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Oxidative stress markers in breast cancer

Meme kanserinde oksidatif stres belirteçleri

Özge Fenercioğlu, Giray Bozkaya, Nuriye Uzuncan, Baha Zengel

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Abstract

Purpose: The aim of this study was to compare serum Total Antioxidant Status (TAS), Total Oxidant Status (TOS), Oxidative Stress Index (OSI) and Ischemia Modified Albumin (IMA) levels in breast cancer patients and healthy women and to determine whether there is a relationship between oxidative stress markers and breast cancer.

Materials and methods: Newly diagnosed 106 breast cancer patients at the Bozyaka Training and Research Hospital General Surgery Clinic and 30 healthy women were included in the study. Serum levels of IMA, TAS, TOS were analyzed by spectrophotometric methods.

Results: IMA, TOS and OSI values were found significantly higher in the breast cancer group. Although the TAS values in patients with breast cancer were lower than the control group, the difference was not statistically significant.

Conclusions: In our study, it was seen that oxidative stress levels increased in breast cancer patients due to decreased TAS, and significantly increased IMA, TOS and OSI results. There was no correlation of histopathologic findings with IMA, TOS and OSI levels in terms of tumor grade and size. Disturbed oxidative stress status and IMA may contribute to the pathogenesis of breast cancer and more comprehensive studies are needed for prognostic value.

Keywords: Breast cancer, biomarkers, ischemia-modified albumin, oxidative stress.

Fenercioglu O, Bozkaya G, Uzuncan N, Zengel B. Oxidative stress markers in breast cancer. Pam Med J 2024;17:402-411.

Öz

Amaç: Çalışmamızda oksidatif stres ile meme kanseri arasında ilişki olup olmadığını araştırmak için, meme kanseri hastalarında ve sağlıklı kadınlarda serumda Total Antioksidan Seviye (TAS), Total Oksidan Seviye (TOS), Oksidatif Stres İndeksi (OSİ) ve İskemi Modifiye Albumin (İMA) değerlerinin karşılaştırması amaçlandı.

Gereç ve yöntem: Bozyaka Eğitim ve Araştırma Hastanesi Genel Cerrahi Kliniği'nde yeni tanı almış ve henüz tedavi almamış 106 meme kanseri hastası ve sağlıklı 30 kadın çalışmaya alındı. Serum TAS, TOS ve İMA düzeyleri spektrofotometrik yöntemle analiz edildi.

Bulgular: Meme kanseri grubunda İMA, TOS ve OSİ düzeyleri, istatistiksel olarak anlamlı yüksek bulundu. TAS değerleri ise, meme kanserli hastalarda kontrol grubuna göre düşük olmasına rağmen, aradaki fark istatistiksel olarak anlamlı değildi.

Sonuç: Çalışmamızda meme kanseri hastalarında azalmış TAS, anlamlı derecede artmış İMA, TOS ve OSİ sonuçları sebebiyle oksidatif stres düzeylerinin arttığı anlaşılmaktadır. IMA, TOS ve OSI meme kanseri patofizyolojisinde önemli bir belirteç olup prognostik değer için daha kapsamlı çalışmalara ihtiyaç duyulmaktadır.

Anahtar kelimeler: Meme kanseri, biyobelirteçler, iskemi modifiye albumin, oksidatif stres.

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Introduction

Breast cancer is known as the most prevalent form of cancer in women, constituting approximately 30% of all cancers in women. Breast cancer is the second leading cause of deaths associated with cancer, following lung cancer [1]. The exact cause of breast cancer is unknown, but many factors such as genetics, environmental factors, age, diet, reproductive features, body weight, physical activity, alcohol use, endogenous and exogenous hormonal factors are thought to play a role in its etiology [2].

Metabolic and physiological processes in the body produce free radicals but they are removed by antioxidant mechanisms. Excessive production of free radicals or insufficient antioxidant system causes oxidative stress which plays a vital role in many health problems such as inflammation, ischemia reperfusion injury, diabetes mellitus, lung diseases, atherosclerosis, muscle diseases, kidney diseases and especially cancer [3-5]. Endogenous and exogenous antioxidants can prevent the development of diseases by neutralizing the free radicals or preventing their effects [6]. Reactive oxygen species (ROS), which are composed of oxygen, are the most important free radicals in biological systems and affect different stages of cancer formation. There are studies showing that ROS has many roles in cancer formation [7, 8].

Under normal physiological conditions, the organism has an antioxidant defense system that combats endogenous or exogenous free radicals and oxidative stress. By-products of free oxygen molecules synthesized in the body endogenously make the biggest contribution to total oxidant capacity [9]. Total antioxidant capacity is composed of mainly from antioxidant molecules in plasma such as uric acid, bilirubin, albumin, ceruloplasmin, transferrin, vitamins E and C, as well as chain-breaking antioxidants that bind free radicals [10]. The N-terminal end of albumin consists of the amino acid sequence aspartate-alanine-histidine-lysine amino acid chain, which is a high affinity binding site for transition metals such as cobalt, copper and nickel. It has been observed that it shows strong binding capacity for cobalt (Co^{+2}) compared to other metal ions. In the case of ischemia and reperfusion, cellular changes, especially due to free radical production and oxidative damage, have been shown to cause structural modifications at the N-terminal end by deletion or N-acetylation of one or more amino acids [11]. These modifications decrease the albumin binding capacity and a new molecule formed is ischemia modified albumin (IMA) [12, 13].

Our aim was to compare the serum IMA, Total Antioxidant Status (TAS), Total Oxidant Status (TOS), Oxidative Stress Index (OSI) levels in women with breast cancer and healthy control group to investigate whether there was any relationship between oxidative stress and breast cancer.

Materials and methods

One hundred and six women who were recently diagnosed as breast cancer and have not yet started medical or surgical treatment and 30 healthy women who did not have any pathology in their annual routine mammography and ultrasonography, were included as control group in the study. Tru-cut biopsies were performed via the same surgeon accompanied with the same radiologist. After the biopsies were taken, histopathological diagnosis was made by the same pathologist to prevent the difference between the observers. Eighty-nine of breast cancer patients were diagnosed as invasive ductal carcinoma. Stage 1 and 2 carcinomas constitute most of the patient group while stage 3 patients are few and stage 4 patients are absent in our study. Patients with cardiovascular disease, hypertension, diabetes mellitus, any active or chronic infectious disease, thyroid conditions, liver diseases, chronic kidney failure, secondary malignancy, those had cerebral stroke or transient ischemic attack, and pregnant women were not included in the study. None of the participants in the present study were using drug medications including vitamins or antioxidant drugs.

The research protocol received approval from the SBU Bozyaka Training and Research Hospital Ethics Committee and all the patients were informed before the research.

Blood samples were obtained from all individuals after fasting. After centrifugation of the blood samples, aliquots of serum were stored at -80°C until the analysis.

Ischemia modified albumin analysis

IMA levels were measured using Bar Or et al. [14] method which is based on the colorimetric detection of the color complex formed by dithiothreitol (DTT) and cobalt that does not bind to albumin. Albumin corrected IMA (A-IMA) levels of all samples were given in absorbance unit (ABSU) with the following formula: A-IMA=IMAx (albumin/albumin median of the group) [15].

Total oxidant status analysis

TOS levels were studied in Beckman Coulter's Olympus AU 680 autoanalyzer (Brea, CA, USA) using a TOS kit (Relassay®, Gaziantep, Türkiye). This automatic colorimetric measurement method developed by Erel et al. [9] was performed. Ferrous iron is oxidized to ferric iron and forms a colored compound. The color intensity in a sample corresponds to the total oxidant molecules present in the sample. Calibration was performed using hydrogen peroxide, and the TOS results are expressed as μmol H_2O_2 Eq/L [9].

Total antioxidant status analysis

TAS levels were also studied in Olympus 680 autoanalyzer using a commercially available TAS kit (Relassay®, Gaziantep, Türkiye). This method was also developed by Erel et al. [9] and is based on antioxidants that cause fading of dark blue green 2,2'-azinobis- 3-ethylbenzothiazoline-6-sulfonic acid. This color change is measured at 660 nm spectrophotometrically and the results are given in mmolTrolox/L.

Oxidative stress index

TAS results were calculated as μmol/L and OSI was determined according to the following formula:

OSI=TOS (μmol H2O2Eq/L)/TAS [μmol Troloks/L)x100

Statistical analysis

For statistical analysis, SPSS 21 statistics program was used. The normality of the variables was analysed by Kolmogorov-Smirnov test. The parameters having normal or abnormal distribution were examined with Student's t test and Mann-Whitney U test, respectively. Mean and standard deviation (SD) values were specified for normally distributed parameters. Median and 25^{th}-75^{th} percentile values were specified for abnormally distributed parameters. Chi-square test was used for categorical data.

The relation between parameters was investigated with Pearson and Spearman correlation analysis according to normal or abnormal distribution, respectively. Cut-off value was determined for the IMA, TOS, and OSI values by the Receiver operating characteristic (ROC) graph. *P* value <0.05 was accepted as a significance level.

Results

The age of the patients and controls were between 15-84 and 29-61 years, respectively. The age of the control and patient group were 47.5±5.6 and 50.9±9.5, respectively. The age between the groups did not exhibit a statistically significant difference (p>0.05).

When the cases in the breast cancer group were questioned about the presence of cancer in their family history, it was observed that the answer was positive in 26.4% (n=28) of the patients. There was a statistically significant distinction between the patient group and the control group concerning family history. The breast cancer group demonstrated a higher utilization of oral contraceptives (OC) (p=0.001) and hormone replacement therapy (HRT) (p=0.041), with a statistically significant difference between the groups. However, no statistically significant differences were observed between the patient and control groups regarding birth and breastfeeding or smoking (p>0.05). Table 1 provides a summary of the demographic information for both the control and patient groups.

The results of the comparison of serum albumin, IMA, TAS, TOS and OSI levels between groups are presented in Table 2. Despite the patient group having a lower serum albumin level, the difference was not statistically significant. Therefore A-IMA levels were not different from IMA levels. The mean level of IMA was 0.559±0.128 in the patient group and was statistically higher than the control group (p<0.001). When the mean TAS values in the patient and control groups were compared; it was seen that TAS was lower in breast cancer patients than healthy women, but the difference was not statistically significant (p>0.05). Mean

values of TOS; 3.78±4.13 μmol H_2O_2 Eq/L in the control group and 2.37±1.45 μmol H2O2 Eq/L in the patient group. TOS demonstrated a statistically significant increase in breast cancer patients compared to healthy women (*p*=0.005). OSI was found to be higher in breast cancer patients than in healthy women. These results exhibited statistically significant (*p*=0.004).

Table 1. Demographic characteristics of the groups

Characteristics	Breast cancer (n=106)	Control (n=30)	*p*
Age (years)	50.92±9.45	47.53±5.63	0.064
BMI (kg/m²)	28.9±5.9	24.3±3.4	<0.001
Menopausal Status			
Premenopause	30 (28.3)	22 (73.3)	<0.001
Postmenopause	76 (71.7)	8 (26.7)	
Family History			
Positive	28 (26.4)	0 (0)	0.002
Negative	78 (73.6)	30 (100)	
Birth			
Positive	94 (88.7)	23 (76.7)	0.132
Negative	12 (11.3)	7 (23.3)	
Breast Feeding			
Positive	85 (80.2)	23 (76.7)	0.674
Negative	21 (19.8)	7 (23.3)	
OC			
Positive	38 (35.8)	0 (0)	0.001
Negative	68 (64.2)	30 (100)	
HRT			
Positive	15 (14.2)	0 (0)	0.041
Negative	91 (85.8)	30 (100)	
Smoking			
Positive	28 (26.4)	6 (20)	0.474
Negative	78 (73.6)	24 (80)	

Data are presented as the (mean±SD) or n (%)
OC, oral contraceptives; HRT, hormone replacement therapy, SD, standard deviation

Table 2. Serum albumin, IMA, TAS, TOS and OSI levels

	Breast cancer (n=106)	Control (n=30)	*p*
Albumin (g/dL)	4.16±0.32	4.22±0.21	0.315
IMA (ABSU)	0.559±0.128	0.466±0.164	0.001
TAS (mmolTroloks/L)	1.23±0.20	1.26±0.15	0.485
TOS (μmol H_2O_2 Eq/L)	2.65 (1.9-4.09)	1.96 (1.65-2.59)	0.005
OSI	0.211 (0.157-0.308)	0.154 (0.129-0.225)	0.004

Data are presented as the (mean±SD) or median (25^{th}-75^{th} percentile)
SD, standard deviation

IMA, TOS and OSI levels were also evaluated with ROC curve analysis. Cut-off values, sensitivity and specificity, for the parameters are demonstrated in Table 3. The cut-off value for IMA was >0.51 ABSU (Figure 1). The cut-off value for TOS was >2.14 μmol H2O2 Equivalent/L (Figure 2). The OSI cut-off value was >0.174 (Figure 3).

Eighty-nine of breast cancer patients were diagnosed with invasive ductal carcinoma. The others were 11 lobular, 3 mucinous, 1 medullary, 1 papillary, and 1 metaplastic carcinoma. Histopathologic findings of breast cancer patients were evaluated (Table 4). Among our patients, patients with Stage 1 and Stage 2 tumors constituted 84.9% of all breast cancer patients. TNM Classifications of patients were as follows; Stage IA: 36 (34%), Stage IB: 3 (2.83%), Stage IIA: 26 (24.5%), StageIIB: 25 (23.6%), Stage IIIA: 6 (5.7%), Stage IIIB: 2 (1.9%), Stage IIIC: 8 (7.5%). No significant correlation was found between tumor stage and tumor size and IMA, TAS, TOS and OSI values of the patients. The difference between estrogen and progesterone receptor positivity, proliferative change, necrosis, elastosis, calcinosis, lymph node positivity, c-erbB2 and oxidative stress parameters were not statistically significant (p>0.05).

Table 3. The receiver operating characteristic (ROC) curve and diagnostic scan values

	Cut-off	Sensitivity %	Specifity %	AUC	*p*
IMA (ABSU)	>0.51	67	76.7	0.737	<0.001
TOS (μmol H_2O_2 Eq/L)	>2.14	64.2	66.7	0.667	0.005
OSI	>0.174	67.9	66.7	0.672	0.004

AUC = area under the curve

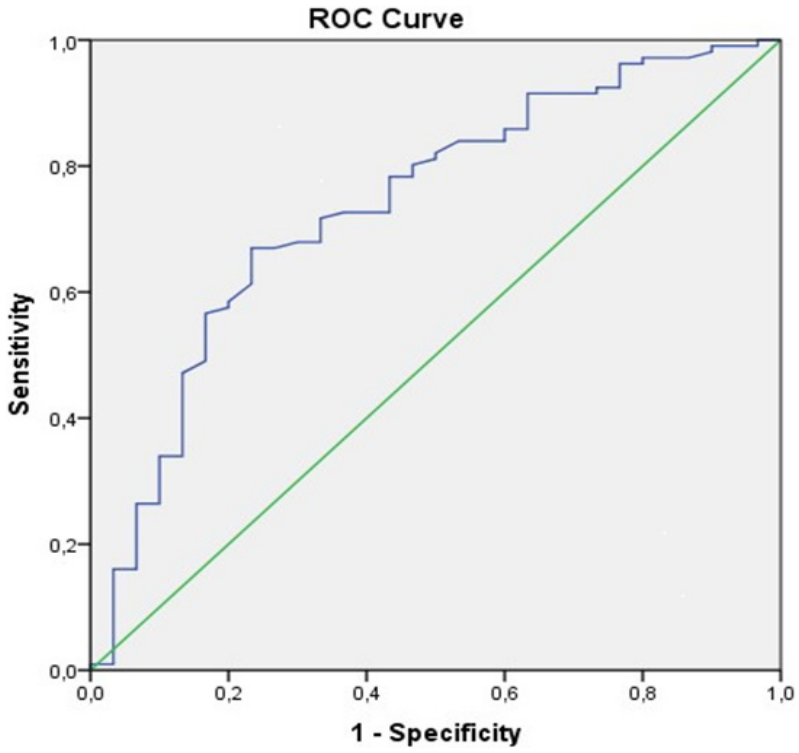

Figure 1. Receiver operating characteristic (ROC) curve analyses of IMA value

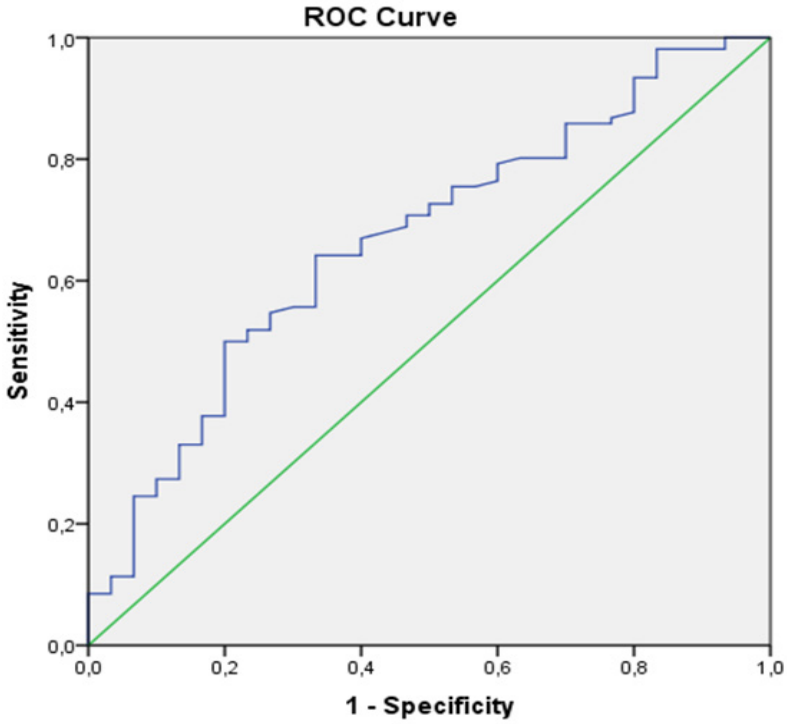

Figure 2. Receiver operating characteristic (ROC) curve analyses of TOS value

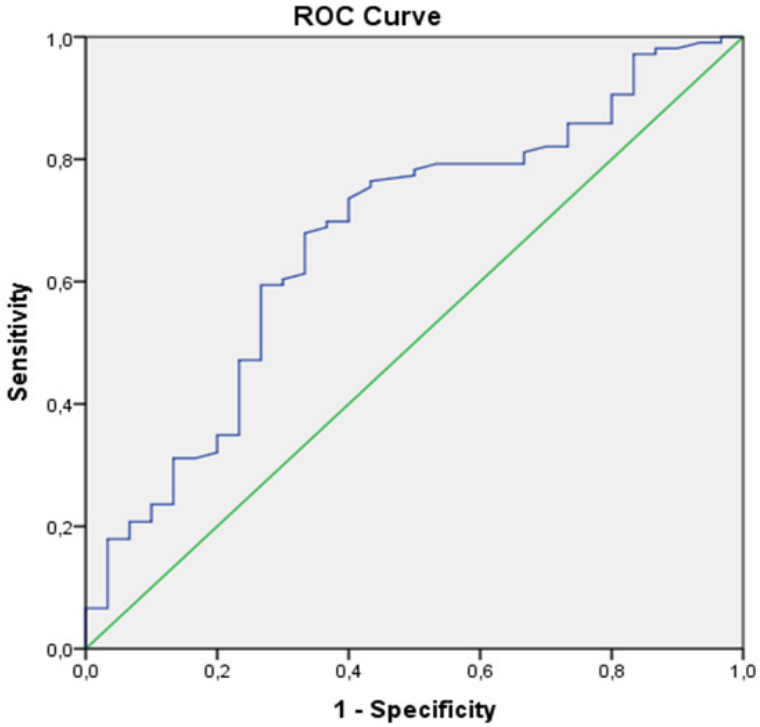

Figure 3. Receiver operating characteristic (ROC) curve analyses of OSI value

Table 4. Histopathologic findings of patients

Characteristics	n (%)
Tumor size	
≤2cm	40 (37.7)
>2cm	66 (62.3)
Lymph Node	
Positive	43 (42.5)
Negative	63 (57.5)
Metastatic Lymph Node	
1-3	32 (30.2)
4-7	4 (3.8)
>7	7 (6.6)
Stage	
1	39 (36.8)
2	51 (48.1)
3	16 (15.1)
Histologic Grade	
1	19 (18.6)
2	62 (60.8)
3	21 (20.6)
Estrogen Receptor	
Positive	85 (80.2)
Negative	21 (19.8)
Progesterone Receptor	
Positive	77 (72.6)
Negative	29 (27.4)
c-erbB2	
Positive	45 (33.1)
Negative	61 (45.9)
e-kaderin	
Positive	83 (78.3)
Negative	23 (21.7)
Proliferative Change	
Positive	60 (56.6)
Negative	46 (43.4)
Necrosis	
Positive	23 (21.7)
Negative	83 (78.3)
Elastosis	
Positive	58 (54.7)
Negative	48 (45.3)
Calcinosis	
Positive	34 (32.1)
Negative	72 (67.9)

Data are presented as the n (%)

Discussion

Breast cancer is one of the most common cancers [1]. Although there are great advances in treatment, the etiology of breast cancer has not been adequately clarified yet [16]. Although some markers have been identified that can guide the treatment or follow-up and predict the prognosis of breast cancer patients, studies are continuing in finding a suitable marker in the diagnosis of breast cancer.

Free radicals and antioxidant defense systems are in balance in healthy individuals. If this balance is disrupted, oxidative stress occurs and causes oxidative damage of the basic structural molecules of our body such as lipid, protein and DNA [17]. Oxidative stress caused by increased reactive oxygen species accelerates mutation and oncogenic transformation, resulting in DNA damage and may eventually lead to cancer development [18]. It was shown that oxidative stress causes cellular damage which in turn takes part in the development of breast cancer [16, 19-21]. In addition to the increase in free radicals, decrease in antioxidant activity also plays an important role in cancer formation. Endogenous and exogenous antioxidants can prevent cancer development by neutralizing or inhibiting cancer-causing free radicals [6].

When the results of our study are evaluated; IMA, TOS and OSI levels were significantly elevated in women with breast cancer compared to healthy women. Despite breast cancer patients having lower TAS values than healthy women, the difference was not statistically significant. These results support other studies indicating that increased oxidative stress formation or insufficient antioxidant capacity can cause breast cancer [16, 19, 22].

IMA is an oxidative stress marker that has been studied heavily in recent years. Bilgili et al. [23] found IMA levels significantly higher in breast cancer in comparison to other groups in their study on breast cancer, fibroadenoma and healthy women and similar to our study, no significant correlation was found between histopathological findings such as tumor size, stage, hormone receptor and IMA levels. Kosova et al. [24] also compared serum IMA levels in breast cancer patients and control group and found IMA levels to be significantly higher in the breast cancer patients. In the study conducted by Hunur et al. [25], IMA levels and their relationship with clinicopathological features in breast cancer patients were examined; IMA levels were significantly higher in the breast cancer patients compared to the healthy women, no significant relationship was found between clinicopathological factors (age, stage, smoking, comorbidity, subtype, menopausal status, body mass index and IMA. In their study conducted by Kundaktepe et al. [26] IMA values in breast cancer patients were significantly higher than in controls. IMA showed a strong positive correlation with tumor size, as well as very strong correlations with TNM stage and the presence of metastasis.

Plasma TAS levels in breast cancer group were significantly lower than control group in some studies [27-29]. They thought that the reduced antioxidant defense system played a role in breast cancer pathogenesis, causing increased ROS and lipid peroxidation products. Zowczak M. et al. [30] found that the TAS levels were low in breast cancer patients compared to the healthy controls. They thought that antioxidant consumption in plasma and the consequent oxidative stress in newly diagnosed breast cancer patients played a role in breast cancer pathogenesis. In our study, TAS levels were low in the patient group, but this difference was not statistically significant.

In the study conducted by Feng et al. [16, 22] TOS and OSI levels were significantly higher and TAS levels were significantly lower in breast cancer patients compared to the control group. These findings are consistent with our study in terms of TOS and OSI.

In conclusion, in patients diagnosed with breast cancer, IMA, TOS and OSI values were found to be significantly higher and TAS value lower than in healthy women. Disturbed oxidative stress status and IMA may contribute to the pathogenesis of breast cancer. There was no correlation of histopathologic findings with IMA, TOS and OSI levels in terms of tumor size, grade, lymph node involvements, histologic grade, c-erbB-2, estrogen and progesteron receptor positivity. We think this may be due to the low number of advanced stage patients. IMA, TOS, OSI values may be a new biomarker that can be used to differentiate patients with

breast cancer and healthy women. Further studies with larger groups are needed for these markers to be used as routine breast cancer markers.

Conflict of interest: No conflict of interest was declared by the authors.

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Authors' contributions to the article

G.B., O.F. constructed the main idea and hypothesis of the study. O.F., B.Z. collected data. G.B., O.F., N.U. developed the theory and arranged the material and method section, have done the evaluation of the data in the results section. Discussion section of the article written by O.F. and G.B., N.U. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

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Frequency of asymptomatic hyperuricemia in advanced-stage symptomatic knee osteoarthritis and its relationship with inflammatory parameters

İleri evre semptomatik diz osteoartritinde asemptomatik hiperürisemi sıklığı ve inflamatuar parametreler ile ilişkisi

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Abstract

Purpose: Hyperuricemia (HU) is thought to be a risk factor in the development and progression of knee osteoarthritis (OA). We sought the frequency of asymptomatic HU in advanced knee OA patients and whether it was related to systemic inflammation.

Materials and methods: This is a single-center, retrospective study including patients with symptomatic stage 3/4 knee OA classified based on Kellgren-Lawrence (K-L) system. Demographic data and serum uric acid (UA), hemogram parameters, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), body mass index (BMI) were recorded. Neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR) and systemic immune-inflammation index (SII)=[(neutrophil count × platelet count)/lymphocyte count] were calculated. Patients with/without hyperuricemia were defined as Group 1 and Group 2, respectively. Demographic and laboratory data were compared between the groups.

Results: Hyperuricemia was present in 51 of 240 patients (21%). There was no significant difference between the groups based on age (Group 1: 70.54±7.02, Group 2: 68.63±6.29, p=0.07) and BMI (Group 1: 34 kg/m^2 (27.7-41), Group 2: 32 kg/m^2 (25-51.5), p=0.107). NLR, PLR, and SII were similar between two groups (p=0.404, p=0.604, p=0.537). While there was no difference in ESR values between the two groups (p=0.051), CRP levels were found to be significantly higher in Group 1 (p=0.007). A positive correlation was detected between serum UA level and CRP (rho=0.243**, p<0.001), SII (rho=0.173*, p=0.017), NLR (rho=0.154*, p=0.035) and PLR (rho=0.166*, p=0.023) in Group 2. No correlation was detected between serum UA level and ESR, CRP, SII, NLR, PLR and BMI in Group 1.

Conclusion: Asymptomatic HU was found 1 in 5 of advanced-stage knee OA patients and may contribute to inflammation in knee OA. Serum UA level may need to be reduced to normal level in these patients.

Keywords: Asymptomatic hyperuricemia, hyperuricemia, knee osteoarthritis, systemic inflammation.

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Öz

Amaç: Hiperüriseminin diz OA gelişmesinde ve ilerlemesinde risk faktörü olduğu düşünülmektedir. Çalışmamızda ileri evre diz OA hastalarında asemptomatik hiperürisemi sıklığı ve sistemik inflamasyonla ilişkili olup olmadığının araştırılması amaçlandı.

Gereç ve yöntem: Semptomatik diz OA olan ve radyografik Kellgren-Lawrence (K-L) sistemine göre derece 3 veya 4 olarak sınıflandırılan hastaları dahil eden tek merkezli, retrospektif bir çalışmadır. Yaş, cinsiyet, komorbiditeler, kullanılan ilaçlar, serum ürik asit (ÜA), hemogram parametreleri, eritrosit sedimantasyon hızı (ESH), C-reaktif protein (CRP) ve vücut kitle indeksi (VKİ) kaydedildi. Nötrofil/lenfosit oranı (NLO), trombosit/lenfosit oranı (TLO) ve sistemik immün-inflamasyon indeksi (Sİİ)=[(nötrofil sayısı × trombosit sayısı)/lenfosit sayısı] hesaplandı. Hiperürisemisi olan ve olmayan hastalar sırasıyla Grup 1 ve Grup 2 olarak tanımlandı ve gruplar arasında demografik ve laboratuvar veriler karşılaştırıldı.

Bulgular: 240 hastanın 51'inde hiperürisemi mevcuttu (%21). Grup 1'de yaş ortalaması 70,54±7,02, BKİ 34 kg/m^2 (27,7-41), Grup 2'de yaş ortalaması 68,63±6,29, BKİ 32 kg/m^2 (25-51,5), gruplar arasında anlamlı fark yoktu. Grup 1 ve Grup 2 arasında NLO, TLO, Sİİ arasında fark yoktu (p=0,404, p=0,604, p=0,537). Her iki grup arasında ESH değerlerinde fark yokken (p=0,051), CRP düzeyleri Grup 1'de anlamlı yüksek saptandı (p=0,007).

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Grup 2'de ÜA düzeyi ile CRP (rho=0,243**, *p*<0,001), Sİİ (rho=0,173*, *p*=0,017), NLO (rho=0,154*, *p*=0,035) ve PLO (rho=0,166*, *p*=0,023) pozitif ilişki tespit edildi. Ancak Grup 1'de ÜA düzeyi ile ESH, CRP, Sİİ, NLO, PLO ve VKİ arasında ilişki yoktu.
Sonuç: Çalışmamızda ileri evre diz OA hastalarının yaklaşık 5'de 1'inin asemptomatik hiperürisemisinin olduğu gözlendi. Asemptomatik HÜ, diz OA'da inflamasyona neden olabilir, sonuç olarak bu hastalarda serum ÜA seviyesinin normale çekilmesi gerekebilir.

Anahtar kelimeler: Asemptomatik hiperürisemi, hiperürisemi, diz osteoartriti, sistemik inflamasyon.

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Introduction

Osteoarthritis (OA) is the most common chronic joint disease worldwide, characterized by cartilage degeneration and subchondral bone damage. The most commonly affected joints are knee, hip, hand and axial joints [1]. In a study conducted in 2020 on the incidence and prevalence of knee osteoarthritis, the global prevalence of knee OA was found 16% and incidence was 203/10,000 person-years [2]. It has begun to be understood that some biochemical factors, as well as mechanical factors, play a role in OA, besides, a subclinical chronic inflammatory process also contributes to the pathogenesis [1].

Uric acid (UA) is the end product of purine metabolism in humans. There may be an increase in serum levels due to the reasons such as lack of excretion or excess production. Different thresholds have been suggested for the definition of hyperuricemia (HU) in different epidemiological studies in the literature [3]. Asymptomatic HU is defined as the presence of serum hyperuricemia in the absence of gout, tophi or urate nephropathy/urolithiasis [4]. HU is the pivotal risk factor in gout and is also suggested to be an important marker in the progression of diseases such as metabolic syndrome, atherosclerotic cardiovascular diseases and ischemic stroke. In the past years, HU was suggested to predict the development of OA in various joints [5]. When serum UA reaches saturation, monosodium urate (MSU) crystals which can accumulate in various tissues such as articular cartilage, synovium, and tendons, begin to form. In vitro studies have shown the anti-oxidant and anti-inflammatory properties of soluble UA [6, 7]. However, MSU crystals formed as a result of the effects of some environmental tissue factors such as low pH and temperature, acquire pro-inflammatory properties. MSU crystals activate NLRP3, an intracellular protein, leading to caspase-1 activation, which causes the formation of the active form of interleukin-1beta (IL-1β) and the production of IL-18, leading to the activation of the innate immune system [8].

In recent years, the relationship between knee OA, the most common type of OA, and hyperuricemia has begun to be investigated due to its frequent association with metabolic syndrome. Xiao et al. [9], examined the MRI findings of knee OA patients with asymptomatic HU and observed that they were positively associated with synovitis and soft tissue swelling. Another study found a significant relationship between radiographic knee OA and asymptomatic HU, independent of confounding factors such as age, gender and body mass index (BMI) [10].

To determine systemic inflammation, in addition to conventional markers such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), neutrophil/lymphocyte ratio (NLR), platelet/lymphocyte ratio (PLR) and systemic immune-inflammation index (SII) obtained from hematological parameters are newly used. They have been studied in many fields in recent years as markers of inflammatory response [11-13]. It has been suggested that NLR may be a predictor of the severity of radiographic knee OA [11, 12].

This study sought the frequency of asymptomatic HU in advanced-stage symptomatic knee OA patients and whether HU was associated with systemic inflammation in these patients.

Materials and methods

This is a retrospective study and approval was obtained from the Faculty of Medicine Non-Interventional Clinical Research Ethics Committee. Patients with symptomatic knee OA at least in one knee and classified as stage 3 or 4 according to Kellgren-Lawrence (K-L) radiographic scale [14] indicated with arthroplasty, who admitted to orthopedics outpatient clinic between 1st of January 2021 and 31th of December 2022, were included in the study. Demographic, laboratory and radiological data of the patients were obtained retrospectively.

Exclusion criteria were history of knee injury-trauma or history of knee surgery, diagnosis of inflammatory arthritis (e.g., rheumatoid arthritis, spondyloarthritis, gout or other crystal arthropathy), kidney disease, chronic inflammatory disease, history of cancer. Additionally, patients who used drugs known to cause hyperuricemia (diuretics, aspirin) in the last two weeks were not included in the study. Serum UA, hemogram parameters, ESR, CRP were recorded. NLR, PLR and SII=[(neutrophil count × platelet count)/lymphocyte count] were calculated. Since no standard threshold level is currently used for hyperuricemia; serum UA levels >6.0 mg/dL, which is the level of the risk of developing gout begins to increase, was considered as hyperuricemia [3]. Patients with hyperuricemia were defined as Group 1, normouricemic patients were defined as Group 2, and demographic data and inflammatory markers were compared between these groups.

The Kolmogorov-Smirnov test was used to assess normality of numerical variables. For normally distributed numerical variables, intergroup comparisons were made with independent samples t test, and descriptive statistics were presented as mean and standard deviation (SD). For numerical variables that were not normally distributed, comparison between groups was made with the Mann-Whitney U test and descriptive statistics were presented as median (minimum-maximum). The chi-square test was used for comparison of categorical data. For categorical variables, data were presented as frequencies and percentages. Spearman correlation test was used for correlation analysis and evaluated with rho correlation coefficient, $p<0.05$ was considered statistically significant.

Results

Asymptomatic hyperuricemia (serum UA >6.0) was present in 21.3% of a total of 240 patients (n=51, Group 1), and 78.7% (n=189, Group 2) was normouricemic. The mean age of the patients was found to be similar (Group 1: 70.5±7.0 years, Group 2: 68.6±6.2 years, $p=0.07$). There was no difference between the two groups in terms of body mass index (BMI) (Group 1: median 34 [27.7-41] kg/m^2, Group 2: median 32 [25-41.5] kg/m^2, $p=0.107$). Gender ratios were female 70% (n=36) and male 30% (n=15) in Group 1, female 88% (n=167), male 12% (n=22) in Group 2, $p>0.05$. Comorbidity rates were also similar in both groups ($p>0.05$); In Group 1, hypertension was 64%, diabetes mellitus was 35%, heart failure was 6%, and renal failure was 2%, while in Group 2, hypertension was 59%, diabetes mellitus was 31%, and heart failure was 2%. Demographic characteristics and laboratory data of the groups are delineated in Table 1.

All inflammatory markers (ESR, CRP, SII, NLR, PLR) were found to be higher in Group 1 compared to Group 2, and there was a statistically significant difference in CRP levels. Inflammatory markers of the patients and differences between groups are listed in Table 1.

A positive correlation was detected between serum UA level and CRP, SII, NLR and PLR but no correlation was found between BMI and UA levels in Group 2. No correlation was detected between serum UA level and ESR, CRP, SII, NLR, PLR and BMI in Group 1 (Table 2).

Table 1. Characteristics of demographic and laboratory data of the groups

	Group 1 (n=51)	Group 2 (n=189)	*p*	t or z
Age, mean±standard deviation	70.54±7.02	68.63±6.29	0.07	**3.505**
UA, mg/dl	6.8 (6.1-9.2)	4.4 (2.2-5.9)	**<0.001**	**-10.958**
ESR, mm/h	32.5 (6-77)	24 (1-86)	0.051	**-1.950**
CRP, mg/dl	2.4 (2-30)	2 (2-23)	**0.007**	**-2.680**
SII	1144.2 (142.7-7258.2)	1055.1 (115.4-7842.4)	0.537	**-0.617**
NLR	4.7 (1.5-25)	4.1 (0.7-33.3)	0.404	**-0.834**
PLR	153.9 (66.4-414.2)	147.2 (41.1-621.4)	0.604	**-0.519**
BMI, kg/m²	34 (27.7-41)	32 (25-51.5)	0.107	**-1.610**

n: number of patients, UA: Uric acid, ESR: Erythrocyte sedimentation rate, CRP: C reactive protein, SII: Systemic immune inflammatory index NLR: Neutrophil lymphocyte ratio, PLR: Platelet lymphocyte ratio, BMI: Body mass index
Mean ± standard deviation was used for normally distributed data, and median (minimum-maximum) was used for non-normally distributed data. Independent samples t test was used to compare normally distributed data. Mann-Whitney U test was used to compare data that did not show normal distribution. t was the test value of the independent samples, z was the test value of the Mann-Whitney U test
p<0.05 was considered statistically significant

Table 2. Correlation between uric acid levels and BMI and inflammatory markers in Group 1 and Group 2

Variables	Uric acid (mg/dl) in Group 1 (n= 59)	Uric acid (mg/dl) in Group 2 (n=181)
ESH (mm/h)	rho=-0.039, *p*=0.783	rho=0.071, *p*=0.335
CRP (mg/L)	rho=-0.059, *p*=0.679	**rho=0.243**, *p*<0.001**
SII	rho=0.025, *p*=0.861	**rho=0.173*, *p*=0.017**
NLR	rho=-0.048, *p*=0.736	**rho=0.154*, *p*=0.035**
PLR	rho=-0.022, *p*=0.876	**rho=0.166*, *p*=0.023**
BMI, kg/m²	rho=0.042, *p*=0.770	rho=-0.109, *p*=0.137

n: number of patients, rho: Spearman correlation coefficient, UA: Uric acid, ESR: Erythrocyte sedimentation rate, CRP: C reactive protein SII: Systemic immune inflammatory index, NLR: Neutrophil lymphocyte ratio, PLR: Platelet lymphocyte ratio, BMI: Body mass index
*The correlation is significant at the level of 0.05, *p*<0.05 was considered statistically significant

Discussion

Osteoarthritis is the most common chronic joint disease worldwide, and it is understood that it develops not only as a result of mechanical damage, but also as a result of complex pathogenetic mechanisms in which subclinical inflammation occurs along with some local and systemic biochemical interactions [1, 15, 16]. Considering the frequency of knee OA, the disability it causes, and the economic burden, revealing the risk factors associated with the progression of the disease is very crucial for the management of the disease. The coexistence of knee OA with metabolic syndrome is common. The association between knee OA and several factors such as obesity, dyslipidemia, and impaired glucose tolerance has been investigated [17], and its relation with uric acid has become a matter of ongoing investigation in recent years [9, 10, 18, 19]. Wang et al. [19], examined adults aged 60 years and older with knee OA using the Third National Health and Nutrition Examination Survey (NHANES III) data set and compared patients with asymptomatic HU with normouricemic controls. They found the prevalence of symptomatic knee OA which are K-L stage 2 and above, was significantly higher in the asymptomatic HU group (17.4% and 10.9%, respectively, *p*=0.04). The NHANES III study reported the frequency of asymptomatic HU as 17.9% in the overall radiographic knee OA (K-L stage ≥2) cohort (n=2213) [19]. In our study, we found the frequency of

asymptomatic HU in all knee OA (K-L stages 3 and 4) patients to be similar, at 21%. On the other hand, rates of up to 40% have been reported by other studies [10, 20], but these studies included a small number of patients. Nevertheless, asymptomatic HU was found to have a significantly higher frequency in knee OA patients than in non-knee OA patients, and after controlling potential risk factors associated with knee OA such as age, gender, BMI, education level, occupational activities (e.g., kneeling and squatting) and hypertension, it was observed that asymptomatic HU continued to have a significant association with knee OA (OR=2.61) [10].

In the pathogenesis of OA, especially IL-1β and tumor necrosis factor (TNF) are involved with the degeneration of the articular cartilage matrix [15]. There are different hypotheses about the role of UA in the pathogenesis of knee OA. It is considered that IL-1β and IL-18, which are formed as a result of activation of the NLRP3 inflammasome complex, accelerate bone and cartilage destruction [15, 21, 22]. In a study including 69 knee OA patients, it was found that UA levels in the synovial fluid were strongly correlated with synovial fluid IL-1β and IL-18 levels, thus it was suggested that synovial fluid UA levels were a marker for the severity of knee OA [20]. In our study, despite similar BMI and mean age in the two groups, systemic inflammation markers were detected to be higher in the group with high UA. UA is defined as an endogenous 'danger signal' that stimulates systemic inflammation [23, 24]. Serum UA was found to be strongly correlated with serum CRP, IL-6 and fibrinogen levels in patients with metabolic syndrome, and was also found to be positively correlated with serum TNF-alpha and IL-6 in patients with cardiovascular disease [24]. In our study, inflammation markers were all higher in the hyperuricemic group, but only CRP reached statistical difference. However, no correlation was observed between serum UA level and ESR, CRP, SII, NLR, PLR in hyperuricemic Group 1. A positive correlation was observed between serum UA level and CRP, SII, NLR, PLR in normouricemic Group 2. These results may be due to the relatively small number of patients in Group 1, moreover, increased levels of inflammatory markers may not always accompany increased levels of serum urate in the patients with asymptomatic hyperuricemia.

The pathogenesis of the relationship between UA and OA is not yet fully elucidated, but increasing evidence suggests that high UA levels may be an important risk factor and/or predictor of disease severity for knee OA [5, 9, 10, 18, 19]. UA may play a role in the development of low-grade inflammatory synovitis in the joint with OA, as well as the increase in IL-1β in the synovial fluid which is thought to be the most responsible cytokine for OA. Studies have determined that the presence of synovitis in knee OA enhances the severity of pain and contributes to the acceleration of joint damage [9, 21].

Our study has some limitations. Since it was thought that subclinical inflammation would be higher in symptomatic patients with advanced-stage OA, the frequency of asymptomatic HU was investigated in these patients, and a comparison was not performed with the patients with early stage knee OA. Due to the retrospective design, clinical measurement tools such as pain scores could not be compared. The level of 6 mg/dl was taken as the hyperuricemia threshold because further imaging methods could not be applied for pre-clinical gout and its effect was tried to be minimized. Obesity may have an effect on serum UA, however, since obesity is common in knee OA patients in our population, the study was not limited to non-obese patients, and the BMI medians were similar between the two groups.

In conclusion, our study showed that one in five patients with advanced-stage knee OA had asymptomatic HU and that asymptomatic HU may be associated with systemic inflammation. It may be plausible to check serum UA levels in advanced-stage knee OA patients scheduled for surgery. The association of serum UA levels with different radiographic stages and pain scores in hyperuricemic patients with knee OA, and whether UA-lowering treatment can help limit OA progression, may be the subjects of new researches.

Conflict of interest: No conflict of interest was declared by the authors.

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Authors' contributions to the article

R.K.C. constructed the main idea and hypothesis of the study. B.B.A. collected the data. V.Y. and F.T.O edited the material and method section. R.K.C. have done the evaluation of the data in the results section. Discussion section of the article written by R.K.C.and B.B.A. R.K.C reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

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The effect of cardiometabolic control and malnutrition on the prevalence and prognosis of diabetic retinopathy in type 2 diabetes

Tip 2 diyabette kardiyometabolik kontrol ve malnütrisyonun diyabetik retinopati prevalansı ve prognozuna etkisi

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Abstract

Purpose: This study aims to investigate the predictive value of cardiometabolic control, gamma glutamyl transferase (GGT) and malnutrition-related inflammation markers for predicting diabetic retinopathy (DR) prevalence and prognosis**.**

Materials and methods: Type 2 Diabetes Mellitus patients who were consecutively admitted to Internal and Ophthalmology outpatient clinics were included in this study. Clinical, haematological and biochemical data were recorded. Cut-off values of GGT, hemoglobin, albumin, lymphocyte and platelet (HALP) score, nutritional risk index (NRI) and prognostic nutritional index (PNI) scores were determined by receiver operator characteristic curve analysis. Univariate and multivariate analyses were performed to determine the association of all variables with DR. We evaluated which of these tests were predictive and prognostic for the development of DR.

Results: This study included 166 patients. Fasting blood glucose (p<0.001), creatinine (p=0.01), HbA1c (p<0.001) and microalbuminuria (p=0.01) were higher in patients with retinopathy. Mean arterial pressure (p=0.01), fasting blood glucose (p=0.03), triglyceride (p=0.008), body mass index (BMI) (p=0.02) and HbA1c (p=0.04) increased significantly as GGT level increased. Contrary to the literature, HALP, PNI and NRI scores were not associated with DR.

Conclusion: Duration of diabetes, cardiometabolic control and GGT level are variables with predictive value for the prognosis of DR. No significant correlation was found between malnutrition-related inflammation markers and DR development and stage.

Keywords: Diabetic retinopathy, cardiometabolic control, GGT, malnutrition releated inflamation markers.

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Öz

Amaç: Bu çalışmanın amacı kardiyometabolik kontrol, gama glutamil transferaz (GGT) ve malnütrisyonla ilişkili inflamasyon belirteçlerinin diyabetik retinopati (DR) prevalansı ve prognozunu öngörmedeki değerini araştırmaktır.

Gereç ve yöntem: Bu çalışmaya Dahiliye ve Göz Hastalıkları polikliniklerine ardışık olarak başvuran Tip 2 Diabetes Mellitus hastaları dahil edilmiştir. Klinik, hematolojik ve biyokimyasal veriler kaydedildi. Alıcı operatör karakteristik eğri analizi ile GGT, hemoglobin, albümin, lenfosit ve trombosit (HALP) skoru, nutrisyonel risk indeksi (NRI) ve prognostik nutrisyonel indeks (PNI) skorlarının cut-off değerleri belirlendi. Tüm değişkenlerin DR ile ilişkisini belirlemek için tek değişkenli ve çok değişkenli analizler yapıldı. Bu testlerden hangilerinin DR gelişimi için öngörücü ve prognostik olduğu değerlendirilmiştir.

Bulgular: Bu çalışmaya 166 Tip 2 Diyabetes Mellitus hastası dahil edildi. Retinopati olanlarda; açlık kan glukozu (p<0,001), kreatinin (p=0,01), HbA1c (p<0,001) ve mikroalbüminüri (p=0,01) yüksek bulundu. Hastaların GGT düzeyi arttıkça ortalama arteriyel basınç (p=0,01), açlık kan glukoz (p=0,03), trigliserit (p=0,008), vücut kütle indeksi (BMI) (p=0,02) ve HbA1c (p=0,04) değerlerinin anlamlı arttığı bulundu. Literatürün aksine HALP, PNI ve NRI skorun DR ile anlamlı ilişki saptanmadı.

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Sonuç: Diyabet süresi, kardiyometabolik kontrol ve GGT düzeyi diyabetik retinopati prognozu için prediktif değeri olan değişkenlerdir. Malnütrisyonla ilişkili inflamasyon belirteçleri ile DR gelişimi ve evresi arasında anlamlı bir ilişki bulunmamıştır.

Anahtar kelimeler: Diyabetik retinopati, kardiyometabolik kontrol, GGT, malnutrisyon ilişkili inflamasyon belirteçleri.

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Introduction

The metabolic disorder of type 2 diabetes mellitus (type 2 DM) is a condition characterised by hyperglycaemia in which peripheral insulin resistance or impaired insulin secretion mechanisms are the cause. It is a chronic metabolic disorder requiring continuous medical care as a result of impaired efficient utilisation of carbohydrates, fats and proteins. In the pathogenesis, environmental factors such as obesity are blamed as well as the influence of genetic factors. It is estimated that diabetes affects 530 million people worldwide and DR is seen in 10.5 per cent of people aged 20-79 years. The majority of diabetes cases consist of type 2 DM patients. The increase in metabolic syndrome and obesity cases over the years also suggests that type 2 DM cases will increase. According to the World Health Organization, the number of people with diabetes is expected to reach 300 million by 2025.

Turkiye is a region where cases are increasing in proportion to its large surface area and increasing population and screening programmes are emphasised more. Increasing life expectancy, changes in lifestyle and deterioration in dietary habits increase the frequency of diabetes and diabetes-related complications. Glycosylation, abnormalities in lipid metabolism, chronic inflammatory state and oxidative system disorders are thought to be the causes of pathogenesis. Microvascular and macrovascular complications are main causes of morbidity and mortality in type 2 DM. Early diagnosis will provide early treatment and rehabilitation of chronic complications. As a result, we believe that the duration without complications due to diabetes will increase [1-3].

In the current literature, duration of diabetes was the strongest predictor of the prevalence of DR [4, 5]. In another study, Henricsson et al. [6] accepted glycaemic control as the risk factor of the most common. And defined other risk factors as high blood pressure, high hyperlipidaemia, increased oxidant stress, renal impairment and chronic inflammation [7]. In the current literature hypertension and dyslipidaemia are also defined as risk factors.

Since DR may be asymptomatic until the end of life, every patient with type 2 DM should undergo annual screening including visual acuity, intraocular pressure measurement, slit lamp and dilated fundus examination [8]. It will be too late as irreversible complications will develop after the symptoms occur. Microvascular and macrovascular complications can be prevented with early diagnosis and treatment.

GGT, which is the subject of internal medicine speciality thesis, is a glycoprotein membrane-bound peptidase enzyme. It is a transporter of gamma-glutamyl groups from gamma-glutamyl peptides to other peptides, water and amino acids. In the literature, studies show that elevated GGT levels negatively predict metabolic syndrome and cardiovascular disease [9-11]. Considering that it may have a predictive value in the prevalence of DR, it was included in this study. The pathogenesis of DR consists of steps including ischemia, cell damage, inflammatory response, disruption of the retinal-blood barrier and neovascularisation. Clinical studies have concluded that inflammation, immune response and nutrition predict the presence of DR [12].

In this study, the full text of the internal medicine speciality thesis on ‘The effect of GGT level and cardiometabolic risk factors on DR staging in type 2 DM patients. This clinical study was organised on the basis of the recorded data of the patients included in the internal medicine thesis, using current knowledge from the literature and predictive markers obtained from the recorded laboratory data. There are no new data records about the patients [11].

As prognostic markers to predict malnutrition, PNI, NRI and HALP scores were calculated. The incidence of microvascular and macrovascular complications increases with malnutrition and hypoglycaemia. The HALP score takes into account malnutrition, inflammation and anaemia. It has been shown in the literature to predict malignancy, cerebrovascular disease and heart failure [13-16]. The power of the PNI and the NRI, which are markers that allow us to measure malnutrition, to predict DR was investigated in this study. The prognostic nutritional index obtained from the serum albumin and the lymphocyte count was called the PNI [7, 17]. The nutritional risk index was calculated from albumin, weight, ideal weight and height. It was called NRI [17]. The relationship between malnutrition and the prevalence and severity of DR is not well understood. In this study, the predictive power of HALP, PNI and NRI score, defined in the literature as markers of malnutrition in clinical trials, to predict DR was calculated.

The predictive value of cardiometabolic control, malnutrition markers (HALP, PNI and NRI) and GGT levels in predicting DR development in type 2 DM was investigated. The etiology of microvascular complications of diabetes has been studied in the literature [18-22]. To the best of our knowledge, there has been no study on this topic and its content, and it is believed that this study will contribute to the literature.

Materials and methods

Study population

In this study, 166 consecutive type 2 DM patients who applied to Internal Medicine and Ophthalmology outpatient clinics between January-July 2014 were included. Ethics committee approval was obtained on 01.08.2013. The Declaration of Helsinki principles were followed throughout. The following were included: type 2 DM, age above 40 years, absence of DR in the control group and presence of DR in the study group. Exclusion criteria were alcohol use, drug use (paracetamol, phenytoin, TAD, phenobarbital), known acute or chronic hepatobiliary disease (cholangitis, cirrhosis), chronic renal failure and retinopathy caused by other causes (collagen tissue disease, radiation induced retinopathy, hypertension, malignancy). Detailed anamnesis was taken from the patients who had the study criteria and physical examination was performed.

Data collection and calculation of prognostic markers

Laboratory results; data within the last 3 months were recorded. Patients without results in the hospital database were excluded. Cardiometabolic control (waist circumference, body mass index, fasting blood glucose, HbA1C, mean arterial pressure and triglyceride) and GGT level were evaluated. HALP, PNI and NRI scores were calculated using haematological and biochemical values (Figure 1). The predictive and prognostic value of these tests in predicting the development of retinopathy in type 2 DM was evaluated.

Eye examinations and retinopathy evaluations

Visual acuity according to Snellen-chart, intraocular pressures with Goldmann applanation tonometry and fundus examination with indirect ophthalmoscope were performed by the same physician. Fundus examinations were performed with Goldman's three-mirror and Quadrospheric (Volk) contact lenses in patients who were deemed necessary by the clinician. Retinopathy staging was performed using the ınternational clinical classification system for diabetic retinopathy (Hoskins Center for Quality Eye Care) [23]. The control group without DR and the patient group consisted of patients with DR. The two groups were compared between clinical and demographic characteristics.

HALP: haemogram (g/L) x Albumin (g/L) x Lymphocyte/Thrombocyte

NRI: 14.87 x albumin (g/L) + 41.7 x weight/ideal weight (kg)
Ideal body weight calculation: 22 x height squared (m)

PNI: Albumin (g/L) + 5 *Lymphocyte (109/L)

Figure 1. Calculation of PNI, NRI and HALP score
HALP score: Haemoglobin, albumin, lymphocyte and platelet ratio, NRI: Nutritional risk index, PNI: Prognostic Nutritional Index

Markers of malnutrition

HALP, PNI and NRI scores calculated using laboratory values within three months were recorded for each patient during routine controls. The cut-off values of these values were found by ROC analysis and their predictive value in predicting the development of DR was calculated.

Statistical analysis

IBM SPSS Statistics for Windows was used for statistical analyses (Version 25.0" (IBM Corp., Armonk/NY/USA). Descriptive statistical methods (mean, SD, median, frequencies, ratios, min, max), the Student t test was used for two-group comparisons of normally distributed parameters and the Mann-Whitney U test for two-group comparisons of non-normally distributed parameters. Pearson and Spearman correlation analysis were used to assess relationships between parameters. Receiver operating characteristic (ROC) analysis was used. The study data were analysed using Kolmogorov-Smirnov normality assumption. Independent t-test and ANOVA test, which are parametric tests, were performed to determine whether there were significant differences between DR and DR stage groups with different variables. To compare categorical variables, the chi-squared test and Fisher's exact test were used. Significance was accepted at $p<0.05$.

Results

Of the 166 patients included in this study, 91 were female (55%) and 75 were male (45%). The age range of the patients was 30 to 75. The average age was 61 years old. Ophthalmological examinations were performed by the same ophthalmologist and standardised. Patients were categorised as having retinopathy (n:108 patients; Proliferative retinopathy (PR): 53 and Nonproliferative retinopathy (NPR): 55) and non-retinopathy (n:58 patients). Clinical and demographic data were analysed. Median age ($p<0.001$), FBG ($p<0.001$), creatinine ($p=0.01$), HbA1c ($p<0.001$) and microalbuminuria ($p=0.01$) were higher in DR patients. In addition, no significant correlation was found between the presence of DR and cholesterol, LDL, triglyceride, HDL, ALT and AST levels ($p>0.05$) (Table 1).

Table 1. Association of clinical and demographic data of the patients with the development of retinopathy

		With retinopathy (n=108)	Without retinopathy (n=58)	*p*
Median age		62±7	57±9	<0.001
Hypertension n (%)		88 (78.5)	23 (21.5)	<0.001
Diabetes Mellitus treatment (n, %)	**Oral Antidiabetic**	71 (58.6)	50 (41.3)	0.005
	Insulin	80 (87.9)	11 (12.1)	<0.001
BMI (body mass index) (kg/m²)		31.2 (27.7-34.0)	29.3 (26.5-35.0)	0.38
Waist circumference (cm)	**Median**	104 (97-114)	96 (92-104)	0.002
	Female	107 (96-120)	98 (92-110)	0.03
	Male	102 (97-110)	94 (93-101)	0.009
Mean arterial pressure (MAP) (mmHg)		93 (87-100)	87 (83-97)	0.05
Fasting blood glucose (mg/dl)		202±76	158±76	<0.001
Triglyceride (mg/dl)		178±104	175±109	0.93
HDL cholesterol (mg/dl)		47±11	49±14	0.41
LDL cholesterol (mg/dl)		123±45	124±39	0.56
HbA1C		8.7±1.8	7.6±2.3	<0.001
Microalbuminuria		470±815	172±184	0.002

$p<0.05$ was considered statistically significant

Mean arterial pressure (p=0.01), fasting blood glucose (p=0.03), triglyceride (p=0.008), BMI (p=0.02) and HbA1c level (p=0.04) were found to increase statistically significantly as GGT level increased. All results support a statistically significant relationship between GGT and metabolic syndrome (Table 2).

The duration of type 2 DM and the development of DR were significantly related (Table 3) (p<0.001). It was found that the group without DR was mostly in the first 10 years, NPR was more common in >10 years and PR was more common in >15 years (p<0.001) (Table 3 and Table 4). The predictive power of HALP, PNI and NRI scores, which are defined as malnutrition markers in the literature, in predicting DR was calculated (Figure 1). A significant correlation was not found between HALP, PNI and NRI levels and DR development and stage (p>0.05) (Table 4).

Table 2. The relationship of GGT interval values to cardiometabolic parameters

	GGT <19 (n:73)	GGT: 19-36 (n:68)	GTT >36 (n:25)	*p*
BMI (body mass index) (kg/m²)	31 (±5)	30 (±5)	33 (±5)	**0.02**
Waist circumference	103.4 (±11.9)	101.5 (±13.1)	109.1 (±4.5)	0.06
Mean Arterial Pressure (mmHg)	88 (±10)	92 (±9)	94 (±9)	**0.01**
Fasting blood glucose (FBG) (mg/dl)	74 (±70)	188 (±86)	220 (±75)	**0.03**
Triglyceride (mg/dl)	152 (±99)	188 (±107)	204 (±111)	**0.008**
LDL cholesterol (mg/dl)	119 (±139)	125 (±41)	135 (±56)	0.59
HbA1C	8.1 (±1.83)	8.31 (±2.18)	9.20 (±2.01)	**0.04**

p<0.05 was considered statistically significant

Table 3. Duration of diabetes and diabetic retinopathy

Diabetes Mellitus duration	With retinopathy (n=108)	Without retinopathy (n=58)	*p*
0-5 year, n (%)	8 (22.2)	28 (77.8)	<0.001
5-10 year, n (%)	12 (36.4)	21 (63.6)	<0.001
10-15 year, n (%)	26 (86.7)	4 (13.3)	<0.001
15-20 year, n (%)	18 (81.8)	4 (18.2)	<0.001
>20 year, n (%)	44 (97.8)	1 (2.2)	<0.001

p<0.05 was considered statistically significant

Table 4. Comparison of diabetic retinopathy stage and malnutrition markers

	Stages of diabetic retinopathy			
Duration of Diabetes Mellitus, n (%)	**No Diabetic Retinopathy (n=58)**	**Non-proliferative retinopathy (n=55)**	**Proliferative retinopathy (n=58)**	*p*
0-10	49 (84.5)	14 (25.5)	5 (9.4)	
10-20	8 (13.8)	24 (43.6)	21 (39.6)	<0.001
>20	1 (1.7)	17 (30.9)	27 (50.0)	
HALP, Mean±SD	2.15±2.73	1.9±0.69	1.64±0.44	0.277
NRI, Mean±SD	65.83±6.1	65.36±4.95	64±5	0.187
PNI, Mean±SD	41.07±8.74	42.6±6.76	42.19±7.52	0.553

HALP: hemoglobin + albumin + lymphocyte + platelet, NRI: nutritional risk index, PNI: prognostic nutritional index
P<0.05 was considered statistically significant

Discussion

In this study, we investigated the predictive value of cardiometabolic control, GGT levels and malnutrition in the prediction of the development of DR in patients with type 2 DM using current data from the literature. Cardiometabolic control (waist circumference, BMI, fasting blood glucose, HbA1C, mean arterial pressure and triglycerides) was evaluated with metabolic syndrome parameters. PNI, NRI and HALP score were calculated as prognostic markers to predict malnutrition. Median age, FBG, creatinine, HbA1c and microalbuminuria were higher in patients with DR. In addition, significant correlation was not found with cholesterol, LDL, triglyceride and HDL levels. MAP, FBG, triglyceride, BMI and HbA1c levels were found to increase significantly as GGT level increased. All results support a statistically significant relationship between GGT and metabolic syndrome. A significant relationship was found between the development of DR and the duration of type 2 DM. It was observed that the group without DR was mostly in the first 10 years, and DR development increased after the tenth year despite the presence of controlled diabetes. No significant correlation was found between DR and HALP, PNI, NRS (p>0.05).

Visual loss due to DR is thought to be caused by macular edema, tractional retinal detachment or neovascular glaucoma. Ischemia and neovascularisation occur in the tissue following impaired vascular permeability and microthrombi. A major role in the pathogenesis is played by biochemical (protein kinase C, glycation and polyol pathways) and angiogenesis-inducing factors such as vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1). Patients are a heterogeneous group with different levels of retinopathy findings. Genetic susceptibility is also very important. Some patients develop minimal retinopathy despite a long period of hyperglycaemic disease and some patients have severe retinopathy despite regular glycaemic control. Genetic susceptibility can be mentioned in these patients [12, 24-27].

In the study conducted by Ren et al. [28] although many causes are blamed for the pathogenesis of DR, research is ongoing. It was emphasised that laboratory parameters measured with patient blood are the primary biomarker of DR. Many studies have been conducted considering that the detection of metabolites passing into the circulation through the impaired blood-retinal barrier due to the pathophysiological process may be diagnostic. The most common proteins have been studied and the only significant correlation was found with HbA1c. In this study, a positive correlation has been found between a number of proteins (FABP4, iNOS, homocysteine, PTX3, ADAM, ANGPTL3, TIMP, MMP) that predict inflammation and increased angiogenesis and DR. However, the availability of these tests in daily practice is limited. There are no detailed clinical studies including prognostic markers that are routinely evaluated by clinicians at each visit, calculated by haemogram and biochemical parameters and predict inflammation.

In studies involving diabetic chronic complications, GGT levels at upper reference range significantly associated with metabolic syndrome, cardiovascular disease, diabetic neuropathy and diabetic nephropathy (p<0.05) [29-33]. In this study, mean arterial pressure (p=0.01), fasting blood glucose (p=0.03), triglyceride (p=0.008), BMI (p=0.02) and HbA1c level (p=0.04) increased with increasing GGT level (19-36U/L) and were statistically significant. All results support a statistically significant relationship between GGT and metabolic syndrome (Table 2).

In patients with uncontrolled diabetes, chronic hyperglycaemia and high mean blood pressure impair autoregulation in retinal blood vessels. Leukocytes adhere to the vascular endothelium. As a result, endothelial integrity is impaired and vascular permeability increases. Neovascularisation is triggered due to retinal ischaemia. Its reflection in the laboratory is an increase in inflammation markers such as platelets, leukocytes and neutrophils [18]. Hyperglycaemia disrupts retinal blood flow by affecting metabolism at cellular level. Neovascularisation related proliferative changes. Increased VEGF and IGF1 trigger the pathogenesis leading to more advanced retinal disease. In this process, the presence of independent metabolic variables such as hypertension accelerates the pathogenesis [19].

In the study conducted by Perais et al. [34] 6391 studies were reviewed and data from 59 studies (35 were prospective cohort studies and

22 were retrospective studies) were included. Nineteen of the studies included type 2 DM. The number of participants was min: 100, max: 71817 and follow-up periods ranged from 1-20 years. Duration of diabetes, diagnosis at an early age, blood pressure, LDL, total cholesterol, HDL, triglycerides, HbA1c, BMI, gender, smoking/alcohol use and socioeconomic status were found to be predictive and prognostic for the development of DR. In addition, in a systematic review analysing data from 19 clinical trials, duration of diabetes, dyslipidaemia, poor glycaemic control, microalbuminuria and hypertension were risk factors for DR. In this study, FBG (p<0.001), creatinine (p=0.01), HbA1c (p<0.001) and microalbuminuria (p=0.01) were found higher in DR patients. In addition, no significant correlation was found with GGT (p=0.16), cholesterol (p=0.63), LDL (p=0.56), triglyceride (p=0.93), HDL (p=0.41), ALT (p=0.81) and AST (p=0.69) levels (Table 1).

In patients trying to live with a chronic disease, good glycaemic control certainly reduces microvascular complications. However, recommending a more aggressive diet by keeping the HbA1c target below 6.5 leads away from being protective from increasing risk of hypoglycaemia and malnutrition. Malnutrition and hypoglycaemia will increase the frequency of microvascular and macrovascular complications [20-22]. The predictive power of HALP, PNI and NRS score, which are defined as malnutrition markers in clinical studies in the literature, in predicting DR was calculated. No statistically significant correlation was found between HALP, PNI and NRI levels and DR development and stage (p>0.05) (Table 4). There was a feeling that prospective studies with larger numbers of patients might have been of value.

In the study conducted by Saini et al. [35] 57 patients with DR were included. A significant correlation was found between DR severity and diabetic nephropathy severity (p<0.05). Consistent with the literature, creatinine elevation (p=0.01) and positive microalbuminuria (p=0.01) was higher in DR patients. The presence of an accompanying chronic complication in patients with DM indicates a high likelihood of other complications. Patients should be informed about chronic complications and measures should be taken for early diagnosis and primary prevention.

In a prospective study of 7458 non-diabetic men with an average follow-up of 12.8 years, 194 men developed type 2 DM. Compared with the rest of the cohort, the levels of GGT were higher in the patients who went on to develop type 2 DM (15.3U/l; 20.9U/l; p<0.0001). In this study by Perry et al. [30] it was emphasised that high GGT level may be a simple marker of increased visceral adipose tissue and hepatic insulin resistance. In this study, GGT was high in all patients with no statistically significant difference between the two groups.

In the prospective study showing that diabetes duration is the strongest indicator of DR prevalence, 627 patients with DM followed for 8-10 years on average. Despite routine follow-up, 39% (n:247) of patients developed DR (1.8% proliferative retinopathy (PR), 36.2% nonproliferative retinopathy (NPR)). Patients without DR had a diabetes duration of less than ten years, which is consistent with our study. Increased BMI, elevated HbA1c and poor glycaemic control have been defined as additional risk factors in patients with DR. In this study, in the absence of additional risk factors, DR was less common in the first decade, whereas NPR and PR developed after the tenth year. Based on all these data, to prevent the development of type 2 DR, close monitoring of patients should ensure glycaemic control, HbA1c within target range, blood pressure control and metabolic control [6].

Zheng et al. [13] took data from the National Health and Nutrition Examination Survey database. 657 patients with a history of cardiovascular disease (CVD) from 2003 to 2018 included. The HALP score (haemoglobin, albumin, lymphocyte and platelet), which includes malnutrition, inflammation and anaemia, shown to predict CVD prognosis. Mortality of all causes were higher in low-HALP patients (p<0.05). In the literature, it was shown to be predictive in the prognosis of malignancy, cerebrovascular disease and heart failure [14-16]. To our knowledge, there is no clinical study in the literature showing the prognostic value of HALP in DR patients. HALP score allows simultaneous assessment of anaemia, malnutrition and inflammation. In this study, the predictive and prognostic relationship between HALP score and DR was evaluated. It was observed that HALP score level decreased when

DR developed, but no significant relationship was found (p=0.277).

Kurtul et al. [7] prognostic nutritional index (PNI) was applied to 128 consecutively recruited patients with type 2 DM. PNI levels of patients without DR significantly higher than with DR (44.49±3.10 and 41.20±4.81; p<0.001). The prevalence of insulin use (63.6% (n:28); 26.2% (n:22); p<0.001) and hypertension (59.1% (n:26); 40.5% (n:34); p=0.045) were higher in patients with DR. Low haemoglobin (12.8±1.8; 13.6±1.6; p=0.009) and low albumin levels (4.11±0.48; 4.44±0.31; p<0.001) were found in DR patients. This result supports the pathogenesis of DR initiated by hypoxia. When multivariate analysis was performed with these significant results, PNI (HR=0.845, 95% CI=0.735-0.971; p=0.017), duration of diabetes (HR=1.135, 95% CI=1.051-1.226; p=0.001) and creatinine (HR=8.468, 95% CI=1.773-40.454, p=0.007) were identified as independent and significant prognostic factors for DR [7]. It can be concluded that inflammation and malnutrition have an important role in causing DR [7, 24].

In the study conducted by Wei et al. [17] 612 type 2 DM patients were included. In patients with DR, malnutrition was found to be common. In order to prevent the development and progression of DR, it was interpreted that malnutrition should be avoided. PNI and NRI scores were used to assess malnutrition. Multivariate analysis showed that the incidence of DR was lower with higher PNI (HR=0.96,95% CI=0.92-1.00; p=0.033) and NRI (HR=0.95,95% CI=0.92-0.99; p=0.007). In this study and in the literature, significant results were obtained with both nutritional scores. Patients with malnutrition had higher mean age and lower BMI, haemoglobin, albumin and lymphocyte levels than patients without malnutrition.

In this study, it was found that diabetes duration, age, FBG, creatinine, HbA1c, microalbuminuria, mean arterial pressure, triglyceride, GGT level and cardiometabolic parameters evaluated together would be predictive for the prognosis of DR in type 2 DM patients. Although statistically significant data could not be obtained with malnutrition markers, we should avoid malnutrition while recommending a diabetic diet. We may unintentionally increase the incidence of DR while trying to control blood glucose. This study had some limitations that may have influenced the results;

(1) Receipt of single centre data,

(2) Relatively small sample size

(3) Lack of a healthy control group (healthy control group was not included because it was considered unethical to perform fundus examination in healthy volunteers without DM when they were asymptomatic)

(4) Since it was a cross-sectional study, causality was not evaluated and subsequent follow-up was not recorded. Similar limitations were present in the studies on DR in the literature.

In conclusion, annual detailed ophthalmological examination should be recommended in patients with type 2 DM. It should be kept in mind that patients may be asymptomatic until irreversible changes occur. The importance of this should be explained in diabetic patient education. Thus, it is thought that this complication that may lead to blindness can be prevented. When additional risk factors were analysed in patients with retinopathy, it was found that diabetes duration, poor glycaemic control, high HbA1c level and increased BMI were important risk factors for DR. Many risk factors for cardiovascular disease, metabolic syndrome and microvascular complications are associated with increased GGT activity at the upper levels of the reference range. Strict glycaemic control, lowering of HbA1c levels and lowering of blood pressure should be known to prevent the development and progression of DR. Proteinuria, elevated urea and creatinine levels were found in patients with DR. The presence of microalbuminuria is a harbinger of imminent development of retinopathy. Statistically significant correlation wasn't found between HALP, PNI and NRS levels, which are defined as malnutrition markers in the literature, and the development and stage of DR. It was thought that studies with a larger number of patients may be guiding. The importance of early diagnosis and treatment as well as primary prevention for diabetic microvascular complications is emphasised. Diabetic patients are susceptible to microvascular and macrovascular complications.

Conflict of interest: No conflict of interest was declared by the authors.

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Authors' contributions to the article

M.O. and M.U. constructed the main idea and hypothesis of the study. M.O. and Y.D. developed the theory and arranged/edited the material and method section. M.O., M.U. and Y.D. has done the evaluation of the data in the Results section. Discussion section of the article written by A.O., M.U., M.O., Y.D., A.A.A., O.A. and B.S. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

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Current approaches, significance and prognostic impact of lumbar ligament flavum preserving surgeries

Ligament flavum koruyucu cerrahinin güncel yaklaşımları, önemi ve prognoza etkisi

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Abstract

Purpose: The aim of this study is to evaluate the use of flavum-preserving surgeries in lumbar disc herniation surgery, compare their clinical outcomes with standard microdiscectomy techniques, and demonstrate the transflaval microdiscectomy technique.

Material and methods: Between 2020 and 2023, a total of 48 patients who underwent transflaval microdiscectomy and 48 patients who underwent standard microdiscectomy at a single center were included in the study. Epidemiologic characteristics of the groups were described, and from the preoperative and postoperative 12 months visual analog scale scores for leg pain (LPVAS) and back pain (BPVAS) were retrospectively analyzed.

Results: There is no significant difference between the groups in preoperative mean LPVAS scores (p=0.474) and there is no significant difference between the groups in postoperative mean LPVAS scores (p=0.598). There is no significant difference between the groups in preoperative mean BPVAS scores (p=0.608). However, there is a significant difference between the groups in postoperative mean BPVAS scores (p<0.001). This result indicates transflaval microdiscectomy surgery shows better clinical outcomes in follow-up in terms of back pain compared to standard microdiscectomy surgery. In Group 1 (transflaval group), recurrence occurred in 3 patients, while in Group 2 (microdiscectomy), 4 patients experienced recurrence. During the reoperation of patients with recurrence, none of the patients in Group 1 exhibited epidural fibrosis, whereas all patients in Group 2 showed signs of epidural fibrosis. Additionally, during reoperation, no patient in Group 1 experienced dural injury, while dural injury occurred in 2 patients in Group 2.

Conclusion: Preserving the ligamentum flavum structure and minimizing its damage during lumbar microdiscectomy surgery results in less axial pain and improved clinical outcomes during follow-ups.

Keywords: Ligamentum flavum, ligamentum flavum preserving surgery, transflaval microdiscectomy.

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Öz

Amaç: Bu çalışmanın amacı, lomber disk hernisi cerrahisinde flavum koruyucu cerrahilerin kullanımını değerlendirmek, klinik sonuçlarını standart mikrodiskektomi teknikleri ile karşılaştırmak ve transflaval mikrodiskektomi tekniğini göstermektir.

Gereç ve yöntem: 2020-2023 yılları arasında tek bir merkezde transflaval mikrodiskektomi uygulanan toplam 48 hasta ve standart mikrodiskektomi uygulanan 48 hasta çalışmaya dahil edildi. Grupların yaş ve cinsiyet özellikleri tanımlandı ve bacak ağrısı (LPVAS) ve sırt ağrısı (BPVAS) için ameliyat öncesi ve sonrasındaki 12 ay boyunca izlenerek VAS skorları retrospektif olarak analiz edildi.

Bulgular: Ameliyat öncesi ortalama LPVAS skorlarında gruplar arasında ve ameliyat sonrası ortalama LPVAS skorlarında anlamlı fark bulunamadı (Sırasıyla p=0,474 ve p=0,598). Ameliyat öncesi ortalama BPVAS skorlarında gruplar arasında da anlamlı bir fark gözlenmedi (p=0,608). Ancak ameliyat sonrası ortalama BPVAS skorlarında gruplar arasında anlamlı bir fark saptandı (p<0,001) Bu sonuç, transflaval mikrodiskektomi ameliyatının standart mikrodiskektomi ameliyatına kıyasla bel ağrısı açısından takipte daha iyi klinik sonuçlar gösterdiğini ortaya koymaktadır.

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Grup 1'de (transflaval cerrahi grubu) 3 hastada nüks görülürken, Grup 2'de (mikrodiskektomi grubu) 4 hastada nüks görüldü. Nüks görülen hastaların yeniden ameliyatı sırasında, Grup 1'deki hiçbir hastada epidural fibrozis görülmezken, Grup 2'deki tüm hastalarda epidural fibrozis belirtileri görüldü. Ayrıca, yeniden ameliyat sırasında Grup 1'deki hiçbir hastada dural yaralanma görülmezken, Grup 2'deki 2 hastada dural yaralanma meydana gelmişti.
Sonuç: Lomber mikrodiskektomi ameliyatı sırasında ligamentum flavum yapısının korunması ve hasarının en aza indirilmesi, takiplerde daha az aksiyel ağrı ve daha iyi klinik sonuçlarla sonuçlanır.

Anahtar kelimeler: Ligamentum flavum, ligamentum flavum koruyucu cerrahi, transflaval microdiskektomi.

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Introduction

Intervertebral disc degeneration is a chronic process that naturally occurs as a consequence of aging, influenced by genetic factors, mechanical stress, and axial loading on the spine [1]. One of the most commonly encountered conditions following lumbar disc degeneration is lumbar disc herniation (LDH). LDH is a condition that presents with symptoms of lower back pain and leg pain and can be treated through conservative or surgical methods [2]. There are several methods and approaches defined and currently in use for the surgical treatment of LDH's. The most common surgical techniques today include standard open discectomy, standard open microscopic discectomy, percutaneous tubular microscopic discectomy, biportal endoscopic discectomy, and uniportal endoscopic discectomy [3-5].

The Ligamentum Flavum (LF) is a ligament that extends between two adjacent laminae and consists of two layers, namely the superficial and deep layers. It attaches to the facet joints on the lateral side, interspinous ligament on the medial side and is a yellow-colored, elastic ligament [6]. LF serves as a ligament that resists hyperflexion movement in the spine, thus limiting this motion and assisting in stabilizing the spine. However, it should be noted that due to this mechanical stress, ligamentum flavum hypertrophy and ossification can develop, which may be a contributing factor to lumbar spinal stenosis [7, 8]. The LF was typically damaged during standard LDH surgery. However, it should be noted that preserving the structure of the LF or minimizing its damage is crucial in preventing post-surgical adhesions and reducing the development of epidural fibrous tissue [9, 10].

There are various methods and techniques defined for performing LDH surgery while preserving the LF. In these techniques, typically following a sharp dissection applied to the LF, a window extending into the epidural space is created by reflecting the LF to one side (laterally or caudally), and the microsurgical discectomy procedure is then carried out through this window [11, 12]. In our LF preserving LDH surgery technique, which we refer to as Transflaval Lumbar discectomy, we create a window using suspension sutures from both sides after making a longitudinal incision on LF. After removing the sequestered disc fragment through the resulting opening, we suture the created LF layers. This way, a transverse incision is not made, and we believe that it better preserves the mechanical resistance against flexion movement of the LF.

Material and methods

Study population

Between 2020 and 2023, a total of 96 patients who underwent LDH surgery at our institution were included in the study. All patients in the study population had undergone surgery due to sequestered disc herniation at the L5-S1 level. Among them, 48 patients (28 males and 20 females) were operated on using the transflaval microdiscectomy technique (Group 1), while 48 patients (27 males and 21 females) underwent surgery using the standard microscopic discectomy technique (Group 2). Group 1 consists of patients who underwent surgery using the transforaminal microdiscectomy method, while Group 2 serves as the control group and consists of patients who underwent standard microdiscectomy.

The age and gender characteristics of all patients, Visual Analog Scale (VAS) scores

recorded 24 hours before surgery, VAS scores at 12 months postoperatively, and recurrence rates at postoperative period were noted. When calculating preoperative and postoperative VAS scores, leg pain VAS (LP-VAS) and back pain VAS (BP-VAS) were calculated for each group.

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics version 22.0 for Windows (IBM, Armonk, NY). The results were evaluated with a 95% confidence interval, and the *p*-value <0.05 was considered significant.

This retrospective study was approved by Ankara University Faculty of Medicine Clinical Research Ethics Committee.

Operative technique for transflaval lumbar discectomy

After ensuring appropriate surgical sterilization conditions and draping in a prone position, a midline skin incision was made to accommodate the L5-S1 disc space. Subsequently, unilateral subperiostal dissection of the muscles attached to the spinous process and lamina was performed. Using a microscope, the ligamentum flavum (LF) structure extending between the laminae was identified without performing laminectomy, and a 1-centimeter incision was made parallel to the LF fibers (Figure 1). The two LF leaves created with this incision were suspended using 4/0 Vicryl sutures, and a window extending into the epidural space was formed (Figure 2). Looking through this window, the traversing S1 root and the fragmentary disc tissue situated laterally to S1 root were identified with the use of a microdissector and a micro nerve hook (Figure 2). Sequestrectomy was performed using microforceps, and after confirming that the root was relieved, the LF defect was sutured with 4/0 Vicryl suture (Figure 3). After suturing the muscles, fascia, and skin in their anatomical planes, the surgery was completed.

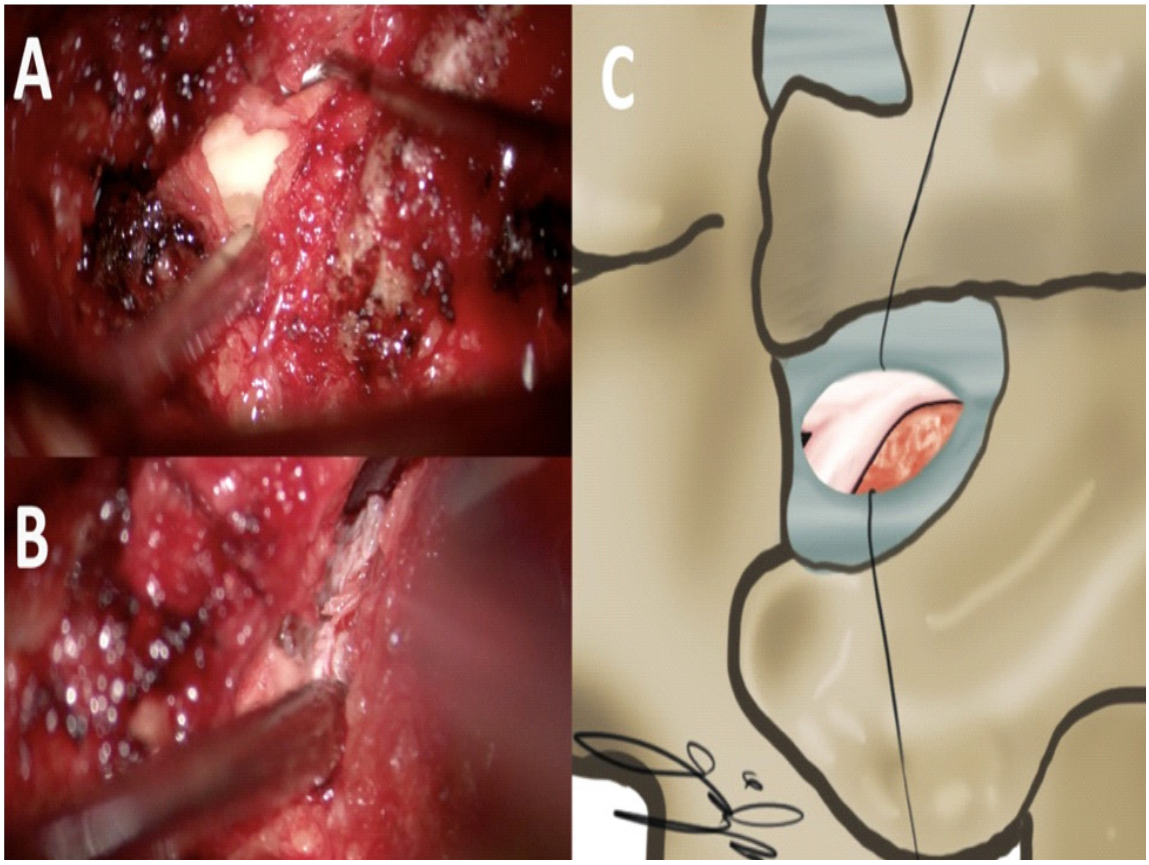

Figure 1. A, demonstration of the Ligamentum Flavum under the surgical microscope, B and C show the surgical image and illustration of Ligamentum Flavum incision, respectively. Laminectomy has not been performed, and thus, no damage has been made to the attachment area of the ligamentum flavum to the lamina

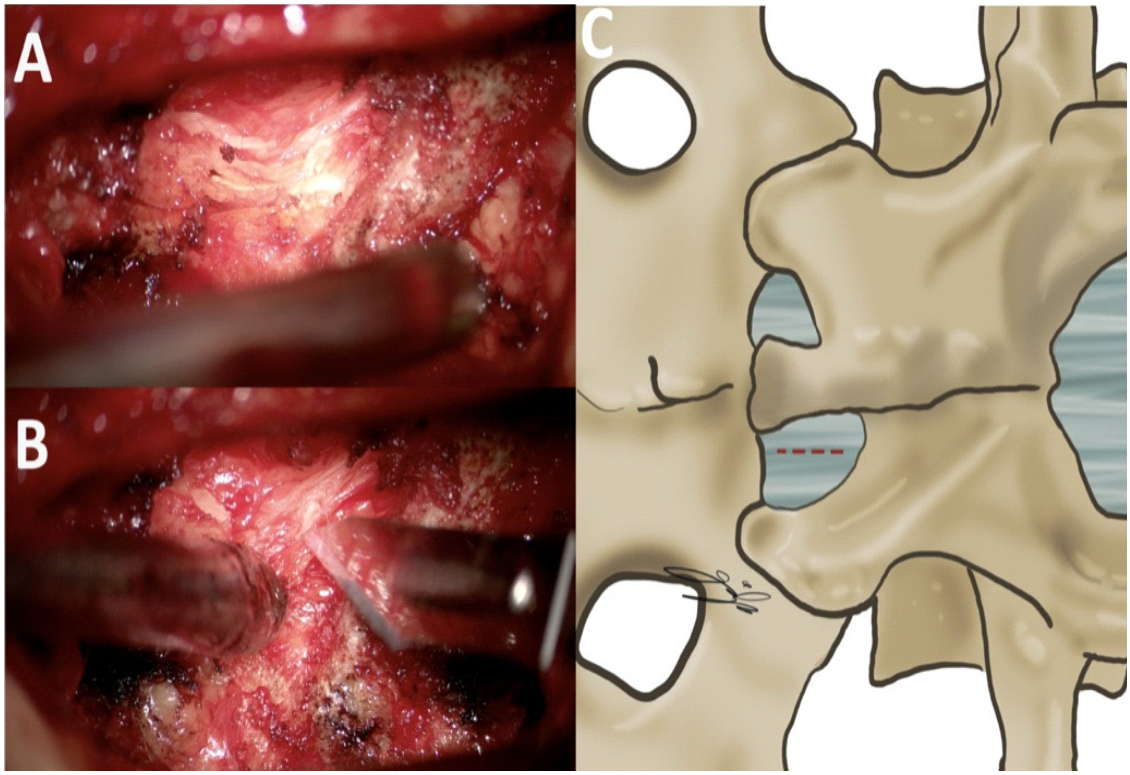

Figure 2. A creation of a window extending into the epidural space after the incision of Ligamentum Flavum and its suspension with sutures. In B, sequestered disc material located laterally to the Root can be observed. C is an illustration showing the creation of a window with sutures after the incision of the Ligamentum Flavum. Sequestered disc fragment located laterally to root is illustrated in red colour

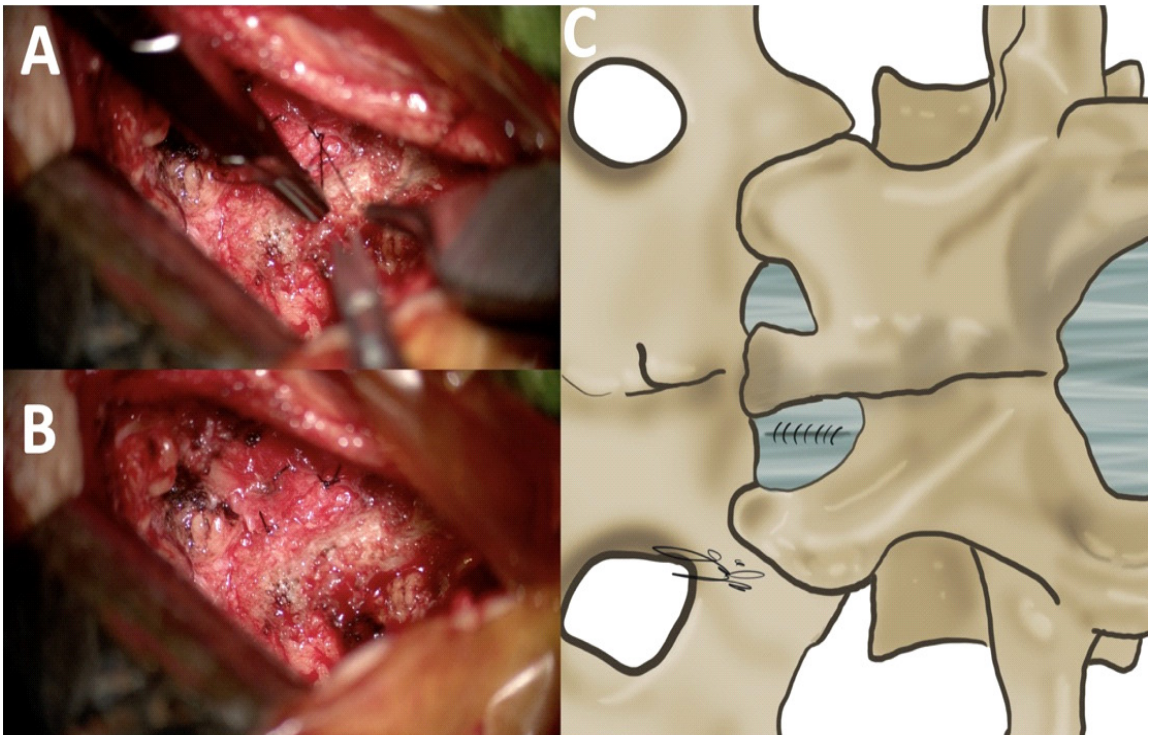

Figure 3. A and B, surgical image demonstrating the suturing of the Ligamentum Flavum after sequestrectomy. C is an illustration depicting after the suturing process

Results

Group 1 consists of 48 patients, 28 males and 20 females, with a mean age of 41.3. Group 2 consists of 48 patients, 27 males and 21 females, with a mean age of 42.8. There is no significant difference between the two groups in terms of gender and age, p=0.837 and p=0.554, respectively.

In Group 1, lumbar disc herniation recurrence occurred in 3 out of the 48 patients (6.25%) during postoperative follow-ups within the first year. Due to recurrence occurring before the 12th month, these cases underwent reoperation and were not included in the postoperative 12-month follow-up. Therefore, preoperative LPVAS and BPVAS scores were calculated for 48 patients in Group 1, whereas postoperative LPVAS and BPVAS scores were calculated for the remaining 45 patients, as three patients were excluded due to recurrence. Similarly, in Group 2, recurrence occurred in 4 patients (8.33%) within the first year, leading to their exclusion from the postoperative 12-month follow-up. Consequently, preoperative scores for Group 2 were calculated for 48 patients, while postoperative scores were calculated for 44 patients, excluding those with recurrence.

In the preoperative period, mean VAS scores (LPVAS) were assessed for Group 1, with a value of 7.27, and for Group 2, with a mean preoperative LPVAS score of 7.13. The mean LPVAS scores evaluated at postoperative 12th month were 1.84 for Group 1 and 1.75 for Group 2. There is no significant difference between the groups in preoperative mean LPVAS scores (p=0.474) and there is no significant difference between the groups in postoperative mean LPVAS scores (p=0.598). This result indicates that the transflaval or standard microdiscectomy method does not show differences in clinical outcomes in postoperative leg pain.

The mean BPVAS score, assessed for the evaluation of severity of back pain, was found to be 4.58 for Group 1 and 4.42 for Group 2 at preoperative period. When the BPVAS score was assessed at postoperative 12th month, it was found to be 1.44 for Group 1 and 2.84 for Group 2. There is no significant difference between the groups in preoperative mean BPVAS scores (p=0.608). However, there is a significant difference between the groups in postoperative mean BPVAS scores (p<0.001). This result indicates that patients who underwent transflaval microdiscectomy surgery show better clinical outcomes in follow-up in terms of back pain compared to patients who underwent standard microdiscectomy surgery.

In Group 1, recurrence occurred in 3 patients within first year, and in Group 2 recurrence occurred in 4 patients within first year. All these patients underwent reoperation with using the standard microdiscectomy method. Among the 3 patients in the first group who underwent reoperation, none showed intraoperative cerebrospinal fluid (CSF) leakage related to dural injury. However, in the second Group, 2 out of the 4 patients who underwent reoperation exhibited CSF leakage associated with intraoperative dural injury. Furthermore, none of the 3 patients in the first group, who experienced a recurrence, exhibited observations of epidural fibrosis during the surgery for recurrence, whereas all 4 patients in the second group exhibited areas of epidural fibrosis during the reoperation procedure.

Discussion

The condition of lumbar disc herniation and its association with leg pain has been a subject of debate since ancient Greek times [13]. Surgical interventions to the lumbar spine began in the 18th century, but the first established and documented lumbar discectomy surgery was performed by Truumees et al. [13] in 1932. From the subsequent years to the present, LDH surgery has undergone significant changes and developments with the introduction of surgical microscopes, and the utilization of tubular retractors as well as the performance of tubular surgeries (Micro-endoscopic discectomy) with Video-assisted techniques by Butler et al. [14].

In lumbar disc surgery, regardless of the surgical approach chosen (microscopic or endoscopic techniques), the preservation of the LF structure has been debated and researched for its potential to reduce postoperative epidural fibrosis, facilitate revision surgeries, and be associated with better postoperative outcomes [10-12]. Studies investigating the relationship between obtaining better clinical outcomes after surgeries with preserved LF structure have shown that epidural fibrotic tissue can cause neural irritation, and depending on the size of

the scar tissue, it can even create mass effects, resulting in both radicular and axial pain [15-17]

In one of the studies conducted to address these problems, it was found that preserving the LF structure during lumbar discectomy surgery is associated with radiologically less development of epidural fibrosis and better postoperative clinical outcomes [12]. In another study on this topic, it was observed that surgeries performed with LF preservation resulted in less epidural fibrosis at postoperative 6-month follow-up evaluations, indicating that this technique acts as a natural physical protective barrier [11]. In a serie that follow-ups were documented for 2 years with using Visual Analog Scale (VAS) scores and Oswestry Disability Index (ODI), it was demonstrated that postoperative clinical outcomes obtained after LF-preserving surgeries were associated with better results when assessed using these scales [18]. In a series of 78 patients in which the LF structure was preserved and an endoscopic approach was used, it was demonstrated that the endoscopic lumbar discectomy technique resulted in better clinical outcomes when performed with the preservation of the LF structure [19].

Various LF preserving surgical techniques have been described in the literature. In a method described as the Ozer et al. [11] technique, the LF is opened using a circular incision, anchored to the sacrum with a needle, and after completing the discectomy, the surgery is completed by performing LF sutures. In another described technique, the LF structure is cut from the lateral part near the facet joint and folded medially to create a window, followed by the discectomy procedure [20]. In another described technique, the LF is lifted as a three-sided flap and, with the help of a retractor, is pulled medially to perform the surgery [10]. In our technique, we divide the LF longitudinally into two pieces with an incision and then suspend those pieces with a suture. We believe that this incision, being parallel to the LF fibers, causes less damage to the LF fibers and is more suitable for preserving their function, as it avoids creating a perpendicular incision on the flexion force arm that resists hyperflexion forces. Moreover, the omission of laminectomy and the preservation of the attachment area of the ligamentum flavum to the lamina contribute to the preservation of resistance against hyperflexion forces.

It is possible to find many articles on epidural fibrosis in the literature as it is a highly studied topic. Especially after microdiscectomy operation, epidural fibrosis rates and methods to reduce these numbers can be found in a large number of articles. For example, in the experimental study conducted by Keskin et al. [21] in rats, it was noticed that the use of berberine in vivo significantly reduced epidural fibrosis. Similarly, Dayanır et al. [22] addressed the same issue through ozone, while many different research teams have studied the effects of systemic or local use of different molecules and pharmaceutical agents on epidural fibrosis [23, 24]. In our series, when comparing flavum-preserving surgery with standard surgery, no comparison has been made radiologically regarding the development of epidural fibrosis. The reason for this is the non-application of control MRI examinations during follow-ups in patients without symptoms that would raise suspicion of recurrence. However, during intraoperative observations in patients with recurrence, it has been observed that epidural fibrosis is more commonly encountered in patients undergoing standard microdiscectomy compared to those undergoing transflaval microdiscectomy. Additionally, due to these adhesions, dural injury has been found to be more frequent in cases with recurrence during reoperation in patients who underwent standard microdiscectomy.

The study's limitations include the relatively short postoperative follow-up period of 12 months and the use of a relatively small patient population. More definitive conclusions can be drawn through investigations with extended follow-up intervals and larger cohorts of patients.

In conclusion, preserving the ligamentum flavum structure and minimizing its damage during lumbar microdiscectomy surgery results in less axial pain, a lower incidence of epidural fibrozis, a reduced risk of dural injury in reoperations following the development of recurrence, and improved clinical outcomes during follow-ups.

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Authors' contributions to the article

B.C.A. and M.Z. constructed the main idea and hypothesis of the study. O.M.O. and E.G. developed the theory and arranged/edited the material and method section. M.B. has done the evaluation of the data in the results section. Discussion section of the article was written by B.C.A.

F.Y. and U.E. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

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Does vitamin D deficiency affect ventricular repolarization in the elderly?

D vitamini eksikliği yaşlılarda ventriküler repolarizasyonu etkiler mi?

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Abstract

Purpose: In this study, we aimed to establish if vitamin D deficiency interacts with the electrocardiographic indices of abnormal ventricular repolarization in elderly patients.

Material and methods: 138 patients were divided into three groups: patients with vitamin D deficiency, patients with vitamin D insufficiency, and patients with adequate vitamin D levels. 12-lead electrocardiography and laboratory data were collected.

Results: The resting heart rate, PR interval, Tp-e interval, QRS duration, Tp-e/QTc, QT dispersion, and Tp-e dispersion were similar among the groups. However, the QTc interval was significantly prolonged in patients with vitamin D deficiency when compared to patients with sufficient vitamin D levels. Vitamin D level was found as the only independent predictor of the QTc interval. The cut-off value of Vitamin D level determining the significant prolongation of QTc interval was found to be 20 ng/ml on ROC analysis (Area Under Curve: 0.629±0.051 (95% CI: 0.530-0.728, *p*=0.013).

Conclusion: Low vitamin D levels are related to QTc prolongation in the elderly. Vitamin D-deficient elderly patients may benefit from routine ECG screening. Timely diagnosis and treatment of vitamin D deficiency may aid in reducing the rate of arrhythmias in this patient population.

Keywords: Vitamin D deficiency, elderly, ventricular repolarization.

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Öz

Amaç: Bu çalışmanın amacı, D vitamini eksikliğinin yaşlı hastalarda anormal ventriküler repolarizasyonun elektrokardiyografik indeksleri ile ilişkili olup olmadığını belirlemektir.

Gereç ve yöntem: 138 hasta üç gruba ayrıldı: D vitamini eksikliği olan hastalar, D vitamini yetersizliği olan hastalar ve yeterli D vitamini düzeyine sahip hastalar. 12 derivasyonlu elektrokardiyografi ve laboratuvar verileri toplandı.

Bulgular: İstirahat kalp hızı, PR aralığı, QRS süresi, QTd ve Tp-e dispersiyonu gruplar arasında benzerdi. Ancak, D vitamini eksikliği olan hastalarda QTc aralığı, D vitamini düzeyi yeterli olan hastalara kıyasla anlamlı derecede uzamıştı. D vitamini düzeyi QTc aralığının tek bağımsız belirleyicisi olarak bulundu. ROC analizinde QTc aralığında anlamlı uzamayı belirleyen D vitamini düzeyinin eşik değeri 20 ng/ml olarak bulundu (Eğri Altındaki Alan: 0,629±0,051 (%95 GA: 0,530-0,728, *p*=0,013).

Sonuç: Düşük D vitamini düzeyleri yaşlılarda QTc uzaması ile ilişkilidir. D vitamini eksikliği olan yaşlı hastalar rutin EKG taramasından fayda görebilir. D vitamini eksikliğinin zamanında teşhis ve tedavisi, bu hasta popülasyonunda aritmi oranını azaltmaya yardımcı olabilir.

Anahtar kelimeler: D vitamini eksikliği, yaşlılık, ventriküler repolarizasyon.

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Introduction

Vitamin D is well-known for its role in calcium and bone metabolism. Nonetheless, owing to its widespread receptors (VDR) throughout the body, it plays a crucial role in several organ systems, including the cardiovascular (CV) system [1]. VDR is expressed abundantly in cardiac muscle [2]. The binding of active vitamin D [1,25-dihydroxyvitamin D; 1,25(OH)2D] to the VDR in cardiomyocytes regulates several vitamin D response genes, hence promoting CV system benefits such as modulation of renin synthesis and improvement in vascular compliance [3, 4]. Not surprisingly, low vitamin D levels are associated with increased CV risk [5]. Vitamin D deficiency has been related to many CV disorders, such as coronary artery disease, hypertension, arrhythmias, and sudden cardiac death [6].

Vitamin D deficiency is highly prevalent worldwide and across all age groups. Moreover, specific risk groups, such as the elderly, are more prone to vitamin D deficiency. Therefore, general screening for vitamin D deficiency is recommended for adults older than 65 years old [7].

Ventricular repolarization (VR) is a complex phase of cardiac electrical activity. Abnormalities in ventricular myocardial repolarization may increase the risk of developing malignant ventricular arrhythmias and, thus, sudden cardiac death [8]. Multiple indices on surface electocardiography have been applied to predict alterations in VR, including QT interval, corrected QT interval (QTc), QT dispersion (QTd), Tp-e interval, Tp-e/QT ratio, and Tp-e/QTc ratio [9, 10].

Numerous studies have revealed that low vitamin D levels were related to disturbances in cardiac repolarization [11-13]. Nonetheless, the association between vitamin D deficiency and VR has not been explicitly evaluated in a geriatric population before.

The purpose of the current study is to establish whether deficient vitamin D levels is accociated with the electrocardiographic indices of abnormal ventricular repolarization in elderly patients.

Material and methods

Study population

In this single-center study, we retrospectively assessed the medical records of a total of 156 patients who were admitted to our institution's geriatrics clinic between June 2021 and October 2021. 46 participants were recruited from another study population that was intended to investigate the octogenarians' clinical traits. The remaining cases were recruited using medical records. Fasting blood samples and 12-lead ECG recordings were obtained from the medical records. 18 patients were excluded due to insufficient electrocardiographic (ECG) data and the use of antipsychotics or antiarrhythmics. As there are a vast number of medications known to prolong the QTc interval, we did not exclude patients who were taking other QTc-prolonging drugs. Eventually, a final total of 138 patients were included in the analysis. The subjects were seperated into three groups:

1st Group: Vitamin D deficiency

2nd Group: Vitamin D insufficiency

3rd Group: Adequate vitamin D levels

Vitamin D deficiency was defined as serum vitamin D levels <20 ng/ml, insufficiency as 20-30 ng/ml, and normal vitamin D levels were considered as >30 ng/ml [7].

Permission was obtained from Ankara University Non-Interventional Clinical Research Ethics Committee for the study and was performed in concordance with ethical rules and principles of the Declaration of Helsinki.

Electrocardiography (ECG)

A standard 12-lead ECG recording was obtained for each patient while in the supine position at 25 mm/s paper speed and 10 mV/mm amplitude, using standard ECG equipment. The ECG strips were scanned and loaded into computer and analyzed with a digital caliper using a software program (EP Calipers, EP Studios, Inc.). Heart rate, PR interval, QRS duration, QT and QTc intervals, QT dispersion (QTd), QTc dispersion (QTc), T wave peak-to-end interval (Tp-e), and Tp-e dispersion (Tpe-d) were measured from the D2 and V5

derivations. All measurements were obtained from the average of five consecutive beats. The PR interval was calculated as the time from the start of the P wave to the beginning of the QRS wave. The QT interval was calculated as the time from the beginning of the QRS wave to the end of the T wave. QT values were corrected by using the Bazett formula (QTc=QT/√RR). QTd was determined by subtracting the minimum QT interval from the maximum QT interval. The Tp-e interval was calculated as the interval from the peak of T wave to the end of T wave. Tpe-d was calculated as the difference between the maximum and minimum Tp-e intervals. Tp-e/QTc was calculated by dividing Tp-e interval to QTc interval.

Statistical analysis

Statistical analyses were conducted with the Statistical Package for Social Sciences (SPSS) software, version 10.0. The Kolmogorov-Smirnov test was used to test the normality of the distribution for continuous variables. The results were given as mean ± standard deviation (SD), whereas categorical ones were given as percentages (%). In order to compare the mean levels of the continuous variables between the groups, ANOVA (1-way analysis of variance) was used. Tukey post-hoc test was employed for multiple comparisons. ROC curve analysis was used to measure the diagnostic accuracy of the determinants used in the study. The Youden index method defined the optimal cut-off points for the studied determinants. The Pearson or Spearmen test was used to assess correlations between the variables. The association between independent variables and the outcome variable was first evaluated with univariate analysis. Linear regression analysis was used to examine the relationship between the QTc interval and clinical parameters. Variables selected with univariate analysis having a *p*-value less than 0.3 were incorporated into the multivariable analysis. A *p*-value of less than 0.05 was considered significant.

Results

The baseline characteristics and biochemical parameters of the 3 groups are presented in Table 1. Patients in group 2 were significantly longer than patients in group 3. No other significant difference was observed between the groups in terms of baseline characteristics.

The ECG characteristics of the groups are shown in Table 2.

The resting heart rate, PR interval, QRS duration, QTd and Tp-e dispersion were similar among the groups. However, QTc interval was meaningfully prolonged in patients with deficient levels of vitamin D, when compared to patients with sufficient vitamin D levels. Potential parameters that may be associated with QTc were evaluated by univariate analysis. Neither the number of drugs used by the patient nor the presence of coronary artery disease were related to the QTc interval in the linear regression analysis (Table 3).

Then a multivariate logistic regression analysis was performed by including all parameters that were associated with QTc in the univariate analysis. Vitamin D level was found as the only independent predictor of the QTc interval (Table 4).

The cut-off value of Vitamin D level determining the significant prolongation of QTc interval was found to be 20 ng/ml on ROC analysis (Area Under Curve: 0.629±0.051 (95% CI: 0.530-0.728, *p*=0.013) (Figure 1).

Table 1. Baseline demographic and characteristics

Variables	Group 1 (vitamin D deficiency) (n=87)	Group 2 (vitamin D insufficiency) (n=27)	Group 3 (Sufficient vitamin D levels) (n=24)	*p* value
Age (years)	75.9±7.2	74.4±6.3	77.8±5.5	0.212
Men n (%)	36 (41.3)	10 (37)	10 (41.6)	0.916
Height (cm)	159.7±8.9	162.4±7.3	156±9.1	**0.036***
Weight (kg)	73.5±13.2	75.2±9.1	68.5±16.3	0.164
Number of drugs (n)	5.3±3.2	5.8±2.8	5.1±3.1	0.647
Co-morbidities (n)				
HT	68	21	17	0.747
DM	40	15	10	0.576
Dementia	10	3	2	0.907
COPD	17	3	7	0.268
AF	14	3	2	0.619
HF	9	3	3	0.970
CAD	26	14	6	0.068
Laboratory values				
eGFR, mg/dl/1.73m^2	66.9±20.5	66.2±23.7	62.9±17.9	0.705
Creatinine, mg/dl	1.01±0.3	1.07±0.5	1.02±0.3	0.821
Hemoglobin, gr/dl	12.4±2.1	13.2±2.5	11.8±2.2	0.085
White blood count, 10^9/L	8±6.6	7.6±2.1	7±1.8	0.705
Vitamin D, ng/ml	9.8±3.8	25.6±6.5	39.3±8.1	**0.000***
Total cholesterol, mg/dl	177.3±49.2	195.9±46.2	162.7±53.6	0.057
Calcium, mg/dl	10.1±8.8	9.3±0.5	9.3±0.5	0.801
Albumin, mg/dl	4.1±0.5	4.1±0.6	4.1±0.5	0.911

HT: hypertension, DM: diabetes mellitus, COPD: chronic obstructive pulmonary disease, AF: atrial fibrillation, HF: heart failure
CAD: coronary artery disease, eGFR: estimated glomerular filtration rate

Table 2. ECG parameters of the patients

ECG Parameters	Group 1 (vitamin D deficiency) (n=87)	Group 2 (vitamin D insufficiency) (n=27)	Group 3 (Sufficient vitamin D levels) (n=24)	*p* value
Resting heart rate (beats/min)	75.6±14.3	75.8±13	78±11.7	0.764
PR interval (msn)	163.7±30.8	165±24.2	155.8±25	0.504
QRS duration (msn)	94.5±20.1	92.9±18.1	92±16.4	0.839
QTc interval (msn)	435.4±35.7(a)	430±28.8	409.2±38.9	**0.01***
QTd (msn)	59±20.6	59.8±21.3	58.7±30.6	0.984
Tp-e	79.8±22.03	85±24.5	80.1±16.3	0.890
Tp-e/QTc	0.16±0.04	0.21±0.06	0.18±0.04	0.193
Tp-e dispersion (msn)	47.2±16.1	48±13.3	41.4±15.5	0.260

The post-hoc analysis revealed a difference between group 1 and group 3

Table 3. Univariate analysis of clinical and laboratory parameters affecting QTc interval

	HR (95% CI)	*p*
Age	0.974 (0.924-1.027)	0.330
Gender	0.605 (0.295-1.240)	0.170
Number of drugs	1.021 (0.913-1.142)	0.716
HT	1.178 (0.507-2.736)	0.703
DM	1.098 (0.543-2.221)	0.794
HF	1.956 (0.544-7.028)	0.304
CAD	1.100 (0.526-2.302)	0.800
AF	1.105 (0.491-2.485)	0.810
Vitamin D level	0.952 (0.922-0.983)	**0.003***
eGFR	1.014 (0.997-1.032)	0.117
Hemoglobin	0.903 (0.711-1.059)	0.211
TSH	0.974 (0.779-1.218)	0.819

HT: hypertension, DM: diabetes mellitus, HF: heart failure, CAD: coronary artery disease
AF: atrial fibrillation, Egfr: estimated glomerular filtration rate

Table 4. Predictors of QTc interval on multivariate analysis

	HR	95% CI	*p*
Age	0.945	0.853-1.048	0.286
Gender	0.736	0.209-2.601	0.635
HF	2.425	0.462-12.734	0.295
Vitamin D level	0.927	0.870-0.989	**0.021***
eGFR	1.014	0.980-1.049	0.424
Hemoglobin	0.854	0.620-1.177	0.336

HF; heart failure, eGFR; estimated glomerular filtration rate

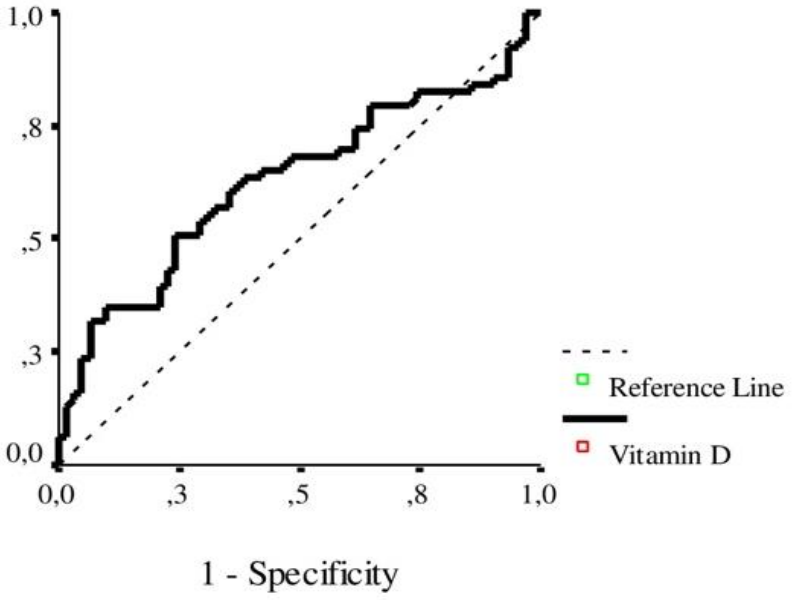

Figure 1. The Receiver operating curves (ROC) of significant prolongation of QTc interval

Vitamin D level AUC=0.629±0.051 (95% CI: 0.530-0.728)

Discussion

In the present study, we aimed to investigate the relationship between vitamin D levels and ventricular repolarization in the elderly population. We revealed that deficient vitamin D levels were independently associated with QTc interval prolongation in elderly patients. QT and Tpe dispersion, Tp-e interval, and Tp-e/QTc ratio as further indices of abnormal ventricular repolarization, were not associated with the vitamin D levels. Additionally, we showed that 20 ng/ml was the optimal cut-off value for vitamin D levels that was associated with significant prolongation of the QTc interval.

The receptors of Vitamin D are extensively present in the cardiovascular system. When combined with its receptor, vitamin D regulates the transcription of genes that control oxidative stress, cell adhesion, cell proliferation, and cell apoptosis in cardiomyocytes [14]. Low vitamin D levels were associated with an increased risk of many cardiovascular diseases such as hypertension, coronary artery disease, and arrhythmias in individuals with vitamin D deficiency [6, 15]. Both vitamin D insufficiency and mortality due to sudden cardiac death are more prevalent among the elderly. Therefore, in the present study we have chosen this patient population in particular.

Numerous studies have investigated the link between atrial fibrillation (AF) and vitamin D deficiency [16-18]. Low vitamin D levels have also been related to repolarization abnormalities, ventricular arrhythmias, and sudden cardiac death. Previously, vitamin D deficiency was revealed to be related to prolongation of the QTc interval [19]. Moreover, lower vitamin D levels were linked to sudden cardiac death in older adults who had no prior cardiovascular disease [20]. A recent study on the pediatric population revealed a correlation between vitamin D levels and surface ECG indicators of ventricular repolarization, including QT interval, QTc interval, QT dispersion, JT interval, JTc interval, Tpeak-to-Tend interval, and Tp-e/QTc [12]. Similarly, Bagrul et al. [13] have shown that adolescents with deficient and insufficient vitamin D levels had a prolonged Tp-e interval compared to patients with sufficient levels of vitamin D. In another study by Yetkin et al. [21], vitamin D deficient diabetic patients had prolonged QTc and higher QTC dispersion, compared to patients with adequate vitamin D levels. Similarly, we found a prolonged QTc interval in vitamin D-deficient patients compared to patients with adequate levels of vitamin D. Nonetheless, QTc intervals were similar between patients with sufficient levels of vitamin D and patients with vitamin D insufficiency. We did not observe a significant relationship between QTd, Tp-e, Tp-e/QTc, and Tpe-d and vitamin D levels.

Ventricular repolarization abnormalities are considered mechanistic for arrhythmogenesis. QTc interval is the most frequently employed marker of ventricular repolarization on the surface ECG. Various studies have indicated that prolonged QTc is associated with an increased risk of ventricular arrhythmias and sudden cardiac death [22, 23]. Vitamin D deficiency may activate aldosterone receptors and thus affect calcium and potassium currents through the cardiomyocytes. Also, the active metabolite of vitamin D may increase the potassium currents in cardiomyocytes. Furthermore, vitamin D deficiency may result in structural and ionic channel remodeling and autonomic function disorders. In conclusion, vitamin D deficiency induces changes in the repolarization of the ventricular myocardium that may increase susceptibility to malignant ventricular arrhythmias and sudden cardiac death [24-26].

The main limitation of our study was that it was a single-center study with a relatively low number of cases. Also, beacuse this was a cross-sectional study, we cannot estalish a casual relationship between vitamin D levels and the QTc interval. Another limitation was the absence of control ECG findings following treatment for vitamin D deficiency. Also, the serum electrolytes of the patients were missing. Moreover, as a parameter that could influence the QTc interval, we simply mentioned the number of medications utilized by the patients. We did not report individual medications. Last but not least, the patients have not been followed up long term for the development of ventricular arrhythmias and sudden cardiac death.

In conclusion, low vitamin D levels are associated with QTc prolongation in the elderly. As vitamin D deficiency is highly prevalent among adults >65 years old, this patient population is at increased risk for ventricular arrhythmias

and sudden cardiac death. Therefore, vitamin D deficient elderly patients may benefit from routine ECG screening. Timely diagnosis and treatment of vitamin D deficiency may aid in reducing the rate of arrhythmias in this patient population. Despite its limitations, our study is the first to reveal the clear relationship between vitamin D deficiency and QTc prolongation in an elderly population. Further studies involving more patients with longer follow-up times are needed in order to reveal the mechanistic relationship between vitamin D and malignant ventricular arrhythmias.

Conflict of interest: No conflict of interest was declared by the authors.

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Authors' contributions to the article

M.A. and B.Y. constructed the main idea and hypothesis of the study. V.A. developed the theory and arranged the material and method section. B.Y. has done the evaluation of the data in the results section. M.A. has written the article. M.V. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

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Anti-CD38 monoclonal antibody daratumumab enhances the overall response rate in patients with multiple myeloma

Multipl miyelomda Anti-CD38 monoklonal antikoru daratumumab tedavisi genel yanıt oranını arttırır

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Abstract

Purpose: New medicines employed in recent years have resulted in significant increases in survival rates for Multiple Myeloma (MM). Daratumumab, a monoclonal antibody against CD38, is utilized in both first-line myeloma treatment and relapsed/refractory illness. Our study aims to assess the clinical features, response to treatment and factors influencing response to treatment in patients who received daratumumab monotherapy or combination therapy at our center.

Materials and methods: In the Pamukkale University Faculty of Medicine Hematology clinic between June 2022 and June 2023, 21 patients who were treated with daratumumab after receiving a multiple myeloma diagnosis were included. Demographic features of the patients, disease stage, prior therapies, characteristics of daratumumab treatment, and response rates to treatments were retrospectively analyzed.

Results: The patients median age was 65±9.7 years (42-80), with a female/male ratio of 11/10. Treatment with daratumumab: 61.9% was used after two lines of therapy, 23.8% was used in first-line therapy, and 14.28% was used in second-line therapy. The average number of cycles was 4.05±5.06. Of the patients treated with daratumumab, 4.76% were treated as a single agent; 61.9% were treated in combination with immunomodulatory medications, cyclophosphamide and/or melphalan; and 33.4% were treated in conjunction with chemotherapy. When the response to treatment was evaluated, 38.1% of the patients passed away, 38.1% had a very good partial response (VGPR) or better, and 23.8% had a partial response (PR). 42.9% of patients who received daratumumab along with chemotherapy died. With daratumumab-containing regimens, overall response rates increased significantly as the number of cycles increased (ORR) (p=0.026).

Conclusion: When daratumumab-containing protocols are used in the treatment of multiple myeloma, it has been observed that overall response rates improve and treatment success increases in direct proportion to the number of cures.

Keywords: Daratumumab, multiple myeloma, treatment.

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Öz

Amaç: Son yıllarda kullanılan yeni ilaçlar Multipl Miyelom (MM) hastalığında sağkalım oranlarında önemli artışlara yol açmıştır. CD38'e karşı geliştirilmiş monoklonal antikor olan Daratumumab hem birinci basamak miyelom tedavisinde hem de nükseden/dirençli hastalıkta kullanılmaktadır. Çalışmamız, merkezimizde daratumumab monoterapisi veya kombinasyon tedavisi alan hastaların klinik özelliklerini, tedaviye yanıt ve yanıtı etkileyen faktörleri değerlendirmeyi amaçlamaktadır.

Gereç ve yöntem: Pamukkale Üniversitesi Tıp Fakültesi Hematoloji kliniğinde Haziran 2022 ile Haziran 2023 tarihleri arasında multipl miyelom tansı ile takip edilen ve daratumumab tedavisi alan 21 hasta çalışmaya dahil edildi. Hastaların demografik özellikleri, evreleri, daha önce aldıkları tedaviler, daratumumab tedavisinin özellikleri ve tedaviyle elde edilen yanıt oranları retrospektif olarak incelendi.

Bulgular: Hastaların ortanca yaşı 65±9,7 yıl (42-80), kadın/erkek oranı 11/10 idi. Daratumumab tedavisi hastaların %61,9'da iki basamak tedavi sonrasında, %23,8'inde birinci basamak tedavide ve %14,28'de ikinci basamak tedavide kullanıldı. Ortalama siklus sayısı 4,05±5,06 idi. Daratumumab ile tedavi edilen hastaların %4,76'sında tek ajan, %61,9'u immünomodülatör ilaçlar, siklofosfamid ve/veya melfalan ile kombinasyon halinde ve %33,4'ü ise kemoterapi ile kombinasyon halinde kullanıldı.

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Tedaviye yanıt değerlendirildiğinde; hastaların %38,1'inin kaybedildiği, %38,1'inin çok iyi kısmi yanıt (ÇİKY) ve üzeri yanıt ile %23,8'inin stabil hastalık (SH) ile tedaviye devam ettiği görüldü. Kemoterapiyle birlikte daratumumab alan hastaların %42,9'u kaybedildi. Daratumumab içeren rejimler ile kür sayısı arttıkça genel yanıt oranlarının anlamlı bir şekilde arttığı görüldü (p=0,026).
Sonuç: Multiple miyelom tedavisinde daratumumab içeren protokoller kullanıldığında kür sayısı ile doğru orantılı olarak genel yanıt oranlarının iyileştiği ve tedavi başarısının arttığı görülmüştür.

Anahtar kelimeler: Daratumumab, multiple miyelom, tedavi.

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Introduction

It is widely accepted, multiple myeloma (MM) is an extremely challenging form of hematological malignancy. Despite the difficulties of treatment, significant gains in response rates have been made with novel drugs employed in recent years, as well as great success in progression-free survival and overall survival [1].

Daratumumab, a monoclonal antibody created against CD38 produced in myeloma cells, is one of the novel treatments used for MM. Daratumumab displays cytotoxicity that is reliant on complement, cytotoxicity that is dependent on antibodies, cellular phagocytosis that is dependent on antibodies, and immunomodulatory effects [2-4]. In view of its shown performance in monotherapy or combination treatment protocols in clinical trials, daratumumab is replacing MM patients' previous treatment methods. Even in patients with poor prognostic features who had received multiple lines of therapy, 20.1-month overall survival was achieved with daratumumab treatment administered as monotherapy [5]. Many studies in newly diagnosed and relapse-refractory patients have shown that triple and quadriple treatment regimens with the inclusion of daratumumab have significant survival successes [6-9]. The aim of this study was to examine the parameters influencing the clinical course and response rates to daratumumab treatment in patients with newly diagnosed or relapsed refractory myeloma (MM) at our clinic.

Materials and methods

The study included patients with multiple myeloma who were followed up in the hematology clinic of Pamukkale University Faculty of Medicine. Retrospective analysis was done on the data of newly diagnosed or relapsed refractory multiple myeloma patients treated with daratumumab as a single agent or in combination regimen. Patients were diagnosed as multiple myeloma according to the diagnostic criteria established by the International Myeloma Study Group (IMWG) [10].

Newly diagnosed or relaps/refractory multiple myeloma patients who received daratumumab as a single agent or combination therapy between June 2022 and June 2023 were included in study. Demographic features of the patients, paraprotein type, laboratory results, stage of the disease according to international staging system (ISS) [10], prior treatments (if any), treatment line and treatment protocol of daratumumab, response to treatment and survival of the patients were obtained from electronic data system. Response to treatment was determined according to IMWG treatment response criteria [10].

Treatments in practice and assessment of response: Daratumumab was infused intravenously at a dose of 16 mg/kg and given weekly infusions in first 8 weeks, then every two weeks, and then monthly infusions according to treatment protocol. First dose of daratumumab was given as splited dose in two consecutive days. Premedication including antihistaminic, dexamethasone and montelukast was administered before infusion of daratumumab. According to manufacturer suggestions, the first infusion was started at 50 ml/h, followed by dose escalation up to 200 ml/h, in the absence of infusion-related reactions (IRRs). Subsequent infusions were diluted in 500 ml and started from 50 ml/h in second infusion or 100 ml/h in subsequent infusions with an increase up to 200 mL/h Infusion-related side effects were graded based on the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 [11].

Treatment responses were assessed in accordance with the IMWG [10]. Serum free kappa and lambda levels, immunoglobulin levels, and serum/urine protein electrophoresis were evaluated every month for response assessment; serum and urine immunoelectrophoresis were evaluated bimonthly. Any elevation in M protein or clinical progression of myeloma-associated end-organ damage during this period was considered treatment resistance. In addition to laboratory and clinical assessment, bone marrow aspiration and biopsy was performed after 3 or 4 cycles of treatment. Furthermore, individuals with initial extramedullary myeloma, lytic bone lesions or plasmocytoma underwent response evaluation with magnetic resonance imaging or 18 Fluorodeoxyglucose Positron Emission Tomography (F-18 FDG PET). Responses to treatment were classified as complete response (CR), very good partial response (VGPR), partial response (PR), stable disease (SD) and progressive disease (PD) according to IMWG response criteria [10].

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Pamukkale University on June 13, 2022, with the reference number 168199, and informed consents were obtained from patients.

Statistical analysis

The data were analysed with SPSS 25.0 package program. Continuous variables were given as mean ± standard deviation and categorical variables as number and percentage. Mann-Whitney U test was used to compare independent group differences. The correlations between continuous variables were analysed by Pearson correlation analyses and the differences between categorical variables were analysed by Chi-square analysis. Logistic regression analysis was used to determine the factors affecting response to treatment.

Results

Data of 21 patients who met the inclusion criteria were analyzed. The median age of the patients was 65±9.7 (42-80) years, with an 11/10 female to male ratio. Among the patients, 66.7% were ISS stage III (advanced stage) and 47.5% had IgG kappa paraproteinaemia. Clinical characteristics of patients were shown in Table 1.

Extramedullary-paraosseous myeloma was present in 42.9% of the patients. Daratumumab is administered as first-line therapy in 23.8% of cases, as second-line therapy in 14.28% of cases, and following second-line treatment in 61.9% of cases. Median follow-up time was 5.15 months (0.36-23). The mean number of cycles was 4.05±5.06.

When we looked at treatment regimens; five patients received daratumumab-bortezomib-thalidomide-dexamethasone (D-VTD); seven patients received D-VTD+chemotherapy (cyclophosphamide-etoposide-cisplatin-doxorubicin; all or some of them); one patient received daratumumab-bortezomib-cyclophosphamide-dexamethasone (D-VCD); one patient received daratumumab-melphalan-prednisolone (D-VMP); five patients received daratumumab-lenalidomide-dexamethasone (D-RD); one patient received daratumumab monotherapy; and one patient received daratumomab-bortezomib-dexamethasone (D-VD).

Analysis of the response rates revealed that the overall response rate (ORR) was 66%; of these, 6 patients (28.6%) had a complete response (CR), 3 patients (14.3%) had a very good partial response (VGPR), 5 patients (23.8%) had a partial response (PR), and 7 patients (33.3%) had progressive disease (PD) (Table 1).

When the factors that may affect response to treatment were analyzed, it was observed that age, gender, stage, presence of extramedullary-paraosseous myeloma, treatment line and response to previous treatments had no statistically significant effect on daratumumab response (p>0.05) (Table 2). All patients who received more than 3 cycles of daratumumab-containing therapy achieved VGPR and better response. In patients who received ≤3 cycles of daratumumab containing therapy only 25% (n:4) of patients achieved VGPR and better response. Comparison of these two groups shows that giving more than 3 cycles of daratumumab-containing therapy significantly increases response rates (p=0.026) (Table 2).

Table 1. Patients' clinical characteristics and treatments

Variable	n (%) or Median
Median Age (years)	65±9.7 (42- 80)
Gender	
Female	11(52.4)
Male	10 (47.6)
ISS stage	
Stage 1	5 (23.8)
Stage 2	2 (9.5)
Stage 3	14 (66.7)
Paraprotein Type	
IgG lambda	6 (28.6)
IgG kappa	10 (47.6)
Lambda mild chain	1 (4.8)
Kappa mild chain	2 (9.5)
IgA lambda	2 (9.5)
Disease status	
New Diagnosis	5 (23.8)
Relapse-Refractory	16 (76.2)
Number of Previous Treatments	
0	5 (23.8)
1	3 (14.3)
2	5 (23.8)
≥3	8 (38.1)
Extramedullary disease	
Yes	9 (42.9)
No	12 (57.1)
Treatment regimes	
D-VTd	5 (23.8)
D-VTd+ Chemotherapy	7 (33.3)
D-VCd	1 (4.8)
D-VMP	1 (4.8)
D-Rd	5 (23.8)
D-Vd	1 (4.8)
Daratumumab monotherapy	1 (4.8)
Daratumumab-related reactions	
Yes	2 (9.5)
No	19 (90.5)
Response Status	
CR	6 (28.6)
VGPR	3 (14.3)
PR	5 (23.8)
PD	7 (33.3)
Mortality	8 (38.1)
Due to disease progression	4 (19.05)
Due to sepsis	4 (19.05)

ISS: International staging system, CR: Complete Response, PR: Partial Response, VGPR: Very Good Partial Response,
D-VTd: Daratumumab-Bortezomib- Thalidomide-Dexamethasone, D-VCd: Daratumumab-Bortezomide-Cyclophosphamide-Dexamethasone
D-VMP: Daratumumab-Bortezomib-Melphalan-Dexamethasone, D-Rd: Daratumumab-Lenalidomide-Dexamethasone
D-Vd: Daratumumab-Bortezomib-Dexamethasone, PD: Progressive Disease

Table 2. Treatment response rates according to clinical characteristics

Variable	Response Rate		*P* value
	≥VGPR n (%)	<VGPR n (%)	
Age			0.256
>65	7 (70)	3 (30)	
≤65	6 (54.5)	5 (45.59)	
Gender			0.056
Female	2 (20)	8 (80)	
Male	7 (63.6)	4 (36.4)	
Stage ISS			0.155
I	1 (20)	4 (80)	
II	0 (0)	2 (80)	
III	6 (42.9)	8 (57.1)	
Number of Cycles			**0.026**
>3	5 (100)	0 (0)	
≤3	4 (25)	12 (75)	
Extramedullary Myeloma			0.445
Yes	3 (33.3)	6(66.7)	
No	6 (50)	6(50)	
PI refractory			0.309
Yes	4 (33.3)	8 (66.7)	
No	5 (55.6)	4 (44.4)	
IMID refractory			0.604
Yes	5 (38.5)	8 (61.5)	
No	4 (50)	4 (50)	

ISS: International Staging System, VGPR: Very Good Partial Response, PI: Proteasome Inhibitor, IMID: Immunomodulatory Drug
$p<0.05$ was considered statistically significant

Drug-related infusion reaction was seen in only 2 patients and treatment was discontinued in one of these patients because of grade 4 reaction. In the other patient, a grade 2 infusion reaction developed, the infusion was interrupted, controlled with an additional dose of dexamethasone, and then resumed.

When the final status of the patients was evaluated, it was observed that 38.1% of patients were died, 38.1% continued treatment with VGPR or higher response, and 23.8% (n:5) continued treatment with PR. Among the 7 patients who received D-VTd+chemotherapy, 3 (42.9%) died due to progressive disease and one patient could not complete the treatment due to acute heart failure that developed during treatment.

Analysis of potential treatment-affecting factors revealed that age, gender, stage, extramedullary-paraosseous myeloma existence, treatment step, and prior therapies did not significantly impact response ($p>0.05$). When patients receive more than 3 cycles of daratumumab, the rate of obtaining very good partial response and better response (VGPR and CR) increased and this effect was found to be statistically significant ($p=0.026$) (Table 2). Neverthless none of these factors appeared to have an impact on the response to treatment, according to multivariate analysis.

Discussion

Multiple myeloma treatment remains difficult even with the new drugs that have been developed and made available recently, as well as the consolidation with autologous bone marrow transplantation—an essential component of treatment for eligible patients. Survival is particularly poor in patients with an aggressive course, high risk and resistant to proteasome inhibitors (PIs) or immunomodulatory agents (IMIDs). Our study showed that Daratumumab containing regimens improve the response rates especially after 3 cycles of therapy. VGPR and CR rates increased when the patients recieved more than 3 cycles of daratumumab containing therapy.

Numerous clinical studies demonstrate the effectiveness of daratumumab, a monoclonal antibody directed against CD38, in the treatment of myeloma through both monotherapy and various combination regimens [5-9]. In the 2016 study by Usmani et al. [5] demonstrating the effectiveness of daratumumab monotherapy in relapsed refractory multiple myeloma patients, the total response rate was 31% among patients who had previously undergone at least two lines of treatment, comprising IMID and/or PI. In this study, patients received daratumumab at a dose of 16mg/kg for the first 8 doses once a week, then 8 doses twice a month and then once a month until progression. As a single agent this success in patients who had previously received multiple lines of treatment, led to studies showing the efficacy of combinations of daratumumab with IMID and/or PI in R/R patients; POLLUX (D-Rd), CASTOR (D-Vd), and in newly diagnosed patients CASSIOPEIA (D-VTd), ALCYONE (D-VMP). As a result, it is now used to treat MM patients who are both transplant-ineligible and transplant-eligible [6-9].

In our study, daratumumab monotherapy and combination therapies were used in 5 newly diagnosed and 16 relapsed/refractory MM patients in our center, and these treatment protocols are similar to those whose efficacy has been shown by clinical studies in the literature; D-VTd, D-VCd, D-VMP, D-Rd, D-Vd and daratumumab monotherapy. Different from the literature, D-VTd+chemotherapy protocol was applied in 7 relapsed/refractory (R/R) patients. Although the protocols of our patients were different, we found that complete response rates (CR) increased significantly if the patient receives more than 3 cycles of daratumumab-containing cycles (p=0.026). According to the results of phase 3 studies in which daratumumab was used as monotherapy and with combination regimens, it is seen that the rates of undetectable minimal residual disease (MRD) and complete response rate increase with the duration of treatment [5-9]. With D-Rd, the response rates after more than three years of follow-up were CR 56.6% and ORR 92.9% in the POLLUX trial, which comprised 559 relapsed refractory MM patients [7]. MRD negative was found in 64% of patients at the 100-day evaluation following autologous stem cell transplantation in the CASSIOPEIA research, which administered the D-VTd regimen to 543 newly diagnosed MM patients [8].

In a retrospective study by Zhou et al. [12], the total survival was determined to be 8.4 months, and the total response rate was 70%. The study included 38 R/R MM patients who were given D-KRd-PACE, of which 30% were found to be nonresponsive. However, in our study, mortality rate was 42.9% in patients who received D-VTd+chemotherapy combination. Patients who underwent D-VTd-chemotherapy had a high mortality rate, which might be explained by the fact that some of them were frail patients who had run out of choices, that their disease was developing quickly, and that they previously had many therapies before receiving daratumumab treatment. Although promising results have been obtained in the treatment of MM with daratumumab, loss of response may be observed due to the development of resistance to daratumumab by different mechanisms. Studies on understanding the mechanisms of resistance development and solutions to be developed for prevention are ongoing [13,14].

The most important limitations of this study were the insufficient number of patients and the analysis of a heterogeneous patient group. Due to the small number of patients, separate statistical evaluation could not be performed in newly diagnosed and relapsed refractory patients. In addition, because our center has

been using regimens including daratumumab for the last several years, the patients' follow-up periods were short, making it unable to undertake a survival study. With multicenter studies that include more patients, offer long-term follow-up, and analyze real-world data, it appears feasible to experience varying clinical outcomes.

In conclusion, it is obvious that different daratumumab combinations play a significant role in the treatment of multiple myeloma, both as salvage therapy for patients who have previously received several lines of treatment and as the first line for newly diagnosed and high-risk patients. Consequently, an increase in response rates can have a substantial impact on the course of treatment as the number of cycles with Daratumomab administration increases.

Conflict of interest: No conflict of interest was declared by the authors.

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The study was previously presented as a paper at the 2nd Leukaemia Lymphoma Myeloma Congress and was awarded the “Best 7 Paper Award”.

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Authors’ contributions to the article

O.E. contributed to data collection, literature review and article writing, N.A.A. contributed to the creation of the study design, checking the accuracy of the data, statistical analysis of the data, literature review and article writing, V.E. and I.C.K. contributed to data collection, and N.G contributed to the creation of the study design and literature review.

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Empowering self-management: exploring self-care practices in heart failure patients

Öz bakım yönetimini güçlendirme: kalp yetersizliği hastalarında öz bakımı etkileyen faktörlerin incelenmesi

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Abstract

Purpose: Heart failure is a complex clinical syndrome in which ventricular filling and beating functions are impaired. HF also causes psychosocial and economic problems and is a significant public health problem affecting the quality of life. Effective self-care is a non-pharmacological method that contributes to treatment, improves the patient's quality of life, and reduces repeated hospitalizations and economic burdens. Our study aimed to evaluate self-care in heart failure patients and to determine the factors affecting self-care.

Materials and methods: A total of 100 patients with heart failure, including 36 women and 64 men, were included in the study. Data were collected by using the Descriptive Information Form and the European Heart Failure Self-Care Behavior Scale in one-on-one interviews with patients under appropriate physical conditions.

Results: Our findings indicate that a range of factors, including age, hypertension, educational status, the total number of comorbid diseases, and the use of certain medications such as SGLT-2 inhibitors, differently impact self-care behaviors. Self-care behaviors were found to be adequate in the study population.

Conclusion: We identified essential factors that affect self-care in heart failure patients. We have identified critical factors such as age, educational status, hypertension, comorbidity, and particularly the use of SGLT2 inhibitors, as key influencers of self-care practices. For this reason patient-centered healthcare models should be developed and considered by medical practitioners.

Keywords: Heart failure, self-care behavior, patient education, SGLT2 inhibitors.

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Öz

Amaç: Kalp yetersizliği, ventriküler dolum ve atım fonksiyonlarının bozulduğu kompleks bir klinik sendromdur. Kalp yetersizliği aynı zamanda psikososyal ve ekonomik sorunlara da yol açarak yaşam kalitesini etkileyen önemli bir halk sağlığı sorunudur. Etkili kişisel bakım, tedaviye katkı sağlayan, hastanın yaşam kalitesini artıran, tekrarlayan hastaneye yatışları ve ekonomik yükleri azaltan, farmakolojik olmayan bir yöntemdir. Çalışmamızda kalp yetersizliği hastalarında öz bakımın değerlendirilmesi ve öz bakımı etkileyen faktörlerin belirlenmesi amaçlanmaktadır.

Gereç ve yöntem: Çalışmaya 36'sı kadın, 64'ü erkek olmak üzere toplam 100 kalp yetersizliği olan hasta dahil edildi. Veriler, uygun fiziksel koşullar altında hastalarla yapılan birebir görüşmelerde Tanımlayıcı Bilgi Formu ve Avrupa Kalp Yetersizliği Öz Bakım Davranış Ölçeği kullanılarak toplanmıştır.

Bulgular: Bulgularımız, yaş, hipertansiyon, eğitim durumu, komorbid hastalıkların toplam sayısı ve SGLT-2 inhibitörleri gibi bazı ilaçların kullanımının öz bakım davranışlarını farklı şekillerde etkilediğini göstermektedir. Öz bakım davranışları, çalışma popülasyonunda yeterli düzeyde bulunmuştur.

Sonuç: KY hastalarında öz bakımı etkileyen temel faktörleri belirledik. Yaş, eğitim durumu, hipertansiyon, komorbidite ve özellikle SGLT2 inhibitörlerinin kullanımı gibi kritik faktörleri, öz bakım uygulamalarının önemli etkileyicileri olarak belirledik. Bu nedenle, hasta merkezli sağlık bakım modelleri geliştirilmeli ve tıp uzmanları tarafından dikkate alınmalıdır.

Anahtar kelimeler: Kalp yetersizliği, öz bakım davranışı, hasta eğitimi, SGLT2 inhibitörleri.

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Introduction

Heart failure (HF), characterized as a complex clinical syndrome, manifests through signs and symptoms arising from either structural or functional impairments in ventricular filling or the effective pumping of blood. This condition notably escalates with advancing age. For instance, in 2012, the prevalence of HF was reported to be approximately 4.3% among individuals aged 65-70. Moreover, projections indicate a steady increase in this prevalence, with expectations of it reaching around 8.5% by the year 2030 [1, 2].

Distinctively, the epidemiology of HF in Türkiye presents a contrast to that observed in Western countries, particularly in terms of the affected age groups. According to the findings of the HAPPY (Heart Failure Prevalence and Predictors in Türkiye) study, the prevalence of HF in the Turkish population was estimated at 6.9%, with a notably higher occurrence in the younger demographic compared to Western counterparts [3].

HF not only impedes the physical activities of those affected but also profoundly impacts their social and psychological well-being. This multifaceted influence leads to a notable decline in patient productivity and a corresponding deterioration in the quality of life for individuals with HF [4, 5]. Self-care (SC) emerges as a crucial factor in this context, significantly affecting the social, psychological, and economic circumstances of patients.

The effective management of HF encompasses an accurate assessment and treatment regime. This includes the optimization of medical therapy, proactive management of early signs and symptoms of decompensation, thorough identification and handling of comorbid conditions, clear determination of end-of-life demands, and consistent continuous monitoring. These components collectively contribute to facilitating SC behaviors in patients. There is a growing body of evidence suggesting that strategies positively impacting SC can significantly reduce the economic burden associated with HF [6].

The concept of SC, initially introduced by Dorothea Orem in 1959, encompasses the actions an individual undertakes to maintain life, health and well-being. This concept posits that SC is a skill honed over time through communication, cultural influences, education and interaction. SC ability or power, refers to the capacity of an individual to carry out self-related activities, essentially enabling the organization and fulfillment of ongoing personal care needs. For effective SC, an individual must possess certain qualities, including the ability to take care of oneself, efficiently use and control physical energy, make informed SC decisions, stay motivated, organize, and maintain consistent actions towards SC and take responsibility for one's health. Adequate SC power leads to sufficient and appropriate fulfillment of SC needs, fostering independence in health management. Conversely, a lack of SC, stemming from failure to engage in related activities, leads to inadequacies in maintaining and improving health, highlighting the importance of SC in overall well-being [7].

In accordance with the guidelines of the European Society of Cardiology (ESC), SC is recognized as a pivotal element in the successful management of HF, exerting a profound impact on patient symptoms, functional capacity, general health status, morbidity, and overall prognosis [8]. Patients diagnosed with HF are required to engage in a range of self-care activities throughout their lifetime. These activities encompass managing complex treatment protocols, adhering to a low-sodium diet, vigilant monitoring of early symptoms such as fluid retention, dyspnea, and fatigue, and executing appropriate interventions when necessary [9]. However, challenges persist as patients often struggle to recognize these symptoms, with the underlying reasons for this difficulty remaining unclear. Despite the acknowledged significance of SC in yielding positive health outcomes, a substantial number of HF patients exhibit inadequate self-care behaviors [10]. Our study aims to investigate the assessment of SC skills in HF patients using the European Heart Failure Self-Care Behavior (EHFScB) Scale, focusing on identifying the determinants that influence SC practices.

Materials and methods

This research was approved by the Pamukkale University Faculty of Medicine Non-Interventional Clinical Research Ethics

Committee and adhered to the principles of the Declaration of Helsinki. A cross-sectional study design was employed, involving 100 patients who attended the Cardiology outpatient clinic of Pamukkale University Medical Faculty Hospital.

Patient selection and administration of the EHFScB scale

The study's inclusion criteria were as follows: a diagnosis of HF; being aged 18 years or older; absence of communication barriers such as visual impairment, decreased hearing, inability to understand or speak Turkish, or psychiatric/ congenital disorders that could hinder participation; and willingness to participate after being informed about the research. Patients who later requested to withdraw were excluded from the study.

Data collection was conducted using a Descriptive Information Form and the European Heart Failure Self-care Behavior (EHFScB) scale. The Descriptive Information Form is an 8-item tool designed to capture the demographic characteristics of the patients. It includes age, gender, marital status, educational status, New York Heart Association (NYHA) functional classification, presence of concomitant diseases, medical treatment, and time since diagnosis.

EHFScB scale description and usage

The European Heart Failure Self-Care Behaviour Scale (EHFScB) was initially developed in 2003 as a 12-item instrument. Subsequently, its validity, reliability, and consistency were established in Turkish through adaptation by Baydemir et al. [11] Jaarsma et al. [12] later streamlined the scale into a more concise 9-item version. For scoring, a Likert-type scale ranging from 1 to 5 was utilized, where each item is rated from 1 (strongly disagree) to 5 (strongly agree) [13].

Given its simplicity and ease of administration, the 9-item version of the EHFScB was chosen for this study. This version comprises 9 items, divided into 2 sub-factors: compliance with treatment and seeking help [14].

The categorization of the scale into these two sub-factors in its Turkish version is based on a principal components analysis, a statistical method employed to ascertain the underlying sub-factor structure of the scale. This analysis elucidates the stronger associations between variables and respective sub-factors following rotation within the dataset, ensuring a balanced distribution of variables.

In the Turkish adaptation of the scale, the principal components analysis led to the distribution of items under these two sub-factors. This categorization was integral to the process of contextualizing the scale for Turkish patients, aiding in a more nuanced understanding and evaluation of SC behaviors in heart failure [14].

The two sub-factors delineated in the 9-Item EHFScB (EHFScB-9) are as follows:

Sub-factor 1: Monitoring/Counseling Behaviors: Encompasses items 2-4, and 6 of the scale.

Sub-factor 2: Adherence to Regimen such as Food and Liquid: Includes items 1, 5, 7, 8, and 9 of the scale.

Data collection and statistical analyses

Prior to the commencement of data collection, all participating patients were assured that their information would be used solely for the purposes of this study, ensuring confidentiality and adherence to ethical standards. Data were gathered through one-on-one interviews conducted under appropriate physical conditions. These interviews utilized the Descriptive Information Form and the European Heart Failure Self-Care Behavior Scale. The responses to the questionnaire and scale were recorded based on the patients' answers.

The statistical analysis of the collected data was performed using SPSS version 22.0 (Statistical Package for Windows, Chicago, Illinois, USA). To further understand the relationships between the variables and the scale scores, the Spearman Correlation Coefficient was utilized. This statistical tool helped in deciphering the associations and dependencies between the variables. Additionally, the Mann-Whitney U Test was applied to assess the effects of variables such as gender, marital status, hypertension, diabetes, COPD, and medication usage on the scale scores.

Results

The study's patient cohort comprised 100 individuals, characterized by notable demographic and clinical diversity. A significant proportion, 33%, were within the 61-70 age range. The cohort predominantly consisted of males (64%) and married individuals (81%), with 55% having completed primary school education.

From a clinical perspective, a majority of the patients, 53.6%, were diagnosed with primary hypertension, while 38.4% had diabetes mellitus (DM). Regarding medication usage, 34.1% of the patients were on beta-blocker treatment. The use of loop diuretics and SGLT2 inhibitors was noted in 18.2% of the patients, and the rate of angiotensin-converting enzyme inhibitors / angiotensin receptor blockers (ACE/ARB) usage stood at 17.3%. Additionally, 10.9% of patients were on Ca channel blockers, and a small fraction, 1.4%, used spironolactone.

The sociodemographic characteristics of the patient population are detailed in Table 1. Table 2 presents the distribution of responses to the European Heart Failure Self-Care Behavior Scale.

Table 1. Distribution of patients' sociodemographic characteristics (n=100)

Features	n=%	Features	n=%
Age		**Educational Status**	
42-50	2	Primary school	55
51-60	13	Middle school	17
61-70	33	High school	21
71-80	24	University	7
81-91	28		
		NYHA	
Gender		Class 1	5
Male	64	Class 2	21
Female	36	Class 3	28
		Class 4	46
Concomitant Disease			
Hypertension	81	**Marital Status**	
Diabetes mellitus	58	Married	81
COPD	10	Single	19
Congenital adrenal hyperplasia	2		
		Time in Diagnosis	
Medical Treatment		1-10	45
ACE inh/ARB	38	11-20	40
Diuretic	40	21-31	15
Beta-blocker	75		
Spironolactone	3		
SGLT2 inhibitor	40		
Calcium channel blocker	24		

COPD: Chronic obstructive pulmonary disease, ACE inh/ARB: Anjiontensin converting enzyme inhibitor/Anjiotensin receptor blocker
SGLT2: Sodium–glucose cotransporter 2 inhibitor

Table 2. Distribution of answers given to the European Heart Failure Self-Care Behavior Scale (EHFScB) questions (n=100)

Questions(1-9)	Strongly disagree		Disagree		Undecided		Agree		Agree strongly	
	n	%	n	%	n	%	n	%	n	%
I weigh every day (SF-2)	50	50	19	19	17	17	7	7	7	7
I let my doctor or nurse know when my shortness of breath increases (SF-1)	42	42	15	15	15	15	14	14	14	14
I inform my doctor or nurse when I have a lot of edema/swelling in my feet and legs (SF-1)	27	27	8	8	20	20	14	4	31	31
I let my doctor or nurse know when I gain weight (SF-1)	76	76	13	13	6	6	5	5	0	0
I limit the amount of fluids I take (SF-2)	58	58	13	13	19	19	8	8	2	2
I let my doctor or nurse know when I'm tired (SF-1)	60	60	15	15	15	15	6	6	4	4
I eat a diet low in salt (SF-2)	12	12	11	11	27	27	14	4	36	36
I take my medication as prescribed (SF-2)	0	0	0	0	2	2	12	2	86	86
I exercise regularly (SF-2)	30	30	22	22	28	28	10	0	10	10

SF: Sub-factor

The comparison of SC behavior scores across different demographic and clinical groups, using the Mann-Whitney U test, is displayed in Table 3. Table 4 showcases the correlation analysis of SC behavior scores with demographic and clinical characteristics, employing Spearman correlation coefficients to examine the relationships between SC behavior scores and various factors, including age, total number of diseases, and education level.

To enhance the interpretability of the EHFScBS-9, a reversed and standardized scoring system ranging from 0 to 100 was developed, where a higher score indicates better SC [15]. Additionally, based on prior research, a threshold of ≥19.8 (≥70) has been established as adequate, while a score of <19.8 (<70) is considered indicative of inadequate SC [16]. In our study total patient population, 22% scored below 19.8 points, while 78% scored above this threshold. This suggests that the majority of patients, with scores over 19.8, demonstrate adequate SC, whereas those scoring under 19.8 exhibit inadequate SC.

When the biochemical parameters of the groups with adequate (n:78) and inadequate self-care (n:22) were compared, no statistically significant difference was observed between the groups in terms of hemoglobin levels, creatinine values, serum electrolytes, and other primary biochemical parameters. Additionally, no significant differences were detected in the economic incomes between the groups. The biochemical parameters are shown in Table 5.

Table 3. Comparison of self-care behavior scores by demographic and clinical characteristics

		Total point		Sub-factor 1		Sub-factor 2	
		Mean±S.D.	**Med (IQR)**	**Mean±S.D.**	**Med (IQR)**	**Mean±S.D.**	**Med (IQR)**
Gender	**Male**	23.08±5.61	23 (19.25-25)	8.63±3.87	8 (6-11)	14.45±3.37	15 (12-16)
	Female	24.08±4.87	23.5 (20-27)	9±3.79	8 (6-12)	15.08±2.32	15 (13-16.75)
	p	**0.31 (z=-1.015)**		**0.621 (z=-0.495)**		**0.272 (z=-1.098)**	
Marital Status	**Married**	23.19±5.28	23 (20-26)	8.58±3.9	8 (6-12)	14.6±3.06	15 (12.5-16)
	Single	24.53±5.68	25 (21-27)	9.53±3.47	8 (7-12)	15±3.02	15 (13-16)
	p	**0.224 (z=-1.216)**		**0.168 (z=-1.379)**		**0.61 (z=-0.51)**	
Hypertension	**No**	20.74±4.56	21 (17-25)	7.16±2.54	7 (5-8)	13.58±3.45	13 (11-15)
	Yes	24.07±5.35	23 (20-26)	9.14±3.99	8 (6-12.5)	14.94±2.9	15 (13-16)
	p	**0.02* (z=-2.33)**		**0.069 (z=-1.821)**		**0.05* (z=-1.937)**	
Diabetes Mellitus	**No**	23.19±5.38	22.5 (20-26)	8.43±3.72	8 (6-11.25)	14.76±2.93	15 (13-16.25)
	Yes	23.62±5.37	23 (20-26)	9±3.92	8 (6-12.25)	14.62±3.14	15 (12.75-16)
	p	**0.716 (z=-0.364)**		**0.482 (z=-0.703)**		**0.841 (z=-0.201)**	
COPD	**No**	23.19±5.22	23 (20-26)	8.53±3.76	8 (6-12)	14.66±3.04	15 (13-16)
	Yes	25.7±6.29	25 (20.75-30.75)	10.8±3.99	10.5 (7.5-14.25)	14.9±3.14	14.5 (13.25-17.5)
	p	**0.172 (z=-1.365)**		**0.074 (z=-1.786)**		**0.867 (z=-0.168)**	
Congenital Adrenal Hyperplasia	**No**	23.44±5.4	23 (20-26)	8.8±3.86	8 (6-12)	14.64±3.05	15 (13-16)
	Yes	23.5±3.54	23.5 (21-0)	7±1.41	7 (6-0)	16.5±2.12	16.5 (15-0)
	p	-		-		-	
ACE inh/ARB	**No**	23.48±5.67	23 (19.75-27)	9.18±3.93	8 (6-12)	14.31±3.12	15 (12-16)
	Yes	23.37±4.87	23 (20-25.25)	8.08±3.6	8 (4.75-10.25)	15.29±2.84	15 (14-16.25)
	p	**0.77 (z=-0.292)**		**0.214 (z=-1.243)**		**0.143 (z=-1.465)**	
Diuretic	**No**	24±5.39	23 (21-26)	9.13±4.03	8 (6-13)	14.87±3.09	15 (13-16)
	Yes	22.6±5.25	22.5 (18.25-26)	8.2±3.48	8 (5-11)	14.4±2.97	14 (12-15)
	p	**0.244 (z=-1.164)**		**0.265 (z=-1.115)**		**0.281 (z=-1.079)**	

Table 3. Comparison of self-care behavior scores by demographic and clinical characteristics (contunied)

		Total point		Sub-factor 1		Sub-factor 2	
		Mean±S.D.	**Med (IQR)**	**Mean±S.D.**	**Med (IQR)**	**Mean±S.D.**	**Med (IQR)**
Beta blocker	**No**	23.4±5.22	25 (19.5-27)	9.16±3.72	8 (6-13)	14.24±3.36	14 (11.5-16.5)
	Yes	23.45±5.43	23 (20-26)	8.63±3.88	8 (6-12)	14.83±2.93	15 (13-16)
	p	**0.649 (z=-0.455)**		**0.452 (z=-0.753)**		**0.354 (z=-0.928)**	
Spironolactone	**No**	23.55±5.32	23 (20-26)	8.85±3.84	8 (6-12)	14.7±3.01	15 (13-16)
	Yes	20±6.56	21 (13-0)	6±2	6 (4-0)	14±4.58	15 (9-0)
	p	-		-		-	
SGLT 2 inhibitor	**No**	22.77±5.39	22 (19-26)	8.2±3.81	7.5 (5-11)	14.57±2.86	14.5 (13-16)
	Yes	24.45±5.2	23.5 (22-26)	9.6±3.75	8 (7-13)	14.85±3.32	15 (13-16)
	p	**0.071 (z=-1.802)**		**0.041* (z=-2.042)**		**0.67 (z=-0.426)**	
Calcium channel blocker	**No**	23.39±5.71	23 (20-26)	8.68±4.06	8 (5-12)	14.71±3.28	15 (12.25-16)
	Yes	23.58±4.13	23.5 (20.25-26.75)	9±3.04	8 (7-11.75)	14.58±2.15	15 (13.25-15.75)
	p	**0.552 (z=-0.595)**		**0.453 (z=-0.751)**		**0.99 (z=-0.012)**	

*$p<0.05$ Statistically significant difference, z: Mann Whitney U test, IQR: Interquartil range, COPD: Chronic obstructive pulmonary disease
ACE inh/ARB: Anjiontensin converting enzyme inhibitor/Anjiotensin receptor blocker, SGLT2: Sodium–glucose cotransporter 2 inhibitor

Table 4. Correlation analysis of self-care behavior scores with demographic and clinical characteristics

		Total point	Sub-factor1	Sub-factor2
Age	r	0.330*	0.368*	0.103
	p	0.001	0.000	0.307
Heart failure duration	r	0.066	0.130	-0.026
	p	0.514	0.197	0.798
NYHA Classification	r	-0.106	-0.038	-0.103
	p	0.293	0.709	0.306
Education Status	r	0.019	-0.210*	0.254*
	p	0.851	0.036	0.011
Number of chronic diseases	r	0.251*	0.264*	0.114
	p	0.012	0.008	0.259
Total number of treatments	r	0.012	-0.044	0.099
	p	0.905	0.666	0.329

*$p<0.05$ Statistically significant correlation; r: Spearman correlation coefficent, NYHA: New York Heart Association

Table 5. Biochemical parameteres between adequate and inadequate self care groups

	Inadequate Self Care (n:22)		Adequate Self Care (n:78)		*p* value
	Mean	S.D.	Mean	S.D.	
Creatinine (mg/dl)	1.25	0.52	1.27	0.39	0.42
Hemoglobin (mmol/L)	13.2	1.73	13	1.61	0.11
Na^+ (mmol/L)	138.40	3.32	137.6	3.82	0.57
K^+ (mmol/L)	4.30	0.47	4.23	0.46	0.16
AST (IU/L)	23.1	11.6	24	8	0.23
ALT (IU/L)	22.4	13.5	23.1	14.5	0.74
TSH (mU/L)	2.92	2.68	2.97	4.8	0.51
HbA1c (%)	6.8	1.2	6.71	1.5	0.61
Fasting glucose (mmol/L)	97.3	26.2	105.9	36	0.12

AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, TSH: Thyroid Stimulating Hormone, HbA1c: Hemoglobin A1c

Discussion

In the present study, we sought to identify the factors influencing SC behaviors among HF patients. Our findings indicate that a range of factors, including age, hypertension, educational status, the total number of comorbid diseases, and the use of certain medications such as SGLT-2 inhibitors, differently impact SC behaviors. Moreover, we observed that specific sub-factors within SC behaviors vary according to each unique patient condition. Significantly, this research is the first to demonstrate a statistically significant association between the use of SGLT2 inhibitors and improved SC behaviors in HF patients.

HF, declared an epidemic in 1997, remains a major health concern affecting a substantial portion of the population, with its incidence and prevalence steadily rising [17]. Managing this disease, which profoundly impacts the quality of life, involves a lengthy, challenging, and expensive process. Hence, placing patients at

the forefront of treatment is crucial. For effective management of HF, practitioners must prioritize evaluating SC behaviors in patients. Effective SC management serves as a non-pharmacological approach that not only enhances treatment but also improves patient quality of life. Additionally, it plays a significant role in reducing recurrent hospitalizations and alleviating the economic burden associated with HF.

In this study focusing on SC behaviors in HF patients, we identified a positive but weak correlation between age and overall SC scores. Notably, an increase in age correlated with improved general SC behaviors. Specifically, a similar positive and weak relationship was observed between age and the scores for sub-factor 1 (Monitoring/Consultation Behaviors), suggesting that older patients are more inclined to engage in monitoring and consultation activities related to heart failure management. However, age did not significantly correlate with sub-factor 2 (Adherence to Diet and Fluid Intake). Contrastingly, Prochota et al. [18] reported an inverse relationship between SC and age in their study. Our results imply that older heart failure patients may demonstrate more effective monitoring and consultation SC behaviors, potentially due to their prolonged experience in managing their condition or a heightened awareness of its severity. The findings specifically highlight that increased age, along with a greater number of illnesses, could enhance engagement in certain SC behaviors, particularly those involving monitoring and consultation.

In our study, the level of education emerged as the only statistically significant factor affecting sub-factor 2 in the univariate analysis. Sub-factor 2 encompasses behaviors related to "adherence to regimen, such as diet and fluid intake" in heart failure SC. We observed that individuals with higher education levels showed greater adherence to these regimens. This pattern suggests that more educated individuals might possess a better capacity to comprehend and implement health-related information, highlighting the pivotal role of education in fostering effective SC behaviors. Kessing et al. [5] similarly reported that patients with lower education levels demonstrated inadequate SC. Another study assessing the link between patient characteristics and SC behaviors in heart failure patients found that education, along with symptom severity, significantly influenced SC [19]. The correlation between higher education and enhanced health literacy implies that more educated individuals can more readily integrate their daily life patterns with health-related behaviors, compared to those with lower education levels. Consequently, individuals with higher education, who are likely to be open to development and change, tend to be more health-conscious and likely exhibit better SC practices.

In our analysis, we also observed a statistically significant yet weak negative correlation between education level and sub-factor 1 scores. Sub-factor 1 represents the 'monitoring and consultation behaviors' associated with HF SC. This finding suggests that individuals with higher education levels may engage less in monitoring and consultation behaviors related to their heart failure management. A possible explanation for this trend could be that more educated individuals, having easier access to health services, might depend more on professional healthcare support rather than solely on SC practices especially on monitoring and consultation behaviors.

In this study, we found a statistically significant, positive, but weak correlation between the scores of sub-factor 1 and the total number of diseases among participants. This suggests that an increase in the number of diseases may boost the likelihood of engaging in such SC behaviors. However, contrasting findings were reported by Buck et al. [20] who observed that an excess of comorbidities led to inadequate SC in patients with heart failure, impacting hospitalizations and quality of life. Similarly, another study reported inadequate SC among patients with both diabetes and heart failure [21]. In our research, while the total number of diseases did not affect sub-factor 2 SC behaviors, it had a positive impact on sub-factor 1. This could be attributed to heightened SC awareness driven by awareness of multiple diseases. Given that sub-factor 1 predominantly involves monitoring and consultation behaviors, the ease of access to hospital services for patients with chronic diseases in our region might have influenced this outcome. These findings imply that the presence of multiple health conditions can increase patients'

attentiveness and propensity to seek medical advice, thus influencing their engagement in specific SC practices for HF.

We observed that the presence of hypertension significantly influenced both the overall SC scores and the scores for sub-factor 2 among heart failure patients. Remarkably, patients diagnosed with hypertension showed higher scores in these areas. This finding suggests that hypertension as a comorbid condition in HF may affect patients' adherence to specific SC regimens particularly those concerning diet and fluid intake which are encompassed in sub-factor 2. Previous studies showed that patient adherence to SC behavior has an impact on lowering blood pressure [22, 23]. This correlation highlights the critical role of managing hypertension in the comprehensive SC framework for heart failure patients.

In our study, a statistically significant difference was noted in the scores of sub-factor 1 between HF patients using SGLT2 inhibitors and those not using them. Specifically, patients treated with SGLT2 inhibitors exhibited higher scores in sub-factor 1, which is associated with monitoring and consultation behaviors. SGLT inhibitors, increasingly recognized for their role in HF treatment, represent a relatively recent addition to the therapeutic arsenal. The initiation of SGLT inhibitor therapy typically involves providing patients with comprehensive information and detailed guidance, which might contribute to their heightened engagement in SC practices, particularly those relating to sub-factor 1 as observed in our study. This link highlights the influential role of medication management in shaping SC behaviors, especially with the introduction of novel treatments in heart failure management. Notably, our research is one of the first to document a significant association between the use of SGLT2 inhibitors and specific SC behaviors in HF patients. This finding adds a valuable dimension to the growing body of knowledge on how new treatment options such as SGLT2 inhibitors, can impact SC practices among these patients.

We observed that SC behaviors among HF patients were mostly adequate. In other studies, there are conflicting results. There have been some studies involving individuals with HF demonstrating low SC agency [24]. In another study, the rate of inadequate SC was 52%, while in our study, this rate was determined to be 22% [25]. These findings suggest that while there are general trends in SC behaviors among HF patients, the extent and nature of these behaviors can significantly vary across different populations and studies. This underscores the importance of considering local and cultural contexts when interpreting SC behaviors in heart failure patients.

There are some limitations of our study; designed as a cross-sectional questionnaire-based investigation, depended heavily on the patients' full cooperation. The quality of responses was contingent upon the patients' education and perception levels, factors that could have influenced the accuracy of the data collected. Additionally, the study population was limited to fully cooperative patients, which may not represent the wider HF patient community. Moreover, the research was confined to the Aegean region in Türkiye, potentially limiting the generalizability of our findings to other geographical areas.

In conclusion, our study sheds light on the multifaceted nature of SC behaviors in HF patients. We have identified critical factors such as age, educational status, hypertension, comorbidity, and particularly the use of SGLT2 inhibitors, as key influencers of SC practices. These findings underscore the importance of considering individual patient profiles when developing management strategies for HF. The positive association of SGLT2 inhibitors with SC behaviors is a novel insight, suggesting the need for further exploration in this area. Ultimately, our study calls for a more personalized approach in educating and managing heart failure patients, taking into account their unique circumstances and the varying healthcare resources available in different regions.

Conflict of interest: No conflict of interest was declared by the authors.

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Author contributions

I.B. was responsible for the medical examination and study planning. Both I.B. and I.T. conducted data collection, analysis, and calculations. I.T. prepared the initial draft, incorporating inputs from all authors. Furthermore, I.T. and I.B. engaged in discussing the results, and jointly reviewed and provided feedback on the manuscript.

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Investigation of laboratory parameters as mortality marker in patients with blunt multi-trauma

Künt çoklu travmalı hastalarda mortalite belirleyicisi olarak laboratuvar parametrelerinin araştırılması

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Abstract

Purpose: In trauma patients, vital signs may not be beneficial in patient management. Therefore, evaluating vital signs, some laboratory parameters, and imaging methods is essential in managing trauma patients. Based on the hypothesis that lactate and base excess (BE) is an early marker of morbidity and mortality in the evaluation in blunt multi-trauma patients, we aimed to investigate the value of lactate albumin ratio and lactate dehydrogenase (LDH) albumin ratio in addition to lactate and BE in predicting mortality in blunt multi-trauma patients.

Materials and methods: This was a single-centre, retrospective study. Prior to data collection, approval was obtained from the local ethics committee. Patients admitted with multi-trauma to the emergency department of a tertiary education and research hospital in Türkiye between January 1, 2018, and December 31, 2021, who did not meet the exclusion criteria, were included in the study. This study examined the predictive value of Lactate, BE, Lactate-to-albumin ratio, and LDH-to-albumin ratio in predicting mortality in blunt multi-trauma patients.

Results: The lactate cut-off value for mortality was 4.2, exhibiting 73.3% sensitivity and 89.2% specificity. The BE cut-off value for mortality was -3, exhibiting 80.0% sensitivity and 76.9% specificity. The lactate-albumin ratio cut-off value for mortality was 0.11, exhibiting 73.3% sensitivity and 90.8% specificity. The LDH-albumin ratio cut-off value for mortality was 7.2, exhibiting 86.7% sensitivity and 58.5% specificity.

Conclusion: Lactate albumin ratio was more specific in predicting mortality than lactate and BE, while LDH albumin ratio was more sensitive in predicting mortality than lactate and BE.

Keywords: Lactate albumin ratio, lactate dehydrogenase albumin ratio, multi-trauma.

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Öz

Amaç: Travma hastalarında vital bulgular hasta yönetiminde yeterli olmayabilir. Bu nedenle travma hastalarının yönetiminde yaşamsal bulguların, bazı laboratuvar parametrelerinin ve görüntüleme yöntemlerinin değerlendirilmesi esastır. Künt multi-travma hastalarının değerlendirilmesinde laktat ve baz fazlalığının (BE) morbidite ve mortalitenin erken bir belirteci olduğu hipotezinden yola çıkarak, künt multi-travma hastalarında mortaliteyi öngörmede laktat ve BE'ye ek olarak laktat albümin oranı ve laktat dehidrogenaz (LDH) albümin oranının değerini araştırmayı amaçladık.

Gereç ve yöntem: Bu tek merkezli, retrospektif bir çalışmadır. Veri toplanmadan önce yerel etik kuruldan onay alınmıştır. Çalışmaya, 1 Ocak 2018 ile 31 Aralık 2021 tarihleri arasında Türkiye'deki üçüncü basamak bir eğitim ve araştırma hastanesinin acil servisine çoklu travma ile başvuran ve dışlama kriterlerini karşılamayan hastalar dahil edildi. Bu çalışmada, künt çoklu travma hastalarında, mortaliteyi öngörmede Laktat, BE, Laktat / albümin oranı ve LDH / albümin oranının tanısal değerliliği hesaplanmıştır.

Bulgular: Mortalite için laktat kesme değeri 4,2 olup %73,3 duyarlılık ve %89,2 özgüllük sergilemektedir. Mortalite için BE kesme değeri -3 olup %80,0 duyarlılık ve %76,9 özgüllük sergilemektedir. Mortalite için laktat albümin oranı kesme değeri 0,11 olup %73,3 duyarlılık ve %90,8 özgüllük sergilemektedir. Mortalite için LDH albümin oranı kesme değeri 7,2 olup %86,7 duyarlılık ve %58,5 özgüllük sergilemektedir.

Sonuç: Laktat albümin oranı mortaliteyi öngörmede laktat ve BE'ye göre daha spesifik iken, LDH albümin oranı mortaliteyi öngörmede laktat ve BE'ye göre daha duyarlıydı.

Anahtar kelimeler: Laktat albumin oranı, laktat dehidrogenaz albumin oranı, çoklu-travma.

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Introduction

Trauma patients represent a significant proportion of emergency department (ED) presentations [1]. Trauma-related deaths are the leading cause of death in young people [2]. Emergency management of trauma patients is based on a general evaluation of anamnesis, physical examination, vital signs, laboratory results, and imaging findings. This patient management is based on advanced trauma life support (ATLS) guidelines [3]. In trauma patients, vital signs may not be beneficial in patient management. Therefore, evaluating vital signs, some laboratory parameters, and imaging methods is essential in managing trauma patients [4]. In this direction, some laboratory parameters may arouse the clinician's suspicion of possible organ damage and play a key role in using appropriate imaging tests in this direction [5]. Laboratory tests performed during follow-up can help prevent morbidity and mortality that can occur after trauma.

Hemorrhagic shock is an important cause of morbidity and mortality in trauma patients. Intravascular volume loss and vasoconstriction due to hemorrhage cause tissue hypoxia and lactic acidosis. This triggers the development of morbidity and mortality. Consequently, it is essential to limit these findings [6, 7]. Accordingly, early detection of hemorrhagic shock should be possible. Laboratory parameters that can be used in the early detection of hemorrhagic shock were investigated. Serum lactate and base excess (BE) are more sensitive markers of morbidity and mortality compared to other measured laboratory parameters in multi-trauma [8-10].

Based on the hypothesis that lactate and base deficit is an early marker of morbidity and mortality in the evaluation of hemorrhagic shock in blunt multi-trauma patients, we aimed to investigate the value of lactate albumin ratio and lactate dehydrogenase albumin ratio in addition to lactate and BE in predicting mortality in blunt multi-trauma patients.

Materials and methods

Study design and population

This was a single-center, retrospective study. Prior to data collection, approval was obtained from the local ethics committee and the principles outlined in the Declaration of Helsinki have been followed.

Patients admitted with multi-trauma to the emergency department of a tertiary education and research hospital in Türkiye between January 1, 2018, and December 31, 2021, who did not meet the exclusion criteria, were included in the study. Patients under 18 years of age, pregnant women, patients with penetrating trauma, patients transferred to another institution, patients not considered as multi-trauma, patients who refused diagnosis and treatment, patients with incomplete laboratory parameters, patients with a history of disease affecting laboratory parameters, and patients admitted to the emergency department with arrest were excluded. The study population (N:450) was constructed considering the inclusion and exclusion criteria. The patient flow chart is shown in Figure 1.

Study protocol

The study population was formed after applying the inclusion and exclusion criteria. Demographic data, trauma mechanisms, laboratory parameters, and clinical outcomes of the patients included in the study were planned to be analyzed. All these patient data were obtained from the hospital's digital archive.

Laboratory parameters at the time of ED admission included hemoglobin (HGB) (mg/dl), Platelets (10^3/uL), Alanine transaminase (ALT) (U/L), Aspartate transaminase (AST) (U/L), Lactate dehydrogenase (LDH) (U/L), Albumin (g/L), Creatine (mg/dL), BE (mmol/L), Lactate (mmol/L). In addition, the lactate albumin ratio and LDH albumin ratio were calculated with laboratory parameters.

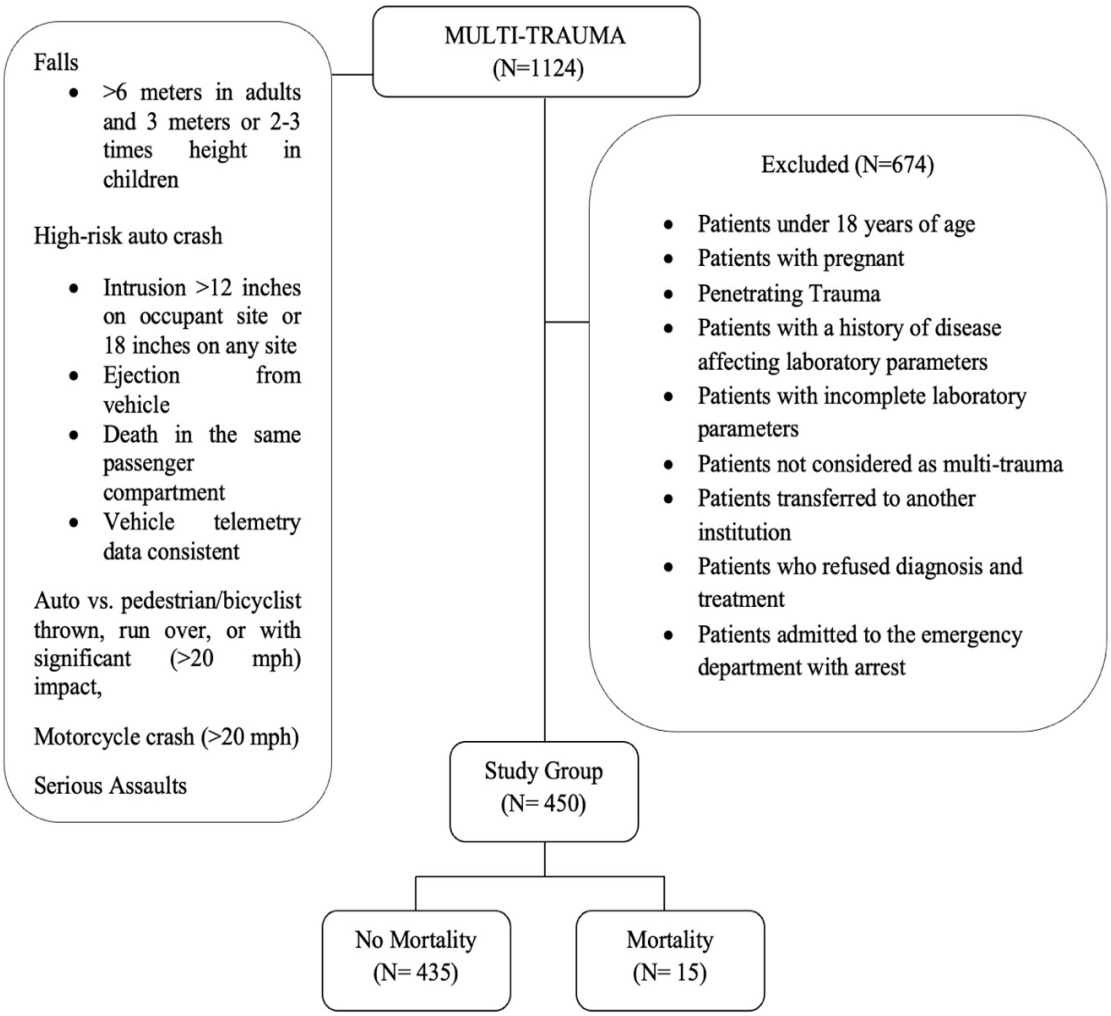

Figure 1. Patient flow chart

Shock staging of the patients included in the study was performed in accordance with ATLS guidelines. Admission Glasgow Coma Score (GCS), hospitalization status and clinical outcome (mortality and non-mortality) were recorded.

Endpoints

The primary endpoint of this study was the predictive value of Lactate, BE, Lactate-to-albumin ratio and LDH-to-albumin ratio in predicting mortality in blunt multi-trauma patients. The secondary endpoint was to evaluate the association of laboratory parameters between the mortality and non-mortality groups.

Statistical analysis

All statistical analyses were performed on Jamovi v. 1.6 software (Jamovi Project Computer Software, version 1.6. Sydney, Australia). Type 1 errors were regarded as 5% for all comparisons. The Shapiro-Wilk test was applied to evaluate normality of data distribution. Non-normally distributed data for continuous variables were expressed as median and interquartile range (IQR) and normally distributed data as mean and minimum-maximum values. Categorical data were expressed as frequency (n) and percentage (%) values. Comparisons of continuous variables were performed using the t-test for normally distributed groups and the Mann-Whitney U test for non-normally distributed groups. A receiver operating curve (ROC) was produced to determine the cut-off levels of the Lactate, BE, Lactate albumin ratio, and LDH albumin ratio value for mortality. Youden's index (maximum value) in ROC analysis was used to select the cut-off value. Finally, sensitivity, specificity, likelihood ratios (+LR and -LR), and positive and negative predictive values were calculated for the Lactate, BE, Lactate albumin ratio, and LDH albumin ratio value.

Results

The study population comprised 450 patients, meeting the inclusion and exclusion criteria. Three hundred thirty-four (74.2) patients were men and 116 (25.8%) women. The patients' median age was 39 years (IQR 28.3-53). The mechanisms of trauma were 279 (62.0%) vehicle traffic accidents, 139 (30.9%) falls and rolls, and 32 (7.1%) accidents caused by objects falling on. While shock was not observed in 359 (79.8%) patients, stage 1 shock was observed in 39 (8.7%), stage 2 shock in 30 (6.7%), stage 3 shock in 14 (3.1%), and stage 4 shock in 8 (1.7%) patients. The median value of the patients' GCS was 15, with a range of 15-15. Of the patients, 155 (3.4%) were hospitalized and 15 (3.3%) died. Demographic data, mechanism of trauma, hospitalization and mortality rates are summarized in Table 1.

Table 1. The patients' demographic data and baseline characteristics

Characteristics, n=450	**Value**
Gender	
Male, n (%)	334 (74.2%)
Female, n (%)	116 (25.8%)
Age (years), median (IQR)	39.0 (IQR 28.3-53.0)
Trauma Mechanisms	
Vehicle Traffic Accident	279 (62.0%)
Fall and Roll	139 (30.9%)
Accident by Object Falling on	32 (7.1%)
Laboratory Indices	
HGB (mg/dl), median (IQR)	13.7 (IQR 12.2-15.2)
Platelet (10^3/uL), median (IQR)	248 (IQR 210-293)
ALT (U/L), median (IQR)	27.0 (IQR 19.0-49.5)
AST (U/L), median (IQR)	33.0 (IQR 25.8-63.0)
LDH (U/L), median (IQR)	300 (IQR 241-398)
Albumin (g/L), median (IQR)	42.0 (IQR 38.8-45.0)
Creatine (mg/dL), median (IQR)	0.90 (IQR 0.76-1.02)
Base Excess (mmol/L), median (IQR)	-1.65 (IQR (-5.5)-0.7)
Lactate (mmol/L), median (IQR)	2.4 (IQR 1.7-3.9)
Lactate/Albumin Ratio (%), median (IQR)	0.06 (IQR 0.03-0.1)
LDH/Albumin Ratio (%), median (IQR)	7.21 (IQR 5.86-9.86)
Shock Stage	
None, n (%)	359 (79.8%)
Stage 1, n (%)	39 (8.7%)
Stage 2, n (%)	30 (6.7%)
Stage 3, n (%)	14 (3.1%)
Stage 4, n (%)	8 (1.7%)
GCS (score), median (IQR)	15 (IQR 15-15)
Hospitalization, n (%)	155 (34.4%)
Mortality, n (%)	15 (3.3%)

IQR: Interquartile Range (25p, 75p), HGB: Hemoglobin, ALT: Alanine Transaminase, AST: Aspartate Transaminase
LDH: Lactate dehydrogenase, GCS: Glasgow Coma Score

Laboratory parameters were analyzed: The median value of the HGB was 13.7 mg/dl (IQR 12.2-15.2); The median value of the platelet was 248 10^3/uL (IQR 210-293); the median value of the ALT was 27.0 U/L (IQR 19.0-49.5); the median value of the AST was 33.0 U/L (IQR 25.8-63.0); the median value of the LDH was 300 U/L (IQR 241-398); the median value of the albumin was 42.0 g/L (IQR 38.8-45.0); the median value of the creatine was 0.90 mg/dL

(IQR 0.76-1.02); the median value of the BE was -1.65 mmol/L (IQR (-5.5)-0.7); the median value of the lactate was 2.4 mmol/L (IQR 1.7-3.9); the median value of the lactate albumin ratio was 0.06 (IQR 0.03-0.1); the median value of the LDH albumin ratio was 7.21 (IQR 5.86-9.86). The median AST, LDH, albumin, creatine, BE, lactate, lactate albumin ratio, and LDH albumin ratio values measured in the included a statistically significant difference at mortality and non-mortality. (p=0.037 for AST, p=0.040 for LDH, p=0.007 for albumin, p=0.005 for creatine, p=0.001 for BE, p=0.001 for lactate, p=0.001 for lactate albumin ratio, and p=0.003 for LDH albumin ratio). An evaluation of the measurements and a statistical summary are shown in Table 1 and Table 2.

The lactate, BE, lactate albumin ratio, and LDH albumin ratio cut-off values were calculated to predict mortality. The Area Under the Curve (AUC) value for lactate was 0.819±0.03, and the cut-off value for mortality was 4.2, exhibiting 73.3% sensitivity and 89.2% specificity. The AUC value for BE was 0.843±0.04, and the cut-off value for mortality was -3, exhibiting 80.0% sensitivity and 76.9% specificity. The AUC value for lactate albumin ratio was 0.829±0.04, and the cut-off value for mortality was 0.11, exhibiting 73.3% sensitivity and 90.8% specificity. The AUC value for LDH albumin ratio was 0.747±0.04, and the cut-off value for mortality was 7.2, exhibiting 86.7% sensitivity and 58.5% specificity. The ROC analysis is shown in Table 3 and Figure 2.

Table 2. Patients' laboratory indices statistics

Laboratory Indices	No Mortality (n=435)	Mortality (n=15)	All Patients (n=450)	*p* Value
HGB (mg/dl)	13.9 (IQR 12.7-15.2)	10.9 (IQR 10.5-15.1)	13.7 (IQR 12.2-15.2)	0.068
Platelet (10^3/uL)	248 (IQR 211-283)	247 (IQR 167-306)	248 (IQR 210-293)	0.763
ALT (U/L)	27 (IQR 18.8-45.5)	35 (IQR 19.5-81.5)	27.0 (IQR 19.0-49.5)	0.468
AST (U/L)	31 (IQR 24-60)	38 (IQR 32-140)	33.0 (IQR 25.8-63.0)	0.037
LDH (U/L)	295 (IQR 237-360)	361 (IQR 282-471)	300 (IQR 241-398)	0.040
Albumin (g/L)	43 (IQR 39-45)	38 (32.5-41)	42.0 (IQR 38.8-45.0)	0.007
Creatine (mg/dL)	0.86 (IQR 0.75-0.98)	1.13 (IQR 0.99-1.23)	0.90 (IQR 0.76-1.02)	0.005
Base Excess (mmol/L)	-0.6 (IQR (-2.9)-0.9)	-6.1 (IQR (-9.75)-(-3.15))	-1.65 (IQR (-5.5)-0.7)	0.001
Lactate (mmol/L)	2.2 (IQR 1.5-3.3)	5.7 (IQR 3.9-7.4)	2.4 (IQR 1.7-3.9)	0.001
Lactate/Albumin Ratio (%)	0.05 (IQR 0.03-0.08)	0.17 (IQR 0.09-0.2)	0.06 (IQR 0.03-0.1)	0.001
LDH/Albumin Ratio (%)	6.7 (IQR 5.6-9.5)	9.8 (IQR 7.4-12.9)	7.2 (IQR 5.86-9.86)	0.003

HGB: Hemoglobin, ALT: Alanine Transaminase, AST: Aspartate Transaminase, LDH: Lactate dehydrogenase

Table 3. The cut-off values of laboratory indices for mortality

	Lactate	Base Excess	Lactate/Albumin Ratio	LDH/Albumin Ratio
AUC + SD	0.819±0.03	0.843±0.04	0.829±0.04	0.747±0.04
Cutoff	4.2	-3	0.11	7.2
Sensitivity (%), 95% CI	73.3 (44.9-92.2)	80.0 (51.9-95.7)	73.3 (44.9-92.2)	86.7 (59.5-98.3)
Specificity (%), 95% CI	89.2 (79.1-95.6)	76.9 (64.8-86.5)	90.8 (81.0-96.5)	58.5 (45.6-70.6)
+ LR, 95% CI	6.8 (3.2-14.6)	3.5 (2.1-5.8)	7.9 (3.5-18.1)	2.1 (1.5-3.0)
- LR, 95% CI	0.3 (0.1-0.7)	0.3 (0.1-0.7)	0.3 (0.1-0.7)	0.2 (0.1-0.9)
PPV (%),95% CI	61.1 (42.3-77.1)	44.4 (32.4-57.1)	64.7 (44.7-80.7)	32.5 (25.3-40.6)
NPV (%),95% CI	93.6 (86.2-97.1)	94.3 (85.7-97.8)	93.7 (86.4-97.2)	95.0 (83.7-98.6)
Accuracy (%), 95% CI	86.3 (76.7-92.9)	77.5 (66.8-86.1)	87.5 (78.2-93.8)	63.8 (52.2-74.2)

AUC: Area Under the Curve, SD: Standard Deviation, LR: Likelihood Ratio, PPV: Positive Predictive Value, NPV: Negative Predictive Value CI: Confidence Interval, LDH: Lactate dehydrogenase

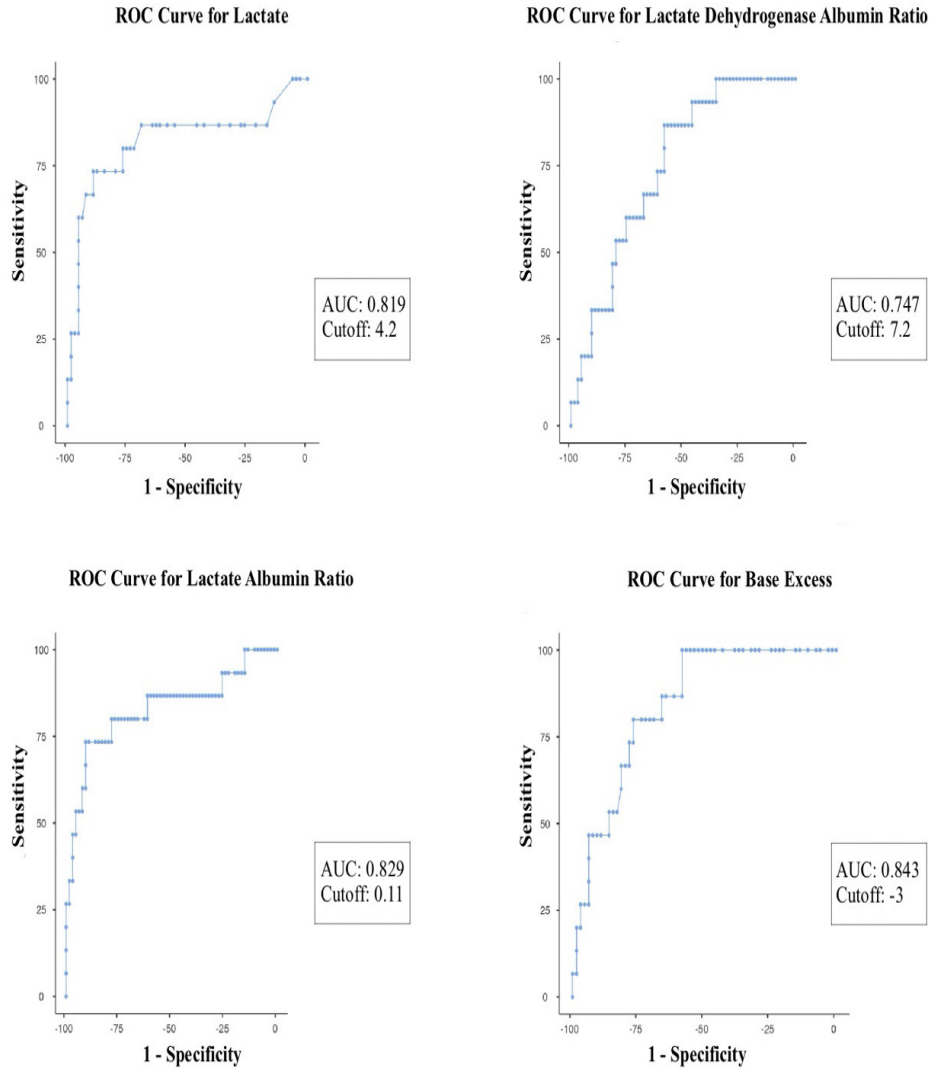

Figure 2. Receiver Operating Curve (ROC) analysis

AUC: Area Under the Curve

Discussion

In trauma patients, anamnesis, physical examination, and vital signs may not be beneficial in patient management. Therefore, evaluating anamnesis, physical examination, vital signs, some laboratory parameters, and imaging methods is essential in managing trauma patients [3, 4]. The use of many laboratory parameters as mortality predictors in trauma has been evaluated. Lactate and BE are widely used because they are the most important laboratory indicators of mortality, especially in hemorrhagic shock. In our study, the diagnostic value of both lactate and BE as mortality indicators in the blunt multitrauma was evaluated. In addition, the diagnostic value of lactate albumin ratio and LDH albumin ratio, which were found to be mortality indicators in many critically ill patients [11-13], were evaluated as mortality indicators in the blunt multitrauma. Lactate albumin ratio was more specific in predicting mortality than lactate and BE, while LDH albumin ratio was more sensitive in predicting mortality than lactate and BE. This did not result in a clear superiority in predicting clinical outcome.

Javali et al. [14] compared the lactate and BE laboratory parameters with vital findings from the blood gas analysis of trauma patients taken at the time of the first admission to the ED and 24 hours of follow-up. They found that a 4 mmol/L increase in lactate (100% sensitive,

85.9 specific) and 12 mEq/L change in BE (85.7% sensitive, 82.6% specific) were more sensitive than vital signs in demonstrating 24-hour mortality. They recommended using lactate and BE as essential indicators for 24-hour mortality, intensive care unit admission, and blood transfusion. Qi et al. [15] evaluated the predictive value of BE, lactate, and pH for 72-hour mortality in patients with multi-trauma. They found that the AUC of BE, lactate and pH were 0.693 (95% CI:0.675-0.712), 0.715 (95% CI:0.697-0.733), 0.670 (95% CI:0.651-0.689), respectively. Jyoti et al. [16] evaluated the relationship between serum lactate concentration, BE and mortality as a prognostic factor in patients with polytrauma. They found that serum lactate and BE were simple, rapid, and independent biochemical predictors of 48-hour mortality in polytrauma patients. In our study, lactate and BE were considered to be predictors of mortality. When mortality and non-mortality groups were analyzed, lactate and BE were found to be statistically significant between the two groups. Also, in the ROC analysis, the lactate cut-off value for mortality was 4.2, exhibiting 73.3% sensitivity and 89.2% specificity. The BE cut-off value for mortality was -3, exhibiting 80.0% sensitivity and 76.9% specificity. In conclusion, similar to the literature, we can say that lactate and BE are strong predictors of mortality in patients with blunt multitrauma.

Wang et al. [17] evaluated the relationship between lactate albumin ratio and mortality in patients with moderate and severe traumatic brain injury. Lactate albumin ratio was found to be a prognostic marker of mortality in patients with moderate and severe traumatic brain injury. In addition, many studies have evaluated the relationship between LDH albumin ratio and mortality. It has been determined that LDH albumin ratio may be a predictor of mortality in sepsis, critically ill patients, and colon cancer [18-20]. In our study, the relationship between lactate albumin ratio and LDH albumin ratio and mortality in blunt multitrauma patients was investigated. Lactate albumin ratio and LDH albumin ratio were found to be predictors of mortality in blunt multitrauma patients. The fact that previous studies have not investigated the predictive value of lactate albumin ratio and LDH albumin ratio for mortality in patients with multi-trauma makes our findings valuable. However, as expected, its predictive sensitivity and specificity are not significantly superior to lactate alone and BE alone.

In our study, we also compared the laboratory parameters at the time of first admission between the groups with and without mortality. AST, LDH, albumin and creatine were found to be statistically related between mortality and non-mortality groups.

This study has some limitations. In particular, the study was small, conducted in a single center, and was retrospective. Patient data cannot be adequately assessed because of the retrospective design. This raises concerns about selection bias, similar to other retrospective studies. However, the study population was designed to address this concern by excluding situations that may cause bias (e.g. Lactate and LDH were excluded in arrest patients because they would be high). Further studies with many patients and more centers are needed to confirm our findings.

In conclusion, the lactate cut-off value for mortality was 4.2, exhibiting 73.3% sensitivity and 89.2% specificity. The BE cut-off value for mortality was -3, exhibiting 80.0% sensitivity and 76.9% specificity. Lactate albumin ratio was more specific in predicting mortality than lactate and BE, while LDH albumin ratio was more sensitive in predicting mortality than lactate and BE.

Conflicts of interest: No conflict of interest was declared by the authors.

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Author contributions

Literature search: O.B., M.M.Y.; Study design: M.M.Y.; Legislative applications: O.B., M.M.Y.; Data collection: M.M.Y., U.S.C., N.P.; Supervision and quality control: O.B.; Statistical data analysis: M.M.Y.; Data interpretation: M.M.Y., N.P., U.S.C.; Drafting the manuscript: M.M.Y., O.B. All authors were involved in the writing and critical revision of the manuscript and approved the final version. M.M.Y. and O.B. take the whole responsibility for the paper.

Research Article

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Can emergency department blood parameters predict coronary artery occlusion in acute myocardial infarction?

Acil servis kan parametreleri akut miyokard infarktüsünde koroner arter oklüzyonunu öngörebilir mi?

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Abstract

Purpose: The aim of this study is to assess the detectability of coronary artery stenosis in patients evaluated for acute myocardial infarction (AMI) in the emergency department and undergoing coronary angiography, based on blood parameters examined in the emergency setting.

Materials and methods: In our single-center prospective observational study, patients diagnosed with AMI in the Emergency Department between September 1 and October 31, 2023, and those who underwent coronary angiography by a single cardiologist were included. The blood parameters applied during routine assessment in the Emergency Department were recorded, and parameters with predictive effects based on the percentages of vessel stenosis after angiography were evaluated.

Results: A total of 64 patients (44 males and 20 females) who met the study criteria were included in our research. Following the evaluation based on the highest percentage of stenosis in any coronary artery after coronary angiography, patients were divided into two groups. Group 1 consisted of 15 patients with mild stenosis (stenosis <50%), and Group 2 comprised 49 patients with severe stenosis (70-99% stenosis). Group 2, a predominance of male gender was observed along with elevated Troponin-I (Tn-I) levels, and lower values of lymphocyte and platelet counts (p=0.010, p=0.004, p=0.042, and p=0.007, respectively).

Conclusion: In males, it has been observed that Tn-I levels are higher in association with coronary stenosis. Alongside atherosclerosis and thrombosis, inflammation may contribute to decreased platelet and lymphocyte counts in cases of severe stenosis. Further prospective, randomized controlled studies with larger sample sizes are needed to confirm these findings.

Keywords: Acute myocardial infarction, cardiac markers, whole blood count, coronary stenosis, coronary angiography.

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Öz

Amaç: Bu çalışmanın amacı acil serviste akut miyokard infarktüsü (AMİ) nedeni ile değerlendirilen ve koroner anjiografi uygulanan hastaların koroner damar darlığının acilde bakılan kan parametreleri ile saptanabilirliğini değerlendirmek.

Gereç ve yöntem: Tek merkez prospektif gözlemsel çalışmamızda 1 Eylül-31 Ekim 2023 tarihleri arasında acil serviste AMİ tanısı alan ve tek kardiyolog tarafından koroner anjiyografi yapılan hastalar dâhil edildi. Acil servis rutin değerlendirmede uygulanan kan parametreleri kayıt edildi ve anjiografi sonrası damar darlık yüzdelerine göre prediktif etkisi olan parametreler değerlendirildi.

Bulgular: Araştırmamıza çalışma kriterlerini karşılayan toplam 64 hasta (44 erkek, 20 kadın) dâhil edildi. Koroner anjiyografi sonrası herhangi bir koroner arterde en yüksek darlık yüzdesine göre yapılan değerlendirme sonrası hastalar 2 gruba ayrıldı. Grup 1 hafif darlık (darlık <%50) saptanan 15 hasta ve grup 2 ciddi darlık (%70-99 darlık) tespit edilen 49 hastadan oluşmaktadır. Grup 2'de erkek cinsiyet baskınlığı ile Troponin-I (Tn-I) düzeyinin yüksek, lenfosit ve trombosit değerlerinin düşük olduğu görüldü (sırasıyla p=0,010, p=0,004, p=0,042 ve p=0,007).

Sonuç: Erkeklerde koroner darlığa bağlı olarak Tn-I düzeylerinin daha yüksek olduğu görülmüştür. Ateroskleroz ve trombozun yanı sıra, şiddetli darlık vakalarında iltihaplanma, trombosit ve lenfosit sayısının azalmasına katkıda bulunabilir. Bu bulguları doğrulamak için daha büyük örneklem büyüklüğüne sahip, ileriye dönük, randomize kontrollü çalışmalara ihtiyaç vardır.

Anahtar kelimeler: Akut miyokard infarktüsü, kardiyak belirteçler, tam kan sayımı, koroner darlık, koroner anjiografi.

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Introduction

Acute myocardial infarction (AMI), commonly known as a heart attack, is a consequence of ischemic heart disease or coronary artery disease. It represents a clinical condition arising from the blockage of one or more coronary arteries by a vulnerable plaque, leading to the inadequate nourishment of the heart with blood [1]. Despite advances in prevention, diagnosis, and treatment strategies, it continues to be a leading cause of worldwide morbidity and mortality [2]. The diagnosis can be established with the presence of at least two characteristics among typical symptoms, a characteristic rise-fall pattern of a cardiac marker (such as creatine kinase MB (CK-MB) isoenzymes), or preferably, the development of Q waves, in accordance with the consensus of the European Society of Cardiology and the American College of Cardiology [3].

Current marker technologies, particularly serum troponins, are capable of detecting very small amounts of myocardial necrosis (<1.0 g) when compared to patients without myocardial infarction in acute coronary syndrome [4]. In addition, CK-MB, a creatine kinase (CK) isoenzyme, serves as a standard marker for AMI. It begins to rise 4-9 hours after myocardial injury, reaching its peak within 24 hours, and returns to the normal range between 48-72 hours [5]. The pathophysiology of AMI involves a complex chain of reactions, including atheroma plaque rupture, platelet activation leading to aggregation, endothelial dysfunction, vasospasm, and revascularization [6]. The primary mechanism in the pathogenesis of AMI is thrombocyte hyperactivation and local platelet activation, with the progression rate of the atherosclerotic event determining the onset timing of symptoms [7, 8].

The aim of this study was to investigate the relationship between the percentage of coronary artery occlusion and cardiac enzymes, as well as hematological parameters, in patients diagnosed with acute myocardial infarction (AMI) upon admission to the emergency department who subsequently underwent coronary angiography.

Materials and methods

This research constitutes a prospective observational study that obtained ethical approval from the Bandırma Onyedi Eylül University Health Sciences Non-Interventional Research Ethics Committee. The study adhered to the guidelines for observational studies outlined by STROBE (www.strobestatement.org).

We included patients diagnosed with non-ST-segment elevation myocardial infarction (NSTEMI) and Unstable angina pectoris (USAP) and undergoing coronary angiography by a single cardiologist at the Emergency Department of Bandırma Training and Research Hospital between September 1 and October 31, 2023, in our study. The diagnosis and management of AMI, as well as the follow-up of coronary angiography procedures, were conducted as follows: Patients presenting to the emergency department underwent diagnostic evaluation by the attending physician, and those diagnosed with non-ST-segment elevation myocardial infarction (NSTEMI) following cardiology consultation were included in the study. Consultation assessments and coronary angiography procedures were performed by the same cardiologist for standardization purposes. We excluded patients referred from external centers, those without NSTEMI, individuals whose blood test results could not be analyzed in our emergency department, those with a history of previous bypass surgery or stent placement, and patients presenting to the emergency department with coronary syndrome who could not undergo coronary angiography within the first 24 hours from the study. In Figure 1, the study design is illustrated in more detail using a flow chart.

After the patients' emergency admissions, electrocardiography, cardiac enzyme markers, and simultaneous hemogram data were recorded. Consultation with the same cardiologist was requested for patients suspected of having AMI. The procedure results of patients undergoing coronary angiography, consistently performed by the same physician using the same methodology and interpretation,

were recorded. In coronary angiography, patients were categorized based on the percentage of stenosis in the Right coronary artery, Left Anterior Descending artery, Left Main Coronary Artery, and Circumflex arteries as follows: mild (<50%), moderate (50-70%), and severe (70-99%) according to the extent of vascular narrowing.

Statistical analysis

The data acquired were recorded using the SPSS 20.0 version (Statistical Package for Social Sciences, Inc., Chicago, IL, USA) statistical program. The normal distribution of the data was evaluated through the Kolmogorov-Smirnov test. Descriptive statistics for continuous variables included standard deviation, mean, median, minimum, and maximum values, while categorical variables were represented using percentages and numbers. Student's t-test was applied to compare normally distributed data between two groups, and the Mann-Whitney U test was employed when the data deviated from a normal distribution. The Chi-square test or Fisher's exact test (used when Chi-square test assumptions were not met due to low expected cell counts) was utilized to compare categorical variables. The cut-off point was chosen based on the ROC (Receiver Operating Characteristic) curve analysis. A significance level of $p<0.05$ was considered statistically significant for all outcomes.

Results

As shown in the flowchart (Figure 1), a total of 108 patients were evaluated by the same cardiologist. During the designated time frame, our research included a total of 64 patients, comprising 44 males and 20 females, who met the study criteria. The median age of the patients was 59.50 years, ranging from a minimum of 47 years to a maximum of 88 years. The results of the blood parameters at the time of admission for the patients are presented in Table 1.

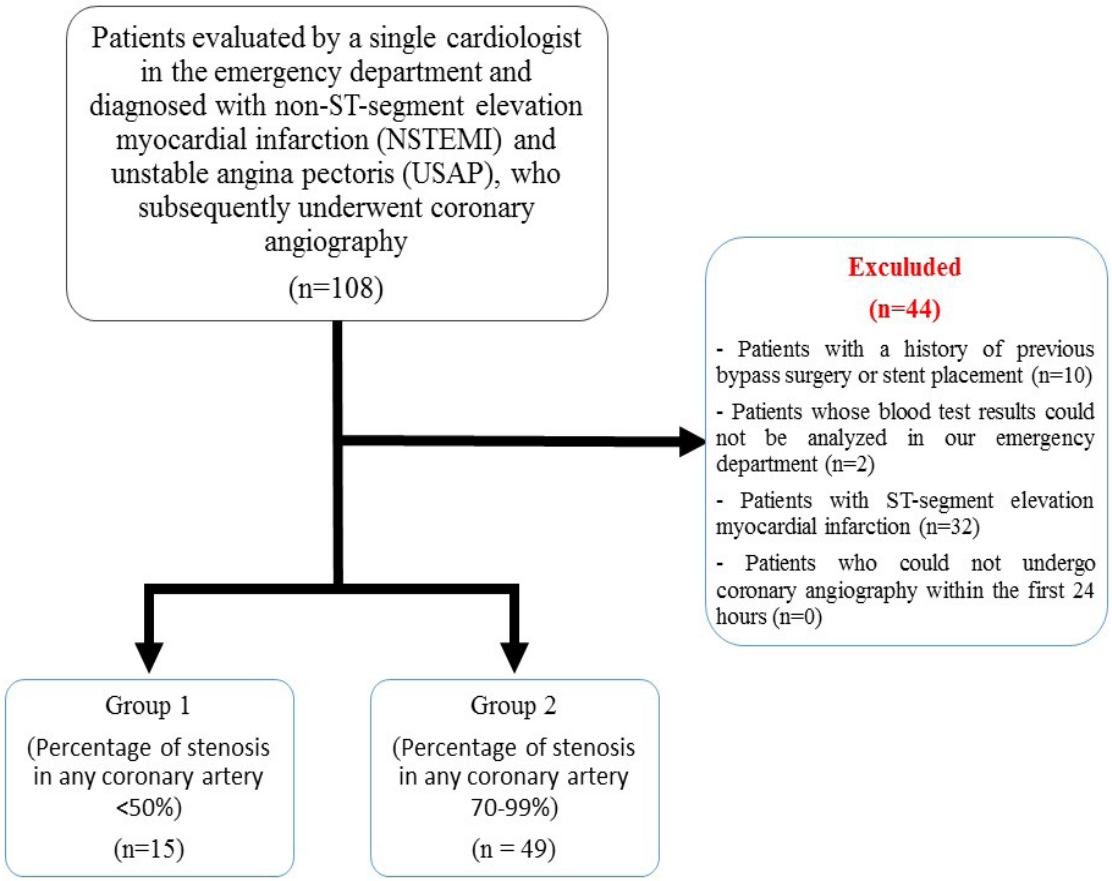

Figure 1. Flow chart for the study design

Table 1. Emergency department admission blood parameter results of the patients

Blood parameters	Median (IQR)
Troponin- I (Tn-I) (pg/ml)	153.80 (2746.5)
Creatine kinase (U/L)	126.50 (109)
Creatine kinase-myocardial band (CK- MB) (U/L)	26 (25)
CRP (mg/dl)	0.50 (0.89)
WBC (10^3 /ul)	11.03 (3.99)
PLT (10^3 /ul)	262 (80)
MPV (fl)	10.05 (0.90)
Neutrophil (10^3 /ul)	6.71 (4.62)
Lymphocyte (10^3 /ul)	2.20 (0.97)
NLR	3.22 (3.06)

IQR: Interquartile Range, CRP: C-Reactive Protein, WBC: White Blood Cell, MPV: Mean Platelet Volume, NLR: Neutrophil Lymphocyte Ratio PLR: Platelet Lymphocyte Ratio, PLT: Platelet

In the assessment based on the highest percentage of stenosis in any coronary artery after coronary angiography, mild stenosis was observed in 15 patients (stenosis <50%) (Group 1), while severe stenosis (stenosis 70-99%) was detected in 49 patients (Group 2). No patient with moderate (50-70%) stenosis was detected. Although the median age of Group 2 (61 years) was higher than Group 1 (51 years), no statistically significant difference was observed. When considering the gender distribution between the groups, a statistically significant higher prevalence of stenosis was observed in males (p=0.010). Among the cardiac enzyme markers examined at the time of emergency admission, only Troponin-I (Tn-I) levels were found to be elevated in Group 2 (p=0.004). No significant difference was observed among CK and CK-MB values (Respectively p=0.584 and p=0.552). Group-wise analysis of hematological parameters revealed that only lymphocyte and platelet (PLT) values were found to be higher in Group 1 (respectively, p=0.042 and p=0.007). The comparison of gender and blood parameters according to coronary artery stenosis percentage groups is presented in Table 2.

The ROC curve was plotted for Tn-I, lymphocyte, and PLT values regarding the severity of coronary artery stenosis. The area under the curve for Tn-I was 0.746 with a standard deviation (SD) of 0.06. The area under the curve was significantly higher than the diagnostic insignificance level of 0.05 (p=0.004). The cutoff value for Tn-I in diagnosing severe coronary artery stenosis was 0.3 pg/ml. It was determined that this value had a sensitivity of 100% and specificity of 100% (Figure 2a). For lymphocyte and PLT values, the areas under the curve were 0.675 (SD=0.8) and 0.733 (SD=0.79), respectively. The areas under the curve were significantly higher than the diagnostic insignificance level of 0.05 (p=0.042 and p=0.007, respectively). The cutoff value for lymphocyte in diagnosing severe coronary artery stenosis was 4.02 10^3/µl. It was found that having a value below this cutoff had a sensitivity of 98% and specificity of 60% for severe coronary stenosis (Figure 2b). The cutoff value for PLT in diagnosing severe coronary artery stenosis was 367 10^3/µl. It was determined that having a value below this cutoff had a sensitivity of 96% and specificity of 87% for severe coronary stenosis (Figure 2b).

Table 2. The comparison of gender and blood parameters according to coronary artery stenosis percentage groups

Blood parameters		Group 1 n=15	Group 2 n=49	*p*
Gender	**Male n (%)**	6 (40)	38 (77.6)	**0.010**
	Female n (%)	9 (60)	11 (22.4)	
Age (years) Median (IQR)		51 (21)	61 (19)	0.085
Troponin- I (Tn-I) (pg/ml) Median (IQR)		39.50 (179.3)	397.70 (2808.8)	**0.004**
Creatine kinase (U/L) Median (IQR)		137 (90)	120 (110)	0.584
Creatine kinase-myocardial band (CK- MB) (U/L) Median (IQR)		27 (25)	25 (30)	0.552
CRP (mg/dl) Median (IQR)		0.50 (1.60)	0.50 (0.89)	0.639
WBC (10^3 /ul) Median (IQR)		11.33 (2.83)	9.89 (6.64)	0.617
PLT (10^3 /ul) Median (IQR)		310 (107)	254 (64)	**0.007**
MPV (fl) Median (IQR)		10 (1.60)	10.10 (1.35)	0.994
Neutrophil (10^3 /ul) Median (IQR)		6.60 (2.15)	6.71 (7.21)	0.981
Lymphocyte (10^3 /ul) Median (IQR)		2.81 (2.50)	2.20 (1.14)	**0.042**
NLR Median (IQR)		2.72 (2.16)	3.43 (5.42)	0.062
PLR Median (IQR)		103.20 (77.38)	115.45 (96.80)	0.396

IQR: Interquartile Range, CRP: C-Reactive Protein, WBC: White Blood Cell, MPV: Mean Platelet Volume, NLR: Neutrophil Lymphocyte Ratio PLR: Platelet Lymphocyte Ratio, PLT: Platelet

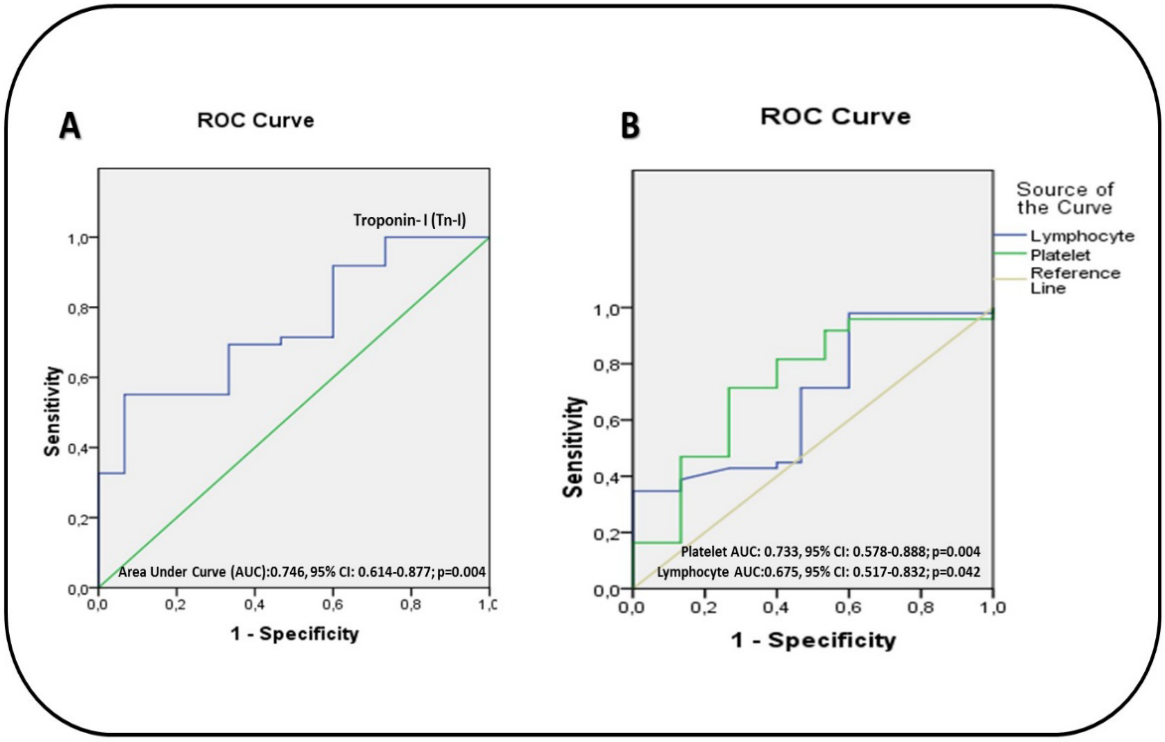

Figure 2. Diagnostic values of troponin-I, lymphocyte and platelet values and the severity of coronary artery stenosis

Discussion

AMI is responsible for approximately 7 million deaths worldwide annually, highlighting the importance of early identification of signs and symptoms, as well as the detection of specific serological markers for a prompt diagnosis and the initiation of potentially life-saving treatment [9]. The specificity of markers used in the diagnosis of AMI and ongoing research on new markers continue to be an active area in contemporary medical studies [9-12]. While studies are specifically focused on achieving a faster and more accurate diagnosis, there is limited research evaluating the correlation with vascular stenosis.

The clinical presentation of AMI involves complex reactions, including atheromatous plaque rupture in coronary arteries, platelet activation leading to thrombus formation, aggregation, endothelial dysfunction, vasospasm, and revascularization [7]. After the onset of atherosclerosis, continuous progression occurs, accompanied by inflammation. In this process, the formed elements of blood and inflammatory markers related to inflammation actively play a role. Particularly during the plaque rupture phase of atherosclerosis and consequently in the progression stages of this process, neutrophils, lymphocytes, and platelets actively participate [13]. Specifically, platelets actively play a role in plaque destabilization, rupture, and the coagulation cascade [13]. In early atherogenesis, the progression of lesions, and ultimately in the thrombotic complications of plaques, it has been shown that inflammatory pathways play a role, and decreased lymphocyte levels suppress the immune response [14]. The mechanism mentioned would lead to an expected positive correlation between the amount of thrombus formation and the PLT and lymphocyte values. Indeed, in our study, PLT and lymphocyte values were found to be lower in patients with severe coronary stenosis compared to those with mild stenosis. The literature contains findings consistent with the results of our study. A decrease in lymphocyte count has been reported to be associated with the progression of atherosclerosis and major cardiac complications [15, 16]. Similarly, Tangjitgamol et al. [17] found significantly low platelet levels in doctors with coronary artery disease. Yüksel et al. [18] also demonstrated that the average Platelet-to-Lymphocyte Ratio (PLR) in the severe atherosclerosis group was significantly higher compared to the mild atherosclerosis and control groups, supporting the mentioned pathophysiology. Liu et al. [19] have also provided evidence supporting the relationship between severe stenosis and the inflammatory process. In the literature, there are publications that express the opposite of this situation. Yücel and Amanvermez Şenarslan [20] investigated the relationship between the progression of atherosclerosis and hematological parameters in patients undergoing Coronary Artery Bypass Graft (CABG) surgery. In their study, they found that platelet counts increased in recurrent stenosis, while lymphocyte counts decreased. Ayaz et al. [21] reported that there was no statistically significant relationship between the severity of coronary artery disease and the number of vascular occlusions with platelet aggregation slope and % amplitude values. Patients monitored for AMI in the emergency department continue to be searched for new rapid markers. Although there is a relationship between coronary artery stenosis and the processes of platelet and inflammation, more detailed studies are needed.

Our study has various limitations. Firstly, it is a single-center study with a small number of patients. Secondly, the inability to assess values such as HDL, Triglycerides, and Low-Density Lipoprotein, as well as comorbid additional diseases and other processes that may affect inflammation, before the procedure, is another limitation. However, values such as HDL, Triglyceride, and Low-Density Lipoprotein could not be evaluated in patients who would undergo urgent angiography in the emergency department, as values such as HDL, Triglyceride, and Low-Density Lipoprotein could not be studied, and previous or post-procedure values were not targeted for the acute situation in our study.

In conclusion, the severity of coronary artery occlusion in patients diagnosed with AMI in the emergency department can be predicted not only by cardiac enzyme markers such as Troponin I but also by using platelet and lymphocyte values. To generalize this condition, there is a need for multicenter randomized controlled prospective studies.

Conflict of interest: No conflict of interest was declared by the authors.

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Ethics committee approval: The study received approval from the Bandırma Onyedi Eylül University Health Sciences Non-Interventional Research Ethics Committee (approval date: 12.01.2023 and no: 2023-2).

Authors contributions

H.Y.B. Concept and design, data collection and processing, analysis and interpretation, literature review, writing, reviewing and revision. A.A. Concept and design, analysis and interpretation, literature review, writing, reviewing and revision, critical review A.B. Concept and design, data collection and processing, analysis and interpretation, literature review, writing, reviewing and revision.

Research Article

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Evaluation of ultrasound screening method and prevalence for developmental hip dysplasia in the central Anatolia

Orta Anadolu'da ultrason tarama yönteminin değerlendirilmesi ve gelişimsel kalça displazisi prevalansı

Oğuzhan Pekince, Ferhat Sayar, Emrah Cevat Ercan, Özkan Köse

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Abstract

Purpose: This study aims to evaluate the incidence and follow-up outcomes of Developmental Hip Dysplasia (DDH) in infants admitted to City Hospital using the Graf classification. Furthermore, it aims to investigate the prevalence of DDH in Central Anatolia and assess the effectiveness of current screening and treatment protocols for detecting and managing DDH in infants.

Materials and methods: A total of 10.650 infants underwent screening for DDH using the Graf USG method as part of the National DDH screening program at City Hospital between August 2020 and September 2022. Infants born at term (38 weeks and above) and screened between 30-90 days of birth were included, while premature infants were excluded. Hips were classified according to the Graf method into Types 1 (normal), 2 (immature), 2A (+), 2A (-), 2B, 2C, D, 3, and 4, based on alpha angles.

Results: The study examined the USG results of 8,695 term infants (52.5% male and 47.5% female) between 2020 and 2022. The mean gestational age of participants at the time of the initial USG examination was approximately 7.94±2.07 weeks. Graf Type 1 was more prevalent in males (97-96.5%), while Graf Type 2 was more common in females (7.2-7.8%). Radiologists tended to recommend a re-examination after one month for Type 2A Graf hips (84.49-82.02%), whereas orthopedic consultation was advised for Type 2B, 2C, and Type 3 hips. The vast majority of infants (93.6%) underwent only one USG screening. Pelvic X-ray was requested for 15.9% of patients, and additional USGs were requested for 5.7% of patients. Pavlik treatment was applied to 4.2% of patients who did not return to normal, Frejka pillow treatment was applied to 1.5% Interestingly, none of the patients who maintained regular USG monitoring and treatment required surgical intervention involving osteotomy.

Conclusion: USG is an early diagnostic method for DDH, which allows for simple treatment options and the prevention of complications. It is a simple, inexpensive, and non-invasive method. Our study supports that regular USG screenings in infants eliminate the need for surgical procedures requiring osteotomy. However, the proportion of individuals who failed to adhere to their follow-up appointments despite receiving abnormal results remains elevated, underscoring the necessity for implementing diverse strategies aimed at augmenting parental awareness in this context.

Keywords: Developmental hip dysplasia, Anatolia, prevalence, ultrasound screening method**.**

Pekince O, Sayar F, Ercan EC, Kose O. Evaluation of ultrasound screening method and prevalence for developmental hip dysplasia in the central Anatolia. Pam Med J 2024;17:486-496.

Öz

Amaç: Bu çalışma, Şehir Hastanesi'ne başvuran bebeklerde Graf sınıflaması kullanılarak Gelişimsel Kalça Displazisi (GKD) insidansını ve takip sonuçlarını değerlendirmeyi amaçlamaktadır. Ayrıca, Orta Anadolu'da GKD prevalansını araştırmayı ve GKD'nin tespit edilmesi ve yönetilmesi için mevcut tarama ve tedavi protokollerinin etkinliğini değerlendirmeyi hedeflemektedir.

Gereç ve yöntem: Ağustos 2020 ile Eylül 2022 tarihleri arasında Şehir Hastanesi'nde Ulusal GKD tarama programının bir parçası olarak toplam 10.650 bebek Graf USG yöntemiyle GKD taramasından geçirilmiştir. Doğum haftası 38 hafta ve üzeri olan ve doğumdan 30-90 gün sonra taranan bebekler dahil edilirken, prematüre bebekler hariç tutulmuştur. Kalçalar alfa açılarına dayanarak Graf yöntemine göre Tip 1 (normal), 2 (olgunlaşmamış), 2A (+), 2A (-), 2B, 2C, D, 3 ve 4 şeklinde sınıflandırılmıştır.

Bulgular: Çalışma, 2020-2022 yılları arasında 8.695 term bebek (%52,5 erkek ve %47,5 dişi) USG sonuçlarını incelemiştir. Katılımcıların ilk USG muayenesi sırasındaki ortalama gebelik haftası yaklaşık 7,94±2,07 hafta idi. Graf Tip 1 erkeklerde daha yaygındı (%97-96,5), Graf Tip 2 ise kadınlarda daha yaygındı (%7,2-7,8).

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Graf Tip 2A kalçalar için radyologlar genellikle bir ay sonra tekrar muayene önerirken (%84,49-82,02), Tip 2B, 2C ve Tip-3 kalçalar için ortopedik danışma önerilmiştir. Bebeklerin büyük çoğunluğu (%93,6) yalnızca bir kez USG taramasına tabi tutulmuştur. Hastaların %15,9'una pelvis grafisi istenmiş ve %5,7'sine ek USG'ler istenmiştir. Takiplerinde normale dönmeyen hastaların %4,2'sine Pavlik tedavisi uygulanmış, %1,5'ine Frejka yastığı tedavisi uygulanmıştır. İlginç bir şekilde, düzenli USG takibi ve tedavisi devam eden hastalar arasında hiçbirinin osteotomi gerektiren cerrahi müdahaleye ihtiyaç duymadığı gözlemlenmiştir.
Sonuç: USG, GKD tanısı için erken, basit, ucuz ve invaziv olmayan bir tanı yöntemidir. Sonuçlarında GKD tanısı konulan çocukların basit tedavi modaliteleri ile tedavi edilmelerine ve komplikasyonların önlenmesine olanak sağlar. Çalışmamız, bebeklerde düzenli USG taramalarının osteotomi gerektiren cerrahi işlemlere ihtiyacı ortadan kaldırdığını desteklemektedir. Ancak, anormal sonuçlar almasına rağmen takip randevularına uymayan bireylerin oranı hala yüksektir, bu da ebeveyn farkındalığını artırmaya yönelik çeşitli stratejilerin uygulanması gerekliliğini vurgular.

Anahtar kelimeler: Gelişimsel kalça displazisi, Anadolu, prevalans, ultrason tarama metodu.

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Introduction

Developmental Hip Dysplasia (DDH) is a congenital hip anomaly that can lead to instability, dislocation, and reduced mobility [1]. The incidence of DDH varies based on the population and the screening method used. According to studies, the prevalence of DDH in newborns ranges from 0.06% to 7.61%, with females, breech position infants, firstborn children, and family stories at higher risk [2] Timely diagnosis and treatment are crucial to prevent long-term complications and ensure proper hip joint development [3].

The hip joint's development is a dynamic process that occurs during the neonatal period and is difficult to assess using direct radiography due to the predominance of cartilage. Hip USG using the Graf classification is the gold standard for detecting DDH in newborns, particularly in the first six months of life [4]. In Türkiye, Graf hip USG screening is a standard method for identifying DDH in newborns and is incorporated into routine newborn evaluations to ensure early detection and treatment of DDH.

This study aims to evaluate the incidence and follow-up outcomes of DDH in infants admitted to Konya City Hospital using the Graf classification between August 2020 and September 2022. This research study aimed to investigate the prevalence of DDH in Central Anatolia and assess the effectiveness of the current screening and treatment protocols for detecting and managing DDH in infants.

Materials and methods

Study design

From August 2020 to September 2022, the results of 10,650 infants screened using the Graf USG method under the National DDH screening program at City Hospital were retrospectively examined. Delivery weeks of the infants were analyzed from the hospital database, and infants born at term (38 weeks and above) were included in the study, while premature infants were excluded. Infants who underwent USG between 30-90 days of birth, and whose hip development physiology was standardized during USG, were also included.

The Graf method was used as the screening method, and the infants were placed on their sides with their hips and knees held in 15-20 degrees of internal rotation and semi-flexion. Gel was applied to the hip skin, and coronal sections were obtained with a probe held vertically to the body. The angles were calculated by obtaining appropriate images of all sections of the hip reference points observed on the monitor (the deepest point of the acetabulum, the ilium wing plane and its smooth appearance, and the labrum). After determining the alpha and beta angles, hip dysplasias were classified ultrasonographically according to the Graf method, with Type 1 hips being normal and Type 2 hips being considered "immature" in their development cycles. Type 2 hips are further divided into 2A (+) and 2A (-) based on alpha angles of 55-59 and 50-54, respectively,

while Type 2B hips have alpha angles between 45-49. Type 2C and Type D hips are considered "dysplastic," and Type 3 and 4 hips are considered "dislocations (Table 1).

Statistical analysis

The statistical analyses of the study were conducted using the SPSS 20.0 (IBM Inc, Chicago, IL, USA) program. Descriptive statistics were presented as mean±SD and median; min-max for numerical variables and frequency (percentage) for categorical variables. Monte Carlo-corrected chi-square analysis was used to determine the relationships between categorical variables. A *p*-value of <0.05 was considered statistically significant in the analyses.

Table 1. Graf classification

Graf Clasification	Alpha Angle (Bone Roof)	Beta Angle (Cartilaginous Roof)	Explanation
Type 1	a>60	b<55° <3month	Normal Hip
Type 2A+	a= 55°-60°	b>55° <3month	Pathological Immature Hip
Type 2A-	aaaaa= 50°-55°	b>55° <3month	Pathological Immature Hip
Type 2B	a= 50°-60°	b>55° <3month	Centered Hip Stable
Type 2C	a= 43°-49°	b<77°	Centered Hip Unstable
Type D	a= 43°-49°	b>77°	Decentered Hip
Type 3	a<43°	b>77°	Eccentered Hip
Type 4	a<43°	b>77°	Dislocated Hip

Results

The study included 8,695 term infants (17,390 hips) who underwent developmental hip dysplasia screening using the Graf USG method between 2020 and 2022, within 30-90 days after birth at Konya City Hospital by individuals from Konya and its surrounding areas. Of the patients, slightly over half (52.5%) were male and the remaining were female (47.5%). The mean age of the first USG examination was 55.64±14.49 days, while the mean gestational age at the time of the first USG examination was 7.94±2.07 weeks. The majority of babies (93.6%) underwent USG screening only once. Of the babies, 6.4% underwent a second USG and 0.64% underwent a third USG for control and follow-up purposes based on their first USG examination results. Eight babies required a fourth and two babies required a fifth USG examination (Table 2, 3).

Graf types were evaluated by gender. In the first USG examinations, Graf types showed a significant difference by gender for both right and left hips (p<0.001). The proportion of Type 1 graphs was higher in male infants for both sides (right: 97%, left: 96.5%), while the Type 2A rate was higher in female infants (right: 7.2%, left: 7.8%). Type 2B and C had a slightly higher number (2.2%, 0.8%) of patients in females, and for Type 4 rates were equal, but for the left hip, all Type 4 patients (n=2) were females (Table 2, 4).

The relationships between Graf types and USG decisions were investigated. For both right and left hips, except for patients diagnosed as 'NORMAL' with Type 1 Graf, who had a borderline Graf angle, despite being within normal limits, it was decided to "re-examine after 1 month" (2.67% and 2.35%, respectively). The highest rate of "re-examine after 1 month" was determined for Type 2A Graf types for both right and left hips (84.49% and 82.02%, respectively) (p<0.001). Generally, orthopedic consultation was recommended for Type 2B, 2C, and Type 3 Graf types. The distribution of Graf types was similar for both right and left hips according to the second USG decisions (p<0.001). It was observed that the rate of orthopedic consultation requests increased for Type 2A Graf hips in radiologists alongside control USG compared to the first USG (13.7-20%). Type 2B, 2C, and Type 3 Graf types were generally directed to orthopedic consultation (Table 5, 6).

Table 2. Rate of sex and type of graf

Variable	Category	N (%)	Variable	Category	N (%)
Sex	**Female**	4127 (47.5)			
	Male	4568 (52.5)			
Graf Types			**Graf Types**		
Right Hip 1. USG	**Type 1**	8248 (94.8)	**Left Hip 1. USG**	**Type 1**	8194 (94.2)
	Type 2A	432 (5.0)		**Type 2A**	473 (5.4)
	Type 2B	8 (0.1)		**Type 2B**	10 (0.1)
	Type 2C	4 (0.0)		**Type 2C**	11 (0.1)
	Type 4	2 (0.0)		**Type 4**	4 (0.0)
Right Hip 2. USG	**Type 1**	491 (5.6)	**Left Hip 2. USG**	**Type 1**	476 (5.5)
	Type 2A	52 (0.6)		**Type 2A**	65 (0.7)
	Type 2B	10 (0.1)		**Type 2B**	10 (0.1)
	Type 2C	3 (0.0)		**Type 2C**	5 (0.1)
	Type 3	2 (0.0)		**Type 3**	1 (0.0)
Right Hip 3. USG	**Type 1**	44 (0.5)	**Left Hip 3. USG**	**Type 1**	38 (0.4)
	Type 2A	6 (0.1)		**Type 2A**	5 (0.1)
	Type 2B	7 (0.1)		**Type 2B**	14 (0.2)
Right Hip 4. USG	**Type 1**	6 (0.1)	**Left Hip 4. USG**	**Type 1**	5 (0.1)
	Type 2B	2 (0.0)		**Type 2B**	3 (0.0)
Right Hip 5. USG	**Type 2A**	1 (0.0)	**Left Hip 5. USG**	**Type 1**	2 (0.0)
	Type 2B	1 (0.0)			

Table 3. Ultrasound imaging day

	Mean±SS	Median; Min-Max
Birth Week	38.22±0.65	38; 38-42
Age (Month)	18.26±7.62	17.7; 5.5-34.3
Ultrasound imaging day		
1.USG (n=8695)	55.64±14.49	53; 30-90
2.USG (n=558)	84.23±20.3	82; 41-206
3.USG (n=56)	110.0±18.44	107; 84-194
4.USG (n=8)	126.13±23.73	119; 104-176
5.USG (n=2)	125±1.41	125; 124-126

Table 4. Graf types according to sex

		Sex		
		Female	Male	
Graf Types	**Categories**	**N (%)**	**N (%)**	***p***
1. USG Right Hip	**Type 1**	1819 (92.5)	4429 (97.0)	
	Type 2A	298 (7.2)	134 (2.9)	
	Type 2B	6 (0.1)	2 (0.0)	*<0.001**
	Type 2C	3 (0.1)	1 (0.0)	
	Type 4	1 (0.0)	1 (0.0)	
1. USG Left Hip	**Type 1**	3786 (91.7)	4408 (96.5)	
	Type 2A	320 (7.8)	153 (3.4)	
	Type 2B	7 (0.2)	3 (0.1)	*<0.001**
	Type 2C	9 (0.2)	2 (0.0)	
	Type 3	3 (0.1)	1 (0.0)	
	Type 4	2 (0.0)	0 (0.0)	
2. USG Right Hip	**Type 1**	316 (86.6)	175 (90.7)	
	Type 2A	37 (10.1)	15 (7.8)	
	Type 2B	8 (2.2)	2 (1.0)	*0.256*
	Type 2C	3 (0.8)	0 (0.0)	
	Type 3	1 (0.3)	1 (0.5)	
2. USG Left Hip	**Type 1**	302 (82.7)	174 (90.2)	
	Type 2A	50 (13.7)	15 (7.8)	
	Type 2B	8 (2.2)	2 (1.0)	*0.042**
	Type 2C	3 (0.8)	2 (1.0)	
	Type 2D	1 (0.3)	0 (0.0)	
	Type 3	1 (0.3)	0 (0.0)	
3. USG Right Hip	**Type 1**	32 (74.4)	12 (85.7)	
	Type 2A	5 (11.6)	1 (7.1)	*0.397*
	Type 2B	6 (14.0)	1 (7.1)	
3. USG Left Hip	**Type 1**	27 (62.8)	11 (78.6)	
	Type 2A	4 (9.3)	1 (7.1)	*0.269*
	Type 2B	12 (27.9)	2 (14.3)	
4. USG Right Hip	**Type 1**	6 (85.7)	0 (0.0)	*0.083*
	Type 2B	1 (14.3)	1 (100.0)	
4. USG Left Hip	**Type 1**	5 (71.4)	0 (0.0)	*0.197*
	Type 2B	2 (28.6)	1 (100.0)	
5. USG Right Hip	**Type 2A**	0 (0.0)	1 (100.0)	*0.317*
	Type 2B	1 (100.0)	0 (0.0)	
5. USG Left Hip	**Type 1**	1 (100.0)	1 (100.0)	

**: Significant at the 0.05 level according to chi-square analysis*

Table 5. USG desicions and treatments

Variable	Category	N (%)
1.Desicion	**2 weeks later control**	13 (0.1)
	3 weeks later control	80 (0.9)
	1 month later control	589 (6.8)
	Orthopaedics Consultation	54 (0.6)
	Normal	7959 (91.5)
2.Desicion	**2 weeks later control**	12 (0.1)
	3 weeks later control	13 (0.1)
	1 month later control	56 (0.6)
	Orthopaedics Consultation	34 (0.4)
	Normal	443 (5.1)
3.Desicion	**2 weeks later control**	1 (0.0)
	3 weeks later control	1 (0.0)
	1 month later control	9 (0.1)
	Orthopaedics Consultation	14 (0.2)
	Normal	32 (0.4)
4.Desicion	**1 month later control**	1 (0.0)
	Orthopaedics Consultation	4 (0.0)
	Normal	3 (0.0)
5.Desicion	**1 month later control**	1 (0.0)
	Orthopaedics Consultation	1 (0.0)
Additional Imaging	**Pelvis X-ray**	119 (1.4)
	USG	42 (0.5)
	Out Of Follow Up	197 (2.3)
	No	8337 (95.9)
Treatment	**No**	8448 (97.2)
	Pavlic	34 (0.4)
	Frejka splint	11 (0.1)
	Pelvic Cast	3 (0.01)
	Out Of Follow Up	198 (2.3)
Surgical Treatment	**No**	8490 (97.6)
	Open Reduction	2 (0.002)
	Closed Reduction	5 (0.1)
	Out Of Follow Up	197 (2.3)

Table 6. Graf types and USG decisions according to right/left hip

USG		2 weeks later control	3 weeks later control	1 month later control	Orthopaedics Consultation	Normal	
Graf Types	**Types**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	**N (%)**	***p***
1. Desicion Right Hip	**Type 1**	3 (0.03)	41 (0.49)	221 (2.67)	23 (0.27)	7959 (96.50)	*<0.001**
	Type 2A	10 (2.31)	38 (8.79)	365 (84.49)	19 (4.39)	0 (0.0)	
	Type 2B	0 (0.0)	1 (12.5)	3 (37.5)	4 (50.0)	0 (0.0)	
	Type 2C	0 (0.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	
	Type 4	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	
1. Decision Left Hip	**Type 1**	7 (0.08)	25 (0.3)	193 (2.35)	11 (0.13)	7958 (97.1)	*<0.001**
	Type 2A	6 (1.26)	53 (11.2)	388 (82.02)	25 (5.28)	1 (0.21)	
	Type 2B	0 (0.0)	2 (20.0)	6 (60.0)	2 (20.0)	0 (0.0)	
	Type 2C	0 (0.0)	0 (0.0)	1 (9.1)	10 (90.9)	0 (0.0)	
	Type 3	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	
	Type 4	0 (0.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	
2. Desicion Right Hip	**Type 1**	4 (0.81)	6 (1.22)	24 (4.88)	14 (2.88)	443 (90.22)	*<0.001**
	Type 2A	8 (15.38)	7 (13.46)	28 (53.84)	9 (13.70)	0 (0.0)	
	Type 2B	0 (0.0)	0 (0.0)	2 (20.0)	8 (80.0)	0 (0.0)	
	Type 2C	0 (0.0)	0 (0.0)	0 (0.0)	3 (100.0)	0 (0.0)	
	Type 3	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	
2. Decision Left Hip	**Type 1**	5 (1.05)	5 (1.05)	16 (3.36)	7 (1.47)	443 (93.06)	*<0.001**
	Type 2A	7 (10.76)	8 (12.30)	37 (56.92)	13 (20)	0 (0.0)	
	Type 2B	0 (0.0)	0 (0.0)	2 (20.0)	8 (80.0)	0 (0.0)	
	Type 2C	0 (0.0)	0 (0.0)	0 (0.0)	5 (100.0)	0 (0.0)	
	Type 2D	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	
	Type 3	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	
3. Desicion Right Hip	**Type 1**	1 (2.27)	0 (0.0)	4 (9.09)	7 (15.9)	32 (72.7)	*0.002**
	Type 2A	0 (0.0)	1 (16.67)	3 (50.0)	2 (33.3)	0 (0.0)	
	Type 2B	0 (0.0)	0 (0.0)	2 (28.57)	5 (71.42)	0 (0.0)	
3. Decision Left Hip	**Type 1**	0 (0.0)	1 (2.63)	2 (5.26)	3 (7.89)	32 (84.21)	*<0.001**
	Type 2A	0 (0.0)	0 (0.0)	3 (60.0)	2 (40.0)	0 (0.0)	
	Type 2B	1 (7.14)	0 (0.0)	4 (28.57)	9 (64.28)	0 (0.0)	
		1 month later control		**Orthopaedics Consultation**		**Normal**	
4. Desicion Right Hip	**Type 1**	1 (16.7)		2 (33.3)		3 (50.0)	*0.564*
	Type 2B	0 (0.0)		2 (100.0)		0 (0.0)	
		1 month later control		**Orthopaedics Consultation**		**Normal**	
4. Decision Left Hip	**Type 1**	1 (20.0)		1 (20.0)		3 (60.0)	*0.439*
	Type 2B	0 (0.0)		3 (100.0)		0 (0.0)	
		1 month later control		**Orthopaedics Consultation**			
5. Decision Left Hip	**Type 2A**	1 (100.0)		0 (0.0)			*0.317*
	Type 2B	0 (0.0)		1 (100.0)			
		1 month later control		**Orthopaedics Consultation**			
5. Desicion Left Hip	**Type 1**	1 (50.0)		1 (50.0)			*N/A*

**: Significant at the 0.05 level according to chi-square analysis*

Following the initial evaluation, 48.4% of patients who received abnormal results returned to normal after the second evaluation, 3.7% after the third USG, and 0.4% after the fourth USG. Among patients who did not return to normal regardless of the number of USG, 26.5% (n=195) were evaluated as being out of follow-up because they did not have any health records in the national health database. Pelvic X-ray was requested for 15.9% of patients, and additional USGs were requested for 5.7% of patients beyond our study periods. Pavlik treatment was applied to 4.2% of patients who did not return to normal, Frejka pillow treatment was applied to 1.5%, and only 3 patients received pelvic-cast treatment. Open reduction was applied to 2 patients, while adductor tenotomy and closed reduction surgery were applied to 5 patients (Table 5). No patients who continued their USG control and treatment regularly required surgical treatment requiring osteotomy.

The relationship between the outcomes of patients who did not return to normal and non-surgical treatments and evaluations after USG was evaluated. A significant relationship was found between decisions and treatments after the first evaluation (p<0.001). Most patients who received a normal and control USG did not receive treatment and remained out of follow-up by 27.3%. Pavlik treatment was applied to 3.2% of patients and Frejka pillow treatment was applied to 1.5% of USG control patients. Approximately 73% of patients who were referred for orthopedic consultation did not receive treatment and remained out of follow-up, while the remaining patients received Pavlik treatment (16.7%), Frejka pillow (1.9%), and pelvic-cast treatment (3.7%). At the second follow-up, approximately 59.3-44.1% of patients who were referred for orthopedic consultation did not receive treatment, while Pavlik treatment was applied to 14.8-26.5% of patients and Frejka pillow treatment was applied to 3.7-11.8% of patients. Only Pavlik treatment was observed for children who had fourth and fifth USG controls, and no surgical treatment was needed (Table 7).

Table 7. Follow up of patiens whose USG do not return to normal

Treatment	No	Yes	Pavlic	Frejka Splint	Pelvic Cast	Out of Follow Up	
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	*p*
1.USG desicion							
Control USG	462 (67.7)	1 (0.1)	22 (3.2)	10 (1.5)	1 (0.1)	186 (27.3)	*<0.001**
Orthopaedics Consultation	30 (55.6)	2 (3.7)	9 (16.7)	1 (1.9)	2 (3.7)	10 (18.5)	
Normal	7956 (99.7)	0	1 (0.01)	0	0	2 (0.02)	
2.USG desicion							
Control USG	48 (59.3)	1 (1.2)	12 (14.8)	3 (3.7)	…	17 (21.0)	*<0.001**
Orthopaedics Consultation	15 (44.1)	2 (5.9)	9 (26.5)	4 (11.8)	…	4 (11.8)	
Normal	443 (100.0)	0	0	0	…	0	
3.USG desicion							
Control USG	4 (36.4)	…	3 (27.3)	4 (36.4)	…	0	*<0.001**
Orthopaedics Consultation	4 (28.6)	…	6 (42.9)	2 (14.3)	…	2 (14.3)	
Normal	32 (100.0)	…	0	0	…	0	
4.USG desicion							
Control USG	0	…	1 (100.0)	…	…	…	*0.046*
Orthopaedics Consultation	1 (25.0)	…	3 (75.0)	…	…	…	
Normal	3 (100.0)	…	0	…	…	…	
5.USG desicion							
Control USG	…	…	1 (100.0)	…	…	…	*N/A*
	…	…	1 (100.0)	…	…	…	

**: Significant at the 0.05 level according to chi-square analysis*

Discussion

Ultrasonography represents a straightforward, cost-effective, and non-invasive technique for the early detection of DDH. It not only permits treatment through uncomplicated methods but also aids in averting potential complications. Our research corroborates this standpoint by revealing that none of the infants subjected to routine USG screenings necessitated surgical osteotomy.

In particular, USG is the primary screening method for cases where complete ossification has not occurred in infants under six months of age [5]. Once ossification increases after six months, pelvic X-rays are preferred over USG. Many developed countries routinely use USG-assisted screening methods [6]. In Türkiye, the Graf USG method is part of the free routine screening program for all newborns. This study aims to investigate the incidence of developmental hip dysplasia in the Konya region by analyzing the results of patients who underwent USG within 30 to 90 days after term birth at Konya City Hospital. Additionally, the study aims to explore the outcomes of infants with abnormal results.

Various studies in the literature indicate that developmental hip dysplasia (DDH) incidence varies according to geographical region and cultural habits, ranging from 0.01% to 6.6% [7]. Studies on the incidence of DDH using various physical examination and imaging methods show rates ranging from 0.5% to 1.5% [2]. Dongsheng Zhu's study in China analyzed the results of 9803 infants and reported a DDH incidence rate of 1.19% [8]. Çekiç et al. [9] used the Graf USG method to screen 1162 infants in the western Mediterranean region and found an incidence rate of 1.36%. In 1992, Kutlu et al. [10] conducted a survey of five hospitals in Konya and found a DDH incidence rate of 1.34%. Unilateral hip disorders are more common in the left hip (60%) and less common in the (20%), while bilateral disorders are less frequent (20%). In our study, the results of 8695 term babies who underwent hip USG screening at Konya City Hospital and came from Konya and its surroundings were scanned from the system. The gender distribution was approximately homogeneous. As a result of the first USG, normal hips were found in 94.8% of the and 94.2% of the left hip. Considering Type 2B and higher types, the incidence of GKD was 0.2% for the and 0.4% for the left hip. Additionally, in our study, Type 1 hips were significantly more common in boys, while Type 2A hips were significantly more common in girls. Type 2B, C 4 hips were also more common in girls.

In Graf Type 2A hips, which describe insufficient development of the hip joint, only follow-up was reported to result in 97% improvement [11]. In Roovers et al.'s [12] study, the rate of return to normal was reported as 95% for Type 2A (+) hips and 95% for Type 2A (-) hips. According to a study conducted in Türkiye, Type 2A hip is more common in newborn girls than in boys. Among 431 Type 2A hips, 225 out of 285 hips (79%) that were completely followed up returned to normal. The hips of newborn boys are more likely to spontaneously normalize than those of girls at 6-7 weeks of age [13]. In a study conducted in Mongolia, of 147 infants who continued to be followed up out of 174 babies with Type 2A, 3 hips at the first examination, 121 returned to normal at the second USG and 26 returned to normal at the third ultrasound [14]. In our study, 48.4% of patients who did not have a normal result in the first ultrasound returned to normal in the second USG, and 3.7% returned to normal in the third USG. In 26.5% of babies, no record was found in our hospital or the national health database, so they were considered lost to follow-up.

According to some published reports, Type 2A cases may experience deterioration after the initial USG. A study that examined 201 Type 2A cases found that 4 cases remained stable at first but progressed to Type 2B during later follow-up assessments, while 6 cases deteriorated to Type 2C [9]. Duramaz et al. [15] conducted a study in which routine USG examinations were performed two weeks after the diagnosis of Type 2A in infants. Pavlik bandages were applied to infants with persistent Type 2A on the follow-up USG examination. For hips that reverted to Type 1 with Pavlik, treatment was extended to 12 weeks, while closed/open reduction treatments were performed for stubborn Type 2A cases. In another study conducted in our country, it was reported that 71 of 78 infants who did not miss their follow-up appointments returned to normal without treatment, while 56 infants did not continue their follow-up appointments [16]. In our study, among 273 infants diagnosed with

Type 2A on the right side on their first USG, 16.1% remained Type 2A, 1.8% progressed to Type 2B, and 1.1% deteriorated to Type 2C on their second USG. 81% of the infants were lost to follow-up. Among 301 infants diagnosed with Type 2A on the left side on their first USG, 15.3% remained Type 2A, 3.3% progressed to Type 2B, and 0.3% deteriorated to Type 2C on their second USG. Thirty-two infants were followed and treated with the Pavlik method, and 11 were followed with the Frejka pillow. Closed reduction was required for five patients who showed deterioration or had hips of Type 2B or above, and two patients required open reduction and pelvic-pedal plaster cast treatment. Three patients were treated with closed reduction and pelvic radiography after the follow-up USG examination, as per the surgical preference based on the initial USG results. No patient who received regular ultrasound follow-up required surgery requiring osteotomy.

The main advantage of USG is that it is non-invasive, low-cost, and does not involve radiation. However, the results are dependent on various factors, such as the operator's experience and the equipment used, which may sometimes require orthopedists to perform additional imaging, such as pelvic X-rays. In our study, pelvic X-rays were taken in 117 patients. In the first USG examination, Type 2A was detected in 49.6% of the joints, Type 2B in 5%, and Type 2C or higher in 5.9%. On the left side, Type 2A was detected in 60.5%, Type 2B in 8.4%, and Type 2C or higher in 12.6%. Of these, 68.1% underwent two or more follow-up USG examinations, and 42.4% required treatment such as Pavlik harness, Frejka pillow, or open/closed reduction and pelvic-foot cast. In two patients (0.8%) who underwent pelvic X-rays, pathology-immaturity was detected on USG, but the surgeon made a normal diagnosis and did not follow up with treatment.

It has been observed that in our country, USG screening is mainly followed by primary healthcare facilities and referred to pediatricians. Therefore, the decisions made by radiologists based on USG results are also essential for us as orthopedists. A NORMAL decision is mostly made for patients diagnosed with Type 1 hip in USG, but as the angles are borderline, follow-up USG is recommended. When it comes to Type 2A results, while follow-up USG is recommended, radiologists suggest orthopedic consultation for treatment in Type 2B and above hips. It is observed that the rate of orthopedic consultation for treatment or follow-up of Type 2A hips in the second USG increases from the right/left hip ratio of 4.39%/5.28% to 13.70%/20%, aiming for the orthopedist to perform the treatment or follow-up.

In our study, which examined a substantial population, the lack of recording of intrauterine problems, birth positions, and family histories of babies whose USG results were evaluated, as well as the inability to determine the fate of some babies due to the parents' failure to attend follow-up appointments for various reasons, can be considered limitations of our study. In addition, the fact that the results were based on our hospital's database and that the USG was performed by multiple physicians are also limitations.

In conclusion, screening with USG is a simple, inexpensive, and non-invasive method that enables early diagnosis of DDH, and allows for treatment with simple modalities, as well as the prevention of complications. Our study supports this thesis by showing that none of the babies who underwent regular USG screenings required surgical osteotomy. Nevertheless, the percentage of patients who did not continue with their follow-ups despite abnormal results is still high, highlighting the need for various measures to increase parental awareness in this regard.

Conflict of interest: The author(s) have no conflicts of interest relevant to this article.

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Authors' contributions to the article

O.P. has constructed the main idea and hypothesis of the study. O.P. and O.K. developed the theory and edited the material and method sections. O.P., F.S. and E.C.E. have collected data and evaluated the data in the results. The article was written by O.P. Review and correction has been done by O.K. In addition, all authors discussed the entire study and approved the final version.

Research Article

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Investigation of the apoptotic and cell cycle effects of sorafenib and doxorubicin on URG4/URGCP in leukemia cells

Sorafenib ve doxorubicin'in lösemi hücrelerinde URG4/URGCP üzerindeki apoptotik ve hücre döngüsü etkilerinin incelenmesi

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Abstract

Purpose: The aim of this study is to investigate the effects of anticancer drugs such as Sorafenib (SOR) and Doxorubicin (DOX) on URG4/URGCP mRNA levels in K562 and HL-60 leukemia cells, elucidating their effects on apoptosis and cell cycle. The effects of these drugs on apoptosis and the cell cycle in leukemia cells have been explored. This research aims to understand the cellular effects of drugs used in leukemia treatment and contribute valuable insights to the drug development processes in leukemia therapy.

Materials and methods: DOX and SOR were evaluated for their IC50 values in K562 and HL-60 cell lines using the CellTiter-Glo assay (Promega, USA), based on ATP measurement. Total RNA isolation was performed using Trizol reagent in both control and dose groups of each treated cell line. Following RNA isolation, cDNAs were synthesized using the "Transcriptor High Fidelity cDNA Synthesis Kit". Subsequently, changes in mRNA expression levels were examined using specific primers for URG4/URGCP, Casp-3, Casp-8, Casp-9, FADD, DR4, TRADD, CCDN1, CDK4, CDK6, PTEN, P53, and Rel-A genes.

Results: In the groups treated with Sorafenib, the IC50 dose for HL-60 cell line was calculated as 40 μM at the 24th hour, and for K562 cell line, it was calculated as 40 μM at the 48th hour. In the groups treated with Doxorubicin, the IC50 doses were calculated as 50 μM at the 48th hour for HL-60 cell line, and as 50 μM at the 72nd hour for K562 cell line. Significant increases were observed in the mRNA expression levels of Casp-8, Casp-9, TRADD, DR4, Rel A, and FADD genes in the groups treated with SOR, while a decrease was observed in the mRNA expression levels of URG4/URGCP, CCDN1, CDK4, and CDK6 genes. In the groups treated with DOX, significant increases were observed in the fold changes of Casp-3, Casp-8, P53, and PTEN genes. However, a significant decrease in mRNA expression levels was observed in URG4/URGCP, CCDN1, and CDK4 genes.

Conclusion: As a result, it has been demonstrated that both SOR and DOX may play a role in regulating the mRNA expressions of URG4/URGCP, Casp-3, Casp-8, Casp-9, CDK6, CDK4, CCND1, P53, PTEN, TRADD, DR4, Rel A, and FADD genes in HL-60 and K562 cells.

Keywords: Doxorubicin, sorafenib, cell cycle, apoptosis, URG4/URGCP.

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Öz

Amaç: Çalışmanın amacı, Sorafenib (SOR) ve Doxorubicin (DOX) gibi antikanser ilaçlarının K562 ve HL-60 lösemi hücrelerinde URG4/URGCP mRNA düzeyleri üzerindeki etkilerini inceleyerek, apoptoz ve hücre döngüsü üzerindeki etkilerini açıklığa kavuşturmaktır. Bu ilaçların lösemi hücrelerinde apoptoz ve hücre döngüsü üzerindeki etkileri araştırılmıştır. Bu araştırma, lösemi tedavisinde kullanılan ilaçların hücresel etkilerini anlamak ve lösemi tedavisinde ilaç geliştirme süreçlerine değerli bilgilerle katkıda bulunmayı amaçlamaktadır.

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Gereç ve yöntem: DOX ve SOR'in K562 ve HL-60 hücre hatlarında IC50 ATP ölçümüne dayanan CellTiter-Glo (Promega, ABD) tarafından değerlendirildi. Uygulama yapılan her iki hücre hattının kontrol ve doz gruplarında Trizol kimyasalı kullanılarak total RNA izolasyonu yapıldı. RNA izolasyonunun ardından "Transcriptor High Fidelity cDNA Sentez Kiti" ile cDNA'lar sentezlendi. Daha sonra URG4/URGCP, Casp-3, Casp-8, Casp-9, FADD, DR4, TRADD, CCDN1, CDK4, CDK6, PTEN, P53, Rel-A genlerine özgü primerler kullanılarak mRNA düzeyindeki ekspresyon değişiklikleri incelendi.
Bulgular: Çalışmada Sorafenib uygulanan gruplarda IC50 dozu HL-60 hücre hattı için 24'üncü saatte 40 μM, K562 hücre hattı için ise 48'inci saatte 40 μM olarak hesaplandı. Doksorubisin uygulanan gruplarda ise IC50 dozları, HL-60 hücre hattı için 48'inci saatte 50 μM, K562 hücre hattı için ise 72'inci saatte 50 μM olarak hesaplanmıştır. SOR uygulanan gruplarda Casp-8, Casp-9, TRADD, DR4, Rel A ve FADD genlerin mRNA ekspresyon düzeylerindeki kat değişimlerinde önemli derecede artış gözlemlenirken, URG4/URGCP, CCDN1, CDK4 ve CDK6 genlerinin mRNA ekspresyon düzeylerinde azalma olduğu gözlemlendi. DOX uygulanan gruplarda, Casp-3, Casp-8, P53 ve PTEN genlerinin kat değişimlerinde önemli derecede bir artış olduğu gözlemlenmiştir. Ancak, URG4/URGCP, CCDN1 ve CDK4 genleri üzerinde mRNA ekspresyon düzeylerinde önemli bir azalma meydana gelmiştir.
Sonuç: Sonuç olarak hem SOR hem de DOX' in HL-60 ve K562 hücrelerinde URG4/URGCP, Casp-3, Casp-8, Casp-9, CDK6, CDK4, CCND1, P53, PTEN, TRADD, DR4, Rel A ve FADD genlerin mRNA ekspresyonları düzenlenmesinde rol alabilecekleri gösterilmiştir.

Anahtar kelimeler: Doksorubisin, sorafenib, hücre döngüsü, apoptoz, URG4/URGCP.

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Introduction

Leukemia is a type of cancer caused by abnormal proliferation of blood cells. It is characterized by disrupting the body's normal blood functions by replacing abnormally proliferating blood cells as opposed to normal blood cells found in the bone marrow [1]. This type of cancer usually affects leukocytes, but in different cases it can also affect other blood cells. Leukemia is usually treated with stem cell transplants, radiotherapy and chemotherapy [2]. Chemotherapy is a drug treatment method widely used in cancer treatment [3]. The primary goal of this treatment method is to eliminate cancer cells, but it also causes serious damage to normal healthy cells during the treatment process. When the treatment is administered, the drugs enter the bloodstream and can either stop or hinder cell division during mitosis, causing serious damage to the cells [4, 5]. Chemotherapy employs a variety of anticancer medications, each with distinct mechanisms of action.

SOR is an anti-cancer drug belonging to the class of biaryl urea compounds [6]. While initially identified as a RAF kinase inhibitor, SOR has been found to have a broad spectrum of activity by affecting different cellular signaling pathways [7]. SOR can inhibit "*vascular endothelial growth factor receptors* (VEGFR) *1, 2,* and *3, platelet-derived growth factor receptor β* (PDGFRβ), *RET receptor tyrosine kinases*, *c-Kit protein* (c-Kit), and *FMS-like tyrosine kinase 3* (Flt-3)". Due to this wide spectrum of activity, it has become an effective drug used in cancer treatment [8]. The antiproliferative activity of SOR varies in different tumor types (lung cancer, hepatocellular carcinoma etc.) and is generally associated with oncogenic signaling pathways that regulate tumor cell proliferation. Additionally, SOR initiates the process of programmed cell death (apoptosis) in many tumor cell lines [9, 10].

DOX is an antitumor drug derived from the *bacterium Streptomyces peucetius* [11]. This drug is commonly utilized in chemotherapy for the treatment of breast, ovarian, bladder, acute lymphoblastic leukemia, and acute myeloblastic leukemia [12]. DOX hinders macromolecular biosynthesis by interacting with DNA through intercalation. It also prevents the enzyme topoisomerase II from making progress and loosens supercoils in DNA, which increases the accessibility of DNA for transcription [13]. By breaking DNA strands, it stabilizes the topoisomerase II complex, preventing the DNA double helix from resealing. This mechanism can halt the replication process in cells, ultimately leading to cell death [14].

Oncogenes are genes that control the normal division and growth of cells, but when these genes are mutated they can lead to cancer [15]. Typically, oncogenes encode proteins that play significant roles in foundational cellular processes such as cell proliferation, programmed cell death or cell cycle control [16]. Despite a considerable number of identified genes, there are still new genes that remain unidentified [16, 17]. One such gene recently studied and identified is Up-regulated Gene 4 (URG4) and Up-regulator of Cell Proliferation (URGCP), located on chromosome 7 [17, 18]. When overexpressed, URG4/URGCP proteins stimulate cell growth and initiate DNA synthesis in the cell cycle. [19]. This process occurs through cyclin D1 (CCND1) protein, which is associated with the cell cycle. CCND1 plays a crucial role in regulating the checkpoints of the cell cycle at both the G0/G1 and S phases. CCND1 controls cell proliferation and division by affecting CDK4/CDK6 in the cell cycle. CCND1 forms a complex with CDK4/6, promoting the cell's progression to the S phase [20]. Another protein that suppresses cyclin-dependent kinase activity is encoded by the P21 gene [21]. These genes are typically activated by another protein involved in the cycle, p53, when DNA damage or other stress factors are detected in the cell cycle. The p53 gene detects DNA damage in cells, leading to responses such as halting the cell cycle, initiating DNA repair, or triggering cell apoptosis [22].

Apoptosis is a physiological process known as programmed cell death [22]. When a cell receives an apoptosis stimulus, it moves away from the environment and disconnects from other cells, resulting in chromatin condensation and a pyknotic appearance [23]. Apoptosis, as a vital component, leads to the controlled death of damaged cells under the influence of immune system development, cell cycle regulation and various chemical entities. There are two families of proteins involved in apoptosis. These are proapoptotic (BAX, PUMA, BAK, NOXA, BID, etc.) and antiapoptotic (BCL-XL, BCL-2, etc.) proteins [24, 25]. Apoptosis occurs through two different pathways, internal and external pathways. In the internal pathway, BID proteins inactivate BCL-2 and BAX are activated [24]. By regulating the integrity of mitochondrial membranes, these proteins contribute to the release of pro-apoptotic cytochrome-c (Cyt-C). Cyt-C, upon release into the cytoplasm, collaborates with Apaf-1 to activate caspase-9 (Casp-9), thus initiating the apoptosis process by subsequently activating caspase-3 (Casp-3) [26]. In the extrinsic pathway, death receptors (DR4, DR5, TNFR, FADD, TRADD, etc.) transmit death signals (FasL, TNF-Alpha, etc.). Subsequently, the complex formed by these receptors activates caspase-8 (Casp-8). Casp-8 can directly activate Casp-3 and also activate BID proteins, triggering the intrinsic pathway as well [27, 28].

The aim of this research was to investigate how DOX and SOR impact apoptosis and the cell cycle in K562 and HL-60 leukemia cells through their effects on URG4/URGCP mRNA levels.

Material and method

Chemicals used

Sorafenib (Nexavar, USA) was dissolved in DMSO solution. Doxorubicin (Koçak Farma, TURKEY) was dissolved in distilled water. 100 mM stock solutions were prepared in advance and cell culture treatments were performed.

Cell Culture

In this study, experiments were performed on leukemia cancer HL-60 and K562 cell lines obtained from our stocks. These cell lines were propagated in appropriate culture medium. RPMI 1640 medium, 10% fetal bovine serum, 2 mM L-glutamine, and 1% Penicillin-Streptomycin were used as culture medium. These cells had incubated at 37°C in an oven containing 95% humidity and 5% CO_2.

Cell viability

HL-60 and K562 cell lines were counted using Trypan Blue stain and planted in 96-well plates at $1x10^5$ cells per well. The cytotoxic effect of DOX and SOR on K562 and HL-60 leukemia cell lines was evaluated by CellTiter-Glo (Promega, USA), a luminometric-based method based on ATP measurement [29]. To determine the IC50 dose, both drugs were applied to the cells in the range of 1 µM-50 µM in a manner dependent on both dosage and time.

Determination of transcription level (mRNA) expression of target genes

The effects of SOR and DOX on the mRNA level of cell cycle and apoptosis -related genes were analyzed using quantitative real-time PCR method. Firstly, total RNA isolation was performed from control and dose groups of both cell lines of K562 and HL-60 leukemia cancer using Trizol chemical and cDNAs were synthesized with "*Transcriptor High Fidelity cDNA Synthesis Kit"* after RNA isolation. Then, expression changes at mRNA level were examined by using primers specific for Casp-3, Casp-8, CCDN1, CDK-4, P53, PTEN and URG-4/URGCP genes for DOX and CCDN1, CDK-4, CDK-6, Casp-8, Casp-9, FADD, TRADD, DR-4, Rel-A and URG-4/URGCP genes for SOR in both cell lines. These expression changes were determined using Step one plus real time PCR device (Table 1).

Table 1. The preferred real-time PCR primer sequences in this study

Gene Name	Gene Base Sequence
Casp-3	Forward 5′TGTTTGTGTGCTTCTGAGCC3′
	Reverse 5′CACGCCATGTCATCATCAAC3′
β-Actin	Forward 5′CTGGAACGGTGAAGGTGACA 3′
	Reverse 5′AAGGAACTTCCTTGAACAATGCA3′
Casp-8	Forward 5′AGAGTCTGTGCCCAAATCAAC3′
	Reverse 5′GCTGCTTCTCTCTTTGCTGAA3′
Casp-9	Forward 5′CTGTCTACGGCACAGATGGAT3′
	Reverse 5′GGGACTCGTCTTCAGGGGAA3′
FADD	Forward 5′CTCCTGCGCGAGCTGCTCGC3′
	Reverse 5′GCCTTCTCCAATCTTTCCCCAC3′
DR4	Forward 5′TCCAGCAAATGGTGCTGAC3′
	Reverse 5′GAGTCAAAGGGCACGATGTT3′
TRADD	Forward 5'GCTGTTTGAGTTGCATCCTAGC3'
	Reverse 5'CCGCACTTCAGATTTCGCA3'
CCDN1	Forward 5'AGCTCCTGTGCTGCGAAGTGGAAAC3'
	Reverse 5'AGTGTTCAATGAAATCGTGCGGGGT3'
CDK4	Forward 5'ATGTTGTCCGGCTGATGGA3'
	Reverse 5'CACCAGCGTTACCTTGATCTCCC3'
CDK6	Forward 5'AGACCCAAGAAGCAGTGTGG3'
	Reverse 5'AAGGAGCAAGAGCATTCAGC3'
URG4/URGCP	Forward 5′CGGGAGATGGGACAGTTTTA3′
	Reverse 5′CATGGTGTTGAGGAGTGTGG3′
PTEN	Forward 5'CCCAGACATGACAGCCATC3'
	Reverse 5'TCTGCAGGAAATCCCATAGC3'
P53	Forward 5′ATCTACAAGCAGTCACAGCACA3′
	Reverse 5′GTGGTACAGTCAGAGCCAACC3′
Rel-A	Forward 5'AGCAGCGTGGGGACTACGAC3'
	Reverse 5'AGGCTGGGGTCTGCGTAGGG3'

Statistical analysis

Data analysis was conducted utilized the ΔΔCT method., and these assessments were executed utilizing the online "RT² Profiler™ PCR Array Data Analysis" software. Additionally, Volcano Plot analyses were also carried out using this program.

Results

Cell viability

In the research, cell viability assays were performed employing the CellTiter-Glo kit with SOR on K562and HL-60 cell lines. Evaluations were performed at 24, 48, and 72 hours following treatment application. The effects of SOR were observed to vary dose-dependently and time-dependently in both cell lines. The IC50 dose of SOR in the treated groups was calculated as 40 µM at 24 hours for the HL-60 cell line and at 48 hours for the K562 cell line (Figure 1, Figure 2).

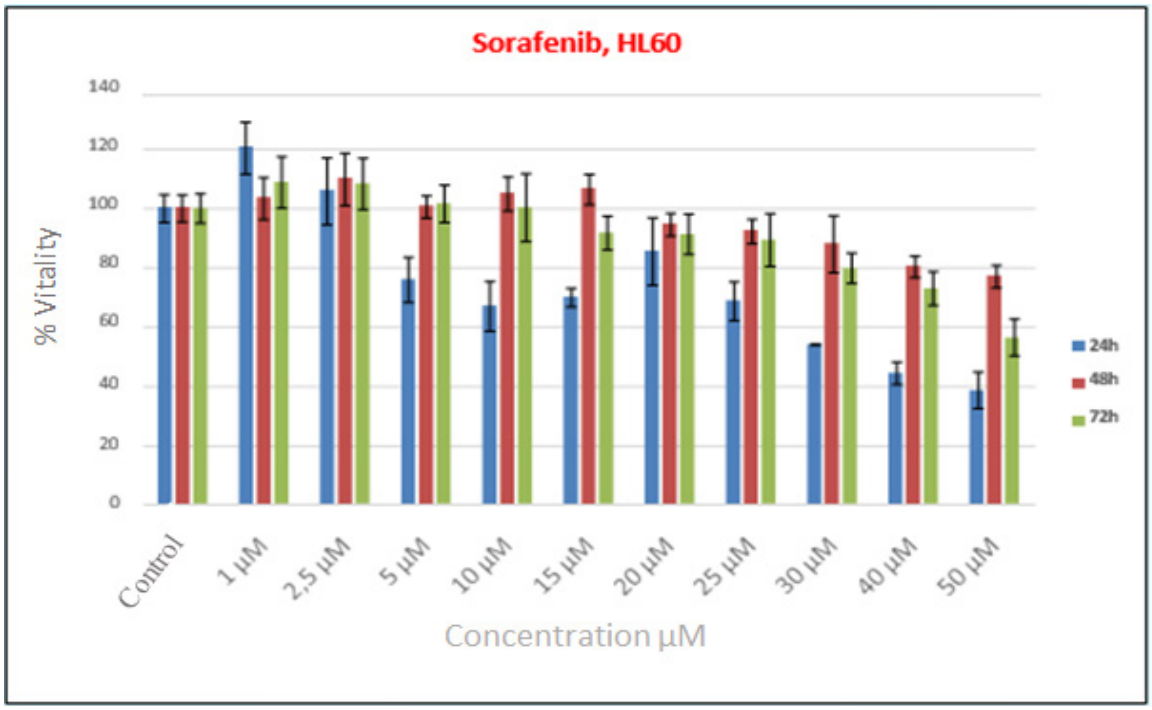


Figure 1. Cell viability percentage of Sorafenib on HL-60 leukemia cell line using the CellTiter-Glo kit

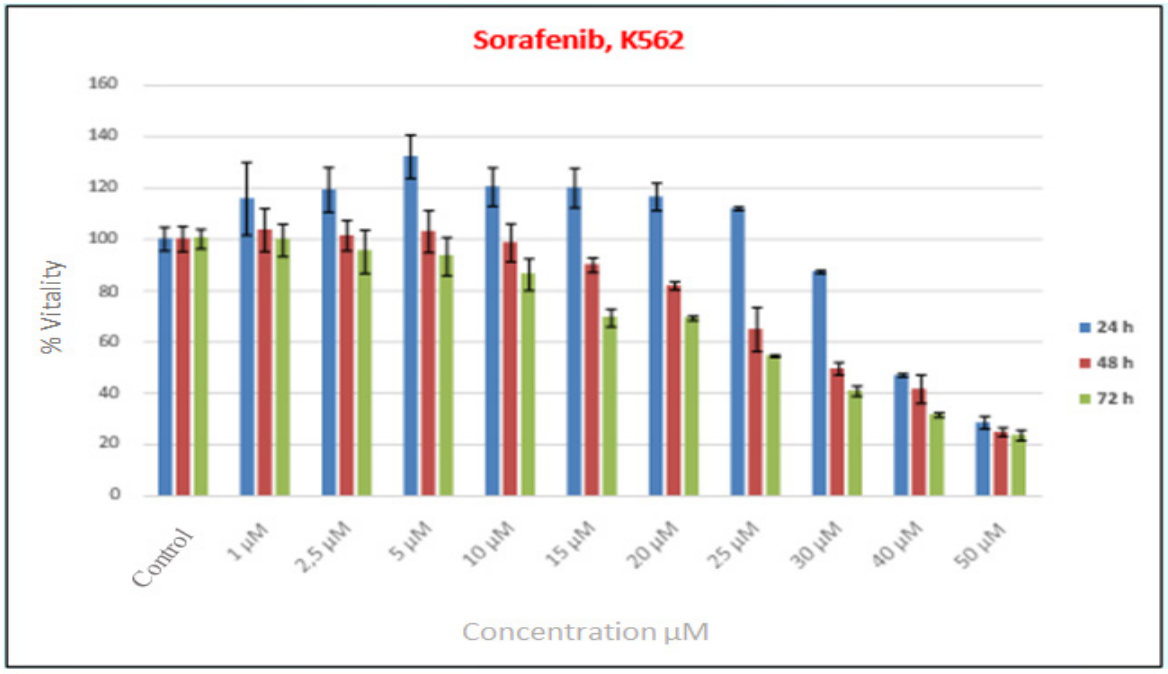


Figure 2. Cell viability percentage of Sorafenib on K562 leukemia cell line using the CellTiter-Glo kit

At the same time, cell viability tests were evaluated using CellTiter-Glo kit in HL-60 and K562 cell lines in DOX-treated groups. Cell measurements were recorded 24, 48, and 72 hours after treatment application. The effects of DOX showed dose- and time-dependent changes in both cell lines. IC50 doses in DOX treated groups were determined as 50 µM at 48 h for HL-60 cell line and 50 µM at 72 h for K562 cell line (Figure 3 and 4).

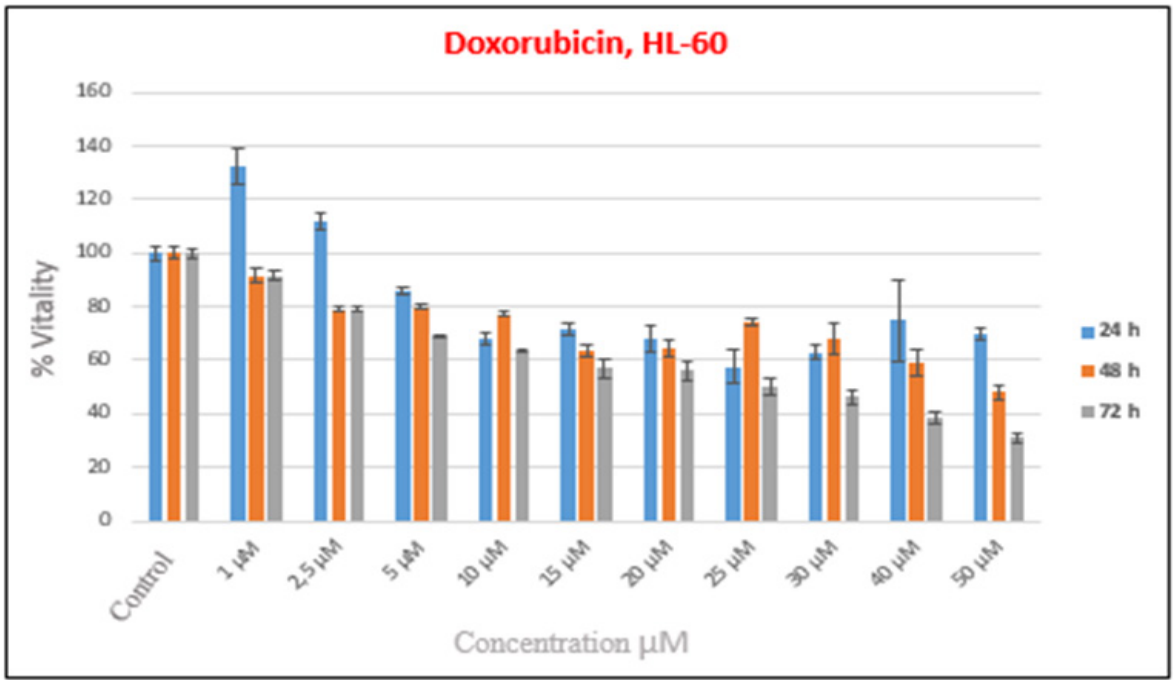


Figure 3. Cell viability percentage of Doxorubicin on HL-60 cell line using the CellTiter-Glo kit

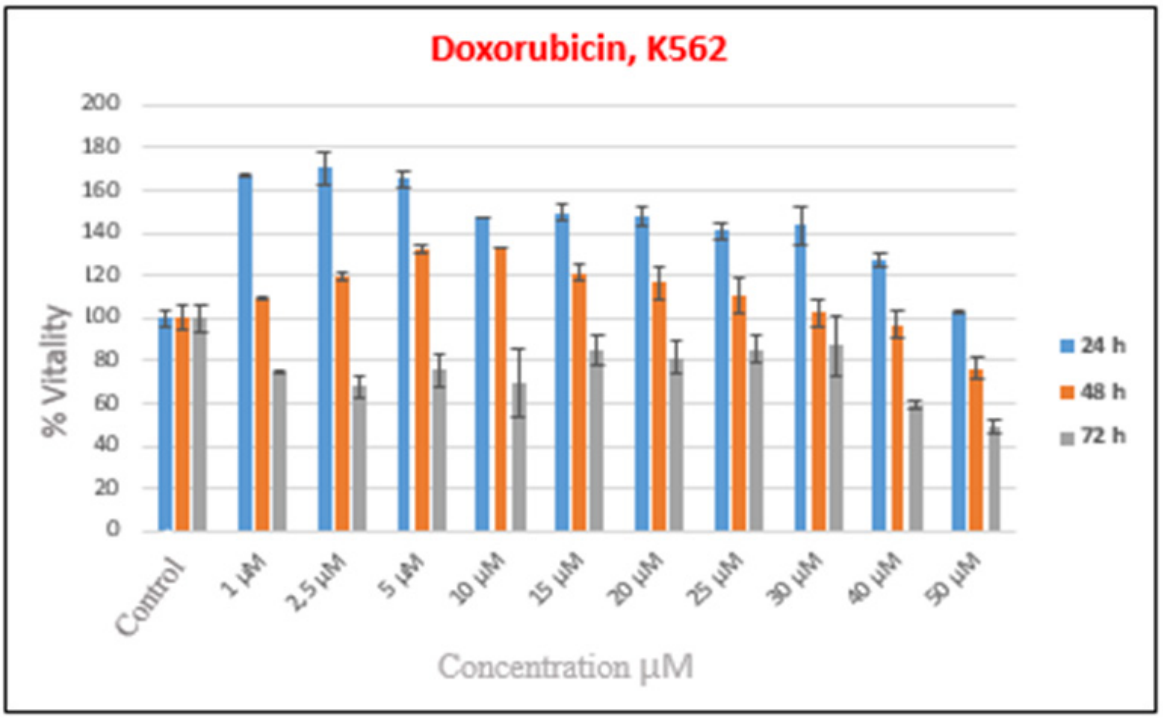
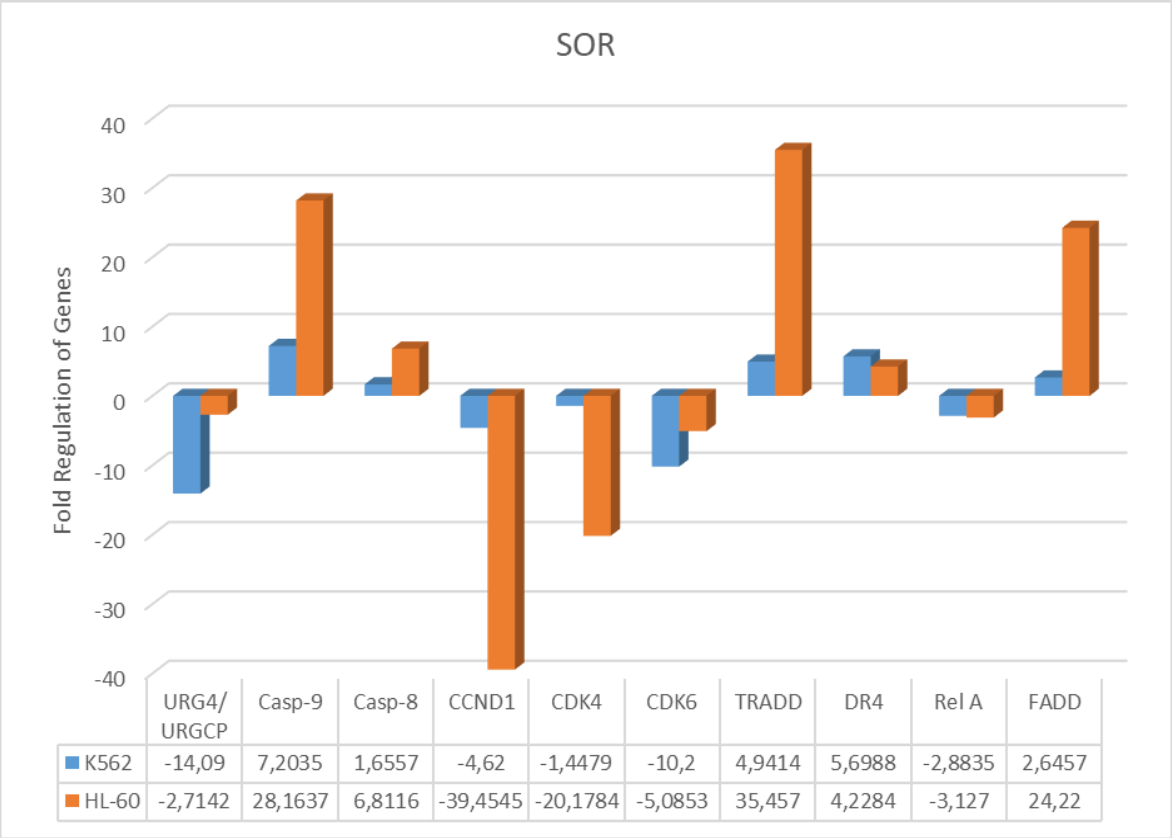


Figure 4. Cell viability percentage of Doxorubicin on K562 cell line using the CellTiter-Glo kit

Real-time PCR analysis

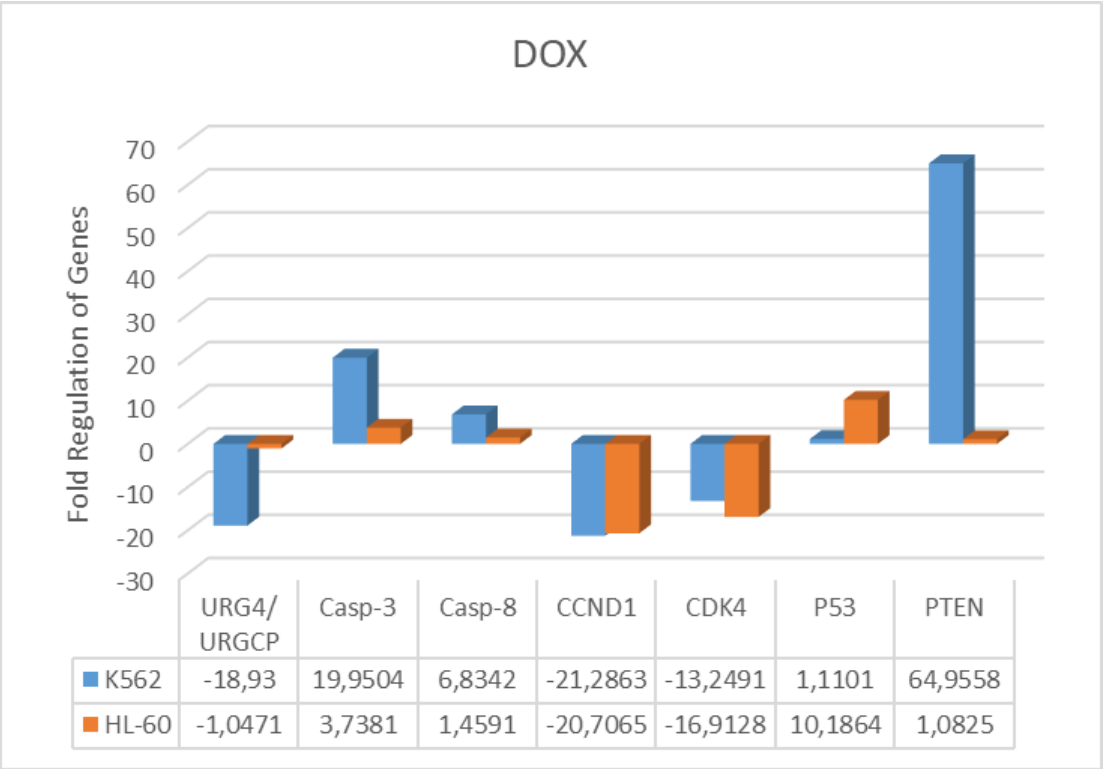
The obtained cDNAs for the SOR-treated groups were synthesized from total RNAs collected from the control and SOR-treated groups. In this research, the Real-Time PCR method was used to study the expression levels of specific mRNAs associated with cell cycle and apoptosis -related genes. Following SOR application to HL-60 and K562 cell lines, it was noted that the expression levels of Casp-8, Casp-9, TRADD, DR4, Rel A, and FADD genes increased, while the expression levels of URG4/URGCP, CCDN1, CDK4, and CDK6 genes decreased (Figure 5).



	URG4/ URGCP	Casp-9	Casp-8	CCND1	CDK4	CDK6	TRADD	DR4	Rel A	FADD
K562	-14,09	7,2035	1,6557	-4,62	-1,4479	-10,2	4,9414	5,6988	-2,8835	2,6457
HL-60	-2,7142	28,1637	6,8116	-39,4545	-20,1784	-5,0853	35,457	4,2284	-3,127	24,22

Figure 5. Fold regulation in mRNA expression levels of URG4/URGCP, Casp-9, Casp-8, CCND1, CDK4, CDK6, TRADD, DR4, Rel A, and FADD genes in K562 and HL-60 cell lines treated with Sorafenib

For the groups treated with DOX, cDNAs were synthesized from total RNAs collected from the control and DOX-treated groups. The Real-Time PCR method was used to analyze the expression levels of specific mRNAs associated with apoptosis and cell cycle-related genes. Within this research, the expression levels of genes related to the cell cycle and apoptosis, URG4/URGCP, Casp-3, Casp-8, CCDN1, CDK4, P53, and PTEN, were assessed in HL-60 and K562 cell lines treated with DOX. It was noted that the mRNA expression levels of Casp-3, Casp-8, P53, and PTEN genes exhibited an increase, whereas there was a notable reduce in the mRNA expression levels of URG4/URGCP, CCDN1, and CDK4 genes (Figure 6).



	URG4/ URGCP	Casp-3	Casp-8	CCND1	CDK4	P53	PTEN
K562	-18,93	19,9504	6,8342	-21,2863	-13,2491	1,1101	64,9558
HL-60	-1,0471	3,7381	1,4591	-20,7065	-16,9128	10,1864	1,0825

Figure 6. Fold regulation in mRNA expression levels of URG4/URGCP, Casp-3, Casp-8, CCND1, CDK4, P53, and PTEN genes in HL-60 and K562 cell lines were treated with DOX

Discussion

Leukemia is a type of blood cancer characterized by the abnormal and uncontrolled proliferation of white blood cells [30]. This disease typically originates in the bone marrow, where immature leukocytes can replace normal blood cells, leading to adverse outcomes [31]. Furthermore, it is a widely prevalent form of cancer globally [32]. Cancer treatments commonly involve radiotherapy, immunotherapy, chemotherapy, or, in the case of leukemia cancer, additional bone marrow transplants (BMT) are administered [33]. However, these treatment methods not only affect rapidly proliferating cancer cells but also impact normally dividing healthy cells, causing damage. Particularly, many drugs used in chemotherapy can halt or hinder cell division in the cell cycle [34]. SOR is known as a multiple kinase inhibitor. This drug generally inhibits VEGFR, PDGFRβ, c-Kit and Flt-3 [35]. It is an active drug used in cancer treatment because it has many different properties. SOR's efficacy may vary in different cancer types. It is usually related to tumor-inducing signaling pathways that control the proliferation of cancer cells and also initiates the apoptosis process [36, 37]. DOX is an anti-cancer drug isolated from the *bacterium Streptomyces peucetius*. This drug is frequently used in chemotherapy in different cancer types [38]. It is known that DOX prevents the DNA double helix from closing during replication, leading to replication arrest and cell death. In short, DOX is utilized as a treatment in chemotherapy, but it has serious side effects such as DNA damage or cell death [39, 40].

The main aim of this study is to research the impacts of DOX and SOR on the mRNA level of K562 and HL-60 leukemia cell lines subsequent to determining suitable dosages. Specifically, we aim to examine their effects on certain genes associated withal the cell cycle and apoptosis. In the study, IC50 doses of cell viability for HL-60 and K562 cell lines treated with SOR and DOX were determined using the CellTiter-Glo kit. Measurements was conducted at 24, 48, and 72 hours post-drug application in both experimental groups. Changes in response to dose and time were observed in both cell lines upon drug application. The IC50 values for SOR-treated groups were found to be 40 μM at 24 hours for HL-60 and 40 μM

at 48 hours for K562 cell lines. In contrast, for DOX-treated groups, the IC50 values were 50 μM at 48 hours for HL-60 and 50 μM at 72 hours for K562 cell lines. After determining the IC50 values for the drug groups, the mRNA expression levels of genes associated withal the cell cycle and apoptosis were analyzed using the Real-Time PCR method. Specifically, in SOR-treated groups, genes affecting both the extrinsic and intrinsic pathways of apoptosis were investigated. A significant increase in the fold regulation in the mRNA expression of Casp-8, Casp-9, TRADD, DR4, Rel A, and FADD genes was observed in SOR-treated groups. Many studies have reported that SOR induces apoptosis and causes cell death [41, 42]. In this study, results supporting the literature were achieved. Simultaneously, it was observed that mRNA expression levels of genes URG4/URGCP, CCDN1, CDK4, and CDK6, known to be associated withal the cell cycle, decreased in SOR-treated groups. According to the results obtained, it can be inferred that SOR induces disruption in the cell cycle, leading to cell cycle arrest. Numerous findings have been documented regarding the impact of SOR on the cell cycle [43, 44]. A notable increase in the fold regulation was noticed in the mRNA expression levels of apoptosis-associated genes, including Casp-3, Casp-8, P53, and PTEN, in the DOX-treated groups. There are various studies in the literature demonstrating that DOX induces apoptosis [45, 46]. In this study, similar results to the literature were obtained regarding the effects of DOX on apoptosis. However, a significant reduce in mRNA expression levels of DOX on URG4/URGCP, CCDN1 and CDK4 genes was found. It supported the results obtained in studies managed in the literature [47, 48]. However, in a source different from the results we found in the literature, Zuryn et al. [49] examined the effects of DOX on HL-60 cell line and found an increase in CCDN1 expression level. They stated that the increase in CCDN1 expression may also be related with the reduce in the some of apoptotic cells correlated with increased hTERT and telomerase activity and the increase in S phase cells.

This study evaluated the effects of SOR and DOX on the cell cycle and apoptotic mechanisms in both cell lines (HL-60, K562). The results obtained demonstrate that both SOR and DOX effective in regulating the mRNA expressions of URG4/URGCP, Casp-3, Casp-8, Casp-9, CDK6, CDK4, CCND1, P53, PTEN, TRADD, DR4, Rel A, and FADD genes in both HL-60 and K562 cells. In this regard, this study carries originality and will provide an important foundation for future research, making a significant present to the literature.

Conflict of interest: No conflict of interest was declared by the authors.

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Ethics committee approval: Since our study is conducted in vitro, it does not require ethics committee approval.

Authors' contributions to the article

Y.D. have constructed the main idea and hypothesis of the study. Y.D., M.S., L.E. and N.D. conducted experiments. Y.D., C.B.A., G.B., and L.S.T. analyzed data. Y.D. and S.S. wrote the manuscript. In addition, all authors discussed the entire study and approved the final version.

Research Article

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Demineralization effects of breast milk, formula milk and cow's milk on the primary teeth. A study of SEM-EDX analysis

Anne sütü, formül süt ve inek sütünün süt dişleri üzerindeki demineralizasyon etkileri. Bir SEM-EDX analizi çalışması

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Abstract

Purpose: It was aimed to investigate demineralization effects of breast milk, formula milk and cow's milk on deciduous teeth with SEM-EDX analysis.

Materials and methods: Demineralization effects of breast milk (Group 1), 3 different formula milks: Aptamil Pronutra-3 (Group 2), Hipp Organic-3 (Group 3), SmA Optipro-2 (Group 4) and cow's milk (Group 5) on newly extracted caries-free primary incisors were investigated on 5 teeth in each group. Each tooth was split in the sagittal direction from the midline and embedded in 2 separate acrylic blocks with their labial surfaces exposed. Half of the teeth was exposed to the milk material for 15 minutes, 4 times a day for 5 days, while the other half was kept in artificial saliva only. After the exposures, Scanning Electron Microscopy (SEM) images of the labial surfaces were taken and Energy Dispersive X-ray Spectroscopy (EDX) analyzes were performed. For the statistical analysis Independent-Samples T test, Man Whitney U and Kruskal-Wallis test were used.

Results: The most increased enamel porosity was seen in Group 2. A significant decrease was observed in the amount of Ca on tooth surfaces exposed to breast milk and formula milks, and a significant increase in the amount of both Ca and P after exposure to cow's milk (p=0.009). The decrease observed in the amount of Ca after exposure to breast milk was significantly higher than that of formula milks (p=0.001).

Conclusion: Formula milk and breast milk are not innocent in terms of causing demineralization, therefore awareness of parents and dentists on this issue should be increased.

Keywords: Breast milk, formula milk, Cow's milk, demineralization, SEM-EDX analysis.

Ertugrul CC. Demineralization effects of breast milk, formula milk and cow's milk on the primary teeth. A study of SEM-EDX analysis. Pam Med J 2024;17:510-519.

Öz

Amaç: Bu araştırmada anne sütü, formül süt ve inek sütünün süt dişleri üzerindeki demineralizasyon etkilerinin SEM-EDX analizi ile araştırılması amaçlanmıştır.

Gereç ve yöntem: Anne sütü (Grup 1), 3 farklı formül süt: Aptamil Pronutra-3 (Grup 2), Hipp Organik-3 (Grup 3), SmA Optipro-2 (Grup 4) ve inek sütünün (Grup 5) demineralizasyon etkileri her bir grupta 5 yeni çekilmiş çürüksüz süt kesici dişi üzerinde incelenmiştir. Her diş orta hattan sagittal yönde ikiye bölünerek labial yüzeyleri açıkta kalacak şekilde 2 ayrı akrilik bloğa gömülmüştür. Açıkta kalan diş yüzeyinin yarısı 5 gün boyunca günde 4 kez 15 dakika süreyle incelenecek süt materyaline maruz bırakılırken, diğer yarısı ise sadece yapay tükürük içerisinde tutulmuştur. Deney materyallerine maruziyet sonrası diş yüzeylerinin Taramalı Elektron Mikroskobu (SEM) görüntüleri alınmış ve Enerji Dağılımlı X-ışını Spektroskopisi (EDX) analizleri yapılmıştır. Verilerin istatistiksel analizinde Independent-Samples T testi, Man Whitney U ve Kruskal-Wallis testi kullanılmıştır.

Bulgular: En fazla artan mine pürüzlülüğü Grup 2'de görülmüştür. Anne sütü ve formül sütlere maruz kalan diş yüzeylerinde Ca miktarında anlamlı azalma, inek sütüne maruz kalan diş yüzeylerinde ise hem Ca hem de P miktarında anlamlı artış gözlenmiştir (p=0,009). Anne sütüne maruz kaldıktan sonra Ca miktarında gözlenen azalma, formül sütlere göre anlamlı olarak daha yüksek bulunmuştur (p=0,001).

Sonuç: Bu araştırmanın sonuçlarına göre bebeklik döneminde yaygın kullanılan formül sütler ve anne sütü süt dişlerinde demineralizasyona neden olması açısından şüphe uyandırmaktadır, bu nedenle ebeveynlerin ve diş hekimlerinin bu konuda farkındalığı arttırılmalıdır.

Anahtar kelimeler: Anne sütü, formül süt, inek sütü, demineralizasyon, SEM-EDX analizi.

Ertuğrul CÇ. Anne sütü, formül süt ve inek sütünün süt dişleri üzerindeki demineralizasyon etkileri. Bir SEM-EDX analizi çalışması. Pam Tıp Derg 2024;17:510-519.

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Introduction

The main cause of early childhood caries (ECC), which may cause severe pain and loss of function, therefore may affect nutrition and general health status of the infants [1, 2] is repetitive and prolonged breast-feeding or bottle-feeding with formula milk, especially during sleep at night [3, 4].

Breast milk which is the most valuable nutrient in infant nutrition, contains protein, fat, lactose and iron, and besides being an important source of energy for brain development, it increases calcium absorption and is beneficial for microorganisms in the body [5]. However, Birkhed et al. [6] reported that lactose can cause dental caries by increasing the adhesion of caries-causing microorganisms to tooth surfaces and causing demineralization. Bowen and Lawrence [7] emphasized that tooth decays are more common in children who take breast milk compared to children who take formula milk, and that oral hygiene practices should be done more carefully in these children. On the other hand, Erickson and Mazhari [2] stated that it is not possible for breast milk to have an effect on early childhood caries.

Formula milks, another nutritional source in infant nutrition, are complex compounds containing fermentable carbohydrates such as sucrose, lactose and glucose, which play a leading role in the etiology of dental caries [8]. Despite the proteins, fats, vitamins and minerals such as calcium and phosphate in their content, their cariogenic effects have been detected and reported in many studies [9-12]. Generally, it is stated that a wide variety of carbohydrates in the structure of formula milk are effective in the formation of dental caries, but on the other hand, peptides, caseins, ions and vitamins in the structure of formula milk can prevent the cariogenic activity of sucrose [8, 10]. Formula milks are generally produced in a similar structure to breast milk. There are similar amounts of lactose in breast milk (6.7 to 7.8 g/dL) and formula milk (8-8,3 g/dL), but there is more protein content in formula milk (2-2,5 g/dL) than in breast milk (0.9-1.2g/dL) [8, 13].

Another commonly consumed nutrient in early childhood nutrition is cow's milk. It is stated that cow's milk protects from dental caries due to its high calcium and phosphorus content and buffering activity of milk proteins [14, 15]. It shows anticariogenic effect thanks to the proteins in the form of casein particles in the cow's milk structure. These particles are calcium phosphate complexes in highly stable form [16]. In addition, it has been stated that the antibacterial enzymes, vitamin D and fluoride in cow's milk also play a role in the caries preventive effect [17, 18]. However, despite these advantages, cow's milk consumption is not recommended in the first year of life [19].

Demineralisation is the process of removing minerals such as calcium and phosphate from the hydroxyapatite crystals of the tooth enamel and is the initial stage for the formation of dental caries. Studies about the demineralisation effect of breast milk, formula milk and cow's milk are few and the results are controversial [20]. In this study, it was aimed to investigate and compare the possible demineralization effects of indispensable infant nutrients such as breast milk, formula milk and cow's milk on deciduous tooth surfaces with SEM-EDX analysis.

Materials and methods

Permission was obtained from Pamukkale University Non-Interventional Clinical Research Ethics Committee for the study.

Preparation of the tooth specimens

In order to be used in in-vitro experiments, a total of 25 newly extracted, caries-free healthy human primary teeth for 5 groups that were planned to be included in the study, with 5 teeth in each were collected. The collected teeth were kept in 0.1% thymol solution at room temperature until the time of the experiment. Before the experiments, the crowns of the teeth were separated from enamel-cement junction with the help of a diamond separe under water cooling. Then, the crown part of the teeth was divided into two from the midline in the sagittal direction, and each half of the teeth was embedded in 2 separate acrylic blocks with their labial surfaces up. Finally, these labial surfaces, which will be exposed to the test material, were polished using green and red color Shofu Super-Snap polishing discs (Shofu Inc., Kyoto, Japan) respectively.

Milk materials used in the experiments

Breast milk samples (Group 1) taken from a volunteer mother who was breastfeeding a 9-12 month-old baby were stored in separate storage bags in a deep freezer at -16°C until the experiment day, and before the each experiment, only the package to be used was thawed and brought to 37°C. Three different formula milks with similar contents of sugar (8-8.3 g/100 mL), calcium (75-76.2 mg/100 mL) and phosphorus (43-52 mg/100 mL) which are commonly found in the market and suitable for 9-12 months old babies: Aptamil Pronutra 3 (Group 2), Hipp Organic 3 (Group 3) and SMA Optipro 2 (Group 4) were freshly prepared in accordance with the manufacturer's instructions before the experiments and used at 37°C. Finally, a brand of Ultra High Temperature (UHT) cow's milk (Pınar Whole Milk) (Group 5), which is easily accessible in the market, was used at 37°C by opening a new package in each experiment.

Endogenous pH measurement of milk materials

Before the experiments, endogenous pH of 5 different test materials was measured with a digital pH meter (WTW InoLab pH 7110, Germany) at room temperature by placing the measuring tip of the device directly into the material. The pH meter accurate to 0.1 was first calibrated according to manufacturer's instructions using buffer standarts of pH 7 and pH 4. As much of 10 mL of each milk material was placed in a beaker, the pH meter was immersed into the milk and the value was recorded.

Preparation of artificial saliva

The artificial human saliva to be used in the research was prepared by a scientist from Department of Biochemistry of the Faculty of Science according to the recipe and method specified in the literature [21] excluding only sorbitol [22]. It contained Methyl-p-hydroxybenzoate 2.00g, carboxymethyl cellulose 10.0 g, KCl 0.625 g, $MgCl_2.6H_2O$ 0.059 g, $CaCl_2.2H_2O$ 0.166 g, K_2HPO_4 0.804 g, KH_2PO_4 0.326 g in 1000 mL of deionized water. The protein and mucin content of the natural saliva was simulated by the first two components, which increased the viscosity of the saliva, while the other ingredients provided the inorganic components at levels comparable with that of natural saliva. The pH of the artificial saliva was adjusted to 6.75 using KOH.

Immersion procedures

- During the experiment, the prepared acrylic blocks were kept into artificial saliva in a 5 mL beaker for each group in an oven (Nüve Oven KD400, İstanbul, Türkiye) at 37°C.
- Half of the divided tooth samples were exposed to the test material (experiment) and the other half was kept in artificial saliva throughout the experiment (control).
- Half teeth in the experimental groups were removed from the artificial saliva 4 times a day and kept in the tested milk material in the oven at 37°C for 15 minutes.
- At the end of the immersion period, the teeth were washed with distilled water and placed in artificial saliva again and kept in the oven until the next experiment.
- The experiments were repeated for 5 days and for each material, a total of 20 applications of 15 minutes were made on the tooth surface.

SEM-EDX analyzes

After the last experiments, the teeth were removed from the artificial saliva and posterior dehydration was performed in an ascending ethanol series (25, 50, 75, 95 and 100%). Following the specimens were mounted on stubs, sputter-coated with a rate of 80% gold and 20% palladium and got ready for scanning electron microscopy imaging.

A total of 10 half-tooth specimens in each group (5 experiments, 5 controls) observed at 20 kV accelerated voltage at 500, 1000, 1500 and 3000 times magnification with an SEM (Zeiss Supra 40 VP, Carl Zeiss SMT Inc., Oberkochen, Germany). SEM images of each experimental and control tooth sample were analyzed qualitatively. Erosion signs such

as loss of integrity, irregular enamel surface, roughness, crater formation, porosity, pitted or cracked surfaces and sporadic rod ends were investigated in the images. In addition, the changes in the mineral level of the enamel surfaces caused by the milk materials were evaluated using energy dispersive X-ray spectroscopy (EDX) (Zeiss Supra 40 VP, Carl Zeiss SMT Inc., Oberkochen, Germany) on a total of 50 tooth samples that 10 half tooth surfaces in each group. As a result of the surface element analysis of each tooth surface, Ca and P values were recorded quantitatively (wt%).

Statistical analyzes

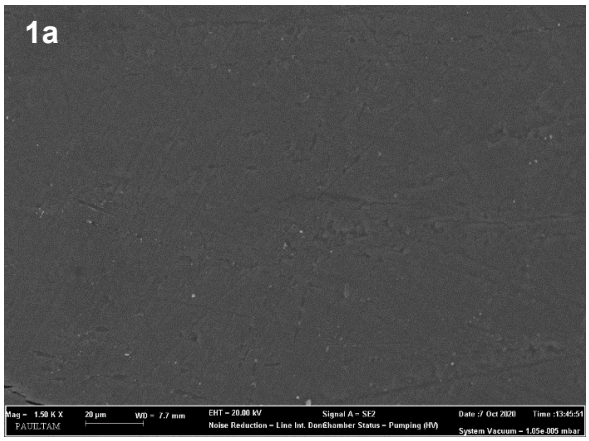
Data were analyzed with the SPSS package program (SPSS v23.0, SPSS Inc., Chicago, IL, USA). For the analysis of the differences intra-groups, the data which meet the prerequisites of parametric tests were analyzed with Independent-Samples T test, and the values which did not meet the prerequisites of parametric tests with the Man Whitney U test. For the analysis of the differences between groups Kruskal-Wallis test was used.

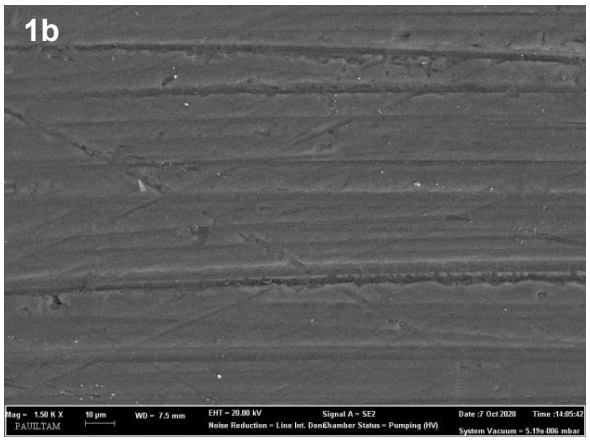
Results

The endogenous pH values of the tested milk materials are shown in Table 1. SEM images of the groups are shown in Figure 1. Qualitative evaluation of SEM images of experimental tooth surfaces in each group are listed in Table 2. A statistically significant decrease was observed in the amount of Ca on tooth surfaces exposed to breast milk and all the formula milks, on the other hand a statistically significant increase was observed in the amount of Ca of the teeth exposed to cow's milk. The amount of phosphorus increased in the Group 1, 3 and 5 after exposure to the tested milk material, and decreased in the Group 2 and 4. The mean SEM-EDX findings of Ca and P minerals of the teeth in each group and the differences within each group are given in Table 3. The decrease observed in the amount of Ca on the tooth surfaces exposured to breast milk was statistically significantly higher than that of formula milks (p=0.001). The differences observed in terms of the mean Ca decrease between the groups are given in Table 4.

Table 1. Endogenous pH values of the milk materials at room temperature

Groups	Milk materials	Endogenous pH value
Group 1	Breast milk	7.22
Group 2	Formula milk (Aptamil Pronutra 3)	7.16
Group 3	Formula milk (Hipp Organic 3)	7.20
Group 4	Formula milk (SMA Optipro 2)	7.19
Group 5	Cow's milk	7.16


1a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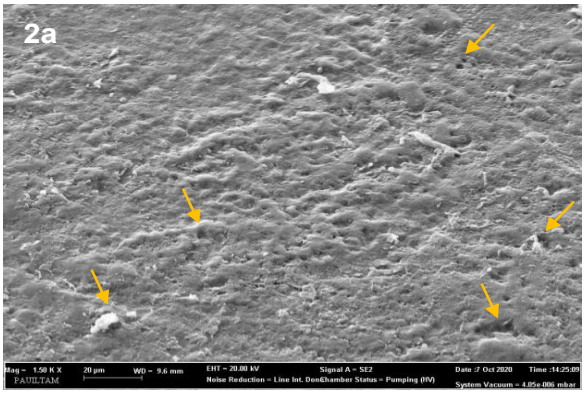
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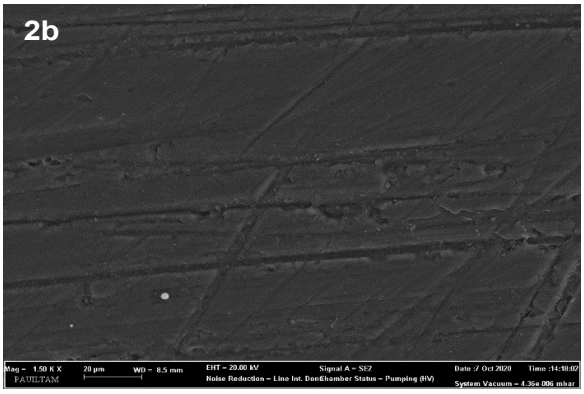
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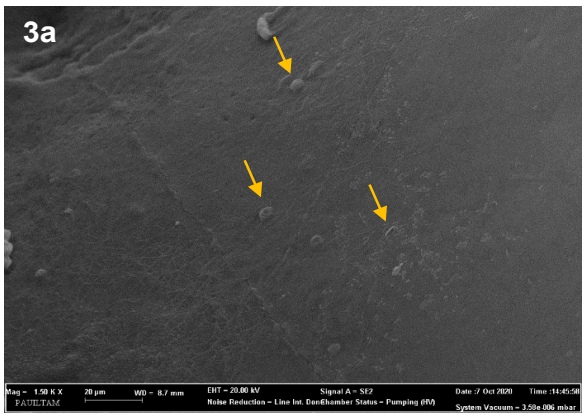
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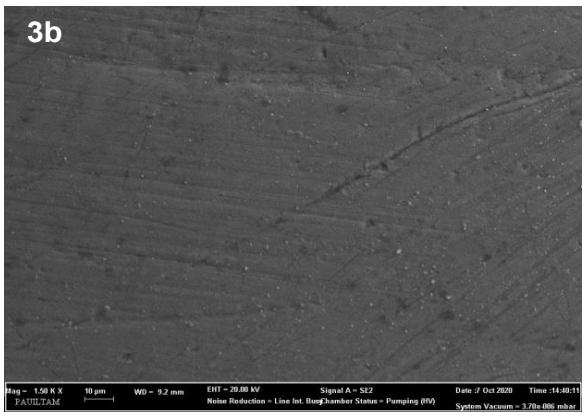
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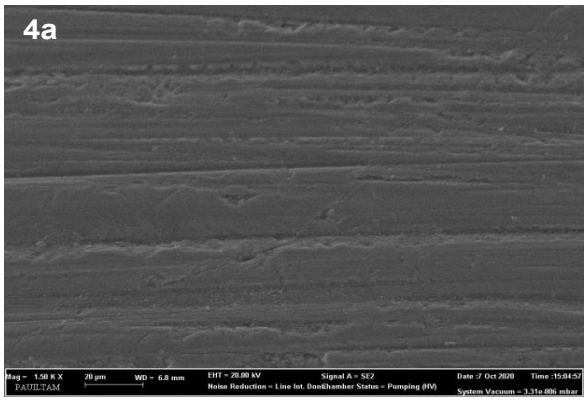
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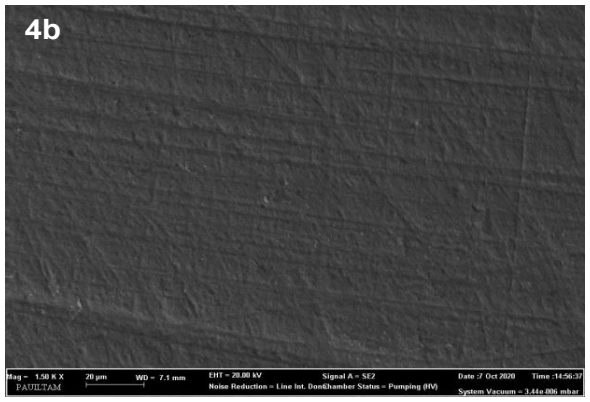
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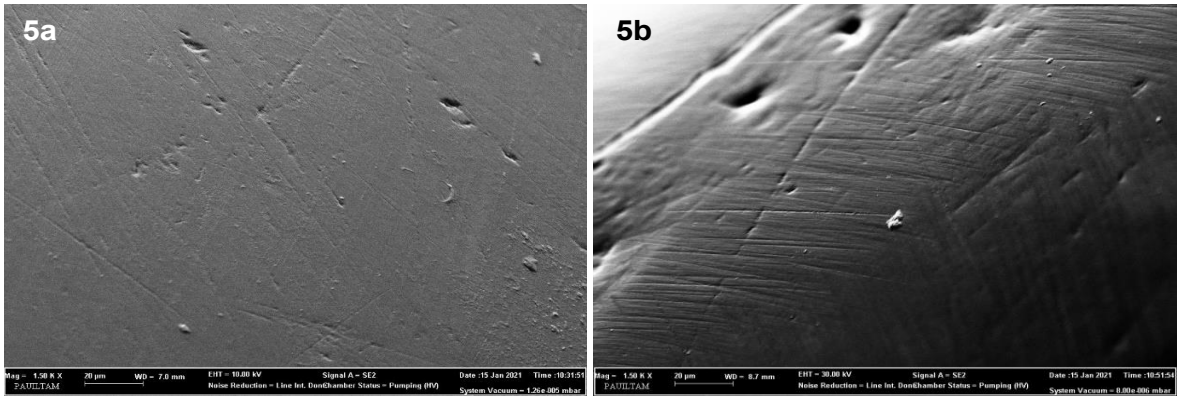

Figure 1. SEM images (1500x and 20.00 kV) of the groups

1a: Image of half tooth surface exposed to breast milk (experiment), **1b:** The other half of the tooth exposed to breast milk which is kept in only artificial saliva (control)
2a: Image of half tooth surface exposed to formula milk Aptamil Pronutra 3 (experiment), **2b:** The other half of the tooth exposed to formula milk Aptamil Pronutra 3 which is kept in only artificial saliva (control)
3a: Image of half tooth surface exposed to formula milk Hipp Organic 3 (experiment), **3b:** The other half of the tooth exposed to formula milk Hipp Organic 3 which is kept in only artificial saliva (control)
4a: Image of half tooth surface exposed to formula milk SmA Optipro 2 (experiment), **4b:** The other half of the tooth exposed to formula milk SmA Optipro 2 which is kept in only artificial saliva (control)
5a: Image of half tooth surface exposed to cow's milk (experiment), **5b:** The other half of the tooth exposed to cow's milk which is kept in only artificial saliva (control)

Table 2. Qualitative evaluation of SEM images of experimental tooth surfaces in each group

Group 1 **Breast milk**	Microstructure and integrity of the enamel is generally preserved. Little porosity is seen. No crater formation or sporadic rod ends.
Group 2 **Formula milk** **(Aptamil Pronutra 3)**	Is the group that the enamel integrity shows the most deterioration. Irregular and porous enamel surface. Crater formation, roughness and etched prism patterns are seen.
Group 3 **Formula milk** **(Hipp Organic 3)**	Increased enamel porosity. No crater formation or etched prism pattern but few sporadic rod ends available.
Group 4 **Formula milk** **(SMA Optipro 2)**	Microstructure and integrity of the enamel is generally preserved. No porosity, crater formation or sporadic rod ends.
Group 5 **Cow's milk**	Smooth enamel surface. No evidence of erosion. Is the group that experimental and control half of tooth sample gives the most similar appearance.

Table 3. SEM-EDX findings of Ca and P minerals of the teeth

Groups	Milk materials	Minerals wt%	Control mean±SD	Experiment mean±SD	*p** value
Group 1	Breast milk	Ca	34.48±0.11	26.99±0.14	0.009
		P	12.91±0.17	14.13±0.17	0.000
Group 2	Formula milk (Aptamil Pronutra 3)	Ca	33.39±0.08	28.90±0.19	0.009
		P	12.99±0.23	10.71±0.20	0.009
Group 3	Formula milk (Hipp Organic 3)	Ca	38.74±0.15	34.52±0.15	0.009
		P	11.98±0.29	14.91±0.15	0.009
Group 4	Formula milk (SMA Optipro 2)	Ca	29.68±0.19	24.95±0.33	0.009
		P	11.23±0.23	10.46±0.14	0.000
Group 5	Cow's milk	Ca	24.67±0.33	30.89±0.22	0.009
		P	9.82±0.40	12.56±0.33	0.009

SEM-EDX: Scanning Electron Microscopy- Energy Dispersive X-ray Spectroscopy
Ca: Calcium, P: Phosphorus, $p \leq 0.05$ values indicate a statistically significant difference
*p** value: differences of the Ca and *P* values between control and experimental groups in each group

Table 4. Intergroup differences in the decrease in calcium values

Groups	Milk materials	Mean decrease in calcium values (wt%)	Mean rank*	*p* value
Group 1	Breast milk	7.49	18.00[a]	**0.001**
Group 2	Formula milk (Aptamil Pronutra 3)	4.49	8.60[bc]	
Group 3	Formula milk (Hipp Organic 3)	4.22	3.40[b]	
Group 4	Formula milk (SMA Optipro 2)	4.73	12.00[cd]	

***** Same superscript lowercase letters show statistical similarity, different lowercases show statistical difference
$p \leq 0.05$ values indicate a statistically significant difference

Discussion

Nutrition type and nutrient intake are important factors in the etiology of early childhood caries. The cariogenic potential of consuming sucrose-containing liquids with a bottle is well known, but studies comparing breast milk, cow's milk, and formula milk are few and the results are controversial [5, 20, 23]. As a result of this in-vitro study investigating the possible demineralization effects of breast milk, commercially available formula milks and cow's milk on primary teeth, among all the tested milk materials with similar pH values, the material that caused the most deterioration quantitatively in the microstructure of the deciduous tooth enamel was the formula milk named Aptamil Pronutra 3, followed by the formula milk named Hipp Organic 3 that caused some porosity on the enamel surface. In the SEM images, the image in which the integrity of the enamel structure was best preserved was observed in the tooth sample exposed to cow's milk. In a similar study including the results of SEM analysis of the tooth surfaces after exposure to formula milk, breast milk, saliva and saline materials, similarly the roughest surface was observed in the formula milk group [5].

According to SEM-EDX results of the present study, the material causing the most Ca loss from deciduous tooth enamel was breast milk, followed by formula milk, but after exposure to cow's milk, the amount of Ca and P in deciduous tooth enamel increased. This result is consistent with a meta-analysis in the literature reporting that the cariogenic potential of breast milk is higher than that of cow's milk [20]. It also overlaps with the opinion that cow's milk is protective against dental caries and has more mineral content than breast milk [14, 15], however it is inconsistent with the finding that formula milk causes more inorganic substance loss than breast milk [5].

Liquids containing lactose can cause sudden decreases in oral pH and thus create an environment prone to caries. However, milk is a complex structure consisting of calcium, phosphorus, protein, vitamins and fats as well as lactose [24]. It has been reported that breast milk has lower mineral content, higher lactose level (7 versus 3%) and lower protein content (1.2 g/100 mL versus 3.3 g/100 mL) compared to cow's milk, but these content differences are not indicative of their cariogenic potential [25]. In this study, not only Ca and P levels decrease in tooth samples exposed to breast milk, but also more microstructural deterioration was observed compared to those exposed with cow's milk. Therefore, the data suggest that the content differences between breast milk and cow's milk directly affect their demineralizing potential.

Formula milk, which is another baby bottle feeding material, can be a cause of tooth decay due to various carbohydrates in it [8]. It has been stated that formula milk taken with a bottle during sleep at night, remains stagnant in the mouth due to low saliva flow and suckle-sleep-suckle cycle, and this causes the enzymatic deterioration of the casein which is the protective protein in its structure [26]. In this research, as a result of SEM analysis, Aptamil Pronutra 3 and Hipp Organic 3 material caused prominent roughness on the surface of the primary teeth, and as a result of the EDX analysis, both materials caused a significant loss of Ca and also a significant decrease in the amount of P on the surfaces of the deciduous teeth exposed to Aptamil Pronutra 3. In a study examining the decrease in plaque pH caused by infant formula milk, it was reported that the formula milk named Aptamil Pronutra 2 was one of the formula milks that caused the most decrease in plaque pH [23]. Researchers stated that this may be due to the lower fat content of this material compared to others [23].

Energy Dispersive X-ray Spectroscopy used in our research is an analytical technique used in conjunction with SEM to perform elemental analysis or examine the chemical characterization of a sample [27]. The principle of operation in this system is the emission of energy in the form of X-ray photons when electrons from external sources collide with atoms of the material. Thus, the X-ray characteristic of each element is formed. The characterization capabilities of this system are mainly due to the unique atomic structure of each element, which allows for the formation of peaks in the X-ray spectrum [28]. In this method, the relative number of X-rays detected by the energy spectrum is obtained and the quantitative determination of the elements can be made using a computer-based program [27, 28].

The milk materials examined in our research are indispensable for infant nutrition. For this reason, it is not possible to give up their consumption in order to prevent dental caries. However, it is not impossible to be protected from the cariogenic effects of these indispensable nutrients. Thanks to such in-vitro studies, it is revealed that nutritional materials such as formula milk and breast milk are not innocent in terms of causing dental caries and the awareness of parents and dentists on this issue should be increased. Dental caries can be prevented with a few simple precautions and lifestyle changes in infants who continue breast-feeding or bottle-feeding with formula milk. In the current guidelines of the AAPD, it is stated that brushing teeth with smear amount of fluoride toothpaste prevents dental caries in children aged 0-3 [29]. Due to the decrease in the flow rate of saliva and thus the protective effect of saliva from dental caries during sleep at night, especially after 1 year of age cessation of feeding during sleep at night is also effective in protecting from early childhood caries [30]. Present results were showing that cow's milk can play a protective role against dental caries, but wrong dietary habits such as adding refined

sugar, honey or various sweeteners into cow's milk drunk in early childhood may negatively change the cariogenic potential of cow's milk.

As a result of this in-vitro study, formula milks can cause deterioration in the microstructure of the enamel of the primary teeth. Breast milk and the examined formula milks cause a significant decrease in Ca amounts in primary tooth enamel, while exposure to cow's milk causes a significant increase in Ca and P amounts.

Conflict of interest: No conflict of interest was declared by the authors.

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Ethics committee approval: Permission was obtained from Pamukkale University Non-Interventional Clinical Research Ethics Committee for the study (permission date: March 03, 2020, number:5).

Authors' contributions to the article

C.C.E. constructed the main idea and hypothesis of the study. C.C.E. developed the theory and arranged/edited the material and method section. C.C.E. has done the evaluation of the data in the Results section. Discussion section of the article was written by C.C.E. and C.C.E. reviewed, corrected and approved the final version.

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Comparison of short-term radiographic outcomes of medial parapatellar, mini-midvastus, and subvastus surgical approaches in fast-track total knee arthroplasty

Fast-track total diz artroplastisinde medial parapatellar, mini-midvastus ve subvastus cerrahi yaklaşımlarının kısa dönem radyografik sonuçlarının karşılaştırılması

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Abstract

Purpose: Due to the difficulties in accessing the knee joint, correct prosthesis placement is of great importance during the implementation of total knee arthroplasty (TKA). This study aimed to compare short-term radiographic X-ray outcomes in patients who underwent fast-track TKA with medial parapatellar (MPP), mini-midvastus (mMV), or subvastus (SV) surgical approaches.

Materials and methods: Between 2018 and 2020, 93 patients operated with MPP, mMV, and SV surgical approaches and who had complete data of radiographic outcomes before and sixth-week postoperative were retrospectively analyzed and patients divided into three groups: MPP (n=31), mMV (n=31), and SV (n=31). The alignments of preoperative and sixth-week postoperative X-ray images of the surgical approaches were measured. The operative time of fast-track TKA implementation with MPP, mMV, and SV surgical approaches was recorded.

Results: The MPP group had a higher preoperative lateral distal femoral angle than the mMV group and a higher preoperative lateral proximal femoral angle than the SV group (p=0.018 and p=0.027, respectively). The mMV group had a higher postoperative proximal medial tibial angle than the SV group (p=0.011). In the postoperative sixth week, the MPP and mMV groups had a lower posterior tibial slope angle than the SV group (p=0.001). The MPP approach had significantly shorter operative time than the mMV and SV approaches (p=0.001).

Conclusion: The outcomes indicate that MPP, mMV, and SV surgical approaches are preferable and feasible in obtaining a satisfactory prosthesis alignment during fast-track TKA. The MPP approach may be preferable because of its shorter operative time and potential advantage in minimizing surgical complication risks.

Keywords: Total knee replacement, surgical approaches, radiography, operative time.

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Öz

Amaç: Diz eklemine erişimdeki zorluklar nedeniyle, total diz artroplastisi (TDA) uygulaması sırasında doğru protez yerleşimi büyük önem taşımaktadır. Bu çalışmanın amacı medial parapatellar (MPP), mini-midvastus (mMV) veya subvastus (SV) cerrahi yaklaşımları ile fast-track TDA uygulanan hastalarda kısa dönem radyografik X-ray sonuçlarını karşılaştırmaktır.

Gereç ve yöntem: 2018-2020 yılları arasında MPP, mMV ve SV cerrahi yaklaşımlarıyla opere edilen ve cerrahi öncesi ve cerrahi sonrası altıncı hafta radyografik sonuçları eksiksiz olan 93 hastanın verisi retrospektif olarak analiz edildi ve hastalar üç gruba ayrıldı: MPP (n=31), mMV (n=31) ve SV (n=31). Cerrahi yaklaşımların cerrahi öncesi ve cerrahi sonrası altıncı hafta X-ray görüntülerine ait dizilimleri ölçüldü. MPP, mMV ve SV cerrahi yaklaşımlarıyla uygulanan fast-track TDA'nın operasyon süresi kaydedildi.

Bulgular: MPP grubu, cerrahi öncesinde mMV grubuna göre daha yüksek lateral distal femoral açıya ve SV grubuna göre daha yüksek lateral proksimal femoral açıya sahipti (sırasıyla p=0,018 ve p=0,027). mMV grubunun cerrahi sonrası proksimal medial tibial açısı SV grubuna göre daha yüksekti (p=0,011). Cerrahi sonrası altıncı haftada, MPP ve mMV grupları SV grubuna göre daha düşük posterior tibial eğim açısına sahipti (p=0,001). MPP yaklaşımı, mMV ve SV yaklaşımlarına göre anlamlı derecede daha kısa operasyon süresine sahipti (p=0,001).

Sonuç: Sonuçlar, MPP, mMV ve SV cerrahi yaklaşımlarının fast-track TDA sırasında memnun edici bir protez dizilimi elde etmede tercih edilebilir ve uygulanabilir olduğuna işaret etmektedir. MPP yaklaşımı daha kısa operasyon süresi ve cerrahi komplikasyon risklerini en aza indirmedeki potansiyel avantajı nedeniyle tercih edilebilir.

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Anahtar kelimeler: Total diz artroplastisi, cerrahi yaklaşımlar, radyografi, operasyon süresi**.**

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Introduction

Total knee arthroplasty (TKA) is a standard surgical treatment for end-stage knee osteoarthritis (KOA) patients [1]. Fast-track procedures in the surgery of TKA include preoperatively informing the patients regarding nutrition and optimal pain control, transition to early postoperative oral nutrition, early mobilization, early rehabilitation, and early discharging [2]. Recent evidence indicates that fast-track surgical procedures support early rehabilitation and recovery after TKA [3]. The outcomes of TKA, as determined mainly by patient-reported scales, are entirely acceptable; however, certain factors may affect patient satisfaction and comfort [4]. Notably, correct alignment of the prosthesis implemented in the knee radiologically and planarly in the desired angle ranges significantly affects knee kinematics, knee range of motion, knee functional scores, and implant failure rates [5].

Various surgical approaches have been utilized for satisfactory postoperative recovery, shortest hospital stay, best joint alignment, and minimal risk of complications [6]. These approaches employed in TKA surgery are the traditional medial parapatellar (MPP), mini-midvastus (mMV), and subvastus (SV) surgical approaches [6, 7]. The MPP surgical approach, generally used in TKA surgery, is considered a simple and standardized approach that visually provides a broad and desired joint opening during surgery [6]. However, the MPP approach is known to carry functional risks, including decreased knee extensor strength, decreased blood supply to the patellar region, and proximal deep venous thrombosis in the short and long term [8-10]. The mMV and SV approaches, considered minimally invasive surgical methods, have advantages and disadvantages [11]. The mMV surgical approach has demonstrated proficiency in preserving the quadriceps tendon and achieving precise component alignment, even in knees with substantial deformities [8, 11, 12]. However, the drawback of the mMV surgical approach is that it requires some splitting of the extensor mechanism [9]. The SV approach is one of the alternative methods used in TKA surgery. The SV approach is an anatomical surgery that protects the medial retinaculum and vastus medialis obliquus muscle and minimizes blood loss [9, 11]. Nevertheless, the SV surgical approach could potentially induce adverse effects on the positioning of prostheses and the alignment of extremities due to constraints within the limited operative space [13].

There is ongoing debate about the preference for MPP or mMV approaches in TKA surgery [8]. In previous studies, MPP and SV surgical approaches [8, 14-16] and mMV and SV surgical approaches [17] were compared regarding radiographic appearance and alignment, and the results were found to be acceptably equivalent. In addition, in a prior investigation comparing the radiologic Hip-knee-ankle (HKA) angle across the MPP, mMV, and SV surgical approaches in TKA, the researchers noted that all three approaches yielded comparable radiologic outcomes [6]. There are concerns that minimally invasive surgical approaches may make achieving correct component alignment during TKA challenging due to difficulty accessing the knee joint during the operation [17]. Although the mMV and SV approaches offer clinical advantages over the traditional MPP approach, such as shorter hospitalization and lower pain levels [6, 9], it is unclear whether they create an advantage or disadvantage regarding component placement and alignment due to the difficulty of arthrotomy [9]. Therefore, this study aimed to compare short-term radiographic X-ray outcomes in patients who underwent fast-track TKA with MPP, mMV, and SV surgical approaches.

Materials and methods

This retrospective study was performed with patients who underwent fast-track TKA surgery with MPP, mMV, and SV surgical approaches for KOA in the Orthopedics and Traumatology Department of Pamukkale University Hospital between January 2018 and January 2020. A written informed consent was obtained from

all patients. Ethics Committee approval was obtained from the author's affiliated ethics committee. The study was conducted under the principles of the Declaration of Helsinki.

Study design

Preoperative and postoperative sixth-week radiographic X-ray outcomes of the patients operated with MPP, mMV, or SV surgical approaches for KOA were retrospectively analyzed. In addition, the duration of fast-track TKA implementation completed with MPP, mMV, or SV surgical approaches was recorded.

Participants

Patients who were admitted to the orthopedics and traumatology clinic of Pamukkale University Hospital for KOA and underwent fast-track TKA with MPP, mMV, or SV surgical approaches and met the inclusion and exclusion criteria were included in the study. A G*power (Version 3.1) analysis program determined the study's sample size. According to the priori power analysis of the F-tests of one-way analysis of variance (ANOVA) test based on a tibial posterior slope (SLOP) angle of the reference study [14], the priori calculated sample size was at least 87 patients (29 per group) with a power of 90% (d=0.39), *a* level of .05. A total of 93 patients with complete demographic and preoperative and sixth-week postoperative radiographic data were divided into three groups: MPP (n=31), mMV (n=31), and SV groups (n=31). Inclusion criteria were as follows: being between 50-75 years of age, undergoing fast-track TKA surgery with MPP, mMV, or SV surgical approaches due to KOA, having radiologic images before fast-track TKA surgery, and at six weeks after surgery, and understanding the verbal and written information given. Exclusion criteria were as follows: revision TKA surgery, American Society of Anesthesiologists (ASA) classification score >3, rheumatoid arthritis, history of previous surgery on the affected extremity, neurologic disease which causes functional disability, psychiatric disorder, uncorrectable hearing or visual impairment, use of hearing aids, and morbid obesity (body mass index >40 kg/m^2).

Surgical approaches

The same institutional fast-track surgical protocol was utilized on all patients as previously described [18]. All patients underwent using MPP, mMV or SV surgical approaches by the same surgical team using the same brand of ligament-cutting fixated TKA (NexGen Legacy® Posterior Stabilized (LPS-Flex) Knee-Fixed Bearing, Zimmer-Biomet Inc., Warsaw, Indiana 46580, USA), the same brand of polymethyl methacrylic acid (PMMA) and bone cement (Oliga- G21 srl-Vias. Pertini,8-41039 San Possodonio (MO)-Italy) and surgical approaches were performed by the same surgical team. All operations were performed without the use of a tourniquet.

During the fast-track TKA surgery, the silicone supports were placed in all patients to give the knee a 90-degree flexion position. In the MPP surgical approach, the vastus medialis muscle was separated proximally with an incision in the quadriceps tendon. Then, the incision was continued along the medial retinaculum and patellar tendon, and the incision was terminated approximately 0.5-1 cm medial to the tibial tuberosity [19, 20]. In the mMV surgical approach, the incision in the arthrotomy stage following the skin incision was applied parallel to the muscle fibers of the vastus medialis. After the attachment site of the vastus medialis muscle to the patella was exposed, it was separated as a split parallel to the muscle fibers. In the arthrotomy stage, the incision was made at the superomedial corner of the patella, then medial to the patellar tendon, and terminated medial to the tibial tuberosity [20]. In the SV surgical approach, after the skin incision, the vastus medialis muscle was advanced along the inferior border of the muscle with blunt dissection proximally without touching the patella and quadriceps tendon attachment sites. Distally, it was terminated medial to the patellar tendon and medial to the tibial tuberosity [21].

Outcome evaluations

The demographic (age, body mass index, and gender) and clinical (dominant extremity, affected extremity, and infection) characteristics of the patients were recorded. Radiographic X-ray outcomes of the knee were measured on the radiographic images of all patients preoperative and six weeks after fast-track TKA surgery. For the alignment analysis, preoperative and postoperative orthorhontgenograms of all patients were obtained using the Materialise OrthoView (OrthoView 7th version, Materialise HQ, Technologelaan 15 3001 Leuven, Belgium)

program. HKA angle, femorotibial (FT) angle, lateral distal femoral (LDF) angle, lateral proximal femoral (LPF) angle, proximal medial tibial (PMT) angle, lateral distal tibial (LDT) angle, and SLOP angles were measured and recorded by a single-blinded physician using appropriate measurement techniques on the radiographic X-ray images (Figure 1).

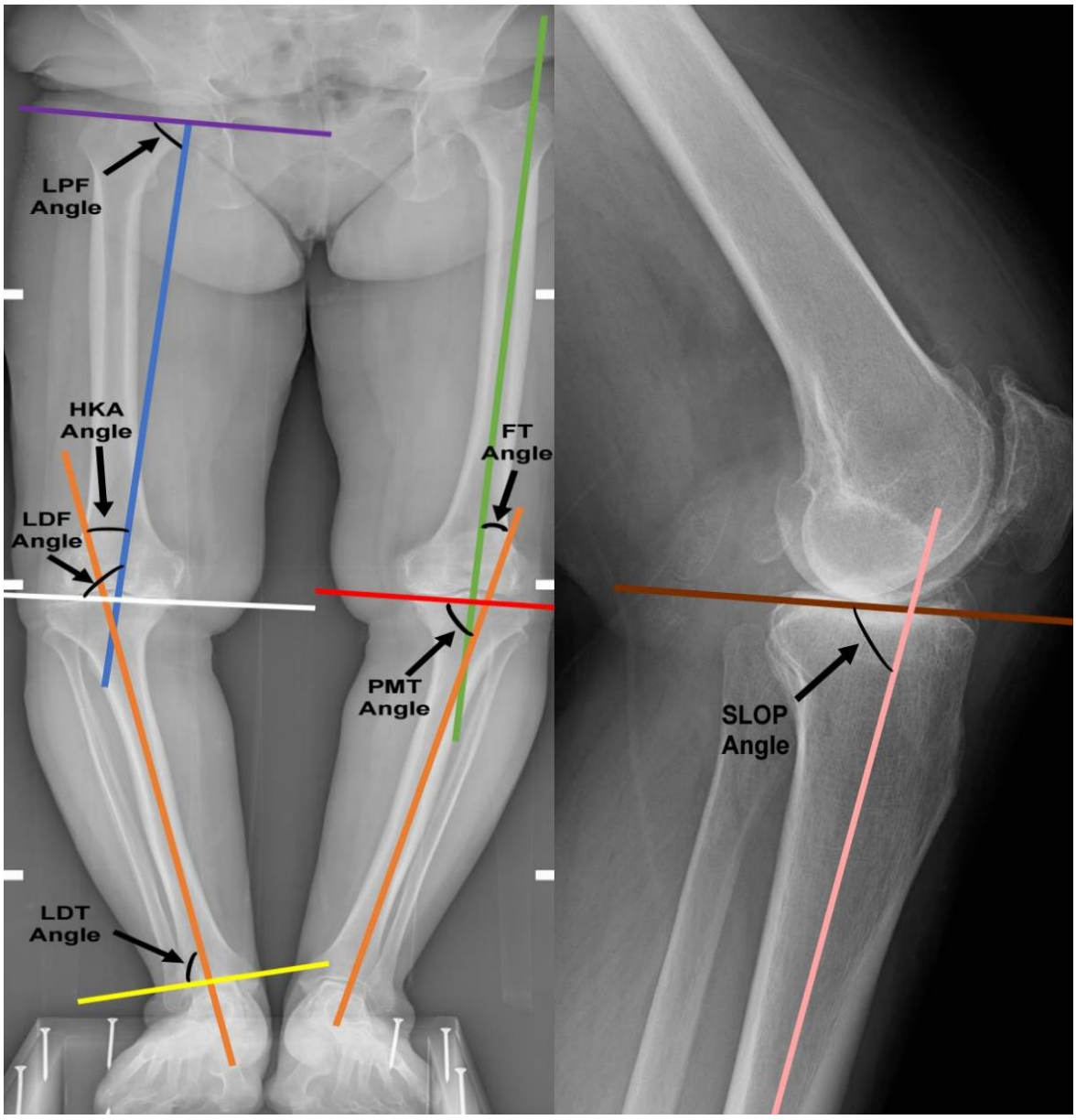

Figure 1. Radiological angles utilized in the study

Orange Line: Mechanical and anatomical axis of the tibia
Blue Line: Mechanical axis of the femur
Green Line: Anatomical axis of the femur
White Line: The distal transcondylar line of the femur
Red Line: Proximal transtibial line of the tibia
Yellow Line: Tibia distal joint line
HKA: Hip-knee-ankle, LDF: Lateral distal femoral, LPF: Lateral proximal femoral
PMT: Proximal medial tibial, LDT: Lateral distal tibial, SLOP: Tibial posterior slope, FT: Femorotibial

Hip-knee-ankle angle: To measure the HKA angle, the femur and the tibia's mechanical axis are drawn as two lines. The angle between these two lines is the HKA angle, defined as a deviation from 180 degrees [22].

Femorotibial angle: FT angle is formed by the intersection of the femur's anatomical axis and the tibia's anatomical axis [23, 24].

Lateral distal femoral angle: The LDF angle is the lateral angle between the distal transcondylar line and the mechanical axis of the femur [23].

Lateral proximal femoral angle: The LPF angle represents the angle formed laterally by the line from the midpoint of the femoral head to the apex of the greater trochanter and the mechanical axis of the femur [25-27].

Proximal medial tibial angle: The PMT angle is the medial angle between the transtibial axis and the mechanical axis of the tibia. The PMT angle was determined by assessing the angle formed between a line drawn from the most proximal medial to proximal lateral points of the tibial component and another line connecting the center of the tibial medullary canal [28, 29].

Lateral distal tibial angle: The LDT angle is the lateral angle between the tibia's anatomical axis and the tibia's distal articular surface line [27, 30].

Tibial posterior slope angle: The SLOP is defined as the angle formed on lateral axis radiographs between a line perpendicular to the long axis of the tibia and a line parallel to the medial tibial plateau [14, 31].

Statistical analysis

The SPSS (Version 25; IBM, Armonk, NY, USA) program was used to evaluate the data obtained statistically. Data were presented as mean and standard deviation. The ANOVA test was used to compare the angular variables of the groups when normal distribution data were provided; Kruskal Wallis tests were used when non-normal distribution data were provided. In intragroup comparisons, the paired samples *t*-test was used when normal distribution data were provided, and the Wilcoxon test was used when non-normal distribution data were provided. The statistical significance level was accepted as $p<0.05$.

Results

The comparisons of the groups, including demographic characteristics and operative time of the surgery, are shown in Table 1. The groups showed similar results in age and body mass index ($p>0.05$). Regarding surgical time, the MPP surgical approach (65.48 minutes) was significantly shorter than the mMV (77.12 minutes) and SV (77.67 minutes) surgical approaches ($p<0.05$). In the MPP group, 90.3% of patients were female, and 9.7% were male; in the mMV group, 96.8% of patients were female, and 3.2% were male; and in the SV group, 87.1% of patients were female, and 12.9% were male. The right extremity was dominant in 93.5% of patients in the MPP group, 96.8% in the mMV group, and 96.8% in the SV group. In the MPP group, 51.6% of patients were operated on the right and 48.4% on the left extremity; in the mMV group, 48.4% of patients were operated on the right and 51.6% on the left extremity; and in the SV group, 41.9% of patients were operated on the right and 58.1% on the left extremity. No infection was observed in any patients (Table 1).

The MPP group had a significantly higher preoperative LDF angle than the mMV group and a significantly higher preoperative LPF angle than the SV group ($p=0.018$ and $p=0.027$, respectively). The mMV group had a significantly higher postoperative PMT angle than the SV group ($p=0.011$). The MPP and mMV groups had significantly lower postoperative SLOP angles than the SV group ($p=0.001$) (Table 2).

The MPP group significantly improved the HKA, FT, LDF, LPF, PMT, and SLOP angles between the preoperative and the sixth week after surgery ($p<0.05$). The mMV group significantly improved the HKA, FT, LDF, LPF, PMT, and SLOP angles between the preoperative and the sixth week after surgery ($p=0.001$). In the SV group, significant improvement was detected in the HKA, FT, LPF, PMT, and SLOP angles between the preoperative and the sixth week after surgery ($p=0.001$) (Table 2).

Table 1. The demographic and clinical characteristics of the groups and the comparison of the operative time of the surgical approaches

Variables	[1]MPP (n=31) Mean (SD)		[2]mMV (n=31) Mean (SD)		[3]SV (n=31) Mean (SD)		*p*
Age (year)	63.51 (6.90)		64.80 (7.45)		64.25 (6.86)		0.772
Body mass index (kg/m²)	30.01 (2.82)		28.57 (3.22)		29.69 (4.27)		0.243
The operative time (minutes)	65.48 (2.61)		77.12 (3.31)		77.67 (3.86)		**0.001***1-2, 1-3
	n	**%**	**n**	**%**	**n**	**%**	
Gender							
Female	28	90.3	30	96.8	27	87.1	0.384
Male	3	9.7	1	3.2	4	12.9	
Dominant extremity							
Right	29	93.5	30	96.8	30	96.8	0.770
Left	2	6.5	1	3.2	1	3.2	
Operated extremity							
Right	16	51.6	15	48.4	13	41.9	0.739
Left	15	48.4	16	51.1	18	58.1	
Infection							
Yes	0	0	0	0	0	0	-
No	31	100	31	100	31	100	

[1]MPP: Medial parapatellar approach, [2]mMV: mini-midvastus approach, [3]SV: Subvastus approach, SD: Standard deviation
kg: kilogram, m: meter
p: Value of the independent group comparison analysis

Table 2. Comparison of the alignments of preoperative and sixth-week postoperative X-ray images of the surgical approaches

Variables (angle)	[1]MPP (n=31) Mean (SD)	[2]mMV (n=31) Mean (SD)	[3]SV (n=31) Mean (SD)	p^1
HKA				
Preoperative	-13.9 (5.25)	-12.66 (4.38)	-12.21 (5.25)	0.387
Sixth-week after TKA	-1.34 (3.53)	-1.23 (2.94)	-1.10 (3.04)	0.956
p^2	**0.001***	**0.001***	**0.001***	
FT				
Preoperative	-7.45 (4.07)	-6.23 (3.79)	-6.83 (4.96)	0.541
Sixth-week after TKA	3.75 (2.57)	4.37 (2.50)	3.62 (3.08)	0.107
p^2	**0.001***	**0.001***	**0.001***	
LDF				
Preoperative	91.61 (2.60)	89.81 (2.97)	90.18 (2.12)	**0.018***[1-2]
Sixth-week after TKA	90.58 (1.74)	90.32 (2.36)	90.60 (1.82)	0.834
p^2	**0.019***	0.234	0.335	
LPF				
Preoperative	88.66 (2.70)	90.47 (3.17)	91.10 (4.73)	**0.027***[1-3]
Sixth-week after TKA	90.36 (3.33)	90.59 (3.24)	90.90 (3.90)	0.836
p^2	**0.001***	**0.001***	**0.001***	
PMT				
Preoperative	85.46 (3.62)	84.74 (3.85)	83.99 (3.47)	0.290
Sixth-week after TKA	89.36 (1.51)	90.38 (2.01)	89.03 (1.83)	**0.011***[2-3]
p^2	**0.001***	**0.001***	**0.001***	
LDT				
Preoperative	88.11 (3.98)	87.81 (3.68)	88.23 (5.02)	0.923
Sixth-week after TKA	87.85 (4.27)	88.45 (3.24)	87.34 (4.02)	0.527
p^2	0.684	0.373	0.203	
SLOP				
Preoperative	7.99 (3.57)	9.21 (2.64)	8.49 (1.60)	0.216
Sixth-week after TKA	6.11 (1.39)	5.54 (1.27)	7.35 (0.27)	**0.001***[1-3, 2-3]
p^2	**0.006***	**0.001***	**0.001***	

[1]MPP: Medial parapatellar approach, [2]mMV: mini-midvastus approach, [3]SV: Subvastus approach, SD: Standard Deviation, FT: Femorotibial HKA: Hip-knee-ankle, LDF: Lateral distal femoral, LPF: Lateral proximal femoral, PMT: Proximal medial tibial, LDTA: Lateral distal tibial SLOP: Tibial posterior slope

p^1: Value of the independent group comparison analysis

p^2: Value of the dependent group comparison analysis

Discussion

This study aimed to compare the radiographic results related to the knee joint in patients who underwent fast-track TKA with MPP, mMV, and SV surgical approaches. The study's results determined that fast-track TKA performed with medial parapatellar and mini-midvastus surgical approaches provided a better prosthesis alignment in the posterior tibial inclination angle after surgery. The mMV group displayed a remarkably higher postoperative PMT angle than the SV group. Moreover, the observed improvements in the normative values in the HKA, FT, LDF, LFTA, PMT, and LDT angular measurements postoperatively across all groups highlight the efficacy of the MPP, mMV, and SV surgical interventions in prosthesis alignment. Regarding operative time, fast-track TKA surgery performed with the MPP surgical approach was completed in a shorter surgery time than mMV and SV surgical approaches.

In recent years, many studies have examined the results of minimally invasive surgical approaches, mMV and SV, compared with the traditional MPP surgical approach [9]. However, the results of these surgical approaches regarding prosthesis alignment after TKA are limited [15, 32]. An earlier investigation reported that the normative values of the HKA angle ranged between 1 and 1.5 degrees [33]. Another study focusing on TKA utilizing the MPP or lateral approaches revealed a mean postoperative HKA angle of 0.976 [5]. Our study observed that all three surgical approaches provided prosthesis alignment in the normative value range between 1-1.5 degrees in patients who underwent fast-track TKA.

TKA aims to provide a normal prosthesis alignment, and the accepted normative range for the FT angle generally falls within approximately 5-7 degrees [24, 28]. Previous investigations utilizing the MPP surgical approach have reported an FT angle of 0.6±3.3 degrees [34] and a mean FT angle of 4 degrees post-TKA surgery [35]. Comparative studies between the MPP and mMV surgical approaches have demonstrated similar postoperative FT angles of 6.1 and 6.5 degrees [35] and 6.6 and 6.4 degrees, respectively, with no significant intergroup differences observed [36]. Our study confirms these findings, revealing mean postoperative FT angles of 3.75 in the MPP group, 4.37 in the mMV group, and 3.62 in the SV group. These values align closely with established normative values and existing literature.

The normative values of LDF, LPF, and PMT angles were determined to be between 85-95 degrees [25, 29]. It is argued that the deviation of the LDF and PMT angles of approximately 5 degrees from 90 degrees after TKA is seriously discussed in terms of outcomes [37]. Similarly, in a previous study, LPF angle was 91.6±0.1 degrees in patients after TKA [26]. In our study, the LDF, LPF, and PMT angles in the MPP, mMV, and SV groups were found to be in the range of 89.03-90.90 degrees, which is within the ranges recommended in the literature and compatible with other literature findings.

The distal tibial articular surface and the anatomical axis of the tibia form an LDT angle. The normative value of the LDT angle ranges between 86 and 92 degrees [25]. A previous study reported a mean LDT angle of 87.3 degrees in patients who underwent TKA with the MPP surgical approach [38]. In our study, all three surgical approaches had LDT angles in the range of 87.34-88.45 degrees, consistent with the normative values in the literature and previous literature findings.

Increasing the SLOP angle, which refers to the tibial slope, widens the already increased flexion deficit due to the incision of the posterior cruciate ligament and, if increased greatly, can result in a posteriorly displaced knee [31]. The normative SLOP values typically range from 0 to 7 degrees [14]. Previous studies examining the SLOP angle after TKA with the MPP surgical approach found SLOP angle values to vary between 7-8.1 degrees [39, 40]. SLOP angles after TKA with MPP and SV surgical approaches were 5.1 and 4.08 degrees, respectively [14]. Similarly, a previous study found that the SLOP angle was 5.3±0.4 degrees in the ligament cutting group after TKA was performed with the MPP surgical approach [31]. Our study found that MPP and mMV surgical approaches (6.11 and 5.54 degrees, respectively) were significantly lower than the SV approach (7.35 degrees). Our results were found to be consistent with the results of previous studies in the literature.

The duration of surgery is one of the critical points in the TKA process. The existing literature shows that surgical operative time is longer in mMV and SV surgical approaches compared to conventional methods [32]. A prior investigation found that the MPP and SV surgical approaches demonstrated similar surgical durations of 80 and 75 minutes, respectively [16]. Regarding the operative time, it is reported that mMV and SV surgical approaches take an average of 18 minutes longer than the MPP surgical approach [32]. Patients who underwent TKA with the SV or MPP surgical approach discovered that the SV surgical approach had a longer surgical time of meanly 13 minutes [7] and more [41]. A previous meta-analysis showed that the mMV surgical approach had a significantly longer duration of surgery than the MPP surgical approach [11]. In our study, the duration of the MPP surgical approach was shorter (65.48 minutes) than the mMV (77.12 minutes) and SV (77.67 minutes) surgical approaches in accordance with the findings in the literature.

Prior investigations highlighted the complications associated with the MPP and

minimally invasive surgical approaches. The MPP surgical approach may bear potential complications such as patellar fracture and patellofemoral instability [8]. The quadriceps-sparing technique exhibited superficial and deep infections, peroneal nerve palsy, and supracondylar fractures attributable to the constrained visual field inherent to this approach [42]. Likewise, a previous study documented that minimally invasive surgical methodologies, such as the mMV and SV approaches, prolonged surgical duration and may be linked with potentially significant complications, including challenges related to the learning curve and mastering difficulties [6]. In the present study, none of these potential complications were encountered among fast-track TKA patients who underwent procedures utilizing the MPP, mMV, and SV surgical approaches, as stated in previous reports [20, 36]. In this study, we think that the routine preference of MPP, mMV, or SV surgical approach utilization in the clinical setting, the minimal learning effect of the experienced surgeon regarding surgical approaches, and the involvement of the same surgical team during surgical operations may be effective in preventing these potential complications.

Our study has several limitations. The first limitation is that we did not evaluate the knee range of motion at preoperative and postoperative week 6. The second limitation is that we should have made long-term radiologic X-ray outcome follow-ups. Lastly, the substantial predominance of female participants in our study (87.1-96.8%) may not accurately reflect the demographic composition of the normal patient distribution in TKA surgery, thereby constituting a limitation of our study.

In conclusion, in this study, which aimed to compare the radiographic results related to the knee joint in patients who underwent fast-track TKA with MPP, mMV, and SV surgical approaches, it was observed that all three approaches were within the radiographic angle ranges recommended by the literature and were compatible with the literature findings. The results obtained from our study indicate that MPP, mMV, and SV surgical approaches are feasible in fast-track TKA and help to obtain a satisfactory prosthesis alignment. The MPP surgical approach might be deemed more suitable and preferable for achieving a shorter operative time, potentially conferring an advantage over the mMV and SV surgical approaches in minimizing surgical complications. In future surgical procedures, patients' postoperative clinical and functional status following MPP, mMV, and SV surgical approaches and their satisfaction with the chosen surgical technique will need to be specifically considered. In addition, further analyses with extended patient populations and long-term radiologic X-ray outcomes are needed.

Conflict of interest: No conflict of interest was declared by the authors.

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Authors' contributions to the article

H.R.G. constructed the main idea and hypothesis of the study. G.B. and H.Z. developed the theory and arranged/edited the material and method section. H.Z. made the measurements and drafted the data. G.B. has done the statistical analysis and evaluation of the data in the results section. The discussion section of the article was written by G.B., H.Z., and H.R.G. and G.B., H.Z., and H.R.G. reviewed, corrected, and approved. In addition, all authors discussed the entire study and approved the final version.

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The role of Odoroside A in immune transformation in RAW264.7 cells

RAW264.7 hücrelerinde Odoroside A'nın immün transformasyondaki rolü

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Abstract

Purpose: Macrophages are one of the most important elements of the immune system and play dual role in inflammation. They regulate both the formation of the immune response in the environment and the suppression. This signaling in the cancer microenvironment affects the progression of cancer. This cancer-promoting or cancer-eliminating process is managed by macrophages. The increase in pro-inflammatory cytokine genes provides suppression of tumor cells in the environment. It was aimed to investigate the effect of Odoroside A on macrophage transformation.

Materials and methods: The non-lethal dose of Odoroside A in macrophage cells was determined by CCK-8 analysis. Then, gene expression analysis of cytokines genes and surface markers in RAW264.7 cells was performed by using qRT-PCR. The protein levels of the cytokines that were significant in the gene expression analysis were examined using the ELISA method.

Results: Odoroside A significantly increased the expression of IL1 and IL6 genes in RAW264.7 cells, while it significantly decreased the expression of IL10 gene. There was no significant change in IL4 and TGFβ gene expressions. IL1 and IL6 gene expressions, which had a statistically significant increase, were also found to increase significantly at the protein level.

Conclusions: When all the data are evaluated together, it has been observed that Odoroside A increases the pro-inflammatory response in RAW264.7 cells, thus providing M1 type macrophage transformation. This suggests that Odoroside A may be effective in tumor elimination of the macrophage-mediated environment in the tumor niche.

Keywords: Odoroside A, macrophage, cytokines.

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Öz

Amaç: Makrofajlar bağışıklık sisteminin en önemli unsurlarından biridir ve inflamasyonda ikili rol oynamaktadır. Hem ortamda immün yanıtın oluşumunu hem de baskılanmasını düzenlemektedir. Kanser mikroçevresindeki bu sinyalleme, kanserin ilerlemesini etkiler. Bu kanseri teşvik eden veya kanseri ortadan kaldıran süreç makrofajlar tarafından yönetilmektedir. Proinflamatuar sitokin genlerinin ifadesindeki artış, ortamdaki tümör hücrelerinin baskılanmasını sağlar. Bu çalışmada Odoroside A'nın makrofaj dönüşümüne etkisinin araştırılması amaçlanmıştır.

Gereç ve yöntem: Makrofaj hücrelerinde öldürücü olmayan Odoroside A dozu CCK-8 analizi ile belirlenmiştir. Daha sonra RAW264.7 hücrelerinde sitokin genlerinin ve yüzey belirteçlerinin qRT-PCR kullanılarak gen ekspresyon analizi yapılmıştır. Gen ekspresyonu analizinde anlamlı çıkan sitokinlerin protein düzeyleri ELISA yöntemi kullanılarak incelenmiştir.

Bulgular: Odoroside A, RAW264.7 hücrelerinde IL1 ve IL6 genlerinin ekspresyonunu önemli ölçüde artırırken, IL10 geninin ekspresyonunu önemli ölçüde azaltmıştır. IL4 ve TGFβ gen ifadelerinde anlamlı bir değişiklik olmamıştır. İstatistiksel olarak anlamlı artış gösteren IL1 ve IL6 gen ekspresyonlarının protein düzeyinde de anlamlı düzeyde arttığı belirlenmiştir.

Sonuç: Tüm veriler birlikte değerlendirildiğinde Odoroside A'nın RAW264.7 hücrelerinde proinflamatuar yanıtı arttırdığı, dolayısıyla M1 tip makrofaj dönüşümünü sağladığı gözlenmiştir. Bu, Odoroside A'nın, tümör nişindeki makrofaj aracılı oluşan proinflamatuar ortamın tümörün ortadan kaldırılmasında etkili olabileceğini düşündürmektedir.

Anahtar kelimeler: Odoroside A, makrofaj, sitokinler.

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Introduction

Macrophages are the first cell group to respond among immune cells. Macrophages can be classified as classically activated M1 (pro-inflammatory) or alternatively activated M2 (anti-inflammatory) phenotypes based on their pro- and anti-inflammatory functions under various stimuli [1]. M1 macrophages play a key role in the positive immune response by presenting antigens, secreting pro-inflammatory cytokines and chemokines, and acting as immune monitors. The primary pro-inflammatory cytokines that are produced by it are TNFα, IL6, and IL12. M2 macrophages secrete a variety of anti-inflammatory cytokines, including TGF-β, IL10, and Arginase-I. These cytokines can reduce inflammation, promote tumor growth, and have an immune-suppressive effect [2]. It is crucial for tissue repair and wound healing. It's interesting to note that these pro- and anti-inflammatory mechanisms could interact. Because injured areas are frequently targets of pathogen invasion, eradicating damage necessitates a balance between macrophage types. As a result, both functioning macrophages and macrophages that fall outside of the M1 and M2 spectrum can be found in these situations [3]. Through the recruitment of pro-immunostimulatory leukocytes and the phagocytosis of tumor cells, M1 cells initiate the production of cytokines inside the tumor microenvironment (TME) and aid in the killing of tumor cells. Nonetheless, other research indicates that M2 macrophages play a crucial part in the dissemination of tumors [4]. According to studies by Quail and Joyce [5], M2 cells have been demonstrated to promote the growth of tumors in both primary and metastatic locations by interfering with the formation and accumulation of basement membranes, angiogenesis, the recruitment of leukocytes, and general immunosuppression.

Cardiac glycosides, which are classified into A and B types, are a class of chemicals having a steroid-like structure that are widely found in angiosperms. They are also referred to as cardiotonic or cardiac glycosides. *Apocynaceae* (Oleaceae oleander, Scutellaria), *Rosaceae* (Pygnus, milkweed), and *Liliaceae* (Lily of the Valley, evergreen) are the families in which type A is primarily found, whereas B type is primarily found in *Liliaceae* (scallop) and *Ranunculaceae* [6]. *Nerium oleander Linn*., a traditional folk remedy, contains an active cardenolide called odoroside A (OA) (*Apocynaceae*). Due to their cardiovascular toxicity, clinically utilized cardiac glycosides have a narrow therapeutic index, which may restrict their use in therapy [7, 8]. The solution to this issue is to look for chemicals derived from cardiac glycosides that can stop cancer cells from proliferating and spreading without endangering the heart. According to *in vitro* research, cardiac glycosides are safe for normal cells at nanomolar doses and may even prevent apoptosis or promote cell growth in them; however, in cancer cells, these medications inhibit cell growth and cause cell death [9-11]. According to previous research, OA reduced cell activity in human cancer cell lines, such as those from the stomach, colon, and cervical regions [12]. Mono glycosidic cardenolides, such as oleandrin and OA that were isolated from *Nerium oleander*, have been shown to have strong anticancer properties [13]. The effects of OA and the underlying processes behind them are less understood than those of oleandrin.

When using anticancer treatments, not only cancer cells but all cell types in that microenvironment are affected. Studies have shown that M2 type macrophages, which are especially abundant in the tumor microenvironment, are tumor promoters. The pro or anti-inflammatory effect created by macrophage transformation in the tumor microenvironment is very critical for tumor development or elimination. The aim of this study is to show how OA affects the transformation in macrophage cells.

Materials and methods

Chemicals

Odoroside A (Merck), CCK-8 kit (Abbkine), DMEM (Gibco), fetal bovine serum (FBS) (Capricorn Scientific), PBS (Biological Industries), penicilin/streptomycin (Biological Industries), QIAzol (Qiagen), cDNA kit (Bio-Rad) and qRT-PCR mix (Solis BioDyne) commercially obtained.

Cell culture

The murine macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC TIB-71™). Proliferation of the murine macrophage cell line RAW 264.7 was achieved in the appropriate culture medium using DMEM, 10% FBS, 2 mM L-glutamine, 1% Penicillin-Streptomycin. Cell proliferation, passage and follow-up processes were monitored with an inverted microscope. It was incubated in an oven with 95% humidity and 5% CO_2 until sufficient growth was achieved.

CCK-8 proliferation analysis

CCK-8 colorimetric experiment was conducted to determine non-toxic doses of OA in RAW264.7 cells. DMSO was used to dissolve OA. In a 96-well plate, $1x10^3$ cells/well were used to seed cells. For 24 and 48 hours, the cells were exposed to OA at different concentrations (100, 200, 500, 1000, 2000, 3000, and 4000 nM). Then, using a microplate reader, levels of cell viabilities were measured at 450 nm to calculate cell viability (%).

Gene expression analysis by qRT-PCR

Following the instructions provided by the manufacturers, QIAzol and the Transcriptor First-Strand cDNA Synthesis Kit were used to isolate total RNA from RAW264.7 cells and synthesize cDNA, respectively. IDT PrimerQuest was used to build the primer sequences for the genes involved in this study's qRT-PCR analysis, and the results were shown in Table 1 [14]. For every gene, a qRT-PCR reaction mix was made. In summary, each reaction contained 4 µl of Solis qRT-PCR master mix, 5 pmol of forward and reverse primer, and 2 µl of cDNA. The steps in the qRT-PCR technique were as follows: 12 minutes of initial denaturation at 95°C, 15 seconds of denaturation at 95°C, 20 seconds of annealing at 60°C, and 20 seconds of extension at 72°C. The PCR reaction was run for 40 cycles. Utilizing the Bio-Rad CFX Connect™ Real-Time System, qRT-PCR analysis was carried out. Melting curve analysis was performed by gradually heating the PCR products from 65°C to 95°C. In the study, GAPDH was used as the reference gene.

Table 1. Primers used in this study

Gene	Forward Primer (5'->3')	Reverse Primer (5'->3')	PCR (bp)
IL1	GATCCCAAACAATACCCAAAGAAG	AGGTGCTGATGTACCAGTTG	118
IL4	TTGAGAGAGATCATCGGCATTT	CTCACTCTCTGTGGTGTTCTTC	111
IL6	CTTCCATCCAGTTGCCTTCT	CTCCGACTTGTGAAGTGGTATAG	134
IL10	CTATGCTGCCTGCTCTTACTG	GGGAAGTGGGTGCAGTTATT	83
TGFβ	CTGAACCAAGGAGACGGAATAC	GGGCTGATCCCGTTGATTT	101
CD86	GGGCTTGGCAATCCTTATCT	CAGCTCACTCAGGCTTATGTT	139
CD163	CAGACTGGTTGGAGGAGAAATC	CAGCTTCCAGAGACAAGTCAA	101
GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA	307

Enzyme-linked immunosorbent assay (ELISA)

Supernatants were extracted from cell cultures. To measure the protein amounts of IL1 and IL6, SunRedBio ELISA kits (Shanghai SunRed Biological Technologies, China) were utilized. After centrifuging the supernatants for 20 minutes at 3000 rpm, 10 µL of samples and 40 µL of dilution buffer were put to 96-well plates. The wells were rinsed five times for 30 seconds each after being incubated for 30 minutes at 37 °C. Each well received 50 µL of HRP-conjugated reagent, which was then incubated for 30 minutes at 37°C. Following the cleaning, each well was filled with 50 µL of chromogen solutions A and B, and the wells were gently shaken. 50 µL of stop solution was added to end the reaction after it had been incubated for 15 minutes at 37°C in the dark. At 450 nm OD, absorbance was determined with an ELISA reader (Epoch, USA).

Statistical analysis

Using the $2^{(-\Delta\Delta Ct)}$ technique, the Ct values of the genes examined in the study were standardized with respect to the reference gene. The GraphPad®Prism version 9.2.0 software was used to assess the groups' cytokine quantities and gene expression levels using the "Multiple t test." Statistics were deemed significant if $p<0.05$.

The study is a cell culture study that does not require ethics committee approval.

Results

In the viability test of OA in RAW264.7 cells, the selected doses were shown to be non-toxic (Figure 1). Since the aim of this study was to see how OA affects cellular transformation in macrophage cells rather than the lethal dose, a dose of 500 nM was used for 24 h.

Expression analysis of OA in inflammatory genes is shown in Figure 2. According to gene expression analysis, OA caused a significant increase in IL1 and IL6 genes and significant decrease in IL10 gene in RAW264.7 cells, while it caused a non-significant change in IL4 and TGFβ.

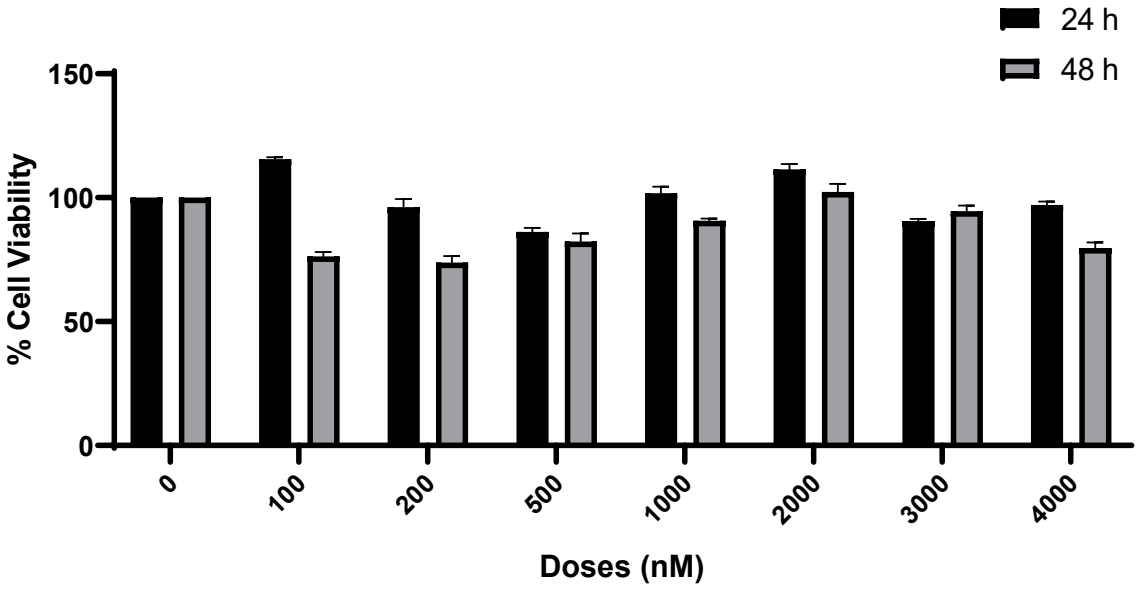

Figure 1. Non-toxic doses range in RAW264.7 cells

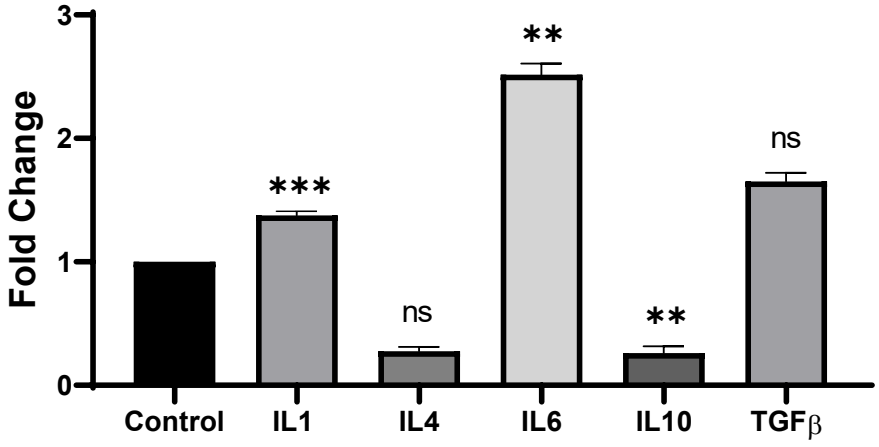

Figure 2. Genes of inflammation expressions

** $p<0.005$, *** $p<0.0005$

The expression levels of CD86 and CD163 markers, which are important markers in the transformation of macrophage cells, are shown in Figure 3. Accordingly, while CD86 expression, which is a pro-inflammatory change, increased significantly, CD163 expression decreased significantly.

ELISA analysis was performed to show how gene expression analyzes change at the protein level. The data obtained according to ELISA analysis were found to be compatible with gene expression analysis (Figure 4).

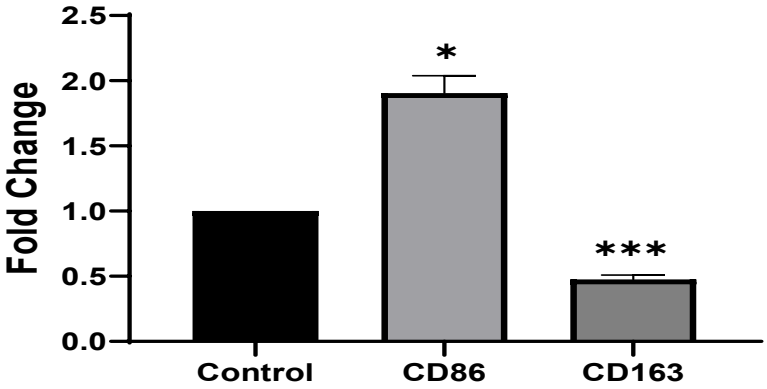

Figure 3. CD86 and CD163 surface markers gene expressions
* $p<0.05$, *** $p<0.0005$

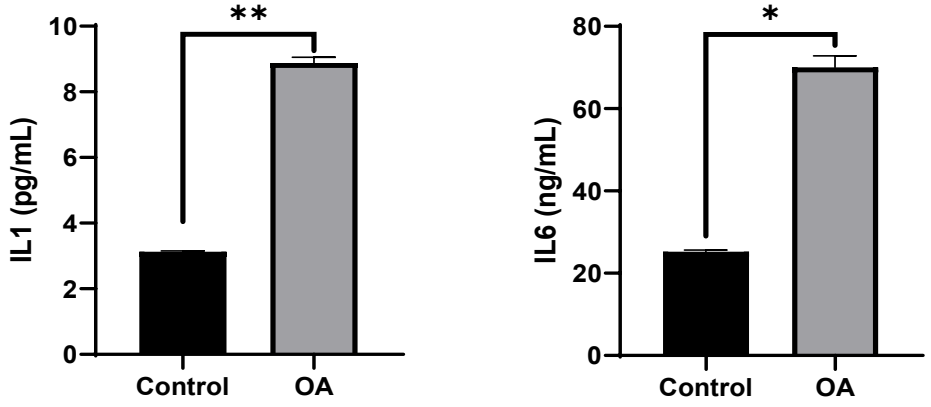

Figure 4. Cytokine amounts expressed by Odoroside A applied cells compared to control

Discussion

Macrophages play a significant part in the control of physiological processes and are found in many tissues devoid of blood vessels [15]. They are useful in the initiation, maintenance, and termination of the inflammatory response because of their great elasticity [16, 17]. Tissue integrity is not ideal when pro-inflammatory cytokines are released in large quantities. According to Gordon and Martinez [18], macrophages aid in the inhibition of the inflammatory response. Tissue purification and the restoration of typical physiological circumstances take place [19]. It is commonly recognized that chronic inflammation is linked to the majority of malignancies (90-95%). As a result, M2 macrophages can promote the growth of tumors, while M1 macrophages can contribute to a mutagenic microenvironment [20, 21]. The role of macrophages in the tumor microenvironment is noteworthy. They are

the most effective elements in creating a pro-inflammatory or anti-inflammatory environment in the tumor microenvironment. In addition to the effectiveness of antitumor chemicals in the elimination of tumor cells, their effects on other types of cells in the tumor microenvironment should be investigated. In our study, the effect of OA, which has been shown to have an antitumor effect, on macrophage cells intertwined with the tumor was investigated.

Transformation of macrophage cells was examined without the lethal effect of OA on RAW264.7 cells. In the gene expression analysis of IL1 and IL6 cytokines, which have pro-inflammatory effects, OA was found to be an enhancer. In addition, anti-inflammatory cytokines IL10 were found to be significantly reduced in gene expression analysis. This showed that OA affects macrophage cells in a way that creates a pro-inflammatory response. The formation of a pro-inflammatory environment in the tumor microenvironment promotes the elimination of tumor cells by the immune system. Anti-inflammatory macrophages (M2 type) are the most common immune cells in the cancer microenvironment [22]. Therefore, the transformation of M2 type macrophages into pro-inflammatory type under the influence of OA may retard cancer development.

Dendritic cells, monocytes, T lymphocytes, and B lymphocytes can express CD86, commonly referred to as B7-2. CD86 is an 80 kDa T lymphocyte activation antigen [23]. CD86 stimulates T cell proliferation and generates IL2 through interactions with its ligands, CD28 and CTLA4 [24]. Likewise, when the expression analysis of the genes belonging to the surface markers that are determinant in the transformation of macrophage cells was examined, it was determined that CD86 gene expression increased significantly. Primarily expressed on the surface of monocytes and macrophages, CD163 is a highly selective M2-type tumor-associated macrophage marker. In addition to its ability to fend against inflammation, CD163, a member of the tumor-related macrophage family, is crucial for the growth and spread of tumors. Research has demonstrated a strong correlation between CD163 and malignant tumors, including bladder, lung, colorectal, and breast cancer [25]. The degree of CD163 infiltration influences the prognosis, invasion, metastasis, and proliferation of tumors. Expression analysis of surface markers varied in accordance with the transformation of macrophages compared to cytokine gene expressions.

TGFβ may show a different expression profile in the immune response to tumor [26]. In our study, it was found that TGFβ expression increased in a manner that was not statistically significant. This may be due to TGFβ causing different immunological responses in different conditions.

All the data obtained were evaluated together, it was shown that OA affected pro-inflammatory response-related cellular pathways in RAW264.7 cells. In conclusion, it can be said that macrophages in the tumor microenvironment of OA may have an effect that helps tumor elimination.

Conflict of interest: No conflict of interest was declared by the authors.

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Ethics committee approval: The study is a cell culture study that does not require ethics committee approval.

Author contributions

F.S.C. constructed the main idea and hypothesis of the study. F.S.C. and C.E.G. developed the theory and arranged/edited the material and method section. F.S.C. has done the evaluation of the data in the Results section. Discussion section of the article was written by F.S.C. and C.E.G.

C.E.G. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

Research Article

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Noonan syndrome: molecular and clinical findings in individuals with PTPN11 pathogenic variants

Noonan sendromu: PTPN11 patojenik varyantları olan bireylerde moleküler ve klinik bulgular

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Abstract

Purpose: RASopathies encompass a spectrum of disorders resulting from pathogenic variants in genes associated with the Ras/mitogen-activated protein kinase (RAS/MAPK) pathway, critical for cellular functions like proliferation, differentiation and survival. Noonan syndrome (NS), the most prevalent form of RASopathies, presents with a myriad of clinical features including characteristic facial dysmorphisms, congenital heart defects, and developmental delays. Despite its clinical recognition, molecular confirmation remains elusive in a notable percentage of cases. In this study, we aimed to investigate the clinical and molecular profiles of six patients diagnosed with NS, focusing on the role of PTPN11 gene mutations.

Materials and methods: Molecular evaluation was performed using PTPN11 gene sequence analysis and whole gene sequencing methods in six patients who were thought to have typical NS phenotypes based on clinical evaluations.

Results: Molecular screening in patients identified four different pathogenic variants in the PTPN11 gene. These variants, all heterozygous, were classified as pathogenic according to established criteria.

Conclusion: Our findings contribute to understanding the genetic landscape of NS and underscore the significance of molecular analysis in confirming diagnoses.

Keywords: Noonan syndrome, PTPN11, pathogenic variant, SHP2.

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Öz

Amaç: RASopatiler, çoğalma, farklılaşma ve hayatta kalma gibi hücresel işlevler için kritik olan, Ras/mitojenle aktifleştirilen protein kinaz (RAS/MAPK) yolu ile ilişkili genlerdeki patojenik varyantlardan kaynaklanan, bir dizi bozukluğu kapsar. RASopatilerin en yaygın şekli olan Noonan sendromu (NS), karakteristik yüz dismorfizmleri, konjenital kalp defektleri ve gelişimsel gecikmeler dahil olmak üzere sayısız klinik özellik ile ortaya çıkar. Klinik olarak tanınmasına rağmen, vakaların kayda değer bir yüzdesinde moleküler doğrulama hala belirsizliğini korumaktadır. Bu çalışmada PTPN11 gen mutasyonu bulunan NS tanısı alan altı hastanın klinik ve moleküler profillerini araştırmayı amaçladık.

Gereç ve yöntem: Klinik değerlendirmelere göre tipik NS fenotipine sahip olduğu düşünülen altı hastada PTPN11 gen dizi analizi ve tam gen dizileme yöntemleri kullanılarak moleküler değerlendirme yapıldı.

Bulgular: Hastalarda yapılan moleküler taramada PTPN11 geninde dört farklı patojenik varyant tespit edildi. Tamamı heterozigot olan bu varyantlar, belirlenen kriterlere göre patojenik olarak sınıflandırıldı.

Sonuç: Bulgularımız NS'nin genetik yapısının anlaşılmasına katkıda bulunmakta ve tanıların doğrulanmasında moleküler analizin öneminin altını çizmektedir.

Anahtar kelimeler: Noonan sendromu, PTPN11, patojenik varyant, SHP2.

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Introduction

RASopathies are a group of diseases caused by pathogenic variants in genes encoding the Ras/mitogen-activated protein kinase (RAS/MAPK) pathway. The RAS/MAPK pathway has been associated with cell differentiation, proliferation, metabolism, cell survival and apoptosis [1]. Noonan syndrome (NS) (OMIM:163950) is the most common form (prevalence rate in livebirths of 1:1000 to 1:2500) of RASopathies, approximately 80% of its cases are associated with abnormal activation of the RAS/MAPK pathway but still 10-20% of clinical diagnoses remain unconfirmed at the molecular level [2, 3]. To date, many genes involved in the RASopathies have been identified; *PTPN11, SOS1, SOS2, KRAS, RAF1, RIT1, NRAS, BRAF, LZTR1, MAP2K1, RRAS2, RASA2, MAP2K2, HRAS, NF1, SHOC2, SPRED1, CBL, PPP1CB, MAPK1* and *MRAS* [4]. In 50% of NS cases mutation is observed in the *PTPN11* (protein tyrosine phosphatase non-receptor type 11) gene on the long arm of chromosome 12q24.1. *SOS1* (20%), *RAF1* (3-17%) and *KRAS* (<5%) mutations are less common [5, 6]. Other well-known causal genes are *RIT1*, *BRAF*, *NRAS* and *LZTR1* [7-9].

NS is a syndrome in which autosomal dominant inheritance has been reported but cases are usually sporadic [10, 11]. Mental retardation is observed in approximately 1/3 of the patients. Typical facial dysmorphism (epicanthus, ptosis, hypertelorism, downward slanting palpebral fissures, flattened nasal root, low ears, prominent upper lip, retrognathia) is the most important criterion for diagnosis. Low nape hairline and short/mane neck, raised chest, cubitus valgus syndrome are other findings that can be observed. The heart defects most commonly associated with NS are pulmonary stenosis, hypertrophic cardiomyopathy and septal defects respectively. Other clinical findings include developmental delay, chest deformities, cryptorchidism in boys, mild mental retardation, lymphatic dysplasia, bleeding diathesis and feeding difficulties in the neonatal period [12, 13].

In this study, the clinical and molecular findings of six patients with a clinical diagnosis of NS were evaluated, and the genotype-phenotype relationship in NS was discussed.

Materials and methods

Clinical evaluation

Six cases with suspicion of Noonan Syndrome aged between 3 months and 26 years were included in this study. Criteria developed by Van der Burgt [10] were applied to all patients. Patients underwent detailed physical and clinical evaluation, including systemic examination for minor and major anomalies. Facial findings of the patients are given in Figure 1.

Figure 1. Facial findings of the patients

All patients were evaluated for short stature, developmental delay, congenital heart defects, history of predisposition to bleeding, history of cryptorchidism, skeletal abnormalities, radiological test results, hearing test results, and karyotype results (Table 1).

Table 1. Phenotypic and genotypic features in cases

Features	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6
Variant	*PTPN11* c.781C>T	*PTPN11* c.184T>G	*PTPN11* c.184T>G	*PTPN11* c.184T>G	*PTPN11* c.836A>G	*PTPN11* c.417G>C
Gender	F	M	M	M	F	F
Age at diagnosis	26	1	4	12	13	7
Weight (kg)	48	8,7	14	25	32	15
Height (cm)	152	68	92	124	141	112
OFD (cm)	54	45	48	50	NA	NA
Karyotype	46, XX	46, XY	46, XY	46, XY	46, XX	46, XX
Facial dysmorphism						
Ears set low and pointing backwards	+	+	+	-	-	+
Hypertelorism	-	+	+	-	+	+
Downslanting palpebral fissures	-	-	-	+	-	-
Sparse eyebrows	+	+	+	+	-	-
Prominent philtrum	+	+	+	+	+	+
Micrognathia	-	+	-	-	+	-
Triangle face	+	-	-	+	+	+
Congenital cardiac findings						
Vascular pulmonary stenosis	-	+	+	-	+	+
Ventricular septal defect (VSD)	-	-	-	-	-	-
Atrial septal defect (ASD)	+	-	+	+	-	+
Chest-neck findings						
Mane neck	+	+	+	+	-	+
Pectus deformity	+	+	+	+	+	+
Broad thorax	+	-	+	+	-	+
Bleeding disorders						
Predisposition to ecchymosis	-	-	-	-	-	-
Other findings						
Cryptorchidism	-	+	+	+	-	-
Developmental delay	-	-	+	-	-	-
Intellectual disability	-	-	+	-	-	-
Hearing loss	-	-	-	-	-	-

F: Female, M: Male

Informed written consent was obtained from participants and their parents for photographs and genomic study. This study complies with the principles of the Declaration of Helsinki. We followed the CARE guidelines to write the case report. Written informed consent was obtained from the legal representatives of the family before blood samples were taken. All clinical tests and researches were done in Dr. Ersin Arslan Training and Research Hospital, Genetic Diseases Diagnosis Center and Pamukkale University Medical Genetics Departments.

Ethics committee approval for this study was received from Pamukkale University Non-Invasive Clinical Research Ethics Committee.

Molecular screening

DNAs were extracted from peripheral blood samples from the patients. Sequence analysis was performed using an automated capillary sequencer method in four patients. Mutations in several genes encoding components of the RAS/MAPK pathway are known to cause the N/CFC/C syndrome disease group, including 5 genes encoding components of the RAS/MAPK pathway [*PTPN11* exons 2-4, 7, 8, 11-14 (NM_002834), *KRAS* isoform B exon 2, 3, 5 (NM_004985), *RAF1* exons 7, 12, 14, 17 (NM_002880), *SHOC2* exon 2 partial (NM_007373), *SOS1* exons 3-11, 13-14, 16 (NM_005633)] exons and flanking intronic regions were amplified by PCR and analyzed by high-resolution melting on a Light Cycler (Roche, LC-480). Amplicons obtained by PCR were sequenced using an automated capillary sequencer. The sequences were compared with reference sequences in the NCBI (National Center for Biotechnology Information) database. *PTPN11* whole gene sequencing was performed on the DNA samples of the other two patients. The 16 exons of the *PTPN11* gene and their flanking intron sequences were amplified by polymerase chain reaction and sequenced with the Illumina MiSeq system. The resulting sequences were aligned to the hg19 genome using Illumina MiSeq Reporter software. Identified variants were checked against those present in 1,000 Genomes, HGMD, ClinVar and dbSNP. ACMG (American Standards and Guidelines for Medical Genetics and Genomics) criteria [14] were used for detection of variant pathogenicity.

Result

As a result of the study, 4 different pathogenic variants were detected in the *PTPN11* gene in 6 cases in which NS-related disease was considered and variant analysis was performed. All of the variants detected in the cases were heterozygous and are shown in Table 2. These variants were identified in HGMD [15] and ClinVar [16] and were interpreted as “Pathogenic” according to the ACMG criteria (PS2, PM1, PM2, PP2, PP3 criteria were applied) [14].

Table 2. Disease-causing variants detected in patients

Case no	Gene	Location	Functional domain of the SHP-2 protein	Nucleotid change	Amino acid change	Mutation type	Reference	Pathogenicity according to ACMG criteria
1	*PTPN11*	Exon 7	PTP	c.781C>T heterozygous	p.L261F	Missense	ClinVar, HGMD	Pathogenic
2	*PTPN11*	Exon 3	N-SH2	c.184T>G heterozygous	p.Y62D	Missense	ClinVar, HGMD	Pathogenic
3	*PTPN11*	Exon 3	N-SH2	c.184T>G heterozygous	p.Y62D	Missense	ClinVar, HGMD	Pathogenic
4	*PTPN11*	Exon 3	N-SH2	c.184T>G heterozygous	p.Y62D	Missense	ClinVar, HGMD	Pathogenic
5	*PTPN11*	Exon 7	PTP	c.836A>G heterozygous	p.Y279C	Missense	ClinVar, HGMD	Pathogenic
6	*PTPN11*	Exon 4	CSH2	c.417G>C heterozygous	p.E139D	Missense	ClinVar, HGMD	Pathogenic

HGMD: Human gene mutation database, ACMG: American College of Medical Genetics and Genomics and Association for Molecular Pathology

Discussion

Noonan syndrome is a very common disease that varies in severity and can involve more than one organ system throughout the patient's life. Approximately half of the cases are sporadic and are mostly inherited in an autosomal dominant inherited [13]. Almost 50% of Noonan patients have a heterozygous missense mutation in the *PTPN11* gene which encodes SHP2 (Src homology region 2). SHP2 is a non-receptor protein tyrosine phosphatase consisting of a catalytic PTP domain and two tandem SH2 domains (N-terminal SH2 and C-terminal SH2) that phosphorylate tyrosine-phosphorylated signaling proteins and a C-terminal hydrophilic tail [17]. The structure and function of this protein, a member of the RAS/MAPK cascade, is evolutionarily well conserved. The majority of missense mutations in the *PTPN11* gene cause activation of the catalytic domain of the protein product that transmits excessive amounts of RAS/MAPK signals [4]. According to the literature, the exons of the *PTPN11* gene containing the most pathogenic variants are exon 3 and exon 8 constitute 62% of *PTPN11* pathogenic variants [17].

As a result of molecular genetic study, variants were detected in 6 patients. All identified variants were previously reported missense pathogenic variants and occurred at conserved positions among vertebrate *PTPN11* orthologous genes [15, 16]. Three of the detected variants are located in exon 3, one in exon 4 and two in exon 7. Exon 8 mutation (c.922A>G) which is defined as the most common mutation seen in NS, was not detected in any of our patients. All variants we found in our patients were heterozygous missense changes.

According to the literature the most frequently detected variant is the c.922A>G (p.Asn308Asp) variant [18]. In studies in our country, the exons with the highest mutation rate are exons 3 and 8 and the most frequently detected variant is c.922A>G (p.Asn308Asp) [19, 20]. On the contrary, we could not detect the c.922A>G (p.Asn308Asp) change in our patients.

To date, no significant correlation has been reported between intellectual disability and *PTPN11* pathogenic variants (the exon where the variants is located - the area in the protein it affects) in patients with NS. Only one of our patients had intellectual disability and had an exon 3 variants. Cognitive abilities and development of the other two patients with the PTPN1 gene exon 3 variant and the patients with exon 4-7 variants were found to be normal. Cryptorchidism has been reported in a range of 44-94% in male patients with Noonan syndrome [17, 18]. We detected cryptorchidism in all of our male cases.

NS is one of the syndromes without chromosomal abnormalities with Turner-like phenotypic features. Cardiovascular pathologies are reported at a rate of 40-50% [21]. The most common cardiac malformation in Noonan syndrome is valvular pulmonary stenosis due to pulmonary valve dysplasia and hypertrophic cardiomyopathy with an incidence of 37.9-39% [22]. Many chromosomal abnormalities; may manifest themselves with findings including short stature, heart defects and developmental delay. Chromosomal analysis was performed using conventional methods in all 6 patients and chromosomal abnormalities were excluded.

In conclusion, this study underscores the clinical and genetic heterogeneity of Noonan syndrome. Despite the predominance of *PTPN11* mutations, the absence of exon 8 mutations in our cohort suggests genetic diversity. Understanding genotype-phenotype correlations aids in diagnosis and management, emphasizing the importance of comprehensive genetic screening in NS patients. Further research is warranted to elucidate the full spectrum of genetic variants contributing to NS pathogenesis.

Overall, our study contributes to advancing our understanding of NS by integrating clinical and molecular data, highlighting the complexity of this disorder and emphasizing the need for multidisciplinary approaches to diagnosis and management. Moving forward, continued research efforts aimed at unraveling the genetic and molecular basis of NS are essential for improving diagnostic accuracy, prognostication, and therapeutic interventions for affected individuals.

Conflict of interest: No conflict of interest was declared by the authors.

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Informed consent: Written informed consent was obtained from the patients.

Ethics committee approval: Permission was obtained from Pamukkale University Non-Invasive Clinical Research Ethics Committee (approval date: December 28, 2023 and approval number: E-60116787-020-468745).

Authors' contributions to the article

D.K., T.D. and K.K. constructed the main idea and hypothesis of the study. D.K. developed the theory and arranged/edited the material and method section. Discussion section of the article was written by D.K., T.D. and K.K. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

Research Article

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Is there any difference between M694V heterozygote and non-exon 10 mutations on symptoms onset and response to colchicine treatment?

M694V heterozigot ve non-ekzon 10 mutasyonları arasında semptomların başlangıcı ve kolşisin tedavisine yanıt açısından fark var mıdır?

Hatice Adıgüzel Dundar, Serkan Türkuçar, Ceyhun Açarı, Özge Altuğ Gücenmez, Balahan Makay Erbil Ünsal

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Abstract

Purpose: Familial Mediterranean fever (FMF) is the most common inherited autoinflammatory syndrome throughout the world. The most frequent genotype-phenotype correlation is in a certain part of exon 10, especially M694V mutation. There are also a group of patients with non-exon 10 mutations, who have a similar clinical spectrum of the disease. We aim to investigate the genotype-phenotype differences between M694V heterozygote mutations and non-exon 10 mutations.

Materials and methods: Data charts of children (n=431) with FMF from two tertiary hospitals were reviewed. Patients were divided into two groups with regard to having M694V heterozygote or non-exon 10 mutations. Genotype-phenotype features and response to treatment were compared.

Results: There were M694V heterozygote mutations in 128 (29.7%) patients and non-exon 10 mutations in 303 (70.3%) patients. The follow-up period was 54.5 (33-105) months. There was no difference between the age of symptoms onset, the age of diagnosis, and the diagnosis delay time. The family history in patients with M694V heterozygote mutation was statistically positive compared to non-exon 10 mutation group (p=0.001). The symptoms of joint involvement as arthritis and PRAS scores were significantly higher in the M694V heterozygote group (p=0.026 and p=0.001). Additionally, biological agent need due to colchicine unresponsiveness was statistically higher in M694V heterozygote group than group with non-exon 10 mutation (p=0.004).

Conclusion: There is a significant difference between children with M694V and non-exon 10 mutations, even when the M694V mutation is present in one allele only. Family history with FMF, musculoskeletal symptoms, and unresponsiveness to colchicine are main parameters.

Key words: Familial Mediterranean Fever, M694V, MEFV mutation, colchicine**.**

Adiguzel Dundar H, Turkucar S, Acari C, Altug Gucenmez O, Makay B, Unsal E. Is there any difference between M694V heterozygote and non-exon 10 mutations on symptoms onset and response to colchicine treatment? Pam Med J 2024;17:550-559.

Öz

Amaç: Ailesel Akdeniz Ateşi (AAA), tüm dünyada en sık görülen kalıtsal otoinflamatuvar sendromdur. En sık genotip-fenotip korelasyonu ekzon 10'un belirli bir kısmında, özellikle M694V mutasyonundadır. Ekzon 10 mutasyonu olmayan ve benzer klinik spektruma sahip bir grup hasta da bulunmaktadır. Amacımız M694V heterozigot mutasyonları ve ekzon 10 dışı mutasyonlar arasındaki genotip-fenotip farklılıklarını araştırmaktır.

Gereç ve yöntem: İki üçüncü basamak hastaneden AAA'lı çocukların (n=431) veri dosyaları incelendi. Hastalar M694V heterozigot veya non-ekson 10 mutasyonuna sahip olma açısından iki gruba ayrıldı. Genotip-fenotip özellikleri ve tedaviye yanıt karşılaştırıldı.

Bulgular: 128 (%29,7) hastada M694V heterozigot mutasyonu ve 303 (%70,3) hastada non-ekson 10 mutasyonu vardı. Takip süresi 54,5 (33-105) aydı. Semptomların başlama yaşı, tanı yaşı ve tanı gecikme süresi arasında fark yoktu. M694V heterozigot mutasyonu olan hastalarda aile öyküsü, ekzon 10 mutasyonu olmayan gruba kıyasla istatistiksel olarak pozitifti (p=0,001). Artrit olarak eklem tutulumu semptomları ve PRAS skorları M694V heterozigot grubunda anlamlı olarak daha yüksekti (p=0,026 ve p=0,001). Ayrıca, kolşisin yanıtsızlığı nedeniyle biyolojik ajan ihtiyacı M694V heterozigot grupta ekzon 10 mutasyonu olmayan gruba göre istatistiksel olarak daha yüksekti (p=0,004).

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Sonuç: M694V mutasyonu sadece bir alelde mevcut olsa bile, M694V ve non-ekson 10 mutasyonu olan çocuklar arasında anlamlı bir fark vardır. AAA ile aile öyküsü, kas-iskelet sistemi semptomları ve kolşisine yanıtsızlık ana parametrelerdir.

Anahtar kelimeler: Ailevi Akdeniz Ateşi, M694V, MEFV mutasyonu, kolşisin.

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Introduction

Familial Mediterranean Fever (FMF) is the most prevalent hereditary autoinflammatory syndrome globally. It is characterized by recurrent episodes of fever and inflammation affecting serous membranes, joints, and skin [1, 2]. The underlying cause of FMF is mutations in the MEFV gene, which encodes the pyrin protein. Pyrin is crucial for regulating inflammation, particularly in the activation of caspase-1 and the subsequent production of interleukin-1β [3, 4]. The MEFV gene is located on chromosome 16p13.3 and comprises 10 exons separated by 9 introns. Mutations are primarily identified in exons 2, 3, 5, and 10. The Infevers online database documents over 398 alleles classified as mutations or polymorphisms in the MEFV gene [5]. Although FMF is traditionally considered an autosomal recessive disorder, the inheritance pattern is more complex. Heterozygotes can exhibit disease phenotypes, likely due to the influence of other modifying genes, environmental factors, or epigenetic changes affecting the MEFV gene's function [6-8].

Among the documented mutations in the MEFV gene, particularly those in exon 10 like the M694V mutation, there is a clear genotype-phenotype correlation. This correlation is marked by a severe disease phenotype with high inflammatory responses and a predisposition to complications such as amyloidosis [9, 10]. Some FMF patients do not carry exon 10 mutations but still exhibit clinical findings similar to those with exon 10 mutations. Despite genetic diversity, these patients show a similar clinical disease spectrum, highlighting the complexity of FMF pathogenesis and its multifactorial clinical presentation [11, 12].

The genotype-phenotype correlation in FMF remains an area of ongoing research and debate. Many studies have emphasized the clinical significance of exon 10 mutations, particularly the homozygote M694V mutation [13-15]. While some mutations, such as M694V, are linked to a more severe disease course, others like V726A and E148Q tend to present with milder symptoms [8, 16]. In a study investigating amyloidosis cases associated with FMF, 10 patients (8.4%) were reported to have the M694V heterozygote mutation, and 4 patients (3.4%) had non-exon 10 mutations [17]. Kandur et al. [18] found that arthritis was more common in patients with the M694V heterozygote and exon 2 mutation, and these children had higher severity scores compared to those with the M694V heterozygote mutation, underscoring the significant impact of non-exon 10 mutations on FMF clinical manifestations. However, the precise relationship between genotype and phenotype, especially in cases without exon 10 mutations, remains unclear, necessitating further research [19].

In addition to genetic heterogeneity, therapeutic management of FMF can be challenging, particularly in colchicine-resistant cases. While colchicine is the gold standard treatment, a subset of patients does not respond adequately and requires alternative therapeutic approaches, such as anti-interleukin-1 therapies [20, 21].

The aim of this study was to comprehensively characterise the clinical features and genetic profiles of a cohort of patients with non-exon 10 mutation and to elucidate differences in disease manifestations and response to treatment compared to patients with heterozygote M694V mutation. By elucidating these differences, we aimed to advance our understanding of FMF pathogenesis and pave the way for more specialised approaches to diagnosis and treatment in the future.

Materials and methods

Study group

Pediatric patients diagnosed with FMF at two tertiary pediatric rheumatology clinic (Dokuz Eylul University childrens' hospital and Dr.B.Uz childrens' hospital) were retrospectively evaluated. Patients were included if they fulfilled at least two of the Ankara clinical diagnostic criteria, including typical fever lasting 6-72 hours, abdominal pain, chest pain, and arthritis attacks, positive family history of AAA, were included [22]. A total of 431 patients previously subjected to MEFV gene analysis were enrolled, encompassing those with heterozygote M694V mutation and heterozygote, homozygote, or compound heterozygote mutations outside of exon 10. The patients were divided into two groups based on the presence of M694V heterozygote mutation and non-exon 10 mutations, allowing for the evaluation of their relationship.

Clinical and demographic data

Demographic and clinical information such as age, gender, body weight, and height at the last visit, age at the first attack, age of colchicine initiation, delay in diagnosis, follow-up period, presence of FMF in the family, and findings during attacks were recorded. Body weight and height values were adjusted for age according to the data of Nevzi et al [23]. Additionally, the presence of joint complaints between attacks, presence of concomitant rheumatological disease, FMF severity scores calculated using the scoring system of Pras et al. [24], and daily colchicine doses were documented. According to the PRAS score, a score of 3-5 was classified as mild, 6-8 as moderate, and >9 as severe disease.

The most recent hemogram parameters, including hemoglobin (Hb), white blood cell (WBC), and platelet (Plt) counts, as well as neutrophil/lymphocyte ratios (NLR), of all patients during the inter-attack period were recorded using data obtained from the electronic patient record system. Concurrent acute phase responses, such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels, liver and kidney function tests (creatinine (Cr) and alanine aminotransferase (ALT)), and urinary protein excretion were also noted.

Colchicine resistance was defined as experiencing more than one typical episode per three months or an increase in at least two out of three acute phase reactants (CRP, ESR, and serum amyloid A) between attacks despite maximal colchicine intake [25, 26].

Genetic analysis

Genetic analyses were performed on DNA samples extracted from peripheral blood using the real-time polymerase chain reaction (RT-PCR) method with the Cobas z480 RT-PCR instrument from Roche, Germany, and the LightSNiP assay kit from TIB Molbiol, Germany. This method identified 20 common mutations associated with AAA: E148Q (exon 2), R202Q (exon 2), M680I (exon 10), M694V (exon 10), M694I (exon 10), K695R (exon 10), V726A (exon 10), R761H (exon 10), A744S (exon 10), P369S (exon 3), D510D (exon 5), F479L (exon 5), R314H (exon 3), E230K (exon 2), R408Q (exon 3), R314R (exon 3), G304R (exon 2), R241K (exon 2), S339F (exon 3) and E167D (exon 2).

The study included only patients with a heterozygote M694V mutation among those with exon 10 mutations and all patients with mutations in exons 2, 3 or 5, as well as patients with negative MEFV gene analysis (including those with negative autoinflammatory panel results). Patients with non-exon 10 mutations who were resistant to colchicine were also tested with the autoinflammatory panel. The autoinflammatory panel was used to screen for mutations in genes for MVK, IL1RN, LPIN2, NLRP3, NOD2, NLRP12, ADA2, PSTPIP1, TNFRSF11A, ELANE and TNFRSF1A.

Patients were divided into two groups based on the presence of the heterozygote M694V mutation and non-exon 10 mutations (including exon 2, 3 and 5 mutations and MEFV-negative cases). These groups were analysed to evaluate their demographic, clinical and laboratory characteristics, PRAS scores and response to treatment.

Ethics committee approval was obtained for the study.

Statistical analysis

Descriptive statistics were used to summarise quantitative process evaluation data: frequency and percentage for categorical variables, and mean, standard deviation, median, minimum and maximum for continuously distributed variables. Normality tests (Kolmogorov-Smirnov test) were performed. The chi-squared test was used to compare categorical variables, and the independent samples t-test was used for continuously distributed variables between two groups. Statistical analyses were performed with IBM SPSS software version 25. A *p*-value of <0.05 was considered statistically significant.

Results

The study included 431 FMF patients with the M694V heterozygote/non-exon 10 mutation. Among these patients, 45.9% were female and the mean age was 128.4±52.3 months. The most common symptoms at disease onset were abdominal pain, fever and arthralgia. Colchicine resistance was observed in 12 (2.8%) patients who subsequently received anti-IL-1 treatment (Table 1). Among the patients, 128 (29.7%) had the heterozygote M694V mutation, 252 (58.4%) had mutations in exons 2, 3 or 5 (36.8% heterozygote, 21.6% compound heterozygote/homozygote) and 51 (11.8%) had a negative MEFV gene analysis. Detailed MEFV gene mutation results for patients with the M694V heterozygote and non-exon 10 mutations are shown in Table 2.

Table 1. Demographic findings of the patients with familial Mediterranean fever (n=461)

Female/Male (n) (%)	198/233 (45.9/54.1)
Age	128.4±52.3 (20-223)
BMI*	18.0±5.1 (10.9-29.5)
Age of symptoms onset*	69.7±48.7 (2-200)
Age of diagnosis*	95.7±50.1 (13-210)
Diagnosis delay time*	25.5±28.0 (3-159)
Follow-up time*	26.6±21.8 (6-144)
Family history, n (%)	154 (35.7)
Symptoms, n (%)	
Fever	275 (63.8)
Abdominal pain	292 (67.7)
Chest pain	26 (6.0)
Erysipelas like rash	12 (2.8)
Arthralgia	165 (38.3)
Arthritis	77 (17.9)
Myalgia	49 (11.4)
Emesis	14 (0.9)
Laboratory*	
WBC (10^3/uL)	7550 (6375-9300)
Hemoglobin (g/dL)	12.8 (12.1-13.5)
Platelet (10^3/uL)	285.5(247.0-335.0)
Neutrophil/Lymphocyte	1.4 (1.0-2.1)
CRP (mg/L)	0.7 (0.3-2.9)
ESR (mm/sa)	6.0 (3.0-12)
PRAS score*	5.8±1.6 (3-11)
PRAS degree*	1.6±0.6 (1-3)
Unresponsiveness to colchicine treatment, n (%)	12 (2.8)

*Mean±standard deviation (minimum-maximum), #Median (25-75%), BMI; body mass index, CRP; C-reaktive protein ESR; erythrocyte sedimentation rate, PRAS; projected retained ability score, WBC; white blood cell. All of ages are months

Table 2. Distribution of patients according to MEFV gene mutation result (n=431)

Non-exon 10 mutation	n (%) 303 (100%)	Exon-10 mutation	n (%) 128 (100)
R202Q	74 (24.4)	M694V	128 (100)
E148Q	72 (23.7)		
R202Q/R202Q	24 (7.9)		
E148Q/P369S	22 (7.2)		
E148Q/R202Q	21 (6.9)		
P369S	7 (2.3)		
E148Q/E148Q	6 (2.0)		
R202Q/P369S	4 (1.3)		
R314H	3 (0.9)		
D510D	2 (0.6)		
R314H/ R314H	2 (0.6)		
R202Q/P369S/R408Q	2 (0.6)		
G304R	1 (0.3)		
D510D/R314R	1 (0.3)		
E148Q/E230K	1 (0.3)		
E148Q/F479L	1 (0.3)		
R202Q/S339F	1 (0.3)		
R241K/E148Q	1 (0.3)		
E148Q/E230K	1 (0.3)		
E148Q/R202Q/P369S	1 (0.3)		
E148Q/P369S/R408Q	1 (0.3)		
E167D/ E167D/F479L/ F479L	1 (0.3)		
E148Q/ E148Q/P369S	1 (0.3)		
R314R/ R314R/E148Q	1 (0.3)		
R314R/R314R/R408Q/P369S	1 (0.3)		
Negative	51 (16.8)		

When comparing the M694V heterozygote and non-exon 10 mutation patient groups, no significant differences were observed with respect to sex distribution, age, age at symptom onset, age at diagnosis, delay in diagnosis, and follow-up periods. However, family history was significantly more common in the M694V heterozygote group (p=0.001). Analysis of symptoms during attacks showed that arthritis and myalgia were significantly more frequent in the M694V heterozygote mutation group (p=0.026 and p=0.005, respectively). There were no significant differences in laboratory findings between the groups, except during attacks. The PRAS score was significantly higher in the M694V heterozygote group (p=0.001),

with severe PRAS scores observed in 18.1% of this group compared to 4.3% in the non-exon 10 mutation group. Colchicine resistance was observed in 6.3% of the M694V heterozygote group compared to 1.3% of the non-exon 10 mutation group. No significant difference was found between the 2 groups regarding concomitant rheumatological diseases (Table 3).

Table 3. Comparison of demographics of patients with heterozygous M694V mutation and patients with non-exon 10 mutation

	Group 1 Non-exon 10 mutation n=303	**Group 2 M694V heterozygote mutation n=128**	***p*¥**
Female/Male (n) (%)	141/162 (46.5/53.5)	57/71 (44.5/55.5)	0.703
BMI*	17.7±3.1(10.9-29.5)	19.1± 4.2 (12.0-29.3)	0.083
Age of symptoms onset*	70.9±50.2 (2-200)	65.7±43.4 (6-196)	0.675
Age of diagnosis*	95.9±50.3 (13-210)	95.1±50.1(21-206)	0.886
Diagnosis delay time*	24.9±26.0 (3-133)	27.8±34.3 (4-159)	0.976
Follow-up time*	24.1±19.4 (6-116)	35.1±30.8 (8-144)	0.112
Family history, n (%)	88 (29.2)	66 (51.6)	0.001
Symptoms, n (%)			
Fever	193 (64.3)	82 (64.6)	0.963
Abdominal pain	206 (68.9)	86 (67.7)	0.810
Chest pain	18 (6)	8 (6.3)	0.906
Erysipelas like rash	10 (3.4)	2 (1.6)	0.310
Arthralgia	109 (36.6)	56 (44.1)	0.146
Arthritis	46 (15.3)	31 (24.4)	0.026
Myalgia	26 (8.7)	23 (18.3)	0.005
Emesis	12 (4)	2 (1.6)	0.198
Laboratory#			
WBC (10^3/uL)	7600 (6300-9750)	7300 (6550-8600)	0.468
Hemoglobin (g/dL)	12.8 (12-13.5)	13.1 (12.3-13.7)	0.116
Platelet (10^3/uL)	288.0 (247.0-341.0)	274.0 (244.5-306.5)	0.217
Neutrophil/Lymphocyte	1.4 (1.0-2.1)	1.5 (1.1-2.0)	0.943
CRP (mg/L)	0.7 (0.3-2.7)	1.4 (0.4-3.9)	0.187
ESR (mm/sa)	6.0 (3.0-13.0)	6.0 (3.0-20.0)	0.664
PRAS score*	5.6±1.6 (3.0-13.0)	6.4±2.0(3.0-13.0)	0.001
PRAS degree n(%)			
Mild	149 (49.2)	46 (36.2)	
Moderate	141 (46.5)	58 (45.7)	0.001
Severe	13 (4.3)	23 (18.1)	
Unresponsiveness to colchicine treatment, n (%)	4 (1.3)	8 (6.3)	0.004
Presence of concomitant rheumatological disease n (%)	26 (8.6)	13 (10.2)	0.610

*Mean±standard deviation (minimum-maximum), #Median (25-75%), BMI; body mass index, ESR; erythrocyte sedimentation rate
¥Chi-square test (categorical variables) and, Independent simple test (continuously distributed variables)
CRP; C-reaktive protein, PRAS; projected retained ability score, WBC; white blood cell. All of ages are months

Patients with negative MEFV gene mutations and those with non-exon 10 mutations who had colchicine resistance had negative results on the autoinflammatory panel. All patients identified with colchicine resistance who received anti-IL1 treatment responded positively to therapy.

Discussion

This study analysed 431 patients with FMF to elucidate genotype-phenotype differences between heterozygote M694V mutations and non-exon 10 mutations in FMF patients. The results show significant differences in clinical characteristics and response to treatment, contributing to a more detailed understanding of the pathogenesis and management of FMF.

In our study, no significant differences were found between the groups in terms of sex distribution, age, age at symptom onset, age at diagnosis, delay in diagnosis and follow-up periods. This finding is consistent with other studies [27, 28]. However, a study by Turkucar et al. [29] comparing patients with homozygote M694V mutations with those with exon 10 and exon 2 mutations showed that only the exon 10 mutation group had a significantly earlier onset of attacks.

The heterozygote M694V mutation is associated with more severe clinical symptoms compared to those without exon 10 mutations. This finding is consistent with numerous studies showing a strong genotype-phenotype correlation for exon 10 mutations, particularly M694V. The M694V mutation is often associated with severe disease manifestations, including a higher risk of amyloidosis and frequent, intense inflammatory episodes [30-33]. Our results also showed that patients with the heterozygote M694V mutation had higher PRAS scores and more pronounced musculoskeletal symptoms such as arthritis and myalgia compared to those without exon 10 mutations. Specifically, arthritis and myalgia were significantly higher in the M694V heterozygote group (24.4% and 18.3%, respectively) compared to the other group (15.3% and 8.7%, respectively). Similarly, in the largest cohort study from Turkey, 22.9% of patients with heterozygote M694V mutations had arthritis and 22.2% had myalgia [28].

A review of studies on patients with exon 2, 3 and 5 mutations found more mutation-specific studies. In the study by Kilic et al. [34], arthritis/arthralgia was observed in 32% of the R202Q heterozygote group, while myalgia was present in 16%. In the study by Turkucar et al. [27], arthralgia was observed in 37.5% and arthritis in 20% of 40 patients with heterozygote and homozygote R202Q mutations. In the study by Kilic et al. [34], arthralgia was observed in 49% and arthritis in 3% of 290 patients with heterozygote M694V mutations, while 93.5% and 66.6% of 171 patients with heterozygote R202Q and E148Q mutations had arthralgia and arthritis, respectively. In our study, arthralgia and arthritis were observed in 36.6% and 15.3% of patients with non-exon 10 mutations, respectively. In another study comparing patients with exon 2 mutations to those with exon 10 mutations, no significant difference in clinical findings during attacks was found [29]. In a study of patients with homozygote E148Q mutations, arthralgia was observed in 50%, arthritis in 6.7% and myalgia in 3.3% [35]. Our study found no significant differences in laboratory findings between the attack periods, which is consistent with the literature [27, 29, 36].

In our study, a severe PRAS score was observed in 18.1% of the M694V heterozygote group and 4.3% of the non-exon 10 mutation group. Similarly, in a study of 30 patients with the E148Q homozygote mutation, a severe PRAS score of 3.3% was found [36]. In a study by Kilic et al. [34] analysing genotype-phenotype characteristics in patients diagnosed with FMF, a severe PRAS score was observed in 18.3% of 290 patients with the heterozygote M694V mutation, while severe PRAS scores of 24% and 41.6% were found in 171 patients with non-exon 10 mutations.

Colchicine resistance was found in 8 patients (6.3%) in the M694V heterozygote group and in 4 patients (1.3%) in the non-exon 10 mutation group. In the study by Kilic et al. [34], 5.1% of 290 patients with the heterozygote M694V mutation were unresponsive to colchicine, whereas 6.4% of 171 patients with the heterozygote E148Q and R202Q mutations were unresponsive to treatment. Topaloglu et al. [36] studied 30 patients with the E148Q homozygote mutation and found a 3.3% rate of non-response to colchicine. In a study reported from Japan, colchicine resistance was found in

7 of 27 FMF patients, including those with exon 10 mutations, exon 2 in 2 patients, exon 3 in 2 patients and MEFV negative in 3 patients [35].

Several studies in the literature have suggested that FMF may predispose individuals to other inflammatory diseases such as PFAPA (periodic fever, aphthous stomatitis, pharyngitis, and adenitis), inflammatory bowel disease (IBD), and vasculitis [37-39]. In our study, we observed that 8.6% of patients witho non-exon 10 mutation and 10.2% of patients with the heterozygote M694V mutation had concomitant rheumatological diseases. Consistent with our study, Otar Yener et al. [38], reported a rate of 13.8% of rheumatological comorbidities in patients with heterozygote M694V mutation. Aktay Ayaz et al. [40]. reported comorbidity in 11.8% of 268 patients with M694V heterozygote mutation.

One of the limitations of this study is that we could not screen for rare mutations. We do not know if the rare mutations are associated with FMF as we were not able to screen all exons. In addition, there are studies suggesting that not only MEFV mutations but also environmental factors, miRNA expressions and microbiota influence the FMF phenotype [41-43]. In this study, only genotypic differences were evaluated.

This study shows that the M694V mutation, the clinical significance of which has been established by numerous previous studies, results in more severe PRAS scores and more pronounced musculoskeletal involvement compared to non-exon 10 mutations, regardless of the mode of inheritance, even in cases of heterozygote inheritance. In addition, the presence of severe PRAS scores and colchicine resistance, although less frequent, in non-exon 10 mutations indicates that these mutations should be taken seriously in clinical practice. Future studies investigating the clinical impact of non-exon 10 mutations will help to clarify these findings.

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Authors' contributions to the article

H.A.D, S.T., C.A., O.A.G., B.M. and E.U. have constructed the main idea and hypothesis of the study. They developed the theory and arranged the material and method section. H.A.D. has done the evaluation of the data in the Results section. Discussion section of the article written by H.A.D. and E.U. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

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The effects of lithium, metformin and everolimus substances on cell growth in 2D and 3D Ishikawa endometrial carcinoma cell culture

Lityum, metformin ve everolimus maddelerinin 2D ve 3D Ishikawa endometrial karsinom hücre kültüründe hücre büyümesi üzerine etkileri

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Abstract

Purpose: Our aim is to study the effects of the single and combined treatments of Everolimus, Metformin, and Lithium Chloride in two-dimensional (2D, monolayer) and three-dimensional (3D, spheroid) cell cultures of Ishikawa cells, which comprise the endometrial cancer cell line.

Materials and methods: As part of the study, the effects of single and combined forms of Everolimus, Metformin, and Lithium Chloride were determined on cell viability, invasion, colony formation and apoptosis, and PI3K/AKT/mTOR pathway. Cell viability was assessed using XTT assay. *CASP3, CASP8, CASP9, FASL, FADD, TNF, TRADD, BAX, TP53, PI3KCA, PI3KCB, PTEN, MTOR, AKT1* genes were evaluated with RT-PCR, apoptosis was evaluated by flow cytometry and 3D spheroid results were evaluated with invert microscope analysis.

Results: Everolimus, metformin, and lithium's IC50 levels were found at 48 hours to be 37.46 nM, 48.59 mM, and 100 μM, respectively. It was determined that the invasive capacities of Ishikawa cells in treatment groups, as well as cell colony formation were significantly reduced. In addition, Ishikawa spheroid cells were significantly suppressed compared with the control groups. RT-PCR results revealed that substances and their combinations affect genes associated with PI3K/AKT/mTOR pathway and apoptosis. Flow cytometry results showed notably increased apoptosis by single and combined treatments.

Conclusion: As a result, the single and combination forms of everolimus, metformin, and lithium have reduced cell proliferation, induced apoptosis, and decreased mTOR activation through various mechanisms in Ishikawa cells. However, our study has shown that Eve alone and triple combination therapy (Eve+Met+Lit) are more effective than other therapies in the treatment of endometrial cancer.

Keywords: Endometrial cancer, PI3K/AKT/mTOR pathway, everolimus, metformin, lithium.

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Öz

Amaç: Amacımız endometrium kanser hücre hattı olan Ishikawa hücrelerinin iki boyutlu (2D, monolayer) ve üç boyutlu (3D, spheroid) hücre kültürlerinde Everolimus, Metformin ve Lityum Klorür'ün tekli ve kombine tedavilerinin etkilerini incelemektir.

Gereç ve yöntem: Çalışma kapsamında, Everolimus, Metformin ve Lityum Klorürün tekli ve kombine formlarının hücre canlılığı, invazyon, koloni oluşumu ve apoptoz ve PI3K/AKT/mTOR yolağı üzerindeki etkileri belirlendi. Hücre canlılığı XTT testi kullanılarak değerlendirilmiştir. *CASP3, CASP8, CASP9, FASL, FADD, TNF, TRADD, BAX, TP53, PI3KCA, PI3KCB, PTEN, MTOR, AKT1* genleri RT-PCR ile, apoptoz flow sitometri ile ve 3D sferoid sonuçları invert mikroskop analizi ile değerlendirildi.

Bulgular: Everolimus, metformin ve lityumun IC50 seviyeleri 48 saatte sırasıyla 37,46 nM, 48,59 mM ve 100 μM olarak bulundu. Tedavi gruplarındaki Ishikawa hücrelerinin invazyon kapasitelerinin yanı sıra hücre koloni oluşumunun da önemli ölçüde azaldığı tespit edilmiştir. Ayrıca, Ishikawa sferoid hücreleri kontrol gruplarına kıyasla önemli ölçüde baskılanmıştır. RT-PCR sonuçları, maddelerin ve kombinasyonlarının PI3K/AKT/mTOR yolağı ve apoptoz ile ilişkili genleri etkilediğini ortaya koymuştur. Flow sitometri sonuçları tekli ve kombine tedavilerin apoptozu belirgin şekilde arttırdığını göstermiştir.

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Sonuç: Sonuç olarak, everolimus, metformin ve lityumun tekli ve kombinasyon formları, Ishikawa hücrelerinde çeşitli mekanizmalar yoluyla hücre çoğalmasını azaltmış, apoptozu indüklemiş ve mTOR aktivasyonunu azaltmıştır. Bununla birlikte, çalışmamız tek başına Eve ve üçlü kombinasyon tedavisinin (Eve+Met+Lit) endometriyal kanser tedavisinde diğer tedavilerden daha etkili olduğunu göstermiştir.

Anahtar kelimeler: Endometriyal kanser, PI3K/AKT/mTOR yolağı, everolimus, metformin, lityum.

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Introduction

Cancers are ranked second after cardiovascular disease-related deaths due to disease. Endometrial cancer is the most common gynecological cancer in developing countries [1-3].

According to biological, molecular, and clinical characteristics endometrial cancers divide into two types. Type I tumors are low-grade International Federation of Gynecology and Obstetrics grades 1 and grade 2 (FIGO grade1, 2). These patients typically develop endometrial hyperplasia. Type II (FIGO grade 3) contains histological subtypes that are not endometrioid. They generally appear on the atrophic endometrium base, are not estrogen-dependent, and have a poor prognosis with a high grade [4-7].

To improve the treatment of endometrial cancer, signaling pathways which may play a role in the development of cancer are targeted. The PI3K/AKT/mTOR is important in endometrial cancers as well as in many other cancers. The PI3K/AKT/mTOR pathway regulates various cellular functions. PI3K/AKT/mTOR overactivation is implicated in developing endometrial cancer [8]. It is important to elucidate the antitumour effects of different PI3K/AKT/mTOR pathway inhibitors and to identify the patient populations in which these inhibitors may be most effective, based on preclinical and clinical studies [4-10].

A number of studies have investigated the role of mTOR inhibition as a single agent in the recurrence of endometrial cancer [10, 11]. Everolimus (Eve) is an effective, selective, and orally active mTOR inhibitor [12]. In previous clinical studies of EC patients with progressive or recurrent phases I and II, Eve was shown to be promising [11-13].

It has been reported that single-agent treatment such as mTOR inhibitors and its analogous activates the negative feedback and as a result, the mechanisms that lead to the development of resistance [14, 15]. Combined treatments have been applied to overcome this problem and to maximally inhibit this pathway.

It has been hypothesised that drugs targeting glucose metabolism may be effective in preventing or treating endometrial cancer because of the association between obesity, diabetes, hyperinsulinemia and endometrial cancer. A prominent drug in this area was Metformin (Met) (1.1-dimethylbiguanid), a first-line oral antihyperglycemic agent used in treating type 2 diabetes [13, 14]. Met has a direct and indirect effect on cell development and metabolism. Its direct effect activates AMP-Activated protein kinase (AMPK) and causes phosphorylation of tuberous sclerosis 2 protein and mTOR inhibition. Its indirect effect increases the glucose intake of cells, thereby reducing insulin circulation. The decrease in IGF-1 and insulin inhibit cell proliferation [15, 16].

With a well-known confidence interval, Lithium (Lit) chloride is used to treat psychotic diseases, particularly bipolar disorder. Studies with Lit have shown that it has antineoplastic effects in various cancers, including colorectal cancer, stomach cancer, and neuroblastoma [17-22]. In addition, limited clinical studies have suggested that Lit may increase the therapeutic efficacy and reduce the side effects of some anti-cancer drugs. Investigation of the antitumor effects of Lit may be important for combination treatment of endometrial cancers. This is the first study to investigate the use of Lit alone or in combination with Eve and Met for the treatment of endometrial cancer.

In this study, we investigated whether Eve, Met and Lit single and combined treatments affect the human endometrial carcinoma cell line (Ishikawa), and their potential mechanisms of action.

Materials and methods

Cell culture

This study was performed using the human endometrial adenocarcinoma cell line (Ishikawa). Cells were cultured in an RPMI 1640 nutrient environment containing 10% fetal bovine serum (FBS), 1% L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL) in a stove under 95% humidity at 37°C and 5% CO_2. Studies were performed when the cell density reached 80-90%, studies were performed. In this study, single, dual, and triple combinations of Eve, Met and Lit were used.

The experimental groups were as follows:

Group1: Control

Group2: Everolimus 37.46 nM IC50 (Eve)

Group3: Metformin 48.59 mM IC50) (Met)

Group4: Lithium 100 µM IC50 (Lit)

Group5: Everolimus IC50 + MetforminIC50 (Eve+Met)

Group6: Everolimus IC50 + Lithium 100 µM IC50) (Eve+Lit)

Group7: Everolimus IC50 + Metformin IC50 + Lithium 100 µM IC50 (Eve+Lit+Met)

Cell viability assay

Cytotoxicity, dose, and time-dependent effects of the substances that we used were studied with the Biological Industries Cell Proliferation Kit XTT based Colorimetric Assay (CellTiter-Glo® luminescent cell viability assay REF:20-300-1000, LOT:2002010). The powdered substances were dissolved in 1/1000 DMSO in an RPMI 1640 nutrient environment containing 10% FBS at various doses, and their effects were investigated by adjusting their concentrations. The selected concentration range was determined according to the test kit protocol, considering information available in the literature. The cells were seeded in 96-well plate within RPMI 1640 with 2,000 Ishikawa cells in each well and were kept 24 hours in the incubator containing 5% CO_2 at 37°C. At the end of 24h, the medium was aspirated. Then, study of following concentrations: 0.1 mM, 1 mM, 5 mM, 10 mM, 20 mM, 50 mM, 100 mM for Met; 50 µM, 100 µM, 200 µM, 400 µM for Lit; 2 nM, 10 nM, 25 nM, 50 nM, 100 nM, 200 nM and 300 nM for Eve were prepared in the medium containing 10% FBS and the effects at 24, 48 and 72h were determined.

After 24 h, 100 µL medium was added to each well, 50 µL reagent solution A, 1 µL XTT activator mixture was prepared, and 150 µL was added per well. The cells were then incubated for 4h in an incubator containing 5% CO_2 at 37°C, and the absorbance levels of the groups were determined in the ELISA. IC50 ratio was calculated as follows:

Cell viability (%)= (Absorbance value of the substance-applied group/Absorbance value of the control group) x 100

Spheroid formation with three-dimensional (3D) cell culture method

The liquid overlay technique was used to create an *in vitro* Ishikawa spheroid model. Ishikawa cells were first seeded in a T75 flask with RPMI 1640 medium and were incubated in a stove with 95% humidity and 5% CO_2 at 37°C. When the cells were 90% confluent, they were washed with PBS, thoroughly purged from dead cells and cell waste, and aspirated. Trypsin was added to allow the cells to leave the flask. In 1500 g, the cell-media mix in the flask was centrifuged for 4 min, and the supernatant was removed. Then cells with 100% viability were cultured using RPMI 1640 to a 3% Noble agar-covered six-well culture plate with 1×10^6 cells in each well, and images of the cells were taken in the inverted microscope in certain ranges and measured in size.

Colony formation analysis

Ishikawa cells were seeded at 6-well plates at $1x10^3$ cell/well. After 24h of incubation, the cells were exposed to the single and combined concentrations of Eve, Met and Lit agents for 48h. The cells were incubated at 5% CO_2, 37°C. At the end of 14 days, the cells were fixed for 10 min with methanol and incubation. The colonies were painted with crystal violet and counted.

Matrigel invasion analysis

Ishikawa cells were seeded at a concentration of $5x10^5$ cells/wells on a Matrigel membrane with 8 µm pores and incubated overnight in a non-serum environment. They were put in RPMI 1640 24 well plates with 10% FBS containing serum. After the cells were incubated overnight, Met, Eve and Lit and combined doses were applied to the medium without serum. The cells passing through the membrane were detected by methanol, painted with crystal violet and counted.

Apoptosis detection with annexin V

The apoptotic index was evaluated using flow cytometric annexin-V-fluorescence isothiocyanate/propidium iodide (Annexin-V-FITC/PI) (BD Pharmingen™ FITC, catolog No:5565447). After the instructions in the kit's manual, the cells were washed twice with PBS and re-suspended with the 0.01 M HEPES, 0.14 mM NaCl, and 2.5 mM $CaCl_2$ containing binding pad. The cells in the cell suspension were incubated with 5 µL Annexin V (BD Pharmingen) FITC-labelled stain and PI for 15 min at room temperature in the dark. PI fluorescence and Annexin V were measured at the same time in a (BD FACS Calibur™, Cat. No:349227) and analyzed with the operating software of the device.

Real-time PCR analysis

RNA isolation was performed in the groups to assess expression at the gene level. Cells were seeded at a density of $3×10^6$ cells/well. After 24h of incubation, Lit, Eve, Met, and their combination were applied to each well, except the control well, and incubated for 48h. Complementary DNA (cDNA) was synthesized using the cDNA Synthesis Kit (WizScript™). *CASP3, CASP8, CASP9, PI3KCA, PI3KCB, FASL, FADD, TNF, TRADD, BAX, TP53, PTEN, mTOR, and AKT* expression analyses were performed using the StepOnePlus quantitative real-time PCR (Thermo Scientific PikoReal 96) according to the expression analysis SYBR Green (Thermo Scientific, USA). Values were normalised to *ACTB* levels for each gene (Table 1).

Statistical evaluation of data

PCR data were analysed by the ΔΔCT method and quantified by a computer programme. The Volcano Plot analysis provided by the RT² Profiler; PCR Array Data Analysis programme was used to compare the groups. Student's t-test was used to statistically evaluate the comparison between groups. SPSS 24.0 package program was used for data analysis. Continuous variables were presented in terms of mean ± standard deviation and medians (minimum and maximum), and categorical variables in terms of numbers and proportions. The Shapiro-Wilk test was used to examine the conformity of the data for normal distribution. The Kruskal-Wallis Analysis of Variance (Post-hoc, Mann-Whitney U-test with Bonferroni adjustment) was used to compare independent group differences (p<0.05, considered statistically significant).

Results

Eve, Met and Lit and their combined forms decreased cell proliferation of Ishikawa cells

In the results of the 24h cell viability test of the applied substances, dose rates at 48h were considered IC50 as cell proliferation did not fall below 50%. The varying dose ranges and overtime effects of Eve, Met, Lit alone, and combined treatments are shown in Figure 1.

Eve, Met and Lit and their combination reduced Ishikawa 3D spheroids

In the 48h images, which represent the IC50 time of the doses, a reduced number of spheroids, shrinking sizes, and border irregularities were detected. The control group presented a large number of spheroids with an average size of 170 µm, ranging from 120 µm to 225 µm. In treatment groups, average of spheroid size in the Eve treatment group was 71.33 µm, average of spheroid size in the Met treatment group was 97.5 µm, in the Lit treatment group was 90 µm, in the Eve+Met treatment group was 96.4 µm, in the Eve+Lit, treatment group was 97 µm, in the Eve+Met+Lit treatment group was found to be 80 µm (Figure 2).

Table 1. Primary list of genes analyzed on RT-PCR

Genes	Primer Sequence
ACTB	F: CACCATTGGCAATGAGCGGTTC R: AGGTCTTTGCGGATGTCCACGT
CASP3	F: GGAAGCGAATCAATGGACTCTGG R: GCATCGACATCTGTACCAGACC
CASP8	F: AGAAGAGGGTCATCCTGGGAGA R: TCAGGACTTCCTTCAAGGCTGC
CASP9	F: GTTTGAGGACCTTCGACCAGCT R: CAACGTACCAGGAGCCACTCTT
MTOR	F: GCTTGATTTGGTTCCCAGGACAGT R: GTGCTGAGTTTGCTGTACCCATGT
AKT1	F: TTCTGCAGCTATGCGCAATGTG R: TGGCCAGCATACCATAGTGAGGTT
PIK3CA	F: GGTTGTCTGTCAATCGGTGACTGT R: GAACTGCAGTGCACCTTTCAAGC
PIK3CB	F: TTGTCTGTCACACTTCTGTAGTT R: AACAGTTCCCATTGGATTCAACA
BAX	F: TCAGGATGCGTCCACCAAGAAG R: TGTGTCCACGGCGGCAATCATC
PTEN	F: TGAGTTCCCTCAGCCGTTACCT R: GAGGTTTCCTCTGGTCCTGGTA
FADD	F: GCTGGCTCGTCAGCTCAAA R: ACTGTTGCGTTCTCCTTCTCT
TRADD	F: GCTGTTTGAGTTGCATCCTAGC R: CCGCACTTCAGATTTCGCA
FASL	F: GGTTCTGGTTGCCTTGGTAGGA R: CTGTGTGCATCTGGCTGGTAGA
TNFα	F: CTCTTCTGCCTGCTGCACTTTG R: ATGGGCTACAGGCTTGTCACTC
TP53	F: ATCTACAAGCAGTCACAGCACAT R: GTGGTACAGTCAGAGCCAACC

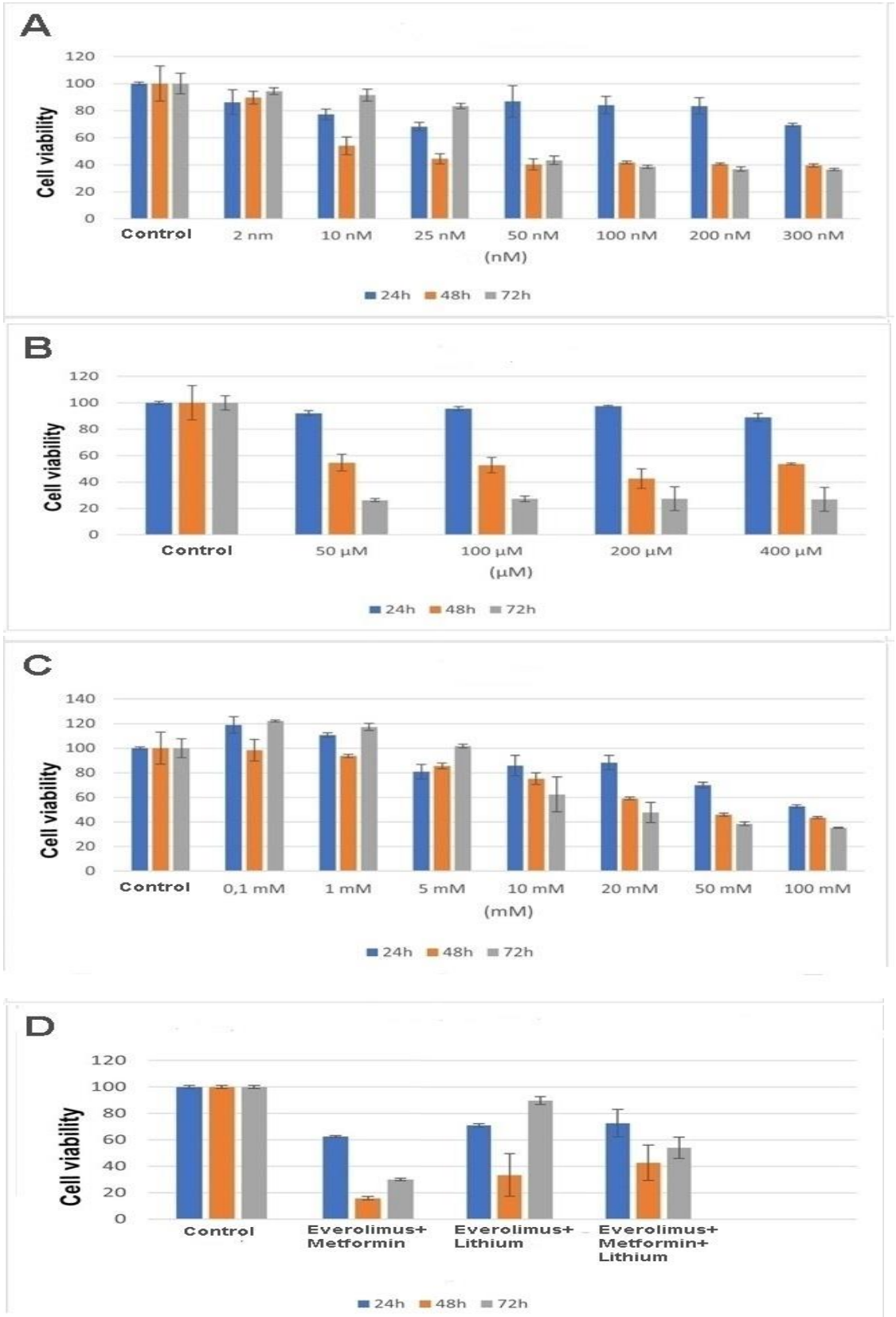

Figure 1. The effect of Everolimus, Lithium, Metformin, and combined doses on Ishikawa cell viability.
A) IC50 doses of Everolimus; Ishikawa cells were detected 37.46 nM at the 48th hour
B) IC50 doses of Metformin; Ishikawa cells were detected 48.59 mM at the 48th hour
C) IC50 doses of Lithium; Ishikawa cells were detected 100 mM at the 48th hour
D) IC50 doses of combined treatment; Ishikawa cells were detected at the 48th hour

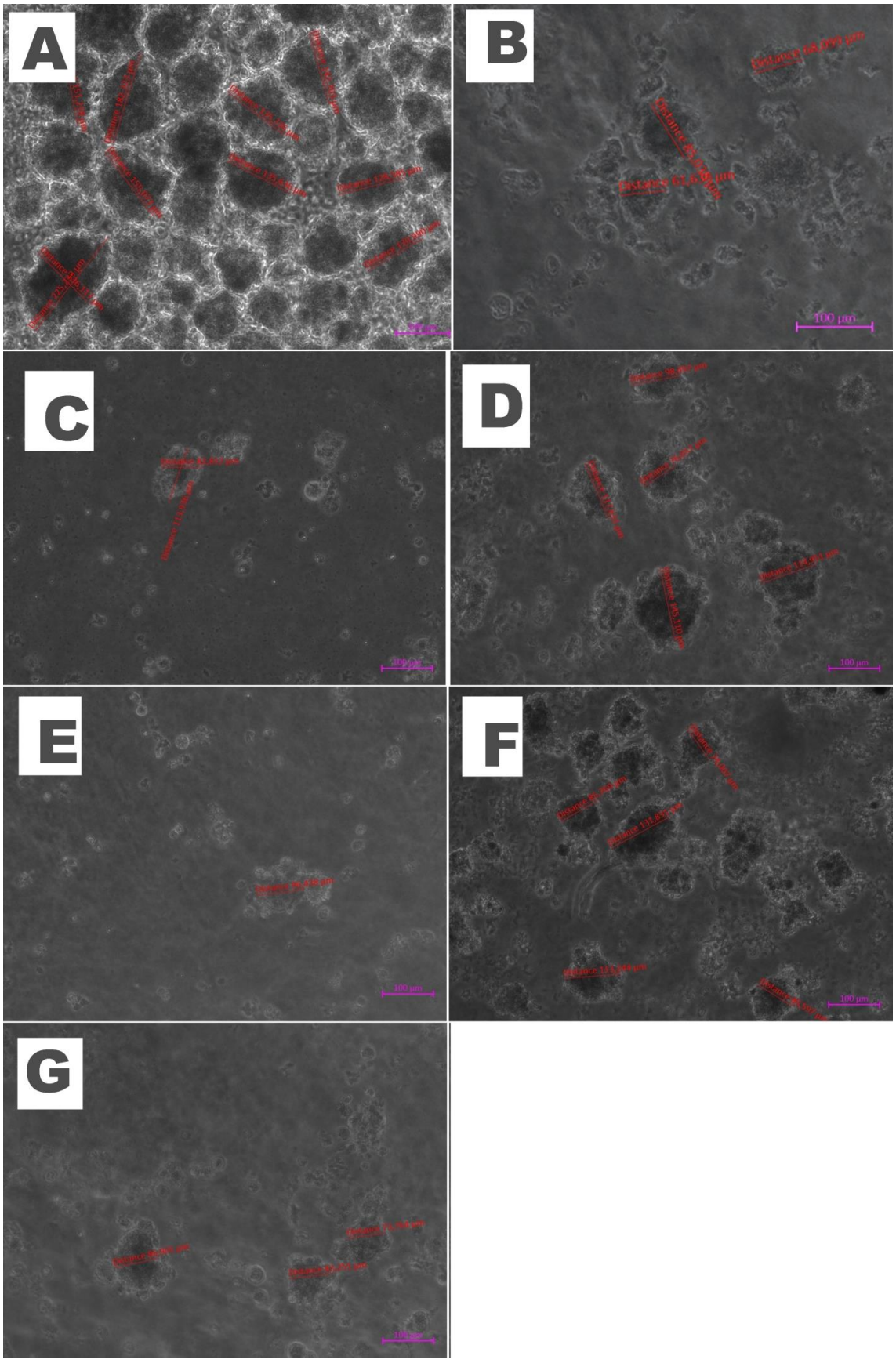

Figure 2. Images of spheroids consisting of endometrial cancer cells. A: Control, B: Everolimus, C: Metformin, D: Lithium, E: Everolimus + Metformin, F: Everolimus + Lithium, G: Everolimus + Metformin + Lithium. Bar:100µm

Eve, Met and Lit and their combined forms reduced colony formation capacity of Ishikawa cells

In all groups colony numbers in Ishikawa cells was significantly reduced with respect to the control group. The colony formation data of the groups in the Ishikawa cell array was shown as follows, respectively: Control (382.33±20.53), Eve (195±13.23), Met (126±7), Lit (251.67±12.58) Eve+Met (48.33±13.01), Eve+Lit (210±43.59) Eve+Met+Lit (62.67±15.37) Eve+Met was the lowest in the colony formation analysis compared to the control group (Figure 3).

Eve, Met and Lit and their combined forms inhibits migration of Ishikawa cells

As a result of the Matrigel invasion experiment, single and combined of Eve, Met and Lit administered Ishikawa cells noticeably decreased according to the control group of the invasion. The invasive data of the groups in the Ishikawa cell array were as follows, respectively: Control (1420±5), Eve (311±3.61), Met (10±0), Lit (47.33±2.52), Eve+Met (10±0), Eve+Lit (210±43.59), Eve+Met+Lit (351±3) (Figure 4).

Eve, Met and Lit and their combined forms induces apoptosis on Ishikawa cells

Flow cytometry results

According to the flow cytometry results, compared to the control group, late apoptosis increased significantly in the treatment groups. The highest rate of late apoptosis was observed in the Eve+Met+Lit and Eve+Lit groups (Figure 5).

Eve, Met and Lit and their combined forms changes mRNA expressions genes

In the group administered Eve, *FASL* and *CASP3* showed a significant increase. In the group administered with Met, *FASL, PTEN, CASP9*, and *TRADD* increased significantly ($p<0.05$), whereas *CASP8* decreased significantly ($p<0.05$) in the Lit administered group. In the group administered Eve+Met, there was a significant increase in *FADD, CASP9, and BAX* values, and the Eve+Lit group showed a significant increase in *FADD* and *TNF* values ($p<0.05$). *PTEN*, *FADD, TNF*, and *TP53* levels increased significantly ($p<0.05$) in the Eve+Met+Lit group (Table 2, 3).

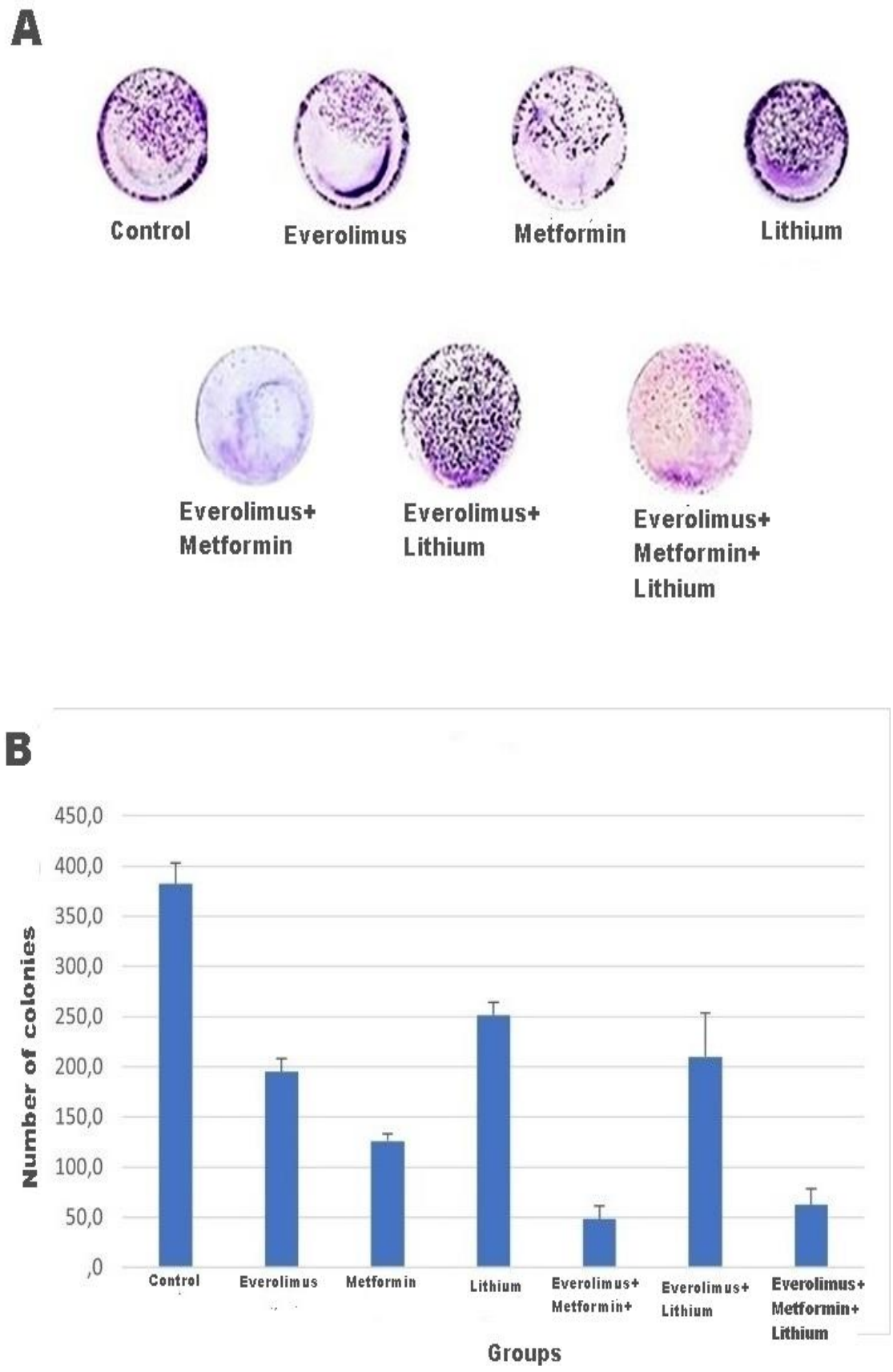

Figure 3. A: Everolimus, Lithium, Metformin and combined doses decrease colony formation in Ishikawa cells. Colonies were stained with crystal violet. B: Data was presented as mean ± SD

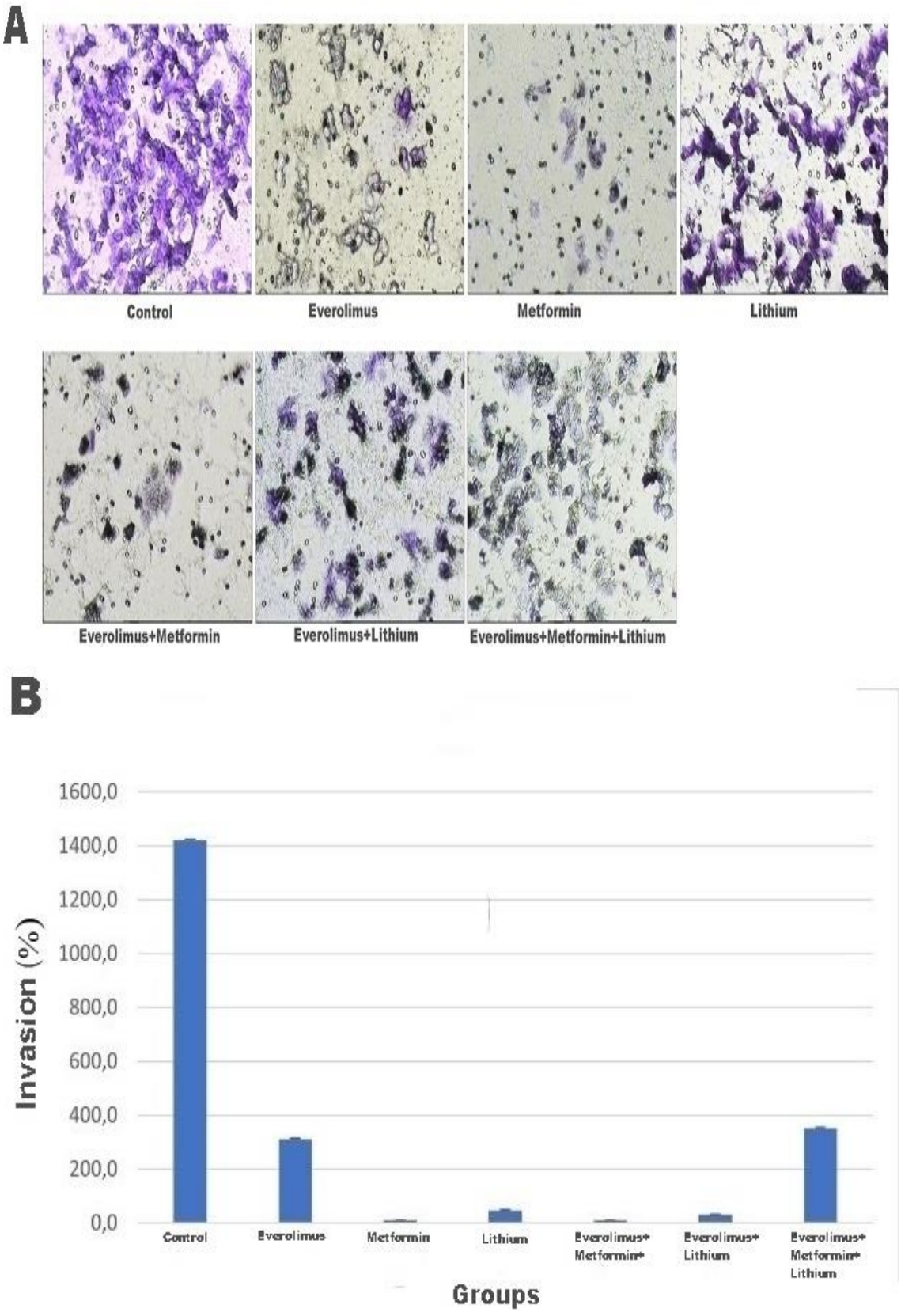

Figure 4. A: Migration and invasion assay results of Ishikawa cells were showed. Cells that passed through the membrane were counted in 10 representative areas. Graph showing invasion for control and dose groups. B: Graph showing invasion for both control and dose groups. Data were presented as mean ± SD

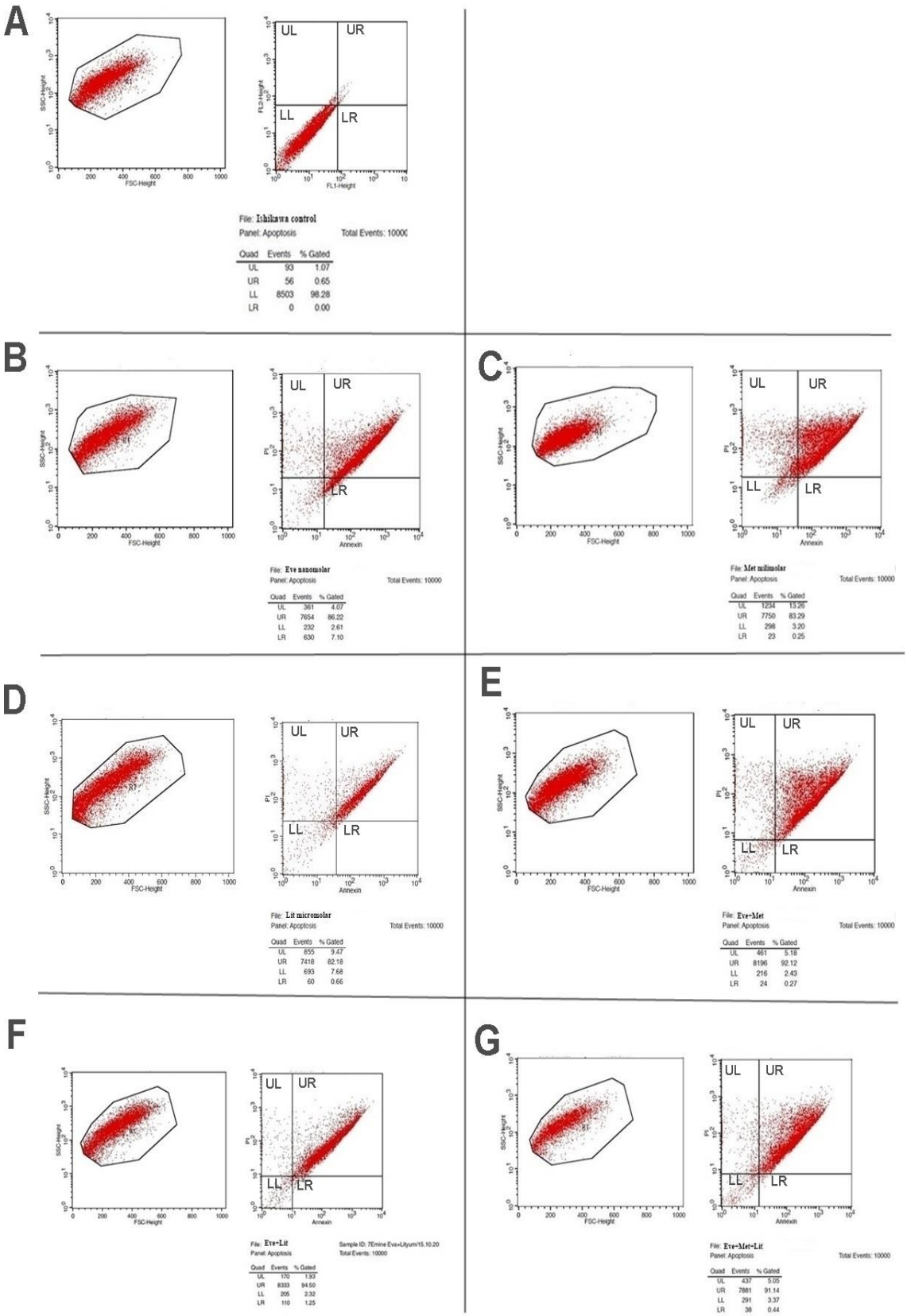

Figure 5. The effect of Everolimus, Metformin, Lithium, and combined doses on apoptosis in Ishikawa cells. A: Control, B: Everolimus, C: Metformin, D: Lithium, E: Everolimus+ Metformin, F: Everolimus +Lithium, G: Everolimus + Metformin + Lithium. UL: Necrotic cell, LL; Live cell, UR: Late apoptotic cell, LR: early apoptotic cell

Table 2. The mRNA expression changes of cell cycle and apoptosis genes in Ishikawa cell line treated with everolimus (Eve), metformin (Met) and lithium (Lit), compared with the control group cells

Gene Symbol	Fold regulation (comparing to the control grup)					
	Eve		Lit		Met	
	Fold regulation	*p*-value	Fold regulation	*p*-value	Fold regulation	*p*-value
ACTB	1.00	nan	1.00	nan	1.00	nan
CASP-8	1.13	0.64	-4.92	0.01	-1.16	0.40
FASL	2.11	0.02	-1.38	0.47	1.87	0.04
FADD	6.88	0.23	23 75	0.10	32.90	0.05
TNF-Alpha	10.51	0.09	5.64	0.19	2.60	0.35
TRADD	-1.71	0.90	3.54	0.15	13.83	0.00
CASP-9	-1.63	0.56	4.75	0.13	2.30	0.00
BAX	-1.45	0.31	3.12	0.14	1.98	0.19
CASP-3	1.65	0.04	1.39	0.46	1.38	0.06
TP53	1.21	0.79	-1.94	0.91	5.75	0.27
PTEN	-1.46	0.05	1.02	0.90	346	0.00
PIK3CA	2.72	0.66	6.93	0.42	40.13	0.13
PIK3CB	-54.07	0.24	-1.64	0.35	2.41	0.76
AKT1	-1.39	0.19	2.73	0.11	2.05	0.20
MTOR	-8.67	0.38	1.50	0.87	1.46	0.42

Table 3. The mRNA expression changes of cell cycle and apoptosis genes in Ishikawa cell line treated with everolimus+metformin (Eve+Met), everolimus+lithium (Eve+Lit), and everolimus+metformin+lithium (Eve+Met+Lit) compared with the control group cells

Gene Symbol	Fold regulation (comparing to the control grup)					
	Eve+Met		Eve+Lit		Eve+Met+Lit	
	Fold regulation	*p*-value	Fold regulation	*p*-value	Fold regulation	*p*-value
ACTB	1.00	nan	1.00	nan	1.00	nan
CASP8	-1.57	0.39	2.80	0.16	-1.13	0.47
FASL	-1.77	0.41	-2.14	0.76	1.47	0.18
FADD	18 55	0.00	15.21	0.03	-2.43	0.00
TNF	7.24	0.31	34.70	0.00	8.42	0.02
TRADD	66.72	0.33	33.59	0.37	-2.12	0.06
CASP9	7.43	0.01	7.91	0.06	-1.31	0.41
BAX	4.41	0.02	2.35	0.25	1.17	0.64
CASP3	2.44	0.21	1.32	0.42	-1.10	0.57
TP53	100.66	0.37	12.35	0.10	4.34	0.03
PTEN	2.79	0.24	1.78	0.11	-2.76	0.00
PIK3CA	21,51	0.37	15.93	0.17	-2.47	0.41
PIK3CB	4.52	0.50	-3.18	0.28	-9.49	0.26
AKT1	2.08	0.19	3.42	0.15	5.51	0.36
MTOR	6.56	0.62	4.77	0.41	1.40	0.42

Discussion

This is the first study to investigate the effects of single and combined forms of Eve, Met and Lit on cell viability, colony formation, cell invasion, spheroid formation, apoptosis, PI3K/Akt/mTOR signaling pathway in cultures of Ishikawa cells. In our study, colony formation and invasion capacity decreased significantly in the single and combined treatment groups compared with the control group. In particular, the combination of Met and Eve and Eve+Met+Lit was significantly more effective than either monotherapy or the other combined therapies in inhibiting cell invasion and the ability of Ishikawa cells to form colonies, a crucial event involved in tumorigenesis. These results were consistent with previously reported findings that the combined use of Met and Eve synergistically augmented anticancer activity in the treatment of breast and cervical cancers [21-25].

3D spheroids geometrically and molecularly mimic tumors *in vivo* compared to monolayer 2D cells [26]. 3D cell culture has become an effective method for drug screening [27]. In our study, the effects of Met, Eve, and Lit alone and in combination on the growth of Ishikawa 3D cells were examined. Interestingly, Eve, Met and Lit alone and in combination blocked the

growth rate of Ishikawa spheroids. However, Eve alone was quite potent in reducing the growth of spheroids compared to other groups, except for the Eve+Met+Lit group. The results in the triple combination group were similar to those in the Eve group.

We examined whether the antineoplastic effects of Met, Eve, and Lit single and combined administration were mediated by the induction of apoptosis. Apoptosis was higher in all treatment groups than that in the control group. However, while early apoptosis was highest in the Eve treatment group, late apoptosis was slightly higher in all combined groups than in the single treatment groups.

When the molecules involved in the extrinsic and intrinsic apoptotic pathways were examined by RT-PCR analysis to elucidate the mechanism of apoptosis, it was determined that both the extrinsic and intrinsic pathways were effective in the single and combined treatments. In addition, another apoptosis-related gene, *TP53,* which plays an important role in the regulation of cell proliferation, DNA repair, apoptosis, was significantly upregulated in the Eve+Met+Lit treatment group.

The PI3K/AKT/mTOR is closely related to various common malignancies and plays a major role in resistance to anticancer drugs in response to treatment [28-32]. In a wide range of tumours, the components of this pathway support irregular and limitless cancer cell growth and proliferation and contribute to the tumor by allowing the avoidance of apoptosis [33, 34]. It is known that the increased mTOR activity supports the PI3K/AKT/mTOR pathway. Supported by preclinical data the important role of mTOR in cancer, resulting in mTOR inhibiting drugs being developed as a therapeutic target [35]. Eve is an inhibitor of rapamycin (mTOR), which has an antitumor effect on cancers and reduces the proliferation of endometrial cancers through mTOR inhibition [36]. According to our PCR results, a decrease in *mTOR* mRNA levels was observed in all the groups.

The *PTEN* gene is a negative regulator of the PI3K/AKT/mTOR pathway, which is the most frequent alteration in endometrioid tumors [14, 15]. In preclinical studies, mutations in various genes have been identified in the molecular analysis of endometrial cancers. *PTEN* and *PI3CA* were the most common genes. *PTEN* loss has been detected in 80% of endometrial tumors [14]. Thus, abnormal cell growth and escape from apoptosis due to loss of PTEN activity [13]. We observed that single-met treatment increased *PTEN* mRNA levels. These results are in line with those reported by other studies that Met increased *PTEN* mRNA levels in Ishikawa cells and advanced and recurrent endometrial cancers [37, 38]. According to the PCR results, the lowest *PTEN* levels were determined in the Eve alone and Eve+Met+Lit combined groups. These results suggest that Eve suppresses the efficacy of Met, and that Eve and Eve+Met+Lit may use different pathways to inhibit mTOR in endometrial cancers.

Increased *PI3K* activity via gain of function has also been shown in several human cancers: *PIK3CA,* with a frequency of up to 33% in Type I endometrial tumors and 15-20% in Type II endometrial tumors, is the second most mutated gene [39-42]. Previous studies have shown that *PIK3CB*, the other catalytic component of PIK, is highly expressed in endometrial cancer cell lines and clinical samples taken at the initial stage of endometrial carcinogenesis. *PIK3CB* mutations are less frequent than *PIK3CA* mutations [43, 36]. Both *PIK3CA and PIK3CB* mRNA levels were low in the Eve+Met+Lit group, indicating that the triple combined treatment was effective against *PIK3CA* and *PIK3CB* mutations. *AKT* is known to regulate signaling events that promote cell survival, proliferation, angiogenesis, and invasion [44]. In our study, no significant change was observed in *AKT* mRNA level in any groups.

This study has some limitations. These include not demonstrating the effects of the tested substances on non-cancerous cell lines by using any healthy normal cells in the study, and also evaluating the study using only one endometrial cancer cell line. It is recommended to improve the study by using additional cell lines and healthy cells.

In conclusion, we determined that the single and combination of Eve, Met, and Lit were effective in inhibiting Ishikawa cell proliferation, promoting apoptosis, and preventing colony formation and invasion capacity, probably acting via different pathways in mTOR inhibition.

Our study showed that single and combined therapies, especially Eve alone and Eve+Met+Lit, were more effective than other therapies in the treatment of endometrial cancer. We believe that Eve+Met+Lit therapy will be an alternative in the treatment of endometrial cancer because of the possibility of resistance in a single application, the high probability of combined treatments to prevent this condition, and the ability to treat it as an anti-carcinogen at lower doses; however, we believe that further preclinical and clinical studies are required on this matter.

Conflict of interest: No conflict of interest was declared by the authors.

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Ethics committee disclosure: In our study “The Effects of Lithium, Metformin and Everolimus Substances on Cell Growth in 2D and 3D Ishikawa Endometrial Carcinoma Cell Culture”, human Endometrial Carcinoma Cell (Ishikawa Cell Line) that we have in stock were used. Cells are cultured in medium. Cells that become confluent between 2 and 3 days are multiplied by changing the medium, and experimental groups of cells that have reached sufficient density are formed. Ethics committee approval is not required for cell culture studies and cells are in our stock.

Contributions of the authors to the article

E.T. and G.A.M. constructed the main idea and hypothesis of the study. E.T. and G.A.M. formulated the research hypothesis, designed the methodology, literature review. N.C. developed the theory and arranged/edited the material and method section. M.S. designed and implemened a 2D cell culture experiment. H.D. designed the 3D cell culture involved in the research. A.B. contributed to the formulation of the research hypothesis. E.K. provided space and personnel for the 3D culture of work. The article was written by E.T., N.C., and G.A.M. reviewed the article and made the necessary corrections and approved it. In addition, all authors discussed the entire study and approved the final version.

Research Article

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Effect of quercetin on oxidative stress in 3T3-L1 mature and hypertrophic cells

Quercetin'in 3T3-L1 olgun ve hipertrofik hücrelerde oksidatif stres üzerine etkisi

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Abstract

Purpose: The process of excessive or abnormal accumulation of fat in the body is called obesity, and its prevalence is increasing globally. The imbalance between antioxidants and free radicals, or oxidative stress, can be caused by or result from obesity. Flavonoids with antioxidant potential may help lower the increased oxidative stress associated with obesity. This study aimed to determine how quercetin affected oxidative stress in hypertrophied and mature 3T3-L1 adipocytes.

Materials and methods: After differentiating, 3T3-L1 adipocytes were treated with insulin and a glucose-containing medium to become mature (10 days) and hypertrophic (18 days). The cells were subsequently incubated with 80 µM quercetin for 24 and 48 hours. ELISA was used to determine the levels of total antioxidant total oxidant capacity (TAS/TOS). Using Oil Red O staining, an accumulation of triglycerides in cells was examined.

Results: The results showed that quercetin molecule only increased TAS level on oxidative stress in mature adipocytes (TAS; M-C: 649.37±1.38; M-Q80: 655.87±1.68 *p*=0.0001), whereas it exerted a prooxidative effect in hypertrophic adipocytes (OSI; H-C: 4.90±0.19; H-Q80: 6.20±0.039 *p*=0.0001).

Conclusion: It is believed that the administration of quercetin at the appropriate dose and duration in different fat cell types is crucial for the antioxidant mechanism of action that produces numerous beneficial effects.

Keywords: Obesity, 3T3-L1 cell, quercetin, oxidative stress, oxidant/antioxidant level.

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Öz

Amaç: Bütün dünyada sıklığı giderek artan obezite, vücutta aşırı veya anormal yağ birikmesi sürecidir. Oksidatif stres; serbest radikaller ve antioksidanlar arasındaki dengesizlik olup, bu durum obezitenin bir sonucu olabileceği gibi aynı zamanda obezitenin bir tetikleyicisi de olabilir. Obezite durumunda artan oksidatif stres, antioksidan kapasiteye sahip flavanoidler tarafından azaltılabilir. Bu araştırmanın amacı, olgun ve hipertrofik hale getirilmiş 3T3-L1 adipositlerde quercetinin oksidatif stres üzerine etkisinin belirlenmesidir.

Gereç ve yöntem: Önce farklılaştırılan ve daha sonra glikoz içeren medyum ve insülin ile muamele edilerek mature (10 gün) ve hipertrofik (18 gün) hale getirilen 3T3-L1 adipositleri 80 µM dozda quercetin (24saat, 48saat) ile inkübe edildi. Total antioksidan total oksidan kapasite (TAS/TOS) düzeyleri ELISA aracığılıyla ölçüldü. Oil Red O boyama ile hücrelerde oluşan trigliserit birikimi analiz edildi.

Bulgular: Sonuçlar quercetin molekülünün olgun adipositlerde oksidatif stres üzerine yalnızca TAS düzeyini arttırdığını gösterdi (TAS; M-C: 649,37±1,38; M-Q80: 655,87±1,68 *p*=0,0001), hipertrofik adipositlerde ise prooksidatif etki yaptığını gösterdi (OSI; H-C: 4,90±0,19; H-Q80: 6,20±0,039 *p*=0,0001).

Sonuç: Quercetin'in farklı yağ hücre türlerinde uygun doz ve sürede uygulanmasının çok sayıda yararlı etki üreten antioksidan etki mekanizması için belirleyici olduğuna inanılmaktadır.

Anahtar kelimeler: Obezite, 3T3-L1 cell, quercetin, oksidatif stres, oksidan/antioksidan seviye.

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Introductıon

Around the world, there is a rising prevalence of obesity and disorders associated with it. Adipose tissue enlargement, or obesity, is closely linked to long-term metabolic conditions such type 2 diabetes mellitus (T2DM) and cardiovascular illnesses [1]. The combination of hyperplasia and hypertrophy results in an increase in adipose tissue, which is characterized by an excessive accumulation of lipids and ultimately leads to adipose tissue dysfunction [2, 3]. Adipogenesis is the process by which a preadipocyte differentiates into a mature adipocyte through the accumulation of lipid droplets; however, obesity is the result of excessive lipid accumulation [4]. Obese people have high levels of genotoxic stress. Hyperlipidemia is linked to alterations in lipoprotein distribution and systemic oxidative stress in both humans [5] and lab animals [6].

The fundamental mechanism behind the chronic state of systemic inflammation that these individuals display is not entirely understood, however it is commonly related to cellular stress [7]. The methods used today for preventing obesity include increased energy expenditure through physical exercise, medication, bariatric surgery, and calorie restriction by dietary modifications [8]. Quercetin, a flavonoid, is frequently found in onion peels, wine, and tea [9]. Recent studies have demonstrated that Quercetin has the potential to reduce body fat. The suggested mechanism of action for its favorable effects includes its impact on lipolysis, apoptosis, fatty acid absorption, inhibition of adipogenesis, and reduction of lipogenesis [10, 11]. Treatment with quercetin in adipocytes triggers the apoptosis pathway by regulating AMP-activated protein kinase (AMPK) [11]. Alternatively, it inhibits the expression of transcription factors involved in adipocyte differentiation, such as peroxisomal proliferator-activated receptors (PPAR)-γ, CCAAT/enhancer-binding protein (C/EBP) α, fatty acid-binding protein 4, and adipocyte protein 2 (aP2) [12]. The 3T3-L1 preadipocyte cell line is an important tool for investigating the in vitro processes by which obesogens can impact lipid accumulation and adipocyte development [13, 14]. This study aimed to assess the impact of quercetin on oxidative stress in hypertrophic 3T3L-1 cells caused by high glucose.

Therefore, this study may make an important contribution to the literature in terms of revealing the fine details of the antioxidant or prooxidant potential of quercetin.

Materials and methods

Cell culture

In our study, 3T3-L1 cell line (ATCC® CL173™), a fibroblast cell line obtained from mouse (Mus musculus) embryo, which is also known as preadipocyte, was used. Mouse 3T3-L1 fibroblasts were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA, Waltham) containing 25 mM glucose supplemented with 10% calf serum (Cegrogen, Germany) 100 U/ml penicillin (Wisent, Saint-Jean-Baptiste, Canada) 100 lg/ml streptomycin (Wisent, Saint-Jean-Baptiste, Canada) and 2 mM L-glutamine at 37°C in a humidified atmosphere of 5% CO_2. The cells were propagated by transferring them to new culture dishes every 3-4 days until they reached around 80% coverage.

Differentiation of the 3T3-L1 cell line

In brief, cells were first cultured in low-glucose medium to create the experimental groups. Cells were followed with the same medium until confluent. When the cells reached approximately 80% density in the culture dishes, a medium containing a differentiation cocktail 3-Isobutyl-1-methylxanthine (IBMX, Sigma I5879, USA) dexamethasone (DEX, Sigma D4902, USA), insulin (INS) (Sigma I6634, USA) fetal bovine serum (FBS, Biowest, South America) was added to the medium to induce the differentiation of preadipocytes into adipocytes, and the cells were incubated in this medium (MD1) for 48 hours.

Mature/Hypertrophic adipocyte model

After incubation, the medium containing (Gibco, USA, Waltham) glucose and INS (MD2) was changed every other day for 10 days mature and 18 days for hypertrophic and the cells were monitored. Thus, the diameter and number of lipid droplets in adipocytes increased.

Oil Red O staining

To confirm the transformation of preadipocytes into mature (day 10) and hypertrophic (day 18), they were analysed microscopically using the Oil Red O staining kit [Biovision Lipid (Oil Red O) Staining Kit (Catalog # K580-24)]. Differentiated 3T3-L1 adipocytes were fixed with 10% formalin in Phosphate Buffered Saline (PBS, Wisent, Saint-Jean-Baptiste, Canada) for 1 h and washed twice with 60% isopropanol. The fixed cells were then stained using Oil Red O solution for 30 min and washed with distilled water. After drying, the cells were imaged by scanner. The Oil Red O solution taken up by the cells was then extracted using 100% isopropanol and its optical intensity was measured at 490 nm. A microscope was used to see the triglyserid accumulation at 20X and 40X magnifications.

Administration of quercetin

Quercetin 80 μM [15] dose was applied to mature 3T3-L1 cells at day 10 post-differentiation for 24 hours and hypertrophic 3T3-L1 cells at day 18 post-differentiation for 48 hours. Details of the experimental timeline are shown in Figure 1.

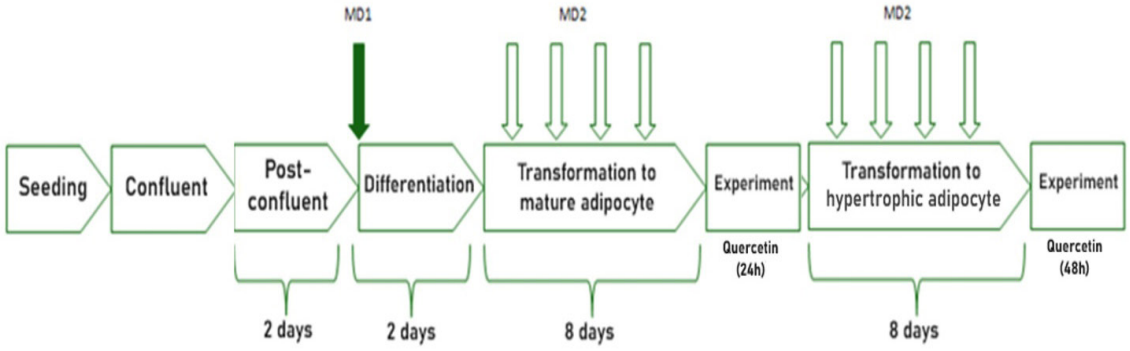

Figure 1. Illustration of experimental design.Quercetin was administered 80 μM dose

For medium 1, DMEM containing high glucose (4.5 g/L) was prepared by adding 0.5 mM IBMX, 1 μM DEX, 10 μg/mL INS, 10% FBS, 1% P/S solution. For medium 2, DMEM containing high glucose (4.5 g/L) was prepared by adding 10μg/ml INS, 10% Fetal bovine serum (FBS), 1% P/S solution. IBMX; 3-Isobutyl-1-methylxanthine, DEX; dexamethasone, INS; insulin, FBS; fetal bovine serum, P/S; penicilin/streptomycin, DMEM; Dulbecco's Modified Eagle Medium

Measurements of total antioxidant status

Following a treatment of 3T3-L1 mature and hypertrophic cells with 80 μM Q for 24 and 48 hours, the cell culture media was removed. The Fenton reaction generates the most powerful biological radical, namely the hydroxyl radical. The hydroxyl radical reacted with the colorless substrate o-dianisidine to produce the diansyl radical, which exhibits a prominent yellowish-brown hue. This approach is utilized to assess the overall antioxidant response to potent free radical reactions. When a sample of cell culture medium is introduced into the reaction mixture, the antioxidant components hinder the oxidative processes initiated by the hydroxyl radicals in the reaction mixture, hence preventing any alteration in color and offering a reliable method to measure the overall level of antioxidant capacity (TAS). The assay results are reported in mmol Trolox Equiv./L (RelAssay, Türkiye, Gaziantep).

Measurements of total oxidant status

The colorimetric examine was used to measure the amounts of total oxidant status (TOS). The ferrous ion-o-dianisidine complex underwent oxidation to form the ferric ion. The presence of glycerol molecules enhanced the oxidation reaction, which was inadequate in the reaction media. At the conclusion of the reaction, an intense complex formed between xylenol orange and the ferric ion in the acidic

environment. The concentration of oxidant molecules in the sample is directly proportional to the intensity of its color, which is quantified using spectrophotometry. The calibrations were conducted utilizing hydrogen peroxide, and the results were expressed in micromoles of H_2O_2 equivalent per liter (RelAssay, Türkiye, Gaziantep).

Determination of oxidative stress index

The oxidative stress index (OSI) was calculated by the following formula: OSI (arbitrary unit) = TOS (μmol)/TAS (mmol)×100.

Statistical analysis

The data were analyzed with the software package SPSS 25.0. Continuous variables are expressed as the mean±standard deviation. Shapiro Wilk test was used for normality testing. Independent samples t test was used for comparing independent groups. In all analyses, $p \leq 0.05$ was considered statistically significant.

Results

In order to analyze the suitability of the experimental model, an Oil Red O was performed on mature and hypertrophic adipocytes, and the results were evaluated qualitatively. It was shown that lipid accumulation increased towards hypertrophic adipocytes. The visual difference between preadipocytes, mature adipocytes, and hypertrophic adipocytes confirmed the suitability of the model for lipid accumulation (Figure 2).

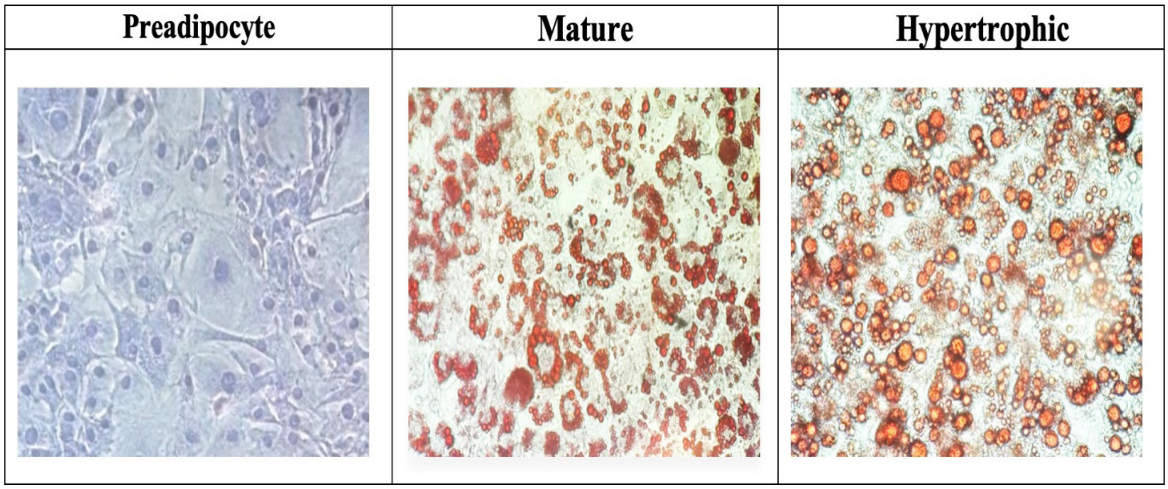

Figure 2. Representative inverted microscopy images (20× and 40× magnification)

Mature and hypertrophic cells before differentiation, after 10 and 18 days of differentiation stained with Oil Red O

The oxidant and antioxidant responses obtained after the application of 80 μM quercetin were examined in mature and hypertrophic 3T3-L1 cells. TAS level was significantly higher in the M-Q80 (655.87±1.68) group compared to the M-C (649.37±1.38) (p=0.0001; t=-6.683) group. No significant difference was seen between groups in terms of total oxidant status (TOS, M-C: 50.39±1.54; M-Q80: 50.62±1.63, p=0.828, t=-0.225), and oxidative stress index (OSI, M-C: 7.76±0.24; M-Q80: 7.72±0.25, p=0.791, t=0.275) levels after a 24-hour treatment with quercetin (Figure 3-5). After a 48-hour application of quercetin to hypertrophic cells, the TAS level was significantly lower in the H-Q80 (804.62±1.85) group compared to the H-C (884.48±1.26) (p=0.0001; t=79.907) group, while the TOS and OSI levels were significantly higher in the H-Q80 (TOS: 49.91±71.15; p=0.0001, t=-7.065; OSI: 6.20±0.39, p=0.0001, t=0.688) group compared to the H-C (TOS: 43.36±1.72, p=0.0001; OSI: 4.90±0.19, p=0.0001).

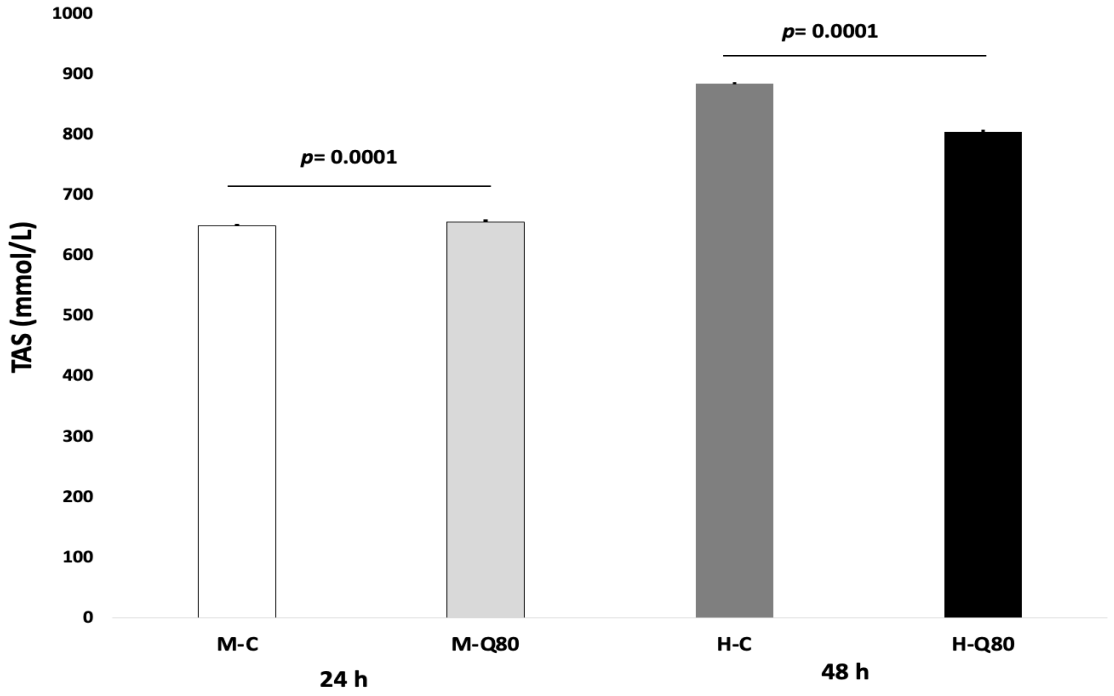

Figure 3. TAS results in mature (24 h) and hypertrophic (48 h) 3T3L-1 cells treated with quercetin

Arithmetic mean and the standard deviation is used to express the results. $p \leq 0.05$ is considered statistically significant. Independent samples t test was used to analyze the data. M-C: Mature control group, M-Q80: Mature 80μM quercetin group, H-C: Hypertrophic control group H-Q80: Hypertrophic 80μM quercetin group

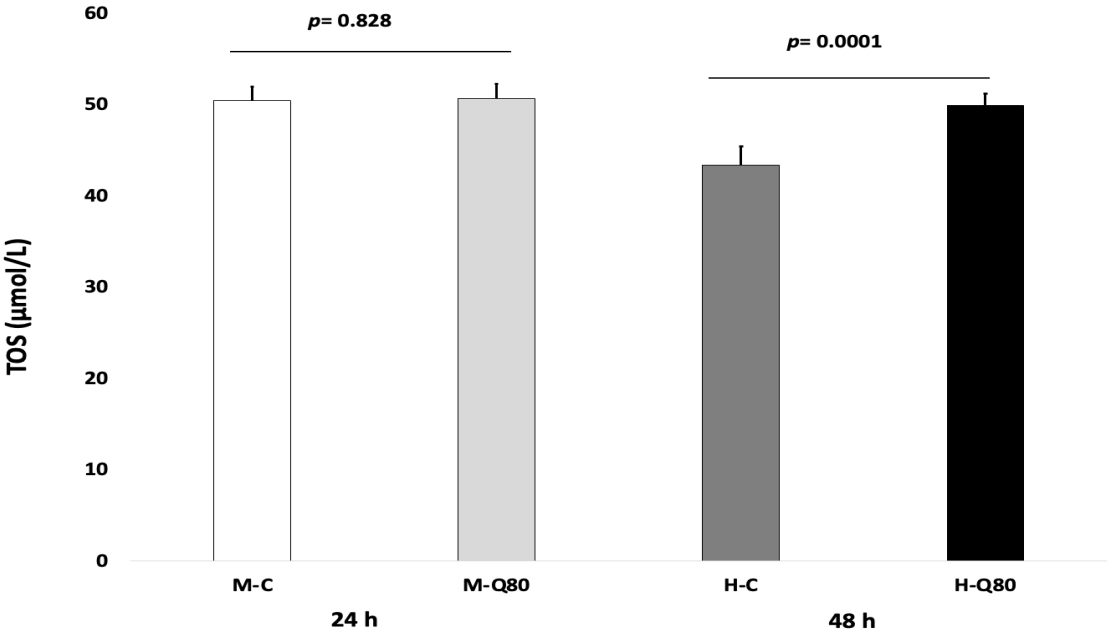

Figure 4. TOS results in mature (24 h) and hypertrophic (48 h) 3T3L-1 cells treated with quercetin

Arithmetic mean and the standard deviation is used to express the results. $p \leq 0.05$ is considered statistically significant. Independent samples t test was used to analyze the data. M-C: Mature control group, M-Q80: Mature 80μM quercetin group, H-C: Hypertrophic control group H-Q80: Hypertrophic 80μM quercetin group

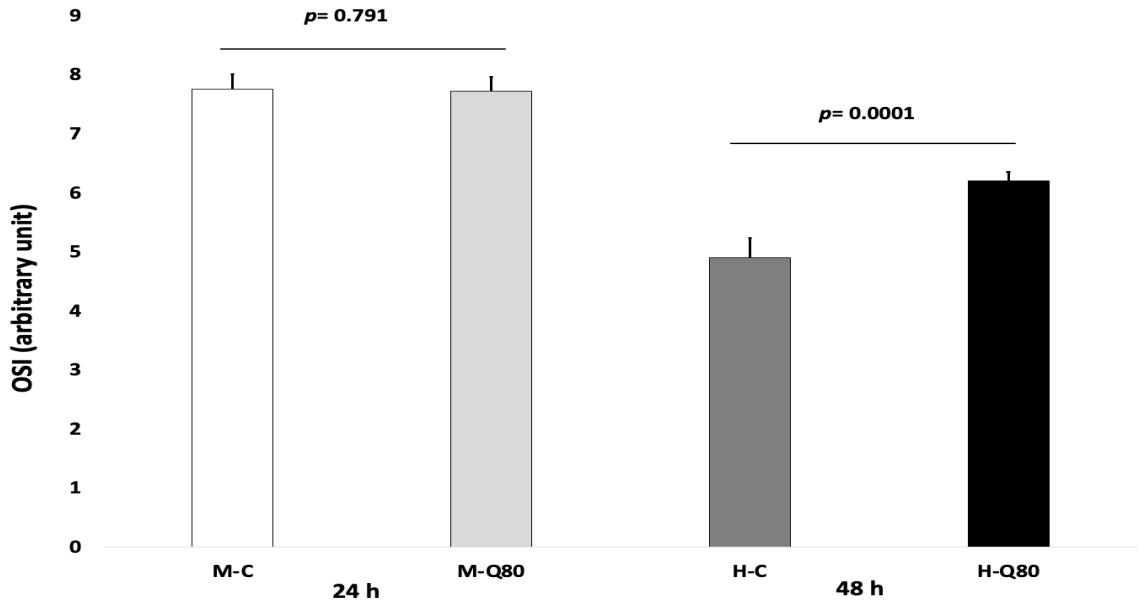

Figure 5. OSI results in mature (24 hours) and hypertrophic (48 hours) 3T3L-1 cells treated with quercetin

Arithmetic mean and the standard deviation is used to express the results. $p \leq 0.05$ is considered statistically significant. Independent samples t test was used to analyze the data. M-C: Mature control group, M-Q80: Mature 80 µM quercetin group, H-C: Hypertrophic control group H-Q80: hypertrophic 80 µM quercetin group

Discussion

Reactive oxygen species (ROS) and the cell's antioxidant defense system are out of balance, which leads to oxidative stress, one of the causes contributing to obesity [16]. It is highly interesting to study the biological effects of naturally occurring micronutrients that may enhance the body's antioxidant capacity because oxidative stress is an important component in obesity and related chronic diseases like T2DM. These substances, known as polyphenols, have demonstrated anti-inflammatory activity both in vitro and in vivo, making them useful as therapeutic tools in a variety of acute and chronic illnesses [17, 18]. In addition, their anti-oxidant features, including scavenging reactive oxygen species, these chemical compounds also have a role in regulating inflammatory signaling by modifying the expression of many pro-inflammatory genes, including lipoxygenase, nitric oxide synthases, and different cytokines [19, 20]. Additionally, they block several of the enzymes that cause ROS to develop [21]. Quercetin is one of these polyphenols. Numerous beneficial effects of quercetin, including anti-poliferative, anti-oxidant, anti-diabetic, and anti-obesity properties, have been documented in the literature through different mechanisms [22, 23]. In light of this, we aimed to examine how quercetin affected oxidative stress in hypertrophic and mature 3T3-L1 adipocyte cells. This study showed that quercetin has a pro-inflammatory effect in hypertrophic adipocytes, but may have a marked effect on oxidative stress by increasing only the total antioxidant level in 3T3-L1 mature adipocyte cells.

In our work, 3T3-L1 preadipocytes were stimulated to transform into mature, hypertrophic adipocytes by use of differentiation media. In matching previous research, the Oil Red O data confirmed an accumulation of lipids in these adipocytes [24, 25]. Quercetin increased antioxidant activity in mature cells compared to control cells. Oxidative stress is determined by the balance between ROS and antioxidants. Optimal redox conditions for most cells are achieved when high levels of antioxidants are present to quench and keep ROS at low levels, and a state of balance is achieved [26]. This

effect is referred to as the oxidant-antioxidant balance. According to this information, it was determined that although quercetin did not have a significant effect on the TOS level in mature cells, it could affect this balance. Quercetin has demonstrated strong antioxidant properties and the ability to scavenge free radicals in vitro studies [27]. Quercetin's antioxidant potential has been revealed through animal experiments. For instance, when quercetin is given to rodents, it results in increased antioxidant activity [28] and reduced lipid peroxidation [29]. A few small-scale human studies on quercetin supplementation have provided inconsistent results about its potential antioxidant properties. Several human investigations have indicated that quercetin does not have any impact on different indicators of antioxidant capacity and oxidative stress [30, 31].

Administration of quercetin to hypertrophic 3T3-L1 adipocytes was found to enhance the oxidative stress index. In cases of obesity and comorbidities, there is an increase in reactive oxygen species (ROS) and a decrease in antioxidants in fat cells. This imbalance leads to oxidative stress and other issues associated with obesity, such as insulin resistance and diabetes [26]. According to reports, quercetin has been found to enhance the activity of free radical scavengers such as superoxide dismutase (SOD), catalase, and glutathione in cases of obesity and related disorders [32, 33].

In vitro experiments on quercetin showed that the dosage and duration of administration of this flavonoid were different. In the study, Boadi et al. [34] demonstrated that administering quercetin (at concentrations ranging from 0 to 25 micromolar) for 24 hours increased the activity of glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rx), and superoxide dismutase (SOD) in oxidatively damaged 3T3L-1 cells. Noh et al. [35] demonstrated that giving 2, 10 micromolar quercetin to 3T3-L1-adipocytes for 6 and 16 hours effectively suppressed the production of macrophage inflammatory protein (MIP)-1α from hypertrophic adipocytes, which is a known trigger for adipose tissue inflammation. According to a report, this result was found to be beneficial in preventing inflammation of adipose tissue caused by obesity. Another study shown that high concentrations of quercetin led to a gradual decrease in the total antioxidant capacity of cell extracts [36]. In this study, we believe that the administration of 80 μM quercetin dose over 48 hours in these cells has a time- and dose-dependent prooxidative effect.

In conclusion, although the mechanisms of action of polyphenols are still unclear, they are seen as an innovative approach for the prevention and treatment of metabolic diseases, including obesity. The use of these polyphenols at the right dose and duration is very important for a clearer explanation of their mechanisms. Future studies should investigate the dose-duration relationship in more detail to better understand the effects of quercetin at the cellular level and determine the optimal conditions for the use of quercetin in cell models. Furthermore, clinical studies evaluating the potential therapeutic effects of quercetin on metabolic health are needed. Such studies may contribute to our understanding of the biological effects of quercetin and clarify the role of this natural compound in health.

This study has limitations. The results of the study are based on ELISA results. These results need to be supported by other experiments (DNA/RNA damage, ROS levels).

Conflict of interest: No conflict of interest was declared by the authors.

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Ethics committee approval: The study is a cell culture study that does not require ethics committee approval.

Author contributions

Conceptualization, M.T.A., E.K.T. and G.A. Data curation, M.T.A., E.K.T. and G.A. Formal analysis, E.K.T. and G.A. Investigation, M.T.A., E.K.T. and G.A. Methodology, M.T.A., E.K.T. and G.A. Supervision, E.K.T. Validation, M.T.A., E.K.T and G.A. Visualization, M.T.A. and G.A. Writing – original draft, M.T.A. and G.A. Reviewing, M.T.A. and G.A. All authors have read and agree to the published version of the manuscript.

Case Report

Pamukkale Medical Journal
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Management of ischemic priapism in a 14 year old patient

14 yaşındaki hastada iskemik priapizmin yönetimi

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Abstract

Priapism can occur in all age groups, including neonatales, children and adolescents. Pediatric priapism treatment is similar to adult priapism treatment, but there are no guidelines for the management of priapism, which is not common in children. We present a pediatric low-flow priapism case who was unresponsive to conservative methods and underwent T-shunt operation. In the present case, we wanted to emphasize when distal shunt surgery, which is rarely performed in pediatric patients, is necessary and how it is managed.

Keywords: Pediatric ischemic priapism, T-shunt surgery, pediatric priapism case report.

Simsek A, Butun S, Duran MB, Kucuker K, Celen S, Ozlulerden Y. Management of ischemic priapism in a 14 year old patient. Pam Med J 2024;17:588-594.

Öz

Priapizm yenidoğan, çocuk ve ergenler de dahil olmak üzere tüm yaş gruplarında ortaya çıkabilir. Pediatrik priapizm tedavisi erişkin priapizm tedavisine benzer ancak çocuklarda yaygın olmayan priapizmin tedavisine yönelik bir kılavuz bulunmamaktadır. Konservatif yöntemlere yanıt vermeyen ve T-şant ameliyatı geçiren pediatrik düşük akımlı priapizm olgusunu sunmayı amaçladık. Bu olguda çocuk hastalarda nadiren uygulanan distal şant ameliyatının ne zaman gerekli olduğunu ve nasıl yönetildiğini vurgulamak istedik.

Anahtar kelimeler: Pediatrik iskemik priapizm, T-şant ameliyatı, pediatric priapizm olgu sunumu.

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Introduction

Priapism is defined as having a full or partial penile erection for more than 4 hours without sexual stimulation [1]. Priapism can occur in all age groups, including neonatales, but it is rarely observed in children and adolescents. Its incidence in all ages is estimated to be 0.3–5.3/100,000 per year, with the 5th decade being the most common [2-4]. Data on the prevalence of the disease in children are insufficient [5].

Hematological diseases, infections, drugs, trauma, and iatrogenic causes are the most common causes of priapism in children [6]. Most cases of low-flow pediatric priapism occur in boys with sickle cell anemia, leukemia, and other hematological disorders [7]. Low-flow priapism, which can cause temporary or irreversible erectile dysfunction, penile deformity, and psychological sequelae, is a urological emergency [8]. High-flow non-ischemic priapism is most commonly observed as a complication of penile trauma, with up to 62% of these cases resolving spontaneously [9].

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Pediatric priapism treatment is similar to adult priapism treatment. First, conservative methods such as exercise, cold application, urination, mechanical compression, and masturbation are applied. When those treatment fails, corporal aspiration, blood transfusion, and shunt surgeries are applied [8]. There are no guidelines for the management of priapism, which is not common in children. Therefore, the treatments applied can help to guide disease management.

Case presentation

A 14-year-old patient presented to the emergency department with a painful erection that had been going on for a week. It was discovered that the erection developed spontaneously, and the patient had never experienced such a situation before. The patient did not have dysuria, hematuria, abdominal pain, fever, vomiting, diarrhea, or constipation. Moreover, there was no history of trauma. The patient was on a usual, normal diet, and his urine output was normal. There was no hematological disease, malignancy, or drug history. The body mass index was 38.06. Genital examination revealed that the penis was painful and rigid, the corpus cavernosum was hard, and the glans penis and corpus spongiosum were soft. No signs of trauma or ecchymosis were observed. Although the patient had a long-term erection, no signs of penile necrosis were observed. The patient's complete blood count and bleeding parameters were normal. Penile Doppler ultrasonography revealed that no significant arterial flow was observed in both cavernous bodies, indicating ischemic priapism.

As the first-line therapy, corporal aspiration accompanied with analgesia was applied. Corporal aspiration was performed with a 20-gauge butterfly needle from the corpus cavernosum at the 3–9 o'clock position, close to the root of the penis. Aspiration was not successful, and appropriate blood gas could not be obtained. A cavernosal saline infusion and a 1/1,000,000-mg epinephrine infusion were administered four times at 10-minute intervals. Penile rigidity partially decreased after the infusion. A cold application was applied after compression dressing with Coban. The penis became rigid again in the follow-up the next day (Figure 1), and the patient was scheduled for distal shunt surgery.

During the operation, a No.11 scalpel was used to perform a T-shunt technique from the glans on the right lateral side of the urethral meatus. After the incision, milking was performed. Because sufficient detumescence was provided in the penis, no intervention in the left cavernosal body was needed (Figure 2).

Blood gas was taken perioperatively after milking, and partial oxygen pressure was 44 mmHg, partial carbon dioxide pressure was 42 mmHg, and pH was 7.29. Then, a 6-fr feeding tube was placed and secured in the right corpus cavernosum (Figure 3).

As the penile rigidity did not recur, the feeding tube was removed on the 2nd postoperative day and the patient was discharged. The patient was consulted to pediatric hematology, and the hemoglobin electrophoresis result was normal. No hematological diseases, such as sickle cell anemia or hemolytic anemia, were detected. Three months after the procedure, penile erections were normal and there was no recurrence of priapism.

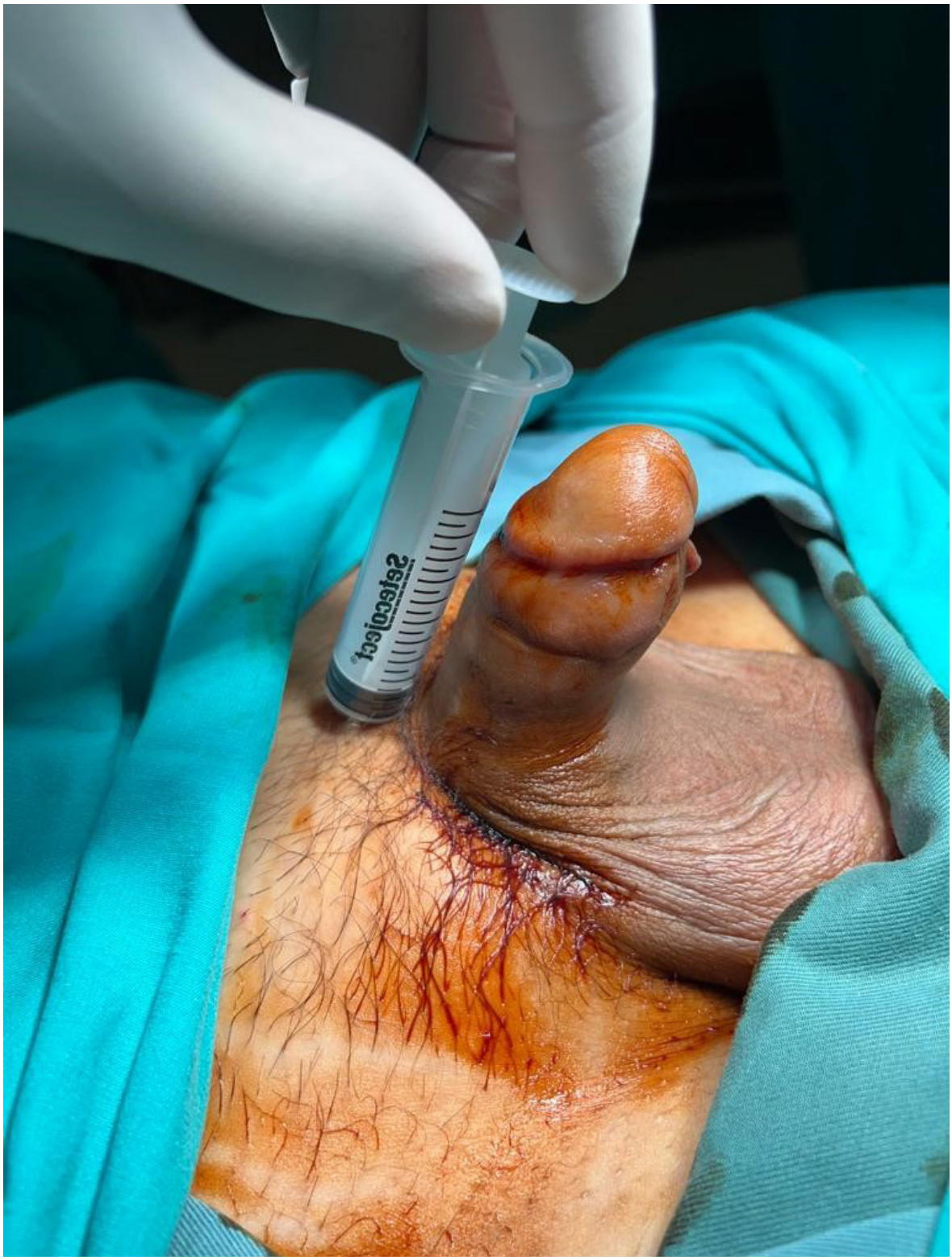

Figure 1. Rigid penis prior to distal shunt surgery

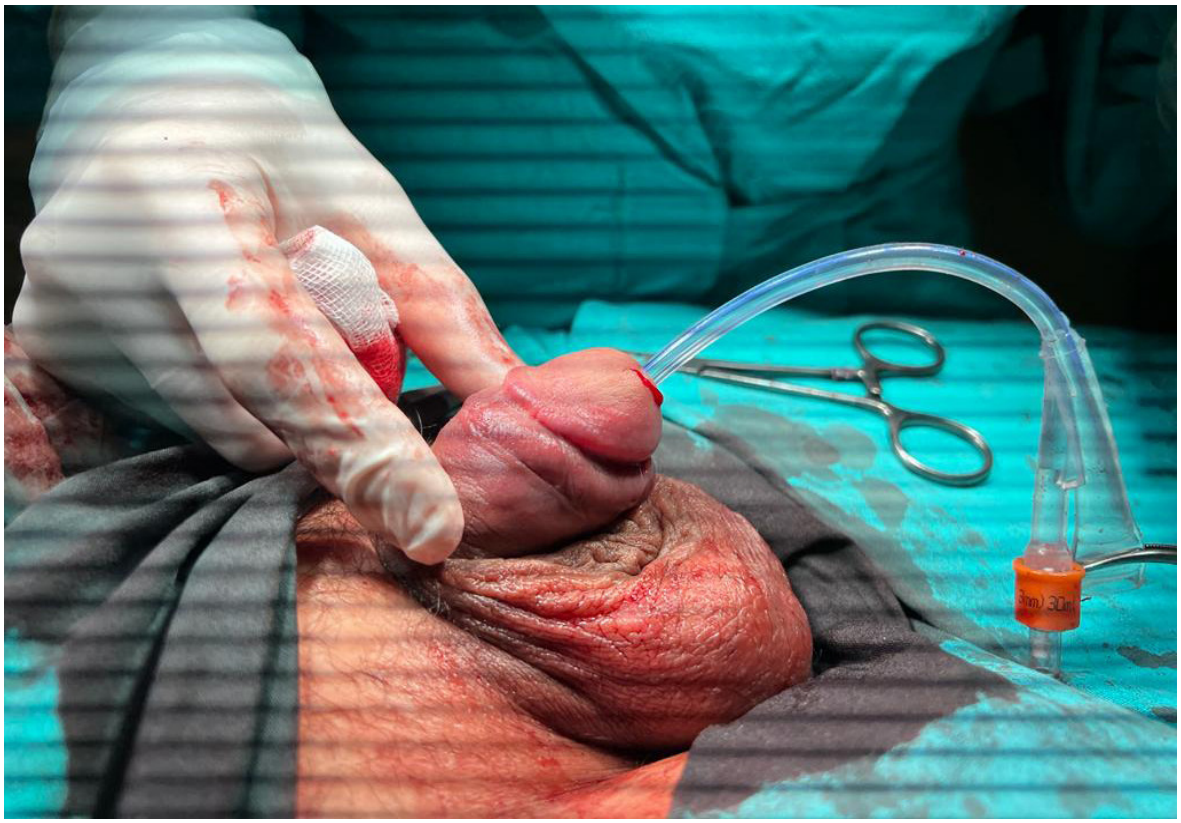

Figure 2. Detumescent penis after distal shunt surgery and milking

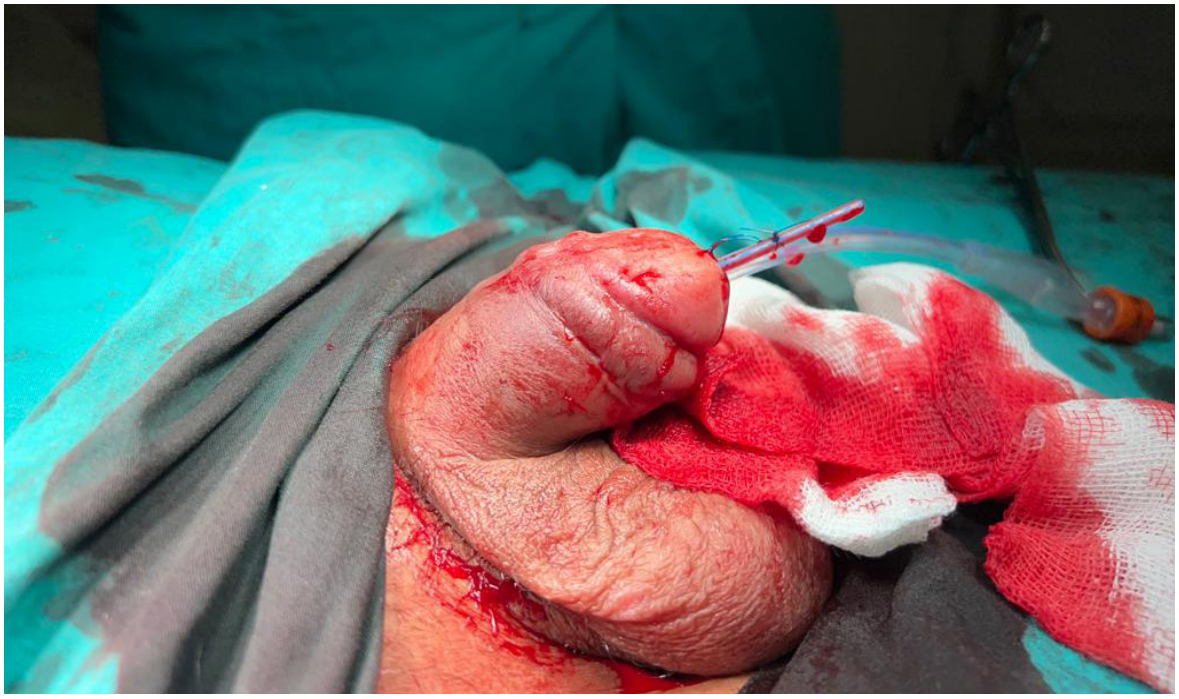

Figure 3. A feeding tube was placed in the penis after detumescence

Discussion

Priapism should be treated promptly because it can cause serious, irreversible complications after a prolonged erection. However, in different ethnic, religious, and social environments, the disease may be diagnosed late owing to family members' feelings of shame and stigma.

Various treatment modalities, such as mechanical (continuous perineal compression and cold application), pharmacological (intracavernous, venous, or oral drug therapy), radiological (selective transcatheter embolization therapy), and surgical (arterial ligation or arteriovenous shunts), are used in the treatment of priapism. The use of noninvasive, conservative methods with high success rates is reduces the need for surgical intervention [10].

Especially before invasive methods are applied, it should be determined whether the priapism is low or high flow. Intracavernosal blood gas analysis and penile Doppler ultrasonography are widely used to distinguish between low- and high-flow priapism. High-flow priapism, which rarely causes pain, is usually caused by excessive arterial flow due to perineal or penile trauma, which creates a fistula between the cavernosal artery and corpus cavernosum. If conventional treatments fail, arterial embolization and ligation treatments are used [11].

Low-flow priapism, which is more common in pediatric patients, is caused by stasis in the cavernosum due to hematological, vascular, or neurological diseases or infectious, drug-related causes. Tissue ischemia caused by stasis causes cavernosal smooth muscle ischemia, pain, and corporal smooth muscle necrosis over time. Tissue fibrosis and permanent impotence may develop over time. Therefore, it is a urological emergency that requires rapid diagnosis and treatment [12]. Erectile dysfunction was observed in 14% of pediatric patients in whom detumescence could not be achieved [13]. In the present case, there was no hematological disease, malignancy, trauma, or drug use history that could cause priapism, and no complications were encountered during the 3rd month of follow-up.

In low-flow priapism, the aim is to provide venous outflow for the arterial blood supply of the corpus cavernosa with surgical treatment. Among the initial treatments, blood aspiration from the corpus cavernosum, saline irrigation applied when necessary, and intracavernosal phenylephrine/epinephrine injection were successful in 77% of the cases [9]. If those treatments fail, shunt surgery should be considered. In a previous case report, a 14-year-old male patient was successfully treated with intracavernosal tissue plasminogen activator after shunt surgery was unsuccessful [14].

Four shunt procedures have been described as a percutaneous distal (corporaglanular) shunts (Winter, Ebbehoj, Lue), open distal (corporoglanular) shunts (Al-Ghorab, Burnett), open proximal (corporospongiosal) shunts (Quackles, Sacher), and vein anastomoses/ shunts (Grayhack, Barry) [15]. In cases of priapism lasting more than 36 hours, the erectile tissue is generally impaired both structurally and functionally. These four shunt methods used to prevent structural deterioration have not been shown to be superior in terms of erectile function to each other [16]. The surgeon's experience and familiarity with the technique are important factors in deciding which shunt procedure to use. However, distal shunts are recommended because they are easier to apply and have fewer complications [9].

In a study of 13 adult male patients who underwent T-shunt surgery for priapism, 6 of them had a history of unsuccessful distal or proximal shunt surgery. After the operation, pain was reduced and penile detumescence was achieved in all patients, but only two patients' erectile function could not be preserved during follow-up. In this study, T-shunt was defined as an easy-to-use, reliable technique that provides rapid resolution of penile pain and rigidity [17].

In a 7-year-old patient with cerebral palsy who underwent surgery for an extra finger incision and developed propofol-induced priapism, corporal aspiration and intracavernosal epinephrine were used, and then distal shunt surgery was performed on the next day because of a persistent painful erection. The patient was followed up for 1 year after surgery, and penile erection was normal [12]. In another case report, a 7-year-old male patient with no comorbidity, drug history, or trauma history presented to the emergency department with a painful erection that began during the night, and abdominal direct X-ray revealed non-obstructive

gas and a dense stool pattern along the colon and rectum. After constipation treatment, the patient began to have bowel movements and after defecation, the erection spontaneously regressed due the resolution of the obstruction in the pelvic blood vessels, and no additional treatment was needed [18]. In the case report of Brönimann et al. [19], the complete blood count and bleeding parameters of a 12-year-old boy who had a painful erection for 24 hours were found to be normal. The patient was previously diagnosed with COVID-19 7 weeks ago and was in the subacute period. The COVID-19 PCR test result performed at admission was also positive; corporal aspiration was performed twice because of the recurrence of erection after 3 days, and detumescence was achieved. The patient was followed up after 8 weeks, and penile erection was found to be normal [19]. In the present case, the patient had a painful erection for approximately 1 week. Blood gas analysis could not be performed on cavernosal blood that had thickened due to long-term priapism. Doppler ultrasonography revealed low-flow priapism, and corporal aspiration was performed. However, distal shunt surgery, which is rarely performed in children, was performed owing to the development of penile rigidity the next day, and detumescence in the penis was achieved. The patient was followed up 3 months after surgery, and no loss of erection or recurrence of priapism was observed. The patient expressed that he was satisfied with his treatment because he did not have priapism and erection problems again.

Primary treatments are usually sufficient and successful in pediatric patients with priapism, but distal shunt surgery is rarely used in patients with prolonged and resistant priapism. In the present case, we wanted to emphasize when distal shunt surgery, which is rarely performed in pediatric patients, is necessary and how it is managed. Considering its serious potential complications, rapid differential diagnosis of high- vs. low-flow priapism and early intervention for low-flow priapism are necessary to reduce the rate of permanent sequelae. Informing physicians and families, especially in cases of recurrent priapism, is important to prevent delayed diagnosis and treatment.

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Informed consent: The patient and his parents gave informed consent for the publication.

Authors' contributions to the article

A.S. constructed the main idea and hypothesis of the study. S.B developed the theory and arranged/edited the material and method section. Discussion section of the article was written by M.B.D. and K.K.

Y.O. and S.C. reviewed, corrected and approved. In addition, all authors discussed the entire study and approved the final version.

Review

Pamukkale Medical Journal
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Hypocalcemia in cancer treatment

Kanser hastalarında hipokalsemi

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Abstract

The primary objective of this review is to provide a concise summary and critical appraisal of the current literature on the differential diagnosis and management of hypocalcemia. Calcium plays a crucial role in muscle function and neurotransmitter release. However, hypocalcemia, defined as serum calcium levels below 8 mg/dL (2.12 mmol/L), can affect various organs and systems and lead to a range of clinical symptoms. This condition can range from being completely asymptomatic to life-threatening situations.

Disorders responsible for hypocalcemia can be divided into two groups: those influenced by parathyroid hormone (PTH) and those not affected. In non-surgical and PTH-mediated forms, more comprehensive investigation is necessary to identify the underlying cause and determine appropriate treatment.

In cases of acute hypocalcemia, intravenous calcium infusion is required to rapidly increase calcium levels and correct symptoms. On the other hand, treatment of chronic hypocalcemia generally involves oral calcium and/or vitamin D supplementation.

In conclusion, this review specifically emphasizes iatrogenic (treatment-related) hypocalcemia while assessing the causes, dimensions, and management of hypocalcemia in cancer patients. Physicians' familiarity with these conditions is crucial in treatment management.

Keywords: Hypocalcemia, hypomagnesemia, cancer treatment, chemotherapy, cisplatin.

Akay S, Unal OU. Hypocalcemia in cancer treatment. Pam Med J 2024;17:596-599.

Öz

Bu derlemenin temel amacı, hipokalseminin ayırıcı tanısı ve yönetimi ile ilgili mevcut literatürün kısa bir özetini ve eleştirel bir değerlendirmesini sunmaktır. Kalsiyum, kas fonksiyonu ve nörotransmitter salınımında önemli bir rol oynar. Bununla birlikte, 8 mg/dL'nin (2,12 mmol/L) altındaki serum kalsiyum seviyeleri olarak tanımlanan hipokalsemi, çeşitli organ ve sistemleri etkileyebilir ve çeşitli klinik belirtilere yol açabilir. Bu tablo tamamen asemptomatik olabileceği gibi hayatı tehdit eden durumlara da yol açabilir.

Hipokalsemiden sorumlu bozukluklar iki gruba ayrılabilir: paratiroid hormonundan (PTH) etkilenenler ve etkilenmeyenler. Cerrahi nedenlerden kaynaklanmayan ve PTH aracılı olmayan formlarda, altta yatan nedeni tanımlamak ve uygun tedaviyi belirlemek için daha kapsamlı bir araştırma gereklidir.

Akut hipokalsemi vakalarında, intravenöz kalsiyum infüzyonu kalsiyum seviyelerini hızla artırmak ve semptomları düzeltmek gereklidir. Öte yandan, kronik hipokalsemi tedavisinde oral kalsiyum ve/veya D vitamini takviyesi genellikle yeterlidir.

Sonuç olarak, bu derleme kanser hastalarında hipokalseminin nedenlerini, boyutlarını ve yönetimini değerlendirirken tedavi ilişkili (iatrojenik) hipokalsemiye özellikle vurgu yapmaktadır. Hekimlerin bu durumları iyi tanıması tedavi yönetiminde önemlidir.

Anahtar kelimeler: Hipokalsemi, hipomagnezemi, kanser tedavisi, kemoterapi, sisplatin.

Akay S, Ünal OÜ. Kanser hastalarında hipokalsemi. Pam Tıp Derg 2024;17:596-599.

Introduction

Calcium is an essential molecule for muscle contraction and neurotransmitter release [1]. Hypercalcemia in cancer patients is considered a poor prognostic marker but there’s no prognostic statement for hypocalcemia in this population [2].

Hypocalcemia is defined as a serum level of calcium under 8 mg/dl or 2.12 mmol/L. Measurement of ionized calcium is preferred as serum calcium levels are easily affected by serum protein levels [3].

While calcium metabolism proceeds in a balance between intestinal absorption and

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renal excretion in a process in which vitamin D and parathormone play an active role, finding the cause of hypocalcemia in our patients will relieve the patient from complaints and findings. Patients may be asymptomatic, or have nonspecific complaints such as fatigue, irritability, tetany, anxiety, or depression; and even bronchospasm and cardiac arrhythmias as a result of prolonged QT that may result in death [3]. Muscular irritability may be presented with cramps, numbness, and paresthesias. Clinical hypocalcemia can affect almost all organ systems and can have fatal consequences. We are therefore lucky if we have time to investigate the etiology of hypocalcemia. In acute hypocalcemia, the first thing to do is to alleviate or, if possible, even eliminate the symptoms with intravenous calcium replacement. In chronic hypocalcemia treatment, oral calcium and vitamin D replacement are often sufficient [4].

The most common clinical etiology for hypocalcemia is vitamin D insufficiency which may be prevalent as high as 50% [5]. Surgical causes follow, often as a result of loss of the parathyroid gland after thyroidectomy. Hypocalcemia can be seen in up to 38% of patients following thyroid surgery [6].

Surgical causes can be easily recognizable both from the patient's history and the observation of the incision scar during physical examination. However, hypocalcemia due to medical causes should be meticulously investigated. In this context, the patient's medication use should be questioned well. Bisphosphonates, cisplatin, antiepileptics, aminoglycosides, and proton pump inhibitors well-known agents to cause hypocalcemia in cancer patients [7].

We frequently see hypocalcemia in cancer patients after bone-sparing therapies [8]. For this reason, we administer calcium and vitamin D replacement after treatment with RANKL inhibitors or bisphosphonates and even without evident hypocalcemia [9]. Monitoring serum vitamin D levels in these patients taking vitamin D supplements is questioned [10]. However, calcium levels need to be monitored periodically. A high vitamin D level before treatment is considered protective against hypocalcemia [11]. Treatment-related hypocalcemia rates for denosumab and bisphosphonates are similar, although hypocalcemia is not expected with replaced patients [12]. Chronic kidney disease, malabsorption syndromes, or hypoparathyroidism may predispose to hypocalcemia in this group of patients [13]. During continued denosumab treatment, there is a risk of experiencing hypocalcemia for each administered dose [13]. However, once hypocalcemia occurs, denosumab should not be continued until the calcium level is normalized [14].

In immunotherapy, one of the new oncologic treatment approaches, TLS may occur after CAR-T cell-associated target cell damage [15]. Furthermore, PD1 inhibitors may rarely cause acute kidney injury and hypocalcemia, but the mechanism is unclear [16, 17].

Conditions such as massive hydration, intensive diuretic use, and massive blood transfusions may be among the iatrogenic causes. In a study that reviewed TLSs, the rate of solid cancers was found to be 38%, which is not insignificant.

Cases of asymptomatic hypocalcemia have also been reported after hyperthermic intraperitoneal chemotherapy (HIPEC), which is a modern treatment modality used in peritoneal involvement of gastrointestinal and gynecological malignancies [18, 19].

Hypocalcemia may appear as a component of tumor lysis syndrome (TLS) which is characterized by at least two of the following abnormalities; hyperuricemia, hyperpotassemia, hyperphosphatemia, and hypocalcemia [20]. Hyperphosphatemia can cause secondary hypocalcemia and calcium can precipitate as phosphate crystals in organs such as the kidney [21]. TLS may occur following a biopsy, radiotherapy, or chemotherapy, or it may develop spontaneously [22]. TLS is more common in hematologic malignancies, but patients with solid malignancies may be prone to TLS in such situations as extrinsic compression of the genitourinary tract leading to renal dysfunction, high tumor burden, large tumors, high sensitivity to chemotherapy, and patient-related factors such as dehydration, hypotension, nephrotoxic agent intake, and obstructive uropathy [23, 24].

Another important point that concerns us as medical oncologists is hypocalcemia, which is seen after chemotherapy and is confusing in etiology because it is a rare condition. Here, magnesium level should be questioned as an underlying factor. Hypomagnesemia leads to parathormone resistance and causes hypocalcemia. This occurs in the setting of severe hypomagnesemia, in which the serum magnesium level drops to 0.8 mg/dL or under. Calcium level does not normalize without magnesium replacement. The most common chemotherapy drug causing this is cisplatin [25]. In a study by Komurcuoglu et al. [26] among 35 patients receiving cisplatin-based chemotherapy, hypomagnesemia was observed in half of the patients, while hypocalcemia was described in only one patient. Hypomagnesemia is observed in approximately 10% of patients who received cisplatin and may persist even months to years after treatment [27].

Hypomagnesemia can also be seen with EGFR inhibitors cetuximab and panitumumab [28].

Tyrosine kinase inhibitors (TKIs), which are better tolerated than chemotherapy, have many effects on almost all endocrine glands as well as on bone metabolism. They decrease osteoclastogenesis and bone turnover, which eventually results in hyperparathyroidism. Hypocalcemia has been reported as a side effect of treatment with lenvatinib and vandetanib [29, 30].

Recognizing hypocalcemia, which we rarely see in the management of cancer patients, should be promptly treated especially when any symptoms such as muscle cramps, numbness or tingling in the fingers or around the mouth, or seizures are present. It requires a multidisciplinary approach and a complex process, which can be life-saving beyond eliminating complaints that reduce the quality of life such as weakness and fatigue.

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Authors' contributions to the article

S.A. have constructed the main idea of the paper. She developed the theory, pooled the information and arranged/edited the text. Written by S.A., and reviewed, corrected and approved by O.U.U. In addition, all authors discussed the entire paper and approved the final version.

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The relationship between salivary flow rate, oral health, and malnutrition in the elderly: a cross-sectional study "about"

Yaşlılarda tükürük akış hızı, ağız sağlığı ve malnütrisyon arasındaki ilişki; kesitsel bir çalışma "hakkında"

Deniz Mut Sürmeli

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Dear Editor,

I am writing regarding the article titled " The relationship between salivary flow rate, oral health, and malnutrition in the elderly: a cross-sectional study" published in the Pamukkale Medical Journal [1]. As a geriatrician, I extend my thanks to the authors for their valuable contribution to this significant area of geriatrics. Malnutrition, with a prevalence of 19% among community-dwelling older adults in Türkiye, is a prevalent and critical geriatric syndrome due to its adverse outcomes on older adults [2]. Understanding all of its triggers is vital for the elimination and prevention of its development. Sometimes, inquiries about the oral health, taste perception, or satisfaction with eating of older individuals in outpatient clinics may be overlooked or neglected, despite these factors being major contributors to decreased oral intake and malnutrition. This study is important for highlighting this issue and also for reflecting Türkiye's data.

The study revealed no significant relationship between salivary flow rate and dental/oral health and malnutrition. However, a significantly negative correlation was observed between BMI and salivary flow rate among older adults (r=-0.291, p=0.021). In the methods section, it was mentioned that individuals with diseases that could affect salivary flow rate (such as Sjögren's syndrome, rheumatoid arthritis, systemic lupus erythematosus, Parkinson's disease, diabetes mellitus, and other endocrine diseases, Crohn's disease, inflammatory bowel disease, periodontitis, and mucositis), as well as individuals who received radiotherapy to the head and neck region, and those who were fasting, were not included in the study. However, another important consideration, medications, especially those with anticholinergic effects, was not addressed in this study. Medications that have anticholinergic effects inhibit salivary function either directly by blocking acetylcholine binding to muscarinic receptors in the salivary glands or indirectly through effects in the central nervous system, resulting in decreased salivary flow and dry mouth [3].

Anticholinergic drug use is a significant concern among older adults, with a notably high prevalence. Studies indicate that approximately 57% of individuals aged 75 and above use at least one anticholinergic drug [4]. Many drugs in these categories have anticholinergic effects, such as diuretics, antihypertensive agents, antidepressants, antipsychotics, antihistamines, anxiolytics, muscle relaxants, anticonvulsants, antiparkinsonian drugs, anti-incontinence agents, and medications for insomnia. The list of such medications is extensive, with even nonsteroidal anti-inflammatory drugs like ibuprofen and naproxen also included [5].

According to the "drug burden index," which measures exposure to drugs with anticholinergic and sedative effects, medications are categorized into groups based on their anticholinergic activities. Some antidepressants, medications used to treat urinary incontinence, and those used for insomnia have the highest anticholinergic side effects, and they are relatively common among older adults [6]. Therefore, although the individuals selected for this study were functionally independent and had relatively intact cognition, it is possible that the participants may have been using anticholinergic drugs for common reasons such as depression, urinary incontinence, insomnia or pain, which are prevalent in the older adults.

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On the other hand, the study indicates that individuals with obesity tend to have lower salivary flow rates. Obesity is frequently associated with comorbid conditions such as diabetes, cardiovascular disease, metabolic syndrome, depression, anxiety, urinary incontinence, and certain types of pain, which are more prevalent in this population [7-9]. Some anticholinergic medications may be used to treat these conditions, potentially resulting in higher anticholinergic usage among obese individuals. Therefore, this situation could also contribute to a decrease in salivary flow rate among obese individuals.

In conclusion, it would have been preferable to record the medications used by the patients included in the study and to exclude those who use medications with anticholinergic side effects. Alternatively, patients could have been grouped based on whether they used medications with high anticholinergic effects or not, allowing for a comparison of salivary flow rates between these two groups. It is important to exclude potential confounders in such studies because they could undermine the level of evidence obtained. These suggestions are made with the aim of guiding future studies that will explore these relationships further.

Geriatric medicine relies on teamwork, and dietitians and dentists play crucial roles as important colleagues of geriatricians. I am glad to see that they are already engaged and interested in addressing specific aspects of geriatric care, such as malnutrition and dental/oral health, respectively. The authors have conducted a study that involved considerable effort. I would like to express my appreciation for their efforts in conducting the first study to elucidate this issue in community-dwelling older adults in Türkiye, and I look forward to seeing an increase in new studies on this topic.

Thank you for the opportunity to provide feedback.

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