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Adıyaman Üniversitesi Sağlık Bilimleri Dergisi
Journal of Health Sciences of Adıyaman University

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Research Article/Özgün Araştırma

In silico and in vitro evaluation of oxypeucedanin-induced anticancer activity: Mitotoxicity?

Oksipösedanin kaynaklı antikanser aktivitenin *in siliko* ve in vitro değerlendirilmesi: Mitotoksisite?

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Abstract

Aim: This study aims to evaluate the alterations in Oxypeucedanin (OXY)-mediated anticancer activity in different media. Second aim is to predict the affinity of OXY to electron transfer chain (ETC) complexes.

Materials and Methods: MTT and LDH leakage assays were performed with OXY. Molecular docking studies were also conducted to predict the affinity of OXY to ETC complexes.

Results: 250 µM OXY reduced viability in glucose media. ≥50 µM OXY decreased viability in galactose media. ≥50 µM OXY increased membrane disruption in galactose media. Molecular docking studies also showed that OXY might possess the capacity to bind to the inhibition sites of Complex I and IV.

Conclusion: Galactose-conditioned media exacerbated the OXY-mediated cytotoxicity. Preliminary results suggested that mitotoxicity might take part in anticancer activity. Furthermore, OXY might cause ETC dysfunctions due to selective inhibition of Complex I and IV.

Keywords: Oxypeucedanin; Mitotoxicity; Anticancer activity; *In silico*.

Öz

Amaç: Çalışmanın amacı, farklı ortamlarda Oksipösedanin (OKS) aracılı antikanser aktivitedeki değişiklikleri değerlendirmektir. İkinci amaç, OKS'inin elektron transfer zincirine (ETZ) karşı afinitesini öngörmektir.

Gereç ve Yöntem: MTT ve LDH sızma deneyleri OKS ile gerçekleştirilmiştir. Ayrıca, OKS'inin ETZ komplekslerine karşı afinitesini öngörmek için moleküler kenetlenme çalışmaları uygulanmıştır.

Bulgular: Glukoz içeren ortamda 250 µM OKS canlılığı azaltmıştır. Galaktoz içeren ortamda ≥50 µM OKS hücre canlılığını azalmıştır. Galaktoz içeren ortamda ≥50 µM OKS membran parçalanmasını artırmıştır. Moleküler kenetlenme çalışmaları, OKS'inin Kompleks I ve IV'ün inhibisyon bölgelerine bağlanma kapasitesine sahip olabileceğini göstermektedir.

Sonuç: Galaktoz içeren ortam, OKS aracılı sitotoksisiteyi artırmıştır. Ön sonuçlar, antikanser aktivitede mitotoksisitenin yer alabileceğini göstermektedir. Ayrıca OKS, Kompleks I ve IV'ün seçici inhibisyonu nedeni ile ETZ disfonksiyonuna neden olabilmektedir.

Anahtar Kelimeler: Oksipösedanin; Mitotoksisite; Antikanser aktivite; *İn siliko*.

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Introduction

Natural plants are sources of phytochemicals such as bioactive secondary metabolites. Pharmacologically active phytochemicals have been used since ancient times in order to treat various diseases with the advantages of effectiveness and low occurrence of adverse effects. A wide range of phytochemicals have been isolated from medicinal plants to suppress several diseases, including cancer progression and development.[1,2] OXY is a derivative of furanocoumarin extracted and isolated from *Angelica*, *Ferulago*, and *Prangos* species. Over 50% of OXY has been isolated and characterized from the roots.[3,4] OXY was reported to have anti-mutagenic, cytotoxic, and antiproliferative activities against several cancer cells, including colon, breast, liver, and lung cancers.[5-7]

Cancer cells need more significant biosynthetic components and building blocks, including amino acids and nucleotides, than normal cells due to their uncontrolled and highly proliferative cell division characteristics. Also, the expression of proapoptotic proteins is lower in cancer cells than in normal cells, which makes cancer cells more resistant to anti-cancer treatments and molecules.[8,9] Mitochondria are one of the most targeted organelles for cancer treatment in drug discovery and development processes, as the mitochondria clearly play a pivotal role in cancer cells in that they take part in tumor initiation and promotion, regulation of energy homeostasis, intrinsic apoptosis, and the synthesis of biomass and building blocks.[9,10] The primary purpose of anticancer treatment relies on killing of cancer cells. Investigating the role of mitotoxicity in phytochemical-mediated anti-cancer activity sheds light on novel pathways and molecules for cancer treatment.[9-11]

It is complicated for scientists to investigate mitotoxicity directly. *In vivo* models, including in-bred rodent models and transgenic mice, are not properly effective in reflecting the mechanism of mitotoxicity.[12-14] *In vitro* studies are more likely to reveal the mechanism of mitotoxicity compared to *in vivo* models. Standard *in vitro* models use high glucose-conditioned media for cancer cells to uncover the mechanisms of mitotoxicity. However, cancer cells produce more than 50% of their energy via glycolytic pathway apart from oxidative phosphorylation (OXPHOS) due to the Crabtree effect, which reduces their sensitivity to mitochondrial toxicants (mitotoxicants).[15,16] Marroquin et al. (2007) proposed a model for HepG2 cells by replacing glucose with galactose. This model allows cancer cells to use galactose inefficiently via glycolysis, blocking ATP generation in the cytosol, and forcing the cell to produce ATP via OXPHOS.[17] This model was adopted by many *in vitro* studies in order to figure out the mitotoxicity by using several cell types.[18-20]

Previous results showed that OXY caused selective inhibition in a wide range of human cancer cells.[7,21-23] Nevertheless, the mechanism of OXY-mediated mitotoxicity has yet to be precisely uncovered. Furthermore, previous studies, including *in vitro* assays, used standard glucose-conditioned media, which can not fully indicate mitotoxicity due to the Crabtree effect. Thus, observing alterations in OXY-mediated anticancer activity as well as to possible mitotoxicity by comparing both glucose, and galactose conditions matters to investigate. The present study aims to investigate two primary purposes: i) to figure out the alterations in OXY-mediated anticancer activity in HepG2 cells made vulnerable to mitotoxicants by using either glucose- or galactose-conditioned media. ii) to predict the possible affinity of OXY to the ETC, which takes part in the inner membrane of mitochondria as structural and functional components, using molecular docking studies.

Materials and Methods

Materials and chemical reagents

All chemicals were obtained from Sigma-Aldrich (Darmstadt, Germany) except for cell culture reagents. Cell culture reagents were purchased from Thermo-Fisher Scientific (Loughborough, UK).

Cell line and cell culture

HepG2 cells were purchased from American Type Culture Collection (ATTC,

HB-8065, USA). HepG2 cells were maintained under high glucose and galactose conditions as described previously.[17] The passage numbers for HepG2 cells were maintained between 7 and 15.

Isolation and characterization of oxypeucedanin

The OXY used in this study was obtained from previous study.[24] The compound was isolated from the roots of *Prangos heyniae* H.Duman & M.F. Watson, an endemic species in Türkiye. The roots were collected from Hadim/ Konya city of Türkiye in 2016. The air-dried and crushed roots were sequentially extracted with *n*-hexane, chloroform, and methanol in an ultrasonic water bath for 24 h. The extracts were filtrated and evaporated to dryness separately at 40°C under low pressure, yielding *n*-hexane (25g), chloroform (9g), and methanol (39g) extracts. Column chromatography was used for purification studies. After several chromatographic column studies with the chloroform extract, oxypeucedanin (100 mg), was isolated and identified using 1D NMR and MALDI-TOF-MS.[24] The compound was stored at 20°C as frozen form.

MTT assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to evaluate cell viability in HepG2 cells exposed to OXY in a dose-dependent manner in high glucose or galactose conditions as described in previous studies with minor modifications.[17,25] In brief, HepG2 cells (10^4 cells/well) exposed to OXY (6.25, 12.5, 25, 50, 100, and 250 µM) were incubated for 24 h at 37˚C with 5% CO_2. Final dimethyl sulfoxide (DMSO, solvent control) and Triton X-100 (positive control) concentrations were 1%. After treatment, MTT solution (5 mg/mL in phosphate buffer solution) was added to each well, and the well plate was incubated for 4 h. After incubation, formazan crystals were solubilized by using DMSO, and the color intensity was measured by a multi-plate reader. IC_{50} values were calculated as previously described in our study.[25]

LDH leakage assay

Lactate dehydrogenase (LDH) leakage assay was used to observe alterations in HepG2 cells exposed to OXY in a dose-dependent manner in high glucose or galactose conditions as described in previous studies with minor modifications.[17,26] In brief, HepG2 cells (10^4 cells/well) were exposed to OXY (6.25, 12.5, 25, 50, 100, and 250 µM) for 24 h at 37˚C with 5% CO_2. Final DMSO (solvent control) and Triton X-100 (positive control) concentrations were 1%. After treatment, LDH activity was determined by diluting media with pH 7.4 phosphate buffer (1:2) at 37 °C. Then, NADH (300 µM, final concentration) and sodium pyruvate (770 µM, final concentration) were added to the media. Absorbances of the media were measured by a multi-plate reader at 340 nm for 4 minutes as previously described in our study.[26] IC_{50} values were calculated as described in our previous study.[25]

Docking simulation

An *in silico* docking analysis of OXY with ETC complexes was carried out in this study using MOE 2020 (Molecular Operating Environment 2020). The structure of OXY was drawn in the ChemDraw 19.1 (Perkin Elmer Informatics) program, optimized by MOE, and subjected to energy minimization using the MMFF 94x (Merck Molecular Force Field) package program. The RCSB website (http://www.rcsb.org/pdb) was used to obtain ETC complex structures in PDB format [Complex I (PDB ID: 5XTD)[27], Complex II (PDB ID: 8GS8)[28], Complex III (PDB ID: 5XTE)[27], and Complex IV (PDB ID: 5Z62)[29]]. Since the human crystal structure of Complex V was not found, it was not used in this study. Crystal ligands and water molecules were removed from the enzyme complexes before docking. The surfaces of the complexes were scanned to identify the active sites of the enzymes. Hydrogen atoms and charges were added, while default values for other properties were used. The docking score was used to compare the capacity of affinity of OXY to ETC Complexes. Since a well-known inhibitor with organic structure of Complex IV was not found by molecular modeling study, Complex I inhibitor, Rotenone (ROT), was used as a

positive control[30] ROT was used as a reference molecule for molecular docking studies.

Statistics

The data were shown as the mean ± SD from three experiments (n:3). Data were analyzed by Mann-Whitney U test using GraphPad Prism version 8.4.2 for Windows. Statistical significance was accepted when $p \leq 0.05$.

Results

MTT assay demonstrated that lower than 250 µM concentrations did not cause any alterations in cell viability in high glucose-conditioned media (Figure 1). 250 µM OXY reduced cell viability to 79% compared to control. Predicted IC_{50} value for OXY-induced cytotoxicty in glucose conditioned media was 548 ± 16 µM (Table 1). In galactose-conditioned media, 6.25, 12.5, and 25 µM OXY did not cause any cytotoxicity, however 50 µM and higher concentrations of OXY decreased cell viability by 21, 39, and 55% compared to control (Figure 1). IC_{50} value for OXY-induced cytotoxicty in galactose conditioned media was 211 ± 8 µM (Table 1). 50, 100, and 250 µM OXY in galactose-conditioned media gave rise to significant decrease of cell viability compared to glucose-conditioned media (Figure 1). Triton X-100 used as a positive control reduced cell viability by 92 and 93% in high glucose and galactose-conditioned media, respectively (data not shown).

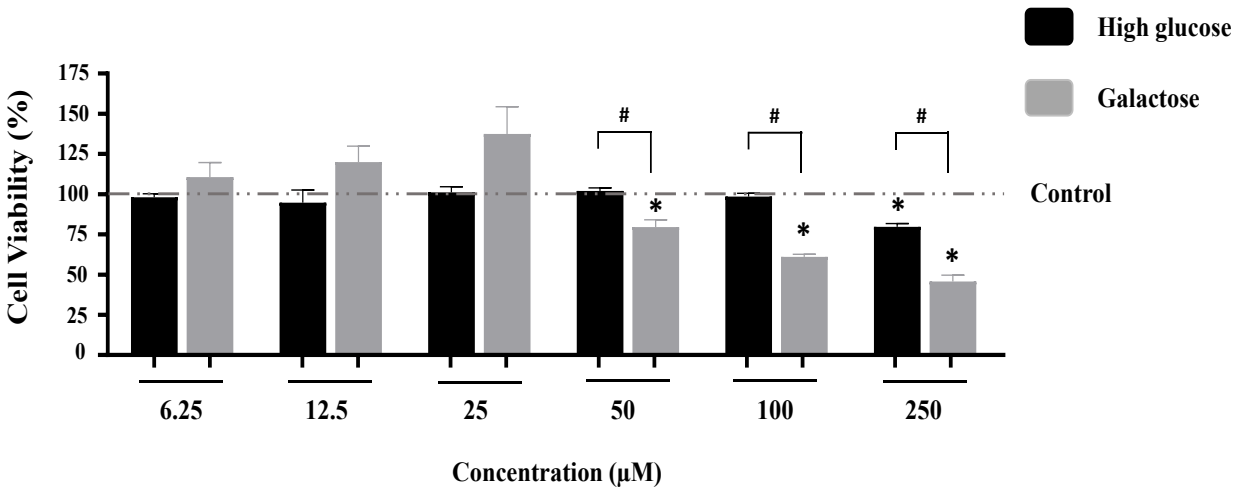

Figure 1. Cell viability in HepG2 cells exposed to OXY. MTT assay was performed in order to determine the cytotoxicity of HepG2 cells exposed to OXY in a dose-dependent manner in high glucose (black) or galactose (gray) conditioned media after 24 hours of incubation. Values are the mean ± SD from three independent experiments (n:3). The data were expressed as a percent of the solvent (1% DMSO) control. (*) significantly different ($p<0.05$) than the solvent control (1% DMSO)

Table 1. IC_{50} values (µM) ± SD of OXY against HepG2 cells cultured in glucose and galactose conditions for 24 h.

	IC50 (µM)	
Assay	**Glucose**	**Galactose**
MTT	548 ± 16#	211 ± 8*
LDH Leakage	744± 24#	227± 9*

IC_{50}: The concentrations (µM) that inhibited 50% of cell viability and increased 50% of LDH enzyme activity for MTT, and LDH leakage assays, respectively.
#: Predicted IC_{50} values.
*: IC_{50} value is significantly different ($p<0.05$) than glucose.

As shown in Figure 2, OXY did not cause any increase in LDH activity in high glucose-conditioned media. Predicted IC_{50} value for OXY-induced membrane damage in glucose conditioned media was 744 ± 24 µM (Table 1). In galactose-conditioned media, 6.25, 12.5, and 25 µM OXY did not increase the LDH activity (Figure 2). 50 µM and higher concentrations of OXY led to an increase in LDH activity and membrane disruption compared to control. IC_{50} value for OXY-induced membrane damage in galactose conditioned media was 227 ± 9 µM (Table 1). In addition, 50, 100, and 250 µM OXY increased membrane damage in galactose-conditioned media compared to glucose-conditioned media (Figure 2). Triton X-100 is a kind of detergent and is used as a positive control for membrane disruption. Triton X-100 increased membrane damage by 47 and 138% in high glucose and galactose-conditioned media, respectively (data not shown).

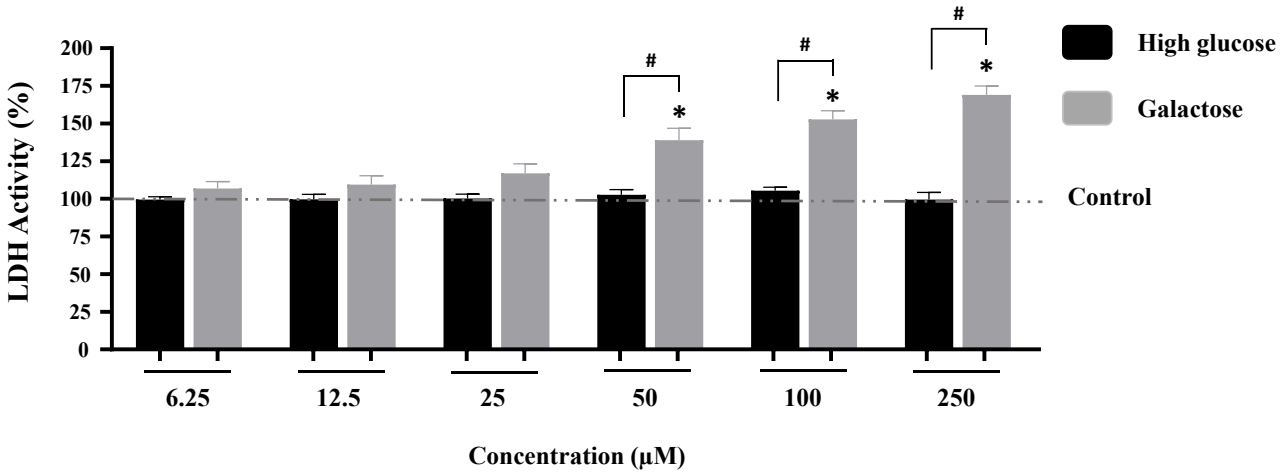

Figure 2. LDH activity resulting from membrane damage in HepG2 cells exposed to OXY. LDH leakage assay was performed in order to observe the membrane of HepG2 cells exposed to OXY in a dose-dependent manner in high glucose (black) or galactose (gray) conditioned media after 24 hours of incubation. Values are the mean ± SD from three independent experiments (n:3). The data were expressed as a percent of the solvent (1% DMSO) control. (*) significantly different ($p<0.05$) than the solvent control (1% DMSO).

In silico binding affinity of OXY with ETC complexes showed that docking scores for Complex I, Complex II, Complex III, and Complex IV ranged from - 6.46 to - 7.3 kcal/mol. OXY showed significant docking score with Complex IV (-7.3 kcal/mol, RMSD: 1.1646), Complex III (-6.94 kcal/mol, RMSD: 1.5761), Complex I (-6.79 kcal/mol, RMSD: 1.1327) and Complex II (-6.46 kcal/mol, RMSD: 1.6117) (Table 2). The highest docking score resulted from combination of OXY and Complex IV (-7.3 kcal/mol), indicating that it was properly positioned inside the Complex IV binding site. Table 2 and Figure 3 demonstrated that this enzyme possessed a greater affinity for OXY. The Aren (π)-H, Aren (π)- Aren (π), and H-Aren (π) interactions with the residues (Trp 126, Tyr 129, Trp 236, His 291, and Phe 377) led to the establishment of the maximum binding energy between OXY and Complex IV (Table 2 and Figure 3). Positive control ROT showed remarkable docking score with Complex I (-7.49 kcal/mol, RMSD: 0.9158) (data not shown).

Table 2. Docking result of OXY with the ETC complexes.

	Ligand = Oxypeucedanin			
Targets	Binding energy (kcal/mol)	RMSD values	Binding site amino acids	Interactions
Complex I (5XTD)	-6.79	1.1327	Phe 64, Gly 63, Asp 205	Aren (π)-H, H-bond acceptor, Ligand exposure
Complex II (8GS8)	-6.46	1.6117	Asn 81, Arg 512, Leu 513, Gln 516	Aren (π)-H, Aren (π)-cation, H-bond acceptor, H-bond donör, Ligand exposure
Complex III (5XTE)	-6.94	1.5761	Ala 84, Gly 130, Tyr 131	Aren (π)-H, Ligand exposure,
Complex IV (5Z62)	-7.3	1.1646	Trp 126, Tyr 129, Trp 236, His 291, Phe 377	Aren (π)-H, Aren (π)- Aren (π), H-Aren (π), Ligand exposure

Discussion

Mitochondria play a pivotal role in maintaining biomass synthesis including nucleotides, fatty acids, and amino acids, in highly proliferative cells such as cancer. Mitochondria also control programmed cell death or apoptosis. However, apoptosis is inhibited in cancer cells. Therefore, mitochondrial dysfunction is one of the most targeted mechanisms in the treatment of cancer.[9] In addition to synthetic drugs or chemicals, pharmacologically active phytochemicals are also used to lead to mitochondrial dysfunction and consequently mitotoxicity to destroy the cancer cells.[31,32]

Nevertheless, most studies estimated the Crabtree effect and used high glucose-conditioned media required for cancer cells to investigate the mitotoxicity.[7,21-23]

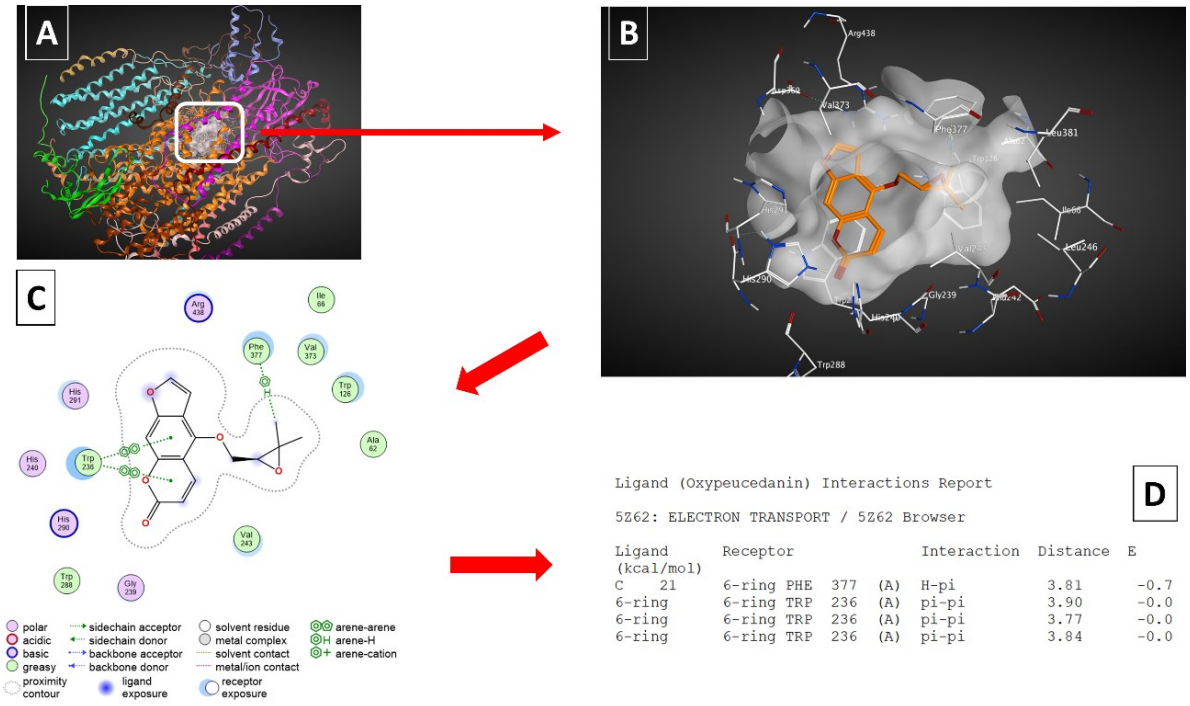

Figure 3. 2D binding pose of OXY with the human Complex IV [cytochrome c oxidase (PDB ID: 5Z62)] active site. Receptor cites (A), binding site amino acids (B), interactions (C), and ligand interaction report (D).

Several *in vitro* studies have been reported for isolation, and antiproliferative activities of OXY. Kim et al. (2007) isolated OXY from the root of *Angelica dahurica* and determined antitumor properties by using sulforhodamine B (SRB) assay in various cell lines. IC_{50} values for A549 (human lung carcinoma), SK-OV-3 (human ovarian cancer), SK-MEL-2 (human melanoma cancer), XF498 (human central nervous system) and HCT-15 (human colon adenocarcinoma) found as approximately 32, 68, 58, 57, and 12 µM, respectively.[22] In another study Mottaghipisheh et al. (2018) isolated the OXY and other furocoumarins from flower, leaves and stem of *Ducrosia anethifolia*. Furocoumarins were subjected to MTT assay for anticancer activities by using L5178Y mouse T-cell lymphoma cells (IC_{50}: 26 µM), ABCB1-expressing L5178Y cell line (IC_{50}: 29 µM).[23] Tavakoli et al. (2017) isolated a wide range of OXY and its analogs from the root of *Ferulago trifida* Boiss and antitumor potantial was also investigated using MTT assay. IC_{50} values for MDA-MB-231 (human breast adenocarcinoma), A-549, HT-29 (human colon adenocarcinoma), and MRC-5 (human fetal lung fibroblast) were reported as 1190, 800, 1280, and 1790 µM, respectively.[21] A recent study isolated OXY from the root of *Angelica dahurica* and evaluated anticancer activity by using SRB assay. This study indicated that OXY led to selective inhibiton towards SK-Hep-1 (human hepatic adenocarcinoma, IC_{50}: 32.4 µM) and HepG2 (IC_{50}: 43.8 µM) cells rather than MDA-MB-231 (IC_{50}: 50.8 µM), T47D (ductal carcinoma, IC_{50}: 95.5 µM), SNU-638 (gastric carcinoma, IC_{50}: 50.4 µM), A549 (IC_{50}: 46.3 µM).[7]

In addition to anticancer and antiproliferative activities, OXY was also found to have protective effects towards drug-induced cytotoxicity. OXY isolated from the root of *Angelica dahurica* reversed Tacrin-mediated cytotoxicity in HepG2 cells (EC_{50}: 286 µM).[5] Another study revealed that 10 µM OXY alleviated Sunitinib induced apoptosis.[33] 280 µM OXY was also suggested to inhibit doxorubicin-induced apoptosis in PC12 (rat adrenal pheochromocytoma) cells. In same study, MTT assay displayed that 350 µM, the highest dose, OXY did not cause any cytotoxicity in PC12 cells [34]

Even though OXY was reported to display anticancer, antiproliferative, and protective

activities, limited mechanisms have been proposed to uncover the mechanism of cytotoxicity and mitotoxicity in OXY-mediated anticancer activity in hepatoma cells. Park et al. (2020) reported that OXY-mediated anticancer activity might be result from induction of cell cycle arrest and p53-mediated signaling.[7] However, there is no study applied in galactose-conditioned media. For this reason, experiments must also be performed in galactose-conditioned media to make cells more sensitive to mitotoxicity as well as to high glucose-conditioned media. Hence, we first planned to investigate and compare the alterations of anticancer and cytotoxic activities of OXY in HepG2 cells by utilizing frequently used end-point assays (MTT and LDH leakage) in glucose and galactose-conditioned media; second, molecular docking studies were performed to predict the possible affinity for OXY in ETC Complexes. MTT and LDH leakage assays displayed that galactose-conditioned media altered response of HepG2 cells exposed to OXY. 50, 100, and 250 µM OXY in galactose-conditioned media gave rise to significant decrease of cell viability, and increase of membrane disruption compared to glucose-conditioned media (Figure 1 and 2). These preliminary data propose that anticancer activity of OXY might depend on mitotoxicity in HepG2 cells.

ETC (Complex I-V) is a functional and structural components in mitochondria. In addition to the production of energy and membrane potential, ETC also maintains the synthesis of enzymes and intermediates including aspartase and pyrimidine, in highly proliferative cells such as cancer. Therefore, ETC inhibition is one of the most commonly used mechanisms in mitotoxicity to reduce cancer cell proliferation and growth.[35] Since no molecular modeling study indicating the possible interactions with OXY and ETC complexes existed, molecular docking study was also applied to predict the affinity of OXY to ETC complexes. Although OXY showed high affinity for the inhibition site of Complex IV (Table 2 and Figure 3), it was also found that RMSD values for Complex I (RMSD: 1.1327) and IV (RMSD: 1.1646) were close for OXY. Furthermore, OXY (-6.79 kcal/mol, RMSD: 1.1327) showed close activity to ROT (-7.49 kcal/mol, RMSD: 0.9158) for Complex I thanks to high score and low RMSD value. This data might suggest that OXY have a potential for Complex I and Complex IV inhibition. Consequently, inhibition of Complex I and IV by OXY might result in collapse of proton gradient and energy production.[36] This data need to be supported with enzymatic assays to claim that OXY is a Complex I and IV inhibitor.

Conclusion

Our study showed that significant alterations in OXY-mediated anticancer activity were observed in glucose, and galactose-conditions. Our preliminary data suggest that mitotoxicity might take part in OXY-mediated anticancer activity. Also, *in silico* studies supported our hypothesis. Molecular docking studies proposed that OXY might show high affinity to complex I and IV, and OXY might be a potential candidate for Complex inhibition. Further studies including oxygene consumption assay, measurement of cellular and mitochondrial energy status, membrane potential, complex activity assay require to make certain of the role of mitotoxicity in OXY-mediated anticancer activity in glucose and galactose conditioned media.

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Ethics Committee Approval

There was no data obtained from animal or human experiments for this article.

Informed Consent

The consents were obtained from all of the authors for this article.

Author Contributions

All of the authors contributed at every stage of the study.

Conflict of Interest

The authors declare that there is no conflict of interest for this article.

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Research Article/Özgün Araştırma

Preliminary *in vitro* assessment of cytotoxic and genotoxic effects of avocado (*Persea Americano*) oil in breast cancer cell line (MCF-7)

Avokado (*Persea Americano*) yağının meme kanseri hücre hattı (MCF-7) üzerindeki sitotoksik ve genotoksik etkilerinin *in vitro* ön değerlendirmesi

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Abstract

Aim: Toxicological evaluation is required to understand the safety of avocado (*Persea Americana*) oil for use as a food supplement. In this study, cytotoxic and genotoxic effects of avocado oil in MCF-7 cell line were evaluated.

Materials and Methods: In this study, the MCF-7 was exposed to avocado oil (1, 10, 25 and 100 ppm) for 24, 48 and 72 hrs to assess the cytotoxic and genotoxic effects.

Results: IC_{50} of avocado oil were found to be 68.1, 62.8 and 64.3 ppm for 24, 48 and 72 hrs, respectively. There was a statistically significant decrease in cell polferation between the control and exposed groups (p<0.05). Micronucleus frequency was significantly increased compared with negative control (p<0.005).

Conclusion: Results of the study, avocado oil had cytotoxic and genotoxic effects in a time and concentration dependent manner. Regular use of avocado oil as a dietary supplement has been shown to have a protective effect.

Keywords: Avocado oil, Cytotoxicity, Genotoxicity, MCF-7, Cytokinesis-block micronucleus assay.

Öz

Amaç: Avokado (*Persea Americana)* yağının gıda takviyesi olarak kullanımında güvenliğinin anlaşılması için toksikolojik değerlendirilme yapılması gerekmektedir. Planlanan bu çalışmada avokado yağının MCF-7 hücre hattındaki sitotoksik ve genotoksik etkileri değerlendirilmiştir.

Gereç ve Yöntem: Bu çalışmada MCF-7 hücre hattı, avokado yağına (1, 10, 25 ve 100 ppm) ile 24, 48 ve 72 maruz bırakılarak sitotoksik ve genotoksik etkisi değerlendirilmiştir.

Bulgular: Avokado yağının IC_{50} değerleri 24, 48 ve 72 saat için sırasıyla; 68.1, 62.8 ve 64.3 ppm olarak bulunmuştur. Avokado yağının bütün maruziyet sürelerinde kontrol grubu ile maruziyet grupları arasında hücre poliferasyonundaki azalma istatiksel olarak anlamlı bulunmuştur (p<0,05). Avokado yağına maruziyetine bağlı mikroçekirdek frekansında, tüm dozlarda negatif kontrole göre önemli artış görülmüştür (p<0,005).

Sonuç: Çalışmanın sonucunda avokado yağının MCF-7 hücre hattında zamana ve konstrasyona bağımlı olarak sitotoksik ve genotoksik etkilerinin olduğu görülmüştür. Avokado yağının gıda takviyesi olarak düzenli kullanılması sonucunda koruyucu etkisinin olabileceği görülmüştür.

Anahtar Kelimeler: Avokado yağı, Sitotoksisite, Genotoksisite, MCF-7, Sitokinezin durdurulduğu mikroçekirdek yöntemi.

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Introduction

Avocado (*Persea Americana, Mill*) is a fruit that grows in temperate and subtropical regions worldwide, including Turkey. This fruit's pulp involves approximately 60% oil, 7% peel, and 2% seed. Avocado is a nutritious source of protein, fiber, vitamins (A, E, and C), and other critical compounds (potassium, magnesium minerals and carotenoids, phenolics, phytosterols, terpenoids which are known to exert antioxidant actions)[1,3] Many African countries use *Persea americana* fruit, leaves, and seeds in traditional medicine.[2]

Multiple studies of avocado seeds have been reported such as antioxidant, antihypertensive, larvicidal, fungicidal, bactericidal hypolipidemic, and recently amoebicidal and giardicidal activities.[3-12] Avocado oil have also been shown to effectively treat symptomatic osteoarthritis,[13] periodontal illnesses,[14] and skin problems.[15,16] Avocado is employed in various industries, including food, cosmetics, and medicine. Typically, pulp or avocado oil is utilized for these reasons. Avocado oil is used to marinate salads, sauces, and meat preparations. Compared to olive oil, the usage of cold-pressed avocado oil in cooking is relatively recent.[17]

Breast cancer is the most frequent cancer in women, accounting for 18% of all malignancies [18]. Breast cancer is difficult to cure because there are different classes of tumors that respond differently to treatment.[18-19]

Foods, in addition to medications, play an essential part in cancer treatment. Avocado-based food production and consumption are expanding globally due to rising studies on avocados' nutritional advantages and health benefits.[18-20] Raises questions about using avocado to supplement other foods for cancer treatment.[20]

This study examines the cytotoxic and genotoxic profile of avocado oil, which is widely used today on MCF-7 cells. While the cytotoxic effects of avocado oil in the MCF-7 cell line were evaluated using the xCELLigence system, the genotoxic effect of avocado oil was evaluated using *in vitro* the cytokinesis-block micronucleus (CBMN).

Materials and Methods

Materials and chemical reagents

The chemicals used as follows: A brand of avocado oil sold as a food supplement was commercially available obtained from a local shop; dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium, ethanol, fetal bovine serum (FBS), hydrogen peroxide (35%) (H_2O_2), Giemsa stain, trypsin-EDTA, RPMI 1640 medium, Dulbecco's phosphate buffered saline (PBS) from Sigma (St. Louis, MO, USA); Millipore filters from Millipore (Billerica, MA, USA).

Cell culture

Human breast adenocarcinoma cell line MCF-7 (HTB-22) was obtained from ATCC MCF-7 cells were raised in RPMI-1640 medium with 10% heat-inactivated FBS, 1% penicillin-streptomycin solution (10,000 units penicillin and 10 mg streptomycin in 0.9% NaCl), and 2 mM L-glutamine. The medium was replaced every 2-3 days. The xCELLigence system was used to assess the cytotoxicity of avocado oil in the MCF-7 cell line. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Avocado oil cytotoxicity on MCF-7 via xCELLigence

The xCELLigence manufacturer's instructions was followed for cytoxicity analyses and MCF-7 cell line was seeded reaching the cell number as 1×10^4 cells/well on 16-well plates. Subsequently, cell growth was then observed at a fifteen-minute interval and analysed using RTCA Software 1.2. After 24 hours of transplanted, cells in the 'logarithmic development phase' were treated to varied concentrations of avocado oil (1, 10, 25, and 100 ppm) and examined in real-time for 24, 48, and 72 hours. For positive control 20 mM H_2O_2 was used. As a negative control, untreated cells grown in growth medium were used.

All samples were administered in quadruplicate and all processes were carried out in the dark to avoid additional light-induced cellular damage. Using absorbance-

concentration curve, the 50% inhibitory concentration (IC_{50}) was determined. After the values of IC_{50} were determined, the genotoxic profiles of Avocado oil on the MCF-7 were evaluated for 24,48 and 72 hrs.

Avocado oil genotoxicity on MCF-7 via cytokinesis-block micronucleus assay (CBMN)

In vitro Mammalian Cell Micronucleus Test (OECD Test 487) was performed with minor modifications.[21] MCF-7 cells were seeded at a density of 5×10^4 cells per well in a T-25 flask and exposed to (1, 10, 25, and 100 ppm) avocado oil for 24, 48, and 72 hrs. 3 µg/mL Cytochalasin B was added to inhibit cytoplasmic division in 38th hours. As previously described, 2000 binucleated cells for each sample were examined microscopically and evaluated toxicity by classifying cells according to the number of micronucleus (MN) compared to the negative control.[22,23]

Statistics

The Kolmogorov-Smirnov test was used to determine the normality of the data distribution. The means of the data were compared using the One-way variance analysis test, and the least significant difference test was used for post hoc analysis of group differences. The results were displayed as the mean and standard deviation from three experiments in triplicate. GraphPad Prism Software version 5.0.1 (San Diego, CA, USA) for Windows was used for statistical analyses. A *p*-value of under 0.05 was determined to be statistically significant.

Results

The avocado oil used in the study was purchased from a national producer and the company's analytical characterisation values were accepted. The characterisation values obtained are shown in Table 1.

The xCELLigence technique analyzes the net adhesion of cells on a specifically designed gold electrode as the impedance of electricity fluctuates to quantify cellular growth in real-time. As a result, it provides better pre-sized data regarding the viability over the long term for cell screening that minimizes erroneous responses caused by material-dye interactions.[24] For the cytotoxicity study, the xCELLigence method was preferred. Unlike MTT, this approach provides more information regarding the long-term viability of cell assessment and avoids incorrect responses based on material-dye interaction.[24]

Table 1. Avocado oil content components.

Components	%
Myristic Acid	0.01
Palmitic Acid	18.33
Palmitoleic Acid	9.27
Steraic Acid	0.61
Oleic Acid	56.89
Linoleic Acid	13.16
Linolenic Acid	0.86
Eicosanoic Acid	0.08
11- Eicosanoic Acid	0.16

This study evaluates the cytotoxic response of the MCF-7 cell line to avocado oil application. The time-dependent graph of proliferation curves acquired from the real-time cell analyser was displayed. The device's software digitized the collected data according to the definition of the cell value. According to these graphic drawings, by looking at the r^2 values, a cell index (IC_{50}) was found for avocado oil. IC_{50} value correlates with the viability of cells. The results were obtained by taking logarithms of all administered dose groups at 72 hrs and plotting a graph against cell index values. IC_{50} values; 68.1 for 24 hrs; 62.8 ppm for 48 hrs and 64.3 ppm for 72 hrs.

The doses in all 24, 48, and 72 hours of incubation were statistically significant compared to the negative control(p>0.05). After 48 and 72 hours of incubation, the doses were statistically significant within themselves, while no significant difference was observed between 25 ppm and 50 ppm in 24 hours of exposure. Furthermore, the viability of cells decreases with duration in all exposure durations. Figure 1 shows the standardized cell index of MCF-7 cells treated with varied doses of avocado oil (1, 25, 50, 100 ppm) in contrast to medium as the negative control.

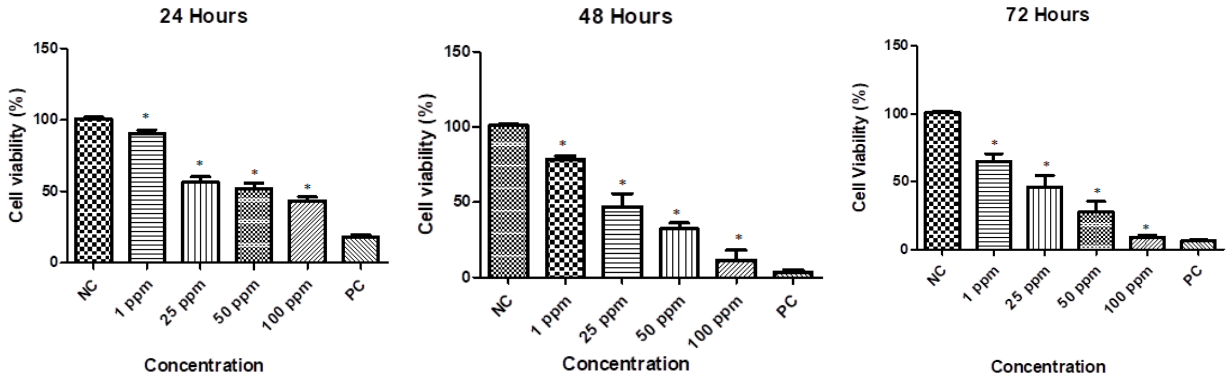

Figure 1. Effects of avocado oil on the cell viability of MCF-7 cells for 24 h, 48 h and 72 h. *Significant difference as compared to the negative control (p<0.05).

Avocado oil significantly increased the frequency of MN regardless of doses to the negative control after 24, 48, and 72 hours of exposure (p< 0.005).

When the MN frequencies were found at the most in the 48 hrs evaluated depending on time. In addition, the MN frequency with the avocado oil increased compared to the negative control, statistical difference was seen only at 100 ppm (p<0.005). According to doses, the MN frequency is evaluated, the MN increases with each exposure time depending on the concentration (Table 2).

Table 2. Changes in micronucleus frequencies in MCF-7 cell line treated with different concentrations of avocado oil depending on the exposure time 24, 48 and 72 h.

Groups		24 hours	48 hours	72 hours
	Concentration(ppm)	MN (Mean ± SD)	MN (Mean ± SD)	MN (Mean ± SD)
NC		3.83 ± 1.04	4.83 ± 1.04	4.67 ± 0.76
PC		18.50 ± 0.50*	17.50 ± 0.50*	18.00 ± 0.01*
Doses	**1**	7.83 ± 0.76	7.67 ± 0.29	7.67 ± 0.76
	25	10.33 ± 0.58	11.67 ± 0.29	11.00 ± 0.50
	50	13.00 ± 0.50	13.50 ± 0.87	11.17 ± 0.29
	100	15.33 ± 0.58	18 ± 0.50*	14.33 ± 1.04

MN: Micronucleus. SD: Standart deviation.NC: Negative Control. PC: Positive Control. *Significant difference as compared to the negative control (p<0.05). Negative control (1% PBS). positive control (50 µM H_2O_2).

Discussion

This study investigates the cytotoxicity and genotoxicity profile of avocado oil in the MCF-7 cell line as a function of exposure and time.

Several biological activities of the avocado seed have been reported such as antioxidant, antihypertensive, larvicidal, fungicidal, hypolipidemic, and recently amoebicidal and giardicidal activities.[25-28] Treatment of MDA-MB-231 human breast cancer cells with a methanolic extract of avocado seed led to induction of apoptosis as measured by increased caspase-3, caspase-7, and poly(ADPribose) polymerase (PARP) cleavage and increased DNA laddering.[27] Abubakar, Achmadi, & Suparto (2017) isolated a triterpenoid fraction from an ethanolic extract of avocado seeds and studied its cytotoxic effects in MCF-7 breast cells.[28] They found that the triterpenoid fraction and the whole extract had IC_{50} values of 80.1 μg/mL and 99.7 μg/mL, respectively. Kristanty, Suriawati, & Sulistiyo (2014) found that the cytotoxicity of aqueous and ethanolic extract of avocado seeds inhibited T47D breast cancer cell line with IC_{50} values of 560.2 μg/mL and 107.2 μg/mL, respectively.[29]

Our study investigated the cytotoxicity of avocado oil and according to our results, our IC_{50} values were found 68.1 for 24 hrs, 62.8 ppm for 48 hrs, and 64.3 ppm for 72 hrs in the MCF-7 cell line, respectively. Additionally, several studies have focused on the evaluation of acute toxicity of the fruit and leaves.[29] Avocado leaves showed cardiotoxic effects in mammals and birds.[30-33] Queiroz Junior et. al., (2021) found that avocado extract and oil in the presence of rotenone increased cellular viability at all tested concentrations compared to cells exposed only to rotenone. In addition,

extract and avocado oil exhibited antioxidant action as evidenced by decreased levels of reactive oxygen species (ROS), superoxide ion, and lipid peroxidation, generated by rotenone.[34]

Kulkarni et al.[35] showed that the extracts of both avocado fruit and leaves can potentially cause genomic instability and some genetic damage *in vitro* human lymphocytes. Padilla-Camberos et al.[36] showed that the genotoxic potential of an ethanolic seed extract of *Persea americana* in rats using a MN test. There were no differences in the incidence of micronuclei in rodent groups given an avocado seed extract against the negative control.[36] This is the first study of avocado oil genotoxicity in the MCF-7 cell line using CBMN. According to our results, the MN frequency increases with each exposure time depending on the concentration against the negative control.

The cytokinesis-block micronucleus (CBMN) assay according to the OECD 487 guideline was the preferred method for measuring MN in MCF-7 cell line.[21] In addition to its reliability, the CBMN assay has evolved into one of the industry-standard cytogenetic techniques for genetic toxicity testing *in vivo* and *in vitro* research. MN is an effective genotoxic biomarker; [37,38] hence, the staining procedure with Giemsa dye assists in differentiating micronucleated cells. Examining micronucleus frequencies *in vitro* is one of the significant genotoxicity assays regulatory organizations suggest for product safety evaluation.[37]

According to toxicity data, many plants used as food or in traditional medicine contain cytotoxic, mutagenic, and genotoxic characteristics.[39,40] The results highlight the need to comprehend thetoxicological effects of substances that come into contact, either directly or indirectly, with humans.[40] Other cell lines and toxicity tests must be investigated to complete the toxicological evaluation of avocado oil.

Conclusion

The study results shows that avocado oil has both cytotoxic and genotoxic effects in MCF-7 cell line. Genotoxicity results was found statistically significant, especially for 100 ppm dose of avocado oil at 48th hour.

Recently, new alternative agents for treating and preventing breast cancer have been investigated. The focus has been on therapeutically effective foods based on the induction of apoptosis in cancer cells, especially those containing natural products or herbs. This study will provide a new perspective on the mechanisms between the use of avocado oil as a food supplement and breast cancer and new approaches to forming potential cancer drugs for managing breast cancer.

However, the study was only conducted in the MCF-7 cancer cell line. Avocado oil concentrations should also be conducted in healthy cell lines to better evaluate the results obtained and in order to verify our results, we believe it is important to conduct a safety study with *in vivo* experimental animals in further studies.

Ethics Committee Approval

In the current work, there was no animal or human experiments conducted.

Informed Consent

Informed consent forms were obtained from all participants.

Author Contributions

All of the authors contributed at every stage of the study.

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Conflict of Interest

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

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Statements

These results have not been presented anywhere previously.

Peer-review

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Research Article/Özgün Araştırma

Investigation of the germline *PALB2* variants in cancer patients using the next-generation sequencing in Türkiye

Türkiye'deki kanser hastalarında kalıtsal *PALB2* gen varyantlarının yeni nesil dizileme yöntemiyle araştırılması

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Abstract

Aim: The study aimed to investigate germline *PALB2* gene variants in 1056 cancer patients in Türkiye, selected based on the National Comprehensive Cancer Network guidelines for genetic/familial high-risk assessment related to breast, ovarian, and pancreatic cancer.

Materials and Methods: The next-generation sequencing analysis of genomic DNA was performed using a Sophia Hereditary Cancer Solutions Panel for *PALB2* gene mutation screening.

Results: The *PALB2* genetic variants were detected in 48 patients, including 20 patients with pathogenic or likely pathogenic variants and 28 patients with variants of uncertain significance. The most common *PALB2* mutations were the frameshift mutations c.557dupA p.(Asn186Lysfs*4) and c.509_510del p.(Arg170Ilefs*14), found in 0.57% and 0.28% of patients, respectively.

Conclusion: The findings of the study emphasize the importance of *PALB2* gene analysis for breast cancer predisposition in Türkiye.

Keywords: *PALB2*, Germline mutations, Hereditary cancer risk factor.

Öz

Amaç: Çalışmada, meme, yumurtalık ve pankreas kanseri ile ilgili genetik/ailesel yüksek risk değerlendirmesi için Ulusal Kapsamlı Kanser Ağı kılavuzlarına göre seçilen, Türkiye'deki 1056 kanser hastasında germline *PALB2* geni varyantlarının araştırılması amaçlandı.

Gereç ve Yöntem: *PALB2* geni mutasyon taraması için Sophia Kalıtsal Kanser Çözümleri Paneli kullanılarak genomik DNA'nın yeni nesil dizileme analizi gerçekleştirildi.

Bulgular: *PALB2* genetik varyantları, 20 hastada patojenik veya muhtemel patojenik varyant ve 28 hastada belirsiz öneme sahip varyantlara sahip olmak üzere toplam 48 hastada tespit edildi. En yaygın *PALB2* mutasyonları, hastaların sırasıyla %0,57 ve %0,28'inde bulunan c.557dupA p.(Asn186Lysfs*4) ve c.509_510del p.(Arg170Ilefs*14) çerçeve kayması mutasyonlarıydı.

Sonuç: Araştırma bulguları, Türkiye'de meme kanseri yatkınlığı açısından *PALB2* gen analizinin önemini vurgulamaktadır.

Anahtar Kelimeler: *PALB2*; Germline mutasyonlar; Kalıtsal kanser risk faktörü.

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Introduction

Repairing of the DNA double-strand breaks (DSBs) by homologous recombination (HR) prevents cancer development. Hereditary pathogenic variants (PV) and likely pathogenic variants (LPV) of *BRCA1* and *BRCA2* are the major genetic causes of increased risk of breast, ovarian, and pancreatic cancers[1]. Genetic mutations in these genes are responsible for 20% of the inherited breast cancer[2]. *ATM, CHEK2,* and *PALB2* are involved in DNA damage response (DDR) which causes hereditary breast and ovarian cancer (HBOC)[2].

The *PALB2*, has 1186 amino acids, including a core coiled-coil motif and amino-terminal WD40 repeats[3], and is known as a BRCA-interacting protein[4]. It acts as a scaffold in forming the 'BRCA complex' involved in homologous recombination repair[5]. Cells with defective *BRCA1-PALB2* interaction display impaired homologous recombination[5]. Impaired homologous recombination repair causes genomic instability and carcinogenesis in *BRCA1, BRCA2,* and *PALB2* mutation carriers.

Biallelic mutations in *PALB2*, similar to *BRCA2*, are associated with Fanconi anemia[6], whereas monoallelic truncating mutations increase the risk of developing pancreatic, breast, and ovarian cancer[7]. Early research has indicated that individuals having pathogenic germline variants in the *PALB2* gene are at higher risk for breast cancer, with estimated penetrance up to 70% based on family history and diagnosis age[8,9]. Also, germline pathogenic variants (PVs) in *PALB2* have been detected in individuals with ovarian and pancreatic cancer[10,11].

The germline PV/LPV spectrum of the *PALB2* gene may differ among various global regions due to variations in ethnicity, lifestyle, and reproductive behaviors. These differences have sparked our curiosity to thoroughly comprehend the occurrence and diversity of *PALB2* gene variants within the Turkish cancer cohort. However, the range of *PALB2* mutations in Türkiye is still poorly understood. Therefore, in the present study, we aimed to investigate the PV/LPV and the variant of unsignificance (VUS) in *PALB2* genes in Turkish cancer patients which were selected based on the inclusion criteria established in the NCCN Guidelines for Genetic/Familial High-Risk Assessment concerning breast, ovarian, and pancreatic cancer[12]. The discovery of the recurrent *PALB2* PV/LPV and VUS may improve our understanding of their role in various cancer risks. This data can be used to develop optimal prevention and treatment strategies for *PALB2* mutation carriers in Türkiye.

Materials and Methods

Selection/Description of the patients

The Clinical Research Ethics Committee of Istanbul University authorized the current research on 17.03.2023 with the approval number 2023/500 following the Declaration of Helsinki[13]. The pathology report evaluated for tumor parameters such as diagnosis, receptor status, and histological grades. Before the study, all patients signed an informed written consent form. The study included 1056 cancer patients and was presented by the Department of Cancer Genetics at Istanbul University, Türkiye. The NCCN Guidelines Genetic/Familial High-Risk Assessment: Breast, Ovarian, and Pancreatic[12] were used as inclusion criteria in the study.

Technical information

PALB2 mutation screening

The blood samples were first processed using the Ficoll (Sigma-Aldrich, Darmstadt, Germany) procedures for lymphocyte isolation. The DNA of lymphocyte pellets was assessed using the QIAamp DNA micro kit (Qiagen, Hilden, Germany) in accordance with the kit protocol. The DNA concentration was assessed using the NanoDrop 2000c Spectrophotometer (NanoDropT, DE, USA). Illumina's MiSeq® platform (Illumina, Ca, USA) was used to screen all coding exons of the *PALB2* gene to summarize the patterns of genetic variations and frequencies of the gene, and the Sophia Genetics DDM analysis (Illumina, CA, USA) was used. For library construction, the Sophia Hereditary Cancer Solutions 59 gene (Sophia Genetics, Boston, USA) kit was used in accordance with the

manufacturer's instructions. The next-generation sequencing (NGS) technique was applied via the MiSeq platform by Illumina.

The next generation sequencing

Illumina's MiSeq® platform (Illumina, Ca, USA) was used to screen all coding exons of the *PALB2* gene to summarize the patterns of genetic variations and frequencies of the gene and Sophia Genetics DDM analysis (Sophia Genetics, Boston, USA). During the research, the NGS pipeline utilized the Illumina MiSeq platform and Sophia Genetics DDM analysis, both of which were previously established methods (Illumina, San Diego, CA, USA)[14].

Sequencing

DNA libraries were prepared and subjected to NGS during the study using the Illumina MiSeq platform (San Diego, California, USA). The Illumina MiSeq Reagent Kit v3 (600-cycle) was used for the sequencing. For library construction, the Sophia Hereditary Cancer Solutions 59 gene (Sophia Genetics, Boston, USA) kit was used following the manufacturer's instructions. The DNA was denatured and diluted with 0.2 N NaOH at a concentration of 2 nM. The library was then further diluted to a final concentration of 10 pM using a Prechilled HT1 buffer. Additionally, 6% of PhiX Control v3 (Ilumina, San Diego, CA, USA) was added to create a spiked library.

Genetic analysis

The genetic analysis was performed using the Sophia DDM analysis program. For variant calling and alignment of sequences to the reference genome (GRCh37/hg19), the Sophia Genomic Alignment and Variant Calling software was utilized. Specifically, Sophia DDM software (Sophia Genetics, Ecublens, Switzerland) was employed for independent read alignment and variant calling. The variant call files generated were further analyzed and filtered using VariantStudio software by Illumina and Sophia DDM software.

Genome interpretation using in silico predictors

The web-based algorithms were employed to assess the potential impact of identified nonsynonymous *PALB2* germline variants on protein function. These algorithms included the databases such as dbSNP[15], G1000[16], GnomAD[17], SIFT[18], POLYPHEN2[19], MUTATION TASTER[20], ClinVar[21], and HGMD[22].

Variant classification

The classification of variants involved an assessment of findings from the ClinVar[21] and HGMD[22] databases, alongside adherence to the sequencing/sequence variants classification guidelines set forth by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP)[23]". In the ACMG/AMP guidelines, the only criterion designated with very strong strength level for pathogenicity is defined as "null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss-of-function (LoF) is a known mechanism of disease"[23]. In this study, the identified causal variants were categorized into three groups: variant of unsignificance, likely pathogenic, and pathogenic based on the ACMG criteria. The missense mutations obtained in the study were suggested to have disease-causing effects based on in silico analysis programs. However, they were classified as VUS due to insufficient evidence supporting their disease-causing effects according to the ACMG criteria. Conducting functional studies on the detected VUS and evaluating their impact in terms of benign or pathogenicity will significantly enhance the accuracy of variant classification.

Clinicopathologic features

We evaluated the clinicopathologic characteristics by referencing the pathology reports in the patient's clinical records. These reports provided data on the clinical stage and histologic grade of cancer patients.

Results

NGS analysis

In the present study, *PALB2* variant analysis was conducted among *BRCA1/2* non-mutant 1056 patients who presented to our clinic for a genetic testing (828 breast cancer patients, 97 ovarian cancer patients, 19 endometrial cancer

patients, 26 pancreatic cancer patients, 56 colon cancer patients and 30 prostate cancer patients).

Among the investigated patients, PV/LPV or VUS were detected in the *PALB2* gene in forty-one breast cancer patients (41/828), four ovarian cancer patients (4/97), one endometrial cancer patient (1/19), one pancreatic cancer patient (1/26), one prostate cancer patient (1/30).

The causal variants found in the study were classified following the variant classification guidelines determined by the ACMG. In total, 20 patients (20/1056; ~1.9%) had at least one PV/LPV variant (13 different mutations) (Table 1), and 28 patients (28/1056; ~2.7%) had a VUS (23 different variants) (Table 2). In terms of *PALB2* VUS, we found 28 patients with 23 different *PALB2* VUSs.

Table 1. Pathogenic and likely pathogenic *PALB2* variants and their risk assessment among cancer patients in Türkiye in this study **(***PALB2* / TRANSCRIPT: NM_024675.3 / REFERENCE GENOM: GRCh37/hg19 Chromosome:16)

Nucleotide substitution	Amino acid change	Impact	dbSNP number	GnomAD Freq.	POLYPHEN2	SIFT	Mut. Tast.	ClinVar Clinical Sig.	HGMD
c.1692_1698 dup	p.(His567Lysfs*13)	frameshift	N/A	N/A	N/A	N/A	N/A	No Data	Not reported
c.1704_1707 delAAAA	p.(Lys569Argfs*29)	frameshift	rs1060502759	N/A	N/A	N/A	N/A	pathogenic	Disease causing mutation Breast cancer risk
c.172_175 delTTGT	p.(Gln60Argfs*7)	frameshift	rs180177143	0.000036	N/A	N/A	N/A	pathogenic	Disease causing mutation Pancreatic cancer risk
c.1960_1961 insC	p.(Ile654Thrfs*9)	frameshift	N/A	N/A	N/A	N/A	N/A	No Data	Not reported
c.1967dupC	p.(Glu657Argfs*6)	frameshift	N/A	N/A	N/A	N/A	N/A	No Data	Not reported
c.211+1G>T	p.(?)	splice_donor +1	rs1555462026	N/A	N/A	N/A	1.0	likely pathogenic	Disease causing mutation Breast cancer risk
c.2368C>T	p.(Gln790*)	nonsense	rs886039480	N/A	N/A	N/A	1.0	pathogenic	Not reported
c.2587-1G>C	p.(?)	splice_acceptor-1	rs761214886	0.000004	N/A	N/A	1.0	likely pathogenic	Disease causing mutation Breast and/or ovarian cancer risk
c.3256 C>T	p.(Arg1086*)	nonsense	rs587776527	0.00002	N/A	N/A	1.0	pathogenic	Disease causing mutation? Pancreatic cancer risk
c.390_391insT	p.(Arg131*)	nonsense	N/A	N/A	N/A	N/A	N/A	likely pathogenic	Disease causing mutation Breast cancer risk
c.481_482del	p.(Asp161Leufs*6)	frameshift	rs1597099149	N/A	N/A	N/A	N/A	pathogenic	Disease causing mutation Breast and/or ovarian cancer risk
c.509_510del	p.(Arg170Ilefs*14)	frameshift	rs515726123	0.000014	N/A	N/A	N/A	pathogenic	Disease causing mutation Breast Cancer Risk
c.557dupA	p.(Asn186Lysfs*4)	frameshift	rs1555461727	N/A	N/A	N/A	N/A	pathogenic	Disease causing mutation Breast Cancer risk

Freq: Frequency, Mut.Tast: Mutation Taster, Sig: Significance, HGMD: The Human Gene Mutation Database, N/A: Not Applicable

Table 2. *PALB2* variants of unsignificance (VUS) and their risk assessment among cancer patients in Türkiye in this study.

Nucleotide substitution	Amino acid change	Impact	dbSNP number	GnomAD Freq.	POLYPHEN2	SIFT	Mut. Tast.	ClinVar Clinical Sig.	HGMD
c.1001A>G	p.(Tyr334Cys)	missense	rs200620434	0.00006	0.03	0.83	0.0	uncertain sig.	Disease-causing mutation? Colorectal cancer suscept.
c.1163C>T	p.(Pro388Leu)	missense	rs1597096898	N/A	0.04	0.9	0.0	uncertain sig.	Not reported
c.121G>A	p.(Ala41Thr)	missense	N/A	N/A	1.0	1.0	0.76	No Data	Not reported
c.1298T>C	p.(Leu433Ser)	missense	rs1597096465	N/A	0.797	0.999	0.094	uncertain sig.	Not reported
c.13C>T	p.(Pro5Ser)	missense	rs377085677	0.00004	0.027	0.423	0.0	uncertain sig.	Disease-causing mutation? Breast cancer risk
c.1408A>G	p.(Thr470Ala)	missense	rs150636811	0.00001	0.006	0.551	0.0	uncertain sig.	Not reported
c.1448C>T	p.(Ser483Leu)	missense	rs1057520736	0.00001	0.999	0.883	0.004	uncertain sig.	Disease causing mutation? Cancer pred. syndrome
c.1867A>G	p.(Lys623Glu)	missense	rs1966864669	N/A	0.927	1.0	0.125	uncertain sig.	Not reported
c.194C>T	p.(Pro65Leu)	missense	rs62625272	0.00004	0.003	0.505	N/A	uncertain sig.	Disease causing mutation? Breast cancer risk
c.2113T>A	p.Tyr705Asn	missense	N/A	N/A	0.253	1.0	0.022	uncertain sig.	Not reported
c.2974A>C	p.(Met992Leu)	missense	rs1555459522	N/A	0.013	0.755	0.04	uncertain sig.	Not reported
c.307G>C	p.(Gly103Arg)	missense	N/A	N/A	0.053	0.973	0.0	No Data	Not reported
c.3073G>A	p.(Ala1025Thr)	missense	rs746872839	0.00001	0.403	0.953	0.999	uncertain sig.	Disease causing mutation? Cancer pred. syndrome
c.3122A>C	p.(Lys1041Thr)	missense	rs781663559	N/A	0.17	0.986	0.9954	uncertain sig.	Disease causing mutation? Cancer pred. syndrome
c.315G>C	p.(Glu105Asp)	missense	rs515726108	N/A	0.027	0.978	0.0	uncertain sig.	Disease causing mutation? Breast cancer risk, male
c.3201+4del	p.(?)	splice_donor +4	rs1555458807	N/A	N/A	N/A	0.0	uncertain sig.	Not reported
c.3203G>A	p.(Gly1068Glu)	missense	rs759587160	N/A	1.0	0.997	0.999	uncertain sig.	Not reported

c.3306C>G	p.(Ser1102Arg)	missense	rs515726112	N/A	0.609	0.989	0.0	uncertain sig.	Disease causing mutation? Breast cancer risk
c.3529G>A	p.(Asp1177Asn)	missense	N/A	N/A	0.252	0.753	0.94	No Data	Not reported
c.758T>C	p.(Leu253Pro)	missense	N/A	N/A	0.0	0.939	0.0	uncertain sig.	Not Reported
c.814G>A	p.(Glu272Lys)	missense	rs515726127	0.00001	0.107	0.646	0.0	uncertain sig.	Disease causing mutation? Breast and/or ovarian cancer risk
c.833_834 delinsAT	p.(Leu278His)	missense	rs587778582	N/A	N/A	N/A	N/A	uncertain sig.	Disease causing mutation? Cancer pred. syndrome
c.91A>G	p.(Thr31Ala)	missense	rs1967110664	N/A	0.997	1.0	0.585	uncertain sig.	Not Reported

Freq: Frequency, Mut.Tast: Mutation Taster, Sig: Significance, HGMD: The Human Gene Mutation Database, N/A: Not Applicable, pred: predisposition, sust: susceptibility

All breast cancer patients with *PALB2* mutation, had invasive-ductal breast cancer (100%), with 85% being hormone receptor-positive. Triple-negative histology was 15% among PV/LPV carriers. In terms of tumor grade, patients had grade 1 (5%) or grade III (30%) tumors, and the majority were at stage II (65%). Except for one patient, all investigated patients who were found to contain the PV/LPV had cancer in the first/second/third-degree relatives. The clinical features of *PALB2* mutant breast cancer patients are presented in Table 3. Additionally, 95% of *PALB2* mutation-carrier breast cancer patients had at least one relative diagnosed with cancer (Table 4).

Table 3. Clinico-pathologic features of Turkish breast cancer patients with *PALB2* PV/LPV detected in this study.

Nucleotide substitution	Age at Diag.	St.	Gr.	His. Sub.	ER	PR	HER2	Node Inv.	TNBC	Met.	Status
c.1692_1698dup	38	III	3	IDC	Pos.	Neg.	Neg.	Yes	No	Yes	Alive
c.1704_1707del	42	II	2	IDC	Pos.	Pos.	Neg.	Yes	No	No	Alive
c.172_175del	39	II	2	IDC	Pos.	Pos.	Neg.	No	No	No	Alive
c.1960_1961insC	43	III	3	IDC	Pos.	Pos.	Neg.	No	No	No	Alive
c.1967dupC	42	II	2	IDC	Pos.	Pos.	Pos.	Yes	No	No	Alive
c.211+1G>T	67	II	2	IDC	Neg.	Neg.	Neg.	Yes	Yes	No	Alive
c.2368C>T	44	II	2	IDC	Pos.	Pos.	Neg.	No	No	No	Alive
c.2587-1G>C	40	III	3	IDC	Neg.	Neg.	Neg.	No	Yes	No	Alive
c.3256 C>T	40	II	2	IDC	Pos.	Pos.	Neg.	No	No	No	Alive
c.390_391insT	51	II	3	IDC	Pos.	Neg.	Pos.	No	No	No	Alive
c.481_482del	39	III	3	IDC	Pos.	Pos.	Neg.	No	No	Yes	Alive
c.509_510del	50	II	2	IDC	Pos.	Pos.	Pos.	Yes	No	Yes	Alive
c.509_510del	36	II	2	IDC	Pos.	Pos.	Neg.	Yes	No	Yes	Alive
c.509_510del	24	I	1	IDC	Pos.	Pos.	Pos.	No	No	No	Alive
c.557dupA	45	II	1	IDC	Pos.	Pos.	Pos.	No	No	No	Alive
c.557dupA	31	III	3	IDC	Neg.	Neg.	Neg.	Yes	Yes	Yes	Alive

c.557dupA	41	II	2	IDC	Pos.	Pos.	Neg.	No	No	No	Alive
c.557dupA	51	II	2	IDC	Pos.	Pos.	Pos.	Yes	No	Yes	Alive
c.557dupA	36	II	2	IDC	Pos.	Pos.	Neg.	No	No	Yes	Alive
c.557dupA	40	III	3	IDC	Pos.	Pos.	Neg.	Yes	No	Yes	Alive

Diag: Diagnosis, St: Stage, Gr: Grade, His Sub: Histologic Subtype, ER: Estrogen Receptor, PR: Progesterone Receptor, HER2: Human Epidermal Growth Factor Receptor 2, Node Inv: Node Involvement, TNBC: Triple-negative Breast Cancer, Met: Metastasis, Pos: Positive, Neg: Negative

Table 4. Frequency of PV/LPV and family history of tested individuals in this study.

Exon	Nucleotide substitution	Amino acid change	Age at Diagnosis & cancer type	Family history
5	c.1692_1698dup	p.(His567Lysfs*13)	38y/44y Bilateral Breast Ca	Esophageal Ca Ovarian Ca
5	c.1704_1707delAAAA	p.(Lys569Argfs*29)	42y Unilateral Breast Ca	Breast Ca Stomach Ca
3	c.172_175delTTGT	p.(Gln60Argfs*7)	39y Unilateral Breast Ca	Breast Ca
5	c.1960_1961insC	p.(Ile654Thrfs*9)	43y Unilateral Breast Ca	Breast Ca Cervix Ca
5	c.1967dupC	p.(Glu657Argfs*6)	42y Bilateral Breast Ca	Lung Ca Breast Ca
3	c.211+1G>T	p.(?)	67y Breast Ca	Ovarian Ca Cervix Ca Stomach Ca Endometrial Ca
5	c.2368C>T	p.(Gln790*)	44y Unilateral Breast Ca	Thyroid Ca Stomach Ca
7	c.2587-1G>C	p.(?)	40y Bilateral Breast Ca	None
12	c.3256 C>T	p.(Arg1086*)	40y/56y Bilateral Breast Ca	Bladder Ca Breast Ca
4	c.390_391insT	p.(Ala1025Thr)	51y Unilateral Breast Ca	Prostate Ca Ovarian Ca Breast Ca
4	c.481_482del	p.(Asp161Leufs*6)	39y/53y Bilateral Breast Ca	Cervix Ca Breast Ca Prostat Ca
4	c.509_510del	p.(Arg170Ilefs*14)	50y Unilateral Breast Ca	Breast Ca Cervix Ca Non-Hodgkin lymphoma. Prostat Ca Uterus Ca

4	c.509_510del	p.(Arg170Ilefs*14)	24y Unilateral Breast Ca	Breast Ca
4	c.509_510del	p.(Arg170Ilefs*14)	36y/52y Bilateral Breast Ca	Breast Ca Cervix Ca
4	c.557dupA	p.(Asn186Lysfs*4)	36y Unilateral Breast Ca	Breast Ca Lung Ca
4	c.557dupA	p.(Asn186Lysfs*4)	40y Unilateral Breast Ca	Pancreas Ca Thyroid Ca Lung Ca Breast Ca Cervix Ca
4	c.557dupA	p.(Asn186Lysfs*4)	45y/57y Bilateral Breast Ca	Prostat Ca Lung Ca Cervix Ca
4	c.557dupA	p.(Asn186Lysfs*4)	41y Unilateral Breast Ca	Breast Ca
4	c.557dupA	p.(Asn186Lysfs*4)	31y Unilateral Breast Ca	Breast Ca Uterus Ca
4	c.557dupA	p.(Asn186Lysfs*4)	51y Unilateral Breast Ca	Pancreas Ca

Ca: cancer

The eight frame-shift *PALB2* pathogenic mutations: c.1692_1698dup, c.1704_1707delAAAA, c.172_175delTTGT, c.1960_1961insC, c.1967dupC, c.481_482del, c.509_510del, c.557dupA were among breast cancer patients. The mutation median age of mutation carriers of breast cancer was 39.8 years (Table4).

The most common *PALB2* PV/LPV found in the study were: The c.557dupA p.(Asn186Lysfs*4) was identified in six breast cancer patients. c.509_510del p.(Arg170Ilefs*14) in three breast cancer patients.

The frameshift pathogenic mutations were more frequent compared to missense genetic alterations here in our study cohort. The frameshift, non-sense, splice donor and spice acceptor variant frequencies were 61.5%, 23.1%, 7.7%, and 7.7%, respectively (Figure1). Surprisingly, no missense pathogenic genetic alterations were found in our patient groups. All identified PV/LPV resulted in a loss-of-function of the *PALB2* gene.

The two most common PV were PALB2, c.509_510del p.(Arg170Ilefs*14) and c.557dupA p.(Asn186Lysfs*4) variant.

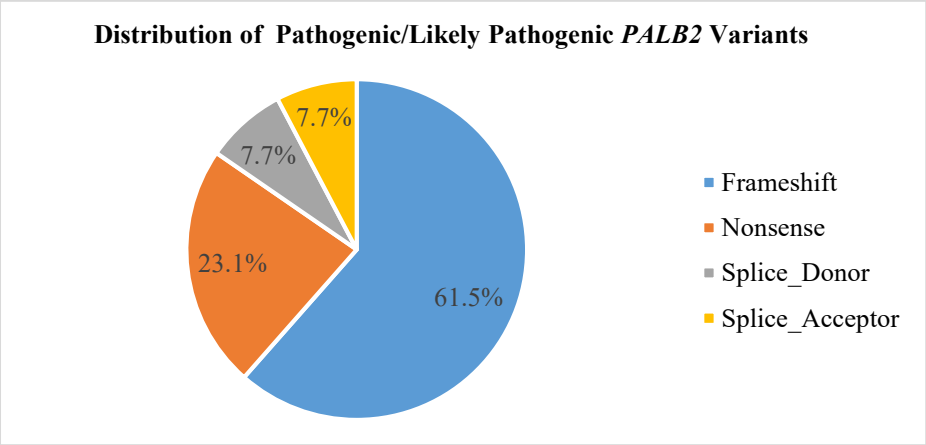

Figure 1. The prevalence of pathogenic/likely pathogenic *PALB2* variant types in cohort of cancer investigated in this study.

Discussion

In this study, we analyzed the *PALB2* PV/LPV and VUS frequencies in the Turkish population. We investigated 1056 cancer patients selected based on the inclusion criteria established in the NCCN Guideline. We found 13 different PV/LPV in 20 patients (20/1056; ~1.9%) and 23 different VUS in 28 patients (28/1056; ~2.7%) in the *PALB2* gene in the entire cohort. We found that the *PALB2* PV/LPV ratio was ~1.9% among patients. In the literature, the detection rate varied from 0.36% to 4.8% overall[24]. The higher prevalence was detected in Finland, which was attributed to the presence of a founder mutation[25] and low incidence was noted in the Jewish Ashkenazi population, in the Netherlands Japan and Ireland studies [26].

Women with germline *PALB2* mutations are at risk of up to 58% for developing breast cancer when they have a positive family history, approximately five-fold higher than the general population[27]. Yang et al. reported that individuals with inherited pathogenic variants in *PALB2* face an increased risk of 7.18 times for breast cancer in women, 2.91 times for ovarian cancer, 2.37 times for pancreatic cancer, and 7.34 times for male breast cancer[28].

The *PALB2* PV/LPV and VUS were identified in forty-one breast cancer patients (41/828), four ovarian cancer patients (4/97), one endometrial cancer patient (1/19), one pancreatic cancer patient (1/26), one prostate cancer patient (1/30).

However, this result should be taken with caution because the number of patients in the endometrial, pancreatic, and prostate cancer cohort is relatively small compared with the breast cancer cohort that was part of this study.

In the current study, the most common recurrent frameshift mutation c.557dupA p.(Asn186Lysfs*4) was detected in six unrelated breast cancer patients 30% (6 of 20 among PV/LPV carriers) diagnosed with early onset cancer breast cancer. The prevalence of *PALB2* germline mutations in patients with early-onset breast cancer has been reported, indicating its potential contribution to hereditary breast cancer similar to our results. The c.557dupA p.(Asn186Lysfs*4) variant in the *PALB2* gene has been extensively studied in the context of cancer susceptibility, particularly in relation to breast cancer. The evidence suggests that *PALB2* plays a significant role in cancer predisposition and has clinical implications for genetic testing and cancer risk assessment. It was determined that a mutation c.557dupA p.(Asn186Lysfs*4) in the *PALB2* gene created a non-sense codon (stop codon) at position 186, leading to a shortening in the length of the protein. In the literature, the effect of this mutation on protein function and cancer risk prediction is yet unknown. The second common recurrent frameshift mutation c.509_510del p.(Arg170Ilefs*14) was detected in 3 unrelated breast cancer patients 15% (3 of 20 among PV/LPV carriers) two were diagnosed at an early age and all patients had familial breast cancer). This mutation is anticipated to result in a significant alteration in the protein structure, potentially affecting its binding sites with *BRCA2*[29] causing an activation of HR for repair of double-strand DNA breaks[8]. *PALB2* gene the PV c.509_510del p.(Arg170Ilefs*14) appears to be a prevalent mutation that has also been observed in other groups[30]. Dansonka-Mieszkowsa discovered the *PALB2*: c.509_510del p.(Arg170Ilefs*14) pathogenic variant in breast/ovarian cancer patients from the southern Polish population[10]. They detected this mutation in 0.6% (4 out of 648) of familial breast cancer patients and 0.08% (1 out of 1310) in the control group, which was s statistically significant. We detected *PALB2*: c.509_510del p.(Arg170Ilefs*14) mutation in 3 breast cancer patients (3/828; 0.36%). More research is necessary to evaluate whether it may be regarded as a founder mutation in the Turkish population.

The most prevalent pathogenic genetic variations among our patients were frameshift, nonsense, and splice variants; and pathogenic missense genetic alterations were not detected. The higher frequency of pathogenic or likely pathogenic loss-of-function mutations, such as frameshift, nonsense, splice, deletions/duplications, compared to missense variants may be attributed to the greater

difficulty in functionally validating missense variants. This difficulty in validating missense variants could lead to a higher number of reported pathogenic or likely pathogenic loss-of-function mutations. In order to address this issue, advanced functional assays such as protein-protein interaction or proficiency testing in homologous recombination repair should be utilized. Despite these efforts, a considerable number of missense variants are still not categorized. As a result, the ClinVar[21] and HGMD[22] have endeavored to offer expert curation on pathogenic/likely pathogenic *PALB2* variants.

The research on *PALB2* has predominantly concentrated on identifying the truncating mutations; however, there were also the documented cases of VUS in patients [31], the presence of these variants poses a challenge for genetic counselors, clinicians, and patients. Although no distinctions were observed in the clinicopathological parameters of *PALB2* VUS carriers in this study, additional functional characterization of *PALB2* VUS could help to differentiate particular VUS with potential pathogenicity, thereby contributing to clinical practice. Until the role of VUS in *PALB2* is elucidated, the ACMG advises against utilizing *PALB2* VUSs to inform the clinical management[32]. However, the functional characterization of *PALB2* VUS have revealed their potential to disrupt DNA repair and lead to functional defects in homologous recombination repair in some studies [33].

Various cancer types were observed among the family members of the patients, carrying this variant in the Turkish cohort. Studies conducted in other populations have reported varying prevalence rates of *PALB2* mutations. Studies have highlighted the significance of *PALB2* as a tumor suppressor gene[33] and its interaction with *BRCA2* in breast cancer susceptibility[34] and the impact of this variant on DNA repair and cancer predisposition[35]. These studies collectively emphasize the importance of investigating the functional consequences of this variant in the context of cancer predisposition and DNA repair mechanisms. To exemplify the *PALB2* mutations accounted for 0.9% of breast cancer cases in the Chinese population[36]. They were similarly truncated *PALB2* mutations detected in 3 out of 96 American patients with familial pancreatic cancer[7]. These findings suggest that *PALB2* mutations may contribute to a small but significant proportion of cancer cases in different populations.

According to the clinicopathologic features of *PALB2* mutation-carrier breast cancer patients detected in this study, all patients had invasive-type ductal cancer. Most cancer patients were classified from intermediate to high-grade types and mostly had hormone receptor-positive expression. Notably, all individuals carrying *PALB2* pathogenic variants were diagnosed at a younger age, most of them aged below 50 years, including six younger than 40 years.

There is no specific study on *PALB2* mutations in a large number of Turkish cancer patients using NGS as in our study. However, researchers in a study aimed to identify the prevalence of *PALB2* variants in *BRCA1/2* and *PALB2*-negative early-onset breast and ovarian cancer patients in a Turkish population[37]. Although the study did not focus solely on *PALB2* mutations, it provides valuable insights into the genetic landscape of hereditary breast and ovarian cancers in Türkiye. Also, in 2016, Cecener et al. investigated all *PALB2* exons in 223 Turkish women with early-onset breast cancer who tested negative for *BRCA1/2* mutations and identified 18 distinct variants by heteroduplex analysis (HDA) and DNA sequencing in Türkiye [38]. However, only a limited number of variants and no conclusively pathogenic variants were detected. Also, Bilen et al. investigated the effects of three different single nucleotide polymorphisms (rs249954, rs249935, and rs16940342) of the *PALB2* gene on Turkish breast cancer predisposition in 2020[39]. Their research aimed only to explore the association between specific single-nucleotide polymorphisms (SNPs) and their impact on breast cancer risk. This study contributes to the growing body of research on the genetic factors influencing breast cancer predisposition and provides valuable insights into the potential role of *PALB2* variants in breast cancer susceptibility.

PALB2 mutations have important clinical implications, particularly regarding cancer risk assessment and genetic testing. It was reported that pathogenic large genomic rearrangements (LGRs) in *PALB2* accounted for 10.3% of pathogenic *PALB2* variants detected in Australian families with familial breast cancer[40], highlighting the importance of considering LGRs in genetic testing for *PALB2* mutations.

Furthermore, *PALB2* mutations have been associated with an increased risk of breast cancer similar to *BRCA2* mutations[41]. Therefore, the inclusion of the *PALB2* in genetic testing panels for high-risk breast and ovarian cancer patients is crucial, as demonstrated in a study on Chinese patients[42].

Of the entire cohort, we identified 13 different PV/LPV in 20 patients, accounting for ~1.9%(20/1056) and 23 different VUS in 28 patients, accounting for ~2.7% (28/1056). However, this result should be taken with caution because the number of patients in the endometrial, pancreatic, and prostate cancer cohort is relatively small compared with the breast cancer cohort that was the part of this study. Overall, the incidence of *PALB2* variants is typically between 0.1% and 1.5%, influenced by the factors such as the study population, the size of the cohort, and the testing methods[43]. The pathogenic *PALB2* variants detected in this study in the Turkish population is about ~1.9% (20/1056 patients), which is slightly higher than the reported frequencies worldwide.

Study Limitations

Firstly, the selection of cancer patients from one hospital for the study may cause bias, and limit the generalizability of the results. Secondly, the small size of the prostate, pancreatic, and colon cancer patients makes it challenging to definitively conclude the non-PALB2 pathogenic variant carriers of cancer patients from the population-based group investigation.

Conclusion

This study successfully determined the *PALB2* variants in cancer patients in Türkiye. The ratio of the *PALB2* variants in cancer patients seems to be slightly higher than the ratio in other populations.

Notably, the recurrent *PALB2* c.557dupA p.(Asn186Lysfs*4) and c.509_510del p.(Arg170Ilefs*14) mutations should be considered as a significant portion of *PALB2* mutation carriers. Recently, the efficacy of PARP inhibitors in *PALB2*-mutated breast cancer patients has been shown, suggesting a possible avenue for targeted therapy that may be helpful for breast cancer patients. Therefore, we recommend that genetic testing for *PALB2* could be integrated into the genetic evaluation of breast cancer patients in Türkiye. This approach might have the potential to make a valuable understanding of breast cancer risks and facilitate the development of prevention and treatment strategies in Türkiye.

Although the number of specific studies on *PALB2* mutations in Turkish cancer patients is scarce, the available evidence from other populations suggests that *PALB2* mutations may contribute to a small but significant proportion of hereditary breast, ovarian, and pancreatic cancers. Further research is needed to determine the prevalence and clinical implications of *PALB2* mutations in the Turkish population.

Ethics Committee Approval

This study was approved by the Clinical Research Ethics Committee of Istanbul Faculty of Medicine in Istanbul University with the decision number of 2023/500 dated 17.03.2023. The study was in compliance with the Helsinki Declaration.

Informed Consent

The written informed consent was obtained from all patients before the study was commenced.

Author Contributions

Seref Bugra Tuncer: Conceptualization, Formal analysis, Investigation, Methodology, Writing-original draft. Seda Kılıc Erciyas: Formal analysis and Investigation. Ozge Sukruoglu Erdogan: Formal analysis, investigation; Betul Celik: Investigation,

Writing-original-draft; Zubeyde Yalnız Kayım; Busra Kurt Gultaslar: Formal analysis.

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Conflict of Interest

There is no conflict of interest to declare.

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Özgün Araştırma/Research Article

Quercetin'in HT-29 ve HCT-116 kolon kanseri hücre hatları üzerine etkisinin RIPK1, RIPK3 ve MLKL genlerinin ekspresyonları ile incelenmesi

The investigation of the effect of quercetin on HT-29 and HCT-116 colon cancer cell lines through the expression of RIPK1, RIPK3, and MLKL genes

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Öz

Amaç: Quercetin kolon kanseri dahil birçok kanser çeşidinde anti-kanser aktivite gösteren bir bileşiktir. Ancak, quercetinin nekroptoz yolağı üzerine etkilerini gösteren çalışmalar kısıtlıdır ve bu nedenle bu çalışmada quercetinin nekroptoz yolağına etkisinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: HT-29 ve HCT-116 kolon kanseri hücreleri kültür edilip farklı konsantrasyondaki quercetinin hücre canlılığına etkisi MTT yöntemi ile belirlendi. Sonrasında quercetinin nekroptoza etkisinin belirlenmesi için RIPK1, RIPK3 ve MLKL genlerinin ekspresyon seviyesi analiz edildi.

Bulgular: HT-29 hücrelerinde quercetinin aktif dozu 50 µM (p=0,0286) olarak bulunurken HCT-116 hücrelerinde 100 µM (p=0,009) bulundu. 50 ve 100 µM quercetin ile maruz bırakılan HT-29 hücrelerinde nekroptoz belirteçlerinin ekspresyon seviyesinde ciddi bir artış tespit edildi.

Sonuç: Bu çalışmanın sonuçları quercetinin nekroptoz yolağının aktif bir düzenleyicisi olabileceğini göstermiştir.

Anahtar Kelimeler: Kolon kanseri, MLKL, Nekroptoz, Quercetin, RIPK1, RIPK3

Abstract

Aim: Quercetin is a compound with anti-cancer activity in many types of cancer. However, studies showing the effects of quercetin on the necroptosis pathway are limited, and therefore, the aim of this study was to determine the effect of quercetin on the necroptosis pathway.

Materials and Methods: Colon cancer cells were cultured and effect of different concentrations of quercetin on cell viability was determined by MTT method. Afterwards, the expression level of RIPK1, RIPK3 and MLKL genes were analyzed to determine the effect of quercetin on necroptosis.

Results: The active dose of quercetin was found to be 50 µM in HT-29 cells (p=0.0286), while 100 µM was found in HCT-116 cells (p=0.009). A significant increase in the expression level of necroptosis markers was detected in HT-29 cells treated with 50 and 100 µM quercetin.

Conclusion: The results of this study showed that quercetin may be an active regulator of the necroptosis pathway.

Keywords: Colon cancer, MLKL, Necroptosis, Quercetin, RIPK1, RIPK3.

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Giriş

Kolon kanseri, Dünya genelinde yaygın görülen bir kanser çeşididir. 2020 Global kanser istatistiklerine göre insidans olarak üçüncü sırada, mortalite olarak ikinci sırada yer almaktadır.[1] Dünya genelinde kolorektal kanserlerin her yıl 1 milyondan fazla insanı, kansere bağlı ölümlerde ise yarım milyondan fazla insanı etkilediği bilinmektedir.[1] Kolon kanserinin gelişiminde ve ilerlemesinde birçok genin önemli olduğu bilinmektedir. Kolon kanserlerinin gelişimi için genetik mutasyonların yanı sıra, diyet, fiziksel aktivite eksikliği, obezite, aşırı alkol tüketimi, sigara, stres ve kırmızı et tüketimi gibi risk faktörleri sıralanabilir.[2] Son yıllarda yapılan çalışmalarda, bol sebze, meyve, tahıl, lif ve vitamin içeren diyetlerin kolon kanseri riskinde önemli bir düşüş ile ilişkili olduğu rapor edilmiştir.[3] Besinlerin içerisindeki polifenolik bileşiklerin en büyük sınıfı olan flavonoidlerin, *in vitro* ve *in vivo* tümör hücresi büyümesini baskıladığı birçok çalışmada gösterilmiştir.[4]

Quercetin; kapari, yaban mersini, dereotu, kişniş, brokoli, soğan, kırmızı meyveler ve çay dahil olmak üzere çeşitli sebze ve meyvelerde bol miktarda bulunan en önemli ve iyi çalışılmış flavonoidlerden biridir.[5] Quercetin birçok bitkinin kabuğunda bulunan kimyasal bir pigmenttir. Vücutta antioksidan olarak işlev gördüklerinden yararlı etkileri fazladır. Her çeşit kırmızı, mor ve yeşil pigmentli bitkiler quercetin içermektedir. Bitkisel kaynaklarda bulunan quercetin miktarı yetiştirilen yere göre, tazeliğine göre ve nasıl hazırlandığına göre değişmektedir.[5]

Quercetinin kolon kanseri dahil birçok kanser çeşidinde anti-kanser etkilerinin olduğu gösterilmiştir. Quercetinin hücre çoğalması, anjiyogenez, apoptoz, inflamasyon, ilaç dirençliliği, hücre göçü, metastaz ve otofaji gibi çeşitli mekanizmalarda etki gösterdiği bildirilmiştir.[2] Örneğin; Caco-2 kolon kanseri hücrelerinde CDC6 (cell division cycle 6), CDK4 (cyclin-dependent kinase 4) ve siklin D1 gibi hücre döngüsü ile ilişkili genlerin ekspresyon seviyesinin düşmesine neden olarak hücre döngüsünü duraksattığı gösterilmiştir.[6] HT-29 hücrelerinde AMPK ve p53'ün fosforilasyonu yoluyla apoptozu uyardığı gösterilmiştir.[7,8] HCT-116 kolon kanseri hücrelerinde ise AMPK sinyal yolağı aracılığı ile apoptoz yolağının uyarılmasında önemli olduğu gösterilmiştir.[9] Bunun yanı sıra, LoVo kolon kanseri hücrelerinde reaktif oksijen türlerinin (ROS) üretimini arttırarak apoptozu uyardığı gösterilmiştir.[10] Quercetinin kolon kanseri hücrelerinde farklı mekanizmalara etki ettiği yukarıda sıralanmıştır. Ancak nekroptoz üzerine etkilerinin gösteren çalışmalar kısıtlıdır. Nekroptoz, apoptoz ve nekrozun izlerini taşıyan bir programlı hücre ölümü çeşididir. Programlı olmasından dolayı apoptoza, hücre ölümü morfolojisi olarak nekroza benzediği için nekroptoz olarak adlandırılmıştır. Nekroptotik sinyalizasyonu, tümör nekrozis faktörü alfa reseptörleri (TNFR1), Toll benzeri reseptörler, interferon reseptörleri veya belirli DNA bağlayıcı proteinlerin aktivasyonunun aşağı akışındaki üç anahtar protein ile uyarılır. Bu proteinler: RIPK1 (receptor interacting serine/threonine kinase 1), RIPK3 ve MLKL (mixed lineage kinase domain-like pseudokinase).[11-15] İlk olarak RIP proteinleri nekrosozomu oluşturur ve sonrasında MLKL'nin bir araya toplanmasını ve fosforilasyonunu sağlar. MLKL'nin RIPK3 ile fosforilasyonu, litik hücre ölümünü uyarmak için membranları geçirgen hale getiren MLKL oligomerlerinin toplanmasını ve plazma zarı translokasyonunu kolaylaştırır.[16]

Quercetinin HT-29[17] ve HCT-116[18] hücrelerinin proliferasyonunu baskıladığı ve apoptozu uyardığı gösterilmiştir. Ancak, yapılan literatür taramaları neticesinde quercetinin kolon kanseri hücrelerinde nekroptoz yolağına etkisini gösteren herhangi bir çalışma bulunmamaktadır. Dolayısıyla bu çalışmada, quercetinin kolon kanseri hücrelerinde nekroptoz yolağına etkisinin araştırılması amaçlanmıştır.

Gereç ve Yöntem

Hücre kültürü

Bu çalışma için HT-29 ve HCT-116 kolon kanseri hücreleri ATCC'den temin edilmiştir. Hücrelerin kültür işlemleri 37 ºC sıcaklıkta, %5 CO_2 ve %95 hava içeren inkübatörde gerçekleştirildi. HT-29 ve HCT-116 hücreleri

%10 fetal sığır serumu (Gibco, Thermo Fisher Scientific, ABD) ve %1 penisilin/streptomisin içeren Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher Scientific, ABD) besiyerinde çoğaltıldı. Flasklarda çoğaltılarak yeterli sayıya ulaşmış hücreler Tripsin-EDTA (Thermo, Thermo Fisher Scientific, ABD) ile kaldırılarak falkon tüpe toplandı. Ardından 2000 rpm'de 5 dk santrifüj edildi ve pelet tekrardan süspanse edilerek Triphan blue ile Thoma lamında hücre sayımı yapıldı.

Quercetinin kolon kanseri hücrelerinde hücre canlılığına etkisinin gösterilmesi

İlk olarak 96-kuyucuklu kültür kaplarına 30.000 hücre/ml ekildi ve 24 saat karbondioksitli inkübatörde inkübe edildi. 24 saatin sonunda hücrelerdeki besi yeri uzaklaştırıp PBS (Phosphate-Buffered Saline) ile yıkama işlemleri yapıldı. Dimetil sülfoksit (Sigma, ABD) içinde çözdürülen quercetin farklı konsantrasyonlara (200-100-50-25-12.5-6.25-0 µM) hazırlandı ve HT-29 ve HCT-166 hücrelerine verilerek 24h inkübe edildi.[19] İnkübasyon sonrasında kuyucuklara 100 µl 1 mg/ml oranında methylthiazolyldiphenyl-tetrazolium bromide (Sigma, ABD) solüsyonu eklendi ve 2-3 saat 37 °C'de inkübe edildi. İnkübasyon sonrasında süpernatant çekilip mavi-mor formazan partiküller DMSO ile çözdürülerek 570 nm dalga boyunda Thermo Scientific Multiskan GO (Thermo Fisher Scientific, ABD) mikroplaka okuyucuda okutuldu.

Real-Time PCR deneyleri

Gene ekspresyonları için ilk olarak hücrelerden RNA izolasyonu yapıldı. Hücreler kaldırıldıktan sonra beta-merkaptoetanol içeren liziz tampon çözeltisi ile çözdürüldü. RNA izolasyonları için GeneJET RNA Purification Kit (Thermo Scientific, ABD) kullanıldı. Kitin prosedürüne göre diğer işlemler aşama aşama takip edildi. Son olarak, elde edilen RNA örneklerinin konsantrasyonlarını belirlemek için RNA'lar NanoDrop 1000 (Thermo Fisher Scientific, ABD) spektrofotometrede ölçüldü ve hızlıca -80 °C dondurucuya kaldırıldı.

İzole edilen RNA örneklerinden tek sarmal cDNA sentezi için RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, ABD) kullanıldı. cDNA sentezi için 500 ng RNA kullanıldı ve üretici firmanın önerisi doğrultusunda belirlenen miktarlarda karışım hazırlandı. Hazılarlanan karışım SensoQuest PCR cihazında uygun termal şartlara tabii tutuldu.

Nekroptoz ile ilişkili genlerin ekspresyon seviyelerini belirlemek için bu genlere spesifik primerler kullanıldı.[20] Bu primerler ve RealQ Plus 2x Master Mix Green Kit (Amplicon, Danimarka) ile genlerin ekspresyon seviyeleri belirlendi. Her bir örnek için hazırlanan bu karışım RotorGene (Qiagen, Almanya) cihazında 95 °C'de 15 dk, 95°C'de 15 s 60°C'de s ve 72 °C'de 30 s (40 döngü) termal şartlara tabii tutuldu. Her reaksiyon sonunda 55-95°C arasında erime eğrisi analizi yapıldı. Reaksiyon sonrasında uygun bir eşik değerde her bir örnek için Ct (cycling threshold) değeri belirlendi. Hesaplanan Ct değerlerine göre gen ekspresyon seviyesi $2^{-\Delta Ct}$ (ΔCt=Cthedef gen–Ctreferans gen) formülüne göre belirlendi.[21] Formüldeki hedef genler RIPK1, RIPK3, MLKL'yi, referans gen ise GAPDH'i ifade etmektedir.

Verilerin analizi

Tüm sonuçların istatistiksel olarak değerlendirilmesi ve grafik olarak gösterilmesinde GraphPad Prism (v.8) programı kullanıldı. Verilerin normalitesini analiz etmek için Shapiro-Wilk testi uygulandı. Sonrasında, ikili grupların karşılaştırılmasında t-testi, ikiden fazla grup karşılaştırmalarında ise One-Way ANOVA analizi kullanıldı. Tüm sonuçlar için %95 güven aralığından $p<0,05$ olan sonuçlar istatistiksel olarak anlamlı kabul edildi.

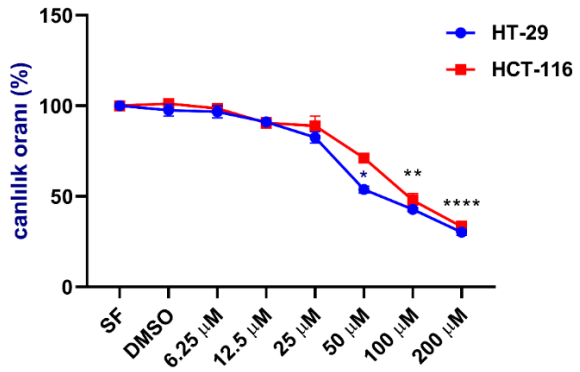
Araştırmanın etik boyutu

Bu çalışma etik kurul onayı gerektirmediğinden etik kurul izni alınmamıştır.

Bulgular

Quercetinin kolon kanseri hücrelerinde hücre canlılığı üzerine etkilerinin belirlenmesi için MTT deneyleri yapıldı. Doza bağlı olarak HT-29 ve HCT-116 hücrelerinde hücre canlılığının azaldığı gösterildi (Şekil 1). HT-29 kolon kanseri hücrelerinde quercetinin

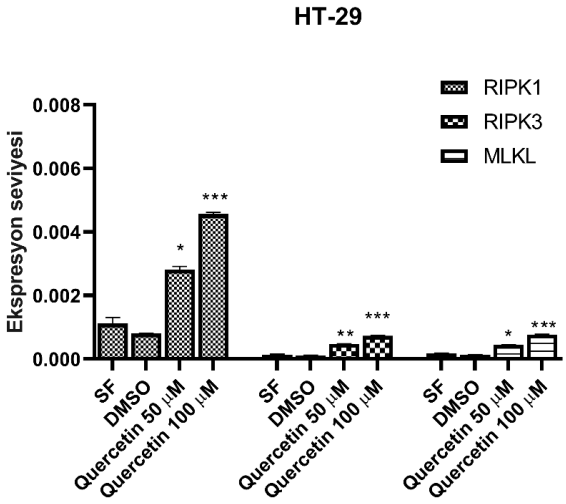
etkin dozu 50 µM olarak belirlendi (p=0,0286). HCT-116 hücrelerinde ise etkin doz 100 µM olarak belirlendi (p=0,009).



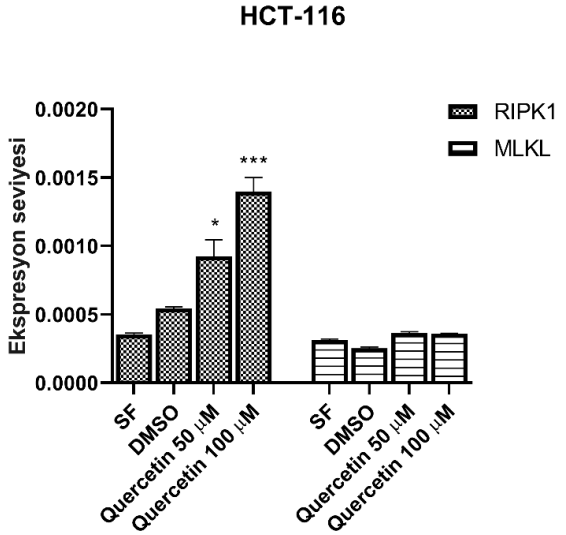
Şekil 1. HT-29 ve HCT-116 kolon kanseri hücrelerinde quercetinin hücre canlılığına etkisi gösterilmiştir. *p<0,05, **p<0,01, ****p<0,0001. SF: serum içermeyen besiyeri, DMSO: dimetilsülfoksit

HT-29 ve HCT-116 hücrelerinde quercetin maruziyeti sonrasında nekroptoz ile ilişkili genlerin ekspresyon seviyesinin belirlenmesi için Real-Time PCR deneyleri yapıldı. Nekroptoz ilişkili genlerin ekspresyon seviyesindeki değişimler iki farklı dozdaki (50-100 µM) quercetin uygulaması sonrasında belirlendi. HT-29 hücrelerinde RIPK1 ekspresyon seviyesi, 50 µM ve 100 µM quercetin uygulanan iki grupta kontrol grubuna kıyasla anlamlı derecede artış gösterdi. 50 µM quercetin uygulanan grupta RIPK1 ekspresyon seviyesi yaklaşık 4 kat artış gösterirken (p=0,023), 100 µM quercetin uygulanan gruptaki RIPK1 seviyesinin yaklaşık 6,5 kat artış gösterdiği bulundu (p=0,001). Bunun yanı sıra, RIPK3 ve MLKL genlerinin ekspresyon seviyesinde de anlamlı artışlar gözlemlendi (Şekil 2).

HCT-116 kolon kanseri hücrelerinde RIPK1 ve MLKL genlerinin ekspresyon seviyesi gösterildi. HCT-116 hücrelerinin RIPK3 eksprese etmediği daha önceki çalışmalarda gösterilmişti.[20,22] HCT-116 hücrelerinin 50 µM ve 100 µM quercetin maruziyeti sonrasında RIPK1 ekspresyon seviyesi kontrol grubuna kıyasla anlamlı bir artış gösterdi. HCT-116 hücrelerinin 50 µM quercetin maruziyeti sonrasında RIPK1 ekspresyon seviyesinin kontrol grubuna kıyasla yaklaşık 2 kat arttığı (p=0,049), 100 µM quercetin maruziyeti sonrasında ise 2,5 kat arttığı bulundu (p=0,001). Ancak MLKL geninin ekspresyon düzeyinde anlamlı bir değişim görülmedi (p=0,136) (Şekil 3).



Şekil 2. HT-29 kolon kanseri hücrelerinin 50 µM ve 100 µM quercetin maruziyeti sonrasında RIPK1, RIPK3 ve MLKL genlerinin ekspresyon sevilerindeki değişimler gösterilmiştir. *p<0,05, **p<0,01, ***p<0,001. SF: serum içermeyen besiyeri, DMSO: dimetilsülfoksit



Şekil 3. HCT-116 kolon kanseri hücrelerinin 50 µM ve 100 µM quercetin maruziyeti sonrasında RIPK1 ve MLKL genlerinin ekspresyon sevilerindeki değişimler gösterilmiştir. *p<0,05, ***p<0,001. SF: serum içermeyen besiyeri, DMSO: dimetilsülfoksit

Tartışma

Nekroptoz, apoptozdan farklı programlanmış bir enflamatuvar hücre ölümü şeklidir. Bu programlı hücre ölümü çeşidi, doku onarımını ve patojenlerin tespitini

desteklemek için gelişmiştir.[23] RIPK1-RIPK3-MLKL'den oluşan kanonik ölüm reseptörü aracılı nekroptotik yolak, TNFR gibi ölüm alanı reseptörlerinin ve Toll benzeri reseptörlerin aşağı akışında tetiklenir.[11]

Doğal ürünler, özellikle flavonoidler düşük toksisiteleri ve çoklu hedefleme teknikleri nedeniyle kanser tedavisi için önemli moleküllerdir. Antioksidan, anti-bakteriyal ve anti-inflamatuar etkileri gibi çeşitli biyolojik aktivitelere sahip bir flavonoid olarak quercetin sebze ve meyvelerde bol miktarda bulunmaktadır. Yapılan çalışmalarda quercetinin farklı kanser türlerinde apoptoza etkisi gösterilmiştir. Bunun yanı sıra, otofaji, hücresel yaşlanma, mitotik felaket, ferroptoz, piroptoz ve nekroptoz gibi apoptoz dışı hücre ölümlerini de etkilediği gösterilmiştir.[24] Az sayıdaki çalışma kanser hücrelerinde quercetinin nekroptozu uyardığını göstermektedir. Estrada-Villaseñor ve arkadaşları, dev hücreli kemik tümöründe quercetin maruziyetinin otofajiye ek olarak RIPK1 ekspresyonunu arttırarak nekroptozun uyarıldığını göstermişlerdir.[25] Buna ek olarak, MCF7 meme kanseri hücrelerinin quercetin ile ZVAD (apoptoz inhibitörü) maruziyetine kıyasla Necrostatin-1 ile maruz bırakıldığında hücre çoğalmasının artmasına neden olduğu gösterilmiştir. Bununla birlikte, Necrostatin-1 varlığında quercetinin Necrostatin-1 yokluğuna kıyasla BAX geninin ekspresyonunu azaltarak apoptozu baskıladığı ve hücre proliferasyonunu arttırdığı gösterilmiştir.[26] Ayrıca, quercetinin sıçanlarda omurilik yaralanmasından sonra RIPK3/MLKL aracılı oligodendrosit nekroptozunu hafiflettiği bildirilmiştir.[27] Bir diğer çalışmada ise, quercetinin tavuk beyninde nekrositoz insidansını önemli ölçüde azalttığı gösterilmiştir.[28]

Bu çalışmada ise quercetinin kolon kanseri hücrelerinde RIPK1, RIPK3 ve MLKL genlerinin ekspresyonunu düzenleyerek nekroptozu gösterilmiştir. Özellikle HT-29 hücrelerinde quercetin maruziyetinin hücrelerde RIPK1, RIPK3 ve MLKL ekspresyonunu uyararak nekroptozu uyardığı gösterilmiştir. RIPK3 eksprese etmeyen HCT-116 hücrelerinde[20,22] ise yalnızca RIPK1 geninin ekspresyon seviyesinde bir artış olduğu gösterilmiştir. Bu hücrelerde RIPK3 ekspresyonunun olmaması alt sinyal yolağındaki MLKL geninin ekspresyon seviyesinin değişmemesine neden olabilir. Bu durumun açıklanması için, RIPK1 ve RIPK3 proteinlerinin seviyesi ve MLKL fosforilasyonunun gösterilmesi gibi ileriki çalışmalara ihtiyaç duyulmaktadır. Dolayısıyla, nekroptoz ilişkili genlerin protein seviyelerinin belirlenememesi çalışmamızın sınırlılıkları arasındadır. Buna ek olarak, elektron mikroskobu görüntüleriyle nekroptoz morfolojisinin gösterilmesi quercetinin nekroptoz yolağındaki rolünün daha net olarak ortaya konmasında büyük önem arz etmektedir. Ayrıca, *in vivo* fare modellerinde quercetinin nekroptoz üzerine etkisinin belirlenmemiş olması da sınırlılıklarımız arasında yer almaktadır.

Araştırmanın Etik Boyutu

Bu çalışma için etik kurul onayı etik kurul izni alınmamıştır.

Yazar Katkıları

ACT: Fikir/Kavram, Tasarım ve Dizayn, Kaynaklar, Veri Toplama ve/veya İşleme, Literatür Taraması, Yazı. **SH:** Tasarım ve Dizayn, Kaynaklar, Veri Toplama ve/veya İşleme. **HB:** Literatür Taraması, Kaynaklar, Eleştirel İnceleme. **EB:** Fikir/Kavram, Denetleme/Danışmanlık, Kaynaklar, Malzemeler, Veri Toplama ve/veya İşleme, Analiz ve/veya Yorum, Literatür Taraması, Yazı, Eleştirel İnceleme

Çıkar Çatışması Beyanı

Yazarlar herhangi bir çıkar çatışması bildirmemiştir. Makalenin içeriğinden ve yazımından yalnızca yazarlar sorumludur.

Araştırma Desteği

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Hakem Değerlendirmesi

Dış bağımsız

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Research Article/Özgün Araştırma

Comparison of brain volume measurements in methamphetamine use disorder with healthy individuals using volbrain method

Metamfetamin kullanım bozukluğunda beyin hacmi ölçümlerinin volbrain yöntemi kullanılarak sağlıklı bireylerle karşılaştırılması

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Abstract

Aim: This study aims to examine brain structures in individuals with methamphetamine use disorder (MUD) and to understand the possible effects of methamphetamine on these structures.

Materials and Methods: The study was retrospectively evaluated in 21 MUD and 21 healthy controls. VolBrain segmentation method was used.

Results: Grey Matter (GM), Cortical GM, Cerebrum total, and GM volumes were found to be less and significantly higher in MUD compared to healthy controls (p<0.01). Accumbens, Basal Forebrain, Caudate, Pallidum, Putamen, and Parietal Lobe volumes were increased in MUD (p<0.01). Amygdala, Hippocampus, Ventral Diencephalone, Frontal Lobe, Posterior Orbital Gyrus, Precentral Gyrus, Temporal Lobe, Calcarine Cortex, Middle Occipital Gyrus, Superior Occipital Gyrus, Limbic Cortex volumes were significantly smaller in MUD compared to healthy controls.

Conclusion: This study helped us better understand MUD's effects on brain structures. It also provided important information for developing effective strategies for treating and preventing MUD.

Keywords: Methamphetamine; Brain; Grey matter; Basal forebrain.

Öz

Amaç: Bu çalışmanın amacı, metamfetamin kullanım bozukluğu (MKB) olan bireylerde beyin yapılarını incelemek ve metamfetaminin bu yapılar üzerindeki olası etkilerini anlamaktır.

Gereç ve Yöntem: Çalışmada 21 MKB ve 21 sağlıklı kontrol retrospektif olarak değerlendirildi. VolBrain segmentasyon yöntemi kullanıldı.

Bulgular: Substantia grisea (SG), kortikal SG serebrum total ve SG hacimleri sağlıklı kontrol grubuna kıyasla daha az ve anlamlı bulunmuştur (p<0,01). Accumbens, pars basalis telencephali, lobus caudatus, globus pallidus, putamen ve lobus parietalis hacimleri MKB'de artmıştır (p<0,01). Amygdala, hippocampus, ventral diensefalon, lobus frontalis, gyrus orbitalis posterior, gyrus precentralis, lobus temporalis, calcarine cortex, gyrus occipitalis medium, gyrus occipitalis superior, lobus limbicus hacimleri MKB'de sağlıklı kontrollere kıyasla anlamlı derecede küçüktü.

Sonuç: Bu çalışma, MKB'nin beyin yapıları üzerindeki etkilerini daha iyi anlamamıza yardımcı oldu. Ayrıca, MKB tedavisi ve önlenmesi için etkili stratejiler geliştirmek için önemli bilgiler sağlamıştır.

Anahtar Kelimeler: Metamfetamin; Beyin; Gri madde; Bazal ön beyin.

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Introduction

Methamphetamine crystal (chalk or ice) is an addictive stimulant that can be administered orally, smoked, snorted, or injected. Smoking or intravenous injection rapidly delivers methamphetamine to the brain, resulting in a sudden and intense euphoria. Methamphetamine use is associated with serious neurological and physical consequences and has become a severe public health problem worldwide[1]. Methamphetamine was discovered in Japan in 1919 and commercialized in 1938 under Pervitin. It was trendy for tired night shift workers and was used by Germany during World War II to treat fatigue in weary army troops. Methamphetamine became widely used in 1943 to treat various disorders, including narcolepsy, depression, obesity, alcoholism, and attention deficit hyperactivity disorder[2]. The euphoric effects of methamphetamine occur due to the release of the neurotransmitter dopamine, which is involved in the experience of pleasure, motivation, and motor function. However, long-term use of methamphetamine causes molecular changes in the dopamine system, contributing to nerve terminal brain damage and impaired motor skills, rapid cognitive decline, increased anxiety, psychotic disorders, violent behavior, hallucinations, delusions, and depression[3,4]. Although drugs of abuse have been shown to alter brain structures over time, there is limited information about how methamphetamine use may affect the brain over time[5]. The existing literature on this matter needs more unequivocal clarity. Within this context, individuals who use methamphetamine exhibit an array of neuroanatomical differences compared to non-users and control participants[6-8]. However, the specific brain regions involved in these disparities vary among studies. While some investigations have reported reduced cortical volumes in methamphetamine users[9,10], other studies have findings, including increased volumes in distinct brain regions such as the basal ganglia and parietal lobe[11].

Moreover, another finding was that methamphetamine use was more common in the male gender[6]. These results within the existing literature underscore the complexity of the impact of methamphetamine use on brain morphology, and they emphasize the need for further research to delineate the precise mechanisms and factors contributing to these observed differences. In addition, a more comprehensive understanding of the neuroanatomical changes associated with methamphetamine use is essential for physicians and nurses, who have active roles in this field, to determine effective prevention and treatment strategies for substance abuse.

To the best of our knowledge, this is the first study in which 238 different brain segments of each participant were measured with the volbrain method in methamphetamine use disorder (MUD). The aim of this study was to obtain information about the course of the disease in individuals diagnosed with MUD and to determine the extent to which the volumes of the brain and other structures related to the disease are affected.

Materials and Methods

Type of the study

This study is cross-sectional and retrospective.

The sample size of the study

This study evaluated 21 male patients who were admitted to the hospital due to MUD, diagnosed with MUD in urinalysis, had no serious systemic disease, did not use alcohol, and was followed up in Atatürk University Psychiatry Clinic. Healthy controls consisted of 21 male participants who were compatible with the patient group among individuals without any health problems[12,13] registered in Atatürk University Archives and were evaluated retrospectively.

Data collection tools

MR protocol: The MR protocol used in the study was as follows. High-resolution T1-weighted 3D Magnetization Prepared Rapid Gradient Echo (MPRAGE) images were used to show the anatomical structure. Sequence=Sagittal, Repeat time=1900 ms/2.84s, Flip angle=15o, Echo time=2.67 ms, FOV=256 mm2, Matrix=256x256, Number of

slices=160, Slice thickness=1 mm, Resolution=1x1x1x1 mm3 isotropic.

VolBrain Method: VolBrain (https://volbrain.net/) is an open access platform for automatic segmentation of various brain structures[14-16]. We used the segmentation method with default VolBrain T1w volume metric images and performed total cerebrum volumetric analysis in the study groups. The Mricloud method is a web-based software developed by Johns Hopkins University. It is used for volume calculation with brain parcellation in MR images. In order to perform volume calculation with VolBrain, MR images must be converted to "gz or rar" format. The process steps to be performed for these calculations are as follows.

A file with the extension "DICOMDIR" is opened through a DICOM viewer software program. To show the anatomical structure, high-resolution T1-weighted 3D MPRAGE images are opened with mricron, and a file with gz extension in compressed FSL format is created. In the next step, the images converted to "gz" format of the exported images are uploaded to the volbrain web page. Registration is done. Gz extension files are uploaded to the system. In approximately 5-10 minutes, the volumes of all regions in the brain are obtained. The results are saved as pdf. Again, images are recorded as native and mni, and a three-dimensional evaluation is made visually with itksnap.[14,15]

In this study, the AssemblyNet partition was selected from VolBrain measurements. AssemblyNet is a large central nervous system ensemble for 3D whole-brain MRI segmentation[16]. Volumetric values of all parts of the brain were measured in cm3 and percentages, and total-right-left ratios were measured. A total of 462 different data were obtained from each participant. white matter (WM), grey matter (GM), subcortical GM, cortical GM, cerebellar GM, cerebro spinal fluid (CSF), brain (WM+GM), intracranial cavity (IC), cerebrum, cerebrum WM, cerebrum GM, cerebellum, cerebellum WM, cerebellum GM, vermis, brainstem were measured. Subcortical structures accumbens, amygdala, basal forebrain, caudate, hippocampus, pallidum, putamen, thalamus, and ventral diencephalon were measured. Among the cortical structures, frontal lobe and frontal lobe parts, the frontal pole, gyrus rectus, opercular inferior frontal gyrus, orbital inferior frontal gyrus, triangular inferior frontal gyrus, medial frontal cortex, middle frontal gyrus, anterior orbital gyrus, lateral orbital gyrus, medial orbital gyrus, posterior orbital gyrus, precentral gyrus, precentral gyrus medial segment, subcallosal area, superior frontal gyrus, superior frontal gyrus medial segment, supplementary motor cortex were measured. Temporal lobe and fusiform gyrus, planum polare, planum temporale, inferior temporal gyrus, middle temporal gyrus, superior temporal gyrus, transverse temporal gyrus, and temporal pole were measured. The parietal lobe and angular gyrus, postcentral gyrus, postcentral gyrus medial segment, precuneus, superior parietal lobule, and supramarginal gyrus were measured. The occipital lobe and calcarine cortex, cuneus, lingual gyrus, occipital fusiform gyrus, inferior occipital gyrus, middle occipital gyrus, superior occipital gyrus, and occipital pole were measured. The limbic cortex and entorhinal area, anterior cingulate gyrus, middle cingulate gyrus, posterior cingulate gyrus, and parahippocampal gyrus were measured. The insular and insular cortex parts, anterior insula, posterior insula, central operculum, frontal operculum, and parietal operculum were measured. CSF, inferior lateral ventricle, lateral ventricle, third ventricle, fourth ventricle, and external CSF were measured (Figure 1).

Data analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 22.0 (IBM Corporation, Armonk, New York, USA). The priori power analysis was performed using the G-Power 3.1.9.4 program to determine that the sample size was sufficient, the effect size was 1.1, and the power was 0.90 at the 95% confidence interval, at a significance level of 0.05[13]. These values indicate that the sample size is at the desired level. Values were presented as mean and standard deviation. Mann-Whitney U test was used to evaluate

differences between groups. $p<0.05$ was considered statistically significant.

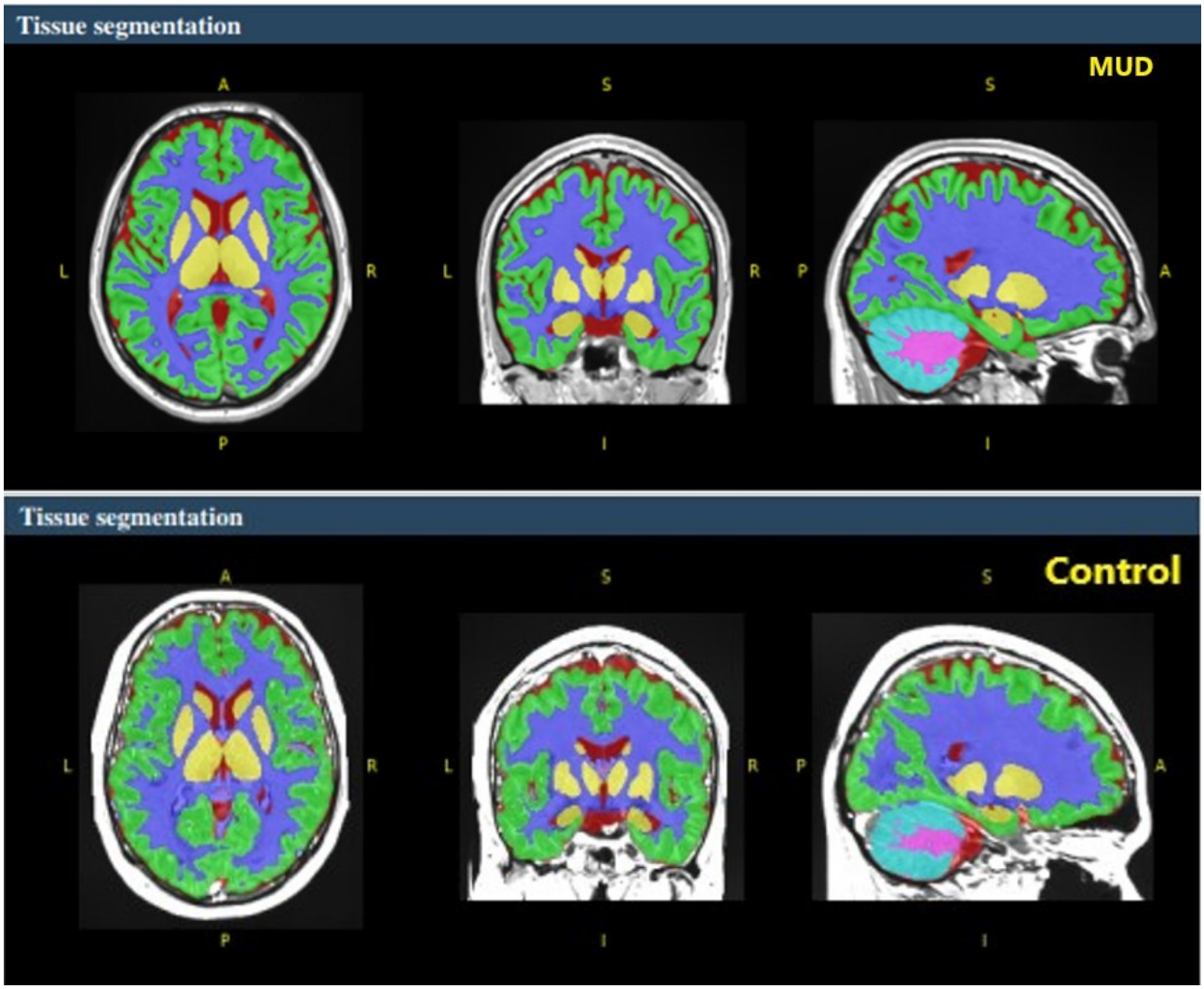

Figure 1. The top image shows brain volume measurements in individuals with methamphetamine use disorder, and the bottom image shows brain volume measurements in healthy controls.

Ethics committee approval

Ataturk University Faculty of Medicine Clinical Research Ethics Committee with the ethics committee decision numbered B.30.2.ATA.0.01.00/128 and dated 26.01.2023. This study conformed to the Helsinki Declaration.

Results

Since all MUD in the study were male, the control group was also selected from male healthy individuals. The mean age of MUD was 40.14±6.82 years, and the mean age of the control group was 41.33±5.0 years (p=0.33). The body mass index was 22.03±2.03 in MUD and 22.66±1.65 in the control group (p=0.48).

The volumes of WM, GM, subcortical GM, cortical GM, cerebellar GM, WM+GM, IC, cerebrum total, cerebrum WM, and cerebrum GM were decreased in MUD compared to healthy controls. In addition, it was found that GM, cortical GM, WM+GM, IC, cerebrum total, cerebrum total, and cerebrum total GM volumes decreased statistically significantly in MUD (Table 1). In MUD, the volume measurements of the total, right, and left parts of the cerebellum, cerebellum WM, cerebellum GM, vermis, and brainstem sections were less than in healthy individuals. However, no statistically significant difference was found. In addition, accumbens volume increased in MUD, while hippocampus, thalamus and ventral diencephalon volumes decreased in healthy controls. In addition, statistically significant differences were found in the accumbens, hippocampus, and ventral diencephalon volume measurements of MUD

and healthy individuals (Table 1). Amygdala, one of the subcortical structures, decreased in MUD, while basal forebrain, caudate, pallidum, and putamen volume measurements increased significantly (Table 2). In addition, the frontal lobe, temporal lobe, occipital lobe, limbic cortex, and insular cortex volume measurements were significantly reduced in MUD. Parietal lobe volume increased, but no statistically significant difference was found (Table 2, Table 3). In MUD, inferior lateral ventricle, lateral ventricle, third ventricle, and fourth ventricle volume measurements from CSF sections were measured less, and it was found that the third ventricle volume measurement was statistically significantly decreased compared to healthy individuals (Figure 2).

Table 1. Comparison of brain volume (Cerebrum, cerebellum, vermis, brainstem, accumbens hippocampus. Thalamus and ventral diencephalon) measurements in methamphetamine use disorder and healthy individuals.

	Methamphetamine Mean±SD	**Control Mean±SD**	***p***
White Matter cm^3	450.83±58.70	523.12±165.35	0.308
Grey Matter cm^3	643.96±117.14	749.52±71.872	**0.004****
Subcortical cm^3	29.94-15.81	34.18±12.85	0.296
Cortical GM cm^3	512.61±94.13	608.80±72.31	**0.004****
Cerebellar GM cm^3	101.88±15.50	106.53±19.48	0.458
Cerebro Spinal Fluid cm^3	216.01±98.75	208.39±146.51	0.372
Brain (WM+GM) cm^3	1094.81±141.58	1272.65±203.13	**0.006****
Intracranial Cavity cm^3	1325.28±142.79	1495.87±323.69	**0.051**
Cerebrum total cm^3	973.18±123.71	1141.24±201.18	**0.004****
Cerebrum right cm^3	515.60±61.67	572.75±102.271	0.128
Cerebrum left cm^3	457.57±103.14	568.46±100.79	**0.009****
Cerebrum total WM cm^3	430.63±59.33	481.62±193.91	0.678
Cerebrum right WM cm^3	232.01±42.54	250.37±87.54	0.811
Cerebrum left WM cm^3	198.61±47.91	247.83±79.84	0.064
Cerebrum total GM cm^3	542.55±105.55	642.98±70.17	**0.005****
Cerebrum right GM cm^3	283.58±65.01	322.33±31.98	0.110
Cerebrum leftt GM cm^3	258.96±63.76	320.65±42.77	**0.004****
Cerebellum total cm^3	111.35±19.29	120.30±18.01	0.162
Cerebellum right cm^3	58.44±10.64	63.75±10.19	0.178
Cerebellum left cm^3	52.91±13.17	56.55±10.51	0.345
Cerebellum WM total cm^3	20.20±9.08	24.86±4.77	0.128
Cerebellum WM right cm^3	10.70±4.80	12.79±2.79	0.421
Cerebellum WM left cm^3	9.51±5.64	12.07±2.24	0.263
Cerebellum GM total cm^3	91.14±12.51	95.44±18.35	0.489
Cerebellum GM right cm^3	47.73±6.73	50.96±11.35	0.513
Cerebellum GM left cm^3	43.41±9.31	44.47±9.51	0.930
Vermis cm^3	10.27±3.22	11.09±2.04	0.588
Brainstem cm^3	14.48±5.18	14.83±4.37	0.772
Accumbens total cm^3	0.84±0.12	0.35±0.32	**0.001****
Accumbens right cm^3	0.38±0.07	0.18±0.15	**0.001****
Accumbens left cm^3	0.45±0.06	0.21±0.19	**0.001****
Hippocampus total cm^3	3.70±2.82	6.78±1.62	**0.001****
Hippocampus right cm^3	2.04±1.41	3.40±0.78	**0.001****
Hippocampus left cm^3	1.65±1.52	3.37±0.90	**0.001****
Thalamus total cm3	12.32±5.24	14.16±3.29	0.443
Thalamus right cm3	6.57±2.91	7.25±1.17	0.489
Thalamus left cm3	5.74±3.09	6.91±2.16	0.273
Ventral Diencephalon total cm3	7.38±3.59	9.72±3.15	**0.044***
Ventral Diencephalon right cm3	3.83±1.85	5.24±2.11	0.222
Ventral Diencephalon left cm3	3.55±1.91	4.71±1.46	**0.048***

SD: Standard Deviation, WM: White Matter, GM: Grey Matter. **: $p<0.01$, *: $p<0.05$, Mann Whitney U Test was used.

Table 2. Comparison of volume measurements of amygdala, basal forebrain, caudate, pallidum and putamen from subcortical structures, limbic cortex and ınsular cortex in methamphetamine use disorder and healthy ındividual.

	Methamphetamine Mean±SD	Control Mean±SD	*p*
Amygdala total cm3	0.76±0.74	1.94±0.47	**0.001****
Amygdala right cm3	0.41±0.40	0.98±0.23	**0.001****
Amygdala left cm3	0.34±0.36	0.94±0.24	**0.001****
Basal forebrain total cm^3	0.56±0.18	0.36±0.21	**0.003****
Basal forebrain right cm^3	0.26±0.08	0.17±0.11	**0.019***
Basal forebrain left cm^3	0.31±0.11	0.18±0.12	**0.002****
Caudate total cm^3	4.92±1.84	3.11±2.71	**0.016***
Caudate right cm^3	2.55±0.86	1.49±1.26	**0.005****
Caudate left cm^3	2.36±1.06	1.61±1.47	**0.054***
Pallidum total cm^3	2.39±0.76	1.27±1.11	**0.004****
Pallidum right cm^3	1.31±0.31	0.66±0.55	**0.001****
Pallidum left cm^3	1.08±0.54	0.62±0.56	**0.039***
Putamen total cm^3	7.02±2.24	4.56±2.86	**0.005****
Putamen right cm^3	3.93±0.71	2.41±1.48	**0.001****
Putamen left cm^3	3.08±1.71	2.16±1.43	**0.054***
Limbic cortex	34.70±11.33	45.05±5.97	**0.003****
Entorhinal area	2.99±1.43	3.47±1.51	0.273
Anterior cingulate gyrus	9.16±4.28	10.79±3.03	0.059
Middle cingulate gyrus	7.55±2.95	11.29±1.82	**0.001****
Posterior cingulate gyrus	8.83±3.73	13.24±6.92	0.07
Parahippocampal gyrus	6.16±1.50	6.20±2.58	0.263
Insular cortex	20.93±11.56	25.39±11.78	0.385
Anterior insula	6.10±3.27	7.49±3.05	0.358
Posterior insula	3.14±1.77	3.94±1.82	0.089
Central operculum	5.95±2.86	7.68±2.63	**0.038***
Frontal operculum	2.59±1.70	3.35±1.54	0.178
Parietal operculum	3.14±2.21	3.93±2.01	0.182

SD: Standard Deviation, **: $p<0.01$, *: $p<0.05$, Mann Whitney U Test was used.

Table 3. Comparison of volume measurements of frontal lobe, temporal lobe, occipital lobe, limbic cortex, and insular cortex in methamphetamine use disorder and healthy individuals.

Cortical	Methamphetamine Mean±SD	Control Mean±SD	*p*
Frontal lobe	164.24±32.415	188.63±51.31	**0.017***
Frontal pole	6.60±1.62	89.62±26.81	0.076
Gyrus rectus	2.65±1.94	3.30±1.72	0.473
Opercular inf. frontal gyrus	5.24±2.24	6.21±2.29	0.186
Orbital inf. frontal gyrus	2.24±0.97	3.24±0.90	**0.002****
Triangular inf. frontal gyrus	6.52±1.77	7.95±2.06	**0.043***
Medial frontal cortex	2.27±1.63	3.28±1.41	**0.036***
Middle frontal gyrus	35.25±7.32	45.29±16.91	**0.031***
Anterior orbital gyrus	3.33±0.98	3.53±1.50	0.606
Lateral orbital gyrus	3.86±1.38	4.27±1.56	0.505
Medial orbital gyrus	6.75±2.62	8.18±1.55	0.151
Posterior orbital gyrus	5.46±1.81	7.96±2.21	**0.001****
Precentral gyrus	24.01±5.82	28.23±3.12	**0.021***
Precentral gyrus medial seg.	4.47±2.49	5.96±1.11	0.148
Subcallosal area	1.80±0.79	2.32±0.75	**0.043***
Sup. frontal gyrus	30.47±4.92	36.04±11.41	0.162
Sup. frontal gyrus medial seg.	12.93±5.12	13.10±3.67	0.85
Supplementary motor cortex	10.13±3.87	12.32±1.30	**0.036***
Temporal lobe	103.67±18.51	116.69±13.44	**0.033***
Fusiform gyrus	13.41±4.38	16.05±2.81	0.186
Planum polare	2.85±1.45	3.93±1.88	**0.017***
Planum temporale	2.47±1.70	3.26±1.89	0.186
Inf. temporal gyrus	24.22±3.38	23.21±4.87	0.473
Middle temporal gyrus	28.60±9.71	33.23±4.08	**0.011***
Sup. temporal gyrus	13.24±3.97	16.12±2.29	**0.02***
Transverse temporal gyrus	2.12±1.42	2.43±1.30	0.458

Temporal pole	16.73±4.14	18.42±3.41	0.213
Parietal lobe	117.78±35.49	115.08±17.27	0.93
Angular gyrus	27.64±15.58	25.04±4.60	0.99
Postcentral gyrus	19.82±5.07	23.49±5.62	**0.024***
Postcentral gyrus medial seg.	1.64±0.86	1.77±0.86	0.562
Precuneus	24.36±8.28	22.14±7.03	0.371
Sup. parietal lobule	28.26±9.63	24.17±3.73	0.057
Supramarginal gyrus	16.04±4.43	17.99±3.53	0.195
Occipital lobe	77.65±13.07	84.55±17.51	0.213
Calcarine cortex	5.42±2.80	7.11±2.54	**0.038***
Cuneus	7.34±4.09	9.03±3.63	0.195
Lingual gyrus	17.02±6.27	16.54±5.19	0.473
Occipital fusiform gyrus	8.77±1.64	9.32±1.76	0.358
Inf. occipital gyrus	14.83±4.25	16.09±5.24	0.372
Middle occipital gyrus	10.27±2.48	11.86±2.57	**0.005****
Sup. occipital gyrus	8.64±1.73	10.05±2.01	**0.011***
Occipital pole	4.96±1.72	5.00±2.49	0.93

SD: Standard Deviation, **: $p<0.01$, *: $p<0.05$, Mann Whitney U Test was used.

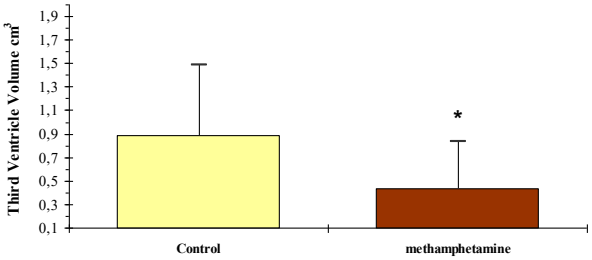

Figure 2. Third ventricle volume values (cm^3) *p=0.022.

Discussion

Substance use disorder is an increasing problem in our country and the world[17]. Methamphetamine, a potent sympathomimetic substance, shows its stimulant effect by causing the release of dopamine and norepinephrine from dopaminergic and noradrenergic nerve endings. Methamphetamine is more dangerous than other stimulants due to its acute complications, long-term neurotoxicity, and high addictive potential. In addition to being addictive, Methamphetamine causes many complications. These include epilepsy, vasculitis, severe headache, hypertension, tachycardia, hyperthermia, increased respiratory rate, and hemorrhagic and ischemic stroke, the most important of which may cause permanent damage to the central nervous system[18]. For this reason, studies on methamphetamine have always remained up-to-date in the literature[19,20]. In the studies on methamphetamine, some of the brain areas have been evaluated, and affected areas have been reported, but not all brain structures have been examined as a whole. In this study, 238 different regions of the brain in MUD and healthy controls were analyzed with the volbrain method, making it one of the first studies. Our study aimed to shed light on the potential changes in brain structure associated with methamphetamine consumption.

One of the most striking findings in studies on is the significant decrease in GM volume in methamphetamine users. It has been suggested that methamphetamine use may lead to structural changes in the brain, especially in decision-making, emotion regulation, and cognitive control[5,21-23] In this study, similar to the literature, decreases in GM volume were found in the measurements performed on MUD. The observed decrease in GM volume may reflect neuron loss and changes in neuron density due to the neurotoxic effects of methamphetamine. Cortical GM, also known as the cerebral cortex, is rich in cell bodies, dendrites, and synapses and plays a vital role in various cognitive, sensory, and motor functions in the brain. Methamphetamine disrupts the delicate balance of neurotransmitters in individuals and triggers neurotoxic effects[24,25]. Prior investigations in the field have consistently reported a notable decline in GM volume among individuals afflicted with MUD[6,24]. In this study, aligning with this existing body of literature, yielded compelling evidence of significantly diminished cortical GM volume, as well as reduced total GM volume within the cerebrum, in individuals diagnosed with MUD. The observed reduction in GM volume can be attributed to several factors, including neuron

loss and alterations in neuron density resulting from the neurotoxic effects of methamphetamine. These structural changes in the cerebral cortex, a dense region with cell bodies, dendrites and synapses, have a profound effect on cognitive, sensory and motor functions in the brain. The disruption of the intricate equilibrium of neurotransmitters induced by methamphetamine usage plays a pivotal role in triggering these neurotoxic effects. Such structural changes in GM may lead to a range of debilitating consequences for affected individuals. Specifically, the documented GM reduction is associated with cognitive deficits, impaired emotional regulation, and compromised executive functions. These impairments collectively underscore the complexity of the challenges faced by individuals grappling with MUD and emphasize the pressing need for comprehensive interventions and targeted treatments to address these profound structural alterations within the brain[24].

Located behind the bulbus and pons, below the tentorium cerebelli, the cerebellum is the most significant part of the rhombencephalon. The cerebellum is a compactly organized structure with many functions: movement, emotional memory, planning, and perception. In the literature, it has been reported that the cerebellum volume decreased in MUD[25-27]. In our study, cerebellum total, cerebellum WM, and cerebellum GM volumes were measured less in MUD. However, it was not statistically significant.

The accumbens is a crucial brain region that mediates various behaviors, including reward and satisfaction. Jernigan et al[11]. conducted a study in which they observed the impact of methamphetamine use on the nucleus accumbens, explicitly focusing on alterations in dopamine release and the volume of this brain region. Their findings revealed that methamphetamine use led to a significant increase in the volume of the nucleus accumbens.

In this study, the researchers assessed the volume of the nucleus accumbens in three specific dimensions: right, left, and total volumes. Their results demonstrated statistically significant increases in the volumes of the right, left, and total nucleus accumbens in individuals with MUD. These findings shed light on the neurobiological changes associated with chronic methamphetamine use, highlighting the profound impact of this substance on the structure and function of the nucleus accumbens, a vital component of the brain's reward system.

The hippocampus which has a role in the limbic system, memory, and especially short-term memory, is known to undergo hippocampal neurodegeneration in methamphetamine exposure[28]. Thompson et al[12] reported that the hippocampus volumes of MUD were 7.8% smaller than healthy subjects. Warton et al[29] found that methamphetamine exposure in the prenatal period was associated with decreased thalamus volume. Similarly, the hippocampus volume was smaller in this study compared to healthy subjects. The thalamus, one of the parts of the diencephalon, was also among the affected parts in MUD. The thalamus, an intermediate station for all sensory stimuli except odor, was volumetrically less in MUD[24]. In this study, the volume of the diencephalon and thalamus was also found to be less in MUD.

The amygdala, one of the subcortical structures, is responsible for controlling emotions, especially fear, and is involved in the formation and storage of memory related to emotional events and activation of the nervous system. Orikabe et al[30] found significant volume reductions in both amygdala and hippocampus in MUD compared to healthy controls. The degree of volume reduction was significantly greater in the amygdala than in the hippocampus. In the present study, amygdala volumes were significantly reduced in MUD.

The basal forebrain, caudate, pallidum, and putamen control many different functions, such as body movement planning, eye movements, and cognitive and emotional functions. These structures have been extensively investigated in studies on addiction. Jan et al[31] found increased putamen volume in MUD. Roos et al[32] found increased putamen volume in children prenatally exposed to methamphetamine. Lin et al[33]

reported that diffusion indices increased in the basal forebrain regions of methamphetamine users but emphasized that this increase was not significant. Berman et al[24] conducted a detailed study on methamphetamine users, reporting that pallidum, putamen, and caudate volumes increased in MUD. In this study, basal forebrain, caudate, pallidum, and putamen volumes were significantly increased in MUD compared to healthy controls.

Studies investigating individuals with MUD have consistently reported significant alterations in cortical brain volumes. Specifically, these investigations have highlighted volumetric changes within distinct cortical regions. For instance, Jia et al[4] documented a reduction in the volume of the Frontal Lobe in individuals with MUD. In concurrence with these findings, Aoki et al[34], Bartzokis et al[35] reported not only decreased Frontal Lobe volumes but also reduced volumes in the Temporal Lobe among MUD-afflicted individuals. Furthermore, Thompson et al[12] observed a diminished volume within the Limbic cortex, further emphasizing the widespread impact of methamphetamine on cortical brain regions. Interestingly, in the literature, there are studies by Jernigan et al[11] reporting an increase in parietal lobe volume in individuals with MUD, as well as studies reporting a decrease in parietal lobe volume[9]. This discrepancy underscores the complexity of structural alterations within the brain in response to methamphetamine use. In our study, we sought to contribute to this body of knowledge by investigating a broad spectrum of cortical regions, in line with the existing literature. Our findings align with prior research in detecting reduced volumes in several cortical regions, namely the Frontal Lobe, Temporal Lobe, Occipital Lobe, Limbic cortex, and Insular cortex among individuals with MUD. This concordance with previous research underscores the consistent nature of cortical volume reduction in MUD. Remarkably, our study also revealed an increase in Parietal lobe volume in individuals with MUD. This unique observation highlights the complexity of the impact of methamphetamine on cortical regions and further emphasizes the need for comprehensive investigations to elucidate the intricacies of structural changes within the brain in response to methamphetamine use.

Limitations

The limitation of this study is that the sample consisted only of male participants. This is due to the lack of female patients in the study's institution. Having only male participants prevented comparison between genders. The strength of our study is that it has significant strength in that it comprehensively examined the effects of MUD on brain structures, including 238 different brain regions. This provides a broader perspective and helps us understand which areas are particularly affected.

Conclusion

In conclusion, findings from studies on individuals with MUD reveal significant structural changes in the brain. MUD is associated with significant changes in the brain, including a decrease in cortical GM. Prolonged and excessive use of methamphetamine disrupts the delicate balance of neurotransmitters and triggers neurotoxic effects in the cortex. This vital region of the brain, responsible for decision-making, impulse control, and judgment, experiences a significant decrease in GM volume. As volume reduction occurs in the frontal lobe, temporal lobe, occipital lobe, limbic cortex, and insular cortex, cognitive deficits, emotional dysregulation, and impaired executive function can occur in individuals struggling with MUD. Understanding these structural changes is crucial not only to explain the mechanisms underlying MUD but also to inform targeted interventions and treatment strategies. The complex interplay between affected brain regions highlights the complexity of MUD and underscores the importance of addressing it as a multifaceted public health problem. Further research is needed to explore these structural changes' functional implications and develop comprehensive approaches to preventing and rehabilitating MUD.

Ethics Committee Approval

Ataturk University Faculty of Medicine Clinical Research Ethics Committee with the ethics committee decision numbered B.30.2.ATA.0.01.00/128 and dated 26.01.2023. This study conformed to the Helsinki Declaration.

Author Contributions

Study concept/design, data collecting: HÖ., GD., data analysis and interpretation GD. NA. NK, literature review, writers: GD., NA, NK, The final version of this article was read and approved by all authors.

Conflict of Interest

There is no conflict of interest to declare.

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Peer-review

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Research Article/Özgün Araştırma

Prognostic factors influencing regorafenib treatment outcomes in metastatic colorectal cancer

Metastatik kolorektal kanserde regorafenib tedavi sonuçlarını etkileyen prognostik faktörler

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Abstract

Aim: We aimed to determine the efficacy and prognostic factors of Regorafenib in advanced colorectal cancer patients.

Materials and Methods: This study was designed as single-center and retrospective. The study included 72 patients with metastatic colorectal cancer treated with Regorafenib. Univariate and multivariate analyses of factors affecting survival were generated by Cox Regression Models.

Results: Twenty-three (31.9%) of the patients were female, the median age was 65 years. The median progression-free survival (PFS) and overall survival (OS) were 4.13 and 8.7 months, respectively. The carcinoembryonic antigen (CEA) level (p=0.001), and Eastern Cooperative Oncology Group (ECOG) score (p<0.001) were found to be prognostic in the multivariate model for PFS. ECOG (p<0.001), CEA level (p<0.001), dose reduction (p=0.003), and side of the primary tumor (p=0.037) were prognostic for OS.

Conclusion: Our study revealed that ECOG, requiring dose reduction during the treatment, and lower baseline CEA levels were found to be prognostic.

Keywords: Regorafenib; Advanced colorectal cancer; Survival.

Öz

Amaç: Metastatik kolorektal kanserli hastalarda Regorafenib'in etkinliğini ve prognostik faktörlerini belirlemeyi amaçladık.

Gereç ve Yöntem: Bu çalışma tek merkezli ve retrospektif olarak tasarlandı. Çalışmaya Regorafenib ile tedavi edilen 72 metastatik kolorektal kanserli hasta dahil edildi. Sağkalımı etkileyen faktörlerin tek değişkenli ve çok değişkenli analizleri Cox Regresyon Modelleri ile oluşturuldu.

Bulgular: Hastaların yirmi üçü (%31,9) kadındı ve medyan yaş 65 idi. Hastalara ait medyan progresyonsuz sağkalım (PFS) ve toplam sağkalım (OS) sırasıyla 4,13 ay ve 8,7 aydı. Karsinoembriyonik antijen (CEA) seviyesi (p=0.001) ve Eastern Cooperative Oncology Group (ECOG) Skoru (p<0,001) PFS için çok değişkenli Cox-regresyon modelinde prognostik bulunmuştur. OS için yapılan çok değişkenli modelde ECOG (p<0,001), CEA (p<0,001), doz azaltımı (p=0,003), primer tümörün olduğu taraf (p=0,037) prognostik olarak bulundu.

Sonuç: Çalışmamız, ECOG skoru, tedavi sırasında doz azaltımı, ve daha düşük başlangıç CEA seviyelerinin OS için prognostik olduğunu ortaya koydu.

Anahtar Kelimeler: Regorafenib; İleri-Evre kolorektal kanser; Sağkalım.

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Introduction

Colorectal cancer (CRC) is a common and lethal disease. According to 2023 cancer statistics data, CRC is the third most diagnosed cancer in men and the second most common in women.[1] Incidence and mortality rates are substantially higher among men than among women. In the United States and many other countries, CRC mortality rates have steadily declined since the mid-1980s. This improvement can be attributed to the earlier detection of CRC and the increased efficacy of primary and adjuvant therapies.[2,3] However, approximately a quarter of newly diagnosed colorectal cancers have an advanced-stage disease at presentation, and some others may develop metastatic disease after potentially curative treatment of localized disease. In the era of fluorouracil as the only active agent, overall survival was approximately 11 to 12 months, but nowadays the average median survival is approaching three years.[4]

Regorafenib is an alternative treatment for metastatic colorectal cancer (mCRC) patients who have been previously treated and failed with chemotherapy, and who are willing to receive additional cancer treatment. Regorafenib provides anti-angiogenesis by activating multi-kinase VEGF receptor inhibition.[5,6] For patients with treatment-refractory mCRC, advanced gastrointestinal stromal tumors after imatinib and sunitinib, and unresectable hepatocellular carcinoma following sorafenib, Regorafenib is an approved alternative medication.[7] Effectiveness in refractory mCRC was first reported in the CORRECT study, where patients who progressed after multiple standard therapies were assigned to regorafenib (160 mg orally once daily, three times every four weeks) or placebo in addition to best supportive care.[8] As shown in the CORRECT study, the efficacy of regorafenib was subsequently verified in the multicenter CONCUR study, in which 204 Asian patients with mCRC who had progressed after standard therapies were randomly assigned to regorafenib or placebo.[9]

We aimed to elucidate the effect of regorafenib on survival as well as prognostic factors affecting the duration of response in mCRC.

Materials and Methods

This study was designed as a single-center and retrospective study. The study included patients with metastatic colorectal cancer who were treated with Regorafenib between 2012 and 2022. The following patients were included in the study: *1)* patients with pathologically proven colorectal cancer; *2)* 18 years of age or older; *3)* with at least one comparable metastatic site confirmed using imaging methods; *4)* no history of concomitant or prior malignancy. Patients receiving immunotherapy were excluded.

All patients received standard chemotherapy for metastatic disease and disease progression during or after the last treatment. Standard imaging modalities (computed tomography, magnetic resonance imaging, and positron emission tomography) used in the center were considered to assess response to treatment. Patients' characteristics such as age, sex, side of the primary tumor, Eastern Cooperative Oncology Group Performance (ECOG) score, initial presentation (de-novo or recurrent), RAS mutation results, anti-vascular endothelial growth factor (VEGF), and anti-epidermal growth factor receptor (EGFR) treatment, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19.9) measurement prior the Regorafenib treatment were recorded from the hospital electronic data record system. The institutional ethics committee approved this study, which was conducted in accordance with the ethical standards of the Declaration of Helsinki.

Statistics

Progression-free survival (PFS) was defined as the time from the start of Regorafenib until any documented clinical progression, relapse, or death from any cause. Overall survival (OS) was defined as the time from the start of Regorafenib treatment until death from any cause. SPSS version 26.0 package program was used for statistical analyses. Survival plots were performed using the Kaplan-Meier curves. Univariate and multivariate analyses of factors affecting

survival were generated by Cox Regression Models. CEA and CA 19.9 levels prior to the Regorafenib treatment were categorized into two groups according to median level. Statistical significance was defined as a *p-value* <0.05.

Results

A total of 72 patients with mCRC were included in this study. Twenty-three (31.9%) of the patients were female, the median age was 65 years and the number of patients with ECOG score ≥2 was 18 (25%). Colon cancer and rectal cancer rates in the patient population were equal. While 8 patients (11.1%) had right-side tumors, the rate of ras mutant patients was 47.2%. Thirty-one patients (43.1%) were de-novo metastatic at baseline, while 52 patients (72.2%) underwent surgery for the primary tumor. The number of patients receiving anti-VEGF therapy was 59 (81.9%), while the percentage of patients receiving anti-EGFR therapy was 52.8%. Patients who received regorafenib treatment at the 4th line or more were 10 (13.9%). Prior to Regorafenib treatment, the median CEA value was 58 mg/dL, while the median CA 19.9 level was 74 mg/dL (Table 1).

Table 1. Clinical-pathological characteristics.

Variable	n (%)
Age	
<65	35 (48.6)
≥65	37 (51.4)
Sex	
male	49 (68.1)
female	23 (31.9)
ECOG	
0-1	54 (75)
≥2	18 (25)
Type of tumor	
colon	36 (50)
rectum	36 (50)
Side of primary tumor	
right side	8 (11.1)
left side	64 (88.9)
Ras Mutation	
yes	34 (47.2)
no	38 (52.8)
Presentation at initial diagnosis	
de-novo metastatic	31 (43.1)
recurrent metastatic	41 (56.9)
Surgery for primary tumor	
yes	52 (72.2)
no	20 (27.8)
Radiotherapy for primary tumor	
yes	18 (25)
no	54 (75)
Anti-VEGF treatment	
yes	59 (81.9)
no	13 (18.1)
Anti-EGFR treatment	
yes	38 (52.8)
no	34 (47.2)
Line of regorafenib treatment	
3rd	62 (86.1)
4th or above	10 (13.9)
Best response to Regorafenib	
Partial Response	2 (2.8)
Stable Disease	22 (30.6)
Progressive Disease	48 (66.7)
Dose reduction	
yes	33 (45.8)
no	39 (54.2)
CEA	
≥58	35 (48.6)
<58	37 (51.4)
CA 19.9	
≥74	36 (50)
<74	36 (50)

%: percent, ECOG: Eastern Cooperative Oncology Group, VEGF: Vascular endothelial growth factor receptor, EGFR: Epidermal growth factor receptor

Progression and survival time

The median PFS and OS for regorafenib-treated patients were 4.13 months and 8.7 months, respectively (Figure 1).

In the univariate analysis for PFS; age (<65 vs. ≥65), sex (female vs male), type of tumor (colon vs rectum), side of the primary tumor (right vs left), Ras mutation (yes vs no), anti-VEGF treatment (yes vs no), anti-EGFR treatment (yes vs no), line of regorafenib treatment (3rd vs 4th or above), dose reduction (yes vs no) showed no significant difference, while ECOG PS (p<0.001), presentation at initial diagnosis (p=0.015), surgery for primary tumor (p=0.033), CEA level (p=0.014), CA 19.9 level (p=0.024) were found to be statistically significant (Table 2). The CEA level {Hazard Ratio (HR)=5.70, 95% Confidence Interval (CI): 1.46-10.60, p=0.001}, and ECOG score (HR=2.46, 95% CI: 1.46-4.16, p<0.001) remained statistically prognostic in the multivariate Cox-regression model for PFS (Table 3).

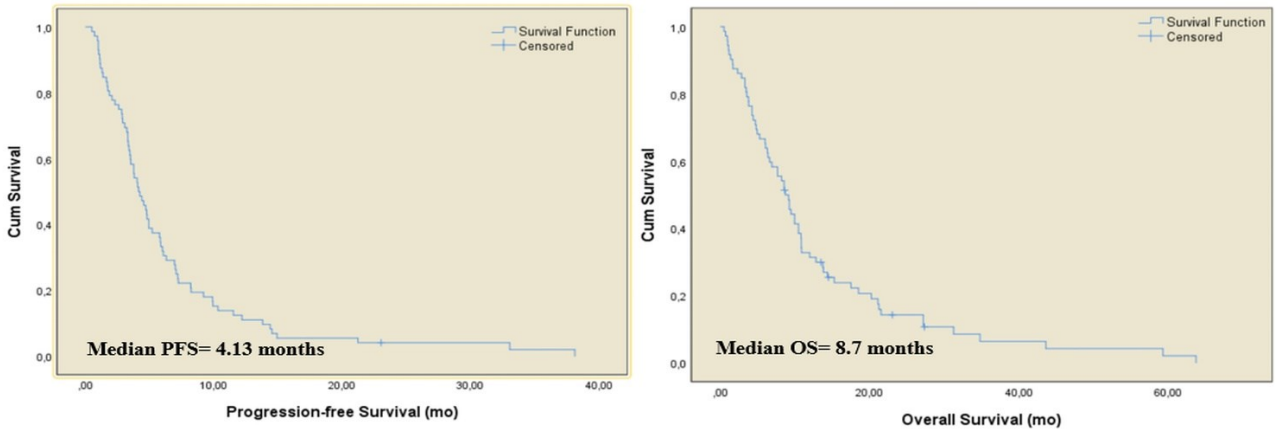

Figure 1. Kaplan-Meier curve of progression-free survival and Overall Survival.

Table 2. Univariate analysis of factors for Progression Free Survival and Overall Survival.

Variable	Progression Free Survival (months)	*p*	Overall Survival (months)	*p*
Age				
<65	4.53 (3.80-5.27)	0.814	9.13 (7.19-11.08)	0.520
≥65	3.77 (2.81-4.72)		8.70 (4.85-12.55)	
Sex				
female	4.70 (2.51-6.89)	0.866	9.23 (4.28-14.19)	0.230
male	4.07 (3.20-4.93)		8.70 (6.60-10.80)	
ECOG PS				
0-1	4.93 (3.65-6.21)	**<0.001**	10.47 (9.06-11.87)	**<0.001**
≥2	1.63 (0.94-2.33)		1.70 (0.31 - 3.09)	
Type of tumor				
colon	3.80 (3.02-4.58)	0.771	7.67 (5.12-10.22)	0.456
rectum	4.77 (3.44-6.09)		10.7 (8.06-12.87)	
Side of primary tumor				
right side	3.40 (2.01-4.79)	0.334	4.93 (2.08-10.75)	**0.047**
left side	4.23 (3.25-5.21)		9.23 (7.08-11.38)	
Ras Mutation				
yes	4.23 (2.81-5.66)	0.717	8.53 (5.96-11.11)	0.442
no	4.03 (2.99-5.08)		9.13 (6.85-11.42)	
Presentation at initial diagnosis				
de-novo metastatic	4.23 (2.92-5.54)	**0.015**	6.60 (2.34-10.86)	0.212
recurrent metastatic	4.13 (1.54-6.73)		9.23 (7.48-10.99)	
Surgery for primary tumor				
yes	4.13 (2.45-5.82)	**0.033**	9.23 (7.78-10.69)	0.720
no	3.80 (2.78-4.82)		6.07 (3.66- 8.48)	
Anti-VEGF treatment				
yes	4.07 (3.14-5.00)	0.220	8.53 (6.37-10.70)	0.222
no	6.00(3.61-8.39)		10.47 (2.56-18-37)	
Anti-EGFR treatment				
yes	4.53 (3.58-5.49)	0.382	8.53 (5.18-11.89)	0.227
no	3.77 (2.96-4.57)		8.70 (6.72-10.68)	
Line of regorafenib treatment				
3rd	4.07 (3.07-5.06)	0.563	8.53 (6.53-10.53)	0.053
4th or above	5.83 (2.58-9.09)		10.83 (2.80-24.91)	
Dose reduction				
yes	4.77 (2.85-6.68)	0.398	10.83 (8.68-12.99)	**0.003**
no	3.77 (2.99-4.54)		6.37 (3.02 - 9.71)	
CEA				
≥58	3.77 (2.92-4.62)	**0.014**	6.37 (4.47 - 8.26)	**0.001**
<58	4.77 (2.46-7.07)		12.80 (8.35-17.25)	
CA 19.9				
≥74	3.77 (3.18-4.35)	**0.024**	6.30 (4.34 - 8.26)	**0.001**
<74	4.77 (3.59-5.94)		10.47 (9.10-11.84)	

ECOG PS: Eastern Cooperative Oncology Group Performance Score, VEGF: Vascular endothelial growth factor receptor, EGFR: Epidermal growth factor receptor, CEA: Carcinoembryonic antigen, CA 19.9: Cancer antigen 19-9

Table 3. Multivariate analyses of factors for Progression Free Survival and Overall Survival

		Progression Free Survival (months)		Overall Survival (months)	
Variable	**Category**	HR (95% CI)	***P*** [f]	HR (95% CI)	***P*** [f]
CEA	<58 vs ≥58	5.70 (1.46-10.60)	**0.001**	3.16 (1.80-5.52)	**<0.001**
ECOG	0-1 vs ≥*2*	2.46 (1.46-4.16)	**<0.001**	6.17 (3.27-11.64)	**<0.001**
Side of primary tumor	right vs left	-	-	0.44 (0.20-0.95)	**0.037**
Dose reduction	yes vs no	-	-	0.43 (0.25-0.75)	**0.003**

[s] Significant values are indicated in bold. P[f]: Forward: LR method.

In univariate analysis established for OS; ECOG score (p<0.001), side of the primary tumor (p=0.047), dose reduction (p=0.*003*), CEA level (p=0.001), and CA 19.9 level (*p=0.001*) were found to be statistically significant (Table 2). The multivariate Cox-regression model revealed that the ECOG score (HR=6.17, 95% CI: 3.27-11.64, p<0.001), the CEA level (HR=3.16, 95% CI: 1.80-5.52, p<0.001), the dose reduction (HR=0.43, 95% CI: 0.25-0.75, p=0.003), the side of the primary tumor (HR=0.44, 95% CI: 0.20-0.95, p=0.037) were found to be prognostic for OS (Table 3).

Discussion

This study elaborated on the survival effect of Regorafenib in mCRC patients and the prognostic factors affecting the duration of response to Regorafenib treatment as a real-life, single-center experience. In our study, we found that Regorafenib can be the preferable treatment for patients who have used prior therapies. Our analyses showed that ECOG score and CEA levels were independently prognostic for PFS, while ECOG score and CEA levels as well as the side of the primary tumor, and the dose reduction were prognostic for OS.

In the CORRECT study, which included 760 patients who progressed after multiple therapies, demonstrated the efficacy of regorafenib in mCRC and received approval, the median OS was 6.4. This study also showed a statistically modest statistically significant improvement in PFS (1.9 months) in patients receiving Regorafenib compared to placebo. In the phase 3 CONCUR study, which evaluated the CORRECT study in a larger Asian patient population, the mOS was 8.8 months. This study, too, demonstrated the OS benefit of Regorafenib vs. placebo. In another large randomized trial, patients receiving regorafenib in later-line therapy for mCRC had a mOS of 5.6 months, and the 12-month survival rate was 22% (10). In our study, median OS and PFS in patients receiving Regorafenib were 4.13, and 8.7 months, respectively.

The REBECCA study, which is one of the real-life studies evaluating the efficacy of Regorafenib used in later-line treatment for mCRC, revealed that OS was unfavorably associated with the following factors: poorer performance status, a shorter time from diagnosis to start of regorafenib treatment, lower regorafenib dose (< 160 mg), > 3 metastatic sites, having liver metastases, and presence of KRAS mutations.[10] In another real-world study, OS was significantly different in subgroups according to ECOG score (ECOG 0/1 vs. 2) and time since initial diagnosis (<18/≥18 months).[11] With the OS benefit of treatment with regorafenib, several studies have been conducted to identify predictive/prognostic markers. In one of these studies, Komori et al. determined CEA and CA19-9 as prognostic markers of PFS. Relationships between treatment outcomes and other laboratory parameters such as high platelet count/high neutrophil/lymphocyte ratio (related to worse OS), or higher lymphocyte count (related to better OS) were also reported in the literature.[12,13] In our study, however, ECOG score, the side of the primary tumor, the dose reduction, CEA, and CA 19.9 were shown as independent prognostic factors for Overall Survival.

Regorafenib can cause adverse events in using mCRC similar to its use in other indications.[14,15] In the CORRECT study, side effects were reported in 93% of patients, which generally improved with dose reduction and drug interruption. Adverse reactions usually seen with regorafenib were hand-foot skin reaction (HFSR), asthenia/fatigue, diarrhea, decreased appetite and food intake,

hypertension, and infections; however, side effects such as severe liver damage, bleeding, and gastrointestinal perforation may also occur.[16] Regorafenib as a small-molecule multiple kinase inhibitor, as can be seen with other drugs in this class, side effects may also be associated with better OS.[17] The CORRECT study suggested that patients who had hand-foot skin reactions had a greater OS. A study of 102 patients with mCRC treated with Regorafenib found that better OS was significantly ($p<0.05$) associated with HFSR and rash, neutropenia, and AST elevations.[18]

Study limitation

This study has several limitations. The main limitation of our study is the retrospective design and the smaller patient population than other studies in the literature. The strength of the study is that it shows real-life data on patients receiving Regorafenib for mCRC.

Conclusion

Regorafenib treatment is a preferable medication in resistant mCRC. Our study revealed that patients with better ECOG score, requiring dose reduction during the treatment, and lower levels of initial CEA were found to be prognostic for OS. It can be used in patients with mCRC who have failed after standard therapies and are willing to receive treatment.

Ethics Committee Approval

The present study was performed in line with the principles of the Declaration of Helsinki. The Tekirdag Namik Kemal University Ethics Committee granted formal approval to this study (approval no: 2023.72.08. 20 on April 25th, 2023).

Informed Consent

Not Applicable.

Authors' contributions

All of the authors contributed at every stage of the study.

Acknowledgments

Not Applicable.

Conflicts of interest/Competing interests

There is no conflict of interest to declare.

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Research Article/Özgün Araştırma

Effects of hydroxychloroquine and azithromycin use on ECG parameters due to COVID-19 in pediatric patient population

Pediatrik hasta popülasyonunda COVİD-19 sebebiyle hidroksiklorokin ve azitromisin kullanımın EKG parametreleri üzerine etkileri

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Abstract

Aim: Due to COVID-19 infection, the use of two drugs, hydroxychloroquine and azithromycin, with a high potential for arrhythmia, came to the fore in the pediatric patient group at the beginning of 2020, during the search for treatment. The aim is to reveal the synergistic arrhythmic effects of these two drugs in prolonging the QT interval on the ECG.

Materials and Methods: First of all, patients taking hydroxychloroquine were identified. Demographic data of these patients were recorded. In addition to hydroxychloroquine, azithromycin and other treatments they used were also recorded. Those with ECG data were selected. Transmyocardial repolarization parameters calculated by ECG were calculated retrospectively (QT, QTc, Tpe, Tpe/QT, Tpe/QTc). Then, laboratory findings and radiological imaging of these patients were recorded.

Results: Twenty-three pediatric patients who met the study criteria were identified. All of the patients were asymptomatic or mild disease. When initial and post-drug ECG parameters were compared; It was observed that the drugs did not have a significant arrhythmogenic effect on ECG parameters, especially QT interval and QTc.

Conclusion: Unlike the literature showing arrhythmic effects of these drugs in adult COVID-19 disease, hydroxychloroquine and azithromycin did not show such an effect in the pediatric population.

Keywords: COVID-19; ECG; QTc; Pediatrics; Arrhythmia.

Öz

Amaç: COVID-19 enfeksiyonu nedeniyle, 2020 yılının başında tedavi arayışı sırasında aritmi potansiyeli yüksek hidroksiklorokin ve azitromisin isimli iki ilacın kullanımı pediatrik hasta grubunda gündeme geldi. Amaç bu iki ilacın EKG üzerinde QT intervalini uzatmadaki sinerjistik aritmik etkilerini ortaya koymaktır.

Gereç ve yöntem: Öncelikle hidroksiklorokin alan hastalar tespit edildi. Bu hastaların demografik verileri kaydedildi. Hidroksiklorokine ilaveten kullandıkları azitromisin ve diğer tedavileri de kayıt altına alındı. EKG verileri olanlar seçildi. EKG ile hesaplanan transmiyokardiyal repolarizasyon parametreleri retrospektif olarak (QT, QTc, Tpe Tpe/QT, Tpe/QTc) hesaplandı. Ardından bu hastaların laboratuvar bulguları ve radyolojik görüntülemeleri kaydedildi.

Bulgular: Çalışma kriterlerine uygun 23 pediatrik hasta tespit edildi. Hastaların tamamı asemptomatik ya da hafif hastalık tablosundaydı. Başlangıç ve ilaç sonrası EKG parametreleri karşılaştırıldığında; başta QT intervali ve QTc olmak üzere EKG parametreleri üzerine ilaçların belirgin bir aritmojenik etkisi olmadığı görüldü.

Sonuç: Erişkin COVİD-19 hastalığında bu ilaçların aritmik etkilerini gösteren literatüründen farklı olarak, hidroksiklorokin ve azitromisin böyle bir etkiyi pediatrik popülasyonda göstermediler.

Anahtar kelimeler: COVİD-19; EKG; QTc; Pediatri; Aritmi.

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Introduction

COVID-19 infection has been a rapidly spreading infection worldwide since the beginning of 2020, and with its acceptance as a pandemic, various treatments have been sought. Treatments for COVID-19 infection in the early stages of the pandemic, either chloroquine (CQ) and hydroxychloroquine (HCQ) alone or in combination with azithromycin (AZT), have been recommended. CQ and HCQ are used in chronic inflammatory diseases such as rheumatoid arthritis and systemic lupus erythromatosus. It has been suggested that it could potentially inhibit virus entry into cells, particularly via the endosomal pathway, by inhibiting glycosylation of host receptors, proteolytic processing, and endosomal acidification in COVID-19 infection.[1]

These drugs, when used alone or in combination with AZT, can prolong the QT interval (QT) pathologically due to genetic or acquired reasons and cause malignant ventricular arrhythmias. Evaluation of QT and corrected QT (QTc) in electrocardiography (ECG) examination is important to reduce and prevent drug-related mortality and morbidity. Indiscriminate use may produce malignant arrhythmias such as “torsades de pointes” or ventricular fibrillation as a result of drug-induced long QT.

CQ and HCQ are drugs included in the aminoquinoline group. They prolong the QT by inhibiting voltage-gated sodium and potassium channels. The most important known side effects are QT prolongation and malignant ventricular arrhythmia. HCQ; It inhibits this channel by binding to the K channel protein, product of the KCNH2 gene. It results in prolongation of repolarization. In cases of congenital long QT, hypokalemia and hypomagnasemia where repolarization is prolonged; It constitutes an important risk factor for severe ventricular arrhythmia, “torsades de pointes”.[2] Inducible risk factors were stated in another study as hypocalcemia, hypokalemia, hypomagnesemia, use of drugs that prolong QT.[3] Use of more than one drug that prolongs QT at the same time increases the risk of arrhythmias.

Adverse cardiovascular side effects have been described, especially in adults.[4] SARS Cov 2 virus, which is the cause of COVID-19, uses the ACE 2 receptor to enter the cell.[4] This receptor is a regulator of two opposite pathways of angiotensin 2 in the renin angiotensin system. The ACE 2 receptor has important cardiac functions. These; It can be listed as a negative regulator on myocardial hypertrophy, fibrosis and diastolic dysfunction. SARS Cov 2 is assumed to cause damage to the heart as well as the respiratory tract via the ACE 2 receptor. The ACE-Angiotensin-II-AT1R axis has been suggested to be the likely mechanism of more severe SARS Cov 2 infection. Stimulation of this axis triggers inflammation, thrombosis, fibrosis, and vasoconstriction.[5]

Acute infection in the pediatric population showed a milder course than the adult population. However, the effects of the COVID-19 infection appeared after the acute period. Kawasaki-like disease in the pediatric age group caused a group of hyperinflammatory diseases called MIS-C in the adolescent age group. These diseases, with their adverse cardiovascular effects, appeared on average 4-6 weeks after the transmission of the infection.

However, there is a potential for arrhythmia in the acute phase of COVID-19 infection. The prevalence of arrhythmia in hospitalized children was 5.5%.[6] This prevalence is the prevalence of the disease itself, regardless of the drug. An increase in the prevalence of arrhythmia can be predicted if drugs that prolong the QT, such as HCQ and AZT, are used.

Pharmacological form: HCQ: Hydroxychloroquine sulfate is produced as 200 mg tablets. The drug has a long half-life. The mean duration has been reported to be 20 days. In case of high dose use in a short time, it can lead to a cumulative toxicity.

AZT: It is a macrolide antibiotic. It has been used in treatment because of its antiviral activity. After entering the cell, it achieves this effect by alkalizing the inside of the cell. It prevents viral phagocytosis through the endosome. Because AZT prolongs QTc, there

is the potential for ventricular arrhythmias such as “torsades de pointes”.

Literature on HCQ use in childhood; mostly consists of adult case series. There is a modest literature on childhood. There is little research in the pediatric literature regarding the electrophysiological effects of HCQ and CQ use over a therapeutic dosage range. We present the data we have obtained in this area to the attention of the reader.

The study investigates the effects of asymptomatic and mildly symptomatic COVID-19 infection on transmyocardial repolarization parameters (QT, QTc, Tpe, Tpe/QT, Tpe/QTc parameters ventricular repolarization parameters) in children receiving HCQ therapy. In this way, we aimed to analyze the data in the group that does not require intensive care follow-up in case of mild disease.

Materials and Methods

Type of research

This study was carried out with the permission of the Turkish Ministry of Health. The study was carried out in a third step hospital, which is the only reference hospital of the city. This is a retrospective cohort study of pediatric patients (0-18 years) using HCQ alone and/or combination therapy with AZT for the treatment of COVID-19 between March 28, 2020 and May 25, 2020.

Study population (research universe)

In our hospital, the diagnosis of COVID-19 was confirmed by PCR tests studied from nasopharyngeal and oropharyngeal swab samples from all patients. ECG monitoring was performed daily in accordance with the drug protocol. Liver kidney functions and electrolytes were also monitored. The patients were hospitalized and did not require organ support treatment.

As a result of the archive scanning, a total of 42 patients who received HCQ treatment were identified among the patients diagnosed with COVID-19. In the archive records, patients with daily ECG follow-ups at the beginning and after the hospitalization were selected. A total of 26 patients were identified. While at least two ECGs were evaluated, those with a minimum of 3 days between the ECG recording dates were included in the study. Three patients with missing ECG data were excluded from the study. ECG data of 23 patients were obtained.

Exclusion criteria:

- Patients without initial ECG in their follow-up. Patients who had an ECG at the beginning but did not have an ECG on the 3rd day at least, and patients who did not complete the requirement to complete ECG examinations at the end of the treatment,
- Patients with QTc>470 millisecond (msec) in initial investigations,
- Patients with central cyanosis and dyspnea; patients with oxygen-free saturation <92%
- Patients requiring intensive care follow-up

Medication dosage: HCQ dose: It was given in accordance with the Turkish Ministry of Health guidelines. [7] On day 1 of treatment: 6,5 mg/kg dose (maximum dose 400 mg) was given twice daily. On days 2-5, half of this dose (maximum dose 200 mg) was given 2 times a day.

AZT dosage: 10 mg/kg once daily (maximum dose 500 mg) on day 1 in children older than 6 months. On days 2-5, a dose of 5 mg/kg was given once a day (maximum dose 250 mg).

Data collection tools

Age, gender, admission complaint, history of comorbid disease, treatments used were recorded as demographic parameters. Laboratory results were scanned. Complete blood count, liver-kidney function tests, electrolyte measurements, enzymes showing cardiac damage were listed as parameters. Radiological records (lung x-rays and thorax computed tomography) were evaluated by the pediatric infectious disease physician. In order to evaluate cardiotoxicity, QRS duration, QT, QTc, Tpe, Tpe/QT, Tpe/QTc parameters measured in ECG were measured by a pediatric cardiologist.

ECG recordings were performed using Nihon Kohden Cardiofax S device with 25 mm/sec and 10 mm/mV 12 leads. QT is the

time between the onset of activation and the end of its repolarization of the ventricular myocardium, represented by the onset of the QRS and the end of the T wave, respectively. The measurement was made in lead II and V5 or V6 with the longest measured value. The heart rate corrected QT (QTc) interval was calculated using Bazett's formula (QT/√R-R). As a general reference, patients with a prolonged resting QTc of ≥ 470 ms, regardless of cause (congenital or acquired), were considered a risk marker for "torsades de pointes" or ventricular fibrillation. Tpe; It was calculated as the time interval between the peak value of the T wave and the end of the t wave.

Echocardiography was not performed in our patients due to the risk of contamination.

Among the laboratory parameters, hypokalemia, hypomagnesemia and various factors that could prolong QT and QTc were taken into consideration. Inducible risk factors were defined as hypocalcemia, hypokalemia, and hypomagnesemia.

Among the treatment options, it was observed that many patients received additional treatments, especially AZT, in addition to HCQ treatment.

Analysis of data

Parametric numerical values were expressed as mean±standard deviation. Age, platelet, lymphocyte count, polymorphonuclear lymphocyte, urea, AST, ALT, albümin, total protein, alkaline phosphatase, lactate dehydrogenase (LDH), INR, aPTT, Troponin I, fibrinogen and electrolytes were laboratory parameters with a statistically normal distribution. All ECG numerical parameters showed a statistically normal distribution. Student`s t-test was used to compare variables. Chi-square test was used for categorical variables. p value <0.05 was considered statistically significant. Pearson correlation analysis was used.

Ethics comittee Approval

Our study was carried out in accordance with the Declaration of Helsinki Principles. Our study was approved by the local ethics committee (Ethics Committee Approval Date and Number: 2020: 8/11)

Results

Between March 28, 2020 and May 25, 2020, a total of 23 patients, 9 boys (40%) and 14 girls (60%) who used HCQ therapy and whose ECG follow-up were performed, were identified (Table 1). The mean age was 13.3±2.8 years.

Most of the patients did not have a comorbid disease. One patient was receiving epilepsy treatment, and one patient had Down syndrome. Asymptomatic patients were 22%. The most common symptom was cough (26%). Chest radiography consistent with viral pneumonia was seen in 26%. Chest X-ray was evaluated as normal in 17% of the patients. All patients were using HCQ. The number of patients who received the combination with AZT was 5. Patients receiving triple therapy in combination with AZT and oseltamivir comprised 52% of the entire study population (Table 1).

Numerical laboratory parameters such as hemoglobin, white blood cell count (WBC), GGT, D-dimer, ferritin, creatine kinase (CK), creatine kinase-myocardial band (CK-MB) and lactate values were parameters that did not show a statistically normal distribution (Table 2). These parameters were expressed in the Table 2 with min-max values, unlike other parameters. All ECG measurements and other laboratory parameters showed statistically normal distribution. These parameters are included in the Table 2 with their mean and standard deviation values.

WBC, neutrophil, lymphocyte and hemogram numerical values obtained from the patients were recorded to be within normal laboratory values.

Parameters showing cardiac damage: Cardiac Troponin I, CK-MB and D-dimer values were recorded within normal ranges.

Calcium, magnesium, potassium and sodium values were found to be normal in the evaluation in terms of inducible risk factors.

Table 1. Summary of pediatric COVID-19 infections characteristics.

Parameters		**Patients**	
		Number/n	**Percent /%**
Gender	Girl	14	60
	Boy	9	40
Complaint	None	5	22
	Weakness	4	18
	Fever and headache	5	22
	Cough	6	26
	Diarrhea vomiting	1	4
	Facial paralysis	1	4
	Sensory Loss	1	4
Comorbid Condition	None	21	92
	Epilepsy	1	4
	Down syndrome	1	4
Lung X-ray	Normal	4	17
	Mild infiltration	6	31
	Paracardiac infiltration	**7**	26
	Compatible with viral pneumonia	**7**	26
Torax BT	None	11	48
	Normal	5	22
	Ground glass densities of viral pneumonia	7	30
Treatment	HCQ	2	9
	HCQ+ AZT	5	22
	HCQ+ AZT +Oseltamivir	12	52
	HCQ+ Acyclovir	1	5
	HCQ+ Favipavir	3	12

Table 2. Summary of pediatric COVID-19 infections demographic and laboratory findings.

Numeric parameter	**n**	**Mean**	**Standard deviation (±)**
Age (years)	23	13.3	2.8
Platelet ($x10^3$)	23	247.4	72.1
Lymphocyte Count ($x10^3$)	23	1892.8	636
Polymorphonuclear lymphocyte ($x10^3$)	23	3660.6	2021.4
Urea (mg/dl)	23	20.5	4.9
AST (U/L)	23	19.8	5
ALT (U/L)	23	16.1	5
Albumin (g/L)	23	4	0.4
Total Protein (g/L)	10	7.6	0.4
Alkaline Phosphatase (U/L)	22	179.4	84.5
LDH (U/L)	23	215.5	74.8
INR	21	1.1	0.4
aPTT (sec)	21	30.1	4.1
Troponin I (ng/L)	22	0.01	0
Fibrinogen (mg/dl)	22	317.3	139.1
Sodium (mmol/lt)	23	139.4	3
Potassium (mmol/lt)	23	4.2	0.4
Calcium (mg/dl)	23	9.3	0.7
Magnesium (mg/dl)	14	2	0.1
Numeric parameter	**n**	**Min**	**Max**
Hemoglobin (g/dl)	23	9.2	15.9
WBC	23	2745	11440
GGT (U/L)	23	7	66
D-dimer (ng/ml)	22	84	3620
Ferritin (µg/L)	21	5.4	405
CK (µg/L)	22	29	194
CK-MB (µg/L)	21	2	13.1
Lactat (mg/dl)	16	0.9	374

While there were 2 patients who received HCQ treatment alone, combination therapy was used in the other 21 patients. (Table 1). Only one patient had a QTc value of 450 msec after HCQ treatment. None of the patients required inotropic therapy. No patient developed ventricular arrhythmias or "torsades de pointes" with treatment. All patients were in sinus rhythm.

A 20 msec prolongation of the QT was noted at the next value after drug use compared to the baseline value. No significant prolongation was observed in QTc measurements. No difference was observed between Tpe values. A statistical difference in Tpe/QT ratio was recorded in the measurements of Tpe/QT and Tpe/QTc. However, this difference was not clinically significant (Table 3). In addition, the last QTc value was detected as 400 msec in the epilepsy patient using levatiracetam.

Table 3. Summary of pediatric COVID-19 infections ECG findings.

ECG parametrers	n	Mean	Standard deviation (±)	*p* value
QT baseline value	23	354 (msec)	29.2	**QT baseline value - QT final value: 0.012**
QT final value	23	374.4 (msec)	26.3	
QTc baseline value	23	411.4 (msec)	16.6	QTc baseline value-QTc final value : 0.354
QTc final value	23	414.8 (msec)	17.9	
Tpe first value	23	70 (msec)	9.4	Tpe fisrt value- Tpe last value 0.464
Tpe last value	23	68.1 (msec)	8.9	
Tpe/QT first value	23	0.19	0.027	**Tpe/QT first value- Tpe/QT last value: 0.02**
Tpe/QT final value	23	0.18	0.026	
Tpe/QTc first value	23	0.17	0.02	Tpe/QTc first value- Tpe/QTc last value: 0.372
Tpe/QTc final value	23	0.16	0.01	

Discussion

This study aims to examine the reliability of the combined use of HCQ and AZT; planned to determine arrhythmia potentials.

The ratio of asymptomatic patients to the patient population in our study is similar to that in larger series.[8]

T wave shows ventricular repolarization. Transmyocardial parameters are measurements based on the T wave. Measurement of these parameters indicates the risk of ventricular arrhythmia. These parameters are: Tpe, QT, QTc, Tpe/QT and Tpe/QT. Transmyocardial repolarization parameters including Tpe, QT interval, QTc, and Tpe/QT ratio have been reported to be associated with increased risk of cardiac arrhythmia.[9]

A study by Ece et al. included a population of pediatric patients infected with COVID-19.[9] Patients not taking QT prolonging drugs were included. The arrhythmia potential of the COVID-19 infection itself was evaluated. QT and QTc dispersions Tpe parameters were found to be significantly higher in the patient group compared to the healthy pediatric population. This study suggested that with the disease itself, drugs such as HCQ and AZT to be used to treat would increase the potential for arrhythmia.

Arrhythmia has been documented with short-term use of high-dose HCQ in patients diagnosed with critical COVID-19 infection in the adult review.[10] Risk reduction strategies for arrhythmia by ECG monitoring have been recommended in all patients. Although ECG monitoring is helpful in preventing "torsades de pointes", post-baseline ECG monitoring in pediatric patients was unnecessary in terms of reducing exposure to infected patients in repeated follow-ups. Although QT prolongation is statistically significant in studies, clinical arrhythmia is extremely rare, as reported in many other studies.

Timing of QTc prolongation; A study reported that QTc prolongation was recorded maximum 3-4 days after starting HCQ and AZT drugs.[11] In our study, the minimum period between ECG recording dates was 3 days, which coincided with the period specified in the mentioned article. In this way, we evaluated the potential for ventricular arrhythmias. We calculated the prolongation in QT and QTc.

Effects of antimalarial use on the cardiovascular system: In a meta-analysis review conducted in 2018, no side effects were found in the evaluation of cardiovascular side effects in patients using antimalarial therapy (mostly young).[12] In that review, we see that there are 7 studies of children using CQ. In these, most of the children did not have comorbid disease except malaria.

Factors determining QT prolongation differ from adults: The most important determinant of risk in patients with QT prolongation has been shown to be severe COVID-19 infection and use of QT prolonging drugs for adult patients. Our study targeted a population of less sick children who did not require intensive care monitoring. Studies were conducted in adults with severe disease under the influence of a cytokine storm or myocarditis. Receptors to which SARS Cov 2 binds in adult patients cause a different course than children. In underlying comorbid conditions such as diabetes, obesity and chronic lung disease, the behavior of this pathway changes in adults, leading to a severe course of the disease in adults. The disease is mild in children. Our patient group consists of cases with asymptomatic or mild disease. A possible risk factor for QT prolongation in our study is drug use. However, no significant QT prolongation was found in our study results. Another inducible risk factor in adult studies is electrolyte abnormalities. [13] In our study, there was no patient with severe electrolyte problems. The susceptibility to electrolyte problems is higher in adults. Electrolyte abnormalities can also be seen with renal effects and hyperinflammation-cytokine storm through the receptor to which SARS Cov 2 binds.[5]

Studies by the amount of QT prolongation: There are reviews reporting that the use of AZT and HCQ prolongs the QT and QTc by 40-60 msec.[14] In our study, unlike the literature, we did not detect any obvious QT and QTc prolongation in any group. In a study conducted in the healthy group, the drug alone prolonged the QTc by an average of 16 msec.[15] This finding is consistent with our study. No significant prolongation was recorded even in combination with other drugs in the case that did not require intensive care follow-up.

In a retrospective population study, it was reported that the combined use of HCQ and AZT increases cardiovascular morbidity and reveals the potential for heart failure.[16] In this study, which included a very large population, it was determined that the use of HCQ in combination with amoxocillin or sulfasalazine had no effect on cardiovascular mortality. This study shows that adverse side effects occur synergistically with the use of the two drugs. In our study, 52% of the patients used this combination. We did not find any significant arrhythmia potential among our findings in the smaller patient population without inducible risk factors.

In a meta-analysis of the combination of HCQ with AZT, the drug itself was shown to be effective in increasing the QTc above 500 msec.[17] It is also reported in this study that the incidence of arrhythmia is lower than previously stated. In order to make a similar observation in our study, we excluded patients with QT>470 msec on initial ECG. At the same time, there was no patient with QT>450 msec in the initial ECG among our patients.

Pediatric population studies: In the study of Samuel et al., 36 participants were divided into 3 groups. The groups were as follows; those taking HCQ alone, those taking the HCQ-AZT combination, and those not taking either drug. A statistically significant QT prolongation was found in the group receiving HCQ alone.[18] Significant arrhythmia was noted in 6 patients (17%). Significant ECG findings (longest daily measured QTc and baseline ECG abnormalities) were not significantly associated with arrhythmias. Our study is similar in this aspect.

In a single-center retrospective study by Tuncer et al., HCQ was used with or without AZT.[19] A total of 21 patients were included in the study. They reported that children in this patient group consisted of children who were not severely affected by COVID-19 infection. In this patient population, a serious arrhythmia did not develop as in our population. Our study also introduced additional parameters to the literature to assess the potential for arrhythmia.

In the review study of Parthasarathy et al. in the pediatric population, they reported that the literature on HCQ and QTc prolongation was variable. [20] They reported that QTc prolongation generally occurred on the 1st-4th day of drug use in studies.

In another study involving pediatric cases, data from 20 centers were evaluated. Treatment containing HCQ was started in 78 patients. ECG abnormality was not detected in any of these patients.[8] 59 patients used combination therapy containing HCQ and AZT. No significant arrhythmia developed in this group either. There was no serious illness in this population. This study supports our study in terms of study population and findings.

Conclusion

In general, there is a lack of information about the cardiac rhythm effects of therapeutic HCQ/AZT use in pediatric populations. We think that it will fill the knowledge gap in the literature on this subject. In cases of possible viral epidemics, upper respiratory tract pathogens show pathogenic characteristics with similar mechanisms. In such a case, reuse of these drugs in large patient populations may come to the fore due to their generally accepted antiviral activities. The effects of these drugs on myocardial action potential should be kept in mind. The drugs themselves are unlikely to prolong the QT and predispose to malignant ventricular arrhythmias, although they do exist.

Limitations of this article

The study was retrospective and carried out in a single center. Children who applied to the center and required hospitalization are few in number. These drugs were started in the early stages of the pandemic with the possibility of being effective in the treatment of the disease. In the treatment of COVID-19 infection, HCQ and AZT treatment were suspended in the next period.

The patient group consisted of the pediatric population. In this respect, data were collected in a limited area in a relatively less patient population (patient group that did not require intensive care follow-up). Considering the effects that can be seen in the use of these drugs other than intensive care; patients in this group will represent a larger number of people across the population. We hope that it will shed light on the future if it is used in indications arising from various requirements in the future.

Ethics Committee Approval

Ethics committee approval was obtained with the decision of the Ethics Committee for Non-Interventional Procedures of Adıyaman University, dated and numbered 2020: 8/11. The study was conducted under the principles of the Declaration of Helsinki.

Informed Consent

Data concerning the study were collected with the permission of the Adıyaman Provincial Health Directorate.

Authors Contrubituons

All of the authors contributed at every stage of the study

Conflict of Interests

There is no conflict of interest to declare.

Financial Disclosure

No person/organization is supporting this study financially.

Statements

These research results have yet to be presented anywhere previously. Data related to the study is available on request.

Peer-review

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Research Article/Özgün Araştırma

The impact of maternal age distribution on pregnancy-related complications and neonatal outcomes: a single-center retrospective experience

Anne yaşı dağılımının gebelikle ilişkili komplikasyonlar ve neonatal sonuçlar üzerindeki etkisi: tek merkezli retrospektif bir deneyim

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Abstract

Aim: To determine possible risks for mother and baby in advanced age pregnancies.

Materials and Methods: This study is a retrospective archive review examining 14192 cases who gave live births between 24 and 42 weeks between 2020-2023.

Results: The frequency of preeclampsia, gestational hypertension, gestational diabetes mellitus, rupture of membranes and possible miscarriage was high in advanced-age pregnant women compared to other groups. When the groups were compared according to neonatal outcomes, the frequency of low birth weight in adolescence was high than in advanced-age pregnant women. When the groups were compared in terms of macrosomia, the frequency of macrosomia was high in the older age group than in the other groups.

Conclusion: It should be known that pregnancies at an advanced-age can be more complicated for both mother and baby, and pregnancy follow-up should be done more carefully.

Keywords: Advanced age pregnancy; Adolescent pregnancy; Pregnancy complications; Premature rupture of membranes; Neonatal outcomes.

Öz

Amaç: İleri yaş gebeliklerde anne ve bebek için olası riskleri belirlemektir.

Gereç ve Yöntem: Bu çalışma, 2020-2023 yılları arasında 24 ila 42 haftalar arasında canlı doğum yapmış 14192 vakayı inceleyen retrospektif bir arşiv taramasıdır.

Bulgular: İleri yaş gebelerde preeklampsi, gestasyonel hipertansiyon, gestasyonel diabetes mellitus, membran rüptürü ve olası düşük sıklığı diğer gruplara göre yüksekti. Neonatal sonuçlara göre gruplar karşılaştırıldığında Adölesan yaşta düşük doğum ağırlığı sıklığı ileri yaş gebelere göre daha yüksekti. Makrozomi açısından gruplar karşılaştırıldığında makrozomi sıklığı ileri yaş grubunda diğer gruplara göre daha yüksekti.

Sonuç: İleri yaştaki gebeliklerin hem anne hem de bebek için daha komplike olabileceği bilinmeli ve gebelik takipleri daha dikkatli yapılmalıdır.

Anahtar Kelimeler: İleri yaş gebelik; Adölesan gebelik; Gebelik komplikasyonları; Erken membran rüptürü; Neonatal sonuçlar.

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Introduction

In recent years, there have been significant changes in the age of pregnancy, the number of births, and the rate of pregnancy-related complications in women. In the United States alone, while a total of 3,613,647 births were recorded in 2020, there was a 4% decrease compared to 2019. Also, there has been an 8% decline in the number of births in the adolescent female population aged 15-19. The number of births for women aged fifty and over has generally increased since 1997.[1] Globally, while there were 64.5 births per 1000 women in the adolescent age group in 2000, this number decreased to 41.3 births per 1000 women in 2023. This decline shows a general decrease, although it varies proportionally in countries with different socioeconomic levels.[2] According to data from the General Directorate of Population and Citizenship of Turkey, 2.3% of all women giving birth in 2009 were aged 40 and over, while this rate rose to 2.8% in 2014.[3]

The decrease in birth rates and the postponement of the age of pregnancy are associated with the increase in education level, the awareness of protection methods, and the increase in women playing an active role in the workforce. Family planning strategies developed to prevent grandmultiparity, adolescent pregnancies, and related complications, as well as assisted reproductive techniques (ART) implemented for advanced-age infertile groups, also contribute to this process. However, in societies where grand multiparity is common due to socio-cultural structuring, beliefs, the existence of inadequate education level, and low economic development, where adolescent girls are married at a young age and are not sufficiently knowledgeable about birth control methods, advanced-age pregnancies are also frequently encountered. There are many studies in the literature that reveal the relationship of maternal morbidity and mortality with these parameters.[4-6]

The postponement of the age of pregnancy has increased the rate of advanced-age pregnancies; hence, the investigation of complications developing due to advanced-age pregnancies has recently come to the fore. Maternal complications include gestational diabetes mellitus (GDM), gestational hypertension (GHT), and cesarean delivery, while adverse perinatal outcomes include a high rate of chromosomal abnormalities, miscarriage, threatened preterm labour (TPTL), admissions to the neonatal intensive care unit (NICU), and stillbirth.[7] Although there are many studies investigating the effect of advanced maternal age on prenatal and postnatal outcomes, the results are contradictory. Again, studies investigating the complications caused by advanced maternal age, which arises independently of the postponement of pregnancy and ART, due to socio-cultural, and socio-economic conditions, are limited. The aim of this study is to reveal and compare the peri-postpartum complications and neonatal outcomes occurring in pregnant women who gave birth in the same socio-economic level region without resorting to family planning and assisted reproductive techniques in different age groups. To evaluate the complications in advanced age and adolescent pregnancies in the most homogeneous way.

Materials and Methods

The adolescent pregnancy age was determined as 18 and under; advanced maternal age was considered 35 and over in line with the literature.11 Patients were divided into three groups 18 and under (Group 1), between 19 and 34 (Group 2), and 35 and over (Group 3) and these parameters were compared and analyzed using statistical methods. Verbal consent was acquired from the participants or their legal representatives in the study, which was managed in accordance with the principles of the Declaration of Helsinki. The primary aim was to determine maternal complications associated with age, and the secondary purpose was to reveal neonatal outcomes related to maternal age.

Type of the study

The study is a retrospective archive review.

The sample size of the study

A total of 14192 pregnant women who had a live normal vaginal delivery between 24-43 weeks of gestation in Van Regional Training

and Research Hospital between 2020-2023 were included in the study.

Data collection tools

Multiple pregnancies, patients who had cesarean deliveries, patients who got pregnant through ART, intrauterine dead fetuses, and stillbirths were excluded from the study. The demographic characteristics of the patients (age, body mass index, gravida, antepartum and postpartum haemoglobin values, socio-economic status information); pregnancy-related diseases (GDM, GHT, preeclampsia, PROM, EDT, post-term pregnancy, abortus imminens, hyperemesis gravidarum); neonatal outcomes (estimated fetal weight, 1st and 5th minute APGAR scores, need for intensive care, low birth weight evaluated as below the 10th percentile, macrozomy referring to birth weight of 4000 gr and above) were collected from the hospital database. We excluded patients with fetal death from the study, as the neonatal results of birth weight and week of birth may affect apgar results

Patients' socio-economic statuses were separated as low, medium, and high. This classification was determined based on the household income-expenditure level of the patients. Due to the variable income level of our country and the inflation rate not being constant, those with an income level lower than the expenditure level were evaluated as low-income level, those equal to the expenditure level as medium, and those with an income level high than the expenditure level as high economic level. We considered the education level from demographic data as the social level and evaluated it accordingly. The diagnosis of GDM was made when the fasting glucose value was above 92mg/dL; the 1st-hour postprandial glucose value was above 180mg/dL; 2nd-hour postprandial glucose value was above 153mg/dL in patients who had a 75mg OGTT (oral glucose tolerance test) applied between 24-28 gestational weeks.8 The diagnosis of GHT was made when the systolic blood pressure was 140mmHg/ diastolic blood pressure was 90mmHg and above measured at least 4 hours apart from the 20th gestational week. The diagnosis of preeclampsia was made when proteinuria accompanied hypertension or when end-organ effects appeared (platelet count in the blood being below 100 X 109/L, serum creatinine level being above 1.1 mg/dL or the development of renal failure with a two-fold increase from the start, impaired liver function tests with transaminases increased more than twice the normal, pulmonary oedema, the presence of new-onset headache with visual symptoms).[9] The diagnosis of PROM was made when the gestational membrane rupture was observed before the 37th gestational week.[10]

Ethics committee approval

The study received non-interventional ethics committee approval from the Ethics Committee of Van Regional Training and Research Hospital. The approval number is 2023/01-05.

Data analysis

Data analysis was done with a licensed SPSS 22.0 program. ANOVA test was used to compare more than three normally distributed groups. Tukey's HSD post hoc test was used to determine the differentiations between the groups. Paired sample t-test was used to compare dependent groups. Fisher's Exact test was used to compare categorical variables. The statistical significance level was determined as α=0.05.

Results

A total of 14,192 individuals were included in the study: 1,185 (8.4%) from Group 1, 11,147 (78.5%) from Group 2, and 1,860 (13.1%) from Group 3. The average age of the pregnant individuals was 26.89±5.94 years.

The distribution of age groups according to maternal characteristics is presented in Table 1. There was a significant differentiation between the groups in terms of gravida, antepartum and postpartum haemoglobin values. The numbers of parity and gravida were similar in Groups 2 and 3 and significantly high than in Group 1. There was a significant differentiation between the groups in terms of antepartum and postpartum haemoglobin values. Haemoglobin values decrease from Group 1 to Group 3. There was no significant differentiation between the groups in terms of body mass index. No

statistically significant differentiation was between the groups in terms of socioeconomic status.

Table 1. Distribution of groups according to maternal characteristics.

		Grup 1 n:1185 (%)	Grup 2 n:11147 (%)	Grup 3 n:1860 (%)	*p*
Gravida (Mean±SD)		1.00±0.0[a]	1.42±1.52[b]	1.44±1.04[b]	**0.001****
Parity (Mean±SD)		0.00±0.0 [a]	2.27±1.44 [b]	2.32±1.15 [b]	**0.001****
Antepartum Hemoglobin (Mean±SD) (g/dL)		13.14±1.19[a]	12.73±1.37[b]	11.23±1.28[c]	**0.01****
Postpartum Hemoglobin (Mean±SD) (g/dL)		12.14±1.3[a]	11.47±1.46[b]	10.39±1.39[c]	**0.03****
Differentiation Of Antepartum And Postpartum Hemoglobin (Mean±SD) (g/dL)		1.00±0.53[a]	1.26±0.71[b]	0.84±0.35[c]	**0.001****
Economic status	**Low**	281	2356	387	0.345*
	Medium	769	7729	1273	
	High	135	1062	200	
Body mass index (BMI) (kg/m²)	**25 and below**	252 (21.3)	2341 (21.0)	404 (21.7)	0.237
	Over 25	933 (78.7) [a]	8806 (79.0) [a]	1456 (78.3) [a]	

Abbreviations: SD; Standard deviation. * Fisher exact test ** ANOVA test, Values in bold represent statistically significant results. Column percentages are given. [a,b, c] shows the differentiations between the groups.
Patients were divided into three groups 18 and under (Group 1), between 19 and 34 (Group 2), and 35 and over (Group 3). Subgroup comparisons are indicated in superscript (a, b, c). The same letters indicate that the groups are similar, and different letters indicate that the groups are different

The distribution of age groups according to maternal complications is presented in Table 2. There was a significant differentiation between the groups in terms of GDM. The frequency of GDM was highest in Group 3 (p<0.05). There was a significant difference between the 1st and 2nd groups and the 3rd group in terms of GHT and premature rupture of membranes (PROM). The frequency of PROM and GHT was highest in Group 3 (p <0.05). There was a significant differentiation between the groups in terms of TPTL and post-term pregnancy. The frequency of TPTL and post-term pregnancy was high in Group 1 (p<0.05). There was a significant differentiation between the groups in terms of preeclampsia. The frequency of preeclampsia in Group 3 was significantly high than in the other groups (p<0.05). There was also a significant differentiation between the groups in terms of abortus imminens. The frequency of threatened miscarriage was significantly high in Group 3 (p<0.05). However, there was no differentiation between the groups in terms of hyperemesis gravidarum (p>0.05).

Table 2. Distribution of groups according to maternal complications.

		Grup 1 n:1185 (%)	Grup 2 n:11147 (%)	Grup 3 n:1860 (%)	*p*
Gestational Diabetes n(%)	**Yes**	32 (2.7) a	346 (3.1) a	337 (18.1)b	**0.001***
Gestational Hypertension n(%)	**Yes**	5 (0.04) a	56 (0.05) a	87 (4.7) b	**0.001***
Premature rupture of membranes n(%)	**Yes**	37 (3.1) [a]	479 (4.3) [a]	478 (25.7) [b]	**0.001***
Premature birth threat n(%)	**Yes**	84 (7.1) [a]	123 (1.1) [b]	22 (1.2) [b]	**0.009***
Post-term pregnancy n(%)	**Yes**	254 (21.4) [a]	134 (1.4) [b]	28 (1.5) [b]	**0.001***
Preeclampsia n(%)	**Yes**	3 (0.3) [a]	42 (0.4) [a]	507 (27.3) [b]	**0.001***
Abortus imminens n(%)	**Yes**	18 (1.5) [a]	212 (1.9) [a]	422 (22.7) [b]	**0.001***
Hyperemezis gravidarum n(%)	**Yes**	28 (2.4)	256 (2.3)	41 (2.2)	0.240*

Abbreviations: SD; Standard deviation. * Fisher exact test ** ANOVA test, Values in bold represent statistically significant results. Column percentages are given. a,b, c shows the differentiations between the groups.
Patients were divided into three groups 18 and under (Group 1), between 19 and 34 (Group 2), and 35 and over (Group 3).

The distribution of groups according to newborn characteristics is presented in Table 3. There is a significant difference between the groups in terms of estimated birth weight. The average estimated birth weight also increases from group 1 to group 3. While there is no difference between group 1 and group 2 in terms of intensive care needs, group 3 is different from these two groups. The frequency of need for intensive care is higher in Group 3.

There is significant differentiation between the groups regarding low birth weight. Groups 1 and 2 are similar, but they have a high frequency of babies with low birth weight compared to Group 3. Significant differentiation was found in terms of macrosomia between the groups. Group 3 has a high frequency of having macrosomic babies than Groups 1 and 2. However, there is no significant differentiation between the groups when considering APGAR scores

Table 3. Distribution of groups according to neonatal outcomes.

		Grup 1 n:1185	Grup 2 n:11147	Grup 3 n:1860	*p*
Estimated fetal weight		2969.29±350.90 [a]	3079.58±610.47 [a]	3273.18±351.69 [b]	**0.003****
APGAR 1 min		7.71±0.58	7.72±0.59	7.77±0.51	0.718**
APGAR 5 min		9.05±0.29	9.03±0.36	9.05±0.35	0.760**
NICU) n(%)	**Yes**	108 (9.1) [a]	1215 (10.9) [a]	432 (23.2) [b]	**0.026***
Low birth weight n(%)	**Yes**	63 (5.3) [a]	613 (5.5) [a]	233 (12.5) [b]	**0.001***
Macrosomia n(%)	**Yes**	122 (10.3) [a]	1092 (9.8) [a]	294 (15.8) [b]	**0.001***

Abbreviations: NICU; Neonatal intensive care unit, , SD; Standard deviation. * Fisher exact test ** ANOVA test, Values in bold represent statistically significant results. Column percentages are given. [a,b, c] shows the differentiations between the groups
Patients were divided into three groups 18 and under (Group 1), between 19 and 34 (Group 2), and 35 and over (Group 3).

Discussion

The significant correlation between maternal age at conception and pregnancy outcomes, as well as maternal health, has been well established over time. Numerous studies have been conducted on the maternal and neonatal outcomes of adolescent pregnancies. In these studies, advanced maternal age is typically examined within groups using assisted reproductive technologies, while adolescent pregnancy studies are usually carried out in regions with lower socioeconomic status. As a result, no homogeneous studies have been conducted. Our aim is to evaluate the maternal complications of both advanced maternal age and adolescent pregnancies in a homogeneous population, with the goal of predicting the prognosis and treatment approach for these patients.

In a study conducted in Japan analyzing the outcomes of 325 adolescent pregnancies and 2029 pregnancies in women aged 28-30, it was found that adolescent pregnancy did not have a correlation with adverse obstetric outcomes, aside from TPTL. [12] In alignment with previous literature, our study also identified that the risk of TPTL was significantly high in the adolescent pregnancy group (p=0.009). Furthermore, the rate of post-term pregnancy was also found to be significantly high in the adolescent group in comparison to other groups (p=0.001).

In a 2019 study investigating pregnancy-related complications of advanced maternal age, the risk of GDM and preeclampsia was found to be significantly high in the advanced maternal age group compared to the control group. The incidence of TPTL was also found to be statistically high in the advanced maternal age group .[13] Similarly, in our study, we found that the incidence of GDM, GHT, preeclampsia, and abortus imminens was statistically high in the advanced maternal age group compared to the adolescent and control groups (p=0.001, p=0.001, p=0.026, respectively). When our study evaluated hyperemesis gravidarum, no significant differentiation was identified between the groups (p=0.240).

In a large population study by Althabe et al. encompassing seven middle-income countries, they reported no increase in the risk of adverse maternal outcomes in adolescent pregnancies compared to adults. However, the risk of TPTL and low birth weight was reported to be statistically high in the adolescent group, with the highest risk in the group under 15 years of age.[14] In a cohort study examining the maternal risk factors of low birth weight in neonatal complications, it was found that women under the age of 19 and over the age of 39 had a 4% and 14% high rate of having low birth weight babies compared to mothers aged 19-34.[15] In another study related to low birth weight, it was reported that this risk was high in pregnant women over the age of 45. The incidence of macrosomia independent of parity was also

statistically significantly increased with advanced maternal age.[13] In our study, we found that the incidence of low birth weight was significantly high in Group 3 (p=0.001). Unlike previous literature, we did not find a statistically significant differentiation when comparing the rates of low birth weight babies between Group 1 and Group 2.

In a study on the neonatal outcomes of advanced maternal age, the incidence of macrosomia was found to be significantly high in pregnancies over the age of 40.[16] In our study, while no differentiation was found between Groups 1 and 2, the incidence of macrosomia was significantly high in Group 3 (p<0.05).

In the literature, studies evaluating the relationship between advanced maternal age and the APGAR scores and need for NICU in neonates generally found no significant differentiation. Contrary to the literature, in a meta-analysis published in 2019, it was reported that the incidence of GDM, GHT, TPTL, low birth weight, and NICU need to increase in the advanced maternal age group.[17] In our study, when we compared Group 3 with other groups, we found that the incidence of NICU need for newborns was statistically significantly high (p=0.001, p=0.001). However, there was no significant differentiation between the groups in terms of APGAR scores of newborns at 1 and 5 minutes (p=0.718, p=0.760, respectively).

Several hypotheses have been proposed to explain the adverse effects of advanced maternal age on the later periods of newborn life. However, these hypotheses are not supported by clinical and epidemiological evidence.[18] Considering the pathophysiology of GDM, GHT, and preeclampsia, we believe that advanced-age mothers adapt poorly to the physiological changes of pregnancy. Therefore, we think that pregnancy and potential complications progress worse in mothers of advanced age. In our study, we found the incidence of abortus imminens in Group 3 to be high than in other groups. We attribute this situation to several reasons.

1. As the maternal age progresses, the endometrial condition, which is an important factor in the settlement and development of the fetus, and hormonal support may physiologically be insufficient in the advanced age group,
2. Increased accumulation of environmental pollutants in the body,
3. Disruption of cellular anabolic and catabolic balance

Also, when we look at the reasons for abortus imminens, the most common reason is chromosomal abnormalities. The frequency of chromosomal anomalies in embryos will increase as maternal age increases.

For PROM, maternal risk factors can be listed as GHT, preeclampsia, short cervical length (history of conization), and autoimmune diseases.[19] There is no study in the literature that reveals the relation between maternal age and PROM development. In our study, we observed that the incidence of PROM in Group 3 significantly increased compared to other groups.

Our study has some limitations, primarily its retrospective design and the imbalanced patient count according to age distribution. However, it holds significant strengths in its unique approach. Contrary to many studies in the field, our research incorporates socioeconomic status. This allows for a deeper exploration into traditional childbearing roles in the context of prospective mothers' educational and professional backgrounds, which could offer insights into the societal influences potentially leading to spontaneous pregnancies at an advanced maternal age with a high birth rate in the highlighted region.

This investigation specifically targets pregnancies that occurred spontaneously amongst women of advanced maternal age who, due to sociocultural factors, did not employ contraception or family planning measures. It intentionally excludes patients who opted for assisted reproductive techniques, thus postponing motherhood. By focusing solely on birth data from a specific province, our study provides a unique perspective on the complications associated with advanced maternal age within a homogeneous society sharing a common sociocultural environment.

To the best of our knowledge, this research also marks a first in the literature by elucidating the association between preterm rupture of membranes (PROM) – a recognized maternal and neonatal complication – and advanced maternal age, setting it apart from other studies.

Conclusion

Advanced maternal age is considered a parameter associated with maternal and neonatal complications. It should be acknowledged that pregnancies at later ages, formed with postponed pregnancies and assisted reproductive techniques, could be more complicated for both mother and baby, and these pregnancies should be monitored more closely. Examinations of advanced-age mother candidates in terms of systemic diseases prior to pregnancy will assist in the early diagnosis and management of complications that may arise during the pregnancy process. Middle-aged women should be informed that pregnancies occurring at advanced maternal age can be more complicated for both mother and baby when receiving family planning counselling. We believe that this information could lead to a decrease in the rate of pregnancies at advanced ages.

Ethics Committee Approval

The study received non-interventional ethics committee approval from the Ethics Committee of Van Regional Training and Research Hospital. The approval number is 2023/01-05. The study was managed in accordance with the principles of the Declaration of Helsinki.

Informed Consent

Verbal consent was acquired from the study participants or their legal representatives.

Authors Contributions

All of the authors contributed at every stage of the study

Conflict of Interests

There is no conflict of interest to declare.

Financial Disclosure

No person/organization is supporting this study financially.

Statements

These research results have yet to be presented anywhere previously. Data related to the study is available on request.

Peer-review

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Research Article/Özgün Araştırma

Distribution of epidemiological and clinical involvement of extrapulmonary tuberculosis patients in the infectious disease's outpatient clinic by years

Enfeksiyon hastalıkları polikliniğinde ekstrapulmoner tüberküloz hastalarının epidemiyolojik ve klinik tutulumlarının yıllara göre dağılımı

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Abstract

Aim: It was aimed to examine the patients diagnosed with extrapulmonary tuberculosis (EPTB) in terms of diagnostic methods and demographic characteristics.

Materials and Methods: The files of patients with EPTB who were followed up in the infectious disease's outpatient clinic between 2012 and 2022 in our study were retrospectively reviewed.

Results: Of the patients diagnosed with EPTB, 70.8% (102) were female and 29.2% (42) were male. The ages of the patients ranged from 20 to 88, the mean age of women was 54.2, and the mean age of men was 55. The most common site of involvement in these patients was lymph node involvement. This was followed by bone-joint, peritoneal, central nervous system and genitourinary system involvement, respectively. Histopathological methods were used most frequently in 81 (56.5%) of the patients.

Conclusion: The signs and symptoms of EPTB differ according to the organs and tissues involved in the body. We believe that EPTB should be considered in the differential diagnosis in endemic regions.

Keywords: Extrapulmonary tuberculosis; Lymph node; Mycobacterium tuberculosis; Histopathology, Infectious diseases outpatient clinic.

Öz

Amaç: Ekstrapulmoner tüberküloz (EPTB) tanısı alan hastaların tanı yöntemleri ve demografik özellikleri açısından incelenmesi amaçlandı.

Gereç ve Yöntemler: Çalışmamıza 2012-2022 yılları arasında enfeksiyon hastalıkları polikliniğinde takip edilen EPTB tanılı hastaların dosyaları retrospektif olarak incelendi.

Bulgular: EPTB tanısı alan hastaların %70,8'i (102) kadın, %29,2'si (42) erkek olarak saptandı. Hastaların yaşları 20 ile 88 arasında değişmekte, kadınların yaş ortalaması 54,2, erkeklerin yaş ortalaması ise 55 olarak gözlendi. Bu hastalarda en sık lenf bezi tutulumu görülmüştür. Bunu sırasıyla kemik-eklem, periton, santral sinir sistemi ve genitoüriner sistem tutulumu izlemiştir. Hastaların tanısında en sık histopatolojik yöntemler 81(%56,5) kullanılmıştır.

Sonuç: EPTB'nin belirti ve bulguları vücutta tutulan organ ve dokulara gore farklılıklar göstermektedir. Endemik bölgelerde ayırıcı tanıda EPTB hastalığının düşünülmesi gerektiği kanaatindeyiz.

Anahtar Kelimeler: Ekstrapulmoner tüberküloz; Lenf nodu; Mycobacterium tuberculosis; Histopatoloji, Enfeksiyon hastalıkları polikliniğinde.

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Introduction

Tuberculosis (TB) remains a serious global public health problem. According to the World Health Organisation (WHO) report (global TB report 2021), an estimated 10 million people are diagnosed with TB each year and approximately 1.5 million people die from TB annually.[1] Although the majority of cases are diagnosed with pumonary tuberculosis (PTB); pleural tuberculosis, lymph nodes, musculoskeletal system, gastrointestinal tract, meninges, etc. Involvement of other organs and organ systems is classified as extrapulmonary tuberculosis (EPTB).[2,3]

EPTB causes 15 to 20 % of all TB cases in humans, and this number is reported to have increased in the last decade.[4] Tuberculin skin test (TST), interferon gamma release test, polymerase chain reaction test, acid-fast bacilli (AFB) smear and culture (gold standard) and radiological imaging, methods are used in the diagnosis of EPTB.[5,6] There are difficulties in the diagnosis, treatment and follow-up of EPTB due to the fact that it can be seen in almost all organs, has a wide range of clinical symptoms, affects body fluids and settles in hard-to-reach areas in the body.[7] Due to these difficulties in diagnosis and follow-up, it is thought that EPTB cases are more than previously diagnosed.

The aim of this study was to draw attention to the importance of extrapulmonary tuberculosis, which is an important public health problem, by showing the distribution of organ involvement, demographic characteristics and epidemiological evaluation between 2012-2022 in Adıyaman.

Materials and Methods

Onehundredfortyfour patients diagnosed with EPTB who applied to our outpatient clinic between 2012 and 2022 were included in the study and examined retrospectively. The infectious diseases polyclinic serves a city with a population of six hundred thousand. Cases with at least one of the following criteria were accepted for the diagnosis of EPTB:

- Direct examination of the material taken from the extrapulmonary focus (peritoneal fluid, urine, gastric juice, lymph node puncture material, etc.) shows the presence or culture growth of AFB.
- Positive TST in patients with caseating granuloma on biopsy.
- The biopsy does not show caseification, granulomatous inflammation is detected and there is clinical findings compatible with TB and other diagnoses are excluded.
- Clinical findings compatible with TB, positive tuberculin skin test and response to treatment.
- TST induration diameter of 10 mm or more in those without BCG scar and 15 mm or more in those with BCG scar were considered positive.

EPTB cases were analysed according to age, gender, organ or organ system involved and distribution rates according to years.

Type of the study

The study is retrospective.

The sample size of the study

Onehundredfortyfour patients aged 20-88 years applied to the infection disease out patient clinic.

Data collection tools

Datas were taken retrospectively.

Data analysis

Analyzes were evaluated in 22 pack age programs of SPSS (Statistical Pack age for Social Sciences; SPSS Inc., Chicago, IL). In the study, descriptive data are shown as n and % values in categorical data and mean±standard deviation (mean±SD) and median (minimum-maximum) values in continuous data. Chi-square analysis (PearsonChi-square) was used to compare categorical variables between groups. The statistical significance level in the analysis was accepted as $p<0.05$.

Ethics committee approval

Ethics committee approval was obtained with the decision of the Ethics Committee for Non-Interventional Procedures of Adıyaman University, dated 13.12.2022, and numbered 2022/9-5. The principles of the Declaration of Helsinki conducted the research.

Results

During the 10-year period of the study, 144 EPTB patients were identified. Among the patients diagnosed with EPTB, 70.8% (102) were female and 29.2% (42) were male. Although the ages of the patients ranged between 20 and 88 years, the mean age of women was 54.2 years and the mean age of men was 55 years. Considering the annual number of patients followed up, a significant decrease was observed between 2019 and 2021, and it was thought that the reason for this was that patients could not apply to the hospital due to the global COVID-19 pandemic in the specified years. The number distribution of the detected cases according to years is shown in Figure 1.

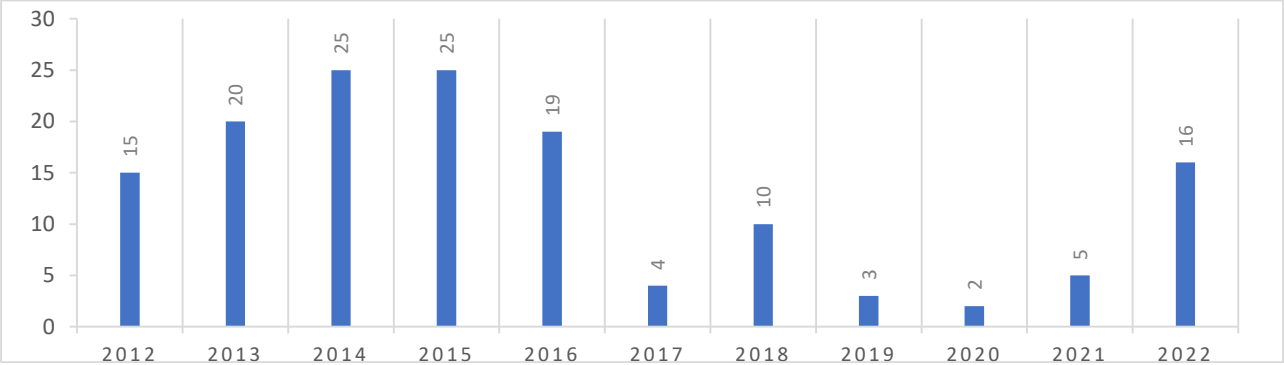

Figure 1. Number of EPTB patients by years.

Among the cases included in the study, histopathological examination was used in the diagnosis of 81 cases, molecular and histopathological diagnosis was made in 7 cases, molecular diagnosis was made in 4 cases, microbiological diagnosis was made in 3 cases and other methods were used in the remaining 49 cases. Diagnostic methods used in EPTB cases are shown in table 1.

Table 1. Methods used in the diagnosis of EPTB.

Diagnosismethod	Number of patients(n)	%
Histopathological	81	56.5
Microbiological and histopathological	3	2
Molecular	4	2.7
Molecular and histopathological	7	4.8
Other examinations	49	34

*Other examinations: Radiological examinations, TST, liquid adenosine deaminase, lymphocyte-rich fluid, etc.

When the organ types involved were examined, the most frequently involved organ was the lymph node with a rate of 54.8% (79). This was followed by bone-joint with 18.7% (27), peritoneum with 10.4% (15), and central nervous system with 4.1% (6). Genitourinary system, gastrointestinal system, breast and skin were seen in 2.7% (4). Among the cases, there was one EPTB (0.6%) in the pharynx (Pleural tuberculosis patients were not included in the study because they were followed by chest diseases departments).

Cervical lymph nodes were most commonly involved in cervical involvement and vertebrae were most commonly involved in bone joint involvement. The organ distribution of the cases is shown in table 2.

Table 2. Involved organ and severity

Organ Involved (n)	Patient (n)(%)
LymphNodes	**79 (54.8)**
Cervical	53 (67)
Axillary	9 (11.3)
Mediastinal	9 (11.3)
Intra-abdominal	6 (7.5)
Submandibular	2 (1.3)
Peritoneum	15 (10.4)
Genitourinary	**4 (2.7)**
Ovary	1 (0.6)
Bladder	3 (2)
Central nervoussystem	6 (4.1)
Pharynx	1 (0.6)
Bone Joint	**27 (18.7)**
Vertebra	13 (9)
Hip	3 (2)
Spondilodiscite	3 (2)
Femur	1 (0.6)
Foot	2 (1.3)
Clavicle	1 (0.6)
Other	4 (2.7)
Gastrointestinal	4 (2.7)
Breast	4 (2.7)
Skin	4 (2.7)

Discussion

In this study, patients who were followed up with the diagnosis of EPTB were analysed. The mean age was 54.4 years and 70.8% of the patients were women. Among the previous studies conducted in Turkey, the mean age was 52.2 years and the female rate was 31% in the study by Sünnetçioğlu et al, the mean age was 64.6 years and the female rate was 40.7% in the study by Binici İ., and the mean age was 52 years and the female rate was 39.2% in the study by Şengül A. et al.[8,9,10] In a study conducted in Afghanistan, Fader T et al. found that the mean age of the patients was 31.5 years and the female gender ratio was 50.73%.[11] In Iran, Fallah et al. observed a different gender ratio of 50.3% females and the mean age was 43.6 years.[12] According to the Tuberculosis War 2020 report in Turkey, the EPTB rate was 47.8% in women and 24.4% in men.[13] It was thought that the higher average age in our study compared to other studies may be due to the level of development between countries or the fact that young patients did not apply to the hospital.

When the subtypes of EPTB cases were evaluated, lymph node involvement (54.8%) was found most frequently in our study. These results are in agreement with similar studies in the literature.[8,9,10] In addition, in a study conducted by Lee Jy.in Korea, it was reported that lymph node involvement was the most common after pleura.[14] Similar results were observed by Fader T et al. in Afghanistan, Arega B. et al. in Ethiopia, and Gaifer Z. in Oman.[11,12,15] In a study conducted by LI L. et al. in Guangxi Zhuang Self-Governing Region, unlike our findings, it was observed that the most common involvement of the pleura followed by the skeletal system and then the lymphatic system in EPTB cases.[16]

In our study, cervical lymph node involvement was the most common with 53 (67%) patients, followed by axillary and mediastinal lymph node involvement with 9 (11.39%) patients, intraabdominal lymph node involvement with 6 (7.59%) patients and submandibular lymph node involvement with 2 (2.54%) patients. In Turkey, Sünnetçioğlu A et al. found cervical lymph node involvement in 39.4%, Binici İ. et al. 48.98%, Taşbakan et al. 61.4%.[8,9,17] In a meta-analysis study conducted by Mekonnen et al. in Africa on tuberculous lymphadenitis patients, it was reported that cervical lymph node involvement was between 47-98% and cervical lymph nodes were the most commonly involved region.[18] In studies conducted in Guangxi Zhuang Self-Governing Region, Ethiopia, Tunisia and India, cervical lymph node involvement was observed most frequently, similar to our study.[15,19,20,21]

In our study, bone-joint involvement (18.7%) and peritoneal involvement (10.4%) were the most common after lymph node involvement among EPTB cases.In the study of Binici İ. et al. it was found that peritoneal (13.4%), pleural (9.9%) and spondylitis (9.2%) involvement followed lymph node involvement, respectively (9).In a study conducted by Raval A et al. in India, it was observed that vertebral (20.6%), abdominal (7.84%) and central nervous system (7.35%) involvement followed by lymph node (41.67%) involvement.[21]

Among the methods used in the diagnosis of EPTB cases in our study, histopathological methods (56.5%) were used most frequently, followed by other methods (radiological examinations, TST, liquid adenosine deaminase, lymphocyte-rich fluid, etc.) (34%) and molecular and histopathological methods (4.8%). Similar to our study, the diagnostic methods used in the study by Şengül A et al. were histopathological (68.9%), other tests (adenosine deaminase in fluid, lymphocyte-rich fluid, TST, radiological tests, etc.) (24.8%), microbiological (5.1%), microbiological and histopathological (1.2%).[10] The diagnostic methods used in the study by Raval A et al. were histopathology (48.04%), radiological methods (53.3%), microbiological methods (19.12%).[21]

Conclusion

The signs and symptoms of extrapulmonary tuberculosis (EPTB) vary according to the organs and tissues involved in the body. Epidemiological data on the distribution of EPTB cases were examined. As a result, lymph node tuberculosis and bone joint tuberculosis are the most common forms of EPTB. In our

country where the incidence of tuberculosis is high, tuberculosis can be controlled, and its spread can be prevented by tracking the epidemiological data of tuberculosis cases and their changes over the years.

The limitations of our study are that the study was retrospective, limited to Adıyaman province, there may be missing data in the files, patients travelling outside the province were excluded from the study, and pleural tuberculosis cases were not followed up in the infectious disease's outpatient clinic.

Ethics Committee Approval

Ethics committee approval was obtained with the decision of the Ethics Committee for Non-Interventional Procedures of Adıyaman University, dated 13.12.2022, and numbered 2022/9-5. The principles of the Declaration of Helsinki conducted the research. The study was conducted under the principles of the Declaration of Helsinki.

Informed Consent

Informed consent was obtained from the individuals participating in the study.

Authors Contrubituons

All of the authors contributed at every stage of thestudy

Conflict of Interests

There is no conflict of interest to declare.

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Statements

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Peer-review

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Research Article/Özgün Araştırma

Comparison of anesthesia results in Turkish and immigrant patients who underwent cesarean section

Sezaryen yapılan Türk ve göçmen hastalarda anestezi sonuçlarının karşılaştırılması

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Abstract

Aim: Our study aims to compare anesthesia complications between immigrant and Turkish patients thus better knowledge to clinicians and anesthetists for the management of cesarean operative delivery among different race obstetric populations.

Materials and Methods: Between 06.2018-08.2018, cesarean anesthesia forms were examined retrospectively. Age, gestational week, indication of surgery, anesthesia method applied, complications in mother (hypotension, bradycardia, bleeding, emesis) recorded.

Results: 143 Turkish and 145 immigrant patients were recruited for our study. ASA II score, emergency cesarean (CS) rate, emesis incidence, hypotension rate of patients were statistically higher in immigrant patients than in Turkish patients (p<0.05). There was statically no significant difference found between the two groups of patients on behalf of bradycardia.

Conclusion: We highlight the barriers to emergency cesarean section operations in the un-monitored obstetric population, so it is vital to raise awareness of both obstetricians and anesthesiologists on this issue.

Keywords: Cesarean; Anesthesia; Immigrant.

Öz

Amaç: Çalışmamız göçmen ve Türk hastalar arasındaki anestezi komplikasyonlarını karşılaştırmayı, böylece klinisyenlere ve anestezistlere farklı ırktan obstetrik popülasyonlarda sezaryenle operatif doğum yönetimi konusunda daha fazla bilgi vermeyi amaçlamaktadır.

Gereç ve Yöntem: 06.2018-08.2018 tarihleri arasında sezaryen anestezi formları retrospektif olarak incelenerek çalışma gerçekleştrildi. Yaş, gebelik haftası, ameliyat endikasyonu, uygulanan anestezi yöntemi, annedeki komplikasyonlar (hipotansiyon, bradikardi, kanama, kusma) kaydedildi.

Bulgular: Çalışmamıza 143 Türk ve 145 göçmen hasta alındı. Göçmen hastalarda hastaların ASA II skoru, acil sezaryen (CS) oranı, kusma, hipotansiyon insidansı, Türk hastalara göre istatistiksel olarak daha yüksekti (p<0,05). İki hasta grubu arasında bradikardi adına istatistiksel olarak anlamlı fark bulunmadı.

Sonuç: Takipsiz obstetrik popülasyonda acil sezaryen operasyonlarının önündeki engelleri vurguluyoruz, bu nedenle hem kadın doğum uzmanlarının hem de anestezistlerin bu konudaki farkındalığının artırılması hayati önem taşıyor.

Anahtar Kelimeler: Sezeryan; Anestezi; Göçmen.

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Introduction

Cesarean birth frequency continues to increase worldwide[1]. Although cesarean section (CS) has become very reliable over the years, is still accompanied by poor perinatal and maternal outcomes compared to vaginal delivery[2]. The overall CS-associated postoperative surgery and anesthesia-related morbidity rate is 35.7%[3].

Although general anesthesia for CS has many advantages, such as cardiovascular stability, better and good control over ventilation, lower incidence of hypotension than regional anesthesia and faster induction in case of emergency; anesthetic drugs that are used during CS, can cross the placental barrier may affect neonatal wellbeing by respiratory depression[4]. It has been reported that during general anesthesia, complications such as difficult intubation, intubation failure, and aspiration of gastric contents may contribute to maternal mortality[5,6].

In spinal anesthesia main disadvantage is maternal hypotension; as it may cause vomiting and nausea in pregnancy and is the main cause of emesis during regional anesthesia and may result in a decreased level of consciousness and vertigo, which occurs less often when the drop in blood pressure is immediately treated[7], fetal acidosis may develop which may lead to fetal bradycardia and cardiovascular collapse in severe cases by a decrease in the uteroplacental blood flow (available at: https://www.nysora.com). It has been hypothesized that along with severity, the duration of hypotension is a major risk factor in maternal and fetal well-being.

An abundant body of research demonstrates that language and cultural barriers negatively affect care for the estimated 9% of the population or more than 21 million people who have limited language proficiency resulting in reduced access, higher hospitalization rates, lack of knowledge about doctors, increased risk of permanent damage, and limited health knowledge from communication difficulties[8]. Although treatment costs were not frequently considered as barriers, access to outpatient clinics remains a major issue with low utilization of hospital services, with daycare treatment.

Our study aims to compare anesthesia complications between immigrant and Turkish patients thus better knowledge to clinicians and anesthetists for the management of cesarean operative delivery among different race obstetric populations. The secondary aim was a better understanding of anesthesia complications in low-income, unmonitored obstetric populations since compared to Turkish patients, immigrant obstetric patients do not have scheduled visits to the hospital periodically throughout their pregnancy.

Materials and Methods

Between 01.06.2018-31.08.2018, the anesthesia forms of pregnant women who underwent cesarean section in Istanbul Training and Research Hospital were examined retrospectively after the approval of the Local Human Ethical Commitee (07.02.2020-2170). Name, age, gestational week, indication of surgery, whether emergency or elective, anesthesia method applied. Complications in the mother (hypotension, bradycardia, bleeding, emesis) were recorded.

Inclusion criteria: Pregnant women between 18-45 years old, American Society of Anesthesiologists (ASA) scores I, II patients, patients who received general and spinal anesthesia

Exclusion criteria: patients with known psychiatric illness, a history of taking any antidepressant or antianxiety drugs and having absolute or relative contraindication for either regional or general anesthesia.

In our study 166 Turkish and 153 immigrant patients were included. 21 out of 166 Turkish patients who were emergency and not under our department's provision were excluded. 10 out of 153 immigrant patients who had scheduled visits to our department were excluded. All the patients had intravenous access. Standard monitoring was performed routinely (electrocardiogram monitoring, noninvasive peripheral oxygen saturation, and arterial blood pressure follow-up). 6–8 hour of fasting was expected before all elective CS

patients. As recommended by the American College of Obstetricians and Gynecologists guidelines antibiotic prophylaxis is administered within 1 hour of surgery[9]. Pre-operative hemoglobin and hematocrit values were determined within 1 month in elective CS vs immediately after hospitalization in the emergency CS group of patients.

Indication of general anesthesia over spinal anesthesia includes; immediate threat to the life of the pregnant woman or fetus (placental abruption, umbilical cord prolapse, acute and massive bleeding from placenta previa)

Before the operation, only in elective CS group of patients had a crystalloid solution, for 20 minutes at the rate of 15 ml/kg rapidly before the operation. Afterward, in the sitting position 25-gauge needle from the L3-4 or L4-5 subarachnoid space entered after proper skin cleansing.

0.5% hyperbaric bupivacaine (2.2 ml) was administered into the intervertebral space after observation of cerebral spinal fluid (CSF) flow. Patients were lateralized for 5-10 minutes in a fully supine position with their heads elevated to 30 degrees, for proper positioning.

Bromage scale was used for the determination of motor block level, while the hot/cold test, as a dermatome level, was used for the sensory block level. The operation started after the T4-T5 level sensory block level reached sufficient. When required midazolam is used for sedation for patients after the delivery of the newborn. In both Turkish and immigrant patients, when hypotension occurred following the anesthesia (mean arterial blood pressure falling below 60 mmHg of baseline), ephedrine hydrochloride (10 mg; IV) was administered if hypotension continued. In case of continuation additional 10 mg ephedrine hydrochloride is added to the regimen until the patient stabilizes. In addition to ephedrine a rapid crystalloid infusion was given to all hypotensive patients.

Bradycardia was defined as a falling of heart rate below 50 beats per minute during anesthesia. For patients who developed bradycardia, the issue was resolved by the administration of IV atropine sulfate (0.5 mg).

The study was carried out in consonance with the Declaration of Helsinki. The Ethics Committee approved our study protocol of Istanbul Training and Research Hospital (file number: 2170, date: 07.02.2020). As our study is retrospective, we could not get the informed consent of patients.

Type of the study

The study was planned as a descriptive retrospective study.

The sample size of the study

A total of 288 patients were included to the study that performed between 01.06.2018-31.08.2018.

Data collection tools

All the files of patients' who undergone cesarean section in Istanbul Training and Research Hospital 01.06.2018-31.08.2018 were examined.

Data analysis

IBM SPSS Statistics 25.0 for Mac (SPSS, Chicago, IL, USA) was used for performing statistical analyses. Descriptive statistics were stated as standard deviation, frequency, mean and percentage. Continuous numeric variables and categorical variables like ASA score, hypotension, emesis, bradycardia and emergency or elective c-section rates between Turkish and immigrant patients were performed by using Student's t-test (because of random sampling and seen from the histogram of each of the two groups) and the chi-square test statistical analyses performed. Statistical significance was defined as $p< 0.05$.

Ethics Committee Approval

Ethics committee approval was obtained with the decision of the Ethics Committee for Non-Interventional Procedures of Istanbul Training and Research Hospital, dated 07.02.2020, and numbered 2170. The principles of the Declaration of Helsinki conducted the research.

Results

143 Turkish and 145 immigrant patients were recruited for our study.

Cesarean indications among 143 Turkish patients and 145 immigrant patients were listed in Table 1. In both groups, the main CS delivery indication was previous CS operation followed by cephalopelvic disproportion and fetal compromise.

Table 1. Cesarean delivery indications in Turkish and immigrant patients.

	Turkish patients n=143	Immigrant patients n=145
Breech presentation	9	11
Cephalopelvic disproportion	17	16
Fetal compromise	18	16
Multiple gestation	5	6
Previous CS	82	71
Cord prolapsus	1	
Preeclampsia	11	11
Ablatio placenta		5
Premature rupture of membranes		4
Fetal macrosomia		1
Fetal transverse lie		1
Placenta previa		3

CS: Cesarean section

As shown in Table 2, 143 Turkish and 145 immigrant patients were recruited for our study. The mean age of Turkish and immigrant patients was 28.8 and 26.1 years old respectively. Preoperative hematocrit (Hct) values of Turkish and immigrant patients were 33.8 and 33.4 g/dl respectively There was no statistical difference between the patient's age and pre-operative hematocrit values (p>0.2). ASA II score and emergency CS rate of patients were statistically higher in immigrant patients than in Turkish patients (p<0.05). ASA I score and elective CS rate of patients were statistically higher in Turkish patients than in immigrant patients (p<0.05). 11 out of 143 Turkish patients had general anesthesia versus 18 out of 145 immigrant patients. There was no statistically significant difference between the two groups of patients. (p=0.18).

Table 2. Operative data of patients.

	Turkish patients	Immigrant patients	*p*-value*
Number of patients (n)	143	145	
Age (years)	28.8 ± 6.01	26.1 ± 6.44	0.2*
Pre-operative Hct (g/dl)	33.8 ± 3.13	33.4 ± 3.39	0.16*
ASA I (n)	26	6	0.0001**
ASA II (n)	117	139	0.0001**
General Anesthesia (n)	11	18	0.18**
Regional Anesthesia (n)	132	127	0.18**
Emergency CS (n)	66 ±	122	0.0001**
Elective CS (n)	77 ±	23	0.0001**

*: Student T test; **Chi-square test; Hct: Hematocrit; ASA: American Society of Anesthesiologists; CS: Cesarean section

In all patients, the incidence of emesis was not related with the age of patients. 163 patients had emesis and the mean age of patients were 27.2 years old versus 125 patients with no emesis, the mean ages of patients was 27.5 years old (p=0.35).

When analyzing all 288 patients, 60 out of 184 emergency CS patients versus 33 out of 96 patients in elective CS had bradycardia CS (p=0.04). Emergency CS patients had a statistically higher bradycardia rate than elective CS.

When analyzing all 288 patients, 11 out of 32 ASA I patients versus 81out of 246 ASA II patients had bradycardia (p=0.98). There was no statistically significant difference found in ASA I and ASA II patients on behalf of bradycardia.

Sixty-five out of 143 patients had emesis in Turkish patients versus 98 out of 145 patients in immigrant patients. There was a statically significantly higher emesis rate in immigrant patients than Turkish patients (p<0.05).

Ninety-one out of 143 patients had hypotension in the Turkish group versus 108 out of 145 patients in immigrant patients. There was a statically significant difference between immigrant and Turkish patients on behalf of hypotension (p=0.046).

Fifty-three out of 143 patients had bradycardia in the Turkish group versus 40 out of 145 patients in immigrant patients. There

was statically no significant difference between immigrant and Turkish patients on behalf of bradycardia (p=0.091)

Discussion

In both Turkish and immigrant patients, the indications of cesarean delivery remain similar (Table 1). Although guidelines for anesthesia recommend regional anesthesia for a cesarean section because of the higher risk of intraoperative blood loss, aspiration, failed intubation, and awareness of non-regional anesthesia, maternal request for general anesthesia is still high. In our study, although not reaching the level of statistical significance, the general anesthesia ratio was higher in immigrant patients than in Turkish patients (Table 2). Thus, for avoiding both fetal and maternal complications, the preferred anesthetic technique has now regional anesthesia as recent rates of CS using general anesthesia decreasing[10,11]. As for obstetric reasons, immigrant patients have more likely to have emergency operations thus complications of general anesthesia are more likely to occur.

One of the most important etiological factors for intraoperative nausea and vomiting is hypotension occurring during regional anesthesia. We found a higher incidence of emesis in immigrant patients (Figure 1) since the rate of emergency surgery and hypotension (Figure 2) were significantly higher in immigrant patients (Table 2), which may cause full stomach and inadequate fasting time that aggregates nausea and vomiting. In a recent study, intra-operative nausea was observed less frequently with advanced maternal age, which they attributed to decreased estrogen levels[12], we could not examine this correlation in our study, since both emesis and no emesis patients groups had similar age distribution.

In a study by Balki et al.[13] optimizing the use of i.v. and neuraxial opioids, cautious administration of uterotonic agents, minimizing surgical stimulus, improving the quality of block, and controlling hypotension, emesis can be prevented despite that prophylactic antiemetic usage during cesarean sections advocated by some clinicians. Thus in our clinic, we do not use prophylactic antiemetics. We reserved antiemetics (metoclopramide 20 mg) for the treatment of vomiting and nausea not responding to routine approaches.

Maternal hypotension is common with labor epidural anesthesia procedures, complicating 5-17% of cases[14]. If maternal hypotension is uncorrected during regional anesthesia, decreased uteroplacental perfusion can cause during labor bleeding complication[15]. During spinal anesthesia, there are a certain number of proven risk factors for the development of hypotension. We found hypotension was statistically significantly higher in immigrant patients (Figure 2). One of the reasons for this situation was the high rates of emergency CS rate in immigrant patients. Lack of receiving standard 1000-mL crystalloid intravenous fluid bolus immediately before the operation to achieve adequate volume preloading before regional anesthesia placement in immigrant patients with no proper appointment for CS may cause the problem.

Sun and Huang conclude that hypotension after regional anesthesia is affected by effective circulating blood volume and preoperative sympathetic activity[16]. Since Turkish and immigrant patient groups had the same mean Hct values, we conclude that sympathetic activity is more effective in the etiopathogenesis of maternal hypotension.

It is important to be able to discover and predict maternal hypotension during CS, highlighted by Olang et al. who conclude that the impact of maternal hypotension that occurred less than two minutes, affects the incidence of neonatal acidemia and five-minute Apgar scores of neonates minimally[17].

Pereira et al. found in their study that factors associated with hypotension were age, type of anesthesia, and patient gender[18]. When compared to patients younger than 41 years of age, the probability of an individual developing hypotension was found 1.51 times higher at ages between 41 and 60 years and 2.80 times higher in the age group >61 years. As shown in Table 2, even though the immigrant patients' ages were slightly lower

than Turkish patients, there was no statistically significant difference between the groups.

Contrary to our results (higher ASA II and equal rate of bradycardia in immigrant patients) (Figure 3), Pereira et al. found in their study that, the probability of developing sinus bradycardia was greater in ASA I patients compared to higher ASA score (ASA II, III, and IV) patients, they conclude that, these findings most likely because, in younger patients, vagal tonus is more pronounced[18]. They found in their study that, emergency or urgent CS patients had a less frequent bradycardia rate than routine anesthesia patients. These data differ from those reported in the literature, as well from our study, as we found a higher rate of bradycardia in the emergency CS group, we conclude that sinus bradycardia is more frequent in patients undergoing urgent and emergency CS anesthesia that might have pre-existent, inadequately treated or non-diagnosed underlying diseases.

As we mentioned above communication issues are the major problem in immigrant pregnancies. Karaca et al. attribute this issue with them not to benefit effectively benefit from the healthcare system.[19] This problem is not only for immigrants but also for health professionals. In their study, the immigrant patient who underwent emergency cesarean section is 596 (12.2%). Failure to communicate is one of the relative contraindications of spinal anesthesia. Since an interpreter is available 24 hours a day in goverment hospitals there was less difficulty in communicating with patients. Again, spinalwas the most commonly performed anesthesia method in these patients.

It was independent of the presence of any known risk factors or usual clinical indications, suggesting that cultural background influences the mode of delivery and/or anesthesia overcoming the expected standard of care and outcomes in public health services.

In immigrant patients with inadequate preparation for CS, major complications in cesarean anesthesia such as hypotension, emesis, and/or bradycardia, arise; the ASA score and age of patients have either no or little value since the obstetric population is relatively young age. Thus, training, tools, and resources to support potential referrers in detecting to help increase the proportion of referrals to obstetric & gynecology clinics might help in managing those patients.

Conclusion

In our study, we highlight obstacles associated with emergency CS operations since the number of immigrant pregnancies is equal if not higher than Turkish pregnancies in our country thus it is vital to increase the awareness of both obstetricians and anesthetists on this issue.

Ethics Committee Approval

Ethics committee approval was obtained with the decision of the Ethics Committee for Non-Interventional Procedures of Istanbul Training and Research Hospital, dated 07.02.2020, and numbered 2170. The principles of the Declaration of Helsinki conducted the research.

Informed Consent

Data concerning the study were collected with the permission of the Istanbul Training and Research Hospital.

Author Contribution

All of the authors contributed at every stage of the study

Conflict of Interests

There is no conflict of interest to declare.

Financial Disclosure

No person/organization is supporting this study financially.

Statements

These research results have yet to be presented anywhere previously. Data related to the study is available on request.

Peer-review

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Research Article/Özgün Araştırma

The relationship between febrile seizure and hematological parameters in children

Çocuklarda febril nöbet ile hematolojik parametreler arasındaki ilişki

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Abstract

Aim: The aim of this study was to investigate whether hematological parameters play a significant role in the relationship between hematological parameters and seizure occurrence in children with febrile seizures (FS) by comparing them to a healthy control group with no fever or seizures.

Materials and Methods: One-hundred forty-one patients diagnosed with FS and with available a complete blood count results and a control group of 143 children were finally enrolled.

Results: The study group consisted of 141 patients, 57 girls (40.4%) and 84 boys (59.6%) (M/F=1.4). Mean age at the time of first FS was 22.89 ± 13.95 months. Ninety-two (65.2%) of the study group were diagnosed with simple FS, 32 (22.7%) with complex FS, and 17 (12.1%) with febrile status epilepticus (FSE).

Conclusion: Since our neutrophil, lymphocyte, eosinophil, and mean platelet volume (MPV) results were statistically significant in patients with FS, it is thought that these markers may represent potential predictive parameters in that condition.

Keywords: Eosinophil; Febrile seizure; Lymphocyte; MPV; Neutrophil.

Öz

Amaç: Bu çalışmanın amacı, ateşli nöbet (FN) geçiren çocuklarda hematolojik parametreler ile nöbet oluşumu arasındaki ilişkide hematolojik parametrelerin önemli bir rol oynayıp oynamadığını, ateşi ve nöbeti olmayan sağlıklı bir kontrol grubu ile karşılaştırarak araştırmaktır.

Gereç ve Yöntem: FN tanısı almış ve hemogram sonuçları bulunan 141 hasta ve 143 sağlıklı çocuk kontrol grubu olarak çalışmaya dahil edildi.

Bulgular: Çalışma grubu 57 Kız (%40,4), 84 Erkek (59,6) olmak üzere 141 hastadan (E/K=1,4) oluşmaktadır. Hastaların ilk FN geçirme yaş aralığı 22,89 ± 13,95 aydır. Hasta grubunun 92'si (%65,2) basit FK, 32'si (%22,7) komplike FN ve 17'si (%12,1) febril status epileptikus (FSE) tanısı almıştı.

Sonuç: Çalışmamızda FN'li hastalarda nötrofil, lenfosit, eozinofil ve ortalama trombosit hacmi (MPV) sonuçlarının istatistiksel olarak anlamlı tespit edilmesi nedeniyle bu belirteçlerin FN'de öngörücü parametreler olabileceği düşünülmektedir.

Anahtar Kelimeler: Eozinofil; Febril nöbet; Lenfosit; MPV; Nötrofil.

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Introduction

Febrile seizure (FS) is an age-dependent event that emerges with fever, exhibits a generally benign course, and represents the most frequent seizure type in childhood. The International League Against Epilepsy (ILAE) defines FS as a seizure occurring in childhood after one month of age, associated with a febrile illness not caused by an infection of the central nervous system, without previous neonatal seizures or a previous unprovoked seizure, and not meeting the criteria for other acute symptomatic seizures.[1] FS is more frequently seen in early childhood when the seizure threshold is low, the fever response is more pronounced, and susceptibility to infections is more common. FS is seen between the ages of three months and six years and generally emerges with fever during the course of viral or bacterial infections. The highest incidence is seen between 12 and 18 months.[2] Although the gender difference is not pronounced, it is reported to be more common in boys.[3] There are two subtypes, simple and complex. Simple FS involves generalized seizures less than 15 min in duration and not recurring within 24 h, while complex FS refers to seizures that are generally focal in nature and last longer than 15 min, and that may be observed more than once in 24 h. In FSE, seizures persist without restoration of consciousness or last for 30 min or longer. However, in 2015 FSE was defined as seizures lasting 5 min or longer.[4]

Although the etiology of FS is not yet fully understood, age, high body temperature, viral infections, immunization, and family history have been implicated as risk factors. Complete blood laboratory tests, which are available in all hospitals and widely employed, are requested to assist with status determination in children presenting with acute fever. Complete blood count tests are simple and easily available and help to determine cell numbers and ratios in blood. They are used in the control and follow-up of numerous diseases. Similar to other diseases, they are an important and useful test in FS, particularly in differential diagnosis. This study was intended to determine whether or not hematological markers can represent a predictive parameter in FS.

Materials and Methods

Type of the study

This is an original research study including the patients diagnosed with FS at the Adıyaman University Training and Research Hospital pediatric emergency and pediatric neurology clinics, Turkey, between July 2014 and May 2022, and with available complete blood count results.

Population and sample of the study

Inclusion criteria were diagnosis of FS, being within the FS age range, body temperature elevation, absence of central nervous system infection, exclusion of other causes of seizure, and absence of any other disease capable of causing neuromotor retardation. It has been determined that the minimum participant size should be 87, with a confidence level of 95%.

Data collection tools

All patients' files were reviewed retrospectively, and age at first FS, diagnosis of simple or complex FS or FSE, and neutrophil, lymphocyte, eosinophil, platelet, and MPV findings were retrieved and recorded. Patients were classified into three groups, simple FS, complex FS, and FSE. The groups were established for the purpose of identifying and prognostic differences.

Analysis of data

The study data were analyzed on Statistical Package for the Social Sciences (SPSS) version 22 software. The Independent sample t-test was applied in the comparison of two independent groups when normal distribution assumptions were met, while the Mann-Whitney U test was employed when these were not met. The chi-square test was applied to investigate differences between categorical variables, with exact test results being considered in case of expected frequency percentages being lower than 25%. Sensitivity and specificity for neutrophil, lymphocyte, eosinophil, and MPV values were determined using ROC analysis. Continuous variables were expressed as both mean ± standard deviation and median [minimum-maximum] values. Categorical variables were

summarized as numbers and percentages. *p* levels <0.05 were regarded as statistically significant.

Ethics committee approval

All procedures were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Ethical approval for the study was obtained from the institutional review board (no.2022/7-53).

Results

The records of 191 patients presenting to the Adıyaman University Training and Research Hospital pediatric neurology clinic between July 2014 and May 2022 and diagnosed with FS were examined retrospectively. Fifty patients with risk factors affecting neurological development, with afebrile seizures, or with inadequate file data were excluded. One-hundred forty-one patients diagnosed with FS and with available a complete blood count results and 143 healthy children were finally enrolled. No significant differences were observed between the patient and control groups in terms of age or sex (p>0.05).

The study group consisted of 141 patients, 57 girls (40.4%) and 84 boys (59.6%), with a F/M ratio of 1.4. The mean age at first FS was 22.89 ± 13.95 months. Ninety-two (65.2%) of the patient group were diagnosed with simple FS, 32 (22.7%) with complex FS, and 17 (12.1%) with FSE. No significant associations were determined between the disease groups and patients' hematological parameters.

Statistically significant differences were determined between the study groups in terms of neutrophil (p<0.001), MPV (p<0.001), eosinophil (p=0.001), and lymphocyte (p=0.001) values, but no significant difference was observed in platelet (p=0.115) values (Table 1). Lymphocyte values were lower in the patient group, while neutrophil, eosinophil, and MPV values were higher than in the control group.

Table 1. Differences in hematological parameter results between the patient and study groups

	Group		*p*-value
	Patient (n=141) Mean±SD	Control (n=143) Mean±SD	
Neutrophil	5.94±4.45	3.02±2.17	<0.001*
Lymphocyte	4.12±2.40	4.88±1.90	=0.001*
Eosinophil	0.25±0.50	0.23±0.21	=0.001*
Platelet	310.15±108.87	329.52±97.55	=0.115+
MPV	6.82±1.36	6.09±1.16	<0.001+

* Independent Sample t test, +Mann Whitney U test

An optimal cut=off value of 3.03 for neutrophil count at ROC analysis (AUC: 0.763) exhibited 70.6% specificity and 72% sensitivity for FS (p<0.001), an optimal cut-off value of 4.24 for lymphocyte count (AUC: 0.383) exhibited 41.3% specificity and 44% sensitivity for FS (p=0.001), an optimal cut-off value of 0.135 for eosinophil count (AUC: 0.424) exhibited 41.3% specificity and 42.6% sensitivity for FS (p=0.028), and an optimal cut-off value of 6.25 for MPV (AUC: 0.665) exhibited 62.9% specificity and 64.7% sensitivity for FS (p<0.001) (Figure 1)

Discussion

FS is the most common form of seizure in childhood, affecting 2-5% of children. It is reported to be more frequent in boys.[3,5] Our study group being made up of 57 girls (40.4%) and 84 boys (59.6%) is consistent with the existing literature. Different age ranges for FS have been reported in previous studies, although the mean age at first seizure in the present research was 22.9 months. Sharawat et al. reported a figure of 24.9 months and Gontko–Romanowska et al. one of 22 months.[6,7] Simple FS is more common than complicated FS. FSE is less frequently seen. In their study of 428 children presenting with first seizure, Berg et al. reported that FS was the most common form, followed by complicated FS at 35% and FSE at 5%.[8] Simple FS was determined in 92 (65.2%) of the 141 patients in the present study, followed by complicated FS in 32 (22.7%), and FSE in 17 (12.1%). No statistically significant difference was

observed in terms of hematological parameters between the subgroups in the study group.

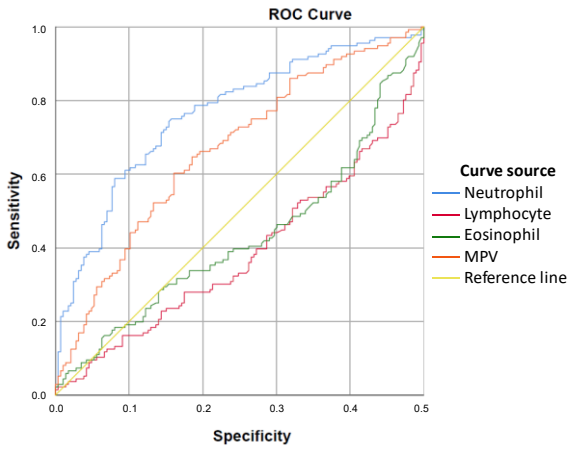

Figure 1. ROC curve analysis results for neutrophil, lymphocyte, eosinophil, and MPV values

Although the pathophysiology of FS is not yet entirely understood, studies have reported a relationship between FS and inflammation.[9,10] Pathogenic micro-organisms that enter the body trigger the release of inflammatory mediators by directing white blood cells and macrophages to the blood and infected tissues. These represent the first condition that precipitates fever and inflammation. The pyrogens that then form affect the hypothalamus by being released from white blood cells and macrophages. Uncontrolled temperature creates a major risk for FS.[11] Although there is a great variety of causes leading to seizure, the complete blood count is one of the tests that assist in the identification of the underlying condition. Among the hematological markers examined in the present study, lymphocyte counts were lower than in the control group, while neutrophil, eosinophil, and MPV values were higher. Gontko–Romanowska et al. reported a significant difference in neutrophil and lymphocyte counts in patients with FS compared to a control group.[7] In another study of patients with FS, Güneş et al. observed a significantly higher neutrophil count and a significantly lower lymphocyte count compared to a control group.[12] Additionally, Liu et al. observed a significant association between elevated neutrophil and low lymphocyte values.[13]

Specific viral infections such as human herpes virus-6, herpes simplex virus-1, respiratory syncytial virus, influenza, adenovirus, and cytomegalovirus have been linked to FS.[14] Exposure to viral infections in the early periods of life can represent a risk in terms of airway hypersensitivity and atopy. It is important for the cellular mechanisms underlying an atopic disposition to be understood. Inflammatory cells, such as antigen-presenting cells, mast cells, eosinophils, basophils, and lymphocytes, associated with asthma are known to precipitate or exacerbate airway hypersensitivity by releasing cytokines through viral infections.[15]

One of the first parameters frequently investigated in the diagnosis of allergic diseases is the blood eosinophil count. Although eosinophils are known to play an important role in allergic inflammation, there is still no evidence of an association with FS.[15] Very few studies have suggested that children undergoing FS may be at an increased risk of asthma in the future. Lin et al. emphasized the relationship between asthma and FS.[16] Eosinophils in the airways have an advanced inflammatory capacity in bronchial asthma.[17] The significant difference in eosinophil counts between the study and control groups in the present research suggests that eosinophils may be used as a marker for FS. In addition, an optimal cut-off value of 0.135 for eosinophil count at ROC analysis (AUC: 0.424) exhibited 41.3% specificity for FS and 42.6% sensitivity. To the best of our knowledge, this is the first report of a relationship between FS and the hematological parameter of the eosinophil count.

Eosinophilia can arise from both infectious and non-infectious conditions, many of which have no distinguishing clinical features. Eosinophilia can stem from parasitic infections, as well as allergies, autoimmune diseases, malignancies or other underlying conditions.[18] Studies have shown that peripheral eosinophil count is suppressed in patients during acute bacterial and viral infections.[19,20] Therefore, the presence of eosinophilia in the context of an acute illness indicates a non-infectious (such as

autoimmune), parasitic, or fungal origin as the underlying cause of the disease. The detection of eosinophilia in our study was statistically significant and indicates that this marker could be a predictive parameter in FS.

High MPV may indicate large, more reactive platelets resulting from increased platelet turnover and can be employed as a marker of platelet activation and inflammation.[21] MPV values in the present study were significantly higher than in the control group. In addition to Liu et al.'s study[13], Abuhandan et al. also reported a similar association to that in the present research.[22] Studies have also investigated the relationship between MPV and other systemic diseases.[23] The optimal cut-off value for MPV of 6.25 at ROC analysis in the present study (AUC: 0.665) exhibited 62.9% specificity and 64.7% sensitivity for FS. Additionally, the optimal cut-off value of 3.03 for the neutrophil count (AUC: 0.763), another of the hematological parameters investigated, exhibited 70.6% specificity for FS and 72% sensitivity, while the optimal cut-off value for the lymphocyte count of 4.24 (AUC: 0.383) exhibited 41.3% specificity for FS and 44% sensitivity. This shows an independent risk factor for FS.

Numerous studies in the literature have compared the laboratory results of individuals with febrile seizures to those of a control group with fever but no seizures.[7,12] In addition, it should be stated that there are studies incorporating research comparing the laboratory results of individuals experiencing febrile seizures and those in the healthy control group who are not experiencing seizures and fever. Although Tang and Chen found that the MPV value was significantly higher in patients with febrile seizures than in patients with fever but not febrile seizures and healthy control group without fever, no statistically significant difference was observed between the compared control groups.[24] Additionally, there are studies in the literature, but a limited number, comparing patients experiencing febrile seizures to healthy controls without a fever. In the study of Aydın et al., a significant difference was observed in the MPV value of patients with febrile seizures compared to the healthy control group without fever and seizure.[25]

The principal limitations of this study are the low case numbers, its retrospective nature, and the fact that the research employed the hospital database. Further prospective studies with wider population-based case series are now needed to elicit more detailed results. The lack of a second control group of similar age and gender with fever and no seizure for comparison is also a notable deficiency, as it would have allowed for a clearer examination of whether the parameters discussed in this study were affected by seizure, infection, or both. The authors believe that including two control groups, one containing patients with fever but no seizures and the other with patients experiencing neither fever nor seizures, would have yielded more informative data.

Conclusions

In conclusion, the neutrophil, lymphocyte, eosinophil, and MPV levels of the patients with FS differed significantly from those of the control group. The ROC curve analysis demonstrated that these values may be used as a possible assistant parameter to predict FS. The relationship between eosinophils and FS may be considered another important finding of this study. We suggest that inflammation resulting from eosinophil activation is important in bronchial asthma and that due to the relationship between eosinophils and FS in this study, children undergoing FS should be evaluated and followed up in terms of allergic diseases.

Ethics Committee Approval

Ethical approval for the study was obtained from the institutional review board (no.2022/7-53). This study conformed to the Helsinki Declaration.

Informed Consent

Data concerning the study were collected with the permission of the Adıyaman Provincial Health Directorate.

Authors Contributions

All of the authors contributed at every stage of the study

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Financial Disclosure

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Statements

These research results have yet to be presented anywhere previously. Data related to the study is available on request.

Peer-review

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Research Article/Özgün Araştırma

Assessment of bruxism and temporomandibular disorder in mothers of children with cerebral palsy

Serebral palsili çocuğu olan annelerde bruksizm ve temporomandibular rahatsızlığın değerlendirilmesi

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Abstract

Aim: The aim of this study was to assess bruxism and temporomandibular disorder in mothers of children with cerebral palsy (CP).

Materials and Methods: 18 mothers of children with CP and 18 mothers of healthy children were included in study. The pressure pain threshold of the masticatory muscles and the upper trapezius were measured with a digital dynamometer. Maximum mouth opening was assessed with a digital caliper. Bruxism was evaluated by a non-instrumental method. Sleep quality, depression, neck disability, and temporomandibular disorder were evaluated with the Pittsburgh Sleep Quality Index, Beck Depression Index, Neck Disability Index, and Fonseca Questionnaire.

Results: Pain thresholds were lower (p<0.05), sleep disturbance, depression, neck disability, and temporomandibular disorder were higher in the mothers of children with CP (p<0.05).

Conclusion: Our study showed that bruxism and temporomandibular disorder are highly observed in mothers of children with CP.

Keywords: Pain threshold; Bruxism; Depression.

Öz

Amaç: Bu çalışmanın amacı serebral palsili çocuğu olan annelerde bruksizmi ve temporomandibular rahatsızlığı değerlendirmekti.

Gereç ve Yöntem: Çalışmaya serebral palsili çocuğu olan 18 anne ve sağlıklı çocuğu olan 18 anne dahil edildi. Çiğneme kasları ve üst trapez kasının ağrı eşiği dijital dinamometre ile ölçüldü. Maksimum ağız açma mesafesi dijital kaliper ile ölçüldü. Bruksizm non-enstrümental yöntemle değerlendirildi. Uyku kalitesi, depresyon, boyun özürlülüğü ve temporomandibular rahatsızlık; Pittsburgh Uyku Kalitesi İndeksi, Beck Depresyon Envanteri, Boyun Özürlülük İndeksi ve Fonseka Anketiyle değerlendirildi.

Bulgular: Serebral palsili çocuğu olan annelerde kas ağrı eşikleri düşüktü (p<0,05), uyku bozukluğu, depresyon, boyun özürlülüğü ve temporomandibular rahatsızlıksa daha yüksekti (p<0,05).

Sonuç: Çalışmamız serebral palsili çocuğu olan annelerde bruksizm ve temporomandibular rahatsızlığın yüksek oranda görüldüğünü gösterdi.

Anahtar Kelimeler: Ağrı eşiği; Bruksizm; Depresyon.

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Introduction

Cerebral palsy (CP) is a group of disorders affecting infant brain development and characterized by motor and functional impairment.[1] Although the definition of CP specifies children, mothers whose children were diagnosed with CP are one of the individuals that have been considered to be deeply affected by CP.[2] Upon the diagnosis, mothers' hope gives way to frustration, and they try to make sense of the circumstances that they are facing.[3] After that, they comprehend the situation by experiencing it daily and realize that their responsibility is much heavier than that of the mothers of healthy children because their children look up to them for their daily living activities.[4] At this point, mothers must balance the scale between the needs of their children and their other duties.[4] In this endeavor, they make sacrifices like quitting their job, giving up their social life, and even leaving aside their personal care.[5] Unfortunately, the sacrifices mentioned above are only one dimension. They sacrifice their psychological health, sleep quality, and physical health as well.[6-8] More than half of the mothers whose children were diagnosed with CP had depression, neck disability, and sleep impairments.[6-8]

Interestingly, individuals with bruxism and patients with temporomandibular disorder (TMD) harbor similar symptoms with mothers whose children were diagnosed with CP. Poor sleep quality is correlated with bruxism and TMD, and nearly half of the patients with TMD experience sleep impairments.[9] Depression is commonly observed in patients with TMD,[10] and bruxism aggravates the severity of depression in patients with TMD.[11] TMD and bruxism are correlated with neck disability.[12]

Considering the common symptom characteristic observed in mothers whose children were diagnosed with CP and bruxers and patients with TMD, the following question comes to mind: Do mothers whose children were diagnosed with CP have bruxism or TMD? To our knowledge the answer to this question is not directly addressed in the literature. From this point of view, this study aimed to assess the TMD and bruxism in mothers whose children were diagnosed with CP. In this direction, the hypothesis of the study was a high rate of bruxism and TMD observed in mothers whose children were diagnosed with CP.

Materials and Methods

This study was performed at Bahçesaray Special Education and Rehabilitation Center between August 15, 2022, and November 24, 2022, once the ethical approval was obtained. The control group was recruited from the mothers of healthy children who are the residents of Bitlis and Van provinces. Before enrollment mothers were verbally informed, and then their written approval was acquired. Mothers aged 18 to 65 who have children under the age of 18 and diagnosed with CP were included in the study group. Mothers of healthy children under the age of 18 were included in the control group. Mothers using sleeping pills, diagnosed with any psychiatric, neurodegenerative, or neurological disease or fibromyalgia, having a history of jaw, head, or neck surgery, and already being treated for bruxism were excluded from both groups. A total of 41 mothers were excluded from the study (Figure 1).

Type of the study

The study is a cross-sectional study.

The sample size of the study

Considering no study exists related to the topic, we performed a post hoc power analysis based on the bruxism questionnaire score of mothers included in the study using G Power 3.1.9.5. The study had very large size effect (1.08).[13] Then, the power of the study was analyzed, and the power of the study was found to be 88 %.

Data collection tools

Data related to the characteristics of CP was collected by one author (İ.H.S.), and other data related to bruxism and TMD was collected by two authors (Ö.D. and E.D.). The evaluation methods used in the study are mentioned below.

The predominant motor type of the CP was categorized as spastic, ataxic, and dyskinetic.[14]

Spastic types are further subtyped as diplegic, hemiplegic, and quadriplegic.[15]

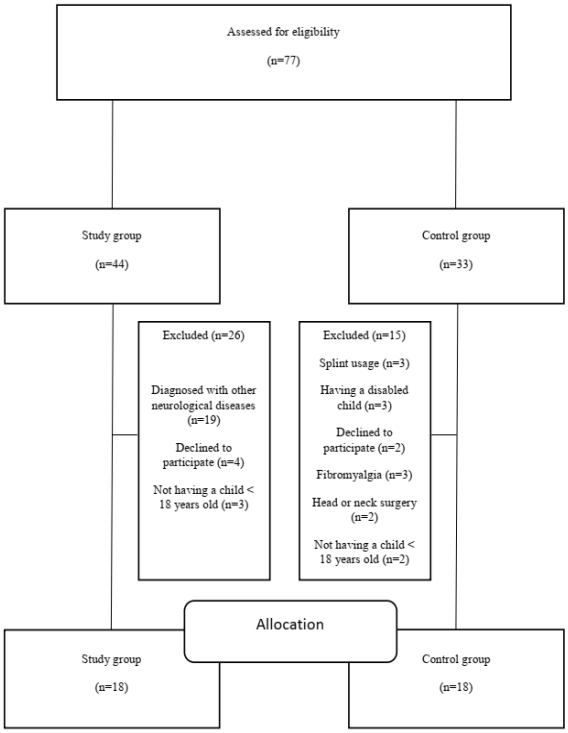

Figure 1. Study flow chart.

The gross motor function classification system (GMFCS) was used to determine the severity of the motor disability. GMFCS consists of five levels, from one to five. The children's age-specific activity competence is questioned to determine the appropriate level. From levels one to five, a decline in the activity competence of the children is observed.[16]

Bruxism's existence was determined by a questionnaire developed by Pintado et al.[17] The questionnaire consists of six questions assessing the daytime and nighttime grinding or clenching and the symptoms caused by the bruxism, such as fatigue, headache, and soreness. Mothers who have answered at least two of the questions with a yes are considered to have probable bruxism.

The maximum mouth-opening distance of the mothers was evaluated with a digital caliper. Mothers were asked to open their jaws as much as possible, and then the vertical distance was measured.[18]

TMD was assessed with the Fonseca Anamnestic Index (FAI). The index consists of ten questions that can be answered as no, sometimes, or yes. The total score is categorized into four levels: no TMD (0-15 points), mild TMD (20-40 points), moderate TMD (45-65 points), and severe TMD (70-100 points).[19]

The Neck Disability Index (NDI) was used for the evaluation of the cervical area-related disabilities of the mothers. The index has ten questions with six possible choices ranging from zero to five points. The total score of the index is 50 points. A higher score indicates a higher neck disability.[20]

Pittsburgh Sleep Quality Index (PSQI) was used to evaluate sleep quality. The index consists of 19 questions and seven subdivisions. Four questions are open-ended and self-rated. Questions in the subdivisions have four answers and are scored between zero and four. The total score of the index is 21 points. A higher score indicates low sleep quality.[21]

Depression level was evaluated with the Beck Depression Inventory (BDI). The index has 21 questions with four possible choices, scored from zero to three points. The total score of the inventory is 63 points, and high scores indicate a high depression level.[22]

The pain thresholds of the masticatory muscles and the upper trapezius muscle were measured with a digital dynamometer. Masticatory muscles and the upper trapezius muscle were measured.[23,24] Measurements were taken four times for each point. Because the first measurement value is generally high, the average of the other measurements was recorded.[23,25]

Data analysis

The data were given as mean, standard deviation, or median, minimum, and maximum for continuous variables. Frequency and percentage were given for the categorical variables. The normal distribution of the data was evaluated with the Shapiro-Wilk test. Intergroup comparison of the variables was performed by independent t-test, Mann-Whitney U test, and chi-square test. Correlation analysis was performed by Spearman and Pearson correlation tests. Statistical analysis was performed with the SPSS 25 program, and the significance level was adjusted as $p<0.05$.

Ethics committee approval

The study was approved by the Clinical Research Ethics Committee of the Van Training and Research Hospital (Approval date: 06.07.2022, Approval number: 2022/15-05) and conducted in accordance with the Declaration of Helsinki.

Results

Both groups' baseline physical and sociodemographic characteristics were similar (p>0.05). More than half of the children diagnosed with CP were non-ambulant. Spastic type CP was the most commonly seen predominant motor type in children with CP (Table 1).

Table 1. CP characteristics and intergroup comparison of the physical and sociodemographic characteristics.

	Study group	Control group		
	X±SD	X±SD	t	*p*
Age (y)	38.83±10.26	35.44±8.03	1.103	0.278
Height (cm)	161.33±5.45	163.05±7.74	0.771	0.446
Body weight (kg)	77.63±12.11	75±16.45	0.548	0.587
BMI (kg/m^2)	29.93±4.71	28.30±6.25	0.884	0.383
GMFCS score	n	%		
I	3	17		
II	5	28		
III	-	-		
IV	-	-		
V	10	55		
Types of CP				
Ataxic	2	11		
Quadriplegic	11	61		
Hemiplegic	4	22		
Diplegic	1	6		

*p<0.05 statistical significance, independent t test, CP: Cerebral palsy, BMI: Body mass index, GMFCS: Gross motor function classification system

Bruxism was highly observed in the study group (p<0.05). Nearly three-quarters of the study group had bruxism. Similarly, TMD was highly prevalent in the study group (p<0.05). About one-fifth of the mothers in the control group had bruxism, and two-fifths had TMD (Table 2).

Table 2. Intergroup comparison of bruxism and TMD.

		Control group		Study group			
		n	%	n	%	x^2	*p*
Bruxism	Yes	3	17	13	72	11.25	**0.001***
	No	15	83	5	28		
TMD	Yes	7	39	17	95	12.5	**<0.001***
	No	11	61	1	5		

*p<0.05 statistical significance, chi square test, TMD: Temporomandibular disorder

Mothers in the study group had poorer sleep quality, higher depression, and neck disability compared to mothers in the control group (p<0.05). The FAI score of the mothers in the study group was higher as well (p<0.05) (Table 3).

Table 3. Intergroup comparison of FAI, NDI, PSQI, BDI and bruxism questionnaire scores.

	Control group	Study group		
	Median Min-Max	Median Min-Max	u	*p*
FAI	4 (1-14)	29 (5-38)	-3.707	**<0.001***
NDI	15 (5-75)	42.5 (10-70)	-4.439	**<0.001***
PSQI	7.5 (5-16)	13 (5-17)	-2.96	**0.003***
BDI	5 (0-23)	31.5 (6-49)	-4.562	**<0.001***
Bruxism Questionnaire Score	0 (0-5)	3 (0-5)	-2.952	**0.004***

*p<0.05 statistical significance, Mann Whitney U test, FAI: Fonseca Anamnestic Index, NDI: Neck Disability Index, PSQI: Pittsburgh Sleep Quality Index, BDI: Beck Depression Inventory

Pressure pain thresholds of the masticatory and upper trapezius muscles were lower in the study group compared to the control group (p<0.05) (Table 4). While mothers in the study group had limited mouth opening, mouth opening of the mothers in the control group had acceptable interincisal distance (Table 4).

Table 4. Intergroup comparison of pressure pain thresholds and maximum mouth opening.

		Control group	Study group		
		X±SD	X±SD	t	*p*
Maximum mouth opening (cm)		41.99±4.90	39.26±5.38	1.591	0.121
Right	Masseter anterior (kg/cm^2)	1.46±0.32	1.11±0.23	3.789	**0.001***
	Masseter inferior (kg/cm^2)	1.48±0.31	1.17±0.22	3.421	**0.002***
	Temporalis anterior (kg/cm^2)	1.99±0.30	1.55±0.34	-3.386	**0.001***
	Temporalis middle (kg/cm^2)	2.18±0.32	1.67±0.30	4.848	**<0.001***
	Upper trapezius (kg/cm^2)	2.25±0.46	1.70±0.35	3.975	**<0.001***
Left	Masseter anterior (kg/cm^2)	1.37±0.29	1.09±0.24	3.081	**0.004***
	Masseter inferior (kg/cm^2)	1.46±0.31	1.22±0.21	2.668	**0.012***
	Temporalis anterior (kg/cm^2)	1.98±0.25	1.56±0.33	4.372	**<0.001***
	Temporalis middle (kg/cm^2)	2.12±0.28	1.65±0.32	4.759	**<0.001***
	Upper trapezius (kg/cm^2)	2.28±0.39	1.69±0.31	4.893	**<0.001***

*p<0.05 statistical significance, independent t test

While a significant positive correlation was found between PSQI score and BDI, NDI, and FAI scores (p<0.05), no significant correlation was found between PSQI score and bruxism questionnaire score in the study group (p>0.05). A significant positive correlation was found between BDI score and PSQI, NDI, the bruxism questionnaire, and FAI scores in the study group (p<0.05). A significant positive correlation was found between NDI score and PSQI, BDI, the bruxism questionnaire, and FAI scores in the study group (p<0.05). While a significant positive correlation was found between bruxism questionnaire score and BDI, NDI, and FAI scores (p<0.05), no significant correlation was found between bruxism questionnaire score and FAI score in the study group (p>0.05). A significant positive correlation was found between the FAI score and the PSQI, BDI, NDI, and bruxism questionnaire scores in the study group (p<0.05) (Table 5).

Table 5. Correlation between the FAI, NDI, PSQI, BDI and bruxism questionnaire scores of the study group

	PSQI	BDI	NDI	FAI	Bruxism assessment questionnaire
PSQI		**0.479***	**0.647****	**0.542***	0.148
BDI	**0.479***		**0.765****	**0.714****	**0.676****
NDI	**0.647****	**0.765****		**0.705****	**0.733****
FAI	**0.542***	**0.714****	**0.705****		**0.651****[a]
Bruxism assessment questionnaire	0.148	**0.676****	**0.733****	**0.651****[a]	

*p<0.05, ** p<0.01 statistical significance, Spearman correlation test, [a] Pearson correlation test, FAI: Fonseca Anamnestic Index, NDI: Neck Disability Index, PSQI: Pittsburgh Sleep Quality Index, BDI: Beck Depression Inventory

Discussion

This study revealed that mothers whose children were diagnosed with CP may tend to develop bruxism and TMD.

TMD in the study group was characterized with limited mouth opening and lower mechanical sensitivity of masticatory muscles, which are the cardinal symptoms of TMD.[26] While almost all the mothers in the study group had TMD, nearly two-fifths of the mothers in the control group (39%) had TMD. It was reported that one-third of the population (31%) develops TMD.[27] Considering the prevalence of TMD, both groups in our study had a high rate of TMD. TMD most commonly develops in females aged 20 to 40 years.[28] Considering the control group's average age and gender, these factors might play a role in the high rate of TMD in the control group. However, a high rate of TMD in the study group cannot be explained by risk factors for TMD. At this point, bruxism might have caused the development of TMD in the study group. Bruxism is a rhythmic grinding and clenching masticatory muscle activity.[29] Constant overloading of the temporomandibular joint due to bruxism causes biochemical changes in

the synovial fluid, triggers the inflammatory process, and results in adhesions.[30] Ciancaglini et al.[31] reported that bruxers experience difficulties in mouth opening. Likewise, mothers whose children were diagnosed with CP had limitations in mouth opening. Repetitive muscle activity in bruxism results in microtraumas that might trigger chronic pain by inducing firing in low-frequency muscle nociceptors.[32] In addition, repetitive contraction of masticatory muscles causes hyperirritable spot formation.[33] These sensitive spots might have developed in the study group. Poor sleep quality is another factor causing a lower pressure pain threshold in the masticatory and upper trapezius muscles. A reduction in sleep quality causes a reduction in descending pain inhibition, which results in a central pain modulation deficiency.[34] Considering the effect of reduced sleep quality on pain modulation, poor sleep quality observed in the study group might have a role in lowering pressure pain threshold in the masticatory and upper trapezius muscles.

In this study, 17 % of the mothers in the control group and 72 % of the mothers in the study group had bruxism. Bruxism prevalence in adults is between 8 % and 31.4 %.[35] Although the rate of bruxism in the control group is in line with the reported prevalence, bruxism in the study group was relatively higher than the reported prevalence. Depression might be the primary reason for such a high bruxism rate. A study by Çebi et al.[36] emphasizes that bruxers had a higher BDI score than healthy individuals. Similarly, in our study, a positive correlation was found between the BDI score and the bruxism questionnaire score (p=0.002, r=0.676).

Apart from the cardinal symptoms of TMD, the study group had a high prevalence of secondary symptoms accompanying TMD as well. This study characterized these with high PSQI index, NDI, and BDI score.

Poor sleep quality in mothers whose children were diagnosed with CP was remarkable. Several studies report that there is a relationship between depression and sleep deterioration.[37,38] Nutt et al.[38] report that nearly three-quarters of individuals with depression had sleep deprivation. In line with the study of Nutt et al.[38], there was a positive correlation between the PSQI score and the BDI score (p=0.044, r=0.479). Pain might be another factor affecting the sleep quality of mothers whose children were diagnosed with CP. Sarıpınarlı and Takinacı report that there is a significant positive correlation between PSQI global score and NDI score.[39] In our study, a significant positive correlation was found between the NDI score, which assesses the disability caused by neck pain, and the PSQI score (p=0.004, r=0.647). It was reported that individuals with bruxism had poor sleep quality.[40] Yet, there was no correlation between bruxism and sleep quality in our study. Mothers whose children were diagnosed with CP without having bruxism had poor sleep quality as well. In this regard, the effect of bruxism on sleep quality might be overshadowed by the effect of depression on sleep quality.

Depression is commonly seen in mothers whose children were diagnosed with CP.[41] In our study, similar to previous studies, mothers whose children were diagnosed with CP had a higher depression rate. It was reported that having a child with CP is already enough to trigger depression in mothers. Sajedi et al.[41] reported that having a child with CP increases the risk of depression by 2.12-fold. Poor sleep quality might be another contributing factor to depression. A study by Hu et al.[42] points out an association between poor sleep quality and depression. Accordingly, the PSQI and BDI scores of the study group were positively correlated (p=0.044, r=0.479). Another factor causing depression might be neck disability.[43] In our study, there was a correlation between NDI and BDI scores (p<0.001, r=0.765).

Musculoskeletal problems are observed in mothers whose children were diagnosed with CP.[44] In our study, neck pain was characterized by a lower pressure pain threshold in the upper trapezius and a higher NDI score. In our study, mothers whose children were diagnosed with CP were the primary caregivers of the children. Caregiving includes a variety of activities, ranging from bathing to transfer activities. These activities may result in musculoskeletal problems. During the assessment of the mothers of children, we had the opportunity to

observe the mothers carrying their children for the rehabilitation session. As a result of these activities, neck disability might develop in mothers whose children were diagnosed with CP. Bruxism might be another factor in the development of neck disability in mothers whose children were diagnosed with CP. Neck pain is reported to be one of the symptoms developed due to bruxism. [45] There is a close relationship between neck muscles and masticatory muscles, as mentioned in the study by Giannakopoulos et al.[46] In their study, Giannakopoulos et al. report that during maximum isometric contraction, co-contraction occurs in the neck muscles with up to 11% of their maximum voluntary contraction. This supports our hypothesis, considering the reported close neurophysiological relationship between orofacial and cervical regions.[47] This close neurophysiological relationship emerged in studies as the dynamic interplay between orofacial pain and neck pain.[48] Piekartz et al.[12] found that bruxism and TMD are correlated with neck disability. Accordingly, positive correlations were found between NDI score and FAI score (p=0.001, r=0.705) and NDI score and bruxism questionnaire score (p=0.001, r=0.733).

Limitations

Our study has several limitations. A self-reported questionnaire determined bruxism, so we were only able to prove the existence of probable bruxism in mothers whose children were diagnosed with CP. FAI is used to determine the existence and severity of the TMD; for this reason, we could not determine the subtypes of TMD. For further studies, the use of instrumental assessment methods to diagnose bruxism and the use of DC/TMD to determine the subtypes of the TMD would be much suitable.

Conclusion

Our study showed that mothers whose children were diagnosed with CP may tend to develop TMD and bruxism and once again emphasized the dynamic relationship between factors that might have a role in the development of bruxism and TMD. In clinical settings, mothers whose children were diagnosed with CP should not be ignored, and approaches and assessments should be performed to improve their overall health status as well. In this point of view, TMD and bruxism should be evaluated in these individuals in the context of preventive health services.

Ethics Committee Approval

The study was approved by the Clinical Research Ethics Committee of the Van Training and Research Hospital (Approval date: 06.07.2022, Approval number: 2022/15-05) and conducted in accordance with the Declaration of Helsinki.

Informed Consent

Before enrollment, participants were verbally informed, and their written approval was acquired.

Author Contributions

All authors contributed to every stage of the study.

Conflict of Interest

None.

Financial Disclosure

The authors funded the study.

Peer-review

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Özgün Araştırma/Research Article

Preterm doğum sonrası germinal matriks kanaması olan yenidoğanların retrospektif değerlendirilmesi

Retrospective evaluation of newborns with germinal matrix hemorrhage after preterm delivery

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Öz

Amaç: Bu çalışmada germinal matriks kanaması olan hastaların klinik seyri ve tedavi sonuçlarını değerlendirmek amaçlanmıştır.

Gereç ve Yöntem: Ocak 2018 – Mart 2020 tarihleri arasında germinal matriks kanaması olan hastalar retrospektif olarak incelenmiştir.

Bulgular: Toplam 66 hasta germinal matriks kanaması nedeni ile takip edildi. Hastaların 34'ü kadın, 32'si erkekti. On sekiz hastanın evre-1, 22 hastanın evre-2, 16 hastanın evre-3 ve 10 hastanın evre-4 kanaması vardı. Yirmi altı hastaya ventriküler tap yapıldı. On üç hastaya eksternal ventriküler drenaj takıldı. Bir hastaya ventriküler rezervuar ve bir hastaya ventrikülosubgaleal şant takıldı. Takiplerinde sekiz hastaya ventrikülöperitoneal şant takıldı. Otuz altı hasta exitus oldu. Yirmi beş hasta taburcu edildi. Beş hasta dış merkeze sevk edildi.

Sonuç: Preterm doğum sonrası germinal matriks kanamaları sık görülmekte ve asemptomatik olabilmektedir. Hastaların transfontanel ultrasonografi ile değerlendirilmeleri erken tanı ve tedavi olanağı sağlamaktadır. Bu hastaların tedavileri konusunda ortak bir algoritma henüz bulunmamaktadır.

Anahtar Kelimeler: Preterm; Germinal matriks; Posthemorajik hidrosefali.

Abstract

Aim: This study aimed to evaluate the clinical course and treatment results of patients with germinal matrix hemorrhage.

Materials and Methods: Patients who have germinal matrix hemorrhage, between January 2018 and March 2020 were retrospectively analysed.

Results: A total of 66 patients were followed up due to germinal matrix hemorrhage. Thirty-four of the patients were girls and 32 were boys. Eighteen patients had stage-1, 22 stage-2, 16 stage-3 and 10 stage-4 hemorrhages. Ventricular tap was performed in 26 patients. External ventricular drainage was used in 13 patients. Ventricular reservoir, and ventriculosubgaleal shunt were used in one patient each. Eight patients underwent ventriculoperitoneal shunt surgery. Thirty-six patients died. Twenty-five patients were discharged. Five patients were referred to an external center.

Conclusions: Germinal matrix hemorrhages are common after preterm delivery and may be asymptomatic. Evaluation of these patients with transfontanel ultrasonography provides the opportunity for early diagnosis and treatment. There is no common algorithm for the treatment of these patients yet.

Keywords: Preterm; Germinal matrix; Posthemorrhagic hydrocephalus.

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Giriş

Dünya Sağlık Örgütü verilerine göre yılda yaklaşık 15 milyon bebek prematür doğmakta ve yılda bir milyon preterm, yenidoğan dönemini bitiremeden hayatını kaybetmektedir.[1,2] Prematür doğum oranları ülkelerin gelişmişlik düzeyleri ile ters orantı gösterir. Bu oran bazı Avrupa ülkelerinde %5 iken, bazı Afrika ülkelerinde %18'lere çıkmaktadır.[3] Günümüzde yenidoğan yoğun bakım ünitelerinin şartlarının iyileşmesi, uzmanların klinik tecrübelerinin artması sayesinde prematür doğan bebeklerin mortalitesi azalmıştır. Bu durum klinikte, nöral gelişime ciddi zarar verebilen germinal matriks kanamalarının daha sık görülmesini de beraberinde getirmiştir.[4] Germinal matriks kanamaları çoğunlukla hayatın ilk üç günü içerisinde gelişmekte ve insidansı %15 ile %40 arasında değişmektedir. Serebral palsi ve mental retardasyonun en önemli nedenlerinden biridir.[5,6] Doğum haftası, düşük doğum ağırlığı, düşük APGAR skoru, doğumda hipoksi öyküsü, mekanik ventilatör desteği, sepsis gibi birçok faktörün germinal matriks kanaması ile ilişkili olduğu bildirilmiştir.[6,7]

Transfontanel Ultrasonografi (USG) hasta başı uygulanabilmesi, iyonize radyasyon içermemesi ve güvenilirliği ile prematürlerde germinal matriks kanamalarını değerlendirmede yaygın olarak kullanılmaktadır.[8] Günümüzde germinal matriks kanamalarının cerrahi tedavisine ilişkin ortak bir algoritma bulunmamaktadır.

Çalışmamızda preterm doğan ve yenidoğan yoğun bakım ünitesinde takiplerinde germinal matriks kanaması tanısı alan hastalar retrospektif olarak değerlendirilmiştir.

Gereç ve Yöntem

Araştırmanın tipi

Bu çalışma Adıyaman Üniversitesi Eğitim ve Araştırma Hastanesi Beyin ve Sinir Cerrahisi Anabilim Dalı ve Yenidoğan Yoğun Bakım Ünitesinde düzenlenen retrospektif bir çalışmadır.

Araştırmanın evreni ve örneklemi

Bu çalışmaya Ocak 2018–Mart 2020 tarihleri arasında yenidoğan yoğun bakım ünitesine yatırılarak takip edilen, prematür doğum öyküsü bulunan ve takiplerinde germinal matriks kanaması tanısı alan hastalar dahil edilmiştir. Prematür doğum öyküsü olmayan, farklı etiyolojiye bağlı gelişen germinal matriks kanaması olan hastalar çalışmadan dışlanmıştır.

Veri toplama araçları

Hasta verileri için hasta dosyaları retrospektif olarak tarandı. Hastaların demografik karakteristikleri, ameliyat notları, hastane progres notları, yapılan radyolojik görüntüleme tetkikleri, poliklinik takip notları incelendi.

Verilerin analizi

Bütün hastaların gestasyonel yaşı, doğum ağırlığı, doğum şekli, cinsiyeti, baş çevresi belirlenip kaydedildi. Germinal matriks kanamaları Papile Evrelemesine[9] göre sınıflandırıldı. Cerrahi yolla tedavi edilen hastalarda operasyon ilk yazar (Özen A.) tarafından yapıldı.

Araştırmanın etik boyutu

Bu çalışma için ilgili üniversitenin girişimsel olmayan klinik çalışmalar etik kurulundan izin alınmıştır (Tarih: 15.11.2022, Karar Sayısı: 2022/8-10). Araştırma süreci Helsinki Bildirgesi ilkelerine uygun olarak yürütülmüştür.

Bulgular

Ocak 2018 – Mart 2020 tarihleri arasında prematür doğum nedeni ile yenidoğan yoğun bakım ünitesinde takip edilen hastalar gestasyonel yaşına göre geç preterm (34-36 hafta), orta preterm (32-34 hafta) ve erken preterm (<32 hafta) olarak üç gruba bölünerek tarandı. Bu hastalardan germinal matriks kanaması olan hastalar retrospektif olarak incelendi. Preterm doğum öyküsü olan hastalara ilk üç gün boyunca her gün, sonrasında gün aşırı yatak başı transfontanel USG yapıldı. Orta preterm ve geç preterm doğum öyküsü olan infantlarda germinal matriks kanaması tanısı alan hasta yoktu. Erken preterm doğum öyküsü olan 304 hastanın 66'sında (%21,7) germinal matriks

kanaması gözlendi. Bu hastalarda ortalama gestasyonel yaş 26.1 (22-32) hafta idi. Hastaların 48'i (%73) sezaryen doğum ile 18'i (%27) normal vajinal yol ile doğmuştu. Ortalama doğum ağırlığı 901 (370-1500) gramdı. Hastaların 34'ü (%51,5) kadın ve 32'si (%48,5) erkekti. Hastalara ait demografik veriler Tablo 1'de özetlenmiştir.

Tablo 1. Demografik veriler.

Cinsiyet	**n (%)**
Erkek	32 (%48,5)
Kadın	34 (%51,5)
Gestasyonel Yaş (hafta)	26.1 (22-32)
Doğum Şekli	**n (%)**
Sezaryen	48 (%73)
Normal Vajinal Yol	18 (%27)
Doğum Ağırlığı (gram)	901 (370-1500)

Hastaların hepsinde tanı yatak başı yapılan transfontanel USG ile kondu. Papile evrelemesine göre hastaların 18'inde (%27) evre-1, 22'sinde (%33) evre-2, 16'sında (%24) evre-3 ve 10'unda (%16) evre-4 germinal matriks kanaması gözlendi.

Hastaların hepsi tanı konduktan sonra nöroşirurji bölümü tarafından değerlendirildi ve takibe alındı. Tanı sonrası hastalara günlük baş çevresi takibi yapıldı. Hastalar aynı zamanda gün aşırı transfontanel USG ile takip edildi. Hastaların takiplerinde baş çevresi Levene'in[10] geliştirdiği ventriküler indeks değerlerine göre incelendi. Bu indeks değerlerine göre, baş çevresi 97 persantil değerini 4 mm aşan hastalar hidrosefalik olarak değerlendirildi ve Beyin Omurilik Sıvısı (BOS) boşaltıldı. BOS boşaltılması için hastalarda ilk olarak ventriküler tap uygulandı. Takiplerinde günde birden fazla ventriküler tap ihtiyacı olan ve üç gün üst üste ventriküler tap ihtiyacı olan hastalara cerrahi tedavi uygulandı.

Evre-1 ve evre-2 kanaması olan toplam kırk hastanın takiplerinde hidrosefali gelişmedi ve BOS boşaltılması ihtiyacı olmadı. Bu hastaların 24'ü takiplerinde farklı etiyolojiler sonucu multiorgan yetmezliği nedeni ile exitus oldu.

Evre-3 germinal matriks kanaması olan hastaların hepsine tanı konduktan sonra ventriküler tap yapılarak BOS boşaltıldı. Bu hastaların altısında ventriküler tap sonrası sebat eden hidrosefalisi olmadı ve ek bir girişime gerek duyulmadı. Evre-3 kanaması olan diğer 10 hastada ardışık ventriküler tap yapılmasına rağmen hızlı baş çevresi büyümesinin durmaması nedeni ile cerrahi işlem yapıldı. Bu hastaların sekizine Eksternal Ventriküler Drenaj (EVD) sistemi takılırken, bir hastaya ventrikülosubgaleal şant ve bir hastaya ventriküler rezervuar takıldı. Ventrikülosubgaleal şant takılan hastanın takiplerinde hidrosefalisi sebat etti ve hastada multikistik hidrosefali gelişti. Bu hastaya ağırlığı 2500 gramı geçtikten, BOS sterilizasyonundan emin olduktan ve BOS protein düzeyi 1.5 gr/L altına indikten sonra endoskopik kist fenestrasyonu ve aynı seansta ventrikülöperitoneal şant takılması operasyonu yapıldı. Aynı hastanın yoğun bakım sonrası birinci yıl takiplerinde şant disfonksiyonu saptanması üzerine tekrar endoskopik kist fenestrasyonu ve şant revizyonu operasyonu yapıldı. Ventriküler rezervuar takılan hastanın hidrosefalisi sebat etti ve uygun şartlar sağlandıktan sonra ventriküler rezervuar çıkartılarak, ventrikülöperitoneal şant takıldı. Bu hastanın uzun dönem takiplerinde şant enfeksiyonu ya da disfonksiyonu görülmedi. EVD sistemi takılan sekiz hastanın dördünde menenjit gelişti ve antibiyoterapi başlandı. EVD sistemi takılan sekiz hastanın altısında hidrosefalinin sebat etmesi üzerine uygun şartlar sağlandıktan sonra ventrikülöperitoneal şant takıldı. Evre-3 kanaması olan hastalardan ikisi sepsise bağlı multiorgan yetmezliği nedeni ile exitus oldu.

Evre-4 germinal matriks kanaması olan hastaların takiplerinde, ventriküler tap yapılmasına rağmen baş çevresinde hızlı büyüme devam eden beş hastaya EVD sistemi takıldı. Bu hastaların takiplerinde hepsinde EVD sistemine bağlı menenjt gelişti ve antibiyoterapileri başlandı. Evre-4 germinal matriks kanaması olan hastaların hepsi farklı etiyolojiler nedeni ile multiorgan yetmezliği sonucu exitus oldu.

Toplamda germinal matriks kanaması nedeni ile takip edilen 66 hastanın 13'üne EVD sistemi takıldı. EVD sistemi takılan hastaların dokuzunda (%70) menenjit gelişti. EVD sistemi takılan evre-3 kanaması olan sekiz hastanın altısında hidrosefalinin sebat

etmesi nedeni ile ventriküloperitoneal şant takıldı. EVD sistemi takılan evre-4 kanaması olan beş hastanın hepsi exitus oldu. Ventrikülosubgaleal şant ve ventriküler rezervuar takılan iki hastada da hidrosefalinin sebat etmesi üzerine ventriküloperitoneal şant takıldı. Toplamda 66 germinal matriks kanaması olan hastanın 8'ine (%12) ventriküloperitoneal şant takılmış oldu.

Germinal matriks kanaması nedeni ile takip edilen 66 hastanın 36'sı (%54,5) takiplerinde farklı etiyolojiler nedeni ile multiorgan yetmezliğine girerek exitus oldu. Yirmi beş hasta (%38) yenidoğan yoğun bakım ünitesinden şifa ile taburcu oldu. Beş hasta (%7,5) ise ailesinin isteği üzerine farklı merkezlere transfer edildi. Hastaların germinal matriks kanama evreleri ve uygulanan tedaviler Tablo 2'de özetlenmiştir.

Tablo 2. Germinal matriks kanama evreleri ve uygulanan tedaviler.

Tedavi	Evre-1 (18 hasta)	Evre-2 (22 hasta)	Evre-3 (16 hasta)	Evre-4 (10 hasta)
Eksternal Ventriküler Drenaj	-	-	8 hasta	5 hasta
Ventrikülosubgaleal Şant	-	-	1 hasta	-
Ventriküler rezervuar	-	-	1 hasta	-
Sebat eden hidrosefali nedeni ile ventriküloperitoneal şant takılması	-	-	8 hasta (%50)	-(Hastaların hepsi exitus oldu.)

Tartışma

Düşük doğum ağırlıklı prematürelerin %80'inde germinal matriks kanamaları ilk iki gün içerisinde görülür ve genellikle asemptomatiktir. Kliniğimizde de preterm doğum öyküsü olan hastaların hepsinde ilk üç gün günlük yatak başı transfontanel USG yapılmaktadır. Hastaların hepsi rutin USG taramalarında tanı almıştır. Germinal matriks kaynaklı intraventriküler kanamalar transfontanel USG sınıflamasına göre 4 evrede değerlendirilir. Evre-1: germinal matriks ile sınırlı kanamaları, evre-2: ventriküler dilatasyon olmadan intraventriküler kanamaları, evre-3: ventriküler dilatasyonun eşlik ettiği intraventriküler kanamaları, evre-4: ventriküler dilatasyona ek olarak intraparankimal kanamaları ifade etmektedir. Evre-1 ve evre-2 kanamalar, bebeklerde ilerleyen dönemde gelişim yetersizliği riski oluştururken, evre-3 ve evre-4 kanamalar hidrosefali, serebral palsi, mental retardasyon gibi ciddi komplikasyonlara neden olabilir.[11–13] Çalışmamızda 18 hastada evre-1, 22 hastada evre-2, 16 hastada evre-3 ve 10 hastada evre-4 germinal matriks kanaması saptandı. Toplamda 66 germinal matriks kanaması olan hastanın 36'sının takiplerinde exitus olması ve taburcu olan hastaların COVID-19 pandemisi nedeni ile uzun dönem takip verilerine ulaşılamaması nedeni ile, hastaların uzun dönem mental motor gelişim durumları değerlendirilemedi.

Patogenezde temel sorun, immatür vasküler bir ağa sahip germinal matriksten olan kanamadır.[14] Germinal matriks fetal ventriküler sistemi çevreler ve zamanla küçülerek term dönemde kaybolur. Germinal matriks glial ve nöronal prekürsör hücrelerin kaynağıdır. Hızla prolifere olan bu hücreler nedeni ile bu alan çok vasküler bir alandır. İrregüler, geniş, immatür ve frajil damarlardan oluşmuş ince duvarlı kapiller bir ağa sahiptir.[15] Temel sorun bu kapiller ağdan olan kanamadır. Bu bölgede tanımlanmış fibrinolitik aktivite de kanamanın yayılmasında rol oynamaktadır.[16]

Germinal matriks kanamalarının tanısı transfontanel USG ile konur. Erken preterm bebekler mutlaka, orta ve geç preterm bebekler ise stabil değillerse transfontanel USG ile taranmalıdır.[17] Yenidoğan yoğun bakım ünitemizde tüm preterm bebekler ilk üç gün transfontanel USG ile taranmaktadır. Kanama saptanan hastaların takipleri yine transfontanel USG ile yapılmaktadır. Ventriküler dilatasyon görülen ve BOS boşaltılması gereken hastalarda kranial bilgisayarlı tomografi çekilmektedir.

Germinal matriks kanaması olan hastaların yaklaşık %50'si asemptomatiktir ve rutin yapılan transfontanel USG sırasında tanı alır. Semptomatik hastalarda ise klinikte bilinç değişiklikleri, kardiyorespiratuar

değişiklikler, ani kan şeker düzey değişiklikleri, ani hematokrit düşüşleri görülebilir. Germinal matriks kanaması olan hastaların fizik muayenesinde gergin fontanel, hipotonik görünüm, letarji ve anormal göz hareketleri görülebilir. Hastaların klinik takipleri sırasında epileptik atak geçirme riski vardır.[18] Çalışmamızda hastaların hepsi rutin yapılan transfontanel USG sırasında tanı almıştır. Tanı aldıktan sonraki takiplerinde BOS boşaltılması gereken hastaların hepsinde ön fontanel gergin olarak muayene edilmiştir. Tanı sonrası tüm hastalardan kontrol hemogram düzeylerine bakılmıştır. Hastaların hepsinde hematokrit düzeyinde düşüş gözlenmiş fakat hiçbirinde kan ürünü replasmanı ihtiyacı olmamıştır.

Germinal matriks kanaması nedeni ile takip edilen hastalarda günlük baş çevresi takibi yapılmalıdır. Normalde 26-32 haftalık pretermlerde 1 mm/gün, 32-40 haftalık pretermlerde 0.7 mm/gün baş çevresi artışı görülebilir. Günlük 2 mm baş çevresi artışı veya iki günde toplam 4 mm artış anormal olarak kabul edilir. Bu hastalarda beyaz cevher kaybına bağlı ventriküler dilatasyonu, hidrosefaliye bağlı ventriküler dilatasyondan ayırt etmek önemlidir.[19] Çalışmamızda tedavi kararı günlük baş çevresi ölçümlerine ve yapılan transfontanel USG ölçümlerine göre verilmiştir. Germinal matriks kanamalarında tedavi kararı lateral ventrikül genişliğinin (midkoronal düzeyde orta hattan laterale olan mesafe ölçülür) yaş için 97 persentilin 4 mm üzerine çıkması olarak kabul edilmiştir.[20] Ancak bazen ventriküler genişleme laterale doğru olmaz ve posteriora veya anteriora doğru genişleme olabilir. Bu durumda tedavi kararı için Davies ve ark.[21] tarafından tanımlanan anterior horn genişliği, talamo-oksipital genişlik ve üçüncü ventrikül genişliği ölçümleri kullanılabilir. Tedavi kararı için bu ölçümlerde her üç ölçümün 95 persentil değerinin 1 mm üzerinde olması gerekmektedir.

Germinal matriks kanamasına bağlı posthemorajik ventriküler dilatasyonun tedavisinde birçok yöntem denenmiştir. Günümüzde halen etkinliği ve güvenilirliği kanıtlanmış, nörolojik prognozu olumlu etkileyen bir tedaviden söz etmek mümkün değildir. Tekrarlayan lomber ponksiyon ve/veya ventriküler tap ile BOS boşaltılabilir. Bu tedavide minimum 10 ml/kg BOS boşaltılmalıdır. Bir defada en fazla 20 ml/kg BOS boşaltılabilir ve BOS boşaltma hızı 1 ml/kg/dakikayı aşmamalıdır.[19] Yapılan çalışmalar ventriküler tap veya lomber ponksiyonla BOS boşaltılmasının, şant ihtiyacını azaltma ve nörolojik hasarı azaltma konusunda yardımcı olmadığını göstermiştir. Yine bu hastalarda seri lomber ponksiyon veya ventriküler tap ile BOS boşaltılmasında, iki yöntem arasında bir fark belirtilmeksizin %7 oranında enfeksiyon ihtimali olduğu gösterilmiştir. Aynı zamanda her girişim nöral yapılara zarar verme ve kanama riski taşımaktadır.[22] Whitelaw ve Aquilina[19] çalışmalarında; yenidoğan döneminde fontanel açık olduğu için ve bu hastalarda ventrikülomegali olmasına rağmen kafa içi basınç yüksekliği fazla olmadığı için kendi klinik tecrübelerince seri lomber ponksiyon ile yeterli miktarda ve efektif BOS boşaltılmasının sağlanamadığını ve ventriküler tap tercih edildiğini belirtmişlerdir. Hastalarımızda da ilk tedavi seçeneği olarak ventriküler tap uygulanmıştır. Her defasında 10 ml/kg BOS boşaltılmıştır. Ardışık ventriküler tap ihtiyacı olan hastalara cerrahi tedavi uygulanmıştır. Ventriküler tap uygulanan hastalarımızda kanama ya da enfeksiyon görülmemiştir. Ventriküler tap yapılan evre-3 ve evre-4 toplam 26 hastanın takiplerinde, 15 hastada ventriküler tap ihtiyacının artması nedeni ile cerrahi girişime gidilmiştir. Bu hastalardan 13'üne EVD sistemi takılmıştır. Bir hastaya ventriküler rezervuar ve bir hastaya ventrikülosubgaleal şant takılmıştır.

EVD sistemi devamlı olarak BOS boşaltılmasını sağlayarak kafa içi basıncın düşük tutulmasını sağlar. Ventriküler genişlemeyi engeller. Aynı zamanda bu hastalarda ventriküllerde bulunan kanı uzaklaştırır.[23] Germinal matriks kanaması sonrası ventriküler dilatasyonda EVD sistemi her ne kadar sık kullanılsa da güvenilirliği ile ilgili kontrollü çalışmalar yoktur. Uzun dönem kullanılması yüksek enfeksiyon riski taşımaktadır. Uzun süre kullanımlarda yenilenmesi gerekmektedir.[19] Çalışmamızda

toplamda 13 hastaya EVD sistemi takılmıştır. Bu hastaların 9'unda (%70) menenjit gelişmiştir. Çalışmamızda literatüre benzer şekilde EVD sistemi takılan hastalarda yüksek oranda enfeksiyon görülmüştür. EVD sistemi takılan evre-3 kanaması olan sekiz hastanın altısında hidrosefalinin sebat etmesi nedeni ile ventriküloperitoneal şant takıldı. İki hastanın takiplerinde hidrosefalisi gelişmediği için ek bir cerrahiye ihtiyacı olmadı. EVD takılan ve evre-4 kanaması olan beş hastanın hepsi takiplerinde exitus oldu.

Ventrikülosubgaleal şant, subgaeal boşluk ile ventriküllerin bir tüp sistemi aracılığı ile birleştirilmesi ile bu boşluğa BOS drenajının sağlanması ile çalışır. Subgaleal boşluk geniş bir şekilde açılmalıdır. Ek bir girişim gerekmeksizin sürekli BOS drenajını sağlaması açısından avantajlıdır. Yapılan bir çalışmada ventriküler rezervuar ile karşılaştırılmış ve şant gereksinimi ve enfeksiyon açısından anlamlı farklılık saptanmamıştır.[24] Farklı bir çalışmada ise şant gereksinimi açısından ventriküler rezervuardan daha üstün bulunmuştur.[25] Köksal ve ark.[26] yaptığı çalışmada ventrikülosubgaleal şant kullanılan grupta mortalite ve morbiditeyi daha düşük bulmuştur. Çalışmamızda bir hastaya ventrikülosubgaleal şant takılmıştır. Bu hastanın takiplerinde subgaleal mesafede aşırı BOS birikimi ve emilim yetersizliği nedeni ile 2 defa ponksiyonla subgaleal mesafede biriken BOS boşaltılmıştır. Biriken BOS sonucu kalvaryal şekil bozukluğu görülmüştür. Takiplerinde bu hastada multikistik hidrosefali gelişmiş ve endoskopik kist fenestrasyonu sonrası ventrikülöperitoneal şant takılmıştır. Hastanın takiplerinde menenjit görülmemiştir. Fakat birinci yıl takiplerinde şant disfonksiyonu nedeni ile tekrar endoskopik kist fenestrasyonu ve şant revizyonu yapılmıştır.

Ventriküler rezervuar, basit, her defasında ventrikülü ponksiyone etmeden BOS boşaltılmasını sağlayan bir araçtır. Günümüzde bu hastalarda standart tedavi olması gerektiği bildirilmiştir. Bu tedavi yöntemi de randomize kontrollü çalışmalar ile değerlendirilmemiştir.[19] Yine ventriküler rezervuar takılması sonrası cilt nekrozu, BOS kaçağı, katater migrasyonu ve enfeksiyon gibi komplikasyonlar bildirilmiştir.[27,28] Çalışmamızda bir hastaya ventriküler rezervuar takılmıştır. Bu hastanın takiplerinde hidrosefalisi sebat etmiştir. Hastaya ventrikülöperitoneal şant takılmıştır. Uzun dönem takiplerinde şant enfeksiyonu ya da disfonksiyonu görülmemiştir.

Posthemorajik hidrosefali hastalarının %60'ında ventriküler dilatasyon spontan olarak veya tedavi ile durmaktadır. Bu hastaların %15'ine takiplerinde ventrikülöperitoneal şant takılması gerekmektedir. Hastaların %5'i aylar sonra geç progresif hidrosefali geliştirebilmektedir. Bu açıdan olgular bir yıl izlenmelidir. Şant gereksinimi olan ve şant takılan hastalarda serebral palsi gelişimi ve kötü nörogelişimsel prognoz, şant gereksinimi olmayan hastalara göre daha yüksek oranda bulunmuştur.[29] Germinal matriks kanaması sonrası hidrosefalisi olan hastalarda hastanın çok küçük olması, ventriküllerde kan olması nedeni ile erken dönemde şant takılması mümkün olmamaktadır. Bu hastalarda 35 günlükten önce takılan şantlarda yüksek oranda enfeksiyon ve disfonksiyon bildirilmiştir.[30] Yine BOS protein miktarının 1.5 gr/L'nin üzerine çıkmasının yüksek şant disfonksiyonu ile ilişkili olduğunu gösteren çalışmalar mevcuttur.[19] Kliniğimizde bu hastalara ventrikülöperitoneal şant takılması için, hastaların 2500 grama ulaşmaları beklenmektedir. Şant takılması öncesi mutlaka üremesiz BOS kültürü ve BOS proteininin 1.5 gr/L altında olması beklenmektedir. Çalışmamızda toplam sekiz hastaya (%12) ventrikülöperitoneal şant takılmıştır. Bu hastalardan hiçbirinde takiplerinde şant enfeksiyonu görülmemiştir. Bir hastada şant disfonksiyonu nedeni ile revizyon yapılmıştır. Çalışma grubumuzda uzun dönem takiplerde kalıcı şant gereksinimi oranının düşük olmasını, çalışma grubumuzun erken preterm doğan ve düşük doğum ağırlıklı bebeklerden oluşmasına ve ek sorunlar nedeni ile mortalite oranının yüksek olmasına bağlamaktayız.

Araştırmanın kısıtlılıkları

Araştırma kapsamında prematür doğan ve germinal matriks kanaması tanısı alan 66

hastanın olması çalışma grubunun küçüklüğü açısından çalışmanın kısıtlılıklarından biridir. Çalışmayı sınırlandıran faktörlerden diğeri ise, bu hastaların hepsinin erken preterm doğan ve düşük doğum ağırlıklı bebekler olması nedeni ile takiplerde ek sorunlar nedeni ile mortalitenin yüksek olması ve exitus olan hastaların posthemorajik ventriküler dilatasyon açısından uzun dönem takip edilememiş olmasıdır.

Sonuç

Preterm doğum olan hastalarda germinal matriks kanamaları çoğunlukla ilk üç gün içerisinde olmaktadır. Bu yüzden bu hastaların transfontanel USG ile takip edilmeleri, erken tanı ve tedavi açısından önemlidir. Ventriküler dilatasyon gelişen hastalarda henüz ortak bir tedavi algoritması bulunmamaktadır. Aralıklı ventriküler tap, EVD sistemi takılması, ventriküler rezervuar, ventrikülosubgaleal şant takılması tedavileri uygulanabilir. EVD sistemi takılması yüksek oranda enfeksiyon riski barındırmaktadır.

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Bilgilendirilmiş Onam

Çalışma retrospektif bir çalışmadır.

Yazar Katkıları

Fikir/Kavram A.Ö.; Tasarım A.Ö., S.A.; Veri Toplama ve/veya İşleme A.Ö., S.A.; Analiz ve/veya Yorum A.Ö., S.A.; Literatür Taraması A.Ö., S.A.; Makale Yazımı A.Ö., S.A.; Eleştirel İnceleme A.Ö., S.A.

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Yazarların herhangi bir çıkara dayalı ilişkisi yoktur.

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Dış bağımsız

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