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ORIGINAL ARTICLE

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Investigation of Sensitivity to AZD7762 in Triple-Negative Breast Cancer (TNBC) with *RBFOX2* Gene Expression as a Biomarker

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ABSTRACT

Objective: Triple negative breast cancer (TNBC) is one of the most metastatic, aggressive with poor prognosis types of breast cancers. There is currently no standard molecular-targeted treatment for TNBC. Therefore, new therapeutics should be developed. The aim of this study was to determine the effect of AZD7762 on several breast cancer cell lines and evaluate the *RBFOX2* gene expression levels as a marker to show sensitivity to this drug.

Materials and Methods: The cytotoxic effect of AZD7762 on breast cancer cell lines was determined by sulforhodamine B method. The expression levels of *RBFOX2* gene were determined by quantitative real-time polymerase chain reaction (qRT-PCR). The association between the IC50 values of the selected drug AZD7762 and *RBFOX2* expression levels was evaluated by using Pearson r correlation analysis.

Results: Although there was no significant difference between the IC50 values of TNBC and non-TNBC groups, it was determined that TNBC cell lines tended to be more sensitive to AZD7762. In addition, it was obvious that increasing levels of *RBFOX2* expression were detected in cells that showed more sensitivity to AZD7762.

Conclusion: It was concluded that the *RBFOX2* gene can be used as a biomarker to show AZD7762 efficiency. Further studies are needed to investigate the potential signaling mechanisms that are associated with the effect of AZD7762.

Keywords: Triple negative breast cancer, AZD7762, *RBFOX2*, biomarker

INTRODUCTION

Breast cancer was the most frequently diagnosed cancer type (2.26 million) among women worldwide in 2020. Moreover, according to a 2022 report, 290,560 new cases were diagnosed in USA resulting in roughly 43,780 deaths (1, 2). Breast cancer is also the most frequently detected cancer type among Turkish women, and according to Globocan 2020 data, 24,175 new cases were reported while the incidence was 46.6/100,000 (3). TNBC is a form of breast cancer in which the progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth receptor-2 (HER-2) are lacking. TNBC accounts for 15-20% of all invasive breast cancer patients (4). 13.1 per 100,000 new TNBC cases among women were reported in 2021 around the world (5). 10.6% (22,375) of breast cancer cases were determined as TNBC in 2018 in Turkey (6). Compared to other breast cancer subgroups, TNBC is characterized by its unique molecular profile, aggressive behavior, differential propensity for metastasis, and unsuitability for targeted therapies (7). A specific treatment approach against TNBC is not yet available. Therefore, different combinations of current chemotherapeutic agents and the same dose of ionizing radiation (IR) are used (8).

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AZD7762 is a check point kinase inhibitor. It is applied in combination with chemotherapeutic (gemcitabine, topotecan, irinotecan, cisplatin, paclitaxel) or radiotherapeutic agents that damage DNA (9-11). In addition, there are many studies that have tried to overcome treatment resistance in triple negative breast cancers that are resistant to chemotherapeutics or radiotherapy, and significant results have been achieved (12-15).

In this study, it was aimed to find a drug with a specific effect against TNBC and to define a potential biomarker to determine the effectiveness of this drug.

MATERIALS AND METHODS

In Silico Drug and Biomarker Selection

Garnett et al. have tested 130 anticancer drugs in 368 different cancer cell lines, such as breast cancer, renal cell carcinoma, pancreatic cancer, etc. (16). Among these, we selected 24 breast cancer cell lines and classified them as TNBC and non-TNBC, according to the clinicopathological classification of Kao et al. (17). Moreover, we compared IC50 values of these 130 anticancer drugs between these 24 TNBC and non-TNBC cell lines. To determine the marker gene, we performed correlation analysis between Garnett's gene expression data and IC50 values of selected drugs in 24 breast cancer cell lines.

Cell Culture

MDA-MB-231, MDA-MB-157, and BT-20 cell lines were used for TNBC trials, and T47D, MCF-7, and MDA-MB-453 cell lines were used for non-TNBC trials. Cell lines were kindly provided by Bilkent University, Department of Molecular Biology and Genetics. There was no need to obtain ethics committee approval.

DMEM was used for cell lines MDA-MB-231, MCF-7, BT-20, and MDA-MB-453; for MDA-MB-157 and T47D cell lines, media prepared with RPMI 1640, 1% penicillin/streptomycin antibiotic, and 10% FBS was used, and all cells were incubated at 37°C and 5% CO_2.

96-well plates were used for ionizing radiation (IR) and cytotoxicity tests, and optimization was performed to determine the number of cells to be seeded in each well. As a result, 6400 cells/mL for BT-20, 10000 cells/mL for MDA-MB-157, 8000 cells/mL for MDA-MB-231, 6000 cells/mL for MCF-7, 2500 cells/mL for MDA-MB-453 /mL, and 9000 cells/mL for T47D were seeded into the wells.

Ionizing Radiation (IR) Application

Eight 96-well plates were used for each cell line. Each plate was stained with sulforhodamine B (SRB) at time zero (24 hours after cell cultivation) and measured spectrometrically. One plate was used as a control group and incubated for 96 hours without any drug application. The remaining 6 plates were given 1Gy/1min IR 24 hours after the cells were seeded. A dose of 1 Gy causes less than 10% apoptosis in MCF-7 and MDA-MB-231 cell lines (18). Many studies stated that, to increase the effectiveness of AZD7762, which is a Chk1/2 inhibitor, it was necessary to give an IR or chemotherapeutic agent that creates DNA breaks (19-21).

AZD7762 Drug Application

Different drug concentrations (0.63µM, 1.25µM, 2.5µM, 5µM, 10µM, 20µM) were applied 24 hours after IR was given to 6 plates, and 72 hours after drug addition, they were stained with SRB together with the control group (no drug added) and their IC50 values were determined.

Cytotoxicity Assay (sulforhodamine B method) and IC50 Calculation

After drug and IR applications, fixation was performed with 10% trichloroacetic acid (TCA). After washing with distilled water, the plates were left to dry for 1 day to remove all the water. SRB dye dissolved with acetic acid was applied to each well and then washed with 10% acetic acid. The plates were then allowed to dry for a short time. Then, after 30 minutes of incubation with 300 mM Tris base in a shaker, OD 515 values were measured, and standard deviations were calculated.

RNA Isolation and cDNA Synthesis

RNA isolations were performed using TRIzol (Thermo Fisher Scientific, USA) 72 hours after application of IR and drug. Nanodrop (Thermo Fisher Scientific, USA) was used to assess RNA quality and quantity. cDNA synthesis was performed using 100 ng total RNA and iScript cDNA synthesis kit (Biorad, USA) according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA binding protein fox-1 homolog 2 (RBFOX2) gene expression levels were analyzed by qRT-PCR method using TaqMan Gene Expression Assays (Applied Biosystems (AB by life technologies)) in a Biorad T100 thermalcycler device. The relative expression levels of *RBFOX2* and the β-actin reference gene were calculated using the ΔΔCt method.

Statistical Analyses

TNBC and non-TNBC cell lines were classified by using a one way ANOVA test. Drug selection was performed by Student's t-test. Candidate biomarker gene was determined by Pearson r correlation analysis. All statistical calculations were done using Graphpad prism 5 (GraphPad Software, USA). The statistical significance of the data determined as $p<0.05$ was acceptable.

RESULTS

In Silico Drug and Biomarker Selection

AZD7762 and RDEA119 were found significantly more effective on TNBC than non-TNBC (p: 0.01 and p: 0.04, respectively). AZD7762 (AstraZenecaDrug 7762) was selected as a potential biomarker due to the more significant p-value.

As a result of the correlation analysis between the IC50 values of the AZD7762 of all breast cancer cell lines used by Garnett et al. (16), and the gene expression levels of these cell lines, the *RBFOX2* gene, which was found to be 70% inversely correlated with the IC50 values of AZD7762, was selected as a potential biomarker candidate gene (p: 0.02607).

RBFOX2 Gene Expression

For *in vitro* validation studies, the ΔΔCt method was used together with the Ct values of the beta-actin gene in order to calculate the expression value of the *RBFOX2* gene in cell lines according to the qRT-PCR results, and the relative quantification calculation is given in Figure 1. When the *RBFOX2* gene expression levels of the two groups: TNBC and non-TNBC were compared, no significant difference was found (p: 0.5947; Figure 1).

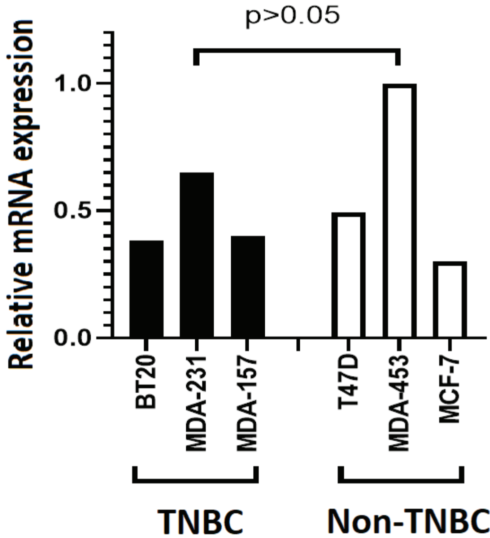

Figure 1. Relative quantification results of *RBFOX2* among TNBC and non-TNBC groups. TNBC: Triple negative breast cancer.

In addition, MDA-MB-231 and MDA-MB-453 cell lines were found to have the highest *RBFOX2* expression among both TNBC and non-TNBC groups. Significant differences in *RBFOX2* expression between two groups are shown in Figure 1.

SRB Cytotoxicity and IC50 Values

The drug concentrations of each cell line and the cytotoxicity values obtained after the SRB method and IC50 values were calculated. There was no statistically significant difference between the IC50 values of the cell lines in the TNBC and non-TNBC groups in our investigation (p: 0.6965; Figure 2). However, on average, a sensitivity to this drug was observed in TNBC cell lines.

Correlation Analysis

There was no significant correlation between *RBFOX2* expression levels and IC50 values in our study (p: 0.8101; Figure 3). However, as *RBFOX2* expression increases, drug resistance increases; in other words, there is an inverse correlation between IC50 and *RBFOX2*.

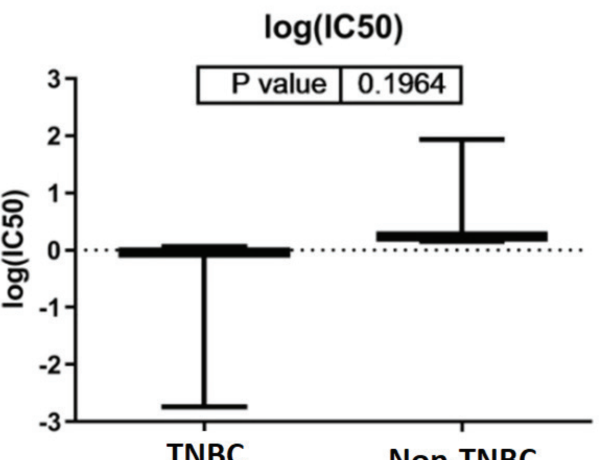

Figure 2. Comparison of log (IC50) between TNBC and non-TNBC groups. TNBC: Triple negative breast cancer.

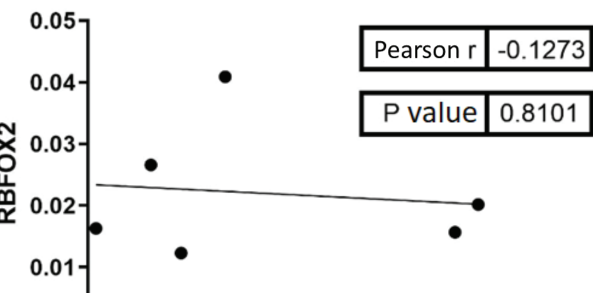

Figure 3. Correlation analysis results between *RBFOX2* gene expression and IC50 values. TNBC: Triple negative breast cancer, *RBFOX2:* RNA binding fox-1 homolog 2, IC50: half maximal inhibitory concentration.

DISCUSSION

DNA breaks created by ultraviolet (UV) and chemotherapeutic agents used in cancer treatments lead the cell to apoptosis. However, breast cancer and especially TNBC cells develop resistance to chemotherapeutic agents. They provide this resistance by evading checkpoints and by DNA repair. Therefore, in order to break drug resistance in TNBC, that is, to increase sensitivity to chemotherapeutic agents and UV, it has been an important strategy recently to eliminate kinase activity in these control contacts and direct the cell to apoptosis without giving DNA repair opportunity. Thus, when Chk1/2 inhibitors are used in combination with agents that cause DNA damage (radio and chemotherapeutic agents), they increase the anti-tumor effect and response. The drug AZD7762 is also a Chk1/2 inhibitor and, therefore, has an anti-tumor effect (22-25).

In many studies, it was shown that AZD7762 in combination with gencitabine or cisplatin in solid tumors, urethral carcinoma, lung carcinoma, and osteocarcinoma causes increased sensitivity to radiation or chemotherapeutic agents by inhibiting the G2/M transition, thus promoting apoptosis and mitotic catastrophe. In addition, AZD7762 has been shown to inhibit growth in radiotherapy-resistant breast cancer cells (21, 25-29).

Moreover, TNBC frequently harbors *TP53* mutations, resulting in the loss of the G1 checkpoint, thereby relying on Chk1 to recruit cells in response to DNA damage. Therefore, agents targeting Chk1 might be more specific for TNBC in particular (16, 23, 26, 30).

Studies have been focused on indication of whether AZD7762 is a suitable therapeutic option that might be used in the treatment of TNBC, and that it might also be used as a biomarker at the expression level of pChk1 (s296), showing a therapeutic synergy when used in combination with gemcitabine (23, 26, 28, 30).

According to the literature, many studies have strong statements about AZD7762 that it is a candidate drug that can be used in combination in the treatment of breast cancer. However, associating the usability of this drug with a genetic marker has not yet been specified in the current literature. In this study, it was aimed to find a drug that has a specific effect on TNBC and to define a potential marker to determine the effectiveness of AZD7762 drug, which was predicted to be sensitive to TNBC cell lines, as a result of the analysis made using the raw data of Garnett et al. (16). In consideration of this study, the drug AZD7762, which is predicted to be sensitive to TNBC cell lines, was selected, and its cytotoxic effect on MCF-7, T47D, BT-20, MDA-MB-157, MDA-MB-231, and MDA-MB-453 cell lines was investigated. In addition, the expression levels of *RBFOX2*, which can be a candidate marker, was examined by correlation analysis.

The fact that *RBFOX2* is associated with TNBC cells in preliminary analysis and its expression is relatively higher in TNBC cells in the experimental results reveals the relationship of this gene with breast cancer cells. In breast cancer, the alternative splicing mechanism plays a very important role in tumor progression by the Endothelial mesenchymal transition (EMT) and is associated with metastasis (24-27). In early prognostic marker identification and association studies, it has been shown that *RBFOX2*, one of the alternative splicing factors, is associated with EMT (especially in the formation of EMT in breast cancer) (24-26). In a polymorphism study of *RBFOX2*, it was shown that *RBFOX2* is highly associated with estrogen negative (ER-) breast cancer subtype, and it has been reported that *RBFOX2* plays a role in the development and progression of breast cancer (27). According to this information, the *RBFOX2* gene was selected as the biomarker candidate in our study, which showed a 70% correlation with the IC50 values of the AZD7762 drug (p: 0.02607) as a result of our correlation analysis.

In our experiments, we applied IR to the cells. This is because Chk1/2 is a checkpoint kinase that is activated (triggered) by breaking the DNA by chemical or IR agents (28,29). For this reason, in order to show the effectiveness of AZD7762, which is a Chk1/2 inhibitor, the amount of IR that would not kill the cells but would cause DNA breakage was given (1 Gy/min), and the Chk1/2 kinase was activated (A dose of 1 Gy causes less than 10% apoptosis in MDA-MB-231 and MCF-7 cell lines (18)). Then, the Chk1/2 inhibitor, AZD7762, was added. Thus, the cells were sensitized to IR.

Although there was no significant difference in the IC50 values of the TNBC and non-TNBC groups in the validation studies (p: 0.6965), it was determined that TNBC cells tended to be more sensitive to AZD7762 compared to non-TNBC. In addition, although there was no significance in the correlation analysis between *RBFOX2* gene expression and IC50 values (p: 0.8101), a relationship between increased *RBFOX2* expression and decreased IC50 value was observed between TNBC and non-TNBC groups.

As a conclusion, although no significant results were found between cytotoxicity tests and gene expression levels in our study, it was determined that *RBFOX2* gene expression and IC50 values were different between the TNBC and non-TNBC groups, that TNBC cells tended to be more sensitive to the drug, and that *RBFOX2* gene expression was higher. Thus, the *RBFOX2* gene expression for TNBC was associated for the first time with AZD7762 activity in the preliminary analysis and experiments performed in our study.

There may be several reasons why *in silico*-detected relationships could not be verified *in vitro*. One of the most important reasons may be the transcript variants of the gene of interest in microarrays used for *in silico* studies can be different from those produced by qRT-PCR primers.

Studying with the various breast cancer cell lines could support the usage of *RBFOX2* gene as a biomarker to detect the effect of cancer drugs. Moreover, the protein levels of *RBFOX2* gene can be analyzed. Further studies are needed to detect the association between *RBFOX2* gene and applicability of AZD7762 to support our results.

Ethics Committee Approval: Ethics committee approval is not required because of no material or experimental animal that would require permission.

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REFERENCES

1. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, et al. Cancer statistics for the year 2020: An overview. Int J Cancer 2021; 149(4): 778-89.
2. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. CA Cancer J Clin.2022; 72(1): 7-33. [CrossRef]
3. World Health Organization, Globocan 2020 database, Turkey cancer statistics. (Available from: https://gco.iarc.fr/today/data/factsheets/populations/792-turkey-fact-sheets.pdf)
4. Karagoz Eren S, Arslan A, Akay E, Özhan N, Dönder Y. Assessing Clinicopathological features and prognosis of triple-negative breast cancer patients: A single-center study in Turkey. Arch Breast Cancer 2021; 8(4): 297-304. [CrossRef]
5. Landry I, Sumbly V, Vest M. Advancements in the treatment of triple-negative breast cancer: A narrative review of the literature. Cureus 2022; 14(2).
6. Odemis DA, Celik B, Erciyas SK, Erdogan OS, Tuncer SB, Gultaslar BK, et al. Evaluation of BRCA1/2 gene mutations in patients with high-risk breast and/or ovarian cancer in Turkey. Turk J Biochem 2022. [CrossRef]
7. Wahba HA, El-Hadaad HA. Current approaches in treatment of triple-negative breast cancer. Cancer Biol Med 2015; 12(2): 106-16.
8. Arslan C, Dizdar O, Altundag K. Pharmacotherapy of triple-negative breast cancer. Expert Opin Pharmacother 2009; 10(13): 2081-93. [CrossRef]
9. Ma CX, Cai S, Li S, Ryan CE, Guo Z, Schaiff WT, et al. Targeting Chk1 in p53-deficient triple-negative breast cancer is therapeutically beneficial in human-in-mouse tumor models. J Clin Invest 2012; 122(4): 1541-52. [CrossRef]
10. Isono M, Hoffmann MJ, Pinkerneil M, Sato A, Michaelis M, Cinatl J, et al. Checkpoint kinase inhibitor AZD7762 strongly sensitises urothelial carcinoma cells to gemcitabine. J Exp Clin Cancer Res 2017; 36(1): 1-12. [CrossRef]
11. Sausville E, LoRusso P, Carducci M, Carter J, Quinn MF, Malburg L, et al. Phase I dose-escalation study of AZD7762, a checkpoint kinase inhibitor, in combination with gemcitabine in US patients with advanced solid tumors. Cancer Chemother Pharmacol 2014; 73(3): 539-49. [CrossRef]
12. Bryant C, Rawlinson R, Massey AJ. Chk1 Inhibition as a novel therapeutic strategy for treating triple-negative breast and ovarian cancers. BMC Cancer 2014; 14(1): 1-14. [CrossRef]
13. Zhu J, Zou H, Yu W, Huang Y, Liu B, Li T, et al. Checkpoint kinase inhibitor AZD7762 enhance cisplatin-induced apoptosis in osteosarcoma cells. Cancer Cell Int 2019; 19(1): 1-11. [CrossRef]
14. Zhang Y, Lai J, Du Z, Gao J, Yang S, Gorityala S, et al. Targeting radioresistant breast cancer cells by single agent CHK1 inhibitor via enhancing replication stress. Oncotarget 2016; 7(23): 34688-702. [CrossRef]
15. Min DJ, He S, Green JE. Birinapant (TL32711) improves responses to GEM/AZD7762 combination therapy in triple-negative breast cancer cell lines. Anticancer Res 2016; 36(6): 2649-57.
16. Garnett MJ, Edelman EJ, Heidorn SJ, Greenman CD, Dastur A, Lau KW, et al. Systematic identification of genomic markers of drug sensitivity in cancer cells. Nature 2012; 483(7391): 570-5. [CrossRef]
17. Kao J, Salari K, Bocanegra M, Choi Y La, Girard L, Gandhi J, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. PLoS ONE 2009; 4(7): e6146.
18. Liu X, Sun C, Jin X, Li P, Ye F, Zhao T, et al. Genistein enhances the radiosensitivity of breast cancer cells via G2/M cell cycle arrest and apoptosis. Molecules 2013;18(11): 13200-17. [CrossRef]
19. Ma Z, Yao G, Zhou B, Fan Y, Gao S, Feng X. The Chk1 inhibitor AZD7762 sensitises p53 mutant breast cancer cells to radiation in vitro and in vivo. Mol Med Rep 2012; 6(4): 897-903. [CrossRef]
20. Zhu J, Zou H, Yu W, Huang Y, Liu B, Li T, et al. Checkpoint kinase inhibitor AZD7762 enhance cisplatin-induced apoptosis in osteosarcoma cells. Cancer Cell Int 2019; 19(1): 195.
21. Liu Y, Li Y, Wang X, Liu F, Gao P, Quinn MM, et al. Gemcitabine and Chk1 inhibitor AZD7762 synergistically suppress the growth of Lkb1-deficient lung adenocarcinoma. Cancer Res 2017; 77(18): 5068-76. [CrossRef]
22. Bennett CN, Tomlinson CC, Michalowski AM, Chu IM, Luger D, Mittereder LR, et al. Cross-species genomic and functional analyses identify a combination therapy using a CHK1 inhibitor and a ribonucleotide reductase inhibitor to treat triple-negative breast cancer. Breast Cancer Res 2012; 14(4): R109.
23. Park JS, Lee CH, Kim HK, Kim D, Son JB, Ko E, et al. Suppression of the metastatic spread of breast cancer by DN10764 (AZD7762)-mediated inhibition of AXL signaling. Oncotarget 2016; 7(50): 83308-18. [CrossRef]
24. Zhang Y, Lai J, Du Z, Gao J, Yang S, Gorityala S, et al. Targeting radioresistant breast cancer cells by single agent CHK1 inhibitor via enhancing replication stress. Oncotarget 2016; 7(23): 34688-702.
25. Fici P, Gallerani G, Morel AP, Mercatali L, Ibrahim T, Scarpi E, et al. Splicing factor ratio as an index of epithelial-mesenchymal transition and tumor aggressiveness in breast cancer. Oncotarget 2017; 8(2): 2423-36. [CrossRef]
26. Meng X, Yang S, Zhang J, Yu H. Contribution of alternative splicing to breast cancer metastasis. J Cancer Metastasis Treat 2019; 5: 21. [CrossRef]
27. Braeutigam C, Rago L, Rolke A, Waldmeier L, Christofori G, Winter J. The RNA-binding protein Rbfox2: An essential regulator of EMT-driven alternative splicing and a mediator of cellular invasion. Oncogene 2014; 33(9): 1082-92. [CrossRef]
28. Cen YL, Qi ML, Li HG, Su Y, Chen LJ, Lin Y, et al. Associations of polymorphisms in the genes of FGFR2, FGF1, and RBFOX2 with breast cancer risk by estrogen/progesterone receptor status. Mol Carcinog 2013; 52(SUPPL1): E52-9.
29. Oza V, Ashwell S, Almeida L, Brassil P, Breed J, Deng C, et al. Discovery of checkpoint kinase inhibitor (S)-5-(3-fluorophenyl)- N -(piperidin-3-yl)-3-ureidothiophene-2-carboxamide (AZD7762) by structure-based design and optimization of thiophenecarboxamide ureas. J Med Chem 2012; 55(11): 5130-42.
30. Mitchell JB, Choudhuri R, Fabre K, Sowers AL, Citrin D, Zabludoff SD, et al. In vitro and in vivo radiation sensitization of human tumor cells by a novel checkpoint kinase inhibitor, AZD7762. Clin Cancer Res 2010; 16(7): 2076-84. [CrossRef]

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ORIGINAL ARTICLE

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The Protective Effects of S-Methyl Methionine Sulfonium Chloride on Brain Tissue Damage in D-Galactosamine-Induced Hepatotoxicity

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ABSTRACT

Objective: The objective of the current work is to examine the protective effects of S-methyl methionine sulfonium chloride (MMSC) on brain in galactosamine (GalN)-induced hepatotoxicity in rats.

Materials and Methods: A total of twenty two female Sprague-Dawley rats were randomly assigned into four groups as follows: Group I (n=5), intact control animals; Group II (n=6), animals that received 50 mg/kg/day of MMSC by gavage technique for 3 consecutive days; Group III (n=5), animals injected with a single dose of 500 mg/kg of GalN intraperitoneally (i.p.); and Group IV (n=6) are animals injected with the same dose of GalN (i.p.) 1 hour after MMSC treatment. At the end of the experiments (after 6 hours of the last GalN treatment), all animals were sacrificed under anaesthesia, and brain tissues were dissected out.

Results: A statistically remarkable increase in lipid peroxidation, hydroxyproline, and nitric oxide levels, was detected while a notable decline in the activities of sodium/potassium ATPase was observed in GalN group in comparison with control rats. In contrast, all alterations observed were reversed when MMSC was given to GalN groups.

Conclusion: Consequently, it may be considered that MMSC has a protective role on brain in GalN-induced hepatotoxicity in rats.

Keywords: Galactosamine, S-methyl methionine sulfonium chloride, brain, antioxidant effect, oxidative stress

INTRODUCTION

Galactosamine (GalN) is a derivative of six-carbon amino sugar (galactose) whose extreme accumulation, or formation in organisms give rise to liver injury. The usage of GalN, as a well-known hepatotoxin, alone [or in combination with lipopolysaccharide (LPS)] has been shown to not only triggering depletion of uracil-containing nucleotides, but also disruption of the biosynthesis of uridylate nucleotides in hepatocytes, thereby causing hepatotoxicity/liver dysfunction in animal models (1). Given that the liver is the main organ responsible for the detoxification/biotransformation of chemicals/drugs and foods, damage to the liver by chemicals such as GalN may cause unwanted effects in other major organs (2). In the hepatic and brain cells of mice, the chronic administration of LPS/GalN has been revealed to cause oxidative stress, by disrupting oxidant/antioxidant balance in favour of oxidant substances [e.g., reactive oxygen species, (ROS)], along with inducing clear DNA migration (3). On the other hand, the structure and function of biomolecules (e.g., nucleic acids, lipids, carbohydrates, and proteins) may easily change when oxidative stress conditions emerge (4). Given that the brain consumes vast amount of oxygen and is characterized by being rich in polyunsaturated fatty acids (PUFAs) content, its regions (especially the hippocampus, amygdala, and

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cerebellar cells) are prone to detrimental effects of oxidative stress and ROS damage as well (5).

S-methyl methionine sulfonium chloride (MMSC), also known as vitamin U, has a sulfonium group containing derivative of the essential amino acid L-methionine, that the vegetable source is Brassica species such as white cabbage, Brussel sprouts, kohlrabi, and kale (6). It is also called a vitamin because of its vitamin-like effects. It is being studied as a source of anti-peptic ulcer dietary factors. As of its clinical importance, MMSC had several biofunctions such as cytoprotective agents against gastrointestinal disorders (6) and anti-epileptic effects (7). Besides, MMSC plays an important role by acting as a radical scavenger, as well as anti-fibrotic and anti-inflammatory agent under oxidative stress conditions (8).

The main objective of the present work designed due to limited published articles on the effect of GalN on the brain was to assess the potential protective roles of MMSC on brain injury GalN-induced hepatotoxicity in rats.

MATERIALS AND METHODS

Chemicals and Apparatus

All chemicals were of the highest purity. They were obtained commercially from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) and/or Merck Chemical Company (Darmstadt, Germany). The GalN (catalog number 48250) and MMSC (catalog number 64382) were supplied by Fluka Chemie AG (Buchs, Switzerland).

Animals and Animal Grouping

Female Sprague-Dawley rats (about 200-250 gr and 26-28 weeks old) were chosen for the study. They were acclimated to standard laboratory conditions. Fresh tap water and standard pellet chow were supplied. A total of twenty two rats were randomly assigned into four groups as follows: Group I (n=5), intact control animals; Group II (n=6), animals that received 50 mg/kg/day of MMSC by gavage technique for three consecutive days; Group III (n=5), animals injected a single dose of 500 mg/kg of GalN intraperitoneally (i.p.) (9); Group IV (n=6), animals injected with the same dose of GalN (i.p.) 1 hour after MMSC treatment. At the end of the experiments (after 6 hours of last GalN treatment), all rats were sacrificed under anaesthesia, and brain tissues were dissected out. The study was carried out according to the permission of the Animal Experimentation Local Ethics Committee of Marmara University (Protocol/Approval Number: 053.2020.mar).

Biochemical Analyses

Tissue samples were homogenized in cold physiologic saline using a glass apparatus to obtain a homogenate (1/10 w/v). After centrifugation (at 10,000 xg at +4°C for 10 min), clear supernatants were obtained, which were thereafter used for biochemical analyses.

Lipid peroxidation (LPO) levels were estimated according to Ledwozyw et al. (10). Briefly, appropriate volumes of tissue homogenate and trichloroacetic acid solution were mixed and allowed to stand at room temperature for 15 min. The thiobarbituric acid solution was pipetted to the reaction medium and then boiled in a water bath at 95°C for 30 min. The resultant mixture was mixed with the appropriate volume of n-butanol solution to extract organic phase. The absorbance of the organic phase at 532 nm was then monitored using a spectrophotometer (Shimadzu UV-Mini-1240, Kyoto, Japan) in terms of brain malondialdehyde (MDA), which is undertaken as an index of LPO. Results were expressed as nmol MDA/mg protein.

The hydroxyproline levels were determined by the method of Reddy and Enwemeka (11). This assay is based on alkaline hydrolysis of tissue homogenates thereafter subsequent determination of free hydroxyproline content in hydrolysates. After the hydrolyzation process was done at 110°C for 3 h, hydrolysates were mixed with chloramine-T solution and the oxidation of free hydroxyproline contents was allowed to proceed for 25 min at room temperature. Ehrlich's reagent [*p*-dimethylaminobenzaldehyde in n-propanol/perchloric acid (2:1 ratio v/v) solution] was added to reaction media and the chromophore was developed by incubating the samples at 65°C for 20 min. The absorbance of the samples were then recorded at 550 nm using a spectrophotometer. Results were expressed as µg hydroxyproline/mg protein.

Nitric oxide (NO) levels were carried out by spectrophotometric method of Miranda et al. (12). The principle of this assay is reduction of nitrate to nitrite by VCl_3 in an acidic reaction media containing Griess reagent [mixed an equal volume of sulfanilamide (2% w/v) and *N*-(1-Naphtyl) ethylenediamine dihydrochloride (0.1% w/v)]. After the coloured diazonium complex completed, absorbance of the resultant mixture was measured at 540 nm by a spectrophotometer, and the results were expressed as nmol NO/mg protein.

The sodium/potassium adenosine triphosphatase (Na^+/K^+-ATPase) activities in the brain tissue homogenates were determined by the method developed by Ridderstap and Bonting (13). The main principle of this method was based on determination of inorganic phosphate (P_i) after the hydrolyzation of ATP when the homogenates were incubated with the appropriate amount of ATP. First, total ATPase activities were assayed thereafter Mg^{2+}-ATPase activities were determined in the presence of ouabain. The Na^+/K^+-ATPase activities were calculated by subtraction from the total ATPase of Mg^{2+}-ATPase. The obtained results were expressed as micromoles of P_i/mg protein/h.

Total protein levels in the homogenates were estimated according to the method of Lowry et al. (14). Briefly, proteins were reacted with Cu^{2+} ions in alkaline medium (2% Na_2CO_3 in 0.1N NaOH) and reduced by the Folin-Ciocalteu reagent. The absorbance of the blue-coloured product that colour intensity is proportional to the amount of protein in the sample was evaluated at 500 nm. Bovine serum albumin was used as a standard for determination of protein levels.

Statistical Analyses

To analyse the obtained data, an unpaired t-test and one-way analysis of variance (ANOVA) were carried out, followed by Tukey's test as a post hoc test for multiple comparisons with the aid of the GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) statistical program. The values were illustrated as the mean±standard deviation (SD). Differences were regarded as significant when the p value is less than 0.05.

RESULTS

The LPO levels of brain tissues are depicted in Figure 1. LPO levels in the GalN group were remarkably elevated by 3.02-fold in comparison with the control group (p<0.0001). Pretreatment with MMSC in the GalN group resulted in an approximately 2.5-fold reduction in LPO levels (p<0.0001; Figure 1).

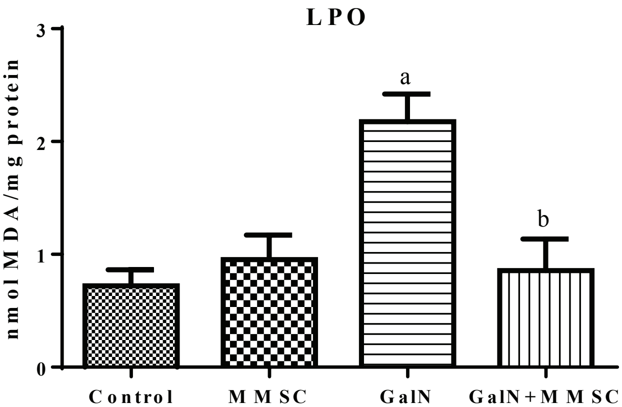

Figure 1. The effect of MMSC on brain tissues lipid peroxidation levels of all rats. Data were presented as mean±SD. Group I (n=5), intact control animals; Group II (n=6), MMSC given group; Group III (n=5), GalN given group; Group IV (n=6), GalN+MMSC given group.

Abbreviations: SD, standard deviation; ANOVA, analysis of variance; LPO, lipid peroxidation; MDA, malondialdehyde; MMSC, S-methyl methionine sulfonium chloride; GalN, galactosamine. To analyze data, an unpaired t-test and one-way ANOVA was carried out, followed by Tukey's as a post hoc test for multiple comparisons. [a]p<0.0001 vs control; [b]p<0.0001 vs GalN group.

Alteration of hydroxyproline levels of all groups is shown in Figure 2. More than 2.5-fold increase in hydroxyproline levels were detected in the GalN group as compared to the intact rats (p<0.0001). Treatment with MMSC to the GalN group led to 8.7-fold decline of hydroxyproline levels in the brain (p<0.0001; Figure 2).

Levels of NO of all groups are presented in Figure 3. NO levels were considerably higher (about 3.5-fold) in the MMSC pretreated group and the GalN group (3.6-fold) (p<0.0001) in comparison with intact rats (Figure 3). By contrast, a statistically significant (p<0.0001) diminishment (approximately 1.5-fold)

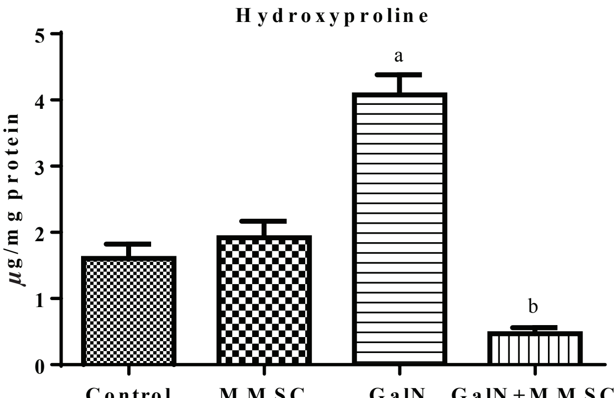

Figure 2. The effect of MMSC on brain tissues hydroxyproline levels of all rats. Data were presented as mean±SD. Group I (n=5), intact control animals; Group II (n=6), MMSC given group; Group III (n=5), GalN given group; Group IV (n=6), GalN+MMSC given group.

Abbreviations: SD, standard deviation; ANOVA, analysis of variance; MMSC, S-methyl methionine sulfonium chloride; GalN, galactosamine. To analyze data, an unpaired t-test and one-way ANOVA was carried out, followed by Tukey's as a post hoc test for multiple comparisons. [a]p<0.0001 vs control; [b]p<0.0001 vs GalN group.

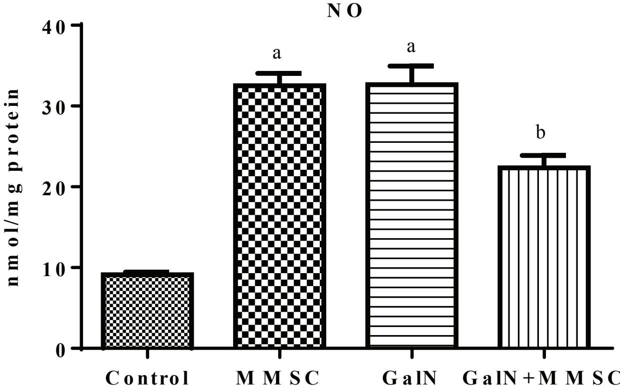

Figure 3. The effect of MMSC on brain tissues nitric oxide levels of all rats. Data were presented as mean±SD. Group I (n=5), intact control animals; Group II (n=6), MMSC given group; Group III (n=5), GalN given group; Group IV (n=6), GalN+MMSC given group.

Abbreviations: SD, standard deviation; ANOVA, analysis of variance; NO, nitric oxide; MMSC, S-methyl methionine sulfonium chloride; GalN, galactosamine. To analyze data, an unpaired t-test and one-way ANOVA was carried out, followed by Tukey's as a post hoc test for multiple comparisons. [a]p<0.0001 vs control; [b]p<0.0001 vs GalN group.

in the levels of NO was detected in brain tissues in the GalN+MMSC group compared to that of the GalN group (Figure 3).

Activities of Na^+/K^+-ATPase of experimental groups are given in Figure 4. A remarkable (more than 2.4-fold) decline in Na^+/K^+-ATPase activities was observed in the GalN group (p<0.0001) in comparison with the control group (Figure 4). On the other hand, a considerable elevation (more than 2.1-fold) of Na^+/K^+-ATPase activities in brain tissues in the GalN+MMSC group was detected when MMSC was pretreated with the GalN group (p<0.0001; Figure 4).

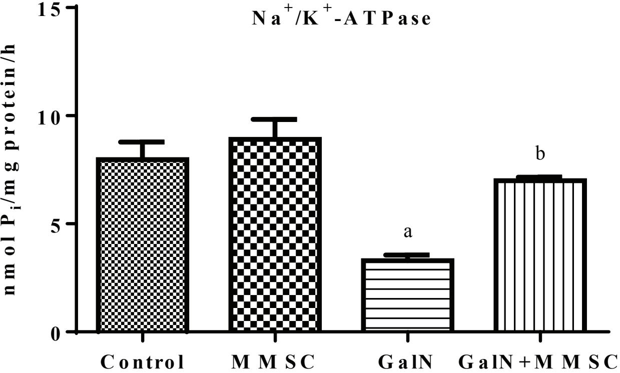

Figure 4. The effect of MMSC on brain tissues sodium/potassium ATPase activities of all rats. Data were presented as mean±SD. Group I (n=5), intact control animals; Group II (n=6), MMSC given group; Group III (n=5), GalN given group; Group IV (n=6), GalN+MMSC given group.

Abbreviations: SD, standard deviation; ANOVA, analysis of variance; Na^+/K^+-ATPase, sodium/potassium ATPase; MMSC, S-methyl methionine sulfonium chloride; GalN, galactosamine. To analyze data, an unpaired t-test and one-way ANOVA was carried out, followed by Tukey's as a post hoc test for multiple comparisons. [a]p<0.0001 vs control; [b]p<0.0001 vs GalN group.

DISCUSSION

The current outcomes revealed that GalN-induced injury resulted in alterations not only in the levels of LPO, hydroxyproline, and NO, but also in the activity of Na^+/K^+-ATPase enzyme in the rat's brain. These defects are associated with an increase in ROS formation.

The hippocampus, amygdala, and brain cells are highly sensitive to the harmful effects of oxidative stress-mediated tissue injury. This is because the brain is an organ containing high amounts of lipids and their derivatives and utilizes high total basal oxygen levels and consequently forms ROS (5). When ROS attack the PUFAs of membrane lipids, the abstraction of hydrogen from PUFA gives rise to the formation of carbon-centered lipid radicals, thus rapidly interacting with the O_2 to form lipid peroxyl radicals. This in turn, leads to the initiation of a chain reaction known as LPO. The lipid peroxides formed in this way are converted into highly reactive biochemical products such as acrolein, 4-hydroxynonenal, and MDA in organisms. MDA is an important biochemical marker that is frequently determined in the assessment of LPO (15). In the present study, LPO level in the GalN given group was found to be statistically higher than those of intact rats. This might be associated with a potential effect of ROS-mediated brain injury. An increase in LPO levels may be owing to the consequences of GalN-induced oxidative stress via an upsurge of ROS in the brain tissues of rats. In several studies related to central nervous system (CNS) diseases, rise in the levels of LPO has been accepted to play a crucial role in ROS-mediated brain damage (16). The reversed LPO levels may be linked to not only ROS mopping effect, but also antioxidant potential of the MMSC (8).

In general, hydroxyproline (an imino acid) is synthesized by post-translational modification of collagen (viz., hydroxylation of proline), and is one of the main components of the collagen protein. Amino acid sequences of collagen are regularly ordered as Gly-Pro-X or Gly-X-Hyp. In possible two motifs, the sequence of the Gly–Pro–Hyp occurs usually. Collagen contains approximately 99.8% of the hydroxyproline stored in the body and is therefore used as an important biomarker in the diagnosis of collagen and hydroxyproline-related diseases (17). The elevation of oxidative stress levels as well as neuroinflammation was shown to cause modification of extracellular matrix components which may cause Parkinson's disease (18). In the current study, hydroxyproline levels in brain homogenates were determined because it is an important diagnostic indicator of the severity of liver hepatitis caused by GalN (19). According to the findings, hydroxyproline levels in the GalN given group were statistically higher than that of control rats. This alteration might be associated with GalN-mediated toxicity, which causes oxidative stress as well as disruption of membrane integrity via high LPO levels. On the other hand, Ganai and his colleagues (20) reported that 250 mg/kg GalN injection to rats for 12 weeks (twice a week) resulted in the formation of fibrosis/cirrhosis in the liver, with hydroxyproline levels increasing more than 7 times compared to the control group.

NO, a short-lived, small, and freely diffusible gas molecule, is a highly reactive inorganic free radical. The high reactivity of this molecule is due to its small size, high diffusion rate and lipophilic character rather than the unpaired electrons in its structure (21). In the biological system, it is formed as a by-product of the gradual conversion of L-arginine to citrulline via hydroxylation reaction catalysed by NO synthase NOS. NO molecule is formed by the action of the NOS enzyme, which has three isoforms: neural, inducible, and endothelial. Moreover, this enzyme has very important functions in the cardiovascular and CNS (22). Apart from that, NO reacts with other molecules (e.g., superoxide, free sulfhydryl groups, and oxygen) to form reactive products known as nitrites, peroxynitrite, and nitrosothiols. Furthermore, NO has diverse functions depending on its physiologic concentration. The formation of this molecule at a low concentration may protect cells, while at higher concentrations it can act as a cytotoxin that plays a role in tumor an-

giogenesis and progression (23). More so, keeping NO levels in balance, which can have both neuroprotective and neurotoxic effects, is important in the CNS. This is because NO acts as an intracellular signal molecule by stimulating the cyclic guanosine monophosphate cascade (24). On the other hand, a rise in the production of NO has been reported to be responsible for the development of several neurodegenerative disorders (i.e., multiple sclerosis, Parkinson's and Alzheimer's diseases, and ischemia) (25). It thus can be called a "double-edged sword" that has key roles in both physiological and pathological processes (21). In the current study, NO levels in the brain tissue of rats were determined by using Griess reagent. NO levels in the MMSC group were unexpectedly found to be statistically higher than that of control animals. This might be associated with the duration of administration of MMSC (three consecutive days). Oktay et al., stated that the administration of MMSC (50 mg/kg/day) to intact animals for 7 days caused an insignificant elevation of NO levels (7). In GalN injected groups, the levels of NO were remarkably elevated as compared to intact rats. Like GalN, LPS (or in combination with GalN) is an agent that is widely used for the induction of multiple organ damage (2). Abdel-Salam et al., revealed that nitrite levels increased in LPS-induced liver and brain injury (26). On the other hand, pretreatment with MMSC gave rise to a notable decrease in NO levels in the GalN given group, which may be because of the ameliorative effect of MMSC, since NO has been shown to exert neurotoxic effects (27).

Na^+/K^+-ATPase, also known as Na^+/K^+-ion pump, is a cation transport protein that is localized in the plasma membrane of various eukaryotic cells. It has an indispensable role in balancing osmotic equilibrium and electrochemical membrane potential, by exporting three Na^+ ions concomitant to importing two K^+ ions across the membranes of neurons as well as other cells [by use free the energy released during the hydrolysis of one ATP molecule to ADP and P_i]. Furthermore, this enzyme is responsible for maintenance of osmoregulation of both Na^+ and K^+ in hyper- and hypotonic environment, regulating cytosolic pH and Ca^{2+} levels, transmission of nerve impulses through neuron and triggering of intracellular signalling (28). Oligomeric proteins of Na^+/K^+-ATPase are vital for its overall activity, which consist of the alpha (ten transmembrane helix subunits), beta (single transmembrane subunit), and family of small membrane proteins (FXYD) subunits (29). The alpha subunit is mainly responsible for its catalytic activity, while the glycosylated beta subunit regulates the activity and conformational stability of the alpha subunit. Also, interaction between the beta subunit and the alpha subunit is required to complete ion transport. On the other hand, the FXYD subunit is divided into two subgroups namely FXYD1 (mainly found in the heart, skeletal muscle, and brain) and FXYD2 (highly expressed in the kidney), respectively (29). In the current study, injection of GalN caused a remarkable decline in Na^+/K^+-ATPase activities. The deterioration of membrane integrity owing to the increase in LPO levels in the GalN given group may be considered as the reason for the decline in Na^+/K^+-ATPase activities. Additionally, it has been put forward that activity of this enzyme in the brain tissue decreases in conditions such as ischemia, epilepsy attacks and hypoglycemia, as well as in oxidative damage caused by the administration of toxins such as GalN (30). In parallel, decreased Na^+/K^+-ATPase activities have been reported in GalN-mediated oxidative stress in lung tissue of rats (9). According to the present findings, administration of MMSC to the GalN given group led to a notable recovery of Na^+/K^+-ATPase activities. The present research apparently revealed for the first time that treatment with MMSC before GalN injection sharply restored Na^+/K^+-ATPase activities. More so, MMSC had protective effects on the brains of GalN-injected rats. Similar outcome was previously reported by Gezginci-Oktayoglu et al. (8).

The limitation of the study is that in order to fully understand the protective effects of MMSC on brain biochemical parameters, different brain disease models need to develop and the effect of MMSC examined on these models.

CONCLUSION

A limited number of published articles on the harmful effects of GalN against brain damage encouraged the design and execution of the present estudy. The findings obtained from the current work demonstrate that GalN-mediated oxidative stress leads to an increase in LPO, hydroxyproline, and NO levels, and a decrease in Na^+/K^+-ATPase activities. In contrast, all alterations observed were reverted when MMSC was administered to the GalN groups. In the light of the current findings, it may be concluded that MMSC has therapeutic effects against GalN-induced brain toxicity in rats.

Ethics Committee Approval: The study was carried out according to the permission of the Animal Experimentation Local Ethics Committee of Marmara University (Protocol/Approval Number: 053.2020.mar).

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REFERENCES

1. Singh G, Sharma R. Animal models for hepatoxicity. In: Sobti RC, editor. Advances in Animal Experimentation and Modeling. Academic Press: London; 2022.p.195-203.
2. Rouf A, Das AK, Khatoon MSTM, Mitu NL, Shahriar M. Evaluation of hepatoprotective effects of arogyavardhini against D-galactosamine-induced hepatotoxicity in rats. J Pharmacog Phytochem 2021; 10(1): 12-9.

3. Dong W, Song E, Song Y. Co-administration of lipopolysaccharide and D-galactosamine induces genotoxicity in mouse liver. Sci Rep 2021; 11: 1733. [CrossRef]
4. Hajam YA, Rani R, Ganie SY, Sheikh TA, Javaid D, Qadri SS, et al. Oxidative stress in human pathology and aging: molecular mechanisms and perspectives. Cells 2022; 11(3): 552. [CrossRef]
5. Anwar MM. Oxidative stress-a direct bridge to central nervous system homeostatic dysfunction and Alzheimer's disease. Cell Biochem Funct 2022; 40(1): 17-27. [CrossRef]
6. Choi EH, Lee SB, Lee DY, Kim GT, Shim SM, Park TS. Increased intestinal absorption of vitamin U in steamed graviola leaf extract activates nicotine detoxification. Nutrients 2019; 11(6): 1334. [CrossRef]
7. Oktay S, Bayrak G, Alev B, Ipekci H, Ustundag UV, Turkyilmaz IB, et al. The effect of vitamin U on the lung tissue of pentylenetetrazole-induced seizures in rats. Naunyn-Schmiedeberg's Arch Pharmacol 2018; 391(2): 177-84. [CrossRef]
8. Gezginci-Oktayoglu S, Turkyilmaz IB, Ercin M, Yanardag R, Bolkent S. Vitamin U has a protective effect on valproic acid-induced renal damage due to its anti-oxidant, anti-inflammatory, and anti-fibrotic properties. Protoplasma 2016; 253(1): 127-35. [CrossRef]
9. Bayrak BB, Catal T, Oztay F, Yanardag R, Bolkent S. Efficacy of antioxidant vitamins (vitamin C, vitamin E, beta-carotene) and selenium supplement on D-galactosamine-induced lung injury. IUFS J Biol 2016; 75(1): 11-8.
10. Ledwozyw A, Michalak J, Stepień A, Kadziołka A. The relationship between plasma triglycerides, cholesterol, total lipids and lipid peroxidation products during human atherosclerosis. Clin Chim Acta 1986; 155: 275-83. [CrossRef]
11. Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 1996; 29: 225-29. [CrossRef]
12. Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 2001; 5: 62-71. [CrossRef]
13. Ridderstap AS, Bonting SL, Na^{+}-K^{+}-activated ATPase and exocrine pancreatic secretion in vitro. Am J Physiol 1969; 217: 1721-7. [CrossRef]
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-75. [CrossRef]
15. Jaganjac M, Cindrić M, Jakovčević A, Žarković K, Žarković N. Lipid peroxidation in brain tumors. Neurochem Int 2021; 149: 105118. [CrossRef]
16. Guglielmotto M, Tamagno E, Danni O. Oxidative stress and hypoxia contribute to Alzheimer's disease pathogenesis: two sides of the same coin. Sci World J 2009; 9(1): 781-91. [CrossRef]
17. Albaugh VL, Mukherjee K, Barbul A. Proline precursors and collagen synthesis: biochemical challenges of nutrient supplementation and wound healing. J Nutr 2017; 147(11): 2011-7. [CrossRef]
18. Freitas A, Aroso M, Barros A, Fernández M, Conde-Sousa E, Leite M, et al. Characterization of the striatal extracellular matrix in a mouse model of Parkinson's disease. Antioxidants (Basel) 2021; 10(7): 1095. [CrossRef]
19. Qiu B, Wei F, Sun X, Wang X, Duan B, Shi C, et al. Measurement of hydroxyproline in collagen with three different methods. Mol Med Rep 2014; 10(2): 1157-63. [CrossRef]
20. Ganai AA, Ganaie IA, Verma N, Farooqi H. Regression of fibrosis/cirrhosis by glycine propionyl-l-carnitine treatment in D-galactosamine induced chronic liver damage. Chemico-Biol Interact 2016; 260: 117-28. [CrossRef]
21. Ramesh KSV, Swetha P, Nirnmal M, Alla RK. Nitrous oxide - "Double edged sword". Trends Biomater Artific Organs 2014; 28(1): 37.
22. Morales-Medina JC, Aguilar-Alonso P, Di Cerbo A, Iannitti T, Flores G. New insights on nitric oxide: focus on animal models of schizophrenia. Behav Brain Res 2021; 409: 113304. [CrossRef]
23. Paradise WA, Vesper BJ, Goel A, Waltonen JD, Altman KW, Haines GK, et al. Nitric oxide: perspectives and emerging studies of a well known cytotoxin. Int J Mol Sci 2010; 11(7): 2715-45. [CrossRef]
24. Mónica FZ, Bian K, Murad F. The endothelium-dependent nitric oxide-cGMP pathway. Adv Pharmacol 2016; 77: 1-27. [CrossRef]
25. Subedi L, Gaire BP, Parveen A, Kim SY. Nitric oxide as a target for phytochemicals in anti-neuroinflammatory prevention therapy. Int J Mol Sci 2021; 22(9): 4771. [CrossRef]
26. Abdel-Salam OME, Youness ER, Mohammed NA, Morsy SMY, Omara EA, Slemm AA. Citric acid effects on brain and liver oxidative stress in lipopolysaccharide-treated mice. J Med Food 2014; 17(5): 588-98. [CrossRef]
27. Esmekaya MA, Tuysuz MZ, Tomruk A, Canseven AG, Yücel E, Aktuna Z, et al. Effects of cell phone radiation on lipid peroxidation, glutathione and nitric oxide levels in mouse brain during epileptic seizure. J Chem Neuroanat 2016; 75: 111-5. [CrossRef]
28. Lichtstein D, Ilani A, Rosen H, Horesh N, Singh SV, Buzaglo N, et al. Na^{+}, K^{+}-ATPase signaling and bipolar disorder. Int J Mol Sci 2018; 19(8): 2314. [CrossRef]
29. Clausen MV, Hilbers F, Poulsen H. The structure and function of Na,K-ATPase isoforms in health and disease. Front Physiol 2017; 8: 371. [CrossRef]
30. Arnaiz GRL. Na^{+}, K^{+}-ATPase in the brain: structure and function. In: Lajtha A, Reith MEA, editors. Handbook of Neurochemistry and Molecular Neurobiology. Springer: Boston MA; 2007.p.209-24.

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Impact of COVID-19 "Stay at home!" Restrictions on the Prevalence of Deep Vein Thrombosis in the Geriatric Population: A Retrospective Controlled Study

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ABSTRACT

Objective: The restrictions during the coronavirus disease 2019 (COVID-19) pandemic period may have caused an increase in the incidence of deep vein thrombosis (DVT) in the elderly population. We aimed to evaluate whether the frequency of DVT increased in the geriatric population due to the restrictions of COVID-19 pandemic period and to emphasize the importance of exercise in this population.

Materials and Methods: Patients over the age of 65 who applied to our hospital, who underwent lower extremity venous doppler ultrasonography in the last year before the pandemic and in the first year with pandemic restrictions, were retrospectively analyzed from the database. The records of a total of 1531 patients were scanned. Patients with DVT were recorded.

Results: Forty-nine DVT cases in the last year before the pandemic, and 53 DVT cases in the first year of the restrictions were detected. The incidence of DVT in the elderly was found to be higher in the restrictions period.

Conclusion: Although the "Stay at home!" restrictions reduced the rate of spread of the disease, it may also have prepared the ground for serious conditions such as DVT in this population. Home exercise programs are important especially for the elderly population during the pandemic period.

Keywords: COVID-19, geriatrics, deep vein thrombosis, exercise

INTRODUCTION

Venous thrombosis (VT) is the general definition for thrombosis occurring in the venous system and is most commonly detected in the deep veins of the lower extremities. The most important life-threatening clinical finding of VT is the pulmonary embolism (PE) (1). VT development is based on blood-flow stasis, hypercoagulability, and endothelial damage as the components of the Virchow Triad (2). Factors such as age, genetic factors, immobilization, sedentary life, obesity, malignancy, and smoking are blamed as risk factors, and age increases the risk of VT independently of other risk factors (3-9).

The coronavirus disease 2019 (COVID-19) pandemic has become a global problem since the end of January 2020 and the spread of the virus is affecting public, economic, and private life rapidly and strongly all around the world. Comprehensive government restriction policies were implemented all over the world to keep the pandemic under control. The slogan "Stay at home!" was conveyed to everyone all around the world. Strict measures have led to social isolation as well as restriction of movement in vulnerable groups, such as the geriatric population (10, 11). This is important because the effects of an immobile lifestyle may be lower risk for children and young adults but much higher risk for elderly adults, who should stay at home. Elderly

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adults should stay at home but avoid a completely sedentary lifestyle as they are at higher risk of contracting COVID-19 infection and death. Physical activity is particularly important for the elderly population to maintain levels of independence, mental health, and well-being (12, 13). Physical inactivity is one of the major risk factors for mortality worldwide and a significant contributor to disability in elderly adults (14). People who do not engage in regular physical activity have higher risks of functional decline (15). Therefore, it is necessary to maintain mobility in old age as loss of independence can be predicted in elderly adults (16,17). Inadequate physical activity in quarantine period might also have detrimental effects on the mental and emotional health of elderly adults (17).

In this study, it was aimed to examine whether the restrictions during the pandemic period caused an increase in the incidence of deep vein thrombosis (DVT) in the elderly population and to emphasize the importance of mobility in this age group.

MATERIALS AND METHODS

Ethical approval was obtained from Adıyaman University Non-Invasive Clinical Research Ethics Committee before starting the study (19.01.2021/01-23). All patients over the age of 65 who applied to any outpatient clinic in the last year before the pandemic and within the first year of the pandemic period and who were requested lower extremity venous doppler ultrasonography (US) with a preliminary diagnosis of DVT were scanned from the database of our hospital. Patients over the age of 65 and who were diagnosed with DVT by doppler US were included in the study. Patients diagnosed with COVID-19 serologically, radiologically, or clinically; who were hospitalized for any other reason; who had a surgical operation with the diagnosis of hip fracture; who had a history of cerebrovascular disease and/or a history of malignancy; who had had severe heart and/or kidney failure; or who had any inflammatory disease were not included in the study.

The data of a total of 970 patients who applied to outpatient clinics between 01.03.2019 and 29.02.2020 and had undergone lower extremity venous doppler US before the start of the pandemic in our country were scanned. Forty-nine patients who met the inclusion criteria and who had thrombus on doppler US were included in the study. The data of 561 patients, who underwent lower extremity venous doppler US with outpatient admission between 01.03.2020 and 28.02.2021, when the pandemic started in our country and restrictions were placed, were also scanned. For this period, 53 patients who met the inclusion criteria and were diagnosed with DVT by doppler US were identified.

Statistical Analyses

The SPSS version 22.0 package software (SPSS Inc., Chicago, Il USA) was used for the analysis of the data. Mean and standard deviation (SD) values were used in descriptive statistics, the Chi-square test was used to compare categorical variables, and the Mann Whitney U test was used to compare the DVT numbers according to gender.

RESULTS

It was determined that 970 of the 1531 patients included in our study were requested to do lower extremity venous doppler US with a preliminary diagnosis of DVT when they applied to our hospital's outpatient clinics in the last one-year period before the pandemic, and for 508 in the first year of the pandemic. The mean age of patients with DVT in the pandemic period was 76.5±12.6, and the mean age of patients with DVT in the pre-pandemic period was 77.6±11.6 ($p>0.05$). The numbers of patients are shown in Table 1.

Table 1. DVT in the pre-restriction period and during the restriction period.

		DVT			
		Yes	No	Total	p
Restrictions	Yes	53	508	561	<0.001
	No	49	921	970	
Total		102	1429	1531	

p<0.05, statistically significant; DVT, Deep vein thrombosis

Whether the detection of DVT in the pre-restriction period and during the restriction period was independent of each other was tested with the Chi-square test. According to the test results, the number of patients with DVT was not independent of the pandemic process (χ^2=11.045; $p<0.001$). Also, the difference between DVT rates in the pre-restriction period and during the restriction period was tested with the Z-test. Accordingly, the rate of DVT development increased at significant levels during the pandemic process ($p<0.001$).

The distribution of DVT count according to gender in the pre-restriction period and during the restriction is given in Table 2.

Table 2. Distribution of patients with DVT according to gender.

		Gender			
		Female	Male	Total	p
Restrictions	Yes	21	32	53	>0.05
	No	25	24	49	
Total		46	56	102	

p<0.05, statistically significant

Whether the distribution of DVT according to gender in the pre-restriction period and during the restriction period was independent of each other was tested with the Chi-square test. According to the test results, DVT and gender were independent of each other (χ^2=1.336; $p>0.05$). Also, although the rate

of men who had DVT during the restriction period compared to the pre-pandemic period increased from 0.49 to 0.60, the rate of women decreased from 0.51 to 0.40. The difference between these rates was tested with the Z-test. In this respect, the rate of men and women who had DVT in the pre-pandemic period and during the pandemic period did not change at significant levels (p>0.05).

DISCUSSION

Based on our review of the subject, this is the first study to investigate the prevalence of DVT in the geriatric patient population without any other risk factors of "Stay at home!" directives and social isolation. This study is important in that it shows how important the problem of immobility is in geriatric patients and how immobility increases the rates of DVT, which can result in life-threatening problems for this age group. Although all causes other than limitation of motion, which might cause DVT, were excluded in our study, it was found that "Stay at home!" restrictions were higher in the geriatric patient population at significant levels during the pandemic period compared to the last year before the pandemic.

Since the COVID-19 pandemic is more lethal for the geriatric population, it caused some restrictions to be applied by governments all over the world, especially for people who are over the age of 65 (18). Schools, public places, and workplaces were closed as part of the curfew and quarantines in many countries to ensure social distance and reduce infection. Although this strategy was reported to be effective in controlling the COVID-19 outbreak, quarantine might be associated with some undesirable effects because of changing social habits. The "Stay at home!" practices might have also caused negative changes, such as obesity, diabetes mellitus, cardiovascular diseases, muscle atrophy, bone loss, impaired immune system, and decreased aerobic capacity as well as psychological effects, such as anxiety, posttraumatic stress symptoms, and confusion (17,19). In the study conducted by Mauger et al. in Italy, it was reported that physical activity decreased at significant levels during the pandemic compared to the pre-pandemic period, which caused psychological problems, such as depression and anxiety (20). The literature described the positive roles of physical activity in improving overall health to have effects on the heart, circulation, and respiration as well as immune function at a great deal (21,22). Therefore, establishing or maintaining regular physical activity habits has the potential to reduce the effects of the pandemic on a personal and societal level. It was proven to be difficult to form and maintain regular physical activity habits. It was reported by the Department of Health and Human Services that only 24% of adults meet the guidelines defined for them (23). The effects of restrictions on human psychology were evaluated in the literature, and it was reported that the importance of exercise in preventing this must be emphasized (20,24). A small number of case reports were detected in which DVT developed only with inactivity during the pandemic period. Blum et al. reported that a completely healthy 84-year-old woman developed DVT because of inactivity in the quarantine period (25). In our study, a significant difference was found in terms of DVT development when other risk factors were excluded and when patients who developed DVT in the last year before the pandemic and the first year of the pandemic were compared. This shows the negative outcomes of the "Stay at home!" restriction. When it is considered that our hospital was the only one other than private ones in our city, these results were found to be important in terms of showing how much the risks increased on a provincial basis. Therefore, prevention and protection methods are important to avoid these problems in the COVID-19 epidemic, especially in geriatric patients who are at great risk in this respect (26,27). If necessary, this negative situation must be prevented by home exercise instructions, viable mobility programs on public television or digital health applications, information to be provided in the form of public announcements, brochures to emphasize the importance of exercise, and the media. Exercise can prevent metabolic disorders, bone, muscle, joint pathologies and neurodegenerative diseases. Also, regarding the respiratory tract infection caused by COVID-19, regular exercise was shown to prevent the development of pathological consequences that cause cell necrosis and damage by inducing the secretion of stress hormones and anti-inflammatory cytokines responsible for decreasing excessive local inflammation in the airways (28,29). Thus, exercise can also avoid COVID-19 infection from becoming more severe in geriatric patients by strengthening the immune system. The opportunity for physical activity outdoors is limited, and it is recommended that people stay active by exercising at home in the current pandemic. For this purpose, various exercises can be recommended, including aerobic exercises, which can be performed by using a stationary bike, arm ergometer, or treadmill, bodyweight strengthening exercises, dance-based exercises, and active video games. Aerobic exercise training is defined as the exercises producing light, moderate, or high cardiovascular loads, which can be performed with a stationary bike, arm ergometer, treadmill, or various types of dance and gymnastics. The World Health Organization recommends 75-minute vigorous-intensity physical activity per week or 150 minutes of moderate-intensity physical activity per week to be combined with muscle-strengthening training twice a week for adults and the elderly (30). While there is no significant increase in the total number of patients with DVT, the statistical difference might have arisen from the lower number of admissions to the hospitals during the pandemic. In the early times of the pandemic, there was a serious decrease in the number of applications to hospitals, due to the fear of transmission of the virus, potential risks in hospitals and general public restrictions. It has been reported that there has been a serious decrease in hospital admissions with the emergence of the pandemic in the United States and that most hospitals are operating below 50 percent of capacity. In March 2022, it was reported that there was a serious decrease in the number of admissions to hospitals, even due to serious health problems such as myocardial infarction and stroke (31-33). In a study conducted in Norway, it was reported that there were significant decreases in the number of admissions to hospitals

in the first months of the pandemic and that these decreases were not different in terms of age and gender (34). In our study, although the number of hospital admissions decreased during the pandemic period, the incidence of DVT was found to be higher than in the pre-pandemic period.

The limitations of our study are that the study design was retrospective, the number of patients was small, the physical activity levels of the patients were not known, and the lower extremity venous doppler US evaluation was performed by different radiologists.

CONCLUSION

In conclusion, it can be said that the quarantine and "Stay at home!" practices applied to prevent the pandemic might cause negative consequences such as DVT, especially in the geriatric population, and it can be said that programs that emphasize the importance of exercise in the scope of public health practices should be increased to prevent this situation.

Ethics Committee Approval: The Adıyaman University's Ethics Committee gave approval to the study's procedures (2021/01-23).

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REFERENCES

1. Segal JB, Eng J, Tamariz LJ, Bass EB. Review of the Evidence on Diagnosis of Deep Venous Thrombosis and Pulmonary Embolism. Ann Fam Med 2007; 5(1): 63-73. [CrossRef]
2. Ogonda L, Hill J, Doran E, Dennison J, Stevenson M, Beverland D. Aspirin for thromboprophylaxis after primary lower limb arthroplasty: early thromboembolic events and 90-day mortality in 11,459 patients. Bone Joint J 2016; 98(3): 341-8. [CrossRef]
3. Anderson FA, Wheeler HB, Goldberg RJ, Hosmer DW, Patwardhan NA, Jovanovic B, et al. A population based perspective of the hospital incidence and case-fatality rates of deep vein thrombosis and pulmonary embolism. The Worcester DVT Study. Arch Intern Med 1991; 151(5): 933-8. [CrossRef]
4. Caprini JA, Arcelus JI, Reyna JJ. Effective risk stratification of surgical and nonsurgical patients for venous thromboembolic disease. Semin Hematol 2001; 3(2): 12-9. [CrossRef]
5. Warlow C, Ogston D, Douglas AS. Venous thrombosis following strokes. Lancet 1972; 299(776)4: 1305-6. [CrossRef]
6. Samama MM. Epidemiology of risk factors of deep venous thrombosis (DVT) of the lower limbs in community practice: the SIRIUS study. Thromb Haemost 1993; 69: 763.
7. Heit JA, Silverstein MD, Mohr DN, Petterson TM, O'Fallon WM, Melton LJ 3rd. Risk factors for deep vein thrombosis and pulmonary embolism: A population-based case-control study. Arch Intern Med 2000; 160(6): 809-15. [CrossRef]
8. Rahr HB, Sorensen JV. Venous thromboembolism and cancer. Blood Coagul Fibrinolysis 1992; 3(4): 451-60. [CrossRef]
9. Pomp ER, Rosendaal FR, Doggen CJ. Smoking increases the risk of venous thrombosis and acts synergistically with oral contraceptive use. Am J Hematol 2008; 83(2): 97-102. [CrossRef]
10. Markowitz J. Virtual treatment and social distancing. The Lancet Psychiatry 2020; 7(5): 388-9. [CrossRef]
11. Guan H, Okely AD, Aguilar-Farias N, del Pozo Cruz B, Draper CE, El Hamdouchi A, et al. Promoting healthy movement behaviours among children during the COVID-19 pandemic. Lancet Child Adolesc Health 2020; 4(6): 416-8. [CrossRef]
12. Sun F, Norman IJ, While AE. Physical activity in older people: a systematic review. BMC Public Health 2013; 13: 449. [CrossRef]
13. Lautenschlager NT, Almeida OP, Flicker L, Janca A. Can physical activity improve the mental health of older adults? Ann Gen Hosp Psychiatry 2004;3(1):1-5. [CrossRef]
14. Gomes M, Figueiredo D, Teixeira L, Poveda V, Paúl C, Santos-Silva A, et al. Physical inactivity among older adults across Europe based on the SHARE database. Age Ageing 2017; 46(1): 71-7. [CrossRef]
15. Guralnik JM, Ferrucci L, Simonsick EM, Salive ME, Wallace RB. Lower-extremity function in persons over the age of 70 years as a predictor of subsequent disability. N Engl J Med 1995; 332(9): 556-61. [CrossRef]
16. Hirvensalo M, Rantanen T, Heikkinen E. Mobility difficulties and physical activity as predictors of mortality and loss of independence in the community-living older population. J Am Geriatr Soc 2000; 48(5): 493-8. [CrossRef]
17. Brooks SK, Webster RK, Smith LE, Woodland L, Wessely S, Greenberg N, et al. The psychological impact of quarantine and how to reduce it: rapid review of the evidence. Lancet 2020; 395(10227): 912-20. [CrossRef]
18. Coronavirus disease 2019 (COVID-19)-Situation report, 72. World Health Organization 2020 (Available from: https://apps.who.int/iris/handle/10665/331685).
19. Bortz II WM. The disuse syndrome. West J Med 1984; 141(5): 691-4.
20. Maugeri G, Castrogiovanni P, Battaglia G, Pippi R, D'Agata V, Palma A, et al. The impact of physical activity on psychological health during Covid-19 pandemic in Italy. Heliyon 2020; 6(6): e04315. [CrossRef]
21. Romeo J, Wärnberg J, Pozo T, Marcos A. Physical activity, immunity and infection. Proc Nutr Soc 2010; 69(3): 390-9. [CrossRef]
22. Wu N, Bredin SS, Guan Y, et al. Cardiovascular health benefits of exercise training in persons living with type 1 diabetes: a systematic review and meta-analysis. J Clin Med 2019; 8(2): 253. [CrossRef]
23. Whitfield GP, Carlson SA, Ussery EN, Fulton JE, Galuska DA, Petersen R. Trends in meeting physical activity guidelines among urban and rural dwelling adults-United States, 2008-2017. MMWR Morb Mortal Wkly Rep 2019; 68(23): 513-8. [CrossRef]
24. Naguib MM, Ellström P, Järhult JD, Lundkvist Å, Olsen B. Towards pandemic preparedness beyond COVID-19. Lancet Microbe 2020; 1(5): 185-6. [CrossRef]
25. Blum E, Abdelwahed YS, Spiess E, Mueller-Werdan U, Leistner DM, Rosada A. COVID-19 #StayAtHome Restrictions and Deep Vein Thrombosis: Case Report. Interact J Med Res 2021; 10(1): e23443. [CrossRef]
26. Goethals L, Barth N, Guyot J, Hupin D, Celarier T, Bongue B. Impact of Home Quarantine on Physical Activity Among Older Adults Living at Home During the COVID-19 Pandemic: Qualitative Interview Study. JMIR Aging 2020; 3(1): e19007. [CrossRef]

27. Parmet WE, Sinha MS. Covid-19-The Law and Limits of Quarantine. N Engl J Med 2020; 382(15): e28. [CrossRef]
28. Bachireddy C, Chen C, Dar M. Securing the Safety Net and Protecting Public Health During a Pandemic: Medicaid's Response to COVID-19. JAMA 2020; 323(20): 2009-10. [CrossRef]
29. Inglesby TV. Public Health Measures and the Reproduction Number of SARS-CoV-2. JAMA 2020; 323(21): 2186-7. [CrossRef]
30. World Health Organization. Global recommendations on physical activity for health. World Health Organization 2010 (Available from: https://apps.who.int/iris/handle/10665/44399).
31. Cox C, Kamal R, McDermott D. How have healthcare utilization and spending changed so far during the coronavirus pandemic? San Francisco (CA): Peterson-KFF Health System Tracker; 2020 (Available from: https://www.healthsystemtracker.org/chart-collection/how-have-healthcare-utilization-and-spending-changed-so-far-during-the-coronavirus-pandemic/.
32. De Rosa S, Spaccarotella C, Basso C, Calabrò MP, Curcio A, Filardi PP, et al. Reduction of hospitalizations for myocardial infarction in Italy in the COVID-19 era. Eur Heart J 2020; 41(22): 2083-8. [CrossRef]
33. Siegler JE, Heslin ME, Thau L, Smith A, Jovin TG. Falling stroke rates during COVID-19 pandemic at a comprehensive stroke center. J Stroke Cerebrovasc Dis 2020; 29(8): 104953. [CrossRef]
34. Helgeland J, Telle KE, Grøsland M, Huseby BM, Håberg S, Lindman AS. Admissions to Norwegian hospitals during the COVID-19 pandemic. Scand J Public Health 2021; 49(7): 681-88. [CrossRef]

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The Study of Whole Genome Sequencing in Monozygotic Twins with Autism Spectrum Disorder

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ABSTRACT

Objective: Autism spectrum disorder (ASD), the most well-known type of neurodevelopmental disorder, is a mental development disorder. Since there is no definitive biomarker for ASD, diagnosis is made based on the assessment of the patient's behavior. In addition to behavioral and social disorders, genetic factors are also important in ASD.

Materials and Methods: In the study, variant analyses were performed by whole genome sequencing (WGS) method, as well as evaluating the clinical features of two monozygotic twin couples (one discordant and the other concordant).

Results: According to the WGS results, thirteen high pathogenic variants were detected in twenty-nine novel candidate genes. Candidate genes include *MEAF6, OR2T8, ABI2, PDE4D, GLIS3, DRD4, LPXN, FAM186A, NEK3, GOLGA8A, SSC5D, ARMCX4, ADAR, LRP1B, DAP, LYRM7, MUC12, CNTNAP3B, TCP11L1, OR8B3, KLRC3,* and *DPP9*.

Conclusion: We speculate that clinical evaluations and examination of genetic changes are important for understanding the disease in individuals with ASD and their families.

Keywords: Autism spectrum disorder, MZ twins, whole-genome-sequencing (WGS), genetics

INTRODUCTION

Autism spectrum disorder (ASD) is a type of mental developmental disorder with characteristic features such as limitations in social communication, repetitive behaviors, insistence on sameness, and limited interests (1). The prevalence of ASD is below 1.0% worldwide. However, this rate is thought to be higher in developed countries (2). In a study that included eleven regions in the United States, the prevalence of the disease was determined as 18.5:1000 (for 8-year-old children). It has been revealed that the incidence of ASD in boys is 4.5 times higher than in girls. Symptoms of the disease appear in the early period (at the age of 1-2 years) (3, 4). Individuals with ASD have difficulties in social behavior, emotional and non-verbal communication, and relationship building. Additionally, restricted areas of interest and repetitive behavior patterns are common clinical features. Examination of social communication, limited interests, and repetitive behavioral symptoms are particularly important in diagnosis of autism (5, 6). Emotional symptoms such as depression, anxiety and attention problems, behavioral conditions such as aggression, and challenging behaviors can be noticeable in individuals with autism (7). Although imaging techniques such as magnetoencephalography (MEG), and magnetic resonance imaging (MRI), positron emission tomography (PET), single-photon emission computerized tomography (SPECT), and electroencephalography (EEG) are neuroimaging techniques that can be used in brain imaging with autism, the diagnosis of the disease is usually made routinely with clinical evaluations (8). Neuroanatomical differences in various parts of the brain are thought to be associated with behavioral and cognitive abnormalities, especially in individuals with ASD aged 2-3 years (9). Gastrointestinal problems, attention

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deficit hyperactivity disorder (ADHD), bipolar disorder, Tourette syndrome and tic disorders, Childhood-onset schizophrenia and epilepsy, and conditions such as sleeping, feeding, and toilet problems have been identified as comorbidities that are associated with ASD. Studies have shown that the prevalence of ASD in epilepsy is high and that it has similar etiological aspects (6,10,11). There are limited treatment options for improving the symptoms seen and for the accompanying mental or clinical manifestations that may increase the severity of the disease in ASD. As in numerous other diseases, personalized treatment and precision medicine approaches are thought to be effective in treatment of ASD.

As a result of studies on twins, it was revealed that genetic and environmental factors were related to the etiology of psychiatric diseases, including autism, and it was determined that the concordance in monozygotic (MZ) twins were higher than in dizygotic (DZ) twins. In addition, it has been stated that genetic factors may influence brain size, curvature, and subcortical gray matter in brains with ASD while environmental factors may have an effect on brain regions such as cortical thickness and cerebellar white matter (12-15). Numerous single-nucleotide polymorphisms (SNPs) or copy number variations (CNVs) have been identified in protein-coding genes, which participate in events such as neuronal development and synapse formation, in approximately 25-35% of individuals, in consequence of many studies conducted with genome sequencing analysis in ASD. In these studies, about one thousand genes were thought to be associated with the disease have been identified. When ASD-related genes are examined, it is thought that changes in many human genes such as *SHANK3, CDH8, CDH9, CDH10, CSMD1, SCNA2, CNTNAP2, MACROD2, SLC9A9*, and *BCKDK* were associated with ASD. With these analyses, it has been detected that CNVs in regions (16q11.2), (15q11-13), (Xp22.3), (15q13.1–13.2), (3p26.3 and 2p12) (16-18).

In this study, we aimed to learn more about the genetic background of the disease and to examine its contribution to the ASD phenotype by performing whole genome sequencing (WGS) analysis on two MZ twins. Besides, we examined the effects of the clinical and psychological conditions of the parents on individuals with autism by applying tests that are used to measure autism status, depression, mood, and quality of life to the families of a couple of concordant and discordant twins.

MATERIALS AND METHODS

Participants

Our study was conducted with concordant and discordant twins diagnosed with ASD at the Umraniye Training and Research Hospital, Child and Adolescent Psychiatry Clinic. Ethical approval of the project was taken from University of Health Sciences, Umraniye Training and Research Hospital, Clinical Research Ethics Committee (B.10.1.TKH.4.34.H.G.P.0.0.1/167, 19.12.2018). In the study, the clinical data of two twin couples, one discordant (Twin couple 1; twin 1.1 and 1.2) and the other concordant (Twin couple 2; twin 2.1 and 2.2), were examined. Then, WGS analysis was performed to investigate the genetic differences and their relationship with the disease. Toronto Alexithymia Scale (TAS-20) and Autism Spectrum Quotient (AQ) questionnaires were applied to the mothers and fathers to measure autism status, depression, mood, and quality of life in the parents of twin couples. In addition, Beck Depression Inventory (BDI) and World Health Organization Quality of Life (WHOQOL- BREF) questionnaires were completed.

Genomic Sample Collection and Preparation

Peripheral blood samples (~2 ml) were collected into a tube with EDTA. The total DNA was extracted from 200 µL blood samples according to the manufacturer's instructions (Cat. No. 11796828001, Roche Applied Sciences, Germany), and the DNAs were kept in a freezer at -20^{0}C until sequencing. Through spectrophotometric analysis (DENOVIX DS-11 FX, USA), the concentration of the samples was determined as 200 ng/µL. DNA fragments were ligated with adaptor oligonucleotides to form paired-end DNA libraries with an insert size of 500 base pair (bp).

Whole Genome Sequencing (WGS)

The samples were run on the Illumina Novaseq platform (NovaSeq™ 6000 Sequencing System, Cat. No. 20012850, US) on S1 flow cell that has 2 lanes; the data is from the two lanes. In the current study, which used the Illumina NovaSeq6000 system, an average length of 100 bp, a sequence depth of 12 Gb per sample and 100×10^6 paired end were read. A total of 265,815 unigenes were detected with an average contig length of 201 bp.

Bioinformatic Analysis

The pool has been created as two forward fast adaptive shrinkage thresholding algorithm and quality (FASTQ) files and two reverse FASTQ files for each sample (as each sample has 4 FASTQ files: 2 forward and 2 reverse (paired-end)). Variant call format (VCF) and PLINK files were created. In preparing the VCF files, we included all possible variants right after standard genome analysis toolkit (GATK, 4.2.0.0) bioinformatics analyses on purpose, in which only a minimum of standard quality control was applied. These VCF files were intended to provide a comprehensive pool of variants, from which further quality controls can be applied manually to filter for higher quality variants. The effects (mutations) and classifications (localization) of variants in genome wide were annotated by ANNOVAR (Annotate Variation). Assuming that the disease is caused by different genotypes between affected and unaffected individuals, MZ couples were compared among themselves to identify differences (variants). Subsequently, overlapping of identified variants shared by the two families was found. The data was analyzed using R Bioconductor (V.3.13; it works with R V.4.1.0). In the study, filtering was performed so that the quality deep (QD) value was between 27-33.

RESULTS

Clinical, Developmental and Diagnostic Evaluation of Twins

When the clinical characteristics of twin individuals were examined, it was observed that while all individuals were found to

have an early birth time and a low birth weight, none of them had epilepsy (Table 1). According to the developmental evaluations of the twins, it was observed that only the individual with severe autism (Twin 2.2) did not speak, and it was determined that walking was delayed in the twin 2 couple, and they did not have toilet training (Table 2). Diagnostic features were divided into social disability, communicative limitation, and repetitive interests and limitations categories and evaluated in three individuals with autism other than the healthy individual. As a conclusion, it was determined that the symptoms were directly proportional to the severity of autism, and it was noticed that the regression of the symptoms was more pronounced with special education, especially in twin 1.1. When the autism-behavior-checklist (ABC) and childhood-autism–rating-scale (CARS) scores were examined in individuals with autism, it was revealed that these values were increased with the severity of autism (Table 3).

Parental Information

While the parents of the twin couple were alive and married, it was determined that there was no consanguinity between the parents. While there was no individual with any psychological illness in the family and relatives of twin 1, it was stated that one of the relatives of twin 2 had a late speaking individual. As a result of the TAS-20 evaluation, possible alexithymia was detected only in the mother of twin 1 (59 points), while alexithymia was detected in other parents (Twin couple 1 father, 75 points; twin couple 2 mother, 71 points; twin couple 2 father, 71 points). According to BDI examinations, mothers of twin couple 1 (24 points) and 2 (29 points) had moderate depression in both. As a result of the WHOQOL-BREF test, the psychological evaluation of both mothers was below 50% (Twin couple 1 mother, 45,8%; twin couple 2 mother, 41,7%). However, in the mother of twin 2, the value of all categories was below 50%.

Genetic Assessment of WGS

According to the identical variants between the twins were examined, there were seven high pathogenic variants out of 64,867 variants, of which 17,936 were genic, when the identical alleles were examined, out of 23,362 variants, of which 5,823 were genic, six high pathogenic variants were detected. As a result of the comparison of the variants of the twin couples among themselves, fifteen of the 265,815 variants, of which 45,626 were genic in the discordant twin couple (Twin couple 1), were determined as high pathogenic. According to the examination of the different variants in the concordant twin couple (Twin couple 2), it was determined that fourteen out of 268,928 variants, 45,521 of which were genic variants, were high pathogenic variants. After filtering data of twins, *MEAF6, OR2T8, ABI2, PDE4D, GLIS3, DRD4, LPXN, FAM186A,*

Table 1. Clinical characteristics of twins.

	Twin 1.1	Twin 1.2	Twin 2.1	Twin 2.2
Age	15		6	
Gender	Female		Female	
Diagnosis-Severity Mild Moderate Severe Healthy	Mild	Healthy	Moderate	Severe
Birth Time Pre-term (< Week 37) Term (Week 37-41) Post-term (≥ Week 42)	Preterm	Preterm	Preterm	Preterm
Birth Weight (g) Very low (<1500 g) Low (<2500 g) Normal (2500-3999 g) High (>4000 g)	Very low	Very low	Very low	Very low
Epilepsy Yes No	No	No	No	No
History of Incubator Yes No	Yes	No	Yes	Yes

Table 2. Developmental information of twins.

	Twin 1.1	Twin 1.2	Twin 2.1	Twin 2.2
Unsupported Sitting Early (<month 7) In time (month 7-9) Late (>month 7-9)	In time	In time	In time	In time
Babbling Early (<month 3) In time (month 3) Late (>month 3) No Babbling	In time	Early	In time	In time
Teething Early (<month 6) In time (month 6-8) Late (>month 6-8)	Late	Late	Late	Late
Walking Early (<month 11) In time (month 11-15) Late (>month 11-15) No Walking	In time	In time	Late	Late
Talking Yes No Regression	Yes	Yes	Yes	No
Toilet Training Early (<years 2-3) Normal (years 2-3) Late (≥years 4) No Toilet Training	Normal	Normal	No Toilet Training	No Toilet Training

Table 3. Total CARS and ABC scores of twins with autism.

	Twin 1.1	Twin 2.1	Twin 2.1
CARS Score	23.5	36	47.5
ABC Scores			
Sensory	7	13	26
Relating	20	26	38
Stereotypes and object use	4	34	34
Language	0	26	18
Self-Help And Social	7	15	18
Total Score	**38**	**114**	**134**

CARS, Childhood Autism Rating Scale; ABC, Autism Behavior Checklist.

NEK3, GOLGA8A, SSC5D, ARMCX4, ADAR, LRP1B, DAP, LYRM7, MUC12, CNTNAP3B, TCP11L1, OR8B3, KLRC3, and *DPP9* genes have been identified as candidate genes in ASD (Figure 1 and 2, Table 4).

DISCUSSION

In the large-scale association studies, genetic heterogeneity and environmental factors make it difficult to reach clear conclusions for disease etiology, especially for psychiatric diseases.

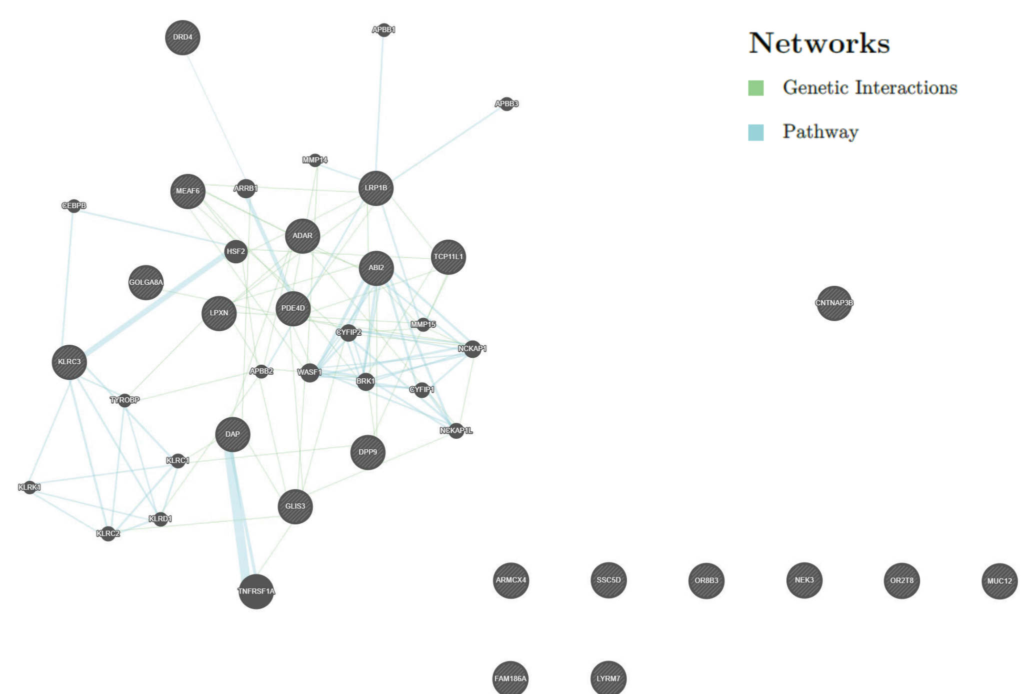

Figure 1. Genetic interaction and pathway networks of candidate genes obtained by WGS analysis. Green lines show genetic interaction, blue lines show pathway networks (https://genemania.org/).

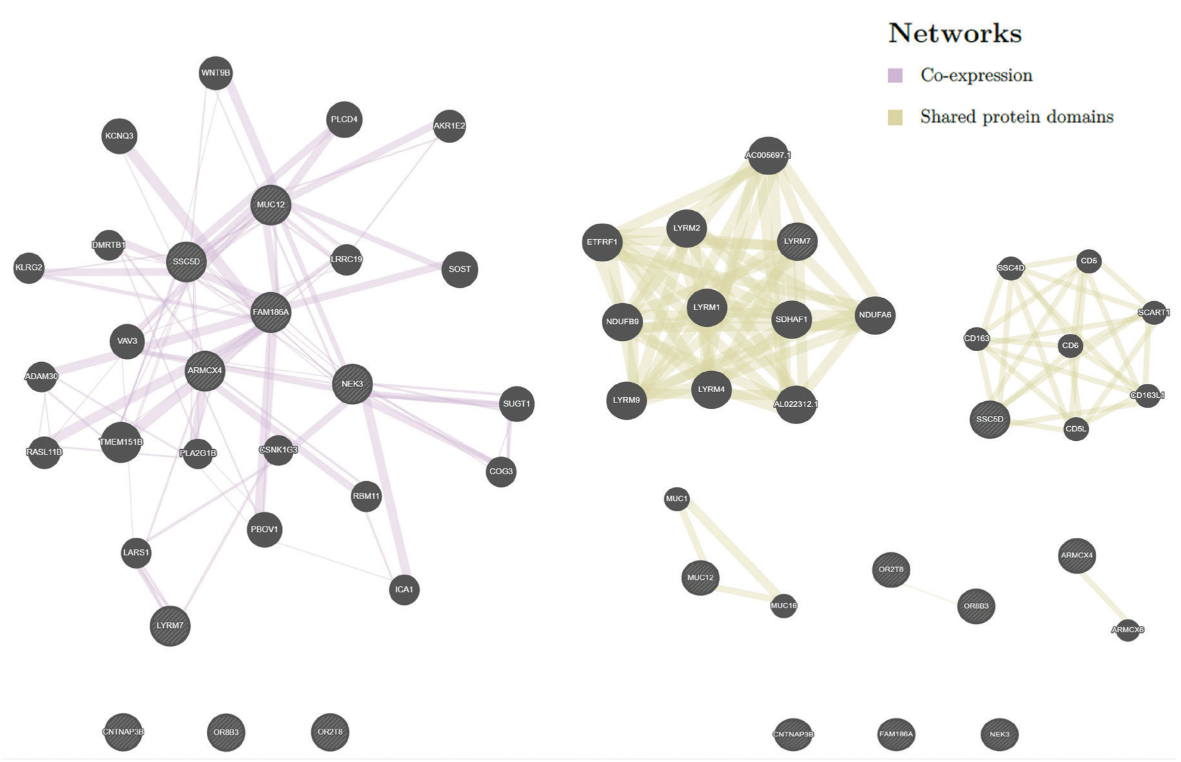

Figure 2. Co-expression and shared protein domains networks of candidate genes obtained by WGS analysis. Violet lines show co-expression, yellow lines show shared protein domains networks (https://genemania.org/).

Table 4. Comparison results of variants obtained by WGS analysis.

<table>
<tr><th></th><th>CHROM: POSX</th><th>REF</th><th>ALT</th><th>GT1</th><th>GT2</th><th>AF</th><th>CH</th><th>EFF</th><th>CD</th><th>AT</th><th>RS</th><th>GENE INFO</th></tr>
<tr><td rowspan="10">Twin Couple 1</td><td>chr1:37498409</td><td>TTTA</td><td>T,*</td><td>2/2</td><td>0/0</td><td>0.25,
0.25</td><td>T</td><td>Moderate</td><td>Protein coding, downstream gene variant, pseudogene, intron variant</td><td>c.534-1676_534-1674del
|n.*2523_*2525delTAA
c.534-1676_534-1674del
c.534-2494_534-2492del
c.533+3392_533+3394del
n.607-1676_607-1674del
n.607-1808_607-1806del
n.696-1676_696-1674del</td><td>35576307</td><td>MEAF6:64769</td></tr>
<tr><td>chr1:247921552</td><td>A</td><td>G</td><td>1/1</td><td>0/1</td><td>0.625</td><td>G</td><td>Moderate</td><td>Protein coding</td><td>c.535A>G|p.Thr179Ala</td><td>4584426</td><td>OR2T8:343172</td></tr>
<tr><td>chr1:247921607</td><td>T</td><td>G</td><td>1/1</td><td>0/1</td><td>0.625</td><td>G</td><td>Moderate</td><td>Protein coding</td><td>c.590T>G|p.Met197Arg</td><td>4474294</td><td>OR2T8:343172</td></tr>
<tr><td>chr2:203395448</td><td>T</td><td>TATATATATATACAC,
TATATACACACAC,
TAC</td><td>0/0</td><td>0/1</td><td>0.125,
0.25,
0.25</td><td>TAC</td><td>Moderate</td><td>Protein coding, intron variant</td><td>c.708-208_708-207ins
c.708-208_708-207ins
c.726-207_726-206ins
c.726-172_726-171dup
c.708-207_708-206ins
c.708-172_708-171dup
c.540-207_540-206ins
c.540-172_540-171dup
c.708-207_708-206ins
c.708-172_708-171dup
c.573-207_573-206ins
c.573-207_573-206ins
c.573-172_573-171dup</td><td>750379504</td><td>ABI2:10152</td></tr>
<tr><td>chr5:59266270</td><td>AATAT</td><td>A,AAT</td><td>2/2</td><td>1/2</td><td>0.375,
0.625</td><td>A</td><td>Moderate</td><td>Protein coding, upstream gene variant, intron variant</td><td>c.90-50306_90-50303del
c.90-60156del
c.-722_-721del
c.-722del
c.456-60156_456-60155del
c.456-60156del
c.90-60156_90-60155del
c.90-60156del
c.48-60156_48-60155del
c.264-60156_264-60155del
c.264-60156del
c.273-60156_273-60155del
c.273-60156del</td><td>150096498</td><td>PDE4D:5144</td></tr>
<tr><td>chr5:59276122</td><td>GAA</td><td>GA,G</td><td>1/1</td><td>0/1</td><td>0.625,
0.25</td><td>G</td><td>Moderate</td><td>Protein coding, intron variant</td><td>c.90-60156_90-60155del</td><td>753205660</td><td>PDE4D:5144</td></tr>
<tr><td>chr7:100999201</td><td>G</td><td>A</td><td>1/1</td><td>0/0</td><td>0.25</td><td>A</td><td>Moderate</td><td>Protein coding, upstream gene variant, intron variant</td><td>c.8638G>A|p.Glu2880Lys</td><td>.</td><td>.</td></tr>
<tr><td>chr9:4118111</td><td>G</td><td>T</td><td>0/1</td><td>1/1</td><td>0.875</td><td>T</td><td>Moderate</td><td>Protein coding</td><td>c.1367C>A|p.Pro456Gln
c.902C>A|p.Pro301Gln</td><td>6415788</td><td>GLIS3:169792</td></tr>
<tr><td>chr11:640090</td><td>G</td><td>C</td><td>1/1</td><td>0/1</td><td>0.75</td><td>C</td><td>Moderate</td><td>Protein coding</td><td>c.841G>C|p.Ala281Pro
c.*4460C>G
c.*4460C>G</td><td>3889692</td><td>DRD4:1815</td></tr>
<tr><td>chr11:1095015</td><td>T</td><td>C</td><td>1/1</td><td>0/1</td><td>0.75</td><td>C</td><td>Moderate</td><td>Protein coding</td><td>c.4772T>C|p.Ile1591Thr</td><td>.</td><td>.</td></tr>
</table>

Table 4. Comparison results of variants obtained by WGS analysis. (Countinued)

	CHROM: POSX	REF	ALT	GT1	GT2	AF	CH	EFF	CD	AT	RS	GENE INFO
Twin Couple 1	chr11:58555768	GCACA	G,GCA	1/2	0/2	0.375, 0.5	G	Moderate	Protein coding	|c.234-832_234-829del c.234-832_234-831del c.219-832_219-829del c.219-832_219-831del c.234-832_234-829del c.234-832_234-831del c.219-832_219-829del c.219-832_219-831del c.159-832_159-829del c.159-832_159-831del	71454340	LPXN:9404
	chr12:50352315	A	G	0/1	1/1	0.875	G	Moderate	Protein coding	c.4517T>C|p.Leu1506Pro	10876022	FAM186A:121006
	chr13:52133615	T	TCACACA	1/1	0/1	0.625	TCACACA	Moderate	Protein coding, upstream gene variant, pseudogene, intron variant	c.1436+68_1436+73dup c.1385+68_1385+73dup c.-6139_-6134dup n.*892_*893ins c.1436+68_1436+73dup c.1436+68_1436+73dup c.1385+68_1385+73dup n.1811+68_1811+73dup	3831081	NEK3:4752
	chr15:34386710	C	G	0/1	1/1	0.875	G	Moderate	Protein coding, non-coding transcript exon variant, upstream gene variant, pseudogene	c.200G>C|p.Arg67Pro n.-4560G>C n.-4560G>C n.2571G>C	147828722	GOLGA8A:23015
	chr16:4920371	G	GAAAGAA AGAAAGA AAGAAAG AAAGAAA GAAAGAA AGAAAGA AA, GAAAGAA AGAAAGA AAGAAAG AAAGAAA GAAAGAA AGAAAGA AAGAAA,*	1/1	0/1	0.375, 0.125, 0.125	GAAAGA AAGAAAG AAAGAAA GAAAGAA AGAAAGA AAGAAAG AAA	Moderate	Protein coding	c.63-9423_63-9422ins c.63-9423_63-9422ins c.63-9423_63-9422ins c.63-9423_63-9422ins	.	.
	chr19:55518098	G	A	1/1	0/1	0.875	A	Moderate	Protein coding	c.3822G>A|p.Met1274Ile	4801331	SSC5D:284297
	chrX:101494140	A	G	0/1	1/1	0.75	G	Moderate	Protein coding, intron variant, pseudogene	c.5551A>G|p.Ile1851Val n.1549-4005A>G n.1440-4005A>G n.574-4005A>G n.1482-4005A>G	5951336	ARMCX4:100131755

Table 4. Comparison results of variants obtained by WGS analysis. (Countinued)

	CHROM: POSX	REF	ALT	GT1	GT2	AF	CH	EFF	CD	AT	RS	GENE INFO
Twin Couple 2	chr1:154593135	CA	C	0/1	1/1	0.875	C	Moderate	Protein coding, intron variant	c.2271-2727del c.1386-2727del c.2271-2727del c.2214-2727del c.1386-2727del	556341696	ADAR:103,ADAR:103
	chr2:140272258	AACACACACAC	A, AACACAC ACACAC, AACACAC ACACACA CACAC	0/3	2/3	0.25, 0.375, 0.25	A	Moderate	Protein coding, intron variant	c.13143-1922_13143-1913del c.13143-1924_13143-1923dup c.13143-1922_13143-1913del c.13143-1924_13143-1923dup c.13143-1930_13143-1923dup	138326343	LRP1B:53353
	chr4:2042401	C	T	1/1	0/1	0.875	T	Moderate	Protein coding	c.149C>T|p.Pro50Leu c.50C>T|p.Pro17Leu	570712	C4orf48:401115|NELFA:7469
	chr5:10731085	G	T	0/1	1/1	0.625	T	Moderate	Protein coding, intron variant	c.152+17090C>A c.152+17090C>A c.152+17090C>A	93417	DAP:1611
	chr5:10739201	CAAA	C	0/0	1/1	0.5	C	Moderate	Protein coding, intron variant	c.152+8971_152+8973del c.152+8971_152+8973del c.152+8971_152+8973del	558306009	DAP:1611
	chr5:131197846	CTGTG	C,CTG	1/1	0/1	0.625, 0.25	C	Moderate	Protein coding, intron variant, pseudogene	c.245-1684_245-1681del c.245-1682_245-1681del c.245-1649_245-1646del c.245-1647_245-1646del c.163-1649_163-1646del c.163-1647_163-1646del n.199-1649_199-1646del	72182125	LYRM7:90624,LYRM7:90624
	chr6:51804371	GGGC	G,*	0/0	1/1	0.25, 0.25	G	Moderate	Protein coding, intron variant	c.8303-13001_8303-12999del c.8303-13001_8303-12999del c.8303-13001_8303-12999del c.8303-13001_8303-12999del	.	.
	chr7:101004095	C	T	0/1	1/1	0.5	T	Moderate	Protein coding	c.13532C>T|p.Thr4511Ile	201694075	MUC12:10071
	chr9:41938349	G	T	1/1	0/1	0.875	T	Moderate	Protein coding	c.2132C>A|p.Ser711Tyr	62536540	CNTNAP3B:728577
	chr11:1095015	T	C	0/1	1/1	0.75	C	Moderate	Protein coding	c.4772T>C|p.Ile1591Thr	.	.
	chr11:33047216	G	GA	0/1	1/1	0.875	GA	Moderate	Protein coding, intron variant	c.163+3280_163+3281ins c.163+3293dup c.163+3293dup	11385765	TCP11L1:55346,TCP11L1:55346
	chr11:124397010	C	T	0/1	1/1	0.625	T	Moderate	Protein coding	c.342G>A|p.Met114Ile	530992	OR8B3:390271
	chr12:10420546	C	T	0/1	1/1	0.625	T	Moderate	Protein coding	c.5G>A|p.Ser2Asn	2682489	KLRC3:3823

Table 4. Comparison results of variants obtained by WGS analysis. (Countinued)

	CHROM: POSX	REF	ALT	GT1	GT2	AF	CH	EFF	CD	AT	RS	GENE INFO
Twin Couple 2	chr16:4920371	G	GAAAGAA AGAAAGA AAGAAAG AAAGAAA GAAAGAA AGAAAGA AA, GAAAGAA AGAAAGA AAGAAAG AAAGAAA GAAAGAA AGAAAGA AAGAAA,*	0/2	0/3	0.375, 0.125, 0.125	GAAAGA AAGAAAG AAAGAAA GAAAGAA AGAAAGA AAGAAAG AAA	Moderate	Protein coding, intron variant	c.63-9423_63-9422ins c.63-9423_63-9422ins |c.63-9423_63-9422ins	.	.
	chr19:4719326	TTAAATAAA	T, TTAAATA AATAAA	2/2	0/2	0.25, 0.375	T	Moderate	Protein coding, intron variant	c.56+517_56+524del c.56+513_56+516dup c.56+517_56+524del c.56+513_56+516dup	150534589	DPP9:91039
	chrX:101494140	A	G	0/1	1/1	0.75	G	Moderate	Protein coding, intron variant, pseudogene	c.5551A>G|p.Ile1851Val n.1549-4005A>G n.568-4005A>G n.1440-4005A>G n.574-4005A>G n.1482-4005A>G	5951336	ARMCX4:100131755

* CHROM & POSX, Chromosome and position; REF, Reference Allele; ALT, Alternate Allele; GT 1, Genotype for 1. twin; GT 2, Genotype for 2. twin; AF, Allele Frequency; CH, Changing; EFF, Effect; CD, Protein Coding or non-coding region; AT, Alteration; RS, dbSNP ID; GENE INFO, Pairs each of gene symbol, gene id.
* CHROM & POSX, Chromosome and position; REF, Reference Allele; ALT, Alternate Allele; GT 1, Genotype for 1. twin; GT 2, Genotype for 2. twin; AF, Allele Frequency; CH, Changing; EFF, Effect; CD, Protein Coding or non-coding region; AT, Alteration; RS, dbSNP ID; GENE INFO, Pairs each of gene symbol, gene id.

However, it may be possible to obtain definitive findings with genetic or epigenetic studies with twins. Twin studies have some advantages over studies with non-twins. Twin studies can sometimes be developed into longitudinal studies. Before and after diagnosis, severity of disease, speaking, age of onset, the profile of symptoms, response to a variety of drugs might potentially need to be considered. With next-generation sequencing technology, it is possible to match clinical features with genetic changes and to detect epigenetic differences. Twin studies, with this technology, make it possible to detect differences in several aspects such as somatic mutations, DNA methylation, histone modifications, CNVs, single nucleotide variants (SNVs), changes in introns, synapse formation, and regulation of neural networks including microglia (12). However, the detection of discordant twins also play a considerable role in understanding the etiology of psychiatric diseases.

Not a little evidence demonstrates the importance of complex genetic factors in ASD development. Examples of candidate genes in our study include *Abl Interactor 2* (*ABI2*), a protein coding gene. *ABI2* is a gene associated with autosomal recessive limb-girdle muscular dystrophy type 2H. In a study investigating the genetic background of autism, it was seen that the *ABI2* gene was among the genes with *de novo* missense mutations discovered in consequence of the evaluation of the exome sequencing results (19). As for that to a study conducted with 192 relatives with non-syndromic intellectual disability, homozygous loss-of-function mutations were found in nine genes, including the *ABI2* gene, in 43 families (20). *MYST/Esa1 Associated Factor 6* (*MEAF6*) is a gene that encodes a nuclear protein involved in transcriptional activation, with a pseudogene for this gene on chromosome 2. This gene aberration was observed in our study. Genes expressed at various levels in schizophrenia and schizoaffective disorder were investigated with microarray datasets; it was determined that *MEAF6* expression levels were low in parvalbumin positive neurons of the 3rd layer of the dorsolateral prefrontal cortex in patients (21). *Mucin 12 (MUC12)* gene encodes an integral membrane glycoprotein that play a crucial role in forming protective mucous barriers on epithelial surfaces and have been implicated in epithelial regeneration and differentiation. In an exome sequencing study conducted with individuals with autism, a *de novo* nonsense variant in the *MUC12* gene was identified in a case with ASD (20). In a different study, in which postzygotic mutations were analyzed with whole exome sequencing (WES) in ASD, six non-synonymous postzygotic mosaic mutations (PZM) were identified in the *MUC12* gene (22). *Dopamine Receptor D4 (DRD4)* gene encodes the D4 subtype of the dopamine receptor. The D4 subtype is a G-protein coupled receptor which inhibits adenylyl cyclase. This gene contains a polymorphic number (2-10 copies) of tandem 48 nt. repeats; the sequence shown contains four repeats. A high prevalence of rare *DRD4* alleles in children diagnosed with ADHD was reported. As to examining whether the *DRD4* alleles overlap in ADHD and ASD, it was reported that rare variants were not observed in individuals with ASD (23). It is known that dopamine receptors are involved in the control of behav-

ior-related signals and are associated with attention disorders. Although the *DRD4* gene, which is associated with the post-synaptic effect of the dopamine hormone, participates in many neural pathways, these gene polymorphisms are thought to be associated with psychiatric disorders (24).

Considering the relationship of the exon 3-7 repeat allele in the *DRD4* gene with autistic symptoms in twins with ADHD was investigated, it was suggested that high repeat alleles may increase the risk of autistic symptoms. As a result, it was reported that this gene may be associated with the possibility of autistic features in the phenotype (25). According to another study, it was stated that *DRD4* polymorphisms of oppositional defiant disorder, separation anxiety disorder, obsession-compulsions, and repetitive behaviors may be related to the severity of the symptoms of the disease. It has been reported that the symptoms are more severe in the phenotype. In addition, it has been determined that oppositional defiant disorder symptoms are more severe in patients who are homozygous with the *DAT1* 10-repeat allele and who are carriers of the *DRD4* 7-repeat allele. These results support the idea that *DRD4* polymorphisms may be a candidate biomarker associated with autism severity (26). In another study, the *DRD4* gene repeat allele was examined in ASD individuals, as well as in their parents, and the children were examined in terms of opposition, separation anxiety, and repetitive behaviors (27). Consequently, the 7-repeat genotypes were found to be associated with oppositional defiant disorder, obsessive-compulsive disorder, and tic severity, it was concluded that genotype research in families could help to establish biomarkers for the evaluation of prognosis for behavioral disorders in patients with ASD.

Phosphodiesterase 4D (PDE4D) regulates cyclic adenosine monophosphate (cAMP) signaling and plays a crucial role in sex-specific signaling regulation in ASD. In a study investigating the behavioral and biochemical effects of *CC2D1A* deficiency in male and female mice in intellectual disability and autism spectrum disorder, it was shown that, unlike females, *PDE4D* was hyperactive in *CC2D1A*-deficient male mice, resulting in a decrease in cAMP response element-binding protein signaling (28).

Armadillo Repeat Containing X-Linked 4 (ARMCX4) is a member of the ARMadillo repeat-containing proteins gene family on the X chromosome (29). In a study examining genetic aberrations with WES in childhood-onset schizophrenia (COS) patients, variants of this gene were identified in twelve male (30). To date, there is no study describing the relationship of this gene with autism. However, this gene variant (c.5551A>G|p.Ile1851Val) was detected in our study.

ADAR enzymes are important in the healthy development of the brain. *ADAR* gene has been linked with Fragile X and ASD (31). In a study in which ASD-related genes were examined by transcriptome analysis, it was suggested that *ADAR* enzymes may cause deterioration in the cells due to insufficient regulation in inhibitory neurons (32).

Low-density lipoprotein (LDL) receptor related protein 1B (LRP1B) gene belongs to the receptor gene family. These receptors play a wide variety of roles in normal cell function and development due to their interactions with multiple ligands. A study conducted with array-comparative genomic hybridization (aCGH) analysis examined a 23-year-old individual with episodes of unexplained severe mental retardation, autism spectrum disorder, congenital malformations including hypospadia and omphalocele, and episodes of high blood pressure. In the study, mutations in eight genes, including the *LRP1B* gene, were detected in an individual with autism accompanied by mental retardation (33).

Death associated protein (DAP) gene has been found associated with ASD as a biomarker by Carvalho et al. (34). Also, in the Center for Health Assessment of Mothers and Children of Salinas [CHAMACOS], associations of prenatal *DAP*s with lower IQ, poorer attention (35), and other genes, such as *GLIS3, LPXN, FAM186A, NEK3, GOLGA8A, SSC5D, LYRM7, CNTNAP3B, TCP11L1, OR8B3, KLRC3*, and *DPP9* had been reported. As a consequence of studies on ASD up to now, indels, SNVs, and CNVs in many genes have been found to be associated with the disease. Genetic variations revealed by whole genome sequencing and whole exome sequencing studies on concordant and discordant twins are important in understanding the genetic background of the disease. Even with great strides in understanding the genetic basis of ASD by sequencing of multiple cohorts in todays, many causes underlying autism remains undiscovered.

CONCLUSION

Our study provides evidence that WGS data can aid in the detection and clinical evaluation of individuals with ASD and their families. According to analysis of sequence, rs5951336 variant in ARMCX4 gene were detected in our MZ twins. The diagnostic yield and clinical utility should increase as more undetected structural genetic variants are discovered and characterized and as additional individuals with ASD are studied. Several genome sequences may help to resolve the role of common variants in ASD, and integrating these data with those on rare variants will aid understanding of penetrance, variable expressivity, and pleiotropic effects. As a result, genetic susceptibility to ASD may be different for each individual. This makes that individual a prime candidate for the precision medicine era.

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Drafting Manuscript - E.C., O.B.G.; Critical Revision of Manuscript - E.C., O.B.G.; Final Approval and Accountability - E.C., O.B.G.;

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REFERENCES

1. American Psychiatric Association. Diagnostic and statistical manual of mental disorders: DSM-5. Arlington, VA; 2013.
2. Lord C, Brugha TS, Charman T, Cusack J, Dumas G, Frazier T, et al. Autism spectrum disorder. Nat Rev Dis Primers 2020; 6(1): 5. [CrossRef]
3. Maenner MJ, Shaw KA, Baio J, Washington A, Patrick M, DiRienzo M, et al. Prevalence of autism spectrum disorder among children aged 8 years autism and developmental disabilities monitoring network, 11 sites, United States, 2016. MMWR Surveill Summ 2020; 69(4): 1-12. Erratum in: MMWR Morb Mortal Wkly Rep. 2020 Apr 24; 69(16): 503.
4. Tan C, Frewer V, Cox G, Williams K, Ure A. Prevalence and age of onset of regression in children with autism spectrum disorder: A systematic review and meta-analytical update. Autism Res 2021; 14(3): 582-98. [CrossRef]
5. Lord C, Elsabbagh M, Baird G, Veenstra-Vanderweele J. Autism spectrum disorder. Lancet 2018; 392(10146): 508-20. [CrossRef]
6. Sharma SR, Gonda X, Tarazi FI. Autism Spectrum Disorder: Classification, diagnosis and therapy. Pharmacol Ther 2018; 190: 91-104. [CrossRef]
7. Tsai CH, Chen KL, Li HJ, Chen KH, Hsu CW, Lu CH, et al. The symptoms of autism including social communication deficits and repetitive and restricted behaviors are associated with different emotional and behavioral problems. Sci Rep 2020; 10(1): 20509. [CrossRef]
8. Spence SJ, Sharifi P, Wiznitzer M. Autism spectrum disorder: screening, diagnosis, and medical evaluation. Semin Pediatr Neurol 2004; 11(3): 186-95. [CrossRef]
9. Xiao Z, Qiu T, Ke X, Xiao X, Xiao T, Liang F, et al. Autism spectrum disorder as early neurodevelopmental disorder: evidence from the brain imaging abnormalities in 2-3 years old toddlers. J Autism Dev Disord 2014; 44(7): 1633-40. [CrossRef]
10. Mannion A, Leader G. Comorbidity in autism spectrum disorder: A literature review. Res Autism Spectr Disord 2013; 7(12): 1595-616. [CrossRef]
11. Holmes H, Sawer F, Clark M. Autism spectrum disorders and epilepsy in children: A commentary on the occurrence of autism in epilepsy; how it can present differently and the challenges associated with diagnosis. Epilepsy Behav 2021; 117: 107813. [CrossRef]
12. Imamura A, Morimoto Y, Ono S, Kurotaki N, Kanegae S, Yamamoto N, et al. Genetic and environmental factors of schizophrenia and autism spectrum disorder: insights from twin studies. J Neural Transm (Vienna) 2020; 127(11): 1501-15. [CrossRef]
13. Colvert E, Tick B, McEwen F, Stewart C, Curran SR, Woodhouse E, et al. Heritability of Autism Spectrum Disorder in a UK population-based twin sample. JAMA Psychiatry 2015; 72(5): 415-23. [CrossRef]
14. Hegarty JP 2nd, Pegoraro LFL, Lazzeroni LC, Raman MM, Hallmayer JF, Monterrey JC, et al. Genetic and environmental influences on structural brain measures in twins with autism spectrum disorder. Mol Psychiatry 2020; 25(10): 2556-66. [CrossRef]
15. Taylor MJ, Rosenqvist MA, Larsson H, Gillberg C, D'Onofrio BM, Lichtenstein P, et al. Etiology of Autism Spectrum Disorders and Autistic Traits Over Time. JAMA Psychiatry 2020; 77(9): 936-43. [CrossRef]
16. Wiśniowiecka-Kowalnik B, Nowakowska BA. Genetics and epigenetics of autism spectrum disorder-current evidence in the field. J Appl Genet 2019; 60(1): 37-47. [CrossRef]
17. Ramaswami G, Geschwind DH. Genetics of autism spectrum disorder. Handb Clin Neurol 2018; 147: 321-9. [CrossRef]
18. Guo H, Peng Y, Hu Z, Li Y, Xun G, Ou J, et al. Genome-wide copy number variation analysis in a Chinese autism spectrum disorder cohort. Sci Rep 2017; 7: 44155. [CrossRef]
19. De Rubeis S, He X, Goldberg AP, Poultney CS, Samocha K, Cicek AE, et al. Synaptic, transcriptional and chromatin genes disrupted in autism. Nature 2014; 515(7526): 209-15. [CrossRef]
20. Harripaul R, Vasli N, Mikhailov A, Rafiq MA, Mittal K, Windpassinger C, et al. Mapping autosomal recessive intellectual disability: combined microarray and exome sequencing identifies 26 novel candidate genes in 192 consanguineous families. Mol Psychiatry 2018; 23(4): 973-84. [CrossRef]
21. Mamoor S. Meaf6 is differentially expressed in the brains of patients with schizophrenia 2020. [CrossRef]
22. Lim ET, Uddin M, De Rubeis S, Chan Y, Kamumbu AS, Zhang X, et al. Rates, distribution and implications of postzygotic mosaic mutations in autism spectrum disorder. Nat Neurosci 2017; 20(9): 1217-24. Erratum in: Nat Neurosci 2020 Sep; 23(9): 1176.
23. Grady DL, Harxhi A, Smith M, Flodman P, Spence MA, Swanson JM, et al. Sequence variants of the DRD4 gene in autism: further evidence that rare DRD4 7R haplotypes are ADHD specific. Am J Med Genet B Neuropsychiatr Genet 2005; 136B(1): 33-5.
24. Ptácek R, Kuzelová H, Stefano GB. Dopamine D4 receptor gene DRD4 and its association with psychiatric disorders. Med Sci Monit 2011; 17(9): RA215-20.
25. Reiersen AM, Todorov AA. Association between DRD4 genotype and Autistic Symptoms in DSM-IV ADHD. J Can Acad Child Adolesc Psychiatry 2011; 20(1): 15-21.
26. Gadow KD, DeVincent CJ, Olvet DM, Pisarevskaya V, Hatchwell E. Association of DRD4 polymorphism with severity of oppositional defiant disorder, separation anxiety disorder and repetitive behaviors in children with autism spectrum disorder. Eur J Neurosci 2010; 32(6): 1058-65. [CrossRef]
27. Gadow KD, DeVincent CJ, Pisarevskaya V, Olvet DM, Xu W, Mendell NR, et al. Parent-child DRD4 genotype as a potential biomarker for oppositional, anxiety, and repetitive behaviors in children with autism spectrum disorder. Prog Neuropsychopharmacol Biol Psychiatry 2010; 34(7): 1208-14. [CrossRef]
28. Zamarbide M, Mossa A, Muñoz-Llancao P, Wilkinson MK, Pond HL, Oaks AW, et al. Male-Specific cAMP Signaling in the Hippocampus Controls Spatial Memory Deficits in a Mouse Model of Autism and Intellectual Disability. Biol Psychiatry 2019; 85(9): 760-8. [CrossRef]
29. Kaeffer J, Zeder-Lutz G, Simonin F, Lecat S. GPRASP/ARMCX protein family: potential involvement in health and diseases revealed by their novel interacting partners. Curr Top Med Chem 2021; 21(3): 227-54. [CrossRef]
30. Ambalavanan A, Chaumette B, Zhou S, Xie P, He Q, Spiegelman D, et al. Exome sequencing of sporadic childhood-onset schizophrenia suggests the contribution of X-linked genes in males. Am J Med Genet B Neuropsychiatr Genet 2019; 180(6): 335-40. [CrossRef]
31. Tran SS, Jun HI, Bahn JH, Azghadi A, Ramaswami G, Van Nostrand EL, et al. Widespread RNA editing dysregulation in brains from autistic individuals. Nat Neurosci 2019; 22(1): 25-36. [CrossRef]

32. Ansell BRE, Thomas SN, Bonelli R, Munro JE, Freytag S, Bahlo M. A survey of RNA editing at single-cell resolution links interneurons to schizophrenia and autism. RNA 2021; 27(12): 1482-96. [CrossRef]
33. Mulatinho MV, de Carvalho Serao CL, Scalco F, Hardekopf D, Pekova S, Mrasek K, et al. Severe intellectual disability, omphalocele, hypospadia and high blood pressure associated to a deletion at 2q22.1q22.3: case report. Mol Cytogenet 2012; 5(1): 30. [CrossRef]
34. Carvalho AF, Solmi M, Sanches M, Machado MO, Stubbs B, Ajnakina O, et al. Evidence-based umbrella review of 162 peripheral biomarkers for major mental disorders. Transl Psychiatry 2020; 10(1): 152. [CrossRef]
35. Bouchard MF, Chevrier J, Harley KG, Kogut K, Vedar M, Calderon N, et al. Prenatal exposure to organophosphate pesticides and IQ in 7-year-old children. Environ Health Perspect 2011; 119(8): 1189-95. [CrossRef]

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The Impact of the COVID-19 Pandemic and Vaccination on Dental Restorative Practices in the Geriatric Population

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ABSTRACT

Objective: Vaccines were considered as one of the most important public health measures during the coronavirus disease 2019 (COVID-19) pandemic. The aim of this study was to quantify and compare restorative dental practices of geriatric patients during pre-pandemic, pre-vaccination, and post-vaccination periods.

Materials and Methods: Dental records of patients aged older than 65 who presented to Bursa Oral and Dental Health Training and Research Hospital were included in the study. Restorative dental practices during the three periods: pre-pandemic period (01.01.2019–10.03.2020), pre-vaccination period (11.03.2020–13.02.2021) and post-vaccination period (14.02.2021–01.09.2021) were reviewed according to the patient's age (young-old, middle-old and old-old), sex, tooth number (anterior and posterior), restoration type (occlusal, proximal, or cervical) and the type of restoration material (amalgam or composite).

Results: Anterior restorations accounted for more than half (53%) of all restorations in the pre-COVID-19 pandemic period and 46.5% and 55.4% of restorations in the pre- and post-vaccination periods, respectively. The distribution of posterior amalgam restorations in the pre-pandemic period (31.5%) was higher than the pandemic period; in contrast, the composite restorations were higher during the pandemic pre-vaccination (73.9%) and post-vaccination (76.3%) periods ($p<0.001$). The males (odds ratio (OR): 1.17, 95% CI: 1.03-1.34, $p=0.02$) and middle-old (OR: 0.79, 95% CI: 0.64-0.96, $p=0.019$) were more likely to use restorative dental services as compared to females and young-old age, respectively.

Conclusion: Vaccination had a noticeable effect on increased admissions of geriatric patients and restorative dental treatments compared to the pre-vaccination period. In addition, in the post-COVID-19 vaccination period, composite restorations seem to have replaced amalgam restorations.

Keywords: COVID-19, geriatrics, dental health services

INTRODUCTION

After coronavirus disease 2019 (COVID-19) was detected in Wuhan, China on December 2019 (1, 2), the virus quickly spread worldwide, leading to a COVID-19 pandemic. The first recorded case in Turkey was on March 11, 2020 (3). Shortly after that, on April 1, 2020, the Ministry of Health in Türkiye published a circular recommending that all elective procedures, except emergency treatment should be delayed in dental clinics (4). The circular included instructions aimed at preventing horizontal transmission of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and included environmental disinfection and regulations. After the virus is transmitted to a person, infection with SARS-CoV-2 activates the immune system. Vaccines can help to prevent the spread of infection by stimulating the body's immune response (5). Vaccines are one of the most important and effective public health practices in terms of their cost, reliability, and ability to prevent infectious diseases (6).

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In early 2021, the China National Medical Products Administration authorized the use of CoronaVac, a two-dose β-propiolactone-inactivated, aluminum hydroxide-adjuvanted vaccine, for the prevention of COVID-19. Since its authorization, 260 million doses have been distributed to domestic and overseas markets, including Türkiye. In Türkiye, a vaccination priority was given to healthcare workers in public and private healthcare facilities on January 14, 2021, and to geriatric individuals older than 65 years on February 11, 2021.

With the commencement of the vaccination, a period called the 'new normal' emerged in Türkiye, during which COVID-19 prevention measures continued in health care facilities, but the range of available treatments, including dental restorations, expanded (7). In the pre-vaccination period of the pandemic, it was recommended that only emergency dental treatment (endodontic and surgical characterized by pain and swelling) could be performed (4, 8). After the commencement of the vaccination program, restorative dental treatments and other elective procedures were resumed. Restorative dental treatments include restoration of hard tissue affected by dental caries using restorative materials, such as amalgam and composites. The main purpose of restorative dental treatment is to improve patient aesthetics and function (9, 10). The aim of this study was to quantify and compare restorative dental practices of geriatric patients during pre-pandemic, pre-vaccination, and post-vaccination periods.

MATERIALS AND METHODS

Ethical approval was obtained from the ethics committee of Bursa Uludag University (2021-12/26). Prior to commencement, this study was approved by the Ministry of Health Turkiye (Prot No: 2021-08-26T13_55_16) and the Bursa Governorship Provincial Health Directorate (Prot. No: E-67508481-799).

The survey population was comprised of adults older than 65 who presented to Bursa Oral and Dental Health Training and Research Hospital for general dental treatment. The initial data consisted of the medical records of all geriatric patients who presented between 01.01.2019 and 01.09.2021. The non-dental records and dental procedures (endodontic, prosthodontic, surgical, radiological, periodontal, and orthodontic) that did not match within the definition of restorative dental practices according to the Healthcare Implementation Communique were excluded.

Data on the patient's age, sex, tooth number, cavity type, and type of restoration material were reviewed retrospectively. Based on the criteria of the World Health Organization, individuals aged 65–74, 75–84 and ≥ 85 years were defined as young-old, middle-old and old-old, respectively.

Study flowcharts are demonstrated in Figure 1.

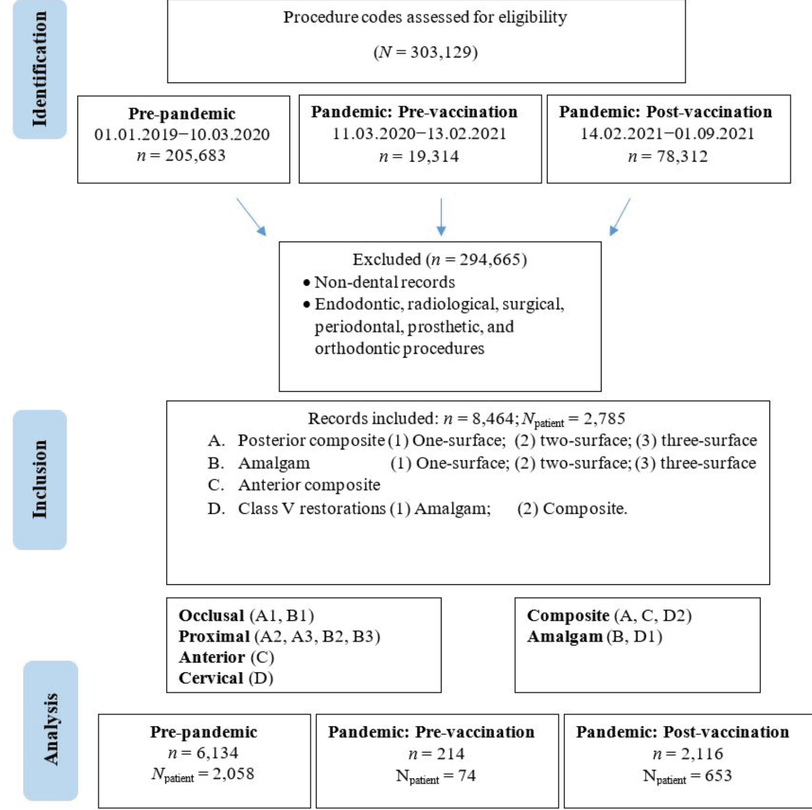

Figure 1. The study flow chart in line with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (http://www.strobestatement.org).

Following assessment of eligibility, a total of 2,058 patients with 6,134 restorations during the pre-pandemic period; 74 patients with 214 restorations during the pandemic pre-vaccination period; and 653 patients with 2,116 restorations during the pandemic vaccination period were analyzed retrospectively.

Statistical Analyses

Statistical analysis was performed using the IBM SPSS ver. 23.0 (IBM Corp., Armonk, NY). The results are presented as frequencies and percentages. Binary logistic regression was performed, and crude odds ratios (ORs), along with their 95% confidence intervals (CIs), are reported. $p<0.05$ was considered significant.

RESULTS

Table 1 provides information on the patient population and restorative treatments according to the patient presentation dates. In all three periods, more males (n=1138, 55.3%; n=41, 55.4%, n=387, 59.3%) than females and more young-olds n=1778, 85.8%; n=63, 86.4%; n=565, 86.5%) presented for treatment. In this study, young-olds (65-74 year olds) accounted for the majority (85.9%) of the patient population.

Anterior teeth were distributed for slightly more than half (53%) of all restorations in the pre-pandemic period, whereas they accounted for 46.5% and 55.4% of restorations after the pandemic in the pre-vaccination and post-vaccination periods, respectively (Table 1). Besides, a majority of posterior restorations were performed on proximal cavities in all periods ($p<0.001$).

In the year before COVID-19 pandemic, 1,474 (31.5%) posterior amalgam restorations were performed. After the pandemic, there were 29 (26.1%) and 216 (23.7%) posterior amalgam restorations performed in the pre-vaccination period and post-vaccination periods, respectively. The opposite was found for composite restorations, where the number increased to 3,207 (68.5%), 82 (73.9%), and 695 (76.3%) in the pre-pandemic, pre-vaccination, and post-vaccination periods, respectively (Figure 2). The difference in the number of amalgam versus composite restorations performed in the pre-pandemic period versus the other two periods (pre- and post-vaccination) was statistically significant ($p<0.001$).

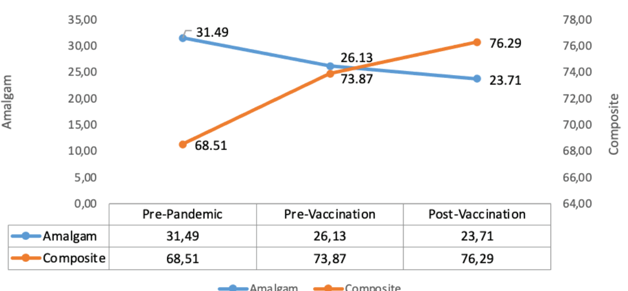

Figure 2. Trajectory of posterior amalgam and composite restorations in the geriatric patient population before and during the COVID-19 pandemic.

Considering the number of dental visits of all geriatric patients in a year, males (OR: 1.17, 95% CI: 1.03-1.34, p=0.02) and middle-olds (OR 0.79, 95% CI: 0.64-0.96, p=0.019) were more likely to use restorative dental services than were females and young-olds.

DISCUSSION

Elderly individuals have an increased risk of caries due to the inability to perform oral hygiene and the prolongation of expo-

Table 1. Patient population and dental restorations in the three periods.

				COVID-19 Pandemic		
			Pre-pandemic n=2,058	Pre-vaccination n=74	Post-vaccination n=653	p
Patients *n* (%)	Sex	Male	1,138 (55.3)	41 (55.4)	387 (59.3)	0.390
		Female	920 (44.7)	33 (44.6)	266 (40.7)	
	Age	65–74	1,778 (85.8)	63 (86.4)	565 (86.5)	0.668
		75–84	269 (13.1)	10 (13.5)	83 (12.7)	
		≥ 85	11 (0.5)	1 (1.4)	5 (0.8)	
			n=6,134	**n=214**	**n=2,116**	
Restorative dental practices *n* (%)	Anterior		3,253 (53.0)	100 (46.7)*	1,173 (55.4)	<0.001
	Posterior	Occlusal	563 (9.2)	24 (11.2)	183 (8.6)	<0.001
		Proximal	2,203 (35.9)*	87 (40.7)*	728 (34.4)*	
		Cervical	115 (1.9)	3 (1.4)	32 (1.5)	

* indicates statistical significance.

sure to cariogenic factors with advancing age, as well as physiological changes caused by aging or general health problems (11, 12). The main purpose of restorative dental treatments is considered as rehabilitating dental hard tissues affected by caries. After COVID-19 was defined as a pandemic, dental elective procedures, except emergency treatments were delayed until the vaccination period. The present study investigated restorative dental practices of geriatric patients during the pre-pandemic, pre-vaccination, and post-vaccination periods.

During the post-vaccination period of the COVID-19 pandemic, there was approximately a 10-fold increase in the number of patients who presented for restorative dental treatments compared to the pre-vaccination period. In this study, we detected a marked decrease in all types of restorative dental practices during the COVID-19 pandemic (pre- and post-vaccination periods) in accordance with the literature (13-15).

The number of anterior composite restorations performed prior to the COVID-19 pandemic was higher than that during the pandemic (pre- and post-vaccination periods). The number decreased to 46.7% during the pre-vaccination period. Considering that the majority of anterior restorations are performed for aesthetic purposes (16), this decline could be explained by the restrictions placed on elective procedures during the pandemic (4). Another finding of this study was the observed increase of approximately 10% in anterior restorations performed in the post-vaccination period. This finding may have resulted from patients' expectations in terms of aesthetics (16). It may also reflect dental-related characteristics of the geriatric patient population. These characteristics include a shortened dental arch (17) and limited posterior teeth to restore.

The choice of restorative material depends on patients' expectations in terms of aesthetics and caries risk, in addition to the location and severity of caries. According to the findings of this study, a composite was preferred as restorative material, both before and after the COVID-19 pandemic. Previous research concluded that cavity preparation of composites was less invasive than amalgam (18). The decrease in amalgam restorations from 31% to 23% during the pandemic may be associated with the preference for the use of less-aerosol generating techniques during this period.

The main factor underlying the preference for composite resins is the potential risk of mercury in amalgams (19, 20). Other factors are the preservation of more tooth structure and the aesthetic benefits of composite resin materials versus those achievable by amalgam restorations (19,20). Opdam et al. reported that the performance of composite restorations for large cavities was superior to that of amalgam restorations (21). In our study, proximal restorations were significantly higher when compared to occlusal. Consistent with our findings, other studies found that geriatric patients tended to present with extensively restored tooth structures and often larger restorations (9, 10, 22).

As found in our study, male patients (OR: 1.17, 95% CI: 1.03-1.34, p=0.02) were more likely than female patients were to present for restorative dental treatments. This finding supports the results of an epidemiological study conducted by Gökalp et al. in Türkiye, in which the prevalence of restored teeth in males and females aged 65–74 years was 17.6 and 12.4, respectively (23). In this study, the majority of the patients requiring dental restorations were aged between 65–74 years (Table 1). The age profile of our patients is consistent with that of another study conducted in Türkiye (24). The reason for the higher number of dental visits among middle-old (74–85 year) (OR: 0.79, 95% CI: 0.64-0.96) may be associated with increased dental health problems and oral health conditions associated with aging (24, 25).

The results of our analysis of a comprehensive dataset suggest that the vaccination resulted in a marked increase in the number of geriatric patients presenting for dental restorative treatment and in composite restorations. Vaccination seems to hold promise as a means to offset the consequences of the current devastating pandemic in terms of restorative dental services provided to geriatric patients.

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REFERENCES

1. Lu H, Stratton CW, Tang YW. Outbreak of pneumonia of unknown etiology in Wuhan, China: The mystery and the miracle. J Med Virol 2020; 92(4): 401-2. [CrossRef]
2. Sohrabi C, Alsafi Z, O'Neill N, Khan M, Kerwan A, Al-Jabir A, et al. World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID- 19). Int J Surg 2020; 76: 71-6. [CrossRef]
3. Ince F, Evcil F. The first three weeks of the COVID-19 in Turkey. Suleyman Demirel Uni J Health Sci 2020; 11: 236-41.
4. Çelik H, Çelik ZC, Yanikoglu F Tağtekin D, Hıdıroğlu S. The Importance of Novel Coronavirus 2019 (COVID-19) for Oral and Dental Professionals and Treatments. Turkiye Klinikleri J Dental Sci 2021; 27(2): 318-23. [CrossRef]
5. Sell S. How vaccines work: immune effector mechanisms and designer vaccines. Expert Rev Vaccines 2019; 18(10): 993-1015. [CrossRef]
6. Xing K, Tu XY, Liu M, Liang ZW, Chen JN, Li JJ, et al. Efficacy and safety of COVID-19 vaccines: a systematic review. Zhongguo Dang Dai Er Ke Za Zhi 2021; 23(3): 221-8.

7. Kantrowitz-Gordon I. A New Normal After the COVID-19 Pandemic. J Midwifery Womens Health 2021; 66(3): 293-4. [CrossRef]
8. Peskersoy C, Gurlek O. Dental precautions and emergencies during COVID-19 outbreak: A review of the current literature. EÜ Dişhek Fak Derg. 2020; 41(1): 27-36. [CrossRef]
9. Huang Y, Song B, Zhou X, Chen H, Wang H, Cheng L. Dental restorative materials for elderly populations. Polymers (Basel) 2021; 13(5): 828. [CrossRef]
10. Jablonski RY, Barber MW. Restorative dentistry for the older patient cohort. Br Dent J 2015; 218(6): 337-42. [CrossRef]
11. Saunders RH, Meyerowitz C. Dental caries in older adults. Dent Clin North Am 2005; 49(2): 293-308. [CrossRef]
12. Allen PF, Da Mata C, Hayes M. Minimal intervention dentistry for partially dentate older adults. Gerodontology 2019; 36(2): 92-8. [CrossRef]
13. Ahmadi H, Ebrahimi A, Ghorbani F. The impact of COVID-19 pandemic on dental practice in Iran: A questionnaire-based report. BMC Oral Health 2020; 20(1): 354. [CrossRef]
14. Farooq I, Ali S. COVID-19 outbreak and its monetary implications for dental practices, hospitals and healthcare workers. Postgrad Med J 2020; 96(1142): 791-2.
15. Erdinç G, Yilmaz Çirakoğlu N. COVID-19 pandemic and its effects on dentistry: A retrospective study. Clin Exp Health Sci 2021; 11(4): 819-24. [CrossRef]
16. LeSage BP. Aesthetic anterior composite restorations: a guide to direct placement. Dent Clin North Am 2007; 51(2): 359-78. [CrossRef]
17. Khan S, Musekiwa A, Chikte UM, Omar R. Differences in functional outcomes for adult patients with prosthodontically-treated and -untreated shortened dental arches: A systematic review. PLoS ONE 2014; 9(7): e101143.
18. Anguswamy S, Adeni KM. Changing concepts and trends from dental amalgam to composites. J Global Oral Health 2021; 4(1): 38-41. [CrossRef]
19. Demarco FF, Corrêa MB, Cenci MS, Moraes RR, Opdam NJ. Longevity of posterior composite restorations: Not only a matter of materials. Dental Materials 2011; (28): 87-101.
20. Roeters FJ, Opdam NJ, Loomans BA. The amalgam-free dental school. J Dent 2004; (32): 371-7.
21. Opdam NJ, Bronkhorst EM, Loomans BA, Huysmans MC.12-year survival of composite vs. amalgam restorations. J Dent Res 2010; (89): 1063-7.
22. Rodrigues JA, Lussi A, Seemann R, Neuhaus KW. Prevention of crown and root caries in adults. Periodontol 2000 2011; 55(1): 231-49. [CrossRef]
23. Gökalp S, Doğan BG, Tekçiçek M, Berberoglu A, Unluer S. The oral health profile of adults and elderly, Turkey-2004. J Hacettepe Fac Dent 2007; 31(4): 11-8.
24. Ozsoy HE, Atalay S, Azak AN. Assessment of oral and general health status in older adults. J Clin Med Kaz 2020; 3(57): 24-8. [CrossRef]
25. Razak PA, Richard KM, Thankachan RP, Hafiz KA, Kumar KN, Sameer KM. Geriatric oral health: a review article. J Int Oral Health 2014; 6(6): 110-6.

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The Effect of Rectal Ozone Use on Bacterial Translocation and Oxidative Stress in Experimental Colitis Model

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ABSTRACT

Objective: The aim of this study was to examine the effect of rectal ozone on the histopathological healing of the colonic mucosa, tissue oxidative stress, and bacterial translocation in the experimental colitis model.

Materials and Methods: Three groups of rats were randomly formed [Group 1: Sham group, group 2: Control group, group 3: Ozone treatment group]. Microscopic and macroscopic scoring were done histopathologically in all groups. Glutathione-s-transferase (GST), superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) levels were measured in the colon and liver tissue. Blood cultures were taken for the detection of bacterial translocation.

Results: Microscopic damage scores were found as 0.0 (0.0-2.0) in the sham group, 3.0 (3.0-3.0) in the control group, 2.0 (0.0-2.0) in the ozone treatment group (p=0.001). Macroscopic damage scores were found as 0.0 (0.0-1.0) in the sham group, 3.0 (0.0-4.0) in the control group, 0.0 (0.0-1.0) in the ozone treatment group; the scores of ozone treatment and sham groups were found to be statistically different (p=0.004). Compared to the control group, the bacterial translocation in the liver, mesenteric lymph node, portal vein, spleen, and systemic blood was fewer in the ozone treatment group. Statistically significant differences were also observed between the groups' SOD and GST levels in colon tissue and MDA, SOD, and GST levels in liver tissue. Regarding MDA values in the liver tissue of the groups, it was 1.95±0.43 in Group I, 3.63±0.81 in Group II, and 1.19±0.72 U/mg in Group III (p=0.017). When the liver tissue SOD levels of the groups were examined, it was 8.21±0.76 U/mg in Group I, 4.57±0.58 U/mg in Group II, and 8.62±1.13 U/mg in Group III (p=0.029). When GST values in liver tissue belonging to the groups were examined, 0.35±0.16 in Group 1, 0.23±0.03 in Group II, 0.49±0.13 U/mg in Group III (p=0.036).

Conclusion: Rectal ozone application plays a role in increasing the organism's antioxidant activity and has an effective role in increasing the enzyme activities of antioxidant defenses. In addition, rectal ozone application has a positive effect on wound healing at a histopathological level and reduces bacterial translocation in various tissues.

Keywords: Experimental colitis, rectal ozone, oxidative stress

INTRODUCTION

Ulcerative colitis is a recurrent inflammatory disease seen only in the colon part of the digestive system; it is characterized by superficial and continuous ulcers starting from the rectum and ascending towards the proximal colon (1). In ulcerative colitis, pathological changes such as mucosal erosion and ulceration in the colon wall, deterioration of the colon gland structure, edema and inflammatory cell infiltration occur in the submucosa and mucosa (2,3). Although cellular damage has an important place in the

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pathogenesis of ulcerative colitis, the pathogenesis of the event has not been fully explained yet. One of the factors triggering or having etiological potential on ulcerative colitis is oxidative stress (4). Oxidative stress causes variations in oxidative parameters, including myeloperoxidase (MPO), glutathione (GSH) and malondialdehyde (MDA), during the inflammatory response (5). Another factor playing a significant role in the pathogenesis of ulcerative colitis is intestinal microflora (6). The resident microflora is an important building block for the host, with some metabolic functions, trophic effects on the intestinal epithelium, and the barrier function it provides through bacterial antagonism (7). With the presence of robust microflora, the settlement of pathogenic microorganisms is eliminated. This is provided by low luminal pH which is provided by microflora and the consumption of trace molecules necessary for pathogenic microorganisms. Thus, invasion of enteroinvasive microorganisms into intestinal epithelial cells is prevented. Intestinal lesions occur as a result of the inflammatory activity between the microflora and the colonic mucosa. Barrier function of the mucosa is impaired with local mucosal damage and inflammation (8), which causes bacterial translocation that occurs due to the spread of bacteria (alive or dead) in the intestine and the emergence of their toxic products in the liver, mesenteric lymph nodes, spleen and systemic circulation (9). Factors such as changes in normal intestinal flora, problems with the immune system, and disruption of the mucosal barrier have a significant role in the formation of bacterial translocation (10, 11).

Ozone is a molecule containing three atoms and is more unstable than oxygen. Therefore, it is more effective than oxygen in generating a biological response. With its strong oxidant feature, it can react chemically with all oxidizable organic compounds (12). The effect of ozone on metabolism varies depending on its concentration and dose (13). The dose to be selected to obtain a stimulating or suppressive effect varies according to the oxidative load and antioxidant capacity of the tissue to be treated. In applications below the required dose, no response will be obtained, and carbohydrates, enzymes, DNA and RNA may be affected by reactions, since the antioxidant capacity will be exceeded in the use of high doses. In order to apply medical ozone therapy, an ozone-oxygen mixture is obtained from pure oxygen with the help of a generator. The predominantly used systemic treatment dose range is 20-80 µg/ml, and the local treatment dose range is 1-20 µg/ml. Although there are many ozone application methods, the rectal route is among the frequently applied ones.

During rectal administration, ozone reacts with water in the colonic mucosa, secreted antioxidants, mucoproteins and glycocalyx, leading to the release of NO from the endothelium and releasing stem cells, erythrocytes containing high amounts of antioxidant enzymes and 2,3-diphosphoglycerate (2,3-DPG) from bone marrow. Rectal ozone also increases the concentration of oxidative shock proteins and antioxidant enzymes in cells. However, ozone increases oxygen transport and glycolysis capacity in erythrocytes, provides immune activation in leukocytes, and causes the secretion of growth factors from platelets (14, 15).

The present study planned to investigate the effect of ozone, whose antioxidative and anti-inflammatory properties on the healing of the colonic mucosa, tissue oxidative stress parameters, and bacterial translocation were previously reported by the rectal administration in an experimental colitis model created with acetic acid.

MATERIALS AND METHODS

Wistar Albino rats [a total of 24 rats weighting 230-250 grams were used in the 3 groups (n=8)] were acquired from Cumhuriyet University's Animal Center Laboratory (Sivas, Türkiye), and kept in standard conditions: light and dark cycles of 12 hours (turning on the lights at 08:00 a.m.), *ad libitum* food and water were given at the 22±2°C. The experiments were carried out, between 09:00 a.m.-17:00 p.m. The procedures were handled following the National Institute of Health guidelines of "Principles of animal laboratory care". Cumhuriyet University Animal Ethics Committee approved the experimental protocols (Approval date and number: 25.04.2019-65202830-050.04.04-282).

3 groups of rats were formed randomly, which are;

Group I (Sham group) (n=8): Normal rats, nothing has been administered on them.

Group II (Control group) (n=8): For colitis formation, after anesthesia, a 5 cm thin catheter was entered into the rectum in the supine position and 1 mL of 4% acetic acid was administered intrarectally. After 30 seconds it was washed with 1.5 mL of phosphate buffer. In the group with colitis, 4 days i.v. 0.09% saline was applied from the tail vein (Image1).

Group III (Ozone treatment group) (n=8): In the group with colitis, an ozone application at a dose of 20 µg/mL was applied intrarectally for 7 days in the supine position.

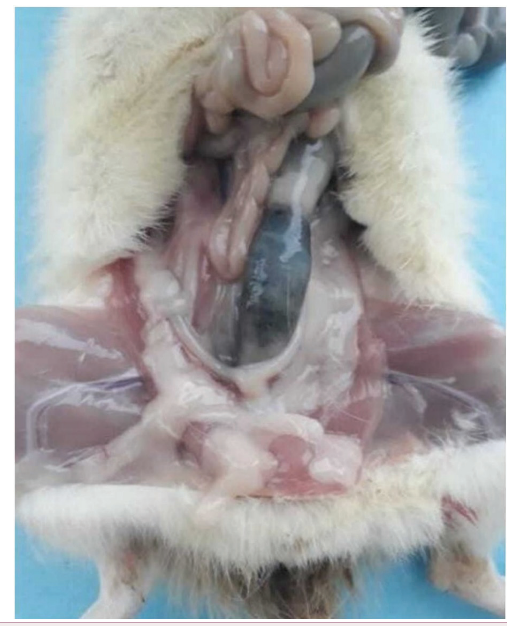

Image 1. Colitis model in rats.

Surgical Procedure

On the 7th day of colitis induction, midline abdominal shaving was carried out in the colitis, sham, and treatment group rats under anesthesia with 8 mg/kg xylazine and intraperitoneal 50 mg/kg ketamine. First site cleaning was performed with an antiseptic solution (10% povidone iodine) than midline laparotomy was performed.

To analyze the blood culture, 4 mL of blood was taken from the vena cava, they were then placed in biochemistry tubes of 2-3 mL. To detect bacterial translocation, 1-2 mL of blood was put into Bact Alert aerobic culture bottles, and attention was paid to sterility. The lymph nodes in the liver, mesentery, and spleen were disintegrated using sterile forceps, homogenized, and transferred into the prepared medium. Then, the colon was resected from the middle of the transverse colon to the rectum.

Rats were anaesthetized by inhalation of lethal doses of ether. Blood samples were placed at -80°C after being centrifuged. Colon resection material was opened longitudinally to measure Glutathione-s-transferase (GST), superoxide dismutase (SOD), GSH, and MDA levels in the colon tissue and to make macroscopic and microscopic evaluation, and the fecal content was cleaned with 0.9% saline. The samples required to measure SOD, MDA, GSH, and GST levels from liver tissue were stored at -80°C for homogenization. The other parts of the tissue were kept in 10% formaldehyde for macroscopic and microscopic evaluations.

Histopathological Evaluation

After keeping the tissue samples in 10% formaldehyde for 24 hours, they were scored macroscopically in the laboratory of the Department of Pathology by the only pathologist who did not know which group they belonged to (Table 1). The classification of the macroscopic changes in the colonic mucosa was made in five different ways, according to the description of Campos et al. (16). Macroscopic morphology scores are given in Table 1.

For microscopic investigation, samples of 2 mm thickness were taken from the column pieces. With the Sakura brand tissue tracking device, dehydration, transparency, and tissue hardening were performed by passing through alcohol, xylene, and paraffin stages in the tissue monitoring process that lasted for 15 hours. Afterwards, the tissues were turned into blocks by embedding paraffin in a Thermo Shandon brand tissue embedding device. After the blocks were cooled in the refrigerator, 3-micron thick sections were taken in the Leica brand microtome device. The slice slides were placed in an oven for 1.5 hours at 65 degrees for deparaffinization. The preparations to be stained with hematoxylin-eosin were taken out of the oven and kept in xylene for 20 minutes. In ethyl alcohol series (100%, 90%, 80%) it was kept for 10 minutes and washed in tap water. After washing, it was left in Hematoxylin paint (Facepath brand) for 2.5 minutes. After washing it in tap water again, it was immersed in acid alcohol solution once and taken back into tap water. It was immersed in ammonia water 3 times and washed again in tap water. After washing, it was left in the eosin solution for 1.5 minutes. The painting process was completed by dipping 4-5 times in alcohol series (80%, 90%, 100%). The preparations whose staining was completed were taken into the oven and left for 10 minutes to become transparent in xylene. Entellan was dropped onto the preparations extracted from xylene and a coverslip was placed on the slide. After the closing process, a microscopic examination was started.

The microscopic changes in the mucosa were evaluated according to the suggestion of Yamamoto et al. The grades between 0 and 3 were given to the microscopic changes in the colonic mucosa as displayed in Table 2 (17).

Bacterial Translocation Detection

The samples placed into a Bact Alert bottle for blood culture were evaluated in the automated culture system. Planted bottles are incubated at 35–37ºC, shaken constantly and mon-

Table 1. Classification of macroscopic findings in colonic mucosa.

Degree	Findings
0	Preserved, normal looking mucosa
1	Erythematous areas without edema, congestion, and superficial ulceration
2	Linear superficial ulcerations (7-15 mm), depressed erythematous or darker mucosa appearance, granular ground
3	Ulcerous area (14-45 mm) on uneven ground, normal islets around ulcerated mucosa or edematous mucosa
4	Diffuse irregular and multiple ulcers (>45 mm), thinned bowel wall, granular, irregular base

Table 2. Classification of microscopic findings in colonic mucosa.

Degree	Findings
0	Normal epithelium
1	Single epithelial cell loss, moderate epithelial swelling, single inflammatory cell infiltration in the crypts, mild monocyte-neutrophil infiltration
2	Multiple epithelial cell loss, epithelial flattening, crypt formation and moderate monocyte-neutrophil infiltration
3	Marked epithelial ulceration, crypt abscesses, and marked increase in monocyte and neutrophil levels

itored at 10-minute intervals. The CO_2 produced in the liquid culture bottles was continuously measured according to the colorimetric principle and each cell of the device was monitored by reflectometers. The detection of a positive bottle was reported by audiovisual messages. In this system, eosin-methylene-blue agar plates and blood agar were transferred from the positive bottles and incubated at 37ºC for 24 hours. Then, traditional methods were employed to identify the microorganism's growth. Samples lacking a positive signal within 7-day were taken as negative. Tissue samples placed in the brain-heart infusion medium in sterile glass bottles were incubated for 24-48 hours at 37ºC. Then passages were made on eosin-methylene-blue agar and blood agar from the liquid medium and incubated at 37ºC for 24 hours. Reproducing microorganisms were identified by traditional microbiological methods.

The Collection and Preparation of the Samples for the Analysis

After the sacrification of the experimental animals, liver and intestine tissues were collected. Liver and intestinal tissues extracted from rats were first weighed. Tissues were washed with cold phosphate buffered solution before and during homogenization. They were then centrifuged with external phosphate buffer (pH: 7.4) in ice water at +4°C at 40,000xg for 60 minutes and homogenization was carried out. Supernatants were transferred to eppendorf tubes for MDA, GST, GSH, and protein determination. Supernatants from liver and intestinal tissue samples were put into a chloroform/ethanol mixture of equal volume (3/5 v/v), then vortexed and centrifuged at 6000xg for 10 minutes at +4°C and the upper phase was left to determine. Thus, the extraction for determining SOD activities was completed and the upper phase was used for analysis. All unused samples were kept at -80°C till the analysis.

Biochemical Analysis

MDA analysis in tissue samples were performed as implied by Yoshioka et al. (18). GST and GSH liver tissue protein analysis in cytoplasmic fraction were carried out spectrophotometrically according to the method suggested by Warholm et al. (19). In the study, SOD activity was determined spectrophotometrically as proposed by Sun et al. (20).

Statistical Analyses

The Statistical Package for Social Sciences, ver. 17.0 was used in statistical analysis of the data (SPSS, Inc., Chicago, IL, USA). The Mann Whitney U-test was used as a post-hoc test and the Kruskal Wallis test was employed for analysis of macroscopic scores, microscopic scores, and biochemical parameters between groups. Data are given as mean±standard deviation (SD). Statistical significance was taken at $p<0.05$ level.

RESULTS

The Results of Histopathological Evaluation

In the current study, the scores of macroscopic damages in colon tissue were reported as mean±SD. They were found as 0.13±0.35 in the sham group, 2.88±1.25 in the control group, 1.0±0.53 in the ozone treatment group; the scores of ozone treatment and sham groups were found to be statistically different ($p=0.004$). The inflammation that occurred significantly in the control group was significantly decreased in the treatment group ($p=0.004$).

In our study, the microscopic damage score values in the colon tissue were statistically expressed as the mean±SD value. It was found as 0.38±0.74 in the sham group, 3.0±0.0 in the control group, 1.75±0.71 in the ozone treatment group, and it was determined that the values in the groups were statistically differ-

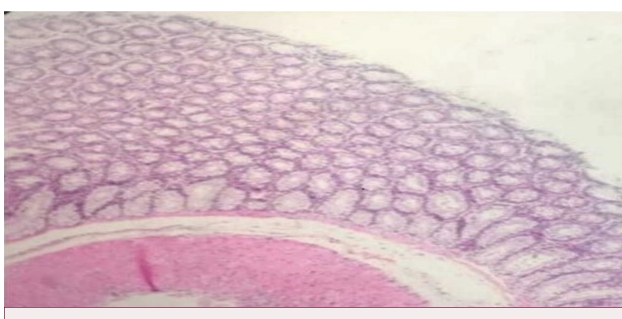

Image 2a. Sham group; normal colonic mucosa.

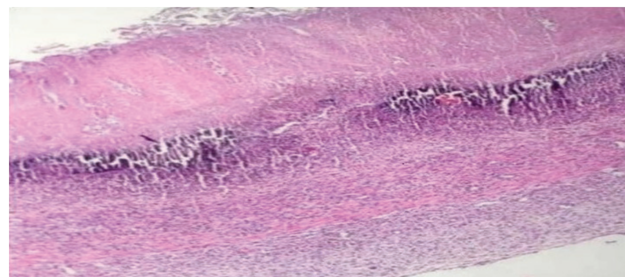

Image 2b. Control group; prominent epithelial ulceration (HEX200).

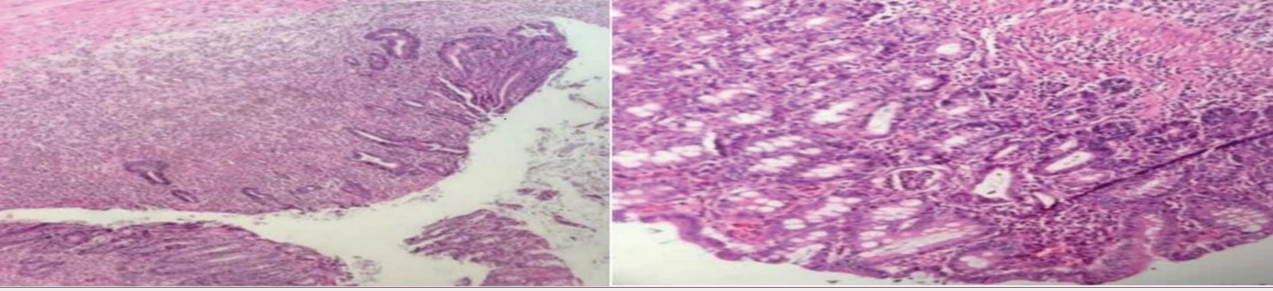

Image 2c -2d. Ozone therapy group; linear superficial ulcerations, loss of single epithelial cells, moderate epithelial swelling (HEX100).

ent from each other (p=0.001). The histopathological images belonging to the groups are shown in Images 2a, 2b, 2c and 2d.

The Results of Bacterial Translocation

No bacterial growth was detected in the sham group. The bacterial translocation frequency was observed to increase significantly in the liver, mesenteric lymph node, spleen, and portal vein blood of the group with acetic acid in which the colitis was formed. The bacterial translocation in the liver, spleen, mesenteric lymph node, systemic blood, and portal vein were observed to decrease in the ozone-treated group compared to control group. The bacterial translocation incidences observed in each group are displayed in Table 3. The translocated bacteria were determined as *Escherichia coli, Enterococcus spp, Proteus spp,* and *Klebsiella pneumoniae*.

Table 3. Bacterial translocation by groups.

Tissues	Sham Group	Control Group	Ozone-treated Group
MLN	0	4	2
Spleen	0	4	2
Liver	0	3	1
Portal Blood	0	4	2
Systemic Blood	0	3	1

Table 4. Biochemical findings.

		Min-Max	Mean±SD	p
Colon tissue MDA	**Group 1**	3.36-9.72	5.91±2.39	0.823
	Group 2	2.39-9.21	6.53±3.64	
	Group 3	3.07-7.73	5.54±1.71	
Liver tissue MDA	**Group 1**	1.60-2.54	1.95±0.43	0.017*
	Group 2	2.73-4.55	3.63±0.81	
	Group 3	0.28-1.85	1.19±0.72	
Colon tissue SOD	**Group 1**	1.50-8.75	5.19±2.63	0.046*
	Group 2	2.72-4.10	3.20±0.63	
	Group 3	6.41-9.02	7.34±1.46	
Liver tissue SOD	**Group 1**	7.38-8.88	8.21±0.76	0.029*
	Group 2	3.75-5.02	4.57±0.58	
	Group 3	7.18-9.56	8.62±1.13	
Colon tissue GSH	**Group 1**	362.07-545.73	453.11±84.64	0.779
	Group 2	344.55-525.76	430.61±89.89	
	Group 3	381.28-643.58	474.77±115.89	
Liver tissue GSH	**Group 1**	362.07-553.71	454.77±89.37	0.334
	Group 2	316.34-488.28	405.63±87.54	
	Group 3	403.36-634.62	500.47±99.13	
Colon tissue GST	**Group 1**	0.34-0.88	0.53±0.23	0.049*
	Group 2	0.19-0.29	0.22±0.06	
	Group 3	0.31-0.81	0.61±0.27	
Liver tissue GST	**Group 1**	0.19-0.54	0.35±0.16	0.036*
	Group 2	0.20-0.26	0.23±0.03	
	Group 3	0.31-0.67	0.49±0.13	

* indicates p<0.05, values shown as U/mg

Two ratio tests, which tested the number of positive tests, were used to compare bacterial translocation in all animals (8 rat-groups; p<0.05).

When the SOD levels in the colon tissue of the groups were examined, it was 5.19±2.63 U/mg in group I, 3.20±0.63 U/mg in group II, and 7.34±1.46 U/mg in group III. A statistically significant difference was observed between the colon tissue SOD levels of the groups (p=0.046). When the liver tissue SOD levels of the groups were examined, it was 8.21±0.76 U/mg in group I, 4.57±0.58 U/mg in group II, and 8.62±1.13 U/mg in group III. A statistically significant difference was also observed between the SOD levels in the liver tissue of the groups (p=0.029; Table 4). Colon and liver tissues SOD levels belonging to the groups were shown in Figure 1.

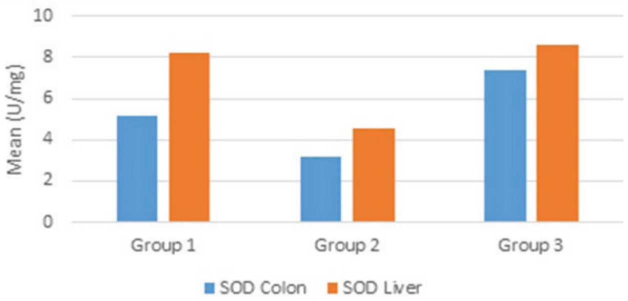

Figure 1. SOD values in colon and liver tissue.

When the values of the MDA belonging to the colon tissue of the groups were examined, it was 5.91±2.39 in group I, 6.53±3.64 in group II, and 5.54±1.71 U/mg in group III. The difference between MDA values in the colon tissues was statistically insignificant (p=0.823). Regarding MDA values in the liver tissue of the groups, it was 1.95±0.43 in group I, 3.63±0.81 in group II, and 1.19±0.72 U/mg in group III. A statistically significant difference was observed between MDA levels in the liver tissue of the groups (p=0.017). Colon and liver tissues MDA values belonging to the groups were shown in Figure 2.

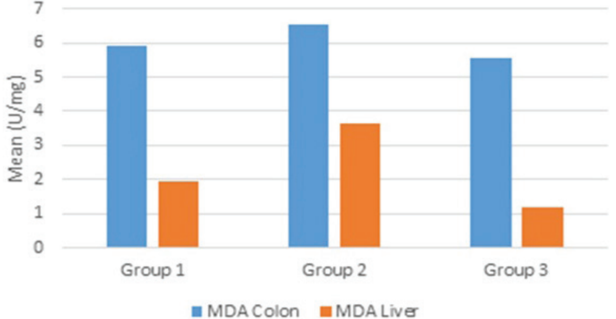

Figure 2. MDA values in colon and liver tissue.

When the GSH values in the colon tissue of the groups were examined, it was 453.11±84.64 in group I, 430.61±89.89 in group II, and 474.77±115.89 U/mg in group III. The difference between GSH values in colon tissue of the groups was insignificant (p=0.779). Regarding GSH values in the liver tissue of the groups, it was 454.77±89.37 in group I, 405.63±87.54 in group II, and 500.47±99.13 U/mg in group III. The considerable differences between GSH values in the liver tissue of the groups was insignificant (p=0.334). Colon and liver tissue GSH values belonging to the groups are shown in Figure 3.

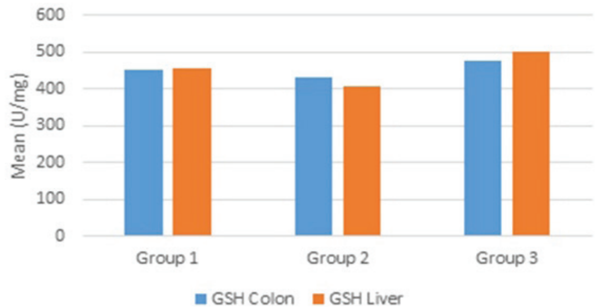

Figure 3. GSH values in colon and liver tissue.

When the GST values in the colon tissue of the groups were examined, it was 0.53±0.23 in group I, 0.22±0.06 in group II, and 0.61±0.27 U/mg in group III. A statistically significant difference was observed between GSH values (p=0.049). When GST values in the liver tissue belonging to the groups are examined, 0.35±0.16 in group 1, 0.23±0.03 in group II, 0.49±0.13 U/mg in group III. A statistically considerable difference was observed between the GST values in the liver tissue of the groups (p=0.036). GST values in colon and liver tissue belonging to the groups are shown in Figure 4.

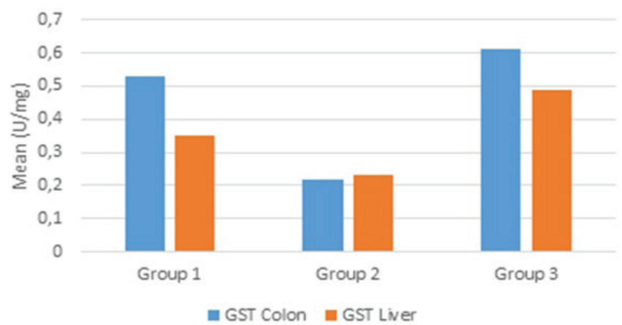

Figure 4. GST values in colon and liver tissue.

DISCUSSION

According to the results of our study, rectal ozone application caused a significant improvement in the colonic mucosa of rats with ulcerative colitis. The decrease in MDA levels in tissues and enzyme changes related to other oxidative reactions indicate that ozone therapy protects the colonic mucosa from oxidative stress. In addition to oxidative stress-related enzyme changes, a significant improvement was observed in the colonic mucosa of the control group with ozone treatment in the histopathological classification of microscopic and macroscopic tissue samples.

Lipid peroxidation occurs due to the free oxygen radicals is one of the most significant factors that causes oxidative damage in the cell membrane containing unsaturated fatty acids. The decrease in MDA in the tissue indicates that ozone therapy protects the colon tissue from oxidative stress. In the studies of Delgado-Roche et al. (21) when the effect of rectal ozone therapy on cellular redox was examined in multiple sclerosis patients, 20 µg/mL ozone was administered by rectal insufflation three times a week for one month. Immunoenzymatic and spectrophotometric tests were used to address the effect of ozone therapy on the biomarkers of inflammation and oxidative stress. Medical ozone was observed to improve the activity of antioxidant enzymes significantly and to increase the cellular reduced glutathione level. Accordingly, a significant decrease was observed in oxidative damage on lipids and proteins in the patients treated with rectal ozone.

In an animal study of Aslaner et al., medical ozone treatment was studied in the acute distal colitis; they have found its possible effect is by means of decreasing inflammation, edema, and affecting the proliferation and the vascularization to treat acute distal colitis (22). We think that the underlying mechanism of medical ozone treatment's anti-inflammatory effect can be a decrease in oxidative stress. In our study, GST, SOD, and GSH levels from colon and liver tissue increased in the rectal ozone group when compared to the control group. Regarding the ozone treatment group, the decrease in the MDA level from colon and liver tissue showed that ozone therapy plays an active role in protecting against oxidative stress. Eliakim et al. (23) studied the effect of an ozonated water enema on inflamed and normal mouse colon mucosa. The ozonated water (20 µg/mL) was prepared with an ozone generator and given intrarectally every day (0.5 mL). The rats were sacrificed on 1, 3 and 7 days after the administration of the rectal ozonated water, and their colons were resected. Damage was evaluated macroscopically and microscopically. Tissue myeloperoxidase and nitric oxide synthase activity was examined, and they found that ozone therapy did not cause macroscopic damage, but induced microscopic colitis accompanied by an increase in nitric oxide activity and myeloperoxidase as well as prostaglandin E2 formation. The groups in our study were evaluated histopathologically and while significant epithelial ulcerations were observed in the control group, linear superficial ulcerations, single epithelial cell loss and moderate epithelial swelling were observed in the ozone treatment group. Macroscopic and microscopic damage scoring results were also found to be better in the ozone treatment group. The study of Guanche et al. (24) was planned with a random distribution in four groups of 5 animals in each group. Group 1 was marked as the control group and received only oxygen; the remaining three groups (group 2, 3 and 4) were treated with a mixture of ozone/oxygen (O_3/O_2) by rectal insufflation with 600, 1400 and 2600 µg dosages in the volumes of 30, 40 and 50 mL, respectively. The study showed that O_3 increased pro-oxidant biomarkers and caused a high activation of the major enzymes of the antioxidant system. In the study, the increase of the antioxidant activity which went up 42 units, caused by the activity of superoxide dismutase, catalase, and glutathione peroxidase enzymes, was significant. The animal studies of León et al., in which carbon tetrachloride (CCl_4) was used as an oxidative threat, demonstrated that an adaptation to oxidative stress can be induced by repeated ozone administration at non-toxic doses, which allows animals to preserve hepatocellular integrity after CCl_4 poisoning (25).

The lack of antibiotic application was a limitation of the study. In conclusion, rectal ozone therapy in the experimental colitis model reduced colon and liver MDA levels significantly compared to the control group; it also improved the histopathological changes in the tissue, microscopic and macroscopic damage scoring results. In addition, the effect of ozone therapy is concluded to be associated with an increase in SOD, GSH, and GST levels in colon and liver tissue.

CONCLUSION

The findings of this study showed that, rectal ozone application plays a role in increasing the antioxidant activity of the organism and the enzyme activities of antioxidant defense systems. Moreover, rectal ozone applications have a positive effect on healing wounds at histopathological level and reduces bacterial translocation in various tissues.

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REFERENCES

1. Liang J, Sha SM, Wu KC. Role of the intestinal microbiota and fecal transplantation in inflammatory bowel diseases. J Dig Dis 2014; 15: 641-6.
2. Kaur, A., Goggolidou, P. Ulcerative colitis: understanding its cellular pathology could provide insights into novel therapies. J Inflamm 2020; 17: 15. [CrossRef]
3. Porter R.J., Kalla R., Ho GT. Ulcerative colitis: Recent advances in the understanding of disease pathogenesis. F1000Research 2020; 9: F1000 Faculty Rev-294.
4. Rezaie A, Parker RD, Abdollahi M. Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? Digest Dis Sci 2007; 52(9): 2015-21.

5. Podolsky DK. Inflammatory bowel disease. New Eng J of Med 1991; 325: 1008-16. [CrossRef]
6. Guarner F, Malagelada JR. Gut flora in health and disease. The Lancet 2003; 361: 512-9. [CrossRef]
7. Miyazawa E, Iwabuchi A, Yoshida T. Phytate breakdown and apparent absorption of phosphorus, calcium and magnesium in germfree and conventionalized rats. Nutrit Res 1996; 16: 603-13. [CrossRef]
8. Farrell RJ, LaMont JT. Microbial factors in inflammatory bowel disease. Gastroenterol Clin North Am 2002; 31: 41. [CrossRef]
9. Brooks SG, May J, Sedman P, Tring I, Johnstone D. Translocation of enteric bacteria in humans. Br J Surg1993; 80: 901-2.
10. Pain JA, Bailey ME. Prevention of endotoxaemia in obstructive jaundice-a comparative study of bile salts. HPB Surg 1988; 1: 21-7. [CrossRef]
11. Aldemir M, Geyik MF, Kökoğlu OF, Büyükbayram H, Hoşoğlu S, Yağmur Y. Effects of ursodeoxycholic acid, glutamine and polyclonal immunoglobulins on bacterial translocation in common bile duct ligated rats. ANZ J Surg 2003; 73: 722-6.
12. Bocci V. Ozone: A New Medical Drug, 2nd ed. Dordrecht Heildelberg New York: Springer Science+Business Media 2011.
13. Bocci V. Ozone A new medical drug. The Netherlands: Springer 2005; 1-295.
14. Bocci V. How does ozone act? How and why can we avoid ozone toxicity? Ozone 2010; 17-26.
15. Bocci V. Oxygen-ozone therapy: a critical evaluation. Springer Science+Business Media 2002.
16. Campos FG, Waitzberg DL, Logulo AF, Cukier C, Soares SR, Oliveira TS, et al. Technical and histological standardization of experimental colitis with trinitrobenzenosulfonic acid (TNBS). Rev Hosp Clin Fac Med Sao Paulo 1997; 52(4): 180-6.
17. Yamamoto M, Yoshizaki K, Kishimoto T, Ito H. IL-6 is required for the development of Th1 cell-mediated murine colitis. J Immunol 2000; 164(9): 4878-82. [CrossRef]
18. Yoshioka T, Kawada K, Shimada T, Mori M. Lipid peroxidation in maternal and cord blood and protective mechanism against activated-oxygen toxicity in the blood. Am J Obstet Gynecol 1979; 135(6): 372-6. [CrossRef]
19. Warholm M, Guthenberg C, von Bahr C, Mannervik B. Glutathione transferases from human liver. Methods Enzymol 1985; 113: 499-504.
20. Sun YI, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin chem 1988; 34: 497-500. [CrossRef]
21. Delgado-Roche L, Riera-Romo M, Mesta F, Hernández-Matos Y, Barrios J.M, Martínez-Sánchez G, et al. Medical ozone promotes Nrf2 phosphorylation reducing oxidative stress and pro-inflammatory cytokines in multiple sclerosis patients. Eur J Pharmacol 2017; 811: 148-54.
22. Aslaner A, Çakır T, Tekeli SÖ, Avcı S, Doğan U, Tekeli F, et al. Medical ozone treatment ameliorates the acute distal colitis in rat. Acta Cir Bras 2016; 31(4): 256-63. [CrossRef]
23. Eliakim R, Karmeli F, Rachmilewitz D, Cohen P, Zimran A. Ozone enema: A model of microscopic colitis in rats. Dig Dis Sci 2001; 46(11): 2515-20.
24. Guanche D, Zamora Z, Hernández F, Mena K, Alonso Y, Roda M, et al. Effect of ozone/oxygen mixture on systemic oxidative stress and organic damage. Toxicol Mech Methods 2010; 20(1): 25-30.
25. León OS, Menéndez S, Merino N, Castillo R, Sam S, Pérez L, et al. Ozone oxidative preconditioning: a protection against cellular damage by free radicals. Mediators Inflamm 1998; 7(4): 289-94. [CrossRef]

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ORIGINAL ARTICLE

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The Effect of Different Storage Conditions on The Migration of Chemicals from Polyethylene Terephthalate and Polycarbonate Bottles to Water

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ABSTRACT

Objective: Polyethylene terephthalate (PET) and polycarbonate (PC) bottles have been used widely in the last years for the consumption of water and the increased use of these chemicals has raised many concerns regarding their adverse effects on health. Phthalates and bisphenol A (BPA) are the main endocrine disrupting chemicals (EDCs) that can migrate from these plastics into potable water.

Materials and Methods: The concentrations of phthalate and BPA were measured in water samples that were stored in PET and PC bottles at different storage conditions. The method of ELISA was used for the determination of phthalate and BPA levels. A standard curve is obtained from the standards prepared at known concentrations of phthalate, BPA, according to their absorbance at 450 nm. The BPA levels of the samples were obtained through the calculation of the absorbance values acquired using the standard curve.

Results: Different storage and heating processes applied on the samples significantly increased the levels of BPA and phthalate. One year of storage led to a statistically significant increase in phthalate levels when compared to the control group. Both BPA and phthalate levels detected in the water samples were higher than the control group depending on the storage conditions including exposure to high temperatures, sunlight and outdoor conditions.

Conclusion: Our results indicate the necessity to establish the environmental conditions that must be ensured during the production, transportation and storage processes of the bottles, on a legal basis with legal regulations.

Keywords: Polyethylene terephthalate, polycarbonate, water, phthalate, bisphenol A

INTRODUCTION

The endocrine system and the nervous system are the two major modulatory systems in physiology of the mammalians. The functions of cells, tissues and organs in the body are regulated by the interaction of various biological messenger systems called endocrine system. The endocrine system plays a major role in modulating functions such as metabolism, reproduction, development, electrolyte and water balance, and behavior (1).

Most of the man-made chemicals used in industry and agriculture are found in the environment as pollutants. These include pesticides, plasticizers, antimicrobial agents

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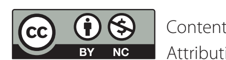
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and rust inhibitors. People are in contact with these chemicals, which are found in the air, water, food, and some personal care products. These chemicals, which are ubiquitous in the environment, act in the body by mimicking our body's complex and delicately regulated hormonal communication system or by antagonizing the effects of endogenous hormones. These chemicals are called endocrine disrupting chemicals (EDC) as a group (2).

Plastics are widely used for food packaging. However, the monomers in the structure of the packaging and various additives used in production can migrate into foods. One of the packaged consumer products is water. Polyethylene terephthalate (PET) and polycarbonate (PC) bottles are widely used in the packaging of potable water. The plasticizer di-(2-ethylhexyl) phthalate (DEHP) is among the chemicals that can migrate from PET bottles to water (3, 4). The building block of PC plastic is bisphenol A (BPA). These compounds have the potential for serious health problems due to their behavior to mimic hormonal activity. The use of DEHP and BPA in children's products under the age of three was banned in Sweden in 2000 and in the USA in 2009 (5). The European Union and the USA have imposed restrictions on the use of DEHP and BPA (5, 6).

Nutrition, inhalation, intravenous injection, and skin absorption are potential routes of EDC exposure. The most likely route of exposure to DEHP is through food. DEHP can migrate from plastics to food during manufacture and storage (7). The DEHP exposure of the population in the USA is thought to be 3–30 µg/kg/day. Since a significant part of this is through nutrition, there is a concern that continuous exposure may exceed the acceptable level, and precautions are being taken to prevent this (8).

BPA, a synthetic estrogen, is one of the highest-produced chemicals worldwide. In the United States alone, BPA has a production volume of 800,000 tons per year, and the five companies that produce it generate revenues of US$6 billion per year (9).

BPA is an EDC and can affect different endocrine pathways. In animal experiments, BPA was shown to mimic the role of estrogen and can inhibit sex differentiation. It may affect reproduction and behavior in some rodents even at doses below the safe exposure limit for humans set at 50µg/kg body weight per day as prescribed by the EPA in 1988 (10, 11).

The amount of EDCs leaking from bottles to drinking water varies according to the environmental conditions they are located in. This reveals the importance of storage conditions. In our study, which we started by considering the health risks of PET and PC bottles stored in adverse conditions, we exposed PET and PC bottle samples of different brands to natural climatic conditions, high temperatures and different levels of microwave applications for various periods of time, and investigated the effects of these processes to the migration of BPA and DEHP from PC and PET bottles to drinking water.

MATERIALS AND METHODS

A total of 85 bottled water samples were used in the study. For the determination of BPA migration, the PC group consisted of 8 groups (7 experimental and 1 control group) each including 5 PC bottles, and a total of 40 bottles. For the determination of DEHP migration, the PET group consisted of 9 groups (8 experimental and 1 control group) each including 5 PET bottles, and a total of 45 bottles. The PC groups are given in Table 1. The PET groups and the processes applied are given in Table 2.

BPA Measurement

BPA analysis was performed by BPA recognition through specific monoclonal antibodies using the Ecologiena Supersensitive BPA ELISA kit. Samples containing BPA and a mixture of BPA-enzyme conjugate (Coloring enzyme labeled BPA) were added to each well of the microplate, and compete for the binding sites of specific antibodies fixed to the surface of the wells. Unbound BPA and excess BPA-enzyme conjugates were washed away. The presence of BPA was evaluated through the addition of a chromogenic substrate. Then the enzyme-labeled BPA, which binds to the BPA antibody, catalyzed substrate con-

Table 1. PC groups and the processes applied.

Groups (n=5)	Processes applied
PC Control	PC bottles of different brands supplied on the day of the experiment
PC1 Group	PC bottles of brand A kept outdoor for 1 year
PC2 Group	PC bottles of brand B kept outdoor for 1 year
PC3 Group	PC bottles of brand A kept outdoor for 2 years
PC4 Group	100^{0}C boiling water poured into plastic containers and cooled
PC5 Group	Water heated in microwave in low-power for 10 min in plastic containers
PC6 Group	Water heated in microwave in medium-power for 10 min in plastic containers
PC7 Group	Water heated in microwave in high-power for 10 min in plastic containers

Table 2. PET groups and the processes applied.	
Groups (n=5)	**Processes applied**
PET Control	PET bottles of different brands supplied on the day of the experiment
PET1 Group	100^{0}C boiling water poured into PET bottles of brand A and cooled
PET2 Group	PET bottles of brand A kept outdoor for 1 month in July
PET3 Group	PET bottles of brand A kept in car for 1 month in July
PET4 Group	PET bottles of brand A kept outdoor for 1 year
PET5 Group	PET bottles of mineral water brand B kept outdoor for 1 year
PET6 Group	PET carboys of brand A kept outdoor for 1 year
PET7 Group	PET carboys of brand B kept outdoor for 1 year
PET8 Group	PET carboys of brand C kept outdoor for 1 year

version to colored product. After the incubation, the reaction was inhibited by adding diluted acid. The intensity of the color formed, hence the measured absorbance, varied inversely with the amount of BPA in the samples. A standard curve was obtained according to the absorbance at 450 nm from BPA standards with known concentrations. The BPA concentration in each sample was calculated using the absorbance values obtained from the standard curve.

Phthalate Measurement

The test is a direct competitive ELISA based on phthalates recognition by specific antibodies by using the Abraxis Phthalates ELISA kit. Phthalate-containing samples and a phthalate-enzyme conjugate were added to the wells of the microplate. The phthalates and phthalate-enzyme conjugate present in the samples competed for the binding sites of the anti-phthalate antibodies in solution. The phthalate antibodies were then bound by a second antibody (goat anti-rabbit), which was fixed to the surface of the wells. After the washing step and substrate solution addition, a color developed. The color reaction was stopped after 20 minutes through addition of a diluted acid. The intensity of the blue color was inversely proportional to the phthalate concentration in the sample. The standard curve was obtained according to the absorbance at 450 nm from phthalate standards with known concentrations. The phthalate concentration in each sample was calculated using the absorbance values obtained from the standard curve.

Statistical Analyses

For all statistical analysis, GraphPad Prism 5.0 (GraphPad Software, San Diego, USA) was used. All data were expressed as mean±standard deviation (SD). Analysis of variance ANOVA test was used for comparison of multiple groups; Tukey test was used for binary comparisons between groups. A value of $p<0.05$ was considered significant.

RESULTS

Results of BPA Analysis of Water Samples Stored in PC Bottles

One way ANOVA test showed that the difference between the means was significant ($p<0.0001$). When the control group and the experimental groups were compared, the mean values of PC1, PC2 and PC5 were found to be 9.35%, 9.43%, and 5.86% lower than the mean of the control group. However, the most dramatic increase was found in the PC3 group. In addition, the mean values of PC4, PC6 and PC7 were found to be 15.69%, 7.13%, and 54.99% higher than the mean of the control group (Figure 1).

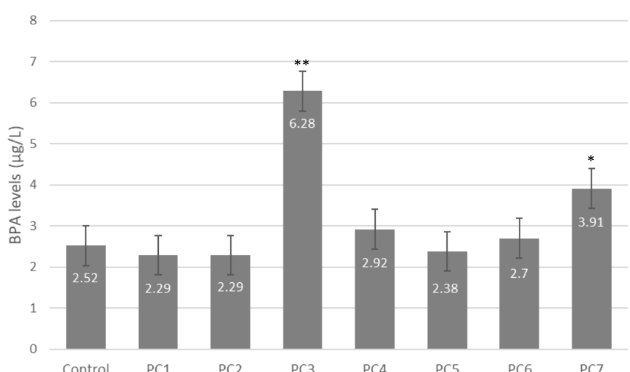

Figure 1. Average BPA levels of the groups. *$p<0.001$; **$p<0.0001$, Significantly different when compared with the control group.

Results of Phthalate Analysis of Water Samples Stored in PET Bottles

One-way ANOVA test showed that the difference between the means of phthalate levels were significant ($p<0.0001$). When the control and experimental groups were compared, it was observed that the phthalate ratio increased in all experimen-

tal groups as 31.39 %, 37.14 %, 181.18%, 187.00% and 163.94% in the PET1, PET3, PET4, PET6, and PET7 groups, respectively. It was observed that the mean phthalate levels of PET8 was 208.07% higher than the mean of the control group. It was determined that the mean phthalate levels of the PET5 group represented the highest increase as 381.77% when compared with the control group. The sample with the least increase was the PET2 sample with an increase of 1.37% (Figure 2).

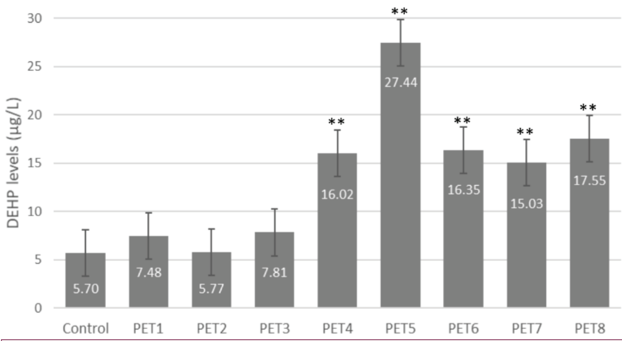

Figure 2. Average DEHP levels of the groups. **p<0.0001, Significantly different when compared with the control group.

DISCUSSION

With the developing technology, the contents of plastic containers are developed by adding various chemicals to improve elasticity, durability and transparency. These plastics contain various chemicals, and are relatively cheaper and less breakable than glass. Especially in the last 10 years, some toxic properties of these chemicals have been discovered, and with the development of determination methods, it has been reported that some of the final product plastics are not toxic, but some monomers used may show toxic properties. The bonds of these monomers may weaken and break due to light, heat, storage time and the contents of the stored liquids, and the monomers separated from the plastic may migrate into the container and pose health risks (12).

Migration from plastic materials that come into contact with water and food poses a danger and risk to public health, primarily in chronic exposures. The chemicals contained in these plastics should not migrate into water and food and should not contain toxic substances. However, it has been shown in the literature that the chemicals in plastic materials can migrate to water and food depending on storage conditions (3, 4, 7).

Today, especially in big cities, bottled water consumption is preferred more than tap water consumption, considering it is safer, pure and of high quality, despite its high price. The preferred water for drinking purposes at homes and workplaces is PET carboys and PET water. This intense demand also focused attention on the effects of phthalates on health. According to the drinking water directives of the World Health Organization (WHO) and the Food and Drug Administration (FDA), the amount of phthalates in bottled water should not exceed 8 µg/L (13, 14).

In our study, when the total phthalate levels of the control group and the PET4 group (water in a 5L PET bottle kept outside for 1 year) were compared, a statistically significant difference was found between them. Interestingly, we also detected phthalates in newly bottled PET bottled water, which we accepted as control. It has been suggested that the reason for this is due to contamination during bottling and water treatment (15) and natural water source including EDC (16). In addition, the EDC values detected in the water samples were found to be higher than the control differed depending on the storage conditions (exposure to high temperatures, sunlight, outdoor conditions, etc.).

Evandri et al. (17) investigated the toxic effects of EDCs transferred from PET bottles to water in different storage conditions and at different times with the *Allium cepa* test. They stated that exposure to high temperatures and sunlight can cause chromosomal changes due to the migration of toxic chemicals that pass from PET bottles to water. Linssen et al. (18) stated that acetaldehyde, which is produced during the condensation and melting of PET plastic bottles at high temperatures, is a volatile and heat degradation product, and showed that this EDC migrates from PET bottles to mineral waters.

In our study, when the phthalate levels of the control group were compared with the phthalate levels of the PET3 group (water in a PET bottle kept in the car in July), no statistically significant difference was found. We associate this insignificance with the fact that PET bottles were kept in the trunk of the car, not in direct sunlight, and the storage period was relatively short. No statistically significant difference was observed between the phthalate levels of the control group and the PET2 group (water in a PET bottle kept outside in July). However, the phthalate contents of the PET3 group were found to be higher than the PET2 group.

Boiling water at 100°C was poured into a PET bottle and stored to cool to investigate whether phthalates could be migrated from the PET bottle to the water. It was observed that boiling water caused increased phthalate levels in the PET bottle, although statistically not significant. In our study, when the amounts of phthalates in the water in carboys of different brands were compared with the control group, a high statistically significant difference was found between each group and the control group. We encountered high phthalate levels due to temperature increase, long-term exposure to ultraviolet rays and the length of the storage period in water stored in PET carboys for 1 year outdoors. However, we observed that this increase was below the levels determined as toxic by the EPA and the WHO. Our findings revealed the necessity of paying attention to the storage conditions of PET bottles, especially during distribution and use. During our research, we determined that phthalates were migrated from PET bottles to mineral waters after 1 year. According to the data obtained, when the mineral waters in the PET bottles were compared with the control group, a statisti-

cally significant difference was found between the two groups. Biscardi et al. (19) investigated DEHP concentrations in mineral waters in PET bottles stored at room temperature for 9 months, and they did not find phthalates in their samples in the first 8 months. DEHP was detected in the ninth month.

Guart et al. (20) found DEP levels to be 20.5 µg/L, and their results are close to the values measured at the end of the 1-year storage period in our study. However, researchers reported that they could not detect DBP and DMP (20). Zaki and Shoeib found the average DEHP concentrations as 0.274 and 0.396 µg/L, respectively, in their measurements after 2 months and 6 months of storage at an average room temperature of 25 degrees, and stated that the increases were 2.6 and 4 times, respectively, compared to the control group. In this study, the amount of phthalate showed a statistically significant increase compared to the control group depending on time (21).

The WHO and the EPA have determined the maximum contaminant level for phthalates in drinking water to be 6-8 µg/L (22, 23). In our study, the control group, PET1, PET2 and PET3 groups were within these limits. However, we observed that phthalate levels rise above this range as the storage conditions deteriorate, that is, the waters are kept outside for a long time such as 1 year and are exposed to seasonal temperature changes. The data we obtained overlap with the results of other researchers (16, 24, 25).

Polycarbonates are formed when the BPA monomer reacts with carbonyl chloride in the presence of sodium hydroxide to form the carbonate bond in the polymer. As a result of the incomplete reactions in the polymer, the unreacted BPA monomers remain in the PC plastic. These monomers migrate to their environment under the influence of heat, light, friction, pH and some minerals. According to the European Community (EC) directives, the specific migration limit of BPA is 3 mg/kg (18, 26).

Maragou et al. (27) investigated whether BPA migrated to the water by putting boiled water in baby bottles made of PC plastic, and showed that the values obtained were in the range of 2.4 to 14.3 µg/kg. Guart et al. (28) reported that BPA levels were between 1.60 and 4.44 µg/L, and the mean concentration was 2.64 µg/L in water samples stored in PC plastic containers. Nerín et al. (29) detected 30 µg/g BPA in their samples in PC plastic storage containers with the HPLC technique. They reported that 6.5 µg/g of this amount migrated to water and nutrients as a result of heating in a microwave oven.

In our experiments, BPA migration was observed, albeit slightly, into water samples exposed to low and medium microwave levels in PC plastic storage containers, although not statistically significant. However, statistically significant BPA migration was detected from PC plastic storage containers into the water at high level microwaves. But, this level is well below the toxic level specified by the WHO.

In our study, we investigated the effects of different storage and usage conditions on the chemical migration from PET and PC bottles and storage containers to water, which are frequently used by the public. While designing and performing our study, we tried to consider the habits of the producer and consumer of using plastic containers. As a result of our study, the highest BPA level found in the analysis of water samples in PC bottles was 6.28 µg/L. When we evaluate our study according to the report of European Food Safety Authority (EFSA), the level of BPA that people living in our country are exposed to in terms of drinking water is at an acceptable level. In our water samples analysis in PET bottles, the highest phthalate level was found to be 17.55 µg/L in drinking water and 27.44 µg/L in mineral waters. We found that most of the data we obtained confirms and supports previous studies showing the migration of BPA from PC plastics and phthalates from PET plastics. The results are well below the toxic limits for both endocrine disruptors. On the other hand, food packaging, medical supplies, kitchen utensils, toys, cosmetic products, detergents, in short, tools and consumer products found in all areas of our daily life also contribute to BPA and phthalates exposure. Considering all these potential sources of EDC, the amount of daily EDC taken into the body increases. When our study is evaluated in this direction, it has been concluded that the levels of EDC that can be exposed from drinking water should be kept as low as possible. For this purpose, it is necessary to establish the environmental conditions that must be ensured during the production, transportation and storage processes of the bottles, and legal regulations should be applied.

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REFERENCES

1. Guyton AC, Hall JE. Introduction to Endocrinology. Hall JE. Textbook of Medical Physiology. Philadelphia: Saunders, 2011; 881.
2. Jarvis E, Campbell NA, Reece JB. Hormones and the Endocrine System. Wilbur B. Biology. San Francisco: Pearson Education, 2005; 945-6.
3. Cao XL, Corriveau J. Migration of bisphenol a from polycarbonate baby and water bottles into water under severe conditions. J Agric Food Chem 2008; 56(15): 6378-81. [CrossRef]
4. Sax L. Polyethylene terephthalate may yield endocrine disruptors. Environ Health Perspect 2010; 118(4): 445-8. [CrossRef]

5. Calafat AM, Ye X, Wong LY, Reidy JA, Needham LL. Exposure of the U.S. population to bisphenol A and 4-tertiary-octylphenol: 2003-2004. Environ Health Perspect 2008; 116(1): 39-44. [CrossRef]
6. Vom Saal FS, Akingbemi BT, Belcher SM, Birnbaum LS, Crain DA, Eriksen M et al. Chapel Hill bisphenol A expert panel consensus statement: Integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. Reprod Toxicol 2007; 24(2): 131. [CrossRef]
7. Agency for Toxic Substances and Disease Registry (ATSDR). Toxicological Profile for Di(2-ethylhexyl)phthalate. Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA. 2002.
8. Latini G. Monitoring phthalate exposure in humans. Clinica Chimica Acta 2005; 361(1-2): 20-9. [CrossRef]
9. Borrell B. Toxicology: the big test for bisphenol A. Nature 2010; 464: 1122-4. [CrossRef]
10. Khetan SK. Environmental chemicals targeting estrogen signaling pathways. Endocrine Disruptors in the Environment, First Edition. Hoboken, New Jersey: John Wiley & Sons, Inc., 2014; 67-8.
11. Vandenberg LN, Maffni MV, Sonnenschein C, Rubin BS, Soto AM. Bisphenol-A and the great divide: A review of controversies in the field of endocrine disruption. Endocr Rev 2009; 30(1): 75-95. [CrossRef]
12. Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, Kaattari S et al. Research needs for the risk assessment of health and environmental effect of endocrine disruptors: a report of the U.S. EPA-sponsored workshop, Environ Health Perspect 1996; 104(Suppl 4): 715-40. [CrossRef]
13. World Health Organization (WHO). Guidelines for Drinking-Water Quality. Fourth Edition. Malta, 2011.
14. U.S. Food and Drug Administration (FDA). Beverages: bottled water quality standard; establishing an allowable level for di (2- ethylhexyl) phthalate. Small entity compliance guide. Rockville, 2012.
15. Amiridou D, Voutsa D. Alkylphenols and phthalates in bottled waters. Jour. Hazard Mater 2011; 185(1): 281-6. [CrossRef]
16. Keresztes S, Tatar E, Czegeny Z, Zaray G, Mihucz VG. Study on the leaching of phthalates from polyethylene terephthalate bottles into mineral water. Sci Total Environ 2013; 458-460: 451-8. [CrossRef]
17. Evandri MG, Tucci P, Bolle P. Toxicological evaluation of commercial mineral water bottled in polyethylene terephthalate: a cytogenetic approach with Allium cepa. Food Addit Contam 2000; 17(12): 1037-45. [CrossRef]
18. Linssen J, Reitsma H, Cozijsen J. Static headspace gas chromatography of acetaldehyde in aqueous foods and polythene terephthalate. Z Lebensm Unters Forsch 1995; 201(3): 253-5. [CrossRef]
19. Biscardi D, Monarca S, De Fusco R, Senatore F, Poli P, Buschini A et al. Evaluation of the migration of mutagens/carcinogens from PET bottles into mineral water by Tradescantia/micronuclei test, Comet assay on leukocytes and GC/MS. Sci Total Environ. 2003; 302(1-3): 101-8. [CrossRef]
20. Guart A, Bono-Blay F, Borrell A, Lacorte S. Effect of bottling and storage on the migration of plastic constituents in Spanish bottled waters. Food Chem 2014; 156: 73-80. [CrossRef]
21. Zaki G, Shoeib T. Concentrations of several phthalates contaminants in Egyptian bottled water: Effects of storage conditions and estimate of human exposure. Sci Total Environ 2018; 618: 142-50. [CrossRef]
22. https://www.epa.gov/sites/production/files/2015-10/documents/20110503-11-p- 0215.pdf (20.04.2018).
23. WHO Guidelines for drinking-water quality. Third edition. World Health Organization, Geneva, 2008; 1.
24. Amiridou D, Voutsa D. Alkylphenols and phthalates in bottled waters. Jour Hazard Mater 2011; 185(1): 281-86. [CrossRef]
25. Greifenstein M, White DW, Stubner A, Hout J, Whelton AJ. Impact of temperature and storage duration on the chemical and odor quality of military packaged water in polyethylene terephthalate bottles. Sci Total Environ 2013; 456-457: 376-83. [CrossRef]
26. Commission directive 2002/72/EC of 6 August 2002 relating to plastic materials and articles intended to come into contact with foodstuffs. Off Jour Eur Commun 2002; 220:18-58.
27. Maragou NC, Makri A, Lampi EN, Thomaidis NS, Koupparis MA. Migration of bisphenol A from polycarbonate baby bottles under real use conditions. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2008; 25(3): 373-83. [CrossRef]
28. Guart A, Bono-Blay F, Borrell A, Lacorte S. Migration of plasticizer phthalates, bisphenol A and alkylphenols from plastic containers and evaluation of risk. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2011; 28(5): 676-85. [CrossRef]
29. Nerin C, Fernandez C, Domeno C, Salafranca J. Determination of potential migrants in polycarbonate containers used for microwave ovens by high- performance liquid chromatography with ultraviolet and fluorescence detection. J Agric Food Chem 2003; 51(19): 5647-53. [CrossRef]

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ORIGINAL ARTICLE

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Evaluation of the Anti-Inflammatory Impact of Vitamin D on Polycystic Ovary Syndrome and Endometriosis

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ABSTRACT

Objective: Although numerous studies demonstrate the link between vitamin D and its anti-inflammatory effects, the correlations could not be fully explained. Platelet-lymphocyte ratio (PLR) and neutrophil-lymphocyte ratio (NLR) are investigated as biomarkers for diagnosis and follow-up in various obstetrics and gynecological studies. We aimed to evaluate the correlation between the anti-inflammatory effect of vitamin D and inflammatory parameters NLR and PLR in women with polycystic ovary syndrome (PCOS) and endometriosis.

Materials and Methods: Serum 25-hydroxy vitamin D (25(OH)D) levels, complete blood count (CBC) parameters, NLR, PLR, Vitamin B_{12}, and thyroid stimulating hormone (TSH) levels of 140 patients who applied to the Department of Obstetrics and Gynecology between 2010-2019 were evaluated. In our study, the levels of 25(OH)D, TSH, vitamin B_{12} and CBC measurements were analyzed simultaneously. SPSS 22.0 Windows version software was utilized in the data analysis, and p<0.05 was accepted as statistical significance level.

Results: There were significant differences in NLR (p=0.026) and lymphocytes count between PCOS and endometriosis groups (p=0.010). When we examined PCOS and endometriosis patients as a patient group; according to 25(OH)D vitamin levels, we found that NLR was significantly higher in the group with 25(OH)D vitamin levels below 20 ng/ml (p=0.017), vit B_{12} levels were significantly lower (p=0.034). Bivariate correlation analysis showed that vitamin D was negatively correlated with NLR (r=-0.196, p=0.050) in the patients with PCOS but not with endometriosis.

Conclusion: This retrospective study assessed the association between vitamin D levels and the new inflammation biomarkers in patients with PCOS and endometriosis. The CBC and neutrophil/lymphocyte ratios are accessible and easily measurable biomarkers, therefore, evaluating the anti-inflammatory effect of vitamin D together with NLR is valuable for both PCOS and endometriosis.

Keywords: Vitamin D insufficiency, inflammation, neutrophil-lymphocyte ratio, platelet-lymphocyte ratio, PCOS, endometriosis

INTRODUCTION

Vitamin D has a crucial role in maintaining the calcium and mineralization balance of the skeletal system and has anti-inflammatory and antimicrobial properties in reducing the production of T helper 1 (Th1) cells and in the formation of inflammatory interleukins (1, 2).

It has a strong anti-inflammatory impact on account of directly restricting the production of interleukin-2 and gamma interferon (IFN-y). Even though it is accepted that vitamin D is potent for the immune system, especially through the T-helper pathway, the principal mechanisms have not been explained until now. Considering the significance of inflammation, especially in the prognosis of the disease, vitamin D deficiency is thought to be responsible for this inflammation (3).

Vitamin D deficiency may affect disrupt placentation, decrease placental blood flow, and increase the inflammatory

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response (4). Vitamin D is also essential for the ovary and testis function in both females and males, respectively (5).

Polycystic ovary syndrome (PCOS) is one of the endocrine disorders characterized by chronic ovulation and androgen elevation widespread at approximately 5-10% of the women's period of reproductive age. Many studies have highlighted that chronic inflammation in the uterus with PCOS may lead to pregnancy complications (6). Studies have shown elevated inflammatory ingredients such as ferritin, C-reactive protein, interleukin (IL)-6), IL-8, tumor necrosis factors (TNFs) (7) and leukocyte levels in women with PCOS (8).

Studies have suggested that androgen elevation in women with PCOS may be responsible for the increase in leukocyte count and the development of inflammation (9).

Endometriosis is a multifactorial estrogen-dependent chronic inflammatory disease that affects women of reproductive age, especially between the ages of 25 and 35, with a prevalence of 5-10% (10). The immune system as well as the genetic and environmental factors have an effective role in the etiology of endometriosis (11). The study indicated that endometriosis elevated inflammatory cytokines, neutrophils, macrophages, and TNFs in the peritoneal fluid (12).

Regarded as inflammation markers, neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR) are also the potential biomarkers for follow-up in the gynecology and perinatology disorders as in the other chronic diseases and cancer (13,14). Increased leukocyte counts have been found to be a marker and prognostic factor in the development of inflammation and atherosclerosis (15).

Easily measurable and easily accessible biomarkers that can reflect the severity of systemic inflammation in obstetrics and gynecology patients in whom inflammation is important in the etiology will greatly relieve the clinician and the patient. Therefore, whole blood cell counts can reflect the immune system response and the inflammation in endometriosis and PCOS.

The aim of our study was to evaluate the correlation between the anti-inflammatory effect of vitamin D and the inflammatory parameters NLR and PLR in women with PCOS and endometriosis.

MATERIALS AND METHODS

Research Plan, Data Collection, and Methods

Our study retrospectively analyzed 140 females, 39 diagnosed with endometriosis and 101 diagnosed with PCOS between 2010 and 2019 applied to the Department of Obstetrics and Gynecology. Gaziantep University Faculty of Medicine Ethics Committee approved our research with the order dated 23.09.2020 (reference numbered 2020/290).

Outcomes of simultaneous achieved measurements of 25(OH) D levels, thyroid-stimulating hormone (TSH) levels, vitamin B_{12} levels, and complete blood count (CBC) were recorded.

CBC was measured by fluorescent flow cytometry method on a Sysmex XN-9000 fully automatic blood count device (Sysmex XN-9000 automated analyzers, Kobe, Japan).

Serum vitamin B_{12} levels, 25(OH)D levels and TSH levels of patients were measured by chemiluminescence assay Beckman Coulter Access Unicel DXI 800 (UniCelDXi 800 immunochemical assay, Beckman Coulter, Fullerton, CA, USA) tool.

Serum vitamin D levels were measured with the most sensitive and specific method as chemiluminescence immunological 25(OH)D test (Beckman Coulter Access Total 25(OH)D).

25(OH)D level below the 20 ng/mL is defined as vitamin D deficiency and 25(OH)D level upper 20 ng/mL is defined as vitamin D sufficient (16).

Statistical Analyses

The data was analyzed using the SPSS 22.0 Windows version software system. The Shapiro Wilk test was used to determine whether numerical variables were suitable for standard dispersion. The student's t-test was used to compare the normal distribution parameters of the two groups. The Mann-Whitney U test was used to compare the not normally distributed parameters between the two groups. The Spearman rank correlation coefficient was used to examine the association between improperly distributed numerical parameters. $p<0.05$ was considered significant.

RESULTS

The main characteristics and laboratory data of the endometriosis and PCOS disease groups are given in Table I. There was a significant difference in age between the PCOS and the endometriosis groups (p=0.001). After the results in the patient group were adjusted according to age, the results were not changed. NLR and lymphocyte count had a significant difference between the PCOS and the endometriosis group (p=0.026 and p=0.010; respectively) (Table 1).

When patients were classified into two groups based on 25(OH)D levels (25(OH)D < 20 ng/mL group; 25(OH)D > 20 ng/mL group), there were significant differences in NLR and in vitamin B_{12} (respectively; p=0.017 and p=0.034). NLR was higher in the insufficient vitamin D group than in the sufficient vitamin D group (p=0.017). Vitamin B_{12} levels were correlated with sufficient 25(OH)D levels (p=0.034; Table 2).

Bivariate correlation analysis showed that vitamin D was negatively correlated with the NLR (r=-0.196, p=0.050) in the patients with PCOS but not with endometriosis. The anti-inflammatory impact of 25(OH)D levels on NLR was a weak negative relation in the PCOS patient group but not in the endometriosis group (Table 3).

Table 1. The characteristics of demographic and laboratory in women with PCOS and endometriosis.

	Endometriosis	PCOS	p-value
Age (year)	31 [24-39]	23 [19-27]	0.001
Platelet (K/µL)	273 [248-356]	308 [270-355]	0.115
Neutrophil (/mm^3)	4.33 [3.52-5.23]	4.3 [3.36-5.27]	0.480
Lymphocytes (/mm^3)	2.19 [1.81-2.67]	2.63 [2.16-3.12]	0.010
PLR	128.96 [93.2-169.68]	117.13 [99.2-143.64]	0.269
NLR	1.87 [1.51-2.54]	1.52 [1.28-2.18]	0.026
25(OH)D level (ng/mL)	15.11 [10-25.31]	16.88 [11.63-23.9]	0.532
Vitamin B_{12} level (ng/mL)	219.5 [174.5-265.5]	215 [158-307]	0.722
TSH level (µIU/mL)	1.79 [1.31-2.36]	1.86 [1.4-2.64]	0.464

All values presented as median (25%-75%)

Table 2. The comparison of data based on vitamin D levels in the group consisting of PCOS and endometriosis.

	25(OH)D<20(ng/mL)	25(OH)D>20(ng/mL)	p-value
Age (year)	24 [21-30]	24 [21-33]	0.941
Platelet (K/µL)	292.5 [262-359]	312 [252-350]	0.879
Neutrophil (/mm^3)	4.41 [3.36-5.3]	4.06 [3.4-5.16]	0.453
Lymphocytes (/mm^3)	2.47 [1.82-2.97]	2.64 [2.16-3.01]	0.153
PLR	126.31 [98.95-160.67]	118.18 [90.35-135.9]	0.104
NLR	1.78 [1.41-2.34]	1.45 [1.27-1.87]	0.017*
Vit B_{12}(ng/mL)	212.5 [157.5-257]	277 [177-348]	0.034*
TSH (µIU/mL)	1.89 [1.38-2.58]	1.73 [1.28-2.65]	0.912

All values presented as median (25%-75%); statistical significance is shown with *: $p<0.05$

Table 3. Correlation analysis between 25(OH)D levels and complete blood count parameters in women with PCOS and endometriosis.

			Platelet (K/µL)	Neutrophil (/mm^3)	Lymphocytes (/mm^3)	PLR	NLR
Endometriosis	25(OH)D (ng/mL)	r	-0.124	-0.239	0.050	-0.088	-0.196
		p	0.464	0.153	0.767	0.596	0.232
		n	37	37	37	39	39
PCOS	25(OH)D (ng/mL)	r	0.018	0.021	0.167	-0.154	-0.196
		p	0.865	0.846	0.113	0.124	0.050*
		n	91	91	91	101	101

*: $p<0.05$

DISCUSSION

This retrospective study assessed the association between vitamin D levels and the new inflammation biomarkers NLR and PLR in women with PCOS and endometriosis.

We obtained three significant findings as the results of our study. Firstly, NLR, the inflammatory parameter of CBC, has importantly higher in the patient group with insufficient 25(OH) D levels. Secondly, the anti-inflammatory action of 25(OH) D levels on NLR has a weak negative correlation in the PCOS group. Finally, the observed correlation between vitamin B_{12} and vitamin D represents that vitamin B_{12} levels should also be controlled in the insufficient 25(OH)D levels.

Vitamin D insufficiency is still a widespread and important global health problem (17). Vitamin D insufficiency has been originated to be related to severe pregnancy results such as repeated pregnancy losses and the possibility of preeclampsia (18). T cells, B cells, and antigen-presenting cells provide vitamin D synthesis via signaling (19), suggesting that vitamin D deficiency may be responsible for the inactive immune system.

Although numerous studies demonstrated the relationship between vitamin D and its anti-inflammatory effects, their correlations could not be completely explained (20).

$1,25(OH)_2D_3$'s *in vivo* suppression mechanism of autoimmune diseases, namely its anti-inflammatory effect, involves inhibiting the development and function of Th1 cells and increasing IL-4 production from Th2 cells (21).

The ratio of neutrophil to lymphocyte is considered as an easy and rapidly measurable parameter of systemic inflammation in severe diseases (22).

In this study, we found vitamin D levels to be significantly lower in the PCOS and endometriosis patient groups. We observed that plasma 25(OH)D level and NLR, which are accepted as inflammatory markers, and lymphocyte count increased in PCOS and endometriosis patients. The low 25(OH)D levels may support a pro-inflammatory environment in PCOS and endometriosis (3).

Numerous studies suggest that increased inflammation in PCOS patients affects obesity and insulin resistance (23) and that there is an association between high androgen concentration and leukocytes (15).

Administration of a synthetic vitamin D derivative to experimental animals has been shown to result in the development of endometriosis and reduction of peritoneal inflammation, indicating the immunomodulatory effect of vitamin D (24). Harris et al. found an opposite correlation between plasma 25(OH) D levels and endometriosis (25). Also, we indicated a negative correlation between plasma 25(OH)D levels and PCOS, but no negative correlation could be demonstrated between endometriosis and vitamin D.

A study in 2018 examining the relationship between vitamin levels and low-grade inflammation in overweight and obese individuals reported that serum vitamin D and vitamin B_{12} increased together, while serum C-Reactive Protein (CRP), the parameter indicating inflammation, decreased (26). A negative correlation was detected between both vitamin B_{12} and vitamin D levels and autoimmune disease (27).

We observed a direct proportional association between vitamin B_{12} levels and 25(OH)D levels because vitamin B_{12} levels were also insufficient in the group with inadequate vitamin D.

As the limitations of our study, since it is a retrospective study, data such as season, obesity, and BMI that may affect vitamin D levels could not be evaluated. Also, we consider that the correlation of vitamin D with inflammation through the immune system should have been explored at the cellular and molecular levels.

Ethics Committee Approval: Ethics committee approval was obtained from Gaziantep University Faculty of Medicine Ethics Committee (Reference number: 2020/290).

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REFERENCES

1. Holick MF. High prevalence of vitamin D inadequacy and implications for health. Mayo Clin Proc 2006; 81(3): 353-73. [CrossRef]
2. Aygun H. Vitamin D can prevent COVID-19 infection-induced multiple organ damage. Naunyn Schmiedebergs Arch Pharmacol 2020; 393(7): 1157-60. [CrossRef]
3. Grudet C, Malm J, Westrin A, Brundin L. Suicidal patients are deficient in vitamin D, associated with a pro-inflammatory status in the blood. Psychoneuroendocrinology 2014; 50: 210-9. [CrossRef]
4. Schröder-Heurich B, Springer CJP, von Versen-Höynck F. Vitamin D effects on the immune system from periconception through pregnancy. Nutrients 2020; 12(5). [CrossRef]
5. Kinuta K, Tanaka H, Moriwake T, Aya K, Kato S, Seino Y. Vitamin D is an important factor in estrogen biosynthesis of both female and male gonads. Endocrinology 2000; 141(4): 1317-24. [CrossRef]
6. Rudnicka E, Suchta K, Grymowicz M, Calik-Ksepka A, Smolarczyk K, Duszewska AM, et al. Chronic low grade inflammation in pathogenesis of PCOS. Int J Mol Sci 2021; 22(7): 3789. [CrossRef]
7. Xiong YL, Liang XY, Yang X, Li Y, Wei LN. Low-grade chronic inflammation in the peripheral blood and ovaries of women with polycystic ovarian syndrome. Eur J Obstet Gynecol Reprod Biol 2011; 159(1): 148-50. [CrossRef]
8. Franik G, Sadlocha M, Madej P, Owczarek A, Skrzypulec-Plinta V, Plinta R, et al. Circulating omentin-1 levels and inflammation in polycystic ovary syndrome. Ginekol Pol 2020; 91(6): 308-12. [CrossRef]

9. Wiernik PH. Androgen therapy for acute myeloid and hairy cell leukemia. Curr Treat Options Oncol 2018; 19(1): 4. [CrossRef]
10. Viganò P, Parazzini F, Somigliana E, Vercellini P. Endometriosis: epidemiology and aetiological factors. Best Pract Res Clin Obstet Gynaecol. 2004; 18(2): 177-200. [CrossRef]
11. Viganó P, Somigliana E, Chiodo I, Abbiati A, Vercellini P. Molecular mechanisms and biological plausibility underlying the malignant transformation of endometriosis: a critical analysis. Hum Reprod Update 2006; 12(1): 77-89. [CrossRef]
12. Tosti C, Pinzauti S, Santulli P, Chapron C, Petraglia F. Pathogenetic mechanisms of deep infiltrating endometriosis. Reprod Sci 2015; 22(9): 1053-9. [CrossRef]
13. Wang D, Yang JX, Cao DY, Wan XR, Feng FZ, Huang HF, et al. Preoperative neutrophil-lymphocyte and platelet-lymphocyte ratios as independent predictors of cervical stromal involvement in surgically treated endometrioid adenocarcinoma. Onco Targets Ther 2013; 6: 211-6. [CrossRef]
14. Örgül G, Aydın Haklı D, Özten G, Fadiloğlu E, Tanacan A, Beksaç MS. First trimester complete blood cell indices in early and late onset preeclampsia. Turk J Obstet Gynecol 2019; 16(2): 112-7. [CrossRef]
15. Phelan N, O'Connor A, Kyaw Tun T, Correia N, Boran G, Roche HM, et al. Leucocytosis in women with polycystic ovary syndrome (PCOS) is incompletely explained by obesity and insulin resistance. Clin Endocrinol (Oxf) 2013; 78(1): 107-13. [CrossRef]
16. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 2011; 96(7): 1911-30. [CrossRef]
17. Mithal A, Wahl DA, Bonjour JP, Burckhardt P, Dawson-Hughes B, Eisman JA, et al. Global vitamin D status and determinants of hypovitaminosis D. Osteoporos Int 2009; 20(11): 1807-20. [CrossRef]
18. Ji JL, Muyayalo KP, Zhang YH, Hu XH, Liao AH. Immunological function of vitamin D during human pregnancy. Am J Reprod Immunol 2017; 78(2). [CrossRef]
19. Aranow C. Vitamin D and the immune system. J Investig Med 2011; 59(6): 881-6. [CrossRef]
20. Shab-Bidar S, Neyestani TR, Djazayery A, Eshraghian MR, Houshiarrad A, Kalayi A, et al. Improvement of vitamin D status resulted in amelioration of biomarkers of systemic inflammation in the subjects with type 2 diabetes. Diabetes Metab Res Rev 2012; 28(5): 424-30. [CrossRef]
21. Cantorna MT, Zhu Y, Froicu M, Wittke A. Vitamin D status, 1,25-dihydroxyvitamin D3, and the immune system. Am J Clin Nutr 2004; 80(6 Suppl): 1717s-20s. [CrossRef]
22. Zahorec R. Ratio of neutrophil to lymphocyte counts--rapid and simple parameter of systemic inflammation and stress in critically ill. Bratisl Lek Listy 2001; 102(1): 5-14.
23. González F, Considine RV, Abdelhadi OA, Acton AJ. Inflammation Triggered by Saturated Fat Ingestion Is Linked to Insulin Resistance and Hyperandrogenism in Polycystic Ovary Syndrome. J Clin Endocrinol Metab 2020; 105(6): e2152-67. [CrossRef]
24. Mariani M, Viganò P, Gentilini D, Camisa B, Caporizzo E, Di Lucia P, et al. The selective vitamin D receptor agonist, elocalcitol, reduces endometriosis development in a mouse model by inhibiting peritoneal inflammation. Hum Reprod 2012; 27(7): 2010-9. [CrossRef]
25. Harris HR, Chavarro JE, Malspeis S, Willett WC, Missmer SA. Dairy-food, calcium, magnesium, and vitamin D intake and endometriosis: a prospective cohort study. Am J Epidemiol 2013; 177(5): 420-30. [CrossRef]
26. Geiker NRW, Veller M, Kjoelbaek L, Jakobsen J, Ritz C, Raben A, et al. Effect of low energy diet for eight weeks to adults with overweight or obesity on folate, retinol, vitamin B(12), D and E status and the degree of inflammation: a post hoc analysis of a randomized intervention trial. Nutr Metab (Lond) 2018; 15: 24. [CrossRef]
27. Aktaş H. Vitamin B_{12} and Vitamin D levels in patients with autoimmune hypothyroidism and their correlation with anti-thyroid peroxidase antibodies. Med Princ Pract 2020; 29(4): 364-70. [CrossRef]

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Cross-interactions between Norepinephrine, Methicillin-Resistant *Staphylococcus aureus* and Human Osteoblast Cells in Culture Conditions

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ABSTRACT

Objective: The role of norepinephrine (NE) on growth, adhesion and invasion of methicillin-resistant *Staphylococcus aureus* (MRSA) ATTC 43300 was examined in human osteoblast (HOB) cells. The effects of NE and/or MRSA on the viability and cell death pathways of HOB cells were also investigated. Furthermore, the alterations of bacterial response to oxidative stress (H_2O_2) were analyzed in the presence/absence of NE.

Materials and Methods: Bacterial growth was detected spectrophotometrically. The colony counting method was examined for adhesion-invasion. The alteration of HOB cell viability was determined by methyl thiazolyl diphenyl-tetrazolium bromide (MTT) assay. The death pathways of HOB cells were examined microscopically using acridine orange-ethidium bromide dual staining and dichlorofluorescein-diacetate (DCF-DA) dye. The bacterial response to H_2O_2 was investigated by agar dilution.

Results: The growth of bacterium was not affected in the presence of NE. Bacterial adhesion was decreased by NE ($p<0.0001$) while high-level NE induced invasion ($p=0.013$). HOB cell viability was reduced by MRSA and/or NE ($p<0.001$). MRSA and co-existence of MRSA and NE caused necrosis more than apoptosis in HOB cells ($p<0.05$). NE did not alter the bacterial response to oxidative stress.

Conclusion: Norepinephrine has different effects on the biological properties of both MRSA and HOB cells.

Keywords: Norepinephrine, MRSA, growth, adhesion-invasion, oxidative stress response, HOB cell viability, apoptosis-necrosis

INTRODUCTION

During an infection process, a microorganism enters the host tissues whereby host factors including hormones are accepted as the microbe's environment. Microbial endocrinology provides a new avenue of approach to infection biology via understanding bi-directional interactions between host and microorganisms. It appears that host hormones can be recognized by microorganisms and regulate their behaviors such as growth, virulence, antimicrobial susceptibilities and gene expressions (1–3). The effects of stress hormones on microbial behaviors during the infectious process have been investigated in many studies (1, 4-12).

Staphylococcus aureus is responsible for a number of conditions ranging from minor skin infections to serious ones including septicemia, endocarditis, pneumonia, or bone joint infections including osteomyelitis (13). In the present study, human osteoblast cell line (HOB) was used as

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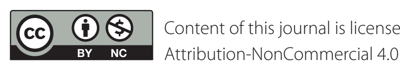
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an infection model. In this model, we investigated the roles of norepinephrine (NE) in the regulation of growth, adhesion and invasion properties of a methicillin resistant *Staphylococcus aureus* (MRSA) strain, viability and cell death pathways of HOB cells. Moreover, the response of MRSA to oxidative stress (in the existence of hydrogen peroxide; H_2O_2) was examined in tryptic soy agar (TSA) medium in the presence of NE.

MATERIALS AND METHODS

Bacterium, Medium and Hormone

MRSA ATCC 43300 strain was used in our experiments. The overnight culture of the bacterium was prepared in tryptic soy broth and incubated at 37°C for 24 hours. The bacterial suspension was prepared to arrange the initial bacterial count to 10^7 CFU/mL.

Physiological levels of norepinephrine (NE); (Sigma) in a healthy individual were taken into consideration to decide the two concentrations (low concentration: 0.0017 µg/mL and high concentration: 0.04 µg/mL).

Human Osteoblast Cell Culture

Due to the fact that *Staphylococcus aureus* may cause bone destruction and loss (14), we used HOB cell line (Sigma-Aldrich) in our experiments. High-glucose Dulbecco's modified eagle medium (DMEM; Sigma) was used for the cell culture. We added 10% fetal bovine serum (FBS; Biowest), 1% 2mM L-glutamine (Biological Industries) and 1% penicillin/streptomycin (Biological Industries) into DMEM.

For detecting bacterial growth, HOB cell viability and HOB cell death pathway, HOB cells were seeded at 1×10^4 density per well; for bacterial adhesion and invasion assays, the seeding density was 5×10^4 per well.

All plates were incubated to obtain confluent monolayers with cell densities 4×10^4 in 96-well plate and 2.4×10^5 in 24-well plate.

Bacterial Inoculation Into HOB Cell Line

DMEM medium was aspirated and replaced with an antimicrobial solution-free medium. Two concentrations of NE were added to each well (20µL for each well/96-well plate and 50µL for each well/24-well plate) for the experiments. Cell cultures without hormones were used as controls.

After hormone addition, the microplates were incubated at 37°C for one hour. Human osteoblast cell culture was inoculated with an overnight culture of MRSA and bacterium was grown at 37°C for different periods in HOB cells. The length of the period depended on the experiment where three, six and 24 hours were given for bacterial growth, 1 hour for bacterial adhesion, three hours for bacterial invasion assays, and four hours for HOB cell viability and HOB cell death pathway at 37°C in CO_2 (15-18).

Bacterial Growth Assay

The wells containing HOB cells with/without NE at two different concentrations were inoculated with MRSA strain (bacterial counts as 10^7 CFU/mL) and incubated for three, six and 24 hours. The effect of NE was determined by comparing the absorbance of growth (600nm) in infected HOB cell culture with/without hormone by the spectrophotometric method at 600 nm (19).

All experiments were repeated independently three times and conditions were analyzed thrice.

Bacterial Adhesion and Invasion Assays

Bacterial adhesion and invasion stages were followed as reported previously (15). After inoculation for one hour at 37°C, phosphate buffer saline (PBS) was used to wash the HOB cells to distract unbound bacteria. The cells were lysed with 500 µL Triton X-100 (0.025%) and the culture dishes were incubated for 5 minutes at 37°C under 5% CO_2 conditions. After homogenization, TSA was used for inoculation of cell lysates and incubated for 24 hours at 37°C. The adhered-bacteria numbers were detected by colony counting.

Invasive bacterial numbers were detected as mentioned above with minor variation. PBS was added into the wells for washing, after HOB cells were incubated with bacteria at 37°C for three hours. To destroy extracellular bacteria, a fresh medium containing 200 µg/mL gentamycin was added. The microplates were incubated at 37°C for one hour. For quantification of invasive bacteria, Triton X-100 was used for lysing of HOB cells. Homogenized cell lysates were inoculated as mentioned above.

The colony counting method was used to detect the adhered and invasive bacterial counts (as colony forming units (CFU)/mL) obtained from inoculated HOB cell lysates.

All conditions were repeated three times and each experiment was performed thrice.

HOB Cell Viability Assay

We used methyl thiazolyl diphenyl-tetrazolium bromide (MTT) to detect HOB cell viability. MTT stock solution (12 mM; Neofrox 3580 MTT) described by Mosmann was used (16). The contents were aspirated from the wells after 4 hours of incubation at 37°C, and 70µL of dimethyl sulfoxide (DMSO) was added to the wells for 10 minutes. The composed formazan crystals showing the metabolic activity of HOB cells were measured spectrophotometrically at 540 nm. Then, cell viability was calculated according to the equation: Viability % = (Treated cells absorbance / Non-treated cells absorbance) × 100.

The effects of MRSA and/or NE at different concentrations on HOB cell viability were detected by comparing control conditions' absorbance data.

Each experiment was repeated twice and all samples were evaluated in duplicates.

HOB Cell Death Assays

Acridine Orange/Ethidium Bromide (AO/EB) Dual Staining

After labeling all nuclei with acridine orange (AO) and ethidium bromide (EB), the frequency of cell death (apoptotic or necrotic

mechanism) was determined by fluorescent microscopy as described by Tapajós et al. (17). The dye was applied directly onto the cells without removing them by trypsinization from the wells. AO/EB staining solution (1µL) (dye mixture containing 100 µg/mL AO and 100 µg/mL EB) was added to the cells. H_2O_2 (0.2 mM) treated cells were used as the positive control for four hours.

After the addition of AO/EB dye (20 minutes), the cell morphology was examined under fluorescent microscope (Carl-Zeiss/Axio Observer 3, Zen 2.3 Blue Edition software).

Visual analysis were used to assess apoptosis or necrosis as well as cell survival according to criteria described previously, namely, alive cells have a homogeneous green nucleus and red-orange cytoplasm; apoptotic cells have chromatin condensation and an uneven green nucleus; and necrotic cells have no cell membrane (uniformly red-stained cell nuclei) (17).

At least 200 cells were counted for statistical analysis. The experiments were repeated independently three times.

Dichlorofluorescein-Diacetate (DCF-DA) Staining

By staining with DCF-DA, the degree of reactive oxygen species (ROS) was determined [DCF-DA (D6883) 50mg; Sigma-Aldrich] under a fluorescence microscope. The amount of green fluorescence is proportional to the amount of ROS. This approach for measuring ROS in living cells is frequently utilized (18). It is known that ROS can cause apoptosis in different kinds of cell types. Therefore, to determine whether generation of ROS has any role in cell death, the degree of ROS was measured.

HOB cells were treated as explained above for four hours. After the treatment, the cells were rinsed with PBS (pH 7.4). The positive control was H_2O_2 (0.2 mM) treated cells.

DCF-DA stock solution (20 mM) was prepared and then diluted in DMSO (0.1 mM culture medium). DCF-DA staining was performed as described previously by application of the dye directly onto the cells without removing them by trypsinization from the wells (18).

The cells were then stained with DCF-DA (0.1 mM) and covered with aluminum foil for 30 minutes at 37°C. Finally, the cells were rinsed in PBS and were examined using fluorescent microscope (Carl-Zeiss/Axio Observer 3, Zen 2.3 Blue Edition software).

A single 300 ms exposure was used to capture fluorescence photos, followed by differential interference contrast (DIC) images in the same field of view. The each cell's fluorescence intensities were measured in a 60 x 60-pixel box by Zen 2.3 Blue Edition software, and the average of at least 100 cells per well was taken (18).

Bacterial Response to Oxidative Stress

To detect the influence of NE on the sensitivity of MRSA strain to H_2O_2, an agar dilution method was used as previously described (20). The initial suspension of MRSA strain was adjusted to $2x10^8$ CFU/mL from the overnight culture.

TSA including 2, 1, 0.5, 0.2 and 0.1 mM H_2O_2 and/or two different concentrations of NE were prepared. The spot inoculation (3 µL) method was used and agar plates were incubated for 24 hours at 37°C. The results were evaluated considering the growth/no growth of MRSA.

Statistical Analyses

Differences between the results of experimental and control conditions were statistically analyzed. The growth alterations were assessed using one-way ANOVA (Dunnett's multiple comparisons test). Two-way ANOVA followed by Dunnett's multiple comparisons test was used for the analysis of adhesion and invasion results. Multiple comparisons were examined by Tukey's procedure for MTT and microscopic analyzed on GraphPad Prism 8.3.0. All results were presented as mean±standart deviation. The significant differences were considered as p values, less than 0.05.

RESULTS

The Alteration of MRSA Growth in the Presence of NE

Norepinephrine, at two concentrations, was shown to have no effect ($p>0.05$) on the growth of MRSA strain at three-, six- and 24-hour periods in cell culture conditions (Figure 1). Even though the alterations were not statistically significant, the growth of bacterium was decreased in three and six hours of incubation. However, if the incubation was prolonged to 24 hours, the growth was shown to be increased in the presence of both two NE concentrations.

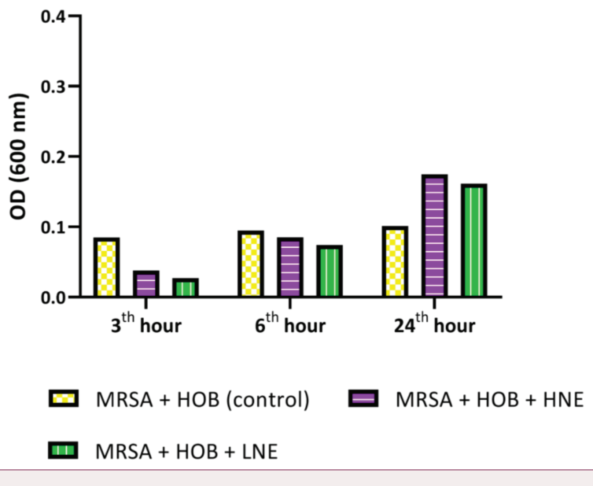

Figure 1. The alteration of MRSA growth in the presence of NE.

The growth of MRSA in HOB cell culture with/without NE was analyzed using one-way ANOVA (Dunnett's multiple comparisons test). HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL)

The Alteration of Adhesion-Invasion of MRSA in the Presence of NE

Both concentrations of NE statistically significantly ($p<0.0001$) decreased the adhesion of MRSA. However, the invasion of MRSA was increased (p: 0.013) only in the presence of high-level NE (Figure 2).

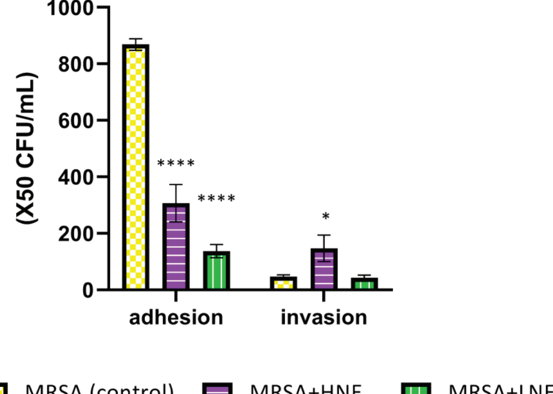

Figure 2. The alteration of MRSA adhesion and invasion in the presence of NE.

The adhesion and invasion of MRSA to HOB cell culture with/without NE were examined using two-way ANOVA (Dunnett's multiple comparisons test). HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL) (****, *p<0.0001 and p=0.013, respectively)

The Alteration of HOB Cell Viability in the Presence of NE and/or MRSA

The MTT experiment revealed that the HOB cell viability was reduced in the presence of both NE concentrations and/or MRSA (p<0.001; Figure 3).

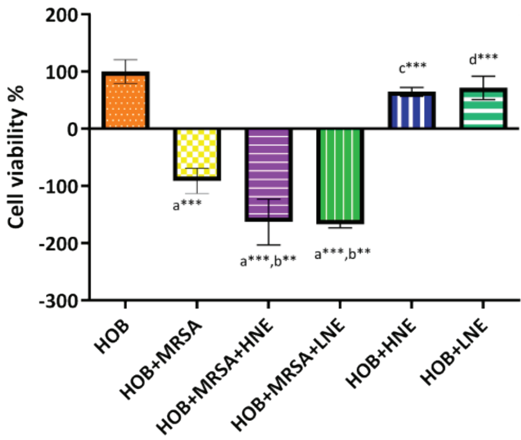

Figure 3. The alteration of cell viability in the presence of NE and/or MRSA.

The cell viability with/without NE and/or MRSA were examined using one-way ANOVA (Tukey multiple comparison tests) (**, ***p<0.0001). HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL) The characters showed comparions of experimental groups' statistically significant results as mentioned in Figure 3 are: **a:** HOB (non-treated negative control), **b:** HOB-HOB+MRSA inoculated group, **c:** HOB- HOB+MRSA+HNE inoculated group, **d:** HOB- HOB+MRSA+LNE inoculated group

Cell Death in the Presence of NE and/or MRSA

As shown in Figure 4, AO/EB staining demonstrated that MRSA and MRSA+HNE/LNE caused mainly necrosis in HOB cells (necrosis: 98.33±1.16% by MRSA; 98.67±1.15% by MRSA+HNE; 97.67±0.58% by MRSA+LNE) when compared to the negative control (0.55±0.33) (mean±standart deviation) (p<0.001). Besides, there was no significant difference in the percentage of apoptotic and necrotic cells between HOB+MRSA (apoptotic: 1.66±1.15; necrotic: 98.33±1.16), HOB+MRSA+HNE (apoptotic: 1.33±0.88; necrotic: 98.67±1.15) and HOB+MRSA+LNE (apoptotic: 2.33±0.57; necrotic: 97.67±0.58).

However, the presence of NE at two concentrations did not cause cell death (neither apoptosis nor necrosis) significantly (p>0.05). There was also no significant difference in the percentage of apoptotic and necrotic cells between HOB+HNE (apoptotic: 5±1; necrotic: 0.58±0.33) and HOB+LNE (apoptotic: 2.67±1.5; necrotic: 0.58±0.33). Microscopic images stained by AO/EB are shown in Figure 5.

As illustrated in Figure 6, according to DCF-DA experiment results, it was found that MRSA and/or NE did not cause ROS production in HOB cells significantly (p< 0.05).

Figure 7 shows representative microscope images from the DCF-DA assay sample.

Bacterial Response to Oxidative Stress in the Presence of NE

MRSA strain was resistant to all H_2O_2 concentrations tested. The presence of both two NE concentrations did not alter the susceptibilities.

DISCUSSION

Many studies have shown the effects of mammalian hormones on the regulation of physiological properties of both mammalian cells and microorganisms. Microbial endocrinology concept has provided a basic understanding of the inter-kingdom interactions. It is well known that catecholamines can regulate the immune system. Norepinephrine is known as a neurotransmitter that can affect the inflammatory process directly or indirectly (21). Furthermore, the level of norepinephrine secretion can be stimulated under stress conditions (22). As previously reported, up-taking of iron in limited conditions, hormone-mediated induction of auto-inducers, acting as quorum sensing compounds have been reported to be the possible action mechanisms of NE (1-3). At present, it is commonly known that during the evolutionary process, microorganisms improved their abilities to modulate the growth, metabolic pathways, virulence and antimicrobial susceptibilities in a host (1-3).

Considering this view, this study determined the role of norepinephrine in the modulation of some biological properties (growth, adhesion, invasion and response to oxidative stress) of methicillin-resistant *Staphylococcus aureus* strain inoculated into human osteoblast cell culture imitating the host environment as much as possible. Furthermore, we investigated the influences of MRSA infections and/or NE on HOB cell viability and cell death pathway.

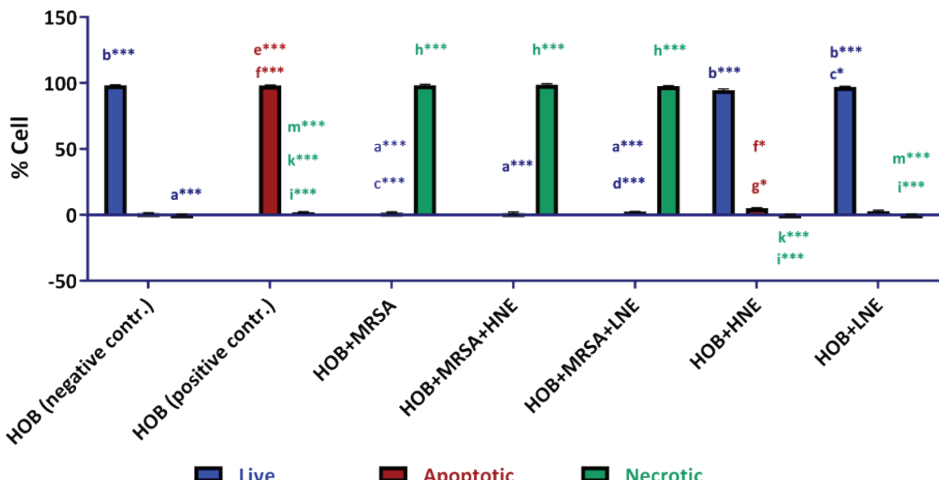

Figure 4. The alteration of cell death frequency in the presence of NE and/or MRSA.

MRSA and/or NE induce necrosis. The percentage change of the proportion of necrotic cells and the untreated negative control was compared, and one-way ANOVA (Tukey multiple comparison test) was used.
(*, **, *** $p<0.05$, indicates a significant difference compared with the negative control, n=3).
H_2O_2 (0.2 mM)- treated HOB cells (positive control), non-treated HOB cells (negative control)
HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL)
The characters showed comparions of experimental groups' statistically significant results as mentioned in figure 4 are:
a: HOB live cell numbers (non-treated negative control), **b:** HOB live cell numbers (H_2O_2-treated positive control), **c:** the live cell numbers of HOB+HNE, **d:** the live cell numbers of HOB+LNE, **e:** apoptotic cell numbers of H_2O_2-treated positive control, **f:** apoptotic cell numbers of non-treated negative control, **g:** apoptotic cell numbers of HOB+MRSA+HNE, **h:** necrotic cell numbers of non-treated negative control, **i:** necrotic cell numbers of HOB+MRSA, **k:** necrotic cell numbers of HOB+MRSA+HNE, **m:** necrotic cell numbers of HOB+MRSA+LNE.

According to some previous studies, norepinephrine decreased the growth of various microorganisms such as *Prevotella intermedia, Porphyromonas gingivalis,* some *Actinomyces* strains, *Escherichia coli (E. coli)* in microbiological culture media (4, 5). However, others showed that the growth of *Staphylococcus epidermidis, Helicobacter pylori, Streptococcus (S) pneumoniae, Vibrio harveyi* and *Campylobacter jejuni (C. jejuni)* was stimulated by NE (1, 6-8). In some studies, there was no alteration of microbial growths in the presence of NE (2, 23, 24).

In the present study, NE did not affect the growth of MRSA independently from the incubation period and the concentration of the hormone in HOB cell culture.

It has also been reported that host hormones can influence some infectious stages such as adhesion and invasion. It seems that in the presence of NE, the adhesion of EHEC O157:H7, adherent-invasive *E. coli, C. jejuni* and *Enterococcus (E) faecalis* strains to various host tissues (HeLa cells, Caco-2/TC-7 cells and Caco-2 cells) have been induced (8-11) but consistent with our results, the adhesion of *S. pneumoniae* to A549 cells is reported to be reduced (12). Most of the studies showed that NE acts as an inducer for the adhesion stage of different kinds of microorganisms as mentioned above, but in the frame of our results, it is possible to suggest that NE plays an inhibitory role in MRSA adhesion to HOB cells.

There are limited number of studies investigating the roles of NE on the invasion to host cells showing that NE acted commonly as an inducer for invasion of adherent-invasive *E. coli and C. jejuni* (8, 9). Our results show that HNE concentration stimulated MRSA invasion of HOB cells correlated with previous studies.

Some studies have reported that cell viability decreases with the increase of NE concentration and high hormone concentrations may cause an increase in bone destruction by inhibiting osteoblast differentiation (25, 26). Suzuki et. reported that NE has a stimulator role for MC3T3-E1 osteoblast-like cells via DNA synthesis, dependent on the increasing of NE dose (27). In our study, the administration of NE inhibited HOB cell viability is consistent with Xue-Min et al. and Grassel's findings (25, 26).

Similary, MRSA significantly decreased the cell viability, consistent with the results of a previous study (28).

To our knowledge, there are no studies to determine the influences of NE on MRSA-infected HOB cells. Furthermore, Beata et al. indicated that NE has effects on the interaction between adherent-invasive *E. coli* and Caco-2 cells which strengthens bacterial virulence (9).

In the present study, when NE was added to MRSA-infected HOB cells, the viability was also reduced significantly. In sum-

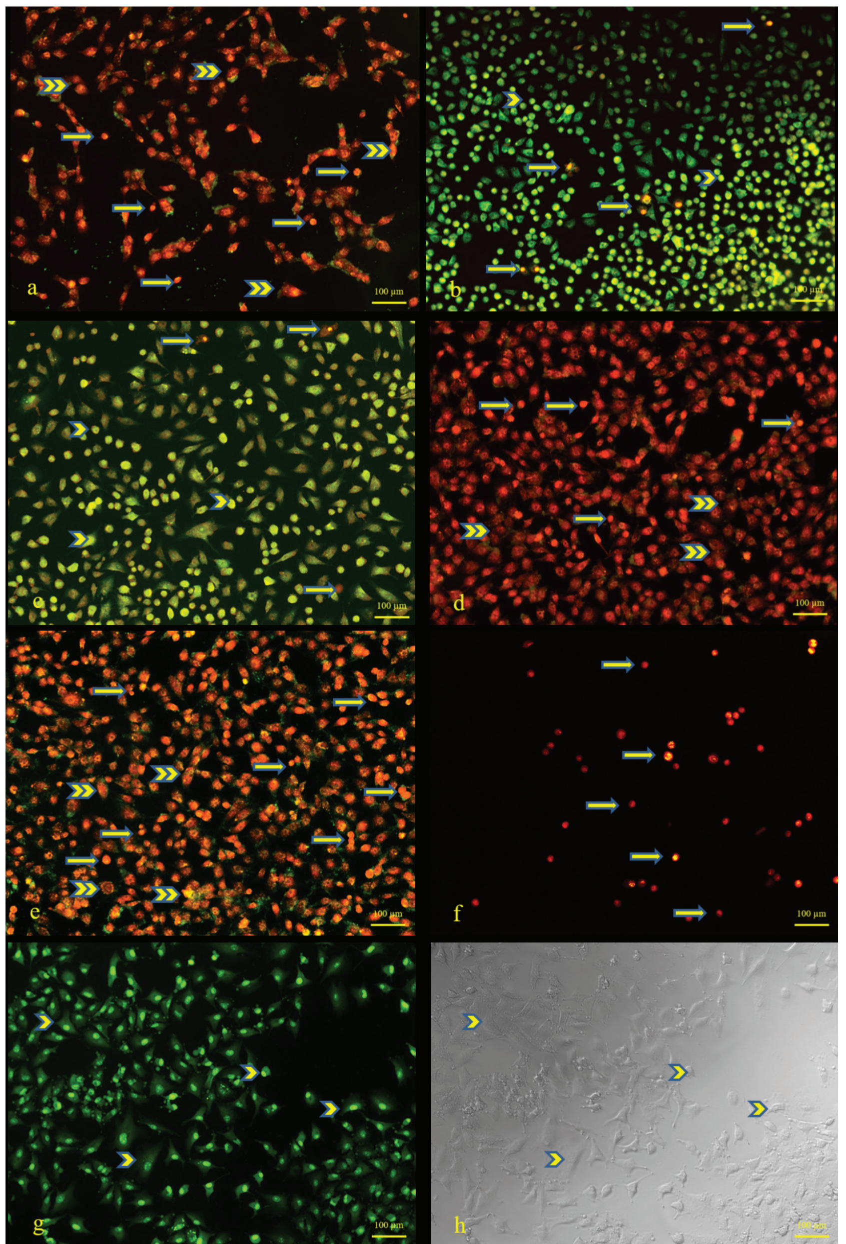

Figure 5. AO/EB dual staining of HOB cells under the microscope.

a) HOB+MRSA b) HOB+HNE **c)** HOB+LNE **d)** HOB+MRSA+HNE **e)** HOB+MRSA+LNE **f)** H_2O_2 (0.2 mM)-treated HOB cells (positive control); **g)** non-treated HOB cells (negative control) **h)** image **g**'s brightfield (Magnifications: 10x).
HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL); Apoptotic cells are shown by arrows, necrotic cells by arrowheads, and living cells by double arrowheads.

mary, our results showed that NE and/or MRSA decreased the viability of HOB cells. It is well known that NE acts as an anti-inflammatory for the host. Thus, it is possible to suggest that both NE and the presence of MRSA infection may be responsible for reducing HOB cell viability.

It was reported that NE modulates cell proliferation and apoptosis pathway on chondrocytes and reduces the rate of apoptosis (29). Ning et al. found that *Staphylococcus aureus* caused apoptosis in HOB cells during *in vitro* conditions (30). However, Choi et al. showed that MRSA had distinct effects on bone cells (*in vitro* or *in vivo*). Detecting significant necrosis rates in rats, they suggested that bone necrosis was dose dependent (31). According to our AO/EB dual staining results, cell death increased significantly in the presence of MRSA, MRSA+HNE and MRSA+LNE and this cell death occurred more with the necrosis

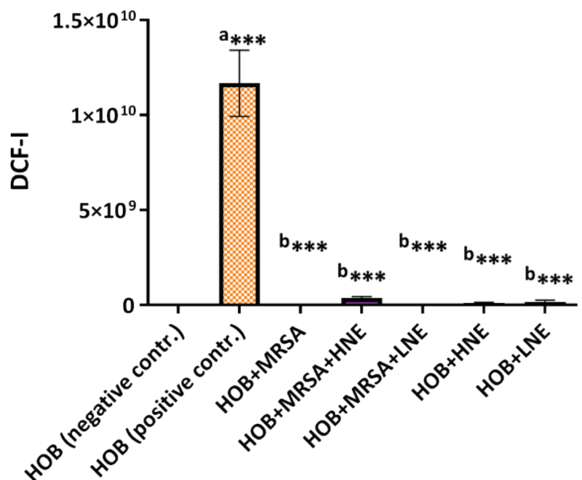

Figure 6. Cellular ROS production of HOB cells was examined by DCF-DA showing ROS detection.

DCF-I: ROS intensity averages for at least 100 cells from each well of each study group.
Used one-way ANOVA (Tukey's multiple comparison test) to analyze statistical significance [***p<0.0001 indicates a significant difference compared with control groups (H_2O_2 (0.2 mM)- treated HOB cells as positive control, non-treated HOB cells as negative control)].
HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL)

rather than apoptosis pathway (necrosis: 98.33±1.16% by MRSA; 98.67±1.15% by MRSA+HNE; 97.67±0.58% by MRSA+LNE). However, the induced pathway leading to necrosis and details of this mechanism is another subject worth investigating.

According to our ROS detection results, there was no significant increase in ROS production in the presence of NE and/or MRSA which confirms that cell death does not occur via the apoptosis pathway. In fact, it was previously reported that MRSA strains did not induce oxidative stress in osteoblast-like cells (32). However, some studies show that infection of osteoblast cells with MRSA induces apoptosis pathway that can disrupt host cells' defense barrier, actin cytoskeleton reorganization and cell proliferation (33, 34).

When an infection occurs, microorganisms encounter several environmental stress factors; one of them is H_2O_2. Oxidative molecules, such as H_2O_2, released by immune system cells destroy microorganisms (35, 36). Some microorganisms resist oxidative stress and some are susceptible. In our study, MRSA was resistant to H_2O_2 and NE did not alter its resistance.

In our study, the most important finding was the significant inhibition of HOB cell viability and induction of cell death via necrosis, by NE and/or MRSA in cell culture.

In conclusion, this study provides the knowledge about the possible influences of NE on the biological properties of bacteria and host cells, hence on the infectious process.

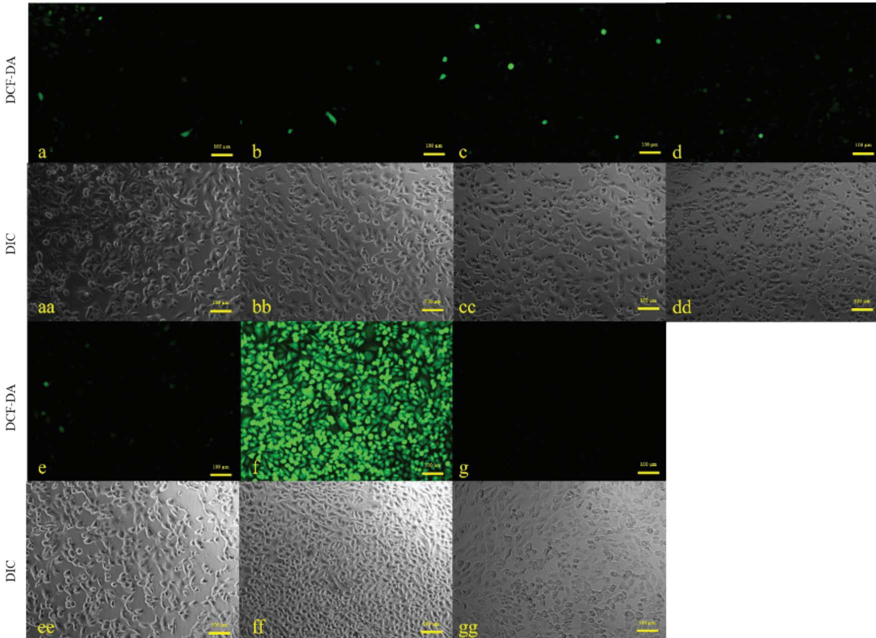

Figure 7. Representative microscope images from DCF-DA assay with fluorescence filter.

DIC brightfield's are included to show the same field with the presence of all the cells in the well.
a) HOB+MRSA **b)** HOB+HNE **c)** HOB+LNE **d)** HOB+MRSA+HNE **e)** HOB+MRSA+LNE **f)** H_2O_2 (0.2 mM)- treated HOB cells (positive control) **g)** non-treated HOB cells (negative control)
HNE: High concentration of NE (0.04 µg/mL), LNE: Low concentration of NE (0.0017 µg/mL)
All images of brightfield are given as DIC (aa, bb, …gg). The amount of green fluorescence is proportional to the quantity of ROS (Magnifications: 10x).

Ethics Committee Approval: This study was performed in cell culture and using a standard bacterial strain. For this reason, ethics committee approval was not obtained.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study - F.K.Y., D.G., G.İ.G.; Data Acquisition - F.K.Y., D.G., G.İ.G., A.U.Ö., N.E.; Data Analysis/Interpretation - F.K.Y., D.G., G.İ.G.; Drafting Manuscript - F.K.Y., D.G., G.İ.G., M.A.; Critical Revision of Manuscript - F.K.Y., D.G., G.İ.G., M.A.; Final Approval and Accountability - F.K.Y., D.G., G.İ.G., M.A.

Conflict of Interest: The authors have no conflict of interest to declare.

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REFERENCES

1. Lyte M, PPE Freestone. Microbial Endocrinology. 1st ed. New York: Springer; 2010. [CrossRef]
2. Lyte M, JF Cryan. Microbial Endocrinology: The microbiota-gut-brain axis in health and disease. 1st ed. New York: Springer; 2014. [CrossRef]
3. Freestone P. Communication between bacteria and their hosts. Scientifica 2013; 2013: 1-15. [CrossRef]
4. Boyanova L. Stress hormone epinephrine (adrenaline) and norepinephrine (noradrenaline) effects on the anaerobic bacteria. Anaerobe 2017; 44: 13-9. [CrossRef]
5. Gümüş D, Kalaycı Yüksek F, Sefer Ö, Yörük E, Uz G, Anğ Küçüker M. The roles of hormones in the modulation of growth and virulence genes' expressions in UPEC strains. Microb Pathog 2019; 132: 319-24. [CrossRef]
6. Doherty NC, Tobias A, Watson S, Atherton JC. The effect of the human gut-signalling hormone, norepinephrine, on the growth of the gastric pathogen *Helicobacter pylori.* Helicobacter 2009; 14(3): 223-30. [CrossRef]
7. Yang Q, Anh NDQ, Bossier P, Defoirdt T. Norepinephrine and dopamine increase motility, biofilm formation, and virulence of *Vibrio harveyi*. Front Microbiol 2014; 5: 1-12. [CrossRef]
8. Xu F, Wu C, Guo F, Cui G, Zeng X, Yang B, et al. Transcriptomic analysis of *Campylobacter jejuni* NCTC 11168 in response to epinephrine and norepinephrine. Front Microbiol 2015; 6: 1-11. [CrossRef]
9. Sobieszczańska B, Turniak M, Olbromski M, Walczuk U, Marcin C, Tukiendorf A. Norepinephrine affects the interaction of adherent-invasive *Escherichia coli* with intestinal epithelial cells. Virulence 2021; 12(1): 630-7. [CrossRef]
10. Bansal T, Englert D, Lee J, Hegde M, Wood TK, Jayaraman A, et al. Differential effects of epinephrine, norepinephrine, and indole on *Escherichia coli* O157:H7 chemotaxis, colonization, and gene expression. Infect Immun 2007; 75(9): 4597–607. [CrossRef]
11. Cambronel M, Nilly F, Mesguida O, Boukerb AM, Racine PJ, Baccouri O, et al. Influence of catecholamines (Epinephrine/Norepinephrine) on biofilm formation and adhesion in pathogenic and probiotic strains of *Enterococcus faecalis*. Front Microbiol 2020; 11: 1–13. [CrossRef]
12. Gonzales XF, Castillo-Rojas G, Castillo-Rodal AI, Tuomanen E, López-Vidal Y. Catecholamine norepinephrine diminishes lung epithelial cell adhesion of Streptococcus pneumoniae by binding iron. Microbiology (United Kingdom) 2013; 159: 2333-41. [CrossRef]
13. Nicolas A, Deplanche M, Commere P-H, Diot A, Genthon C, Marques da Silva W, et al. Transcriptome architecture of osteoblastic cells infected with *Staphylococcus aureus* reveals strong inflammatory responses and signatures of metabolic and epigenetic dysregulation. Front Cell Infect Microbiol 2022; 12: 1-20. [CrossRef]
14. Widaa A, Claro T, Foster TJ, O'Brien FJ, Kerrigan SW. *Staphylococcus aureus* protein A plays a critical role in mediating bone destruction and bone loss in osteomyelitis. PLoS One 2012; 7(7): e40586. [CrossRef]
15. Castilho IG, Dantas STA, Langoni H, Araújo JP, Fernandes A, Alvarenga FCL, et al. Host-pathogen interactions in bovine mammary epithelial cells and HeLa cells by *Staphylococcus aureus* isolated from subclinical bovine mastitis. J Dairy Sci 2017; 100(8): 6414-21. [CrossRef]
16. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55-63. [CrossRef]
17. Tapajós ECC, Longo JP, Simioni AR, Lacava ZGM, Santos MFMA, Morais PC, et al. In vitro photodynamic therapy on human oral keratinocytes using chloroaluminum-phthalocyanine. Oral Oncol 2008; 44(11): 1073-9. [CrossRef]
18. Jiang H, Ren Y, Zhao J, Feng J. Parkin protects human dopaminergic neuroblastoma cells against dopamine-induced apoptosis. Hum Mol Genet 2004; 13(16): 1745-54. [CrossRef]
19. Hugo AA, Kakisu E, De Antoni GL, Pérez PF. Lactobacilli antagonize biological effects of enterohaemorrhagic *Escherichia coli in vitro*. Lett Appl Microbiol 2008; 46(6): 613-9. [CrossRef]
20. Liao H, Zhong X, Xu L, Ma Q, Wang Y, Cai Y, et al. Quorum-sensing systems trigger catalase expression to reverse the oxyR deletion-mediated VBNC state in *Salmonella typhimurium*. Res Microbiol 2019; 170(2): 65-73. [CrossRef]
21. Szelényi J and Vizi E. S. The catecholamine-cytokine balance: Interaction between the brain and the immune system. Ann N Y Acad Sci 2007; 1113: 311-24. [CrossRef]
22. Osier DN and Dixon C. Catecholaminergic based therapies for functional recovery after TBI. Brain Res 2016; 1640: 15-35. [CrossRef]
23. Gümüş D, Kalaycı-Yüksek F, Uz G, Bilgin M, Anğ-Küçüker M. The Possible effects of different hormones on growth rate and ability of biofilm formation in different types of microorganisms. Acta Biol Marisiensis 2018; 34(1): 47-51.
24. Gümüş D, Kalaycı Yüksek F, Gündoğan Gİ, Anğ Küçüker M. Farklı hormonların ve hücre hatlarının bir üropatojen *E . coli* suşunun üremesi üzerine etkileri The alterations on growth of a uropathogenic *E . coli* with the effects of both different hormones and cell lines. Kocaeli Med J 2020; 9(2): 68-76. [CrossRef]
25. Xue-Min Z, Shu-Shan S, Ya-Fei L. Proliferative activity of normal human osteoblasts cultured with neuropeptides at different concentrations. J Clin Rehabil Tissue Engin Res 2010; 14(50): 9325-28.
26. Grässel S. The role of peripheral nerve fibers and their neurotransmitters in cartilage and bone physiology and pathophysiology. Arthritis Res Ther 2014; 6(485): 1-13. [CrossRef]
27. Suzuki A, Palmer G, Bonjour JP, Caverzasio J. Catecholamines stimulate the proliferation and alkaline phosphatase activity of MC3T3-E1 osteoblast-like cells. Bone 1998; 23(3): 197-203. [CrossRef]
28. Stracquadanio S, Musso N, Costantino A, Lazzaro LM, Stefani S, Bongiorno D. *Staphylococcus aureus* internalization in osteoblast cells: Mechanisms, interactions and biochemical processes. what did we learn from experimental models? Pathogens 2021; 10(2): 1-24. [CrossRef]

29. Opolka A, Straub RH, Pasoldt A, Grifka J, Grässel S. Substance P and norepinephrine modulate murine chondrocyte proliferation and apoptosis. Arthritis Rheum 2012; 64(3): 729-39. [CrossRef]
30. Ning R-de, Zhang X-Long, Li Q-Tian, Guo XK. The effect of Staphylococcus aureus on apoptosis of cultured human osteoblasts. Orthop Surg 2011; 3(3): 199-204. [CrossRef]
31. Choi YS, Ham DS, Lim JY, Lee YK. Validation of the Osteomyelitis induced by methicillin-resistant *Staphylococcus aureus* (MRSA) on rat model with calvaria defect. Tissue Eng Regen Med 2021; 18(4): 671-83. [CrossRef]
32. Musso N, Caruso G, Bongiorno D, Grasso M, Bivona DA, Campanile F, et al. Different modulatory effects of four methicillin-resistant *Staphylococcus aureus* clones on MG-63 osteoblast-like cells. Biomol 2021; 11(1): 1-18. [CrossRef]
33. Hermann I, Räth S, Ziesemer S, Volksdorf T, Dress RJ, Gutjahr M, et al. *Staphylococcus aureus* hemolysin A disrupts cell-matrix adhesions in human airway epithelial cells. Am J Respir Cell Mol Biol 2015; 52(1): 14-24. [CrossRef]
34. Alexander EH, Rivera FA, Marriott I, Anguita J, Bost KL, Hudson MC. *Staphylococcus aureus* - induced tumor necrosis factor - related apoptosis - inducing ligand expression mediates apoptosis and caspase-8 activation in infected osteoblasts. BMC Microbiol 2003; 3: 1-11. [CrossRef]
35. Hausladen A, Privalle CT, Keng T, DeAngelo J, Stamler JS. Nitrosative stress: Activation of the transcription factor OxyR. Cell 1996; 86(5): 719-29. [CrossRef]
36. Fields JA and Thompson SA. *Campylobacter jejuni* CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion. J Bacteriol 2008; 190(9): 3411-6. [CrossRef]

 ORIGINAL ARTICLE

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Effects of Thymoquinone (TQ) on the Molecular Structure and Total Antioxidant Capacity of Cerebellum Tissue of Healthy Rats

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ABSTRACT

Objective: The neuroprotective effects of thymoquinone (TQ), the active compound of *Nigella sativa*, have been reported in accordance with the anti-inflammatory and antioxidant features of this drug. It has been suggested that the cerebellum plays a considerable role in neurodegenerative processes. In the current study, the possible effects of TQ on the structure and composition of cerebellum tissues and its total antioxidant capacity were studied dose-dependently.

Materials and Methods: Fifteen adult Long Evans female rats were divided into groups as follows: G1: Control, G2: 10 mg/kg TQ treatment, G3: 20 mg/kg TQ treatment. TQ was injected into the rats intraperitoneally for two weeks. The control group only received corn oil used for the dissolving of TQ. Fourier-transform infrared spectroscopy (FTIR) studies and total protein, and antioxidant capacity measurements were carried out with cerebellum tissues which were removed following the decapitation of rats.

Results and Conclusion: 10 mg/kg TQ treatment improved the saturated and unsaturated lipid and protein content in addition to decreasing nucleic acid content and lipid peroxidation and increasing the total antioxidant capacity of cerebellum tissues. However, 20 mg/kg TQ treatment did not have any significant effect.

Keywords: Thymoquinone, cerebellum, total antioxidant capacity, FTIR spectroscopy

INTRODUCTION

In recent years, many neurodegenerative disorders have been treated by plants commonly used in traditional medicine. These plants are preferred for easy access, collection and minimal side effects among the public (1). *Nigella sativa* (NS) which is a member of *Ranunculaceae* family, is known as black seed or cumin. NS has been proven to have anti-inflammatory and antioxidative effects besides its neuroprotective role (2), and its active compound thymoquinone (TQ) has been indicated to play an important role on oxidative stress and inflammation processes in the body as well (1).

In previous studies, the neuroprotective effects of TQ on neurological disorders were reported in both cell culture and animal studies (3-7). This neuroprotective effect results from its anti-inflammatory and antioxidant properties. One possible mechanism is its inhibitory effect on acetylcholinesterase enzyme which breaks down the neurotransmitter acetylcholine in the neuromuscular junction (6). The other mechanisms include the reduction of pro-inflammatory cytokines and chemokines besides the inhibition of nitric oxide synthase enzyme (3). TQ's antioxidant effect includes the reduction of reactive oxygen species (ROS) produced in oxidative stress. In neurodegenerative diseases, it has also been reported that an abundant value of ROS was produced by the activation of microglial cells as in Alzheimer's disease (8).

The cerebellum, which is located near the brainstem, is a primary component of the hindbrain. The main function of the

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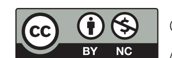
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cerebellum is maintaining motor coordination for movement and balance of the body (9). Structural and functional alterations in cerebellum tissue were reported in different neurodegenerative disorders including Alzheimer's disease, (10), Parkinson disease (11), and multiple system atrophies (12). Although the role of the cerebellum in the clinical condition of neurodegenerative diseases is still unclear, disruption in the structure and function of the brain, including the cerebellum, is well documented. A recent study showed that the cerebellum shares the same pathology with the cerebral cortex in neurodegenerative disorders (9).

In a study conducted by Ismail et al. (13), the protective effect of TQ for the β-amyloid peptide$_{1\text{-}40}$-induced neurotoxicity was investigated by *in vitro* studies using neuron cells. According to the results of this study, TQ has a neuroprotective effect on the apoptosis of cerebellar granule neurons (CGNs) due to β-amyloid peptide$_{1\text{-}40}$ (13). In another recent study, the antioxidant effect of TQ was investigated in the cerebellum of rats who had consumed a high-fat diet (HFD) (1). Since HFD is accepted as a major problem for neuronal damage, oxidative stress markers were measured in the serum of those rats to investigate the meliorative effect of TQ after 4 weeks of HFD supplementation. Alrafiah reported that the treatment with TQ reduced the inflammation and ameliorated the antioxidant enzymes implying an improvement in the HFD-induced neuronal damage in cerebellum tissues (1).

The cerebellum plays a considerable role in neurodegenerative processes, and revealing the molecular and antioxidant effects of TQ in the structure, composition and function of the cerebellum is promising for the prevention of neuronal damage. In the light of previous studies, the aim of the current study was to investigate the dose-dependent effects of TQ on the structure and composition of cerebellum tissues. Besides the structural changes, the effects of TQ in the antioxidant levels of the cerebellum were also studied in the current study.

In recent years, the biomolecular alterations in different parts of brain tissue (14, 15) and other hard (16-18) and soft tissues (19, 20) were studied by our group with attenuated total reflectance attenuated total reflection-fourier-transform infrared (ATR-FTIR) spectroscopy and FTIR microspectroscopy techniques. The TQ-induced variations on the content and confirmation of the cerebellum proteins, lipids and nucleic acids were also studied by ATR-FTIR spectroscopy in the current study.

MATERIALS AND METHODS

Animal Studies

In the current study, adult Long Evans female rats (400-450 g) were divided into 3 groups:

G1: Control group (n=5)

G2: 10 mg/kg TQ treatment group (n=5)

G3: 20 mg/kg TQ treatment group (n=5)

Ad libitum standard lab chow diet besides tap water were provided for the rats. The rats were kept in a 12-h light/12-h dark cycle at room temperature (21°C). TQ dissolved in corn oil (2-2,5 mL) was given to the rats by intraperitoneal (ip) injection every day for 14 days. The control group was only given corn oil with ip injection for the same period.

The doses used in the current study were selected according to previous *in vivo* studies in the literature which investigated the antioxidant effects of TQ in different tissues (21-23). Those effects were commonly seen in TQ doses of between 10 and 20 mg/kg.

Throughout the experiment process guidelines provided by "The Guide for the Care and Use of Laboratory Animals" were followed. All the experimental processes of the study were confirmed by the Scientific Ethical Committee of Bezmialem Vakif University (2016/317).

After two weeks, the rats were decapitated, and the cerebellum tissues were collected from the rats. They were kept at -80°C until ATR-FTIR spectroscopy and biochemical studies.

ATR-FTIR Spectroscopy Studies

The cerebellum tissues were first washed with PBS (0.01M, pH 7.4) to remove any blood surrounding the tissue. Tissue the size of a needle head was put on a crystal plate made up of diamond/zinc selenide (Di/ZnSe) on ATR attachment. The rest of the tissues were homogenized for biochemical studies given in the next sections. The spectra of the samples were recorded using 4 cm^{-1} resolution with 128 scan number between 4000-650 cm^{-1} by Bruker Alpha II 100 FTIR spectrometer (Bruker, Berlin, Germany). Three spectra were recorded from each sample as replicates. The average of those three spectra was calculated to be used in the following spectral analysis.

For the detailed spectral analysis, band wavenumber, band area's ratio and band's width values of the target bands were measured using OPUS v8.5 software (Bruker Optics GmbH Co., Ettlingen, Germany). In this way, the molecular variations in the proteins, lipids and nucleic acids of the cerebellum tissue due to TQ treatment were investigated in the current study.

Total Protein Measurement

After removing the surrounding blood from the cerebellum tissue by washing with PBS solution, 250 mg of tissue were homogenized with lysis buffer for the isolation of cerebellum proteins. For the lysis buffer, the buffer found in ReadyPrep™ Protein Extraction Kit (Bio-Rad Laboratories, California, USA) was used. For the homogenization 2.5 µL lysis buffer was used for every 1 mg of tissue. After mixing the tissue with an appropriate amount of lysis buffer, the homogenization was done with a homogeniser (VELP O5, IKA Process Technology, Germany). Homogenized tissue was centrifuged at 4°C for 10 minutes at 14000g (Eppendorf 5810R, Hamburg, Germany). After the centrifugation, the supernatants were collected.

The total protein of the cerebellum tissue was calculated using Bradford assay with coomassie plus reagent and as a standard bovine serum albumin (Pierce, Thermo Fisher Scientific, Roskilde, Denmark). Optimization for the dilution of samples were done and it was decided that a 1/50 dilution for the supernatants would be the amount of dilution for the measurement.

Five microliters of each diluted supernatant were mixed with 245 µL of Bradford Reagent in 98-well microplate. Seven standards with different protein concentrations (125, 250, 500, 750, 1000, 1200, 1500 µg/mL) were used in the colorimetric measurement with microplate reader (Biochrome EZ Read 400, Biochrome LTD., Cambridge, UK) at 595 nm. The total protein concentrations of the samples were calculated using the standard curve plotted after the measurement. Three replicates for each sample were measured, the average of which was used for the evaluation of the results and the statistical analysis.

Total Antioxidant Capacity Measurement

Hundred mg of cerebellum tissues were homogenized with PBS (0.01M, pH 7.4) at a ratio of 1:9 (the weight of the tissue in milligrams: the volume of homogenized medium in microliters) using homogenizer (VELP O5, IKA Process Technology, Germany). Homogenized tissue was centrifuged at 4°C for 10 minutes at 10000g (Eppendorf 5810R, Hamburg, Germany). Supernatans were used to carry out the total antioxidant capacity measurement with a colorimetric kit (T-AOC Colorimetric Assay Kit, E-BC-K136S) according to recommendations of the manufacturer. Samples were studied in two replicates and the average of these two values were used for the evaluation of the results and the statistical analysis.

Statistical Analyses

The GraphPad Prism 9.0 (GraphPad Software, Inc., San Diego, California, USA) was used to test the significance of the variation between the groups. After testing for normal distribution with Kolmogorov Smirnov test, One-way ANOVA with the addition of Tukey's post-hoc test was chosen for the comparison of the groups. The mean values and the standard deviation values were calculated and used to summarize the data for each of the groups. p values less or equal to 0.05 were accepted as statistically significant in the comparison of the rat groups.

RESULTS

In the current study, dose-dependent effects of TQ on the molecular content and antioxidant capacity of cerebellum tissues of rats were investigated.

Figure 1 shows the representative average spectra of cerebellum tissue of the healthy group. As can be seen from the figure, there are various bands belonging to the several functional groups as a part of tissue proteins, saturated besides unsaturated lipids, phospholipids and nucleic acids. The band assignment table for the labelled bands in Figure 1 is given in Table 1. The band due to

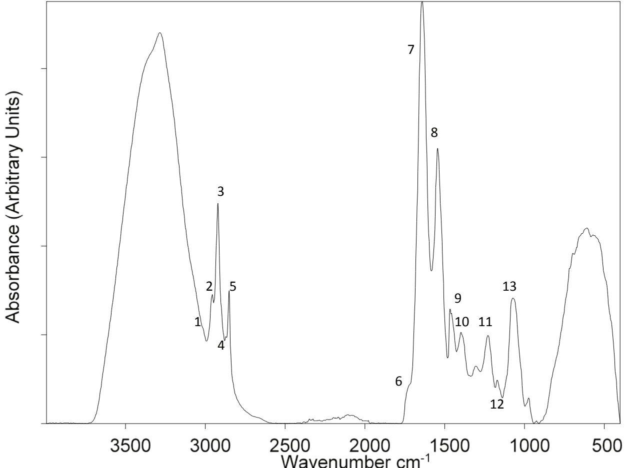

Figure 1. The representative spectrum of control cerebellum tissue at 3800-450 cm^{-1}. (*$p\leq0.05$; **$p\leq0.01$, ***$p\leq0.001$, ****$p\leq0.0001$)

Table 1. General band assignment of a cerebellum tissue between 3700-900 cm^{-1} wavenumber region (15).

Band #	Wavenumber value (cm^{-1})	Band assignment
1	3012	Olefinic HC=CH stretch, due to unsaturated lipids
2	2963	CH_3 antisym stretch, due to proteins and lipids
3	2927	CH_2 antisym stretch, due to primarily lipids
4	2873	CH_3 sym stretch, due to primarily proteins
5	2854	CH_2 sym stretch, due to primarily lipids
6	1742	Ester C=O stretching due to triglyceride and cholesterol esters
7	1638	Amide I primarily C=O stretch, vibrations of amide groups of proteins
8	1545	Amide II primarily N-H bending with C-N stretch, vibrations of amide groups of proteins
9	1454	CH_2 bending due to lipids
10	1398	Fatty acids and amino acids
11	1235	PO_2^- asym stretch, due to primarily nucleic acid with slight contribution of phospholipids
12	1152	Glycogen
13	1080	PO_2^- sym stretch, due to phospholipids

olefinic only appears in the second derivative spectra.

The spectral band area/intensity values supply information on the amounts of the functional groups related to the relative molecules; for example, a rise in the band area values correlate to higher concentrations of the molecules allotted to the spectral bands (24). Band area ratios are used instead of separate band area values in order not to be affected by any difference coming from the changes in tissue thickness during the experiment (19). Band area and intensity ratios were used to get information about total lipid and protein contents, saturated and unsaturated lipid contents besides carbonyl and nucleic acid contents.

The value of the band area/intensity ratios of the calculated bands is given in Table 2.

The band area/intensity ratios of various lipid bands located in CH region and the C=O stretching vibrations of carbonyl ester groups were used to analyse the variations in lipid structure and composition. The calculated ratios for analysing the variations in lipid's structure and composition were as follows: olefinic =CH/total lipid; CH_2 antisym /CH_3 antisym; CH_2 antisym / total lipid and carbonyl /total lipid.

Total lipid amounts were determined using lipid bands in the CH stretching wavenumber region (3030–2800 cm^{-1}) namely CH_2 antisym and CH_2 sym (15). The total lipid content which is obtained by getting the band area ratio of CH_2 antisym (2927 cm^{-1}) to CH_2 antisym + CH_2 sym (2854 cm^{-1}) bands, was significantly higher in the 10 mg/kg TQ treatment group compared with the control, while there was no significant change in the 20 mg/kg TQ treatment group (Table 2 and Figure 2A). This increase was also confirmed by the increase in CH_2 symmetric stretching band, while taking the normalization to CH_2 antisymmetric band seen in Figure 3.

The olefinic and carbonyl ester contents of cerebellum tissues were calculated from the band intensity ratios from second derivative spectra since these bands can be clearly seen in the second derivative of the spectrum but are difficult to see in the first derivative (Figure 4). The band intensity ratio of olefinic band located at 3014cm^{-1} to the total of CH_2 antisym + CH_2 sym bands was significantly increased only in the 10 mg/kg TQ treatment group in comparison to the control (Table 2 and Figure 2B). Since this ratio gives information related to the unsaturation degree of lipids, the result referred to an increase in unsaturated lipid content in treated groups especially in the 10 mg/kg TQ treatment group (19). In addition, the band area ratio of carbonyl band at 1743 cm^{-1} to the total of CH_2 antisym + CH_2 sym bands was slightly increased in the 10 mg/kg treated group implying a higher carbonyl content in the cerebellum of this group compared with the control group (Table 2 and Figure 2C) (20).

The major protein bands: Amide I (at 1640 cm^{-1}) and amide II (at 1545 cm^{-1}) were used for the calculation of total protein content by taking the band area's ratios of amide I to amide I + amide II (25). There was a significant and a slight increase in protein content of the 10 mg/kg and 20 mg/kg TQ treatment groups, respectively, in comparison to the control (Table 2 and Figure 2D). This result was also confirmed by the increase in these bands seen in Figure 5.

The total protein content of the cerebellum tissues were also calculated by Bradford assay. This result supported the results of ATR-FTIR spectroscopy studies by showing an increase of

Table 2. The band area/intensity ratios of the control and treatment groups.

Band area/intensity ratio	Control	TQ 10mg/kg	TQ 20mg/kg
Total Lipid Content Band area ratio of CH_2 antisym stretch. to CH_2 antisym stretch. + CH_2 sym stretch.	0.605±0.03	0.613±0.07 ↑*	0.605±0.03
Unsaturated Lipid Content Band intensity ratio of Olefinic HC=CH stretch. to CH_2 antisym stretch. + CH_2 sym stretch.	0.313±0.053	0.421±0.060 ↑**	0.314±0.043
Carbonyl Content Band area ratio of Ester C=O stretch. to CH_2 antisym stretch. + CH_2 sym stretch.	0.183±0.027	0.217±0.028 ↑	0.182±0.017
Total Protein Content Band area ratio of Amide I to Amide I + Amide II	0.568±0.004	0.602±0.008 ↑****	0.574±0.010 ↑
Nucleic Acid Content Band area ratio of PO_2^- antisym stretch. + PO_2^- sym stretch. to Amide I + Amide II	0.591±0.073	0.583±0.044 ↓	0.632±0.025 ↑

The arrows pointing upward and downward represent a relative increase and decrease, respectively, in the treatment groups compared to the control group.
*: p≤0.05; **: p≤0.01; ****: p≤0.0001

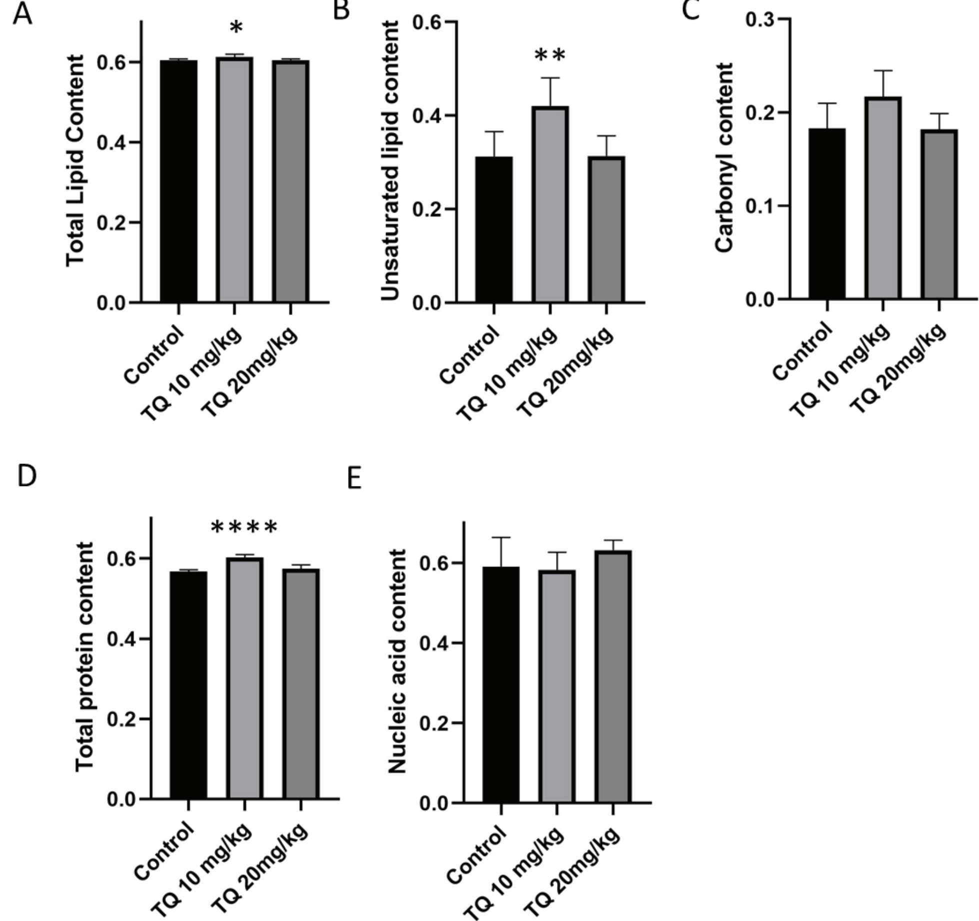

Figure 2. The band area/intensity ratio of different bands for the control, 10 mg/kg TQ treatment and 20 mg/kg TQ treatment groups. The significance compared to the control is indicated with a star symbol (*). *: p≤0.05; **: p≤0.01; ****: p≤0.0001

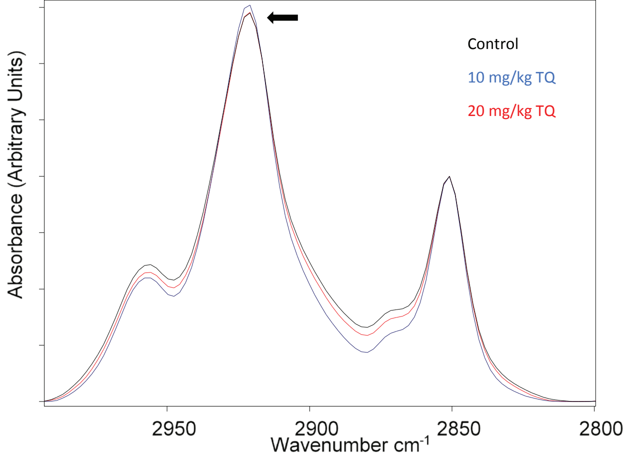

Figure 3. Average ATR-FTIR spectra of cerebellum tissues belonging to control (black), 10 mg/kg TQ treatment (blue) and 20 mg/kg TQ treatment (red) rat groups in 3800-2800 cm^{-1} wavenumber region (The spectrum was normalized to CH_2 sym stretch, band at 2852 cm^{-1}).

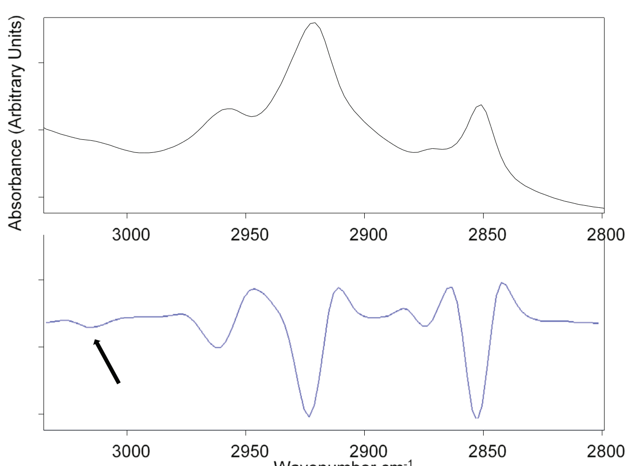

Figure 4. ATR-FTIR first and second derivative spectra of the control cerebellum tissue between 3100-2800 cm^{-1} spectral region, showing olefinic band at 3011 cm^{-1} labelled with an arrow.

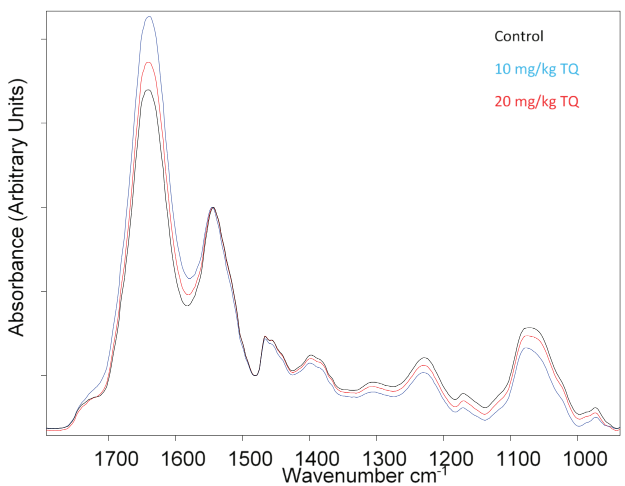

Figure 5. Average ATR-FTIR spectra of cerebellum tissues belonging to control (black), 10 mg/kg TQ treatment (blue) and 20 mg/kg TQ treatment (red) rat groups in 1800-900 cm^{-1} wavenumber region (The spectrum was normalized to amide II at 1544 cm^{-1}).

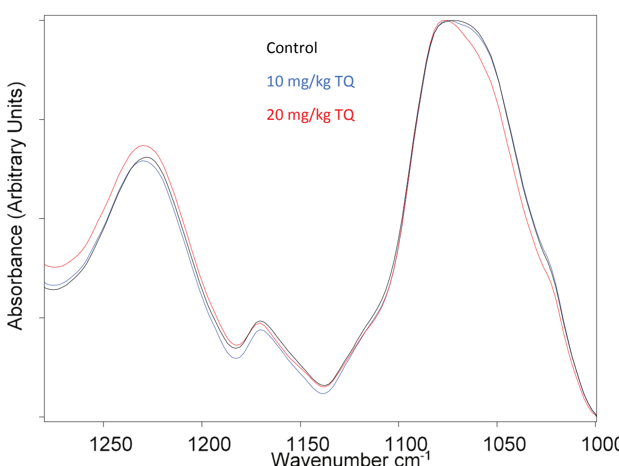

Figure 7. Average ATR-FTIR spectra of the cerebellum tissues belonging to control (black), 10 mg/kg TQ treatment (blue) and 20 mg/kg TQ treatment (red) rat groups in 1300-1000 cm^{-1} wavenumber region (The spectrum was normalized to phosphate sym stretch. band at 1081 cm^{-1}).

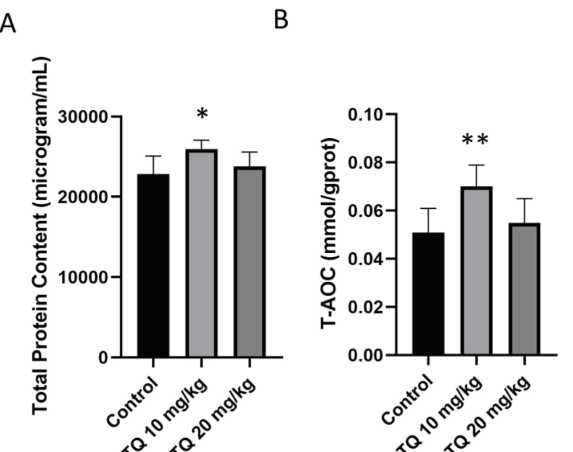

Figure 6. The total protein content (A) and total antioxidant capacity (B) of control, 10 mg/kg TQ treatment and 20 mg/kg TQ treatment groups. The significance compared to the control is indicated with a star symbol (*). *: p≤0.05; **: p≤0.01

total protein amount in the TQ-treated groups compared with the control group as can be seen from Figure 6A.

Bands at 1238 cm^{-1} and 1080 cm^{-1} in the spectra of cerebellum tissues come from the absorbance of P=O bonds of phosphate (PO_2^-) groups in nucleic acids/phospholipids (25). The nucleic acid contents were measured by taking the band area's ratio of phosphate anti-sym stretching (1238 cm^{-1}) + phosphate sym stretching (1081 cm^{-1}) to amide I + amide II (19). The nucleic acid content was decreased in the 10 mg/kg TQ treatment group, while increased in the 20 mg/kg TQ treatment group in comparison with the control (Table 2, Figure 2E and Figure 7).

The effects of two different doses of TQ treatment on the total antioxidant capacity of cerebellum tissues were also evaluated in the present study. The total antioxidant capacity was found to be significantly increased in the cerebellum tissues of the 10 mg/kg TQ treatment group with a slight increase in the 20 mg/kg TQ treatment group compared to the control (Figure 6B).

In summary , the 10 mg/kg TQ treatment caused significant alteration in the composition of cerebellum tissue lipids, proteins and nucleic acids besides increasing the total antioxidant capacity of the tissue. Moreover, the 20 mg/kg TQ treatment did not cause any significant changes in the biomolecular composition of cerebellum tissue and its antioxidant capacity.

DISCUSSION

The cerebellum, which is responsible for the motor coordination and the balance of the body, was also found to be significant in the formation of neurodegenerative diseases by an alteration in its structure and function (10). TQ was reported to have neuroprotective effects in neurological disorders by its role in oxidative stress and inflammation processes (1). However, dose-dependent effects of TQ on the molecular structure and function of cerebellum tissue is still unclear. In the current study, the possible effects of two distinct doses of TQ treatment on the molecular composition, structure and the total antioxidant capacity of cerebellum tissue were studied by ATR-FTIR spectroscopy and biochemical techniques.

Protein, lipid and nucleic acid composition of tissues with the degree of saturation/unsaturation of tissue lipids might be accepted as the basic parameters for the analysis of structure

and function of the tissues. Since they are related to the proper functioning of the tissues, they could indirectly show the disorder/disease state of the tissue.

The total lipid content of cerebellum tissue belonging to the 10 mg/kg rat group was significantly increased compared with the control group, though there was no change in the 20 mg/kg treated group. Elevated serum levels of total cholesterol, low density lipoprotein (LDL) and triglyceride (TG) with less high density lipoprotein (HDL) level is known as hyperlipidemia. The condition of hyperlipidemia is found to be related to neurodegenerative diseases including Alzheimer's disease (26). In our study, the increase in total lipid content in the 10 mg/kg treated group might be mainly due to the increase levels of HDL which has antioxidant properties besides anti-inflammatory features. It was reported that HDL induce the production of M2 polarized macrophages reducing inflammation in the brain tissue (27). The increase in HDL level was also associated with the improvement of cognitive function in aging and also in neurodegenerative diseases (28).

The band area's ratio of the olefinic to total lipid which provides information about unsaturated lipid content, is utilized as an indicator of lipid peroxidation (19). A significant increase in unsaturated lipid content in the 10 mg/kg TQ group compared to the control group was observed in the present study. The study showed that unsaturated fatty acids decrease inflammatory responses, saturated fatty acid-induced cytotoxicity, and ROS production in various models of brain damage and neurodegenerative diseases (29). Moreover, total antioxidant capacity of cerebellum tissues of rats treated with 10 mg/kg TQ was significantly increased when compared with the control according to the present study's results. This implies that 10 mg/kg TQ improves cerebellar antioxidant capacity which is important for protection against the oxidative stress, accumulation of ROS in the brain and, as a result, formation of neurodegenerative diseases. In previous studies in the literature, it was reported that 15 mg/kg TQ treatment for 3 days increased heart and brain total antioxidant capacity in prilocaine (a local anaesthetic)-treated rats with a decrease in ROS formation in different parts of the brain including cerebellum and cerebral cortex (30). In the study of Alrafiah (1), rats were fed with HFD with the supplementation of 300 mg/kg for 4 weeks duration. It was reported that malondialdehyde as an indicator of lipid peroxidation was elevated in the HFD group while it was reduced when it was supported with TQ. The improvement in the levels of antioxidant enzymes with the supplementation of TQ was also reported in the same study (1). It was concluded that TQ treatment can reduce the neural damage with the inflammation induced by HFD by increasing the antioxidant enzyme levels in those rats. The other studies in the literature also reported neuroprotective and neuromodulatory effects of TQ between 2.5-10 mg/kg doses in the brain tissue (5, 31, 32).

According to the results of the current study, 10 mg/kg TQ treatment induces significant changes in the saturated and unsaturated lipid content with an increased total antioxidant capacity of cerebellum tissue while 20 mg/kg TQ treatment did not cause a significant change in those parameters.

Total protein content which was calculated in both FTIR study and Bradford assay, was significantly and slightly increased in the 10 mg/kg and 20 mg/kg TQ treatment groups compared to the control, respectively. This increase may be due to the induction of the levels of antioxidant enzymes and other proteins that have antioxidant property in the cerebellum tissue since increased total antioxidant capacity can arise from an increase into the system of any molecule with antioxidant properties. Increased levels of brain glutathione and superoxide dismutase with the treatment of TQ were reported in the study of Alrafiah (1).

The nucleic acid content of cerebellum tissue was affected in opposite ways in different doses of TQ treatment. There was a slight decrease in the 10 mg/kg TQ treatment group conversely with a slight increase in the 20 mg/kg TQ treatment rat group in comparison to the control. High levels of nucleic acids in the blood were reported in different states including ageing and age-related neurodegenerative disorders, inflammatory conditions, and autoimmune diseases. This high level of nucleic acids can interact with the misfolded proteins causing protein misfolding disorders (PMDs) e.g. Alzheimer's disease, prion diseases or Parkinson disease (33). Although the decrease in nucleic acid content of cerebellum in the 10 mg/kg TQ treatment rat group may be the part of the antioxidant system induced by TQ, both the increase and decrease of this content were not much to boast about.

According to the previous study, 20 mg/kg TQ can be accepted as a high dose for female rats when given by intraperitoneal injection (34). In the study of Abukhader, it was reported that males and females can tolerate 22.5 mg/kg and 15 mg/kg respectively (34). However, both can tolerate 250 mg/kg TQ when given by intragastric intubation because of the elimination of it in the digestive tract (34). Since 20 mg/kg TQ dose is a higher dose for female rats to be tolerated, the improvement effect of 10 mg/kg could not be seen in those rat groups. Besides that, it is not a toxic dose and there was no toxic effect on the cerebellum tissues of rats.

In conclusion, 10 mg/kg TQ treatment improves the saturated and unsaturated lipid, protein and nucleic acid content besides decreasing lipid peroxidation and increasing the total antioxidant capacity of cerebellum tissues. However, 20 mg/kg TQ treatment did not show any significant effect on the molecular composition or the antioxidant capacity of cerebellum tissues. Moreover, ATR-FTIR spectroscopy is a rapid, sensitive, non-invasive method to detect the molecular and compositional alterations in the tissues in disease states and/or supplementation as a part of traditional medicine.

Ethics Committee Approval: All the experimental processes of the study were confirmed by the Scientific Ethical Committee of Bezmi-

alem Vakif University (2016/317).

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REFERENCES

1. Alrafiah A. Thymoquinone protects neurons in the cerebellum of rats through mitigating oxidative stress and inflammation following high-fat diet supplementation. Biomolecules 2021; 11(2): 165. [CrossRef]
2. Farkhondeh T, Samarghandian S, Shahri AMP, Samini F. The neuroprotective effects of thymoquinone: A review. Dose Response 2018; 16: 1559325818761455. [CrossRef]
3. Pottoo FH, Ibrahim AM, Alammar A, Alsinan R, Aleid M, Alshehhi A, et al. Thymoquinone: review of its potential in the treatment of neurological diseases. Pharmaceuticals (Basel, Switzerland) 2022; 15(4): 408. [CrossRef]
4. Isaev NK, Chetverikov NS, Stelmashook EV, Genrikhs EE, Khaspekov LG, Illarioshkin SN. Thymoquinone as a potential neuroprotector in acute and chronic forms of cerebral pathology. Biochemistry. Biokhimiia 2020; 85(2): 167-76. [CrossRef]
5. Cascella M, Bimonte S, Barbieri A, Del Vecchio V, Muzio MR, Vitale A, et al. Dissecting the potential roles of nigella sativa and its constituent thymoquinone on the prevention and on the progression of Alzheimer's Disease. Front Aging Neurosci 2018; 10: 16. [CrossRef]
6. Samarghandian S, Farkhondeh T, Samini F. A review on possible therapeutic effect of *Nigella sativa* and thymoquinone in neurodegenerative diseases. CNS Neurol Disord Drug Targets 2018; 17: 412-20. [CrossRef]
7. Khazdair MR. The protective effects of *Nigella sativa* and its constituents on induced neurotoxicity. J Toxicol 2015; 841823. [CrossRef]
8. Solleiro-Villavicencio H, Rivas-Arancibia S. Effect of chronic oxidative stress on neuroinflammatory response mediated by $CD4^{+}T$ cells in neurodegenerative diseases. Front Cell Neurosci 2018; 12: 114. [CrossRef]
9. Azizi SA. Role of the cerebellum in the phenotype of neurodegenerative diseases: Mitigate or exacerbate? Neurosci Lett 2021; 760; 136105. [CrossRef]
10. Jacobs HIL, Hopkins DA, Mayrhofer HC, Bruner E, van Leeuwen FW, Raaijmakers W, et al. The cerebellum in Alzheimer's disease: evaluating its role in cognitive decline. Brain 2018; 141(1): 37-47. [CrossRef]
11. Wu T, Hallett M. The cerebellum in Parkinson's disease. Brain 2013; 136: 696-709. [CrossRef]
12. Ozawa T, Paviour D, Quinn NP, Josephs KA, Sangha H, Kilford L, et al. The spectrum of pathological involvement of the striatonigral and olivopontocerebellar systems in multiple system atrophy: clinicopathological correlations. Brain 2004; 127(Pt 12): 2657-71. [CrossRef]
13. Ismail N, Ismail M, Mazlan M, Latiff LA, Imam MU, Iqbal S, et al. Thymoquinone prevents β-amyloid neurotoxicity in primary cultured cerebellar granule neurons. Cell Mol Neurobiol 2013; 33(8): 1159-69. [CrossRef]
14. Rakib F, Al-Saad K, Ustaoglu SG, Ullah E, Mall R, Thompson R, et al. Fourier transform infrared imaging-a novel approach to monitor bio molecular changes in subacute mild traumatic brain injury. Brain Sci 2021; 11(7): 918. [CrossRef]
15. Ustaoglu SG, Ali M, Rakib F, Blezer E, Van Heijningen CL, Dijkhuizen RM, et al. Biomolecular changes and subsequent time-dependent recovery in hippocampal tissue after experimental mild traumatic brain injury. Sci Rep 2021; 11(1): 12468. [CrossRef]
16. Ustaoglu SG, Evis Z, Ilbay G, Boskey A, Severcan F. Side-effects of convulsive seizures and anti-seizure therapy on bone in a rat model of epilepsy. Appl Spectrosc 2018; 72(5): 689-705. [CrossRef]
17. Garip S, Sahin D, Severcan F. Epileptic seizures-induced structural and functional changes in rat femur and tibia bone tissues: An FTIR imaging Study. J Biomed Opt 2013; 18(11): 111409. [CrossRef]
18. Garip S, Severcan F. Determination of simvastatin-induced changes in bone composition and structure by Fourier transform infrared spectroscopy in rat animal model. J Pharm Biomed Anal 2010; 52(4): 580-8. [CrossRef]
19. Garip S, Bayari SH, Severcan M, Abbas S, Lednev IK, Severcan F. Structural effects of simvastatin on liver rat [corrected] tissue: Fourier transform infrared and Raman microspectroscopic studies. J Biomed Opt 2016; 21(2): 25008. [CrossRef]
20. Kucuk Baloglu F, Garip S, Heise S, Brockmann G, Severcan F. FTIR imaging of structural changes in visceral and subcutaneous adiposity and brown to white adipocyte transdifferentiation. The Analyst 2015; 140(7): 2205-14. [CrossRef]
21. Shao Y, Feng Y, Xie Y, Luo Q, Chen L, Li B, et al. Protective effects of thymoquinone against convulsant activity induced by lithium-pilocarpine in a model of status epilepticus. Neurochem Res 2016; 41(12): 3399-406. [CrossRef]
22. Aquib M, Najmi AK, Akhtar M. Antidepressant effect of thymoquinone in animal models of depression. Drug Res (Stuttg) 2015; 65(9): 490-4. [CrossRef]
23. Radad K, Hassanein K, Al-Shraim M, Moldzio R, Rausch WD. Thymoquinone ameliorates lead-induced brain damage in Sprague Dawley rats. Exp Toxicol Pathol 2014; 66(1): 13-7. [CrossRef]
24. Demir P, Akkas SB, Severcan M, Zorlu F, Severcan F. Ionizing radiation induces structural and functional damage on the molecules of rat brain homogenate membranes: a Fourier transform infrared (FT-IR) spectroscopic study. Appl Spectrosc 2015; 69(1): 154-64. [CrossRef]
25. Cakmak-Arslan G, Emir S, Goc-Rasgele P, Kekecoglu M. Investigation of the toxic effects of rhododendron honey on mouse cardiac muscle tissue lipids at molecular level. Kafkas Univ Vet Fak Derg 2020; 26(2): 287-94.
26. Yang W, Shi H, Zhang J., Shen Z, Zhou G, Hu M. Effects of the duration of hyperlipidemia on cerebral lipids, vessels and neurons in rats. Lipids Health Dis 2017; 16: 26. [CrossRef]
27. Vitali C, Wellington CL, Calabresi L. HDL and cholesterol handling in the brain. Cardiovasc Res 2014; 103(3): 405-13. [CrossRef]
28. Hottman DA, Chernick D, Cheng S, Wang Z, Li L. HDL and cognition in neurodegenerative disorders. Neurobiol Dis 2014; 72 Pt A: 22-36. [CrossRef]
29. Vesga-Jiménez DJ, Martin C, Barreto GE, Aristizábal-Pachón AF, Pinzón A, González J. Fatty acids: An insight into the pathogenesis of neurodegenerative diseases and therapeutic potential. Int J Mol Sci 2022; 23(5): 2577. [CrossRef]
30. Akgül B, Aycan İÖ, Hidişoğlu E, Afşar E, Yıldırım S, Tanrıöver G, et al. Alleviation of prilocaine-induced epileptiform activity

and cardiotoxicity by thymoquinone. Daru 2021; 29(1): 85-99. [CrossRef]
31. Mehri S, Shahi M, Razavi BM, Hassani FV, Hosseinzadeh H. Neuroprotective effect of thymoquinone in acrylamide-induced neurotoxicity in Wistar rats. Iran J Basic Med Sci 2014; 17(12): 1007-11.
32. Üstün N, Aras M, Özgür T, Bayraktar HS, Sefil F, Özden R, et al. Thymoquinone attenuates trauma induced spinal cord damage in an animal model. Ulus Travma Acil Cerrahi Derg 2014; 20(5): 328-32. [CrossRef]
33. Cordeiro Y, Macedo B, Silva JL, Gomes M. Pathological implications of nucleic acid interactions with proteins associated with neurodegenerative diseases. Biophys Rev 2014; 6(1): 97-110. [CrossRef]
34. Abukhader MM. The effect of route of administration in thymoquinone toxicity in male and female rats. Indian J Pharm Sci 2012; 74(3): 195-200. [CrossRef]

AIMS AND SCOPE

Experimed is an international, scientific, open access periodical published in accordance with independent, unbiased, and double-blinded peer-review principles. The journal is the official online-only publication of Istanbul University Aziz Sancar Institute of Experimental Medicine and it is published triannually on April, August, and December. As of 2022 the publication language of the journal is only English. The manuscripts submitted for publication in the journal must be scientific work in English.

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INSTRUCTIONS TO AUTHORS

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Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

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Table 1. Limitations for each manuscript type

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Review Article	5000	200	50	6	10 or total of 20 images
Case Report	1000	200	15	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	5	No tables	No media

dimensions: 100 × 100 mm). Figure legends should be listed at the end of the main document.

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When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: “Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)”

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Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

References

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Journal Article: Rankovic A, Rancic N, Jovanovic M, Ivanović M, Gajović O, Lazić Z, et al. Impact of imaging diagnostics on the budget – Are we spending too much? Vojnosanit Pregl 2013; 70: 709-11.

Book Section: Suh KN, Keystone JS. Malaria and babesiosis. Gorbach SL, Barlett JG, Blacklow NR, editors. Infectious Diseases. Philadelphia: Lippincott Williams; 2004.p.2290-308.

Books with a Single Author: Sweetman SC. Martindale the Complete Drug Reference. 34th ed. London: Pharmaceutical Press; 2005.

Editor(s) as Author: Huizing EH, de Groot JAM, editors. Functional reconstructive nasal surgery. Stuttgart-New York: Thieme; 2003.

Conference Proceedings: Bengisson S. Sothemin BG. Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics; 1992 Sept 6-10; Geneva, Switzerland. Amsterdam: North-Holland; 1992. pp.1561-5.

Scientific or Technical Report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, et al. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Early Treatment Diabetic Retinopathy Study Kidney Int: 2004. Report No: 26.

Thesis: Yılmaz B. Ankara Üniversitesindeki Öğrencilerin Beslenme Durumları, Fiziksel Aktiviteleri ve Beden Kitle İndeksleri Kan Lipidleri Arasındaki Ilişkiler. H.Ü. Sağlık Bilimleri Enstitüsü, Doktora Tezi. 2007.

Manuscripts Accepted for Publication, Not Published Yet: Slots J. The microflora of black stain on human primary teeth. Scand J Dent Res. 1974.

Epub Ahead of Print Articles: Cai L, Yeh BM, Westphalen AC, Roberts JP, Wang ZJ. Adult living donor liver imaging. Diagn Interv Radiol. 2016 Feb 24. doi: 10.5152/dir.2016.15323. [Epub ahead of print].

Manuscripts Published in Electronic Format: Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) 1995 Jan-Mar (cited 1996 June 5): 1(1): (24 screens). Available from: URL: http:/ www.cdc.gov/ncidodlEID/cid.htm.

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