

The results are stated as median (minimum-maximum). SPSS for Windows v.16.0 (SPSS Inc., Chicago, IL, USA) program was used to evaluate the data. Wilcoxon test was used for statistical evaluation of preoperative and postoperative data. Statistical significance level was accepted as $p < 0.05$.

3. Results

Preoperative active shoulder flexion, abduction, internal and external rotation degrees were 65, 30, 40 and 50 degrees, respectively, post-operatively they were measured as 105, 95, 30, 57.5 degrees, respectively (Fig. 2, Tables 1, 2). Constant score was 20.5 (14-63) preoperatively, and 54.5 (38-64) in the final controls ($p<0.05$). While the preoperative VAS was 7 (3-9), it was found to be 1.5 (1-3) in the final controls ($p<0.05$) (Table 2). As complications, none of the patients had hematoma, infection, instability, acromial insufficiency, glenoid and humeral component loosening. Scapular notching was observed in one patient. In this patient, radiologically notching had not affected the lateral edge of the scapula and reached the lower screw (Fig. 3). Scapular notching was classified as grade 1 according to the Sirveaux classification (Sirveaux et al., 2004). In this patient who was followed up conservatively with physical therapy, it was found that the range of motion was not affected despite the scapular notching.

4. Discussion

RSA is not a very new concept and was found as an alternative to other shoulder prostheses in the 1970s. However, due to the lateralization of the shoulder joint rotation center in early designs, the load on the glenoid increased, and early loosening occurred (Ramirez et al., 2012). In 1987, Paul Grammont created a new prosthesis concept, and in his biomechanical studies, it was shown that medialization of the rotation center by 10 mm would increase the deltoid abduction moment by 20%, and the rotation center by 10 mm inferior would increase the deltoid abduction moment by 30% (Baulot et al., 2011; Ramirez et al., 2012). Although the main indication of the reverse shoulder prosthesis is massive rotator cuff tear arthropathy, its indications have widened over time, it has found use in failed shoulder arthroplasty or internal fixation revisions, shoulder involvement of rheumatological diseases, tumor reconstructions, multi-fragmentary proximal humerus fractures (Boileau et al., 2006; Guery et al., 2006; Matsen et al., 2007; Levy and Badman, 2011; Melis et al., 2012; Shafritz and Flieger, 2012 Schwarz et al., 2021).

In our study, the etiologic diagnosis of patients was rotator cuff tear arthropathy, multi-fragmentary proximal humerus fracture, and hemiarthroplasty revision. When the results of our study were evaluated, shoulder abduction and flexion increased in the rotator cuff tear group patients. It was observed that there was no significant change in internal and external rotation values in patients operated with a diagnosis of fracture and revision. Although the number of patients was low for statistical comparison, we found the best functional outcome in the rotator cuff tear group. The functional outcome of RSA for the rotator cuff tear arthropathy has been reported better than revision arthroplasty and posttraumatic arthritis (Boileau et al., 2006; Wall et al., 2007). However, some authors reported similar functional outcomes of RSA for rotator cuff tear

arthropathy and proximal humerus fracture with lower satisfaction in patients with an acute fracture (Sebastia-Forcada et al., 2020). The determinant of internal and external rotation in RSA applied in fracture etiology depends on the anatomical restoration and healing of tuberculum majus and minus (Levy and Badman, 2011; Gunst et al., 2021).

Table 1. Demographic information and study data of patients

Patient	Age	Gender	Diagnosis	Follow-up (month)	Preoperative					Last control				
					Flexion*	Abduction*	External rotation*	Constant score	VAS	Flexion*	Abduction*	External rotation*	Constant score	VAS
1	65	F	CTA	11	45	20	60	14	8	170	110	75	59	1
2	73	M	CTA	8	50	30	50	27	7	165	100	80	64	1
3	83	F	CTA	10	150	140	70	63	3	70	45	90	49	1
4	80	M	PHF	6	-	-	-	-	-	90	90	10	38	1
5	85	F	PHF	3	-	-	-	-	-	80	100	10	39	3
6	64	F	CTA	31	40	30	55	16	9	145	100	80	63	1
7	68	M	HR	20	70	60	20	19	6	100	90	30	46	2
8	78	F	CTA	25	80	20	40	22	8	130	100	70	52	2
9	72	F	CTA	35	60	30	40	17	6	100	80	45	62	3
10	75	M	CTA	34	70	20	50	27	7	110	70	45	57	2

CTA; Cuff Tear Arthropathy**,** PHF; Proximal humerus fracture, HR; Hemiarthroplasty revision, *; Degree, F, Female, M; male

Fig. 2. Sixty-five-year-old patient with rotator cuff arthropathy and a reverse shoulder prosthesis, functional images of the 6th month after surgery

Although the transacromial approach used by Grammont is less popular now, deltopectoral and anterosuperior approaches are the most used for RSA. Both approaches have advantages and disadvantages. The deltopectoral approach allows an easier approach to the glenoid lower pole and proximal humerus inferior, with the preservation of the deltoid muscle and active external rotation. With this approach, it helps to better position the glenoid component by reducing inferior compression and loosening. However, the disruption of the integrity of the subscapularis tendon is the risk of developing instability with wide capsular relaxation and the disability of the posterior cuff muscles and access to the glenoid (Ladermann et al., 2011).

The anterosuperior approach provides advantages such as simple and easy preparation of the humerus, easier approach to the glenoid, and preservation of the integrity of the subscapularis muscle. With this approach, the weakening of the anterior deltoid and the difficulty of the glenoid component placement are its disadvantages (Mole et al., 2011). The functional superiority of both approaches has not been reported (Ladermann et al., 2011). In all of our patients, we applied RSA with a deltopectoral approach.

Table 2. Comparison of patients' preoperative and final control data

	Preoperative	Last control	P*
Active flexion (°)	65 (40-150)	105(70-170)	≥0.05
Active abduction (°)	30 (20-140)	95 (45-110)	≥0.05
Internal rotation (°)	40 (20-90)	30 (10-90)	≥0.05
External rotation (°)	50 (20-70)	57.5 (10-90)	<0.05
Constant score	20.5 (14-63)	54.5 (38-64)	<0.05
VAS	7 (3-9)	1.5 (1-3)	<0.05

*Wilcoxon test

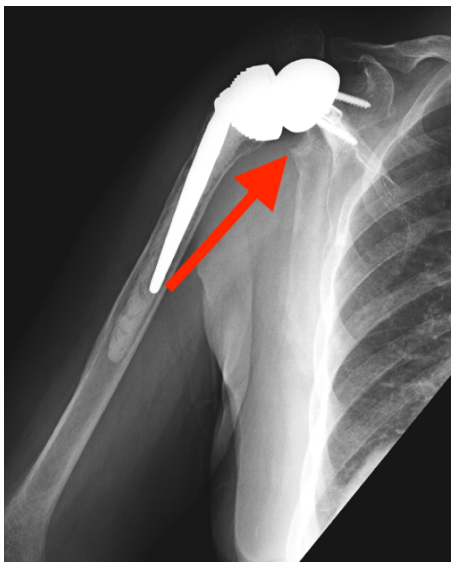

Fig. 3. 25-month control anteroposterior radiograph of a 78-year-old patient with an RSA for rotator cuff arthropathy. Scapular notching is indicated by the arrow

Complications of RSA have been reported as glenoid and humeral component loosening, dislocation, infection, scapular notching, periprosthetic fracture, neurological problems, acromion fracture, hematoma (Sirveaux et al., 2004; Werner et al., 2005; Boileau et al., 2006; Wall et al., 2007; Boileau et al., 2009; Cheung et al., 2011). Complication rates were higher in the RSA series performed as revision surgery (Wall et al., 2007). Dislocation has been reported as the most common complication (Cheung et al., 2011). In different studies, the incidence of scapular notching has been reported to be 44-96% (Vanhove and Beugnies, 2004; Werner et al., 2005; Simovitch et al., 2007; Wall et al., 2007; Levigne et al., 2008; Cazeneuve and Cristofari, 2010). Scapular notching was reported to be progressive and follow-up time and frequency were in direct proportion (Levigne et al., 2008). Different opinions have been reported on the clinical effect of scapular notching. Some authors report that notching does not affect shoulder range of motion or functional scores, but some report it as an unsuccessful result and independent risk factor for glenoid component loosening (Vanhove and Beugnies, 2004; Simovitch et al., 2007; Cazeneuve and Cristofari, 2010; Levigne et al., 2011). Cazeneuve and Cristofari (2010) observed a series of 36 patients with a RSA due to the proximal humerus fracture, with an average of 6.6 years follow-up in 23 patients with glenoid component loosening and 19 patients with scapular notching. Levigne et al. (2008) reported that notching was associated with preoperative rotator cuff arthropathy, but there was no relationship between the degree of notching and range of motion and Constant score. To prevent scapular notching, it is recommended to use eccentric gleno-sphere, to place the glenoid component downward and not to give superior tilt (Levigne et al., 2008; Levigne et al., 2011; Mizuno et al., 2012). However, there are authors who reported that placement of the glenoid component with the inferior tilt position does not decrease the rate of scapular notching (Edwards et al., 2012). The most effective methods of preventing inferior scapular notching were reported to use deltopectoral approach, the use of lateralized large-scale glenoid spheres and the insertion of components according to the patient's natural retroversion (Berhouet et al., 2014; Friedman et al., 2019). In patients in our study, we placed the glenoid sphere component inferiorly, preventing contact between the humeral component and the glenoid inferior. However, we found grade 1 scapular notching in one patient due to the superior tilt position of the glenoid. We did not find any complications in any of the patients except this patient.

The limitations of our study are that the patients with different diagnoses and the total number of patients are low and our follow-up time is short.

In conclusion, early results are good and complication rate is low in the treatment of RSA especially in patients with rotator cuff arthropathy. We recommend paying attention to the glenoid component location to avoid scapular notching. We continue our patients' follow-up to evaluate complications and functional results in the long term.

Conflict of interest

None to declare.

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Evaluation of the association between bilateral calcaneal fractures and suicide attempts: Findings from 4 different trauma centers in Turkey

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Abstract

In this study we aimed to determine the association between bilateral calcaneal fractures and suicide attempt and some socio-demographic characteristics. This study was conducted among patients admitted to the emergency departments of four trauma centers with bilateral calcaneal fractures between January 2014 and March 2017. The Sociodemographic Data Form, Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV) and Axis I Disorders (SCID-I) were applied to participants. Comparisons about age and gender was made between suicidal and non-suicidal patients with bilateral calcaneal fractures. Twenty-three patients with bilateral calcaneus fractures were included in the study. Of the 23 patients with bilateral calcaneal fractures, 14 (61%) were evaluated as suicidal and nine (39%) were evaluated as not suicidal. The majority of suicidal jumpers were female (n = 11, 78.6%). Suicidal patients were younger compared with non-suicidal fallers. As a result of SCID I interview, nine (64.3%) of 14 suicidal jumpers were diagnosed with serious psychiatric diagnoses: One patient was diagnosed with first episode psychotic disorder, one with schizophrenia, one with Schizoaffective Disorder, one with Bipolar Disorder, two with Major Depressive Disorder and three with Adjustment Disorder. Based on our study results we determined that suicidal jumpers are frequently diagnosed with serious psychiatric disorders. Therefore, the psychiatric dimension should be taken into consideration in the treatment plan for patients with bilateral calcaneal fractures and the multidisciplinary approach should be taken.

Keywords: bilateral calcaneus fractures, suicide attempts, suicidal high falls, psychiatric disorders

1. Introduction

Suicide usually occurs as a result of feeling hopeless due to psychiatric disorder that has been diagnosed or has not yet been diagnosed before the attempt. It is estimated that 1 million people lose their lives every year due to suicide and this number is expected to reach 1.5 million by 2020 (Wasserman et al., 2012). Suicide is ranked 10th among the causes of death (American Foundation for Suicide Prevention (AFSP). Suicide statistics., 2018). Thus, prevention of suicide is an important public health problem (Weber et al., 2017).

A fall from a height is a significant cause of morbidity and mortality (Reynolds et al., 1971). The trauma that occurs as a result of a fall or jump from a height is a good example of high-energy blunt trauma, resulting in injuries that have been shown in various previous studies (Katz et al., 1988; Richter et al., 1996; Teh et al., 2003). The majority of falls from height are accidental, while others are the result of suicide (Katz et al., 1988). Jumping from a height constitutes 3 to 15% of the average 140000 suicide attempts per year in the United Kingdom (Gore-Jones and O'Callaghan 2012; Rocos et al., 2015).

The height of the jumping place, the properties of the floor, as well as the characteristics of the body area that falls on it, determine the severity and level of the fractures (Beale et al., 2000; Rocos et al., 2015; Wirbel et al., 1998). For example, the patients falling on their legs have more serious injuries than those of falling on their left or right sides (Katz et al., 1988). Also, limbs are the most vulnerable body areas to severe injuries because of jumping. Calcaneal fractures occur due to the axial loading associated with high-energy trauma, most often with a fall from a height onto the heels, and less often in traffic accidents within a vehicle (Daftary et al., 2005; Mcbride and Laing et al., 2005; Mitchell et al., 2009). A higher rate of calcaneal fractures has been reported in patients jumping from a height and intending to commit suicide compared with

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patients who have fallen from a height (The et al., 2003). In addition, the fractures of limbs tend to be more bilateral in patients who jump from height than those of fall from height (Rocos et al., 2015). Hahn et al. (1995) reported that the majority of limb fractures in jumping from height were calcaneus (65%) and ankle fractures (27%). Also, Li and Smialek (1994) revealed 28% rate of limb fracture in jumpers, while Katz et al. (1988) reported that lower extremity fractures were the most commonly reported skeletal injuries in suicidal jumpers. In another study, it was reported that tarsal, metatarsal and calcaneus fractures were detected in 18 of 30 suicidal jumpers (Wirbel et al., 1998).

Unlike other methods like poisoning, hanging or firearms, jumping from a height does not require any equipment and it can cause serious injuries and mortality in the survivors (Wirbel et al., 1998; Beale et al., 2000). Another important issue is that 19-97% of people who attempt suicide by jumping from height have a psychiatric diagnosis and that the most common diagnosis is schizophrenia. These patients usually have more serious mental health problems than those who attempted suicide by using other methods (Richter et al., 1996; de Moore and Robertson, 1999; Nielssen et al., 2010; Gore-Jones and O'Callaghan 2012; Rocos et al., 2015). This necessitates the cooperation with psychiatry in this patient group and multidisciplinary approach in the treatment and follow-up of patients.

Based on a scan of the literature, we did not find any research specifically about the relationship between bilateral calcaneal fractures and suicide attempt. Therefore, we aimed to investigate the relationship between bilateral calcaneal fractures and suicide attempt to identify the psychiatric diagnoses and to provide the appropriate psychiatric support during the early treatment period in these patients.

2. Materials and methods

This study was conducted on four hospitals functioning as trauma centers between January 2014 and March 2017 (Table 1). The study was approved by a local University Ethics Committee for Clinical Trials. A written informed consent was obtained from each patient. The study was conducted in accordance with the principles of the Declaration of Helsinki. Patients who died prior to admission were excluded. Blood toxicology reports were not sought in any cases as these are not routinely performed unless criminality is suspected.

This research included patients>16-year-old who presented to the emergency department with calcaneal fractures. The patients were first evaluated by the emergency department physicians with respect to their trauma localizations. The first step in the assessment was to obtain radiological findings and then if necessary, the relevant consultations were made.

After the patient was clinically stabilized prior the surgical treatment via analgesic and if necessary anesthetic drug, first evaluation for suicidal attempt or suicidal thought was made by an orthopedic surgeon through a semi-structured clinical interview. All the orthopedists in this study were trained about asking and managing about suicide in order to make the first evaluation of the patient by a psychiatrist experienced in psychological trauma. In this initial evaluation if it was learned that the fractures were as a result of suicidal jumping from height, psychiatric consultation was made before taking the patients to the orthopedics clinics. During the hospitalization in orthopedics clinics, a psychiatric consultation was requested again to provide the appropriate psychiatric support and treatment. When any psychiatric diagnosis was made, medical treatment was performed considering the interaction of analgesic and anesthetic drugs. Patients who are at risk of a second suicide attempt are frequently observed, carefully monitored, kept away from potentially dangerous means that can be used to self-harm, and detailed information has been provided to their relatives. Patients with a major psychiatric diagnosis and/or ongoing suicide attempt plan or thought were taken over to the psychiatry inpatient clinics.

2.1. Instruments

Sociodemographic data form: In this form, the participants were asked about personal information such as age, gender and any other fracures.

Structured clinical interview for diagnostic and statistical manual of mental disorders-fourth edition (DSM-IV) (SCID-I): This is a semi-structured clinical interview developed for use of mental health professionals (Bell, 1994). Apart from clinical diagnosis, family history, age of onset of disease, presence of stressful life events can be evaluated (Spitzer et al., 1996). The reliability and validity of the Turkish version is available (Çorapçıoğlu et al., 1999). The SCID-I allows to diagnose both present and life-time Axis I mental disorders. In this study, we used modules for depression and anxiety disorders only and focused on the current diagnoses.

2.2. Statistical Analysis

The data obtained in this study were recorded and analyzed using SPSS version 20.0 software for Windows. The numerical variables were stated as the mean ± standard deviation (SD) and the categorical variables were reported as a number (n) and percentage (%). A frequency analysis was also applied. A Welch's t test was used for the comparisons between the groups and a proportion test was used for the intragroup comparisons. A p value of < 0.05 was considered statistically significant.

3. Results

From a total of 239 patients who were admitted to four hospitals with calcaneal fractures between January 2014 and March 2017, 216 cases of unilateral fractures were excluded. Overall, this study included a total of 23 patients with bilateral calcaneal fractures. Number of patients with calcaneal fractures according to each hospital are presented at Table 1.

Table 1. Distribution of patients according to four different trauma centers

Hospital	Number of patients with bilateral calcaneus fractures (n)	Number of patients with unilateral calcaneus fracture (n)	Number of total calcaneus fractures (n)
Hospital 1	7	55	62
Hospital 2	6	82	88
Hospital 3	5	51	56
Hospital 4	5	28	33
Total	23	216	239

In 14 (61%) of 23 patients with bilateral calcaneal fractures, it was learned that the fractures occurred as a result of suicidal jump from height, while in nine patients (39%) the fractures were as a result of non-suicidal falls. No statistically significant difference was found between the number of patients with non-suicidal falls and suicidal jumpers (p = 0.404). The suicidal patient group was comprised of 11 (78.6%) females and 3 (21.4%) males, and the gender difference was determined to be statistically significant (proportion test, p <0.001) (Table 2). Non-suicidal faller group was comprised of 1 (11.1%) female and 8 (88.9%) males. There was a statistically significant difference between gender of non-suicidal fallers (proportion test, p <0.001, Table 2).

Table 2. Gender distribution

			N (%)	p
Gender	Non-suicidal fall from height	Female	1(11.1)	p<0.001
		Male	8(88.9)	
	Suicidal jump from height	Female	11(78.6)	p<0.001
		Male	3(21.4)	
	Total	Female	11(47.8)	
		Male	12(522)	

proportion test; p significant at .05 level

The average age of the patient group with bilateral calcaneal fractures was 33.2 (range 17-52) years. The average age of the suicidal jumpers was 28.2 years (range 17-42) years while the average age of the non-suicidal fallers was 41 (range 27-52) years. There was a statistically significant difference between the age of suicidal jumpers and non-suicidal fallers (Welch's t test, p<0.001, Table 3).

No statistically significant difference was determined between the number of patients who stated that they were suicidal during the first interview by orthopedists in the emergency department (n=6, 42.9%) and those who denied attempting suicide during the first interview and admitted to it later during the psychiatric interview (n=8, 57.1%) (proportion test, p=0.418). When the male attempted suicide cases were examined, it was seen that during the first interview by orthopedic surgeons in the emergency department, all 3 had denied the suicidal attempt. The suicidal attempt was later revealed by psychiatric interviews. Of the 11 female patients, only 5 (45.5%) had denied the suicidal attempt during the first interview. There was a statistically significant difference between the genders in terms of denying (Welch's t test, p<0.001). The mean time to a request a psychiatric consultation for those patients who had not reported a suicide attempt during the first interview was 4 days with the range of 2 and 7 days.

Table 3. Data regarding mean age of patients with suicidal and non-suicidal patients

Age	Non-suicidal fall from height	Suicidal jump from height	Total	p
Mean age	41	28.2	33.2	<0.001
Range of age	27-52	17-42	17-52	<0.001

Welch's t test; p significant at .05 level

As a result of SCID I psychiatric interview, 9 (64.3%) of 14 suicidal jumpers were diagnosed with serious psychiatric diagnoses while five (35.7) were not diagnosed with any psychiatric diagnoses. Three of 14 suicidal patients were diagnosed with psychosis according to SCID-I interview. One of these three psychotic patients was diagnosed with first episode psychosis (FEP). The patient who was diagnosed with FEP during our study was also the male patient who denied the suicidal attempt during the first interview and attempted suicide second time during hospitalization. During the psychiatric SCID-I interview, it was learned that he had auditory hallucinations ordering him to jump from a height. One of these psychotic patients was a female patient with a diagnosis of Schizoaffective Disorder who was followed up with olanzapine 20 mg /day, risperidone 6 mg / day and valproate 1000 mg /day. Another patient was a schizophrenic male patient who was diagnosed 3 years ago.

Table 4. Comparison of patients in terms of concomitant fractures

Injury	Non-suicidal fall from height N (%)	Suicidal jump from height N (%)	p
Isolated bilateral calcaneal fracture	8(57)	1(11)	<0.001
Bilateral calcaneal and concomittant other fractures	6(43)	8(89)	<0.001

Welch's t test; p significant at .05 level

Three of 14 suicidal patients were diagnosed with Affective Disorder while one had Bipolar Disorder and two had Major Depressive Disorder (MDD). Also three of 14 suicidal patients were diagnosed with Adjustment Disorder because of serious stressors. While nine of 14 suicidal patients were diagnosed with any psychiatric disorder, five were not diagnosed with any psychiatric disorder. It was learned that 2 of 5 patients who had no major psychopathology were receiving pregabalin treatment for severe pain due to diabetic neuropathy for many years. A statistically significant difference was determined between the suicidal (42.8%) and non-suicidal (89%) groups with respect to the rate of fractures other than the bilateral calcaneal fractures (Welch's t test, p<0.001, Table 4).

4. Discussion

Searching the relevant studies investigating the relationship between jumping from height and suicide, we determined that almost all of the studies had been designed to investigate the patterns of injuries of the jumpers (Katz et al., 1988; Rocos and Chesser, 2016; Wirbel et al., 1998). We did not find any study investigating the prevalence of suicide in patients with a specific fracture such as bilateral calcaneal fractures.

In the current study, of the 23 patients with bilateral calcaneal fractures, 14 (61%) were found to be suicidal and 9 (39%) were not. Although no statistically significant difference was found, the rate of suicide attempt in patients with bilateral calcaneal fractures was found to be higher. Our results emphasize the importance of considering the suicide attempt as an important etiological factor in patients who are admitted to trauma centers with bilateral calcaneal fractures due to jumping from height. We also obtained important results in terms of underlining the necessity of multidisciplinary approach to patients with bilateral calcaneus fractures. Describing the sociodemographic characteristics of patients who attempted suicide by jumping from height has become of the subject of various studies (Li et al., 1994; Gore-Jones and O'Callaghan 2012; Rocos et al., 2015). Unfortunately, there have been conflicting results with regard to gender in this area (Li et al., 1994; Värnik et al., 2008; Värnik et al., 2009). The results of our study showed that majority (78.6%) of the suicidal jumpers were female. While this result is consistent with some studies (Värnik et al., 2008; Värnik et al., 2009; de Pourtalès et al., 2010), it is different from the findings of studies in which males are more present (Bennewith et al., 2007; Kontaxakis et al., 1988; Li et al., 1994). Similar with our results, in a study conducted by Katz et al. (1988) it was reported that 18 of 28 suicidal jumpers were female. In contrast to our results, in a study conducted by Kontaxakis et al. (1988) it was reported that suicide attempt by jumping from height was higher in males and in advanced ages. The difference between studies may be related to sociocultural and socioeconomical factors in the countries where the study has been conducted.

In our study we revealed that the suicidal jumpers were younger than the non-suicidal fallers. This is consistent with the results of many studies (Li et al., 1994; Gore-Jones and O'Callaghan 2012; Rocos et al., 2015). In some studies, conducted in USA and UK, this group was reported to be in the 25-30 age group (Li et al., 1994; Rocos et al., 2015). In a study conducted by Rocos et al. (2015) the medical records of the patients admitting to a regional trauma center due to jumping from height were evaluated retrospectively between 2012-2016 and the average age of the patients was found to be 32. Similar with our results Li and Smialek (1994) reported that the majority of those who attempted suicide by jumping from height were in the 20-30 age group. Our study emphasizes the necessity of questioning the suicidal attempt in patients with bilateral calcaneal fractures, especially in younger patients.

It is important to know the patterns of injury specific to jumps during the evaluation of these patients, although there is no diagnostic fracture for jumpers (Katz et al., 1988; Teh et al., 2003; Rocos et al., 2015). Rocos et al. (2015) revealed that 13 of 41 patients with the history of jumping from height, have been reported to have calcaneal fractures and the possibility of calcaneus fracture has been reported as 0.32. In another study, conducted to determine the pattern of injuries in 28 suicidal jumpres, researchers revealed calcaneal fractures in 12 of these patients (Katz et al., 1988). Teh et al. (2003) reported that calcaneal fractures were seen more frequently in suicidal jumpers (31%) compared to accidentally fallers (5%). Also, the rate of isolated injuries was found to be higher in suicidal jumpers compared with accidental fallers consistent with our results (Richter et al., 1996; Teh et al., 2003).

Patients attempting suicide by jumping from a height, were frequently diagnosed with serious psychiatric disorders like schizophrenia (Katz et al., 1988; Kontaxakis et al., 1988; Hahn et al., 1995; Rocos et al., 2015). Nine of four-teen patients in our study have been diagnosed with psychotic or affective disorder consistent with the literature (Katz et al., 1988; Rocos et al., 2015). In some case series it has been reported that psychotic disorders like schizophrenia, first episode psychosis are common in suicidal jumpers (Katz et al., 1988; Kontaxakis et al., 1988; Nielssen et al., 2010; Rocos et al., 2015). Although the diagnosis of psychosis can be made before or after the jump, psychosis is typically found to be associated with attempting to jump from height (Katz et al., 1988; Rocos et al., 2015). In addition, the first episode psychosis without having any psychiatric treatment was reported to be associated with suicide by jumping from height (Nielssen et al., 2010). In our study, one male patient was diagnosed with first episode psychosis and he did not have any history of psychiatric treatment. Also, this patient did not share his suicidal attempt in the first evaluation. The schizophrenic male patient denied the suicidal attempt during the first interview too. After the psychiatric interview, evaluating the medical records and interviewing with the relatives, it was learned that he was diagnosed with schizophrenia three years ago and he did not receive antipsychotics treatment because of delusions of persecution as thinking that doctors and his family were poisoning him with the drugs. The third female psychotic patient was diagnosed with Schizoaffective Disorder, one of the serious psychotic disorders. Although she did not deny the suicide attempt during the first interview, she had delusions of reference and severe depressive symptoms as a result of discontinuing the psychiatric treatment. These results emphasize the importance of collaboration with psychiatrists even if the patients deny the suicide. Thus, as a result of detailed psychiatric evaluation these patients should be identified and treated to allow the early mobilization of fractures and transfer to psychiatry department as soon as possible.

Also, in our study, one patient was diagnosed with bipolar disorder and two were diagnosed with MDD consistent with the literature (Kontaxakis et al., 1988). Although two patients were not diagnosed with major psychiatric diagnosis, these two patients had severe pain symptoms due to diabetic neuropathy and their pain could not be controlled despite the use of pregabalin. Kontaxakis et al. (1988) revealed that major psychiatric diagnoses, including psychotic depression and schizophrenia, and serious physical disorders were more frequent in the patients who attempted suicide by jumping from height compared with the patients who attempted suicide by taking overdose drugs.

Psychiatric symptoms are subjective distress for these patients and in some cases may impair or even prevent medical and surgical care. Psychiatric conditions, especially risk of suicide, should be evaluated and treated as early as possible during hospitalization. Treatment management requires both psychopharmacological and psychotherapeutic interventions. It should be directed towards symptomatic treatment and, if possible, remission of primary psychiatric disorder.

Psychiatric re-evaluation of the patients during orthopedic hospitalization is also important. Relevant studies reported that patients who had attempted suicide but did not share or denied it may tend to have second suicide attempt and this may end with death (Hawton and Fagg, 1988; Suokas et al., 2001). In our study, one of the patients who initially denied the suicide attempted suicide again during the hospitalization period. Small and Rosenbaum (1984) reported that suicide was more important problem among inpatients. In a study conducted in UK, jumping from height is defined as a more common method of suicide attempt among inpatients (Gunnell and Nowers, 1997). Also, this patient group is usually young, has long been social isolated with a history of traumatic events and suicide attempts (Gunnell et al., 1997; Suokas et al., 2001). Therefore, it is recommended that the history of suicide attempts and traumatic life events should be evaluated in detail in psychiatric interviews. In our study, the patient who had suicide attempt by a similar method, was diagnosed with first episode psychosis after psychiatric interview. In the detailed psychiatric re-evaluation of this patient, it was learned that he had auditory hallucinations telling him to kill himself and therefore, he had a social isolation over than one month.

Furthermore, the treatment of these patients in surgical services can be difficult because of the lack of experience of the nurses and health personnel in the management of these patients (Katz et al., 1988). The patient group included in our study was followed up with daily psychiatric evaluations during orthopedics hospitalization. An important role of psychiatric consultation is to help to manage the necessary orthopedic treatment and rehabilitation by increasing cooperation with the patients, reducing agitation and preventing anger outbursts.

Considering that this group of patients is frequently composed of working age groups in low- and middle-income countries, management of these patients is especially important because of the means of treating the social and economic results. In addition to determining the sociodemographic characteristics of these patients, it is also important to evaluate the type of injuries and psychiatric history and to question the previous suicide attempt and to provide appropriate psychiatric support in order to decrease the mental and social problems. Management of the patients with the history of suicide attempts by jumping from height can be achieved through multidisciplinary approaches (Rocos and Chesser, 2016). Further studies are needed to contribute to the literature in this area.

Conflict of interest

The author declares that there is no conflict of interest.

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The effect of sex and age on cortical grey and white matter volumes of frontal lobe

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Abstract

Grey matter (GM) of the frontal lobe carries out its complex function such as muscle control, speech, decision making, and self-control; while the white matter (WM) of frontal lobe is a major contributor to human brain enlargement and higher structural connectivity. This study was conducted to assess the effect of sex and age on grey and white matter volumes (GMV and WMV) of the frontal lobe and its gyri in healthy young adult Sudanese. The present study included 139 healthy Sudanese subjects (80 males and 59 females). Participant's ages were ranging between 20-40 years. T1-weighted MR brain images with thickness 1mm were obtained. MR images of the subjects were analyzed using the automatic segmentation software (BrainSuite). GMV and WMV of frontal lobe and its gyri were estimated using the output data of the process of software. Males had greater GMV and WMV of the frontal lobes (251.18±31.80 cm^3 and 143.48±22.92 cm^3, respectively) and most of frontal lobe gyri ($P<0.05$). Change with age in GMV has been reported in the frontal lobes, superior frontal gyrus, pars orbitalis, orbitofrontal, and cingulate gyrus ($P<0.05$). Change with age in WM volumes has been found in the cingulate, precentral, and paracentral gyri ($P<0.05$). Sex has prominent effect on GMV and WMV of the frontal lobes and most of frontal lobe gyri, thus sex is a major contributor to GMV and WMV differences between individuals. Age effect GMV and WMV of most frontal lobe gyri, these findings confirm the continuation of maturation of frontal lobe until fourth decade. Assessing effect of sex and age on GM and WM in healthy adult has major importance to distinguish the normal brain from diseased.

Keywords: grey matter volume, white matter volume, BrainSuite

1. Introduction

Frontal lobe occupies an exalted position in neuroscience and become one of the most important area of research in human brain due to its functional characteristics. Frontal lobe is responsible for planning and execution of the movement, formation of words, problem solving, decision making, cognitive flexibility, and working memory (Elaine N. Marieb, 2007; Welsh et al., 1991).

The frontal lobe parenchyma consists mainly of grey matter (GM) and white matter (WM), that differ in the structural and functional. GM consists mainly of nerve cell bodies; where almost all interactions between neurons take place and processes the complex functioning of the frontal lobe (Miller, 1980). WM composes mainly of nerve fibers (Snell, 2010); which proposes as a major contributor to human brain enlargement and higher structural connectivity (Schoenemann et al., 2005). On the basis of MR image, the brain tissue can be classified into GM, WM, and CSF (cerebro-spinal fluid). This classification can be done by using the manual segmentation, but it is hard, time consuming, and subjective (Kasiri et al., 2010). Therefore, there are strong demands to perform the reliable and accurate automated segmentation of brain MR images as a prerequisite for the comprehensive brain analysis (Kasiri et al., 2010). In this study, an automated segmentation software called Brain-Suite was used, it provides a comprehensive approach to extracting a surface multi representation of the cerebral cortex from T1-weighted MR images and segments and labels grey and white matter structures. Although, the effect of sex and age on grey matter has been widely studied; the results regarding young adults were not analyzed separately and mixed with other age groups (younger or older). Therefore, the variations observed could not be applied specifically to individuals in the second and third decade.

In the present study, the grey and white matter volumes of the frontal lobes and their gyri were measured from T1-weighted MR brain images by applying automatic segmentation software (Brain-Suite); to assess effect of sex and age in cortical grey and white matter volumes of the frontal lobe and its gyri in sample of 139 healthy young adult Sudanese ranging from age 20 to 40 years.

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2. Materials and methods

2.1. Subjects

The current study included 139 healthy young adult Sudanese subjects; age of participants was ranging between 20-40 years. Sexes were match in their age (age of males and females were 28±5.72 and 28±6.00 years, respectively) and body mass index (BMI) (BMI of males and females were 23.93±3.6 and 24.89±5.07kg/cm^2, respectively).

The participants were excluded if they are drug abuse; had head trauma, neurological diseases, psychiatric diseases, and congenital malformation related to the brain. The study was approved by the Ethical Committee of the National Ribat University.

2.2. Magnetic resonance imaging

Structural MRI was done in radiology department, Doctors' Clinic. MR imaging was performed on 1.5 Tesla Philips scanner, Version: 3.2.1. T1-weighted MR brain images obtained using three-dimensional acquisition by Magnetization Prepared Rapid Acquisition (MP-RAGE), which produces good grey/white matter contrast in coronal section, acquisition time (5 minutes and 18 seconds), slice distance is 1.0mm, the field of view is 250 read, 192 mm phase, TR=1657ms, TE=2.95ms, bandwidth 180Hz/pixel, flip angle 15°, ECHO spacing=7.5ms, phase resolution=100%, and slice resolution=50%.

2.3. MR images analysis

MR images of the subjects were analyzed using automatic segmentation software (BrainSuite 13a). BrainSuite is "a collection of software tools that enable largely automated processing of magnetic resonance images (MRI) of the human brain". BrainSuite software was performed on a Toshiba computer Core i3, 2.10 GHz, 6GB. The software analyzed each MR image in two stages: the first stage is cortical surface extraction sequence, which take 30 minutes to run; while the second stage two is long and last two hours and 15 minute, which is surface and volume registration.

2.4. Cortical surface extraction sequence (CSE)

The first step in the CSE is the skull stripping, which include removing of the skull and scalp from MR image. After completion of this stage, be sure that the edge detection line surrounded only the brain tissue, if not you have to adjust it by changing the diffusion iterations, diffusion constant, and edge constant from the skull stripping dialog. The next step is non-uniformity correction, which corrects the skull stripped MR image from shading artifacts. These shading artifacts can produced by imperfections in the field coils used in the systems. Then, tissue classification and cerebrum Labelling, during these steps, the software classifies the tissue into cerebrospinal fluid (CSF), grey and white matter; and labelling the brain volume into cerebrum, cerebellum, and brainstem. After that the BrainSuite running Cortex Masks Selection and topology correction that include extraction of the cerebral cortex and correction of any errors that may occurs during this process. The last stages in the cortical surface extraction include the generation of the pial surface and separation of the cortical surface into left and right hemispheres and displays them with different color (Fig. 1).

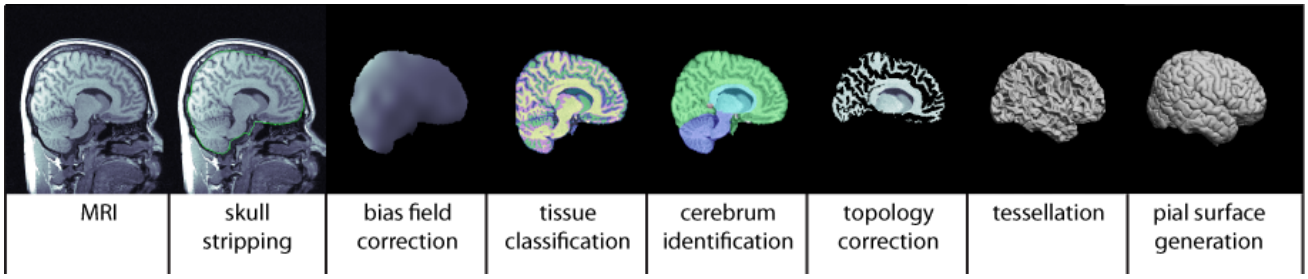

Fig. 1. Cortical surface extraction sequence

2.5. Surface and volume registration (SVReg)

Registering images to a common space also allows for automatic labeling of cortical and subcortical structures, allowing for analysis of ROIs to be automated. BrainSuite "comes with SVReg, a program that registers the surfaces and volumes generated by the Cortical Surface Extraction Sequence to a brain atlas manually labeled by an expert neuroanatomist". Stages of SVReg: Surface Smoothing and Coarse Alignment of Smoothed Surfaces, during these stages the software smoothing the cortical surfaces mesh and producing alignment of the cortex; then aligning these smoothed surfaces in 3D space. After that is atlas to subject registration, this stage needs for Curvature-Based Alignment, which is applying of sulcal curves and cortical labels from the atlas to the subject's cortical surface. The next steps are Refinement of the labels and sulcal curves and Harmonic Extension, the software performed volumetric spatial alignment of brains by extending the surface registration to the entire volume. Finally, is Elastic Deformation, the output of this process is a point correspondence between the two brain volumes based on the deformation required to map one to the other. Once the atlas volume is registered to the subject's volume, the atlas' warped label volume acts as a label volume for the subject .

The results of SVReg were labelled inner, pial and mid cortical surfaces of cerebrum and spread sheet of statistic include measurement of the cortical and subcortical structures (Fig. 2). BrainSuite automatically calculate cortical structures of gyri of the cerebral hemispheres. To calculate cortical structures of cerebral hemisphere the following formulas (1-5) were used in Microsoft excel worksheet.

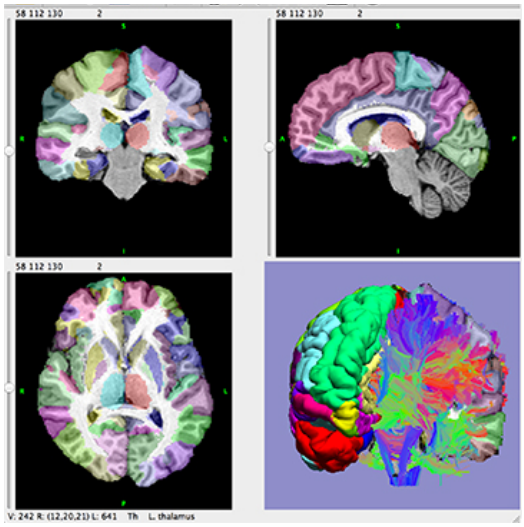

Fig. 2. MR image analyzed by BrainSuite

2.6. Statistical analysis

Data were analyzed using Statistical Package of Social Science (SPSS) version 21.0. Independent sample t. test was performed to compare mean values of grey and white matter between males and female. Bivariate correlation was used to evaluate the relationship of grey and white matter with age. P value equal or less than 0.05 was considered statistically different.

3. Results

3.1. Sex differences in grey matter volume (GMV)

Quantitative data of GMV of the frontal lobes and frontal lobe gyri are shown in Table 1. Males had greater GMV in the right, left, and total frontal lobes; all right frontal lobe gyri; and left and total middle frontal gyri, pars orbitalis, and cingulate gyri; the total precentral and transverse frontal gyri (P<0.05). Conversely, there were no differences between genders in GMV of the other frontal lobe gyri (P>0.05) (Table 1).

Table 1. Grey matter volumes of frontal lobes and frontal lobe gyri

Frontal lobe Gyri	Grey Matter Volume (cm^3)					
	Right		Left		Total	
	Males	Females	Males	Females	Males	Females
Frontal Lobe	132.04±15.9	117.23±11.73*	131.79±15.7	116.81±11.31*	263.82±31.60	234.05±22.95*
Superior Frontal Gyrus	38.54±4.96	34.10±3.72*	38.43±5.55	37.17±4.70	75.74±10.36	72.94±9.18
Middle Frontal Gyrus	19.08±3.70	16.74±2.96*	19.73±3.62	17.92±2.99*	38.20±7.34	35.50±5.43*
Pars Opercularis	4.99±0.90	4.46±0.89*	4.66±0.88	4.57±0.83	9.53±1.63	9.19±1.51
Pars Triangularis	9.11±2.17	7.96±1.68*	6.93±1.30	6.79±1.18	15.69±3.07	15.24±2.623
Pars Orbitalis	1.84±0.36	1.67±0.36*	2.50±0.35	2.30±0.34*	4.29±0.64	4.02±0.56*
Precentral Gyrus	14.79±2.38	13.59±1.70*	14.07±2.29	13.46±1.92	28.71±4.32	27.26±3.49*
Paracentral gyrus	4.78±0.77	4.15±0.56*	4.22±0.72	4.08±0.59	8.79±1.45	8.51±1.11
Transverse Frontal Gyrus	3.14±0.55	2.79±0.61*	2.73±0.55	2.56±0.51	5.85±1.02	5.39±0.90*
Cingulate Gyrus	15.81±2.24	13.88±1.76*	16.53±2.65	15.64±2.26*	31.82±4.91	30.23±4.25*
Subcallosal Area	0.32±0.08	0.29±0.07*	0.34±0.09	0.34±0.09	0.65±0.15	0.64±0.13
Orbito-Frontal Gyri	19.62±2.41	17.23±1.80*	17.65±2.40	16.97±2.19	36.51±4.82	35.23±4.43

*, p≤0.05

3.2. Sex differences in white matter volume

Quantitative data of WMV of frontal lobes and frontal lobe gyri are shown in table 2. Males had greater WMV in the right, left, and total frontal lobes; all right frontal lobe except pars orbitalis and subcallosal area; the left and total precentral gyri; the left middle frontal and cingulate gyri and pars orbitalis (P<0.05). Conversely, there were no differences between genders in WMV of the other frontal lobe gyri (p>0.05).

3.3. Correlation between age and grey matter volume

There was negative correlation between age and GMV of the right, left and total frontal lobes; the right superior frontal gyrus in males and females; the right pars orbitalis and orbitofrontal gyri in males; and the right cingulate gyrus in females (Figs. 4-10). However, there was no correlation between age and GMV of the right, left, and total pars opercularis, pars triangularis, middle frontal, precentral, paracentral, transverse frontal, and subcallosal area (P>0.05).

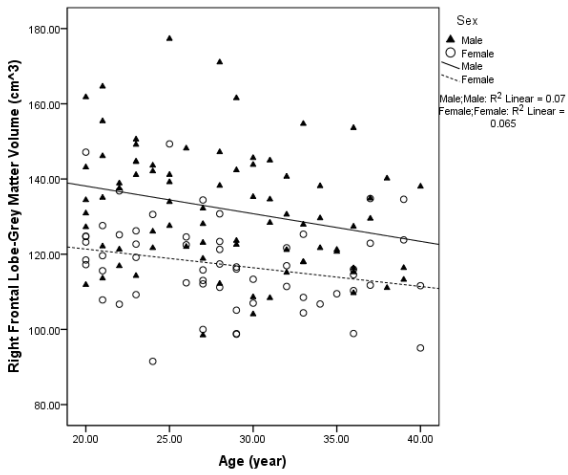

Fig. 3. Correlation between age and grey matter volume of right (RT) frontal lob

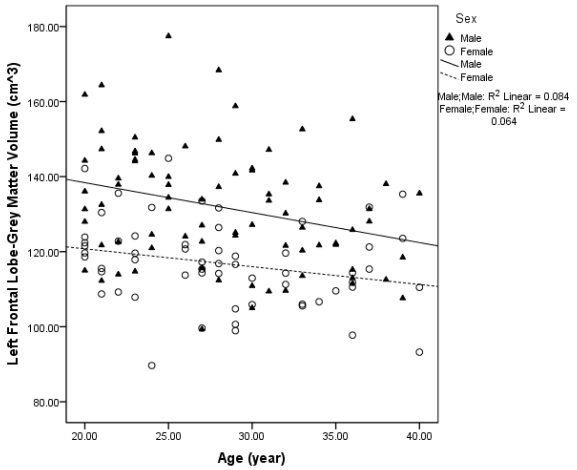

Fig. 4. Correlation between age and grey matter volume of left (LT) frontal lobe

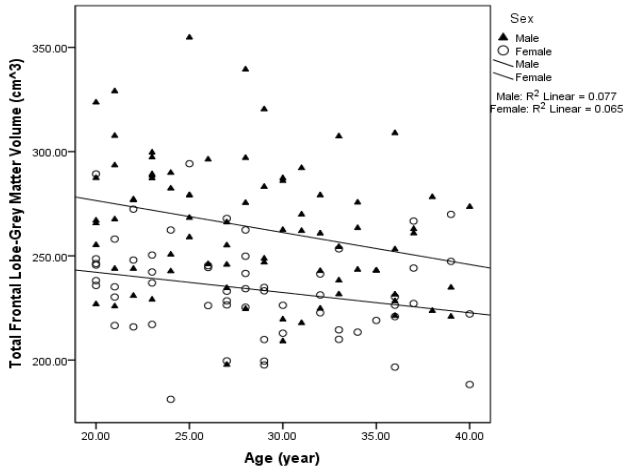

Fig. 5. Correlation between age and grey matter volume of total frontal lobe

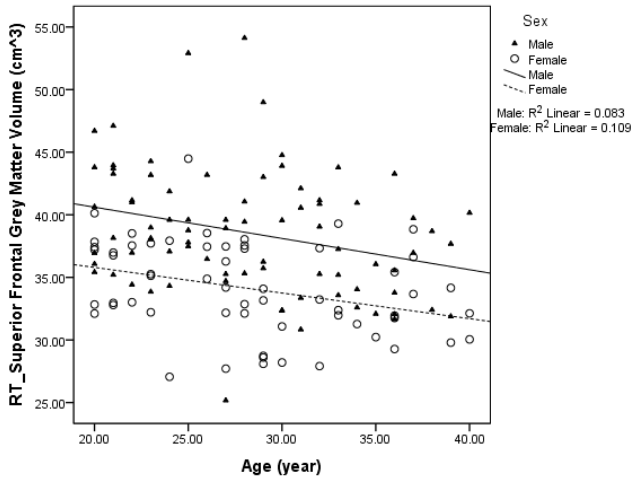

Fig. 6. Correlation between age and grey matter volume of right (RT) superior frontal gyrus

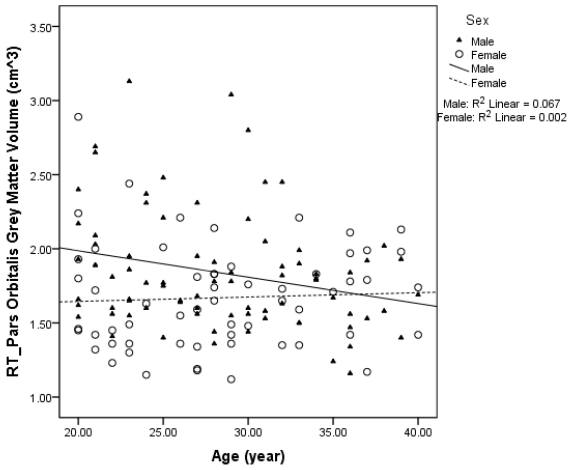

Fig. 7. Correlation between age and grey matter volume of right (RT) pars orbitalis

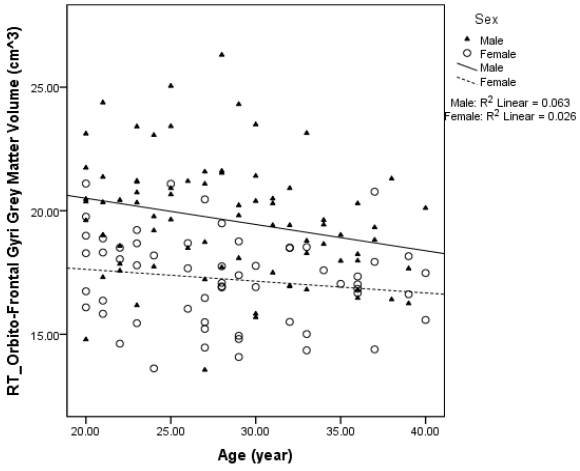

Fig. 8. Correlation between age and grey matter volume of right (RT) orbito-frontal gyri

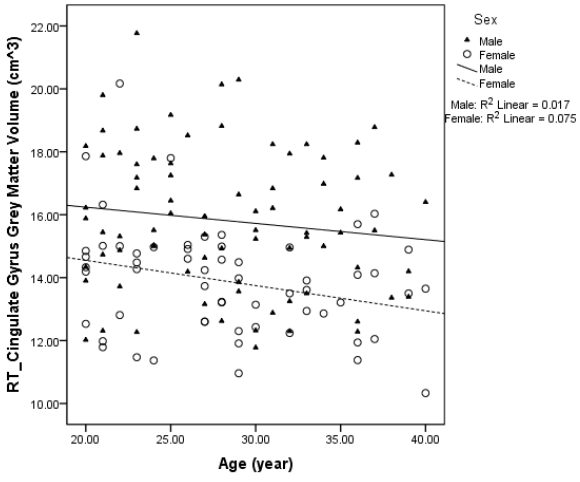

Fig. 9. Correlation between age and grey matter volume of right (RT) cingulate gyrus

3.4. Correlation between age and white matter volume

There was positive correlation between age and WMV of the left and total cingulate gyri in females and right paracentral gyrus in males; but a negative correlation between age and WMV of the total precentral gyri in males (P<0.05, Figs. 10-13). However, there was no correlation between age and WMV of the right, left, and total superior frontal gyri, middle frontal gyri, pars opercularis, pars triangularis, pars orbitalis, transverse frontal gyri, orbito-frontal gyri, and subcallosal areas in males and females (P>0.05).

Table 2. White matter volumes of frontal lobes and frontal lobe gyri

Frontal lobe Gyri	White Matter Volume (Mean±SD cm3)					
	Right		Left		Total	
	Males	Females	Males	Females	Males	Females
Frontal Lobe	75.08±10.51	65.38±9.69	77.02±11.58	66.42±9.30	152.10±21.95	131.80±18.79
Superior Frontal Gyrus	21.82±3.26	18.91±3.22*	24.11±4.41	24.04±3.89	44.80±7.76	44.49±7.17
Middle Frontal Gyrus	10.06±2.16	8.72±1.88*	9.99±2.32	9.04±2.20*	19.65±4.32	18.30±3.90
Pars Opercularis	2.44±0.59	2.19±0.58*	2.92±0.82	2.80±0.71	5.30±1.33	5.07±1.12
Pars Triangularis	5.41±1.48	4.67±1.24*	3.75±0.95	3.63±0.76	8.95±2.19	8.57±1.80
Pars Orbitalis	0.77±0.27	0.73±0.27	0.93±0.19	0.86±0.21*	1.70±0.36	1.60±0.33
Precentral Gyrus	16.23±2.53	14.12±1.81*	14.13±2.46	13.35±2.08*	29.83±4.93	28.20±4.07*
Paracentral gyrus	3.10±0.58	2.58±0.48*	3.90±0.73	3.76±0.62	6.82±1.23	6.57±1.08
Transverse Frontal Gyrus	0.89±0.27	0.76±0.25*	1.02±0.37	0.96±0.36	1.87±0.59	1.78±0.58
Cingulate Gyrus	6.20±1.18	5.35±1.25*	6.43±1.40	5.98±1.10*	12.41±2.75	11.62±2.08
Subcallosal Area	0.03±0.03	0.02±0.02	0.04±0.03	0.04±0.03	0.07±0.03	0.07±0.00
Orbito-Frontal Gyri	8.14±1.28	7.11±0.99*	6.41±1.22	6.31±1.05	14.14±2.48	13.96±2.17

*, p≤0.05

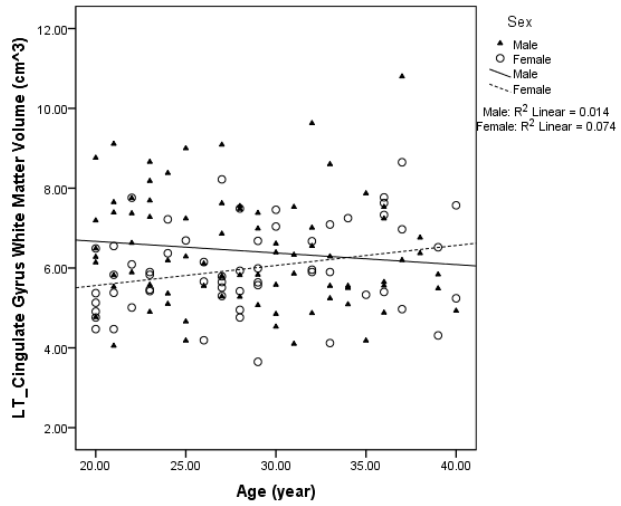

Fig. 10. Correlation between age and white matter volume of left (LT) cingulate gyrus

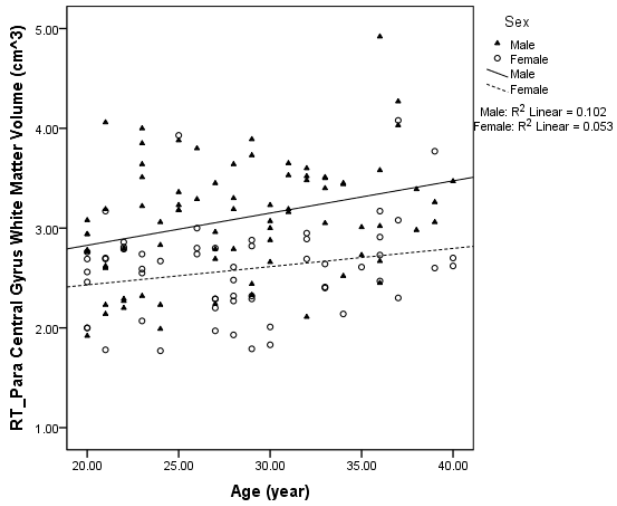

Fig. 12. Correlation between age and white matter volume of right (RT) paracentral gyrus

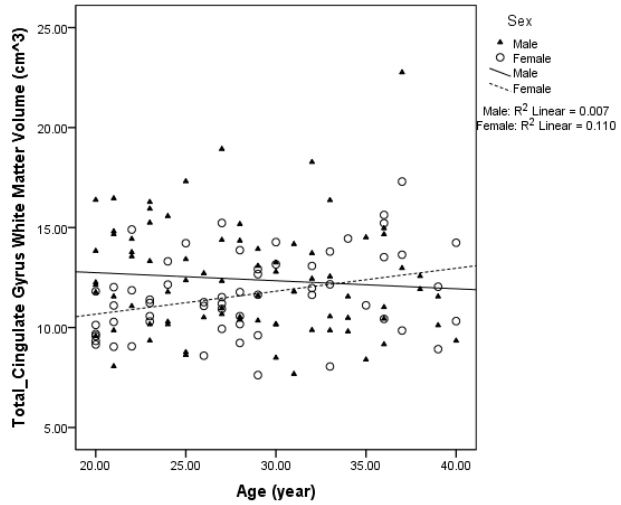

Fig. 11. Correlation between age and white matter volume of total cingulate gyri

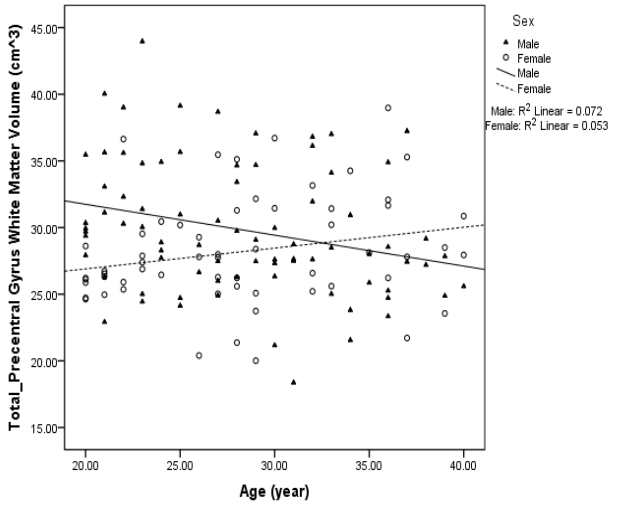

Fig. 13. Correlation between age and white matter volume of total precentral gyri

4. Discussion

The present study provided a detailed description of the effect of sex and age on grey and white matter volumes of frontal lobe and its gyri. Firstly, it was compared mean values of grey and white matter volumes between males and females, to determine sex differences. Secondly, it was correlated grey and white matters volumes to age; to find out the correlations.

According to findings of the presents study, males had significantly greater GMV in the frontal lobe and most of the frontal lobe gyri. Our result in agree with other studies that found greater GMV in males in the left cingulate (Allen et al., 2003), right precentral, middle frontal, and transverse frontal gyri (Brabera et al., 2013). In contrast, other studies found greater GMV in males in the left pars opercularis (Witte et al., 2010); and in females in the left pars triangularis (Witte et al., 2010), right paracentral gyrus, right inferior frontal gyrus, and right and left cingulate gyri (Brabera et al., 2013), precentral gyrus and orbitofrontal cortex. The reasons for these diverging findings may be related to differences in sample size, composition of examined brains, methods to quantify the compartmental volumes, or lack of neuropsychological control.

The possible reasons for the sex differences in grey matter volume (GMV) is the influence of sex hormones. The recent study found a connection between GMV and hormone levels of testosterone, estrogen or progesterone (Witte et al., 2010); but it hasn't yet cleared the effect of these hormones in the brain(Van Amelsvoort et al., 2001).

Sex differences in the regional GMV might develop as a result of differential environmental and social influences. Interestingly, use-dependent influences like lifelong intensive training in specific cognitive or motor skills have been suggested to modulate regional GMV (Draganski et al., 2004).

Our results revealed that males had significantly greater WMV in the frontal lobes and most of frontal lobe gyri. Literatures of cortical white matter volumes (WMV) are not widely available; WMV was measured by other method rather than MR imaging such as diffusion tensor imaging. Our result in agree with other study found males had greater white matter volume in the lateral prefrontal cortex (Kennedy et al., 2009).

The white matter volume (WMV) is determined by the number of axons, their caliber and the thickness of myelin sheath produced by the oligodendrocytes (Paus and Toro, 2009). WM connect various grey matter areas of the brain to each other, and carry nerve impulses between neurons. Increase WMV in males compared to females probably means that more connections among neurons. The increase in WMV might also mean greater communication between the frontal lobe gyri and other parts of the brain (Schoenemann et al., 2005).

In the brain there are many receptors for sex hormones, which affect the neurodevelopment. The hormonal effects on the white matter development have been discussed based on findings from animal studies, which show that testosterone is associated with myelogenesis (Martini and Melcangi, 1991) and estradiol effects synaptogenesis and reduces the rate of myelination (Woolley et al., 1996).

An interesting result was sex differences in GMV and WMV were more prominent in the right than left frontal lobe gyri; these differences may relate to differences in distributions of sex hormones.

According to current findings, there was significant negative correlation between age and GMV of the right, left, and total frontal lobes and the right superior frontal gyrus in males and females; the right pars orbitalis and orbitofrontal gyri in males; and the cingulate gyrus in females.

The results of the present study consistent with the voxel-based morphometric (VBM) studies found the reduction in GMV with age in superior and inferior frontal gyrus (Taki et al., 2011b), cingulate, and orbitofrontal gyri (Terribilli et al., 2011). In contrast, MRI studies using Statistical Parametric Mapping 2 (SPM2) on 1460 healthy subject aged 20-69 years, showed grater decline in GMV of precentral and middle frontal gyri (Taki et al., 2011b). Voxel based morphometric study on 223 aged between 8-79 years detected GMV loss in dorsolateral frontal cortex and precentral gyrus (Grieve et al., 2005). This discrepancy may possibly relate to age of subjects. In the compared study, the inclusion of children and adolescents on one side and elderly subjects on the other probably led to an overlapping of maturation and neurodegenerative processes, which would have been differentially influenced by hormonal effects across separate age ranges.

The reduction in the GMV observed in young adulthood is thought to reflect primarily the maturation changes (Taki et al., 2011b); and is not directly connected with decreases in specific brain function (Taki et al., 2011a). Brain maturation, consisting of both regressive cellular events, such as synaptic pruning, and progressive cellular events, such as myelination, occurs simultaneously in the brain during childhood, adolescence, and young adulthood; and this events result in decline of GMV (Taki et al., 2011b).

An interesting result, GMV of the left frontal lobe gyri didn't decline with age, whereas GMV of the right frontal lobe gyri decline by age, this divergence is possibly related to differences in the time course of the myelination and synaptic pruning maturational processes.

The effect of age on WMV was not homogenous; there was significant positive correlation between age and WMV of the left and total cingulate gyri in females and the right paracentral gyrus in males; but significant negative correlation between age and WMV of the total precentral gyri in males.

Results regarding effect of age on WMV were inconstant. Some studies showed that the WMV increases until the 5th

decade of life (Bartzokis et al., 2001; Taki et al., 2011a). In contrast, other studies showed that WMV remain relatively stable in adults until the age of 60–70 years and decline thereafter (Jernigan et al., 2001; Miller, 1980).

The increase in the WMV in the cingulate and paracentral gyri is likely associated with continued myelination and axonal growth (Webb et al., 2001) and possibly reflecting increasing the complexity in connectivity with functional and structural development (Pfefferbaum et al., 2013). An increase in myelination and/or interconnectivity could facilitate the synchronous integration of information across many regions (Gould et al., 1999). The speed of neural transmission depends on the structural properties of the connecting fibers, including axon diameter and the thickness of the insulating myelin sheath (Happe et al., 1998).

The decrease in WMV of precentral gyrus may result from microscopic structural changes, which include dilatation of perivascular spaces (Fazekas et al., 1993) and gliosis (Grafton et al., 1991). Regarding the fact that decrease in white matter volume not start until late in life, this finding regarding total and/ or cerebral white matter but not regional white matter volumes.

This study included only the young adult to clarify the impact of sex and age on grey matter volume (GMV) and white matter volume (WMV) of frontal lobes and their gyri. Sex has prominent effect on GMV and WMV of frontal lobe and most of frontal lobe gyri, with males had greater GMV and WMV of the frontal lobes and most of the frontal lobe gyri. Sex differences in GMV and WMV may have functional significant and/ or constitute sex-dependent redistributions of tissue volume. Further studies are important to find out the relationship between cognitive function and GMV and WMV.

Decrease in GMV by age has been reported in the right, left, and total frontal lobe; and right superior frontal, pars orbitalis, orbitofrontal gyri, and cingulate gyri. Increase in WMV by age has been reported in the left and total cingulate and right paracentral gyri. The findings of the present study demonstrate continuation of maturation of frontal lobe until third decade of age. These data are useful as baseline data for tracking diseases that cause progressive changes in GM and WM volumes.

Conflict of interest

None to declare.

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Evaluation of metabolic and hormonal parameters in women with PCOS living in Black Sea Region

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Abstract

Polycystic ovary syndrome (PCOS) is the most common endocrinopathy which is frequently associated with metabolic syndrome. The aim of this study is to compare the clinical, biochemical and hormonal characteristics of healthy women and women with PCOS in Black Sea Region. 91 healthy women (Group 1) and 109 PCOS (Group 2) patients were included in this retrospective study. This study was conducted in Samsun Women and Children's Health Research and Training Hospital between October 2019-May 2020.The anthropometric, clinical and laboratory characteristics of the women were recorded. The Institutional Review Board of the hospital approved the study. IBM SPSS Statistics 22 program was used for statistical analysis. Variables were expressed as mean ± standard deviation or as a number (percentage), and statistical significance was defined as a p value of less than 0.05. In this study, no statistically significant difference was found between patients with PCOS and control subjects in age, height, BMI, waist circumference and waist-to-hip ratio. Weight, BP systolic, BP diastolic and FG scores, were significantly higher in patients with PCOS compared to control subjects. In this study, no statistically significant difference was found between patients with PCOS and control subjects in FSH, estradiol prolactin, TSH, fT3, fT4 and DHEASO4 levels. But, LH, cortisol, total and free testosterone and 17-OH progesteron levels were significantly higher in patients with PCOS compared to control subjects. Also in this study, vitamin B12 and the 25-OH-D levels were significantly lower in patients with PCOS compared to control subjects. HbA1C,fasting blood glucose levels, fasting insulin levels and HOMA-IR were significantly higher in patients with PCOS compared to control subjects . Total-C, LDL-C and TG levels were significantly higher in women with PCOS compared to control subjects. Also, HDL-C levels were significantly lower in women with PCOS compared to control subjects. As a result, the risk of metabolic syndrome is increased in PCOS patients because of high androgen levels, obesity and insulin resistance.

Keywords: hormone profile, lipid profile, metabolic syndrome, polycystic ovarian syndrome

1. Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder of reproductive-aged women. Although Stein and Leventhal first described it in 1935, its etiology is still unclear (Stein and Leventhal, 1931). Menstrual irregularities, hyperandrogenism, hirsutism, and fertility problems are the main clinical features of PCOS. The prevalence of PCOS is 5-10%, and it depends on environmental and genetic factors and ethnicity (Abbot et al., 2002). This syndrome's diagnosis requires the exclusion of manifestations with similar clinical presentation, such as congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumors, and other causes of androgen excess. Three available criteria used to diagnose PCOS: (i) the 1990 National Institutes of Health (NIH) and National Institute of Child Health and Human Development (NICHD) criteria; (ii) the 2003 Rotterdam criteria; and (iii) the 2006 Androgen Excess Society criteria. The most widely used one is Rotterdam criteria, and according to the 2003 Rotterdam criteria, it is usually defined by the presence of at least two out of the following three features: i) oligoovulation or anovulation, ii) clinical and biochemical signs of hyperandrogenism (presence of at least two signs) or iii) polycystic ovaries (ovaries with many small cysts no larger than 8-10 mm) (Rotterdam ESHRE/ASRM, 2004). The increasing levels of luteinizing hormone (LH) during the menstrual cycle is responsible for several changes, including the synthesis of androgens by the theca cells, resulting in hirsutism, acne, and androgenic alopecia. Most PCOS patients also present with peripheral

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insulin resistance (IR) and hyperinsulinemia. Insulin plays a critical role in the clinical manifestations of PCOS by increasing the synthesis of androgens by the theca cells and inhibiting the hepatic sex hormone-binding globulin (SHBG). In this way, it increases the fraction of free testosterone to bind with its receptor. Obesity is also very common in PCOS patients, including a higher waist-hip ratio (visceral obesity). This contributes to the exacerbation of menstrual irregularities and many other metabolic alterations (Poretsky and Piper, 1984). A metabolic syndrome is a group of metabolic disorders including insulin resistance, impaired glucose metabolism, abdominal obesity, hypertension, and dyslipidemia. It increases the risk of Type 2 diabetes mellitus (DMT2), coronary heart disease (CHD) and endometrial cancer. Patients with PCOS are at high risk for cardiovascular diseases because of hyperandrogenism, insulin resistance, type II diabetes, and obesity which are commonly seen in PCOS (Dunaif et al., 1989). Adipose tissue dysfunction contributes to insulin resistance in women with PCOS. However, a substantial number of lean women affected by PCOS have insulin resistance as well, independent of obesity (Ketel et al., 2011). Vitamin B12 is important in the remethylation of homocysteine to methionine, and hyperhomocysteinemia is a characteristic of vitamin B12 deficiency (McCarty, 2000). Insulin resistance in women with PCOS is associated with high plasma homocysteine levels (Schacter et al., 2003). Hyperhomocysteinaemia is associated with increased cardiovascular disease risk in type 2 diabetic patients. Vitamin D also plays an essential role in metabolic pathways affected by PCOS, and vitamin D receptor (VDR) polymorphisms are associated with some of the patterns presented by PCOS (Manousopoulou et al., 2015). This study compares the clinical, biochemical, and hormonal characteristics of healthy women and women with PCOS living in the Black Sea Region.

2. Materials and methods

This retrospective, cross-sectional study was conducted with 200 women aged 15-44 years who underwent gynecological examinations in the outpatient clinic of a state hospital between June 2019 and May 2020. This study followed the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Health Sciences University Samsun Research and Education Hospital 2020/15/8. The study was carried out with the permission of the Local Institutional Review Board. Among them, 91 women were healthy volunteers with normal menstrual cycles who had no features of hyperandrogenism and 109 women with the diagnosis of PCOS according to revised Rotterdam Criteria ESHRE/ASRM criteria (2004). All data were abstracted from the electronic medical records, histories, and physicals. A detailed medical, reproductive, and family history had been taken. Also, anthropometric measurements were performed, including height, weight, body mass index (BMI), waist circumference, hip circumference, waist-hip ratio (WHR), systolic and diastolic blood pressure, and the level of hirsutism measured with the use of the Ferriman Gallwey (FG) score. Weight and height were measured in light clothing without shoes. BMI was calculated as weight (kg) divided by height (m) squared. BMI values of ≥25 kg/m^2 were considered as overweight. BMI values of ≥30 kg/m^2 were deemed to be obese. Waist circumference was measured at the narrowest level between the costal margin and iliac crest, and the hip circumference was measured at the most comprehensive level over the buttocks while the subjects were standing and breathing normally. The waist-to-hip ratio (WHR) was calculated. A WHR >0.72 was considered abnormal (Ashwell et al., 1982). Clinical and biochemical hyperandrogenism was defined as an FG score > eight and a free androgen index (FAI: [(T/SHBG) x 100] >4.5). Subsequently, a systematic transvaginal or pelvic ultrasonography was performed to assess ovarian volume, endometrial thickness, and the total number of antral follicles measuring 2-10 mm. Finally, an extensive metabolic and endocrine profile was assessed.

2.1. Assessment of metabolic profile

Regarding the cardiovascular profile, BMI and systolic and diastolic blood pressure were evaluated in both PCOS and healthy women. In all women, waist circumference, fasting glucose, insulin, and lipid profile (i.e., total cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol) were additionally measured. Insulin resistance was assessed by using the homeostasis model assessment (HOMA-IR: [fasting glucose (mmol/L) x fasting insulin [mU/L]]/22.5) (Matthews et al., 1985). Metabolic syndrome was defined according to the National Cholesterol Education Program (NCEP) ADP III criteria (Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2002). According to this definition at least three of the following five features should be present (i) Waist circumference of > 88 cm (80 cm for Asians) (ii) Fasting blood glucose of ≥ 100 mg/dl (iii)Triglycerides ≥ 150 mg/dl (iv) HDL < 40 mg/dl (v) Blood pressure > 130/ 85 mm Hg. Thyroid-stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, dehydroepiandrosterone sulfate (DHEAS), 17-hydroxyprogesterone (17-OHP), and total and free testosterone were checked in patients with PCOS and control subjects. Blood samples were collected at 8:30-9:30 a.m. after 12-h overnight fasting, in the early follicular phase (2nd-3rd days) after spontaneous or gestagen-induced menses in women. These parameters were quantified using a 7600-110 Automatic Analyzer (Hitachi Inc., Tokyo, Japan). The low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula. Women with thyroid diseases, hyperprolactinemia, Cushing's disease, or congenital adrenal hyperplasia and those who were administered agents such as hormonal agents, ovulation-inducing agents, glucocorticoids, anti-androgens, or anti-hypertensives over the last three months prior to the study were excluded from the study.

2.2. Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences for Windows 22.0 software (SPSS, Chicago, IL., USA). Descriptive statistics were inferred as the mean, standard deviation, frequency, and percentage. Shapiro- Wilk test was utilized to check the normality of continuous variables. Parametric comparisons were performed using Student's t-test for normally distributed continuous data, and non-parametric comparisons were performed using the Mann-Whitney U test for non-normal continuous data distribution. Categorical variables were compared using chi-square or Fisher's exact tests, as appropriate. The Pearson Chi-square test was used to compare qualitative data. Spearman's Correlation Analysis was applied to evaluate the relationships between variables. A value of $p<0.05$ was accepted as statistically significant.

3. Results

The demographic data of the PCOS and control groups are presented in Table 1. In this study, no statistically significant difference was found between patients with PCOS and control subjects in age, height, BMI, waist circumference, and waist-to-hip ratio (p:0.428, p:0.995, p:0.073, p:0.313, and p:0.514, respectively)

Table 1. Baseline characteristics of control group and PCOS women

	Control (n=91)	PCOS (n=109)	*p*
Age (years)	25.48±5.36	24.96±5.27	0.428
Weight(kg)	73.35±17.91	82.31±24.81	**0.041**
Height(cm)	160.01±6.70	160.06±6.19	0.995
BMI (kg/m^2)	28.89±7.76	32.27±10.27	0.073
WC (cm)	83.45±20.80	87.13±22,31	0.313
Waist/hip	0.78 ± 0.26	0.83 ± 0.26	0.514
SBP (mmHg)	111 ± 13	118 ± 13	**0.013**
DBP (mmHg)	71 ± 9	78 ± 12	**0.028**
FG score	5.26± 2.1	10.23± 2.14	**0.004**

BMI: body mass index; WC: waist circumference, SBP: systolic blood pressure; DBP: diastolic blood pressure. Results are expressed as median (range) or mean±SD according to distribution. $p<0.05$, significant difference between groups

Weight, blood pressure (BP), systolic and diastolic, and also FG (Ferriman Gallway) scores were significantly higher in patients with PCOS compared to control subjects (p:0.041, p:0.013, p:0.028 and p 0.004, respectively, Table 1). In this study, no statistically significant difference was found between patients with PCOS and control subjects in FSH, estradiol prolactin, TSH, fT3, fT4, and DHEASO4 levels (p:0.483, p:0.223, p:0.623, p:0.231, p:0.129, p:0.250 and p:0.274 respectively). But, LH, cortisol, total and free testosterone, and 17-OH progesterone levels were significantly higher in patients with PCOS than control subjects (p:0.000, p:0.003, p:0.005, p:0.003 and p:0.008 respectively, Table 2). Also, in this study, vitamin B12 and the 25-OH vitamin D3 levels significantly lower in patients with PCOS compared to control subjects (p:0.048 and p:0.046 respectively). HbA1C, fasting blood glucose levels, fasting insulin levels and HOMA-IR were significantly higher in patients with PCOS compared to control subjects (p:0.012, p:0.044, p:0.011 and p:0.006 respectively, Table 2). Total-C, LDL-C, and TG levels were significantly higher in women with PCOS than control subjects (p:0.041, p:0.035, and p:0.039, respectively). Also, HDL-C levels were significantly lower in women with PCOS compared to control subjects(p:0.034).

Table 2. Hormonal and metabolic profile in the control group and PCOS women

	Control (n=91)	PCOS (n=109)	*p*
FSH (IU/L)	6.12 ± 1.32	5.98 ± 1.50	0.483
LH (IU/L)	7.35 ± 4.06	9.88 ± 4.89	**0.000**
Estradiol (pg/ml)	56.41±17.1	53.47±16.8	0.223
Prolactin (ng/ml)	16.39±7.23	16.12±7.31	0.623
TSH (mIU/L)	2.11±1.11	2.39±1.23	0.231
fT_3 (mIU/L)	3.06±0.53	2.79±0.68	0.129
fT_4 (mIU/L)	1.35±0.41	1.42±0.41	0.250
Cortisol (nmol/L)	14.29±6.68	16.81±7.36	**0.003**
TT (ng/dL)	37.56±35.0	61.20±28.2	**0.005**
fT (ng/dL)	1.57±0.43	2.32±1.37	**0.003**
$DHEASO_4$ (nmol/L)	253.4±106.0	258.8±87.6	0.274
17-OH progesteron (ng/dl)	0.88±0.22	1.08±0.30	**0.008**
Vitamin B_{12} (pg/ml)	364.71±127.9	324.0±189.3	**0.048**
25-OH-D (nmol/L)	12.50±6.26	10.53±6.96	**0.046**
HbA1C	5.09±1.32	5.44±1.36	**0.012**
Fasting glucose (mmol/L)	88.04±16.2	93.26±20.9	**0.044**
Fasting insulin (µIU/Ml)	13.42±8.66	18.12±14.0	**0.011**
HOMA-IR	3.09±2.73	4.52±4.37	**0.006**
TC (mmol/L)	167.29±43.6	178.34±31.97	**0.041**
LDL-C (mmol/L	92.48±29.59	99.28±26.3	**0.035**
HDL-C (mmol/L)	57.22±12.37	53.69±12.1	**0.034**
TG (mmol/L)	123.92±51.25	139.6±44.1	**0.039**

FSH: follicle stimulating hormone; LH: luteinizing hormone; FT_3: Free triiodothyronine; FT_4: Free thyroxine; TT: total testosterone; freeT: bioavailable testosterone; $DHEASO_4$: dehydroepiandrostenedionesulphate; 25OHD: 25-hydroxyvitamin D; HOMA-IR: homeostatic model assessment of insulin resistance; TC: total cholesterol; LDL-C: low density lipoprotein cholesterol; HDL: high density lipoprotein cholesterol; TG: triglycerides; FAI: free androgen index; SHBG: sex hormone binding globulin; Results are expressed as median (range) or mean±SD according to distribution. $p < 0.05$, significant difference between groups

4. Discussion

This retrospective, cross-sectional study investigated whether the serum hormone, lipid, and vitamin levels were different in healthy women and in women with PCOS in the Black Sea region. Today most clinicians only focus on infertility in women with PCOS, and underestimate the risk of metabolic syndrome and other long-term effects. PCOS is not only a gynecologic condition affecting women of reproductive age, but also a part of metabolic syndrome with a variety of associated metabolic changes such as vitamin deficiencies, insulin resistance and dyslipidemia. Dokras et al. (2005) showed that 11-fold increase in the prevalence of metabolic

syndrome in PCOS patients compared with age-matched controls.

Chronic LH stimulation in PCOS reduces FSH receptor mRNA expression, which can induce the antral follicles to lose the FSH response (Orisaka et al., 2013). Decreased FSH reduces the expression of CYP19A1, which belongs to the cytochrome P450 family, by inactivating the cAMP-PKA-CREB pathway, thus leading to arrested follicular development (Gu et al., 2018). In the preovulatory follicular wave, acute and elevated insulin can decrease the percentage of large follicles that are ovulated, which reduces ovulatory oocyte fertilization. The effect becomes more obvious when insulin is combined with increased LH (Cadagan et al., 2016). Besides, the effect of insulin on follicle development may involve the insulin-like growth factor (IGF) system. Hyperinsulinemia can reduce IGF-binding proteins (IGFBP) synthesis to increase the free IGF-1 content (Stanek et al., 2007), which is the direct target of miR-323-3p (Wang et al., 2019). Decreased miRNA-323-3p can accelerate granulosa cell (GC) apoptosis via IGF-1 to inhibit folliculogenesis (Wang et al., 2019). Visceral adiposity a common occurrence in women with PCOS is strongly associated with insulin resistance and hyperlipidemia, both of which are harbingers of cardiovascular disease (Zheng and Li, 2016). Similar to the literature, in our study, body weights were heavier, and insulin resistance was found in the PCOS group. Many studies reported an increased risk for cardiovascular diseases in women with PCOS; however, the reason for this increased cardiovascular risk has not been clarified yet. PCOS is a chronic inflammatory disease, and an excess of visceral fat plays an important role in cardiovascular disorders. The inflammatory components of visceral obesity also affect atherosclerosis development (Mathieu et al., 2008). In our study, HbA1C, fasting blood glucose levels, fasting insulin levels, and HOMA-IR were significantly higher in patients with PCOS than in corresponding groups of healthy controls as in the literature (Legro et al., 1999; Hoffman and Ehrman, 2008). Insulin resistance and blunted carbohydrates oxidation were common features in all included women; thus, a common pathway of inappropriate energy oxidation, metabolic inflexibility, and response to insulin in women with PCOS is apparent (Rimmer et al., 2020). Insulin resistance is a risk factor for cardiovascular diseases, type II diabetes, hypertension, and dyslipidemia. Also, our results related to lipid profile in PCOS patients were compatible with the literature (Fulghesu et al., 2010).The most common lipid abnormalities among women with PCOS are elevated levels of triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), very-low-density lipoprotein cholesterol (VLDL-C), and low high-density lipoprotein cholesterol (HDL-C) levels(24) Similar to the literature, total-C, LDL-C, and TG levels were significantly higher in women with PCOS compared to control subjects in our study. Also, in our study, a low HDL-C level was found in the PCOS group. These lipid abnormalities are associated with insulin resistance and predict the development of CVD, such as myocardial infarction (MI) (Wild et al., 2011). Obesity increases the risks of insulin resistance and cardiovascular disease and impairs reproductive functions (Bou et al., 2019). Obesity is commonly characterized by systemic and tissue-specific adipogenesis with increased cholesterol levels and lipid accumulation, which lead to inflammation, oxidative stress, and dysfunction in the ovaries (Araujo et al., 2018). Although obesity is the most critical marker of metabolic dysfunction, excess androgen levels contribute to increased metabolic syndrome risk independent of obesity (Rossi et al., 2008). In this regard, many studies have been conducted to investigate the close relationship between PCOS and metabolic syndrome. The mechanisms by which obesity is associated with dyslipidemia in women with PCOS include insulin resistance, overproduction of VLDL, abnormal lipoprotein lipase-mediated lipolysis, and a defect in the insulin-signaling pathway mediated by overexpression of PI3KR1 gene (Diamanti-Kandarakis et al., 2007).Testosterone induces dyslipidemia in women with PCOS through androgen receptor-mediated insulin resistance and the upregulation of genes responsible for the catabolism of HDL (Diamanti-Kandarakis et al., 2008). Clinical manifestations of MetS such as dyslipidemia develop from insulin resistance through increased secretion of non-esterified fatty acids and increased synthesis of TGs. In contrast, hypertension develops through endothelial damage and reduced nitric oxide bioavailability (Lim et al., 2019). Hypertension develops in women with PCOS from hyperaldosteronism via the activation of the renin-angiotensin system (Cascella et al., 2006). One cross-sectional study in Brazil of 233 women with PCOS and 70 controls found a higher prevalence of hypertension among the PCOS group (Marchesan et al., 2019) as in our study. Additionally, insulin resistance-related compensatory hyperinsulinemia has been implicated in the occurrence of hypertension in women with PCOS through an imbalance in the autonomic nervous system, increased renal sodium reabsorption, as well as a reduction in the production of nitric oxide (Marchesan et al., 2019). Although several studies have suggested that lower vitamin D levels are associated with an increased risk of insulin resistance and metabolic disturbance among women with PCOS the current findings are inconsistent (Van der Schueren et al., 2012). In our study, women with PCOS also had low vitamin D levels. The prevalence of Vitamin D deficiency (VDD) is 20%-48% among the general adult population, but a relatively higher prevalence of VDD is observed among women with PCOS, approximately 67%-85% of women with PCOS have VDD (Thompson et al., 2012). Several studies have confirmed an association between vitamin D status with cardiovascular diseases and diabetes-related outcomes (Camargo, 2011). Because vitamin D has anti-inflammatory effects, it is not surprising that it has beneficial effects on improving islet-cell functions, insulin release, and decreasing insulin resistance (Sung et al., 2012). PCOS presents alterations in metabolic pathways affected by Vitamin D, such as the insulin pathway, sex hormone production, and calcium

homeostasis (Li et al., 2011; Hassan et al., 2012). Patients with metabolic syndrome have low serum 25(OH)D levels, and restoration of serum 25(OH)D levels improves insulin resistance (Takiishi et al., 2012). One study in Tehran, however, did not find any difference in vitamin D levels in women with and without PCOS (Moini et al., 2015). In our study, serum vitamin B12 concentrations were significantly lower in PCOS women in comparison with control women as in the literature (Kaya et al., 2009). The low concentrations of vitamin B12 found in PCOS women with IR suggest the involvement of vitamin B12 in hyperinsulinemia, insulin resistance, and hyperhomocysteinemia. The importance of vitamin B12 in the remethylation of homocysteine to methionine is well recognized, and hyperhomocysteinemia is also a feature of vitamin B12 deficiency (Fonseca et al.,1999). The management of PCOS and metabolic syndrome includes lifestyle changes, especially for weight optimization, which has multiple clinical and personal benefits. Weight loss in overweight women could reduce the risk of suffering diabetes and death from cardiovascular causes by 28% (Feldman et al., 2017) and 21% (Look AHEAD Research Group, 2016), respectively. The low number of patients and retrospective study design were among the limitations of our study.

In this study, we found significant differences between patients with PCOS and control groups for hormone, lipid, and vitamin profiles. PCOS is an excess androgen disorder with different degrees of reproductive and metabolic dysfunctions. These effects are lifetime long, and lifestyle changes consisting of increased physical activity and caloric restriction can improve both metabolic and reproductive outcomes. As a result, effective diagnosis and treatment approaches are needed for protecting patients with PCOS from diabetes and cardiovascular diseases.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Screening for family functioning and mental health in adolescents with non-cardiac chest pain: A comparison study for a frequently encountered problem

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Abstract

Chest pain is highly prevalent in adolescence, represents a considerable burden for health services, and rarely associated with cardiac disease. Since psychosocial factors are related to chest pain there is a need for exploring the accompanying family functioning and mental health problems in adolescents with non-cardiac chest pain. Here, we assessed these determinants in 68 adolescents with non-cardiac chest pain and 68 age and-sex matched control participants using the 4-item Patient Health Questionnaire-4 for a brief screening for anxiety and depression and the Family APGAR Scale to assess the perception of family functioning. Adolescents with non-cardiac chest pain reported more depression, anxiety and experienced impaired family functioning ($p=0.003$, $p<0.001$, $p<0.001$). The results indicated that non-cardiac chest pain is a warning sign of an underlying mental health problem with accompanying family issues and it deserves further psychosocial investigation including anxiety, depression, and impaired satisfaction of the adolescents' family's responsiveness.

Keywords: adolescent, anxiety, chest pain, depression

1. Introduction

Chest pain in adolescents is one of the common causes of admission to the pediatric outpatient clinics and emergency department (Avison and McAlpine, 1992). Despite this significant burden, the natural history of non-cardiac chest pain in the community has not been clearly explained yet (Bass and Mayou, 2002).

In contrast to its presentation in adults, chest pain in children and adolescents is rarely a sign of cardiac disease (Bass and Wade, 1984). Psychogenic factors are the broadest area of non-cardiac causes of chest pain. Although non-cardiac medical causes (e.g., musculoskeletal, gastrointestinal, asthma) are sometimes identified, the majority of cases (25 to 85%) have no clear medical etiology (Avison and McAlpine, 1992; Bekhuis et al., 2015). About half of the children with psychogenic chest pain also have a history of other somatic complaints or have a family member with similar symptoms. Other findings consistent with psychogenic chest pain include stressful life events and sleep disturbances. Kashani, Lababidi, and Jones (1982) have suggested that chest pain can be a physical manifestation of depression in children. Porter, Fein, and Ginsburg (1997) reported similar findings, 22% of depressed adolescents describing the somatic complaint of chest pain on arrival at the emergency department (Avison and McAlpine, 1992). Non-cardiac chest pain can be established thorough history, physical examination and proper laboratory investigations after all other possible etiologies are ruled out (Cole and Rehm, 1986). Chest pain without any organic reason has been named with different labels such as unexplained, idiopathic, and non-cardiac (Cole and Rehm, 1986).

Since at least half of the new referrals to cardiac outpatient clinics with the presenting complaint of chest pain are found not to have heart disease or other serious physical disorders. Despite a normal expectation of life and physical prognosis, most cases continue to experience symptoms, worry about heart disease, restrict their activities and seek medical help (Eliacik et al., 2017; Demircia and Ekşib, 2018). In the adolescence period, depression appears to be associated with family environments characterized by an absence of supportive and facilitative interactions and, conversely, by elevated levels of conflictual, critical, and angry interactions. The most widely reported finding is that depression is inversely related to the level of support and approval provided by the family

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environment (Eslick et al., 2003; Juli-anne et al., 2000). Therefore, we aimed to screen anxiety, depression besides the patient's satisfaction with his or her family's responsiveness to need in this clinical group.

2. Materials and methods

A total of 89 subjects aged 13-18 years were selected sequentially who presented to pediatric outpatient cardiology clinics and diagnosed with non-cardiac chest pain were included in this study. Adolescents who had a chronic disease (gastroesophageal reflux disease, cardiovascular disease, pulmonary disease, etc.) and who were not native Turkish speakers were ruled out from the study. During the survey, 21 adolescents who did not want to participate or have a lack of data were excluded from the study. After the exclusion, 68 participants with chest pain were included in data analysis. The control group comprised of 68 age-and sex-matched volunteer adolescents who visited pediatric cardiology outpatient clinics for pre-participation cardiovascular screening before joining a sports club. The volunteer adolescents were invited to fill the forms including age, sex, Patient Health Questionnaire-4 (PHQ-4) and Family APGAR (Adaptation, Partnership, Growth, Affection, Resolve) Scale. The PHQ-4 is a screening instrument for depression and anxiety for the first step of assessment in patients with medical diseases. The PHQ-4 is an ultra-short screening instrument for depression and anxiety. The items of the PHQ-4 measure core symptoms of depressive disorders (loss of interest, depressed mood) and generalized anxiety disorder (feeling nervous and anxious, difficulty to stop or control worrying). Thereby this 4-item self-report questionnaire consists of two 2-item subscales, the depression scale PHQ-2 and the anxiety scale GAD-2 (General Anxiety Disorder-2). The PHQ–2 consists of two core criteria for depressive disorders. The GAD–2 consists of the two core criteria for generalized anxiety disorder that have also been shown to be good screening items for panic, social anxiety, and posttraumatic stress disorders. The questionnaire starts with the general question "Over the last two weeks, how often have you been bothered by the following problems?" and continues with asking for the four symptoms which are rated on a 4-point scale from 0 ('not at all') to 3 ('nearly every day'). The total sum score ranges from 0 to 12 with a range of 0 to 6 for each of the two subscales. An additional single item that is not included in any of the scale sum scores asks for the extent of the respondent's subjective psychosocial symptom-related impairment. A PHQ-2 score ≥ 3 indicates clinically significant depression, and a GAD-2 score≥3 indicates clinically significant anxiety. A PHQ-4 total score cut-off point ≥ 6 has been recommended as an indicator of the presence of a depressive or an anxiety disorder (Fröjd et al., 2007; Kerper et al., 2014). The validity and reliability of the instrument in Turkish was shown by Demirci et al. with an internal consistency reliability coefficient of 0.83 for overall scale (Khairandish et al., 2017).

Family APGAR is a brief screening questionnaire, which is designed to elicit a database that will reflect a patient's view of the functional state of his or her family. It will also discuss ways of assessing family resources and the use of a family problem-oriented record that may be used by the physician to improve family study, diagnosis, and management. It is a questionnaire that features five closed-ended questions, is introduced by the author as a screening test to give a rapid overview of the components of family function. The APGAR acronym has been applied since it is felt that the familiarity that physicians have with the Apgar evaluation of the newborn will encourage them to remember a similar format that scores the functional status of a family. Field tests with the Family APGAR are presently being conducted and early results are promising. The questions in the Family APGAR are designed to permit qualitative measurement of the family member's satisfaction with each of the five basic components of family function (Kocis, 1999; Kroenke et al., 2009). These components are described below:

Adaptation: How resources are shared, or the degree to which a member is satisfied with the assistance received when family resources are needed.

Partnership: How decisions are shared, or the member's satisfaction with mutuality in family communication and problem-solving.

Growth: How nurturing is shared, or the member's satisfaction with the freedom available within the family to change roles and attain physical and emotional growth or maturation.

Affection: How emotional experiences are shared, or the member's satisfaction with the intimacy and emotional interaction that exists in a family.

Resolve: How time (and space and money*) is shared, or the member's satisfaction with the time commitment that has been made to the family by its members (Kocis, 1999).

Permission for this study was granted by the local ethical committee. Informed consent was received from all patients and controls.

2.1. Data analysis

Statistical Package for Social Sciences Software (SPSS v.24, Chicago, IL, USA) was used for data analysis. Descriptive statics were used to describe the socio-demographic information of the study and control groups. The Student's t-test was used to compare the median of PHQ-4 and APGAR scores between the groups. Spearman correlation coefficients of subscale values were computed in order to quantify the strength of the relationship between the subscale scores of PHQ-4 and different dimensions of APGAR. The cut-off level for statistical significance was usually taken at 0.05. In correlation analysis, a second cut-off was set at 0.01.

3. Results

In terms of gender distribution, the female gender was dominant in both groups and no difference was found in the comparison. 57% of the case group and 51% of the control group were female. The density of adolescents in middle adolescence was remarkable in both groups without any significant difference (Table 1).

Tablo 1. Distribution of groups by gender

Gender	Chest Pain	Control	p
Male	29	33	0.491*
Female	39	35	
Total	68	68	
Age	15.20±1.36	15.04±1.34	0.488**

*Student's t-test, ** Chi-square test

In the comparison of Patient Health Questionnaire-4 scale results, both the anxiety and depression subscale scores were higher in the non-cardiac chest pain group with a statistically significance (Table 2).

Table 2. Comparison of the patient health questionnaire-4 and family apgar scale total and subscale scores between the groups

	Chest Pain (Mean ±sd)	Control (Mean ±sd)	p
Anxiety	2.58±1.69	1.76±1.43	0.003
Depression	3.17±1.37	2.14±0.99	**<0.001**
PHQ Total Score	5.76±2.77	3.91±2.06	**<0.001**
Adaptation	1.16±0.66	1.47±0.53	0.003
Partnership	0.85±0.65	1.47±0.65	**<0.001**
Growth	1.01±0.74	1.42±0.63	0.001
Affect	0.88±0.56	1.10±0.67	0.040
Resolve	1.05±0.64	1.47±0.55	**<0.001**
Total APGAR Score	4.95±2.04	6.94±1.62	**<0.001**

*Student's t-test; PHQ: Patient Health Questionnaire; APGAR: Adaptation, Partnership, Growth, Affection, Resolve

With regard to the Spearman correlation analysis negative reciprocal relations were found between the PHQ-4 and Family APGAR subscale scores (Table 3).

4. Discussion

In the current study, anxiety, depression and all determinants of family functioning were found to be worse in the non-cardiac chest pain group compared to the controls. Concerning the limited data in the literature, the adolescents with non-cardiac chest pain have shown a significant breakdown considering mental health (Bolat et al., 2018, Lieb et al., 2007; Lipsitz et al., 2005; McFarlane et al., 1994). However, this is the first study that showed the impaired family functioning with a brief mental health screening in this clinical group.

Among our participants, the majority of adolescents with non-cardiac chest pain were female. It has been reported that somatic complaints and internalization problems are more common among girls than boys. There are only two studies on non-cardiac chest pain in the adolescent age group. In the first study, no difference was found between the genders, whereas in the second study, the excess female sex ratio was noteworthy (McFarlane et al., 1994; Messer and Gross, 1995).

The mean age of the patients with non-cardiac chest pain in our study was 15.2±1.36 years and consisted mainly of the middle adolescence period. Adolescence period is divided into three as early adolescence (10-13 years), middle adolescence (14-16 years) and late adolescence (17-19 years). The psychosocial development of these three periods varies. In the middle adolescence, psychosocial development steps such as becoming emotionally independent from the family, the development of self-expression ability, and adopting traditional moral rules are accelerating. The problematic capacity of the adolescents to cope with the problems and the ability to respond to emotional problems may explain the increased incidence of non-cardiac chest pain in this group (Milov and Kantor, 1990).

In our study, anxiety, and depression symptoms were significantly higher in the non-cardiac chest pain group. The co-occurrence of depressive and anxiety disorders with somatic symptoms is associated with more functional disability, higher medical care use, and higher costs than the pathologies apart (Eliacık et al., 2020; Ohayon and Schatzberg, 2003). In a study conducted in Iran, where the psychosocial status of all adolescent chest pains was examined without exclusion of organic causes, anxiety and depression levels have shown to be significantly different in almost all patients (Messer and Gross, 1995). Following this study in the literature, in a study conducted in our center where all organic causes were excluded, and only adolescents evaluated, we found that anxiety scores were slightly higher, but there were significantly higher depression scores and more suicidal thoughts (McFarlane et al., 1994). Somatic symptoms may mask depression and delay accurate diagnosis and treatment (Özcan et al., 2011). Moreover, somatic complaints appear to be predictors of poor prognosis in depression. Studies have shown that depressive individuals with physical symptoms tend to have a longer period of illness (Lee et al., 2013).

In our study, the Family APGAR scale scores evaluating the family functioning of adolescents were found to be significantly impaired in the non-cardiac chest pain group. A family is a psychosocial group consisting of the patient and one or more persons, children or adults, in which there is a commitment for members to nurture each other. To establish the parameters by which a family's functional health can be measured, five basic components of family function were chosen. These components appear to represent common themes in the social science literature that deals with families (Khairandish et al., 2017). Regarding the results of our correlation analysis between the PHQ-4 and the Family APGAR scores, perhaps unsurprisingly, all the family functioning subscale scores were in a negative correlation with anxiety and depression.

Table 3. The reciprocal relation between the phq-4 and family apgar subscales scores

		phqtop	aa1	aa2	aa3	aa4	aa5	apgartop	anxiety
phqtop	Pearson Correlation	1	-.438**	-.419**	-.381**	-.287**	-.481**	-.639**	.915**
	Sig. (2-tailed)		.000	.000	.000	.001	.000	.000	.000
	N	136	136	136	136	136	135	135	136
aa1	Pearson Correlation	-.438**	1	.283**	.277**	.083	.281**	.601**	-.397**
	Sig. (2-tailed)	.000		.001	.001	.338	.001	.000	.000
	N	136	136	136	136	136	135	135	136
aa2	Pearson Correlation	-.419**	.283**	1	.202*	.232**	.358**	.679**	-.316**
	Sig. (2-tailed)	.000	.001		.018	.007	.000	.000	.000
	N	136	136	136	136	136	135	135	136
aa3	Pearson Correlation	-.381**	.277**	.202*	1	.152	.315**	.639**	-.340**
	Sig. (2-tailed)	.000	.001	.018		.077	.000	.000	.000
	N	136	136	136	136	136	135	135	136
aa4	Pearson Correlation	-.287**	.083	.232**	.152	1	.226**	.533**	-.225**
	Sig. (2-tailed)	.001	.338	.007	.077		.009	.000	.008
	N	136	136	136	136	136	135	135	136
aa5	Pearson Correlation	-.481**	.281**	.358**	.315**	.226**	1	.687**	-.409**
	Sig. (2-tailed)	.000	.001	.000	.000	.009		.000	.000
	N	135	135	135	135	135	135	135	135
apgartop	Pearson Correlation	-.639**	.601**	.679**	.639**	.533**	.687**	1	-.537**
	Sig. (2-tailed)	.000	.000	.000	.000	.000	.000		.000
	N	135	135	135	135	135	135	135	135
anxiety	Pearson Correlation	.915**	-.397**	-.316**	-.340**	-.225**	-.409**	-.537**	1
	Sig. (2-tailed)	.000	.000	.000	.000	.008	.000	.000	
	N	136	136	136	136	136	135	135	136
collapse	Pearson Correlation	.865**	-.383**	-.446**	-.340**	-.294**	-.455**	-.613**	.588**
	Sig. (2-tailed)	.000	.000	.000	.000	.001	.000	.000	.000
	N	136	136	136	136	136	135	135	136

**, Correlation is significant at the 0.01 level (2-tailed); *, Correlation is significant at the 0.05 level (2-tailed)

Family functioning is disrupted in families of youth with elevated depressive symptomatology (Tunaoglu et al., 1995). In particular, depression appears to be associated with family environments characterized by an absence of supportive and facilitative interactions. The most widely reported finding is that depression is inversely related to the level of support and approval provided by the family environment. In observational studies of families with adolescent children, parents of depressed children displayed less positive, rewarding, and supportive behaviors than did parents in comparison families (Sartorius, 2003; Selbst, 2010).

Although thorough medical evaluation is appropriate and necessary in chest pain, ongoing tests or treatments are often not found to be beneficial after the absence of a medical etiology of pain, and even patients have the potential to raise serious illness concerns (Smilkstein, 1978). Therefore, the necessity of rapidly evaluating psychosocial health in adolescents with chest pain after the exclusion of organic causes was shown in this study.

This study had several important limitations to consider. First, the cross-sectional nature of the study prevented the ability to detect direct causal relationships between the variables. Although it was not evaluated in our study, the loss of labor of parents, anxiety that occurs in the parents, on the other hand, the transportation costs, frequent hospital admissions, and therefore high hospital costs are stated as significant losses in the unresolved chest pain (Kroenke et al., 2009). As these problems increased in this cycle, we suggested that family functioning may be adversely affected and may lead to a vicious cycle of non-cardiac chest pain. Second, this study obtained anxiety, depression, and family functioning scores based on self-report questionnaires, which may be subject to bias due to patient self-concept and perception. However, the value of PHQ-4 and Family APGAR scales are largely related to the perception of the individual, and therefore this limitation should not impact findings given the value of understanding one's own report of mental health and family functioning. To shed more light on adolescent chest pain, which is a common cause of hospital admissions, longitudinal, prospective studies are needed. Other social problems (absenteeism, stigmatization in the form of heart disease, the financial loss experienced by the family, and the cost of social security institution due to frequent visits to the cardiology outpatient clinic), which did not include in this study, may be the subject of future studies.

The results of the study indicated a strong association between anxiety, depression, impaired family dysfunction, and adolescent non-cardiac chest pain. Since chest pain is a common referral to pediatric health services, there is a need for a brief psychosocial evaluation that comprised of mental health screening and family functioning that will prevent unnecessary medical investigations and expenses. The results suggested a need of a collaboration between pediatric emergency physicians, pediatric cardiologists, pediatricians, adolescents medicine physicians and psychiatrists for the adolescent non-cardiac chest pain.

Conflict of interest

None of the authors have any conflict of interest.

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Relationship between arylesterase activity and pulse pressure index in patients with an acute ischemic stroke

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Abstract

Stroke is a multifactorial disease. Arylesterase (ARE) activities have been considered as an anti-atherosclerosis factor. Increased pulse pressure (PP) may lead to a high risk of coronary artery disease and neurovascular morbidity and mortality. Nevertheless, there are limitations for PP as an evaluation index. In order to overcome the defects of PP, there is a novel parameter, "pulse pressure/systolic pressure" named "pulse pressure index (PPI)" for evaluation of cardiovascular effect. We researched the relationship between ARE activities and pulse pressure index (PPI) in acute ischemic stroke patients. We evaluated and compared the ARE activity and PPI in 87 ischemic stroke patients and 48 control patients. ARE activity was measured with the ultraviolet (UV) spectrophotometric method by using "Rel Assay Diagnostic" kits. Pulse pressure was measured by subtraction of diastolic blood pressure from systolic blood pressure. PPI was calculated as "pulse pressure / systolic pressure". Hypertension, age, diabetes mellitus, dyslipidemia importantly higher in ischemic stroke patients than the control group ($p<0.05$). PPI was significantly higher in ischemic stroke patients than the control group (0.486±0.075 and 0.417±0.051, $p<0.05$). ARE activity was significantly lower in ischemic stroke patients than in the control group (511.59 ± 68.51 and 584.16 ± 81.74 $p=0.019$). This study demonstrated that ARE activity is lower and PPI is higher in acute ischemic stroke patients than control subjects. Our results suggested that, ARE activity and PPI are important risk factors in acute ischemic stroke patients.

Keywords: arylesterase, pulse pressure, index, stroke

1. Introduction

The relationship between neurological disease and free radical activity have been researched in clinical trials. Cerebrovascular diseases are a multifactorial etiology originating from genetic and environmental predisposing factors (Kim et al., 2007). The pathophysiology of acute neurovascular diseases is carotid arter atherosclerosis (Aydin et al., 2019). Free radical activity and hypertension were associated with atherosclerotic neurovascular and coronary heart diseases. The generation of free oxidant activity is a significant pathophysiologic mechanism of causing to cerebral damage in the ischemic neurovascular disease (Michalak et al., 2011). Oxidized low density lipoproteins (LDL) in the arterial vascular wall are a significant pathophysiology of neurovascular atherosclerosis. ARE is a carboxylesterase. ARE enzyme catalyzes of fatty acids. ARE has got antioxidant property. This enzyme prevents oxidation of blood lipids. ARE has got an anti-atherosclerotic property (Mackness et al., 1991). ARE is preventing the oxidation of lipoproteins (Rozenberg et al., 2003). Therefore, ARE can decrease the progression of atherosclerotic disease. Also, ARE activity can influence atherosclerotic cerebrovascular disease. Increased vascular free radical activity is an important pathophysiology of arterial hypertension (Miyajima et al., 2007). In arterial hypertension, reactive oxygen radicals (ROR) can influence the antioxidant activity of enzymes. The importance of ROR in arterial vascular endothelial function and the pathophysiology of arterial hypertension have been lately investigated (Touyz and Schiffrin, 2004).

Elevated pulse pressure (PP) is a major clinical risk factor for the coroner and neurovascular morbidity and mortality (Assmann et al., 2005). Cardiovascular factors are affecting PP. Prior clinical trial findings on PP demonstrated that PP is a major risk factor for coronary artery disease, and mortality, genes may affect PP and gender may affect PP, cardiovascular disease and mortality (Lynch et al., 2007). Nevertheless, there is drawback for PP as an assessment for the index. On account of to get over the drawbacks of PP, there is

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a novel parameter, "pulse pressure/systolic pressure" termed "pulse pressure index (PPI)" for evaluation of cardiovascular consequences (Cai et al., 2015). We investigated the association between ARE activities and pulse pressure index (PPI) in acute ischemic stroke patients.

2. Materials and methods

This cross-sectional study included 87 adult patients (Females, 51; males, 36; mean age, 67.5 ± 12.7 years; range 40–89 years) with acute ischemic stroke (≤ 24 hours of symptom onset) admitted to the neurology care unit, between November 2013 and December 2014 and 48 control patients. They were classified into 2 groups: Ischemic stroke patients (Group 1, n=87), control patients (Group 2, n=48). Demographic, clinical characteristics, laboratory parameters of patients, including stroke severity assessment with National Institutes of Health Stroke Scale (NIHSS) on admission to the neurology care unit were recorded. Patient clinical data, history of cardiac and vascular predisposing risk factors, and the onset of acute stroke were established, and neurological physical examination was conducted at the time of admission to the care unit.

The diagnosis of acute ischemic stroke was made to attribute the neurological physical examination and cranial imaging within 24 hours of symptom onset. Patients with a well-defined time of symptom onset of acute ischemic stroke were included in this study. Patients who have any prior history of transient ischemic attack or cerebrovascular disease, intracranial hemorrhage were excluded. Exclusion criteria from the study are diseases that affect ARE activities. These diseases are chronic heart disease, diabetes mellitus, chronic renal disorder and malignancy. Admission stroke severity was determined using the NIHSS score (Lyden, 2017).

All patients taken immediate cranial computed tomography (Philips Brilliance 64, Royal Philips, Netherlands) after admission to the emergency care unit. Troponin values were calculated, and the electrocardiogram (ECG) was taken after admission to the neurology care unit. Echocardiographic examination (Vivid system 5, GE, Horten, Norway) was performed within the first 48 hours of admission to the neurology clinic. The NIHSS assessment and echocardiographic analysis were performed by blinded investigators. The study was accepted by the Ethics Committee of Dicle University Faculty of Medicine (the committee's date: 30-10-2013, Reference number: 411). Informed written consent to participate in this study was obtained from participants or their parents. The study was performed in proper with the guideline of the Helsinki Declaration.

Venous blood samples were obtained from the patients in admission to the neurology care unit. Blood samples were taken from the antecubital vein into blood tubes. Then blood samples were separated from the cells by centrifugation at 5000 rpm for 5 min. Serum samples were aliquoted and stored at -70°C until analysis. ARE activities were calculated with the UV spectrophotometric method by using "Rel Assay Diagnostic" kits. Arterial blood pressure calculations were performed in all stroke patients within 10 minutes after admission to the neurology care unit. Blood pressure was measured with a digital blood pressure measuring device after five minutes of rest, as recommended by the Joint National Committee-7 report (Chobanian et al., 2003). Patients were seated or lying on the bed with their arm bared and supported at heart level. Two readings, separated by 2 minutes, were obtained and averaged. Additional blood pressure measurements were obtained if these measurements differed by > 5 mm Hg. Pulse pressure was measured by subtraction of diastolic blood pressure from systolic blood pressure. PPI was calculated by "pulse pressure / systolic pressure".

Statistical analysis was carried out with the SPSS statistical package (Version 12.0; SPSS Inc., Chicago, IL, USA). All baseline clinical parameters were analyzed. Continuous variables were expressed as the mean ± SD; and categorical variables were expressed as percentages. Independent Sample T-Test, Mann-Whitney U test and Chi-square test, Pearson's Correlation test and regression analysis were used for comparison of data as appropriate. p values of < 0.05 were considered statistically significant.

3. Results

Clinical characteristics of patients are summarized in Table 1. Age, hypertension, systolic blood pressure (BP), diastolic BP (DBP), heart rate, PP, PPI, diabetes mellitus, dyslipidemia, glucose, creatinine, LDL cholesterol were significantly higher in ischemic stroke patients than control group ($p < 0.05$). ARE activity and HDL cholesterol being significantly lower in acute ischemic stroke patients than control group ($p < 0.05$).

Table 1. Clinical characteristics of patients

Variables	Group 1 (Ischemic Stroke) n=87	Group 2 (Control Group) n=48	p
Age (years)	67.5±12.7	44.3±17.2	0.023
Gender (F/M), n	51 / 36	26 / 22	0.321
Hypertension, n %	54 (63%)	10 (20%)	0.027
SBP (mmHg)	161.4 ± 18.7	122.8 ± 12.5	0.042
DBP (mmHg)	95.6 ± 11.9	71.9 ± 8.6	0.039
Heart Rate (bpm)	119.2 ± 19.5	85.1 ± 11.7	0.029
PP, mm-Hg	62.4±17.2	45.1±11.9	0.036
PPI	0.486±0.05	0.417±0.051	0.043
Diabetes Mellitus, %	28 (32 %)	3 (7 %)	0.014
Smoking, n %	19 (22 %)	4 (8 %)	0.627
Dyslipidemia, n %	23 (27%)	2 (4 %)	0.006
Glucose (mg/dl)	165.3 ± 35.6	103.4 ± 24.3	0.029
HbA1c	9.8 ± 2.5	5.7 ± 1.8	0.039
Creatinine (mg/dL)	1.7±0.6	0.8±0.2	0.029
ARE activity	511.59 ± 68.51	584.16 ± 81.74	0.019
LDL cholesterol (mg/dL)	141.4 ± 28.7	105.8 ± 15.9	0.029
HDL cholesterol (mg/dL)	33.8 ± 8.1	41.3 ± 8.3	0.041

F: Female, M: Male, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure, PP: Pulse Pressure, PPI: Pulse Pressure Index, ARE: Arylesterase, LDL: Low Density Lipoprotein, HDL: High Density Lipoprotein

Echocardiographic findings are summarized in Table 2. Left ventricle (LV) septal thickness, LV diastolic diameter, LV posterior wall thickness, and E/e' values were significantly higher in Group 1 patients than in Group 2 patients ($p < 0.05$). LVEF was significantly lower in Group 1 patients than in Group 2 patients ($p < 0.05$).

Table 2. Echocardiographic parameters of patients

Variables	Group 1 (Ischemic Stroke) n=87	Group 2 (Control Group) n=48	p
LV septal thickness	12.1±1.3	10.4±0.8	0.024
LVDd (mm)	54.8±6.3	49.1±4.3	0.045
LV posterior wall thickness, mm	11.4±1.2	10.6±0.7	0.032
LVDs (mm)	43.2±4.2	38.3±3.5	0.367
LAD (mm)	42.4±3.9	37.1±3.6	0.645
RAD (mm)	34.1±3.1	32.4±2.7	0.692
RVDd (mm)	29.8±2.6	27.9 ± 2.2	0.451
LVEF (%)	51.5±6.2	59.6±5.7	0.036
E/e'	10.3±3.6	7.1 ±2.75	0.027

LV: Left ventricle, LVDd: Left ventricular diastolic diameter, LVDs: Left ventricular systolic diameter, LAD: Left atrial diameter, RAD: Right atrial diameter, RVDd: Right ventricular diastolic diameter, LVEF: Left ventricular ejection fraction

Correlation analysis was performed to research the relationship between PPI and clinical parameters showed a negative correlation between the PPI and LVEF (left ventricular ejection fraction), ARE. In addition, there was a positive correlation between the PPI and E/e', age, heart rate, systolic blood pressure (SBP) (Table 3).

Table 3. Correlation of patients' characteristics and pulse pressure index

Parameters	Pearson's correlation coefficient (r value)	p
LVEF	-0.459	0.034
E/e'	0.376	0.038
ARE	-0.457	0.029
Age	0.435	0.045
SBP	0.483	0.038
Heart rate	0.324	0.041

LVEF: Left ventricular ejection fraction, ARE: Arylesterase, SBP: Systolic Blood Pressure

Logistic regression analysis was performed to identify the potential predictors for PPI. Results of the multivariate analysis revealed that age, SBP, LVEF, ARE and heart rate were a powerful predictor of PPI (Table 4).

Table 4. Multivariate logistic regression analysis between PPI and clinical parameters

Parameters	OR	95 % CI	p
SBP	0.647	0.523-0.614	0.028
LVEF	0.789	0.651-0.915	0.036
ARE	0.562	0.376-0.829	0.043
Age	0.482	0.287-0.792	0.037
Heart rate	0.381	0.256-0.691	0.035

SBP: Systolic Blood pressure, ARE: Arylesterase, LVEF: Left ventricular ejection fraction

4. Discussion

Acute stroke is identified as one of the significant reasons for mortality and morbidity (Feigin et al., 2014). Stroke is a multifactorial disease. This may account for why the incidence of acute stroke demonstrates racial and regional variations. There is a proof for ROR relevance in the pathogenesis of a lot of diseases, with a specific point on those related to atherosclerosis, such as diabetes, cardiovascular disease, acute stroke, and chronic renal diseases. ARE acts as a significant component of the enzymatic antioxidant system with PON-1, which has the same functions. Moreover, both PON-1 and ARE interact to form a significant component of the enzymatic plasma antioxidant system (Sahin et al., 2019). Shenhar TS and colleagues found that ARE was significantly lower in acute stroke patients who had any degree of white matter lesion (WML) than WML-free patients (Shenhar-Tsarfaty et al., 2013). Strokes are associated with changes in serum ARE activity (Can Demirdöğen et al., 2008). The present study is the first study in the literature that evaluates the relationship between ARE activity and PPI in acute ischemic stroke patients. The main finding of our study is that ARE activity is significantly decreased in acute ischemic stroke patients than in control subjects. Also, Chawhan and colleagues (2015) suggested that ARE enzyme activity is substantially reduced in ischemic stroke patients compared to healthy controls. They are suggested that, ARE is a risk factor for ischemic stroke. Demirdogen and colleagues demonstrated that the arylesterase activities of acute ischemic stroke patients were lower than those of control subjects, regardless of the genotype group they belong to (Can Demirdöğen et al., 2008). In the opinion of Wannamethee and colleagues, low HDL-C value is a significant predisposing component for the initiation of stroke (Wannamethee et al., 2000). In this research, we observed that HDL is importantly lower in acute ischemic stroke patients than in healthy controls. Also, we observed that LDL cholesterol was importantly higher in acute ischemic stroke patients than in healthy controls.

Acute stroke is characterized by profound autonomic dysregulation, including changes in the autonomic reflex pathways, central autonomic neuroanatomical locations and hormonal factors. According to prior investigations on BP changes during the early period of stroke, there is higher increase of SBP than DBP during the acute stroke (Morfis et al., 1997). The acute stress response to stroke events increased sympathetic tone, and compensatory response to cerebral ischemia might account these evidences (Castillo et al., 2004). As a result, an increase of PP could have occurred if there was a more prominent elevation of SBP than DBP during the acute ischemic stroke period. In this study, we found that SBP, DBP, heart rate, PP, PPI were significantly higher in acute ischemic stroke patients than in healthy controls. Lee and colleagues suggested that PP when calculated in the early period of acute ischemic stroke has relationships with major cerebrovascular and cardiovascular events and recurrent stroke. Also, they are

suggested that PP has an important predictive power than other usually used BP parameters (Lee et al., 2018). For ischemic stroke events, other analyses based on the REGARDS population demonstrated that PP was an independent predictor of stroke event after adjusting for DBP or mean arterial pressure, but not after adjusting for SBP (Glasser et al., 2015). A meta-analysis of 16 cohort clinical studies in Japan suggested that a substantial association between PP and ischemic stroke in men (Miura et al., 2009). On the other hand, SBP and DBP were not compared with PP in this meta-analysis. Recently, Chang JJ and colleagues reported that PP as an independent predictor for in hospital mortality in patients with spontaneous intracerebral hemorrhage (Chang et al., 2017).

Hypertension, age, hyperlipidemia and Diabetes Mellitus are major risk factors for atherosclerotic cerebrovascular disease (Wu et al., 2010). In our study, hypertension was importantly more common in acute ischemic stroke patients. Age was significantly higher in acute ischemic stroke patients than in healthy controls. Stroke-related sympathetic activation is high in patients with acute ischemic stroke. Irrespective of prior cardiovascular status, an acute phase of stroke markedly influences LV function, and biochemical parameters (Glucose, troponin, creatinine) (Ripoll et al., 2018). In this research, we observed that LVEF was importantly lower in acute ischemic stroke patients than in healthy controls. Öztürk and Ozturk (2019) found that the severe acute ischemic stroke patients had lower LVEF.

Hendrix and colleagues (2019) found that diabetes mellitus history is an important predictor of stroke severity. Lindsberg and colleagues suggested that increased blood glucose is frequent in the acute period of stroke (Lindsberg and Roine, 2004). In our study blood glucose and HbA1c levels were significantly in acute ischemic stroke patients than in healthy controls. Lindsberg and Roine (2004) reported that diabetes is frequent in severe acute ischemic patients. But, stress related hyperglycemia is more common in these patients. In this research, we observed that E/é value was importantly higher in severe stroke patients. Ryu and colleagues (2018) suggested that E/e' ratios were associated with carotid arterial occlusion in AF-related stroke and may play a role in identifying patients at high risk of severe stroke. In this research, we observed that creatinine levels were importantly higher in acute ischemic stroke patients. Mostofsky and colleagues (2009) suggesting that shared risk factors underlying vascular diseases including age, diabetes mellitus, hypertension, left ventricular hypertrophy may indicate vascular pathogenesis resulting from reduced renal clearance. Renal function predicts survival in patients with acute ischemic stroke.

In conclusion, this study demonstrated that ARE activity is lower and PPI is higher in acute ischemic stroke patients than control subjects. Our results suggested that, ARE activity and PPI are important risk factors in acute ischemic stroke patients.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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The clinical and prognostic value of the neutrophil lymphocyte ratio, the platelet lymphocyte ratio and mean platelet volume in tinnitus patients

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Abstract

There are high number of studies about the relationship between inflammation and tinnitus. The aim of this study was to evaluate the prognostic importance of the use of the neutrophil-lymphocyte ratio (NLR), the platelet lymphocyte ratio (PLR), and the mean platelet volume (MPV) in patients with tinnitus. This prospective study included 74 patients and 65 healthy control group. The severity of the tinnitus was classified using the Tinnitus Handicap Index (THI). The neutrophil and lymphocyte counts, NLR, PLR and MPV values were recorded and compared between the groups. MPV, and MCV median values were determined statistically significantly higher in the patient group than in the control group (p:0.001, p:0.000). No statistically significant difference was determined between the groups in respect of the NLR and PLR (p>0.05). The mean THI score was calculated as 51.5±21.1 (range, 12.0- 90.0). No statistically significant correlations were determined between the THI and NLR and PLR (r:0.03, p:0.75, and r:0.04, p:0.70). The MPV and MCV values were higher in tinnitus patients than in the control group. However, NLR and PLR were not determined to have any statistically significant relationship with tinnitus or the THI scores.

Keywords: mean platelet volume, neutrophil-lymphocyte ratio, platelet-lymphocyte ratio, tinnitus, tinnitus handicap index

1. Introduction

Tinnitus is the perception of sound when there is no acoustic stimulus in the environment. The sound perceived may be a pure tone or include more than one frequency. It can be pulsatile or fixed at a high or low pitch, in the form of a bell ringing, buzzing, murmuring, clicking, hissing, or a rough sound. As tinnitus is not a disease but a symptom, the ideal approach requires a comprehensive evaluation and the appropriate application of treatment tools and strategies (Jonas and Byron, 2013).

The incidence of tinnitus in the general population varies between 12% and 15%, and for 1-2% of patients, quality of life is severely affected (Kemal et al., 2016). The incidence is increasing in the 40-70 years age range, rising to 33% in patients aged >60 years. It is seen more frequently in males than females, but is rare in children (Jonas and Byron, 2013; Kemal et al., 2016).

Complete blood count (CBC) is an important and commonly used blood test in clinical practice. Several studies in recent years have used the neutrophil-lymphocyte ratio (NLR), the platelet lymphocyte ratio (PLR), and the mean platelet volume (MPV), obtained from CBC values as markers in inflammatory processes. These rates are obtained from neutrophil and platelet values, which are markers of inflammation, and as a result of synthesis with lymphopenia which is a marker of psychogenic stress (Aydoğdu et al., 2017). Sudden sensorineural hearing loss, Bell's palsy, vestibular neuritis, squamous cell carcinoma of the head and neck, and tinnitus are some of the pathological conditions in ear, nose and throat (ENT) practice that have been determined to be related to NLR and PLR.

The mean platelet volume (MPV) is an inflammation marker showing the activation and function of platelets and can be easily determined from peripheral blood. A high MPV level is a sign that thrombocyte activity has increased causing more intense inflammation (Aydoğdu et al., 2017). An elevated MPV level has been found to be related with various pathological states including cardiovascular and cerebrovascular impairments and primarily deep vein thrombosis (Kemal et al., 2016).

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It has been shown in literature that NLR, PLR and MPV as prognostic factors are high in patients with tinnitus and could be used as a marker (Ozbay et al., 2015; Sarıkaya et al., 2016; Kemal et al., 2016; Ulusoy et al., 2018; Kemal et al., 2019). The aim of this study was to evaluate the prognostic importance of NLR, PLR, and MPV values as clinical markers in patients presenting at our clinic with the complaint of tinnitus.

2. Materials and methods

A prospective examination was made of the findings of 74 patients between August 2016 and August 2018 with the complaint of tinnitus. The patients included were those who had the complaint of tinnitus for at least two weeks, with no systemic disease, no recent history of infection, and no findings of active ear infection.

All patients underwent a detailed ENT examination and audiometric tests to rule out any related pathological cause of the tinnitus. Those with moderate or severe hearing loss were excluded from the study because the hearing loss would have affected their ability to perceive tinnitus. Tinnitus patients with systemic diseases, malignancy or any inflammatory disease that could alter the neutrophil-to-lymphocyte ratio were also excluded from the study. Approval for the study was granted by the Local Ethics Committee of Ondokuz Mayıs University School of Medicine, Samsun, Turkey (Decision no: 2016/418). A control group was formed of the data of 65 age and gender-matched healthy individuals with no systemic disease and no recent history of infection, with data recorded in the clinic archives.

From the data obtained from the CBC of the patients; the MPV, neutrophil, lymphocyte, NLR, PLR and MCV values were determined. These values were compared with the data obtained from the control group. Evaluation was also made of whether there was any correlation between these values and the severity of tinnitus. To determine the subjective severity of tinnitus, the Tinnitus Handicap Index (THI), which is a standard scale, was used. The THI is a form comprising 25 items which evaluate the emotional, catastrophic and functional effects of tinnitus and measures the effects on the daily life of the patients (Table 1).

Responses are given as three alternatives of "Yes, No and Sometimes". Scoring is applied as Yes = 4 points, sometimes = 2, and No =0, and thus the total points can range from 0 to 100 (Newman et al., 1996; Meikle et al, 2012). The THI was applied to all the patients in the study to determine the subjective severity of tinnitus and the correlations between THI and the NLR, and PLR were evaluated.

2.1. Statistical analysis

All data were analyzed statistically using SPSS version 15 software (SPSS Inc., Chicago, IL, USA). Continuous variables were stated as mean ± standard deviation (SD), minimum and maximum values, and frequency data as number (n) and percentage (%). Conformity of the data to normal distribution was assessed with the Kolmogorov-Smirnov test. Data obtained from the counts were evaluated with the Chi-square test and continuous variables not showing normal distribution, with the Mann Whitney U-test. Relationships between the THI, NLR and PLR were evaluated with the Spearman Correlation test. A value of $p<0.05$ was accepted as statistically significant.

Table 1. Tinnitus handikap inventory (beta version)

Questions	Points		
	4	0	2
Because of your tinnitus is it difficult for you to concentrate?	Y	N	S
Does the loudness of your tinnitus meke it difficult for you to hear people?	Y	N	S
Does your tinnitus make you angry?	Y	N	S
Does your tinnitus make you confused?	Y	N	S
Because of your tinnitus are you desperate?	Y	N	S
Dou you complain a great deal about your tinnitus?	Y	N	S
Because of your tinnitus do you have trouble falling asleep at night?	Y	N	S
Do you feel as though you cannot escape from your tinnitus?	Y	N	S
Does your tinnitus interfere with your ability to enjoy social activites (Such as going out to dinner, to the cinema)?	Y	N	S
Because of your tinnitus do you feel frustrated?	Y	N	S
Because of your tinnitus do you feel that you have a terrible disease?	Y	N	S
Does your tinnitus make it difficult to enjoy life?	Y	N	S
Does your tinnitus interfere with your job or household responsibilites?	Y	N	S
Because of your tinnitus do you find that you are often irritable?	Y	N	S
Because of your tinnitus is it difficult for you to read?	Y	N	S
Does your tinnitus make you upset?	Y	N	S
Do you feel that your tinnitus has placed stress on your relationships with members of your family and friends?	Y	N	S
Do you find it difficult to focus your attention away from your tinnitus and on to other things?	Y	N	S
Do you feel that you have no control over your tinnitus?	Y	N	S
Because of your tinnitus do you often feel tired?	Y	N	S
Because of your tinnitus do you often feel depressed?	Y	N	S
Does your tinnitus make you feel anxious?	Y	N	S
Do you feel you can no longer cope with your tinnitus?	Y	N	S
Does your tinnitus get worse when you are under stress?	Y	N	S
Does your tinnitus make you insecure?	Y	N	S
Total Score Per Column			
Y, Yes; N, No; S, Sometimes			

3. Results

The statistical evaluations of the gender distribution and the NLR, PLR, MPV and MCV values of the patient and control groups are shown in Table 2.

The patient group had 62.1% males and 37.9% females with a mean age of 52.4±13.4 years where the control group had 52.3% males and 47.7% females with a mean age of 50.6±14.8 years. No statistically significant difference was determined between the groups in respect of age or gender (p>0.05, p>0.05).

The MPV and MCV median values were determined to be statistically significantly higher in the patient group than in the control group (p:0.001, p:0.000). NLR and PLR parameters were not statistically significant between two groups (p>0.05). In the evaluations applied, no significant difference was determined between the groups in respect of the other variables (Table 2).

Table 2. Demographic data and laboratory test results of the tinnitus group and the control group

	Tinnitus Group mean±sd (min-max)	Control Group mean±sd (min-max)	p
Female/male	28/46	30/33	0.326
Haemoglobin (g/dl)	13.8±1.4 (10.0-16.4)	13.7±1.8 (8.3-18.5)	0.974
MCV	87.0±5.5 (71.8-101.1)	83.7±5.8 (60.8-96.4)	0.001
Red cell distribution width (µm)	13.6±1.9 (11.8-24.3)	13.3±1.5 (11.6-20.3)	0.457
Mean platelet volume (µm3)	10.3±0.8 (8.7-13.1)	8.3±0.8 (6.4-10.9)	0.000
NLR	2.2±1.1 (0.9-7.3)	2.2±1.1 (0.6-6.3)	0.501
PLR	118.8±45.8 (5.9-332.2)	130.9±76.6 (60.-583.0)	0.489

The THI score of the whole patient group was determined as mean 51.5±21.1 (range, 12.0-90.0), as mean 58.6±24.2 (range, 14.0-88.0) for females and mean 47.2±18.0 (range, 12.0-90.0) for males, with no statistically significant difference determined between the genders (p:0.08). Correlations between the THI and NLR and PLR were evaluated, and no statistically significant correlations were determined (r:0.03, p:0.75 and r:0.04, p:0.70, respectively).

3. Discussion

Tinnitus, which is frequently encountered in ENT practice, can be defined as the perception of sound without any sound stimulus (Eğilmez et al., 2014).

The sound perceived by the patient in objective tinnitus originates in any region of the body and forms as a result of turbulent blood flow in particular and of muscle contractions in the head and neck region. The sound perceived by the patient in subjective tinnitus is formed without any known sound source and is only heard by the patient. While a cause may be determined as a physical examination finding or radiologically in objective tinnitus, if no cause can be shown, it can be referred to as subjective tinnitus (Eğilmez et al., 2014).

Although various studies have been conducted on the etiology of tinnitus, the etiology has not been fully clarified. It may originate from impairments in the outer, middle or inner ear or in the auditory nerve. Tinnitus has been reported to be related to several potential factors such as audiological, neurological, cardiovascular, metabolic, infectious, drug-related, dental, psychological and physiological impairments (Jonas and Byron, 2013; Kemal et al., 2016).

Neutrophils and lymphocytes are known to be responsible for inflammation. MPV is another marker reflecting thrombocyte activity and has been shown to be related to inflammation and the severity of inflammation. Neutrophil, platelet and monocyte concentrations increase in the inflammatory response and lymphocyte concentrations in the peripheral blood flow decrease (Zahorec, 2001; Aydoğdu et al., 2017).

NLR and PLR can be easily calculated from the peripheral blood and are new biomarkers of subclinical inflammation. This has become an increasingly widely used, inexpensive, easy and practical method used in the diagnosis and prognosis of various diseases, as these are just as valuable as the inflammatory markers of IL-6, IL-1β, IL-8 and TNF-α, which have high costs (Seo et al., 2014; Aydoğdu et al., 2017).

In recent years, NLR and PLR have been used as prognostic markers in atherosclerosis and have been reported to be useful in the determination of peripheral artery obstructive diseases. NLR and PLR have been shown to be increased in systemic inflammation, in some gynaecological and gastrointestinal cancers, brain tumours and some cardiovascular diseases. It has also been shown that NLR could be useful in the determination of short and long-term mortality in acute coronary syndrome (Aydoğdu et al., 2017; Yersal et al., 2018).

In the current study, it was aimed to determine whether or not there was any relationship between tinnitus and NLR, PLR and MPV. In studies by Kemal et al. (2016) and Sarikaya et al. (2016) which examined the relationship between tinnitus and MPV, it was reported that the MPV value was statistically significantly higher in tinnitus patients than in a control group. Özbay et al. (2015) conducted a study to determine the relationship between NLR and inflammation in patients with severe tinnitus. The mean NLR was found to be significantly higher in patients than in the control group and it was concluded that NLR should be considered as a potential marker when evaluating tinnitus patients.

Ulusoy et al. (2018) examined the NLR, PLR and MPV values in tinnitus patients and found a significant difference between the patient and control groups in respect of the MPV value, but no significant difference was determined in respect of the other parameters.

In another study by Bayram et al. (2015) which examined the relationship between tinnitus and NLR, PLR and MPV, no significant relationships were determined and the authors concluded that NLR, PLR and MPV may not be useful

parameters in the routine clinical evaluation of tinnitus patients. Bucak et al. (2014) compared neutrophil count and NLR in Bell's palsy patients and a control group and reported that both neutrophils and the NLR were statistically significantly higher in the patient group than in the control group. In a study of patients with sudden hearing loss, Aydoğdu et al. (2017) examined the mean NLR and PLR and found that both ratios were statistically significantly higher in the hearing loss patients compared to the control group. In addition, as the NLR and PLR levels were higher in patients who recovered compared to those who did not recover, these ratios were evaluated as a poor prognostic factor.

The NLR was also found to be statistically significantly higher in sudden hearing loss patients compared to a control group in a study by Ulu et al. (2013) In the same study, the response to treatment was seen to be lower in patients with high NLR, and thus it was reported as a poor prognostic factor. In contrast, Karli et al. (2013) and Blaha et al. (2015) examined the relationship between MPV and the extent of sudden hearing loss and found no statistically significant correlation. In our study, MPV and MCV values were statistically significant in patients with tinnitus compared to control patients, but there was no significant difference between the two groups in terms of NLR and PLO. According to these findings, MPV and MCV may be used as a cheap prognostic criterion in tinnitus. But a prognostic cut off value should be determined.

In conclusion, the data obtained in this study were found to be consistent with previous findings in literature, in that MPV and MCV values were determined to be higher in patients with tinnitus. These findings support the view that inflammation has a role in the etiology of tinnitus. However, the other parameters examined of NLR and PLR, were not determined to have any statistically significant relationship with tinnitus or the THI scores.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Research Article

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Clinical deterioration of Alzheimer's disease patients during the Covid-19 pandemic and caregiver burden

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Abstract

To investigate a possible deterioration of the symptoms of Alzheimer's disease (AD) patients during a quarantine period of 60 days, and the impact on caregiver burden. This study consisted of 54 individuals (36 female, 18 male) aged 60 years and over who had been diagnosed with AD. During the quarantine period, caregivers of the patients (child, spouse, relatives) who were confined to their homes were contacted by phone and administered a survey composed of questions for neuropsychiatric symptoms and caregiver burden. Patients' mean age was 77.18±6.96 years. The most worsening symptom was forgetfulness (35.2%) and agitation (24.1%). Incoherent speech, introversion, sleep disturbance, hallucinations, carry on with hobbies, reduced personal care, changes in appetite, and incontinence were less deteriorated. Of the caregivers, 61.1% thought that something terrible would happen to the patient, and 38.9% felt they could not find time for themselves. Drug rejection increased the burden twofold. Telemedicine methods, providing opportunities for the caregiver and the patient to walk outside, and giving medication that dissolves in the mouth or epidermal patches may be useful to prevent disease progression during the lockdown.

Keywords: Alzheimer's Disease; caregiver burden; Covid-19; neuropsychiatric symptoms; quarantine

1. Introduction

Alzheimer's disease (AD) is the most commonly seen type of dementia in persons aged 65 years and above (Alzheimer's Association et al., 2013). As the condition significantly affects activities of everyday life, the provision of care by a patient relative or a professional caregiver is required. The provision of caregiver continues for years and may cause psychological and physical problems for the carer. The size of the burden for the caregiver is directly proportional to the carer being female, having a low level of education, depression, social isolation, material problems, the lack of alternatives to being anyone else than the particular patient's carer, the severity of the patient's cognitive impairment, and the number of hours of care provided (Adelman et al., 2014; Yu et al., 2015).

The COVID-19 infection presents with greater severity in the older patient group (Li et al., 2020), and the mortality from pneumonia is high (Du et al., 2020). For this reason, in Turkey, as in the rest of the world, quarantine measures due to the COVID-19 pandemic continue to be applied mainly for the older age group. However, while trying to protect AD patients from the risk of infection, inevitably, new mental and physical problems arise. In a study by Lara et al. (2020) with a group of 40 AD patients, agitation, apathy, and a worsening of aberrant motor activity were observed over a five-week quarantine period. In 30% of patients and 40% of carers, deterioration in the state of health was reported (Lara et al., 2020). Considering the uncertainty regarding drugs and vaccines, it is not yet possible to predict how much longer the COVID-19 pandemic will last. In this study, we aimed to establish possible deterioration of AD symptoms during a 60-day quarantine period among AD patients followed by our clinic and investigate the reasons for worsening and the burden for caregivers.

2. Materials and methods

Study participants were 54 persons aged 60 years and over (36 females, 18 males) with a diagnosis of AD according to DSM-IV-TR and NINCDS-ADRDA criteria. Approval of the ethic committee was obtained (10840098-604.01.01-e.19388). Patients with diagnoses of mixed-type dementia or other types of dementia were excluded. Patients' duration of education, the duration of AD, civil status, number of children, type of caregiver (child, spouse, relative, nurse), and sex of caregiver were recorded.

Caregivers of the patients who had stayed home during a 60-day quarantine (April 1st-May 30rd 2020) procedure were contacted by phone and administered a survey prepared by the

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neurology department of Medipol University Istanbul. All caregivers gave consent to participate the study. In the survey, the individuals caring for the patients were asked about deteriorating symptoms in 11 items (forgetfulness, rejection of medication, incoherent speech, agitation, introversion, sleep status, hallucinations, continued pursuit of their hobbies, personal hygiene, change in appetite, incontinence) for the last 2 months.

The caregiver burden was investigated with five questions:

Has the time you set aside for yourself diminished? Yes/No

1. Do you have the wish to leave the patient and go away? Yes/No
2. Are you more irritable than you used to be? Yes/No
3. Are you worried that something terrible might happen to the patient? Yes/No
4. Do you feel your health being affected? Yes/No

Any "Yes" answer to these questions was given a score of 1 point, "No," a score of 0. The sum of points from this survey was calculated as the caregiver burden score.

The reason for deterioration for the last 2 months during the quarantine procedure was investigated with five items:

1- I could not procure the medication for AD.
2- I was left to my own, could not call anyone for help in this period.
3- I could not take the patient to the doctor for a check-up.
4- I used to go out regularly with the patient, could not go out in this situation
5- I am agitated because COVID-19 can affect the patient.

2.1. Statistical analysis

Data were assessed using IBM SPSS Statistics, Version 25.0. In the evaluation of descriptive data, frequency, percentage, mean, standard deviation, and minimum-maximum values were used. Categorical variables were evaluated with chi-square analysis. In the evaluation of binary and non-parametrically distributed mean scores, the Mann-Whitney U test was used. A value of $p<0.05$ was considered statistically significant. To evaluate the effect of variables on the mean caregiver burden scores, a linear regression model was built with the variable that was significant in the preliminary analysis.

3. Results

Patients' mean age was 77.18±6.96 (60-94) years, mean duration of education 6.01±4.74 (0-18), and mean duration of illness 3.83±2.78 (1-14) years. Thirty persons were married, 24 were widowed. The average number of children was 3.77±0.50 (0-13). Of all patients, 72.2% received care from their child, 14.8% from their spouse, and 13.0% from another relative (nephew/niece, daughter-in-law). Sixty-three percent of the caregivers were female, 37% were male.

Before the quarantine, six patients (11.1%) had refused medication, 23 persons (42.6%) had been talking incoherently, 28 (51.9%) had shown agitation, 26 (48.1%) introversion, 23 individuals (42.6%) sleep disorder, 13 (24.1%) hallucinations, 24 persons (44.4%) were unable to pursue their hobbies, 14 individuals (25.9%) had issues with personal care, 24 (44.4%) had a change in appetite, and 22 patients (40.7%) were incontinent.

These are the rates of deterioration in clinical findings during the quarantine period:

Worsening of forgetfulness was reported for 19 persons (35.2%), two persons (3.7%) had more severe drug compliance problems, nine individuals (16.7%) spoke more incoherently, 13 patients (24.1%) were more agitated, 11 persons (20.4%) more introverted, 8 (14.8%) suffered sleep disturbance, seven (13.0%) had hallucinations, 5 (9.3%) could not carry out their hobbies, 2 (3.7%) showed reduced personal care, 9 (16.7%) had less appetite, and 6 (11.1%) had more problems with incontinence.

Of the caregivers, 27 (50.0%) felt they could not find time for themselves, 8 (14.8%) wished to leave the patient and go away, 21 (38.9%) said they were irritable, 33 (61.1%) expressed their fear that something terrible would happen to the patient, and 17 (31.5%) felt their health had suffered. In the range between 0 and 5 points, the average caregiver burden score was 1.98±1.38.

Regarding the reasons for deterioration:

1- I could not procure the medication for AD: 0 (0.0%)
2- I was left to my own, could not call anyone for help in this period: 1 (1.9%)
3- I could not take the patient to the doctor for a check-up: 3 (5.6%)
4- I used to go out regularly with the patient, could not go out in this situation: 18 (33.3%)
5- I am agitated because COVID-19 can affect the patient: 2 (3.7%)

While a lower rate of deterioration in the pursuit of hobbies was found for patients who were cared for by their children ($p=0.003$), no statistically significant relationship was found between degrees of relationship and deterioration in clinical characteristics ($p>0.05$ in each category).

Caregiver burden scores were significantly higher in the case of patients refusing to take their medication ($p=0.035$). For other clinical characteristics, caregiver burden scores were similar between patients who showed deterioration and those who did not ($p>0.05$ in each category).

In the linear regression model using the only variable to be significant in binary analyses, it was found that rejection of drugs increased the caregiver burden score about two-fold.

Table 1. Distribution of degrees of relationship for caregivers by state of deterioration of patients' clinical status

		Carer child n (%)	Carer spouse n (%)	Carer other relative n (%)	Test value χ^2; p
Forgetfulness	**No deterioration**	24 (61.5)	5 (62.5)	6 (85.7)	1.543; 0.462
	Deterioration	15 (38.5)	3 (37.5)	1 (14.3)	
Drug rejection	**No deterioration**	38 (97.4)	8 (100.0)	6 (85.7)	2.648; 0.266
	Deterioration	1 (2.6)	0 (0.0)	1 (14.3)	
Incoherent speech	**No deterioration**	32 (82.1)	7 (87.5)	6 (85.7)	0.175; 0.916
	Deterioration	7 (17.9)	1 (12.5)	1 (14.3)	
Agitation	**No deterioration**	31 (79.5)	6 (75.0)	4 (57.1)	1.625; 0.444
	Deterioration	8 (20.5)	2 (25.0)	3 (42.9)	
Introversion	**No deterioration**	31 (79.5)	7 (87.5)	5 (71.4)	0.596; 0.742
	Deterioration	8 (20.5)	1 (12.5)	2 (28.6)	
Sleeping status	**No deterioration**	35 (89.7)	7 (87.5)	4 (57.1)	5.038; 0.081
	Deterioration	4 (10.3)	1 (12.5)	3 (42.9)	
Hallucination	**No deterioration**	34 (87.2)	8 (100.0)	5 (71.4)	2.704; 0.259
	Deterioration	5 (12.8)	0 (0.0)	2 (28.6)	
Hobbies	**No deterioration**	38 (97.4)	7 (87.5)	4 (57.1)	**11.585; 0.003**
	Deterioration	1 (2.6)	1 (12.5)	3 (42.9)	
Personal hygiene	**No deterioration**	38 (97.4)	8 (100.0)	6 (85.7)	2.648; 0.266
	Deterioration	1 (2.6)	0 (0.0)	1 (14.3)	
Appetite	**No deterioration**	33 (84.6)	6 (75.0)	6 (85.7)	0.475; 0.789
	Deterioration	6 (15.4)	2 (25.0)	1 (14.3)	
Incontinence	**No deterioration**	33 (84.6)	8 (100.0)	7 (100.0)	2.596; 0.273
	Deterioration	6 (15.4)	0 (0.0)	0 (0.0)	

Table 2. Distribution of caregiver burden scores according to deterioration of patients' clinical characteristics

		n (%)	Total caregiver score mean±SD (min-max)	Test value Z; p
Forgetfulness	**No deterioration**	35 (64.8)	1.80 ±1.41 (0.00-5.00)	1.374; 0.170
	Deterioration	19 (35.2)	2.32±1.29 (0.00-4.00)	
Drug rejection	**No deterioration**	52 (96.3)	1.90±1.35 (0.00-5.00)	**2.065; 0.035**
	Deterioration	2 (3.7)	4.00±0.00 (4.00-4.00)	
Incoherent speech	**No deterioration**	45 (83.3)	1.89 ±1.40 (0.00-5.00)	1.154; 0.249
	Deterioration	9 (16.7)	2.44 ±1.24 (1.00-4.00)	
Agitation	**No deterioration**	41 (75.9)	1.95±1.36 (0.00-5.00)	0.280; 0.780
	Deterioration	13 (24.1)	2.08±1.50 (0.00-4.00)	
Introversion	**No deterioration**	43 (79.6)	1.98±1.46 (0.00-5.00)	0.132; 0.895
	Deterioration	11 (20.4)	2.00±1.10 (1.00-4.00)	
Sleeping status	**No deterioration**	46 (85.2)	2.07±1.44 (0.00-5.00)	0.986; 0.343
	Deterioration	8 (14.8)	1.50±0.93 (0.00-3.00)	
Hallucination	**No deterioration**	47 (87.0)	1.89±1.37 (0.00-5.00)	1.240; 0.232
	Deterioration	7 (13.0)	2.57±1.40 (1.00-4.00)	
Hobbies	**No deterioration**	49 (90.7)	1.92±1.37 (0.00-5.00)	1.040; 0.321
	Deterioration	5 (9.3)	2.60±1.52 (1.00-4.00)	
Personal hygiene	**No deterioration**	52 (96.3)	1.98±1.35 (0.00-5.00)	0.961; 0.981
	Deterioration	2 (3.7)	2.00±2.83 (0.00-4.00)	
Appetite	**No deterioration**	45 (83.3)	1.96±1.45 (0.00-5.00)	0.416; 0.677
	Deterioration	9 (16.7)	2.11±1.05 (1.00-4.00)	
Incontinence	**No deterioration**	48 (88.9)	1.90±1.36 (0.00-5.00)	1.283; 0.216
	Deterioration	6 (11.1)	2.67±1.51 (1.00-4.00)	

Table 3. Linear regression model showing the effect of changes in patients' clinical characteristics on caregiver burden score

	B (95% CI)	t	Sig.
Rejection of medication (binary)	2.096 (0.167-4.025)	2.181	**0.034**
(Constant)	1.904 (1.533-2.275)	10.292	0.000

4. Discussion

In our study, which included AD patients in the early-to-intermediate stage of the disease, neuropsychiatric symptoms were seen in the foreground. Before the quarantine, the most common symptom had been agitation (in about half the patients); issues about carrying on with their hobbies and appetite problems had also been common. Insomnia, incoherent speech and incontinence were found in 40% of patients. As patients with these symptoms remained isolated in

their homes together with their caregivers during quarantine, we investigated how this situation affected the course of AD and the burden on caregivers. The most significant change in patients was an increase in forgetfulness (35.2%), while deterioration of agitation symptoms (24.1%) was found in second place. Incoherent speech, introversion, rejection of medication, and sleep problems also deteriorated in one-fifth of patients. The deterioration in continuing with their hobbies was significantly lower in patients receiving care from their child compared to patients with other caregivers. Reasons for this observation may include the younger age of the patient's child compared to their spouse and the child's more considerable attention in comparison with other family members.

Studies by Peters et al. (2015) showed agitation and psychotic symptoms to be the most relevant predictors for the progression of dementia. Increased agitation and forgetfulness caused by the lack of opportunities to leave the house during quarantine may have led to the deterioration of all other symptoms. There are only a limited number of studies on this issue found in the literature. Research by Boutoleau-Brotonniere et al. (2020) with 38 AD patients quarantined for two months during the COVID-19 pandemic found deteriorating neuropsychiatric symptoms and a significant increase in forgetfulness in 10 individuals (26.3%); however, they showed this situation not to be correlated with caregivers' stress and the duration of the quarantine. In a study by Lara et al. (2020), five weeks of quarantine AD patients showed significant deterioration only in agitation, apathy, and aberrant motor activity, while no significant deterioration was found in neuropsychiatric symptoms like appetite, hallucinations, anxiety, and depression.

Physical activity is known to improve cognition (Jia et al., 2019) and neuropsychiatric symptoms in AD patients (Hoffmann et al., 2016). Remarkably, when we asked caregivers about the etiology of the reasons for patients' deterioration, they most commonly (33.3%) attributed the observed deterioration to the impossibility of taking the patients out for walks. We found no problems with the provision of drugs, nor did being on their own or not being able to take patients to medical check-ups cause concerns, and patient relatives were not overly worried about COVID-19, either.

Considering these aspects one by one, potential problems with drug procurement had been prevented by measures of the Turkish Ministry of Health facilitating the provision of medication to chronically ill patients during the pandemic. Due to COVID-19 infection risk, restrictions were applied to relatives and friends other than the caregiver visiting patients at home (Azarpazhooh et al., 2020), which may have caused carer and patient to feel even more lonely. However, in our study, only one person included this aspect among the reasons for the deterioration in the patient's symptoms. As for all persons living with a chronic illness, medical check-ups and management of pharmacotherapy are essential for AD patients, too. Nevertheless, only 5% of the caregivers considered the impossibility of seeing a doctor for check-up over two months during the pandemic a cause for deterioration.

COVID-19 affects mental health in 25% of the population (Choi et al., 2020). In a study in Turkey, the rate of anxiety during the COVID-19 pandemic increased to around 45% (Özdin and Bayrak Özdin, 2020). Government advice to stay home because of COVID-19 may cause increased stress, depression, and anxiety rates in society (Ozamiz-Etxebarria et al., 2020). However, patient relatives participating in our study reported a relatively low proportion of stress and anxiety (3.7%).

Caring for AD patients is difficult due to the progressive course of the disease and the presence of neuropsychiatric symptoms (Germain et al., 2009). Factors increasing the caregiver's burden have been researched in earlier studies. Anxiety, agitation, disinhibition, aggressive behavior, sleep problems, the carer being female and the patient's spouse, and social isolation are among the factors that contribute most to an increase in the carer's load (Isik et al., 2019). While a study made in care homes found emotional exhaustion in 25% of caregivers (Yıldızhan et al., 2019), this rate increased to 40% when care was provided by a family member (Truzzi et al., 2012). Many adult children in Turkey caring for their parents suffering from AD feel a responsibility towards their parents, do not trust care homes, and prefer providing care themselves (Ar and Karanci, 2019). In our study, around 63% of caregivers were female; 72% were children and 14% spouses. There was no caregiver who was not related to the patient. All of the caregivers had spent the 60 days in social isolation in their homes together with the patient.

Our study found that the disease progressed faster than expected and we also investigated the disease-related caregiver burden in addition. Half of the caregivers stated not to have enough time for themselves during the pandemic, while 61% expressed their worries that something terrible might happen to the patient. Around one-third of caregivers felt more irritable, and again one-third observed that their health had been affected. During this period, only eight persons expressed a wish to leave the patient and go away. The only parameter for an increase in the burden of care that we could establish was a lack of compliance with medication (twofold).

Among the limitations of our study are the inability to administer detailed neuropsychiatric tests before and after the pandemic, the impracticability of carrying out more detailed surveys to investigate the caregiver burden over the phone, and a low number of patients in our sample. Also we cannot obtain the information about the drugs of the patients as anti-dementia drugs and antipsychotic medication can affect the symptoms positively.

During a 60-day quarantine period, the symptoms most commonly found to deteriorate in AD patients were forgetfulness and agitation. In the caregivers' opinion, the most critical reason for deterioration was the impossibility of going out for walks. The caregiver burden increased during this period, mainly due to patients' problems being persuaded to take their medication more than other commonly seen neuropsychiatric symptoms.

The more frequent use of telemedicine through digital devices may help monitor neuropsychiatric symptoms of patients with dementia in general, not only during a pandemic, to solve problems that may emerge in the context of the disease (Cuffaro et al., 2020). Other contributions to addressing these issues might include allowing patients to go out at certain times, finding ways to reduce the caregivers' load, and prescribing medication that dissolves in the mouth or epidermal patches rather than tablets that may be hard to swallow

Conflict of interest

None to declare.

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None to declare.

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Evaluation of the change in liver stiffness after biliary drainage in patients with extrahepatic cholestasis

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Abstract

Increased liver stiffness (LS) due to extrahepatic cholestasis has been reported to reduce after biliary drainage. As far as we know, it has not been evaluated whether the method of drainage makes a difference in the change in liver stiffness until now. The aim of the study was to answer this question. The patients planned for endoscopic biliary drainage (EBD) or percutaneous biliary drainage (PBD) were enrolled for over an 18-months' time period. In those without chronic liver disease or liver tumor, liver stiffness was measured before and 10 days after the intervention, using acoustic radiation force impulse (ARFI) elastography. A total of 30 patients were included in the study excluding the ones not fulfilling the criteria and were divided into two groups: EBD group (n = 15) and PBD group (n = 15). The measurements were above the cut-off value for severe fibrosis (1.55 m/s) in all patients with a mean of 2.50 ± 0.72 m/s before drainage. The pre-drainage values of the PBD group were significantly higher than of the EBD group (2.79 ± 0.58 m/s vs 2.22 ± 0.74 m/s, $p = 0.02$). In all patients except 11, a significant reduction was observed in the stiffness values. The reduction with PBD was more significant than with EBD ($p = 0.04$). Percutaneous biliary drainage provided a more significant reduction in liver stiffness in patients with extrahepatic cholestasis. However, this result appears to be related to the nature of obstruction rather than the method of drainage, making the comparison weaker than expected from the original design.

Keywords: ARFI elastography, biliary drainage, extrahepatic cholestasis, liver stiffness

1. Introduction

Extrahepatic cholestasis (EHC) has been shown to increase liver stiffness (LS) irrespective of fibrosis. Distension of the bile ducts due to obstructed bile flow has been mainly accused of being the reason behind the elevation. It has also been demonstrated that such an increase usually becomes normal thanks to biliary drainage decompressing the obstruction (Millonig et al., 2008; Harata et al., 2011; Trifan et al., 2011; Yashima et al., 2011; Attia et al., 2014; Pfeifer et al., 2014; Kubo et al., 2016; Darweesh et al., 2020). The information and data about the change in LS, evaluated by quantitative US elastography methods including transient elastography (TE) and acoustic radiation force impulse (ARFI) elastography, have mostly been provided by the studies in which endoscopic biliary drainage (EBD) has been used as the primary treatment modality in the setting of EHC. Percutaneous biliary drainage (PBD) is the method that has not been compared with EBD with respect to its effect on LS yet, as far as we know. The aim of this prospective study was to assess whether the method of drainage has an associated impact on LS in patients with EHC, using ARFI elastography.

2. Materials and methods

2.1. Patients

Between March 2018 and October 2019, patients with EHC planned for biliary drainage either by endoscopic or percutaneous method were enrolled in this prospective study. Among them, those without dilated biliary radicles (<2 mm in diameter) or those having a serum total bilirubin level lesser than 2 mg/dl were not included in the study. In addition, patients with chronic liver disease or liver tumors were excluded. Liver stiffness measurement using ARFI elastography was made in all patients immediately before and on the 10th day after biliary drainage which was performed either by endoscopic or percutaneous method. Patients were excluded if biliary drainage was not feasible or biliary drainage was not successful in reducing serum bilirubin level. Additionally, patients who missed the post-drainage measurement, i.e. for whom only the pre-drainage measurement was available, were excluded. Patients who underwent biliary drainage more than one time within the 10 days period were also excluded.

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Fifty-two patients underwent ARFI elastography before drainage. Out of 52 patients, 22 were excluded from the study: 17 patients missed the post-drainage measurement, the attempts to perform EBD were failed in three patients, the biliary drainage with PBD was not successful in reducing the serum bilirubin level in one patient and one patient had to undergo PBD a few days after EBD. As a result, the study population included a total of 30 patients, who were divided into endoscopic biliary drainage (EBD; n=15) and percutaneous biliary drainage (PBD; n=15) groups (Fig. 1). This study was approved by the local Ethical Committee (Approval protocol number: 2018/3). Informed consent was obtained from all patients.

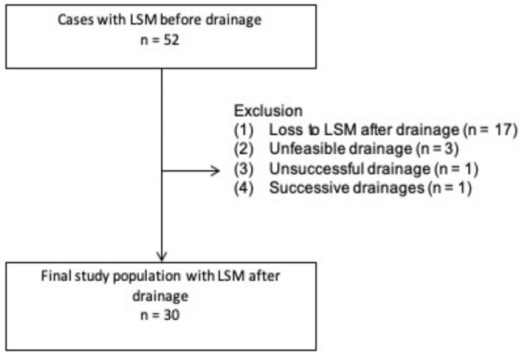

Fig. 1. Flow chart showing the selection of the study population. Twenty-two patients are excluded on the basis of the exclusion criteria, and a total of 30 patients are selected for statistical analysis. *(LSM liver stiffness measurement)*

2.2. Liver stiffness measurement

Liver stiffness was assessed by a single operator (F.U.) in order to avoid operator-related factors, complying with the current EFSUMB guidelines (Sporea, 2017). Using ARFI elastography (Virtual Touch™ Tissue Quantification; Siemens Medical Solutions) integrated in an ACUSON S2000® ultrasound platform (Siemens Medical Solutions, Erlangen, Germany) with a curvilinear array transducer (6C1 HD; Siemens Medical Solutions) at a frequency of 4 MHz, point shear wave speed measurement was performed corresponding with the quantitative value of LS. The measurements using ARFI elastography were performed in a supine position with an intercostal approach to the right liver lobe during relaxed breathing period. The region of interest (6 × 5 mm) was placed in the liver parenchyma with a distance of at least 2 cm from the liver capsule while ensuring not to include visible vessels and biliary radicles.

At least 10 validated measurements were performed in each patient. Results were expressed as the mean value of all measurements, in metres per second (m/s). The results were considered to be reliable when a success rate of measurement of greater than 60% (i.e., the ratio between validated and total measurements) was obtained. Measurements were successful in all patients.

2.3. Biliary drainage

The EBD procedures were performed by two experienced operators (I.G. and A.B.) using a duodenal endoscope (TJF-160VR, Olympus, Tokyo, Japan). The procedures included sphincterotomy, balloon sweeping and stenting, either alone or in combination. Two patients had a sphincterotomy, three patients had plastic stenting, seven patients had a sphincterotomy with balloon sweeping, one patient had a sphincterotomy with plastic stenting, and two patients treated with a combination of all three techniques. The PBD procedures were performed by two experienced operators (F.U. and A.I.S.) under fluoroscopic guidance (Artis Zee, Siemens Medical Solutions, Erlangen, Germany). In all PBD procedures except one, an external/internal biliary drainage catheter (Flexima, Boston Scientific, USA) was placed after crossing the obstruction. Only one patient had a procedure using external drainage due to fixed stenosis.

2.4. Serum cholestasis markers

Serum bilirubin, alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT) and alanine aminotransferase (ALT) were recorded before biliary drainage and at 10 days post-biliary drainage. Other well-known markers of cholestasis such as serum bile acids were not obtained as they were not routinely measured in our clinical setting.

2.5. Sample size

In this study, we aimed to compare the effect of endoscopic and percutaneous biliary drainage on LS, using ARFI elastography. Based on previously published data (Pfeifer et al., 2014), this study must have enrolled 15 individuals for each group to have 90% power with 5% type I error to detect a minimum clinically significant difference of 0.89 units, when the average expected value of the LS measurements before drainage was 2.96, with a standard deviation of 0.95, using a paired Student's *t*-test. Assuming dropouts, we set our enrollment goal as 40 patients.

2.6. Statistical analysis

Statistical Package for Social Sciences for Windows, version 21.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. All data were presented as mean ± standard deviation (SD). Repeated-measures analysis of variance (ANOVA) was used for comparison of means regarding the LS measurements and laboratory data. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Patient characteristics

The study included a total of 30 patients with EHC. A summary of the basic patient characteristics was presented in Table 1. Most of the patients in the PBD group had cholestasis due to malignant biliary obstruction (11/15), whereas the EBD group mostly included patients with benign causes (13/15). The most common cause for biliary obstruction in the whole study population was choledocholithiasis (40%).

Table 1. Patient characteristics

	EBD group[a] n = 15	PBD group[b] n = 15	*p*
Male/Female	10/5	10/5	1.0[#]
Age, mean (range)	54.9 (24–82)	65.9 (40–89)	0.09*
Causative diseases (benign/malignant)	13/2	4/11	**0.03**[#]
Benign			
Stenosis of biliodigestive anastomosis	1	2	
Choledocholithiasis	10	2	
Periampullary diverticulum	1	None	
Autoimmune pancreatitis	1	None	
Malignant			
Pancreas carcinoma	None	4	
Periampullary tumor	2	4	
Extrahepatic biliary obstruction by metastatic carcinoma	None	3	

[a] Patients treated with endoscopic biliary drainage
[b] Patients treated with percutaneous biliary drainage
[#]*p*-values are calculated with chi-square test, and **p*-value with Mann-Whitney U test. Boldface type indicates statistical significance

3.2. Changes in liver stiffness and serum cholestasis markers

Liver stiffness measurements by ARFI elastography and laboratory data before biliary drainage (Day 0) and on the 10th day after biliary drainage (Day 10) were presented in Table 2.

Table 2. Liver stiffness measurements by ARFI elastography and laboratory data of the patients

	EBD group[a] n = 15		PBD group[b] n = 15		*p*		
					Treat	Time	Int
	Day 0	Day 10	Day 0	Day 10			
LS (m/s)	2.2±0.7	2.0±0.7	2.7±0.5	2.4±0.8	**0.04**	**0.03**	0.55
T-bil (mg/dl)	8.1±7.2	2.3±2.7	12.6±6.7	5.1±5.5	0.07	**<0.001**	0.31
ALP (IU/L)	343±245	175±111	517±378	242±122	0.12	**<0.001**	0.23
GGT (IU/L)	479±439	164±126	607±513	213±196	0.44	**<0.001**	0.53
ALT (IU/L)	257±145	69±47	151±107	54±32	**0.04**	**<0.001**	**0.03**

All data are shown as means ± SD.
LS liver stiffness, *T-bil* total bilirubin, *ALP* alkaline phosphatase, *GGT* gamma-glutamyl transferase, *ALT* alanine aminotransferase
[a] Patients treated with endoscopic biliary drainage
[b] Patients treated with percutaneous biliary drainage
p-values are calculated with repeated measures ANOVA model. Boldface type indicates statistical significance.

The measurements before biliary drainage were above the previously determined cut-off value for severe fibrosis (1.55 m/s) in all patients with a mean of 2.50 ± 0.72 m/s (Friedrich-Rust et al., 2012). Additionally, the mean of the measurements in the PBD group was significantly higher than the EBD group (2.79 ± 0.58 m/s vs 2.22 ± 0.74 m/s, $p = 0.02$, respectively). In all patients except 11, a significant reduction was observed in the measurements secondary to biliary drainage (from 2.52 ± 0.63 m/s to 1.92 ± 0.49 m/s, $p < 0.001$). This reduction in the PBD group was more significant than in the EBD group (Fig. 2).

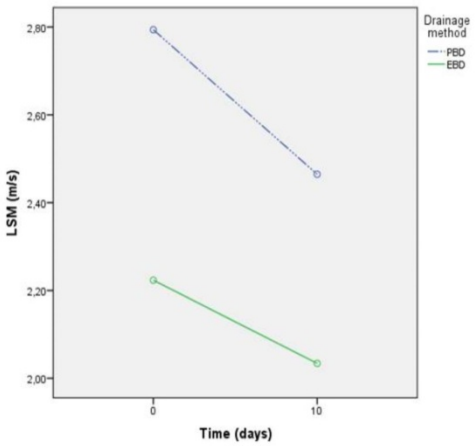

Fig. 2. Graphic showing the change in liver stiffness vs time. The reduction gradient obtained by each drainage method is represented as colored lines. The dashed dark blue line represents PBD and the solid green line EBD. It is obviously seen that the reduction gradient caused by PBD is steeper than by EBD (*LSM* liver stiffness measurement, *PBD* percutaneous biliary drainage, *EBD* endoscopic biliary drainage)

In a subgroup of 11 patients, it was found that LS kept increasing despite successful biliary drainage (from 2.57 ± 0.93 m/s to 2.95 ± 0.97 m/s). Of the 11 patients in this subgroup, six underwent EBD and five underwent PBD. The basic characteristics of this subgroup of patients was presented in Table 3.

In both two groups, it was found that pre-drainage levels of serum bilirubin, ALP, GGT and ALT were all elevated, as expected. Among them, only the levels of ALT in the EBD group were higher than the PBD group before drainage. A remarkable reduction was detected in the levels of all markers after drainage. The reduction in serum total bilirubin levels in the PBD group was considered as more significant than in the EBD group, even though the *p*-value was slightly greater than the significance level (p=0.07). The reduction in ALT levels was significantly higher in the EBD group than in the PBD group, and there was a significant time-treatment (i.e. biliary drainage) interaction, as well. However, the difference between the groups concerning the reduction in ALP as well as GGT levels was not statistically significant (Figs. 3, 4, 5 and 6). Finally, in the subgroup of patients whose LS kept increasing after biliary drainage, post-drainage levels of the serum cholestasis markers were all found to be decreased compared to the baseline levels, which was regarded as the evidence of successful biliary drainage.

Table 3. Characteristics of the patients whose liver stiffness kept increasing after biliary drainage

Patient	Age	Gender	Causative diseases	Drainage method	LS before drainage (m/s)	LS after drainage (m/s)
1	82	M	Choledocholithiasis	EBD	1.99 ± 0.36	2.09 ± 0.39
2	49	M	Choledocholithiasis	EBD	1.59 ± 0.16	1.71 ± 0.48
3	56	M	Choledocholithiasis	EBD	4.01 ± 0.52	4.39 ± 0.34
4	53	M	Choledocholithiasis	EBD	1.57 ± 0.48	2.85 ± 0.45
5	24	M	Autoimmune pancreatitis	EBD	1.58 ± 0.27	1.85 ± 0.37
6	72	M	Periampullary tumor	EBD	1.72 ± 0.13	1.78 ± 0.19
7	79	M	Stenosis of biliodigestive anastomosis	PBD	3.07 ± 0.78	3.28 ± 0.47
8	67	F	Stenosis of biliodigestive anastomosis	PBD	3.19 ± 0.44	3.34 ± 0.82
9	89	M	Pancreas carcinoma	PBD	2.41 ± 0.41	2.53 ± 0.45
10	40	F	Choledocholithiasis	PBD	3.61 ± 0.73	4.25 ± 0.37
11	68	M	Extrahepatic biliary obstruction by metastatic carcinoma	PBD	2.56 ± 0.83	2.80 ± 0.46

All data are shown as means ± SD. *LS* liver stiffness, *EBD* endoscopic biliary drainage, *PBD* percutaneous biliary drainage

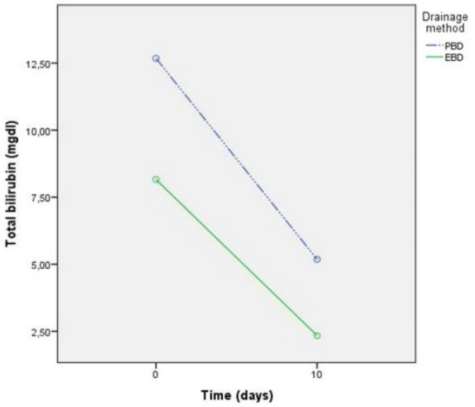

Fig. 3. Graphic showing the change in total bilirubin level vs time. Reduction gradients obtained by each drainage method are represented as colored lines. The dashed dark blue line represents PBD and the solid green line EBD. Although the reduction in serum total bilirubin levels obtained by PBD is considered as more significant than by EBD (please see the results chapter), the reduction gradients are almost similar (*PBD* percutaneous biliary drainage, *EBD* endoscopic biliary drainage)

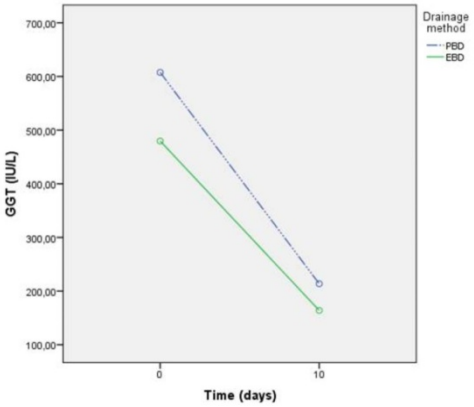

Fig. 5. Graphic showing the change in GGT level vs time. Reduction gradients obtained by each drainage method are represented as colored lines. The dashed dark blue line represents PBD and the solid green line EBD. There is not a significant difference in the reduction gradients caused by PBD and EBD, regarding the change in GGT level (*PBD* percutaneous biliary drainage, *EBD* endoscopic biliary drainage, *GGT* gamma-glutamyl transpeptidase*)*

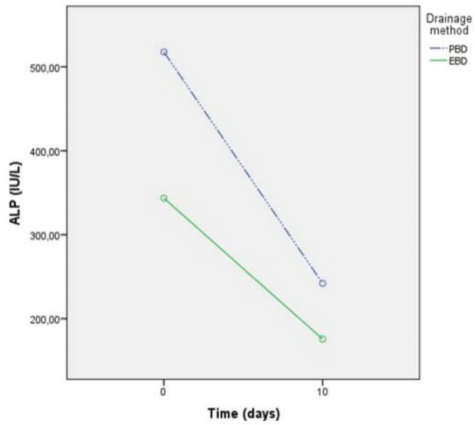

Fig. 4. Graphic showing the change in ALP level vs time. Reduction gradients obtained by each drainage method are represented as colored lines. The dashed dark blue line represents PBD and the solid green line EBD. There is not a significant difference in the reduction gradients caused by PBD and EBD, regarding the change in ALP level (*PBD* percutaneous biliary drainage, *EBD* endoscopic biliary drainage, *ALP* alkaline phosphatase*)*

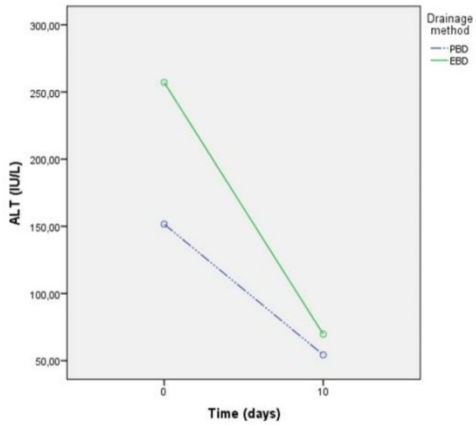

Fig. 6. Graphic showing the change in ALT level vs time. Reduction gradients obtained by each drainage method are represented as colored lines. The dashed dark blue line represents PBD and the solid green line EBD. Regarding the change in ALT level, it is seen that the reduction gradient caused by EBD is steeper than by PBD (*PBD* percutaneous biliary drainage, *EBD* endoscopic biliary drainage, *ALT* alanine aminotransferase*)*

4. Discussion

The main finding of this study is that malignant biliary obstruction causes LS to increase more significantly than benign obstruction. In the patients treated with PBD, the higher LS values as well as the higher serum bilirubin levels at baseline seem to be related to the increased severity of obstruction. In this group, the main reason for EHC was malignant diseases which typically pursue a progressive course, and which finally result in a complete obstruction. However, the patients treated with EBD mostly had EHC due to benign etiologies which are more likely to cause partial obstruction. Therefore, PBD may have yielded a more significant reduction in LS as well as serum bilirubin levels compared to EBD because of the worse cholestatic profile at baseline.

The present study confirmed that successful biliary drainage, either performed by endoscopically or percutaneously, does not necessarily reduce LS to the normal values. While improvement of the cholestatic laboratory profile was observed, the LS measurements did not decrease even after drainage in more than one-third of the patients in our study population. In contrast, an increase emerged when compared to baseline values. This result was slightly different from previously published reports. For instance, in the study reported by Harata et al. (2011), LS did not return to the normal levels in more than half of the patients. Another study revealing a similar result was reported by Attia et al. (2014), in which nearly half of the patients did not reach normal LS values at the end of the follow-up period, although the stiffness was found to be somewhat reduced. In the newly published report (Darweesh et al., 2020), it was noted that a reduction in LS was observed after adequate biliary drainage, even though the resultant values were slightly above the normal limits, measured using both TE and ARFI elastography. Failure to reach normal stiffness values indicates the impact of cofactors on LS. The mechanism behind the elevation of LS due to impaired bile flow was mainly attributed to the increased hepatic hydrostatic pressure (Millonig et al., 2008; Harata et al., 2011; Trifan et al., 2011; Yashima et al., 2011, Attia et al., 2014, Pfeifer et al., 2014; Kubo et al., 2016). Several studies have reported that impaired bile flow also causes cholestatic injury to the liver including hepatocellular necrosis, proliferation of the bile duct epithelial cells and liver fibrosis (Yashima et al., 2011, Kubo et al., 2016). Such changes in the tissue composition secondary to superimposed necroinflammatory damage may cause LS to increase even more (Gujral et al., 2003; Harata et al., 2011; Yashima et al., 2011; Yoon et al., 2012; Attia et al., 2014; Kubo et al., 2016). As a result, a delay may occur in the recovery of LS after an intervention. It seems likely that in the subgroup of patients whose LS kept increasing after drainage, the post-drainage measurements coincided with the secondary inflammatory and fibrotic changes. However, because of its invasiveness, liver biopsy was not performed to rule out probable secondary liver fibrosis formation. Except for this probability, the method of drainage did not appear to be effective on this consequence, because there was almost no difference in the frequency of each method of drainage in this subgroup.

It was previously suggested that LS negatively correlates with ALT levels in patients with EHC. The negative correlation between two variables was attributed to the leakage of bile flow from the bile duct lumen into adjacent potential spaces, with two outcomes (Harata et al., 2011). First, decompression of the bile ducts results in a reduction of hepatic hydrostatic pressure, which eventually causes LS to decrease. Second, ALT levels increase due to hepatocyte injury. Such a tableau is quite different from that observed in acute hepatitis, in which LS positively correlates with ALT levels because the inflammatory liver injury is dominant over increased hepatic hydrostatic pressure (Seo et al., 2010; Harata et al., 2011; Trifan et al., 2011; Bota et al., 2013; Choi et al., 2014). Although we did not analyze the correlation between LS and biochemical parameters, we observed that the patients in the EBD group had lower stiffness values, but had higher ALT levels than that of the patients in the PBD group. Considering the characteristics of the patients entered in the present study, the main difference between two groups was the etiology of biliary obstruction, which may have contributed to this situation. Because benign obstructions usually pursue a rapid course and can trigger acute inflammation, the ALT may have reached higher levels much earlier than LS in the EBD group. In contrast, because malignant obstructions typically pursue a prolonged course and damaged hepatocytes are capable of self-renewal over time, the baseline ALT levels may have been measured relatively less in the PBD group. The significant time-treatment interaction for the changes in ALT levels highlights the course of biliary obstruction, indicating a more rapid amelioration of liver functions in benign disorders.

There are several limitations to our study that need to be considered. First and most importantly, we could not compare the effect of both drainage methods on LS irrespective of the causative disease since the cases did not show a balanced distribution between the treatment groups. If that was possible, we could have clarified whether the method of drainage has an associated impact on the change in LS. This is a single-center study, in which there is an inherent bias in the treatment selection due to the obstructive jaundice patterns. Because of its invasiveness, PBD is not the preferred method of treatment for patients with EHC which EBD has the precedence. Secondly, relatively small sample size of the study may have limited the detection of a significant association between the change in serum bilirubin levels and the method of drainage. Finally, the correlation between LS and biochemical parameters was not analyzed separately. We do believe that a multicenter and larger randomized trial enrolling patient with similar obstruction patterns would be helpful to identify these issues.

In conclusion, our study clearly showed that malignant biliary obstruction increases LS more significantly than benign causes of EHC. Although a more significant reduction in LS was observed in the patients treated percutaneously than in the patients treated endoscopically, this consequence appears to be independent of the method of drainage. Additionally, it was confirmed that successful biliary drainage does not necessarily reduce LS, suggesting superimposed factors such as secondary liver fibrosis impairing the resolution. Finally, as previously defined, the negative correlation between LS and ALT levels was verified.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication that could have influenced its outcome.

Acknowledgments

None to declare.

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The investigation of the changes in the surface glycoconjugates using two different spheroid models of breast cancer cells and availability assessment of these spheroid models for rapid diagnosis

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Abstract

The importance of early cancer diagnosis has led to development of many different diagnostic methods. In this context, the studies investigating the presence and amount of sugar residues to use as indicators in the identification of cancer cell type have become prominent. In the present study, sialic acids found on the membrane surfaces of ER (+) MCF-7 and ER (-) MDA-MB-231 breast cancer cell lines were labeled using three-dimensional (3D) cell culture (Spheroid) model as the closest method to the patient sample, thus its natural environment, among the cell culture methods. These sugar units that play a role in regulation of important immune characteristics such as recognition, binding and metastasis were made visualizable by applying fluorescent-labeled lectins such as FITC-(Wheat Germ Agglutinin) specifically binding to sialic acid units (GlcNAc, Neu5Ac) including particularly ß-GlcNAc and FITC-(*Maackia Amurensis*-Lectin-1) specifically binding to Galß4GlcNAc type sialic acids. These glycan units were specifically labeled with FITC-(*Maackia Amurensis*-Lectin-1) and FITC- (Wheat Germ Agglutinin) and radiation intensities were analyzed relatively. The two different breast cancer cell cultures were compared with respect to change in the amounts of sialic acid residues containing α-2,3- and α-2,6 bonds using fluorescent-labeled lectins. In the present study, we have performed a precise, accurate and rapid determination of the sugar content in the different breast cancer cell surface lines by means of fluorescent-labeled lectins and carried out a relative comparison between the micrographs.

Keywords: breast cancer, lectins, sialic acid, spheroid

1. Introduction

Breast cancer is the most frequently diagnosed cancer type in women with estimated 1.38 million new cases per year worldwide. Totally 458,000 deaths occur due to breast cancer in both developed and developing countries each year. This number of deaths indicates that cancer is the most common cause of death in women (Eccles et al., 2013). Normal cells have a regular life cycle and limited number of cell divisions. Like all other cancer cells, breast cancer cells are the cells that altered cell division characteristics due to various mutations. This alteration is caused by the differentiated pathways of cell division. These differentiations result from the changes in the cell surface glycans (Kohnz et al., 2016). Sialic acids on the end units of the outer cell surface glycans play an essential role in cellular recognition and interaction processes with other cells and microenvironment. High concentrations of sialic acids may also lead to structural changes both in normal and cancer cell surfaces (Samraj et al., 2014).

The change in the amounts of sialic acid residues particularly containing α-2,3- and α-2,6 bonds located on the cell surfaces leads to development of avascular multicellular tumoral formations in normal and cancer cells. It has been demonstrated that they have a capability to form in vivo xenograft tumors particularly in the cancer cell lines. It is considered that the obtainable data will provide a supportive information infrastructure in selection of the appropriate cells, administration of the oriented treatment and establishment of the early diagnosis (Akasov et al., 2016).

Pathological tests are gold standard for diagnosis of cancer. These tests have an important role in identification of etiology and pathogenesis of cancer as well as assessment of clinical correlation and symptoms of the disease. The most novel molecular technologies, particularly high-throughput technologies, have detected that even morphologically similar subtypes of breast cancer may show molecular heterogeneity. For instance, infiltrating ductal carcinoma was found to have at least 4 molecular subtypes. These subtypes were classified

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as luminal (ER+, PR+, Her-2/neu-), Her-2 overexpressing (ER-, PR-, Her-2+), basal-like (ER+, PR-, Her-2-, CK5/6+, EGFR+) and normal breast-like (ER-, PR-, Her-2-) breast carcinomas. Each type shows different clinical results. The proliferative gene expression of these subtypes is detected by immunohistochemical markers including very expensive molecular tests such as Ki67, Her-2, PR and ER (Anthony et al., 2011). Beside all these tests and diagnostic techniques, the importance of glycobiology in cancer research studies has progressively increased in the recent years. Glycosylation may function as a regulator mechanism that can control various pathological processes. The glycosylation disorders in humans may lead to diseases. Human glycomics involves a substantial amount of biological information (Pinho and Reis, 2015). Among the cancer researches carried on cancer cells, the studies investigating the presence and amount of sugar residues to use as indicators in the identification of cancer cell type have become prominent. These studies are conducted using scan techniques such as PET-SCAN and their results are assessed for confirmation by comparing with lectin-labeling (WGA, SNA, MAL etc.) methods (Martinez-Duncker et al., 2011).

Lectins are the glycoproteins with a particular glycan-specific affinity. Glycoprotein enrichment through lectin-affinity is one of the main techniques that utilize specificity of lectins to a particular glycan residue or ligand type. Currently, common use of two different glycoprotein/glycopeptides are accepted as enrichment techniques. For instance, Wheat Germ Agglutinin (WGA) mainly interact with N-Acetyl Glucosamine (GlcNAc) residues that have essentially have a glycan structure. The enriched glycan structures located on the cancer cell surfaces can be visualized by means of the lectins that specifically bind to these glycans (Varki et al., 2017; Schaurer et al., 2018). On the other hand, a multilectin mixture has been used to bind and enrich many various glycans. Lectin enrichment technique were effectively used to analyze the glycoproteomic changes associated with different cancer types including lung, breast, and liver cancers (Song and Mechref, 2015).

In the two-dimensional (2D) cancer cell culture systems, cells are cultured as a single layer on a flat and rigid surface with lacking cell-cell and cell-matrix interactions of natural tumors. These cells with artificial polarity are exposed to a cell skeleton regulation different from original tumor cell microenvironment. That causes abnormal gene and protein expression. Contrarily, three-dimensional (3D) cell culture systems allow culturing cancer cells solely or together with various cell types. This procedure can closely mimic the natural environment of tumors and promote cell-cell and cell-matric interactions. The cells cultured by three-dimensional (3D) models in this way gain morphological and cellular characteristics similarly with in vivo tumors and thereby the closest cancer cell modelling directly to the patient samples can be directly realized. By means of this method, the impact of the microenvironment on the tumor can be evaluated most closely (Nath and Devi, 2016). For this purpose, the amounts of sugar residues were investigated by implementing immunofluorescent-labeled lectins FITC-[WGA] and FITC-[MAL-I] to the spheroids obtained from the MCF-7 [ER (+)] and MDA-MB-231 [ER (-)] spheroid (3D) cancer tissue/cell lines. By this was, it was aimed to demonstrate the availability of this labeling technique in the closest 3D cell model to the patient cancer tissue. Thus, it would be possible to determine the difference between the sugar profiles of the breast cancer cells.

Sugar residues were primarily preferred in this determination since these units control the recognition and binding mechanisms of the cell. That would facilitate for researchers identifying and clarifying the cells that were obtained from different sources and that have different characteristics. For this purpose, the fluorescent-labeled lectins applied to specifically identify the cell surface sugar lines will be visualized applying the modified staining techniques (Mater and Özdaş, 2018; Demircan and Mater; 2019) as in our previous studies. Thanks to the method used in this breast cancer cell model, a fast, specific and new labeling technique with the help of lectins has been developed. Thus, it was concluded that a more specific and rapid method can be created in the diagnosis of breast cancer.

2. Materials and methods

2.1. The preparation of 3D spheroid culture model for breast cancer cells

MCF-7 (ATCC®HTB-22™) and MDA-MB-231 (ATCC®HTB-26™) cell strains supplied from American Type Culture Collection (ATCC) were used in our study. The cells were incubated at 37°C, 5% CO_2 concentration and 1 atm pressure in their medium containing 10% inactivated fetal bovine serum (FCS, Gibco, Invitrogen Life Science, Paisley, UK)), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, St Louis, MO, USA). Leibovitz's L-15 Medium and Eagle's Minimum Essential Medium were used for MDA-MB-213 and MCF-7 cell strains, respectively.

The cells in semi-confluent flasks were harvested using 0.05% trypsin (Sigma-Aldrich), centrifuge (Nuve NF200, Laboratory and Sterilization Technology, Ankara, Turkey) following the addition of FCS for trypsin inactivation and then resuspended in culture medium. The cells counted for 3-dimensional (3D) spheroid model cell culture were seeded so that each well will contain a cell suspension of 1×10^3 cells/ml in the six-well microplates. The wells of the plates were coated with 3% Noble Agar (Difco Laboratories Inc., BD Diagnostic Systems Detroit, MI, USA) to form a non-adhesive surface. Two weeks after onset, the plates were examined regarding development of spheroid (colonies or spheres). The spheroids from each well were counted under microscope and measured, representative fields were photographed. Labeling was performed using fluorescent-labeled lectins for characterization of the spheroids.

2.2. Labeling procedure of the spheroids using lectins

Fluorescent-labeled lectins were purchased from Vector Laboratories (Burlingame, CA, USA) and prepared to be used according to the manufacturer's usage protocol. In the labeling process, the mediums of the spheroids were removed to maintain spheroids in the six-well plates. The spheroids were washed with PBS after removing mediums. Following this step, spheroids were incubated with fluorescent-labeled lectins for an appropriate period. After incubation, fluorescent-containing lectin was removed from the spheroids by washing with PBS and spheroids were left to dry for 10 minutes. Following this step, a special mounting solution (Fluoroshield Mounting Medium with DAPI, ab 104139, Abcam) containing DAPI (4' 6-diamidino-2-phenylindole) was dropped onto the spheroids and the samples were examined using Nikon-Eclipse-80i-Fluorescence-Microscope. The obtained micrographs were visualized to be evaluated using NIS Elements BR 3.0 Software provided by the same device.

3. Results

MCF-7 and MDA-MB 231 breast cancer cell lines were grown in the three-dimensional (3D) cell culture and spheroids were created in our study (Figs. 1A, 2A). In the next step, MCF-7 [ER (+)] and MDA-MB-231 [ER (-)] spheroids were stained using fluorescent-labeled lectins (Figs. 1B, 1C and Figs. 2B, 2C). Accordingly, sialic acid units (GlcNAc, Neu5Ac) and also glycan units containing particularly ß-GlcNAc type sialic acids were visualized by using FITC-[WGA] while Galß4GlcNAc type glycan units were displayed by applying FITC-[MAL-I] as the specifically binding fluorescent-labeled lectins.

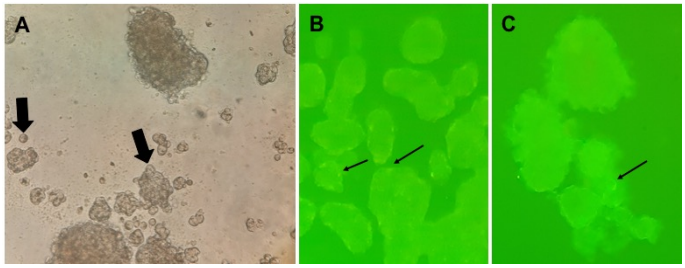

Fig. 1. Cells produced in MCF-7 three-dimensional (3D) cell line, spheroids. Control cells and spheroids [thick black arrow] (1A) and residues specifically stained with fluorescent-labeled WGA (1B) and MAL-I (1C) [thin black arrow] [200X]

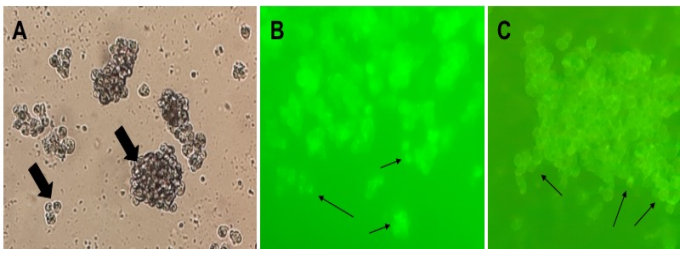

Fig. 2. Cells produced in MDA-MB-231 three-dimensional (3D) cell line, spheroids. Control cells and spheroids. Control cells and spheroids [thick black arrow] (1A) and residues specifically stained with fluorescent-labeled WGA (1B) and MAL-I (1C) [thin black arrow] [200X]

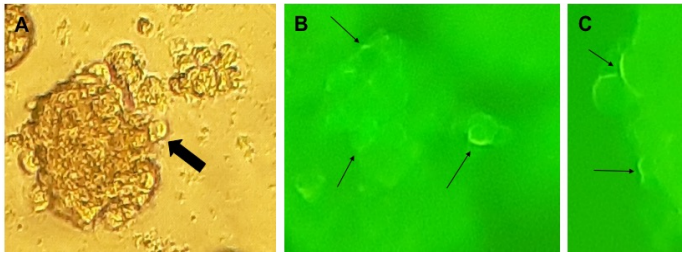

Fig. 3. Cells produced in MCF-7 three-dimensional (3D) cell line, spheroids. Control cells and spheroids [thick black arrow] (1A) and residues specifically stained with fluorescent-labeled WGA (1B) and MAL-I (1C) [thin black arrow] [400X]

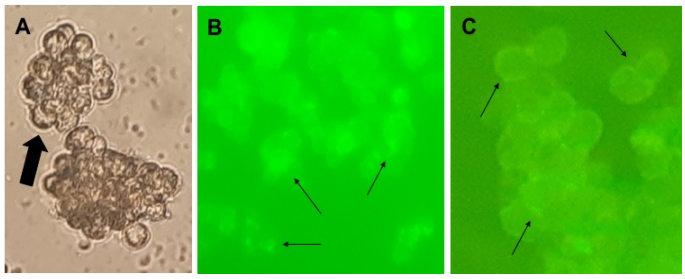

Fig. 4. Cells produced in MDA-MB-231 three-dimensional (3D) cell line, spheroids. Control cells and spheroids. Control cells and spheroids [thick black arrow] (1A) and residues specifically stained with fluorescent-labeled WGA (1B) and MAL-I (1C) [thin black arrow] [200X]

The internal evaluation between Fig. 1 and Fig. 3 revealed that MCF-7 [ER (+)] spheroids were intensely labeled with FITC-[WGA] lectin, however, this labeling was more intense at the surface level of the cells. We can state in other words that GlcNAc and Neu5Ac sialic acid units beside glycan units containing particularly ß-GlcNAc type sialic acid were found to be intense on the cell membranes of the 3D cancer cell lines.

The internal evaluation between Fig. 2 and Fig. 4 demonstrated that MDA-MB-231 [ER (-)] spheroids were intensely labeled by both FITC-[WGA] FITC-[MAL-I] lectins. The obvious difference on these images was that both GlcNAc and Neu5Ac sialic acid units beside glycan units containing particularly ß-GlcNAc type sialic acid specifically labeled by FITC-[WGA] lectin and also glycan units containing particularly Galß4GlcNAc type glycan units labeled by FITC-[MAL-I] were not only focused on the cell membranes of the 3D cancer cell lines, but also, they were intensely encountered in the cytoplasm and functioning in particularly cell adherence and division. This result indicates that MDA-MB-231 [ER (-)] breast cancer cell line is a much more aggressive or metastatic cancer type. In addition, these outcomes obtained using spheroid cell culture method as the closest model to the patient sample suggest that the present study carried out based on different staining and radiation intensities with a two-hour lasting short staining procedure can be implemented as an easy, sensitive, and fast method in differentiation of two cancer types.

As a consequence, sialic acid (GlcNAc, Neu5Ac) units, particularly ß-GlcNAc type sialic acid glycan units and Galß4GlcNAc type glycan units were sensitively and

specifically labeled by FITC-[WGA] and FITC-[MAL-I], and the results were illustrated. Thereby, a modified immunofluorescent staining method that can perform a fast, sensitive, and specific labeling was tested and developed in one of the closest cell models to the patient samples.

4. Discussion

Oligosaccharides and polysaccharides defined as glycans are the cell surface molecules located at the surfaces of all the cells including embryonic and pluripotent stem cells and constitute an intense glycocalyx layer as usually added to proteins and lipids. It has been determined that investigations conducted in the field of glycobiology have detected that these glycoconjugates have various and complicated biological functions. The cells respond to the environment circumstances thanks to this layer while this layer also regulates the interactions with neighboring cells. Beside these, glycoconjugates play an effective role in determination of cellular positioning (Lanctot and Varki, 2007; Patil et al., 2012; Yu et al., 2014). The change in the amounts of sialic acid residues particularly containing α-2,3- and α-2,6 bonds located on the cell surfaces leads to development of avascular multicellular tumoral formations in normal and cancer cells. It has been demonstrated that they have a capability to form in vivo xenograft tumors particularly in the cancer cell lines. In the light of these data; it may be concluded that the ability to specifically label these xenograft tumor formations to differentiate cancer cells from the normal cells is highly important (Akasov et al., 2016).

Several studies have been carried out on specifically labeling of glycoconjugates using fluorescent-labeled lectins (Kataoka and Tavassoli, 1985; Fujitani et al., 2013; Ishihara et al., 2013), appropriate selection of the cells (Lawrence et al., 2014, Kawabe et al., 2013), their use in the oriented treatment (Sethuraman and Stadheim 2006, Abdelkrim et al., 2009) and treatment of many metabolic disorders as adjuvant therapies (Gangaram-Panday et al., 2007; Varki, 2008) and these studies are currently continuing.

Whelan et al. (2009) have reported their study on N-linked membrane glycoproteomics obtained from three breast cancer lines using hydrazide chemistry-based enrichment. They used ER and PR (+) and Her2 (-) MCF-7, ER and PR (-) and Her2 (+) MDAMB-453 and ER (-); PR (-) and Her2 (-) (triple negative) MDA-MB-468 cell strains. In that study, the cells that constitute ER (+) MCF-7 and ER (-) MDAMB-231 breast cancer cell lines were applied fluorescent (FITC)-labeled Maackia amurensis lectin-1 (FITC-[MAL-I]) that can specifically bind to a kind of galactose (Galß4GlcNAc) and fluorescent (FITC)-labeled Wheat Germ Aglutinin (FITC-Triticum vulgaris [WGA]) that can specifically bind to some sialic acid types (GlcNAc, Neu5Ac) and particularly ß-type N-Acetyl Glucosamine (ß-GlcNAc) sialic acid glycan. By this process, the differences between the amounts of sugar residues detected on membrane surfaces of mentioned ER (+) MCF-7 and ER (-) MDAMB-231 breast cancer cell lines were specifically labeled and radiation intensities was relatively compared.

In the light of this information, the differences between the amounts of sugar residues detected on membrane surfaces of mentioned ER (+) MCF-7 and ER (-) MDAMB-231 breast cancer cell lines in our previous studies we have performed using 2D cell culture model (Mater and Özdaş 2018; Demircan and Mater, 2019) were specifically labeled with FITC-[WGA] and FITC-[MAL-I] and radiation intensities was relatively compared (Figs. 1-4). Accordingly, three-dimensional (3D) spheroid models were created from the MCF-7 and MDA-MB 231 breast cancer cell lines (Fig. 1A and Fig. 2A). MCF-7 [ER (+)] and MDA-MB-231 [ER (-)] spheroids were stained with fluorescent-labeled lectins (Fig. 1B-C and Fig. 2B-C). Correspondingly; sialic acid units (GlcNAc, Neu5Ac) and also glycan units containing particularly ß-GlcNAc type sialic acids were visualized by using FITC-[WGA] while Galß4GlcNAc type glycan units were displayed by applying FITC-[MAL-I] as the specifically binding fluorescent-labeled lectins. Their binding ability and the fact that this method was applicable also in the 3D cell model as the closest design to the patient samples were manifested (Figs. 1 and 2).

The internal evaluation of the breast cancer cell lines with different characteristics (Figs. 1 and 3) revealed that spheroids created by MCF-7 [ER (+)] cells were intensely labeled by FITC-[WGA] lectin, however, this labeling was mostly concentrated on the membrane surfaces of the cells (Figure 1B and 3B). We can state in other words that GlcNAc, Neu5Ac sialic acid units and particularly glycan units containing ß-GlcNAc type sialic acid specifically labeled with FITC-[WGA] lectin was intensely found in (3D) breast cancer cell lines. Contrarily, Galß4GlcNAc type glycan units specifically labeled applying FITC-[MAL-I] were detected on the cell membrane in the same manner, however, their amount was found to be very much lower than the residues specifically labeled with FITC-[WGA] lectin (Figs. 1C and 3C). On the other hand, the internal evaluation between the spheroids created by MDA-MB-231 [ER (-)] cells (Figs. 2 and 4) demonstrated that spheroids were intensely stained by both FITC-[WGA] (Figs. 2B and 4B) and FITC-[MAL-I] lectins (Figs. 2C and 4C). The obvious difference on these images was that both GlcNAc and Neu5Ac sialic acid units beside glycan units containing particularly ß-GlcNAc type sialic acid specifically labeled by FITC-[WGA] lectin and also glycan units containing particularly Galß4GlcNAc type glycan units labeled by FITC-[MAL-I] were not only focused on the cell membranes of the 3D cancer cell lines, but also they were intensely encountered in the cytoplasm and functioning in particularly cell adherence and division (Figs. 2B-C and 4B-C). This result indicates by the micrographs that MDA-MB-231 [ER (-)] breast cancer cell line included much more sialic acid units and is a much more aggressive or metastatic cancer type.

In addition, these outcomes obtained using spheroid cell culture method as the closest model to the patient sample proved once more that assessment of the sugar units can be implemented as a relatively easier, absolutely sensitive and fast method in differentiation of two cancer types thanks to the present study carried out based on different staining and radiation intensities with a two-hour lasting short staining procedure.

It has been exhibited by the present study that it would be possible to label the sugar units found on the surfaces of breast cancer cells with specifically binding lectins and thus to identify the sugar profiles of the normal and different breast cancer cells. Additionally, it has been also clarified that it would possible to determine the structure of the monomer units that constitute these specifically detected residues and that the impact of the sugar molecules that have a vital function in recognition-adherence mechanism of the cells on the breast cancer cell types may be analyzed visually and by micrographs.

3D cell culture models applying successfully designed and improved lectin-labeling method will allow visualization with fluorescent microscopes as well as achievement and evaluation of the closest accurate results to the living tissue. Our present and further studies applying improved and improvable specific labeling methods will provide a precious contribution to the research and healthcare sector in “development of fast-resulting alternative immunohistochemical labeling method/methods”.

As a conclusion, the most crucial feature of the present study is that the differences between sugar units on the cell surface were identified in the three-dimensional spheroid cell culture model that shows the closest similarity to the living tissue using fluorescent-labeled lectin for the fast, sensitive, and specific labeling method (Mater and Özdaş, 2018; Demircan and Mater, 2019) of which preliminary studies were performed and modified by us. The surface sugar profiles of two different breast cancer cell lines were analyzed by this method. The inability to specifically label the residues have provided guiding information in both early diagnosis of breast cancer and also oriented drug studies. Thus, collection of essential information for a study has been started to develop a technique allowing a fast, sensitive and specific labeling to be implemented to the closest cell form regarding living tissue and to obtain kit and patent right.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Ultrastructural examination of testicular tissues and evaluation of ZO-1 protein levels obtained from azoospermic cases

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Abstract

Spermatogenesis is a process that involves cellular and biochemical processes. The purpose of this study is to understand the role of tight junctions in spermatogenesis by evaluating Zonula occludens-1 (ZO-1) protein levels of cells from the testicular sperm extraction (TESE) biopsies in patients with azoospermia. The study included 40 infertile men with azoospermia. They were divided into two groups, as spermatozoa positive (n=20) and spermatozoa negative (n=20). Testis tissues were examined morphologically, immunohistochemically and ultrastructurally by using transmission electron microscopy (TEM). The structure of seminiferous tubules was deteriorated, degenerated and hyalinized in spermatozoa negative group. ZO-1 expression level was normal in blood-testis barrier of spermatozoa positive group in comparing to lateral surfaces of Sertoli cells spermatozoa negative group. The ultrastructural results were also supported with light microscopy findings. These findings suggest that decrease of the ZO-1 protein expression level in Sertoli cells might be one of the factors involved in the impairment of spermatogenesis in spermatozoa negative group. Therefore, we suggest that tight junctions may have crucial roles in spermatogenesis.

Keywords: azoospermia, male infertility, TESE, tight junction's protein ZO-1

1. Introduction

Infertility is seen in 10-15% of married couples, and nearly 50% of the cases are male related. One of the main causes of male infertility is azoospermia, which is defined as having no sperm cells in the ejaculate and found in 1% of men (Kumar and Singh, 2015). The quality and quantity of the spermatozoa directly affect the reproduction (Sapanidou et al., 2015). Apoptosis is one of the important processes that provide quality and quantity of germ cells in the testicle (Hikim et al., 2003). Apoptosis may decrease the number of germ cells and their viability (Kang et al., 2017). Accordingly, sufficient number of healthy spermatozoa can't be produced in the azoospermia and this leads to infertility (Lee and Cheng, 2008). The treatment of these cases is performed by surgical operations including 'testicular sperm extraction' in case of sperm cell absence in the tissue (Kumar and Singh, 2015).

The blood-testis barrier (BTB) is located between adjacent Sertoli cells in the testis where it provides a unique microenvironment for the development and maturation of meiotic and post-meiotic germ cells in seminiferous tubules. It compounds junctional proteinous structures, such as tight junctions (TJs), adhesion junctions and gap junctions (GJs). Some of the junctional proteins function as structural proteins of BTB and some have regulatory roles. Deletion or functional silencing of coding genes of these junctional proteins may disrupt the BTB, and concordantly this may cause immunological or other damages to meiotic and postmeiotic cells, this eventually leads to spermatogenic arrest and infertility (Jiang et al., 2014). The intercellular tight junction dynamics induce the formation of blood-testis barrier which play a crucial role in spermatogenesis (Gao et al., 2017). The tight junctions between cells in the testicular tissue, zonula occludens-1 (ZO-1) and regular actin network affect sperm production (Lee and Cheng, 2003). The aim of this study is to understand the role of tight junctions by comparing ZO-1 expression levels between azoospermia testicular tissue sperm positive and negative samples which are obtained by TESE.

2. Materials and methods

2.1. Ethics committee approval

All related material has been obtained in accordance with the Helsinki declaration and all procedures have been approved by İstanbul Şişli Etfal Training and Research Hospital Ethics committee 18.12.2012 date and 2012/133 number. This study

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2.2. Testicular sperm extraction

All patients referred for testicular biopsy to infertility center for diagnosis or infertility treatment. TESE was performed under general anesthesia. The scrotal skin, tunica albuginea and one or two pieces of the extruding testicular tissue were excised using a pair of curved scissors. The specimen was then transferred into a Petri dish (Falcon; Becton-Dickinson, Aalst, Belgium) filled with ~2 ml modified HEPES-buffered heparin 0.4% (H 3149; Sigma, St Louis, MO, USA). The biopsy specimen was shredded into small pieces with two sterile glass microscope slides under a stereomicroscope to release spermatozoa from the seminiferous tubules into the medium. The presence of spermatozoa was examined by using inverted microscope. The testicular incision was continued until the spermatozoa could be distinguishable from the debris, immature cells, red blood cells or Sertoli cells.

In the case of spermatozoa absence in the tissue, up to three biopsies were performed on different areas of each testis. The shredded biopsy tissue was gently homogenized by repeated aspiration and spilling out maneuvers into a tuberculin syringe. The effluent and the minced tissue were transferred into a Falcon tube (Becton-Dickinson) and incubated for 2–3 h in 5% CO_2 containing atmosphere at 37°C. Then, the fluid was aspirated, put into another tube and centrifuged at 300 g for 5 min., after the centrifuge the pellet gently re-suspended by following the removal of supernatant. Finally, droplets were aspirated from the suspension as described in the ESP for searching of spermatozoa.

2.3. Histological and immunohistochemical analysis

In this study, a total of 40 men ages 25-55 were 2012-2013 between evaluated for persistent azoospermia. Testicular biopsy samples were divided into two groups as spermatozoa positive and spermatozoa negative for azoospermia men. The patients were enrolled to study who have diagnosed with non-obstructive azoospermia, could not have a child after one year of unprotected sexual intercourse in their first TESE, and never receive any medication and hormone replacement therapy before TESE procedure, plus was not treated for chronic disease. Testicular samples were evaluated by morphological, immunohistochemical and ultrastructural analysis.

Testicular samples were fixed in Bouin's solution for 6h. After fixation, tissues were incubated with 1% lithium carbonate. After carbonate incubation tissues were transferred to the alcohol. Then samples were embedded in paraffin. 3 µm thick sections were prepared and stained with hematoxylin-eosin and Masson's trichrome. Sections were observed under light microscopy and images were taken (Olympus Bx53, Japan).

2.4. Immunohistochemical analysis

Sections (3µm) were deparaffinized with xylene and rehydrated with decreasing percentages of ethanol and finally with water. Surroundings of the sections were marked with a PAP pen (Pappen, Dako). The endogenous peroxidase activity was blocked with 3% H_2O_2 for 20 min at room temperature and later rinsed with distilled water and phosphate buffered saline (PBS). Antigen retrieval was accomplished by Decloacking chamber (Bicare Medical DC2008) in aEDTA (1/10) for 20 min at 110°C. Blocking reagent (Invitrogen Blocking Solution REF: 859043) was applied to each slide followed by 5 min incubation at room temperature in a humid chamber. Sections were incubated for overnight at 4°C with anti ZO-1 rabbit polyclonal primary antibody (1:100, Millipore, AB2272), ki67 mouse anti rat (1:100 Dako, M7248). Antibodies were diluted in a large volume of UltrAb Diluent (Thermo Scientific, TA-125-UD).

The sections were biotinylated and incubated with goat anti-rabbit antibodies (Invitrogen Broad Spectrum REF: 859043). After slides were washed in PBS, the streptavidin peroxidase label reagent (Invitrogen HRP–Streptavidin REF: 859043) was applied for 30 min at room temperature in a humid chamber. The colored product was developed by incubation with AEC chromogen (Invitrogen, D22187). The slides were counterstained with Mayer's hematoxylin (LabVision, TA-125-MH) and mounted in glycerol gelatin. ZO-1and Ki67 reactions were observed under light microscopy and images were taken (Olympus Bx53, Japan).

2.5. Electron microscopy

Testis tissues were fixed and dissected cut into smaller blocks of 1 mm^3. Tissue samples (1 mm^3) were put in the 2.5% glutaraldehyde with 0.1 M phosphate buffer (pH 7.2) for overnight fixation at +4°C. Then samples were washed again with PBS for 3 times (30 minutes for each wash). Samples were transferred to 1% osmium tetroxide with 0.1 M PBS for 1 h at room temperature (RT) and wash in PBS 3 × 30 min. Samples were dehydrated with increasing concentrations of ethanol (70% Ethanol-5 min. 96% Ethanol 10 min., 100% Ethanol, 2 × 15 min.). Then, dehydrated samples were transferred to the propylene oxide. Tissues were taken in the mixture of propylene oxide and embedding medium (Epon 812) (1:1) at RT for 45 min and in the mixture of propylene oxide and embedding medium (1:3) at RT for 45 min. Then samples were embedded in the fresh medium at +4°C for overnight. Samples were again embedded and polymerized at 60°C for 24–48 h. Ultra-thin sections were taken on ultramicrotome. Semi-thin sections were stained at 60°C with toluidine blue for 1 min. Ultra-thin sections are taken on copper grids and stained with uranyl acetate and Reynolds lead citrate. Tissues were examined in a transmission electron microscope (TEM, JEOL 1200 SX) for more detailed examination and imaging.

3. Results

In TESE (+) group, testis tissues were stained with H&E, the seminiferous tubules were associated with basal membrane area. Cells interacted with each other along the tubular wall at different stages of spermatogenesis. Spermatids were clearly

observable. Cell colonies in different spermatogenic stages and cell debris were found in the lumen of seminiferous tubules. Seminiferous tubules were associated with the basal membrane in the areas stained with Masson's trichrome. Also, oedema and hemorrhage where observed in some of these areas (Fig. 1A, Fig.1C).

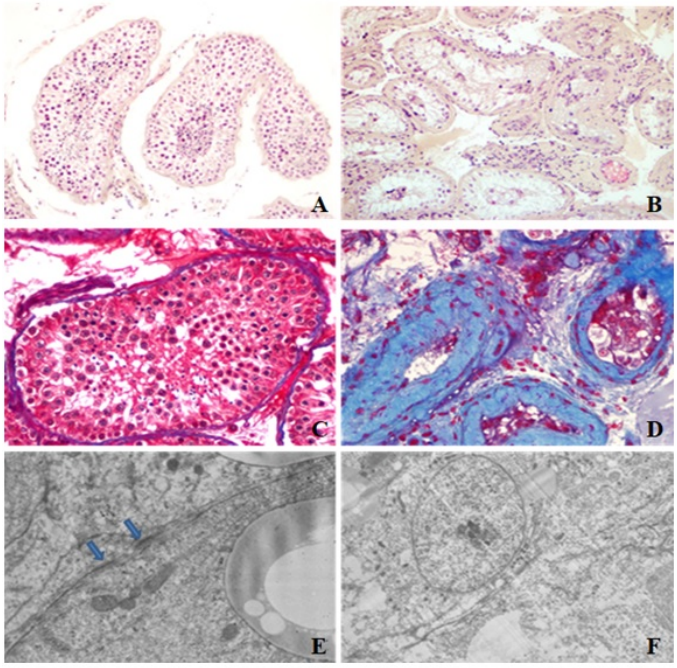

Fig. 1. (A) TESE+and (B) TESE-seminiferous tubules. x200, H&E staining. (C) TESE+and (D) TESE-seminiferous tubules. x400, Masson's trichrome staining. (E) TESE+and (F) TESE-intracellular junctions between germ and Sertoli cells. x5000, TEM

In TESE (-) seminiferous tubules were disordered, and absence of cells in other stages of spermatogenesis were observed. Hyalinated seminiferous tubules with thick basal membrane were observed. Connective tissue deteriorated by interstitial tissue thickening enclosing the seminiferous tubule completely and also hemorrhages area were examined (Fig. 1B, 1D).

Basal membrane which is enclosed to the seminiferous tubules and thin collagen fibril network were examined in TESE (+) group by using transmission electron microscope (TEM). Tight junctions between Sertoli cells within seminiferous tubule were observed (Fig. 1E) In TESE (-) group, peritubular wall was thickened because of increased collagen fibers in the testicle tissue. There were mainly Sertoli cells in the tubular wall enclosed by the basal membrane. Vacuolated hyaline material in Sertoli cell and tight junction were detected along with the whole lateral surface of the Sertoli cell (Fig. 1F). The Ki67 protein is present during all active phases of the cell cycle but it is absent only in resting cells. This finding renders this molecule a proper marker. Correspondingly, in TESE + group we detected Ki67 expression around the seminiferous tubules but not in the TESE (-) group (Fig. 2A and Fig. 2B). In spermatozoa containing testis tissue ZO-1 expression was observed in the blood testes barrier (BTB) with normal localization (Fig. 2C). Spermatozoa-absent group was examined and ZO-1 in the structure of TJs was not only in the basal compartment of the seminiferous epithelium but also observed on the lateral surface covering the adluminal compartment (Fig. 2D). The ultrastructural results were also supported with light microscopy findings.

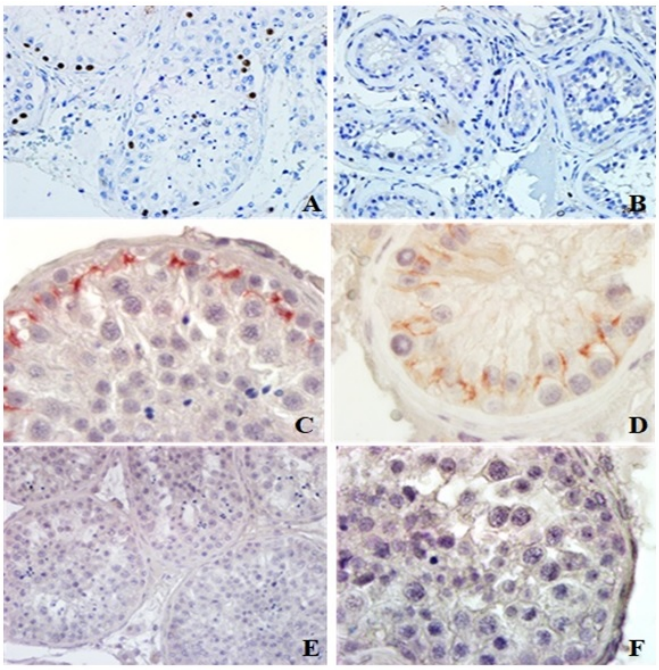

Fig. 2. (A) TESE+and (B) TESE- germ cells in seminiferous tubules. x400, Ki-67 immunohistochemistry. (C) TESE+and (D) TESE-seminiferous tubules. x1000, ZO-1 immunohistochemistry. (E) Ki67, x400 and (F) ZO-1, x1000 negative control sections

4. Discussion

Infertility is a common disorder and nearly 50% of all cases are caused by male factor(s) (Garg and Gard, 2011). In our study we detected that spermatogenesis can be affected by the tight connections between the Sertoli cells and impairment of tight connections is one of the significant causes for infertility. Description of the blood-tissue barrier (BTB) was based on observations which were reported in the early twentieth century. However, the term blood-testis barrier, also known as the Sertoli cell seminiferous epithelium barrier was firstly used by Chiquoine (Chiquoine, 1964) in a study that they examined the effects of cadmium toxicity as it related to testicular necrosis. The number of sperm is effective in fertilization thus, it is important among the sperm parameters. According to World Health Organization (WHO) standards it is considered normal to have $15x10^6$ cell/ml or more. (WHO, 2010) Azoospermia is described as no sperm in the ejaculate, and common in 1% of men. In our study, morphological differences of spermatozoa and non-azoospermic testis tissues were evaluated. In addition, the role of tight junctions between cells in the presence and absence of sperm in the cases of azoospermia also were observed. As supported by the literature, the tight junctions between the cells form the blood-testis barrier within the testis structure and the BTB plays an important role in male infertility (Sun et al., 2013).

The blood-testis barrier (BTB) is one of the tightest blood-tissue barriers in the mammalian body. It divides the seminiferous epithelium into the basal and the apical (adluminal) compartments. Meiosis I and II, spermiogenesis, and spermiation take place in a specialized microenvironment behind the BTB in the apical compartment but spermatogonial renewal and differentiation and cell cycle progression up to the preleptotene spermatocyte stage normally occur outside of the BTB. However, in the azoospermic cases the BTB is distorted. Besides, unexplained male infertility cases involve 30-40% of men with distorted BTB and abnormal semen parameters (Nieschlag et al., 2011). The causes of spermatogenic defects in infertile patients are multifactorial (Skakkebaek et al., 2001). The immunological barrier provided by the BTB even transiently cannot be compromised during the epithelial cycle to avoid the production of antibodies against meiotic and post meiotic germ cells. Studies have demonstrated that some adhesion protein complexes (e.g., occludin-ZO-1, N-cadherin-β-catenin, claudin-5-ZO-1), steroids (e.g., testosterone, estradiol-17β), non-receptor protein kinases (e.g., focal adhesion kinase, c-Src, c-Yes), polarity proteins (e.g., PAR6, Cdc42, 14-3-3), endocytic vesicle proteins (e.g., clathrin, caveolin, dynamin 2), and actin regulatory proteins (e.g., Eps8, Arp2/3 complex), are associated to each other and collaborative with the cytokines. The findings showed that impairment of BTB was responsible for subsequent damage to germ-cell adhesion, thereby leading to germ-cell loss, reduced sperm count, and male infertility or subfertility (Yan Cheng and Dolores, 2012).

According to our microscopic observation, the ZO-1 protein, which provides tight junctions between Sertoli cells in the spermatozoa group, was more intense than the spermatozoa-free group (n:20). In the spermatozoa-containing group (n:20), the ZO-1 staining localized to the expected region between the Sertoli cells, while in the spermatozoa-free group (n:20), the staining took place along the entire lateral surface of the Sertoli cells. Parallel to this, in the spermatozoa group, the tight connections were between the Sertoli cells within the abdominal and basal compartments, while the tight connections in the spermatozoa-free group were along the entire side wall of Sertoli cells. Therefore, ZO-1 plays an effective role in Sertoli cells for the spermatogenesis, as supported with literature.

ZO-1 is suggested to play a role in the regulation of germ cell development and differentiation. Sertoli cell / germ cells in the epithelium of the testis are responsible to preserve the correct ratio and producing vital and efficient sperm by using blood testis barrier protein. Therefore ZO-1 is important to maintain male fertility and pathogenesis (Jiang et al., 2014). Based on this, our electron microscopy observations showed that the number of germ cells in the non-spermatozoa group decreased and the ZO-1 interaction with blood testes barrier was less significant than the group with spermatozoa.

In addition, Ki67 is a non-histon nuclear protein found in all phases of the cell cycle except the G_o phase, it is associated with tight intercellular connections and effective in cell proliferation. Ki67 protein expression is associated with tight binding proteins (E-cadherin, ZO-1, etc.) and involved in cell proliferation, differentiation and apoptosis (Chen et al., 2012). In our study we detected morphological abnormalities in the connective tissue in both groups, suggesting that these changes may also affect the testicular tissue function. The absence of spermatozoa proves that seminiferous tubule structure deteriorates the tubules and hyalinized the spermatogenesis. Ki67 weren't expressed and the absence of cells in different stages of spermatogenesis proved that spermatogenesis never occurred.

In Vitro Fertilization (IVF) applications have also begun to use in male subfertility except for anovulation, tubal factor and unexplained infertility, when classical in vitro fertilization has shown that there may be a solution in male subfertility if there is a sufficient number of motile sperms. However, it is known that in adequacy of sperm parameters in male subfertile caused decreasing of fertilization rate (Rumbold et al., 2019). Consequently, our study emphasizes that BTB integrity is important for spermatogenesis and tight junctions play effective role in regulation of sperm cells. Germ cell transplantation is not yet ready for the human fertility clinic, but it may be eligible for young cancer patients, who have no any other option to preserve their fertility (Vrankovic et al., 2012).

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Mean platelet volume as a diagnostic parameter of respiratory syncytial virus infection

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Abstract

Our aim was to investigate RSV related parameters by comparing clinical findings and physical examination with routinely ordered whole blood counts and biochemical variables in under two age children hospitalized for lower respiratory tract infections. The sample consisted of 193 children [RSV positive (n=85), RSV negative (n=108)] with lower respiratory tract infections from May 2010 to May 2013. Sociodemographic findings, chief complaints and physical examination findings were retrospectively evaluated. RSV positive and negative groups were compared using Chi-square test (% 95 Confidence Interval- %95 CI), Mann-Whitney U test (p=0.05). No significant differences were found in demographic variables and treatment decisions between two groups. In RSV positive group, higher hyponatremia ($p \leq 0.001$) and AST values (p=0.003) were found. Decreased MPV were determined 71.8% in RSV positive group. RSV positive patients had decreased MPV values (OR:10.929, 95% CI). Higher hyponatremia and increased AST values were found to be associated with RSV infection; and decreased MPV was significantly related with RSV infection. This is the second study in literature within our knowledge that found decreased MPV values in RSV positive patients. The mechanism related with decreased MPV, hyponatremia, and increased AST values in RSV infection should be investigated further.

Keywords: RSV, mean platelet volume, bronchiolitis, infection

1. Introduction

According to data from World Health organization (WHO), three million of infants and children die because of lower respiratory tract infections (LRTI) annually. Respiratory Syncytial Virus (RSV) is the most important causative agent of viral LRTIs in infants and children all around the world. The widely use of serologic tests in recent years for diagnosis have shown that RSV infection may occur in any climate and geographic region (Murray et al., 1999). RSV infection is most commonly seen in children under two years of age. Almost half of the children who have infection just involving upper respiratory tract, experienced a progression of infection to lower respiratory tract. Hospitalization rate is about 2%. Mortality rate in hospitalized patients is 0.5-1 % (La Vla et al., 1992; Collins and Chanock, 1996).

Children with a history of prematurity, bronchopulmonary dysplasia, congenital or acquired immune deficiency and haematological malignancies, bone marrow or organ transplantation, cystic fibrosis, oxygen supply at home, neurologic and metabolic diseases are all at higher risk for having RSV infection. The disease is prolonged and causes higher mortality in children with congenital immune deficiency (Walsh et al., 1997; Handforth et al., 2000). The mortality rate is higher in children with congenital heart disease those have already impaired respiratory and cardiac functions. Prognosis worsens in the presence of pulmonary hypertension (Geskey and Cyran, 2012). Almost all infants and children have RSV infection within first two years of life. Reinfections can be seen because of immature host defense (Ohuma et al., 2012). RSV infection is a major public health problem due to high morbidity and mortality in young children and a rapid diagnosis and treatment should be made to prevent spread of disease. Educating health care providers is also an important key point (Prober and Sullender, 1999).

Diagnosis of RSV infection can be made by different tests including isolation of the virus from cell culture or detection of antigens by serologic and immunochromatographic methods. Detection of RSV antigen is made through directly from nasopharyngeal swab, nasopharyngeal aspirate or nasal / nasopharyngeal wash specimens. Nasopharyngeal aspirate method is referred as gold standard method in many literature to detect RSV antigen. Laboratory diagnosis of RSV infection is important for public health and health economics. Rapid

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diagnosis avoids unnecessary use of antibiotics, nosocomial spread and provides early beginning of antiviral treatment for children with serious illness (Luksic et al., 2013). Although rapid diagnosis of RSV is important for prompt treatment, there are no appropriate testing procedures in most of health institutions of our country and many developing countries.

In this study, we aimed to compare clinical findings, complete blood count and biochemical parameters of 1-24 month-old infants, who treated in outpatient clinic or hospitalized for RSV (+) or RSV (-) viral LRTI for setting RSV identifier parameters.

2. Materials and methods

A total of 193 infants aged 1- to 24-month-old were enrolled for study between May 2010 and May 2013 in Turgut Ozal University Faculty of Medicine Hospital with clinical findings of viral LRTI. All patient were tested for RSV antigen by immunochromatographic method. Infants with severe illnesses (sepsis, meningitis), serious neurological and metabolic disorders, immune deficiencies, and suspected bacterial infections were excluded from study.

Demographic data of patients fulfilling the criteria were collected through a computer program. Lacking information was completed by phone calls with families. Detailed history of patients including age, gender, chief complaints, beginning time of the symptoms, number of siblings, presence of sibling ongoing day care or school, comorbidity, smoke exposure, apnea, prematurity and birth season were recorded.

Hospitalization, oxygen requirement, need for antibiotic and other treatment options were extracted from study forms. The patients were followed up in terms of recurrent wheezing through records for a 6 months period after enrollment. Signs and symptoms, laboratory investigations, radiologic findings were recorded. All patients underwent rapid RSV immunochromatographic testing with samples obtained by nasopharyngeal swabs. Patients were assigned into two groups according to recorded test results in study form as RSV antigen positive and negative.

2.1. Statistical analysis

The normal distribution variables were evaluated using Shapiro- Wilk test. Skewed variables were expressed as median (Interquantile ranges). Counts and percentages were used for categorical variables. Comparison of data based on differences between study groups and difference in proportions were analyzed by performing Chi-square test and Likelihood ratio. Crude and adjusted odds ratios (OR) were obtained with 95% confidence intervals (95% CI). Correlation analyses were performed with Spearman Rho correlation coefficient test for variables with nonparametric distribution. The Mann Whitney U-test was used to compare the two groups. All statistical analyses were performed using IBM SPSS Statistics 21.0 (IBM

Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) and MS-Excel 2007. Significance was assigned at $p < 0.05$.

Informed consent for participation in this study was obtained from the patients' parents or guardians. The study protocol was approved by the Ethics Committee of the Turgut Ozal University, Faculty of Medicine.

3. Results

The study group consisted of 183 children, 79 females (40.9%) and 114 males (59.1%). There were 151 infants (78.2%) under the age of one, and 42 toddlers (21.8%) between ages 1 and 2. The median gestational age was 39 weeks. Prematurity rate was 11.9% (n=23). Forty–nine patients (25.4%) had smoke exposure story. Positivity for RSV was 44%, and did not show statistically significant difference with respect to gender. Patients with a history of premature birth rate did not significantly differ for both groups. That is, no significant difference was detected between RSV positivity and all demographic features shown as Table 1 ($p>0.05$).

Table 1. Comparison of demographic features according to rsv positivity

	Negative n(%)	Positive n(%)	χ^2	P
Gender				
Female	41 (51.9)	38 (48.1)	0.894	0.344
Male	67 (58.8)	47 (41.2)		
Age				
<12 month	81 (53.6)	70 (46.4)	1.510	0.219
12-24 month	27 (64.3)	15 (35.7)		
Smoke exposure				
No	79 (54.9)	65 (45.1)	0.277	0.599
Yes	29 (59.2)	20 (40.8)		
Prematurity				
No	99 (58.2)	71 (41.8)	3.000	0.083
Yes	9 (39.1)	14 (60.9)		
Sibling at day care				
No	56 (52.8)	50 (47.2)	0.934	0.334
Yes	52 (59.8)	35 (40.2)		
Breastfeeding				
No	27 (62.8)	16 (37.2)	1.048	0.306
Yes	81 (54.0)	69 (46.0)		
Hospitalization				
No	20 (45.5)	24 (54.5)	2.552	0.110
Yes	88 (59.1)	61 (40.9)		

The median hospitalization duration was 3.5 (IR = 3) in RSV (-) patients, and 4.0 (IR = 2) in RSV (+) patients ($Z=1.878$; $p=0.060$). Need for oxygen, oxygen supplementation duration and gestation week did not significantly differ between groups. Signs and symptoms such as fever, tachypnea, retraction and existence of apnea were also similar for RSV (+) and RSV (-) patients. While the number of patients who have oxygen saturation level below 92% was 45 (52.9%) for RSV (+) group, it was 60 (55.6%) for RSV () patients. Recurrent wheezing episode rate in follow-up period was 28.2% (n=24) for RSV (+) group and 31.5% (n= 34) for RSV (-).

Table 2. The distribution of treatment practices based on RSV positivity and negativity

	Negative n(%)	Positive n(%)	χ^2	P
Antibiotic				
No Yes	30 (27.8) 78 (72.2)	32 (37.6) 53 (62.4)	2.125	0.145
Systemic Steroid				
No Yes	52 (48.1) 56 (51.9)	40 (47.1) 45 (52.9)	0.023	0.880
Inhaler Steroid				
No Yes	17 (15.7) 91 (84.3)	24 (28.2) 61 (71.8)	4.438	**0.035**
Inhaler Beta agonist				
No Yes	13 (12.0) 95 (88.0)	8 (9.4) 77 (90.6)	0.338	0.561
Inhaler hypertonic sodium chloride				
No Yes	101 (93.5) 7 (6.5)	80 (94.1) 5 (5.9)	0.029	0.864
Racemic Epinephrine				
No Yes	97 (89.8) 11 (10.2)	75 (88.2) 10 (11.8)	0.122	0.726
IV Fluid				
No Yes	34 (31.5) 74 (68.5)	29 (34.1) 56 (65.9)	0.150	0.698

Although all patients had a LRTI, antibiotic use was very high in both study groups (62.4 % in RSV (+) group, 72.2 % in RSV (-) group). RSV positivity had no effect on systemic steroid or inhaler beta agonist use (p=0.561). Similarly; inhaler hypertonic sodium chloride, racemic epinephrine use and intravenous fluid administration did not differ significantly between groups (p>0.05). But interestingly, inhaler steroid use rate was 84,3 % (n 91) in RSV (-) group (Table 2). Four patients with RSV bronchiolitis and three from other group developed heart failure and received digoxin. White blood cell and platelet count of 106 patients did not show significantly difference whether the patient is RSV (+) or not (p=0.541). We evaluated serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and sodium (Na) levels of 103 patients. Despite serum AST level was higher in RSV (+) group (p=0.003), ALT level was not different. In about of 31.7 % RSV (+) group serum sodium Na) level was significantly low in (p<0.001), additionally none of group members had increased urine specific gravity in contrast to RSV (-) group (p=0.007) (Table 3). Findings in chest x-ray and C-reactive protein (CRP) levels were similar for both groups (p>0.05). RSV infection was more frequent in infants those born in winter and early (p=0.008).

Table 3. The distribution laboratory investigations based on RSV positivity and negativity

Normal Ranges	Negative n(%)	Positivie n(%)	χ^2	P
MPV				
(7.8 - 11.1) n (%) Low (<7.8) n (%)	86 (81.1) 20 (18.9)	24 (28.2) 61 (71.8)	54.043	**<0.001**
AST				
(≤ 40 U / L) High (> 40 U / L)	78 (75.7) 25 (24.3)	45 (54.9) 37 (45.1)	8.97	**0.003**
Na				
(> 135 mg / dL) Low (<135 mg / dL)	97 (94.2) 6 (5.8)	56 (68.3) 26 (31.7)	21.379	**<0.001**
Urine Specific Gravity				
(1005-1020) High (>1020)	48 (87.3) 7 (12.7)	35 (100) 0 (0.0)	7.267	**0.007****

There was a significant association between decreased mean platelet volume (MPV) and RSV infection as shown on Table 3 (p<0.001). None of study patients had MPV value above limits. Decreased MPV rate was higher in RSV (+) group than RSV (-) group (71.8 % and 18.9 % respectively). The patients with RSV (+) lower respiratory tract infection were about 10 times more prone to have decreased MPV level (OR=10.929; %95 CI). Oxygen saturation and duration of oxygen supply were not related with low hemoglobin level (p=0.507 and p=0.590 respectively). The median mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) values in RSV (+) group were significantly higher than RSV (-) group (p=0.003). RDW median was similar in both groups (p=0.276). Of the twenty-three patients with premature birth story, three infants (17.6 %) had received palivizumab, 5 (%21.7) had needed oxygen supplementation. Palivizumab therapy had no effect on median durations for hospitalization and oxygen need (p=0953 and p=0.801 respectively). Of 60.0 % premature infants those have received palivizumab for palivizumab and 77.8 % who have not received were hospitalized. Data were not enough statistically to conclude about palivizumab effect for RSV infection prophylaxis.

4. Discussion

Acute bronchiolitis is a common LRTI in childhood. Males are more prone to bronchiolitis caused by RSV or other etiologic factors (Boyce et al., 2000; Kristensen et al., 2009). We have observed that a higher proportion of bronchiolitis were in male gender and the male/female ratio was 1.3/1. Since airways of male infants are narrower than female infants, bronchial inflammation caused by LRTI reduces the size of airways further. Their airways become more susceptible to infections (Boyce et al., 2000; Kristensen et al., 2009; Navas et al., 1999). Lamarao et al. conducted a study with 1050 hospitalized infants for acute bronchiolitis and showed that bronchiolitis rate was higher (%55.6) in males (Lamarao et al., 2012). In our

study, we obtained data consistent with previous studies.

Lamarao et al. (2012) also pointed that RSV bronchiolitis was more common in first year of life with a rate of 63.1%. Accordingly, in a study carried out by Xiao et al. (2012) 1165 infants had a diagnosis of viral bronchiolitis in whom were mostly between age of six month and one year. Papenburg et al. (2012) revealed that RSV infection was more common and had a more severe course before age of 5 months. The study was conducted in United Kingdom with 1000 children aged < three years. Consistently with previous reports, we investigated that viral LRTI were more frequent in first year than between ages of 12 and 24 months (Lamarão et al., 2012; Xiao et al., 2012; Papenburg et al., 2012). Besides, Lamarao et al. (2012) detected RSV antigens from nasopharyngeal aspirates in 24% of infants while Xiao et al detected an RSV antigen positivity in 27% of patients and reported rhinovirus in 17% of all subjects as the second most common causative viral agent (Xiao et al., 2012). Papenburg et al. (2012) stated that RSV is the most common causative agent of bronchiolitis while human metapneumovirus was the second. Since patients with clinically high suspicion of RSV infection may be tended to be ordered rapid antigen testing more than others, we found a higher RSV positivity (44%) than previous studies. Hospitalization incidence was 77.2% and it was attributed hospitalization of infants with severe enough bronchiolitis. When compared to mentioned three studies, RSV positivity in our study was higher than previous studies but, results those based on age and gender were similar.

The peak RSV infection season ranges from November to March in temperate climates. Also, infants born in this season are more likely to be infected with RSV (Khor et al., 2012). In a retrospective study of 27 years performed by Khor et al. (2012) data of 10269 infants with RSV infection were analyzed and it was found to be that RSV infection has a peak incidence in especially last months of the year and infants born in this season are riskier to get infection during first year of life (Khor et al., 2012). The infants born in December and January were more prone to RSV infection than others as stated in our study. Although having siblings who attend nursery or school, smoke exposure, being breastfed for a lesser period and prematurity are known risk factors for RSV bronchiolitis, there was no significant difference between two groups based on these variables (Papenburg et al., 2012).

Smoke exposure, larger families with three or more children at home, prematurity (<35-week gestation), congenital heart diseases are determined as risk factors for severity of RSV infection, extension of hospital stay and oxygen requirement by Papenburg et al. (2012). Including almost hospitalized infants with LRTI rather than healthy subjects as control group, describes the difference between literature and our study. No significant difference was found between two groups for duration of hospitalization and oxygen requirement, and low oxygen saturation (hypoxemia) level. However, clinical findings of infants with RSV bronchiolitis such as tachypnea and retraction were similar to those with other viral LRTIs. It is difficult to distinguish bronchiolitis and pneumonia caused by viral agents. Tachypnea, intercostal and subcostal retractions along with nasal flaring, wheezing, rhonchi and crackles are common findings. As confirmed in our study, physical examination does not create distinctive features for etiologic agents (Gruber et al., 2002). Clinical variables such as fever, tachypnea, retractions, level of oxygen saturation and need for hospitalization did not differ among groups according to outcomes. Apnea can occur in especially young infants due to RSV infection. Guinea et al. (2007) reported an apnea incidence of 25.7% in infants age < six week and with severe bronchiolitis among 284 RSV positive subjects. Apnea incidence was 3% (n=3) in RSV (+) group and 1% (n=1) in RSV (-) group. Because of a small study group and cases included older than one month, apnea incidence was not significantly different between two groups. Albeit fever is rare or low-grade in RSV bronchiolitis, it is more common and higher in human metapneumovirus or adenovirus bronchiolitis (Tristram,1997). The fever of RSV (+) patients was low-grade with a rate of 37.6 % that was consistent with literature (Tristram et al., 1997).

RSV bronchiolitis may cause recurrent episodes of wheezing during childhood. Patients were followed up for six months after enrollment. No statistically significant difference was found between RSV (+) group and RSV (-) group for wheezing episodes (28.2% versus 31.5%). In a meta-analysis performed by Daniel et al., it was indicated that Human Rhino Virus (HRV) can also cause recurrent wheezing episodes and asthma as RSV, even more may generate a higher risk (20). Our results supported this data. Hence other causative agents have not been screened, we cannot issue an association between other causative agents and recurrent wheezing episodes and asthma. Hospitalization rate was as high as 77.2% in this study. Therefore, a great proportion of infants received intravenous fluid as expected (65.9% of RSV (+) infants and 68.5% of RSV (-) infants). Avoidance of antibiotic use is offered after detection of viral agents in literature. However rapid tests reduces antibiotic use, antibiotic, systemic steroid, inhaler beta agonist inhaler hypertonic NaCl and racemic epinephrine use were similar for both groups (Luksic et al., 2013). It was attributed to empirical antibiotic treatment for suspected secondary bacterial infections.

If beneficial, bronchodilator agents like inhaler salbuterol or racemic epinephrine are recommended for severe dyspneic infants caused by RSV bronchiolitis or other viral bronchiolitis (Watts, 2007; Langley et al., 2005). Since almost our patients were hospitalized because of severe enough tachypnea and dyspnea, bronchodilator use was so high (90.6% in RSV (+) group and 88% in RSV (-) group). Zhang et al. (2008) has proven improving effect on edema of small airways and facilitating effect on mucus excretion of nebulized hypertonic saline in a meta-analysis conducted with 1095 patients. In

addition to being beneficial and safe, it shortens the hospital stay. Because the first choice was bronchodilators and response of patients to bronchodilators were good, we have not administered hypertonic saline as a main treatment method (5.9% in RSV (+) group and 6.5% in RV (-) group).

Systemic steroid and inhaler steroid are just used for adults and children with recurrent wheezing episodes. Even if they alleviate bronchial secretions and airway obstruction, meta-analysis have proven no effectiveness on infants with bronchiolitis. Patel et al. (2004) found no significant difference between systemic steroid and placebo in terms of hospitalization, oxygen need and dyspnea through a meta-analysis with data of 1198 infants. Bronchodilator and steroid use were quite common in our clinic but our study has lacking data about prognosis after discharge. Thus we could not arrive at any conclusion about this issue. Inhaler steroid use was higher in RSV (-) patients owing to more frequent recurrent wheezing episodes in this group. Only two patients received palivizumab during hospitalization. None of patients has received antiviral therapy.

During RSV bronchiolitis, WBC count and CRP values are almost in normal limits. By a conducted study with 1050 acute bronchiolitis diagnosed infants, Lamaro et al. (2012) showed that WBC count and CRP were significantly lower RSV bronchiolitis group than other group. Conversely, Bicer et al. (2013) determined high levels of CRP and erythrocyte sedimentation in 301 inpatients aged below 9 with viral LRTI. White blood cell counts were increased in children with adenovirus and rhinovirus associated viral LRTI and marked monocytosis was revealed in adenovirus infection when compared to other viruses including RSV (Bicer et al., 2013). An explanation for non-significant difference for WBC count and CRP between two groups in our study is exclusion of patients with suspected bacterial pneumonia. But as expressed in literature, WBC count and CRP values were high in patients with either RSV bronchiolitis or others. While ALT values were in normal limits, AST values had increased in RSV (+) group and were higher than another group (44% in RSV (+) group, 24% in RSV (-) group; p=0.003). Extrapulmonary involvement in RSV infection is rare. However, it may cause convulsion, hyponatremia, hepatitis and heart failure. Nine patients (5%) among all subjects developed heart failure during infection course. Eisenhut et al. (2006) detected aminotransferase increase in 49% of intensive care unit patients who were hospitalized for RSV associated LRTI. The incidence may rise to as high as 80% in children with concurrent congenital heart disease. AST elevation is consistent with those previously reported in our study. Again, in the same study, hyponatremia was determined in 33% RSV (+) patients and thought to be result of SIADH. Four children had seizure because of hyponatremia whose simultaneously serum ADH were increased as a result of SIADH (Eisenhut et al., 2006). Likewise, in our study, hyponatremia was more frequent in RSV (+) patients (31.7% of RSV (+) patients and 5.8% of RSV (-) patients; p<0.001). The possible mechanisms of hyponatremia are SIADH, reduced oral intake and inappropriate fluid replacement (Eisenhut et al., 2006). Since significant increase for urine specific gravity was observed in RSV (+) patients (p=0.007), hyponatremia was correlated to reduced oral intake instead of SIADH. In total, 49% of the urine specimens were obtained, so a prediction could not be made about SIADH incidence fort this study. Only one patient had low platelet count. Although no significant difference was found between platelet count and RSV positivity, MPV was significantly lower in RSV (+) patients than RSV (-) patients (71.8% and 18.9% respectively). Renshaw et al. (2013) performed bronchoscopy to 158 patients with diagnosis of pneumonia or airway obstruction and compared MPV values between RSV (+) patients and healthy controls. The prediction of viruses was made by viral culture through specimens obtained from bronchoalveolar lavage or rapid tests with immunochromatographic method. They also argued that MPV values were significantly lower in RSV (+) patients than randomly selected healthy controls and a MPV value < 9.9 fl had a sensitivity of 71% and specificity of 49% for RSV (Renshaw et al., 2013). Comparing MPV values of RSV (+) and RSV (-) patients with LRTI from same age and similar clinical and demographic features instead of healthy controls was the superiority of our study. Karadağ et al. (2013) observed decreased MPV values in total of 196 children with community acquired pneumonia, either hospitalized (n=108) or out patients (n=88) when compared to healthy subjects. They suggested a cut-off value of 8.1 fL for MPV. Mete et al. (2014) demonstrated no significant difference for MPV between rota virus gastroenteritis and gastroenteritis caused by other viral agents. Nevertheless, there was significant difference between rotavirus gastroenteritis and healthy controls. MPV is associated with platelet age. Previous studies suggested that increased MPV may be associated with diabetes, obesity and alcohol use while platelet count is normal. Furthermore, decreased MPV value may be associated with various infections and may be used as acute phase reactant as well (Renshaw et al., 2013; Karadag et al., 2013; Mete et al., 2014). Whereas a significant difference for MPV was revealed between RSV positive and negative group, there was no significant difference for CRP and white blood cell. Although the mechanism which RSV causes a decrease in MPV is not clear, it can be used as a parameter in differential diagnosis of RSV and non-RSV bronchiolitis. Further investigations are required to identify this mechanism.

RSV infection is more frequent in first two months of life. Additionally, MCV is physiologically increased in same period. Thus, we suggest that, high MCV values are detected as a consequence of physiologic increase in first two months rather than RSV infection. Besides, MCV is physiologically decreased between eight to 12 weeks because of physiologic anemia (BHL, 1981). Our study has a limitation due to the age group of RSV. Since the physiologic anemia of infancy is

detected between 8 to 12 weeks, we couldn't evaluate physiologic anemia and anemia related MPV decrease in both RSV (+) and RSV (-) groups. The count of premature and palivizumab received infants were too low to make a suggestion about RSV (+) and (-) infants for hospital stay and oxygen need. Further investigation is needed for palivizumab use in prevention of RSV.

Conflict of interest

None to declare.

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Comparison of different surgical techniques of sacrococcygeal pilonidal sinus disease

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Abstract

Pilonidal sinus, especially in young males, is one of the most common diseases of the sacrococcygeal region. Although many surgical techniques have been described for pilonidal sinus disease (PSD), high recurrence rates and patient satisfaction are still controversial with regard to the ideal treatment modality. In this study, we aimed to compare the complications in Primary excision, Limberg flap, elliptical rotation flap and karydakis techniques used in patients with pilonidal sinus disease. The patients who were diagnosed with Pilonidal sinus and underwent surgery between January 2012 and January 2017 were retrospectively analysed from our hospital database. Patients in whom the Primary repair, Limberg flap, elliptical rotation flap and karydakis flap repair performed were divided into four groups. The groups were compared in terms of their demographic characteristics, length of hospitalization, seroma formation, surgical site infections, wound dehiscence, flap necrosis, sensory loss and recurrence. There were 107 patients in the primary group, 70 in the Limberg flap group, 72 in the elliptical rotation flap group and 45 in the karydakis flap group. There was a statistically significant difference between the groups in terms of the length of hospitalization ($p < 0.001$). The maximum length of hospitalization period was observed in the Limberg group and was 2.06 ± 0.95 days. The minimum duration of hospitalization was 1.00 ± 0.00 days in the karydakis group. Wound site infection was observed mostly in the primary group with 15% frequency, whereas this higher ratio was significant when compared with Elliptical Rotation flap and Karydakis flap groups ($p = 0.010$, 0.024 respectively). There was no statistically significant difference between the groups in terms of wound dehiscence, flap necrosis, sensory loss and recurrence. There is no consensus on the ideal surgical technique selection for PSD. Our study revealed that wound infection is common in patients undergoing Primary repair and that there is a difference in terms of length of hospitalization for different techniques. We expect the factors we investigate to be a guide for the surgeons.

Keywords: pilonidal sinus, postoperative complications, recurrence, surgical treatment

1. Introduction

Sacrococcygeal Pilonidal Sinus Disease (PSD), especially in young males, is an inflammatory disease that occurs more commonly in the intergluteal region with acute painful episodes or chronically (Okuş et al., 2013; Sondenaa et al., 1995). The incidence is 0.7% and is usually in the 15-25 age group, but can be observed at any age. It is most frequently seen in the period that sex hormones increase after puberty when they affect the pilosebaceous glands and healthy hairs in this region (Sondenaa et al., 1995; Humphries et al., 2010; Duman et al., 2016). It is observed in women at an earlier age, and the possible cause is pubertas praecox. Although the etiology of the disease cannot be determined precisely, it is the most commonly accepted theory that the hair loss from the skin can cause foreign body reaction, chronic irritation and inflammation in the intergluteal region. Risk factors for PSD include obesity, smoking, poor personal hygiene, sedentary lifestyle, family history, deep intergluteal cleft, and excessive body hair in the body (Okuş et al., 2013; Sondenaa et al., 1995; Menteş et al., 2008; Uçar et al., 2015; Vedder, 2006). PSD is often seen as a disease that affects the quality of life adversly in the population of the productive age group, and this leads to labour and material losses. Many conservative and surgical methods have been described in the treatment of PSD, but the standard and optimal surgical method remains controversial (Humphries et al., 2010). The advantages and disadvantages of the treatment modalities, the lack of superiority to each other, led the surgeons to try new methods. Medical treatments include local curettage, phenol injection, silver nitrate applications and electrocauterization of the cavity. Excision-primary repair, excision-marsupialization, de-epithelialization, excision-open left, Bascom operation, Karydakis operation, V-Y-Z plasty, Limberg flap and elliptical rotation flap techniques

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vary depending on clinical experience and are frequently used among the surgical techniques (Sondenaa et al., 1995).

In this study, we aimed to compare the complications and recurrence in patients who underwent primary repair, Limberg flap, elliptical rotation flap, and karydakis due to PSD.

2. Materials and methods

We retrospectively analysed the patients from our hospital database who were admitted to General Surgery outpatient clinic between January 2012 - January 2017, diagnosed with PSD and underwent 4 different surgical techniques, and contacted them for examination. We divided the cases into four groups according to the type of surgery performed: Primary repair, Limberg flap, elliptical rotation flap and karydakis. Main demographic characteristics such as age and gender, hospitalization, seroma formation, surgical site infections, wound healing, flap necrosis, loss of sensation and relapse database were evaluated with physical examination findings and questioning of cases.

2.1. Surgical technique

The patients are hospitalized on the day of surgery and the operative field is shaved. First generation cephalosporin antibiotic prophylaxis is performed 30 minutes before surgery. After spinal anesthesia, prone Jack-knife position is maintained. The plasters from the gluteal region of the cases are adhered to the edges of the operating table, and the intergluteal cleft is exposed and a better view and manipulation environment is created.

2.2. Primary repair

The skin was excised by subcutaneous tissues and total excision to the presacral fascia including all sinus tracts up to the sacral fascia was performed. The skin was primarily closed with 2/0 vicryl suture and was passed through the sacral fascia with 2/0 prolene.

2.3. Limberg and elliptical rotation flap

In order to reveal the pilonidal sinus cavity, each patient was injected into the methylene blue sinus with the help of an injector. First, the pilonidal cyst was excised totally from the 2 cm lateral midline by an elliptical or rhombic incision without injuring the pilonidal sinus and without leaving the sinus appendages until the presacral fascia. In the patients who underwent Limberg flap procedure, the skin was deepened to include the skin, subcutaneous tissue and the gluteal muscle fascia where flap will be performed, and to be the continuation of the rhombus and in equal length to the edges of the rhombus and a Limberg flap was prepared. In cases with elliptical rotation flap, the elliptical rotation flap from the right or left gluteal region was prepared to contain the same tissue layers. After bleeding control, the patches which kept the gluteus in traction were opened for ease of closure, the tension in the flaps was removed and following the insertion of the absorbent drain in the defect, the skin was individually sutured with 2/0 vicryl to include the fascia-subcutaneous tissues together. The skin was approximated using 2/0 polypropylene sutures.

2.4. Karydakis flap technique

The procedure was initiated with an asymmetric ellipsoid incision. If there was a secondary opening and/or a palpable cyst on the lateral line of the midline, the incision edge was shifted to the lesion. If there was no lesion observed, the incision side was randomly selected. The tissue was removed until the presacral fascia after the incision. Then, a flap extending through the entire incision, 1 cm below the edge of the midline, 2 cm inward, was prepared using cautery. The prepared flap was fixed to the other wound edge by the skin and subcutaneous sutures so that the midline was shifted.

2.5. Statistical analysis

All statistical data analyses were performed using SPSS 18.0 version package program for Windows. One way ANOVA and chi-square test were used for descriptive statistics and significance of differences. The level of statistical significance was accepted as $p < 0.05$.

3. Results

Of the 294 cases, 262 (89.12%) were male and 32 (10.88%) were female. The male/female ratio was 8.19. The age distribution of the cases is shown in Table 1. The age difference between the groups was between Limberg and karydakis groups (Table 1).

Table 1. The average age of the groups

	N	Mean ± SD	p
Primary (Group 1)	107	26.65 ± 7.37	0,027*
Limberg flap (Group 2)	70	27.50 ± 7.83	
Elliptical rotation flap (Group 3)	72	26.51 ± 7.28	
Karydakis (Group 4)	45	23.56 ± 3.19	
Total	294	26.35 ± 7.07	

The median duration of re-examination of the cases is (min 1.5 - max 5) years. The follow-up of the patient's postoperative outpatient clinic control was 2.17 ± 0.83 years.

In terms of gender distribution, there were 94 (87.85%) males and 13 (12.15%) females in the primary repair group, 60 (85.71%) males and 10 (14.29%) females in the Limberg flap group, 64 (88.89%) males and 8 (11.11%) females in the elliptical rotation flap group, 44 (97.78%) males and 1 (2.22%) female in karydakis group. No statistically significant difference was observed between the groups in terms of gender distribution ($p = 0.213$).

The durations of hospitalization after surgery are indicated in Table 2. There was a statistically significant difference between the groups in terms of the length of hospitalization ($p < 0.001$). This difference was between limberg and karydakis group, between limberg and primary group, between karydakis and elliptical rotation group, between karydakis and primary, between elliptical rotation and primary ($p < 0.001$). The length of hospitalization after surgery and their order are shown in Table 2. The maximum length of hospitalization was in Limberg group and the minimum length of hospitalization was in karydakis group (Table 2).

Table 2. Hospitalization periods

Operation type	Number of days (Mean ± SD)
Primary (Group 1)	1.41 ± 0.69
Limberg flap (Group 2)	2.06 ± 0.95
Elliptical rotation flap (Group 3)	1.99 ± 0.91
Karydakis (Group 4)	1.00 ± 0.00
p value	p < 0.001

When evaluated in terms of complications, the number of seroma cases was 10 (9.30%) in the primary repair group, 5 (7.10%) in the Limberg flap group, 2 (2.80%) in the elliptical rotation flap group and 1 (2.20%) in the Karydakis group (p = 0.199). There was no statistically significant difference between the groups in terms of seroma accumulation (p = 0.199).

The number of infected cases was 16 (15.00%) in the primary group, 5 (7.10%) in the Limberg flap group, 2 (2.80%) in the elliptical rotation flap group and 1 (2.20%) in the karydakis group (p = 0.009). There was a difference between group 1 and 4 and between groups 1 and 3 in terms of infection (Table 3). Infection was most common in the primary group and the difference was statistically significant compared to karydakis and elliptical rotation groups, but it was not significant compared to limberg (Table 3).

Table 3. The comparisons of p values for the incidence of infection according to the type of operation

	Group 1	Group 2	Group 3
Group 2	0.155		
Group 3	0.010	0.272	
Group 4	0.024	0.401	1.000

Wound dehiscence occurred in 5 (4.70%) cases in the primary repair group, 7 (10.00%) in the Limberg flap group, 2 (2.80%) in the elliptical rotation flap group and 1 (2.20%) in the Karydakis group. There was no statistically significant difference between the groups (p = 0.166). Flap necrosis was not observed in any of the cases.

The sensory loss was 2 (1.90%) in the primary group, 6 (8.60%) in the Limberg flap group and 5 (6.90%) in the elliptical rotation flap group; not in the karydakis group. There was no statistically significant difference between the groups (p = 0.053).

Recurrence was seen in primary group 10 (9.30%), in Limberg flap group 3 (4.30%), in elliptical rotation flap group 1 (1.40%) and 1 (2.20%) in karydakis group. There was no statistically significant difference between the groups (p=0.075). The median time of recurrences is 8 months.

4. Discussion

In our study, pilonidal sinus surgical methods were evaluated in terms of complications; there was no difference between the groups in terms of hospitalization period and wound site infection, while there was no difference between the groups in terms of seroma, wound dehiscence, flap necrosis, sensory loss, and recurrence.

Although PSD has been known for many years and is frequently encountered in clinical settings, there is no consensus in the ideal surgical technique selection to be performed. The frequency of postoperative complications and the variability of recurrence rates have led to many new methods. The long treatment period of the disease causes labour loss and economic losses. The main surgical treatment is excision. Surgeons use different techniques to close the defect after excision. Each method has its own advantages and disadvantages. The common purpose of all methods is to reduce the recurrence of the disease, not to disturb the quality of life, to reduce the length of hospital stay and to return to work early to prevent economic losses.

The incidence of PSD is known as 26/100000 (Okuş et al., 2013; Sondenaa et al., 1995). In this study, the majority of cases operated for PSD consisted of males (262 males (89.12%) and 32 females (10.88%). M/F ratio was determined as 8,19. The fact that the average age range is among the 2-3. Decades coincides with the natural characteristics of PSD (Sondenaa et al., 1995; Humphries et al., 2010; Duman et al., 2016). These findings are consistent with the literature data.

One of the most important details that determines the quality of life in patients operated for PSD is the duration of hospitalization and the other is the duration of work. The duration of hospitalization is shorter in these four methods compared to patients left for secondary recovery. There was a difference between the groups. The maximum hospitalization was in the limberg group. This finding is consistent with literature data (Okuş et al., 2013; Menteş et al., 2008; Uçar et al., 2015).

In our study, there was no statistically significant difference between seroma accumulation in all surgical methods, however, seroma rate was higher in patients who underwent Limberg flap and primary repair.

The feeding of the flaps in PSD surgery is physiologically mediated by macro and microcirculation. Macrocirculation anatomy is used in flap definition and design. Arteriole capillary, venular and arterio-venous anastomoses at the microcirculation level are the location where cellular metabolism occurs and the perfusion is mainly controlled (Vedder, 2006). This circulatory device is partially disrupted by the flap due to Limberg flap with a sharp angle end resulting in a decrease in regional blood circulation. Since the elliptical rotation flap does not have a corner, microcirculation is maintained and a homogeneous feeding is provided throughout the flap. In the light of the fact that seroma accumulation in the flaps, which are generally better fed, will be less, we believe that less seroma was detected in the oval flaps in this study.

Wound site infection is a complication that can be seen postoperatively in PSD cases and has serious consequences. Infection is an important factor in the occurrence of recurrence. Among the factors that cause infection are increased bacterial colonization, the wound area is close to the anal canal, obesity,

localization difficulty and humidity are considered (Duman et al., 2016). In addition, seroma, hematoma, wound site opening accelerates infection formation (Arslan et al., 2016). The resulting infection may endanger the flap viability, adversely affect the life comfort of the cases, cause reoperations and sometimes lead to painful dressings, which will continue for a long time with the patients being left to secondary healing. The number of infected cases was 5 (7.10%) in Limberg flap group, 1 (2.20%) in karydakis group, 2 (2.80%) in the elliptical rotation flap group and 16 (15.00%) in the primary group ($p = 0.009$). In our study, the rate of infection was higher in the primary repaired group than in the other groups. Among the flap methods, there was less seroma formation than the elliptical rotation flap limberge and karydakis flap method. It was found statistically significant. Limberg flap method in patients who underwent flap treatment compared to those with elliptical rotation flaps can be explained by above mentioned microcirculation problems related to Limberg flaps and especially by the contamination of their corners due to their closer to the anal canal than the elliptical rotation flaps.

In fact, suturing sharp distal edges in Limberg flaps can lead to ischemia and necrosis in the thin corners due to reduced circulation. In elliptical rotation flap reconstruction, flap viability is better especially in distant corners (Polat et al., 2011).

Postoperative loss of regional sensation was observed in the same number of cases in both flap groups and it was determined that the cases were not large enough to cause any discomfort to the quality of life. The sensory loss was six (8.60%) in the Limberg flap group, five (6.90%) in the elliptical rotation flap group, and two (1.90%) in the primary group, but not in the karydakis group ($p = 0.053$). We believe that the similarity of pilonidal sinus tissue in both groups is similar in two groups and this results in similar loss rates. Although it is not statistically significant, loss of sensation in elliptical rotation flap is less than limberg method. It is notable that there is no sensory loss in the Karydakis group.

Although it depends on the technique used, the recurrence which is the main problem experienced after PSD surgery is observed 3-46% in the literature (Sondenaa et al., 1995). In this study, Limberg was seen in three (4.30%) cases in Limberg flap group, 1 (2.20%) in karydakis group, one (1.40%) in the Elliptical rotation flap group and 10 (9.30%) in the primary group. Recurrences are known to develop especially in the first year. Although it is not statistically significant in relapse rates, we see that the number is higher in the primary group. The excisional surgery with primary closure is one of the common methods used as it is simple and early wound healing is its advantage. However, because the relapse rate is reported to be relatively higher compared to other techniques, the frequency of application seems to be significantly reduced in recent years (Ocakoğlu and Ünal, 2012). Karydakis reported that the method he developed was an easy technique, the suture line remained lateral, had early healing and early return advantages, and the recurrence rate was as low as 1% (Karydakis, 1973).

The causes of recurrence are generally deep localization and dampness of the region, vacuum effect of hip movements, obesity and bacterial overgrowth due to poor hygiene. Critical points of Limberg flap technique include unwanted cosmetic appearance, necrosis of the flap corner and maceration on the skin in the incision area. We believe that the lower part of the incision in the Limberg flap at the flap and the lower pole of the flap in the intergluteal cleft cause the incision area to remain moist and to create hygiene difficulties due to its closeness to the anal canal, increasing the risk of localized infection and causing wound dehiscence and recurrence. In fact, in many studies carried out in the literature about that, it has been determined that the defect in Limberg flap technique can be closed completely and without tension and therefore the recurrence rates of wound infection in the early period are less and the time to return to daily life is shorter with fast recovery time of the cases (Mentes et al., 2008). We believe that the results obtained here will be more valuable with multicentric, prospective randomized clinical studies with larger populations that will be performed in the future. Although there are many methods in the treatment of PSD, the superiority to each other in terms of the complications and recurrences has not been established clearly.

Conflict of interest

None to declare.

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Nonsynonymous variations of ion channel-related genes as risk factors in epilepsy

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Abstract

Recurrent seizures are characteristic to epilepsy, which often arise due to increased electrical activity. Ligand-gated ion channels are considered as key factors in epilepsy as they regulate and maintain neuronal membrane potential via regulating ion transportation. Therefore, this study aims to identify ion channel-related single nucleotide variations that are considered as risk factors in epilepsy and determine their potential effects on pathogenicity, protein stability and structure using *in silico* methods. For this purpose, ion channel-related mutations linked with epilepsy were retrieved from ClinVar. Pathogenicity scores and protein stability were predicted using FATHMM-XF and MUpro, respectively. Structural alterations were determined via HOPE server. We identified 17 epilepsy-related missense mutations, 11 of which were in ion channel-related genes. Nonsynonymous substitutions of p.E177A, p.D219N, p.A322D, p.R577Q, p.E282K, p.V831M and p.R1072C were determined as pathogenic, while all mutations resulted in varying degrees of decrease in overall protein stability. Furthermore, all variants were annotated with risk for disease and introduction of distinct side chains caused differences in size, charge and hydrophobicity, as well as contact with other proteins and ligands. In conclusion, mutations in ion channel-related genes were previously identified in several genetic association studies while their functional annotations were not addressed. The results of this study provide a functional explanation to the pathogenic effects of ion channel-related gene mutations that are considered as risk factors in epilepsy.

Keywords: ion channels, missense mutation, epilepsy, risk factor

1. Introduction

Epilepsy is a major neurological disease affecting millions worldwide. It is characterized by recurrent and unprovoked seizures that can cause loss of consciousness or abnormal motor behavior depending on the affected brain region (Stafstrom and Carmant, 2015.). The diagnosis begins with seizure type(s) and can then be defined as focal, generalized, combined focal and generalized or unknown epilepsy when sufficient electroclinical information is available (Fisher, 2017; Scheffer et al., 2017). A seizure is defined as an increase in electrical activity in the brain that can be triggered by various factors such as medications (Chen et al., 2016), metabolic changes (Imad et al., 2015) and infections (Zoons et al., 2008). When seizures emerge spontaneously, they are considered epileptic. The main mechanism that triggers epilepsy is the shift of the balance between excitation and inhibition towards the excitation side. In the central nervous system, there are two important paths that mediate this mechanism. The first is the formation of action potential in neurons and its transmission with axonal conduction. Here, voltage-gated sodium and potassium channels play a significant role. The second one is synapses, in which ligand-gated ion channels such as nicotinic acetylcholine receptor (nAChR) channel, GABA and glycine receptor channels, serotonin and glutamate receptor channels are important. Calcium channels, which allow neurotransmitter vesicles to flow into the synaptic space, also play a role. Mutations in these channels cause a change in the neuronal membrane potential, contributing to the disruption of the balance between excitation and inhibition. As a result, epileptic seizures and even epileptogenesis occur (Armijo et al., 2005).

GABAA-mediated inhibition basically affects postsynaptically located GABAA receptors such as GABRD and GABRA1, through GABA release from synaptic vesicles. In the mature central nervous system, it causes chloride ion [Cl^-] influx and an inhibitory postsynaptic potential. Transient receptor activation with high concentrations of GABA at the synapse is called phasic inhibition. Tonic inhibition refers to the condition in which GABA can move away from the synapse at low concentrations in a spatially and temporally constrained GABAA-mediated inhibition (Farrant and Nusser, 2005). CLCN2 encodes the voltage-gated chlorine channel ClC-2 and is expressed in neurons that are postsynaptic to GABAergic inhibitory synapses. Maintenance of low intracellular chloride concentration by CIC-2 is essential for

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the inhibitory response to GABA (Kearney and Meisler, 2009). CACNA1H serves as a member of the T-type calcium channel family. SLC12A5 gene codes for the K^+/Cl^- co-transporter 2 (KCC2), which is the main Cl^- extruder of neurons and functions as a suitable inhibitor for the neurotransmitters γ-aminobutyric acid (GABA) and glycine (Saito et al., 2017). Myoclonin1/EFHC1 is a newly identified microtubule associated protein (MAP) that directly interacts with α-tubulin via the microtubule binding domain located at the N-terminus. EFHC1 acts as a non-ion channel gene, which encodes a protein that contains a Ca^{2+}-binding EF-hand motif, and overexpression of Myoclonin1/EFHC1 induces in vitro neuronal and synaptic apoptosis by increasing R-type voltage-dependent Ca^{2+} channel currents (de Nijs et al., 2009).

Due to their significant roles in the regulating neuronal membrane potential, ion channels have been a center of attention in the field of neurology. In line with this, several studies in the literature have implicated missense mutations of the voltage-gated chlorine channel encoding CLCN2 gene (Kearney and Meisler, 2009), calcium channel CACNA1H (Heron et al., 2007a), GABAA receptor subunits (Ishii et al., 2017; Niturad et al., 2017), the neuron-specific type 2 K^+/Cl^- co-transporter KCC2 encoded by SLC12A5 (Saito et al., 2017) as well as the non-ion channel gene EFHC1 (Bailey et al., 2017) in epilepsy. Therefore, here in this study, we adopted an *in silico*-based approach to identify ion channel-related single nucleotide variations that are considered as risk factors in epilepsy and determine their potential effects on pathogenicity, protein stability and structure.

2. Methods

2.1. Identification of mutations

Ion channel-related mutations that are considered as risk factors in epilepsy are retrieved from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/clinvar/variation/). ClinVar is a repository of public data regarding associations between genetic variations and human phenotypes based on supporting evidence. The parameters used to identify ion channel-related mutations are depicted in Fig. 1. (ClinVar accession numbers: VCV000016212.2, VCV000127232.1, VCV000016214.1, VCV000009039.1, VCV000002701.1, VCV000002702.1, VCV000002703.1, VCV000218378.1, VCV000002068.1, VCV000002066.1 and VCV000002069.1).

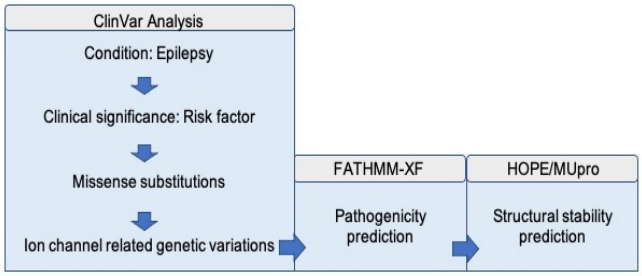

Fig. 1. Flow chart summarizing the methodological approach in the present study

2.2. Prediction of pathogenicity

To determine whether the ion channel-related mutations linked with epilepsy had any deleterious effects, pathogenicity scores were predicted using FATHMM-XF (http://fathmm.biocompute.org.uk/fathmm-xf/index.html) (Rogers et al., 2018). P-values (pathogenicity scores) obtained via FATHMM-XF ranges between 0-1 and values above 0.5 are interpreted as deleterious/pathogenic. FATHMM-XF analysis was run using genomic locations of the mutations (in accordance with genome assembly GRCh38/hg38), which were obtained from ClinVar.

2.3. Analysis of protein stability and structure

Amino acid sequences for the full-length proteins were retrieved from UniProt (GABRD (ID: O14764), GABRA1 (ID: P51788), CLCN2 (ID: P14867), CACNA1H (ID: Q5JVL4), SLC12A5 (ID: O95180), EFHC1 (ID: Q9H2X9)). When more than one isoform was available, the longest isoform, which was chosen as the canonical sequence, was preferred. Analysis of protein stability in relation to the missense amino acid substitutions was performed using MUpro, which is a tool for protein stability prediction (http://mupro.proteomics.ics.uci.edu) (Cheng et al., 2006).

Structural alterations due to identified pathogenic mutations were determined via HOPE server (https://www3.cmbi.umcn.nl/hope) (Venselaar et al., 2010) using amino acid sequences obtained from UniProt.

3. Results

3.1. Ion channel mutations as risk factors for epilepsy

ClinVar revealed 23977 genetic variations in total including but not limited to frameshift, missense and nonsense mutations, deletions, duplications and insertions. The majority of these variations originated from germline mutations (n=21935), while the others were either de novo (n=1053) or somatic (n=48). Among them, 26 variations were attributed clinical significance as risk factors. We further narrowed down the list of epilepsy-related genetic variations by missense mutations, which returned 17 results in GABRD, SLC2A1, CLCN2, GABRA1, EFHC1, CILK1, PDX1, CACNA1H and SLC12A5 genes. SLC2A1 is a glucose transporter, CILK1 is an intestinal serine/threonine kinase and PDX1 functions as a pancreatic nuclear factor; therefore, we omitted them in further analyses and focused on the ion channel-related GABRD, CLCN2, GABRA1, EFHC1, CACNA1H and SLC12A5 genes.

We identified 11 missense mutations (Table 1) within these ion channel-related genes that were implicated as risk factors for epilepsy. All of the identified nucleotide variations were associated with different subtypes of epilepsy with distinct seizure properties and/or affected age groups. Specifically, SLC12A5 R1072C substitution and EFHC1 I174V and C259Y substitutions were linked with susceptibility to IGE and JAE (juvenile absence epilepsy), respectively.

Table 1. Ion channel gene variations analyzed in this study

Gene name	Function	AA substitution	Condition	Reference
GABRD	Ligand-gated chloride channel inhibitor	E177A	Generalized epilepsy with febrile seizures plus	Dibbens et al., 2004
GABRA1	Ligand-gated chloride channel inhibitor	D219N, A322D	Epilepsy, juvenile myoclonic	Lachance-Touchette et al., 2011; Ding et al., 2010
CLCN2	Voltage-gated chloride channel	R577Q	Epilepsy with grand mal seizures on awakening	Saint-Martin et al., 2009
CACNA1H	Voltage-dependent calcium channel complex protein	F161L, E282K, V831M	Epilepsy, childhood absence	Chen et al., 2003
SLC12A5	Potassium/chloride transporter	R1072C	Epilepsy, idiopathic generalized (susceptibility to)	Kahle et al., 2014
EFHC1	Calcium ion binding protein	I174V, C259Y	Epilepsy, juvenile absence (susceptibility to)	Stogmann et al., 2006
		D253Y	Myoclonic epilepsy, juvenile	Suzuki et al., 2004

Table 2. *In silico* analysis of pathogenicity and protein stability for the selected ion channel mutations

				Pathogenicity		Protein Stability	
Gene name	**AA change**	**dbSNP ID**	**Allele frequency**	**Prediction**	**Score**	**Prediction**	**ΔΔG**
GABRD	p.E177A	rs121434580	n/a	Pathogenic	0.757931	Decrease	-0.23182591
GABRA1	p.D219N	rs587777364	n/a	Pathogenic	0.929361	Decrease	-1.1316196
GABRA1	p.A322D	rs121434579	n/a	Pathogenic	0.941727	Decrease	-0.76389212
CLCN2	p.R577Q	rs137852682	$3.13e^{-4}$	Pathogenic	0.879425	Decrease	-0.78638535
CACNA1H	p.F161L	rs119454947	$8.81e^{-6}$	Benign	0.095123	Decrease	-0.57233831
CACNA1H	p.E282K	rs119454948	$1.69e^{-5}$	Pathogenic	0.924846	Decrease	-0.73848501
CACNA1H	p.V831M	rs119454949	$1.43e^{-5}$	Pathogenic	0.916787	Decrease	-1.879213
SLC12A5	p.R1072C	rs548424453	$4.95e^{-5}$	Pathogenic	0.745658	Decrease	-1.0625526
EFHC1	p.I174V	rs137852779	$3.98e^{-6}$	Benign	0.171890	Decrease	-0.97574957
EFHC1	p.C259Y	rs137852780	n/a	Benign	0.180000	Decrease	-1.0762814
EFHC1	p.D253Y	rs137852778	$1.99e^{-5}$	Benign	0.416226	Decrease	-0.52608728

3.2. Determination of deleterious mutations

The potential outcomes of ion channel-related missense mutations were predicted using FATHMM-XF, which identified seven mutations, namely p.E177A, p.D219N, p.A322D, p.R577Q, p.E282K, p.V831M and p.R1072C as pathogenic (pathogenicity score>0.5) (Table 2). All missense substitutions of the EFHC1 gene (p.I174V, p.C259Y, p.D253Y) were predicted as benign, along with CACNA1H mutation p.F161L. Pathogenicity scores closer to 1 indicate higher confidence and accuracy; thus, substitutions of p.D219N, p.A322D, p.E282K and p.V831M were identified as the most confident and accurate deleterious mutations (p-values above 0.9).

3.3. Structural effects

In order to evaluate the potential outcomes of these pathogenic mutations on protein structure and function, we performed protein stability prediction via MUpro and determined the structural changes induced by a single amino acid substitution at the tertiary (3D) level using the HOPE server. Analysis of protein stability in relation to the missense amino acid substitutions using MUpro showed that all mutations resulted in varying degrees of decrease in overall protein stability (Table 2). Lower ΔΔG values implicate a greater decrease in protein stability, pointing towards GABRA1 p.D219N, CACNA1H p.V831M and EFHC1 p.C259Y mutations as the top three most destabilizing mutations. Single nucleotide variations resulting in missense substitutions often cause alterations within the overall protein structure. Results of the HOPE server analysis showed that all variants were annotated with risk for disease. The pathogenic ion channel mutations introduced distinct side chains (Fig. 2), causing differences in size, charge, and hydrophobicity, as well as contact with other proteins and ligands. Specifically, E177A substitution resulted in a smaller and more hydrophobic residue with a neutral charge, while the wild type residue had a negative charge. Similarly, the negative charge of the wild type residue was neutralized by the D219N substitution. A322D mutation led to a less hydrophobic residue with a negative charge, as opposed to the neutral wild type residue. The loss of hydrophobicity was predicted to affect the interactions between the GABRA1 protein and the membrane lipids.

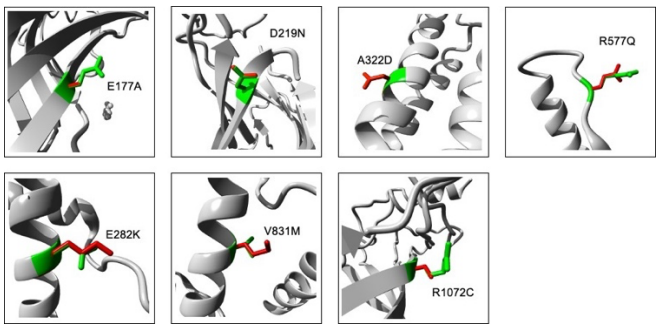

Fig. 2. Structural alterations of the 3D organization resulting from ion channel-related pathogenic missense mutations p.E177A, p.D219N, p.A322D, p.R577Q, p.E282K, p.V831M and p.R1072C. Wild-type amino acids are indicated in green, while mutated amino acids are shown in red

Substitutions of R577Q and R1072C caused the neutralization of the positive charge of the wild type residue. E282K caused the most drastic negative to positive charge difference, which could potentially result in repulsion of other residues in the protein or its ligands. A322D, E282K and V831M introduced larger amino acid side chains into the protein, which were predicted to lead to structural bumps. Furthermore, structural predictions identified E177A, A322D, R577Q and V831M substitutions as damaging as their wild type counterparts were highly conserved.

4. Discussion

Only one third of all epilepsy patients share a medical condition that causes epilepsy such as head trauma, stroke or tumor; of which 70% are diagnosed with idiopathic epilepsy via electrophysiological methods (Gardiner, 2005). On the other hand, genetic factors are considered as more dominant determinants in epilepsy patients. Several subtypes of idiopathic epilepsy emerge with multi-factorial reasons such as the monogenic autosomal dominant idiopathic generalized epilepsy (IGE), certain focal epilepsies and generalized epilepsy with febrile seizures plus (GEFS+), while monogenic epilepsy predominantly occurs with single gene defects (Heron et al., 2007b; Scheffer et al., 2017). One such group of genes that are implicated in familial human epilepsy includes genes encoding for subunits of nicotinic acetylcholine receptors (nAChR), chlorine channel, GABAA receptors, potassium channels, sodium channels and calcium channels. For instance, Genetic epilepsy syndrome (GES) is associated with the mutations in synaptic proteins, which involve neurotransmitter receptors and voltage-gated ion channels creating an abnormal thalamocortical excitability leading to chronic seizures. Monogenetic mutations in GES are generally associated with ion channel mutations (Heyne et al., 2018). In line with the previous work in the literature suggesting significant roles for ion channelopathies in epilepsy, here we identified ion channel-related mutations that are considered as risk factors and determined their potential effects on pathogenicity, protein stability and structure. Our *in-silico* analysis has identified mutations in GABRD, GABRA1, CLCN2, CACNA1H, SLC12A5 and EFHC1 genes as potential risk factors for epilepsy. GABRD and GABRA1 are receptor subunits of GABAA, which acts as an important inhibitor of postsynaptic potential by enabling Cl^- influx. Mutations in the receptor subunits of GABAA often results in the removal this inhibition and causes epileptic discharges. Previous studies have identified single nucleotide variations in several genes encoding GABAA receptors. In particular, E177A mutation was shown to alter cell surface expression and single-channel gating properties of GABRD and was proposed as a susceptibility factor for GEFS+ and JME (Feng et al., 2006). Similarly, A322D and D219N mutations were reported to change gating kinetic properties of GABRA1 (Lachance-Touchette et al., 2011). Moreover, A322D affected GABAergic bouton and dendritic spine formation as well as miniature inhibitory postsynaptic current amplitude in pyramidal neurons, while D219N did not (Ding et al., 2010; Lachance-Touchette et al., 2014).

Voltage-gated chlorine channel ClC-2 is encoded by CLCN2 gene, which is associated with several IGE subtypes including JME, CAE and EGMA (grand mal epilepsy) (Gardiner, 2005). Mutations in CLCN2 were shown to result in reduced ClC-2 activity and overstimulation of postsynaptic cells at GABAergic synapses, which might lead to epileptic seizures (Kearney and Meisler, 2009). *In vitro* studies using recombinant ClC2 channels suggested a loss-of-function role for R577Q and showed that it causes deactivation of the voltage-gated chlorine channel (Saint-Martin et al., 2009). Intracellular [Cl^-] concentration is mainly regulated by the K^+/Cl^- co-transporter KCC2, which is encoded by SLC12A5. Disruptions in [Cl^-] homeostasis, possibly due to KCC2 dysfunction, are implicated in the pathogenesis of common neurodevelopmental disorders including mental disability, autism spectrum disorder, schizophrenia and epilepsy (Bozzi et al., 2012). Therefore, variants of SLC12A5 can potentially be pathogenic for neurological and psychiatric disorders by interfering with the excitation-inhibition balance. In support of this, studies identified SLC12A5 variants as risk factors for seizure disorders, detected pathogenic SLC12A5 variants in patients with neurodevelopmental diseases (Merner et al., 2015) and studies on human genetics associated mutations in KCC2 with the development of epilepsy (Saito et al., 2017).

JME is a lifelong disease, comprises 3-30% of all epilepsies and has no definitive treatment. EFHC1 variants are the most common mutations observed in hereditary myoclonic and grand mal clonic-tonic-clonic convulsions of JME (Bailey et al., 2017). Myoclonin1/EFHC1 is actually a microtubule-associated protein involved in the regulation of cell division and it affects neuroblast migration and synapse/dendrite formation (Grisar et al., 2012). Impairment of EFHC1 in rats was shown to disrupt the radial migration of projection nodes by affecting the development of the neocortex through ex vivo and utero electroporation, the mitosis and cell cycle output of cortical progenitors, radial glial structure (scaffold)

organization and the movement of postmitotic neurons (de Nijs et al., 2009). Furthermore, EFHC1 mutations were associated with increased sensitivity to myoclonic and grand mal seizures and disruption of apoptosis in neurons/synapses where R-type voltage-dependent calcium channel function changes (Grisar et al., 2012). The calcium channel CACNA1H was first defined in the "Genetic Absence Epilepsy Rats of Strasbourg" model, which exhibited increased T-type currents with age and reflected the temporal profile of epilepsy development (Rajakulendran and Hanna, 2016). Mutations in CACNA1H were identified in patients with IGE and CAE, and changes in canal function similar to those due to CAE-related mutations were observed (Chen et al., 2003; Heron et al., 2007a). F161L, E282K and V831M mutations in CACNA1H were reported to cause shifts in membrane polarization and activation potentials (Khosravani et al., 2004). Among these mutations, V831M that we characterized as pathogenic, had a more significant effect. Overall, these results suggest that, in humans, CACNA1H might increase neuronal firing by decreasing the threshold value in neurons, thus leading to overstimulation. However, the role of CACNA1H in epilepsy has been controversial as no seizures were observed in CACNA1H knockout mice (Chen et al., 2003). Likewise, the ClinGen study group recently classified CACNA1H as a controversial gene and claimed that it provides poor genetic support in terms of clinical value (Helbig et al., 2018).

In conclusion, it is clear that ion channels and other related genes are key factors in epilepsy due to their critical importance for the regulation and maintenance of neuronal membrane potential via controlling ion transportation. Although single nucleotide variations of these genes have been widely studied, previous works have mainly consisted of genetic association studies and lacked establishment of functional links. In this paper, we identified a short-list of nonsynonymous mutations in ion channel-related genes, some of which were previously addressed by others in the context of epilepsy while the rest were analyzed for the first time in this study. Furthermore, we determined their potential effects of on pathogenicity, protein stability and structure and provided a functional explanation to the pathogenic effects of these mutations that were observed previously in genetic association studies.

Conflict of interest

The authors declare no financial or non-financial conflict of interest.

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Antitumoral effects of *Santolina chameacyparissus* on non-small cell lung cancer cells

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Abstract

Santolina chameacyparissus (Santo) is an evergreen plant which is traditionally used for its anti-inflammatory effects in various diseases. In this study, we aimed to explore the effects of Santo in non-small cell lung cancer cells. We extracted volatile oil from the plant and evaluated cytotoxicity, apoptosis, and motility effects of the extract on two non-small cell lung cancer (NSCLC) cell lines; one is a patient derived and the other one is a commercially available A549 cells. We also identified its components via GC/MS and investigated possible targets of the major components of the plant using qPCR and docking studies. Cytotoxicity tests showed dose dependent cell killing activity and flow cytometry assays exposed apoptotic effects of Santo. The essential oil also remarkably decreased migration rate of A549 cells, therefore we evaluated the expression levels of epithelial to mesenchymal transition related genes E-cadherin and Vimentin ratio, *ZEB1* and *SNAIL* and another motility related gene *Ezrin*. Santo did not change the expression of EMT related genes but decreased *Ezrin* levels. According to the results of the GS/MS analysis, Artemisia ketone and Camphor were identified as major molecules of the extract. Docking analysis also revealed that artemisia ketone, the major component of the Santo extract, potentially showed strong binding to the active site of ezrin protein and both artemisia ketone and camphor had ability to bind DNA. The results of the present study indicate that Santo and its components artemisia ketone and camphor are promising anti-cancer agents, and their potential in targeting DNA and oncogenic proteins in the lung cancer cells seems worth to focus on this plant in cancer related drug discovery science.

Keywords: essential oil, lung cancer, antitumor agents, ezrin, santolina chameacyparissus, molecular docking, artemisia ketone, camphor

1. Introduction

Aromatic plants have been used since ancient times for their medicinal properties. These properties can be partially or wholly related to their volatile oil fractions which can be also referred as essential oil (Merle et al., 2004). Various essential oils have been used to treat illnesses at different periods in the history can now be reached at the market and subject to regulation in most countries (Baser and Buchbauer, 2010). The diverse therapeutic potentials of essential oils have attracted the attention of many researchers to investigate their anticancer activity because of the limitations and adverse effects of common antitumor agents.

Santolina chameacyparissus also known as Cotton Lavender or Gray Santolina, is an evergreen plant that belongs to the Asteraceae family which has grayish leaves and yellow flowers. It is an aromatic plant, mostly found in Mediterranean region and traditionally used for pain release of premenstrual syndrome, treatment for infections and infestations and digestive disorders due to its anti-inflammatory effects (Mete, 2019). Studies in the literature from Tunisia, Algeria and India also support antimicrobic and antiparasitic activities of the plant (Suresh et al., 1997; Boudoukha et al., 2016, Bel Hadj Salah-Fatnassi et al., 2017) and recently, possible cytotoxic effects of Santo's on cancer cells have also been shown (Elsharkawy, 2014). Even though the components of the essential oils are different between these studies, oxygenated monoterpenes and sesquiterpenes consist most of the essential oil derived from Santo. Among monoterpenes, limonene, peryllil alcohol have been shown to prevent breast, liver, lung, and other cancers (Crowell, 1999), and p-cymene and thymoquinone contribute to autophagic cell death in ovarian, breast and colon adenocarcinoma cells (Ashrafizadeh et al.,

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2019). There are other individual studies focusing on the effects of other components of essential oils derived from various herbals such as β-pinnen, myrcene, artemisia but there still little is known about the effects of Santo's anti-tumoral activity and which of its compounds might have which of the effect remains uncertain.

In this study, to explore anticancer effects of *Santolina chameacyparissus,* we derived essential oil from the plant, from southern part (Osmaniye city) of Turkey and identified its components via GS/MS. We used two non-small cell lung cancer (NSCLC) cell line; one was a patient derived and the other one was a commercially available, and evaluated the antitumoral activity of *Santolina chamaecyparissus* on these cells. Via docking studies, we speculate Ezrin is a possible target of Artemisia ketone which came forward as the major component of the plant.

2. Materials and methods

2.1. Isolation of the essential oil

On April 2018, leaves were collected from the *S. chamaecyparissus* plant and tree of *C. aurantium* grown in the Karacaoglan campus of Osmaniye Korkut Ata University, Osmaniye, which is 20 km away from the East Mediterranean Sea (37°02'303"N; 36°13'0.91"E) and 150 m above sea level. The plant samples were diagnosed by Dr. Fuat BOZOK with the Flora of Turkey and the East Aegean Islands (Davis, 1982). The shade-dried samples were powdered in a Waring blender and hydrodistillated for three hours using a Clevenger according to the method recommended in the current European Pharmacopoeia to obtain essential oils (European Pharmacopoeia, 2008). The essential oils were dried over anhydrous sodium sulphate and stored in an amber vial in the dark at 4ºC for further use.

2.2. Gas chromatography-mass spectrophotometry analysis

The components and the quality of the extracted essential oil was investigated using gas chromatography (GC, Agilent Technologies 6890N Network system) with flame ionization detection (FID) detector GC/FID and gas chromatography combined with a mass selective detector GC/MS (Ulukanli, et al., 2015). Retention indices (RI) calculations were also confirmed with the results obtained from alkanes (C8-C32). Further, RIs of the constituents were compared with the authentic substances. Mass spectra data of the tested complex were crosschecked with the earlier data in the GC/MS databases of the Wiley 2001 library data (NIST 02 version 2.62) and the published references (Adams, 2009).

2.3. Establishment of primary cell line (SA7) and cell culture conditions

Pathologist approved lung squamous cell carcinoma tumor specimens was derived from a 61-year-old male patient who was operated at the Çukurova University Medical Center and given consent to join the research. Ethical approval was obtained from the Local Ethical Board (Number: 2018-73). Small fragments of the tumor tissue (1 mm^3) washed twice in PBS and re-suspended in cell culture medium (RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1%, 200 IU/ml penicillin-streptomycin) then plated in flasks. Cell-culture medium was replaced every three to five days and the cells were cultivated using trypsin to eliminate fibroblasts before starting experiments. A549 cells were provided from Gaziantep University and maintained with the same conditions with SA7 primary cells. Both A549 cells and SA7 cells were cultured under standard conditions at 37°C with 5% CO_2.

2.4. siRNA transfection, western blot, and antibodies

Ezrin siRNA (Ambion Life #s1496) and ON-TARGET plus non-targeting control siRNA (Dharmacon, #D001818-02) were transfected as previously described (Saygideger-Kont et al., 2016) using Lipofectamine 2000. For western blot, cells were lysed with RIPA buffer (50mM Tris HCl pH 7.4, 1%Nonidet p-40, 0.5% Na-deoxycholate, 0.1% SDS, 150mM NaCl, 2mM EDTA, 50mM NaF) supplemented with protease and phosphatase inhibitors and DNAse. For protein quantification, bicinchoninic acid assay was performed and samples were boiled in SDS-PAGE sample buffer as previously described (Saygideger-Kont et al., 2016). Antibodies were as follows: Ezrin (E8897) from Sigma-Aldrich, Actin (C-11) from Cell Signaling, Alexa-Fluor mouse (A21235) secondary and DAPI (D1306) from Thermo Fisher.

2.5. Immunofluorescence (IF) and microscopy

IF staining of A549 cells to confirm Ezrin expression was performed as previously described (Saygideger-Kont et al., 2016). Basically, cells were seeded onto collagen coated coverslips in 12-well plates and fixed with 3.7% paraformaldehyde in PBS, permeabilized and blocked with 1% BSA, 10% goat serum and 0.5% Triton X-100 in PBS before incubation with primary and secondary antibodies and DAPI in the dark. Nikon Eclipse Ti-S microscope with Nikon Intensilight C-HGFI illuminator used to detect images.

2.6. Cell viability

Santo was tested for its cytotoxic effect against two NSCLC cell line, one is a new primary squamous cell lung cancer cell line SA7 and other one is a well-studied lung adenocarcinoma cell line A549, using (MTT) Thiazolyl Blue Tetrazolium Bromide method as previously described (Saygideger-Kont et al., 2016). Briefly, SA7 cells (1.0×10^4/200 mL/well) were cultured in a 96-well plate for overnight at 37ºC, 5% CO_2 and 80% humidity in their respective medium containing 10% FBS and 1% AB. After 24h old medium was removed and the cells were incubated with 0-500 µg/mL concentrations of the oils for 24 and 48h at 37ºC, 5% CO_2. Cells with 0.1% DMSO (vehicle control) and cisplatin (positive control) were also incubated at the same conditions. After incubations, 20µL of MTT solution (5 mg/mL in PBS buffer) was added and the cells were further incubated at 37ºC, 5% CO_2 for 4h to metabolize MTT by viable cells. After MTT treatment, the supernatants were carefully removed, 50µL DMSO was added to each well and then absorbance was measured at 630 nm subtracted from optical density at 570 nm in a multi-well plate reader.

2.7. Flow cytometry

To determine the apoptotic effects of the essential oils on the cells in vitro, Annexin V staining was performed according to the protocol of BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit. For quantitative analysis, 200 µM and 400 µM concentrations of essential oil were tested on A549 cells. Cell suspension (1.0×10^7 cells/mL) in serum-free medium was incubated with the respective compound in 6-well plates in the CO_2 incubator. At the 24th and 48th hour, the cells were harvested and incubated with FITC-Annexin V and PI. The fluorescence emission of FITC-Annexin-V-stained cells was measured at 633 nm (Red laser) in a flow cytometer (Beckman Coulter/CytoFLEX, United States). Dots represent cells as follows: lower left quadrant, normal cells (FITC−/PI−); lower right quadrant, early apoptotic cells (FITC+/PI−); upper left quadrant, necrotic cells (FITC−/PI+); upper right quadrant, late apoptotic cells (FITC+/PI+).

2.8. Scratch assay

A549 cells were plated in 6cm plates in regular growth medium containing 10% FBS. After 24h, a straight-line scratch was made on cell layers using a sterile 1mL disposable pipette tip and washed with PBS. Then, the cells treated either with the essential oil (200 µg /mL) or with PBS in the growth media. Images of cell migration were taken using inverted microscope at 0h and 24h after the scratch. Gap lengths from various points of the scratch were measured using Image J.

2.9. Quantitative Polymerase chain reaction (qPCR) and primers

Total RNA was extracted from A549 cells using RNA isolation kit (Macherey-Nagel, Germany) and reverse transcribed using High-Capacity cDNA Reverse Transcription kit (ThermoFisher Sci.) according to their protocols. qPCR was performed on Light Cycler 480 Instrument (Roche) in 96 well-plate in triple form for each condition using SYBR green mix (Ampliqoon). 18S rRNA was used as internal control gene and fold differences were calculated using $2^{-(\Delta\Delta CT)}$ method which ΔΔCT symbolize (Normalized control sample CT)-(Normalized treated sample CT) (Table 1).

Table 1. Primers used in the experiments

Gene name		Primer sequence
18S rRNA	F	5′-cttagagggacaagtggcg-3′
	R	5′-acgctgagccagtcagtgta-3′
E-Cadherin	F	5’-gtcattgagcctggcaatttag-3
	R	5’-gttgagactcctccattccttc-3’
Vimentin	F	5’-cagctttcaagtgcctttctg-3
	R	5’-cttgtaggagtgtcggttgtt-3
Ezrin	F	5’-catcactgaggcagagaagaac-3’
	R	5’-tgtcattgtgggtcctcttattc-3’
SNAIL	F	5’-gcgatgcccagtctagaaa-3
	R	5’-ggtaatgtgtgggtccgaata-3
ZEB1	F	5’-cttctcacactctgggtcttattc-3’
	R	5’-cgttcttccgcttctctcttac-3’

2.10. Molecular docking studies

In this process, Discovery Studio (DS) 2019 (Dassault Systèmes, 2019) was used to exert molecular docking for understanding interactions between *3,3,6-Trimethylhepta-1,5-dien-4-one* (Artemisia ketone, AK) and Ezrin. Molecular docking includes three steps. First step, the small compound as ligand and human ezrin model structure were prepared using Gaussian 09 (Frisch et al., 2009) and DS 2019 (Phang et al., 2016) software for molecular docking calculations. The ligand, artemisia ketone was drawn and minimized at DFT/B3LY/6-31G* level by using G09. The protein crystal structure of ezrin (PDB code: 4RM9) (Phang, et al., 2016) was taken from the Protein Data Bank. The target protein subsequently optimized using CHARMm forcefield and the adopted-basis Newton-Raphson (ABNR) method (Chattaraj et al., 2011) available in the DS 2019 protocol until the root mean square deviation (RMSD) gradient was < 0.05 kcal/mol $Å^2$. Second step, the active sites were determined by using define and edit binding site subprotocol of DS software. Lastly, *Dock Ligands* (CDOCKER) was applied using the default settings. The best pose of the related compound was defined based on the docking score, binding energy and root mean square deviation (RMSD) values.

2.11. Statistical analysis

Three replicates for each experiment were performed and values were expressed as the mean and standard deviation. Data were subjected to Prism (v. 8.2) for column analysis, dose-response analysis and for graphics. $P<0.05$ was counted as significant when indicated.

3. Results

3.1. Characterization of the essential oil derived from Santo

The oil extracted from *S. chamaecyparissus* by hydro distillation had a pale-yellow color and fragrant pleasant odor. The components, percentages, and retention index of the constituent of the essential oil of Santo is shown in Table 2. Artemisia ketone (33.36%), and camphor (20.86%) were found to be the major compounds of the essential oil. The other increased components were determined as alpha-bisabolol (6.65%), β-Phellandrene (5.61%), camphene (4.63%), sabinene (2.12%), β-pinene (1.77%), myrcene (2.13%), santolina triene (1.4%), and endo-borneol (2.02%).

Table 2. Chemical composition of the essential oil from *Santolina chamaecyparissus* assessed by GC-MS

Name	% Area
Santolina triene	1.488
Camphene	4.639
Sabinene	2.123
beta-Pinene	1.772
Myrcene	2.129
β-Thujene/beta phellandrene	5.611
Artemisia ketone	33.360
Artemisia alcohol	1.930
Camphor	20.864
alpha-bisabolol	6.658
endo-Borneol	2.023

The components of the local essential oil were different from previously published Santo oils (Bel Hadj Salah-Fatnassi et al., 2017; Suresh et al., 1997; Ortiz de Elguea-Culebras et al., 2018; Djeddi et al., 2012) that major component was Artemisia ketone (AK) in the present study. AK is an enone and was found effective in malarial and other parasitic diseases (Radulovic et al., 2013). The plant part and harvesting period usually effect the composition of the corresponding essential oil. However, the quality of an essential oil also depends on several factors which must be taken into consideration such as plant age, vegetation cycle, geographic situation, mode of extraction (Lota et al., 1999; Ferhat et al., 2006) and methods of analysis (Mondello et al., 1995; Boussaada and Chemli, 2007). The differences between the results of this study and previous reports may be attributed to these variables.

3.2. Santo had cytotoxic effect on lung cancer cells inducing apoptosis

In cell viability assay we tested different concentrations (0 to 1000 µg/mL) of *Santo* on lung cancer cells A549 and SA7 for 24h and 48h. Compound had cytotoxic effect on the cells and IC50 values at 24th hour was calculated between 92 to 100µg/mL for SA7 and 200 to 240µg/mL for A549 in replicated experiments (Fig. 1). At the 48th hour, IC50 values did not differ with 24h.

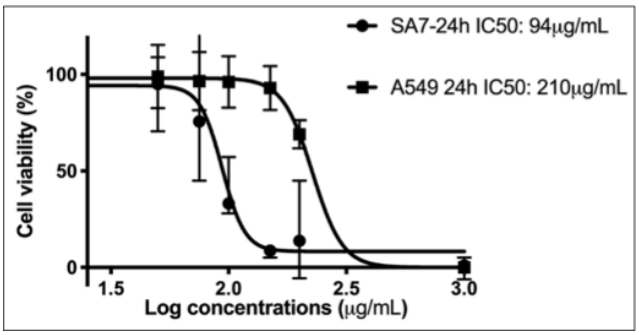

Fig. 1. Cell viability assay. Evaluation of the cytotoxic effect of Santo on SA7 and A549 cells was performed via MTT test with increasing concentrations of Santo (0 to 1000 µg/mL) for 24 hours. Complex had cytotoxic effects on both cells and IC50 values were calculated using Prism V8

We further evaluated the induction of apoptosis in A549 cells for 24h and 48h. We treated cells with 200 µg/mL and 400 µg/mL concentrations of essential oils and used FITC-Annexin V/PI flow cytometry assay to analyze apoptotic cell ratio. The percentage of apoptosis was found to be higher in the increased dosages and prolonged treatments (Figs. 2A and 2B) and we have concluded that compatible with the cytotoxicity experiments, the essential oil had dose-dependent cytotoxic effects. In addition, the results revealed that Santo had its cytotoxic effect in the apoptotic way. Different studies evaluated Santo extract on cancer cell lines and found different results ranging between high cytotoxicity (has triterpene activity) (Elsharkawy and Aljohar, 2016), to low cytotoxicity against A549 cells (Elsharkawy, 2014). The letter, the extract consisted mainly of oxygenated monoterpene compounds rich in Curcumin, Thujol, limonene oxide, Bornyl, Eucalyptol, alpha-bisabolene epoxide, Carveol, caryophllene oxide and Camphor while in our study main component of Santo was artemisia ketone and camphor which might explain the differences between the results.

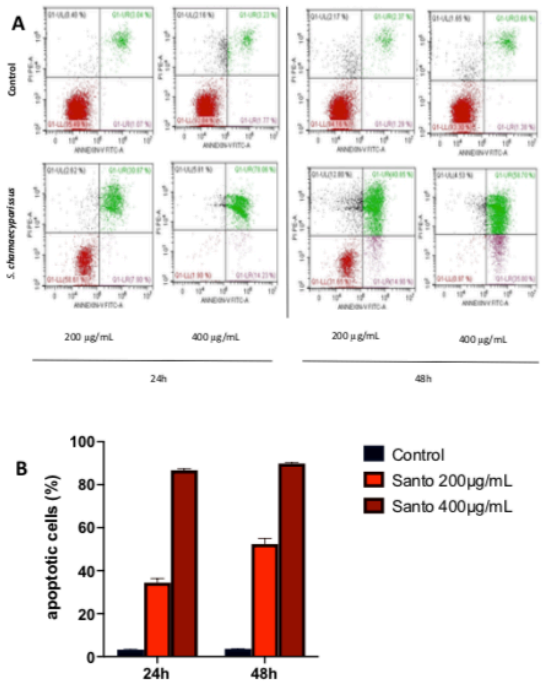

Fig. 2. Flow cytometry analysis of Santo treated A549 cells. Cells were treated with 200 and 400 µg/mL Santo and equal amounts of DMSO as control. Flow cytometry was performed at the 24th and 48th hours of the treatment. In each square image of flow cytometry, upper (late apoptotic cells/green) and lower (early apoptotic cells/purple) right panels show the apoptotic cells of the population (A). Total apoptotic cell rates are given with the bar graph (B)

3.3. Santo suppressed cell motility in A549 cells

We investigated the role of Santo, on the motility of A549 cells via scratch assay. The measurements showed a decreased gap closure on with *Santo* treatment at the end of the 24th hour, comparing to the control cells (Fig. 3A). The percentage of the migrations are shown in Fig. 3B and were significantly different between the two groups ($p<0.0001$). The results suggested that, suppressing cell motility in A549 cells, Santo also had another anti-tumoral effect in lung cancer cells.

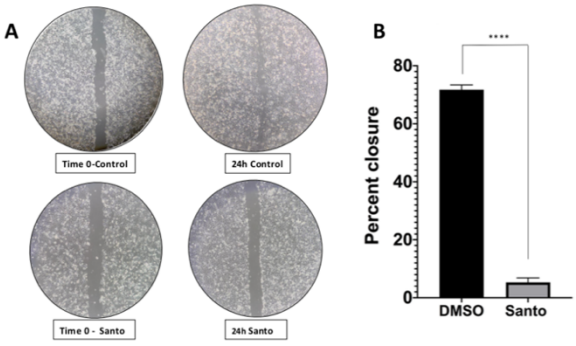

Fig. 3. Effects of Santo on A549 cell migration. Representative images from the scratch assay at time 0, and 24h in the presence of 200 µg/mL Santo (A). The migratory level (%) of A549 cells were quantified by the gap closure and analyzed via Prism (**** $p<0.001$) (B)

3.4. Santo did not change *E-Cadherin / Vimentin* mRNA ratio but suppressed Ezrin expression

To evaluate the underlying mechanisms of Santo's inhibiting cell motility, we evaluated mRNA expression of Epithelial to Mesenchymal Transition (EMT) related genes *E-Cadherin*, *Vimentin*, *Snail* and *Zeb1*, and additionally *Ezrin* which codes a cell membrane-cell skeleton connected protein and takes place in cell motility in cancer cells including A549 and claimed to control TGF-β1 induced EMT in these cells (Chen MJ et al., 2014). The qPCR results regarding with EMT related genes were non-specific in repeated experiments and *E-Cadherin* / *Vimentin* ratio did not change after Santo treatment in A549 cells which did not explain the motility suppression after the essential oil treatment (Figs. 4A and 4B). On the other hand, Ezrin expression was significantly down regulated (p=0.04) with Santo treatment which might be the responsible for the inhibition of cell motility in A549 cells (Fig. 4A).

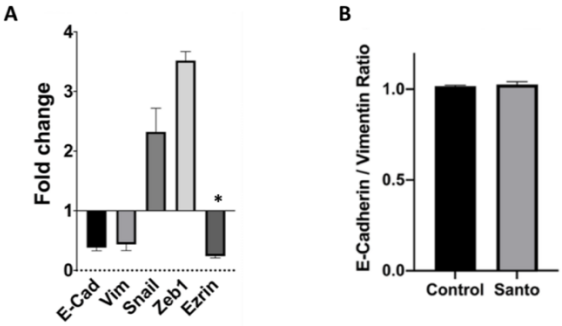

Fig. 4. qPCR analysis of Ezrin and EMT related gene expression. E-Cadherin, Vimentin, Snail and Zeb1 and additionally Ezrin primers used for qPCR analysis. 18S used as internal control. The mRNA expression of EMT related genes were non relevant that both epithelial marker E-Cadherin and mesenchymal marker Vimentin down regulated after Santo treatment while Snail and Zeb1 expressions were increased. Interestingly, Santo significantly suppressed Ezrin mRNA expression (p=0.04) (A). E-cadherin/Vimentin ratio did not differ between Santo treated or DMSO treated A549 cells (B)

3.5. A549 cells expressed ezrin protein and knockdown of Ezrin suppressed cell motility

After analyzing qPCR results, we decided to knock-down Ezrin in A549 cells to see if the motility effect would be like Santo treated cells. Before knock-down experiments, we evaluated ezrin expression in regularly growing A549 cells with immunofluorescent staining and showed that the cells expressed considerable amount of ezrin especially located in and behind the cell membrane (Fig. 5A). We then knock-downed Ezrin via siRNA technique and performed scratch assay in both Ezrin siRNA and scrambled RNA treated cells. The results revealed that Ezrin down regulation suppressed cell motility in A549 cells alike with Santo treatment (Figs. 5B and 5C). We used western blot to confirm Ezrin knock down (Fig. 5D).

3.6. Docking Studies showed potential strong arthemisia ketone- Ezrin binding

In the section 3.1, we have demonstrated that the two major component of Santo used in this research were Artemisia Ketone (AK) and Camphor. Since Santo consisted of multiple compounds, we focused on AK and camphor to speculate their potential anti-tumor activities and designed docking studies to evaluate the interaction between these two complexes and Ezrin, and DNA.

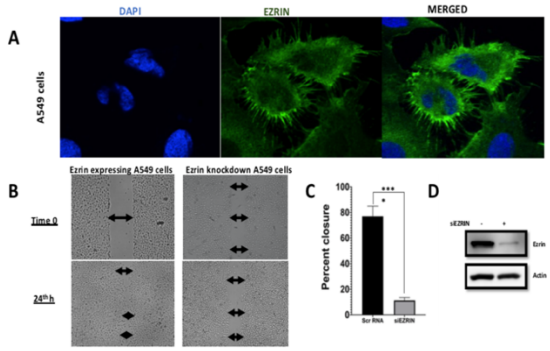

Fig. 5. Ezrin knockdown in A549 cells. We first evaluated Ezrin expression and localization in A549 cells via immunofluorescent staining. A549 cells expressed significant amount of Ezrin and localized in and around the cell membrane as well as cytoplasm (A). After Ezrin knockdown with siRNA, we performed scratch assay (B), and as a result Ezrin knockdown significantly suppressed A549 cell motility ($p<0.001$) multiple gap measurements are made using image J and percent closure is shown in the bar graph (C). Confirmation of Ezrin knockdown via western blot is given (D)

4. Discussion

Among these docking evaluation, AK-Ezrin complex had the strongest interaction on one hydrogen bond at Thr567 (2.798 Å) and had eight hydrophobic interactions with Met558 (4.954 Å), Pro236 (5.00 Å), Lys564 (4.462 Å), Met558 (3.903 Å), Arg562 (4.897 Å) and His554 (4.510, 4.828, 4.526 Å), respectively (Fig. 6). The hydrogen bond at Thr567 might inhibit the activity of Ezrin protein because ezrin is known to change its structure from closed to open form to complete its job in the motility and invasiveness of the cancer cells (Antelmi et al., 2013). The detailed information about the mentioned complex can be seen in Supp. Table 3. The results of the docking calculations between AK and ezrin, comprising of binding energy, docking score and RMSD values are given in Table 3. Specifically, binding energy of any compound shows how the ligand interaction with the target. In this case, the compound, having a low binding energy value to Ezrin (-11.413 kcal / mol) supports a strong binding potential. AK, as mentioned before, is a compound that is effective to inhibit malaria (Radulovic et al., 2013) and a recent research suggest that anti-malarial compounds had ability to bind Ezrin in surface plasmon resonance (SPR) and immunoprecipitation and immunoblotting experiments (Celik et al., 2015). Besides, some of these compounds inhibited cell migration and pulmonary metastasis of osteosarcoma cells in-vitro as well as embryonic development in zebrafish assays in-vivo (Celik et al., 2015). This literature, combining with the findings of this research, support the idea of AK's being a potential ezrin inhibitor in Santo extract.

Table 3. Binding Energy, Docking score and RMSD values of Artemisia ketone and Ezrin

Name	Binding Energy (kcal/mol)	Docking score	RMSD (Å)
Artemisia ketone	-11.4131	-20.1775	0.0890

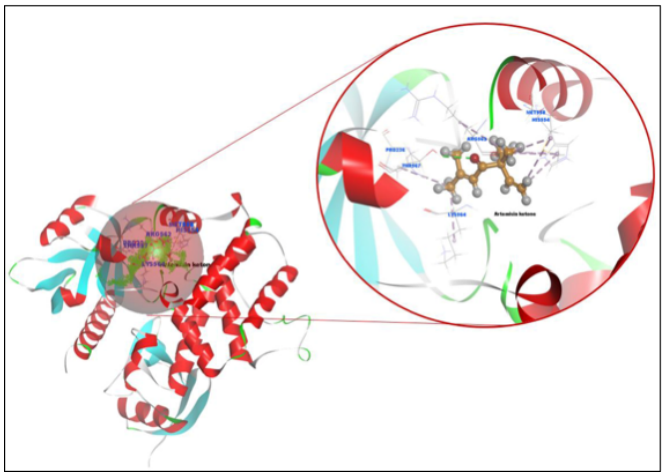

Fig. 6. Top scoring Ezrin along with the crystal structure for compound, Artemisia ketone (orange carbons) shows one H-bonding of the O atom of ketone group to Thr567 (2.798 Å) and eight hydrophobic interactions of the related compound at Met558 (4.954 Å), Pro236 (5.00 Å), Lys564 (4.462 Å), Met558 (3.903 Å), Arg562 (4.897 Å) and His554 (4.510, 4.828, 4.526 Å), respectively

We also studied DNA docking of AK and Camphor which the results indicate weaker binding comparing to AK-Ezrin binding (Fig. 7). Since DNA is a complex biomolecule to be affected from every biochemical change occurring in the cells, the interaction of small compounds with DNA might generate significant effects. The DNA bindings of these two molecules provide that AK-DNA interaction is more stable than Camphor-DNA interaction, based on their calculation of binding energies. In the meantime, these interactions are revealed that the related compounds are intercalating agents on DNA. The results regarding DNA are in Table 4.

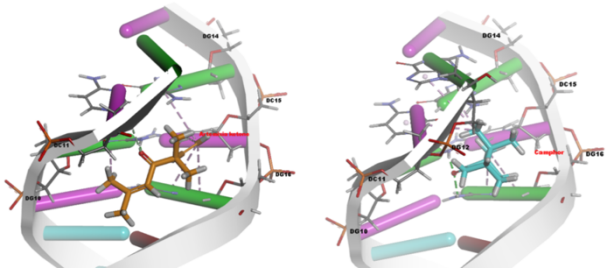

Fig. 7. We also studied DNA docking of AK and Camphor which the results indicate weaker binding comparing to AK-Ezrin binding

Table 4. Binding Energy, Docking score and RMSD values of Artemisia ketone and Camphor against DNA

Name	Binding Energy (kcal/mol)	Docking score	RMS D (Å)
Artemisia ketone-DNA	-5.480	-9.1629	0.0067
Camphor-DNA	-5.410	-34.6270	0.0064

In this research, the essential oil extracted from *Santolina chamaecyparissus,* wildly growing around southern part of Turkey, was found to show in vitro anti-cancer activity on A549 cells. The extract induced apoptosis, inhibited cell motility and downregulated *Ezrin* expression which is a protein that is involved in cell motility and other oncogenic pathways. The major component of the oil Artemisia ketone showed potential strong binding to ezrin protein in the docking studies as potential inhibitor candidate of the protein. Moreover, both major components of Santo are shown to interact with DNA, theoretically. These findings obviously need further investigation to understand the interaction between the Santolina extract and oncogenic proteins such as ezrin in cancer cells as well as *in-vivo* experiments.

Conflict of interest

All authors declare no support, financial or otherwise, from any organization for the submitted work.

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Research Article

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Evaluation of the presence of *AmpC (FOX)* beta-lactamase gene in clinical strains of *Escherichia coli* isolated from hospitalized patients in Tabriz, Iran

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Abstract

Beta-lactamases are the most common cause of bacterial resistance to beta-lactam antibiotics. *AmpC*-type beta-lactamases hydrolyze cephalosporins, penicillins, and cephamycins. Therefore, the study aims was to determine antibiotic resistance and to investigate the presence of *AmpC* beta-lactamase gene in clinical strains of *Escherichia coli* isolated from hospitalized patients in Tabriz. In this cross-sectional descriptive study, 289 *E. coli* specimens were collected from clinical specimens. Disk diffusion method and combined disk method were used to determine the phenotype of extended spectrum β-Lactamase producing (ESBLs) strains. Then PCR was used to evaluate the presence of *AmpC (FOX)* beta-lactamase gene in the strains confirmed in phenotypic tests. Antibiotic resistance was also determined using disk diffusion by the Kibry-Bauer method. A total of 121 isolates were identified as generators of beta-lactamase genes. 72 (59.5 %) isolates producing ESBL and 49 (40.5 %) isolates were identified as *AmpC* generators. In the PCR test, 31 isolates contained the *FOX* gene. The highest resistance was related to the antibiotics amoxicillin (76.12%), ceftazidime (70.24%) and nalidixic acid (65.05%). The results indicate an increase in the prevalence of beta-lactamase genes and increased resistance to beta-lactam antibiotics, which can be the result of improper use of antibiotics and not using antibiotic susceptibility tests before starting treatment. Also, using phenotypic and molecular diagnostic methods such as PCR together can be very useful.

Keywords: extended spectrum β-lactamase producing, *AmpC*, *Escherichia coli, FOX* gene

1. Introduction

Escherichia coli is a major member of the Enterobacteriaceae family and is known to cause many nosocomial infections such as gastroenteritis, neonatal meningitis, sepsis, and urinary tract infections (Eslami and Najar Peerayeh, 2012). *E. coli* is the most common gram-negative bacillus isolated from clinical cases (Jafari Sales et al., 2014; Jafari-Sales and Rasi-Bonab, 2017; Jafari Sales and Mobaiyen, 2017) and is also the cause of more than 80% of cases of community-acquired urinary tract infections (Yazdi et al., 2010). Beta-lactamase is an enzyme that inactivates beta-lactam antibiotics. Penicillinase was the first beta-lactamase to be identified. This enzyme was first isolated from *E. coli* in 1940 (Agrawal et al., 2008). These enzymes inactivate almost all beta-lactam antibiotics by hydrolyzing the amide bond in the beta-lactam ring (Jafari-Sales, 2018; Jafari-Sales and Shadi-Dizaji, 2018; Jafari-Sales et al., 2019; Jafari-Sales and Khaneshpour, 2020). Excessive use of new antibiotics to treat patients and selective pressure on bacteria may affect the production of new beta-lactamases by bacteria (Peter-Getzlaff et al., 2011). In gram-negative pathogens, the production of beta-lactamases is considered to be the most important cause of resistance to beta-lactam antibiotics (Rahimi et al., 2014; Shebani et al., 2010). *E. coli* strains are resistant to beta-lactams through several mechanisms, including changes in outer membrane proteins, overproduction of cephalosporinase (chromosomal and plasmid), or production of an ESBL (Mohamudha et al., 2012). Classification of beta-lactamases functionally began when cephalosporinases were differentiated from penicillinases (Fleming et al., 1963). *AmpC* beta-lactamases were discovered in the late 1970s. *AmpC* beta-lactamases belong to group C or group 1 cephalosporinases, but to some extent have the ability to hydrolyze other beta-lactams. These enzymes hydrolyze broad-spectrum cephalosporins and monobactams but are not inhibited by conventional inhibitors such as clavulanate (Mohamudha et al., 2012). *AmpC* beta-lactamases include *ACC, CMY, FOX, ACT, MOX* and *MIR*. The *ACT, FOX*, and *MOX* genes are resistant to cephalosporins such as ceftazidime, cefotaxime, and aztronam (Philippon et al., 2002). Beta-lactam antibiotics are widely used and the production of *AmpC*-type beta-lactamases results in significant antimicrobial resistance (Peter-Getzlaff et al., 2011). *AmpC* beta-lactamases are generally chromosomal but are easily propagated between other organisms due to motile genetic elements, especially plasmids, and because they are resistant to beta-lactamase inhibitors, especially clavulanic acid, can lead to disruption as a result of confirmatory test ESBL (False negative). Therefore,

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phenotypic diagnosis of *AmpC*-producing microorganisms and ESBLs is difficult (Hanson and Sanders, 1999). The prevalence of *E. coli* strains producing ESBLs in nosocomial infections is increasing and is of concern to the medical community; therefore, this study was performed to evaluate the presence of *AmpC* beta-lactamase gene in clinical strains of *E. coli* isolated from hospitalized patients in Tabriz.

2. Materials and methods

2.1. Sampling

In this descriptive cross-sectional study, 289 *E. coli* samples were collected from clinical specimens including urine, feces, wounds, and blood during six months from March to August 2020 by random sampling method. Clinical samples were collected weekly from hospitalized patients to different wards of the hospital with positive culture for *E. coli*. Then, after obtaining the consent of each patient and filling in the form of their personal and clinical details and observing the statute of the Ethical Commitment Committee regarding the secrecy of the name and details of each person tested, the samples were transferred to the specialized microbiology laboratory of the Islamic Azad University, Ahar branch, and we proceeded to isolate and identify the bacteria causing the infection.

2.2. Isolation and identification

Samples were incubated on Blood agar and McConkey agar for 24-18 hours at 37 °C. The grown colonies were then identified by differential biochemical tests including simon citrate, lysine decarboxylase, urease, TSI, SIM and MR / VP (all media were obtained from Merck, Germany). Then, for further studies, these bacteria were frozen in tryptix medium in broth (Merck, Germany) containing 20% glycerol until further tests at -20 °C (Lin et al., 2010).

2.3. Investigation of drug resistance pattern

Antibiotic resistance pattern of all *E. coli* isolates by Kirby–Bauer standard method and through CLSI (Clinical and Laboratory Standards Institute instructions) and preparation of 0.5 McFarland concentration of bacteria and culture on Mueller-Hinton agar medium against imipenem antibiotics (10 µg), Cefotaxime (30 µg), ceftazidime (30 µg), nalidixic acid (30 µg), amoxicillin (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), norfloxacin (10 µg), and cefxime (30 µg) (Padtan Teb, Iran) was performed. Results were reported according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2013). *E. coli* strain ATCC 25922 was used as quality control of strains for antimicrobial susceptibility testing.

2.4. Phenotypic confirmation of ESBL and *AmpC* producing strains

Combined disk method was used to confirm ESBL-producing bacteria according to CLSI instructions. To confirm the ESBL producing isolates, they were tested using the combined disks of Cefotaxime + Clavulanic acid and Ceftazidime + Clavulanic acid (Jafari et al., 2013). To confirm the phenotypic nature of *AmpC*-producing strains, Cefoxitin sensitivity and Clavulanic acid insensitivity were used and strains with the growth inhibition zone less than 18 mm were selected as possible bla*AmpC*-producing strains (Rostamzad and Padervand, 2016).

2.5. Preparation of samples for molecular analysis

Then, all bacteria were cultured in Loriabertani liquid medium for 24 hours at 37 ° C overnight, and then DNA extraction was performed from the samples using the Invitek Strateg Business kit (made in Canada). The *FOX* gene was then applied together in a PCR reaction based on the specific b-oligonucleotide primers F-AACATGGGGTATCAGGGAGATG and R-CAAAGCGCGTAACCGGATTGG (190 bp) (Mansouri et al., 2014). The final reaction mixture with a volume of 50 µl included 0.1 mmol of each primer, 2.5 µl of 1X PCR buffer, 1.5 mmol of MgCl2, 0.2 mmol of dNTPs, 2 µl of template DNA, 1.5 units of Taq DNA enzyme (Sinagen Iran), which the final volume was increased to 50 µl with sterile deionized water. Thermocycle program (Eppendorf master cycler Germany) 35 cycles as initial denaturation for 3 minutes at 94°C, denaturation stage for 1 minute at 94°C, annealing stage for one minute at 58°C, Extension/elongation stage for one minute at 72 °C, and the final elongation step was performed for 10 minutes at 72°C (Pérez-Pérez and Hanson, 2002). Electrophoresis of PCR products in 0.8% agarose gel was performed in the presence of 100 bp markers and after staining with ethidium bromide, the results were observed with UV. Positive control included (*FOX* gene positive) samples donated by Tabriz University of Medical Sciences. Analyze the data, the twentieth version of SPSS software and Chi-square test was used. $P<0.05$ was considered statistically significant.

3. Results

From 289 samples of *E. coli* in this study, 168 samples of men (58.13%) and 121 samples of women (41.87%) were collected. There were 151 urine samples (52.25%), 70 stool samples (24.22%), 40 blood samples (13.84%) and 28 wound samples (9.69%). The mean age of patients was 44.7±28 years ranging from a minimum of one year to a maximum of 68 years. Antibiogram results show that the highest resistance is related to the antibiotics amoxicillin (76.12%), ceftazidime (70.24%) and nalidixic acid (65.05%) (Figure 1). A total of 121 isolates were identified by the combined disk method as generators of beta-lactamase and *AmpC* genes. 72 isolates producing ESBL and 49 isolates were identified as presumptive producing *AmpC*, which were selected for molecular analysis. In the PCR test, 31 isolates contained the *FOX* gene.

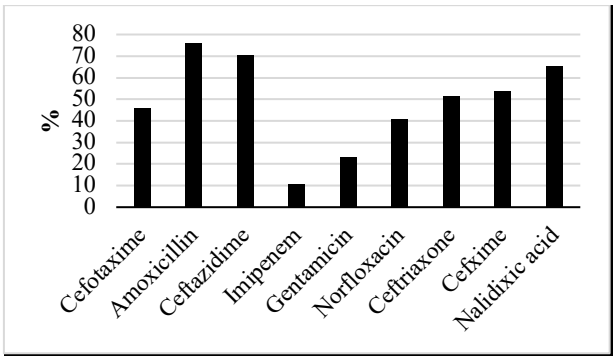

Fig. 1. Percentage of frequency of antibiotic resistance in *E. coli*

4. Discussion

AmpC β-lactamases are dramatically recognized as a growing clinical problem (Den drijver et al., 2018). Detection of *AmpC*-type beta-lactamases in *E.coli* challenges microbiological laboratories. For better treatment, and molecular diagnosis, the use of PCR and other sequencing techniques is necessary, but these techniques are not always available (Kiiru et al., 2012). One of the objectives of this study was to determine the productive strains of beta-lactamase and *AmpC* enzymes.

In the present study, out of 121 isolates, 72 ESBL-producing isolates (59.5%), and 49 *AmpC*-producing isolates (40.49%) were identified. In a study conducted in 2012 in Tehran (Soltan et al., 2013) on clinical specimens including urine, stool, blood and wounds, using a combined disk method, out of 128 samples, 115 isolates (89.8%) were ESBL generators and 13 isolates (10.2%) were *AmpC* generators. The results of this study do not match our study. In a study conducted in 2014 in Kerman (Mansouri et al., 2014) on clinical specimens including urine, blood, and body fluids, using a combination double disk test method, 133 isolates (39.3%) were generators of *AmpC* which is similar to our research and 148 samples (43.76%) were generators of ESBL, which is less than our result. In another study conducted in 2017 in Kerman (Koshesh et al., 2017), out of 105 samples, 42 isolates (40%) were ESBL generators and only 2% of the samples were *AmpC* generators, which does not correspond to our research. As a result of PCR test, 31 isolates contained *AmpC (FOX)* gene. Various studies on the prevalence of the *AmpC* gene, which can give us good information about beta-lactam resistance. In a 2004 study in North America (Deshpande et al., 2006), out of 1429 *E. coli* isolates, 29 samples (2.7%) showed the *AmpC* phenotype and were also found in 3 *FOX* gene isolates, which is much less than our results. In a 2011 study in Pakistan (Hussain et al., 2011), *FOX* gene was detected in 2 samples out of 121 *E. coli* isolates, which is much less than our results. In a study conducted in 2015 in Ilam (Maleki et al., 2015), out of 110 *E.coli* isolates, 28 isolates (40%) were *AmpC*-producing, and finally found in 3 isolates of *FOX* gene. The results of these studies are significantly different from the present study on the prevalence of *AmpC* genes, and it can be concluded that the prevalence of resistance genes in this bacterium is increasing over time, and this issue should be further investigated. It is also estimated from the comparison of the obtained results that the frequency of beta-lactamase enzymes in strain isolated from different countries, and even in hospitals of one country is different, which may be related to differences in infection control and treatment of patients. As long as beta-lactam antibiotics are used in clinical treatments, beta-lactamases play a major role in treatment failure (Maleki et al., 2015). Beta-lactamase-producing bacteria are resistant to various antibiotics, and cause serious problems in the treatment process (Eslami and Najar Peerayeh, 2012). Antibiogram results show that the highest resistance is related to amoxicillin (76.12%), ceftazidime (70.24%), nalidixic acid (65.05%), and the lowest resistance is related to gentamicin (23.18%), and imipenem (10.38%). In a study conducted in 2010 in Tehran (Yazdi et al., 2011) on clinical samples including urine, diarrheal stool, blood, and wounds , using the disk diffusion method, out of 200 samples, the highest resistance was related to amoxicillin (94.5%), cotrimoxazole (80.5%), nalidixic acid (74%) ,which is more than the results of this study, and the lowest resistance is related to imipenem (0.5%) which is less than our study .In a study conducted in 2015 in northwestern Iran (Hasani et al., 2015), among 146 samples, the resistance to cefixime was 94.5%, and nalidixic acid was 80.8% which is more than the results of the present study, and the lowest resistance was to imipenem (5.5%).In another study conducted in 2017 in Kerman (Koshesh et al., 2017), out of 105 *E.coli* isolates, the highest resistance was related to ciprofloxacin (80%), nalidixic acid (71.4%) and the lowest resistance was related to imipenem (3.8%), colistin (3.8%), gentamicin (22.8%), and the results of this study, except for gentamicin resistance, are different from the present study.

Comparing the results of different studies, we find that the prevalence of antibiotic resistance varies in different regions, and this percentage difference can be due to differences in the number of samples, infection control system or history of antibiotic use in each region. One of the limitations of the present study is the time limit in conducting research, and examination of urine, feces, blood, and wound samples of hospitalized patients in all wards of the hospital without classification under study, which can affect the external validity of the results. It is suggested that in future studies, different clinical samples from all wards of the hospital be studied by category, and a pattern of resistance to other antibiotics be determined. Beta-lactamases play an important role in the failure of treatment with beta-lactam antibiotics. In recent years, we have seen an increase in the prevalence of beta-lactamase genes and higher resistance to beta-lactam antibiotics. Therefore, it is better to use antibiotic susceptibility tests before starting treatment to avoid treatment failure and thus the development of resistant strains and it is important to be careful in the use and prescription of antibiotics and to avoid the arbitrary use of antibiotics.

Conflict of interest

The authors declared that there is no conflict of interests.

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Attention-Deficit Hyperactivity disorder in adult primary dysmenorrhea patients

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Abstract

The aim of this study is to evaluate attention-deficit hyperactivity disorder in adult primary dysmenorrhea patients. 62 primary dysmenorrhea patients and 52 control group members are included in the study. Demographic characteristics such as education level and age were noted. Attention-deficit hyperactivity disorder symptoms were identified by the Adult Attention-Deficit/Hyperactivity Disorder Self-Report Scale, Short Form-36 and also Hospital Anxiety and Depression Scale were conducted. According to the Adult Attention-Deficit/Hyperactivity Disorder Self-Report Scale scores primary dysmenorrhea patients had a significantly higher rate compared to the control group. In bivariate analysis, Adult Attention-Deficit/Hyperactivity Disorder Self-Report Scale scores were found to have an association with Short Form-36 physical and mental component scores, anxiety and depression scores. Symptoms of attention-deficit hyperactivity disorder may be confronted in primary dysmenorrhea patients.

Keywords: attention-deficit hyperactivity disorder in adults self-report scale, primary dysmenorrhea, quality of life, anxiety, depression

1. Introduction

Dysmenorrhea is a gynecological problem which is quite common and its characteristics are abdominal and lumbar region pain in the first few days of the menstrual cycle, it may cause limitation of the daily activities. It is categorized as primary and secondary dysmenorrhea, in two groups. Primary dysmenorrhea (PD) is, in the absence of any underlying pelvic pathology, cramps occurring in the uterus during menstruation, whereas secondary dysmenorrhea is menstrual pain that is a result of an underlying pelvic pathologies, for example endometriosis (Sahin et al., 2018; Harel, 2012).

Headache, nausea, vomiting, and fatigue may also be experienced together with dysmenorrhea. 25-50% of the women in reproductive age could be affected by dysmenorrhea (Ju et al., 2014; Zuckerman et al., 2018). Dysmenorrhea can be counted as one of the main factors which disrupt the social activities and quality of life in young women (Nazarpour, 2010; Iacovides et al., 2015). Even if dysmenorrhea is not considered as a morbid situation, it may cause inefficiency and disability (Bajalan et al., 2018). Attention-deficit hyperactivity disorder (ADHD) is a disorder with neurodevelopmental origin, its onset is in childhood and basic characteristics are impaired and inappropriate attention, impulsivity and motor hyperactivity these difficulties often continue to adult ages (Dopheide and Pliszka, 2009), Average prevalence rate of ADHD is about 2.5% 4.9% in general adult population (Simon, 2009).

Adult Attention-Deficit/Hyperactivity Disorder Self-Report Scale is established on the base of criteria for ADHD from the DSM-IV (Kessler et al., 2007). No study investigating the relationship between ADHD and PD has been published yet. This study aims to inspect attention-deficit hyperactivity in adult PD patients.

2. Materials and methods

The study sample was constituted by random patients who registered to Gynecology and Obstetrics Clinics. Current principles of Helsinki Declaration were adopted; institutional ethical committee approval was obtained and patients were informed about the course of study. All patients signed an informed consent form after detailed explanation. Sixty- two adults clinically diagnosed with PD and control group of 52 healthy people were included in this study. The sample size was chosen according to our patients. Studies with common features which published before were also evaluated. Age and education levels were tried to be approximate between volunteer family members, volunteer healthy workers who doesn't have a psychiatric and /or neurodegenerative diseases in their medical history were chosen in control group. Demographic characteristics like age and education level were noted. Exclusion criteria were; history of bipolar disorder, neurodegenerative diseases like Parkinson's disease, psychosis, anxiety disorder, seizure disorder dementia, mental retardation or substance abuse.

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2.1. Attention-Deficit / Hyperactivity Disorder in Adults Self-Report Scale (ASRS)

The ASRS has two subscales, hyperactivity-impulsivity and inattention, each one of them consists of nine items. The criteria of ADHD taken from the DSM-IV constitutes the base of ASRS. The ASRS has a rating scale which expands from 0 to 4: 0 = never, 1 = sometimes, 2 = rarely 3=often, 4 = very often. All items seek answers for the frequency of symptoms in the past 6 months by using a Likert scale with 4 points: 4 for very often, 3 for often, 2 for rarely, 1 for sometimes and 0 for never (Kessler et al., 2007). Dogan et al. performed tests for validation and reliability of the ASRS' s Turkish version (Doğan et al., 2009). Total score of 36 or greater on the ADHD scale was considered as determiner of high ADHD possibility for an individual.

2.2. Quality of life

Short Form (36) Health Survey (SF-36), Version 1.0 (The Health Institute, New England Medical Center) was the tool that used to score Quality of life related with general health (HRQL) in this study. Measurements of the SF-36e are health domains of general health, physical functions, body pain, physical health problems related role limitations, function, vitality, emotional problems related role limitations, and mental health. We can aggregate these domains further into two main groups: physical and mental health summary scores (Ware and Sherbourne, 1992).

2.3. Hospital anxiety and depression (HAD) scale

We used HAD scale to evaluate patients' depression and anxiety levels. There were 14 items which addresses depression (seven items) and anxiety (seven items). Every item had scores from 0 to 3, and total score could change from 0 to 21. The cut-off point for depression and anxiety was 8/21 (Bjelland et al., 2012).

2.4. Statistical analysis

We analyzed the results by a computer software (SPSS version 17.0). In all tests, a $p<0.05$ was regarded as statistical significance. In titer of mean and ±SD, continuous variables were presented. For all variables, normality of distribution was evaluated by using the Kolmogorov–Smirnov test. While Mann–Whitney U test was used for abnormally distributed variables, student's t- test was used for variables with normal distribution and for categorical parameters, Fisher's exact test and Chi-square test was used. Pearson's correlation was used to perform bivariate analyses.

3. Results

PD patients and control group's characteristics are shown in Table 1. Education level and age distribution were approximate in two groups. PD patients showed ASRS scores, depressive scores and anxiety scores with higher significance, in comparison with control group (Table 1).

Mean SF-36 health and physical summary scores of patients with PD were found to be significantly lower than control group members (Table 1). ASRS scores showed negative correlation with SF-36 health and physical summary scores in patients with PD (Table 2). ASRS scores and depression and anxiety scores were positively correlated in patients with PD (Table 2).

Table 1. The characteristics of patients with Primary dysmenorrhea and control group

	Patients with PD (n:62)	Control group (n:52)	P
Age, years	21.24±2.59	21.57±2.32	0.43
Education levels			
Primary school	1	2	
High school	16	20	0.23
University	45	30	
ASRS Inattention	15.54±8.15	8.15±3.50	<0.01
ASRS Hyperactivity-Impulsivity	32.12±6.48	17.13±5.86	<0.01
ASRS total	47.67±9.02	25.28±8.18	<0.01
SF-36 physical component	66.96±20.05	79.13±17.21	0.001
SF-36 mental component	50.98±19.18	71.47±16.06	<0.001
Anxiety score	16.40±3.16	14.23±2.46	<0.001
Depression score	16.67±2.03	15.51±2.10	0.04

PD; Primary dysmenorrhea, ASRS; Adult Attention Deficit/Hyperactivity Disorder Self-Report Scale, SF; Short Form

Table 2. Correlates of ASRS scores in patients with Primary dysmenorrhea

	SF-36 –FC		SF-36 –MC		Anxiety score		Depression score	
	r	p	r	p	r	p	r	P
ASRS Total score	-0.3	*0.001*	-0.50	*<0.001*	0.35	*0.001*	0.27	*0.004*

ASRS; Adult Attention Deficit/Hyperactivity Disorder Self-Report Scale, SF-36 –FC; Short Form- 36- physical component, SF-36 –MC; Short Form- 36- mental component

4. Discussion

This is the first study which has investigated the association of PD with ADHD. The results of study demonstrated remarkably higher mean of ADHD scores than control group. Negative correlations between SF-36 scores and ADHD scores were detected in this study. These results indicated the existence of deterioration of quality of life in PD patients who had increased ADHD scores. Impairment of quality of life in women with PD was also suggested by test results of previous studies (Iacovides et al., 2014; Al-Jefout et al., 2015). Positive correlations between depression and anxiety scores and ADHD scores were found in this study. Association of dysmenorrhea with anxiety and depression was shown in many other studies (Sahin et al., 2018; Nazarpour, 2010; Bahrami et al., 2017).

Gagua et al. showed that women with dysmenorrhea were found to have higher depression and anxiety scores (Gagua et al., 2013). Even though the underlying mechanism of the relationship between quality of life and dysmenorrhea and depression and anxiety has not been fully understood yet, we can explain this relationship as the adverse effect of chronic pain which is experienced in dysmenorrhea. Liang et al. also indicated the possible association of chemicals such as vasopressin, phospholipids and prostaglandins released during menstrual pain with anxiety and depression (Liang et al., 2012). We don't know the reason of higher ADHD scores in patients with P. Co-existing psychiatric comorbidities to this mechanism may be possible in both disease

Some limitations were encountered during this study. First one is the small scale of our study group. Second one is limitations of scales, and diagnoses that shouldn't have been made based on these data alone. Interviews with patients is the key factor the clinical evaluation ADHD. Therefore, in order to confirm these results, studies with larger scales are needed.

As a conclusion, adult PD patients may experience ADHD symptoms. According to findings we had, diminished quality of life, depression, anxiety and ADHD symptoms could be seen among PD patients. It is suggested for all clinicians to evaluate the patients with PD for ADHD symptoms' possibility and when it seems appropriate to make more detailed assessments. We may gain some hopeful outcomes about recovery of PD comorbidities from future studies which will be conducted to seek treatment of ADHD

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Research Article

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A study on influence of different phases of menstrual cycle on hematological parameters

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Abstract

Menstruation is the most common phenomenon observed in fertile women. Menstrual cycle (MC) is of 3 phases: proliferative phase, secretory phase and menstruation phase. It is controlled by endocrine system. Natural fluctuations in sex steroid hormones during MC causes changes in hematological parameters. The aim of the present study to assess the impact of different phases of MC on hematological parameters. The study was conducted in KIMS, Hubli, from 01st March 2011 to 31st March 2012. Women aged between 20-30 years with regular menstrual cycle of 27-30 days were included in the study. During each visit, the subjects' blood was collected and analyzed using KX-21 SYSMEX for various hematological parameters. A total of 50 healthy young women were included in the study. On statistical comparing of hematological parameters, hematocrit, hemoglobin, neutrophil count and eosinophil count showed a significant difference while no statistically significant difference was observed in RBC, leucocyte count, lymphocyte count, monocyte count, erythrocyte sedimentation rate (ESR) and platelet count between different phases of MC. To conclude, the hematological parameters during the MC are highly dependent on the phasic changes in the immune response mechanism and sex steroid hormones.

Keywords: menstrual cycle; hematological parameters; phases of menstrual cycle

1. Introduction

Menstrual cycle (MC) is a most common phenomenon observed in fertile women and other primates during their normal reproductive age between menarche and menopause for the purpose of procreation. Menstruation is defined as "periodic and cyclic shedding of progestational endometrium accompanied by loss of blood" (Tindall, 1987). The menstruation lasts about 4-5 days with an estimated 20-80 ml of blood loss. During MC, a series of utero-ovarian [follicular or proliferative phase (PP)] and hormonal events [(luteal or secretory phase (SP)] take place over a period of 21-35 days, with a mean of 28 days. MC is caused by complex interaction between hypothalamus, anterior pituitary gland, ovaries and uterus (Thiyagarajan and Jeanmonod, 2019; Treloar et al., 1970). Hormones acts as a key regulatory factors in the MC, where their secretion is highly influenced by the negative and positive feedback during the follicular and luteal phases.

The naturally occurring cyclic and rhythmic fluctuations in the levels of hormones like progesterone, oestrogen, luteinizing and follicle stimulating hormones during the MC not only affect oocyte maturation, the endometrial and vaginal environment but are also interrelated with multiple changes in the female body both biochemically and physiologically (Dullo and Vedi, 2008; Usha Rani et al., 2014). These fluctuations largely effect the hemoglobin concentration, platelet count (Alzahrani and Hassan, 2019), other haematological parameters (Usha Rani et al., 2014), immune responses (Northern et al., 1994; Pehlivanoğlu et al., 2001) and serum electrolytes. Changes in oestrogen and progesterone influences Von Willebrand factor concentrations thus effecting platelet function during MC (Drici et al., 1996). Ovulation and menstruation may cause stress induced increase in levels of 17-hydroxycorticosterone which results in eosinopenia. It was reported that fluctuations in ovarian hormones during MC alters immune cells (Pehlivanoğlu et al., 2001). Estimation of leucocyte count helps in women experiencing severe dysmenorrhea to identify underlying reproductive morbidities. Prolonged and excessive bleeding lead to more blood loss (>80 ml) may cause anaemia, making it necessary to estimate red blood cells (RBC), hemoglobin and erythrocyte sedimentation rate (ESR) (Malipatil and Patil, 2013). Numerous studies have been undertaken to examine the changes in various types of blood cell counts and hormonal profile in MC, but the results been variable and contradictory. The present study was aimed to analyse and assess the impact of different phases of MC on hematological parameters.

2. Materials and methods

2.1. Study population

The study was conducted in KIMS, Hubli among 50 healthy

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young women, aged between 20-30 years having regular menstrual cycles from 01st March 2011 to 31st March 2012. The study was approved by Ethical committee of KIMS, Hubli and the study was performed in accordance with declaration of Helsinki. Written informed consent was obtained from patients who participated in this study.

Women with regular menstrual cycle of 27-30 days as by history were included in the study. Women with irregular menstrual cycles, women on any medication or hormonal preparation, any physical illness, women with history of endocrine disorders, bleeding disorders, and excessive bleeding during menstrual phase (MP) were excluded from the study.

2.2. Data collection and analysis

Subjects' history, physical and clinical examination was performed thoroughly before recruiting in the study. Data was collected on pre-approved pro-forma. Subjects were instructed to visit the centre during each phase of menstrual cycle i.e., 1 - 2nd day of MP, 8th -10th day of proliferative phase (PP), and 19th -22nd day of SP.

During each visit, the subjects' blood was collected and analysed using KX-21 SYSMEX – (an automated haematology analyser that uses three detector blocks and two kinds of reagents) for various hematological parameters such as hemoglobin, hematocrit, RBC count, white blood cell (WBC) count, differential count, ESR, and platelet count (Table 1).

Table 1. Hematological parameters analysed using KX-21 SYSMEX

Parameter	Principle	Formula
Whole WBC (white blood cells) count	Optical laser technique	WBC count in 1ml of whole blood
LYM % [WBC-small cell ratio (W-SCR)]	-	Ratio of small cells (lymphocyte) to whole WBC
MXD % [WBC-middle cell ratio (W-MCR)]	-	Ratio of middle cells (basophil + eosinophil + monocytes) to whole WBC
NEUT % [WBC-large cell ratio (W-LCR)]	-	Ratio of large cells (neutrophils) to whole WBC
LYM # [WBC-small cell count (W-SCC)]	-	Absolute count of small cells (lymphocyte) in 1µl
MXD # [WBC-middle cell ratio (W-MCC)]	-	Absolute count of middle cells (basophil + eosinophil + monocytes) in whole blood
NEUT # [WBC-large cell ratio (W-LCC)]	-	Absolute count of large cells (neutrophils) in 1µl
RBC (Red blood cells)	Optical laser technique	RBC count in 1µL of whole blood
HGB (Hemoglobin)	Non cyanide hemoglobin analysis method	Volume (grams) of HGB in 1µL of whole blood
HCT (Hematocrit value)	RBC pulse height detection method	Percentage of whole RBC volume in whole blood
Platelet count	Optical laser technique	Platelet count in 1µL of whole blood

All the hematological parameters during different phases of menstrual cycle were recorded and statistically analysed using the Student's t-test, $p<0.05$ was considered significant.

3. Results

Fifty healthy women, aged between 20-30 years, who volunteered for the study and fits in to inclusion criteria were selected. As shown in Table 2, subjects' hematological parameters such as hemoglobin, hematocrit, RBC count, total leucocyte count, neutrophil count, eosinophil count, lymphocyte count, monocyte count, ESR, and platelet count at various phases of MC were studied and analysed. Among the various phases, ESR (17.08±7.2 mm/at the end of 1st hr) was high in MP, hematocrit (38.13±1.98%), hemoglobin (12.31±0.7 g/dl), RBC count (4.47±0.4 million cells/cu mm), eosinophil count (4.20±1.73%) and monocyte count (1.74±1.77%) was high in PP, and total leucocyte count (7398±1376 cells/cu mm), neutrophil count (60.6±8.74%), lymphocyte count (34.12±7.68%), and platelet count (3.01±0.63 lakh cells/cu mm) was high in SP. On statistical comparison of hematocrit during MP (37.62± 2.14) with that of PP (38.13 ± 1.98), a significant increase was observed during PP ($P<0.05$) while a statistically significant decrease in hemoglobin was observed in SP (12±0.6) compared PP (12.31±0.7; $P<0.05$) and MP (12.23±0.72; $P<0.05$).

Table 2. Hematological parameters in different phases of menstrual cycle

Hematological parameters	Menstrual phase (Mean ± SD)	Proliferative phase (Mean ± SD)	Secretory phase (Mean ± SD)
Hematocrit (%)	37.6162 ± 2.14	38.134 ± 1.98	37.97 ±1.73
Hemoglobin (g/dl)	12.23±0.7	12.31±0.7	12±0.6
RBC count (million cells/cu mm)	4.38±0.36	4.47±0.4	4.37±0.31
ESR (mm/at the end of 1st hr)	17.08±7.2	16±6.1	16.3±9.92
Total leucocyte count (cells/cu mm)	7142±1522	7112±1399	7398±1376
Neutrophil count (% of cells)	59.9±8.49	55.86±7.84	60.6±8.74
Eosinophil count (% of cells)	4.10±2.25	4.20±1.73	3.48±1.32
Lymphocyte count (% of cells)	32.36±7.15	33.72±6.5	34.12±7.68
Monocyte count (% of cells)	1.64±1.77	1.74±1.77	1.38±1.88
Platelet count (lakh cells/cu mm)	2.96±0.68	3.00±0.62	3.01±0.63

In case of neutrophil count, a statistically significant differences was observed on comparing MP (59.9±8.49) with PP (55.86±7.84; P<0.05) and SP (60.6±8.74) with PP (P<0.001) while no statistically significant difference was observed between SP and MP (P>0.05). A statistically significant decrease in eosinophil count was observed in SP (3.48±1.32) compared to PP (4.20±1.73; P<0.05). No statistically significant difference was observed in RBC, leucocyte count, lymphocyte count, monocyte count, ESR and platelet count between different phases of MC. The most significant hematological parameters during different phases of MC were presented in Fig.1.

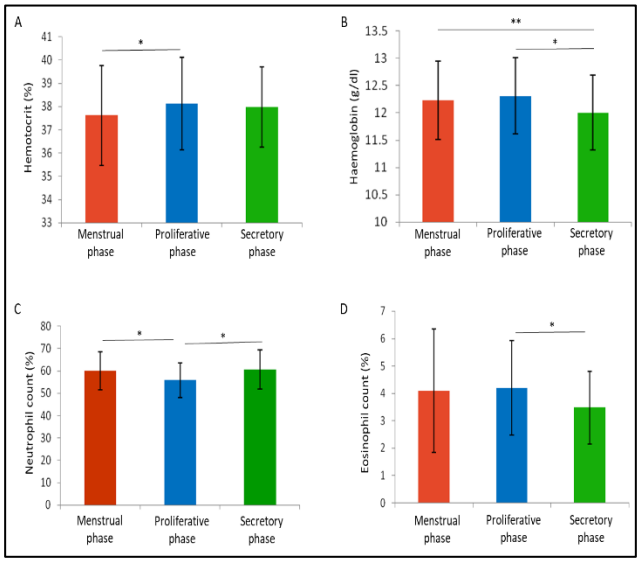

Fig. 1. Comparison of hematological parameters among different phases of menstrual cycle. (A) Hematocrit; (B) Hemoglobin; (C) Neutrophil count; (D) Eosinophil count

4. Discussion

Hematological parameters indicates the status of women health and her reproductive capability. The MC and its interrelated cascade mechanisms during the menstrual phases will largely effect the levels of sex steroid hormones and its associated immune mechanisms. In the present study, hematocrit values were statistically significant (p<0.05) and observed to be high in PP compared to MP and SP which was in accordance with the results of a study by Dapper and Didia (Dapper and Didia, 2002). This might be due to the blood loss during MP of the cycle, leading to a reduction in RBC and fluctuation in oestrogen and progesterone levels (Dapper and Didia, 2002).

Hemoglobin concentration was decreased significantly in SP compared to MP and PP. Similar results were observed in a study conducted by John Reeves et al, where hemoglobin concentration and hematocrit values were found to be lower in secretory phase which may be due to the expansion of plasma volume (Reeves et al., 2001). In par with the previous studies, no significant change was observed in RBC count during the MC (Harewood et al., 2000). During the ovulatory phase of MC, the level of progesterone begins to rise and oestrogen falls. During SP, both hormones rise again but progesterone peaks high compared to low rise in oestrogen. Oestrogen shows direct effect on hemoglobin and hematocrit, however such effects are antagonised by progesterone by the end of SP (Shilpa et al., 2018). An increase in total leukocyte count in SP compared to MP and PP was observed in the present study, which was in agreement with previous studies (Bouman et al., 2001; Tikare et al., 2008). The increased levels of oestradiol and progesterone in the SP might be playing a role in the deviation of immune response toward a type 2 response (Faas et al., 2000). Estradiol also helps in promoting the release of monocytes and granulocytes from bone marrow. IL-4 was significantly increased in T helper cells in SP as compared with the PP of the cycle. Increase in natural killer cells and cytotoxic T cells might also be a reason for increased leukocyte counts (Makinoda et al., 1996; Faas et al., 2000). A statistically significant increase in neutrophil count was found in SP compared to MP and PP. This may be due to oestrogen influence on granulocyte proliferation and may be due to promotion of neutrophil release from bone marrow (Molloy et al., 2003).

Levels of circulating eosinophils highly varies during MC due which both eosinopenia and eosinophilia have been reported (Pathak and Kahali, 1957). A significant increase in eosinophil count was found in PP compared to SP. We found a steady increase in lymphocyte count from MP, PP to SP with no statistical significance. Increase in differential lymphocyte count might be a result of large influx of natural killer cells, cytotoxic T cells, and T helper cells (Faas et al., 2000; Van Den Heuvel et al., 2005). Monocytes play a key role in immune responses and their count is highly dependent on the changes in oestrogen and progesterone levels. These hormones induce mitotic arrest, apoptosis in monocytes, and promotion of monocyte release from bone marrow (Thongngarm et al., 2003; Bouman et al., 2005). Monocyte count was increased in PP and decreased in SP in the present study but none were statistically significant.

ESR was found to decrease in PP and SP compared to MP without any statistical significance. This might due to the increased levels of fibrinogen in MP, because of the sudden loss of sex hormonal support and uterine endometrium necrosis during the process of menstruation (Malipatil and Patil, 2013). Increase in fibrinogen concentrations during MP also influences the rouleaux formation and ESR levels (Dapper and Didia, 2002). In our study, an increase in platelet counts was found in PP and SP compared to MP, showing no statistical significance. Our study is in consistent with previously reported studies, where similar pattern of decrease in platelet count was observed in MP compared to PP and SP (Abbott et al., 2010). Variation of platelet count during MC depends on multiple factors such as corticosteroids or due to an endocrine reaction via spleen on hemopoietic system, or toxic resorption of necrotic material from raw endometrial surface, or because of toxic menotoxin reaction, or variations in the levels of ovarian hormones (Kumar Chawla et al., 2010).

The strengths of the present study is use of automated haematology analyser which helps in reducing manual errors during analysis. The limitation of the study is consideration of comparatively small sample size.

Among all hematological parameters, ESR was high in MP, hematocrit, hemoglobin, RBC, eosinophils, and monocyte count was high in PP, whereas neutrophils, eosinophils, lymphocytes, total leucocytes, and platelet counts were high in SP. This study concludes that the hematological parameters during the MC are highly dependent on the phasic changes in the immune response mechanism and sex steroid hormones

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Research Article

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Effects of ketogenic and western diets on proliferation, vasculogenesis and oxidative stress in the liver

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Abstract

In this study, it was aimed to investigate the effects of different diets on lipid peroxidation, antioxidants, proliferation and vasculogenesis in liver tissue. BALBC female rats (21) were divided as the group fed with standard chow (SC), the group fed with a ketogenic diet (KD) and the group fed with western diet (WD). The rats were fed with tap water and a rat diet specially prepared according to the determined diets for four weeks. Liver tissue oxidative damage, proliferation and vasculogenesis were evaluated using spectrophotometric and immunohistopathological methods. At the end of the experiment, it was found that the highest weight gain was in the WD group and the least weight gain was in the KD group. The mean body weight of the WD group was statistically significantly higher compared to the SC and KD groups ($p<0.05$). MDA levels were found to be lower in the KD group compared to the SC and WD groups. GSH and CAT levels were higher in the KD group compared to the SC and WD groups. In IHC evaluation results, it was seen that Ki-67 percentage in the KD group increased compared to the WD and SC groups ($p = 0.000$), VEGF was close to each other in all three groups and there was no significant difference in the comparisons between groups ($p>0.05$). These results revealed that ketogenic diet prevents tissue damage by decreasing lipid peroxidation in liver tissue as well as inducing cellular proliferation and vasculogenesis.

Keywords: ketogenic diet, oxidative stress, proliferation, vasculogenesis

1. Introduction

Reactive oxygen derivatives (ROS) are consistently produced in low and measurable concentrations in cells and tissues under normal physiological conditions. Their concentrations are determined by the balance between the production and destruction rate of various antioxidant compounds and enzymes (Apel and Hirt, 2004). On the other hand, cells defend themselves against potential damages of ROS through their own antioxidant mechanisms, including multiple enzyme systems, some antioxidant molecules, vitamins and trace elements. There is a strong balance between ROS production and destruction. If this balance is lost, ROS is produced in excess and oxidative damage begins to occur in all tissues (Parihar et al., 2008). In other words, oxidative damage in the cell depends on two factors. The first is the increase in ROS production as a result of chronic diseases or exogenous sources; and the second is the reduction of antioxidant and enzymatic cofactors in the diet. In addition, the dietary composition can affect both conditions (Vetrani et al., 2013).

Ketogenic diet is a diet consisting of high fat, sufficient protein and low carbohydrate that mimics the metabolic changes of hunger in the body (Freeman and Kossoff, 2010). Various studies showed that the ketogenic diet has some effects on epilepsy, obesity, liver, cancer and diabetes. It has been reported to have an effect on various diseases and systems (Wright and Simone, 2016; Newman et al., 2017). Diet improves mitochondrial function in the brain, reduces epileptic seizures, decreases weight, waist circumference, Body Mass Index (BMI), body fat mass, low-density lipoprotein (LDL), triglyceride, HbA1c, fasting insulin; and also increases high-density lipoprotein (HDL) cholesterol, increases insulin sensitivity, inhibits tumor growth in various types of cancer, reduces circulating glucose that causes rapid tumor growth, creates a therapeutic effect in cancer, strengthens memory, affects social behavior. (Boison, 2017; Wright and Simone, 2016). Western diets (WD) are rich in terms of animal fats and food additives; but are poor in other plant-derived molecules such as fiber, vitamins, minerals and antioxidants, and are often consumed in the west and in other countries under the influence of the west (Hariharan et al., 2015). That frequently consumed diet affects processes that have an impact on health such as

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exacerbating the symptoms of kidney failure, obesity, hypertension, colitis; shortening the colon, increasing tumor formation and insulin resistance, causing fatty liver development, increasing liver triglycerides, causing liver damage, increasing Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) levels and changing microbiota composition. (Baena et al., 2017; Wu et al., 2015).

Besides both the ketogenic diet and the western diet have various health consequences indicated above, it is reported especially in recent years that they also affect oxidative stress. While some studies have reported that the ketogenic diet increases oxidative stress (Allen et al., 2013), in others it has been observed that ketones in the ketogenic diet prevent oxidative stress-mediated by mitochondrial dysfunction by providing alternative substrates and antioxidant properties (Greco et al., 2016). Western diet has been reported to increase oxidative stress by the studies examining the effects of Western diet on oxidative stress (Heinonen et al., 2014; Jenkins et al., 2016). Therefore, in this study, the effects of different diets on oxidative stress changes in liver tissue, cellular proliferation and vasculogenesis were examined pathologically and biochemically.

2. Materials and methods

2.1. Ethical approval and drugs

Approval was obtained from Sakarya University Animal Care and Use Ethics Committee for the study (Approval date: 01/07/2020; no: 33). All applications were carried out in Sakarya University Animal Laboratory in accordance with international guidelines. Animals were kept in appropriate wire cages and under standard laboratory conditions (12/12-hour light / dark-light cycle, temperature 22 ° C, humidity 50-60%). All rats were given tap water and specially prepared ad libitum diets for four weeks. Ketamine HCL (Ketalar®, Pfizer, Istanbul) and Xylazine HCL (Rompun®, Bayer, Istanbul) were preferred for anesthesia.

2.2. Study design and creating groups

Twenty-one BALB C female rats (weight 15-19 g and 10-12 weeks) were randomly divided into three groups. *Standard Chow Group (SC);* The rats were fed regular standard rat chow consisting of 77.3% carbohydrates, 2.7% fat and 20% protein of calories. *Western Diet Group (WD);* The rats in this group were fed a western diet consisting of 39.70% of calories from carbohydrates, 39.51% from fat, 19.53% from proteins and 1.26% of other ingredients for 4 weeks. *Ketogenic Diet Group (KD);* This group of rats was fed a ketogenic diet consisting of 4.95% carbohydrates, 74.24% fat, 19.53% proteins and 1.28% other components of calories.

At the end of the experiment, the animals were killed by cervical dislocation while under general anesthesia with 65 mg / kg (i.p) ketamine and 7 mg / kg xylazine (i.p) injection. Samples taken for biochemical analysis were kept at -20 C. Tissue samples reserved for histological evaluations were taken into 10% formaldehyde.

2.3. Biochemical assay

Tissues were washed with 0.9% NaCl solution after weighing. After washing, they were centrifuged (+4 ºC, 3000 RPM, 10 min). They were then homogenized in a cold solution of 1.15% KCl, 0.01M sodium potassium phosphate (pH=7.4). 10% tissue homogenates were prepared. Homogenates were centrifuged at 10.000g for 20 minutes, at + 4°C. Supernatant was taken and used to determine the MDA, GSH and CAT parameters in the tissues. Protein measurement of the samples was performed using the Lowry method (Lowry et al., 1951). The determination of MDA, one of the lipid peroxidation products, in tissue homogenates was carried out using Buege and Aust's (Buege and Aust, 1978) method, based on the principle that MDA reacts with thiobarbituric acid (TBA) to give a colored compound that can be measured at 532 nm wavelength. Results are given as nM / mg protein. For GSH determination, the reaction between 5'5'-Dithiobis 2-nitrobenzoic acid (DTNB) and GSH was used to generate TNB showing maximum absorbance at 412 nm. Results are given as µM / mg protein (Beutler, 1963) CAT activity was determined by a spectrophotometric method based on the degradation of H_2O_2 by CAT. The calculation was made using the absorbance difference that decreases over time as a result of the CAT-peroxide reaction at 240 nm wavelength. Results are given as U / mg protein (Beers and Sizer, 1952).

2.4. Histopathological examination

For histopathological analysis, liver tissues were washed in 10% neutral buffered formaldehyde for 48 hours and in tap water for one day. Then, samples were passed through alcohol series and dehydration process was applied. They were then passed through xylol series to make the tissues transparent and embedded in paraffin blocks. Sections taken by microtome (4µm) were stained with hematoxylin and eosin (H&E). Photographs were taken by examining under a light microscope (Olympus CX31-Japan). Histopathological evaluations were made using Suzuki's quasi-numerical modified scoring system (Suzuki et al., 1993).

2.5. Immunohistochemistry staining

The 4µm paraffin blocks were deparaffinized and were boiled in a microwave oven for 20 minutes. They were kept in 3% hydrogen peroxide for 10 minutes and, UV block was performed for 15 minutes. The primary antibody diluted as 1/300 was dropped and incubated overnight at +4 degrees in a humid environment. Then, they were secondary antibody (Thermo scientific, HRP TP-125-HL-UK) staining following the procedure recommended by the producer company. Finally, they were rehydrated by counter staining with the H&E and the procedure was completed. In our study, the proliferation index of Ki-67 (MS-106-B, Thermo LabVision), which was previously reported in a study, was used to evaluate the groups in terms of proliferation (Hazan et al., 2002). VEGF (Gene TEX-102643) immunohistochemistry application, the staining degree was scored in five randomly selected areas and the area with the highest score was determined. Within both

groups, at least 100 cells were marked in each x40 magnification field. Percentage of stained cells and the degree of staining in sections were taken as criteria. Scoring was done with a semi-quantitative method (Ulloa-Padilla et al., 2020).

2.6. Statistical evaluations

Statistical analyzes were performed using SPSS 22.0 package program (SPSS Inc. and Lead Tech. Inc. Chicago. USA). Shapiro Wilk test was used for the normal distribution of data. One-way ANOVA test was used to compare more than two normally distributed variables. TUKEY HSC test was used for in-group significance. All results are presented as mean ± SD. Results with P <0.05 were considered significant.

3. Results

3.1. Body weight analysis

The change of average body weights of the groups at the beginning and end of the experiment is presented in Fig. 1. Before starting the study, there was no statistically significant difference between all groups in terms of body weight (p> 0.05). Average body weights in body weight analysis performed at the end of the experiment were found to be 17.042 ± 0.237 in the SC group, 17.714 ± 0.397 in the WD group and 16.800 ± 0.509 in the KD group. In the comparisons between the groups, it was observed that the average body weight of the WD group was statistically significantly higher than the SC and KD groups (p = 0.014, p = 0.001, respectively). Although the average body weight of the KD group was lower than the SC group, this difference was not statistically significant (p> 0.05).

3.2. Biochemical Results

As a result of the biochemical measurements, MDA, GSH and CAT levels were found to be significantly different between the groups (Fig. 2). In the comparisons between the groups, MDA levels were found to be statistically significantly lower in the KD group compared to the SC group and the WD group (respectively; p = 0.000, p = 0.000). The mean GSH levels in the KD group were statistically significantly higher compared to the SC and WD groups (p = 0.002, p = 0.019, respectively). When CAT levels were examined, it was found that there was a significant increase in the KD group compared to the SC group (p = 0.020). The average CAT values increased in the KD group compared to the WD group, but this increase was not statistically significant (p> 0.05). When compared with the WD group, there was no statistically significant difference in MDA, GSH and CAT levels of SC group (p> 0.05).

3.3. Histopathological examination results

The preparations were evaluated with sinusoidal occlusion, necrosis, and vascularization evaluation criteria. Vascularization was observed in the SC and WD groups (Fig. 3A-B). When the sinusoidal occlusion and necrosis were examined, it was seen that the SC and WD groups (Fig. 3A-B) had a higher rate compared to the KD group. Portal vein liver sinusoids in the KD group were observed to have more normal cell contours compared to the SC and WD groups (Fig. 3C).

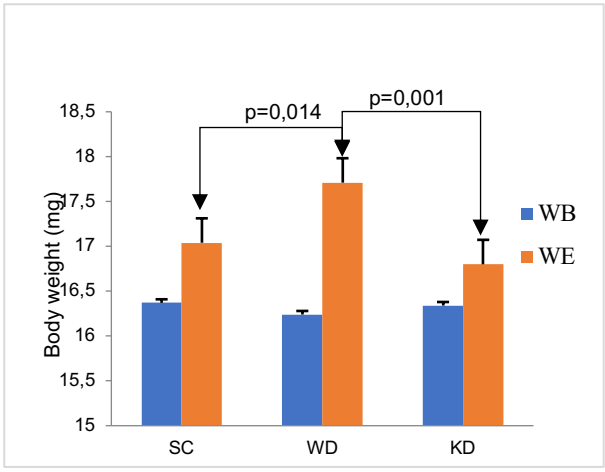

Fig. 1. Evaluation of body weight changes in experimental groups. SC: Standard chow; WD: Western diet; KD: Ketogenic diet; WB: Average body weight at the beginning of the experiment; WE: Average body weight at the end of the experiment; The mean difference is significant at the p <0.05 level

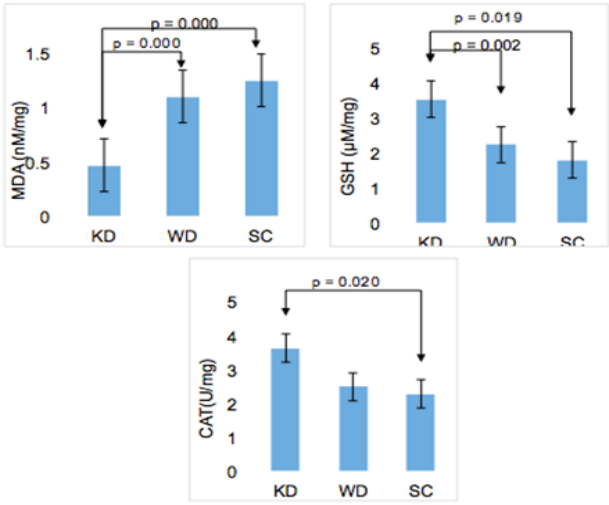

Fig. 2. Evaluation of liver tissue biochemical parameters in experimental groups SC: Standard cow; KD: Ketogenic diet; WD: Western Diet. MDA: Malondialdehyde; GSH: Glutathione; CAT: Catalase; Significance level p <0.05. Results are presented as mean ± SE

3.4. Immunohistochemical staining results

Evaluation results of liver proliferation and vasculogenesis are presented in Fig. 3. It was observed that proliferation (Ki-67) in the KD group increased compared to the WD and SC groups, and vasculogenesis (VEGF) was close to each other in all three groups. Among the groups, the lowest percentage of Ki-67 was detected in the WD group, and this low percentage was significantly lower than the KD group (p = 0.000) (Fig. 3). The highest Ki-67 percentage was found in the KD group. This increase was significant compared to the percentages in all other groups (p = 0.000 for both) (Fig. 3). When VEGF concentrations in the experimental groups were examined, all three groups gave similar results. Among the groups, VEGF was found to be the lowest in SC group and the highest in the KD group. However, there was no significant difference in

comparisons between groups (p> 0.05) (Fig. 4).

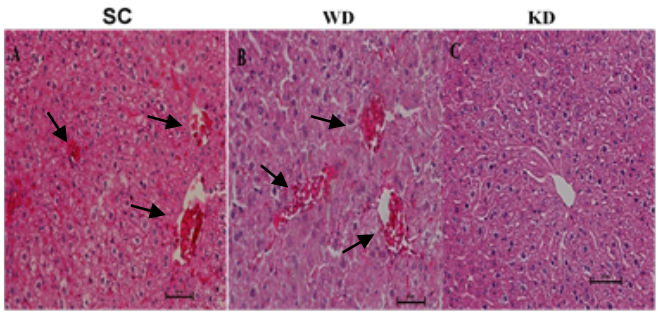

Fig. 3. SC: Standard chow; WD: Western diet; KD: Ketogenic diet. SC Group; Intense congestion of ciosides in the central vein (arrows); WD group; Intense congestion of ciosides in the central vein (arrows); KD group; normal central vein and synosid areas are seen. HE, X200

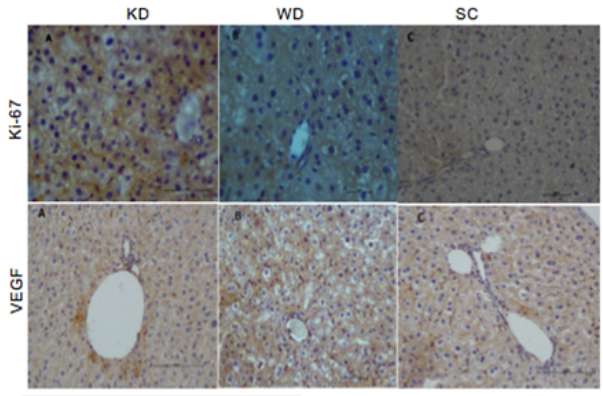

Fig. 4. SC: Standard chow; WD: Western diet; KD: Ketogenic diet. Representative photographs of IHC positivity and statistical evaluation of the effect of diets on liver proliferation and vasculogenesis in rats (400X (50 scale bar)). It is observed that Ki-67 immunopositivity is increased in the KD group compared to WD and SC groups, and VEGF immunopositivity is close to each other in all three groups

4. Discussion

The change in eating habits with modern life causes many metabolic diseases. As a result of the lack of macro or specific micronutrients in the tissues, structural deficiencies in the tissues and dysfunction in the organs occur. It has been suggested that nutritional dietary components may prevent the development of many chronic diseases, as they increase sensitivity to or protect against free radicals (Krenkel and Tacke, 2017). Affecting liver metabolism at the cellular level causes acute and chronic liver diseases. Differences in the diversity and concentrations of nutrients in the content of different diet types are thought to affect human health in different ways (Fleet, 2014). Therefore, in our study, we investigated the effects of different diets (KD, WD and SC) on oxidative stress in the liver tissue of rats, cell proliferation and vasculogenesis with some antioxidant enzymes.

In our study, MDA levels were statistically significantly lower in the KD group compared to the WD group. MDA is the end product of lipid peroxidation and is an essential biochemical parameter used as an oxidative stress marker. Both the ketogenic diet and the western diet were reported to affect oxidative stress (Norton et al., 2020). In the present study, it was observed that feeding with short-term KD caused increases in antioxidant parameters in the liver. Liver GSH levels were significantly higher in the KD group compared to the SC and WD groups. The KD group had the highest average CAT levels. However, there was no significant difference compared to the SC and WD groups. Conflicting results have been reported in dietary studies in the literature. As in some studies, in our study, KD clearly reduced lipid peroxidation, thus oxidative damage. In a study, it was found that short and long-term feeding with KD improved liver oxidative stress markers and feeding with KD significantly increased liver antioxidant capacity and glutathione peroxidase levels compared to those fed with standard rat feed, and decreased liver protein carbonyls (Kephart et al., 2017). In another study, it was shown that long-term feeding with KD (75% kcal fat) regulates GSH biosynthesis in the brain tissue of rats and increases mitochondrial antioxidant capacity (Jarrett et al., 2008). Lu et al. (Lu et al., 2018). found that KD reduces oxidative stress by suppressing some signal pathways after spinal cord injury (Pinto et al., 2018). It has been shown that KD improves mitochondrial function and reduces oxidative stress, and improves mitochondrial respiration by reducing the production of reactive oxygen species of β-hydroxybutyrate. Parry et al. reported that liver SOD and CAT levels were higher in the KD group compared to the SC group, although it was not significant. They noted that the ketogenic diet increased the volume of mitochondria in the liver and the average lifespan in rats, and they reported that the increase in mitochondrial volume occurred without oxidative damage or change in antioxidant protein levels in the liver or brain (Parry et al., 2018).

In studies examining the effects of WDs, it has been shown that their long-term consumption causes weight gain by creating physiopathological changes in lipid and energy metabolism (Wilson et al., 2007). Unlike WDs, short-term (4-6 weeks) and long-term (up to 12 months) studies have shown that KDs cause more fat loss in obese individuals (Harmancey et al., 2010). In our study, it was seen that the highest increase in body weight at the end of the experiment was in the WD group. There are studies in the literature that support our results. It has been shown that consuming a meal of WD causes a decrease in plasma SOD activity in morbidly obese individuals (Mazzoli et al., 2019). Although the physiological mechanisms of these diets on organs are not well understood, both experimental and human studies have shown that obesity is associated with increased oxidative stress markers and lipid peroxidation (Wilson et al., 2007). In the studies examining the effects of feeding with WD; unlike KD, WD has been reported to increase oxidative stress (Jenkins et al., 2016; Norton et al., 2020). Irregularity of hepatic lipogenesis and prolonged exposure of the heart to a high fuel supply have been shown to be important causes of heart failure in rats fed with high-fat WD (Harmancey et al., 2010). In a different study, it

was stated that even though WD increases oxidative stress, vascular cells adapt to this stress by resisting (Norton et al., 2020). In rats fed a diet like a WD diet which is rich in saturated fatty acids and fructose, oxidative stress markers (lipid peroxidation and Nitro-Tyrosine content) were significantly increased, but the antioxidant enzyme CAT decreased (Mazzoli et al., 2019).

In the literature, many markers such as DNA synthesis, mitosis number, cell proliferation and mitochondrial activity have been used to define liver regeneration criteria. Ki-67 antigen in the cell nucleus found by Gerdes et al. and the monoclonal antibody formed against it were described (Garcia-Fuentes et al., 2010). The increase in Ki-67 level is directly proportional to liver recovery and cell renewal. All stages of the cell cycle can be classified since all stages of the Ki-67 cell cycle except the resting stage (G0) can be shown. In our study, we used Ki-67 to show the effects of diets on cellular proliferation in the liver, and the effects on vascularization as IHC using VEGF. In our study, we observed that feeding with KD caused an increase in the percentage of Ki-67 compared to feeding with WD and SC. Thus, in our study, we showed that KD increases cellular regeneration due to cellular proliferation in the liver. VEGF positivity, which is a marker of vasculogenesis, was higher in the KD group, although it did not differ between the groups. In a study, WD was found to cause vascular oxidative stress and a decrease in endothelial function (Jenkins et al., 2016).

The results of our study showed that a low-carbohydrate high-fat diet prevents oxidative damage by decreasing lipid peroxidation in the liver and increasing antioxidant enzymes; also increases cellular proliferation and vasculogenesis. For this reason, we think that feeding with ketogenic diets can prevent the occurrence of diseases and should be preferred as a potential diet (which can be considered as additional treatment) in the treatment of diseases. Besides, changes in DNA and gene transcription levels can be investigated in future studies and their effects on other tissues can be strengthened.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Research Article

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Can histopathology of gastric tissue resected in sleeve gastrectomy be informative about serum iron levels?

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Abstract

One of the most common post-operative deficiencies after bariatric surgery is iron deficiency and one of the important determinants of post-operative iron deficiency is the preoperative condition. In this study, it was aimed to investigate the relationship between iron levels and histopathological findings observed in gastric tissue resected in sleeve gastrectomy. Preoperative and postoperative iron levels were compared with the presence of inflammation, atrophy, *Helicobacter pylori*, intestinal metaplasia, lymphoid follicles, and lymphoid aggregates observed in patients operated due to morbid obesity. The postoperative serum iron levels and preoperative values were compared and a statistically significant increase was found due to the use of iron-containing preparations after the operation. Among the parameters evaluated, inflammation, atrophy, *Helicobacter pylori*, intestinal metaplasia, and the presence of lymphoid follicles were not found to be associated with iron levels, but it was noted that the presence of lymphoid aggregate in all cases and male cases was correlated with preoperative low iron levels (p values 0.047 and 0.015 respectively). In this study, which investigated the role of histopathological findings in the prediction of iron deficiency in sleeve gastrectomies, the relationship between preoperative iron levels, which was reported to be predictive for post-operative iron deficiency, and the presence of lymphoid aggregates was revealed. It is thought that other histopathological findings such as the presence of lymphoid follicle and *Helicobacter pylori* are also important in terms of iron levels but could not be revealed due to the limitations of the study.

Keywords: iron deficiency, sleeve gastrectomy, histopathology, lymphoid aggregate

1. Introduction

Obesity, one of the most important health problems of present and future time, is defined by the World Health Organization (WHO) as abnormal or excessive fat accumulation that poses a risk to the health of the person. (World Health Association, 2020). Obesity, which can be considered pandemic in developed and developing countries; is an important cause of mortality due to increased risk in neoplasms such as breast, endometrium, kidney and colon cancer apart from it's morbidity and accompanying diseases like diabetes mellitus (DM), heart diseases, hypertension, fatty liver disease and sleep apnea syndrome (Sjostrom et al., 2004); Stephenson and Rose, (2003); International Agency for Research on Cancer, 2002). Obesity grading is generally made according to WHO and a body mass index (BMI) above 30 is called obesity. Although there are various treatment options in the treatment of obesity, the method chosen is mostly surgical in patients with BMI above 40, which is called class III obesity. The methods used in obesity surgery are mostly function limiting and are examined under the title of bariatric surgery. Gastric band application, sleeve gastrectomy and gastric bypass are among the methods commonly used today. In sleeve gastrectomy, the stomach is removed along the greater curvature and this resected stomach tissue is sent to the pathology laboratory for histopathological evaluation. Although histopathological evaluation is made according to Sydney classification, additional findings such as the presence of lymphoid follicles and aggregates, and possible benign or malignant neoplasms are also included in the pathology report.

Insufficient intake and malabsorption are two main etiological reasons for nutritional deficiencies. In cases undergoing sleeve gastrectomy or gastric bypass, various vitamin and mineral deficiencies are observed due to absorption defects, as gastric functions decrease or completely disappear (Coupaye et al., 2014; Saif et al., 2012; Al-Mutawa et al., 2018). These deficiencies may be at subclinical levels or, if left untreated, may present with pictures such as anemia or neurological deficits (Al-Mutawa et al., 2018). Vitamin B12 and iron are the most important molecules associated with stomach in terms of absorption. Iron, which is in the structure of hemoglobin and myoglobin, is vitally important. Although

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iron absorption is performed primarily in the duodenum and jejunum, iron absorption is negatively affected if gastric acidity is not sufficient. In this study, we aimed to evaluate the relationship between preoperative and postoperative iron levels and histopathological findings in morbid cases by evaluating resection material after sleeve gastrectomy.

2. Materials and methods

Sixty patients who underwent sleeve gastrectomy in a 3-year period due to morbid obesity were evaluated in the study. Cases without pathology archive material were not included in the study. The sections stained with hematoxylin & eosin (H&E), giemsa and alcian blue were obtained from the pathology preparation archive and re-examined. The presence and activity of gastritis, as well as atrophy, lymphoid follicle and lymphoid aggregate were evaluated in H&E sections. *Helicobacter pylori* presence were evaluated in giemsa stained sections, and intestinal metaplasia in alcian blue stained sections. Demographic data and serum iron levels of the cases were obtained from the hospital information management system. Cases with unknown preoperative and/or postoperative iron levels were excluded from the study. For preoperative iron data, the data closest to the operation date in the same week with the operation date and the data measured at the 6th month postoperatively for postoperative evaluation were used. Histopathological findings and iron levels were compared with the t test for independent variables, and preoperative and postoperative iron values were compared with the t test for dependent variables at 95% confidence interval. SPSS 15.0 package program was used for statistical analysis SPSS Inc. (Released 2006. SPSS for Windows, Version 15.0. Chicago, SPSS Inc.).

3. Results

The mean age of the cases was 35.5 ± 9.3, 45 (75%) cases were female and 15 (25%) cases were male. The mean age of female cases included in the study was 35.4 ± 9.2, and the mean age of male cases was calculated as 35.3 ± 10.3. The frequency of the histopathological findings observed separately in all cases, female and male cases are given in Table 1. A statistically significant increase was found when postoperative iron levels were compared with preoperative values (p = 0.000), since all patients were using a multivitamin tablet containing 14 mg iron once a day after the operation. The same significance was noticed in female and male cases (p values 0.011, 0.000, respectively). Preoperative and postoperative iron values of all cases, male and female cases are given in Table 2.

In the evaluation of all cases, chronic gastritis was observed in 48 cases (80%) and active chronic gastritis in 12 cases (20%). There was no statistical significance between the activity of gastritis and pre and postoperative serum iron values (p values 0.544, 0.617, respectively). When the female and male cases were evaluated separately, the rate of active chronic gastritis was calculated as 22.2% in female cases, while this rate was determined as 13.3% in men. Similar to all cases, there was no statistically significant difference between the activity of gastritis and pre and post-operative serum iron levels in male and female patients (p values 0.200, 0.751, 0.401, 0.291, respectively).

Table 1. The frequency of the histopathological findings observed separately in all cases, male and female cases (columns were evaluated within themselves)

Histopathological findings	All cases (N/%)	Female (N/%)	Male (N/%)
Gastritis	60 (%100)	45 (%100)	15 (%100)
Atrophy	37 (%61.7)	26 (%57.8)	11 (%73.3)
Helicobacter pylori	52 (%86.7)	39 (%86.7)	13 (%86.7)
Intestinal metaplasia	9 (%15)	6 (%13.3)	3 (%20.0)
Lymphoid follicle	31 (%51.7)	24 (%53.3)	7 (%46.7)
Lymphoid agregate	50 (%83.3)	38 (%84.4)	12 (%80.0)

No statistically significant relationship is revealed with preoperative and postoperative serum iron values and histopathologically evaluated parameters as atrophy (p values 0.202 and 0.186, respectively), intestinal metaplasia (p values 0.391 and 0.719, respectively), *Helicobacter pylori* (p values 0.612 and 0.630 respectively) and lymphoid follicle (p values 0.519 and 0.426 respectively). In cases with lymphoid aggregate, preoperative iron values were found to be statistically significantly lower than those without (p = 0.047). The same significance was not observed in the mean postoperative iron values (p = 0.321).

In the analysis of female and male cases in terms of histopathological parameters: the presence of atrophy (p values 0.612 and 0.355, respectively), *Helicobacter pylori* (p values 0.940 and 0.958, respectively), intestinal metaplasia (p values 0.200 and 0.377, respectively), lymphoid follicle (p values 0.791 and 0.318 respectively), lymphoid aggregates (p values 0.179 and 0.412, respectively) and pre-postoperative iron values did not show any statistically difference in female cases. In male cases, the presence of lymphoid aggregate was found to be associated with the mean preoperative low iron values (p = 0.015), similar to that observed in all cases, while the same correlation could not be shown with postoperative iron levels (p = 0.750). Likewise statistically significance was not observed with atrophy (p values 0.430 and 0.293, respectively), intestinal metaplasia (p values 0.566 and 0.389 respectively), *Helicobacter pylori* (p values 0.401 and 0.062 respectively) and lymphoid follicle (p values 0.170 and 0.982 respectively) in male cases.

Table 2. Preoperative and postoperative serum iron values of all cases, female and male cases

	All cases- Means (mcg/dL) /SD	Female- Means (mcg/dL) /SD	Male- Means (mcg/dL) /SD
Preoperative serum iron levels	56.3±27.8	48.7± 24.3	79.1± 25.7
Postoperative serum iron levels	151.2±79.3	146.6± 78.0	165.1± 84.0

4. Discussion

Obesity, which is a global health problem with its rapidly increasing prevalence, brings along an increasing frequency of comorbid diseases with increasing BMI (Guh et al., 2009). Apart from the morbid and mortal situations caused by the disease itself the disease has a serious cost to the society therefore treatment and if possible prevention of obesity has a significant importance. Although non-surgical options such as nutritional change and getting away from sedentary life help to reduce body weight in the short term; surgical approaches provide more benefits in terms of efficient long-term weight loss, maintenance of reduced body weight and reduction of comorbid disease frequency. (Mitka, 2003; Chang et al., 2014). Unfortunately, the methods that are among the surgical approaches also comes out with various problems. Since the stomach and small intestine have an important role in the absorption of nutrients, the loss of tissue in these regions as a result of resections is one of these problems (Mechanick et al., 2013). The main clinical reflection of nutritional deficiencies frequently observed after bariatric surgery is anemia. It is noteworthy that the frequency of anemia is observed at different rates in various bariatric surgery methods. In a study published in 2015, the frequency of anemia after Roux-en-Ygastric bypass method was reported as 4% (Del Villar Madrigal et al., 2015), while in a study conducted on 306298 bariatric surgery cases in France, anemia was reported to be 5.6% in 143733 sleeve gastrectomy cases (Bailly et al., 2008). Theoretically, sleeve gastrectomy causes nutritional deficiencies with a reduction in intake, while gastric bypass has both food intake restriction and malabsorption (Ponsky et al., 2005). In addition, one of the reasons in the pathophysiology of nutritional insufficiency in sleeve gastrectomy is accelerated gastric emptying (Melissas et al., 2008; Braghetto et al., 2009). Moreover, decreased intrinsic factor and hypochloric acid production also negatively affect vitamin B12 and iron absorption, and the use of proton pump inhibitors (PPI) observed in cases worsens this situation (Coupaye et al., 2014).

Most of the studies on hemogram, serum iron and ferritin levels, B12 levels, folate levels, vitamin D and parathyroid hormone levels to assess nutritional deficiencies are aimed at determining the deficiency and its frequency, the breakdown of the time of occurrence, and the times of recovery in levels (Saif et al., 2012; Moizé et al., 2011; Hakeam et al., 2009). Iron is one of the important factors in the etiology of anemia, and studies have shown that serum iron has a wide distribution (Al-Mutawa et al., 2018, Hakeam et al., 2009; Alvarez et al., 2014); therefore, it has been suggested that ferritin may be a more appropriate marker (Al-Mutawa et al., 2018). However, despite all these studies and the theoretical knowledge of the pathopysiology of nutritional deficiencies, the factors to predict the possible deficiency and / or the depth of the deficiency are not clearly known. In one of the few studies conducted for predictive evaluation, Guan et al. (2018) reported that the hemoglobin value, an indicator of nutritional deficiencies, was associated with the post-operative hemoglobin value after adjustment for dependent covariates. In another study, Eroğlu et al. (2019) suggested that the presence of *Helicobacter pylori* in the resected gastric tissue was associated with preoperative red blood cell (RBC), hematocrit (HCT) and hemoglobin (HGB) values, so preoperative evaluation of *Helicobacter pylori* may be predictive in terms of postoperative.

In the present study, postoperative serum iron levels were found to be higher than before the operation, since all patients were using a multivitamin tablet containing iron. Among the histopathological parameters evaluated, there was no statistical relationship between the presence of gastritis and activation status, presence of atrophy and / or intestinal metaplasia, *Helicobacter pylori* status and the presence of lymphoid follicles; and pre- and postoperative iron values. However, it was noted that preoperative iron values were statistically significantly lower in patients with lymphoid aggregate in resected gastric tissue compared to patients without ($p= 0.047$). When the male and female cases were examined separately, it was observed that the mean preoperative iron value decreased in male cases with lymphoid aggregate ($p = 0.015$), while the same difference was not found in female cases. Considering that preoperative iron status is an important indicator of postoperative iron status, it is suggested that the presence of lymphoid aggregates is important histopathologically. Among the main causes of iron deficiency and anemia that develop after sleeve gastrectomies, decreased hydrochloric acid production caused by parietal cell scarcity due to fundus removal plays a role, but this is not the only factor (van Rutte et al., 2014; Snyder-Marlow et al., 2010). There are not many studies evaluating the role of histopathological findings in iron deficiency developing after sleeve gastrectomy, in which preoperative iron deficiency, decreased intake of iron-containing foods, and inefficient use of nutritional supplements for various reasons can be counted as the etiological factors. The fact that the presence of lymphoid aggregate detected in all cases and in male cases is associated with low preoperative iron levels in the present study indicates that the role of *Helicobacter pylori* in this process should be questioned in detail. It is known that lymphoid follicles and / or aggregates are often associated with *Helicobacter pylori*, but may

occasionally be associated with other inflammatory and neoplastic processes (Chakhachiro et al., 2020). Although it is thought that obesity being a low-grade chronic inflammatory condition (Esposito et al., 2003) which may be effective in the formation of lymphoid aggregates, there are also reports that lymphoid clusters that do not contain a germinal center are a normal component of the gastric mucosa without being associated with *Helicobacter pylori* (Carpentieri et al., 2000).

In conclusion, in this study, in which the role of histopathological findings observed in the stomach tissue removed in sleeve gastrectomies in predicting iron deficiency; Preoperative iron levels, which are reported to be predictive for post-operative iron deficiency, have been shown to be associated with the presence of lymphoid aggregates. Our study had limitations such as the small number of cases and the use of iron-containing preparations. It is thought that other histopathological findings such as the presence of lymphoid follicle and *Helicobacter pylori* are also important in terms of iron levels but could not be determined due to the limitations of the study.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Histopathological findings of gastric specimens of patients undergoing sleeve gastrectomy

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Abstract

Sleeve gastrectomy is the most preferred surgical procedure in obese patients. In the literature, the necessity of pathological examination of the specimen is emphasized because of malignant case reports. In this study, we aimed to present the histopathological findings of the specimens of our patients who underwent sleeve gastrectomy due to obesity in the light of the literature. Patients who underwent sleeve gastrectomy due to obesity between 2014-2019 in our clinic were retrospectively screened. Endoscopy was not performed in patients with no symptoms. After the obesity committee, the patients were operated and the specimens were sent for routine histopathological examination. Patients' age, gender, body mass index, surgical procedure information, and histopathological features of resected gastric specimens were analyzed. A total of 411 patients were included in the study and there were 309 (75.2%) female patients. The overall mean age was 35 ± 10.3 and the mean body mass index was 44.7 ± 5.2 kg/m^2. The pathology of 85 (20.7%) of the patients were reported as completely normal. Abnormal pathological findings were detected in 326 (79.3%) patients. No malignant finding was found in the specimen pathology results. The necessity of endoscopic examination before sleeve gastrectomy is controversial. Even if no malignant pathological result was detected in our study, we believe that routine pathological examination is required for medicolegal reasons.

Keywords: laparoscopy, sleeve gastrectomy, stomach, gastrointestinal stromal tumor, adenocarcinoma.

1. Introduction

The number of bariatric operations is gradually increasing with the increasing obesity in the world. According to the estimation of the World Health Organization, there is more than one billion overweight (body mass index ≥25) and over 300 million obese (body mass index ≥30) people worldwide (World Health Organization, 2009). The number of comorbidities such as hypertension, type 2 diabetes mellitus, stroke, cardiovascular diseases, and dyslipidemia is increasing with the increase in the number of obesity. It has been reported that approximately 6700 of 340000 surgical procedures performed worldwide in 2011 were bariatric surgery (Buchwald and Oien, 2013). Laparoscopic sleeve gastrectomy is the most common procedure among obesity procedures. Learning curve and application are easy also are other factors that increase the number of applications is the low morbidity and mortality.

Laparoscopic sleeve gastrectomy is an operation that has restrictive and endocrine effects but no malabsorptive effects. Most of the stomach is removed and the stomach is turned into a tube. Incidental gastrointestinal stromal tumors, dysplasia, and maltoma can be seen as a result of pathological examination of the removed gastric specimen (Canil et al., 2018; Ricci et al., 2018). In this study, we aimed to investigate the presence of incidental precancerous lesions by pathological examination of sleeve gastrectomy specimens applied in our clinic and to present the findings to the literature.

2. Materials and methods

This retrospective study was conducted at Inonu University General Surgery Clinic in accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study protocol was approved by our institutional Ethical Committee (no.2018/16-19). All patients who underwent sleeve gastrectomy due to obesity between June 2014 and August 2019 at Inonu University Faculty of Medicine General Surgery

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Clinic were retrospectively reviewed. Patients with missing data were excluded from the study. 411 patients whose data were available were included in the study. Preoperative informed consent was obtained from all patients in order to examine the resected samples and to use these samples appropriately in the study. Anesthesia consent was obtained after the obesity council made the operation decision. Endoscopy was not performed on patients without symptoms, since it was not in the routine of our clinic. Laparoscopic or open sleeve gastrectomy ± additional surgical procedures were applied to the patients. Patients' age, gender, body mass index (BMI), surgical procedure information, and histopathological features of resected gastric specimens were analyzed. In statistical analysis, quantitative variables were expressed as mean ± SD, median, min-max, and range. Qualitative variables were reported as number and percentage (%).

3. Results

309 (75.2%) of the patients included in the study were female and 102 (24.8) were male. The mean age in the whole group was 35 ± 10.3. While the mean age of men was 34.5 ± 9.8, it was 35.2 ± 10.4 for women. BMI was 44.7 ± 5.2 kg / m^2 in the whole group. BMI of men was 45.5 ± 5.3, and for women, it was 44.4 ± 5.2 (Table 1).

Table 1. Characteristics of 411 sleeve gastrectomy patients

Gender	Number (%)	Age (mean ± SD)	BMI (mean ± SD)
Male	102 (24.8)	34.5 ± 9.8	45.5 ± 5.3
Female	309 (75.2)	35.2 ± 10.4	44.4 ± 5.2

There were patients who underwent additional procedures with sleeve gastrectomy. Only laparoscopic sleeve gastrectomy was performed in 361 patients. Seven of them were anastomosed manually without using a stapler. There were 19, 17, 3, 1, and 1 patient who underwent laparoscopic sleeve gastrectomy with cholecystectomy, jejunoileal bypass (JIB), crurorraphy, JIB + cholecystectomy, and left adrenalectomy, respectively. There were seven patients who underwent open sleeve gastrectomy alone. There was one patient each who underwent cholecystectomy and cholecystectomy + tubal ligation + incisional hernia repair together with open sleeve gastrectomy (Table 2).

No specific pathological finding was observed in 85/411 (20.7%) of the patients and it was reported as normal. Specific pathological findings were present in 326 (79.3%) patients. Helicobacter pylori presence in Giemsa dye was found in 200 (48.7%) patients. Of these, 60/65 (92.3%) were accompanied by chronic active gastritis and 140/257 (54.4%) were accompanied by chronic inactive gastritis. Chronic inactive gastritis 257 (62.5%), Chronic active gastritis 65 (15.8), intestinal metaplasia 17 (4.1%), proton pump inhibitor (PPI) affect 4 (1%), low degree dysplasia 2 (0.5%), Atrophic chronic gastritis 1 (0.2%), ectopic pancreas was reported in 1 (0.2%) patient (Table 3).

Tablo 2. Characteristics of surgery for 411 sleeve gastrectomy patients

Surgery	Number
Laparoscopic sleeve gastrectomy	354
Laparoscopic sleeve gastrectomy + cholecystectomy	19
Laparoscopic sleeve gastrectomy + JIB	17
Laparoscopic sleeve gastrectomy without staple	7
Open sleeve gastrectomy	7
Laparoscopic sleeve gastrectomy + crurorraphy	3
Laparoscopic sleeve gastrectomy + JIB + cholecystectomy	1
Open sleeve gastrectomy + cholecystectomy	1
Open sleeve gastrectomy + cholecystectomy + tubal ligation + incisional hernia repair	1
Laparoscopic sleeve gastrectomy + left adrenalectomy	1

JIB: jejuno-ileal bypass

4. Discussion

Abnormal histopathological findings include a wide spectrum from chronic inactive gastritis to a gastrointestinal stromal tumor. However, the rate of not finding any specific pathological findings in the histopathological examination of the specimen in sleeve gastrectomy has been reported to vary between 0-80.2% (Almazeedi et al., 2013; Miller et al., 2016). In our study, the specimen pathology did not contain specific findings in 85 (20.7%) patients.

Tablo 3. Histopathology of 411 sleeve gastrectomy specimen

Histopathology	Number (%)
Normal (no specific histopathologic change)	85 (20.7)
Abnormal (specific histopathologic change evident)	326 (79.3)
Chronic inactive gastritis	257 (62.5)
Helicobacter pylori	200 (48.7)
In chronic active gastritis	140 (54.4)
In chronic inactive gastritis	60 (92.3)
Chronic active gastritis	65 (15.8)
Intestinal metaplasia	17 (4.1)
Proton pomp inhibitor affect	4 (1.0)
Low grade dysplasia*	2 (0.5)
Atrophic chronic gastritis¶	1 (0.2)
Ectopic pancreas	1 (0.2)

*both low grade dysplasia were accompanied by intestinal metaplasia.
¶ low-grade dysplasia and intestinal metaplasia accompanied atrophic gastritis.

There are two forms of chronic gastritis, active and inactive. In the histopathological examination of sleeve

gastrectomy specimens, the rates of chronic active and inactive gastritis were reported to be 1.6-35% and 12.1-74.5%, respectively (Almazeedi et al., 2013; Ohanessian et al., 2016; Dogan et al., 2017; Raess et al., 2015). In our study, chronic active gastritis was detected in 65/411 (15.8%) patients and chronic inactive gastritis in 257/411 (62.5%) patients. The high presence of *Helicobacter pylori* indicates that many patients need to be treated. However, in the follow-up of these patients, *Helicobacter pylori* infection has been shown to be a disease that does not cause peptic ulcer and cancer. *Helicobacter pylori* was not detected in the majority of urea breath tests performed after surgery. It has been suggested that as a result of removing most of the stomach, the rate of *Helicobacter pylori* decreases as the stomach volume decreases. It has been stated that eradication of this *Helicobacter pylori* infection will cause unnecessary time and cost, especially in non-symptomatic patients (Keren et al., 2009). In the specimen, there were 200 (48.7) patients with signs of *Helicobacter pylori*-associated gastritis. Dysplasia can be seen at a rate of 0.06-0.2% in the histopathological examination of sleeve gastrectomy specimens (Canil et al., 2018; Safaan et al., 2017). These patients need to be followed up strictly. Intestinal metaplasia can be detected at a rate of 0.2-5% and atrophic gastritis with a rate of 0.2-1.8% (Canil et al., 2018; Almazeedi et al., 2013). In our series, low-grade dysplasia was seen in 2 (0.5%) patients, intestinal metaplasia in 17 (4.1%), and atrophic gastritis in 1 (0.5%). One of the two patients with dysplasia had only intestinal metaplasia; the other was accompanied by atrophic gastritis and intestinal metaplasia.

The incidence of gastric cancer after sleeve gastrectomy is very rare (Scozzari et al., 2013). Gastroesophageal reflux is more common in obese patients. It can cause erosive esophagitis, barret esophagitis, and gastroesophageal junction cancer (Nilsson and Lagergren, 2004). However, there are also cases with cancer development in the antrum and corpus of the stomach remaining after sleeve gastrectomy (Seki et al., 2018; Vladimirov et al., 2017; Yamashita et al., 2019). Chronic gastritis, *H.pylori*, and smoking are among the risk factors for stomach cancer (Uemura et al., 2001). Pre-operative eradication of *H.pyronine* can reduce the risk of cancer development. Preoperative endoscopy and removal if there are premalignant lesions are recommended (Lee et al., 2017). Endoscopic removal of premalignant lesions after surgery is not possible due to the narrow area. However, there are centers in the literature that perform preoperative routine endoscopy as well as selective endoscopy (Safaan et al., 2017; Lauti et al., 2016; Solmaz et al., 2018). In our clinic, we do the endoscopy selective rather than routine.

The rate of gastrointestinal stromal tumors in specimen pathologies ranges from 0.1-1.2% (Canil et al., 2018; Kinsinger et al., 2016). In addition, the rate of gastrointestinal stromal tumor detection was found to be 17.3% in one series (Walędziak et al., 2017). The total number of patients in this series was 1252, and only the pathology of 81 patients they deemed suspicious was examined. Therefore, it does not reflect the total incidence since the rate was so high. In our series, 20.9% normal histopathology was detected, and no gastrointestinal stromal tumor was detected. Routine histopathological evaluation is not recommended due to the low incidence of benign and malignant lesions in large clinical series and the majority of lesions. In addition, the histopathological findings found do not correlate with postoperative complications and management of complications. It is thought that performing a microscopic examination of necessary specimens as a result of a careful macroscopic examination will be better in terms of cost and time (AbdullGaffar et al., 2016). In gastric pathology after sleeve gastrectomy, 58 (0.46%) patients had tumors, and one of these patients was found to have adenocarcinoma (Aljerian, 2018).

In our study, gastrointestinal stromal tumors and other malignant tumors were not detected, and we think that the reason for the higher rate of dysplasia compared to the literature may be the low number of patients. The reason for our low number of patients is that patients in our clinic prefer more laparoscopic Roux-N-Y gastric bypass. In some studies, clinics stated that they stopped routine examinations (Dogan et al., 2017; Crouthamel et al., 2015). Sleeve gastrectomy can be performed with increasing frequency and safety. Routine pre-operative endoscopy before these operations is still a controversial issue. Although rare, malignant cases have been reported in the literature as a result of specific pathology. Even if no malignant pathological result was detected in our study, we believe that routine pathological examination is required for medicolegal reasons

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Are AIMS65 and glasgow-blatchford scores useful in predicting health costs in patients admitted to emergency department with acute upper gastrointestinal bleeding: A prospective and observational study

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Abstract

We aimed to investigate the use and superiority of AIMS65 (Albumin, INR, Alteration in mental status, Systolic blood pressure, age) and Glasgow-Blatchford scores (GBS) in predicting hospital health costs in patients admitted to emergency department with upper gastrointestinal bleeding. Patients above the age of 18 who were admitted to the Emergency Department of Ümraniye Training and Research Hospital between 01.06.2018 and 31.05.2019, who were diagnosed with upper gastrointestinal bleeding were included in the study. Patients' calculations of AIMS65 and GBS were recorded. Pearson's Chi-square test was used, and statistical significance was assessed. A total of 151 patients included in the study, 109 (72.2%) were male. Of the patients 2(1.3%) were discharged from the emergency department and 7 (4.6%) were exitus. According to AIMS65 risk scoring, costs of emergency department and inpatient clinics and total clinical costs were higher in high risk group compared to the low risk group ($p=0.007$, $p=0.007$ and $p=0.003$ respectively). The costs of emergency department and inpatient clinic and total costs were found statistically significantly different between GBS groups ($p<0.001$, $p=0.019$, and $p=0.001$ respectively). AIMS65 risk score and GBS have been revealed to be useful in predicting the costs of emergency department and inpatient clinics and total clinical costs for patients with upper gastrointestinal bleeding

Keywords: emergency department, aims65, glasgow-blatchford bleeding score, health cost

1. Introduction

Expenses in healthcare services increase as the need for healthcare services increases, which requires efficient use and improvement of available resources (Zengin et al., 2013). In 2014, the Turkish Ministry of Development prepared a report called "Improvement of Healthcare Services and Fiscal Sustainability" within the scope of ten-year development plan. In this report, the increase in health costs was emphasized and recommendations to provide fiscal sustainability were included (T.C. Kalkınma Bakanlığı Onuncu Kalkınma Planı). The rate of examinations performed in the emergency department is 25.97% in our country (İstatistik, Analiz, Raporlama ve Stratejik Yönetim Dairesi Başkanlığı.) There are only a limited number of studies on cost analyses of emergency departments. It is really hard to calculate quality and cost of the service precisely. Emergency medical interventions, laboratory use, medical equipment and services range from person to person for patients admitted to the emergency departments (Williams, 1996). Upper gastrointestinal bleeding (UGIB) has an important place in admissions to the emergency department. As well as gastrointestinal diseases, the drugs used, and comorbid diseases also affect the duration and severity of upper gastrointestinal bleedings. Meteorological changes also caused more frequent admissions in specific months of the year. Different clinics may have different approaches in predicting clinical costs of upper gastrointestinal bleedings which have an important place in morbidity and mortality, and which may cause hospital stay to extend.

Our study aimed to investigate whether AIMS65 and Glasgow-Blatchford scores (GBS) that were used to predict mortality in patients admitted to the emergency department with upper gastrointestinal bleeding were useful in predicting emergency department, inpatient clinic and total clinical costs and reveal the relationship of these costs with emergency treatments and demographic data.

2. Materials and methods

2.1. Patient population

Patients above the age of 18 who were admitted to the Emergency Department of Ümraniye Training and Research Hospital between 01.06.2018 and 31.05.2019, who were diagnosed with upper gastrointestinal bleeding, who accepted

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to participate in the study (the consents of unconscious patients were provided by their relatives), who were not followed up with a comorbid disease during admission and who were admitted to hospital within the first 24 hours after onset of the symptoms were included in the study. Patients with missing data and patients with a comorbid disease that may affect the treatment were excluded from the study.

2.2. Data collection

Expenses incurred by the health institution for patients were obtained from hospital information system and recorded in the patient forms as costs of emergency department and inpatient clinic in which the patient was hospitalized (ward or intensive care unit) and total clinical costs (total of these two costs). Patients' age, gender, month of admission, complaints and vital parameters during admission, drugs used, emergency department outcome, status and time of blood transfusion in the emergency department or clinics, status and time of endoscopy, laboratory parameters, GBS, AIMS65 score, and emergency department, inpatient clinic and total clinical costs were recorded. Arterial pressure, oxygen saturation and pulse were recorded as vital parameters. Emergency department outcomes were grouped as discharge from the emergency department, hospitalization in ward, hospitalization in intensive care unit, follow-up and mortality during treatment process. The drugs that have a role in the etiology of upper gastrointestinal bleeding were grouped as acetylsalicylic acid (ASA), non-steroidal anti-inflammatory drugs (NSAID), anticoagulant drugs, combination of these drugs and none of these drugs. Complaints of patients during admission to the emergency department were grouped as melena, syncope, black vomit, vomiting blood, dizziness, hematochezia, occult blood in the stool, incidental and coexistence of these. Hemoglobin, INR, and albumin values during admission were recorded as laboratory parameters of the patients.

2.3. Statistical analysis

IBM SPSS Statistics for Mac, version 26 (IBM Corp., Armonk, N.Y., USA) software program was used for statistical analyses of the data obtained in the study. Whether continuous data were normally distributed was determined with Kolmogorov-Smirnov test. Normally distributed continuous data were expressed with mean and standard deviation and non-normally distributed continuous data with median and interquartile range (IQR). Whether there was a difference between two groups was analyzed with Mann Whitney U test for continuous variables. Kruskal-Wallis test was used in the assessment of the relationship between subgroups for non-normally distributed continuous variables. When a statistical significance was found between subgroups in Kruskal-Wallis test Bonferroni correction was used and the significance level was accepted as $p<0.05$.

3. Results

After exclusion criteria were applied a total of 151 patients were included in the study (Fig. 1). Ages of the patients included in the study ranged from 20 to 99 and their median age was 64 (IQR: 45-75). While median age of male patients was 61 (IQR: 42.5-71) years median age of female patients was 69.5 (IQR: 52.7-80.2) years. Of the patients, 109 (72.2%) were male and 42 (27.8%) were female. The most common complaint during admission of the patients was melena (Fig. 2).

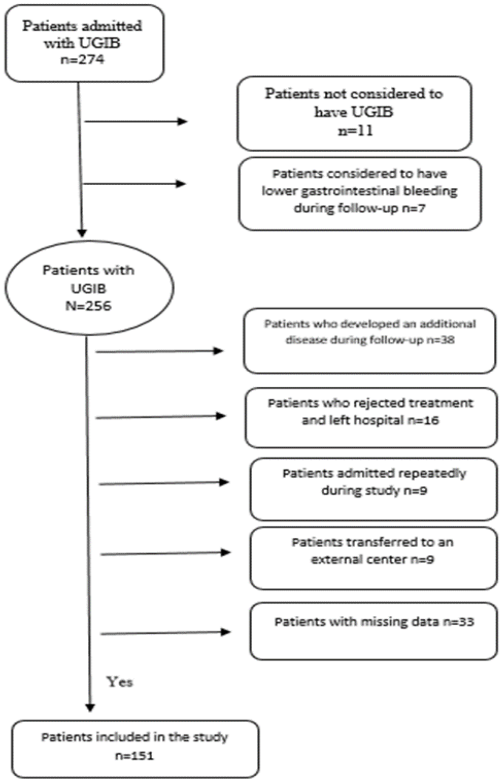

Fig.1. Flow chart

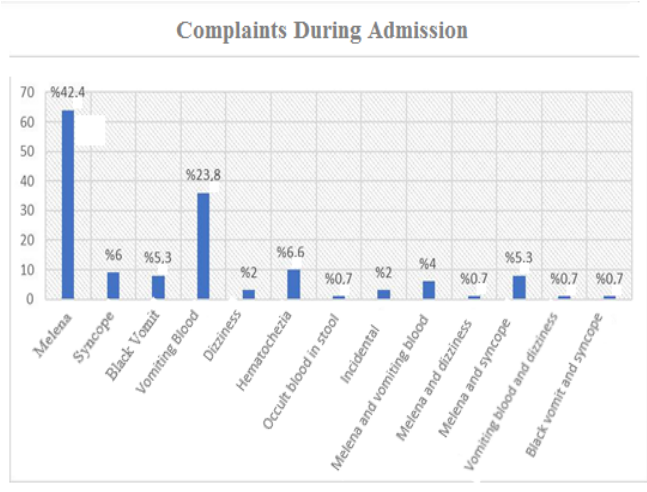

Fig.2. Complaints of patients during the first admission

Distribution of viral and laboratory parameters and AIMS65 scores and GBS of the patients during admission were summarized in Table 1. Of the admissions to emergency department, 92.7% (n=141) resulted in discharge after follow-up on the ward. Mortality rate was 4.6% (n=7) (Fig. 3). In current study, patients' costs in emergency department, costs in inpatient clinics and total costs when they were in the hospital were analyzed as three categories in terms of GBS and AIMS65 score and a statistically significant positivity and

weak correlation were found for both scoring systems (Table 2).

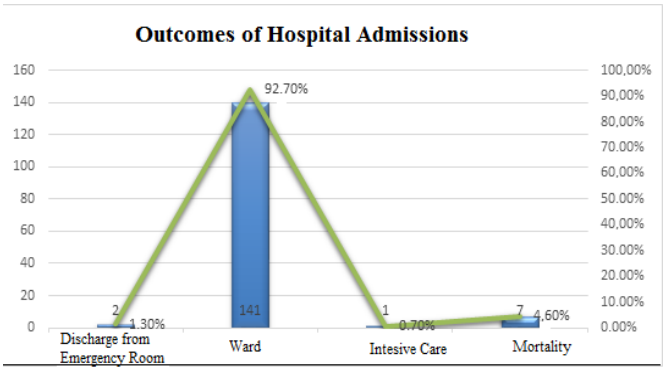

Fig. 3. Outcomes of hospital admissions

Table 1. Viral parameters, test results and scores of patients during admission

Parameters	Values
Age	64 (45-75)
Male	109 (72.2 %)
Level of Consciousness (GCS)	15 (15-15)
SBP (mmHg)	119.38 mmHg (± 26.1 mmHg)
DBP (mmHg)	67.99 mmHg (±15.06 mmHg)
Pulse (bpm)	91 bpm (80-103 bpm)
Saturation 0_2 (%)	98% (96-99)
Hemoglobin (g/dl)	9.41 (±2.82)
BUN (mg/dl)	66.3 (42.8-92.0)
INR	1.1 (1.0-1.2)
Albumin (mg/dl)	3.27 (± 0.59)
AIMS65 Score	1 (0-2)
Glasgow-Blatchford Score	12 (9-14)

Table 2. Correlation analysis of disease scores and patient costs

Cost AIMS65 Score	p	r	Cost GBS	p	r
Emergency Department	0.001	0.26	Emergency Department	<0.001	0.39
Clinic	< 0.001	0.30	Clinic	0.001	0.27
Total	< 0.001	0.32	Total	<0.001	0.35

Table 3. Distribution of costs according to AIMS65 risk groups

Cost	Aims65 Low Risk Median	Aims65 Low Risk Iqr	Aims65 High Risk Median	Aims65 High Risk Iqr	P
Costs of Emergency Department	748.22	375.67 - 1133.32	1171.38	667.91 - 1514.37	0.007
Costs of Non-Emergency Clinics	1499.23	620.90 - 2766.05	2278.56	1379.01 - 4218.99	0.007
Total Clinical Costs	2376.41	1322.32 - 3664.20	3427.76	2515.91 - 5897.06	0.003

According to AIMS65 scores, 68.2% (n=103) of the patients were grouped as low risk (score 0-1) and 31.8% (n=48) as high risk (score 2-5). AIMS65 score was statistically significantly different between low and high-risk groups in terms of emergency department, inpatient clinic, and total clinical costs (p=0.007, p=0.007 and p=0.003 respectively) (Table 3). According to GBS, 35.1% (n=53) of the patients were grouped as low score (score 0-10), 34.4% (n=52) as moderate score (score 11-13) and 30.5% (n=46) as high score (score 14-23) and a statistically significant difference was found between GBS groups in terms of emergency department, inpatient clinic, and total costs (<0.001, p=0.019, and p=0.001 respectively) (Table 4).

When mean costs per patient were assessed according to emergency department, inpatient clinic, and total costs in terms of AIMS65 score an increase was observed in the costs with increasing scores. When statistical significance and clinical correlation were assessed in terms of genders for AIMS65 score and GBS separately a statistically significant positivity and weak correlation were found between AIMS65 score and emergency department, inpatient clinic, and total clinical costs in male gender (p=0.002, r=0.30; p=0.005, r=0.27; p=0.001, r=0.32 respectively) (Spearman's correlation test). While a statistically significantly positive difference and weak correlation were found between GBS of male gender and inpatient clinic and total clinical costs a moderate degree of correlation was observed in terms of emergency department costs (p<0.001, r=0.46; p=0.008, r=0.25; p<0.001, r=0.35 respectively) (Spearman's correlation test). A statistically significant positivity was found in AIMS65 score and GBS of female gender in terms of inpatient clinic and total clinical costs. While AIMS65 score was correlated with inpatient clinic cost at a moderate degree a weak correlation was found in GBS (p=0.005, r=0.43; p=0.44, r=0.32 respectively) (Spearman's correlation test). Total clinical costs were weakly correlated in AIMS65 and GBS systems (r=0.39 and r=0.35 respectively) (Spearman's correlation test). When the correlation of emergency department costs was assessed in female gender no statistically significant result was found in AIMS65 and GBS systems (p>0.263 and p<0.138 respectively).

4. Discussion

We concluded in our study that AIMS65 score could be used in predicting the costs. When we compared the patients in terms of their AIMS65 risk score (score 0-5) they had during admission to the emergency departments, emergency department cost, inpatient clinic cost and total clinical costs a gradually increasing average cost table was obtained in terms of expenses incurred by the health institution within the process from admission to discharge. When patients were divided into low risk (0-1) and high risk (2-5) groups according to their AIMS65 risk score they had during admission the expenses incurred by the health institution significantly increased in terms of emergency department, inpatient clinic and total clinical costs.

Table 4. Distribution of costs according to GBS risk groups

Risk Scoring	Gbs Low Risk Median	Gbs Low Risk Iqr	Gbs Moderate Risk Median	Gbs Moderate Risk Iqr	Gbs High Risk Median	Gbs High Risk Iqr	P
Emergency Department Costs	475.92[a,b]	375.12-816.39	994.59[c]	460.66-1526.08	1164.05	757.22-1446.18	<0.001
Non-Emergency Clinic Costs	1396.39[d,e]	12-2294.99	2579.94[f]	1082.00-3667.56	1795.33	1236.18-2658.01	0.019
Total Clinical Costs	1794.00[x,y]	590.03-3135.45	3605.02[z]	1958.51-4831.60	2895.83	2340.31-3776.15	0.001

a-Low and moderate risk group p value = 0.002, b-Low and high risk group p value <0.001, c- Moderate and high risk group p value>0.05, d-Low and moderate risk group p value= 0.22, e- Low and high risk group p value= 0.017, f- Moderate and high risk group p value>0.05, x- Low and moderate risk group p value= 0.021, y- Low and high risk group p value= 0.002, z- Moderate and high risk group p value>0.05

When we compared patients in terms of their GBS which was another UGIB risk score calculated in our study we observed a statistical increase in emergency department, inpatient clinic and total clinical costs. When this score was divided into three groups as low, moderate and high cost a difference was found between the emergency department and total clinical costs in the low risk group among the subgroups although a statistical significance was found in the three groups in predicting the emergency department, inpatient clinic and total clinical costs. We think this is because of the number of patients and the underlying disease that causes bleeding. As the need for health care services increases the expenses in healthcare service increase as well. Regardless of whether they are seeking profit or not, service costs must be properly defined (Zengin et al., 2013). All expenses (including substructure, personnel, management and food expenditures) must be determined to obtain a real profit and loss statement (Özdemir et al., 2020). Emergency medical interventions, laboratory use, medical equipment and services range from person to person for patients admitted to the emergency departments. Therefore, it is hard to follow up an efficient cost analysis for carrying out emergency services. Williams et al. revealed in their study in which the difference between medical emergency patients and inpatient patients was investigated that the profit per patient was higher for patients who were admitted as a real emergency (Williams et al., 1996). Cremonesi et al. (2015) reported in their studies in which they investigated the cost difference between triage codes that the cost of real emergency patients was higher, but unit income and profit gained from patients were not. In our study, emergency department costs were observed to increase by the increasing risk score. Due to inadequate number of total admissions and absence of patients in each score, we could not calculate emergency department, inpatient clinic, and total clinical costs separately according to patients' GBS calculated during their admission to the emergency department. Therefore, we could not assess how much the costs increased according to GBS of patients.

There are a large number of scoring methods used to determine several risks, such as mortality and duration of hospital stay in a life-threatening disease like UGIB. We believe that AIMS65 which we used in our study will be helpful in predicting the costs in a healthcare institution as it does not contain many scorings. While GBS is useful in calculating an average cost for healthcare costs of patients as it contains many scorings and parameters, we think it is not useful if different calculations will be made for each score. It is better to perform a study with larger number of patients due to limitations in our study. However, to the best of our knowledge, our study is the first study in which efficiency of the scorings used as mortality risk scores in patients with UGIB in predicting the costs that will be incurred by the health institution and utility of these scores in cost analysis have been assessed.

Previous studies revealed that UGIB was more common in male gender (Davarci et al., 2019; Kuşçu et al., 2019). In our study, 72.2% (n=109) of the patients were male and ages of the patients ranged from 20 to 99, which is consistent with studies in literature. Median age of the patients was 64 (IQR: 45-75). Although a statistically significant correlation was found between male and female patients in terms of emergency department, inpatient clinic and total clinical costs in our study this statistically significant relationship may not be clinically significant as correlation coefficients were low. We believe the relationship between genders and emergency department; inpatient clinic and total clinical costs must be revealed with larger study groups.

In a study in which admissions to emergency department with UGIB were retrospectively evaluated, 44.8% of the patients were admitted to the emergency department with melena and the least common reason for admission was syncope (1%). Melena was found in 67.6% of the patients on their rectal examination independent from the complaint during admission (Uysal et al., 2019). In our study, the most common reason for admission to the emergency department was melena (42.4%), which is similar to admission complaints in literature. The number of patients who had melena on rectal examination was 99 (65.6%). In the retrospective study by Uysal et al. (2019) comorbidities of UGIB were reported as hypertension (32.9%), diabetes mellitus (17.4%), coronary artery disease (22.6%), congestive heart failure (6.9%) and liver cirrhosis (25.2%). In a review by Van Leerdam (2008), the rate of severe or life-threatening comorbidities was higher than 40% in 40% of the patients with UGIB. In our study, comorbidities of patients admitted with UGIB to the emergency department were hypertension (35.8%), diabetes

mellitus (17.9%), coronary artery disease (22.5%), congestive heart failure (7.9%), liver failure during admission (7.9%) and diagnosis of liver failure (9.9%).

Several studies have revealed that meteorological factors increase the risk of bleeding in diseases associated with bleeding in cold weather (Lenzen et al., 2017; Prechter et al., 2019). According to the months of admission to the emergency department, the number of admissions was the lowest in summer months (15.2%) in our study, which is similar to findings of other studies. Mortality and morbidity increase in UGIB with ranging prognosis due to late admission of the patient to emergency department, late diagnosis and lack of opportunities for examination (Yalçın et al., 2016). In our study, 92.7% of admissions to the emergency department resulted in discharge after follow-up on the ward. Mortality rate was 4.6%. Some patients with UGIB could not be included in our study due to missing forms and data caused by high density in the emergency department. We did not have any information about the reasons of the costs (e.g. personnel, medical equipment, and consumables). As endoscopic results of the patients were not assessed the relationship between etiology and cost could not be found. Expenses in health services increase by the number of individuals admitted to the health institutions. AIMS65 and GBS are useful in predicting health costs in patients admitted to emergency department with acute upper gastrointestinal bleeding.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Research Article

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The effect of intra-amygdalar leptin administration on anxiety, depression and learning behaviors in rats

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Abstract

Leptin is released by adipose tissue. Leptin can cross the blood–brain barrier and bind to receptors on neurons in brain areas to exert its biological function when released into circulation. This study aimed to determine the influences of intra-amygdalar administration of high and low doses of leptin on anxiety, depression, learning behaviors of rats. In the experimental protocol I, intra-amygdalar injection of high and low doses of leptin (0.1 and 1μg/ kg) and saline were administered 30 min before the behavioral tests. Then, the animals were exposed to open field, elevated plus maze, Porsolt and Morris water maze tests for measuring of behaviors. In experimental protocol 2, the cerebrospinal fluids of all groups of experimental protocol 1 were collected by microdialysis method and then were analyzed by HPLC. The effect of the low dose of leptin was significant on the open field. The effect of the high and low dose of leptin was significant on the elevated plus maze test. The effect of the low dose of leptin was significant on mobility in the center of the Porsolt. A high dose of leptin group had spent less time around the platform than controls in the Morris water maze test. HPLC analysis showed that the amount of serotonin and glutamate in the amygdala region increased after low dose leptin administration. Intra-amygdalar injection of low doses of leptin may decrease anxiety and depression-like behavior in rats by increasing serotonin and glutamate levels in the amygdala.

Keywords: leptin, behavior, amygdala, anxiety, depression

1. Introduction

Leptin is one of the most important adipokines released by adipose tissue (Havel, 2000). When leptin is released into the circulation, it binds to receptors on neurons in brain regions, crossing the blood-brain barrier to perform its biological function (Zhang et al., 1994). Leptin receptors in the central nervous system are widely present especially in the hippocampus, amygdala, and hypothalamus (Krishnan and Nestler, 2010; Patterson, 2011).

Recent studies have shown the effects of leptin on neuronal function. It affects the function of the central nervous system, such as anxiety and depression (Liu et al., 2011; Liu et al., 2015). High levels of leptin and receptors have been detected in many areas of the brain. Evidence that the leptin receptor is present in limbic regions suggests that leptin has a possible role in controlling emotional processes in the limbic region of the brain (Liu et al., 2015).

In rats, hippocampal administration of leptin has been found to significantly reduce depression and affect hippocampal gene expression. Leptin has an antidepressant effect, and this effect has been noted in many studies (Banks et al., 2000; Liu et al., 2011). Leptin acts on the HPA (hypothalamo-pituitary axis) axis (Heiman et al, 1997; Liu et al., 2011). The antidepressant effect of leptin may be associated with the normalization of HPA axis hyperactivity (Banks et al., 2000; De Kloet et al., 2005). HPA axis disorder is the most important factor in the pathogenesis of depression, which causes an increase in corticosteroids in serum. In animal models, leptin has been found to reduce plasma corticosterone (Reseland et al., 2005). Therefore, leptin weakens the HPA axis at various levels (Heiman et al., 1997; Yu et al., 1997; Liu et al., 2011).

Leptin increases serotonin and reverses the increased corticosterone, so that HPA axis hyperactivity is regulated by leptin, which promotes the antidepressant effect of leptin (Hastings, 2002). Leptin has an antidepressant effect in rodents (Lu et al., 2007). Decreased serum leptin levels were found in individuals with major depressive disorder compared to healthy controls (Jow et al., 2006). Other studies in women with MDD found significantly increased plasma leptin levels (Rubin et al., 2002; Esel et al., 2005; Zeman et al., 2009). Similarly, it has been reported in some studies that leptin levels increase or do not change in various ways with antidepressant

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therapy (Kraus et al., 2002). Finally, some studies have suggested that leptin could potentially be a biological marker for depression (Pasco et al., 2008). Hence, this study aims to investigate the effects of intraamygdalar administration of leptin at high and low doses on anxiety, depression, and learning behaviors in rats.

2. Materials and methods

2.1. Animals

All experimental animals have been treated based on the guiding principles approved by the animal Ethical committee of Abant Izzet Baysal University as well as all the treatments comply with recommendations on the Declaration of Helsinki (Registration number 2012/04). The experiment was performed on male Wistar-Albino rats with weighing between 270-300 grams. Animals were housed under a 12 L / 12 D light-dark cycle (light on 07:00h) and room temperature of 22-25 C°. Food and water were ad libitum.

2.2. Experimental protocol

In the first study, rats were selected and randomly divided into the three experimental groups [control-male (i.c.v saline, 2 µl / min., n=10), high dose leptin administration (i.c.v, 1 µg / kg, n=10), and low dose leptin administration (i.c.v, 0.1 µg / kg, n=10)] for 5 min. The leptin and saline treatment were applied thirty minutes before the behavioral tests. All groups were tested by the open field and elevated plus maze tests for measuring anxiety-like behaviors; force swimming test was performed for measuring depression-like behaviors and Morris water maze test was performed for measuring the learning and memory performance. In the control group, animals were applied with the same amount of saline to obtain the same stress conditions as was the case in the experiment groups. In the second study, the cerebrospinal fluids (CSFs) of all groups of experimental protocol 1 were collected by a means of the micro dialysis method. A cannula (CMA 12 elite micro dialysis cannula; CMA Micro dialysis AB, Sweden) was implanted aseptically into the amygdala region (coordinates interior+0.6 mm; at the midline and ventral−7.8 mm; relative to bregma). Following the thirty-minute i.c.v treatments, collecting 7 tubes of CSF from each animal that took thirty minutes per tube and a total of 2.5 hours of time per animals' CSFs were collected. Following the completion of CSF collections, the tubes were saved at -20°C. Then all of the collected CSFs were analyzed using the HPLC method. The Agilent 1100 series HPLC-FLD system (Germany) was used.

2.3. Leptin administration

Leptin (Sigma) was first dissolved in some ethanol and then diluted with saline. Low-dose leptin was set to 0.1 µg / kg, and high-dose leptin to 1 µg / kg. Leptin was injected into the intraamygdalar region at a flow rate of 2 µL / min. The same volume of saline was administered to the control groups.

2.4. HPLC measurements

The Agilent 1100 series HPLC-FLD system (Germany) was used. The analytes were separated on a reversed-phase Inertsil ODS-2 (150 x 4.6 mm i.d., 5 µm) analytical column (Hichrom, England), which was protected by Inertsil ODS-2 (10 x 3.2 mm) guard cartridges (Hichrom, England). Chromatographic conditions, a fluorescence HPLC method for the simultaneous detection of serotonin and noradrenaline in microdialysates from rat brain was used. The separation was performed with a C18 reversed-phase column using isocratic elution.

2.5. Behavioral test

The Open Field Test (OFT)

The OFT was performed after the last day of treatments. A single rat was placed in the center of a black, Plexiglas square measuring 80 cm in length × 80 cm in width × 40 cm in height. The subject was allowed to discover this new environment for 15 minutes, during the training session. After the training session, the rat was exposed to the test for 5 min in the test session. During the test session, the time spent in center arena and edges were monitored by a video camera (Gkb CC-28905S, Commat LTD. ŞTİ., Ankara/Turkey) and recorded by a videotaped interfaced with the EthoVision video tracking system (Noldus Ethovision, Version 6, Netherlands; Commat LTD. ŞTİ. Ankara/Turkey). During the test session, the frequency of the entry to the center area, the number of center entries, and time spent in the center area were recorded for min and recorded data were calculated by the program.

The Elevated Plus Maze Test (EPM)

The EPM is the most thoroughly utilized test to evaluate anxiety-like behavioral state. It is especially sensitive to anxiety-reducing drugs, such as anxiolytic agents. The structure of the EPM apparatus has black color and is composed of the two open arms which are crossed by two closed arms of equal size (55×10 cm) with 41 cm high walls. The maze was elevated to a height of 55 cm above floor level. The rats of each group were placed individually in the central area of the EPM to explore for five minutes. The time spent on open arms, time spent on closed arms, and the number of open and closed arms entries were recorded by the EthoVision video tracking system Noldus Ethovision (Gkb CC-28905S, Commat LTD. ŞTİ., Ankara/Turkey).

The Porsolt test

A method of Porsolt was adopted to perform FST, where animals were allowed to swim in a glass cylinder (diameter: 22.5 cm, height: 30 cm, filled with water (23 ± 2ºC) up to 15 cm height of 15 cm) for six min, having the initial two min for adjustment and recording the immobility time. All behaviors of rats were recorded by the EthoVision video tracking system (Noldus Ethovision; Gkb CC-28905S, Commat LTD. ŞTİ., Ankara/Turkey).

The Morris Water Maze test

The water maze is composed of a black circular tank, 1,5 m in diameter and 60 cm in height, filled with water up to 40 cm in height, and four identical areas for the purpose for analysis. A square black escape platform 2 cm below the water level was situated at the center of one of the four quadrant of the

apparatus. Four different colors and shapes of cues have ensured the animal. The apparatus comprises 4 consecutive trials for five days with a 1-h inter-trial interval. The rats were allowed to explore for 90 s. If they failed to find the platform, they were gently guided to it for waiting 10 s. One day after the last training trial, each rat was exposed to the test trial where they were let to explore the hidden platform for 60 sec. When they found the platform, the rats were allowed to stay for 5 seconds to let them observe the cues around the platform. The time spent (by each animal) for finding the platform on the fifth training trial was measured by EthoVision video tracking system- (Noldus Ethovision, Version 6, Netherlands).

2.6. Statistical analysis

Data were examined by a means of two (the presence of treatment: high and low doses of intra-amygdalar leptin injection × saline injection (control (sham) group)) between ANOVA design Values were considered statistically significant at P <0.05. Data was presented as mean ± standard error after back transforming from ANOVA results.

3. Results

3.1. Open field measurements

The main effect of intra-amygdalar injection of the low doses of leptin hormone was significant in the amount of time spent in the center of the open field (TSCOF), F (2, 57) = 3.97, p = 0.02 (Fig. 1a). The main effect of intra-amygdalar injection of the low doses of leptin hormone was significant in the total frequency of zone transition of the open field (FZTOF) of rats. Compared to the rats in the control groups, anxiety-like behavior has decreased in the group injected with low dose of leptin F (2, 57) = 3.22, p = 0.04 (Fig. 1b). The main effect of intra-amygdalar injection of the high and low doses of leptin hormone was not significant on the mobility in the open field (MOF), F (2, 57) = 1,90, p = 0.16. The main effect of intra-amygdalar injection of the high and low doses of leptin hormone was not significant on the velocity in the open field, F (2, 57) = 0.25, p = 0.78.

3.2. Elevated plus maze measurements

The main effect of intra-amygdalar injection of the high and low doses of leptin hormone was not significant on the total distance travelled, F (2, 55) = 2.12, p = 0.13. The main effect of intra-amygdalar injection of the high and low doses of leptin hormone was significant on the measurement time, F (2, 55) = 6.77, p = 0.002 (Fig. 2a). The main effect of intra-amygdalar injection of the high doses of leptin hormone was significant related to the measurement time, F (2, 55) = 9.97, p =0.001 (Fig. 2b). The main effect of intra-amygdalar injection of the high and low doses of leptin hormone was not significant on the mobility, F (2, 55) = 1.08, p = 0.34. The main effect of intra-amygdalar injection of the high and low doses of leptin hormone was not significant related to the measurement time F (2, 55) = 1 .15, p = 0.33.

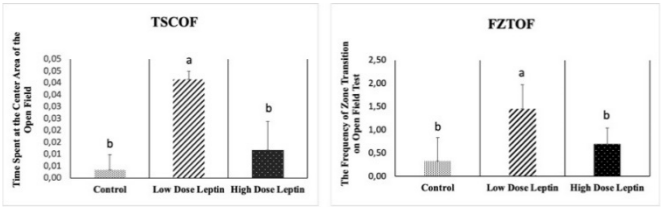

Fig. 1. a) Time spent at the center area of the open field test: control, low dose leptin and high dose leptin; b) Frequency of zone transition on open field test: control, low dose leptin and high dose leptin ($p < 0.05$). [a] $p < 0.05$ vs low dose leptin, [b] $p < 0.05$ vs control

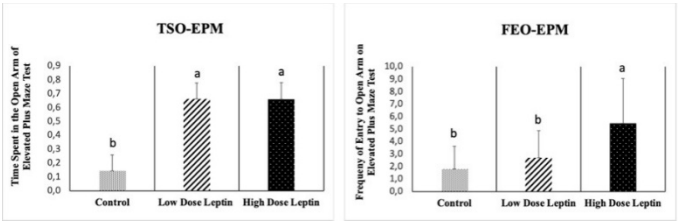

Fig. 2. a) Time spent in the open arm of elevated plus maze test: control, low dose leptin and high dose leptin (mean±SD, n=10) ($p < 0.05$); b) Frequency of entry to open arm on elevated plus maze test: control, low dose leptin and high dose leptin (mean±SD, n=10) ($p < 0.05$). [a] $p < 0.05$ vs low dose leptin, [b] $p < 0.05$ vs control

3.3. Porsolt measurements

The main effect of intraamygdalar injection of the high and low doses of leptin hormone was not significant on the total distance traveled in the Porsolt test, F (2, 55) = 0.25, p = 0.78. The main effect of intra-amygdalar injection of the low doses of leptin hormone was significant in the terms of immobility duration, F (2, 55) = 4.30, p = 0.02 (Fig. 3). The main effect of intra-amygdalar injection of the high and low doses of leptin hormone was not significant on the frequency of mobility, F (2, 160) = 2.91, p = 0.05.

3.4. Morris Water Maze

The effect of leptin hormone injection was not significant on total distance traveled. F (2, 45) = 1.08 p = 0.35. The effect of leptin hormone injection was not significant on the total time to find the platform. F (2, 45) = 0.82 p = 0.45. The effect of leptin hormone injection was significant, F (2, 45) = 3.50 p = 0.04. The subjects exposed to a high dose of leptin (M = 0.05) had less time spent around the platform than controls (M = 0.08). The effect of leptin hormone injection was not significant, F (2, 45) = 0.35 p = 0.70.

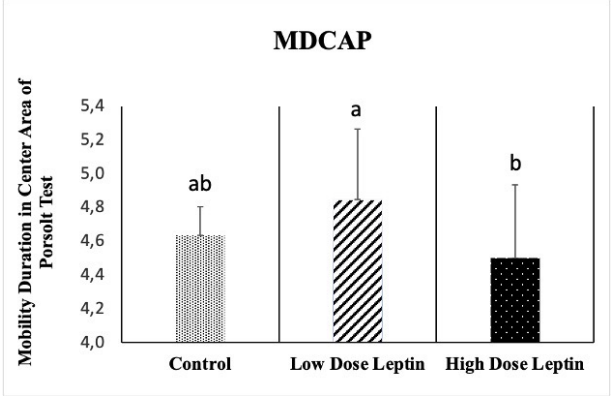

Fig. 3. Mobility duration in center area of Porsolt test. [a] $p < 0.05$ vs low dose leptin, [b] $p < 0.05$ vs control

3.5. Results of HPLC

Serotonin and glutamate levels were observed to have increased after the low dose leptin administration ($p<0.05$). This supports the conclusion that low dose leptin reduces anxiety. GABA, Noradrenaline, and Melatonin concentrations were not determined as their concentrations were below the limits. (Table 1 and 2).

4. Discussion

Leptin is involved in regulating brain development, improves angiogenesis, promotes nerve regeneration, energy homeostasis, reproduction, and cognition (Pasco et al., 2008). Leptin has an antidepressant effect in rodents (Zeman et al., 2009). Moreover, systemic administration of leptin lowers levels of corticosterone (Farr et al., 2006). Decreased serum leptin levels were found in individuals with major depressive disorder compared to healthy controls (Kraus et al., 2002; Farr et al., 2006). Similarly, some studies suggest that leptin levels increase (Kraus et al., 2002; Esel et al., 2005), or do not change with antidepressant treatment in a variety of ways (Kraus et al., 2002; Farr et al., 2006).

Leptin receptor localization has been identified mostly in the arcuate nucleus, dorsomedial hypothalamus, and lateral hypothalamus in the mouse brain. On the other hand, there is a moderate amount of leptin receptors in the amygdala region (Patterson et al., 2011). In this study, there may be a plausible explanation for why low-dose leptin is more effective.

Antidepressant and anxiolytic activity of 5-HT3 receptor antagonists has been proposed in animal models (Schilling et al., 2013, Romanova et al., 2018). The administration of intra-amygdalar leptin leads to modulation of the serotonergic system for antidepressant and anxiolytic effect and the increase in serotonergic neurotransmission. The results of this study showed that low-dose intra-amygdalar leptin administration in anxiety tests reduced anxiety, while in HPLC analysis, serotonin levels were higher in the low-dose leptin group. Considered together, these results support each other.

Intrahippocampal leptin injections improved dose-dependent performance in the T-maze and inhibitory avoidance test (Pasco et al., 2008). High-dose leptin administration did not affect behavior in the avoidance test, possibly indicating the absence of the effect of intrahippocampal leptin reported in Wistar rats (Kurhe et al., 2015). Administration of leptin to the CA1 region of the hippocampus was found to not alter learning and memory in the radial maze test in Wistar rats (Kurhe et al., 2015). Similarly, administration of leptin to the amygdala in this study was the dose dependent.

While leptin administered to the hypothalamus improved appetite, nutrition and spatial memory (Zarrindast et al., 2015), it showed no effect on high and open field tests (Kanoski and Davidson, 2011). No effect of intra-amygdalar leptin was observed on learning and memory in this study; on the other hand, this effect is evident in open field, high plus maze and porsolt tests. According to these results, the effect of leptin depends on both the dose and the area of administration.

Depression is associated to abnormalities in the frontal and limbic neural circuits, including the amygdala (Sharma et al., 2010). In the hippocampus, amygdala, and postrema region, the 5-HT3 receptor is highly expressed in the unique ion channel type in the family of serotonergic receptors (Canli et al., 2005). Leptin increases serotonin in the forebrain region and reverses the increased corticosterone, so that HPA axis hyperactivity is regulated by leptin, supporting the antidepressant effect of leptin (Tecott et al., 1993).

Low-dose leptin (0.1 ul / kg) was found to reduce anxiety and depression in intra-amygdalar leptin administration. In this study, high levels of glutamate and serotonin were found in extracellular fluid collected from the amygdala region in the low-dose leptin group. Moreover, increased glutamate and serotonin expression was found to reduce anxiety and depression. Leptin mediated an increase in glutamate and serotonin. In this study, CSF was collected between 14:30 and 18:30, and these samples were collected in separate tubes at 30-minute intervals. Accordingly, glutamate increased 30 minutes after the administration of leptin, and serotonin increased two hours later. Taken together, intra-amygdalar injection of low-dose leptin can reduce the anxiety and depression-like behavior in male rats by increasing serotonin and glutamate levels in the amygdala. Further studies should investigate why leptin mediates the effects of glutamate and serotonin in order to establish a new strategy for treating anxiety and depression

Conflict of interest

The authors declare that they have no conflict of interest.

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Effect of routine duloxetine administration in the early postoperative period on post-prostatectomy stress incontinence in patients undergoing laparoscopic radical prostatectomy

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Abstract

Post-prostatectomy stress incontinence (PPI) is an important health problem for patients with radical prostatectomy history. Duloxetine is a common drug, used in PPI with the out of indications in most countries. In this study, we aimed to evaluate the prophylactic effect of duloxetine administration in PPI during the early postoperative period in patients undergoing laparoscopic radical prostatectomy (LRP). The retrospective study included 209 patients who underwent LRP. Patients were divided into two groups: Group I (n=96) was initiated on pelvic floor exercises (PFE) + duloxetine in the early postoperative period and continued this regimen for a total of 12 weeks and Group II (n=113) only performed PFE for 12 weeks after surgery. Exclusion criteria were as follows: a history of neuromuscular dysfunction of bladder, post-prostatectomy urge incontinence, receiving adjuvant radiotherapy during the 12-week period, prior anti-incontinence surgery, and post-voiding urine>100 ml. The prevalence rates of urinary incontinence measured at the time of urinary catheter removal (baseline) and at weeks 3, 6, and 12 after surgery and the number of wet pads per day were compared. The study included 209 patients with a mean age of 60.68 ± 7.16 years. Baseline urinary continence rates were similar in Group I and Group II (29.2% *vs.* 35.4%, p=0.338). At 12 weeks, although PPI rates have decreased in both groups, there was no difference between Group I and Group II (15.6% vs. 24.8%, p=0.103). Administration of prophylactic duloxetine in the early postoperative period, which started without regard to the positivity of PPI, is not significantly effective to early postoperative urinary continence recovery.

Keywords: post-prostatectomy incontinence, duloxetine, radical prostatectomy, prostate cancer

1. Introduction

Post-prostatectomy stress incontinence (PPI) is a significant factor leading to reduced quality of life after prostatectomy (Trofimenko et al., 2017). Although it has been reported that the majority of such patients recover continence within three months after surgery, 7-25% of patients have been shown to have persistent urinary incontinence following radical prostatectomy (Holm et al., 2014; Pompe et al., 2017; Borges et al., 2019; Kania et al., 2019).

Pelvic floor exercises (PFE) constitute the primary step in PPI treatment (Sandhu et al., 2019). Patients are recommended to perform these exercises both before and immediately after radical prostatectomy (de Lira et al., 2019). However, for patients that do not benefit from these exercises, there are limited pharmacotherapy options and thus surgical intervention (bulking agents, male slings, or artificial urinary sphincters) may be needed (Nambiar et al., 2018; Nestler et al., 2019). Duloxetine is a selective serotonin (5-HT)-norepinephrine (NE) reuptake inhibitor and exerts its effect by increasing the activity of the urethral sphincter (Neff et al., 2013). This agent has been used in female stress urinary incontinence (SUI) in Europe since 2004 (Boy et al., 2006). Nevertheless, although duloxetine is recommended by some researchers, it has not been licensed in many countries (Fink et al., 2008; Collado et al., 2011; Neff et al., 2013; Gresty et al., 2019).

The present study was designed to evaluate the prophylactic effectiveness of duloxetine administration on PPI during the early postoperative period after laparoscopic radical prostatectomy (LRP) by comparing patients receiving and not receiving duloxetine regardless of their urinary continence status.

2. Patients and methods

2.1. Study design and patients characteristics

The retrospective study included 209 patients with prostate cancer who underwent LRP in Erciyes University Department of Urology between January 2011 and April 2020. The patients were divided into two groups based on the administration of duloxetine: Group I (n=96) was initiated on PFE + prophylactic duloxetine in the early postoperative period (5-7 days before catheter removal) and continued this regimen for a total of 12

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weeks after surgery and Group II (n=113) only performed PFE for 12 weeks after surgery.

In Group 1, duloxetine was administered at a dose of 1x40 mg/day for the first week and at a dose of 2x40 mg/day for the latter weeks. At the end of the 12th postoperative week, duloxetine treatment was discontinued. After waiting 3-4 weeks, duloxetine was re-recommended for patients with urinary incontinence and who wanted to start treatment again. Urinary incontinence data were recorded in both groups throughout the 12-week period. 16 (14.3%) patients who had started duloxetine but had reported adverse events and discontinued the drug were excluded. Other exclusion criteria were as follows: incomplete follow-up records for the 12-week period, discontinuation of duloxetine due to side effects before the completion of the 12-week period, failure to initiate duloxetine therapy 5-7 days before urinary catheter removal, a history of neuromuscular dysfunction of bladder, post-prostatectomy urge incontinence, receiving adjuvant radiotherapy during the 12-week period, history of overactive bladder detected on urodynamic examination, prior anti-incontinence surgery, and post-voiding urine >100 ml.

In our clinic, PFE are routinely initiated in the early postoperative period. Accordingly, the patients that did not start these exercises within this period or performed the exercises irregularly were also excluded from the study. Demographic and clinical characteristics were recorded for each patient. Additionally, the prevalence rates of urinary incontinence measured at the time of urinary catheter removal (baseline) and at weeks 3, 6, and 12 after surgery and the number of wet pads per day were also recorded. In this study, "the need to use a pad or a diaper" is accepted as urinary incontinence and patients were defined according to daily pad need (e.g., 0-1, 2, 3, 4 or more).

2.2. Statistical analysis

Data were analysed using SPSS 22.0 for Windows (IBM Corp. Released 2013, Armonk, USA). Normal distribution of quantitative data was assessed using Shapiro-Wilk test and Histogram plots. Quantitative data with normal distribution were expressed as mean ± standard deviation (SD) and data with non-normal distribution were expressed as median (1st-3rd quartile). Categorical data were expressed as percentages (%). Continuous variables in independent groups were compared using independent samples t-test or Mann-Whitney U test based on their distribution pattern. Categorical variables were compared using chi-square test (Pearson's Chi-square test or Fisher's Exact test). A *p* value of less than 0.05 was considered significant.

This study was approved by the Erciyes University Clinical Research Ethics Committee (Approval number: 2020/078). All participants were informed verbally and in writing before the operations and a written consent was obtained from each of them.

3. Results

The 209 patients had a mean age of 60.68 ± 7.16 years, a median body mass index (BMI) of 27.5 (25.5-29.5) kg/m^2, and a median prostate specific antigen (PSA) level of 0.010 (0.004-0.127) ng/ml (Table 1).

Table 1. Demographic and clinical characteristics of the patients

	Group 1 (n=96)	Group 2 (n=113)	*p*
Age (years)	60.10 ± 7.64	61.17 ± 6.72	0.286
BMI (kg/m2)	28.7 (26.2-29.5)	27.1 (25.6-29.1)	0.062
PSA (ng/ml)	0.011 (0.006-0.145)	0.010 (0.003-0.105)	0.113
Local recurrence (n, %)	21 (21.9%)	17 (15.0%)	0.213
Distant metastasis (n, %)	4 (4.2%)	5 (3.5%)	0.429
Bladder neck contracture (n, %)	2 (2.1%)	2 (1.8%)	0.642

BMI: Body mass index, PSA: Prostate-specific antigen

The mean baseline prevalence rate of urinary incontinence was similar in Group I and II (29.2% vs. 35.4%, p=0.338). The PPI at postoperative weeks 3, 6, and 12 rates were similar in Group I and Group II (Table 2).

Table 2. Postprostatectomy stress incontinence rates of the groups

	Group 1 (n=96)	Group 2 (n=113)	*p*
Baseline (n, %)	28 (29.2%)	40 (35.4%)	0.338
Week 3 (n, %)	20 (20.8%)	36 (31.9%)	0.073
Week 6 (n, %)	16 (16.7%)	31 (27.4%)	0.063
Week 12 (n, %)	15 (15.6%)	28 (24.8%)	0.103

PPI was positive in 43 (20.6%) patients over the 12-week period, including 19 (9.1%) patients with mild PPI (0-1 pads/day), 18 (8.6%) with moderate PPI (2-3 pads/day), and 6 (2.9%) patients with severe PPI (≥4 pads/day). Moreover, no significant difference was found between the two groups with regard to PPI severity. Table 3 presents the patterns and severity of urinary incontinence in PPI-positive patients.

Table 3. Urinary incontinence patterns

	Group 1 (n=96)	Group 2 (n=113)	*p*
Mild PPI (0-1 pads/day)	7 (7.3%)	12 (10.6%)	0.482
Moderate PPI (2-3 pads/day)	7 (7.3%)	11 (9.8%)	0.689
Severe PPI (>4 pads/day)	1 (1%)	5 (4.4%)	0.101

PPI; Post-prostatectomy incontinence

Urinary incontinence developed in two patients in Group-1 after duloxetine treatment was stopped. Thus, after the duloxetine treatment was stopped, the incontinence rates of the groups were 17.7% and 24.8%, respectively (p=0.224).

4. Discussion

The present study evaluated the effect of duloxetine in prostate cancer patients undergoing LRP over a 12-week period and revealed that the administration of prophylactic duloxetine in the early postoperative period did not decrease the prevalence rate of urinary incontinence.

There are several studies in the literature reporting on the efficacy of duloxetine in PPI treatment (Zahariou et al., 2006; Filocamo et al., 2007; Fink et al., 2008; Collado et al., 2011; Cornu et al., 2011; Neff et al., 2013; Alan et al., 2015). Zahariou et al. conducted one of the earliest of these studies in 2006, which evaluated 18 PPI-positive patients and reported that the administration of duloxetine led to significant improvement in urodynamic parameters at the end of a three-month period (Zahariou et al., 2006). A placebo-controlled study conducted by Filocamo et al. (2007) evaluated 112 PPI-positive patients and revealed that the administration of duloxetine allowed early postoperative urinary continence recovery. Another study that was conducted on 49 PPI-positive patients in 2008 showed that the administration of duloxetine twice daily resulted in effective outcomes in the patients (Neff et al., 2013). In our study, unlike the studies abovementioned, duloxetine administration was initiated in the early postoperative period (i.e., 5-7 days before the catheter removal) regardless of the urinary incontinence status of the patients to evaluate the prophylactic effect of duloxetine. The results indicated that the mean baseline, postoperative weeks 3, 6, and 12 prevalence rates of urinary incontinence was similar in Group I and II. In addition, urinary incontinence started only in two patients after discontinuation of duloxetine. According to these data, it is not an effective and rational method to give prophylactic duloxetine treatment to all patients undergoing radical prostatectomy. Pompe et al. (2017) evaluated a large cohort of 8,573 patients undergoing radical prostatectomy due to prostate cancer and found that almost 25% of the patients were PPI-positive at the end of the 12-week period after surgery. However, the authors did not include patients with 0-1 pads/day in the study. In contrast, Holm et al. (2014) reported that the rate of PPI positivity was 74.1%, among whom 40% of the patients used one pad daily. Nevertheless, it is worth noting that no PFE were performed by the patients in that study. Stanford et al. (2000) reported that 14.3% of the patients used three or more pads per day and no bladder control in 5.4% of the patients. Taken together, these findings implicate that there is a wide range of PPI rates reported after radical prostatectomy in the literature. In our study, the rate of SUI was 32.5% in the early postoperative period (i.e. immediately after urinary catheter removal) and decreased consistently over the 12-week period and declined to 15% in the PFE + duloxetine group (Group I) and to 25% in the PFE-only group (Group II) at the end of the 12-week period. These rates were slightly higher than those reported in the literature, which could be attributed to the inclusion of patients who had dribbling urinary incontinence (0-1 pads/day) in the study. When these patients were excluded from the statistics, the overall rate of urinary incontinence with ≥2 pads/day was revealed as 11.4%, which was consistent with the literature. Previous studies indicated that the number of pads per day in PPI-positive cases decreased significantly regardless of duloxetine administration over the periods ranging between three and 12 months (Stanford et al., 2000; Pompe et al., 2017). Filocamo et al. (2007) reported that the administration of duloxetine over a period of 16 weeks led to a significant decrease in the prevalence of PPI. Zahariou et al. (2006) reported that the administration of duloxetine led to significant improvement in urodynamic parameters at the end of a six-month period. In our study, the prevalence of urinary incontinence declined from 29% to 15% in the PFE + duloxetine group at the end of the 12-week period. Similarly, the prevalence of urinary incontinence decreased from 35.4% to 24.8% in the PFE-only group. These reductions were statistically significant and compatible with the literature.

In the literature, the discontinuation rates of duloxetine treatment due to side effects vary between 14-35%. According to a recent meta-analysis, the discontinuation rates of duloxetine treatment due to side effects were reported as 17% (Li et al., 2013). Collado et al. (2011) reported that, 25% of patients stopped the duloxetine treatment because of adverse effects. However, in that study, patients used duloxetine for 9 months. In our study, in accordance with the literature, overall 16 (14.3%) patients reported adverse events and discontinued the drug. Our study had several important limitations. Primarily, the study had a retrospective design and thus the evaluation of SUI was solely based on the numbers of pads used per day. It should be noted that the pet test is a subjective test that can vary from patient to patient. On the contrary, it is commonly known that besides the number of pads, there are some indicators/tools used in the evaluation of SUI including changes in pad weight, bladder diary, questionnaires used for the assessment of quality of life such as Urinary Incontinence Quality of Life Scale (IQOL) and uroflowmetric parameters (maximum flow rate, post-voiding residual urine, etc.). Second, no urodynamic findings were available in the study and thus our results were solely based on subjective evidence. Third, despite being a pharmacological study, the present study had no placebo control group. Fourthly, we did not evaluate the efficacy of duloxetine in PPI positive patients, and therefore, in our study, there is no comment on the effectiveness of duloxetine in PPI positive patients. Finally, the study had a relatively small patient population due to the administration of a large number of exclusion criteria.

In conclusions, our data show that in the early postoperative period, prophylactic administration of duloxetine, which started without regard to the positivity of PPI, is not an effective and rational method in patients undergoing radical prostatectomy. Instead, randomized, prospective and placebo-controlled studies are needed to investigate the efficacy of duloxetine in PPI-positive patients.

Conflict of interest

None to declare.

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The effect of myths about sexuality and the level of knowledge about sexuality on the marital satisfaction in married couples

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Abstract

To look into the effect of sexual myths and level of knowledge about sexuality on marital satisfaction in married couples. The study was carried on with 104 voluntary respondents; 57 of which are married women and 47 are married men. The data has been collected with Personal Information Questionnaire, Marital Adjustment Test, Sexual Myths Analysis Questionnaire and The Golombok Rust Inventory of Sexual Satisfaction (GRISS), data has been analyzed with SPSS 15.0 software package (Statistical Package for Social Sciences). According to these findings, as the belief in sexual myths increases, the sexual satisfaction decreases; yet the increase in the belief in sexual myths does not affect the marital satisfaction. The findings implicate that as the knowledge about sexuality increases, the sexual satisfaction increases while the marital satisfaction decreases. As the number of sexual myths increases, marital adjustment decreases. The findings don't show any significant correlation between sexual satisfaction and marital adjustment.

Keywords: sexuality, sexual myths, level of sexual knowledge, marital satisfaction, sexual satisfaction

1. Introduction

Marriage is the smallest unit that has played a primary role throughout human development as the relational system that is built between two people who have different interests, desires and needs in order to live together, share experiences, give birth and raise children. It is a unity in which sexual needs that are kept out of all social prohibitions are met mutually and it is a contract which depends on mutual solidarity and social validation (Düzgün, 2009).

Sexual satisfaction is feeling happy and content with sexual transmission (Kayır, 2001). Sexual satisfaction defines how content one is with sexual relationship (CETAD, 2009). The main function of sexuality in marriage is sharing pleasure, improving and deepening intimacy and reducing tension resulting from the struggles of marriage and life. Sexual compatibility in marriage is an important part of the marriage process and affects contentment about marriage and marital relationship significantly. While the absence of problems in sexual function has positive impact on marriage, experiencing these problems is claimed to affect marriage negatively, drain positive feelings and marital intimacy (Vural and Temel, 2009). The depression resulting from being unable to satisfy each other sexually will affect the relationship of the married couple negatively in time (Abalı, 2007). Erbek et. al state that in Turkey and all over the world sexual behaviors are highly dominated by religious precepts, prejudices, taboos, traditions, which prevents young people from acquiring knowledge about sexuality and reproduction (Erbek, 2005). The study conducted by the Foundation of Sexuality Education and Research demonstrated that Turkish society sees the most important sources of sexual problems as 62% being uneducated and lacking knowledge (CETAD, 2009).

Sexual education can both raise individuals' awareness about the myths and prejudices they have and help professionals (trainer/therapist) to review their own ideas and beliefs. This kind of education can make sexual issues be discussed easily and prevent them to become taboos (Sungur, 1998).

2. Materials and methods

A signed informed content explaining the study was obtained from all participants.

2.1. Sample

This study aims to understand whether sexual and marital satisfaction are affected by the gender, length of marriage, number of children, level of education, believing in sexual myths and level of knowledge in married couples through

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relational scanning model. All respondents initially applied to a gynecology expert complaining of problems regarding sexual health without any physiological pathology found and thus were further referred to a psychological counseling service center for evaluation and therapy between 2017 and 2019. Socio-demographic characteristics of the respondents are as follows: The average age of the sample is X=33 ± 5. % 39.4 of the respondents are married for 1-5 years, 27.9% for 5-10 years,19.2% for 10- 15 years and 5.8% for 20 and more years. 40.4% of the respondents are childless, 20.2% have one, 29.8% have 2 children, 9.6 %have 3 and more children. Educational level of 104 respondents is as follows: 26.9% graduated from primary school, 12.5% from secondary school, 24% from high school, 30% undergraduate studies and 5.8% from graduate studies.

2.2. Evaluation materials

The data of the study has been collected by Personal Information Questionnaire, Marital Adjustment Scale, Sexual Myths Analysis Questionnaire and Golombok Rust Inventory of Sexual Satisfaction (GRISS).The data has been analyzed with SPSS 15.0 software package.

a- Personal information questionnaire

The questionnaire starts with an informative statement about the objective and content of the study, the identity of the researcher, voluntary based and anonymous nature of the participation to the study. It is followed by a personal information form that asks about the socio-demographical features of the respondents. In this part, respondents are asked to state their gender, age, education level, length of marriage and number of children. Upon literature review, the researchers collated five questions about respondents' sexual lives such as who they talk to about sexual issues, who/where they first received sexual information from, whether they have sexual experience or not.

b- Marital adjustment test

In order to measure adjustment in marriage, the marriage adjustment test has been adapted to Turkish (Ergin, 2008). Marital Adjustment Test is a reliable test with fundamental and distinctive questions which was prepared by Locke and Wallace in 1959 to measure the adjustment in marriage (Açık, 2008). The test evaluates the relationship in terms of general marital satisfaction and characteristics of the marriage, family budget, emotional expression, free-time activities and agreement and disagreement in sexual issues (Açık, 2009).

2.3. Evaluation form for level of knowledge about sexual issues

In the research, the Evaluation Form for Level of Knowledge about Sexual Issues which was prepared by Vural was used (Vural, 2009). This form which was derived from a literature analysis includes 25 statements about male/female reproductive anatomy and physiology, contraceptive methods, sexual life, sexual dysfunction and STD. The score of the tests can vary between 0-100.

2.4. Sexual myths evaluation form

This research uses the sexual myths evaluation form prepared by Zilbergeld both for women and men. This form is composed of 15 statements about various myths. It is a form that includes 15 statements such as 'Masturbation is dirty and harmful', 'The aim of good sex is sexual coitus' 'Men are always ready and eager for action' 'Sex is a natural act and cannot be learnt' (Kayır, 1998).

2.5. 5- Golombok Rust Inventory of Sexual Satisfaction (GRISS)

It was developed to evaluate the quality of sexual relationship and sexual dysfunction for heterosexual couples. The overall score gives a general view about the quality of sexual functions and the sub scores give more detailed information about various aspects of the relationship. Some sub scores can also be used to diagnose a dysfunction (Vural, 2009). The Turkish version of the form was tested for reliability and validity by Tuğrul et al. in 1993 and was standardized to be usable in Turkish culture (Tuğrul et al., 1993; Yılmaz et al., 2010).

3. Results

Table 1 shows the results of Pearson Correlation Coefficient that has been calculated for all tests in the research. The results show a significant negative correlation between GRISS and Sexual Myth Test scores ($r= -0.58$, $p<0.05$). According to that, as the belief in sexual myths increases, the scores of sexual satisfaction decreases. According to results there is a significant positive correlation between GRISS and scores of sexual knowledges ($r=0.47$, $p<0.05$). In other words, as the level of knowledge about sexual issues increases, the scores of sexual satisfactions increase too. The results show a weak negative correlation between marital adjustment and sexual myths inventory scores ($r= -0.32$, $p<0.05$). As the sexual myths increases, marital adjustment decreases. There is a significant negative correlation between marital adjustment and level of sexual knowledge ($r= -0.19$, $p<0.05$). Thus, as the knowledge about sexual issues increases marital adjustment decreases.

Table 1. The correlation results between tests in the research***

GRISS total (n:104)	GRISS Total	Sexual Myth	Marita Adjst.	Sexual Knw.
GRISS	1.000	-.582**	.484**	.473**
Sexual Myth	-.582**	1.00	-.326**	.391**
Marital Adjustment	.484**	.326**	1.00	-.195**
Sexual Knowledge	.473**	.391**	-.195**	1.00

There isn't a significant correlation between GRISS and marital adjustment scores; in other words, between sexual satisfaction and marital adjustment (r=0.48, p>0.05). According to gender variable, independent group t-test applied to the sexual satisfaction total score indicated a significant difference (p<0.01). This difference was in women's favor. The sexual satisfaction scores of women were higher than men's. In addition to that, the sexual myths scores of women were higher, as well (p<0.05). Yet, men's scores in knowledge about sexual issues were higher.

According to ANOVA Test results for Education Level and tests used in the research (GRISS, Sexual Myths Evaluation Form, Evaluation Form for Level of Knowledge about Sexual Issues and Marital Adjustment) there is a significant difference between sexual satisfaction, belief in sexual myths, marital adjustment of respondents and their education level (p<0.01). The sexual satisfaction score of the primary school graduates was (X= 76.24), the score of high school graduates was (X= 68.21), the score of university and higher education graduates was (X=56.02). Taking the results of the correlation between sexual myths and education level, it is seen that the scores of believing in sexual myths is higher in university and higher education graduates (X=25.76) compared to the scores of high school graduates (X=22.84) and scores of the primary school graduates (X=19.04). When the marital adjustment and education level are analysed, the scores of primary school graduates (X=41.16) and high school graduates (X=38.90) were higher than university graduates (X=37.00). The results didn't show any difference between education level and level of knowledge about sexual issues (p>0.05).

According to the results of Scheffi test analyzing the difference for education levels, it is demonstrated that sexual satisfaction scores were higher in primary school graduates compared to the high school and university graduates. Table 2 shows the distribution of sample group according to sexual life information for genders in frequency and percentage.

Table 2. Distribution of individuals according to the characteristics of sexual life

CHARACTERISTICS	Frequency (n)		Percentage (%)	
	F	M	F	M
Importance of Sexuality				
None	11	-	19.3	-
A little	18	7	31.6	14.9
Indecisive	3	3	5.3	6.4
Quite	16	23	28.1	48.9
A lot	9	14	15.8	29.8
Having Knowledge About Sexuality Before Marriage				
None	17	1	29.8	2.1
A little	20	12	35.1	25.5
Indecisive	3	-	5.3	-
Quite	12	24	21.1	51.1
A lot	5	14	8.8	21.3
Being Ashamed of Talking About Sexual Issues				
Yes	30	12	52.6	25.5
No	27	35	47.4	74.5
The Person to Talk to About Sexual Issues				
Girlfriend	39	6	68.4	12.8
Boyfriend	1	27	1.8	57.4
Mother/Father	1	1	1.8	2.1
Sibling	7	2	12.3	4.3
Others	9	11	15.8	23.4
The Source to Learn the First Sexual Information				
School/Lesson	4	3	7	6.4
Books/Magzines	8	9	14	19.1
Tv- Media	-	3	-	6.4
Friends	37	26	64.9	55.3
Mother/Father	2	-	3.5	-
Others	6	6	10.5	12.8
Total	57	47	100	100

For the question 'How important is sexuality for you?' 19.3 % of female respondents said none, 31.6% said a little, 5.3% said indecisive, 28.1% said quite and 15.8% said a lot. 14.9 % of male respondents said a little, 6.4% said indecisive, 48.9% said quite and 29.8% said a lot. To the question 'How knowledgeable were you before you got married?' 29.8% of female respondents said none, 35.1% said a little, 5.3% said indecisive, 21.1% said quite and 8.8% said a lot. 2.1% of male respondents said none, 5.5% said a little, 51.1% said quite and 21.3% said a lot. When asked 'Are you ashamed of talking about sexual issues?' 52.6% of female respondents said yes and 47. 4% said no while 25.5% of male respondents said yes and 75.5 % said no. To the question 'Who do you talk to about sexual issues?'68.4% of female respondents said with my girlfriend,1.8% said with my boyfriend, 1.8 % said with my mother/father,12.3% said with my sibling, 16.8% said others. 2.8% of male respondents said with my girlfriend, 57.4% said with my boyfriend, 2.1% said with my mother/father, 4.3% said with my sibling, 23.4% said others. When asked 'Where did you get your first information about sexuality? 7.0% of female respondents said school, 14.0% said books/magazines, 64.9% said friends, 3.5% said mother/father,10.5% said others.6.4% of male respondents said school,19.1% said books/magazines, 55.3% said friends, 6.4% said media/tv, 12.8% said others According to this, sexuality is more important for male respondents than female respondents. Most of both female and male respondents received their first sexual information from their friends and they both mostly talk to their friends about sexual issues. While most of the female respondents stated that they are ashamed to talk about sexual issues, most of the male respondents stated that they didn't feel ashamed.

4. Discussion

This study has looked into the effect of level of knowledge about sexuality and sexual myths in married couples on sexual satisfaction and the effect of level of knowledge about sexuality and sexual myths in married couples on marital satisfaction. The results have shown that as the scores of knowledge level increase, the scores of marital adjustment increase, as well. These findings are parallel with the previous findings that say that marital satisfaction and sexual satisfaction are directly proportional; if one is bad the other is bad too (Huston and Vangelesti, 1991; Gülsün et al., 2009; Pande et al., 2011). The scores for level of knowledge about sexual issues were higher in men than women. This may be the result of the fact that sexuality is seen as a taboo for women in our country and because of the myths which say that women who are closely interested in these issues will be regarded negatively, which estranges women from the sexuality. This was also suggested by Curtin as a possible problem in certain cultures (Curtin et al., 2011).

Besides, that might be related to the fact that men are interested in sexual issues starting from adolescence and before, and this is seen as normal by the society and they can also access sources and publications easily. According to the results, the sexual satisfaction scores are higher in primary school graduates than high school and university graduates. This can be explained by the fact that as the education and experience level of the individuals increase, their expectations about sexual satisfaction like many other issues increase, too. In the current study, the data has been collected from married individuals, yet their spouses haven't been included in the research. Halloran claims that according to the system approach the spouses can affect each other (Halloran, 1998). Thus, in further studies getting information related to the spouses and incorporating them into the study may enrich the research.

Conflict of interest

None to declare.

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Analysis of occupational accidents and musculoskeletal system problems of butchers in Hatay

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Abstract

Although musculoskeletal system injuries are frequently seen in the area of meat industry, studies on this topic are insufficient. The purpose of this study it to identify the occupational accidents and musculoskeletal problems of butcher shop workers in the city of Hatay. A survey consisting of 32 questions was given to 69 butchers who work in the city of Hatay with 38.20±1.32 in a face-to-face interview. Their social status, activities in the butcher shop, past injuries and activities after their injuries were questioned with the survey. It was determined that the butchers were most frequently injured in their hand and finger area (60.9%). It was determined that 40.6% of the butchers had an accident in the last 5 years; their injuries were mostly non-dominant hand injuries and infections were seen in 15.9% of the butchers after their injuries. It was found that the most frequent injuries took place during the hanging of carcasses; carelessness played an important role in getting injured; use of sharp tools led to injuries in 97.1% of the butchers and that 2.9% of the butchers were left with permanent handicaps after their injuries. It was determined that hand-finger injuries and back pain complaints were frequently seen in butcher shop workers and that they do not use any protective equipment to avoid injuries. Therefore, it was concluded that the necessary analyses need to be done on the butcher shop workers and that training should be provided for them on protective precautions and posture problems.

Keywords: butchers, musculoskeletal problems, industrial injuries, injury

1. Introduction

Musculoskeletal system impairments related to work life or occupations are regarded as a serious laborer health problem throughout the world (Mukhopadhyay and Khan, 2015). Besides bad and inappropriate work order or tools which are not ergonomic, long-term work which physically push the human body are accepted as risk factors for musculoskeletal system impairments. Since these risk factors in question reduce the productivity of workers, decrease work quality and cause economic loss besides giving rise to health problems, they are considered as important factors (Colombini and Occhipinti, 2006; Mukhopadhyay and Khan, 2015). According to the data of American National Electronic Injury Surveillance System Center, 1 million patients each year are reported due to occupational accidents (Dianat et al., 2012). In England, this number is 700 thousand people (Valero et al., 2016). Similarly, it is stated in the annual reports of Finland Occupational Health institute, US Labor Department Occupational Safety Health Administration and Canada occupational Health Safety Center that occupational accidents cause serious economic losses. In addition, it is indicated that they cause loss of labor and subsequently decrease in productivity (Gomez Galan et al., 2017). The first three injuries are waist, back and hand injuries (Dianat et al., 2012; Valero et al., 2016). Occupational injuries take place in many different industries. One of these is the meat industry and it is a highly risky and traumatic area in terms of the injuries workers of this industry face. In a study, it is emphasized that the dimensions and severity of injuries in this sector related to the use of knives, saws and machines have changed (Göçmen, 2016). While some of the hand injuries can be treated with simple medical dressings, others cause deformities in the extremities and even amputations (Dianat et al., 2012; CHIWO, 2013). The high severity level of the injuries delays going back to work and causes personal, social and psychological traumas (Çakır et al., 2014). In a study carried out in England, it was underlined that these types of injuries cause 7.5 million work days to be lost each year (Valero et al., 2016).

The workers' impairment of health and loss of work days do not only affect their well-being and quality of life, but damages the country economy as well. Musculoskeletal System problems caused by accidents impair soft tissue functions such as muscle, joint, tendon and circulatory systems and trigger pain (Valero et al., 2016, Gomez Galan et al., 2017).

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When evaluated within the scope of meat industry, besides posture of the workers and carrying things, activities such as lifting or carrying loads, use of sharp tools such as knives leads to such effects (Dianat et al., 2012; Gomez Galan et al., 2017).

Our hands which are extremely important in our daily life have a sensitive and complex structure in terms of anatomical structure such as veins, nerves and tendons. In hand traumas, recovery after losing anatomical and physiological function can take months (Zhu et al., 2017). Hand injuries and fingertip amputations are frequently seen in particular among workers who use powerful cutting equipment. In addition, infections in the injury area can be seen after injuries. Studies have shown that hand injuries are frequent in the meat industry and that an important part of the injuries cause permanent damages (Rubin et al., 2007). Especially the inappropriate work place of manual meat cutters, use of bad work equipment and long working hours are stated to cause risks in terms of musculoskeletal system impairments (Mukhopadhyay and Khan, 2015; Karltun et al., 2016). In addition, it has been reported that besides the spread of meat integrated facilities in the last decade in Europe and carrying of meat manually are serious risk factors (Göçmen, 2016). It is stated that traumas which can take place both during cutting and carrying can be reduced with ergonomic arrangements and training. Within this scope, it is apparent that the characteristics of cutting and carrying tools, appropriate holding positions and use of protective equipment will play an efficient role in the regulation of working conditions (Göçmen, 2016; Tirlon et al., 2019). Although musculoskeletal system injuries take place in the area of meat industry, there are no studies of national scope. It is know that the education level of butchers working in the city of Hatay is generally low and that their carcass cutting and selling methods are different from each other (Sarıgöz, 2010). The purpose of this study it to identify the occupational accidents and musculoskeletal problems of butcher shop workers in the city of Hatay.

2. Materials and methods

This study was carried out through a survey given to 69 butchers and butcher shop workers who work in the city of Hatay and its districts with a face-to-face interview. The ethical committee approval of our study was given by Hatay Mustafa Kemal University Social and Human Sciences Scientific Research and Publication Ethics Committee (02.05.2019/07). In the 32 question survey prepared within the scope of the study, the butchers' social status, activities in the butcher shop, past injuries and activities after their injuries were questioned. The data obtained in the survey were analyzed with SPSS Statistics 17 software.

3. Results

The data obtained as a result of the study on the social structures of the butchers are given in Table 1. It was determined that the age average of the butchers was 38.20±1.32 and the least seen age group was 36-40. It was determined that most of their mothers were housewives; the butchers mostly continued their fathers' occupation and that their previous job was the same. It was seen that about 90% of the butchers had social security (Table 1).

Table 1. The demographic data and socio-cultural status of the subjects (%)

Age		Mother's occupation	
17-20	2.8	Housewife	79.7
21-25	5.7	Farmer	15.9
26-30	18.8	Retired	2.9
31-35	12.9	Husbandry	1.4
36-40	24.5	Father's occupation	
41-45	14.5	Butcher	40.6
46-50	8.5	Civil servant	20.3
51+	11.3	Self-employed	11.6
Number of siblings		Construction w	4.3
3 and below	18.8	Retired	4.3
4	18.8	Unemployed	4.3
5	15.9	Other	14.6
6	13.0	Education level	
7	8.7	Primary	37.7
8	13.0	Middle-school	34.8
	11.6	High-school	21.7
Occupation before being a butcher		University	4.3
Butcher	44.9	Not literate	1.4
Unemployed	18.8	Social Security	
Other	36.3	Yes	89.9
		No	10.1
Occupational injuries before becoming a butcher			
Yes	43.5	No	56.5

In Table 2, the animals cut by the butchers and the work and processes they perform after the meat cutting process are shown. As it can be seen from the table, butchers who work in the Hatay area mostly cut cattle. The type of animal cut also influences the fact that generally vehicles are used to carry carcasses (%85.5). The number of animals cut daily or weekly is related to the work load of the butchers.

Table 2. Type of animal cut and the use of vehicles in carrying carcass

Type of animal cut		Use of vehicles in carrying carcass	
Cattle	50.7	Yes	85.5
Sheep and goat	13.0	No	14.5
Both	36.2	Number of meat cut per day	
How many kg carcass can you carry?		1 cattle/week	37.7
10-30	16.7	1-2 sheep/day	17.4
31-50	32.6	5-10 sheep/day	13.0
51-70	38.4	5-10 sheep/week	7.2
70+	12.3	2 calves/week	5.8
		Other	18.9

Table 3 evaluates the environment during meat cutting, the equipment used and places of injuries. 34.8% of the workers stated that their work environment is slippery and 98.6% stated that they wear boots during meat cutting. 36.2% of the workers carry the carcasses with pulleys. Although some injuries take place in the transport of carcasses, the most frequently injured areas were reported as the hand and finger area.

Table 3. Meat cutting environment and use of equipment

Slippery work area		Wearing boots while working	
Yes	34.8	Yes	98.6
No	65.2	No	1.4
Style of carrying cattle carcass		**Reason for accident during carcass transport**	
Own power	10.1	Narrow corridor	10.1
Hangar system	23.2	Falling down the stairs	7.2
Pulley	36.2	Collision	11.6
Other	30.4	Other	71.1
Injury area			
Shoulder	4.3	Hand and fingers	60.9
Waist	33.3	Other	1.4

The work environments where butcher shop workers carry out their meat preparation and sales after the cutting of meat, the tools-equipment used during work and injury states are presented in Table 4. In this table, processes such as the preference of sharp tools, injury reasons, use of protective gloves were questioned. It was determined that 40.6% of the subjects had an injury in the last five years, these are mostly non-dominant hand injuries and infections were seen after the injury in 15.9% of the workers. It was determined that injuries took place most frequently during hanging carcasses, carelessness played a major role in getting injured and the injuries took place due to use of sharp tools in the rate of 97.1%. The butchers in the study stated that they use mallets while using the mincing machine in the rate of 88.4% and gloves in the rate of 63.8%. It was determined that 15.9% did not use any protective precautions while working. Another important finding of the study is that the most important reason for injuries is carelessness (66.7%).

Table 4. Work environment and injury process

Experience of injury in the last 5 years		Use of mallets while using the mincing machine	
Yes	40.6	Yes	88.4
No	59.4	No	11.6
Infection formation after injury		Injury in the dominant hand	
Yes	15.9	Yes	37.7
No	84.1	No	62.3
Tool maintenance		Reason for injury	
Yes	98.6	Sharp tools	97.1
No	1.4	Mincing machine	1.4
Injury area		Animal blow	1.4
During carcass hanging	52.2	**Injury period**	
During meat chopping	24.6	Week days	48.6
During meat cutting	17.4	Eid Al-Adha	37.0
In the mincing machine	5.8	Weekend	12.4
Reason for injury		**Protective precautions during work**	
Carelessness	66.7	Use of gloves	63.8
Being tired	13.0	None	15.9
Shaking of the hands	5.8	Boots	8.7
Sleeplessness	4.3	Other	1.4
Irritability	4.3	Gloves and boots	10.1
Other	5.9		

As it can be seen in Table 5, the injuries of the workers generally take place in morning and night hours during the chopping of the meat. While the transfer of the injured workers is done by personal vehicles (91.4%), 4.3 % of the workers stated that they can perform first-aid. The rate of workers who are left with permanent handicaps after injury was determined as 2.9%.

Table 5. Injury period

Permanent handicap after injury	
Yes	2.9
No	97.1
Performing first-aid after injury	
Yes	4.3
No	95.7
Transport to the hospital after injury	
Ambulance	4.3
Private car	91.4
Taxi	4.3
First-aid kit in the work place	
Yes	91.3
No	8.7
Injury hour	
Morning	46.5
Close to end of the shift	47.8
Noon	5.7

4. Discussion

In today's industrial society, more and faster production is targeted. However, it is important that the injury risks of the workers are identified, the workers are protected and corrective precautions are taken in this process (Dianat et al., 2012; Sarıgöz, 2010). In many different occupation groups, in particular spinal impairments in the waist and neck and hand injuries are reported. However, there are very few studies on how sustainable development is affected by work place safety and health proactivity (Sarıgöz, 2010; Jilcha and Kitaw, 2017).

Publications which deal with occupational accidents and musculoskeletal problems of butchers is limited (Rubin et al., 2007; Sarıgöz, 2010; Mukhopadhyay and Khan, 2015; Göçmen, 2016;) and the rate of injuries is not well determined in medical literature. A study carried out in the US showed that the meat packaging industry carried the highest rate of injury in any industry in 5 consecutive years (Becker et al., 1996). Factors which may increase the risk of acute hand injury are associated with activities such as inaccurate work methods and use of inappropriate equipment, use of an out of the ordinary work method, getting distracted or hurrying. Hand injuries take the 3rd place among all occupational injuries in Holland, Denmark and England (Dianat et al., 2012). It was reported that about 40% of hand and wrist injuries in Denmark are due to work accidents which take place in the production industry. In addition, it was reported in 1986 that more frequent and serious hand and finger injuries compared to the other parts of the body took place among Swedish metal workers (Larson, 1986). Although meat integrated facilities became spread especially in Europe in the last decade, carrying of meat manually increases musculoskeletal system impairments (Warriner et al.,

2002). Sarıgöz et. al (2010) carried out in Turkey, it was reported similar to our study that the education level of butchers in the city of Antakya was low and that their occupational methods are far from carcass classification and chopping standards of EU countries and uncontrolled. In the present study, it was determined that butcher shop workers work on slippery areas, carry meat and heavy carcass loads manually (Table 3) and that there is a relationship between their injury areas and hours spent on meat cutting.

According to Evtushenko's study in Ukraine which covered a period from 2003 to 2013, the meat industry is one of the riskiest and most traumatic industries in terms of occupational accidents workers experience (Göçmen, 2016). The scale and the severity of the injuries are determined by the use of knives, saws and the moving parts of the machines (Göçmen, 2016; Karltun et al., 2016). While some of the hand injuries can be treated with simple medical dressings, others cause deformities in the extremities and even amputations. The findings show that meat packaging leads to serious functional losses in the hands and fingers (Becker et al., 1996). The subjects in our study expressed that injuries take place due to carelessness and tiredness (Table 4). It was seen that injury times are morning and night hours (Table 5). It was observed that sharp tools caused injuries in the rate of 90% and hand and finger traumas in the rate of 60%.

The high severity level of the injuries delays going back to work and causes personal, social and psychological traumas (Çakır et al., 2014). It was observed in our study that the butcher shop workers experienced hand-finger injuries the most and backaches afterwards (Table 3). In the literature, it has been indicated that when appropriate hand protection goods and gloves are used to prevent hand and finger injuries, the risk of injury will be reduced (Irzmanska and Tokarski, 2017) and infections will be prevented (Rubin et al., 2007). However, it was determined that butchers do not use butcher gloves or protective equipment against accidents in the city of Hatay. In the butcher occupation, the frequency of repeated technical procedures is associated with postural risk, ergonomic conditions, carpal tunnel syndrome prevalence and hand, tendon and spine pathologies (Ghersi et al., 1996). India has implemented ergonomic arrangements to reduce these types of musculoskeletal problems in the area of meat cutting which is a very important source of income for the country. Within this scope, working conditions, postures of the butchers, the repeated movements of the dominant hand and leg movements and injury risks were analyzed (Mukhopadhyay and Khan, 2015; Karltun et al., 2016). In India, butchers work for 7 days a week and 8-10 hours each day. The areas of the body which most get affected have been reported as; upper back (30%), lower back (25%), upper arm (15%), forearm (15%), shoulder (10%) and neck (5%) (Mukhopadhyay and Khan, 2015). In a study carried out by Buzanello et al., the rate of musculoskeletal impairments in butchers in Brazil was reported as 20% (Buzanello and Moro, 2012). When the most common accidents among butchers is analyzed, it can be seen that the fingers are under greatest risk and that these accidents are related to the use of knives (Kaltun et al., 2016; Warriner et al., 2002). It was seen that hand-finger injuries in the rate of 60% in our study population is much higher to the rate found by Buzanello et al. and that 33% rate of waist area problems determined in this study is parallel to the findings of Vogel et al. (2013). It was observed that restaurant business and butcher occupation in the city of Hatay are quite popular in the industry and that injuries are related to heavy work load (Sarıgöz, 2010). It was determined in our study that butchers mostly get injured while hanging carcasses and using sharp tools and generally on weekdays (Table 4).

Musculoskeletal impairments can take place both while cutting meat and carrying meat. After the meat cutting stage, manually carrying the meat causes a high amount of energy loss. In terms of the use of cranes, pulleys etc., although it was predicted that musculoskeletal system injuries will decrease with controlled carrying methods and suitable ground surface characteristics, it was also indicted that this is not valid in all cases (Larsson, 1990). Carcasses which weight over half a ton also cause injuries and musculoskeletal system impairments in butcher shop/slaughter house workers (Göçmen, 2016). Luger et al. (2019) have found low quality evidence in their study that besides regular and hectic work life, duration of work and number of breaks are not effective on musculoskeletal system aches, impairments and tiredness reported by the participants. In addition, important correlations were identified in the study between education level of the workers and the time slice in which injuries take place ($P<0.05$) and area of injury in the body ($P<0.01$), general reasons for injuries (sleeplessness, irritability, etc.) and having social security ($P<0.01$); number of meat cut per day and method of carrying cattle ($P<0.01$) and place/location of injury ($P<0.01$); slippery ground and then time slice in which injuries take place ($P<0.05$) and injury area in the body ($P<0.01$).

The present study showed that musculoskeletal problems in butchers are related to work load and meat cutting technique. It was found that butchers often carry about 50-70 kg of weight exerting body power and that injuries mostly take place during hanging of the carcasses and cutting of meat. It was also observed that work spaces and corridors are narrow and floors are slippery. Another parameter about getting injured is that there is a significant relationship between long working hours and duration of changing different knives and duration of upper extremity impairments. Meat cutters who change their knives are in the risk group for developing musculoskeletal impairments for this reason (Karltun et al., 2016). In our study, it was seen that the subjects frequently had hand-finger injuries (60%) (Table 3).

More information is needed to understand the workers' education level, their environment and thus determine the accurate movements in the meat cutting procedure (Karltun et

al., 2016). In our study, it was determined that butchers traditionally continued the family occupation, 6 out of every 10 butchers received primary and middle-school education and 4% were university graduates (Table 1). Within this scope, specialized curricula should be formed and implemented. It seems very important to include occupational physicians in the active health surveillance programs with the purpose of identifying critical areas for butcher shop workers and developing efficient preventive precautions (Bonzini et al., 2014). In addition, posture and movement ergonomy analyses should be performed to prevent work related musculoskeletal system impairments. In order to be able to prevent impairments related to biomechanical excessive loads or manual carrying of loads and repetitive movement of the upper extremities, different study topics should be developed. In addition, physiotherapy methods should be developed for the well-being of the trunk and the extremities (Occhipinti et al., 2003).

As a result, it was determined that hand-finger injuries and waist problems are seen frequently in butcher shop workers and that protective equipment against accidents are not used. Therefore, it is considered that the required analyses should be done about the workers and training on protective measures should be given to prevent musculoskeletal system injuries and impairments related to work.

Conflict of interest

None to declare.

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The opinions of specialists and students regarding the use of visual and written expression forms in rhinoplasty education

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Abstract

This research was conducted to investigate the contribution of visual and written expression to nasal surgery education and the preferences of usage. The research was conducted with the observation of cases in the Ear, Nose and Throat (ENT) Surgery Department of Ondokuz Mayıs University Medical Faculty. During the study, the processes of the 'closed dorsum rhinoplasty' cases were observed. The case illustrations made as a result of the observations were presented to the study population comprising 36 medical students from 3 different universities (Ondokuz Mayıs University, Hitit University, İstanbul Medeniyet University). Data were obtained concerning the sampling of the research, the specialization areas of the participants, title groups, number of participants, and the information from the Likert type responses and the non-parametric statistical findings. The results showed that although the use of illustrations was seen to be more important according to the opinion of specialists and non-specialists in the area, sole use was concluded to be insufficient. A total of 43 of 60 ENT specialists and 9 of 11 Plastic Surgery specialists preferred the use of illustrations, photographs and written expression together. Non-specialists were seen to be undecided about their responses while no significant difference was found between specialists. The preferences of the participants were determined to be illustration, photography and written expression, respectively. The results of this study indicate that the use of visual and written expression forms together when they are available is related to the quality of surgery education at a semantic level.

Keywords: rhinoplasty, nasal surgical procedures, education, medical illustration

1. Introduction

The field of medicine is known to have the three main missions of education, healthcare service and scientific research (Songur, 2016). Medical education has no phase at which visual materials are not used. The rapid increase of information in this field has led to a requirement for the use of new illustrations in educational and scientific areas. The use of focused visual tools, such as the illustrations created for this study, has begun to attract attention as an effective and time-efficient tool that can provide a complete overview of a surgical procedure and lay the groundwork for expedited achievement of surgical competence (Crawshaw et al., 2016). It is important that illustrations are both new and of good quality. For an impressive illustration to demonstrate procedures, the methods used by medical illustrators utilizing their talents and expressive ideas can be categorized as 7 different methods: hovering technique; hidden anatomy, ghosted views, or transparency; centrally focused perspective; action techniques to give life to the procedure; use of insets to highlight one part of the drawing; human proportionality using hands or known objects to show relative size; and step-by-step educational process to depict the stages of a procedure (Mavroudis et al., 2020).

The aim of this study was to investigate the contribution of visual and written elements to rhinoplasty education and the usage preferences. The preferability of the visual expression forms among themselves and their advantages were investigated. This study also aimed to determine the effect of medical illustrations on rhinoplasty education during the course of general medicine.

2. Materials and methods

This study is based on the education of surgeons to perform dorsum preservation rhinoplasty. Approval for the study was granted by the Ethics Committee Ondokuz Mayıs University Medical Faculty. A dorsal preservation rhinoplasty operation performed by a senior ENT surgeon was photographed by a medical illustrator. Then the surgeon and the illustrator prepared three descriptive education papers. The first was written only and described the surgery step-by-step. The second paper included step-by-step photographs taken during the surgery. The third paper comprised medical illustrations drawn by an expert medical illustrator.

These three descriptive papers were prepared and then given to the participants in the sequence of first written

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papers, second photographs, and third illustrations. These visual and written expression materials were then presented together (Fig. 1-40). These visuals prepared for the case process were presented to the participants both as (a-photograph, b-illustration) and (c-written expression). Written expression is given as expressed in figure descriptions. Finally, all the papers were examined and then a questionnaire was completed.

Fig. 1. In closed dorsum-sparing surgery, surgery is initiated by marginal septal incision from the caudal end of the septum. Photographic image of the case process

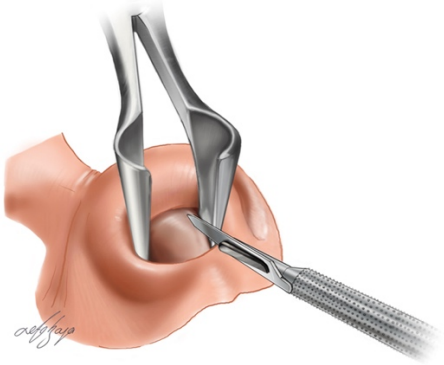

Fig. 2. In closed dorsum-sparing surgery, surgery is initiated by marginal septal incision from the caudal end of the septum. Illustration image as a cross section of the case process

Fig. 3. The incision is removed from the perichondrium and deepened until cartilage is seen. The nasal speculum is used to visualize the incision site. Photographic image of the case process

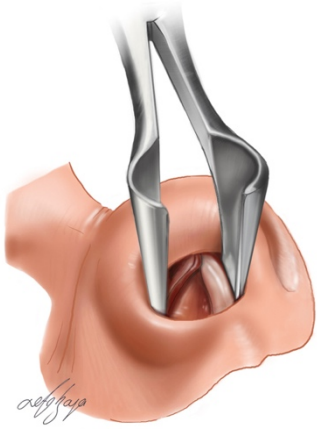

Fig. 4. The incision is removed from the perichondrium and deepened until cartilage is seen. The nasal speculum is used to visualize the incision site. Illustration image as a cross section of the case process

Fig. 5. In closed dorsum-sparing surgery, surgery is initiated by marginal septal incision from the caudal end of the septum. Photographic image of the case process

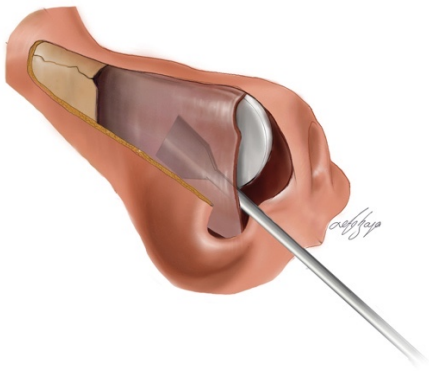

Fig. 6. In closed dorsum-sparing surgery, surgery is initiated by marginal septal incision from the caudal end of the septum. Illustration image as a cross section of the case process

Fig. 7. The incision in the alar wings is made in trans cartilaginous plane. To do this, the incision line is drawn with a surgical pen to leave an approximately 1-2 mm alar rim graft. Photographic image of the case process

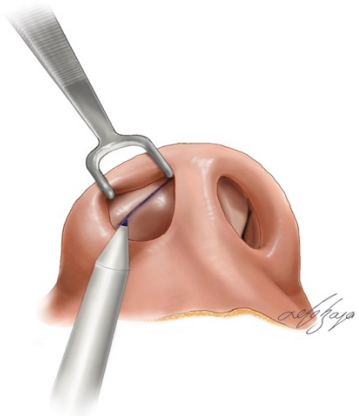

Fig. 8. The incision in the alar wings is made in trans cartilaginous plane. To do this, the incision line is drawn with a surgical pen to leave an approximately 1-2 mm alar rim graft. Illustration image as a cross section of the case process

Fig. 9. The skin incision is done with 15 number scalpel. The incision is terminated after passing the cartilage plan. Photographic image of the case process

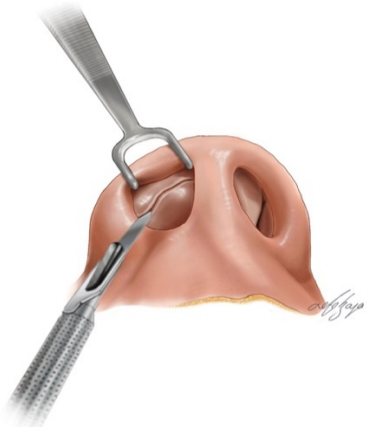

Fig. 10. The skin incision is done with 15 number scalpel. The incision is terminated after passing the cartilage plan. Illustration image as a cross section of the case process

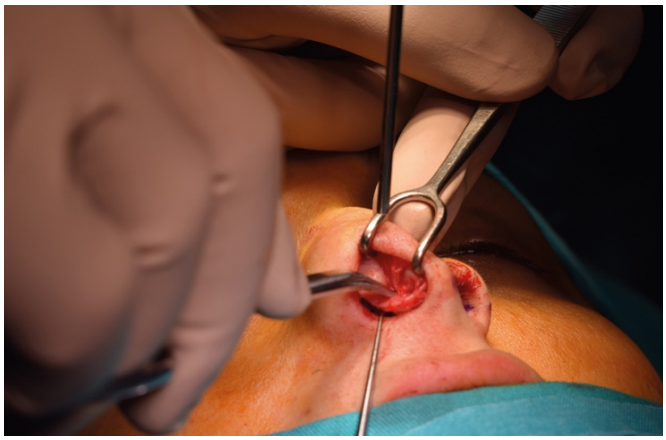

Fig. 11. With the help of elevators, alar cartilages are elevated to the scroll ligament. Photographic image of the case process

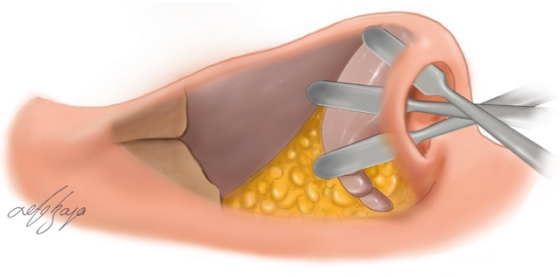

Fig. 12. With the help of elevators, alar cartilages are elevated to the scroll ligament. Illustration image as a cross section of the case process

Fig. 13. If necessary, after the elevation, 1-2 mm cartilage is removed from the posterior part of the septum as vertical strip. Photographic image of the case process

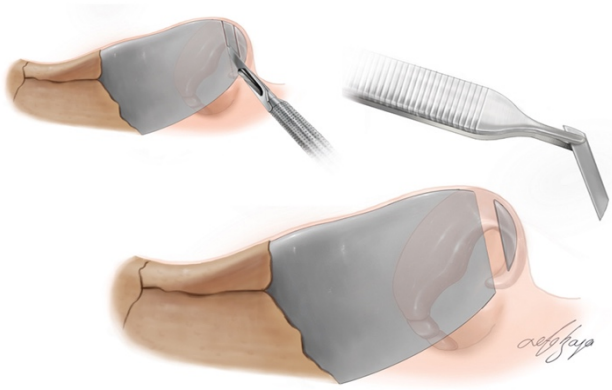

Fig. 14. If necessary, after the elevation, 1-2 mm cartilage is removed from the posterior part of the septum as vertical strip. Illustration image as a cross section of the case process

Fig. 15. Excised septum fragment. Photographic image of the case process

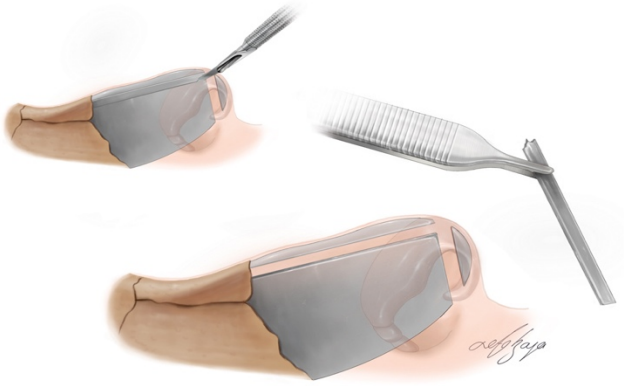

Fig. 16. Excised septum fragment. Illustration image as a cross section of the case process

Fig. 17. Excised septum fragment. Photographic image of the case process

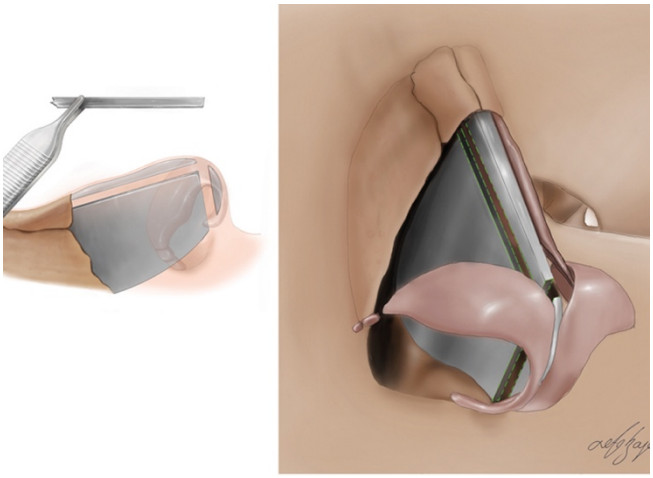

Fig. 18. Excised septum fragment. Illustration image as a cross section of the case process

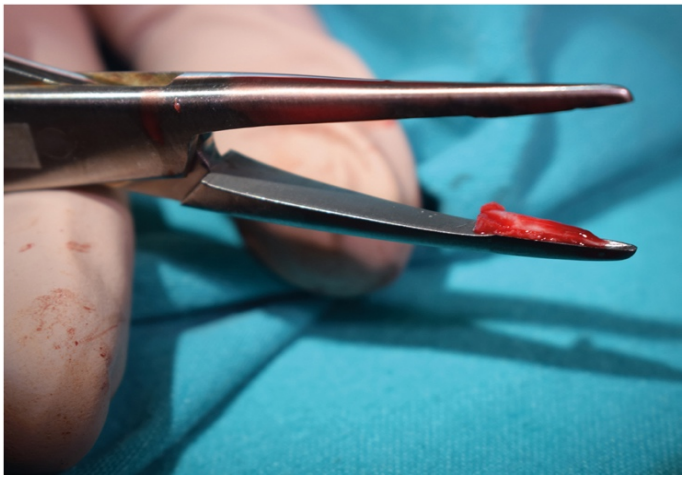

Fig. 19. Once the cartilage is removed, the perpendicular plate of the ethmoid bone is excied using the bone rounger. Photographic image of the case process

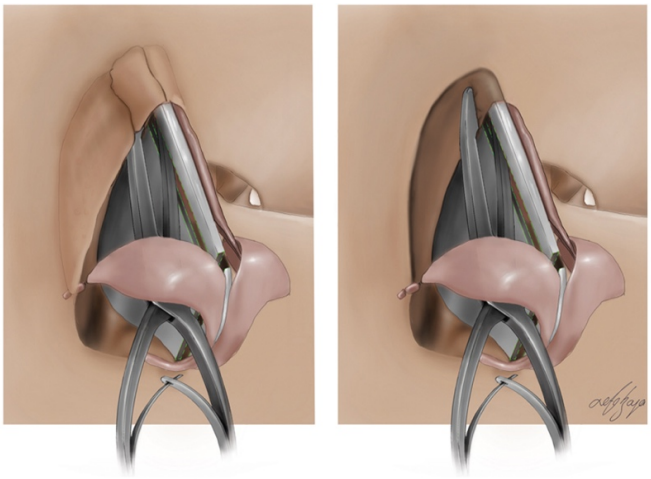

Fig. 20. Once the cartilage is removed, the perpendicular plate of the ethmoid bone is excied using the bone rounger. Illustration image as a cross section of the case process.

Fig. 21. Lateral and medial osteotomies are performed. Although osteotomes and osteotomies are shown in the figüre, instruments such as microtesters and piezzo may also be used for this purpose

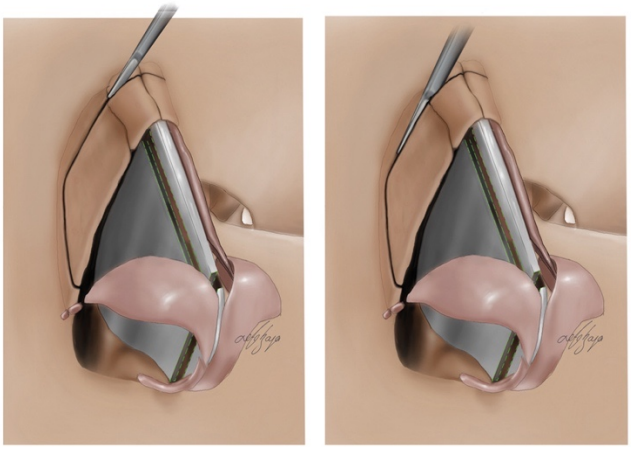

Fig. 22. Lateral and medial osteotomies are performed. Although osteotomes and osteotomies are shown in the figure, instruments such as microtesters and piezzo may also be used for this purpose. Illustration image as a cross section of the case process

Fig. 23. After osteotomies and cartilage resections are completed, the nasal dorsum is pushed and collapsed and piezzo may also be used for this purpose. Photographic image of the case process

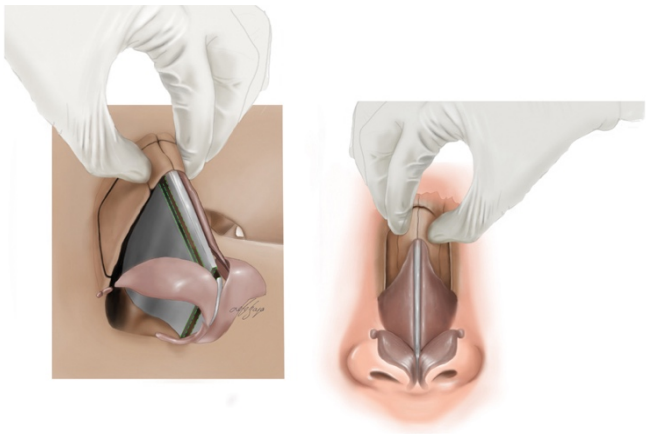

Fig. 24. After osteotomies and cartilage resections are completed, the nasal dorsum is pushed and collapsed and piezzo may also be used for this purpose. Illustration image as a cross section of the case process

Fig. 25. Schematic view of the pushing of the nasal dorsum. Photographic image of the case process

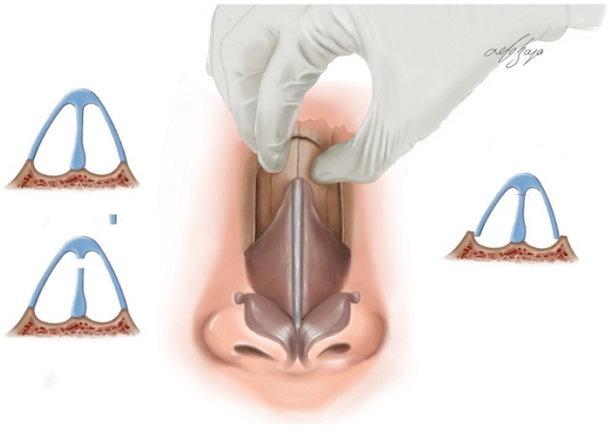

Fig. 26. Schematic view of the pushing of the nasal dorsum. Illustration image as a cross section of the case process

Fig. 27. Resection is performed with a scalpel and the removed part is being seen. Photographic image of the case process

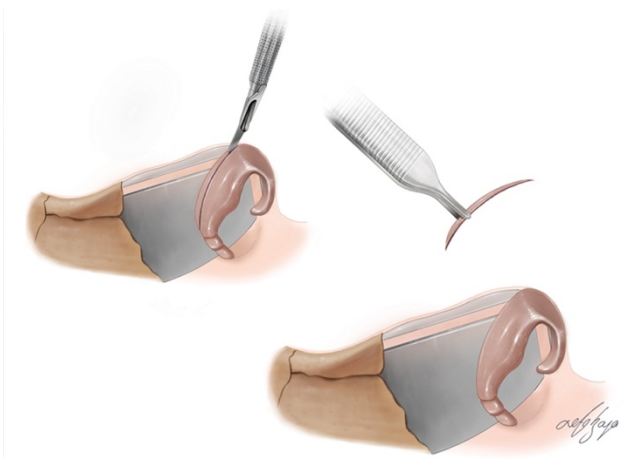

Fig. 28. Resection is performed with a scalpel and the removed part is being seen. Illustration image as a cross section of the case process

Fig. 29. The DOM portions of the alar cartilages are marked. Photographic image of the case process

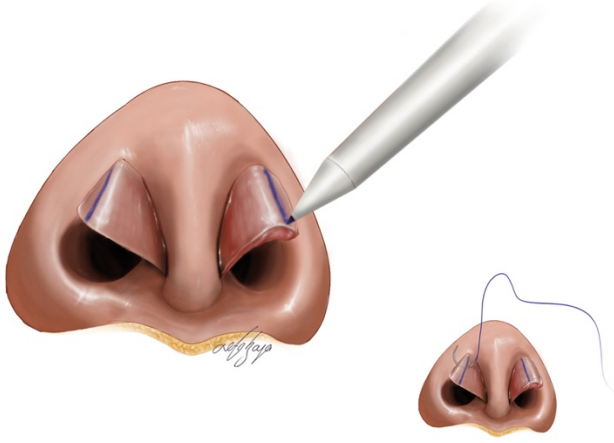

Fig. 30. The DOM portions of the alar cartilages are marked. Illustration image as a cross section of the case process

Fig. 31. Marking the DOM parts of the alar cartilages is being seen. Photographic image of the case process

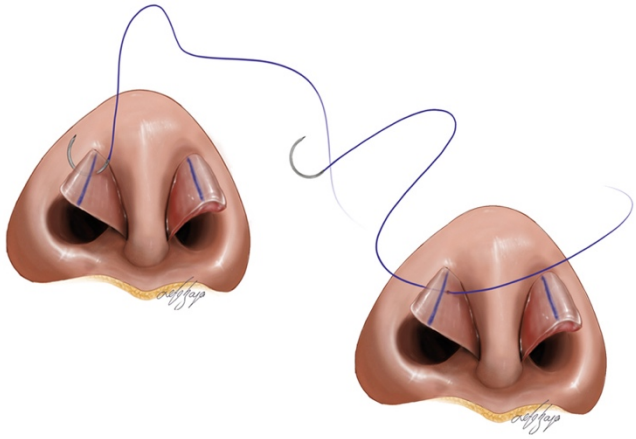

Fig. 32. Marking the DOM parts of the alar cartilages is being seen. Illustration image as a cross section of the case process

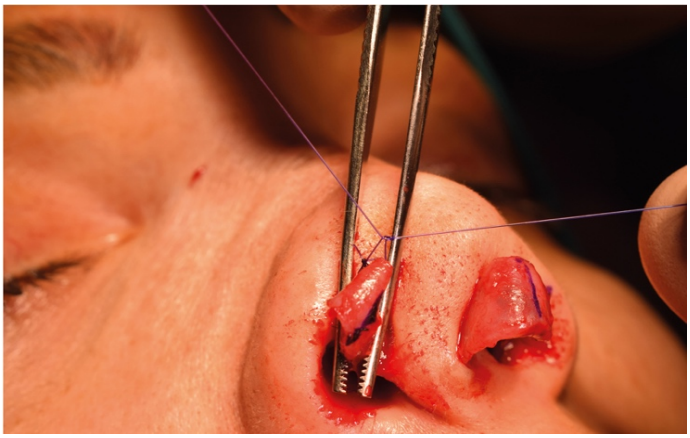

Fig. 33. DOM stealing sutures according to the need of the case after marking the dom portions of the alar cartilages. Photographic image of the case process

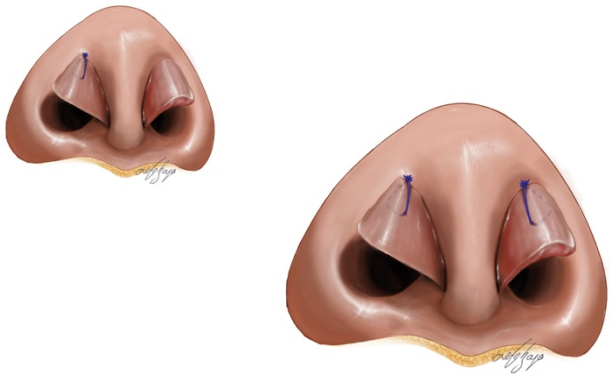

Fig. 34. DOM stealing sutures according to the need of the case after marking the dom portions of the alar cartilages. Illustration image as a cross section of the case process

Fig. 35. It is sutured with at least two points (one of which is the DOM region) with 5/0 PDS. Photographic image of the case process

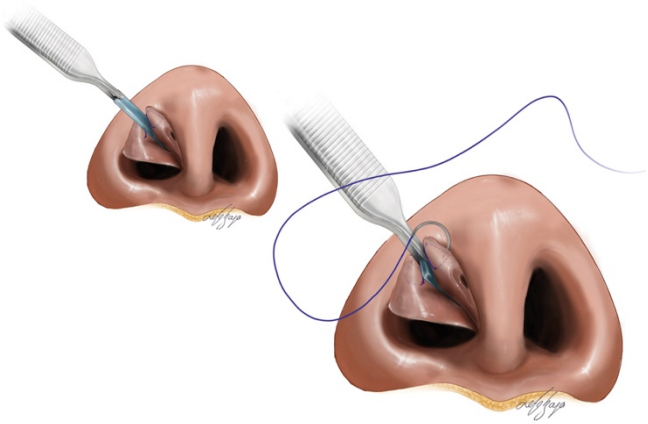

Fig. 36. It is sutured with at least two points (one of which is the DOM region) with 5/0 PDS. Illustration image as a cross section of the case process

Fig. 37. Stabilization of columellar strut to alar cartilages. Photographic image of the case process

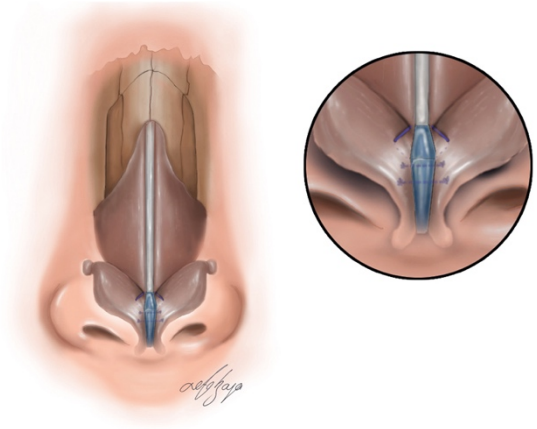

Fig. 38. Stabilization of columellar strut to alar cartilages. Illustration image as a cross section of the case process

Fig. 39. The appearance of sutures after surgery. Photographic image of the case process

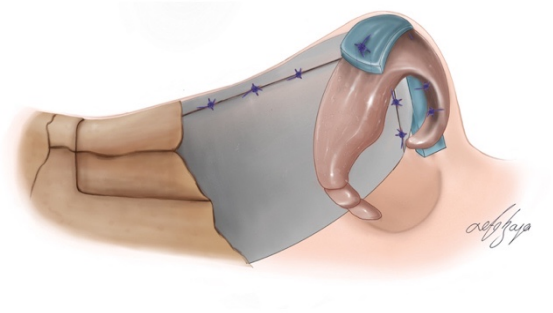

Fig. 40. The appearance of sutures after surgery. Illustration image as a cross section of the case process

3. Results

The study population of 107 participants comprised resident doctors, associate professors, assistant professors, professors, and medical students of Ondokuz Mayis University, Hitit University and İstanbul Medeniyet University. Thus, the population included both surgery experts and medical students, as 71 specialists from the relevant areas and 36 medical students (Table 1).

Table 1. Distribution of the study participants according to specialty, title and number

Specialization	Title					
	Asst. Dr.	Specialist	Asst. Prof.	Assoc. Prof.	Prof.	The number of participants
Ear, Nose and Throat Surgery	5	24	5	12	14	60
Aesthetic, Plastic and Reconstructive Surgery	1	4	0	6	0	11
Medical Students	2	0	0	0	0	36
Total						107

The question, “Do illustrations of the surgical procedures express the subject better than photography and written expression?” had a response of “agree” by 80 (78%) respondents (Table 2). To the question “Do photographs of the surgical procedures express the subject better than illustrations and written expressions?”, the response of “agree” was given by 23 (21.5%) respondents and “disagree” by 51 (30.8%) (Table 2). To the question of “Do written expressions of the surgical procedures express the subject better than photography and illustrations?” 18 (16.8%) respondents answered “agree”, 27 (25.2%) were “undecided” and 62 (57.9%) gave the response of “disagree” (Table 2). The preference for the use of visual and written forms together was expressed by most of the specialists and students with 96 (89.7%) respondents stating “agree”, 6 (4.7%) were “undecided” and 5 (5.6%) disagreed (Table 2). The other questions and responses are shown in Table 2 and Table 3.

4. Discussion

When the development process of visual elements is examined, it can be seen to fulfill the task of transferring knowledge along the communication pathway from past to present. Medicine is one of the fields in which images are used or should be used as messages. Visual material may be medical illustrations or surgical photographs, and in both

eastern and western cultures, medical illustrations have been used since prehistoric times. An increased need and demand for medical illustrations in the field of medicine led to the opening of schools providing medical illustration education. These illustrations have been among the most important sources for describing and archiving surgical techniques and findings, and diagnostic and treatment methods and for the transfer of this knowledge to the next generation.

Table 2. Responses to the questions

	Ear, Nose and Throat Surgery			Aesthetic, Plastic and Reconstructive Surgery			Medical Students		
	Agree	Uncertain	Disagree	Agree	Uncertain	Disagree	Agree	Uncertain	Disagree
Q1	48	6	6	8	0	3	24	5	7
Q2	10	19	31	2	2	7	11	12	13
Q3	6	14	40	3	4	4	9	9	18
Q4	54	3	3	9	0	2	33	3	0
Q5	39	13	8	5	1	5	21	11	4
Q6	32	18	10	7	1	3	9	13	14
Q7	10	22	28	2	3	6	7	12	17
Q8	50	3	7	9	0	2	36	0	0
Q9	56	2	2	10	0	1	34	1	1
Q10	49	6	5	8	2	1	29	4	3
Q11	6	20	34	2	1	8	8	18	10
Q12	59	0	1	11	0	0	33	3	0
Q13	4	14	42	0	1	10	5	9	22

Q1. Illustrations of the surgical procedures express the subject better than photography and written text

Q2. Photographs of the surgical procedures express the subject better than illustrations and written text

Q3. Surgical procedures express the subject better than illustrations and photographs

Q4. Illustrations, photographs, and written text of the surgical procedures should be used together

Q5. The use of illustrations and written text express the subject in the best way

Q6. Illustrations express the subject in detail

Q7. Photographs express the subject in detail

Q8. Illustrations are not sufficient to explain the surgical subject

Q9. Written text is not sufficient to explain the surgical subject

Q10. Illustrations and photography should be used together to explain the surgical subject

Q11. Photographs can explain the surgical subject with the required clarity

Q12. The use of illustrations is important in rhinoplasty education and academic publications

Q13. Photographs are sufficient in rhinoplasty education

Table 3. Responses to the question of the use of which of these forms of expression is important in education

Expression Formats	Specialization			The number of participants
	Ear, Nose and Throat Surgery	Aesthetic, Plastic and Reconstructive Surgery	Medical Students	
Photograph	0	0	0	0
Illustration	5	0	0	5
Written Expression	0	0	0	0
Writing and Illustration	11	2	5	18
Writing and Photograph	1	0	1	2
Photography, Illustration and Written Expression	43	9	30	82
Total				107

According to recent literature, multimedia teaching is more effective than traditional teaching (Motsumi et al., 2019). Studies have demonstrated that visual-based surgical skills training improves the acquisition of surgical skills when combined with standard verbal feedback, compared with standard verbal feedback alone (Farquharson et al., 2013). Visual-based teaching reduces the amount of verbal input needed during the demonstration of surgical skills and reduces learning time. Therefore, the use of medical illustrations on educational platforms, which is an important part of the medical field, can be said to make a positive contribution to the quality of education.

As an interdisciplinary field, medical illustrations can be used in medicine, especially in surgical cases, as illustrations only or together with other forms of expression such as photography and written text. In this study, the field of surgery was limited to nasal surgery to demonstrate competence and the utility of illustrations, photography and written text. The visual and written expression forms were compared and the power to express the subject in terms of surgical training was investigated.

In the opinions stated according to their field of expertise, the ENT surgeons stated that the photographs alone were not sufficient because the frame boundaries could not be well distinguished, and that the use of illustrations together with photographs would be important in resident and student education. The need for photography and recording was considered important to be able to use the procedures performed during surgery for scientific purposes. An ENT Professor Doctor stated that the use of 3D visuals in presentations related to dynamic medical cases and the use of

medical illustrations in the presentations about static cases could provide a better qualified presentation or education / training and that the photographs alone could not be understood sufficiently even by experts in the field. Experts preferred illustrations to see the surgical cases in more visual detail. The students stated that the use of illustrations with photographs and written text together was more memorable, as there are cases in which the photographs are insufficient by themselves and combined use was more efficient in describing three dimensions. It was evident that the medical illustrations may be more sufficient in comparison to photography and written text but they could not provide the desired quality level alone. Therefore, it was concluded that illustrations alone are not sufficient to describe the surgical procedures and should be used together with other forms of expression. Of the 60 respondents from the ENT specialism area, 43 considered it more important to use illustrative, photographic and written expressions together for informational purposes. Title-related variables were not found in the participants' statements.

Aesthetics, Plastic and Reconstructive Surgery specialists showed similar results in their responses. In this area of expertise, although not variable, 9 of 11 participants found it important to use illustrative, photographic and written expressions together for information purposes. The responses of the non-specialist medical faculty students to the scales varied from those of the field specialists. Although it was seen that illustrations expressed the subject better than photographs and written text, an indecisive attitude was mostly taken. It is clear that neither illustrations nor photographic images are sufficient to demonstrate surgical procedures. While the majority of the respondents accepted the importance of illustrations in rhinoplasty education and scientific publications, photographic images were also accepted as important, but were not considered to be as effective as illustrations. The view that a high-quality education / training environment will be provided with the use of visual and written expression forms in rhinoplasty education has not changed. When the results of the analysis were examined, no difference was observed in the responses of the experts. However, indecisiveness and differences were observed in the responses of non-experts. Nevertheless, all participants stated that it is necessary to use illustrations, photographs and written text together in appropriate situations. Consequently, it can be seen that medical illustrations have been used as a necessity in the medical field since the beginning, that they developed parallel to the text and embodied the writing and give meaning to the photographic images. Therefore, it is important that standardized high-quality illustrations are produced by experts in the field. It is a common opinion that illustrations are strong in expression but not sufficient. This was reported in research findings at the University of Texas that when the time factor is kept static, individuals can remember 50% of what they see and hear, 30% of what they see and 10% of what they read (Kinder, 1973). Recent studies have shown the benefits of using visual tools (photographs, illustrations, 3D visuals etc.) in the learning process, specifically converting cognitive input into long-term memory, indicative of learning (Mota et al., 2018). Educational research based on visual learning has shown that this can significantly improve surgical performance. Visual-based training both significantly improves surgical education and can be seen as a reasonable additional tool to be included in surgical education curricula because it leads to improvement in surgical performance (Pape-Kohler et al., 2013). When medical publications are examined, it can be seen that visuals, diagrams, graphics and photographs are used together with written text. These commonly used visual materials contribute to visual perception due to the creation and reinforcement of a visual image. Visual perception is the most effective of the human perception channels. A phenomenon that can be described only by several pages of writing is more easily expressed by a single illustration (Mavroudis et al., 2020). However, the requirement for living tissue to be seen in many cases necessitates supporting the illustration with photographs.

From the findings of this study, it was concluded that illustrations alone are not sufficient and should be supported with photographs. With this reference point, it may be suggested to establish and expand the institutions and organizations serving in the field of clinical and medical photography with examples in various countries. The use of 3D models in the medical and surgical field can create a more interactive educational channel. Illustrations are generally used in crochet training publications. Creating a resource by visualizing new surgical cases can be considered both in terms of education and archives. Future studies should focus on better characterization of the link between the use of surgical visual elements and overall surgical skills. In addition, future studies should give priority to the integration of standardized assessment tools which facilitate the surgical performance and skill of students and residents.

In this study, it has been shown that visual-based education (photographs, illustrations, 3D visuals and the like) can provide substantial benefits in surgical education by reducing the significant barriers that students and residents face in practice. The findings of a systematic review provided fair to good quality studies, which demonstrated significant gains in knowledge compared with traditional teaching (Akgül et al., 2018). Findings of this research has revealed that illustrations are more effective than photographs, but the illustrations should be used together with other forms of visual and written expression. Such an expression is more effective and preferred in both educational and academic publications.

In conclusion, a quality visual-based surgical education method is effective according to the recent findings in the

literature. Visual elements should supplement the standard techniques such as text in surgical education.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Review Article

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Mesenchymal stem cell applications in polycystic ovary syndrome treatment

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Abstract

Mesenchymal stem cells (MSCs) are highly capable of self-renewal and differentiation. They can be isolated from a variety of sources such as adipose tissue, bone marrow, umbilical cord, tooth pulp and can be cultured under in vitro conditions. MSCs have anti-inflammatory, anti-apoptotic, angiogenic, immunomodulatory and many more therapeutic effects because of paracrine factors they secrete. Today, mesenchymal stem cells are used for treatment in more than twenty diseases, from spinal cord injuries to diabetes. However, there is little mention in the literature of the use of these cells in female reproductive system diseases. In this review, a limited number of clinical and experimental studies on the use of mesenchymal stem cells in the treatment of polycystic ovary syndrome, which is quite common in women, were examined and analyzed.

Keywords: BM-MSC, HUC-MSC, AMSC, PCOS

1. Introduction

Polycystic ovary syndrome (PCOS) is a multifactorial metabolic and endocrine disease and has a high incidence in women (Divyashree et al., 2019). Stein and Leventhal wanted to provide information about the morphology and clinical findings of the ovary and for the first time they used the term 'polycystic ovary syndrome' in 1935 (Stein, 1935). In PCOS disease, the growth of early antral follicles is mostly arresting at the early stage, and numerous follicular structures ranging in diameter from 2 to 8 mm appears (Franks et al., 2008). In addition to this, the main complications are abnormal folliculogenesis and decrease in oocyte number (Huang et al., 2013). PCOS affects approximately 5-20% of women of reproductive age, as well as those in the premenopausal period (Yildiz et al., 2012). It is also seen as one of the causes of infertility or failed births in recent years (Corbould 2008, Teede et al., 2010). Based on the diagnostic criteria, the prevalence of PCOS was determined to be approximately 4% - 6.6% according to the NIH 1990 criteria and about 4% -21% according to the Rotterdam 2003 criteria (Lizneva et al., 2016). Treatment method in females with PCOS is determined depending on symptoms such as infertility, menstrual disorders, androgen-induced ovulation dysfunction (Badawy and Elnashar, 2011).

Today, chemical agents are used in almost all PCOS treatments. The most commonly used chemicals in treatment are; clomiphene citrate (Legro et al., 2007), tamoxifen (Messinis and Nillius, 1982; Boostanfar et al., 2001), metformin (Sam and Dunaif, 2003) and various gonadotropin agonists (Artini et al., 1996). Although the therapeutic properties of these chemicals have been demonstrated clinically and experimentally, it has been noted that each of them produces different results in their single or combined use and thus have various advantages and disadvantages (Parsanezhad et al., 2002; Nardo, 2004; Hwu et al., 2005; Elnashar et al., 2006; Legro et al., 2007). In addition, there are also studies that use alternative treatment methods such as acupuncture, but acupuncture treatment has been found to be unsuccessful in women with PCOS cases with high testosterone and insulin levels (Stener-Victorin et al., 2000).

Mesenchymal stem cells (MSCs) were first found in the bone marrow and have been used to support bone healing for nearly 20 years (Hernigou et al., 1997) and later MSCs were detected in adipose tissue (Lin et al., 2011). According to its origins, bone marrow MSCs are called bone marrow stem cells (BMSCs), adipose tissue derived MSCs are called adipose stem cells (ASCs), and perivascular stem cells are called PSCs. In recent years, the effectiveness of MSCs continues to be investigated both experimentally and clinically. Minimum criteria have been set for the identification of mesenchymal stem cells. According to these criteria, MSCs should be cells that can attach plastic surfaces and differentiate into osteoblasts, chondroblasts and adipocytes. They should

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express CD73, CD90 and CD105 surface biomarkers at high levels. However, CD14, CD11b, CD34, CD45, CD19 or CD79a and human leukocyte antigen-D related surface molecules should be expressed at low levels or not at all (Dominici et al., 2006). The areas of use of mesenchymal stem cells obtained from different sources in allogenic or autogenic in vitro and in vivo treatments are increasing day by day. That is; Arthritis-foot fusion, bone fracture, bone tumors, cartilage defects, menisectomy, osteodysplasia, osteogenesis imperfecta, osteonecrosis, periodontitis, spine fusion, cardiomyopathy, heart failure, ischemic heart disease, myocardial infarction, extremity ischemia, kidney disease transplant, lupus nephritis, cirrhosis, familial hypercholesterolemia, chronic obstructive pulmonary disease, multiple system atrophy, neuroblastoma, spinal cord injury, multiple sclerosis, Parkinson disease, ALS, stroke, type 1 diabetes, type 2 diabetes, diabetic wounds, systemic sclerosis, epidermolysis bullosa and many other diseases, the effectiveness of mesenchymal stem cells obtained from various sources was investigated with different injection methods (Ankrum and Karp, 2010). Although it is less in number compared to all these diseases, there are ongoing studies on the use of mesenchymal stem cells in the treatment of reproductive system disorders. The effects of BMSCs, ASCs and stem cells isolated directly from endometrial tissue or menstrual blood on the female reproductive system are investigated (Ding et al., 2011; Mutlu et al., 2015; He et al., 2018). However, when the literature review examined, we see that the studies mostly focus on premature ovarian diseases, endometriosis, endometrial tissue damage, and there are quite a limited number of studies in the use of mesenchymal stem cells directly in PCOS treatment. In this review, current studies investigating the effects of mesenchymal stem cells in PCOS treatment were evaluated.

1.1. Treatment by injecting bone marrow-derived mesenchymal stem cells from the tail vein

In 2018, a study was published showing the therapeutic effect of bone marrow derived stem cells (BM-MSCs) in the PCOS mouse model. In this study, the PCOS model was established using testosterone enanthate in mice. Mice were divided into three groups as control, PCOS and PCOS + BM-MSCs. BM-MSCs are marked with the nucleus dye Hoechst33342. Following the formation of the PCOS model, injections of stem cells from the tail vein were performed on the 1st and 14th days. The animals were sacrificed two weeks after the last injection. As a result, a significant increase in total antral follicle number, oocyte volume and shingles pellucida thickness and a significant decrease in primary and preantral follicle groups were seen in the PCOS + BM-MSC group compared to the PCOS group (Table 1, 2, 3). In addition, there was a significant increase in FSH and TAC serum levels in the PCOS + BM-MSCs group compared to the PCOS group, while there was a significant decrease in testosterone, LH, MDA serum level and TUNEL positive apoptotic cell count. It has been suggested that BM-MSC transplantation improves folliculogenesis in mice with PCOS and may be an operative treatment for PCOS through its anti-inflammatory, antioxidant, antiapoptotic properties (Kalhori et al., 2018).

Table 1. Comparison of the mean total volume of ovary, cortex, and medulla (mm^3) in different Groups of mice post PCOS induction and treatment with BM-MSCs. (Values are means ±SD, One-way analysis of variance and Tukey test; P<0.05, (Kalhori et al., 2018)).

Groups	Volume of ovary	Volume of cortex	Volume of medulla
Control	2.07 ± 0.09	1.88± 0.10	0.19± 0.01
PCOS	1.58± 0.12	1.42± 0.10	0.16± 0.02
PCOS + BM-MSCs	1.85± 0.11	1.68± 0.11	0.17± 0.01

Table 2. Comparison of the mean Number of follicles in different stages of growth in different Groups of mice post PCOS induction and treatment with BM-MSCs. (Values are means ±SD, One-way analysis of variance and Tukey test; P<0.05, (Kalhori et al., 2018)).

Groups	Primordial follicles	Primary follicles	Preantral follicles	Antral Follicles
Control	1761.65± 74.57	524.23± 23.41	339.46± 22.78	130.44± 12.70
PCOS	1813.81± 75.08	658.70± 30.8	510.01± 32.57	75.49± 10.12
PCOS +BM-MSCs	1795.32± 50.43	584.91± 28.53	413.06± 21.79	103.61± 6.86

Table 3. Comparison of the mean thickness of zona pellucida (µm) in pre-antral and Antral follicles, in different Groups of mice post PCOS induction and treatment with BM-MSCs. (Values are means ±SD, One-way analysis of variance and Tukey test; P < 0.05, (Kalhori et al., 2018)).

Groups	Preantral follicles	Antral follicles
Control	12.14± 0.42	17.38± 0.60
PCOS	10.88± 0.32	15.75± 0.51
PCOS + BM-MSCs	11.94± 0.38	17.01± 0.42

1.2. Treatment by injection of mesenchymal stem cells of umbilical cord origin from the tail vein

Chronic inflammation is considered one of the causes of ovarian dysfunction. Increasing evidence in animal studies and preclinical studies have shown that MSCs have immunomodulatory effects by interacting with immune cells (Augello et al., 2005). In the study conducted by Xie et al. In 2019, in the PCOS model induced with dehydroepiandrosterone (DHEA) in mice, the human umbilical cord-derived mesenchymal stem cells (hUC MSC's) were applied by injection from the tail vein. And this practice has been shown to effectively improve pathological changes, including ovarian histopathology and function. As a result of their experiments, hUC reported that MSCs significantly reduce the expression of proinflammatory factors (TNF-a, IL-1β and IFN-y) (Fig. 1). Fibrosis-dependent genes (CTGF) in the ovarian and uterine tissues and affect the systemic inflammatory response (Fig. 2). In the spleen, neutrophils showed that the percentage of M1 macrophages, IFN γ + CD19 + B cell, IFN-γ + CD4 + T cells (Th1) and IL-17 + CD4 + T cells (Th17) decreased significantly in hUC-MSC-treated

mice. With these results, they suggested that hUC-MSC therapy can alleviate ovarian dysfunction by inhibiting local and systemic inflammatory responses (Xie et al., 2019).

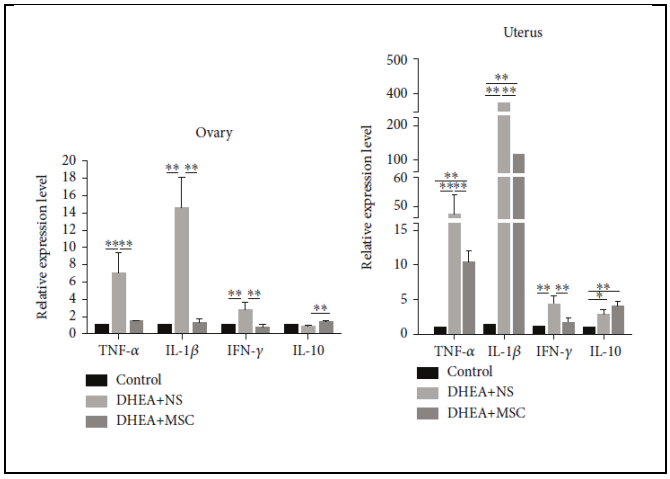

Fig. 1. hUC-MSC treatment alleviates ovarian and uterine local inflammatory response and tissue fibrosis in PCOS mice. Quantitative RT-PCR analysis of the expression of proinflammatory factors (TFN-α, IL-1β, and IFN-γ) and anti-inflammatory factor (IL-10) in the ovaries and uterus (Values are expressed as the means ± SEM. $n = 8$ per group. $*P < 0.05$ and $**P < 0.01$, (Xi et al., 2019)).

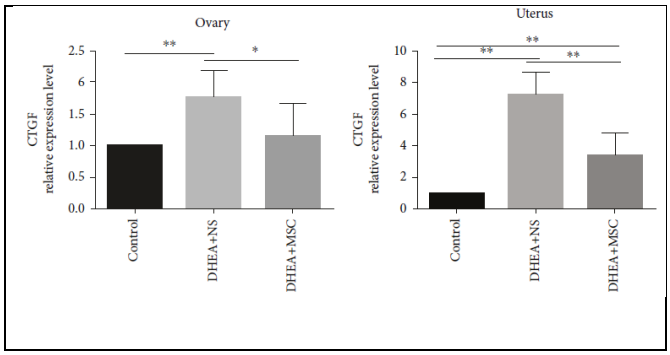

Fig. 2. Quantitative RT-PCR analysis of the expression of connective tissue growth factor (CTGF) in the ovaries and uterus. (Values are expressed as the means ± SEM. $n = 8$ per group, $*P < 0.05$ and $**P < 0.01$, (Xie et al., 2019)).

1.3. The use of mesenchymal stem cells in enrichment of IVM culture medium

Women with PCOS are at high risk of ovarian hyperstimulation syndrome (OHSS) when they receive infertility treatment (Shalom-Paz *et al.* 2012). Therefore, immature oocytes from small antral follicles are collected to prevent OHSS, and their *in vitro* maturation (IVM) is then achieved (Lim *et al.* 2013). However, since the maturation and fertilization rate of oocytes maturing in vitro is not sufficient, the clinical applications of IVM face limitations. To eliminate this, studies examining the effects of cultural media containing different additions are performed (Jee et al., 2008; Demyda and Genero, 2011; Ishizuka et al., 2013; Ellenbogen et al., 2014; Sánchez et al., 2015). MSCs secrete various cytokines and growth factors such as, insulin-like growth factor - 1 (IGF - 1), VEGF, EGF, fibroblast growth factor (FGF), interleukin - 6, leukemia inhibitory factor (LIF), TGF – β (Yoon et al., 2010). EGF and IGF - 1 are thought to play important roles in improving meiotic maturation directly or through cumulus cells (Ling et al., 2008). In addition, female bone marrow MSCs have been shown to differentiate to steroidogenic cells in a culture medium supplemented with high glucose, thereby increasing the potential for estrogen secretion (Li et al., 2015). Thus, in 2018, Jafarzadeh et al. used the medium (hBM - MSC - CM) in which human bone marrow-derived stem cells were grown to improve the IVM culture medium in vitro maturation of oocytes collected from polycystic ovary syndrome mice. In this study, oocytes at germinal vesicle (GV) and metaphase II (MII) stages were collected from dehydroepiandrosterone-induced PCOS mice. GVs were randomly divided into four groups. Classical IVM media (TCM199) used as control group. The dose groups are 25%, 50% and 75% supplemented with TCM199 ((PCOS-CM25E, PCOS-CM50, CM75-PCOS) IVM mediums used, and 24 hours incubated. The results suggest that supplementing the IVM medium with 50% hBM - MSC - CM increases cytoplasmic and nuclear maturation of GVs ($P < 0.001$), as well as fertilization, two cell stage ($P < 0.001$) and blastocyst ($P < 0.001$) formation rate (Fig. 3). In general, considering the result in the PCOS - CM50 group with higher oocyte maturation and fertilization, it has been suggested that enrichment of the IVM medium with hBM - MSC - CM can be considered as a promising approach in improving the IVM of PCOS oocytes (Jafarzadeh et al., 2018).

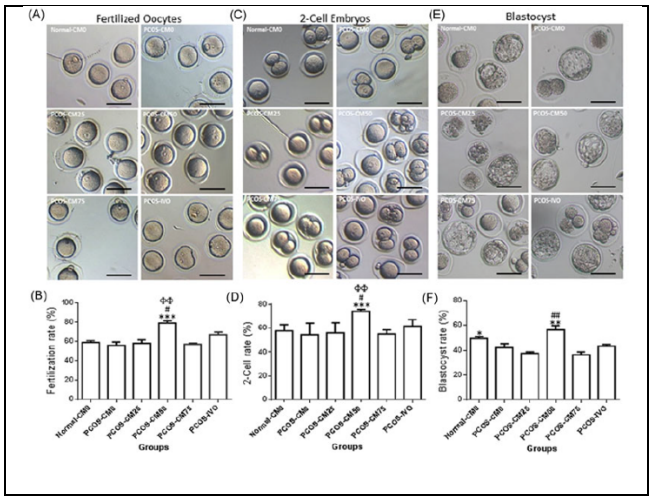

Fig. 3. Fertilization and early embryo development of resultant matured oocyte, supplemented by MSC-CM after IVF. Representative phase-contrast micrographs (A, C, E) and quantitative data for each stage of preimplantation embryo development (B, D, F) are presented. The graphs show that supplementation of IVM medium by 50% of MSC-CM significantly improves the fertilization outcome of PCOS oocytes. These improvements in the PCOS-CM50 group have made the fertilization, two-cell, and blastulation rates to be significantly superior to those of the PCOS-CM0 and PCOS-IVO groups. Data are presented as mean ± SEM. *, Φ, and # are statistically significant compared with the PCOS-CM0, normal-CM0, and PCOS-IVO groups, respectively (* and # = $P < 0.05$, **, ΦΦ, and ## = $P < 0.01$, and *** = $P < 0.001$). CM, conditioned media; IVF, in vitro fertilization; MSC, mesenchymal stromal cell; PCOS, polycystic ovary syndrome; SEM, standard error of mean. (Jafarzadeh et al., 2018).

1.4. The use of exosomes of stem cells originating from adipose in treatment

Exosomes are special nano-sized endocytic vesicles secreted by many cell types (Zomer et al., 2010). Exosomes are important mediators in intercellular communication that enable the transfer of functional miRNAs and proteins (Zhang et al., 2015). Mesenchymal stem cells (AMSCs) derived from adipose tissue can produce many exosomes. This suggests that AMSCs can be used as an agent to transfer miRNAs in exosome-mediated cell interactions (Yeo et al., 2013). AMSCs can communicate with brain parenchymal cells and provide miR-133b transfer via exosomes to regulate neurite growth (Xin et al., 2012). Exosomes produced by AMSCs modified with MiR-181-5P suppress hepatic fibrosis in hepatic stellate cells (Qu et al., 2017). In addition, exosomes derived from AMSCs designed with miR-122 have been reported to cause greater sensitivity to chemotherapeutic treatment in hepatocellular carcinoma cells (Lou et al., 2015). However, the therapeutic approach through exosomes has rarely been reported in PCOS. Recently, miR-323-3p has been reported to play a role in the regulation of steroidogenesis and apoptosis in the cumulus cells (CC) of women with PCOS (Wang et al., 2019). In 2019, Zhao et al conducted a study to determine the effects of exosomal miR-323-3p on cumulus cells (CC) of PCOS patients in the letrazol-induced mouse PCOS model. Exosomal miR-323-3p was isolated from modified AMSCs and collected. Real-time PCR, western blot, MTT, flow cytometry and luciferase analyze were performed to identify exosomal miR-323-3p mechanisms in CCs in PCOS mice. The results showed that miR-323-3p expression was upregulated in AMSCs, exosomes and CCs. The upregulated miR-323-3p promotes cell proliferation in CCs and suppresses apoptosis, while the miR-323-3p inhibitor plays opposite roles in exosome-treated CCs (Fig. 4). They reported that the upregulation of miR-323-3p suppresses the apoptosis of CCs and alleviates PCOS by targeting PDCD4 (Programmed cell death protein 4) (Fig. 5) (Zhao et al*.,* 2019).

With this study, it has been revealed that mesenchymal stem cell exosomes may contribute to the development of new therapeutic strategies as well as it can be understood that their paracrine effects can be benefited by direct injection of mesenchymal stem cells in the treatment of PCOS.

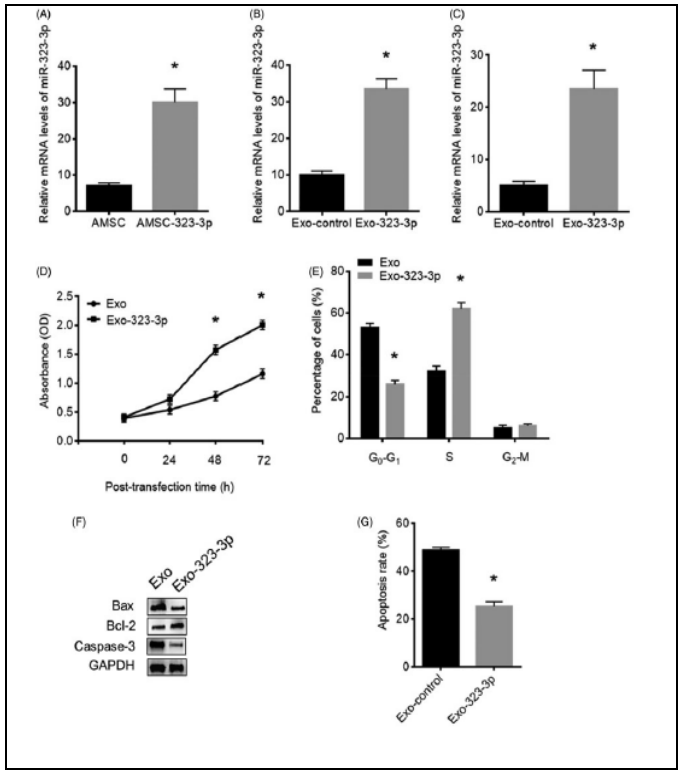

Fig. 4. Upregulation of miR-323-3p promoted cell growth and inhibited apoptosis in cumulus cells (CCs). Detection of miR-323-3p expression in (A) human adipose tissue-derived mesenchymal stem cells (AMSCs), (B) AMSC exosome and (C) CCs. (D) Cell proliferation. (E) Cell cycle distribution. (F) Bax Bcl-2, and caspase-3 protein expression in CCs transfected miR-323-3p-modified exosomes. (G) Apoptosis level in CCs transfected miR-323-3p-modified exosomes. (*) Denotes differences from the control group ($p<.05$). Values are means ± SEM. For each experiment, at least four samples were available for the analysis. (Zhao et al., 2019).

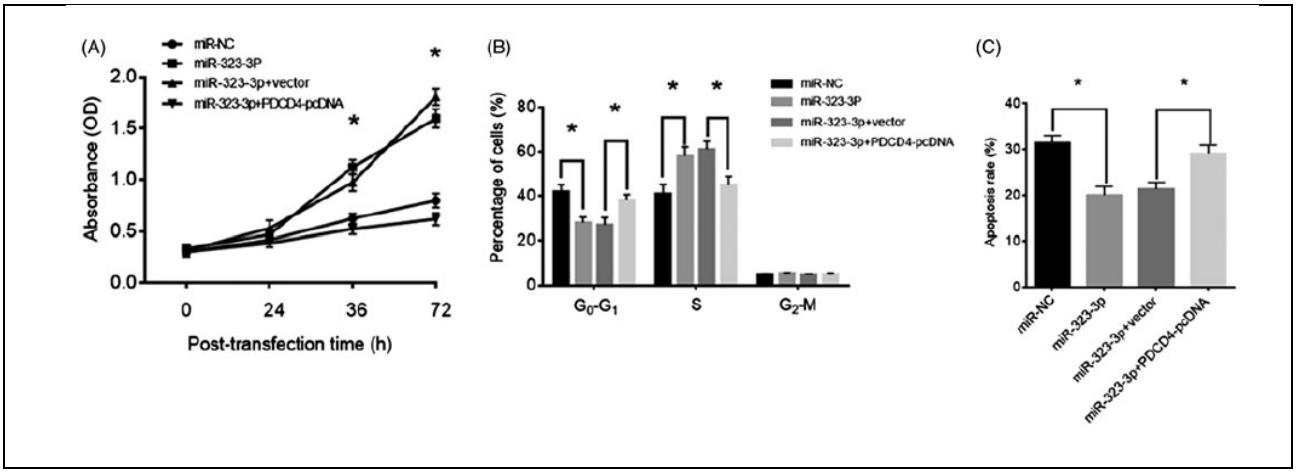

Fig. 5. MiR-323-3p promoted cell growth and inhibited apoptosis in cumulus cells (CCs) by targeting PDCD4. CCs were transfected with miR-323-3p and miR- 323-3p+PDCD4-pcDNA. (A) Cell proliferation. (B) Cell cycle distribution. (C) Apoptosis level. (*) Denotes differences from the control group ($p<.05$). Values are means ± SEM. For each experiment, at least 4 samples were available for the analysis. (Zhao et al*.,* 2019).

2. Discussion

PCOS is a multi-factor reproductive and endocrine system disorder. However, positive results can be obtained in the long term with correct diagnosis and treatment guidelines. In recent years, mesenchymal stem cells are the focus of restorative and regenerative medicine. In many different disease types, the use of mesenchymal stem cells isolated from different sources has positive results. However, a limited number of studies have been conducted in female reproductive system disorders, especially in the treatment of PCOS. This creates a reliability problem in the use of mesenchymal stem cells in the treatment of PCOS. For this reason, experimental and clinical studies on the use of MSCs in the treatment of PCOS should be increased. Thus, according to changing diagnostic criteria, the use of MSCs with the most accurate injection technique and doses has the potential to be a promising new approach in the treatment of PCOS.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Thromboembolic complications in Takotsubo Syndrome during Covid-19 outbreak. A review with case report

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Abstract

New coronavirus (COVID-19) outbreak, which started in Wuhan, China and spread rapidly all over the world, has become the most important cause of the emotional distress for susceptible people. Because the people also remained in fear and anxiety during the period when the elderly population in the whole country was desperate due to the COVID-19 outbreak and was asked to stay at home in fear of death. In addition, the fact that the patients were hospitalized together with COVID-19 patients caused his psychological distress to be experienced even more. Takotsubo Syndrome (TTS) is known as a distress-induced transient left ventricular dysfunction. In our patient with TTS, it was found that a new apical thrombus, which was not seen in the left ventricle, was seen in previous echo and computerized tomographic investigation, and that it was a source of thromboemboli, by stress triggering neurocardiac reactions. While under the medical treatment of viral pneumonia, under the Coronavirus transmission measures, thrombus in the LV was removed by being taken to the emergency open heart surgery. Our case is the first patient in the literature where a new thrombus developed in the LV and splenic infarction in TTS triggered by the pandemic. In this article; We have presented the experience of TTS triggered by pandemic, etiopathogenesis of left ventricular thrombus, open heart surgery indications when COVID-19 is suspected, coronavirus transmission measures in the surgical approach, the postoperative treatment and literature is reviewed.

Keywords: splenic artery occlusion, takotsubo syndrome, left ventricular motile giant thrombus, thromboembolism

1. Introduction

Takotsubo syndrome (TTS) also known as Takotsubo cardiomyopathy or distress cardiomyopathy due to distress was first described in a series of 5 Japanese patients in 1991. Both emotional and physical stimuli that activate the central nervous system can trigger the disastrous response of the sympathetic nervous system and eventually lead to catecholamine-mediated damage to the final organ. However, most of the data support a widely accepted theory that the clinical manifestation of TTS is secondary to the plasma excess of catecholamines associated with increased release of adrenomedullary epinephrine and increased norepinephrine triggered by primary psychological or physical distress with a high sympathetic tone (Templin et al., 2015; Wybraniec et al., 2014).

Novel Coronavirus infection disease (COVID-19) continues its fatal spread globally, demonstrating that it significantly affects the mental health and well-being of individuals. Clinical experience so far shows that there is a relationship between the distress in the COVID-19 pandemic and the involvement and treatment of various diseases (Konstantinos et al., 2020).

Approximately 5.3% of TTS cases are complicated by left ventricular thrombosis (Wybraniec et al., 2014; Kurisu et al., 2011). Recent reports have linked major emotional stress caused by COVID-19 to distress-induced / TTS, which occurs as acute heart failure (Parodi et al, 2007). As the number of COVID-19 cases increases worldwide, it is predicted that there will be an increase in the number of associated cardiovascular complications. Clinicians should be aware of the diversity of cardiovascular complications and develop appropriate strategies for diagnosis and treatment (Minhas et al., 2020). In fact, some researchers have reported that thromboembolic complications occurred in 20-33.3% of TTS cases (de Gregorio et al., 2008; Haghi et al., 2008; Suzuki et al., 2013).

The infarction of the spleen (SI) is caused by thromboembolism or direct thrombotic occlusion of the spleen artery. The spleen has a rich vascular source and takes about 5% of the cardiac output. However, SI is a rare event. In the retrospective analysis of many academic hospitals, only 32 (0.016%) of the patients admitted within 10 years were identified (Schattner et al., 2015).

In this presentation, emergency open cardiac surgery as a suspicion of COVID-19 in our patient with TTS, a recently

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developed mobile thrombus causing splenic artery occlusion in the left ventricle (LV), will be discussed. Due to this phenomenon, we will review the symptomatology, etiopathogenesis and treatment methods of diseases in the light of the literature, and we will present our precautions and experiences in the operating room and cardiovascular surgery intensive care unit.

2. Case report

2.1. The Medical history of a 65-year-old female patient before COVID-19 outbreaks

About 12 years ago, after his 17-year-old son died in a traffic accident, he suffered severe depression, received psychological support to deal with his troubles, and regularly received 60 mg of sertraline (Misol), 1x30 mg of mirtazapine (Zestat), and duloxetine (Duxet) for the past 6 years was learned to use. There were no risk factors such as smoking, alcohol, hyperlipidemia, diabetes mellitus, hypertension or obesity in the background, and there was no family history of ischemic heart disease. Coronary angiography performed 12 years ago when she presented with chest pain while under psychological distress revealed that significant stenosis in the left anterior descending artery (LAD) and treated with percutaneous coronary intervention Echocardiography showed apical akinesia in the LV.

It was triggered by the neurocardiac response of TTS in our patient following her sister's death due to breast cancer in a very short time after diagnosis in 2017. This syndrome was detected when it was brought to our cardiovascular surgery clinic for the first time due to leg pain due to thromboembolic occlusion in the subacute phase in his tibial artery. After being admitted to the hospital, i.v. heparin and oral anticoagulant coumadin were given. Cardiac echocardiography was performed to investigate the source of the embolism, but the embolism outlet was not found. Anticoagulation was continued with the novel oral anticoagulant (NOAC) apixaban 2x5 mg, since effective international normalized ratio (INR) could not be achieved for approximately 6 months.

After a detailed examination at the clinic where she went for breast cancer screening a year ago, the patient's chest pain developed, and the level of troponin increased moderately in the blood analysis. On the coronary angiography, it was reported that there was no stenosis in the coronary arteries and thrombus in the apical region, except for the apical enlargement of the LV.

2.2. The medical history of the last application during the COVID-19 outbreak period

On 13 March 2020, the patient applied to the Samsun Training and Research Hospital emergency department with complaints of headache, fever, shortness of breath, cough, weakness and pain on left upper quadrant of the abdomen. In the thorax Computerized Tomography (CT) performed here, viral pneumonia infiltration was observed in the reticular and ground glass pattern in the lower and upper lobe segments of the bilateral lungs (Fig. 1). However, there was not any thrombotic event in left ventricular cavity and spleen in the thoracic and abdominal CT's (Fig. 2).

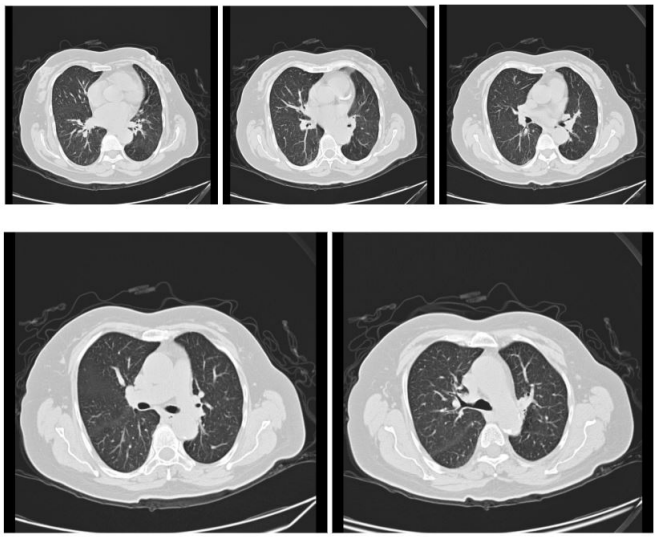

Fig. 1. Virus pneumonia image showing bilateral ground glass appearance in the lungs on chest CT taken in hospitalization of the patient

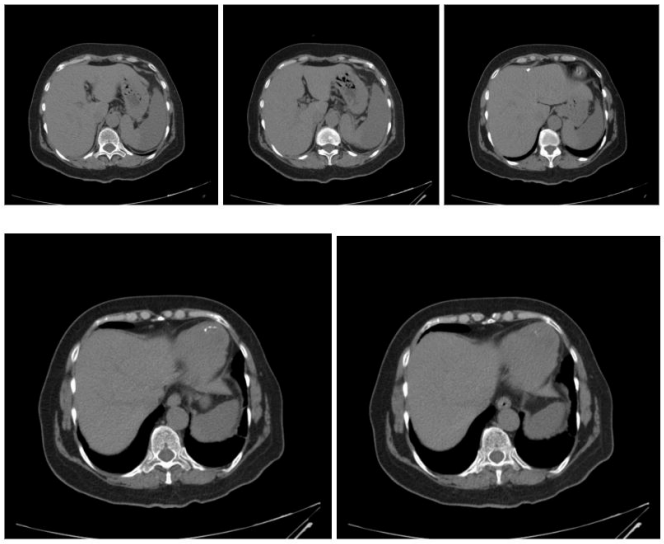

Fig. 2. Normally monitored spleen image in the abdominal CT taken during hospitalization of the patient

Hydroxychloroquine 600 mg / day and Azithromycin 3x250 mg were started with the pre-diagnosis of COVID-19. Samples from the throat and nasal swabs for COVID-19 polymerase chain reaction (PCR) Nucleotide test at two-day intervals had two negative results. Thoracal CT and Echocardiography performed to investigate the thrombus source showed a large thrombus moving in the LV (Fig. 3). In the abdominal CT performed for the cause of the patient's new severe abdominal pain, splenic infarction, occlusion in the splenic artery and thrombosis in the splenic vein were observed (Fig. 4). Bedside transesophageal echo (TTE) showed a large mobile thrombus starting and extending from the akinetic apical wall in addition to normal contraction in the middle and basal segments of the LV (Fig. 5) in different angles of TTE.

In echocardiographic update evaluation, EF measured 24% in measurement with Simpson method, and left ventricular thrombus sizes grew compared to the previous one. Pulmonary pressure was measured at 25 mm Hg. Electrocardiogram (ECG) with ST elevation and anterior T-wave inversions between V3 and V5 revealed frequent premature ventricular contractions and anterior wall infarct in sinus rhythm. Emergency surgery was decided after applying coronary angiography (Fig. 6). An abnormal value was not detected when performing thrombophilia investigation. Troponin T levels were normal.

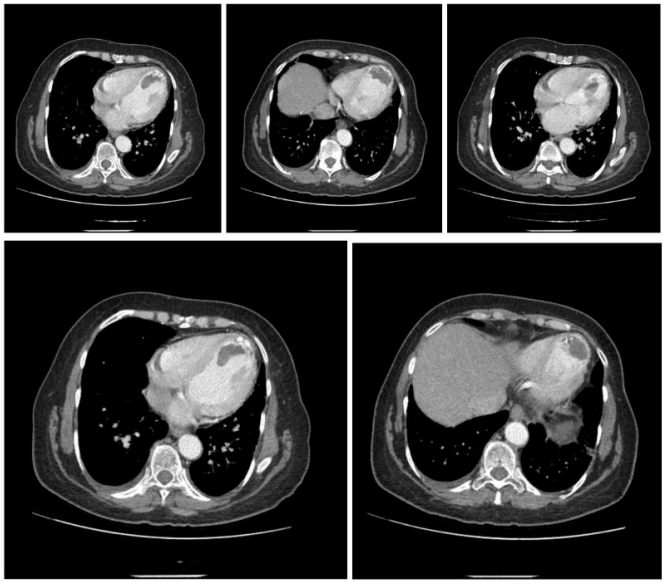

Fig. 3. Chest CT taken on the 10th day of hospitalization; a view of the newly developed thrombus localized to the left ventricular apex

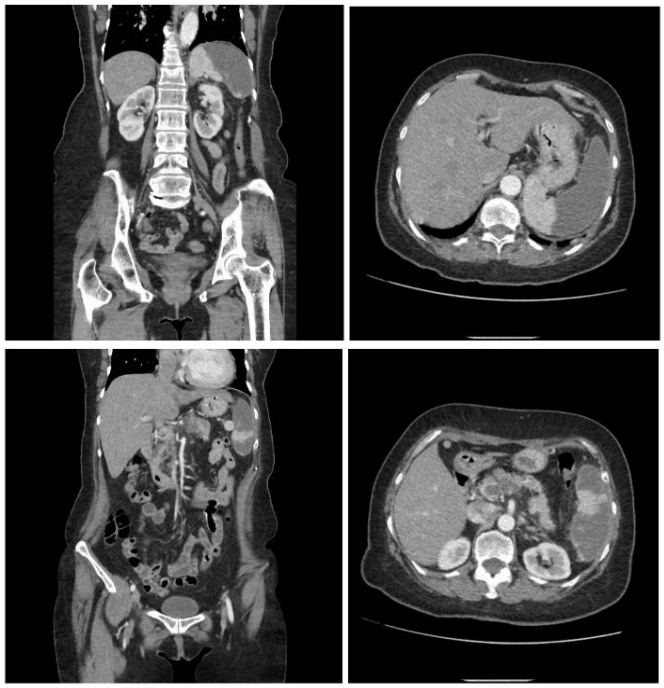

Fig. 4. Splenic infarct images on abdominal CT taken on the 10th day of hospitalization

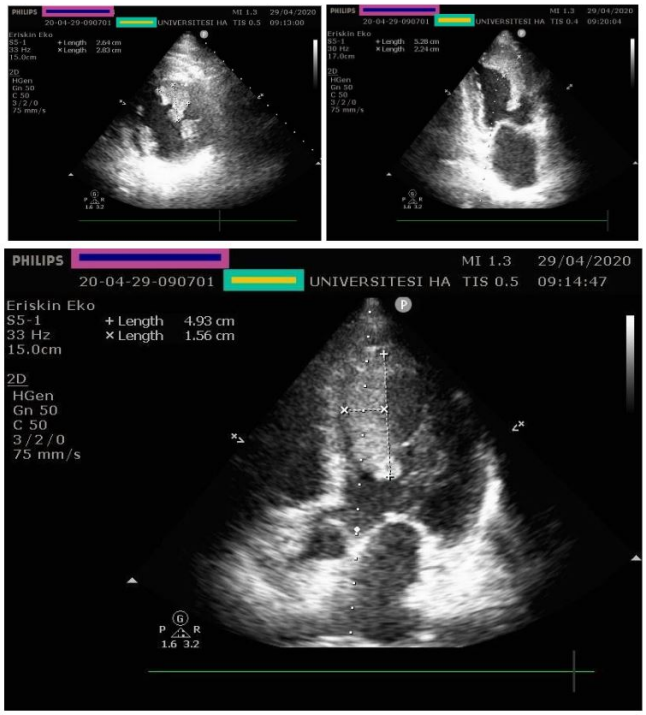

Fig. 5. Preoperative TTE images of the newly developing mobile large-sized thrombus localized in the left ventricular apex

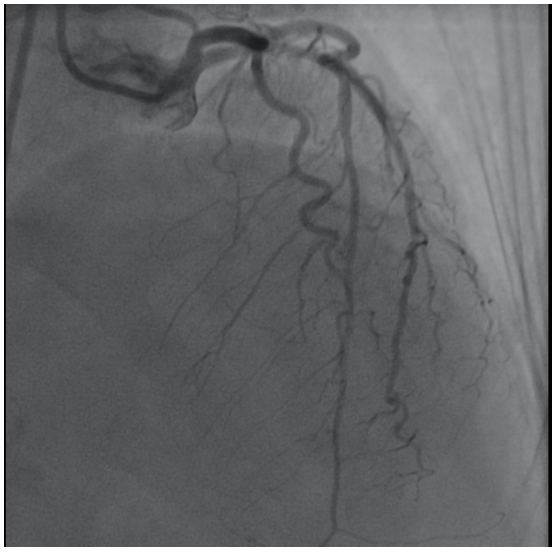

Fig. 6. Preoperative coronary angiogram, left anterior descending artery is open

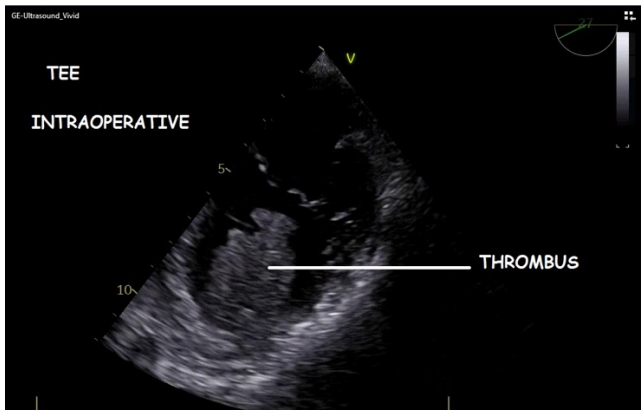

Fig. 7. Preoperative TEE images of newly developing mobile large-sized thrombus localized in the left ventricular apex

Fig. 8. Transparent cage used as an infection measure in anesthesia

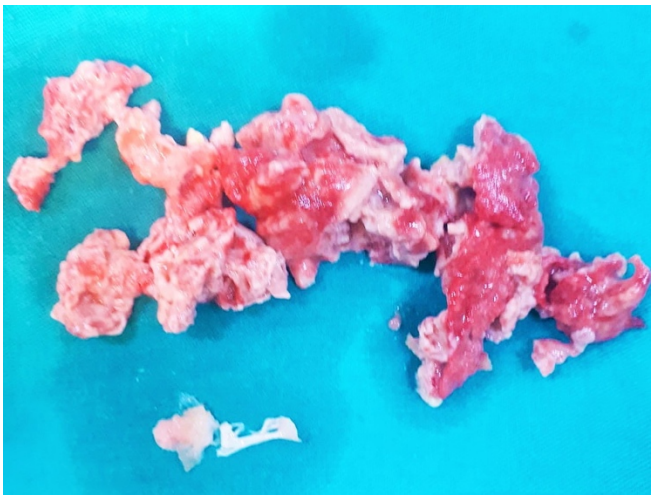

Fig. 9. Thrombus material extracted from the LV

In the context of coronavirus contamination measures, the patient's head was intubated into the lantern and ND95 masking to prevent airway conduction in the isolated operating theater (Fig. 7). Preoperative TEE images of newly developing mobile large-sized thrombus localized in the left ventricular apex (Fig. 8) Cannulation was achieved with a full dose of heparinization. After diastolic arrest was achieved with cold blood cardioplegia given to the aortic root, the left ventricular apex was suspended and a vertical incision was made through the avascular area. The region where the thrombus originated was investigated. The new fresh thrombus between the left ventricular apical trabeculae and the prolonged mass of the thrombus at the sub-acute stage was completely removed (Fig. 9). The presence of residual thrombus was investigated by rigorous observation of the left ventricular cavity with a fiber optic endoscope. Left ventricular air was removed after reduction was applied to the apical dyskinesia area. When the cross clamp was removed, the heart worked spontaneously again in the sinus rhythm. In patients with low left ventricular ejection fraction (LVEF), medication causing catecholamine discharge was avoided and milrinone was used as positive inotropic. While intubated, the patient was taken to the cardiovascular surgery intensive care unit. Supraventricular tachycardia and atrial fibrillation developed at the 6th hour postoperatively returned to sinus rhythm with Cordarone. In the pathological examination, it was reported that the thrombus was in the subacute period. Control echocardiography performed one week later showed normalization of left ventricular dysfunction in the apical segment, and left ventricle ejection fraction (LVEF) increased to 38%, except for hypokinesia. There was no detectable finding of left ventricular thrombus. On the 14th postoperative day, metoprolol succinate, amiodarone hydrochloride, rivaroxaban, and triple antidepressant (Sertraline, mirtazapine and duloxetine) were discharged. It was learned in the first control examination that there were no complaints.

3. Discussion

The COVID-19 epidemic, which started in mid-March in our country, caused intense emotional stressful days with television programs that continued with the fear of not leaving home and showing death to 65 and older. When our patient was hospitalized with a pre-diagnosis of COVID-19, there was a deep anxiety and fear of death. In this period, we think that the root cause of the thromboemboli and the left ventricular thrombosis was provoked coagulation and complication of TTS due to the excessive emotional stress in COVID-19 process. In addition, it is possible that thrombosis in the spleen vein is caused by the thrombophilia phenomenon caused by arterial occlusion secondary to the venous stasis and the excessive stress. When the root cause of coagulation predisposition is investigated, the idea that TTS, COVID-19 and both will be effective is increasingly supported.

In this case, we will try to discuss the specific features of coagulopathy, which is the cause of intracardiac thrombus and splenic embolism, and its relationship with each other.

3.1. Symptoms and signs

Since the symptoms of our patient developed during the COVID-19 outbreak period, the etiology was investigated. The fact that the symptoms did not exactly match those of the classical COVID-19 did not rule out the disease. Indeed, patients with positive PCR tests but with asymptomatic or mild findings have been frequently encountered. For this reason, before the result of swab tests, the diagnosis of COVID-19 was made with thorax CT and the delay in the treatment was prevented in our country. Thus, mortality was reduced to lower levels in patients diagnosed with COVID-19. Despite the fact that viral pneumonia and related changes in the thorax CT of our patient were very similar to COVID-19, although the cardiac findings were similar to the findings in the literature, definitive diagnosis could not be made due to negative PCR tests.

In the COVID-19 outbreaks, it was reported that about 19% of patients hospitalized from many hospital records showed cardiac involvement. Although a recent study reported (Shi et al., 2020) that 12% of patients had COVID-19 associated acute cardiac injury, manifesting as an LVEF decline and all ECGs

were abnormal, with findings compatible with myocardial ischemia, such T-wave depression and inversion, ST-segment depression, and Q waves. Fatal complications in patients were often explained with respiratory and cardiac involvements. The most common ECG finding in TTS is ST segment elevation, typically in the precordial leads (Suzuki et al., 2013; Icli et al., 2016), but ECGs can have normal findings or show T-wave abnormalities or Q waves (Peters et al., 2015). Our patient had an abnormal ECG finding in mention case report above.

3.2. Hyper activation of the coagulation with the stress

Emotional and physical stressors associated with TTS are very much eg. unexpected death of relative or friend, domestic abuse, confrontational argument, catastrophic medical diagnosis, devastating business, armed robbery, gambling losses, surprise party, surprise reunion, car accident, fear of procedure, fear of choking, court appearance, public performance, exacerbated systemic disorders, invasive procedures, cardiac events etc. The high prevalence of emotional triggers in TTS suggests that some individuals are particularly vulnerable to experiencing psychological distress that may result in this syndrome. The prevalence of depression and anxiety disorders in TTS ranges from 21–60 % (Konstantinos et al., 2020; Jeon et al., 2018; Ghadri et al., 2018; Dawson, 2018; Smeijers 2016; Tsamakis et al., 2020). The huge emotional distress at the population level and respiratory infections caused by COVID-19 may represent potential triggers in this context. Myocardial injury, frequently reported in patients with COVID-19, is usually attributed to sepsis and/or hypoxemia and/or underlying coronary artery disease, as well as myocarditis. We are also considered this thought, which TTS can also play an important role in the COVID-19 pandemic (Minhas et al., 2020; Meyer et al., 2020; Moderato et al., 2020).

In any biological disaster fear, uncertainty and stigma prevail and they can act as barriers to proper psychiatric care (Xiang et al., 2020). The distress and uncertainty caused by the lack of endpoint for the COVID-19 pandemic, while treatment is still not in sight, has a significant psychological impact on individuals. Furthermore, the lengthy, massive-scale, unprecedented social distancing and isolation that we are currently undergoing has additive implications; quarantine has been associated with negative psychological effects including emotional disturbance, depression, distress, low mood, irritability, panic attacks, phobic symptoms, insomnia, anger, emotional exhaustion and post-traumatic distress symptoms and in extreme cases, e.g suicide (Brooks et al., 2020). Patients are distressed and reluctant to be transferred to a COVID-19 hospital during the pandemic, which explains the potential delays in treatment for other medical problems (Chadha, 2020). In our case it was most likely a provocative cause the stress concerning ongoing COVID-19 outbreak and her health.

High concentration of norepinephrine in distress cardiomyopathy was undeniably proven in numerous reports (Kurisu et al, 2004; Akashi et al., 2004). Wittstein et al., 2005 documented that concentration of norepinephrine was 3-fold higher in TTS than in acute myocardial infarction (AMI). Furthermore, prolonged stimulation of β1-AR and β2-AR is thought to induce apoptosis of cardiomyocytes (Ueyama et al., 2002; Lyon et al., 2008). In cases with TTS which leads to reversible LV dysfunction following intense physical or emotional distress, rarely formation of a LV thrombus can be seen. In these cases, thrombus has been generally observed during the periods of LV systolic dysfunction (de Gregorio et al., 2008; Prasad et al., 2008; Wiyono et al., 2010). Sasaki et al., 2004 reported formation of LV thrombus with resultant renal artery embolization after normalization of LV function in a patient followed up with the diagnosis of TTS (Kahya Eren et al., 2013; Santoroet al., 2017; Santoro et al., 2016; Vasilieva et al., 2011; Martin, 2010).

Finally, a hypercoagulable state induced by a catecholamine surge may also be present (Santoro et al., 2014). It has been recently shown that patients with TTS show significantly greater values of whole blood viscosity, von Willebrand factor, and lower erythrocyte deformability (Cecchi et al., 2013). von Willebrand factor was 10 times of normal in our patient also. In a study Lodigiani et al., 2020 thromboembolic complications were found 16% of 388 patients in the intensive care patients during the COVID-19 outbreak. Despite All of them used thromboprophylaxis, thromboembolic events corresponded to a cumulative rate of 21%. Half of thromboembolic events were diagnosed within 24 hours of hospitalization. When VTE imaging test was performed in forty-four patients, 16 (36%) VTE was confirmed.

SARS-CoV-2 may damage vascular integrity and cause the myocardial abnormalities observed in TTS. Some authors have reported cases of TTS associated with CMV infection: the virus may mediate coronary endothelial dysfunction with consequent increased expression of endothelial adhesion molecules and trans endothelial migration within the vasculature. SARS-CoV-2 may exert a direct toxic action on myocytes; indeed, the SARS-CoV-2 spike protein shows high-affinity binding to angiotensin-converting enzyme 2 (ACE2), a human cell receptor which is highly expressed in the heart (Roca et al., 2020). Cardiac CT and Cardiac Magnetic Resonance (CMR) should be used for differentiating between distress cardiomyopathy and frequently suspected myocarditis, since TTS is characterized by the absence of late enhancement on gadolinium CMR (Wybraniec et al., 2014; Hombach et al., 2008).

In our case, greater myocardial reactivity to sympathetic stimulation (caused by COVID-19 or TTS) in the apical region combined with abnormal vascular reactivity was thought to cause the left ventricular dysfunction seen in TTS. Therefore, SARS CoV-2 should be considered in patients presenting with TTS to further investigate the role of viral infections in the

pathophysiological mechanisms responsible for this syndrome. As patients with cardiac complications during COVID-19 have a higher mortality rate, we believe that early diagnosis of this disease is important in order to improve patient outcome. In addition, patients infected with TTS and / or COVID-19 appears to be at an increased risk for venous thromboembolism (VTE). There are reports of abnormal coagulation parameters in hospitalized patients with COVID-19 disease (Akar et al., 2020; Tang et al., 2020; Fan et al., 2020). Vascular inflammation may also contribute to the hyper coagulation state and endothelial dysfunction in these patients. Thromboembolic disease should be considered in patients with COVID-19 who have clinically worsening clinical disease, as evidenced by hypoxia or hemodynamic instability (Akashi et al., 2004).

Many conditions have been associated with predisposition to ventricular thrombi. The commonest causes are post myocardial infarct (mural thrombus)1 and dilated cardiomyopathies. Less frequently myocarditis and hypercoagulable states such as protein S and C deficiency and antiphospholipid syndrome have been implicated. Ventricular thrombi have also been noted in muscular dystrophies, Behcet's disease, HIV cardiomyopathy, non-compaction cardiomyopathy and blunt chest trauma. It is rare for a patient to present with a ventricular thrombus in the absence of any underlying disease (Tilling et al., 2007). In our patient, he was using anticoagulant apixaban for prophylactic purposes as well as antidepressant, but it is thought that the development of thrombosis is associated with stress coagulopathy seen in TTS, even though he has never stopped.

We present that may be the first splenic infarct case caused by excessive stress or by itself of COVID-19 process in a TTS patient, in the literature. Genetic predisposition to thrombophilia was investigated because of the arterial occlusion and venous thromboembolism we detected earlier in our patient. There was no evidence to support thrombophilia in methylenetetrahydrofolate reductase (MTHFR), Prothrombin G20210A mutation, Protein C, Protein S and Antithrombin III tests. This also shows that stress induced provocated coagulation is more important than known genetic factors in the thrombosis of the patient with TTS or COVID-19.

3.3. Management of the left ventricular thrombosis

Apical thrombosis complicating TTS was first described in 2003. The majority of the cases had been found female (92%) and above 60 years of age (61%). TTS may be complicated by the formation of left ventricular (LV) thrombus in 1.3-5.3% of patients (Herath et al., 2017). We observed the development of apical akinesia and thromboembolic events in our patient who has been closely monitored for 12 years, as Echocardiographic in routine controls, but intracardiac thrombus was not observed. Our patient had a giant LV thrombus, and his future potential risk for another systemic embolization was thought to be high. Therefore, we performed surgical extirpation of the left ventricular thrombus, because it had morphologic features suggesting a high risk of thromboembolism. Suzuki et al, 2013 recommended surgical management to prevent thromboembolism of mobile and pedunculated left ventricular thrombus related to TTS. However, the area of myocardial infarction does not change the location where the thrombi generally occur. However, the wall abnormalities associated with TTS are usually resolved within a few weeks. Upon normalization of the wall motion, if the left ventricular thrombus remains, the thromboembolic risk rate is being high. We see that the LVEF is improved from %24 to %35 in ECHO in 15th days after operation. Meurin et al., 2005 reported that 73 % of thrombi dissolved with low molecular weight heparin. Niedeggen et al., 2008 reported complete resolution with argatroban. In 57.1% patient's successful lysis was described by Sari et al., 2008 There are reported systemic embolic and high hemorrhagic events after treatment with fibrinolytics (Mallory et al., 1999). Lee et al., 2013 reported the difference between outcomes after anticoagulation, surgical removal, and antiplatelet agents. They could not find a statistically significant difference in event-free survival rate. Mano et al., 2016 demonstrated that left ventricular thrombus can be resolved with apixaban treatment. Apixaban may be an effective alternative to vitamin K antagonist for some patients with acute myocardial infarction complicated by left ventricular thrombus. Bolcal et al, 2019 used Robotic surgery in the surgical treatment of non-pedunculated apical LV thrombus in selected patients.

de Gregorio et al., 2008 reported intracavitary thrombus in 2.5% of the patients with TTS, and stated that 33% of these patients may have thromboembolic complications. However, thromboembolic events may occur even in patients receiving anticoagulant treatment. Our patient was using apixaban 2x5 mg /day for 4 years. Surgical thrombectomy has drawbacks, such as decreasing the LVEF in the early post-surgical period, and the increased risks of anesthesia and operational distress for patients with TTS (Jensen et al., 2006; Niino et al., 2015).

Surgical application is performed atrial or apical ventricular. Since we planned to reduce the akinetic field, intervention from the apical region was preferred. High concentration of norepinephrine in distress cardiomyopathy was undeniably proven in numerous reports (Kurisu et al., 2004; Akashi et al., 2004). Wittstein et al., 2005 documented that concentration of norepinephrine was 3-fold higher in TTS than in AMI. Furthermore, prolonged stimulation of β1-AR and β2-AR is thought to induce apoptosis of cardiomyocytes (Ueyama et al., 2002; Lyon et al., 2008). Conversely, the density of β-AR is higher in the apical segments, inferring from the increased fibrosis of the apical region in response to isoprenaline infusion.

Patients usually do not have any symptoms, and ventricular thrombi are therefore an incidental finding on echocardiography, which has a sensitivity of almost 95 percent

and a specificity of 90 percent. The main concern is that they will embolize, and for this reason anticoagulation is mandated. Surgical thrombectomy may be considered, particularly in the case of a large, protruding, mobile thrombus, after failure of attempts at anticoagulation, or in patients who are unable to take warfarin (Tilling et al., 2007).

There is a few data of NOAC use in TTS patients (Mano et al., 2016; Bolcal et al., 2019; Ohashi et al., 2015). Although many patients have complete resolution with anticoagulant therapy, recurrent embolic phenomena are a common complication, as seen in our patient. Having discussed the relative success of thrombus resolution with anticoagulation, the mere presence of an embolic source in the ventricle poses an immediate threat to a patient's life, and emergent surgical intervention to remove the thrombus and to definitively diagnose the mass seems appropriate. Possible treatment options include anticoagulation and thrombolysis (Tanaka et al., 2014). Given the drug-drug interactions between some antiviral treatments and direct oral anticoagulants, unfractional heparin or enoxaparin may be preferred in all patients with COVID-19 if possible (Akay, 2020). Still, patients with this syndrome should undergo repeated TTE assessment, as dynamic left ventricular outflow tract obstruction may occur in up to 25% of cases (El Mahmoud et al., 2008). Different studies revealed that dynamic, transient intraventricular pressure gradient occurred in about 16–24% of individuals with TTS (Wybraniec et al. 2014). In TTS, approximately 40% of patients can develop left ventricular thrombus (LVT) despite anticoagulation and therapeutic INR (Wybraniec et al., 2008; Vasilieva et al., 2011). Sasaki et al., 2004 reported a case with apical TTS and LVT detected on the 5th day of admission by echocardiography. It was reported that Renal infarction developed in a patient on the 8th day.

3.4. splenic infarction and venous thrombosis

Although splenic artery occlusion is rare, TTS was reported to be among the etiological causes in the literature for the first time in our case. In our case, spleen infarction was seen in abdominal CT. (Fig.4) Spleen infarction (SI) occurs when the spleen artery or 1 or more branches is blocked by an embolus or in situ thrombosis. The spleen has a rich vascular source and makes 5% of the cardiac output sensitive to embolism (cardiogenic, aortic, paradoxical). In addition, it is rarely affected by malignant hematological disorders that increase the risk of thrombosis. However, SI is a rare event. In a retrospective 10-year series with 196.625 patients, only 32 patients with a diagnosis of SI were found - 0.016% of admissions (Schattner et al., 2015).

3.5. Protection for Covid-19 during the surgery

In the first reports of the COVID-19 outbreak, infection rates of up to 29% were identified among healthcare professionals (Tang et al., 2020; Pan et al., 2020; Tang et al., 2020). All procedures for virus infection should be performed by experienced personnel. Patients should be prepared in negative pressure isolation rooms. All laboratory samples should be used carefully. Procedures should be performed with appropriate personal protective equipment for surplus contact measures in the air, including N95/filtering face piece 2 (FFP2) mask, apron, cap, eye protection. The heart-lung machine should be considered as the main source of splash and aerosol production; therefore, a higher level of protection (eg The Association for the Advancement of Medical Instrumentation [AAMI] Level 3 or equivalent) should be considered. Heparinization is recommended from the upper limit. Active clotting time is not a strong indicator at that time. If available, viscoelastic test is the most suitable option. If not, active partial thromboplastin time is a more useful test for monitoring. In patients with multiple thrombosis during cardio pulmonary bypass (CPB) or extracorporeal membrane oxygenation (ECMO) / extracorporeal life support (ECLS), direct thrombin inhibitors may be an alternative in experienced centers. Accumulating evidence suggests that a subgroup of patients with severe COVID-19 may have cytokine storm syndrome. It is recommended identifying and treating hyperinflammation using existing, approved treatments with proven safety profiles to meet the need to immediately reduce rising mortality rates (Günaydın, 2020).

As a result, the rapidly spreading COVID-19 epidemic throughout the world has been the major cause of both emotional and biological distress. It is observed that new neuro cardiac and thromboembolic complications appear in individuals with TTS, in which excessive catecholamine discharge occurs in the face of distress. The operation of the surgical team involved in the treatment of life-threatening complications under special isolation measures requires reducing the patient's emotional distress and adding anticoagulant therapy to the current treatment at a higher dose. Since the biomolecular mechanism of thrombotic reactions occurring under distress is not known exactly, it should be studied in detail.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Case Report

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Gastrointestinal bleeding due to giant gastric bezoar

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Abstract

Gastric bezoars occur in the stomach due to foreign body accumulation with an inability to pass through the pylorus. Major complications of bezoars include intestinal obstruction, gastric ulcer, gastric perforation, and bleeding. Many gastric bezoars can often be treated conservatively. Endoscopy has been commonly used in both the diagnosis and treatment of bezoars. We present a case that complained about abundant gastrointestinal bleeding as well as abdominal distension and was successfully treated with emergency gastric surgery after the failure of bleed control by endoscopic intervention due to giant gastric bezoar.

Keywords: bleeding, gastric bezoar, melena, phytobezoar

1. Introduction

Gastric bezoars occur in the stomach as a result of foreign body accumulation with inability to pass through the pylorus. Major complications of bezoars include intestinal obstruction, gastric ulcer, gastric perforation, and bleeding. The most frequently detected bezoar type is phytobezoars. Foods that lead to their development are Persimmon, orange and other citrus fruits, pulp-rich flesh, and vegetables. Persimmon contains shibutal. When Shibutal reacts with acid in the stomach, an insoluble viscous material is formed. Especially in a stomach with reduced acid secretion and motility due to previous surgery, this viscous material can form a nucleus for bezoar formation. Inadequate oral digestion and chewing due to toothless or damaged tooth structure, decreased gastric acidity, and slowing of gastric emptying as a result of previous gastric surgery. In various series, the stomach surgery rates as high as 55.6% and 70% were reported (Kadian et al., 1978; Ersan et al., 2005; Sumskiene et al., 2009; Khan et al., 2019).

"Gastroparesis diabeticorum" due to diabetic neuropathy has been shown as another factor that causes bezoars in the stomach. It has been suggested that diabetic neuropathy reduces gastric motility and delayed emptying of solid foods with vagotomy. Trichobezoar is seen in children with mental retardation and psychiatric patients. These patients may be paranoid, obsessive, or even with Down syndrome. A special type of trichobezoar is the Rapunzel syndrome. In these cases, apart from the hair mass in the stomach, there are hairballs starting from this gastric mass, passing the entire small intestine, and extending to the right colon. Petrified bodies of the stomach are hard bezoars that become hard and solid in the aqueous environment of the stomach. Most of them occur with the gammalax residue present in furniture polish. It was seen in six people in Nigeria who drink water mixed with industrial cement (Kadian et al., 1978; Ersan et al., 2005; Sumskiene et al., 2009; Khan et al., 2019).

Gastric phytobezoars can often be treated conservatively. Repeated doses of cellulase are the most effective proteolytic enzyme in dissolving stomach phytobezoars. Other proteolytic enzymes such as papain and acetylcysteine are not as effective as cellulase. Endoscopy has been used in both diagnosis and treatment of bezoars. Huge phytobezoars may need to be broken down and fragmented and the fragments removed individually. Sometimes, complete cleansing can be done in 3-4 sessions. There are studies stating that successful results are obtained from the use of "Electrohydraulic Lithotripsy" as an advanced form of endoscopic treatment and combined treatment with cellulase / cystein / metachopramide trio with endoscopic fragmentation (Kadian et al., 1978; Ersan et al., 2005; Sumskiene et al., 2009; Khan et al., 2019). In phytobezoars where conservative treatment is not sufficient, surgical treatment is required in all cement bezoars in most cases of trichobezoar. Surgical treatment can be done laparoscopically or open surgical methods (Kadian et al., 1978;

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Ersan et al., 2005; Sumskiene et al., 2009; Khan et al., 2019). We present a case who was admitted to the emergency department with abundant gastrointestinal bleeding and was successfully treated with emergency gastric surgery, because of gastrointestinal bleeding could not be controlled due to giant gastric bezoar after endoscopic intervention.

2. Case report

A 79-year-old man with complaints of abdominal pain, nausea, hematemesis and black stool was admitted to the emergency room of our university hospital. These symptoms appeared 15 hours before the admission. He had mentioned fullness in the stomach, a complaint lasting for about six months. Past medical history reported peptic ulcer disease with gastric ulcer perforation and surgical ulcer treatment with vagotomy about 20 years before the admission. Physical examination revealed gastrointestinal bleeding (GI) indicating melena. Abdomen was not tight, but painful in the epigastrium Blood analysis showed severe anema with a hemoglobin concentration of 5.4 g/L. Upper GI endoscopy showed a giant foreign body (dimensions; 12x6x4 cm) positioned longitudinally in the stomach and a huge ulcer coated exude formation. General surgery was recommended for the patient who could not be treated endoscopically by the gastroenterologist. Histological examination of biopsy specimens from ulcer and its adjacent area revealed a typical ulcer induced by pressure of bezoar (Fig. 1). There were defects in the mucosal integrity. There were typical changes of superficial ischemic damage, likely due to prolonged bezoar compression. Helicobacter pylori and intestinal metaplasia were not seen.

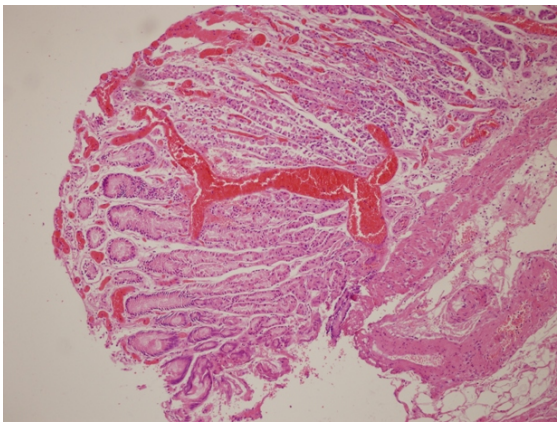

Fig. 1. Histological examination of biopsy specimens from ulcer and its adjacent area revealed a typical ulcer induced by pressure of bezoar

The patient was consulted to a general surgeon for surgical treatment. Abdominal computered tomography was administered. Abdominal CT showed a foreign body which had a contrast defect and a dimension of 12x6x4 cm (Fig. 2). There was an open anastomosis of gastrojejunostomy in the inferior of corpus. Although gastric bezoar was filled into the stomach, there was no obstruction of outlet to distal from the stomach according to CT. The giant bezoar was removed by surgical treatment (Figs. 3 and 4). After appropriate treatment, the patient who had a hemoglobin concentration of 11 g/L was discharged healthily at the seventh day after surgery.

Fig. 2. Abdominal CT showed a foreign body which had a contrast defect and a dimension of 12x6x4 cm

Fig. 3. Removal of bezoar by surgical treatment

Fig. 4. Phytobezoar

3. Discussion

Gastric bezoars are rare (an estimated incidence of 0.3 percent on upper endoscopy), cause nonspecific symptoms, and are usually found incidentally in patients undergoing upper gastrointestinal endoscopy or imaging (Kadian et al., 1978). Our patient had complaints of abdominal pain, nausea and symptoms of gastrointestinal bleeding such as hematemesis and melena. Gastric bezoar was found firstly in GI endoscopy for GI bleeding and confirmed by abdominal CT.

Bezoars are classified according to their composition such as phytobezoars, trichobezoars, pharmacopoeias (e.g., extended release nifedipine, theophylline, enteric-coated aspirin, sodium alginate, and sucralfate) and other type (e.g., tissue paper, fungus, Styrofoam cups, cement, vinyl gloves, and, rarely, milk curd) (Sumskiene et al., 2009). The most

common type of bezoars is phytobezoar and it was also seen in our patient. Several risk factors have been identified in patients with gastric bezoars. Risk factors include gastric dysmotility, gastric outlet obstruction, dehydration, use of anticholinergic agents and opiates and use of medications with an insoluble carrying vehicle (e.g., enteric-coated aspirin and nifedipine) (Krausz et al., 1986). Among patients with gastric bezoars, 70 to 94 percent have had gastric surgery and 54 to 80 percent have undergone vagotomy and pyloroplasty. Patients with gastroparesis appear to be at increased risk of formation of gastric bezoars due to impairment in the grinding mechanism of the stomach and the inter digestive migrating motor complex (Robles et al., 1994; White et al., 2003). Our patient had a history of previous gastric surgery and no medications.

The patients with bezoars are usually asymptomatic and symptom onset is insidious. The most common symptoms include abdominal pain, nausea, vomiting, early satiety, anorexia, and weight loss. Gastrointestinal bleeding due to concurrent gastric ulcers is a common presentation in patients who have previously undergone surgery. The ulcers may be due to peptic ulcer disease or pressure necrosis. Although many bezoars become quite large, gastric outlet obstruction is an uncommon presentation (DeBakey et al., 1938). He had abdominal pain, nausea, hematemesis and black stool. He had a history of peptic ulcer disease and previous gastric surgery. Upper gastrointestinal endoscopy is required to establish the diagnosis of a gastric bezoar and to obtain samples to determine its composition. Endoscopically, a gastric bezoar has the appearance of a dark brown, green, or black ball of amorphous material in the fundus or antrum of the stomach. In our patient, the gastric bezoar was identified by GI endoscopy. It was a dark brown (Figs. 3 and 4) (Gelrud and Gelrud, 2019).

The choice of therapy depends on the type of bezoar and the presence of underlying risk factors. The alternatives of treatment include chemical dissolution, endoscopic removal, adjuvant prokinetics (e.g., metoclopramide10 mg orally before meals and at bedtime) and surgery. Chemical dissolution involves administration of an agent to degrade the gastric bezoar. As compared with endoscopic therapy or surgery, chemical dissolution has the advantage of being noninvasive and inexpensive. However, a potential complication of chemical dissolution therapy is that partially dissolved bezoars may cause small bowel obstruction up to six weeks later. There are no randomized trials comparing these agents (e.g., Coca-Cola, Cellulase, Papain, Acetylcysteine) (Gelrud and Gelrud, 2019). In the literature, cases where chemical resolution was achieved by giving pineapple juice have been reported (Karabıçak et al., 2012). Large gastric bezoars, if pylorus is normal, usually are treated by endoscopy. The bezoar fragmentation can be performed with a large polypectomy snare, electrosurgical knife, lithotripter, drilling, endoscopic laser destruction, and Dormia basket or conventional surgery can be chosen (Sumskiene et al., 2009). Surgical removal should be reserved for patients who fail chemical dissolution and endoscopic therapy and for patients with complications including obstruction and significant bleeding. If a gastrotomy or enterotomy is performed to remove bezoars, the remainder of the small bowel and stomach should be examined to exclude other retained bezoars (Kadian et al., 1978; Sumskiene et al., 2009; Gelrud and Gelrud, 2019). Our patient had a severe anemia and symptoms of acute gastrointestinal bleeding. The GI endoscopy was failed to bleeding control and removal of bezoar. The most appropriate modality for our patient was surgical treatment. The bezoar was removed successfully and no complication was seen after surgery. He was discharged healthily at seventh day of surgery.

Large bezoars may be a cause of gastric ulcer and chronic gastrointestinal bleeding. Large gastric bezoars resulting from gastric dysmotility related to previous surgery have commonly been described, but complications such as ulcer caused by pressure of foreign body and gastrointestinal bleeding are especially rare. Patients with previous gastric surgery should be warned about this preventable complication and dietary advice should be given for these patients.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Lance Adams syndrome following post-hypoxic myoclonic status epilepticus in an adolescent patient with chronic renal failure: A case report

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Abstract

Myoclonic contractions that may occur after hypoxia might be related to post-hypoxic status epilepticus (PHSE) or Lance Adams syndrome (LAS). It is important to distinguish these two myoclonic conditions in terms of treatment and prognosis. In the literature, both PHSE and LAS have not been reported in pediatric age group including adolescents. Here we report an adolescent case with chronic renal failure who developed PHSE and then LAS after a long-term cardiopulmonary resuscitation.

Keywords: Lance Adams syndrome, posthypoxic status epilepticus, myoclonus, movement disorders, cardiopulmonary arrest

1. Introduction

Post-hypoxic myoclonia (PHM) following a cardiac arrest and hypoxia can occur in two different ways, acute and chronic. Acute PHM, called post-hypoxic myoclonic status epilepticus (PHSE), occurs early in patients with severe coma after a cardiac arrest. Chronic PHM, called Lance Adams syndrome (LAS), is characterized by increased myoclonies with stimulations and may occur in patients with significant improvement in consciousness after cardiac arrest. It is important to distinguish LAS from acute PHM. Although it is rarely reported in the literature as case reports in adults, not been reported in childhood. We presented an adolescent case that developed PHSE and then LAS.

2. Case report

A 16-year-old male patient who was with the diagnosis of rapidly progressive glomerulonephritis was brought after a cardiopulmonary arrest. The first treatment was given because of the blood potassium level reached 9 mEq/L, then sent to our hospital for hemodialysis. During the transport, he suffered a cardiac arrest and was brought to our hospital after approximately 40 minutes of resuscitation. He was intubated and unconscious and Glasgow Coma Scale (GCS) was 4. arterial blood pressure was 130/65 mmHg, heart rate was 137/minute, fever was 36.8°C degrees.

In laboratory values, pH:6.98, HCO3:10.6 mmol/L, pCO_2:57.2 mmHg, lactate:7.2 mmol/L, blood urea nitrogen: 99 mg/dL, creatine:12 mg/dL, potassium:9.4 mEq/L, uric acid:9.9 mg/dL. The patient underwent hemodialysis in the pediatric intensive care unit. At the 18th hour after resuscitation, generalized multifocal myoclonic contractions were observed. Midazolam and phenytoin were administered, but myoclonic contractions continued. Although the midazolam infusion dose was increased to 18 mcg/kg/min, his seizures could not be controlled and then thiopental infusion was initiated. MRI was reported as normal except hypoxic changes. In the EEG examination under thiopental infusion, very frequent, generalized, lasting 0.5-1 sec, high- amplitude spike-slow wave discharges and intermittent bursts were observed. On the 5th day of hospitalization, thiopental infusion was discontinued decreasingly, and then myoclonic seizures reappeared.

On the 10th day of hospitalization, thiopental was reduced again and this once it was observed that myoclonic seizures only increased during the patient's examination and stimulation to the patient and consciousness of the patient improved (GCS:10). The infusions were then discontinued, considering that the patient's current condition was compatible with LAS and piracetam was added to phenobarbital and levetiracetam treatments. On the 60th day of his hospitalization, he was conscious, could sit without support, speak single words. He was transferred from the pediatric intensive care unit.

3. Discussion

PHSE is characterized by generalized or multifocal myoclonic contractions and occurs within the first 72 hours of a typical cardiopulmonary arrest and ends within days (Hui et al., 2005). It is thought that it arises from subcortical regions such as brain

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stem. Typically, it is characterized by myoclonic jerks, which can also involve face and neck muscles. Also in our patient, spontaneous myoclonic jerks were generalized and including facial and neck muscles. It continued despite the midazalom infusion until the thiopental infusion was started. Initially, consciousness could not be fully evaluated since his received midazolam and thiopental infusions. PHSE cases are known to be treatment-resistant (Freund et al., 2017). In our patient, myoclonic jerks were partially controlled by thiopental infusion. In the meta-analysis of 65 articles consisting of acute PHSE cases in the literature, 431 cases were reported and EEG findings of these were found to be different. If generalized PHSE cases do not die, vegetative life may develop or they may rarely heal with sequelaes. In some of healing patients may develop LAS (Gupta and Caviness, 2016). Also, in our patient it was detected an improvement in his consciousness within days, and then LAS developed.

LAS was first described in 1963 (Lance and Adams, 1963). Since then, cases have been published, almost all of them in the adult age group. LAS can occur directly after hypoxia and also can be seen in patients who developed PHSE. The form of myoclonus is generalized and multifocal in PHSE, whereas it is action myoclony in LAS. Since our patient was under heavy sedation, we were able to evaluate his state of consciousness after interruption of sedation and infusion of anesthetic drugs. We were only able to diagnose LAS after observing improvement in consciousness and myoclonic jerks, and probably delayed to diagnose it. In treatment, antiepileptic drugs are used because myoclonies are usually of cortex origin. Piracetam, has been found to be useful in the treatment of myoclonies and the efficacy of levetiracetam, which is chemically similar to this agent, has also been reported (Krauss et al., 2001). We found a significant reduction in action myoclonies as a result of the addition of piracetam.

Many hypoxic patients have been kept alive with the developments in pediatric intensive care and the problems which are secondary to hypoxia have been struggled. However, we did not find any cases of PHSE and LAS in childhood. In conclusion, we present this case to emphasize that LAS also can be seen in childhood.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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A case of multiple trauma and lighter gas inhalation

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Abstract

The rate of volatile substance abuse is increasing among young people around the world. A 10-year-old boy was brought to the emergency room by the 112-emergency ambulance service due to fall from the roof of a four-storey building. His general medical condition was moderate-poor; his GCS (Glasgow Coma Scale) was nine, blood pressure: 118/90 mmHg, heart rate: 98/min, O_2 saturation was: 100%. Skin abrasions were present on his jaw and the occipital region of the scalp. The patient was accepted as a multiple trauma patient. When the patient's confusion did not resolve, the patient's father informed us that the child may have inhaled lighter gas. The patient was hospitalized at the pediatric ward and was discharged on the 14th day of admission as his symptoms completely improved. Clinicians should be alert with regard to acute and/or chronic end-organ damages in the cases of gas inhalation without any antidote.

Keywords: confusion, multiple traumas, substance abuse

1. Introduction

The rate of volatile substance abuse is increasing among young people in Turkey, as well as around the world. One of these abuses is lighter gas LPG (liquefied petroleum gas), which is easily available and cheap. Lighter gas contains butane, which is a volatile gas in the aliphatic hydrocarbon group (Altındağ et al., 2001; Öncü et al., 2014). It is sold commercially as propane, butane or propane-butane mixed gas. Propane is an odorless, colorless, flammable gas. Butane is a colorless, flammable gas and has a smell resembling gasoline or natural gas (Ruth, 1986; Büyük et al., 2005). The inhaled gas is absorbed from the alveolar surface and rapidly enters the blood and may cause dizziness, nausea, vomiting, euphoria, confusion, and hallucinations (Broussard, 1999; Öncü et al., 2014). We aimed to present the cigarette lighter gas inhalation case of a 10-year-old boy who was brought to our hospital by the 112-ambulance service due to multiple traumas.

2. Case report

A 10-year-old boy was brought to the emergency room by the 112-emergency ambulance service due to fall from the roof of a 4-storey building. His general medical condition was moderate-poor; he was confused, his GCS (Glasgow Coma Scale) was 9. The blood pressure was 118/90 mmHg, heart rate was 98 bpm, O_2 saturation was 100%, and his four limbs were moving. Skin abrasions were present on his jaw and the occipital region of the scalp. The patient was accepted as a multiple trauma patient. He had no chronic illness in his medical history and no drug or substance abuse either. Intravenous (i.v.) access was achieved and i.v. fluid support treatment was started. Panoramic CT scan was requested. Arterial blood gas (ABG) analysis revealed the serum pH as 7.27, pCO_2: 47 mmHg, HCO_3: 19.6mmol/L, glucose: 153 mg/dL and lactate: 2.9 mmol/l. Cohb:4. Liver and kidney function tests were normal. Hg level was 10.8 g/dl and Htc was 32.1%, which did not decrease during follow-up. ABG follow-up revealed that acidosis had improved. CT scan images were interpreted as normal. When the patient's confusion did not resolve, the patient's father informed us that the child may have inhaled lighter gas. The patient was consulted with brain surgery, orthopedics, and pediatric surgery and no traumatic pathology was detected. The patient was hospitalized at the pediatric ward. Urine buprenorphine and ecstasy levels were found to be low-positive in urine multi-drug analysis test result. On the 3rd day of hospitalization, cranial MRI was requested due to persistent lethargy. Brain edema was observed in the occipital region. Emergency intervention was not considered in the patient who underwent the consultation of brain surgery. Anti-edema treatment and i.v. hypertonic saline infusion was initiated. No pathology was detected on EEG recordings. The patient was discharged on the 14th day of admission as his symptoms completely improved.

3. Discussion

Butane gas has several acute and long-term effects. Acute irritability, cough, rhinorrhea, euphoria, and nausea may be seen in the early period of inhalation. Speech disorder,

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headache, confusion, delirium, hallucinations and decline in reflex responses require attention and close follow-up. Hypoxia, tachycardia, tinnitus, and nystagmus can also be seen at an early stage. Arrhythmias, hypoxia, asphyxia, and cardiac arrest may develop suddenly. While it may have cardiovascular, renal, pulmonary, and hematological effects in long term, it also has neurological and psychiatric long-term effects as well (Seven et al., 2017).

Our patient was brought to the ER due to multiple trauma and no intracerebral hemorrhage or any traumatic cerebral problem was considered on the first examination. Cranial CT imaging was normal and the patient who had undergone a neurosurgery consultation, did not have any acute pathology requiring surgical intervention due to trauma. Follow-up CT scans did not change the clinical approach. The acidosis in the blood gas analysis improved within a few hours after supportive treatment, the patient tended to sleep and he had severe headache at each awakening, which supported substance abuse. Although we questioned him on admission to the hospital, we were informed by his father 2 hours later. We believe that this case is important in terms of demonstrating the need to keep in mind the toxicological events regardless of the pre-diagnosis, especially volatile substance inhalation, in patients admitted with confusion, drowsiness or severe headache. Lighter gas inhalation is known to be more frequent in adolescents, especially in families with low socioeconomic status and psychiatric problems (Carlini-Catrim and Corlini, 1989; Carlini-Catrim, 1995; Frederich et al., 1997; Öncü et al., 2014). Similar features were present in our case. A 14-year-old girl was reported to develop dizziness and confusion after lighter gas inhalation. Since her vital signs and routine biochemical tests were normal, she was discharged 24 hours after admission (Öncü et al., 2014).

A sudden death case of 19-year-old woman was reported, which occurred within 3-4 minutes after lighter gas inhalation. Another case of sudden death resulting from LPG inhalation for suicidal purposes was published in forensic medical journals (Büyük et al., 2005; Akcan et al., 2010). In a postmortem examination, a 15-year-old girl was found to have myocardial necrosis associated with butane gas (Aytül and Taner, 2019). An autopsy report of a 16-year-old boy after sudden death revealed edema in his brain and the cerebellum (Petekkaya et al., 2016). Brain edema detected in our patient two days after admission to ER was not considered because of trauma. Diffuse alveolar hemorrhage was reported in a 21-year-old male due to chronic substance inhalation and encephalopathy was reported in a 15-year-old girl as well (Döring et al., 2002; Yavuz et al., 2018). It is thought that acute ischemia can be seen, although it is rare, secondary to trauma (Kolbaş et al., 2020). Our patient had no acute ischemia on MR imaging. In the previously reported cases and studies, we noticed that a history of lighter gas inhalation was present in the patient's anamnesis. To the best of our knowledge, there is no such case of lighter gas inhalation concomitant with trauma. Furthermore, we did not find any case of children 10 years or younger among reported cases and studies. Clinicians should be alert with regard to acute and/or chronic end-organ damages in the cases of gas inhalation without any antidote, who are treated by supportive treatment. Otherwise, in cases with different preliminary diagnoses, this may lead to follow-up in the emergency department and delay in hospitalization at the related ward.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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A case of pulmonary thromboembolism with ST elevation and literature review

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Abstract

Electrocardiography (ECG) is an auxiliary test applied for differential diagnosis of Pulmonary Thromboembolism (PTE). However, its specificity is low, but it is more often used for differential diagnosis. Although there are some changes on ECG in PTE, ST elevation is not a finding that we expect to see. With this case report, we aimed to draw attention to the issue with the diagnosis of PTE in a patient with ST elevation in ECG. 52-year-old male patient presented to the emergency department with a complaint of convulsion after chest and back pain. During the examination in the emergency department, he had a convulsion and woke up on his own without entering a postictal phase. After that, he had a chest pain. For this reason, ECG was performed to the patient and revealed ST elevation in leads DI, AVL, V1-4. The results of the patient's EEG and head computer tomography (CT) were normal, so coronary CT angiography was performed. And it was observed that the coronary arteries were patent but embolism was detected in his cross-sectional pulmonary arteries. Thus, he was treated with the diagnosis of PTE. By the obtained results in this case that ST elevation may be a finding of PE, although it is rare.

Keywords: electrocardiography, ST segment elevation, pulmonary thromboembolism, emergency department

1. Introduction

Pulmonary thromboembolism (PTE) is a disease that describes the clinical findings resulting from the occlusion of the main pulmonary artery and its branches. Although it is frequently diagnosed and the ratio of mortality is around 10%, its un-diagnosis can result by mortality. To diagnose PTE, the clinician should suspect first. In PTE cases, chest pain, syncope and shortness of breath are the main complaints. As these complaints can be also the symptoms of cardiovascular diseases, the electrocardiography (ECG) must be performed at an early stage. Although it is known that in PTE there are some ECG changes, the sensitivity and specificity are low. Sinus tachycardia, right bundle branch block and S1Q3T3 pattern can frequently be observed as ECG findings for PTE (Siddiqa et al., 2020; Yeh and Chang, 2008; Sinha et al., 2005). Whereas ST elevations are highly suggestive of acute coronary syndrome (ACS), ST elevations associated with PTE have infrequently been reported in literature. With this case report, we aim to take attention to the issue by presenting a PTE case with an ST elevation.

2. Case report

A 52-year-old man presented to the emergency department with complaints of pain in the right side of his chest and back, followed by a convulsion. There were no chronic illnesses in patient's story. The patient's vital findings were: Arterial blood pressure: 110/60 mmHg; Pulse rate: 60/min; sPO_2: 98% and body temperature: 36.7°C. The ECG performed due to the chest pain demonstrated normal sinus rhythm (NSR), heart rate (HR) 60/min, ST elevation in leads V1-V2, DI, AVL, negative T-waves in leads V3-6 (Fig. 1). During the examination in the emergency department, the patient had a convulsion similar to that he had at home. However, neither at home nor at the hospital he didn't have urinary incontinence or the postictal phase afterwards. His convulsion recovered without any medication. The patient described a chest pain after this convulsion-like activity. Therefore, his second ECG was performed. (Fig. 2). By the second ECG, NSR, heart rate of 58/min, ST elevations in leads DI, AVL, V1-4 and ST depression in leads D2, D3 and AVF were detected. The head computer tomography (CT), taken due to convulsion, was interpreted as normal. No pathological finding was observed in the posterior-anterior chest radiography. pH:7,33 pO_2:67 mmHg, pCO_2: 30 mmHg, lactate:3.5 mmol/L were detected in the patient's blood gas. Cardiology consultation was requested for the patient after no pathological finding was detected in complete blood count and biochemistry tests. Echocardiography (ECHO) performed by a cardiologist showed normal left ejection fraction and mild right ventricular dilatation. The highly sensitive troponin test result was 7 (normal reference range 0-14 pg/ml).

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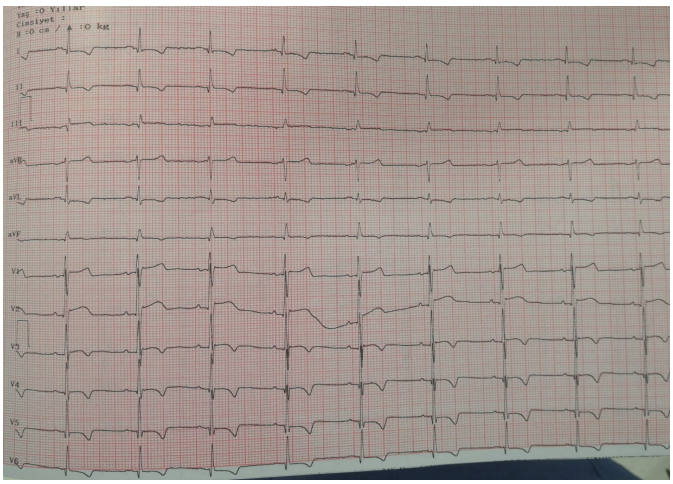

Fig. 1. Patient's 1st ECG done in the emergency department. Patient's electrocardiography (ECG) performed for chest pain showed normal sinus rhythm (NSR), heart rate (HR) 60/min, ST elevation in V1-2 leads, t negative in DI, DII, aVL and V3-6 leads

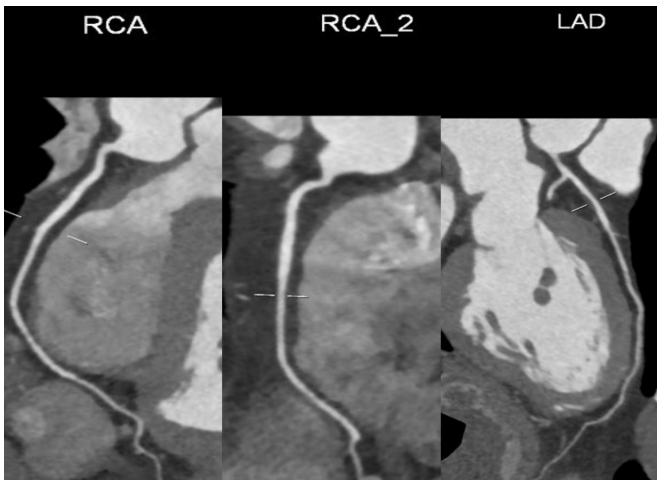

Fig. 3. Coronary Computer tomography (CT) Angiography images of the patient. The patient's CT coronary showed no lesions in the coronary arteries

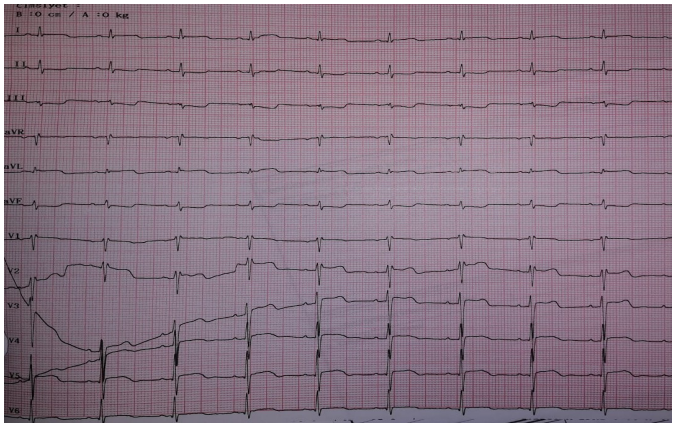

Fig. 2. 2nd ECG done after the patient's convulsion-like activity. Patient's 2nd electrocardiography (ECG) done after the patient's convulsion-like activity: ST elevation was detected in DI, AVL, V1, V2, V3, V4 leads, St depression was detected in DII-III, AVF leads

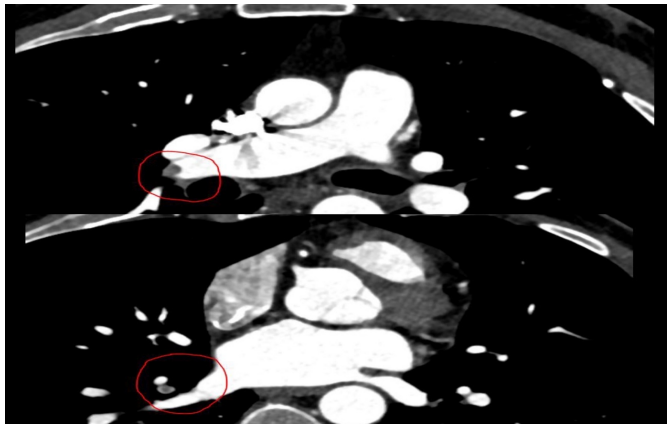

Fig. 4. PTE compatible image seen on coronary Angio computer tomography (CT) of the patient. The patient's coronary CT showed no lesions in the coronary arteries. However, embolism was observed in the right main pulmonary artery and the branch

Table 1. The characteristics of the patients with have ST elevation associated with PTE according to the literature

	Age	Gender	Complaint	Findings suggesting PTE	ECG findings	ECHO findings	Pre-diagnosis
1	32	male	Chest pain, dyspnea	Right leg trauma 5 days ago	Sinus tachycardia, $S_1Q_3T_3$, V3-6 ST elevation	RV dilation, IVS flattening	PTE
2	57	male	Chest pain, syncope	No	Sinus tachycardia, t-negativity in inf. and precordial leads, ST elevation in anteroseptal leads	RV dilation, IVS displaced towards the left ventricle	ACS
3	35	male	Leg pain, syncope	DVT clinic for 4 weeks	RBBB, V1-4 ST elevation, Q (+) in DIII, $S_1Q_3T_3$ (+)	RV dilation, PAB: 40 mmHg	PTE
4	42	female	Shortness of breath, syncope	Oral contraceptive use, 4 months ago abortion history, DVT clinic	V1-3 ST elevation, DI: S (+), DI, AVL, V5-6 ST Depression	RV dilation, IVS displaced towards the left ventricle	PTE
5	62	male	syncope	no	Incomplete RBBB, S1Q3 pattern, V1-4 ST elevation	Not enough information	ACS

The patient was admitted to the coronary intensive care unit with the pre-diagnosis of ACS. Coronary angiography or thrombolytic therapy was not initiated in the first place because the neurological event was not ruled out in the story and ECHO findings were not fully associated with ACS. Coronary CT angiography was performed during his follow-up in the intensive care unit. No significant lesions in the coronary

arteries were detected in the coronary CT. However, an embolism was observed in the right main pulmonary artery and its branch when the sections were examined (Figs. 3 and 4). Therefore, electroencephalogram (EEG) and diffusion cranial magnetic resonance imaging were planned to rule out epileptic seizures for the patient whose treatment had been initiated. No pathology was detected in these examinations. The patient diagnosed with PTE and medicated with Coumadin, was discharged with full recovery on the 7th day of his hospitalization as no additional problems were developed.

3. Discussion

Relationships between PTE and ECG changes have been discussed in several literatures and have been reported that sinus tachycardia, right bundle branch block (RBBB) and S1Q3T3 were frequently observed as ECG findings of PTE (Siddiqa et al., 2020; Yeh and Chang, 2008; Sinha et al., 2005; Boey et al., 2015). The coexistence of PTE and ST elevation is not a common finding (Thomson et al., 2019). In the literature, a small number of cases on PTE associated with ST elevation (Table 1) were found.

When these cases were examined, it was observed that there were other findings suggesting PTE on ECG or clinical findings in addition to ST elevation. For example, Yeh and Chang stated that their 32-year-old male patient had suffered a right leg trauma five days ago before applying to hospital and reported that on the patient's ECG sinus tachycardia and S1Q3T3 pattern in addition to ST elevation in leads V3-6 and RV dilatation in ECHO. Therefore, CT was ordered with the suspect of PTE and confirmed the diagnosis (Yeh and Chang, 2008). In the 57-year-old male case report, Wilson and Schaller (2008) stated that their patient had presented with syncope to the Emergency Department. They described that sinus tachycardia in addition to ST elevation in leads V1-4 on the ECG and RV dilatation in the ECHO were observed. However, t any pathology in PCI was detected, and in order to exclude PTE, the ventilation / perfusion scintigraphy was performed and diagnosed. Lin et al. (2009) stated that their case was a patient scheduled for Doppler ultrasonography (Doppler USG) due to leg pain, and the patient applied to the emergency department after having syncope. Deep vein thrombosis on Doppler USG, RV dilatation on ECHO and PAP: 40 mmHg were detected. On the ECG o, $S_1Q_3T_3$ pattern and right bundle branch block in addition to ST elevation in leads V1-4 were detected. With these clinical findings CT with a pre-diagnosis of PTE was planned and diagnosis was confirmed. The researchers, presenting a 42-year-old female patient with a history of oral contraceptive use, described that their patient applied to the emergency department with complaints of leg pain and shortness of breath (Livaditis et al., 2004). They stated that V1-3 ST elevation on the patient's ECG, S in D1, RV dilatation and leftward displacement of the interventricular septum (IVS) in ECHO were found (Livaditis et al., 2004). In their case of a 62-year-old male without a chronic disease, Falterman et al. (2001) reported that their patient applied to the emergency department due to syncope at home and related head trauma. They stated that the ECG of their patient had S_1Q_3 pattern, incomplete right bundle branch block and V1-4 ST elevation, first suspected of ACS, but later the patient died from a cardiac arrest during PCI, and PTE was detected at autopsy. It was observed that in three of the five cases mentioned above, there were serious risk factors for PTE or ECG changes. Although there were ST elevations in those cases, pulmonary CT angiography was performed primarily for PTE diagnosis. On the other hand, there is no such story in our case. The patient with the complaint of chest pain and shortness of breath, made us think of ACS clinic, except for the convulsion history which left us in doubt. In fact, we thought a loss of consciousness described as a convulsion was syncope. Because the blood gas taken after the loss of consciousness did not have acidosis and the patient did not have a postictal period. However, we still had to exclude epileptic seizures as differential diagnosis.

ECG changes in PTE are thought to observed due to the enlargement in the right structures of heart and the increase in pulmonary artery pressure. From this point on, we can expect to see ST depression in the precordial leads; however, ST elevation is much less common and the cause of ST elevation has not been clearly demonstrated (Ambesh et al., 2019; Cheng, 2005; Van Mieghem et al., 2004). Nevertheless, it is suggested that ST elevation may result from excessive right ventricular dilatation or ischemia, and in this case, it may cause pseudo-infarction findings in the anterior leads (Senthilkumaran et al., 2020; Wang et al., 2003).

In this case, patient also had a mild dilatation of the right ventricle and we can explain the ST elevation with this. In our case the patient wasn't treated with PCI because the ECHO findings didn't suggest STEMI and the patient's relative insisted on convulsion. However, coronary CT angiography was performed on the patient. The coronary arteries were found to be normal and the pulmonary vascular embolism was detected.

As a result, although it is rare, ST elevation might be a sign of PTE. Therefore, PTE should to be ruled out before diagnosing the patient with ACS, especially if clinical findings suggest PTE.

Conflict of interest

None to declare.

Acknowledgments

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Migration of a gastric bezoar to esophagus: A rare cause of acute dysphagia

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Abstract

Sudden onset of dysphagia due to a gastric bezoar migrating to the esophagus is a relatively rare condition. A 72-year-old male patient with known gastric bezoar presented with sudden difficulty swallowing following nausea and vomiting caused by adhesive ileus. Gastroscopic examination showed a bezoar and associated compression ulcers in the esophagus. The bezoar was pushed towards the stomach and extracted by successful endoscopic fragmentation.

Keywords: phytobezoar, esophageal bezoar, dysphagia, ileus

1. Introduction

A bezoar is characterized by a hard mass formed by conglomeration of undigested food in the gastrointestinal tract. Esophagus is an uncommon site for bezoars. Here, we report a case of difficulty swallowing caused by migration of a known gastric bezoar to the esophagus in a patient with nausea, vomiting and sudden onset of dysphagia associated with the esophageal bezoar, bezoar mass and compression ulcers detected by a second endoscopic examination.

2. Case report

A 72-year-old male patient presented to the emergency department with abdominal pain, nausea and vomiting for the last 4-5 days and sudden onset of difficulty swallowing a few days ago. He was advised to admit to a tertiary healthcare facility when a gastric bezoar was detected by endoscopy a week ago. He had a history of diabetes mellitus, essential hypertension, and coronary bypass and abdominal hernia surgeries. A plain abdominal radiograph in standing position (Fig. 1a) and an abdominal CT scan showed adhesions of the small intestine secondary to the hernia surgery and dilation of intestinal loops proximally to the adhesions. An esophagogastroduodenoscopic examination was performed due to difficulty swallowing which revealed a phytobezoar (3x4 cm) localized at 34 cm from the incisors (Fig. 1b). Bezoar was pushed toward the stomach with the aid of endoscope. Deep ulcers with overlying white colored exudates and occasional millimetric hemorrhagic foci caused by compression necrosis were visualized in the esophagus at the site of the bezoar (Fig. 1c). The bezoar pushed into the stomach was endoscopically fragmented by a snare specifically designed for bezoars and extracted with a retrieval net device (Fig. 1d). Parenteral supplementation was given and his ileus manifestations resolved on the fourth day of follow-up.

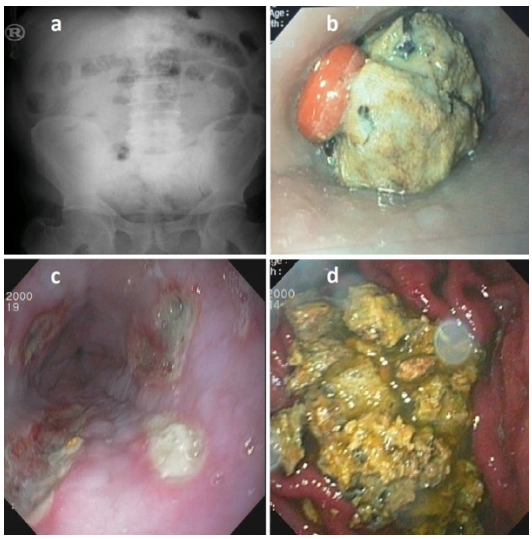

Fig. 1. Dilation of the proximal small intestine loops associated with adhesive ileus was evident on standing plain abdominal radiograph (a), phytobezoar in the esophagus containing the drug (b), ulcers caused by esophageal bezoar (c), pieces of bezoar after snare fragmentation (d)

3. Discussion

Gastric dysmotility, prior gastric surgery, gastric outlet obstruction, dehydration, anticholinergic agents, opiates, medications with insoluble protective coating and water-retaining drugs contribute to the development of gastric

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bezoars medications. Gastroparesis is frequently encountered in patients with a bezoar (Pfau and Hancock, 2016).

Bezoar in the esophagus is a rare condition. Esophageal bezoars can be divided into two groups including primary bezoars that occur in the esophagus and bezoars that are formed in the stomach and migrate to the esophagus (Chaudhry et al., 2013). Cases of esophageal bezoar have been reported in patients with impaired motility due to esophageal diverticula, diffuse esophageal spasm, achalasia, Guillain-Barre syndrome or myasthenia gravis (Chen et al., 2013; Goel et al., 1995; Kim et al., 2010). Our case is one of the rare secondary esophageal bezoars reported in the literature which caused by migration of the gastric bezoar to the esophagus, leading to nausea/vomiting and acute difficulty swallowing in the presence of adhesive ileus.

The narrow lumen of the esophagus and the risk for aspiration from fragments during the intervention make bezoars in the esophagus difficult to treat. Thus, it is more convenient to perform an overtube-assisted endoscopy for an esophageal bezoar and break it down to fragments in the stomach when possible.

Small bezoars can be treated with conservative medical approaches. A success rate of 91.3% was reported in the treatment of gastric bezoars with Coca-Cola lavages and endoscopic procedures (Ladas et al., 2013). On the other hand, larger bezoars may require endoscopic intervention or surgical treatment. Particles of the bezoar broken by endoscopic intervention can be extracted or pushed toward the small intestine. However, it should be borne in mind that fragments of a large bezoar can also result in mechanical ileus in the small intestine.

In conclusion, bezoar should be suspected in patients with relevant risk factors who report one or more symptoms of epigastric discomfort, nausea, vomiting, weight loss and early satiety and it should be considered that bezoar may cause sudden difficulty swallowing.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Skin reaction related to povidone iodine use

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Abstract

Nowadays, 10% povidone-iodine is a topical antiseptic solution that is commonly used in surgical areas' sterilization, wound cleaning and daily wound dressing. Severe complications may arise from povidone-iodine when it is not used carefully and in adequate proportion. Povidone-iodine-induced chemical skin reactions is one of the severe complications. Given the widespread use of povidone-iodine, clinicians need to be aware of this possible adverse drug reaction and of preventive cautions. This report presents two cases about povidone-iodine related skin reaction (these reactions are also called as chemical burns or chemical skin reactions) which are encountered in our hospital (Ondokuz Mayıs University, Samsun, Turkey) to point this important issue.

Keywords: antiseptics, povidone-iodine, contact dermatitis, skin reactions

1. Introduction

Povidone-iodine (PI) is frequently used during surgical preparation, postoperative wound cleaning and dressing. It was described by Shelaski about seventy years ago (Shelanski and Shelanski, 1956). PI consists of the complex structure of povidone, a polymer that is structurally similar to plasma proteins, and the iodine element, which has antimicrobial properties. The function of polyvinylpyrrolidone (PVP) used as a polymer includes increasing iodine solubility and penetration, decreasing iodine ion concentration and keeping iodine bound. However, PVP does not have antimicrobial properties. Shelanski defined this complex structure as 'iodophor' (Shelanski and Shelanski, 1956).

While 99.96% of the iodine element is bound to PVP, a small part of 0.04% is in free form. The spectrum of action of elemental iodine is very wide; it has effects on gram-positive and gram-negative bacteria, viruses, fungi, protozoa, and spores (Ameer et al., 2014). There is almost no resistance development against PVP in organisms. The iodine element separates from the complex structure at certain concentrations, penetrates the microbial cell membrane, interacts with proteins, nucleotides and fatty acids in the cytoplasm, disrupting their structure and functions. It may cause rapid death by affecting the structure of hydrogen bonds. Thus, resistance development is prevented (Ameer et al., 2014).

The Food and Drug Administration (FDA) approved the use of povidone-iodine in December 1986. The pregnancy category is D for topically and vaginally applied products and pregnancy category C for ophthalmic use. It is said that topical or vaginal use during pregnancy may cause temporary hypothyroidism in neonatal if systemic absorption occurs, and ophthalmic use may affect the fetal reproductive system (ScriptSave WellRx, 2021). There are many products, such as povidone iodine-containing solution soap, brush, cream, ointment, gels and ophthalmic drugs. Accidental oral ingestion of these products may cause metabolic acidosis, nausea, vomiting, diarrhea, gastroenteritis, hypotension, sinus tachycardia and cyanosis. Iodine related acute renal failure and renal tubular necrosis are rare but severe clinical presentations.

10% PVP solution contains 10% bound iodine and 1% free iodine molecules (100 mg poly-1-vinyl-2-prolidone with 10% iodine in 1 g) (Mete et al., 2009). It is an effective antiseptic that has fewer irritant properties due to its less amount of free form. However, it may cause irritant contact dermatitis, skin sensitization, allergic dermatitis, urticaria, anaphylaxis, irritation, maceration, necrosis and chemical burns in compression areas in individuals with hypersensitivity (Mark, 1982).

In the postoperative period, severe complications, including skin reactions, contact dermatitis, allergic dermatitis, and chemical burns, related to povidone-iodine use have been reported (Iijima and Kuramochi, 2002; Vandergriff et al., 2006; Kara et al., 2007). In this study, we aimed to present two cases that developed dermatitis after preoperative skin preparation with 10% povidone-iodine. For all photographs,

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written permission was obtained from the families for medical research and education purposes, without sharing the patient's identity information.

2. Case report

2.1. Case 1

A 9-month-old, 9 kg, boy was taken to the operating room for bilateral undescended testicle operation. He had no other disease. The child who was taken to the operating room was taken to the operating table with a heating blanket (Astopad DUO 120, Northern Ireland). The blanket, which was routinely checked and covered with a sterile surgical cover, was set to 36-39°C and the alarm sound was on. There were silicone support and green covers for the operating room on the blanket. After smooth induction and endotracheal intubation, caudal anesthesia was used to provide postoperative analgesia. After the surgical area was prepared with 10% povidone-iodine by the surgical team, the operation was started. The esophageal body temperature measured was between 36.6-36.8°C.

At the end of the operation that lasted for 50 minutes, the patient was awakened without any problem and taken to the recovery room. During the follow-up, a sharp redness was observed in the lower half of the back and the gluteal area (Fig. 1). Silver sulfadiazine 1% cream and 5% lidocaine topical treatment were initiated with the prediagnosis of a first-degree chemical burn to the patient who had no lesions in other body areas. At the visit performed on the first postoperative day, it was observed that the patient's lesions regressed (Fig. 2). The blanket was controlled by the maintenance team and it was learned that there was no problem with its settings.

Fig. 1. Case 1, postoperative 20th-minute examination revealed a markedly demarcated rash spread around the gluteal area, especially the gluteal area

Fig. 2. Case 1, it was observed that the lesions of the patient regressed on the postoperative first day compared to the lesions in the first postoperative hours

2.2. Case 2

A 20-month-old, 14 kg boy was taken to the operating room for the second session of hypospadias. The heating blanket (Astopad DUO 120, Northern Ireland) that was set at 36-39°C and alarms turned on was used to prevent hypothermia; the sterile surgical cover was laid on the blanket to prevent direct contact of child and the blanket. After smooth induction and endotracheal intubation, a caudal block was performed for postoperative analgesia. After the operation area was wiped with 10% povidone-iodine by the surgical team, it was covered with sterile covers, and surgery was initiated. Esophageal body temperature measured was between 36.6-36.8°C. At the end of the operation, which lasted for 75 minutes, the patient was awakened without any problem and taken to the recovery room. Here, a markedly demarcated redness was observed in the gluteal areas, spreading to the perineal and intergluteal areas. Nitrofurazone 0.2% ointment, 5% lidocaine ointment, and 25% pomade for Hamamelis virginiana were initiated for the patient who had no lesions in other body areas, such as back and arms that were exposed to pressure and were in contact with the blanket.

On the postoperative first day visit, it was observed that the lesions regressed (Fig.3). It was learned that there was no problem with the blanket sent for precautionary control.

3. Discussion

Postoperative skin reactions may arise from solutions used, surgical equipment, drugs and heating furnishing. Povidone contained in povidone-iodine is an allergic agent. Contact dermatitis cases caused by some noniodine copolymers of povidone (PVP-eicosene, PVP-hexadecane) have been reported (Constance et al., 2009). The proposed mechanism in the formation of burns is maceration, pressure effect, and skin irritation due to friction (Donna et al., 2006).

Fig. 3. Case 2, the first day of the postoperative period (It was observed that the skin lesions of the patient regressed compared to the first postoperative hour).

Risk factors for skin reactions after using povidone-iodine can be listed as follows (Donna et al., 2006; Chiang et al., 2011):

- Chemical burns caused by not drying the too much PI from the patient's skin and pooling effect due to accumulation of solution on the skin layers

- Increased skin fragility with the reduced age of the patient

- High concentration of alcohol in solution (better disinfectant properties but increased maceration effect)

- Pressure on the compression areas of the patient's body and the possibility of the pooling of the solution can be increased according to operation position (such as Lithotomy).

- High free iodine concentration in PI solution

- Prolonged operation time

To prevent any complications arising from the heating blanket, the blankets should be routinely checked, the alarm intervals should be adjusted appropriately, and alarm tones should be kept. The risk of povidone-iodine-related chemical burns is greater in areas of the body that are exposed to pressure. As a result of tissue trauma caused by the effects of pressure and damage to the protective skin barrier, PI-related chemical burn formation is facilitated (Chiang et al., 2011).

In the literature, there are cases in which the risk of PI-related chemical burns increases due to the pressure effect of the tourniquet used in orthopedic cases (Chiang et al., 2011), and there are cases with PI-related chemical burns in the skin areas exposed to pressure depending on the position in the lithotomy position (Donna et al., 2006). In a one-year-old male patient who underwent Nissen fundoplication, there is a case of contact dermatitis and burn-like lesions with excessive use of PI after four hours of operation (Kara et al., 2007).

Wet skin is also a risk factor for damage. In the operation area that remains wet, the PI solution continuously releases free iodine (it normally has a free iodine concentration of approximately 0.0001% at a stable concentration), and the released iodine molecules cause skin damage (Zamora, 1986). The patient under anesthesia does not respond to painful stimuli caused by skin damage. The local epidermal lipid layer, which is the only defense barrier, weakens with the agents used preoperatively, especially alcohol. Alcohol deesterizes the skin, thus increasing the chemical damage inflicted (Nahlieli et al., 2001).

If the PI solution is planned to be used in surgical area cleaning, the skin should be wetted with water first, and 1ml PI should be used for each 125-200 cm area. It is then recommended to allow the skin to dry (Ellenhorn et al., 2005).

In our cases, the areas cleaned using PI were the lower abdomen, upper abdomen and perineal area. Lesions were seen in areas that included these parts of the body but were more exposed to body pressure. Before the wiping process, no measures were taken to prevent povidone-iodine ponding, the amount used was not taken care of, and the skin was not allowed to dry before sterile dressing. With the adjustment of the alarms of the heaters, burns due to technical problems were ruled out, but the possibility of the lesions caused by the pooling of batticon due to the heating effect and pressure of the body may increase.

Although burns usually develop right after the operation, there are also cases detected the next day (Vandergriff et al., 2006). When conservative treatment is applied, recovery is usually observed with scar tissue after three to four weeks (Donna et al., 2006).

Postoperative skin reaction risk should always be kept in mind in patients who will be taken to the recovery unit at the end of the operation, and the patients should be checked in this regard. PI, which is widely used in operating rooms, is an effective antiseptic agent, but it has side effects, including severe skin reactions. A careful approach to the patient is required to prevent this complication, which can be avoided with simple precautions.

Conflict of interest

The authors declared no conflicts of interest concerning the authorship and/or publication of this article.

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Case Report

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Coexistence of gastrointestinal stromal tumor of the stomach and small bowel adenocarcinoma: A case report

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Abstract

The coexistence of gastrointestinal tumors (GIST's) with other several primary malignant neoplasms has been demonstrated. However, there is no study that reported simultaneous occurrence of primary small bowel adenocarcinoma (SBA) and a GIST located in the stomach. Here, we report the first case of SBA and a synchronous gastric GIST. Surgical resection and clinicopathological characterization were performed for the masses identified through endoscopic and radiologic studies. Computed tomography scan showed a 13 mm mass located at the gastric antrum and another mass lesion partially occluding the jejunum. A biopsy specimen was obtained from the mass at the proximal jejunum by enteroscopy. The tumor was reported as adenocarcinoma. The patient was operated by the general surgery team. The features of the submucosal lesion resected from the antrum were consistent with a diagnosis of a GIST which contained spindle cells and showed positive staining with CD-117 and CD-34. Mitosis was not observed, and Ki 67 index was below 10%. This is the first case of coexistence of GIST of the stomach and SBA to be reported in English-language literature. The prominent characteristics of our case include its antral localization, male sex, absence of mitosis and incidental diagnosis.

Keywords: GIST, small bowel adenocarcinoma, gastric tumor, submucosal lesion

1. Introduction

Gastrointestinal stromal tumors are the most prevalent mesenchymal tumors of the gastrointestinal tract (Corless et al., 2011). Approximately one-third of GIST's are discovered during investigations and treatment procedures undertaken for other disorders (Agaimy et al., 2006). GIST's have been shown to coexist with other type of cancers either synchronously or metachronously (Gonçalves et al., 2010). There are studies which reported the concurrent occurrence of a GIST originating from the small intestine and gastric carcinoma (Karahan et al., 2013).

However, the coexistence of a primary SBA with a GIST located in the stomach has not been reported previously. Here, we report the first case of coexistence of a surgically resected SBA with a gastric GIST incidentally detected in a patient with nausea and vomiting and discuss it in light of clinical, radiological, endoscopic and histopathological findings.

2. Case report

A 79-year-old patient presented with a history of aggravating nausea, vomiting and abdominal bloating in the last 3-4 months. His personal history included essential hypertension. During physical examination, abdominal guarding was not observed, and bowel sounds were normoactive.

Laboratory investigations showed the following results: Haemoglobin 13.40 g/dL, White Blood Cell count 7080/ µL and CRP <3.36 mg/L. Computed tomography (CT) scan revealed a round shaped, heterogeneous mass lesion with a diameter of 13 mm in the posterior wall of gastric antrum which showed a marked contrast uptake and slight protrusion out of the wall and into the lumen (Fig. 1). During upper gastrointestinal endoscopy, a submucosal lesion was detected at the prepyloric antrum. Double balloon enteroscopy showed a mass lesion obstructing the lumen at the proximal jejunum and biopsy specimens were obtained (Fig. 2). Adenocarcinoma was diagnosed by pathological examination (Fig. 3).

The patient was operated by the general surgery team. Histopathological examination showed that the submucosal lesion resected from the antrum was consistent with a gastrointestinal stromal tumor composed of spindle cells. The tumor showed diffuse and strong positive staining with CD-117 and CD-34 and no staining with SMA, Desmin and S100 (Fig. 4). Mitosis was absent and Ki67 index was below 10 percent. The mass obtained from the proximal of jejunum was

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2.3 x 2 x 1.7 cm in size. As a result, the tumor was reported as pT3N1Mx, well-differentiated small bowel adenocarcinoma.

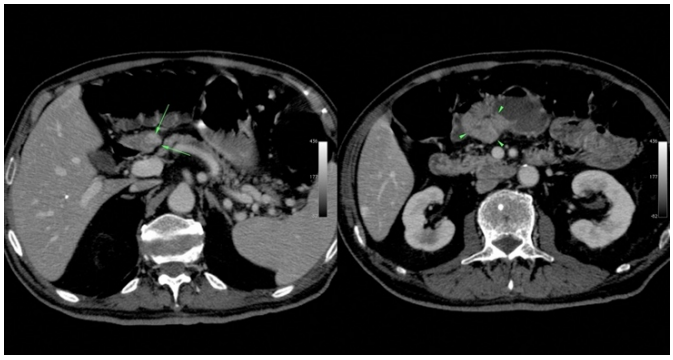

Fig. 1. A solid, round shaped, well-defined mass (arrows) located in the gastric wall and another mass (arrow heads) that resulted in symmetrical thickening of the wall at the level of small bowel in contrast-enhanced sections as shown by abdominal computed tomography

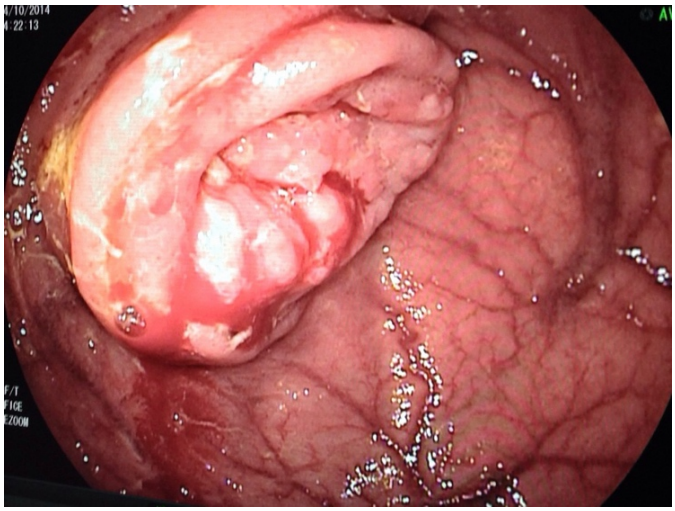

Fig. 2. Double balloon enteroscopy showed a mass lesion obstructing the lumen at the proximal jejunum

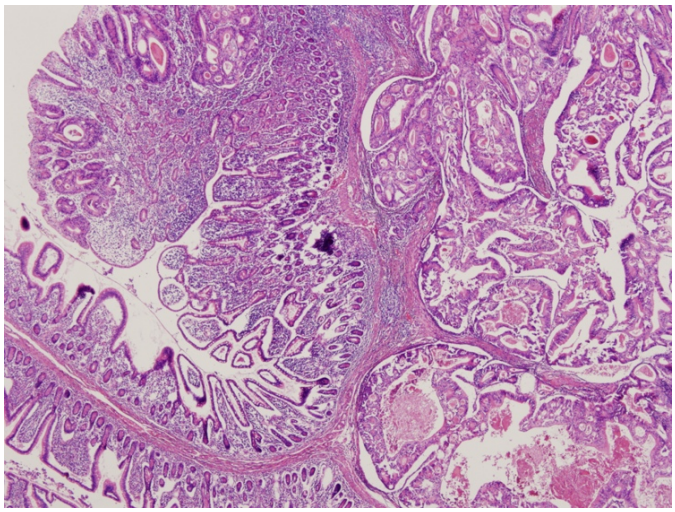

Fig. 3. A well differentiated adenocarcinoma resected from the small bowel (H-Ex40)

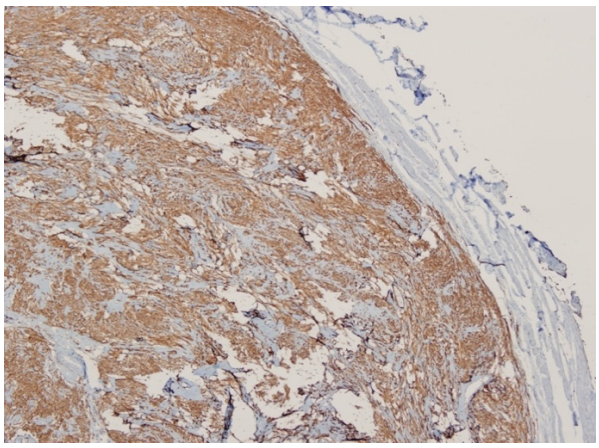

Fig. 4. Immunohistochemical CD-117 positivity shown in a gastrointestinal stromal tumor located in the stomach

3. Discussion

Stromal and mesenchymal tumors typically affect gastrointestinal tract and are commonly located in the stomach (50-60%) and small bowel (30-35%). However, they may occur anywhere along the digestive tract including mesentery, omentum and peritoneum (Corless et al., 2011; Joensuu et al., 2013).

The most common symptoms include bleeding into the intestine or abdominal cavity, anemia and abdominal pain as well as dyspepsia, nausea, vomiting, constipation or diarrhea, frequent urination and fatigue (Mucciarini et al., 2007; Caterino et al., 2011; Bümming et al., 2006).

Morphologically, GIST's are categorized into three groups as spindle cell, epitheloid and mixed by pathological examination (Kindblom et al., 1998; Fletcher et al., 2002). KIT (CD-117) and anoctamin (ANO1) are the two markers with greatest sensitivity and specificity for GIST's (Sarlomo-Rikala et al., 1998; West et al., 2004). Five percent of GIST's are negative for KIT expression but most of these express ANO1 (Medeiros et al., 2004). Masses suspected or diagnosed to be GIST should be removed if they are 2 cm or greater in size. However, smaller tumors may be excised or followed endoscopically at intervals of 6 to 12 months. Complete (R0) resection of GIST's is the primary goal of surgical treatment (Hohenberger et al., 2010).

Primary small bowel malignancies are rare tumors and account for 1-3% of gastrointestinal tumors (Gill et al., 2001). Malignancies that occur in the small bowel include carcinoid tumors, adenocarcinoma, lymphoma and sarcoma (Bilimoria et al., 2009).

Symptomatic tumors usually present at an advanced stage and 50% of cases show metastasis (Talamonti et al., 2002). SBA represents 33% of primary small bowel cancers, most commonly affecting duodenum followed by jejunum and ileum (Bilimoria et al., 2009).

In recent years, an increase has been seen in the diagnosis of GIST with synchronous malignancies mainly as a result of

advances in imaging techniques. Congenital genetic defects, exogenous factors, common pathophysiology and underlying immune suppression have all been implicated to contribute to the development of double primary neoplasms (Dasanu et al., 2011).

We did not identify any articles published in English-language literature that reported on the coexistence of gastric GIST and small bowel adenocarcinoma through PubMed search. In this regard, our case is the first such case to be reported. The lesions were visualized radiologically and endoscopically and characterized by histopathological examination of surgically resected materials. There are several case reports published in literature describing coexistence of GIST with malignancies including gastric carcinoma, gastric lymphoma, colon carcinoma, pancreatic carcinoid tumor and pancreatic adenocarcinoma (Agaimy et al., 2006). These publications reported low mitotic activity in GIST's coexisting with synchronous and other malignancies (Maiorana et al., 2000).

Contrastingly, GIST located in the stomach did not show mitotic activity in our case. The prominent characteristics of our case include its antral localization, male sex, absence of mitosis and incidental diagnosis. As known, gastric localization is frequent among GIST's. However, it should be borne in mind that, although rarely, other primary malignancies located in the gastrointestinal tract may occur concomitantly with GIST.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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A rare case: Renal cell carcinoma metastasis to lower lip

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Abstract

Approximately 15% of renal cell carcinomas metastasize to the head and neck region. Here in, we report a rare case report of a patient who underwent nephrectomy for renal cell carcinoma (RCC) ten years ago and presented with metastatic renal cell carcinoma on her lower lip. A 65-year-old woman presented with a rapidly growing mass on the lower lip. Pathology report resulted in renal cell carcinoma metastasis. Although metastatic renal cell carcinoma to the head and neck is uncommon, metastasis should be considered in the differential diagnosis of a rapidly growing vascular lesion in the head and neck area of patients with renal cell carcinoma.

Keywords: lip, metastasis, renal cell carcinoma, hemorrhage

1. Introduction

Renal cell carcinoma (RCC) is the most common malignant tumor of the kidney, typically affects men between the ages of 30 to 60 years (Gottlieb and Roland, 1998). Renal cell carcinoma usually metastasizes to the lungs, bones, and regional lymph nodes and rarely to the head and neck region (Som et al., 1987). Approximately 15% of RCC metastasis is observed in the paranasal sinuses, larynx, jaws, temporal bones, thyroid gland, and parotid glands (Som et al., 1987). We presented a patient with RCC metastasis in the lower lip who underwent a nephrectomy for RCC previously.

2. Case report

A 65-year-old woman presented with a rapidly growing and intermittently bleeding lower lip mass for three weeks. Ten years ago, the patient underwent a right nephrectomy because of RCC. The patients received chemoradiotherapy three years ago due to lung metastasis on the left side, but treatment was discontinued after the patient was diagnosed with tuberculosis. The physical examination revealed a 2x1 cm hemorrhagic, ulcerated mass on the lower lip' s left side. The mass was excised totally under local anesthesia. Histological examination via immunohistochemical staining identified the tumor as metastatic RCC (Fig. 1 and 2). The closest surgical margin distance of the tumor was 7 mm. Twenty days after the surgical resection patient's general condition was impaired, he was brought to emergency department with unconsciousness. Multiple intracerebral metastases were detected in the brain magnetic resonance imaging, and the patient died in a month.

3. Discussion

RCC, also known as hypernephroma, is the most frequent urological malignancy in adults and has a male predominance (Gottlieb and Roland, 1998). It constitutes 3% of adult malignancies and 90%–95% of neoplasms arising from the kidney (Gottlieb and Roland, 1998). Distant metastases commonly occur in 30%-40% of patients (Pritchyk et al., 2002). The most frequent metastasis sites are the lungs (76%), regional lymph nodes (66%), bone (42%) and liver (41%) (Pritchyk et al., 2002).

RCC is the third most common infraclavicular neoplasm that metastasizes to the head and neck following breast and lung carcinoma (Pritchyk et al., 2002). Approximately 15% of RCC patients have extracranial head and neck metastases (Gottlieb and Roland, 1998). According to the literature, the rare RCC metastasis in head and neck regions include salivary glands, mandible, maxilla, nasal cavity, paranasal sinuses, nasopharynx, palate, orbita, thyroid, and parathyroid glands (Airoldi et al., 1995; Sabo et al., 2001; Pritchyk et al., 2002; Lang et al., 2003; Iesalnieks et al., 2007).

Lips are unusual sites for any metastasis development. A few cases of metastasis to the lips from the lung, gastric adenocarcinoma, breast, lymphoma, and kidneys had been reported previously (Pritchyk et al., 2002; Jatti et al., 2015). Also, intraoral RCC metastases affecting the tongue, palate, buccal mucosa, upper and lower lips, gingiva, and floor of the mouth had been reported previously (Susan et al., 1979; Corsi

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et al., 1994; Airoldi et al., 1995; Ficarra et al., 1996; Pritchyk et al., 2002; Lang et al., 2003; Makos and Psomaderis, 2009). Metastases of RCC are vascular and tend to bleed (Ishak et al., 2010). A clinic biopsy of the lesion could result in uncontrolled hemorrhage (Pritchyk et al., 2002). It was reported that solitary RCC metastatic lesions result in 41% survival at two years and 13% survival at five years, regardless of the time interval between nephrectomy and metastasis (Gttlieb and Roland, 1998). Renal cell carcinoma is traditionally described as a radioresistant tumor.

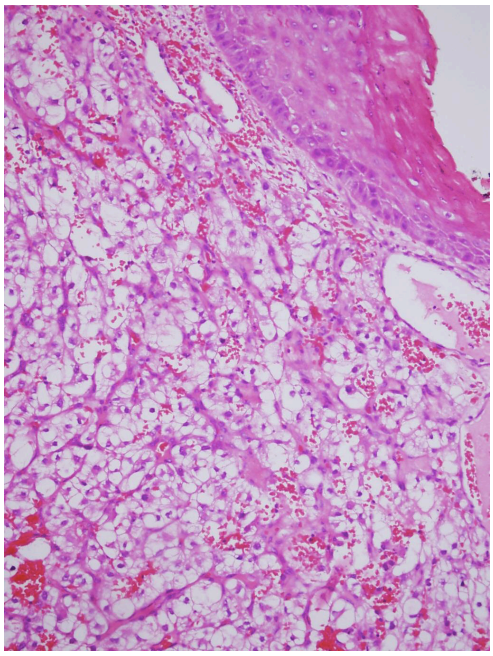

Fig. 1. Solid nest of epithelial cells with clear cytoplasm and small, round hyperchromatic nuclei under mucosa (H&Ex200)

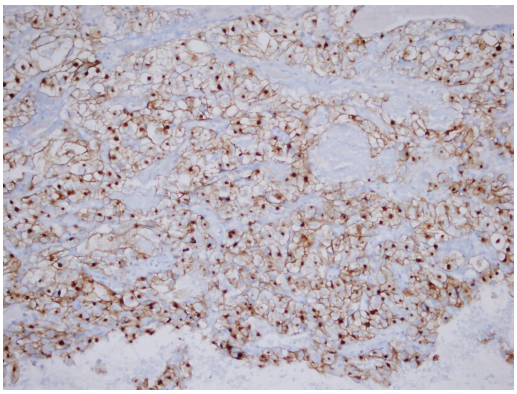

Fig. 2. Tumor cells are positive CD10 (DABx200)

Other forms of treatment have been advocated for metastatic RCC. Immunotherapy with interferon-α and interleukin-2 has been the mainstay of treatment for people with advanced and metastatic RCC. Recent advances in understanding the biology and genetics of RCC have led to several novel target approaches with higher response rates. Treatment with sunitinib, sorafenib, bevacizumab, erlotinib, and CCI-779 provided significant results regarding progression-free survival (Thompson Coon et al., 2009).

Renal cell carcinoma is the third most common infraclavicular neoplasm to metastasize to the head and neck regions. In patients with RCC, metastasis should be considered in the differential diagnosis of rapidly growing vascular lesions in the head and neck area. A biopsy of the lesion could result in uncontrolled hemorrhage. Resection of the lesion with wide margins is the recommended treatment method instead of incisional biopsy due to possible uncontrollable hemorrhage.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Endoscopic vacuum assisted closure of gastrocutaneous fistula after sleeve gastrectomy combined with fibrin sealant

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Abstract

Treatment of gastrointestinal fistulas after staple line leaks is difficult and non-invasive procedures such as endoclips, stents or endoscopic vacuum assisted closure (E-VAC) are sometimes not adequate. Fibrin sealants (FS) may promote healing, although today are used mainly to prevent anastomosis dehiscence within digestive tract. The authors present a 37-year-old female patient who developed a chronic gastrocutaneous fistula after sleeve gastrectomy for severe obesity treated successfully with a combination of E-VAC and a fibrin sealant. Long term peritoneal drainage, total parenteral nutrition, and antibiotic therapy for the next six weeks failed to close the leakage. Ultimately the fistula was closed after 6 changes of E-VAC and final implementation of a fibrin sealant. The duration of treatment was 81 days, including 34 days of E-VAC treatment and FS application. Complete closure of the fistula was visualized on gastroscopy six weeks after the treatment.

Keywords: E-VAC, E-NPWT, tissue sealant, fibrin glue, gastrocutaneous fistula

1. Introduction

Anastomotic leakage after bariatric surgery remains the most severe and a life-threatening complication related to high failure rate. Fistulas resulting from leaks can penetrate into the peritoneum, pleura or other organs and therefore they are a challenge to surgeons (Burgos et al., 2009; Campanile et al., 2013). The priority is effective drainage supported by endoscopic procedures, correction of fluid and electrolyte disturbances, and treatment of sepsis with appropriate antibiotics (Csendes et al., 2005; Campanile et al., 2013). The endoscopic interventions consisting of stent implantation or endoscopic vacuum assisted closure (E-VAC) showed their effectiveness in mild and early leakages (Borejsza-Wysocki et al., 2015; Hwang et al., 2016).

The authors present a patient with a successfully treated gastrocutaneous fistula following anastomotic rupture. That two-stage strategy based on the initial retraction of the fistula using an endoluminal vacuum dressing and then its final closure with fibrin tissue glue. The therapy described was performed in accordance with the principles of medical ethics and good medical practice. Patients provided informed written consent prior to all medical procedures and for the publication of this report.

2. Case report

A 37-year-old female patient was admitted to the authors' surgery clinic due to a late gastric leakage in the form of a gastrocutaneous fistula after a bariatric procedure and after ineffective conservative treatment lasting over six weeks. In February 2019, the patient underwent sleeve gastrectomy for severe obesity and then in five days she required a reoperation due to a subphrenic abscess. During relaparotomy the anastomosis was not visualized due to adhesions and fibrin deposits. Therefore, the peritoneal cavity was only irrigated with a normal saline solution and a drain under the diaphragm was left. In the next two days discharge consisting of pus and gastric content appeared in the drain and Gastrografin swallow confirmed staple line dehiscence close to the cardia. However, despite the drainage of anastomotic dehiscence, total parenteral nutrition, and appropriate antibiotics, a leak in an amount of 200 ml of gastric content and saliva remained. After admission of the patient to the hospital the endoscopic assessment revealed disruption of the esophagogastric anastomosis transforming into a fistula with a diameter less than 1cm (Fig. 1).

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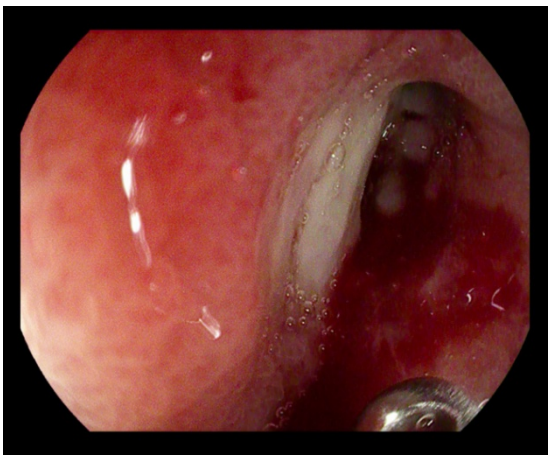

Fig. 1. Dehiscence of the anastomosis with a gastrocutaneous fistula in the suture line

After decision to apply E-VAC therapy a nasogastric catheter wrapped at the tip with a polyurethane foam (Vivano® Med Foam), with a diameter less than the dehiscence and a length of about 2 cm, was introduced with endoscopic measures into the gastrocutaneous fistula filling it almost completely (Fig. 2).

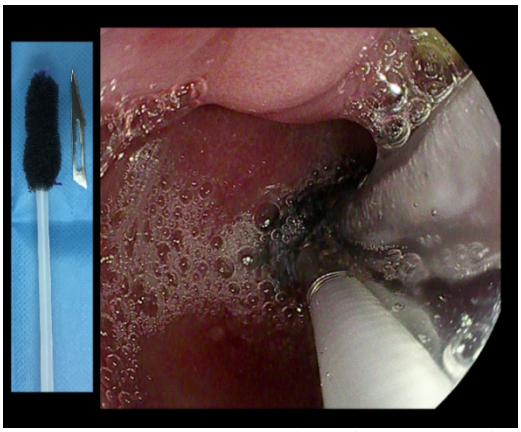

Fig. 2. E-VAC insertion: a nasogastric catheter wrapped with a polyurethane sponge is placed into the gastrocutaneous fistula canal

That catheter was connected with a vacuum generator (Renasys EZ Plus, Smith & Nephew Medical, Hull, UK), and a stable negative pressure of 70 mm Hg was maintained throughout the treatment. E-VAC was changed six times every fifth or sixth day, and during each change either the diameter or length of the sponge was reduced. As a result, shrinkage of the fistula was achieved, and its lumen filled with a fresh granulation tissue. Simultaneously with vacuum therapy a high-pressure Redon drainage of the fistula through the external opening was applied. Thus, after five weeks of treatment, gastric secretion was reduced from around 200 ml per day on admission to 15-20 ml. However, further E-VAC therapy did not result in complete closure of the fistula and still a small amount of discharge maintained. The presence of leakage was confirmed by air passage during endoscopy and an outflow of orally administered dye (Fig. 3). For this reason, seven days after changing the last vacuum dressing, the decision was made to close the fistula with a fibrin sealant (Tisseal, Baxter AG, Viena, Austria).

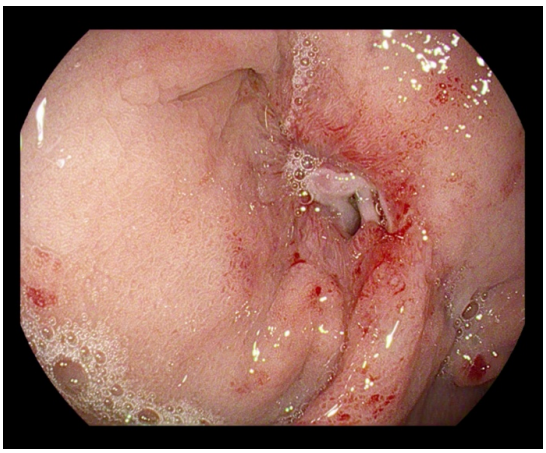

Fig. 3. Endoscopic evaluation after completed E-VAC therapy. Fistula channel significantly reduced and filled with a granulation tissue

A commercially available kit consisted of freeze-dried fibrinogen and aprotinin solution and freeze-dried thrombin and calcium chloride in a pre-filled syringe. After preparation according to the manufacturer's instructions the sealant, in the amount of 2 ml, was introduced into the lumen of the fistula through a single use polyurethane naso-gastric tube (Flocare® PUR ENFit Naso-Gastric Tube), which was previously inserted into the fistula canal (Fig. 4).

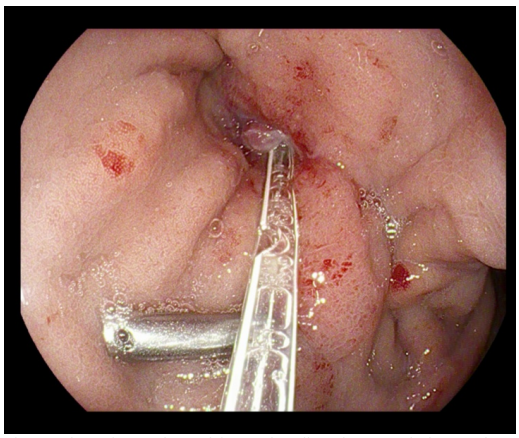

Fig 4. Fibrin sealant introduced into the fistula canal through a nasogastric tube

The conservative surgical treatment was combined with enteral immunomodulatory nutrition through a nasojejunal tube, enriched with L-glutamine, L-arginine and medium chain triglycerides to prevent the patient from hypoalbuminemia and lymphopenia. Basal metabolic rate was evaluated according to European Society for Parenteral and Enteral Nutrition (ESPEN) including anthropometric grade and laboratory tests e.g.: BMI 32, albumin serum range 2.88 g/dL, lymphocyte blood count 0.77 K/uL and TIBC 210 ug/dl. Finally, fistula was completely healed in 81 days, including 34 days of E-VAC treatment combined with final application of fibrin sealant. Patient was put on oral fluids second day after sealant application, and on normal diet the third day after application. Discharge through the Redon abdominal drain ceased just after the sealant introduction and it was removed following ultrasonographic assessment on the third day after the procedure. Endoscopic examination performed in 6 weeks

from sealant application showed complete healing of the fistula (Fig. 5). Weight loss of the patient, from sleeve gastrectomy to the fistula healing, was estimated on 18 kg (14% of body weight).

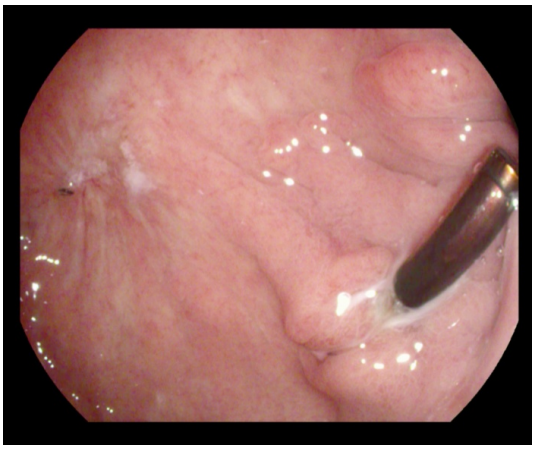

Fig. 5. Follow-up gastroscopy: assessment of the anastomosis line revealed a small scar at the site of the previous fistula

3. Discussion

The rate of leakage after sleeve gastrectomy is estimated from 0% through 20% (Márquez et al., 2010), whereas mortality because of it varies between 0,12 and 0,14% (Chang et al., 2018). The type of leakage and dedicated therapy is determined according to the Csendes et al. (1990) and Burgos et al. (2009) classification dedicated to leaks after gastric surgery or classification of anastomotic leaks within the esophagus according to Consensus for Defining and Reporting Complications After Esophagectomy reached by Esophagectomy Complications Consensus Group (Low et al., 2015). As a result, leak can be classified according to the time of onset, clinical presentation, location, and radiologic signs. Both in type I according to Csendes and in type II according to ECCG, either a stent implantation or endoscopic vacuum assisted closure (E-VAC) is recommended, and supported by percutaneous drainage of pus collections (Low et al., 2015). E-VAC is today a recognized method for the treatment of dehiscences within the digestive tract, showing even its higher effectiveness compared to stenting (Hwang et al., 2016). In turn, application of fibrin sealants for the treatment of digestive tract dehiscences are of experimental nature, and it has been reported in casuistic cases so far. Fibrin sealant was successfully applied according to Papavramidis for gastrocutaneous fistulas after vertical gastroplasty (MacLean procedure) in two patients, and after bilo-pancreatic diversion with duodenal switch (Marceau technique) in three patients. That sealant was introduced in a few sessions via a double-lumen catheter passed through the biopsy channel of a gastroscope (Papavramidis et al., 2008). The primary indications for the use of fibrin sealant are both hemostasis and sealing. It supports the end stage of clotting process as well as promotes the growth of fibroplasts and endothelial cells. As a result, a fresh granulation tissue is formed, rich in collagen and an extracellular matrix that fills tissue defects and deep interstitial spaces. (Limura and Giordano, 2015; Mullens et al., 2019). Due to reduction of drainage volume and edema FS is widely used in reconstructive and plastic surgery as a factor supporting wound healing and fixation of skin grafts (Langer et al., 2015). In gastrointestinal surgery, it has been used since the 1990s mainly to close anal fistulas and to enhance intestinal anastomoses. Tisseal is also beneficial in endoscopy, especially in the treatment of GI fistulas, limited perforations or early anastomotic ruptures (Yang and Chen, 2015). Although, the opponents indicate that FG promotes inflammation and reduces bacterial phagocytosis shrinking, low-out put GI lesions can be successfully treated. (Rotstein et al., 1986; Nordentoft et al., 2015).

In conclusion the presented case shows that E-VAC combined with fibrin sealant application and Redon drainage through the external orifice improve the healing process of an anastomosis dehiscence within the digestive tract. That procedure can be indicated as a treatment of choice for intermediate and late dehiscences and fistulas within the digestive tract. That method however requires further prospective studies in a larger number of patients.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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Case report

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Rare presentation of Crohn's Disease: Massive lower gastrointestinal bleeding

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Abstract

While mild gastrointestinal bleeding is common in Crohn's disease, massive gastrointestinal bleeding is extremely rare. In this case, we present a Crohn's patient who presented with massive lower gastrointestinal bleeding. A literature review of such a rare presentation in Crohn's disease will be discussed.

Keywords: Crohn's disease, bleeding, endoscopy, inflammation

1. Introduction

Crohn's disease (CD) is a chronic inflammatory disease that can affect any part of the gastrointestinal tract from mouth to anus (Veauthier and Hornecker, 2018). Common intestinal complications of Crohn's disease are bleeding, fistula, obstruction and malabsorption

Severe lower gastrointestinal bleeding is a rare but life threatening complication of Crohn's disease. In this setting, bleeding is usually originate from ulcerated areas in colon or intestines (Cirocco et al., 1995). Treatment of the CD is important for mucosal healing and preventing recurrent bleeding. We present a case with massive gastrointestinal bleeding diagnosed with Crohn's disease and successfully treated with prednisolone and azathioprine.

2. Case report

A 21 years old male patient referred to our clinic with lower gastrointestinal bleeding and syncope. The bleeding was first started 14 days ago and he admitted to an emergency department. As hemoglobin levels decreased from 13 g/dl to 8g/dl, he was given three units of erythrocyte suspension. After ressuscitation, esophagogastroduodenoscopy (EGD) and colonoscopy were performed. Both tests were normal and no signs of active bleeding observed. In follow up, hemoglobin levels were stable and bleeding did not recur. So, he was discharged with advice to contact with an advanced center to investigate the bleeding focus.

At the admission, there were no complaints except for mild weakness and occasional constipation. He was not defining any abdominal pain, diarrhea or weight loss. In the past medical history, there was no chronic illness, medication, smoking or alcohol use. Family history was only significant for Crohn's disease in a cousin. There was no lymphadenopathy or organomegalia in physical examination. Rectal examination was also normal. Complete blood count, metabolic panel and liver function tests were performed. Hb level was 12.5 g/dl and hematocrit level was 36%. Erythrocyte sedimentation (ESR) rate and C-reactive protein (CRP) levels were slightly elevated (ESR was 30mm/h and CRP was 10mg/dl). Other tests were normal. Stool examinations were also performed. Microscopic examination and stool cultures were normal. Fecal occult blood test was positive for blood. Fecal calprotectin level was 100 µg/g.

Capsule endoscopy (CE) was performed as the bleeding site could not be detected by EGD and colonoscopy. In the capsule imaging, edematous mucosa with erosions and oozing hemorrhage were seen in the terminal ileum (Fig. 1). Later, colonoscopy and abdominal ultrasonography (USG) were performed to understand the extent of the disease and to take samples for pathologic examination. In the USG, 20 cm segment wall thickening was observed in proximal terminal ileum. In the colonoscopy, a millimeter-sized ulcer was observed on ileocecal valve. Terminal ileum through the ileocecal valve to proximal 50 cm was examined. Distal 40 cm segment of the ileum mucosa was edematous with erosions and milimetric ulcers (Fig. 2). Hypertrophic villis, exudation and "micro cobblestone sign" was prominent in some areas. Multiple samples were taken for tuberculosis and histological examination. Active chronic ileitis with focal involvement and no granuloma was shown in the pathologic examination. Quantiferon test was also performed to exclude tuberculosis

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and it was negative. The findings were consistent with the CD. Prednisolone 40 mg/day and azathioprine (AZA) 50mg/day treatment was started. In two months of follow up, bleeding did not recur and hemoglobin levels were normal.

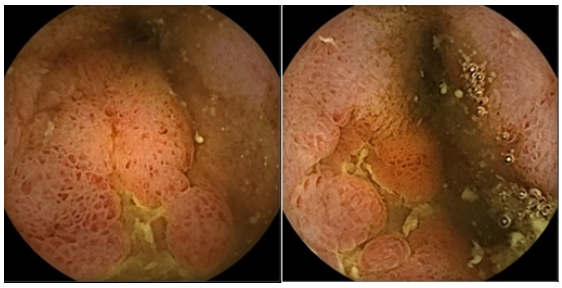

Fig. 1. Capsule endoscopy images

3. Discussion

Presentation with massive bleeding in CD is rare and is detected in only 0.9% to 2.5% of cases (Cirocco et al., 1995). Bleeding due to Crohn's disease was first reported in 1941 (Fallis, 1941). In CD with isolated small intestine involvement, the bleeding rate is 23.5%, while in cases with colon involvement, the bleeding frequency is higher (85%) (Belaiche et al., 1999).

Massive gastrointestinal bleeding is defining as clinical evidence of (1) hemorrhagic shock, (2) syncope related to hematemesis or melena, (3) continued blood loss during the period of one week at no time sufficient to cause syncope or shock but enough to cause a hematocrit reading below 25% and a hemoglobin concentration below 8 g/dl (Atik and Simeone, 1954). So that, hemoglobin decrement of the patient with syncope in the presentation is defined as massive gastrointestinal bleeding. In our patient, there was a syncope history with gastrointestinal bleeding and hemoglobin decrease at the first admission.

Source of bleeding site in our case was not found with EGD and colonoscopy at first. CE is a diagnostic option when the source of bleeding was not found with EGD and colonoscopy have been negative (Pasha et al., 2009). We found the bleeding site in the terminal ileum with capsule endoscopy. Later, we evaluated the ileum with abdominal USG. USG allows early determination of intestinal complications such as abscesses and stenosis, as well as determining the location and size of lesions in CD (Calabrese, 2011). We detected wall thickening in a terminal ileum with USG. We repeated the colonoscopy in order to evaluate this site, as it was possible to reach it by colonoscopy. Colonoscopy findings were consistent with Crohn's disease. Biopsy from the ileum revealed active chronic ileitis with focal involvement, and the patient was diagnosed with Crohn's disease. As they are in the differential diagnosis of the CD, tuberculosis is excluded with negative quantiferon test, intestinal infections are excluded by stool microscopy and culture. There was also no history of non-steroid antinflammatory drug use and no sign of rheumatologic disease in questionnaire and physical examination.

Corticosteroids, azathioprine, and infliximab can be used to reduce the risk of acute bleeding for CD with acute lower gastrointestinal bleeding (Barnacle et al., 2006). Besides medical treatment, embolization therapy can also be effective (Lee et al., 2020). Surgical resection can be performed in hemodynamically unstable patients who are unresponsive to medical treatment and embolization (Alos and Hinojosa, 2008). The recurrence rates of severe bleeding have been reported from 19%-41% (Podugu et al., 2016). In this case, the patient will be followed up with AZA treatment.

In conclusion, CD presenting with massive gastrointestinal bleeding is very rare. It should be kept in mind that Crohn's disease may cause massive bleeding in young patients, alongside diseases such as Meckel diverticulitis, tumors, Dieulafoy lesion, and polyposis. USG, which is well accepted by patients, noninvasive, and low cost, may be the first diagnostic tool, especially in young patients, as it can be used for preliminary diagnostic work-up prior to invasive tests

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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