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The prognostic and predictive value of osteopontin in colon adenocarcinoma

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ABSTRACT

Aim: The identification of cellular pathways in colorectal tumor biology is essential for early diagnosis, treatment, and post-treatment follow-up. Osteopontin is an extracellular matrix protein that has regulatory physiological functions and roles in apoptosis, proliferation, adhesion, invasion, and tumor metastasis. In this study, we aimed to determine the prognostic and predictive value of osteopontin in colon adenocarcinoma. Our study investigated whether osteopontin expression had any prognostic or predictive use in colon adenocarcinoma and also if there were any differences between adenocarcinoma and adenoma.

Methods: Fifty of these colonic specimens were adenocarcinoma, 16 were adenomatous polyps, and 10 were nontumoral colonic tissue that served as a control group. We used a two-tiered evaluation system that examined both the staining intensity and the percentage of staining.

Results: The staining scores of tumors with vascular invasion were significantly higher than those of tumors without vascular invasion. In addition, the tumoral tissues' osteopontin staining scores were significantly higher than the score of polyps.

Conclusion: If future studies support our results, we suggest that osteopontin may be an important biomarker for predicting or detecting vascular invasion in tumors and could be useful in tumor-adenomatous polyp differentiation. Therefore, osteopontin can provide helpful information in the diagnosis and prognosis of colon adenocarcinoma.

Keywords: Osteopontin, colon adenocarcinoma, adenomatous polyp, vascular invasion.

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Introduction

Colorectal carcinoma is the most common carcinoma of the gastrointestinal system and is also the leading cause of death from cancer in some countries [1]. Depending on the incidence, morbidity, and mortality rates, colon carcinoma has a very important place among all

tumors. Cancer development is a complex process that involves combinations of various tumor and tumor stromal-derived growth factors and cytokines. As demonstrated in other types of cancer, the identification of cellular pathways in colorectal tumor biology creates new opportunities for early diagnosis and future treatments. For this reason, many studies are being carried out at the cellular and molecular levels, and molecular level findings in particular play an important role in the prognosis since they assist in predicting the clinical course, patient follow-up, and application of treatment procedures. Some

markers are also used to assess the metastatic potential of a tumor [1-7].

Osteopontin was first isolated from bone tissue in 1979. It is a structural protein of bone tissue and an extracellular matrix protein found in the phosphoglycoprotein structure; osteopontin is also detected in other tissues of the body [2]. This substance plays many roles in cell-matrix interactions, the modulation of cell functions, and carcinogenesis [3]. These cancers include (but are not limited to) malignant breast cancer, osteosarcoma, melanoma, ovarian cancer, endometrial cancer, cervical cancer, renal cancer, oral cancer, esophageal cancer, gastric cancer, colorectal cancer, pancreatic cancer, liver cancer, lung cancer, head and neck cancer, glioblastoma, skin cancer, thyroid cancer, and sarcoma [4-7].

In this study, we researched whether there was a significant relationship between osteopontin expression and prognostic parameters such as age, sex, localization, differentiation grade, lymph node involvement, vascular invasion, and cancer stage in colon adenocarcinomas. We also investigated whether there was any difference between adenocarcinoma and adenoma, which is an accepted precursor lesion of adenocarcinoma, for osteopontin expression.

Materials and Methods

We performed a retrospective review of 50 patients who underwent colon resection and were diagnosed with adenocarcinoma at our hospital and 16 patients with adenoma that were diagnosed by biopsy during colonoscopy. Ethics Committee approval was obtained as Decision Number 25/08. Ten nontumoral colon tissues were selected as the control group. Information and patient records were obtained from the hospital automation system. Hematoxylin and Eosin (H&E) stained slides of

archival paraffin blocks were evaluated. The degrees of differentiation were grouped as follows: well-differentiated if a tumor contained more than 95% of the glandular structure, moderately differentiated if the tumor involved 50-95% of the glandular structure, and poorly differentiated if it contained 5-50% the glandular structure [1]. The tumor stage was assessed using TNM (Tumor-Node-Metastasis) classification. The cases were divided into two groups according to age (<65 or ≥65 years), lateralization (right or left according to embryological development), and positivity (positive or negative) concerning lymph node involvement and the presence of vascular invasion. For each case, slides were selected to show the highest grade of a tumor and also examples of nontumoral tissue.

Immunohistochemical staining method

Immunohistochemistry was performed on the deparaffinized tissue sections obtained from formalin-fixed and paraffin-embedded tissue blocks. We used an automated slide stainer (Leica Bond Max device and osteopontin rabbit polyclonal antibody (RB-9097-R7) (Thermo Scientific) at a 1:50 dilution. The device staining procedure requires boiling the tissue sections in a 10-mM citrate buffer at a pH of 6:0 for 20 minutes, followed by cooling for 20 minutes.

Scoring of the staining method

Stomach adenocarcinoma was used as a positive control, and cytoplasmic staining with osteopontin was confirmed when slight nuclear staining was observed in cells where the staining was intense. We evaluated cytoplasmic staining in tumor epithelial cells. No significant specific staining pattern was observed in stromal structures. To assess the percentage of staining, we used a two-tiered scoring system

that included a combination of the staining intensity and the Allred Scoring System [8]. We developed a grading scale as follows:

Staining intensity

Score of 0: None

Score of 1: Weak

Score of 2: Moderate

Score of 3: Strong

Percentage of staining

Score of 0: 0%

Score of 1: $\leq 5\%$

Score of 2: 6–10%

Score of 3: 11–33%

Score of 4: 34–66%

Score of 5: 67–100%

The sum of these two scorings led to a final score:

0–1: Score of 0

2–3: Score of 1 (+)

4–6: Score of 2 (++)

7–8: Score of 3 (+++)

Statistical analysis

Our statistical analysis was performed using the Statistical Package for the Social Sciences software, version 20.0. Categorical variables were presented as frequencies and percentages. Descriptive statistics were shown as the mean + standard deviation (SD) for numerical cut-off variables, and categorical variables were the number of cases and percentages (%). Pearson's Chi-Square, Fisher's Chi-square, or likelihood ratio tests were used to evaluate the categorical variables. Results with a $p < 0.05$ were considered statistically significant.

Results

The research population consisted of 76 people, 42 (55.3%) males and 34 (44.7%) females. The youngest patient was aged 40, the oldest was 80 years old, and 53% of the entire population were among the geriatric group (65 years and

over). Tumors were found in 65.8% ($n = 50$) of the population, polyps were noted in 21.1% ($n = 16$), and nontumoral tissues were present in 13.2% ($n = 10$; Table 1).

There was low grade dysplasia in 56.3% ($n = 9$) of the polyps and high grade dysplasia in 43.8% ($n = 7$). Of the tumors, 86% ($n = 43$) were moderately differentiated, 8% ($n = 4$) were poorly differentiated and 6% ($n = 3$) were well differentiated. In 54% ($n = 27$) of tumor cases. Lymph node metastasis was present, and 46% ($n = 23$) presented with vascular invasion. According to TNM classification, 64% ($n = 32$) of the cases had invasion into subserosa (T3), 12% ($n = 6$) into muscularis propria (T2), 22% ($n = 11$) into serosa (T4), and 2% ($n = 1$) into both mucosa and submucosa (T1). Gauging the tumors by the AJCC 2017 TNM stage, 36% ($n = 18$) were stage IIIB, 34% ($n = 17$) were stage IIA, 12% ($n = 6$) reached stage IIIC, 10% ($n = 5$) presented as stage I, 4% ($n = 2$) were stage IIB, 2% ($n = 1$) were stage IIIA and 2% ($n = 1$) were stage IVA (Table 1).

In the geriatric age group, 51.4% ($n = 18$) had score 3 staining and 48.6% ($n = 17$) had score 2 staining present. The under 65 age group had score 3 staining present in 74.2% ($n = 23$) and score 2 staining in 25.8% ($n = 8$; $p = 0.059$). Score 3 staining was also present in 81% ($n = 17$) of right localized tumors but was found in only 65.5% ($n = 19$) of left localized tumor cases; these findings were not statistically significant ($p = 0.341$) (Table 2).

A significant relationship could not be found in epidemiological data comparisons, but significant differences were found between diagnoses and tumor characteristics, specifically with the staining score ($p < 0.001$). Accordingly, we found that the score 2 staining rate was higher in adenomatous polyps, and the score 3 staining rate was higher in tumors (Table 2).

Score 3 staining was observed in all poorly differentiated tumors, in 70.3% (n = 26) of moderately differentiated tumors and 66.7% (n = 2) of well-differentiated tumors. An increase in the ratio of Score 3 staining corresponding with decreasing tumor differentiation did not show statistical significance ($p = 0.637$) (Table 2). Score 3 staining was significantly higher in tumors with vascular invasion than in tumors

without it (95.7% / 51.9%, $p = 0.001$). While the staining score was not statistically significant for tumors with and without lymph node metastases, those with lymph node metastases had higher scores than those without (74.1% / 69.6%, $p = 0.726$). No significant difference was found between tumor stage and subgroups ($p=0.801$) or depth of invasion ($p=0.296$) (Table 2), (Figure 1).

Table 1. Demographic features and staining scores of the cases.

| Variables | All population (N=76) N (%) | Tumors (N=50) N (%) | Polyps (N=16) N (%) | Nontumoral Tissues (N=10) N (%) | <i>p</i> |
|-------------------------------|-----------------------------------|---------------------------|---------------------------|--|----------|
| Age (Med±SD) | 66.4±11.2 | 66.1±11.7 | 67.4±9.6 | 0(0) | |
| Unknown | 0(0) | 0(0) | 0(0) | 10(100) | |
| <65 | 31(47) | 24(48) | 7(43.8) | 0(0) | |
| ≥65 | 35(53) | 26(52) | 9(56.3) | 0(0) | 0.993 |
| Gender | | | | | |
| Female | 34(44.7) | 25(50) | 6(37.5) | 3(30) | |
| Male | 42(55.3) | 25(50) | 10(62.5) | 7(70) | 0.404 |
| Diagnosis | | | | | |
| Nontumoral colon tissue | 10(13.2) | 0(0) | 0(0) | 10(100) | |
| Adenocarcinoma | 50(65.8) | 50(100) | 0(0) | 0(0) | - |
| Adenomatous polyps | 16(21.1) | 0(0) | 16(100) | 0(0) | |
| Staining intensity | | | | | |
| Score 0 | 10(13.2) | 0(0) | 0(0) | 10(100) | |
| Score 1 | 17(22.4) | 9(18) | 8(50) | 0(0) | |
| Score 2 | 32(42.1) | 28(56) | 4(25) | 0(0) | |
| Score 3 | 17(22.4) | 13(26) | 4(25) | 0(0) | <0.001* |
| Percentage of staining | | | | | |
| Score 0 | 10(13.2) | 0(0) | 0(0) | 10(100) | |
| Score 3 | 5(6.6) | 3(6) | 2(12.5) | 0(0) | |
| Score 4 | 16(21.1) | 7(14) | 9(56.3) | 0(0) | |
| Score 5 | 45(59.2) | 40(80) | 5(31.3) | 0(0) | <0.001* |
| Final score | | | | | |
| Score 0 | 10(13.2) | 0(0) | 0(0) | 10(100) | |
| Score 2 | 25(32.9) | 14(28.0) | 11(68.8) | 0(0) | |
| Score 3 | 41(53.9) | 36(72.0) | 5(31.2) | 0(0) | <0.001* |

* The results were considered significant at the * $P < 0.05$ level. Med±SD: Median ± Standard Deviation

Table 2. Distribution of final scores according to demographic and clinical findings.

| Variables | Final score | | | p |
|-----------------------------------|------------------|------------------|------------------|---------|
| | Score 0 N (%) | Score 2 N (%) | Score 3 N (%) | |
| Age | | | | |
| Under 65 | 0(0) | 8(25.8) | 23(74.2) | 0.059 |
| 65 and over | 0(0) | 17(48.6) | 18(51.4) | |
| Gender | | | | |
| Female | 3(8.8) | 11(32.4) | 20(58.8) | 0.649 |
| Male | 7(16.7) | 14(33.3) | 21(50) | |
| Diagnosis | | | | |
| Normal colon tissue | 10(100) | 0(0) | 0(0) | <0.001* |
| Adenocarcinoma | 0(0) | 12(27.3) | 32(72.7) | |
| Mucinous Adenocarcinoma | 0(0) | 2(33.3) | 4(66.7) | |
| Tubular Adenoma | 0(0) | 6(75) | 2(25) | |
| Tubulovillous Adenoma | 0(0) | 2(50) | 2(50) | |
| Villous Adenoma | 0(0) | 3(75) | 1(25) | |
| Dysplasia degree in polyps | 0(0) | | | |
| Low | 0(0) | 6(66.7) | 3(33.3) | 0.838 |
| High | 0(0) | 5(71.4) | 2(28.6) | |
| Differentiation | | | | |
| Well | 0(0) | 1(33.3) | 2(66.7) | 0.637 |
| Moderately | 0(0) | 13(30.2) | 30(69.8) | |
| Poorly | 0(0) | 0(0) | 4(100) | |
| Invasion depth | | | | |
| T4 | 0(0) | 1(9.1) | 10(90.9) | 0.296 |
| T3 | 0(0) | 11(34.4) | 21(65.6) | |
| T2-T1 | 0(0) | 2(28.6) | 5(71.4) | |
| Vascular invasion | | | | |
| Negative | 0(0) | 13(48.1) | 14(51.9) | 0.001* |
| Positive | 0(0) | 1(4.3) | 22(95.7) | |
| Lymph node metastasis | | | | |
| Negative | 0(0) | 7(30.4) | 16(69.6) | 0.726 |
| Positive | 0(0) | 7(25.9) | 19(74.1) | |
| Stage | | | | |
| I-II | 0(0) | 7(29.2) | 17(70.8) | 0.860 |
| III- IV | 0(0) | 7(26.92) | 19(73.07) | |
| Stage subgroups | | | | |
| I | 0(0) | 2(40) | 3(60) | 0.963 |
| IIa | 0(0) | 5(29.4) | 12(70.6) | |
| IIb | 0(0) | 0(0) | 2(100) | |
| IIIa | 0(0) | 0(0) | 1(100) | |
| IIIb | 0(0) | 6(33.3) | 12(66.7) | |
| IIIc | 0(0) | 1(16.7) | 5(83.3) | |
| IVa | 0(0) | 0(0) | 1(100) | |
| Localization | | | | |
| Left | 0(0) | 10(34.5) | 19(65.5) | 0.341 |
| Right | 0(0) | 4(19) | 17(81) | |

* The results were considered significant at the $P < 0.05$ level

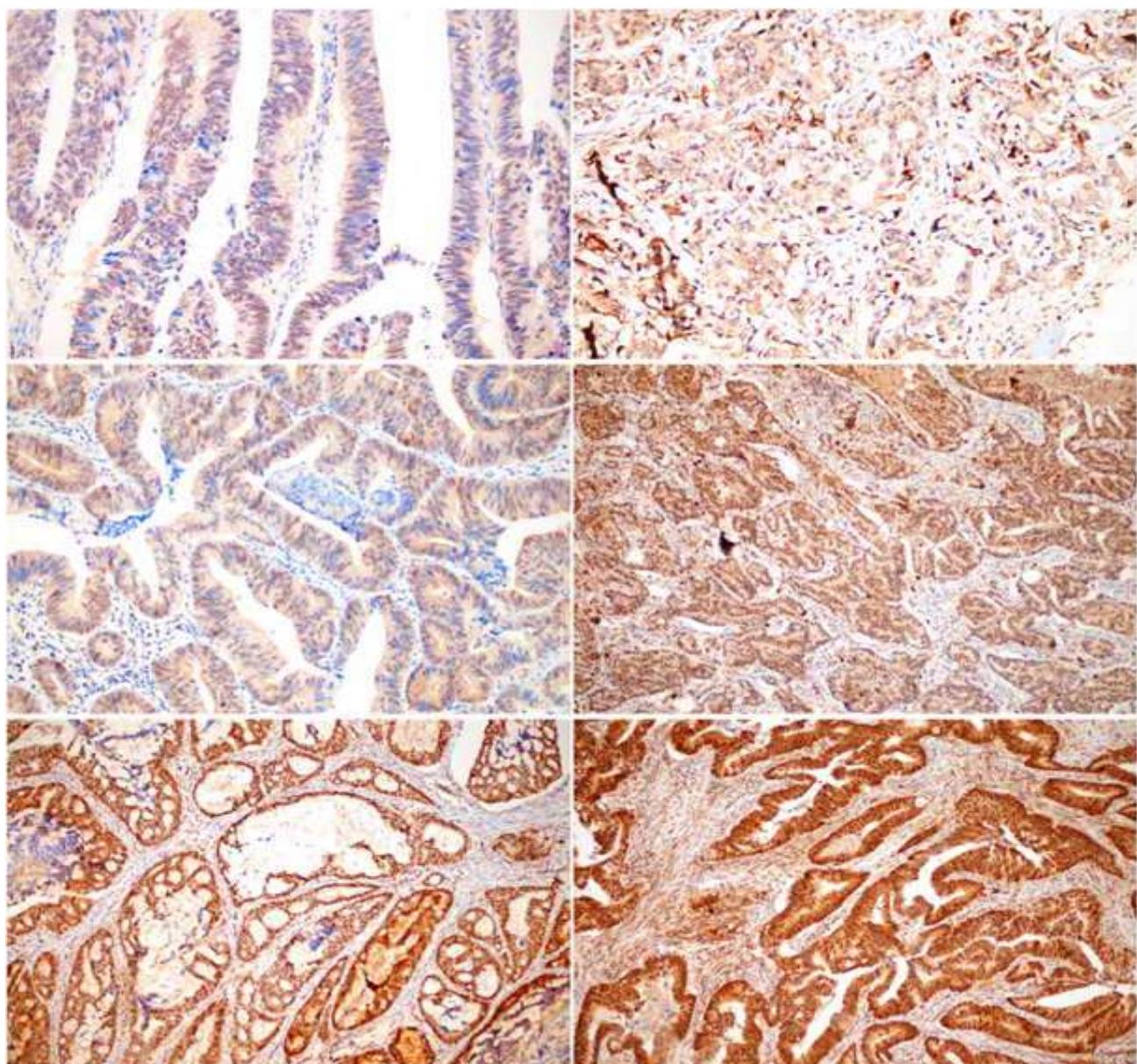


Figure 1. Osteopontin expression; Evaluation for staining intensity; magnifications at x 200 (left to right). **a)** Score 1 cytoplasmic staining at a villous adenoma. **b)** Score 1 cytoplasmic staining at a moderately differentiated adenocarcinoma. **c)** Score 2 cytoplasmic staining at a moderately differentiated adenocarcinoma. **d)** Score 2 cytoplasmic staining at a moderately differentiated adenocarcinoma. **e)** Score 3 cytoplasmic staining at an adenocarcinoma. **f)** Score 3 cytoplasmic staining at a moderately differentiated adenocarcinoma.

Discussion

Osteopontin is a sialic acid-rich phosphoglycoprotein as well as an extracellular matrix protein that binds integrins at the structure [9]. Since its discovery, extensive research has been conducted on its differing roles, as it is considered to be more than a regulatory protein in normal physiological

functions; it has also been shown in various studies to function in apoptosis, proliferation, adhesion, invasion, and tumor metastasis [10,11]. Many studies have shown that osteopontin is highly expressed in various cancers (such as breast, lung, prostate carcinomas, osteosarcoma, glioblastomas, and melanomas), which is useful in different ways

[6,7]. Similarly, there are many reports of increased osteopontin expression in the regulation of cancer invasion, intra-extravasation, and colonization for distant tissues [12,13]. We attempted to obtain meaningful results about the above parameters that would provide useful clues during patient diagnosis and follow-up after surgical resection.

This study found the score 3 staining ratio (72%) to be significantly higher in the malignant population than in the polyp group (31.2%). However, score 2 staining was higher in the polyp group (68.8%) than in the malignant group (28%). In tumoral cases, osteopontin staining intensity was significantly higher in the scoring system than with polyp cases ($p= 0.001$). While insignificant, differences were found according to differentiation grade, with scoring 3 staining patterns present in 66% of well-differentiated tumors rising to universal occurrence among poorly differentiated cases.

Vascular invasion is an established adverse prognostic factor in colorectal and other carcinomas [14]. Wei et al. have identified the top 15 genes (especially OLR1, GPNMB, PRRX1, and BCAT1) that co-upregulate with OPN in human colon cancers specimens, promoting cancer migration and invasion in various types of cancer [15]. A study carried out with hepatocellular carcinoma specimens suggested that OPN overexpression independently correlates with vascular invasion and predicts poor survival in patients undergoing hepatectomy for hepatocellular carcinoma [16]. In our study, we found that score 3 stainings were higher (95.7%) in patients with vascular invasion than those without (51.9%; $p= 0.001$, confirming that hypothesis. In a study with 82 colorectal carcinoma patients, Likui et al. found a

significant correlation between osteopontin mRNA expression and lymph nodes so that venous metastases [17]. We could not find a significant correlation related to lymph node metastasis, but this may be due to having an insufficient number of patients. We did find that OPN staining was not associated with age or gender ($p= 0.059$ and $p= 0.649$, respectively) in tumors, polyps, and normal tissues (Table 2). We also observed significant differences in staining percentage and intensity scores across various parameters of osteopontin expression. However, some of them probably did not reach statistically significant levels, which might also be due to the number of cases investigated. Although the number of polyps in the study group was low, there was a significant difference in staining compared to patients with tumors. To find out whether an intramucosal carcinoma focus is present or has been developing, the widespread and intense staining of osteopontin will provide a meaningful warning sign when evaluating an adenomatous polyp biopsy.

Vascular invasion is a significant negative prognostic factor for all cancers and colon cancer in particular. Pathologists may be unable to observe vascular invasion because of vascular invasion not being present in examined sections. Therefore, intense staining of the osteopontin can be beneficial when detecting vascular invasion.

Conclusions

We suggest that osteopontin could be a beneficial biomarker in predicting vascular invasion. If supported by an adequate case series, it may also provide valuable information regarding invasive-precursor distinction. Hence, high staining score with osteopontin could provide useful information about predicting prognosis and determining the treatment method of colon cancer.

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

Ethical statement: The study was approved by Dışkapi Yıldırım Beyazıt Training and Research Hospital local ethics committee (Decision Number 25/08).

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Effect of vitamin D level and polypharmacy on the risk of falls in the elderly

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ABSTRACT

Aim: To investigate the effects of polypharmacy and vitamin D levels on the risk of falls in the elderly.

Methods: The prospective study included 201 patients (F/M: 155/46) aged 65 years and older who presented with nonspecific musculoskeletal pain. The demographic and laboratory data of the patients, as well as the results of a single leg stance test (SLST), a timed up and go (TUG) test and levels of vitamin D were recorded.

Results: The percentage of patients with polypharmacy is 15.9 percent and 29.4 percent used no medications. The SLST score was the lowest and the TUG test score was significantly higher in the polypharmacy group ($p<0.05$). Vitamin D levels were significantly higher in patients with normal SLST times than those with abnormal SLST times ($p<0.05$). The risk of falls was significantly higher among patients with a previous history of a fall ($p<0.05$). Polypharmacy and the female gender appeared as the most significant factors affecting the risk of falls ($p<0.05$), while vitamin D level was found to have no effect ($p>0.05$).

Conclusion: Medical therapies for the treatment of diseases in the elderly should have a rational basis, as this may reduce falls, particularly in the elderly population, that can have serious consequences, and may even lead to death.

Keywords: Accidental falls, elderly, risk factors, polypharmacy, vitamin D deficiency.

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Introduction

The aging global population has brought problems related to health services, the economy and the environment. Falls among the elderly may result from impaired vision, vestibular and proprioceptive sensation, and muscle strength [1]. Falls can occur as a result of disruption to postural balance, which ensures that the center of gravity remains in contact with the support surface, and can cause

serious mortality and morbidity [2]. Among elderly people aged 65 years and older, approximately 13 percent have a balance disorder and one-third experience at least one fall per year [3]. In case of fall, 20 percent of people are admitted to hospital and 10 percent may have soft tissue or joint damage, or even fractures [4]. Studies have shown that there are many factors other than age that increase the risk of falls, these include multiple chronic disorders (musculoskeletal disorders, neurologic and psychotic disorders, diabetes mellitus, etc.), polypharmacy and vitamin D deficiencies [5,6].

The most common definition of polypharmacy in studies refers to the use of four or more medications in one day [7]. Medications can

have side effects related to the metabolic state already affected by aging, and these side effects may increase the risk of falls. Researchers can be divided into two different groups, based on the results of a meta-analysis on this subject. While some emphasize the importance of a large number of medications, others place more emphasis on the type of medications used (antiarrhythmic, antihypertensive and antiepileptic drugs, etc.) [8]. There are also studies indicating that polypharmacy contributes two times more to falls than any other factor [9], while other studies have suggested that the use of at least one drug that contributes to falls has a more significant impact [10].

Epidemiological studies have identified a possible association between vitamin D level and risk of falling [11], although there is controversy between such studies. Some more recent studies suggest that vitamin D regulates muscle development, its deficiency may cause muscle weakness and may be associated with an increased risk of falls in the elderly [12]. Other studies, in contrast, found no association between falls and vitamin D level [13].

Studies on polypharmacy and vitamin D levels have given conflicting results in terms of their effects on falls. The aim of this study is to investigate whether polypharmacy and vitamin D levels affect the risk of falls in any way.

Materials and Methods

Patients aged 65 years and older who were hospitalized for nonspecific muscle pain within the previous year were prospectively studied. Ethical approval was obtained from Clinical Trials Ethical Board at Bolu Abant Baysal University (Date and decision number: 2015/64). Demographic data of the patients, laboratory and X-ray results, history of falls in the last year, comorbid diseases and the number of medications being used were recorded.

Patients with total blood count, erythrocyte sedimentation rate and C-reactive protein levels within normal ranges are included, whereas those with scoliosis, degenerative and inflammatory joint disease in the lower extremity, total hip and/or knee prosthesis, neuromuscular disease, dementia, mental retardation, traumatic brain damage, cerebrovascular disease, spinal cord injury, severe cardiovascular disease, visual impairment, and patients with alcohol and drug addictions were excluded from the study. Polypharmacy was defined as the use of four or more medications, and vitamin D deficiency was defined as 25(OH)D3 levels below 30 ng/ml, while values of 30 ng/ml and higher were defined as normal [14]. Single-leg stance test (SLST) and the timed up and go (TUG) test results were evaluated to determine the risk of falling. In the SLST, patients were instructed to stand on one leg hands facing down by their sides. The test was terminated when the patient's foot touched the ground, changed position or received support from the ground. The test lasts a maximum of 30 seconds and is performed three times, with the best performance recorded for analysis. A test result of 5 seconds or less indicates a balance disorder. In the TUG test, patients are instructed to get up from a chair without support, walk three meters and turn back, return to the chair, and then sit down. A test result of 14 seconds or higher indicates a balance disorder.

Statistical analysis

The statistical analysis was performed using the SPSS 22.0 statistical software (IBM Corp., Armonk, NY, US). A Shapiro-Wilk Test was used to determine whether the data was normally distributed. Data with a normal distribution was expressed as mean \pm standard deviation, while data without normal distribution was expressed as a median (min,

max). Paired correlations were evaluated from a Spearman's rank correlation coefficient, and a Mann-Whitney U test was used to evaluate the relationship between two independent groups, A Kruskal-Wallis test was used to evaluate the relationship between more than two independent groups, a Chi-Square test to evaluate the relationship between categorical variables, and the proportion of the categorical groups was expressed as a percentage (%). The effects of the quantitative and categorical data on the other groups were evaluated by logistic regression analysis. A p value of <0.05 was considered statistically significant.

Results

The study included 201 patients (F/M: 155/46) with a mean age of 68.6 (65–75) years (Table 1). It was found that 15.9 percent of the study patients used polypharmacy and 29.4 percent were not on any medications. 51.7 percent of the patients using medication used antihypertensive drugs, 6.0 percent used antidiabetic and 2.5 percent used antipsychotic medications. The SLST duration was normal in 49.3 percent and the TUG test was normal in 60.7 percent of the participants. There was a significant negative correlation between the SLST and TUG test results ($r=-.256$, $p<0.05$), and also a significant correlation between the number of drugs and SLST duration ($p<0.05$).

Table 1. Demographic values.

| Variables | Median | Min. | Max. |
|--------------------------------------|--------|------|------|
| Age (year) | 68,6 | 65 | 75 |
| Body mass index (kg/m ²) | 25,9 | 22,1 | 27,5 |
| Vitamin D (IU/ml) | 18,3 | 3 | 97,3 |
| Single-leg distance test (SLDT) (sn) | 12,6 | 4 | 21 |
| Time up & go test (TGT) (sn) | 5,6 | 0 | 15 |

SLST duration was observed to decrease with the increasing number of medications, and SLST durations were found significantly shorter in the polypharmacy group compared to patients who received less than four medications ($p<0.05$), (Figure 1).

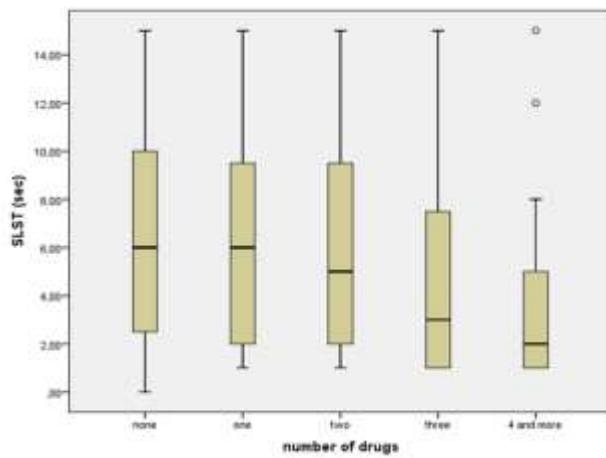


Figure 1. Relationship between SLST duration and number of drugs.

Concomitant use of more than one drug was more common in females than males. Of the study group, 29.8 percent had a remarkable history of falls, and the falling rate was significantly higher in females (88.3%) than in males (72.3%) ($p<0.05$). SLST times were significantly lower and TUG times were higher in patients with a history of falling compared to those without a history of falling ($p<0.05$), (Figure 2 and 3).

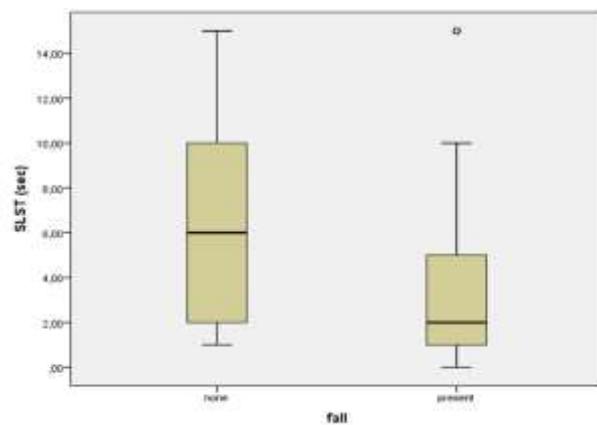


Figure 2. Relationship between falls and SLST duration.

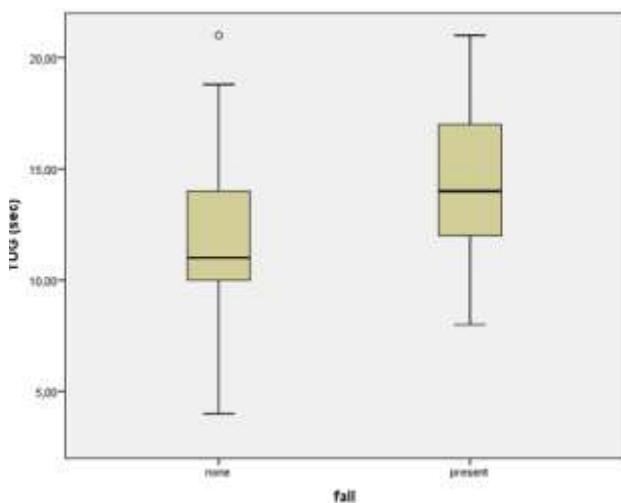


Figure 3. Relationship between falls and TUG duration.

SLST durations were significantly shorter and TUG durations were longer in patients using antipsychotic medications when compared to those who did not use antipsychotic medications ($p<0.05$). Only the SLST time was significantly shorter in patients on antidiabetics when compared to patients who did not take antidiabetic medication ($p<0.05$), (Figure 4).

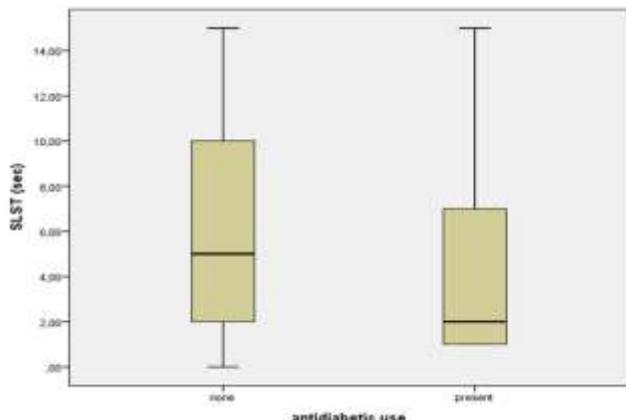


Figure 4. Relationship between antidiabetic use and SLST duration.

SLST durations were significantly lower and TUG durations were higher in female patients compared to male patients ($p<0.05$). There was a significant positive correlation between vitamin D levels and SLST duration ($r: 0.212$ $p<0.05$), and vitamin D levels were

significantly higher in patients with normal SLST times than those with abnormal SLST times ($p<0.05$). In the evaluation of the factors affecting the risk of falls, polypharmacy followed by TUG time and female gender had the most significant positive effects (Table 2) ($p<0.05$).

Table 2. The factors effecting falls.

| Variables | β | 95% Confidence Interval | | p |
|------------------------|---------|-------------------------|-------|-------|
| | | Min. | Max. | |
| Number of drugs | 3,66 | 1,08 | 12,42 | <0,05 |
| Time up & go test (sn) | 1,24 | 1,10 | 1,39 | |
| Women gender | 0,35 | 0,13 | 0,96 | |

Discussion

Showing an increasing prevalence with increased life expectancy, approximately 30–40 percent of elderly people aged 65 years and older experience falls, and 50 percent of these patients have a history of recurrent falls. Preventive measures must be taken due to the increased morbidity and mortality risk in the elderly after falling [15]. In population-based studies, researchers have reported rates of 16.5 percent, 21.8 percent and 45.4 percent for recurrent falls [4,5,9] compared to the recurrent falling rate of 29.9 percent in the this study. Studies have also found that females are three times more likely to experience falls than men, which has been attributed to the difference in muscle mass due to gender, the use of high heels in women and more common household accidents among women. In the present study, recurrent falls were twice as common in women as in men [7].

Due to the potentially serious consequences of falls, studies have focused on determining risk factors and in developing strategies to prevent

falls. Simply, falls occur when an individual loses his/her balance, and therefore balance assessments are the most important and relevant in the prevention of falls. Researchers have developed several tests for balance assessment, including the SLST and TUG tests which observe static and dynamic balance [16]. A three-year study involving 316 volunteers established SLST time as a risk factor for falls, and reported an association between recurrent falls, whereas another study involving patients with a mean age of 73.6 years reported no such relationship [17]. In the present study, the mean age of the study population was 68.6 years and SLST times were significantly lower in patients with a history of recurrent falls. Studies involving patients aged 65 years and above reported a relationship between TUG time and age, and suggested that a high TUG time is associated with recurrent falls [10]. The present study found no significant relationship between TUG time and age, whereas a significant relationship was noted between TUG time and repeated falls.

Hypertension, diabetes, and neurological and psychological disorders are the leading comorbid conditions that increase in parallel with aging. In recent years, there has been a linear increase in the use of antihypertensive, antidiabetic and antipsychotic drugs among the increasing elderly population [10]. Kojima et al. [9] evaluated the two-year fall rate in patients older than 65 years taking medication for chronic diseases, and they found that the concurrent use of multiple medications increased the prevalence of falls. In a longitudinal study, Dhalwani et al. [18] reported a rate of falls of 18 percent in subjects who were on four or more medications, and 50 percent in subjects on 10 or more medications. There are many studies reporting a significant negative correlation between the number of

medications and SLST time, whereas other studies have found that certain types of medication (antihypertensive, antidiabetic, antipsychotic) are more likely to increase falls [8]. These studies define such medications as drugs that increase the risk of falls. The rate of polypharmacy was found to be as high as 79.9 percent in a study involving 293 elderly hospice patients, and polypharmacy was found to increase the rate of fall-related injuries 4.5 times [4], although another study reported that polypharmacy increases the rate of injury 1.5 times [3]. In the present study, the risk of falls increased 3.6 times. Studies have also shown that the use of medications that increase the risk of falls (antihypertensive, antidiabetic, antipsychotic) bring about a further increase in risk when combined with polypharmacy. In a prospective study evaluating patients aged 55 years and above who were admitted to hospital, Ziere et al. [7] reported an increased risk of falls among patients who used four or more medications, and stated that the use of even a single medication increased the rate of falls in the presence of a drug that increased the risk of falls. Laflamme et al. [19] retrospectively evaluated patients aged 65 years and above who presented with fall-related injuries, and found that the risk of falls increased in the presence of polypharmacy, although an increased risk of falls was identified in the presence of medication use that increased the risk of falls regardless of the number of medications. RA balance study involving patients with hypertension found that the use of antihypertensive medications had a negative effect on balance [20], while the present study, in contrast, found no such effect of antihypertensive medication use on the risk of falls, the rate of which was 73 percent in the polypharmacy group. This is attributed to the widespread use of antihypertensive therapies,

while similar studies have found that patients on antidiabetic and antipsychotic medication are more likely to experience falls. Similarly, the present study found a significantly higher rate of antidiabetic and antipsychotic medication use in the polypharmacy group, indicating its negative effect on balance. Taking drug use into consideration rationally would prevent possible drug side effects and would reduce their negative effects on the risk of falls. The musculoskeletal system is another body system that affects balance. Maintaining sufficient level of minerals and vitamins in the body has been found to be associated with overall health status and balance mechanisms, and vitamin D is one of the more important vitamins in this regard [13]. Vitamin D acts as a precursor hormone analogue, and plays an important role in several important mechanisms, including calcium imbalance [13]. Studies have found impaired balance and muscle development in patients with vitamin D deficiency, indicating that vitamin D levels have a positive effect on physical performance in individuals aged 65 years and above [6]. It has been reported that elderly people with normal vitamin D levels perform better than people with vitamin D deficiency, with vitamin D levels below 24 ng/ml in particular have dramatic negative effects on balance, leading to recommendations for careful attention to vitamin D levels [21]. SLST times were higher in individuals aged 60 years and older with normal vitamin D levels than patients with lower levels [13], and the present study found significantly lower TUG durations in patients with vitamin D deficiency, although no effect of vitamin D was observed on falls.

The strengths of the study includes the use of extensive exclusion criteria, the limited age range of the patients and the evaluation of the reviewed data by a specialist. The limitations of

the study include small number of patients, single-center study design, and lack of data on sociocultural aspect of the study patients. Organic factors must be evaluated in conjunction with environmental factors when evaluating fall risk in the elderly. There have been many studies investigating the concurrent use of multiple medications in patients with falls, and in the present study, patients were evaluated with tests that are used to evaluate predispositions to falls, and the findings may be relevant in efforts to come up with measures to decrease the risk of falls. The number of medications used by elderly patients may be reduced through lifestyle modifications, and the type and number of currently used medications must be taken into consideration when prescribing new medications. Monitoring vitamin D levels from early ages, and maintaining optimal levels may be important in maintaining a good neuromuscular structure in advanced ages. More comprehensive studies involving larger populations should be conducted.

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Alcoholic extract of Tarantula cubensis (Theranekron®) induce autophagy on gastric cancer cells

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ABSTRACT

Aim: To evaluate the effects of theranekron in respect of autophagy on gastric cancer that is the fifth leading cancer type worldwide.

Metods: In the present study, metastatic AGS and non-metastatic MKN-45 human gastric cell lines were used together with HEK-293 non-cancer cells as controls. Cytotoxic effect of theranekron besides appropriate treatment time was investigated through cell proliferation by using Cell Proliferation assay Kit (MTT) using different concentrations of the drug. The autophagic effect of the drug was determined using the LC3-GFP translocation assay and western blot analysis. All experiments were performed also using the ethanol since Tarantula cubensis spider was processed and diluted in 60% alcohol to generate as a drug.

Results: MTT assay results demonstrated that the half maximal inhibitory concentration of theranekron was ~100 µM, its effect was found to be significant at 6 hrs, and theranekron decreased the cell viability in all cell lines without specificity in respect to the increasing concentrations. Additionally, a significantly increased GFP accumulation was detected in the autophagosomes of the cells treated with theranekron compared to non-treated cells, indicating the presence of autophagy.

Conclusion: These findings were confirmed by LC3-I to LC3-II conversion with the western blot analysis. The data of ethanol experiments; however, demonstrated that ethanol also induced a cytotoxic effect and autophagic cell death. Our results suggested that theranekron results in cell death and stimulate autophagy process, but it is not specific for cancer cells since it represented similar results on non-cancer control cells. Moreover, the effect of theranekron on cell death might mostly occur through alcohol in which it is extracted.

Keywords: Theranekron, tarantula cubensis, homeopathy, gastric cancer, autophagy.

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Introduction

Gastric cancer is a type of cancer that originates at the mucosa epithelia of the stomach and

expands rapidly to the lining of the stomach. Mostly, it is developed as ulcer. The cancer may spread from the stomach to other parts of the body, particularly to the liver, lungs, bones, lining of the abdomen and lymph nodes through blood [1]. Globally, stomach cancer is the fifth leading cancer type and the third leading cause of death from cancer according to 2012 cancer statistics [2]. It is also fifth leading cancer at

men and sixth leading cancer type at women in Turkey according to 2016 cancer statistics [3]. Gastric cancer occurs most commonly in East Asia especially Japan and South Korea and Eastern Europe. It is observed mostly at ages of 50 to 60 and occurs twice as often in males than females [2].

Theranekron® (Richter Pharma, Austria) is an alcoholic extract of the venom that is provided from a spider known as *tarantula cubensis*. *Tarantula cubensis* is the famous one among the venomous spiders and many therapeutic effects have been reported for its venom [4]. Theranekron is a homeopathic remedy that is used in cattle, horse, sheep, goat and dog. Homeopathy is a treatment method introduced by Dr. Samuel Hahnemann in the end of 18th century with the principle that “any substance causing symptoms of a disease in healthy people will cure similar symptoms in sick people”. Mezger described the homeopathic effects of theranekron first time in 1977 [5]. Theranekron is used as a pharmaceutical compound serving in veterinary medicine with outstanding success for its antiphlogistic, necrotizing, and wound healing effects [4]. In the literature, the drug was studied in various fields such as many types of ulcer and abscess, peripheral nerve healing, as well as treating necrotic or proliferative cases in animals [6-9]. Koch and Stein reported first time that theranekron stimulate the demarcation of mammary gland tumors in dogs [10]. Later, Gultekin et al (2007) reported that theranekron application to the dogs resulted in regression and hardness of benign mammary tumors [11], and tarantula cubensis extract alters the degree of apoptosis and mitosis in canine mammary adenocarcinomas shown in 2015 [12].

Autophagy is a tightly-regulated process involving the degradation of a cell's own components through the lysosomal machinery

[13]. Autophagy plays an important role in the homeostasis of the organelles and protein, and maintains a balance between synthesis and degradation in cells [14]. During the distribution of the homeostasis, autophagy leads to the cell death through activating the signaling pathways. Autophagic cell death is distinct from the apoptotic cell death, but the relationship between autophagy and apoptosis is more complex. It was shown that autophagy can delay apoptotic death following DNA damage [15] as well as can trigger a form of cell death in the absence of apoptosis [16]. The aim of the present study was to evaluate the effects of the alcoholic extract Theranekron on the gastric cancer cells in respect of autophagy.

Materials and Methods

Culture of cells

Metastatic gastric cancer cell line AGS, non-metastatic gastric cell line MKN-45, and non-cancerous cell line HEK-293 were purchased from American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Germany) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany) and 1% penicillin-streptomycin (P/S) antibiotic solution (Sigma-Aldrich, Germany). Cells were grown in a 5% CO₂ incubator at 37°C by replacing the medium every other day, and cells were passaged when they reached confluence.

The half maximal inhibitory concentration and cytotoxic effect of theranekron

AGS, MKN-45, and HEK-293 cells (1×10^4 cells/well) were treated with different concentrations of theranekron (Richter Pharma, Austria) by diluting the drug with culture medium. One group of cells was also treated

with same dilutions of alcohol as control since the drug was extracted in ethanol. Following different time points, the Cell Proliferation Kit I (MTT) experiment was performed. Culture medium was mixed with thiazolyl blue tetrazolium bromide powder (Sigma-Aldrich, Germany) at a ratio of 5 mg/ml, and 10 µl was added to each well. The cells were incubated with the solution for 3 h at 37°C until the color turned into blue. Then, all the solution was taken away from the wells and the formazan salts were dissolved through DMSO about 20 min. The absorbance was measured at 540 nm.

LC3-GFP translocation assay

AGS, MKN-45, and HEK-293 cells were seeded into 6-well plate with 2 mL culture medium and transfected with plasmid coding LC3 fused with green fluorescence protein (GFP). For transfection, first plasmid was diluted in a tube with 125 µl serum free medium at a ratio of 1 µg/100 µl. At the same time, 6 µl Lipofectamine® 2000 (Invitrogen, USA) transfection reagent was diluted with 125 µl serum free medium in another tube. After 5 minutes of incubation time, both tubes were mixed and the mixture was then incubated at room temperature for 20 min to form lipid complexes. 250 µl of mixture was then added into each well. Transfected cells were incubated in CO₂ incubator for 24 h until the cells start to express LC3-GFP. Then, one group of transfected cells was treated with 100 µM theranekron and the other groups were treated with rapamycin as a positive control for autophagy and alcohol as negative control. Treated cells were incubated for 3, 6, and 12 hours for the best result. The cells were analyzed under Epi Fluorescence Microscope (Nikon, Japan).

LC3 protein analysis by western blot

AGS, MKN-45, and HEK-293 cells were treated with theranekron and alcohol for 6 hrs, then they were detached from plate, washed with PBS, and the pellets were lysed with 100–150 µl RIPA lysis buffer (Santa Cruz, USA). The protein concentration of the lysates was determined with Bradford protein assay reagent (Bio-Rad, USA) following the manufacturer's instructions. Each sample (20 µg) was loaded into a 10% separating acrylamide gel and electrophoresis was applied. After blotting, the membrane was incubated with LC3 (Cell Signaling Technology, USA) and β-actin (Santa Cruse Biotechnology, USA) primary antibodies at 4°C. Images of the proteins were captured with a molecular imager (LI-COR Biosciences, USA) at a suitable time (change with respect to the primary antibody) after incubating the membranes with Western ECL Blotting Substrate (Bio-Rad, USA) for 5 min.

Results

Effect of theranekron on cell proliferation

The initial step for evaluating the effect of theranekron on gastric cancer cells was to determine the half maximal inhibitory concentration of the drug. The results of MTT assay showed that the IC₅₀ value of theranekron on these cells was ~100 µM. Moreover, the significant effect of theranekron was found to be at 6 hrs. Therefore, 100 µM theranekron was applied to cells for 6 hrs in the subsequent experiments. After appropriate time and concentration were determined, the viability of cancer and non-cancer cells treated was analyzed for effectiveness and uniqueness. The results showed that theranekron affected similar in both AGS and MKN-45 gastric cells as it affected in control HEK-293 cells (Figure 1).

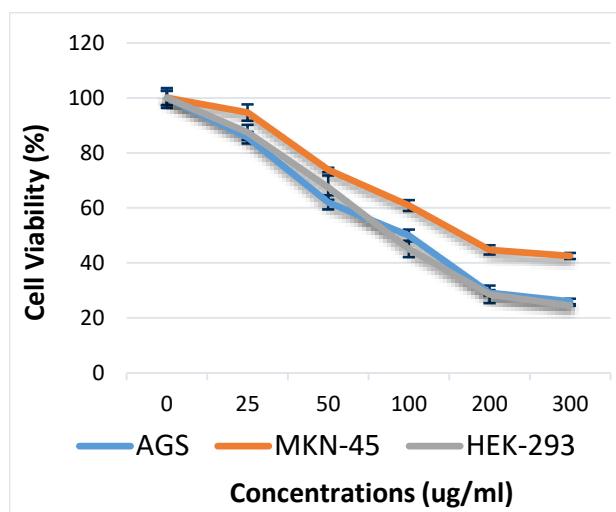


Figure 1. Comparison of theranekron's cytotoxic effects on AGS, MKN-45 gastric cancer and HEK-293 control cells at 6 hrs with different concentrations of the drug.

Moreover, theranekron was provided commercially and it was extracted in 60% ethanol. For this reason, theranekron and alcohol's cytotoxic effects were compared on gastric cancer and non-cancer cells to distinguish theranekron's effect from alcohol. All gastric cancer and control cells were treated with theranekron and ethanol at the same dilutions for 6 hrs. The results of proliferation assay demonstrated that alcohol showed similar results to theranekron (Figure 2).

Theranekron's autophagic effect in respect to the autophagy marker LC3

Since it was observed that theranekron decrease cell viability in all cells, additional experiments

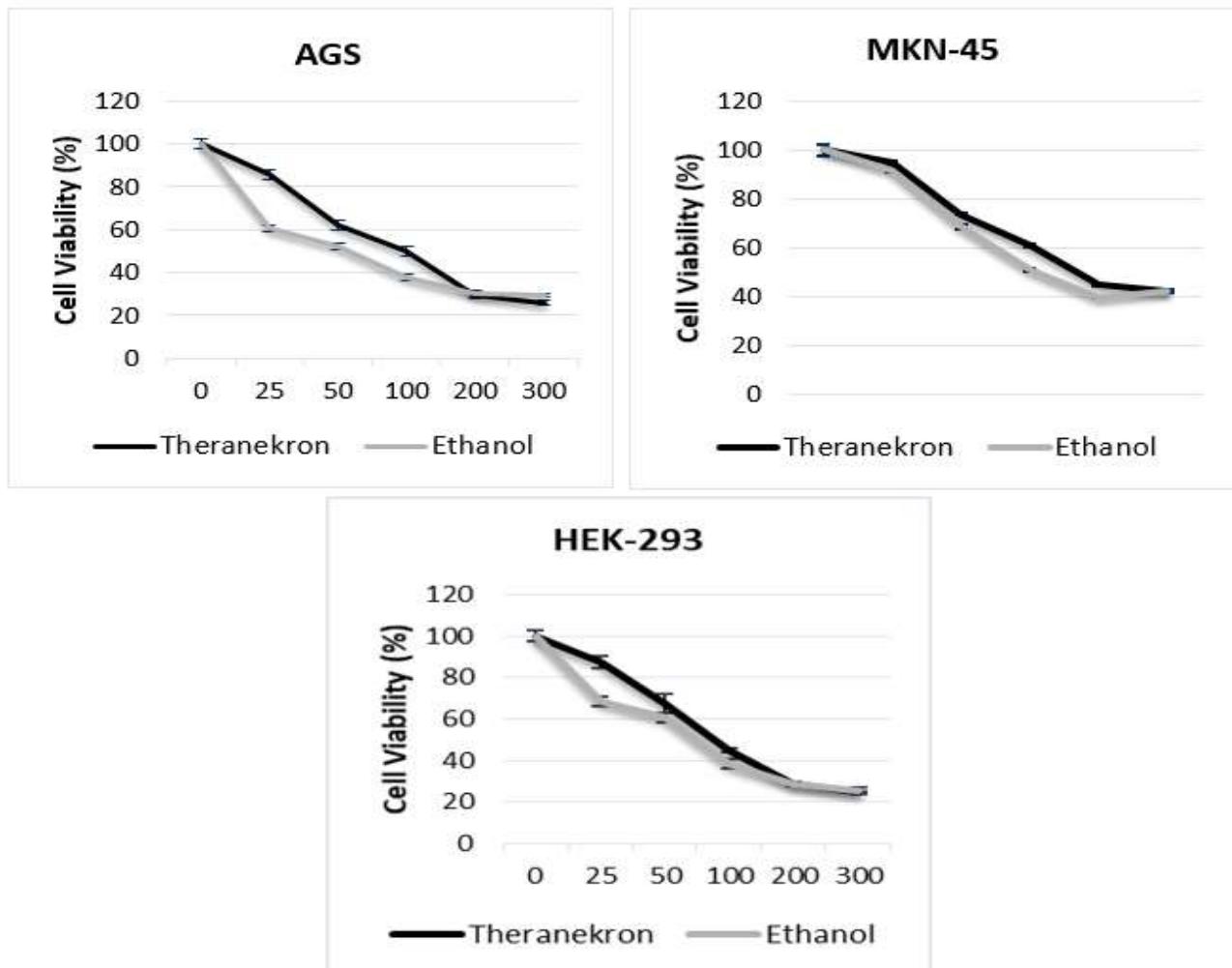


Figure 2. Comparison of theranekron and alcohol effects on AGS, MKN-45 and HEK-293 cells following treatment with theranekron and ethanol.

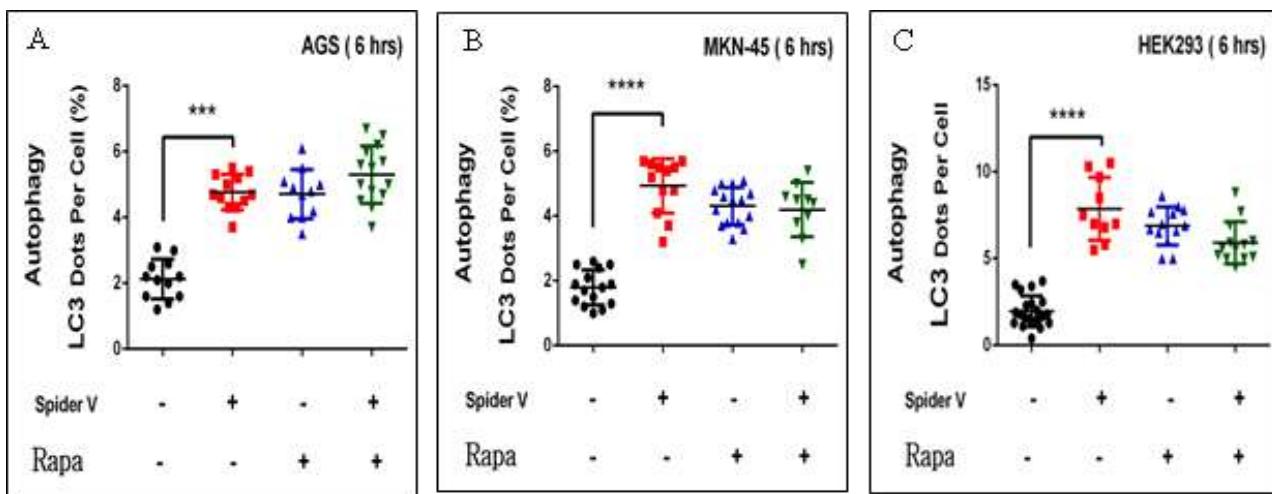


Figure 3. Autophagic effects of theranekron on (A) AGS, (B) MKN-45, and (C) HEK-293 cells displayed by LC3-GFP aggregation in the vacuoles of theranekron treated cells.

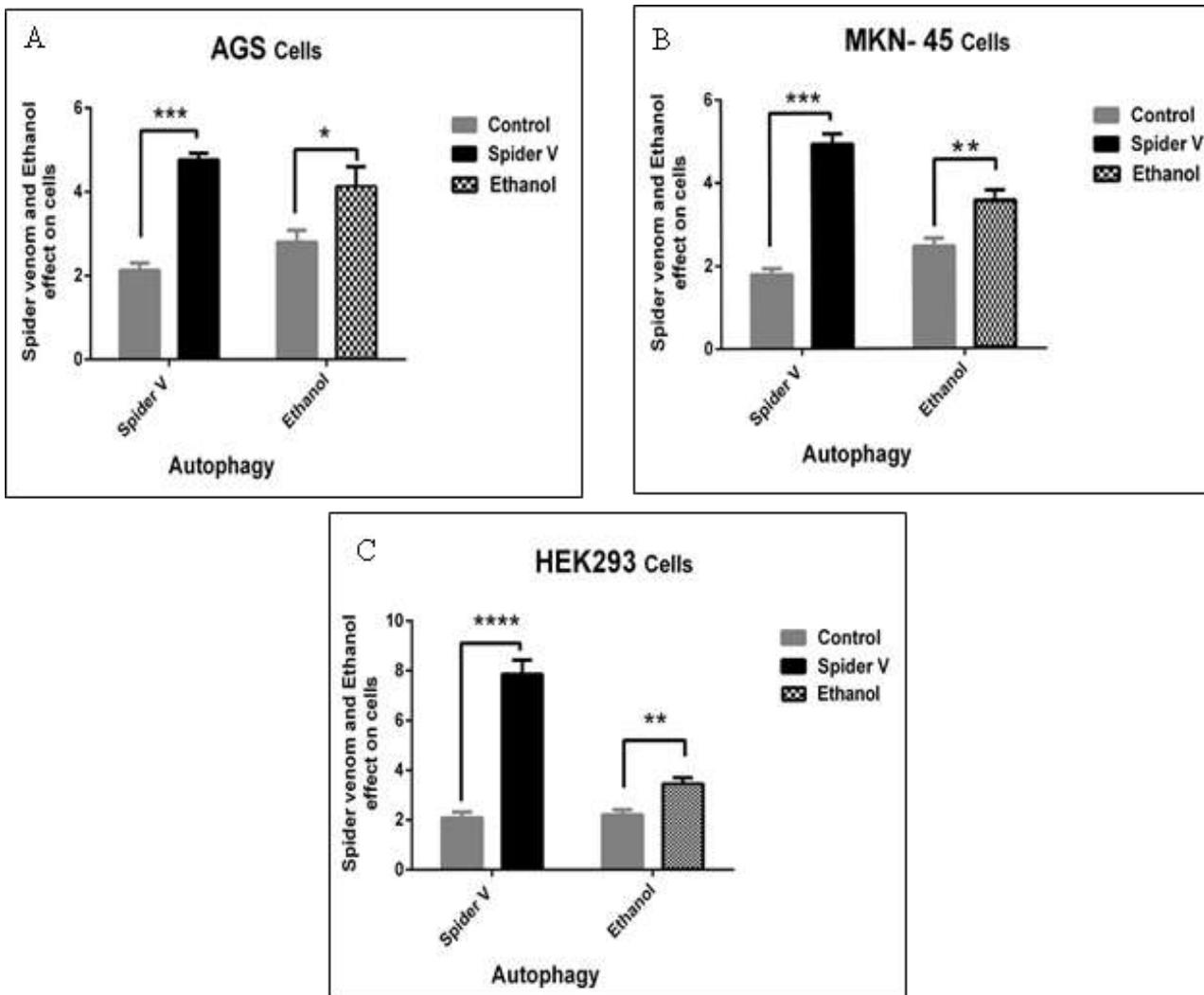


Figure 4. Comparison of autophagy effects of theranekron to ethanol on (A) AGS, (B) MKN-45, and (C) HEK-293 cells displayed by LC3-GFP aggregation in the vacuoles of theranekron and ethanol treated cells.

were carried out to investigate whether theranekron induce cell death through autophagic pathway. Initially, LC3 translocation assay was performed to analyze autophagy in treated cells. The results demonstrated that theranekron induced autophagy in gastric cancer cell lines AGS and MKN-45 when applied for 6 hrs at the similar level with rapamycin that is an autophagy inducer (Figure 3A and 3B). However, it was observed that autophagy was also induced in control HEK-293 cells, so the autophagy induction was not unique to cancer cells (Figure 3C).

In addition to that, alcohol's effect on autophagy was also tested on these cells since the drug was extracted in ethanol. According to the results, ethanol triggered autophagic cell death, but when compared to the effect of drug,

there were less LC3 aggregated vacuoles in ethanol treated cells especially in HEK-293 cells comparing to gastric cancer cells (Figure 4A, 4B, 4C).

To confirm the LC3 translocation assay's results, theranekron treated gastric cancer and non-cancer control cells were analyzed for LC3-I to LC3-II conversion by western blotting, which is known as hallmark of autophagy. The results showed that theranekron induced autophagy in both AGS and MKN-45 gastric cancer cells at 6 h. LC3-I (18 kDa) to LC3-II (16 kDa) conversion was clearly visualized in the treated cells. As in LC3 translocation assay results, theranekron induced autophagy in gastric cancer cells similar to HEK-293 control cells. Moreover, the ethanol treatment resulted in the autophagy in all cell types, but its affect was less than theranekron's

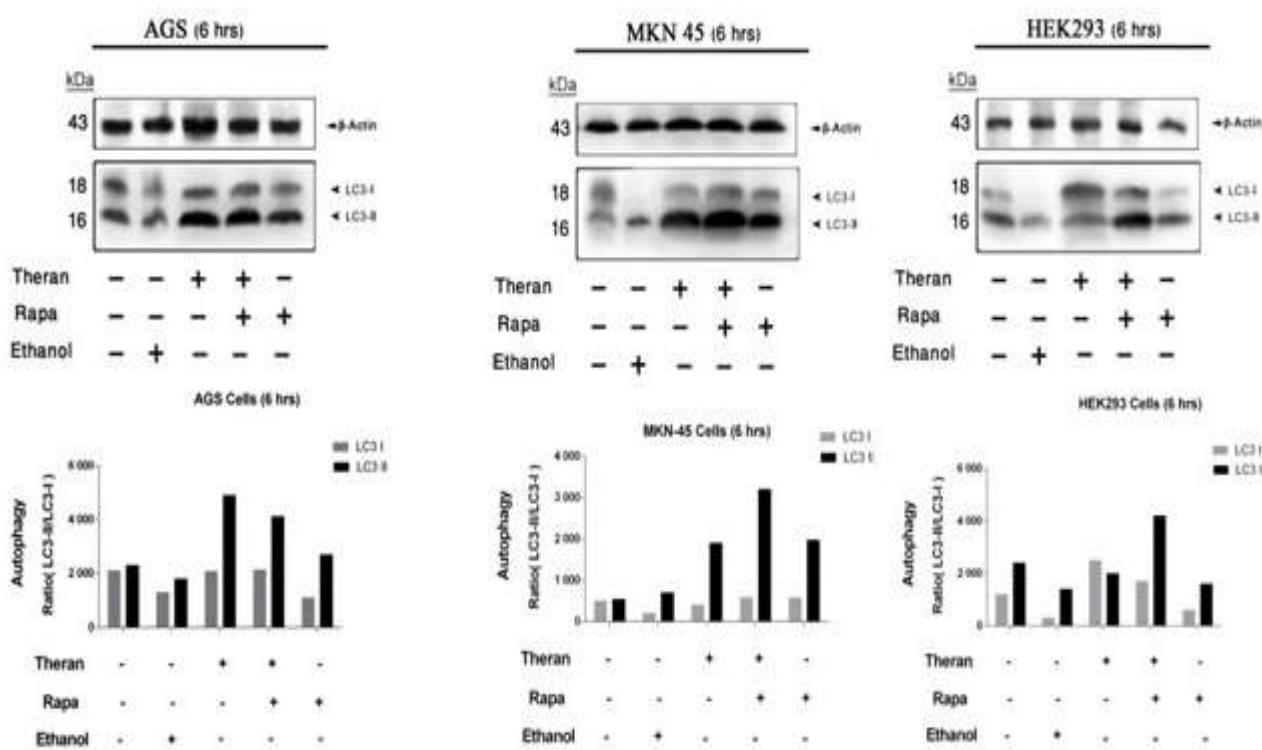


Figure 5. Autophagic effects of theranekron on AGS, MKN-45, and HEK-293 cells demonstrated by LC3-I (18 kDa) to LC3-II (16 kDa) conversion. All cell types were treated with theranekron, ethanol and rapamycin, separately, for comparison.

effect (Figure 5A, 5B, and 5C). This data also showed a consistency with LC3 translocation assay results. These results showed that theranekron induced more extensive autophagic cell death in both cell types when compared to the ethanol treated cells. However, theranekron did not show a significant difference in the gastric cancer cells for autophagic cell death when compared to the HEK-293 control cells.

Discussion

In this study, the effects of theranekron on gastric cancer cells were investigated focusing on autophagic cell death. Gastric cancer is one of the most common gastrointestinal tumors and fifth leading cancer type, globally [2]. Autophagy is a tightly-regulated homeostatic process that involves degradation of cells' own components through the lysosomal machinery. It is a major system to clear the defective or aging organelles and long-lived proteins in eukaryotic cells. It is reported that autophagy plays a dual role as tumor suppressor function at early stages while it plays an oncogenic function once the tumor is formed by providing cancer cells with survival contexts as nutrition [17]. Autophagy plays also tumor-suppressor and tumor-promoter role in gastric cancer [18]. Autophagy-related markers as Beclin1 might be considered as a potential marker of gastric carcinogenesis, aggressiveness and prognostic prediction, and as a target for gene therapy in gastric cancer [19] while autophagy inducers, such as rapamycin, show promise for gastric cancer treatment [20]. It was also reported that long-term *Helicobacter pylori* infection can disrupt autophagy process eventually promoting gastric cancer [21].

Theranekron is a homeopathic remedy that is mostly used in the veterinary medicine. It is an alcoholic extract produced by processing the

whole spider known as Tarantula cubensis and diluting in 60% alcohol. There are many studies carried out with theranekron to present its beneficial effect in the veterinary medicine area such as wound healing [22], anti-inflammatory [23], and necrotizing action [4]. In addition to these studies, antibacterial effect of theranekron was evaluated, but they could not find significant antibacterial property of the venom [24]. Recently, theranekron's effect on peripheral nerve healing was also studied, and it was found that theranekron decreases axonal and myelin damage after sciatic nerve injury [9].

Beside of all these data, there is not much study investigated the theranekron's effect on cancer. For the first time, Koch and Stein published a paper in 1980 about theranekron's effect on dogs' mammary gland tumors [10]. They demonstrated that theranekron stops tumor growth in canine mammary tumors by forming demarcation from surrounding tissue when it is used preoperatively a week for three times with the dosage of 3-6 ml depending on the body weight. It was observed that tumors became smaller, no reoccurrence was observed for years. Then, Gultiken and Vural demonstrated in their 2007 paper that Tarantula cubensis extract applications resulted in regression and hardness of benign mammary tumors while only hardness was detected in malignant mammary tumors in the dogs [11]. The same group then published another study in 2015 about theranekron's apoptotic effect on canine mammary tumors. Pre- and post-treatment tumor tissues were immunohistochemically assessed and they showed that the expression of B-cell lymphoma 2 (Bcl-2) which is considered an important anti-apoptotic protein was found to be higher in pre-treatment compared to post-treatment tissues. They concluded that the apoptotic index was low before treatment and

increased during treatment, so apoptotic cell death increased through theranekron treatment [12]. Beside these results, Ghasemi-Dizgah et al (2017) and Ayse et al (2017) demonstrated in their *in vitro* studies that theranekron increases cell death through apoptosis [25, 26]. In the current study, it was shown that theranekron leaded to cell death, but also presented that the cell death induced by theranekron was also through autophagic pathway. Moreover, it was also shown that the cell death could be triggered by alcohol in which theranekron was diluted. Finally, this study also indicated that alcohol triggered autophagic pathway supporting previous studies in the literature about alcohol induces autophagy [27, 28].

The common ground of all studies performed before current study is that they had been carried out *in vivo*. *In vitro* cancer studies of theranekron is very limited in the literature. Moreover, autophagic effect of theranekron was not examined although its apoptotic effect was shown [25, 26]. In addition, ethanol, the solvent used to extract was not investigated in any study before to eliminate alcohol effect of theranekron. To our knowledge, the present study is the first to reveal the relationship between theranekron and autophagic cell death. Also, this is the first study to show theranekron may cause cell death through alcohol in which it is extracted.

Conclusions

In this study, it was demonstrated that theranekron result in cell death and stimulate the autophagy process, but it is not specific for cancer cells since it represented similar results on non-cancer cells. Moreover, the effect of theranekron on cell death might mostly occur through alcohol effect.

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Ethical statement: Since the study is a cell culture study, ethics committee approval was not obtained.

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The effect of tuberositas tibia osteotomy on patellofemoral joint pressure: An experimental animal study

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ABSTRACT

Aim: To demonstrate the decrease in patellofemoral pressure with an anterior elevation of tuberositas tibia. Therefore, we have performed Maquet's Technique to evaluate the patella pressure on femoral trochlea by biomechanically in an animal experiment model.

Methods: This study includes total of 42 knees of 21 New Zealand rabbits. Animals were divided into two groups. The first group including 21 right knees was designated as the control group. In the second group including 21 left knees, anterior elevation of tuberositas tibia (Maquet's technique) was performed. Pressure measuring film layer "prescala" was placed on the patellofemoral joint under anesthesia in both groups. Mean values of both average and maximal pressure measurements in two groups were compared.

Results: There is a statistically significant difference in between average pressure and maximum pressure in the right and left legs of the rabbits. Average pressure and maximal pressure at rabbit knees performed Maquet's procedure were significantly lower than knees without Maquet's procedure.

Conclusion: Anterior elevation of tuberositas tibia is successful in reducing patellofemoral joint pressure which can be used in cases with patellofemoral pain syndrome non-responding to conservative treatment.

Keywords: Patellofemoral syndrome, patellofemoral pressure, tuberositas tibia, Maquet's Technique, rabbit.

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Introduction

The prevalence of anterior knee pain (AKP) or patellofemoral pain syndrome (PFPS) has been reported as between 15–45% of the population [1]. Asymmetric formations among anatomical structures at the patellofemoral joint lead to

joint discordance and thus predisposes to painful knee clinic including diseases such as osteoarthritis, PFPS and instability. PFPS is a group of symptoms especially negatively affecting the daily life of adults and leading to dysfunction [2].

PFPS is diagnosed in case of pain occurring during activities such as prolonged sitting, climbing upstairs or descending and crouching down unexplained by other pathologies. Patients with AKP are diagnosed patellar chondromalacia for many years, the softening and tissues in cartilage tissue should provide a

diagnosis for chondromalacia. Even though their patellofemoral pain is not chondromalacia, this put them as a candidate for developing chondromalacia in the future [3-5]. The origin and pathogenesis of PFPS is not known precisely. However, most authors have emphasized the theory of increased patellofemoral pressure due to patellar disturbance, with patellar maltracking and dynamic valgus forces being responsible, although it is known to occur in more female patients [6-9]. Among the causes of increasing patellofemoral pressure are; posterior cruciate ligament rupture, hamstring muscle shortening, ankle dorsiflexion weakness, gastrocnemius dominance, quadriceps weakness, increased femoral anteversion, tibial external rotation, genuvalgum and varum, pes planovalgus, lateral condyle hypoplasia, patella alta and patellar subluxation [10].

There are different methods of surgical modalities described in the treatment of PFPS. These methods are; lateral reticular release, medial open wedge high tibial osteotomy and different osteotomy procedures of anterior translation of tuberositas tibia (for example dual osteotomy) [11,12]. In 1963, Maquet described anterior elevation technic of tuberositas tibia to control increased patellofemoral pressure by abnormal muscular and biomechanical factors in the pain of the anterior knee. Vector forces on patella increase the patella pressure on trochlea in case of impaired biomechanical equilibrium. Therefore, he suggested reducing patellofemoral pressure by changing only the direction of vectors, not extension forces on the patella [13].

The starting point of our study is to consider that decreasing patellofemoral pressure would be beneficial in pain control. In our study, the reason for measuring pressure by animal tests is

that the knee joint is dynamic and that the knee could be affected not only by vector changes but also by muscular contractions. We noticed that in the literature, there was no biomechanical animal study about the effect of Maquet's procedure on patellofemoral pressure.

Our study aimed to evaluate biomechanically the patella pressure on femoral trochlea in an animal experiment by Maquet's procedure (anterior elevation of tuberositas tibia).

Materials and methods

Experimental Design

All animal studies were carried out with the approval of the Institutional Animal Care and Use Committee (Date and Decision no: 2010/810-10). Animals were housed at constant temperature (20-22°C) and humidity (50-60%) with a 12-h light and 12-h dark cycle. They were allowed free access to water and standard rat chow.

This study included a total 42 knees of 21 New Zealand rabbits. In rabbits, it is well recognized that skeletal growth is completed at week 28 and reaches to mature adult height at week 34. In respect of this information, animals used in the study were selected among rabbits older than 34 weeks and approximately with 1000-1200 g of weight.

Animals were assigned in two groups. The first group consisted from 21 right knees and surgery for the anterior elevation of tuberositas tibia was not applied, and only pressure measuring film layer "prescala" (Fujifilm, Japan) was placed on the patellofemoral joint (Control group). Second group consisted from 21 left knees. In the second group, anterior elevation of tuberositas tibia (Maquet's technique) was performed on left knees and pressure measuring film layer "prescala"

(Fujifilm, Japan) was placed on patellofemoral joint LLW (Fujifilm, Japan) (super low pressure) 0.5- 2.5 Mpa was used as a film layer (MT Group).

Anesthesia and Analgesia

An injectable mixture of Ketamine- Xylazine was administered to animals for surgical anesthesia. The injection was performed into quadriceps muscle with the tip of syringe toward posterior to prevent sciatic nerve damage. The dose administered for ketamine and Xylazine was 35 mg/kg and 5 mg/kg, respectively. During post-operative care, animals were kept alive and allowed to complete the healing process in a warm and dry and quiet area for 24 hours following the surgical procedure. Feeding was allowed as

soon as the animals were awake to ensure gastrointestinal motility and prevent stasis. Ketoprofen (5mg/kg, sc) was administered for post-operative analgesia and ciprofloxacin (10 mg/kg, Po) was also administered within the first day following surgery as anti-biotic prophylaxis.

Surgical Technique

Preoperative preparations were done on 42 knees of 21 animals before the surgical procedure. For this purpose, knees were kept at extension, shaved and aseptic conditions were obtained by administration of antiseptic (Figure 1A). The midline skin incision was preferred as surgical technic, and then skin and subcutaneous tissue were dissected to the lateral and lateral part of patella and

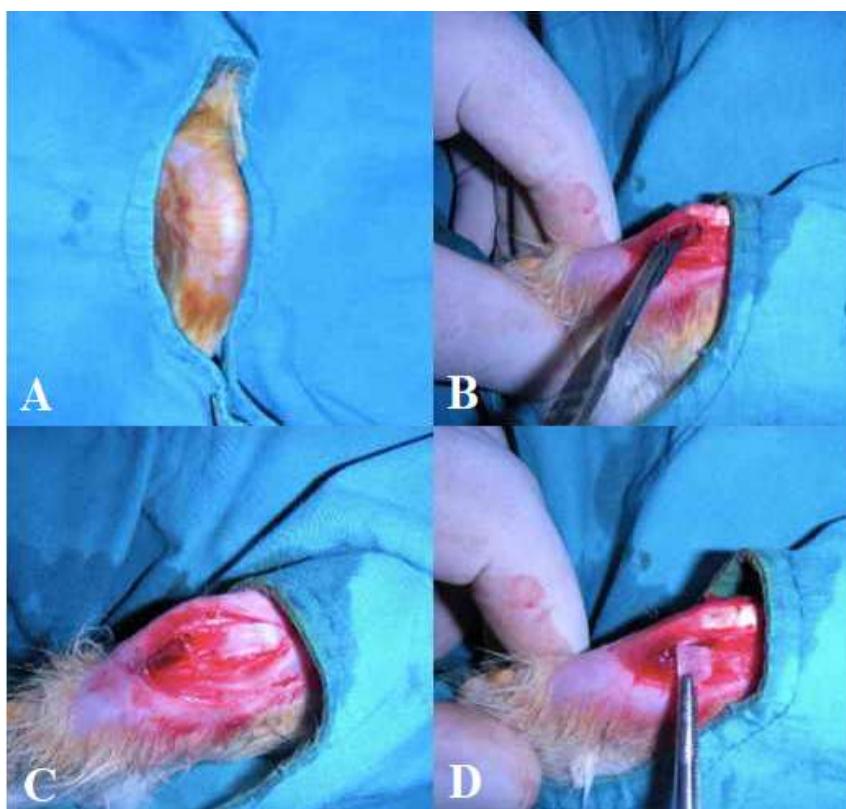


Figure 1. **A)** Pre-operative preparation of rabbit knee. **B)** Opening of knee joint by lateral approach and exposure of patellofemoral joint. **C)** Split elevation of tuberositas tibia. **D)** Anterior elevation of tuberositas tibia for 3 mm and placing sterile polyethylene wedge.

retinaculum were exposed. The lateral patellar approach was used for arthrotomy. Synovial tissue was incised at the lateral margin of the patella and lateral soft tissue providing patellar stability, was cut (Figure 1B). Maquet's procedure was not applied at the right knees of animals; only the patellofemoral groove was visualized.

Maquet's procedure was applied at left knees of animals. In technic described by Maquet in 1963, tuberositas tibia is anteriorly elevated for 2 cm as a split. Autologous bone graft is placed in between without tearing distal attachment [13]. In this study, tuberositas tibia of the left knee was raised as split and anteriorly elevated about 3 mm (In human, when tibia was raised about 30 cm, anterior elevation is 2 cm. we also measured rabbit tibia average 7 cm and proportionally we anteriorly elevated 3 mm) and a sterile polyethylene wedge was placed in between (Figure 1C and D). Maquet's procedure was not applied at the right knee of animals; only the patellofemoral groove was visualized.

Following the surgical procedure, Fuji LLW pressure measuring "prescala" film layer cut as a trochlear groove, was placed in patellofemoral space of both knees. Joint capsule was sutured, and layers were closed regularly. Animals were monitored by a sterile dressing. Following 24 hours of rest after the procedure, film layers were measured by FPD-8010E Fuji Film pressure measuring system. LLW Fuji (Fujifilm, Japan) pressure measuring film layer used in our study is in the form of a trochlear groove. Higher pressure level was observed in certain area of each film layer compared to other areas. Therefore, the average pressure level to calculate total pressure on the whole film layer and maximal pressure on film layers were measured.

Statistical analysis

Data were analyzed using the IBM Statistical Package for Social Sciences v16 (SPSS Inc., Chicago, IL, USA). Parametric tests were applied to data of normal distribution, and non-parametric tests were applied to data of questionably normal distribution. Wilcoxon Signed Ranks Test was used to test the difference between pressures mean. Continuous data were presented as mean \pm standard deviation or median [minimum-maximum], as appropriate. All differences associated with a chance probability of 0.05 or less were considered statistically significant.

Results

Forth two knees of 21 New Zealand rabbits were evaluated. Totally 84 pressure level, including both two average pressure and maximal pressures, were obtained for both knees of 21 rabbits (Table 1). In our study, both average pressure and maximal pressure at rabbit knees subjected to Maquet's procedure were significantly lower than knees without Maquet procedure (Table 2, 3). There is a statistically significant difference in terms of average pressure and maximum pressure between the right and left legs of the rabbits ($p < 0.05$).

Discussion

Our study aimed to evaluate biomechanically the patella pressure on femoral trochlea in an animal experiment by anterior elevation of tuberositas tibia which called Maquet's procedure. We revealed that Maquet's procedure could be suggested as successful in reducing targeted patellofemoral joint pressure to control AKP.

The patellofemoral joint consists of articulation between patella which has an irregular structure and trochlear groove. Contact pattern between

Table 1. Pressure measurement in the study.

| Animals | | Average pressure (psi) | Maximum pressure (psi) |
|------------------|-------------------|------------------------|------------------------|
| Rabbit 1 | Right Knee | 32 | 68 |
| | Left Knee | 31 | 61 |
| Rabbit 2 | Right Knee | 151 | 298 |
| | Left Knee | 150 | 238 |
| Rabbit 3 | Right Knee | 134 | 287 |
| | Left Knee | 94 | 221 |
| Rabbit 4 | Right Knee | 104 | 292 |
| | Left Knee | 89 | 207 |
| Rabbit 5 | Right Knee | 87 | 195 |
| | Left Knee | 52 | 131 |
| Rabbit 6 | Right Knee | 70 | 184 |
| | Left Knee | 66 | 132 |
| Rabbit 7 | Right Knee | 104 | 259 |
| | Left Knee | 64 | 228 |
| Rabbit 8 | Right Knee | 68 | 195 |
| | Left Knee | 56 | 193 |
| Rabbit 9 | Right Knee | 139 | 230 |
| | Left Knee | 136 | 214 |
| Rabbit 10 | Right Knee | 80 | 209 |
| | Left Knee | 52 | 172 |
| Rabbit 11 | Right Knee | 78 | 192 |
| | Left Knee | 56 | 164 |
| Rabbit 12 | Right Knee | 55 | 142 |
| | Left Knee | 54 | 112 |
| Rabbit 13 | Right Knee | 81 | 131 |
| | Left Knee | 74 | 105 |
| Rabbit 14 | Right Knee | 67 | 165 |
| | Left Knee | 53 | 158 |
| Rabbit 15 | Right Knee | 72 | 172 |
| | Left Knee | 70 | 165 |
| Rabbit 16 | Right Knee | 52 | 121 |
| | Left Knee | 48 | 108 |
| Rabbit 17 | Right Knee | 63 | 75 |
| | Left Knee | 60 | 62 |
| Rabbit 18 | Right Knee | 86 | 92 |
| | Left Knee | 71 | 90 |
| Rabbit 19 | Right Knee | 123 | 165 |
| | Left Knee | 114 | 147 |
| Rabbit 20 | Right Knee | 168 | 193 |
| | Left Knee | 155 | 173 |
| Rabbit 21 | Right Knee | 48 | 61 |
| | Left Knee | 46 | 59 |

psi= pounds per inch square (1 psi = 0,068atm).

RK: Right knee; LK: Left knee.

Table 2. Analysis table of both average and maximum pressure of knees subjected to Maquet technique.

| Parameters | N | Mean ± SD | Min - Max |
|-------------------------------------|----|---------------|-----------|
| Right Average pressure (psi) | 21 | 88.6 ± 32.795 | 32 - 168 |
| Right Maximum pressure (psi) | 21 | 177.4 ± 64.36 | 68 - 298 |
| Left Average pressure (psi) | 21 | 75.7 ± 32.42 | 31 - 155 |
| Left Maximum pressure (psi) | 21 | 149.5 ± 51.34 | 59 - 238 |

SD: Standard deviation.

Table 3. There is statistically significant difference between right and left limb of rabbits in respect of average pressure and maximum pressure.

| Parameters | Average pressure right-left | Maximum pressure right-left |
|-------------------------------|-----------------------------|-----------------------------|
| Z value | - 3,411(a) | - 3,411(a) |
| Asymp. Sig. (2-tailed) | 0,001(b) | 0,001(b) |

a: based on positive ranks; b: Wilcoxon Signed Ranks Test.

the patella and trochlear groove changes during movement of the knee joint. According to the neuroanatomic studies, it is reported that the main factor of AKP is tension at lateral retinaculum which causes ischemic process thus forms neural proliferation at nosiseptive axons around vascular structures [14]. The main reasons for AKP have to be identified and after elimination of differential diagnosis “patellofemoral syndrome” can be diagnosed. Although the initial cause and pathogenesis of PFPS are not fully understood; many factors such as acute trauma, injury of knee ligament, instability, over-use, genetic predisposition, impaired alignment of knee extensor

mechanism may be responsible [6-8,15-18]. However, many authors consider the theory of increased patellofemoral pressure due to impairment of patellar alignment. Abnormal muscular and biomechanical factors are considered to change the relationship of the patella with femoral trochlear incisura and thus to increase patellofemoral pressure and lead to pain and dysfunction [19,20-22]. It is also reported that there are surgical methods as conservative methods in the treatment of PFPS. Distal realignment procedures including also Maquet's osteotomy, are demonstrated to have good results in the treatment of misalignment [23]. Shirazi-Adl et al. reported that patellofemoral contact forces decrease with Maquet's osteotomy at low flexion angles, but maximum contact forces increase at 90 degrees of flexion angle in the 3D model biomechanical study [24].

We aimed to evaluate whether this procedure leads to suggested pressure reduction. Thus, we could propose this technic as a safer method in the surgical treatment of cases with PFPS. The goal of this technic is pressure reduction; however, no analytical method could demonstrate the success of this technic. In a study conducted on ten knees from cadavers, the effect of tuberositas tibia straight anterior elevation technic on the pressure of patella on trochlea has been evaluated and a reduction of pressure for 20% to 23% was reported [25]. In 2000, in a computer-modeled study of Farahmand et al., they reported that Maquet's procedure reduced patellofemoral pressure by 70%, 30% and 15% at extension, 30 degrees of flexion and 90 degrees of flexion, respectively [26]. In the literature, there are also studies comparing different technics of the osteotomy and measuring knee pressure [11,27]. In rabbit knee, the Maquet's procedure is more likely to apply compared to other osteotomy technics as

the knee, in this case, is smaller. Although rabbit is a rodent and the knee is at flexion during rest, and it is unlikely to get objective data in an experimental study of knee biomechanics, it is sufficient for applying the technic and providing necessary vector changes.

We are aware that there are clear limitations of the case series presented here. Although the method of pressure measuring film layer used in this study is a quantitative and reliable method, deviations of measurements can occur due to the smaller size of rabbit knee and difficulties in shaping trochlea. Also, another limitation of the study is lack of expected muscular contractions level of rabbits during the post-operative period since the vectors necessary for patellofemoral pressure are due to muscular contractions. However, in our study, we can postulate that standard measurements could be obtained as we operated both knees of animals.

Conclusions

In our study where we used the other knee of the rabbit as control, we detected a significant pressure reduction of patellofemoral joints on knees subjected to Maquet's technique. In conclusion, in our study, Maquet's procedure can be suggested as successful in reducing targeted patellofemoral joint pressure to control AKP. Therefore, Maquet's osteotomy used commonly in the past can be still valid for today and can be safely used in any cases with PFPS non-responding to conservative treatment.

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

Ethical statement: All experiments were approved by the Ege University Animal

Experiments Local Ethics Committee (Date and Decision no: 2010/810-10).

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Postoperative complications and its relationship with the severity of postoperative pain in patients undergoing thoracic surgery

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ABSTRACT

Aim: To investigate the complications that occur in our patients who underwent thoracic surgery, as well as the relationship between postoperative pain and complications.

Method: Of the 117 patients who underwent surgery between January 2018 and December 2018, there were 99 patients with pain and the other parameters whose data's were complete. Medical records of the patients were investigated in terms of age, gender, smoking status and frequency, diagnosis, treatment, length of stay in the hospital, postoperative complications and visual analog scale (VAS). The postoperative complications and VAS values were compared statistically.

Results: The mean age of the patients was 50.52 ± 18.46 years, 26 (26.3%) patients were female and 73 (73.7%) were male. The average length of stay in hospital was 4.08 ± 3.06 days and average pain severity was 3.92 ± 2.07 .

The most common diagnosis in our cases was lung cancer, and the most common complication was prolonged air leakage. There was a significant relationship between the severity of pain and the presence of postoperative complications in our patients ($p=0.001$). However, the correlation relationship was found to be low ($r=0.322$).

Conclusion: The results of our study revealed that optimal postoperative pain control is an important factor for preventing postoperative complications.

Keywords: Thoracic surgery, chest surgery, postoperative complications, pain, visual analog scale.

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Introduction

Since thoracic surgery is one of the most painful surgical procedures, the severity and duration of the pain associated with this surgery is remarkable. The most common causes of pain after thoracic surgery include multilayer intercostal incisions, rib injury or resection,

surgical drains and thoracostomy tube insertion, and suturing technique [1,2]. Acute, moderate to severe pain levels due to this diversity of insults may not significantly decrease during hospital stay or the first month after surgery. In addition, poor management of acute postoperative pain can lead to the development of chronic pain syndromes. Chronic pain can last for years, and even lower pain levels can reduce patient satisfaction [3]. Post-thoracotomy pain syndrome is common in patients undergoing thoracotomy and occurs in approximately 50% of patients. It is estimated

that these patients have persistent pain at 6 months and even 20% may continue to experience pain in 6 to 7 years [4-6]. In a previous study, it was reported that patients described pain one year after thoracotomy and that most continued to report pain even after years [7].

Despite improvements in anesthesia and surgical techniques, the rate of postoperative complications in thoracic procedures is still high, at 27% [8]. Thus, pulmonary and cardiovascular complications after thoracic surgery result in significant morbidity and mortality, and are also the leading cause of prolonged hospital stay with increased overall cost [9,10]. Further, complications compromise return to baseline function, may affect further cancer treatment, and overall quality of life [11]. In addition, there are common patient-specific risk factors such as age, pre-operative adverse pulmonary function tests, cardiovascular comorbidity, smoking status, and chronic obstructive pulmonary disease [12]. The main purpose of this study is to investigate the complications that occur in our patients who underwent thoracic surgery, as well as the relationship between postoperative pain and complications.

Materials and Methods

We retrospectively evaluated patients who underwent classic postero-lateral thoracotomy in our institution (Bolu Abant Izzet Baysal University, Bolu, Turkey). The study protocol was approved by the ethics committee of Bolu Abant Izzet Baysal University Human Research Ethics Committee (2018/298). Of the 117 patients who underwent surgery between January 2018 and December 2018, there were 99 patients with pain and the other parameters whose data's were complete. Medical records of the patients were investigated in terms of age,

gender, smoking status and frequency, diagnosis, treatment, length of stay in the hospital, postoperative complications and visual analog scale (VAS). The postoperative complications and VAS values were compared statistically.

In our clinic, a visual analog scale is used to evaluate the pain intensity of the patients on the first postoperative day. The visual analog scale (VAS) is widely used as an outcome measure, as in this study. It is usually presented as a 100-mm horizontal line on which the patient's pain intensity is represented by a point between the extremes of "no pain at all" and "worst pain imaginable." Its simplicity, reliability, and validity, as well as its ratio scale properties, make the VAS the optimal tool for describing pain severity or intensity [13,14].

Statistical analysis

Data analysis was performed on SPSS version 20.0 software (SPSS Inc., Chicago, USA). Quantitative parametric data were expressed as mean plus standard deviation (SD), and quantitative non-parametric data as median values with minimum and maximum. The Kolmogorov Smirnov and Shapiro-Wilk test was used to analyze the distribution of variables. For parametric data, correlation between groups were performed using the Pearson correlation analysis. The *p*-value <0.05 was significantly considered.

Results

Of the 117 patients who underwent surgery between January 2018 and December 2018, there were 99 patients with complete data regarding pain and the other parameters. The mean age of the patients was 50.52 ± 18.46 years, 26 (26.3%) patients were female and 73 (73.7%) were male. The average length of stay in hospital was 4.08 ± 3.06 days and the average

Table 1. Demographic data of the patients

| Parameters | Cases (n=99) |
|---|--|
| Age (years) (Mean±SD) | 50.52±18.46 |
| Gender, N (%) | 73 (73.7) Woman Male |
| Duration of stay (days) (Mean±SD) | 4.08±3.06 |
| Cigarettes (pack-years) (Mean±SD) | 18.84±22.55 |
| Smoking status, N (%) | Yes No |
| Diagnosis, N (%) | Nodular lesion in the lung Lung cancer Mediastinal LAP Pneumothorax Pleural effusion Pulmonary fibrosis Pleurisy |
| Pain severity (VAS) (Mean±SD) | 3.92±2.07 |
| Postoperative complications, N (%) | Yes No |

pain severity on the visual analog scale (VAS) was 3.92 ± 2.07 . Fifty-nine (59.6%) patients were tobacco smokers (18.84 ± 22.55 pack-years). The demographic data of the individuals included in the study are shown in Table 1. The five most common diagnoses in our patients were as follows: lung cancer (n=25, 25.25%), nodular lesions in the lung (n=20, 20.20%), spontaneous pneumothorax (n=15, 15.15%), pleural effusion (n=9, 9.09%), and hyperhidrosis (n=8, 8.08%) (Table 1). Two patients were revised (air leakage n=1, hemorrhage n=1).

Mortality occurred in one patient after surgery (1.01%).

Five of the most common complications we encountered in our cases were prolonged air leakage (n=4, 14.8%), atrial fibrillation (n=3, 11.1%), hematoma (n=2, 7.4%), hemorrhage (n=2, 7.4%), and tachycardia (n=2, 7.4%). The postoperative complications of the patients are shown in Table 2. There was a significant relationship between the severity of pain and the presence of postoperative complications in our patients ($p=0.001$) (Table 3).

Table 2. Postoperative complications.

| Postoperative complication | N (%) |
|----------------------------|----------|
| Atrial Fibrillation | 3 (11.1) |
| Empyema | 1 (3.7) |
| Apical bull perforation | 1 (3.7) |
| Arintenoid dislocation | 1 (3.7) |
| Atelectasis | 1 (3.7) |
| Delirium | 1 (3.7) |
| Diaphragm elevation | 1 (3.7) |
| Dyspnea | 1 (3.7) |
| Embolism | 1 (3.7) |
| Hematoma | 2 (7.4) |
| Hemorrhage | 2 (7.4) |
| Hypertension | 1 (3.7) |
| Constipation | 1 (3.7) |
| Pericardial effusion | 1 (3.7) |
| Secretion retention | 1 (3.7) |
| Hoarseness | 1 (3.7) |
| Chylothorax | 1 (3.7) |
| Tachycardia | 2 (7.4) |
| Prolonged air leakage | 4 (14.8) |

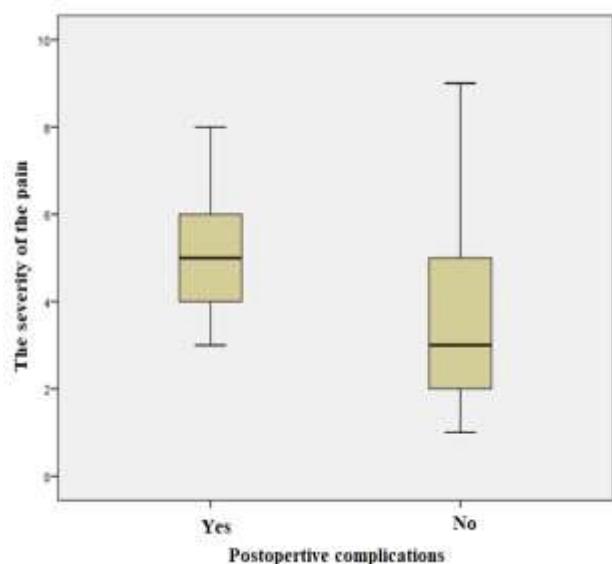


Figure 1. The relationship between the severity of pain and the presence of postoperative complications.

Discussion

In our study, a weak relationship was found between the severity of pain and the presence of postoperative complications. Therefore, at least 10% (r^2) of the pain intensity in the correlation analysis could explain the postoperative complications. In addition, the rate of postoperative complications in the patients included in our study was found to be compatible with the literature [15]. Further, as in our study, Boffa et al. reported an average of 5 days of hospital stay in their extensive study [16].

Thoracic surgery operations are usually performed with one of the classical thoracotomy, minithoracotomy, video-assisted thoracoscopic surgery (VATS) and the latest robot-assisted thoracoscopic surgery (RATS) techniques [1,2,17]. Although classical thoracotomy with a posterolateral incision provides optimal surgical access, it is also a very painful procedure as it involves the division of the latissimus dorsi, serratus anterior, rhomboids, and trapezius muscles. Therefore, thoracotomy intervention requires

good management of both postoperative complications and pain control in thoracic surgery patients. In addition, this painful procedure has been found to be an important risk factor for the development of permanent opioid use [1,2,17].

Pulmonary complications are the most important group affecting morbidity, mortality and long-term hospital stay after thoracic surgery, with an incidence of 30-50% [8]. Complications reported from lungs are prolonged air leak, pneumonia, pulmonary embolism, major atelectasis, adult respiratory distress syndrome, prolonged ventilation and need for tracheostomy [18]. In our study, the most common postoperative complications were prolonged air leakage, atrial fibrillation, tachycardia, hematoma, and hemorrhage. Impaired pulmonary function was the most studied risk factor for lung resection because it is reasonable to assume that a surgeon is the most important factor to consider when weighing the risk of lung resection [19].

Since thoracic surgery operations are one of the most painful surgical procedures, good pain management is crucial for postoperative comfort and increased mortality and morbidity [20]. As we routinely practice in our patients, opioids and NSAIDs are often sufficient to provide optimal pain control in patients undergoing VATS and sternotomy [20]. In addition, performing minimal surgical procedures, epidural analgesia, intercostal block are applications that may be required to provide good analgesia. In a study by Landreneau J et al [21], VATS was applied to 81 patients and thoracotomy to 57 patients. Patients who underwent VATS experienced significantly less postoperative pain and required less post-surgery narcotic analgesia. On the other hand, elderly patients are at a higher risk of postoperative complications, and

the severity of pain in these patients may further increase the complication rate. Therefore, analgesic methods to reduce postoperative complications in this group of patients should be chosen more carefully. In a review about the use of regional analgesia, it was reported that these methods reduce perioperative morbidity, but in addition to the side effects of the drugs used, complications such as epidural hematoma and infection may develop [22]. In our patients, there was a significant relationship between the severity of pain and the presence of postoperative complications. However, the correlation relationship was found to be low. Consequently, for patients scheduled for thoracic surgery, a successful postoperative outcome can be achieved if several conditions are met. These include optimizing patient-related risk factors such as gender, extremely low or extremely high body mass index, nicotine consumption history or pulmonary comorbidities when surgery is indicated, pulmonologist and postoperative intensive care support, choosing the right incision approach, early physical therapy. In addition, the results of our study revealed that optimal postoperative pain control is an important factor in preventing postoperative complications. However, the fact that our study is retrospective is as a limiting factor. Therefore, we believe that more comprehensive new studies should be carried out on this subject.

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Ethical statement: The study protocol was approved by the ethics committee of Bolu Abant Izzet Baysal University Human Research Ethics Committee (2018/298).

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Alginate encapsulation induce colony formation with umbilical cord-derived mesenchymal stem cells

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ABSTRACT

Aim: The umbilical cord (UC) is a rich source of mesenchymal stem cell (MSC) isolation. Since the MSCs isolated from here have high self-renewal capacity and differentiation potential, production through biofabrication is essential for clinical treatments. For the cells to be stored for a long time and presented ready for use, encapsulation is required. In this study, UC-MSC cells were encapsulated with alginate using three different methods: alginate drop, alginate coating, and alginate sphere.

Methods: The cell viability, live/dead cell ratio, and colony formation capacities of the encapsulated cells were examined for 14 days.

Results: In the study, it was found that the most effective method was the alginate sphere form and that the structure of the cells should be preserved by injecting them into biomaterials in encapsulation. Colony formation potential was found to be high in biomaterials with alginate spheres.

Conclusion: As a result, the preservation of UC-MSC cells with alginate sphere encapsulation via biofabrication and their clinical use availability may be beneficial for treating of many diseases.

Keywords: Alginate, hydrogel, mesenchymal stem cell, umbilical cord.

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Introduction

Mesenchymal stem cells (MSCs) are multipotent cell types obtained from a wide variety of tissues such as adipose tissue, bone marrow, dental pulp, placenta, and umbilical cord [1]. MSCs are different from other cells with its features of proliferation, differentiation,

and self-renewal. Although it plays a vital role in development during the embryonic period, they now have the therapeutic potential [2–4]. MSCs have been challenging a role therapeutics in clinical ameliorating from cancer to central nervous system diseases [5,6]. MSCs can inhibit the immune system, increase cell proliferation, induce angiogenesis, or migrate toward damaged tissues. Furthermore, the multifunctional capabilities of MSCs increase their use in both the medical and pharmaceutical industries. In the world, stem cell and cellular therapy companies make

significant investments in obtaining MSCs and keeping them for a long time without disturbing their activity [7]. The umbilical cord (UC) is a non-invasive and ethically trouble-free source of stem cells, as it is isolated from tissue taken at birth. UC contains MSC cells with high availability and high growth capacity of embryonic origin. Compared to cells taken from adult tissues, UC cord cells are a very attractive resource as they have spread faster [8,9].

Isolation processes of MSCs from UC are laborious and higher costly, require a great deal of experience. Isolations can be made differently; the most used methods are enzymatic digestion for separation or explant culture method [10]. After isolation of MSCs, studies are carried out to be stored for a long time and used therapeutically. Biopolymers with high biocompatibility such as alginate, chitosan, agarose, collagen, poly(lactic-co-glycolic acid) (PLGA), poly(ethylene glycol) (PEG), (poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) are used for microencapsulation of MSCs [11]. Alginate is a natural polysaccharide and a biopolymer with high biocompatibility, stability, and non-antigenicity. Alginate shows gelation by crosslinking with calcium ions. These formed hydrogels are used as encapsulation material and can be produced by bio fabrication in various applications. Their use in medicine and pharmacy has been increasing rapidly in recent years due to its non-toxicity and gelling. Microencapsulation of UC-derived MSCs with a highly biocompatible polymer such as alginate after isolation may increase their use in stem cell treatments. New microencapsulation methods should be developed in order for UC-derived MSCs to maintain their rich content and rapid growth capacity without losing their capabilities. In this study, we investigated the

colony formation potential of UC-MSCs in the encapsulation of alginate

Materials and Methods

Cell culture and conditions

Human umbilical cord-derived mesenchymal stem cells (UC-MSC) were provided by ATCC (PCS-500-010). Cells were cultured in DMEM (Dulbecco's Modified Eagle's medium) (Gibco, USA) supplemented with 15% fetal bovine serum (FBS; Gibco), 2 mM L-glutamine, and 100 U/mL penicillin, and 100 µg/mL streptomycin, at 37 °C in a humidified atmosphere containing 5% CO₂. The medium was changed every three days.

Preparation of alginate constructs

Sodium alginate was purchased from Sigma (W201502) and dissolved with sterile PBS (phosphate-buffered saline) solution. Alginate solution was modified prepared different concentration as 0.625%, 1.25% and 2.5% w/v via encapsulation methods [12]. Each solution was passed through 0.45 sterile filters. Sterile calcium chloride solutions (2% w/v) were prepared and used for reducing agent to crosslink the alginate at 25 °C. Calcium and alginate complex was left for 5 minutes to form a hydrogel, and then it was taken into DMEM medium. The alginate encapsulation's rheological behaviors were determined using a rheometer (HAAKE MARS 40 Rheometer, Invitrogen). The viscosity measurements were done at 25°C and calculated by shear rate. Alginate deformation rate was calculated as the change in viscosity over 14 days without cells at 37 °C.

Encapsulation of UC-MSC

Experimental design on encapsulation was included in Figure 1. The encapsulation method

was modified by the amount of alginate and calcium and according to the selected cell [13]. Alginate Drop: Alginate solution (1.25%) and UC-MSC cells (500 cells) were mixed. 10 μ L volume of the mixture was dropped into calcium solution. It was incubated for 5 minutes to crosslink, and after washing with PBS, 100 μ L of cell medium was added into each well of the 96-well plate. Alginate Coating: 50 μ L alginate solution (1.25%) was coated into each well of the 96-well plate, and 50 μ L calcium solution was added to crosslink. After the washing with PBS, UC-MSC cells (500 cells) were seeded with 100 μ L of cell medium was added into each well of the 96-well plate. Alginate Sphere: 10 μ L alginate solution (1.25%) was added into each well of the 96-well plate, and 25 μ L calcium solution was added to the alginate. After the crosslinking, UC-MSC cells (500 cells) were injected into the alginate sphere, and 100 μ L of cell medium was added into each well of the 96-well plate.

Cell viability and live/dead cells

The encapsulated UC-MSC cells were incubated for 7 days and 14 days. At the end of the incubation, alginate encapsulations were dissolved in sodium citrate-EDTA buffer (55 mM Na-citrate, 20 mM EDTA). Each well was incubated with 10 μ L MTT solution (MTT; Vybrant, Invitrogen) for 4 hours at 37°C, 5% CO₂. After incubation, solubilization was done in 100 μ L SDS buffer to formazan precipitates [14]. Color changes were measured at 570 nm using a microplate reader (Epoch, Biotek). Dissolved cells were suspended in PBS and measured in a cell counter (Invitrogen Countess II).

Colony formation

The encapsulated cells were examined morphologically [14] at the end of the 14th day

under an inverted microscope (Zeiss Axiovert, Germany). The areas inside the capsules in the cells were focused. The ratio of colony-forming cells among total cells was calculated in each well.

Statistical analyses

Data were analyzed with GraphPad Prism software (GraphPad Inc., San Diego, CA, USA). All values were presented as mean \pm SD. Between study groups, the obtained data were compared by using a non-paired t-test and two-way ANOVA. Differences were considered statistically significant if the p-value was less than 0.05.

Results

Alginate viscosity was measured in a shear rate range of 1–1000 s⁻¹ by increasing the shear rate every 10 s for 1 min. For 2.5% alginate, the low shear viscosity at 25 °C was found to be 282 mPa s; for 1.25% alginate, the moderate shear viscosity at 25 °C was 365 mPa s; for 0.625% alginate, the high shear viscosity at 25 °C was 687 mPa s (Fig 2a). The effect of shear stress on the viscosity was different for each alginate. In alginate deformation, the deformation rate was significantly higher in the coating of 0.625% alginate on the well surface ($p<0.0001$) while there is no deformation in the other concentration for encapsulation with drop and sphere of alginate (Fig 2b). Cell viability activities of UC-MSC cells were checked on the 7th day, as the adhesion and attachment of stem cells were difficult. A certain number of stem cells were planted in each well, it was observed that the cells maintained their viability in the alginate drop group ($p>0.1350$), but cell viability decreased significantly in the alginate coating group compared to the control ($p<0.0001$). The alginate sphere group

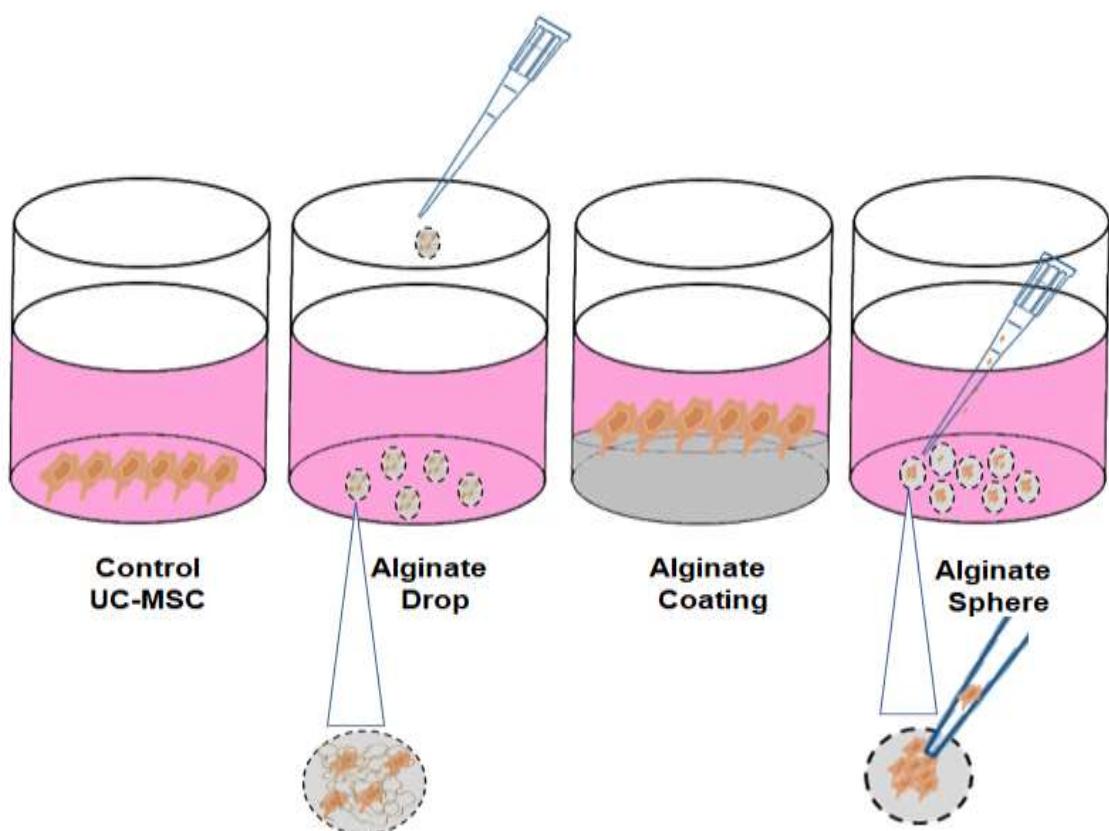


Figure 1. Experimental design for the umbilical cord- mesenchymal stem cells (UC-MSCs).

observed that the cell viability levels increased significantly compared to the control group ($p<0.0098$, Fig 3a). The live and dead cell proportions of the UC-MSC cells after 14 days were evaluated. It was observed that the number of dead cells increased slightly in the alginate drop group compared to the control group ($p<0.0166$), and the change in the number of live cells was not significant ($p>0.0594$). It was observed that the number of dead cells increased significantly in the alginate coating group ($p<0.0001$) and decreased in the alginate coating group of living cells compared to the control group ($p<0.0001$). It was observed that the number of live cells increased significantly in the alginate sphere group compared to the control group ($p<0.0403$), and there was no change in the number of dead cells ($p>0.1708$, Fig 3b).

The colony formation potential of UC-MSC cells after 14 days was evaluated

microscopically (Fig 4). Since cells in the control group tend to adhere to cell culture plates, their colony-forming potential is limited. The colony-forming potential of cells in the alginate drop increased significantly compared to control group cells ($p<0.0002$). When the alginate coating group was compared with the control group, it was observed that there was no change in colony formation potential ($p>0.3780$). The colony-forming potential of UC-MSC cells in the alginate sphere increased significantly compared to control group cells ($p<0.0001$, Fig 4b).

Discussion

MSCs are rich in content, and they interact with other cells through bioactive mediators in their structure, such as hormones, growth factors, cytokines, and extracellular vesicles that exert angiogenic and anti-inflammatory effects. MSCs can show immunosuppressive, anti-

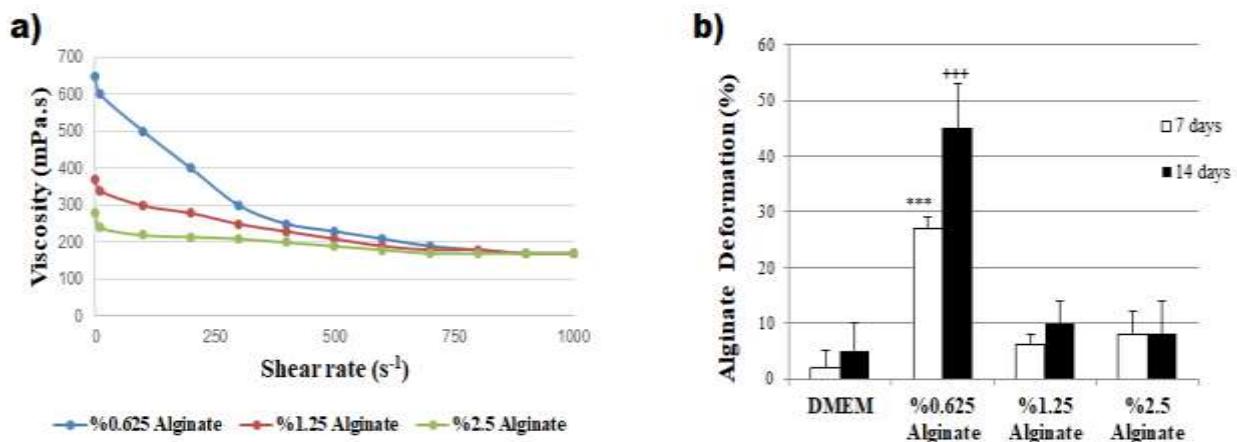


Figure 2. Alginates viscosity and deformation.

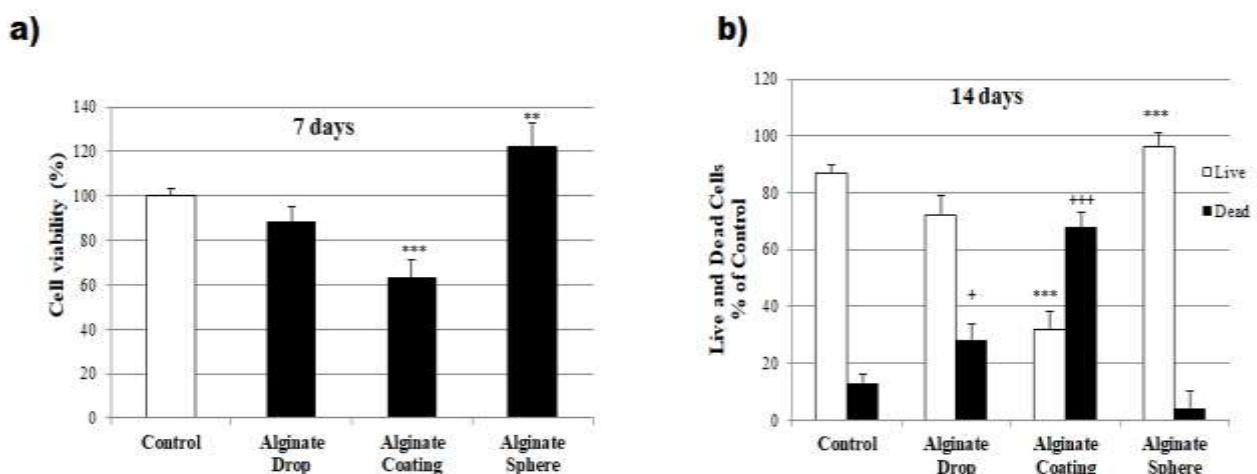


Figure 3. Effects of alginate encapsulation models in cell viability and live/dead cell ratio on UC-MSC.

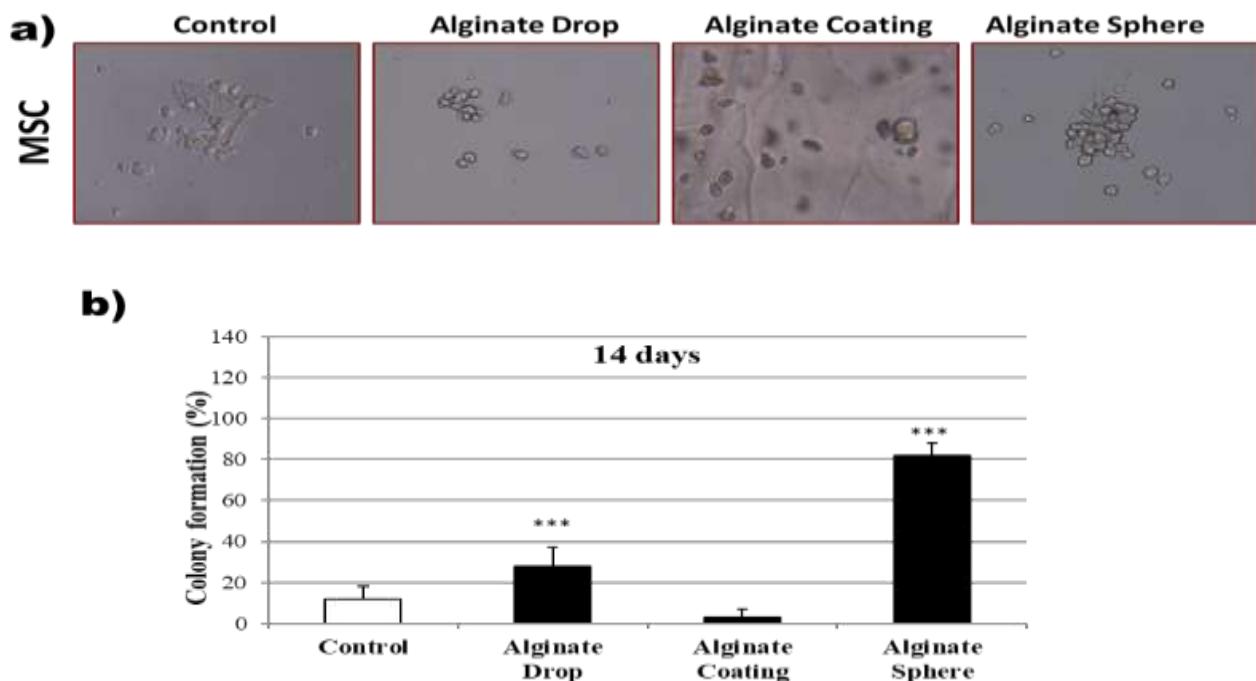


Figure 4. Effects of alginate encapsulation models in cell colony formation on UC-MSC.

apoptotic, anti-fibrotic effects on other cells. To benefit from these potentials MSC cells, are being developed, and new therapeutic dosing studies with encapsulation are carried out in this area for cellular therapy methods [15,16]. For MSC cells to be used therapeutically, there is a need for an encapsulation system that can maintain their viability for a long time. Especially in MSCs, UC-derived cells that are small amount but have the most incredible growth ability and contain important biofactors that are essential. UC-MSCs are isolated from umbilical cord blood or cord tissue. MSC isolation from UC tissue requires a lengthy dissection step, and opening the umbilical cord and manual removal of the vessels before mincing Wharton jelly is time-consuming and increases the risk of contamination. UC-MSCs are essential to be isolated under GMP conditions and stored for long periods [17]. UC-MSCs have significant enhancements, have high proliferation potentials, vast differentiation potentials, and enhanced immune modulation properties [18,19]. For these reasons, UC-MSCs have high therapeutic potential and have an important place in clinical trials worldwide [20,21]. Encapsulation of cells is a technique that enables living cells to be confined with unique biopolymeric materials and use them in the potential treatment of various human diseases [22]. There are many studies on low immunity for cell encapsulation and the development of biomaterials to protect stem cells [23]. It is crucial to develop a method for both the isolation of stem cells and their long-term storage. The properties and shapes of biomaterials during production are essential for clinical applications of cell encapsulation of stem cells [24,25]. Many *in vivo* studies showed that the encapsulation and administration of stem cells produced from different sources (such as bone marrow,

adipose tissue) with alginate [26]. For example, alginate-encapsulated human BM-MSCs have demonstrated a therapeutically curative effect at the infarct site in the rat myocardial infarction model [27]. Another study showed that alginate-encapsulated MSCs remained viable for 30 days and did not lose their therapeutic effect when administered subcutaneously to mice [28]. Similarly, in our study, we found that the alginate sphere group's vitality was high on the 14th day. On the other hand, some suggestions would be beneficial to use a single cell microgel encapsulation approach for MSC. In systemic applications of MSC, it provides diffusion limitations in protecting stem cells against hypoxic effects and facilitating molecules released from the cell [29]. There are still restrictive studies regarding colony formation in stem cells in single-cell encapsulations [30]. Colony formation of mesenchymal stem cells (maybe called spheroidization) exhibits improved therapeutic potential *in vitro*, but if spheroids fail to attach in the environment, they leave control of cell function to the extracellular matrix and potentially limit development time. Using biomaterials is supported by spheroid cell transmission, cell retention and stem cells' functions [31]. In the study using the osteoarthritis model, it was reported that allogeneic rat MSC cells survived longer when encapsulated in alginate and showed metabolic activity for at least eight weeks *in vivo* [32]. In this study, high and low weight alginate and allogenic MSC cells were first mixed in the form of beads and marked with gadolinium for monitoring. It has a similar structure to the drop alginate complexes in our study and is formed with different cells. The production of cell-alginate microcapsules containing MSC cells in a standardized, safe manner must certainly establish the "ready-to-use" potential in clinical

practice [29]. Some researchers, MSC cells isolated from the umbilical cord Wharton jelly were encapsulated with alginate, and differentiation into neuron-like cells was examined. The study showed that alginate could effectively induce neuronal differentiation in a three-dimensional cell culture system by protecting cells [33]. In a different study, MSC cells isolated from Wharton jelly were encapsulated with alginate, and the interleukins, chemokines, growth factors, and soluble forms of adhesion molecules released into the environment were investigated. It has been reported that alginate does not change cells' morphological properties and can provide protein circulation in the general life cycle [34].

Conclusion

For bio fabrication studies, UC-MSC provides dissemination of research on encapsulation. The alginate sphere in our study was created with the logic of microencapsulation. It has been determined that the cells placed in the capsules can form a better colony. When the biodegradable alginate system is opened in the tissues, these colonies' potential to form new cells there will be higher. This method may be more useful for biofabrication studies. For smaller microencapsulation studies, these data will shed light on future studies.

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

Ethical statement: Since it is a cell culture study and commercial line is used, ethics committee permission is not required.

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Inclination and Alsberg angle changes in hip degenerative arthritis

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ABSTRACT

Aim: To compare the inclination and Alsberg angles of both hips in patients with unilateral coxarthrosis and healthy population.

Methods: 89 patients who underwent total hip replacement due to end-stage unilateral idiopathic coxarthrosis between 2016 and 2019 in Bolu Abant Izzet Baysal University Orthopedics and Traumatology Department and had preoperative pelvic anteroposterior radiographs were included in the study. A total of 87 patients with pelvic radiographs taken due to low back pain or minor trauma were included in the study as control group. Patients with bilateral end-stage coxarthrosis, any lower extremity fractures, secondary coxarthrosis caused by any reasons like Perthes disease, trauma, avascular necrosis, hematologic disease etc. and unsuitable radiographs were excluded from the study. Two researchers took the measurements separately and the averages were considered and the results were analyzed statistically.

Results: Statistically significant differences were detected between the right side and the left side in terms of both Alsberg and hip inclination values in individuals of healthy control group. In patients with unilateral osteoarthritis, no significant differences were detected between right and left sides in terms of hip inclination values. Similarly, no significant difference was found between the right and left sides in terms of Alsberg values in patients with unilateral osteoarthritis. The Alsberg angle was found to be higher at statistically significant levels in patients with unilateral hip osteoarthritis, in both the osteoarthritis side and the contralateral hip, compared to the values in healthy individuals.

Conclusion: When compared with healthy individuals, it was concluded that the Alsberg and inclination angles changed with the development of unilateral coxarthrosis. While significant differences were detected between the sides for Alsberg and inclination angle in healthy individuals, this difference between the sides was not observed in the coxarthrosis groups. With the development of unilateral coxarthrosis, it was concluded that the asymmetry between the right and left sides of healthy individuals disappeared and a more symmetrical structure emerged in terms of Alsberg and hip inclination angles.

Keywords: Hip inclination angle, neck – shaft angle, Alsberg angle, osteoarthritis.

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Introduction

Osteoarthritis is a chronic degenerative disease of synovial joints [1] and characterized by deterioration of the articular cartilage, formation of subchondral sclerosis, osteophytes and subchondral cysts in the adjacent bones of joint. Also biochemical and morphological changes are seen in the synovial membrane and

joint capsule [2,3]. It is the most common disease of joints and its frequency increases with age [4,5]. There are many factors responsible in the etiology of osteoarthritis. Especially in hip osteoarthritis, genetic, biomechanical and metabolic factors contribute the destruction of cartilage. Secondary coxarthrosis causes such as trauma, perthes disease, rheumatoid arthritis are common as idiopathic causes [6].

Plain radiographs of the hip are inexpensive, widely available and readily available. Therefore, evaluation of osteoarthritis is not as difficult as other imaging modalities. Anteroposterior (AP) and lateral radiographs of the hip are obtained to examine hip osteoarthritis. As the disease progresses, the joint space narrows and radiological changes occur [7,8]. In the last stage, the anatomy of the femoral head and acetabulum changes. This causes other problems in adjacent joints and spine. The inclination angle (IA) and Alsberg angle (AA) is a decisive factor in determining the strength and stability of the femur [9]. Therefore, we think that degenerative changes in the hip will cause changes in both angles, which will lead to pathologies in neighboring joints.

The purpose of the present study was to compare the IA and AA of both hips in patients who had unilateral coxarthrosis and healthy population.

Materials and Methods

After getting the approval from the Ethics committee of Bolu Abant Izzet Baysal University (2020/79), 89 patients who underwent total hip replacement due to end-stage unilateral idiopathic coxarthrosis between 2016 and 2019 in Bolu Abant Izzet Baysal University Orthopedics and Traumatology Department and had preoperative pelvic AP

radiographs were included in the study. A total of 87 patients with pelvic radiographs taken due to low back pain or minor trauma were included as the control group in the study. Patients who had bilateral end-stage coxarthrosis, any lower extremity fractures, secondary coxarthrosis caused by any reasons like Perthes disease, trauma, avascular necrosis, hematologic disease etc. and unsuitable radiographs were excluded.

Age and gender distributions of the participants were evaluated. Two researchers took the measurements separately and the averages were considered and the results were analyzed statistically.

Radiological evaluation

The IA of hip was calculated by measuring the angle between the line parallel to the femoral head-neck and the line parallel to the femur shaft on pelvic AP radiographs (Figure 1A). The AA was calculated by measuring the the angle between the axis of the femur shaft and the base of epiphyseal plate of femoral head (Figure 1B) [9].

Statistical Analysis

Numerical variables were described with arithmetic mean and standard deviation or median and minimum-maximum values and count (percentage) values were used to summarize categorical variables. Normality of data was assessed using both graphical (histogram, Q-Q plot, etc.) and analytical (Kolmogorov - Smirnov test) approaches. Comparison of numerical variables between groups were made with Student's t test or Mann-Whitney U test for two independent groups, and one-way ANOVA and Kruskal-Wallis test for three or more independent groups according to normality of data. Finally, a post-hoc test was performed to explore

pairwise differences. Dependent groups were compared with paired t-test or Wilcoxon paired test. Statistical Analyses were made with IBM SPSS v.21. Statistical significance was taken as $p<0.05$.

The mean IA value of the left coxarthrosis group is 132.08° for right side and 135.25° for left side. No significant differences were detected between left and right sides for mean IA values of left coxarthrosis group ($p=0.052$).

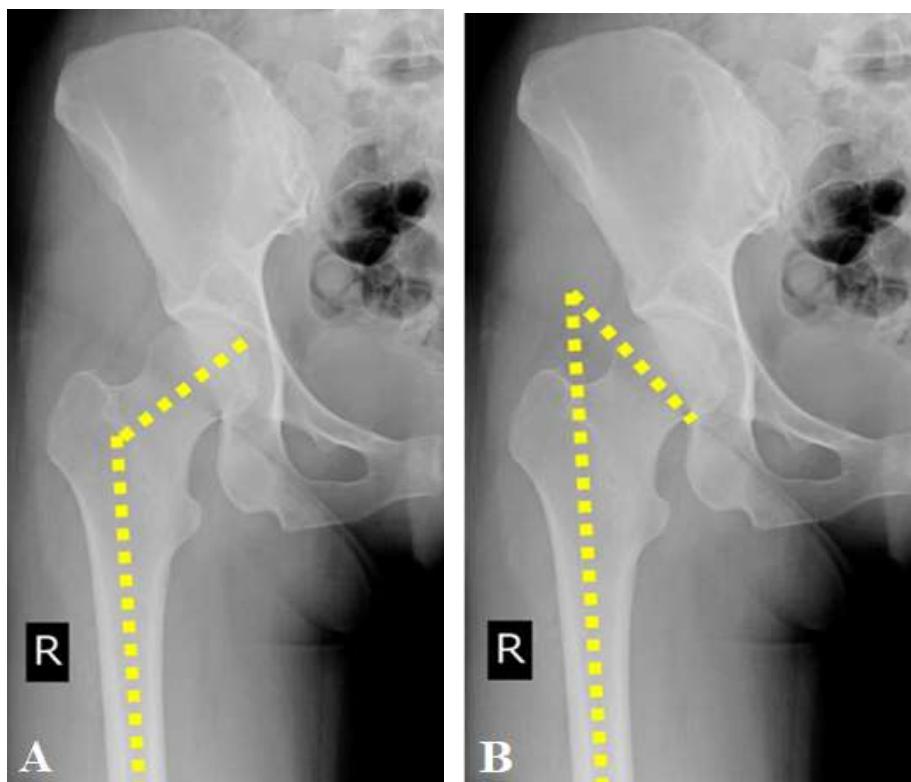


Figure 1. **A)** Inclination angle, the angle between the yellow line parallel to the femoral head-neck and the yellow line parallel to the femur shaft. **B)** The Alsberg angle was calculated by measuring the the angle between the axis of the femur shaft and the base of epiphyseal plate of femoral head.

Results

A total of 176 patients who had a mean age of 69 (ranging from 60 to 81; Standard Deviation: 5.22) were reached. 89 (50.6%) of the patients were females and 87 (49.4%) were males (Table 1).

The mean IA value of the control group is 134.1° for right side and 130.7° for left side. Right IA values of the control group were higher at statistical levels ($p=0.007$). The mean AA value of the control group was 39.5° for right side and 41.9° for left side. Left AA values of the control group were higher at statistical levels ($p=0.022$) (Table 2).

Table 1. Group, gender and age distribution of 176 patients.

| Variables | N (%) |
|--------------------|-------------------|
| Groups | |
| Left coxarthrosis | 44 (25.0%) |
| Right coxarthrosis | 45 (25.6%) |
| Control | 87 (49.4%) |
| Gender | |
| Female | 89 (50.6%) |
| Male | 87 (49.4%) |
| Age (year) | 69.0±5.22 (60-81) |

The mean AA value of left coxarthrosis group is 44.97° for right side and 48.35° for left side. Left AA values of left coxarthrosis group were higher at statistical levels ($p=0.024$) (Table 2). The mean IA value of the group with right coxarthrosis is 130.2° for right side and 128.7° for left side. The mean IA values of the right coxarthrosis group were not significantly different between the right and left sides ($p=0.804$). The average AA value of the group with right coxarthrosis is 50.9° for the right side and 49.6° for the left side. No significant differences were detected between right and left sides for mean AA values of right coxarthrosis group ($p=0.915$) (Table 2).

Significant differences were detected between right coxarthrosis group and control group in terms of mean right IA values ($p=0.004$). Significant differences were also detected

between left coxarthrosis group and control group for mean left IA values ($p<0.001$) (Table 2).

Significant differences were detected between control group and other groups in terms of mean right AA values. Mean right AA of control group was lower than right and left coxarthrosis groups ($p=0.043$). And significant differences were also detected between control group and other groups for mean left AA values. Mean left AA of control group was lower than right and left coxarthrosis groups ($p<0.001$) (Table 2).

No significant differences were detected between operated side and healthy side for IA values ($p=0.238$). No significant differences were detected between operated side and healthy side for AA values ($p = 0.090$) (Table 3).

Table 2. Comparison of the angle measurements by the groups and sides.

| Variables | Inclination angle Median (min.-max.) | | p | Alsberg angle Median (min.-max.) | | p |
|---------------------------|---|---------------------------------------|--------------|---|------------------------------------|--------------|
| | Right | Left | | Right | Left | |
| Left Coxarthrosis | 132.08 ^{a,b} (119.5-148.9) | 135.25 ^a (77-159.9) | 0.052 | 44.97 ^a (31.2-57.1) | 48.35 ^a (13.63-67.3) | 0.024 |
| Right Coxarthrosis | 130.2 ^a (112.3-145.1) | 128.7 ^b (87.2-159) | 0.804 | 50.9 ^a (36-64) | 49.6 ^a (25.8-64.7) | 0.915 |
| Control | 134.1 ^b (115.4-154.1) | 130.7 ^{a,b} (109.6-162.4) | 0.007 | 39.5 ^b (26-58.2) | 41.9 ^b (22.8-55.0) | 0.022 |
| p | 0.004 | <0.001 | | 0.043 | <0.001 | |

According to the post hoc test results, the different groups are shown with different letters (a, b), non-different groups are shown with the same letters (a or b).

Table 3. Comparison of the angle values according to the operation status.

| Variables | Inclination angle Median (min.-max.) | p | Alsberg angle Median (min.-max.) | | p |
|----------------------|---|----------|---|---------------------|--------------|
| | | | Operated side | Healthy side | |
| Operated side | 131.1 (77-159.9) | 0.238 | 49.3 (13.63-67.3) | 46.2 (25.8-65.7) | 0.090 |
| Healthy side | 130.4 (87.2-159) | | | | |

In females, no significant differences were detected in IA values between operated side and healthy side ($p=0.446$). No significant differences were detected in females between operated side and healthy side regarding AA values ($p=0.102$) (Table 4).

No significant differences were detected in IA values between operated side and healthy side in males ($p=0.394$). No significant differences were detected in males between operated side and healthy side regarding AA values ($p=0.473$) (Table 4).

Table 4. Comparison of the angle values according to the operation status in terms of gender.

| Gender | Inclination angle Median (min.-max.) | | <i>p</i> | Alsberg angle Median (min.-max.) | | <i>p</i> |
|--------|---|------------------------|-----------------------|-------------------------------------|----------------------|---------------------|
| | Operated | Healthy | | Operated | Healthy | |
| | Female | 130.2 (112.3-159.9) | 129.9 (87.2-150.7) | 0.446 | 49.7 (32.3-67.3) | 45.5 (25.8-65.0) |
| Male | 131.79 (77-157.14) | 133.05 (96.2-159) | 0.394 | 49.1 (13.63-64.0) | 46.65 (31.2-65.7) | 0.473 |

Table 5. Comparison of the angle measurements according to gender in terms of the patient groups.

| Variables | Inclination angle Mean ± SD | | <i>p</i> | Alsberg angle Mean ± SD | | <i>p</i> |
|-------------------------------|--------------------------------|----------------------------|----------|----------------------------|---------------------------|--------------|
| | Right | Left | | Right | Left | |
| Female | | | | | | |
| Left Coxarthrosis | 131.43±7.47 | 136.06 ^a ±10.57 | 0.055 | 45.86 ^a ±5.94 | 50.68 ^a ±9.26 | 0.006 |
| Right Coxarthrosis | 127.79±8.32 | 128.32 ^b ±12.19 | 0.879 | 47.52 ^a ±6.42 | 47.90 ^a ±10.06 | 0.868 |
| Control | 131.86±6.33 | 129.04 ^b ±7.44 | 0.034 | 39.94 ^b ±5.19 | 40.76 ^b ±5.20 | 0.354 |
| <i>p</i> | 0.082 | 0.011 | | <0.001 | <0.001 | |
| Male | | | | | | |
| Left Coxarthrosis | 132.73 ^{a,b} ±7.09 | 131.74±18.75 | 0.820 | 44.2 ^a ±7.31 | 42.99 ^a ±12.62 | 0.707 |
| Right Coxarthrosis | 130.9 ^a ±6.32 | 130.57±14.64 | 0.915 | 50.73 ^b ±6.92 | 50.15 ^b ±9.43 | 0.731 |
| Control | 136.71 ^b ±7.80 | 135.20±8.64 | 0.178 | 40.45 ^a ±6.38 | 42.61 ^a ±6.03 | 0.020 |
| <i>p</i> | 0.009 | 0.358 | | <0.001 | 0.005 | |

According to the post hoc test results, the different groups are shown with different letters (a, b), non-different groups are shown with the same letters (a or b).

In females, statistically significant differences were detected between right and left sides according to IA values of the control group ($p=0.034$). Right IA values of the control group are higher. In females, no statistically significant differences were detected between the right and left sides of the control group according to the AA values ($p=0.354$) (Table 5). In females, no statistically significant differences were detected between right and left sides of the left coxarthrosis group according to the IA values ($p=0.055$). Statistically significant differences were detected between left and right sides of left coxarthrosis group in females according to the AA values ($p=0.006$). The healthy side values are lower (Table 5). In females, no statistically significant differences were detected between right and left sides according to IA values of right coxarthrosis group ($p=0.879$). In females, no statistically significant differences were detected between right and left sides of right coxarthrosis group according to the AA values ($p=0.868$) (Table 5). In males, no statistically significant differences were detected between right and left sides of left coxarthrosis group according to the IA values ($p=0.820$). There were no statistically significant differences between left and right sides of left coxarthrosis group in males according to the AA values ($p=0.707$) (Table 5). In men, there were no statistically significant differences between right and left sides of right coxarthrosis group according to the IA values ($p=0.915$). There were no statistically significant differences between right and left sides of right coxarthrosis group in males according to the AA values ($p=0.731$) (Table 5).

Discussion

When the literature is reviewed, it is seen that hip IA and AA values differ according to age,

gender and races. In this study the distribution of hip IA and AA in the elder and healthy population was determined since patients without complaints in the hip joint were selected as the control group. In the control group, the mean value of IA was higher in the right hip, while the mean value of the AA was higher in the left hip. In an anatomy study on a younger population (aged 30 to 40) by Oguz et al, [9] the mean IA was measured as 123.7° and 125.9° in the measurements made on 25 right and 25 left femurs, respectively. In the same study, right and left average AA were measured as 39.92° and 40.61° , respectively. There were no statistically significant differences for IA and AA for right and left femur [9]. In our study, when right and left side values of control group were compared, there was an asymmetric condition in terms of both IA and AA. This study with more patients will provide the literature on the values in the elder and healthy population as well. R Sherestha et al., in their study on Nepalese people, classified 148 healthy individuals according to age (21-40, 41-60 and over 60 years) and gender (male - female) and measured the right and left hip IA. They reported the mean of right and left hip IA values as 132.47° and 128.84° , respectively, in individuals over 60 years of age, they reported that the mean IA of the right hip was higher than the left, but there were statistically significant differences [10]. Similarly, in our study, the mean right hip IA value was higher in the control group compared to the left side, but this height was found to be significant on the right side in our study. In the same study, they determined the mean right and left hip IA as 134° and 132.98° , respectively, and they revealed that there were statistically significant differences. They found the mean right and left hip IA in males as 132.96° and 131.54° , respectively, and reported no significant

differences between the two sides. Likewise, in our study, the mean right and left IA values in the measurements performed in females in the control group were found to be 131.86° and 129.04° , respectively, and the right side value was found to be significantly higher than the left side. In the measurements performed in the males in the control group in our study, the mean right and left IA values were found to be 136.71° and 135.20° , respectively, and no statistically significant differences were detected. Our study supports the literature in the light of these data. Tahir A et al., in their study on natives living in the north-east sub-region of Nigeria, reported the mean hip IA values of 200 males and 120 females as 136.7° and 126.65° , respectively, and they reported that the average hip IA in males living in this region was statistically significantly higher than in females [11]. In our study, statistically significant differences were found between genders in terms of hip IA values. However, this value was higher for males on the right side, while it was higher for females on the left side. These similarities and differences seen in studies on hip IA reveal the importance and effect of age range, race and gender of the studied population on these values.

Another important finding is the angle changes in the degenerated hip and healthy hip when unilateral primary coxarthrosis develops. Laforgia R et al. evaluated radiographic variables on normal and osteoarthritic hips and reported that deterioration in hip IA and other variables correlated directly with the development of hip osteoarthritis [12]. Mills HJ et al. examined the relationship between proximal femoral anatomy and coxarthrosis and reported that the hip IA was significantly higher in hips with coxarthrosis [13]. Doherty M et al. stated that the nonspherical femoral head and high IA seen in pistol grip deformity may be the

result of osteoarthritis [14]. In contrast to these studies, Reikeras and Hoiseth reported that there was no significant difference in hip IA between normal hips and hips with osteoarthritis when they compared hip IA of 44 patients with unilateral or bilateral hip idiopathic osteoarthritis with normal values [15]. Our study supports the articles reporting that hip IA have changed with the development of osteoarthritis. We demonstrated that the hip IA was changed as well as the AA. When we look at the mean angle values of right and left sides in control group in our study, we concluded that the IA values were significantly higher on right side, and AA values were higher at significant levels on left side. This situation reveals that there is an asymmetry between the sides in terms of hip IA and AA values in individuals over 60 years of age. With the development of osteoarthritis, we reached the result that this asymmetry disappeared in terms of both AA and IA values, and that the significant difference between the right and left hips disappeared with the development of osteoarthritis. When Labronici et al. compared the contralateral hip angles with osteoarthritis and healthy ones, they reported that there was no significant difference between hip IA [16]. In this study, our results overlap with this study. When we compared the mean IA value of the right hip of the coxarthrosis group and the mean right hip IA value of the control group, we concluded that the value of the control group was significantly higher. However, when we compared the mean left hip IA value of the coxarthrosis group with the left value of the control group, we concluded that the mean IA value of the coxarthrosis group was higher this time. Based on this, it is not possible to say that the IA value generally increases or decreases with the development of osteoarthritis, according to our results. The more striking

result of our study is that, with the development of osteoarthritis, the IA and AA values approach each other with the opposite hip and these angles become more symmetrical. In addition, the AA contributes to the symmetry that occurs with the development of idiopathic osteoarthritis by increasing in both the osteoarthritis developing side and the contralateral hip.

Conclusion

In the present study, statistically significant differences were detected between right side and left side for both AA and hip IA values in individuals over 60 years of age with radiographically and clinically bilateral healthy hip joints. These angles change with the development of osteoarthritis. In patients with unilateral osteoarthritis, no significant differences were detected between right and left sides regarding hip IA values. Similarly, no significant differences were detected between the right and left sides regarding AA values in patients with unilateral osteoarthritis. It was concluded that the angular asymmetry observed in healthy individuals disappeared with the development of osteoarthritis, and the sides took on a more symmetrical structure. The AA was higher at statistically significant levels in patients with unilateral hip osteoarthritis, in both the osteoarthritis side and the contralateral hip, compared to the values in healthy individuals.

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

Ethical statement: The study was approved by the Ethics committee of Bolu Abant Izzet Baysal University (Date and decision no: 2020/79).

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Investigation of the effect of quercetin in an experimental oxygen-induced retinopathy model

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ABSTRACT

Aim: To investigate of the effect of intraperitoneal (IP) quercetin and bevacizumab on oxygen-induced retinopathy (OIR) model in rats.

Methods: In the study, 28 newborn rats were used. The OIR model was performed with the 50/10% oxygen technique. The study consisted of four groups as a control group (Group I) and OIR groups (Group II, III, and IV). IP injection applied to all groups on the postnatal day (PND) 14. Groups I and II were performed 0.9% NaCl, Group III was performed IP bevacizumab, and Group IV was performed IP quercetin. All animals were sacrificed on PND 18.

Results: Based on the data obtained from immunohistochemical and histopathological examinations, the number of vascular endothelial cell (VEC), vascular endothelial growth factor (VEGF), and tumor necrosis factor- α (TNF- α) levels were significantly reduced in Group III and IV compared to Group II. VECs levels were 0 ± 0 , 32.69 ± 5.77 , 2.92 ± 0.63 , and 3.64 ± 0.36 in Group I, Group II, Group III, and Group IV, respectively ($p<0.001$). Likewise, VEGF values were 0.15 ± 0.01 , 7.57 ± 1.80 , 2.45 ± 0.45 , and 2.46 ± 0.49 , respectively ($p<0.001$). As well as TNF- α values were 0.06 ± 0.01 , 8.22 ± 2.24 , 2.32 ± 0.32 , and 2.29 ± 0.26 in Group I, Group II, Group III, and Group IV, respectively ($p<0.001$). There was no significant difference between Group III and Group IV in terms of VEC, VEGF and TNF- α values (range of p values was 0.96-1.00).

Conclusion: The results of the present study showed that quercetin administration significantly reduced the VEC number and suppressed VEGF and TNF- α . Quercetin's anti-inflammatory and anti-angiogenesis effect was found to be similar to bevacizumab.

Keywords: Oxygen-induced retinopathy, neovascularization, bevacizumab, quercetin, VEGF, TNF- α , rats.

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Introduction

Retinopathy of prematurity (ROP) can be mimicked in animals using the oxygen-induced retinopathy (OIR) model. With this model, the vaso-obliterative and neovascularization (NV)

phases of ROP can be simulated with high reproducibility. Therefore, the treatment outcome of anti-angiogenic drugs can be assessed efficiently [1].

Diabetic retinopathy (DRP), ROP, and age-related macular degeneration (AMD) are diseases that are seen as common in ophthalmology clinics that may result in vision loss and are associated with retinal angiogenesis and choroidal angiogenesis [2,3]. Pro- and anti-angiogenic factors are released in

response to hypoxia that occurred in choroid and retina. There are many reasons that trigger the neovascularization (NV) in the eye, but one of the most well-known reasons is vascular endothelial growth factor (VEGF) [4]. Stimulation of VEGF receptor 2 (VEGFR2) via VEGF creates various intracellular signaling in vascular endothelial cells (VECs), including proliferation, migration, morphogenesis and increased permeability [5]. Likewise, another potent angiogenic molecule is tumor necrosis factor- α (TNF- α) that controls the genes encoding adhesion molecules and angiogenic mediators [6].

As a monoclonal antibody, bevacizumab inhibits VEGF, which is an angiogenic cytokine. It prevents pathological angiogenesis through promoting vascular leakage and growth [7]. It has been documented that off-label use of bevacizumab is effective in the treatment of many intraocular vascular proliferation retinal diseases associated with ischaemic retinopathy, such as DRP, ROP, and retinal vein occlusion (RVO) [8,9].

Quercetin (3,3', 4',5,7-penthydroxy flavone) belongs to the polyphenol family, which is one of the most abundant flavonoid in the diet, and is present in many fruits and vegetables, including onions, apples and grapes [10]. Previous in vivo and in vitro studies have shown that quercetin decreases oxidative stress, retinal neurodegeneration, and NV effectively [11,12]. Moreover, VEGF-induced cell proliferation, migration and tube formation was inhibited by quercetin [13]. Likewise, quercetin-treated retinas showed significantly lower levels of TNF- α and other pro-inflammatory cytokines [14].

The impact of quercetin on many organs, including the eye has been extensively studied. However, less is known about the impacts of intraperitoneal (IP) administration of quercetin

on anti-VEGF factors using OIR models. The objective of the present study was therefore to investigate the effect of quercetin on VEGF, TNF- α , and VEC using OIR models and comparing the results with those of bevacizumab.

Materials and methods

This study was conducted in compliance with the recommendations of the ARVO Statement for Animal Use in Ophthalmic and Vision Studies. The research was also accepted by the Bolu Abant Izzet Baysal University Experimental Animal Studies Ethics Committee (Date / decision no: 2018/35).

Creating the OIR Model

In the present study, 28 newborn Sprague Dawley rats were used. All animals were kept in a controlled room with 22-24 °C temperature and 45-60 % relative humidity with 12h light: 12h dark conditions. The food and water were provided *ad libitum*. In the 50/10 OIR model, lactating pups were placed in an oxygen regulated environment with their mothers within 4 h following birth, where they were exposed to 50 % oxygen for 24 h followed by 10% oxygen for 24 h [15]. This cycle was repeated until postnatal day (PND) 14 seven times. Daily monitoring of oxygen levels was performed and was calibrated as needed. Likewise, daily carbon dioxide levels in the cage were also monitored and sufficient gas-flow was maintained through flushing it from the system and adding soda lime. Pups were put into a room with ambient air for 4 days on PND 14.

The experiment was performed with four groups with seven animals per group. The injections were administered intraperitoneally to all animals only once on day 14 of the creation of the OIR model.

Group I: The healthy group administered 0.01 ml ip 0.9% NaCl solution (control group). Group II: OIR group administered 0.01 ml ip 0.9 % NaCl solution (Untreated OIR group). Group III: OIR group treated with 0.01 ml ip bevacizumab (2.5 mg/kg body weight) (Altuzan, Roche, Istanbul, Turkey). Group IV: OIR group treated with 0.01 ml ip quercetin (20 mg/kg body weight) (Sigma-Aldrich, St.Louis, USA) [16]. It is assumed that the OIR pattern is best formed PNDs 18-20. Therefore, on PND 18, all animals were sacrificed with high dose intracardiac anesthesia, and the right eyes were enucleated [17]. The tissues were stored for further histopathological and immunohistochemical analyses.

Haematoxylin and eosin (H&E) staining

The eyes were set in 10 percent neutral buffered formalin in PBS overnight at 4 °C and then embedded in paraffin. The serial sections (4 µm thick) of the whole eye were performed sagittal that was parallel to the optic nerve and stained with H&E. The nuclei of retinal VECs on the vitreal side of the retinal inner limiting membrane (ILM) were counted by an objective observer blind to treatment in ten parts for each eye at 400x magnification and the mean number of nuclei of endothelial cells for each eye was determined for each community [18].

Immunohistochemistry staining

A biotin-streptavidin HRP detection kit (ab93697; Abcam, Cambridge, UK) was used to perform immunohistochemistry. Antigen retrieval was performed with citrate buffer. 3 % H₂O₂ in methanol was used for blocking endogenous peroxidases for 15 min. In order to eliminate non-specific binding, a blocking serum was used for the pretreatment of the sections. Then, the sections were incubated overnight with the following antibodies: anti-

TNF alpha polyclonal antibody (1:250 dilution; ab183896; Abcam) and anti-VEGF monoclonal antibody (1:50 dilution; sc-7269; Santa, Santa Cruz, CA, USA) at 4°C. An appropriate non-immune immunoglobulin G was used as a primary antibody replacement to perform negative control incubations. Then, biotinylated secondary antibodies were used to incubate the tissues. The peroxide complex was visualized using 3,3-diaminobenzidine. Finally, Mayer's hematoxylin (Invitrogen, California, USA) was used to counterstain the tissues, and then they were mounted with Entellan (Merck, Darmstadt, Germany) on glass slides. The images were observed under a light microscope (Leica DM 1000, Germany) and photos were taken using Leica DMC 2900 (CH-9435 Heerbrugg, Germany).

The following scales were used to grade of section: no expression (0), mild (1), moderate (2), strong (3), and very strong expression (4) by two independent observers blind to treatment. The percentage of positive cells was described as (0), < 5%; (1), 6% to 15%; (2), 16% to 50%; (3), 51% to 80%; and (4), > 80% of positive cells [19]. The mean value of the three retinal sections of each rat was determined.

Statistical analyses

In the present study, the data were analyzed using SPSS statistical software package, version 25.0 (SPSS Inc., Chicago, IL, USA). The data were reported as mean ± standard deviations (SDs) for each data set. A statistical significance was considered if $p<0.05$. The homogeneity and normality of the sample distribution of data were determined using Levene's test and Kolmogorov Smirnov test, respectively. The statistical analyses of the data was performed with one-way analysis of variance test and post hoc Tukey's test.

Results

Representative hematoxylin and eosin-stained retinas of all groups are presented in Figure 1. In the untreated OIR group (Group II), abundant longitudinal and transverse aberrant microvessels were noted in ILM. The nuclei of VECs were counted to quantify NV. Bevacizumab (Group III) and Quercetin (Group IV) administered groups showed a reduced number of VECs breaching ILM as well as improvement of vascular tufts and dilated vessels. In Group I (control group), there were no VEC nuclei detected on the vitreal side of the ILM of the retina (Figure 1).

However, the retinal VEC nucleus number was significantly higher in Group II compared with Group III and Group IV (Figure 1). On the other hand, VEC nucleus number were similar between Group III and Group IV ($p=0.960$; Figure 1).

VEGF and TNF- α levels in the control and OIR groups were determined through immunohistochemistry (Table 1). VEGF-positive staining was apparent in the retinas of OIR rats, especially in the GCL and INL layers (Figure 2). The intensity of the VEGF staining was reduced in Group III and Group IV. Semi-

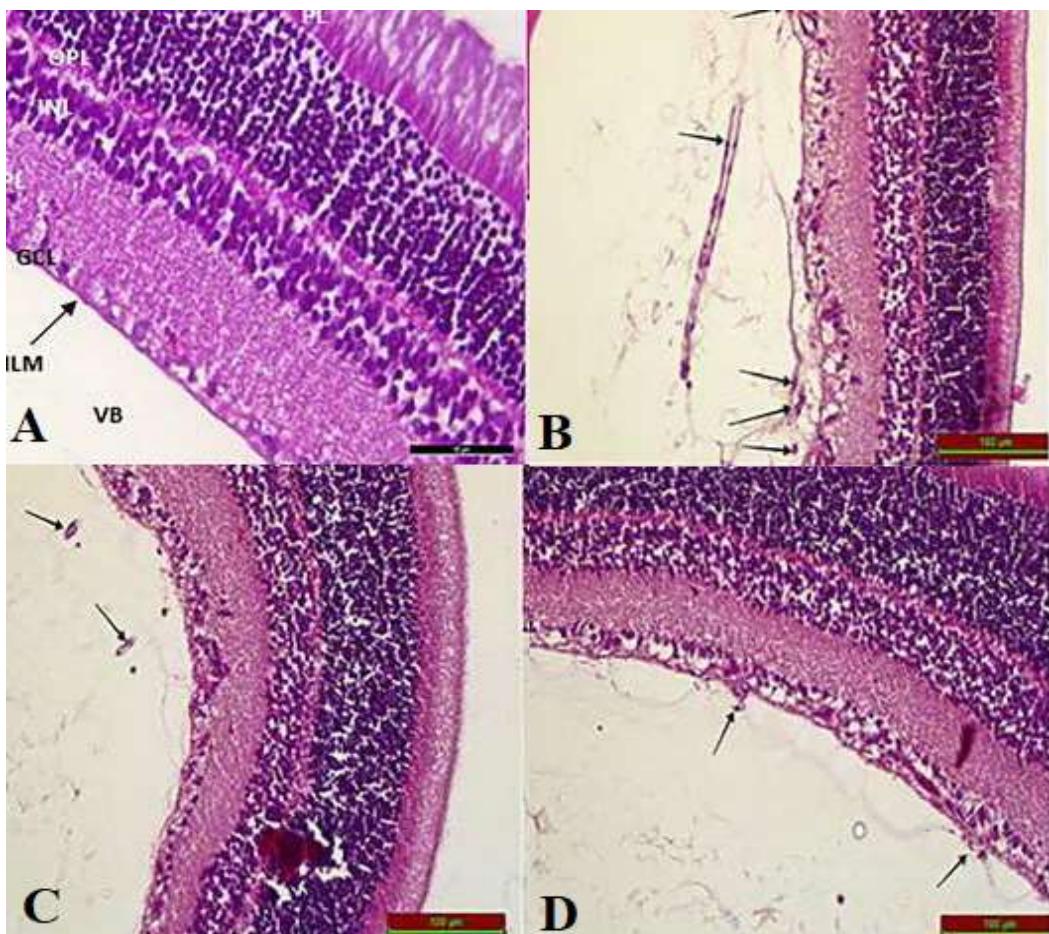


Figure 1. Haematoxylin and Eosin staining of neovascularization in retinal tissues. **A:** Control group (Group I). **B:** Untreated OIR group (Group II). **C:** OIR group treated with intraperitoneal bevacizumab (Group III). **D:** OIR group treated with intraperitoneal quercetin (Group IV). Quantitative analysis was performed by counting the nuclei of retinal vascular endothelial cells (arrows) on the vitreal side of the inner limiting membrane in 10 sections from each eye. GCL: Ganglion cell layer, ILM: Inner limiting membrane, INL: Inner nuclear layer, IPL: Inner plexiform layer, OLM: Outer limiting membrane, ONL: Outer nuclear layer, OPL: Outer plexiform layer, PL: Photoreceptor layer, VB: Vitreous body.

Table 1. The results of H&E and immunohistochemical staining in the OIR model.

| Variables | Group I | Group II | Group III | Group IV | P* |
|-------------------|-------------|--------------|-------------|-------------|--------|
| VEC nuclei | 0 | 32.69 ± 5.77 | 2.92 ± 0.63 | 3.64 ± 0.36 | <0.001 |
| TNF-α | 0.06 ± 0.01 | 8.22 ± 2.24 | 2.32 ± 0.32 | 2.29 ± 0.26 | <0.001 |
| VEGF | 0.15 ± 0.01 | 7.57 ± 1.80 | 2.45 ± 0.45 | 2.46 ± 0.49 | <0.001 |

Values: mean ±SD. H&E: Hematoxylin & eosin. OIR: oxygen-induced retinopathy.

*One-way analysis of variance test

Group I: 0.01 ml intraperitoneal saline solution (0.9% NaCl) was administered without creating an OIR model (control group).

Group II: 0.01 ml intraperitoneal saline solution was administered following the creation of the OIR model (Untreated OIR group).

Group III: 0.01 ml intraperitoneal bevacizumab (2.5 mg/kg body weight) (Altuzan, Roche, Istanbul, Turkey) was administered in the OIR group.

Group IV: 0.01 ml intraperitoneal quercetin (20 mg/kg body weight) (Sigma-Aldrich, St.Louis, USA) was administered in the OIR group.

TNF-α: Tumor necrosis factor-α, VEC: Vascular endothelial cell, VEGF: Vascular endothelial growth factor.

quantification of VEGF immune-intensity demonstrated that VEGF staining was significantly decreased in Group III and Group IV compared with Group II ($p<0.001$ per group compared). Moreover, quercetin treatment reversed the oxygen-induced VEGF elevation that was compatible with bevacizumab.

Semi-quantification of TNF-α immune-intensity demonstrated that TNF-α staining was significantly decreased in Group III and Group IV compared with Group II ($p<0.001$ per group compared). Quercetin treatment reversed the oxygen-induced TNF-α elevation (Figure 3).

Retinal VEGF and TNF-α expression were significantly increased in Group II compared to Group I, III, and IV (Table 1 and Figure 2). On the other hand, levels of VECs, VEGF, and TNF-α were significantly decreased in Group III and Group IV compared to Group II ($p<0.001$; Table 1).

Post hoc test results of VECs, VEGF and TNF-α in Group II were significantly higher compared to Group I, III, and IV ($p<0.001$ per group compared). While the nucleus number of VECs was similar between Group I and III, VEGF and TNF-α levels were found to be significantly reduced in Group III ($p=0.30$, $p=0.001$, and $p=0.008$, respectively). Likewise, the nucleus number of VEC was similar between Group I and IV ($p=0.14$). In addition, VEGF and TNF levels were significantly reduced in Group IV ($p=0.001$ and $p=0.009$, respectively). The nucleus number of VEC, as well as VEGF and TNF levels, were similar between Group III and IV ($p=0.96$, $p=1.000$, and $p=1.000$, respectively).

Discussion

Retinal NVs are the primary reasons for vision loss in patients, including neovascular AMD,

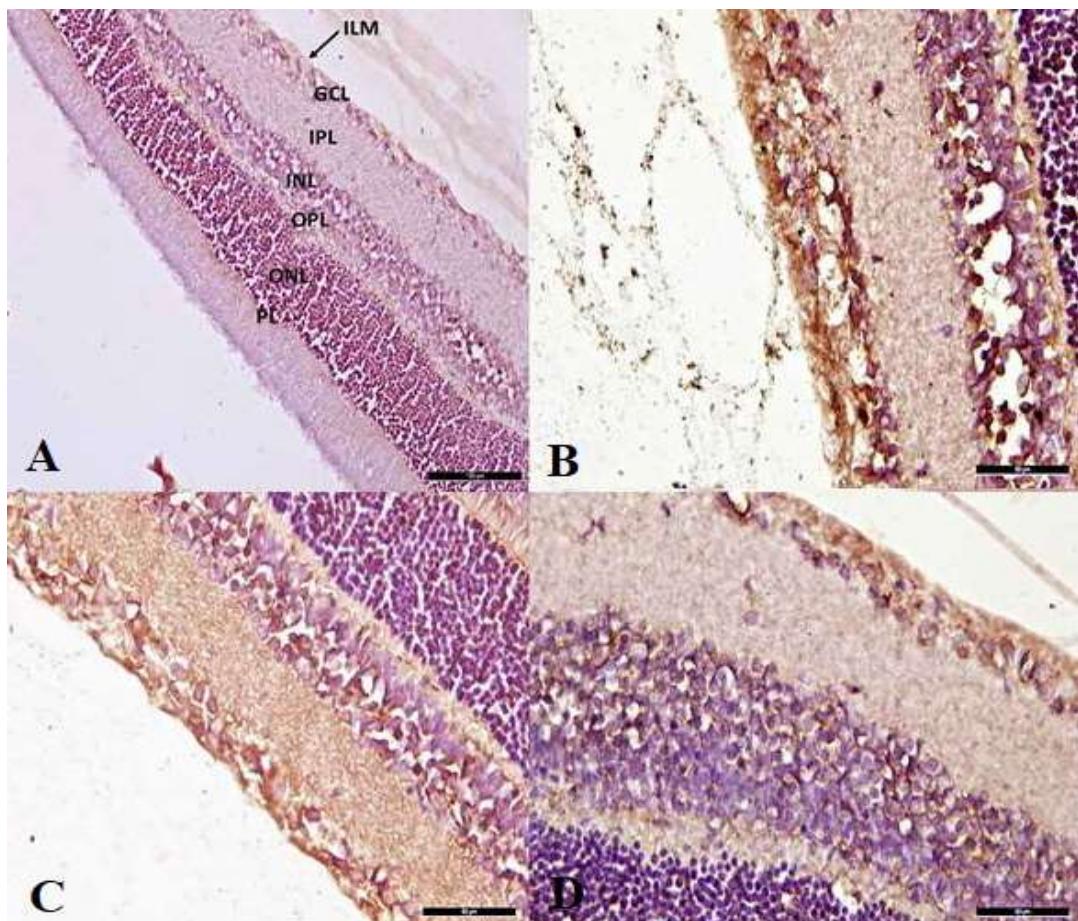


Figure 2. VEGF expressions were determined by immunohistochemistry in non-treated OIR, and OIR rats treated with quercetin or bevacizumab. **A:** Control group (Group I). **B:** Untreated OIR group (Group II). **C:** OIR group treated with intraperitoneal bevacizumab (Group III). **D:** OIR group treated with intraperitoneal quercetin (Group IV). GCL: Ganglion cell layer, ILM: Inner limiting membrane, INL: Inner nuclear layer, IPL: Inner plexiform layer, ONL: Outer nuclear layer, OPL: Outer plexiform layer, PL: Photoreceptor layer.

proliferative DRP, RVO, and ROP. The results of the present study demonstrated that ip administration of quercetin following an OIR model histopathologically suppressed the retinal NVs and inflammatory cell infiltration as well as significantly reduced the number of VECs, VEGF, and TNF- α levels compared to the non-treated OIR groups. The data obtained with quercetin administration were comparable to that of the bevacizumab administration. Stimulation of VECs in the case of retinal hypoxia could increase the mRNA transcript abundance of various genes, including growth factor associated genes, such as VEGF and

TNF- α [20]. Chen et. al. suggested that various stages of angiogenesis, including proliferation, migration, and tube formation of choroidal and retinal VECs, were inhibited following quercetin administration *in vitro* [21]. Likewise, Li et al. found that VEGF-induced choroidal and retinal angiogenesis were inhibited by *in vitro* quercetin administration [13]. It was reported that quercetin could inhibit blood vessel development and reduce cell viability, cell proliferation, and tube formation [22]. Similar to the previously published studies, our results suggested that quercetin significantly reduced VECs in the experimental

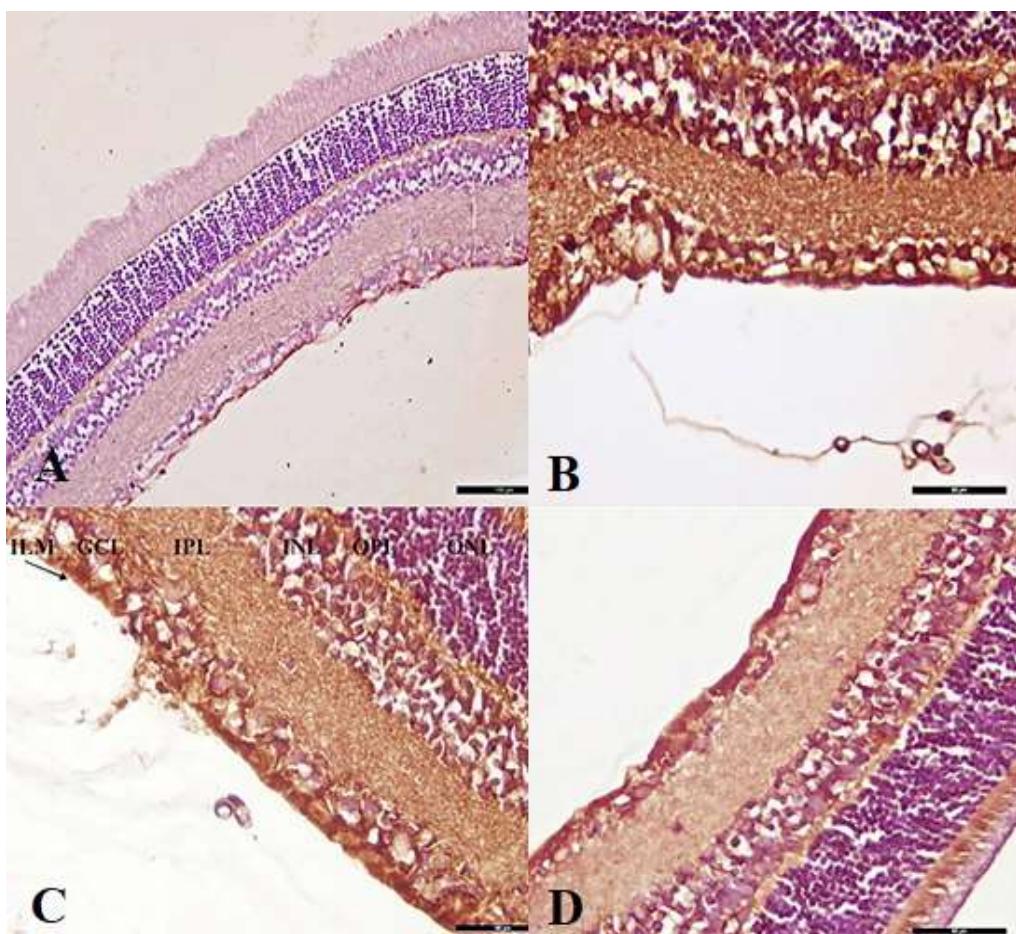


Figure 3. TNF- α expressions were determined by immunohistochemistry in non-treated OIR, and OIR rats treated with quercetin or bevacizumab. **A:** Control group (Group I). **B:** Untreated OIR group (Group II). **C:** OIR group treated with intraperitoneal bevacizumab (Group III). **D:** OIR group treated with intraperitoneal quercetin (Group IV). GCL: Ganglion cell layer, ILM: Inner limiting membrane, INL: Inner nuclear layer, IPL: Inner plexiform layer, ONL: Outer nuclear layer, OPL: Outer plexiform layer.

OIR model, and suppressed the synthesis of VEGF and TNF- α .

Currently, intravitreal bevacizumab has been successfully used for the treatment of DRP, ROP, RVO, and AMD. Many studies have reported that it has strong anti-VEGF properties and similar efficiency compared to the other intravitreal agents (such as aflibercept and ranibizumab) [7,8]. Significant improvement was shown in the zone I ROP following treatment with intravitreal bevacizumab as an anti-VEGF monotherapy and was comparable to the conventional laser therapy [23]. In the current study, the administration of quercetin and bevacizumab showed strong anti-VEGF

properties compared to the non-treated OTNF- α is an important mediator of retinal neuroinflammation and neurodegeneration. It has previously demonstrated that the cytokines and chemokines were strongly suppressed by bevacizumab in the experimental OIR model [24]. Likewise, cytokine levels were significantly reduced in quercetin-treated retinas compared to the proliferative diabetic retinas [25]. To the best of our knowledge, there are no previous studies examining the impact of quercetin administration on TNF- α levels using experimental OIR models. The results of the current study were comparable to that of DRP models. Similar to the bevacizumab

administration, quercetin treatment in the OIR model significantly suppressed TNF- α compared to the non-treated OIR group.

The single-dose i.p. administration of quercetin in the OIR model and examination of only VECs, VEGF and TNF- α limited the strength of the study. In addition, dose-response relationship, pharmacokinetic and functional analyses were not evaluated in the current study. Therefore, future studies are required to determine the optimal dosing protocol, especially for intravitreal application. Although the short-term effects were quite promising, future studies should be performed to confirm the long-term effects. Despite all these limitations, it was found in the present study that quercetin has comparable efficacy to bevacizumab, the anti-VEGF agent currently used in routine therapy.

In conclusion, the results of the present study demonstrated that quercetin administration as a therapeutic agent may ameliorate the severity of OIR in a rat model. Moreover, the effects obtained by quercetin treatment using the OIR model were comparable to the effects obtained following bevacizumab administration. Therefore, quercetin administration is promising for the treatment of diseases causing vision loss in patients, including ROP, DRP, RVO, and AMD. However, more studies are required in the future for a better conclusion.

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

Ethical statement: The study was approved by the Ethics committee of Bolu Abant Izzet Baysal

University Experimental Animal Studies (Date / decision no: 2018/35).

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Evaluation of electrocardiographic ventricular repolarization parameters in stable coronary artery disease

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ABSTRACT

Aim: To examine the relationship between the SYNTAX score (SS) and cardiac repolarization parameters such as cQTd and Tp-e values, and Tp-e/QT, Tp-e/JT, Tp-e/QTc, and Tp-e/JTc ratios in patients who have stable angina pectoris.

Methods: 12-lead resting electrocardiograms (ECGs) and SS of 160 patients (51 female and 109 male) undergoing coronary angiography with the pre-diagnosis of stable angina pectoris were evaluated. Patients with a SS below 22 were classified as Group 1 (low-SYNTAX), and those above 22 (high-SYNTAX) as Group 2. Forty-four patients with normal coronary angiography were included in Group 3.

Results: Mean age of the patients was 62.4 ± 9.1 years. The heart rate, QRS, QT, cQT and JT durations between the groups were similar. In addition, relatively recent ventricular repolarization indices such as Tp-e interval and Tp-e/QT, Tp-e/JT, Tp-e/QTc, and Tp-e/JTc ratios were also not substantially different between groups.

Conclusion: Several surface ECG predictors of ventricular arrhythmias, including QTd, JT and Tp-e intervals and their ratios to QT and JT, are not significantly correlated with SYNTAX score-assessed CAD severity in patients with stable angina pectoris.

Keywords: Arrhythmia, coronary artery disease, SYNTAX score, Tp-e interval, Tp-e/QTc ratio, Tp-e/JTc ratio.

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Introduction

The leading cause of death in developing countries is coronary artery disease (CAD) [1]. In 20-25 percent of patients, sudden cardiac death (SCD) can be the first clinical appearance of ischemic heart disease. About 75% to 80% of all SCDs are due to ischemic heart disease [2,3]. SCD is generally associated with life-threatening ventricular arrhythmias that may cause irregular electrophysiological substrates

for myocardial ischemia. It is controversial to classify high risk patients by surface electrocardiogram (ECG) measures [4,5].

In order to predict people at high risk for ventricular arrhythmias, many surface ECG markers such as T wave peak-to-end interval (Tp-e) and JT interval (JT), QT interval (QT) and QT dispersion (QTd) were suggested [6,7]. Another ventricular repolarization (VR) index that remains stable despite dynamic changes in the heart rate (HR) is the Tp-e/QT ratio [8,9]. The SYNTAX score (SS) was created as a combination of several validated angiographic classifications to grade the coronary anatomy in terms of the number of lesions and their functional effects, position, and complexity

[10]. Initially the SS has been suggested as an aid to decision making for percutaneous coronary intervention rather than a predictive tool for percutaneous coronary intervention. On the other hand, its prognostic utility has been validated in various settings [10-13]. We investigated the relationship between the SS and the repolarization indices of 12-lead surface ECG in this report.

Materials and methods

Between March 2019 and January 2020, this retrospective study was performed at Bolu Abant Izzet Baysal University Faculty of Medicine Hospital. The study procedure has been approved by the Ethics Committee and written informed consent was given by each participant (Date and decision number: 2020/06). The study was conducted in accordance with the principles of the Declaration of Helsinki. All patients and the institution were informed about the study and their written consents were obtained.

For the study, a total of 171 patients who underwent coronary angiography with the pre-diagnosis of stable angina pectoris (SAP) were examined. Medical history of patients with percutaneous intervention (PCI) or bypass operation, coronary artery disease, antiarrhythmic substance usage, cardiac failure, serious renal failure, and chronic obstructive pulmonary disease were excluded. Seven patients were excluded from the study because of atrial fibrillation; three patients were excluded due to the left branch block of the bundle and one patient was excluded due to the right branch block of the ECG bundle. As a result, the study included 160 participants. For patients with coronary angiography lesions of 50 percent or more, the SS was computed. Patients with a SS below 22 was classified as Group 1 (low-SYNTAX) those above 22 (high-

SYNTAX) as Group 2. Group 3 included forty-four patients with normal coronary angiography.

Electrocardiography

After 10 minutes of rest, twelve-lead ECGs were obtained with an amplitude of 10mm/mV and a rate of 25mm/s in the supine position using the available machines (Nihon Kohden Cardio fax ECG-1950 VET). ECGs were measured manually with a magnifying glass (TorQ 150 mm Optical Caliper LCD) by two blinded cardiologists. QT intervals were measured from the onset of the QRS complex to the end of the T wave, which was described as its return to the TP baseline. JT interval was defined as the distance between the end of the QRS complex (J point) and the end of the T wave. The R-R interval was calculated and used with Bazett's formula to calculate the HR and to calculate QTc and JTc [14]. The Tp-e interval was calculated as the distance between the peak and end of the T wave. The difference between the maximum and minimum QT interval in various leads was defined as QTd. Also ratios of Tp-e to QT, Tp-e to JT, Tp-e to QTc and Tp-e to JTc were calculated. For measurements, the intraobserver and interobserver differences were less than 10 percent.

Statistical analysis

Windows Operating System Statistical Package Program SPSS 15.0 (SPSS Inc, Chicago, Illinois, USA) was used. Quantitative variables were expressed as mean±standard deviation (SD), median and interquartile range. To determine the discrepancies between groups we used the Anova and Student t-test for normally distributed variables and Kruskal Wallis test and the Mann-Whitney U-test for variables without normal distribution. Chi-square test was used for qualitative variables. A two-tailed P value of less than 0.05 was accepted to be significant.

Table 1. General characteristics of the study groups.

| Baseline characteristics | Group 1 (n=66) | Group 2 (n=50) | Group 3 (n=44) | P value |
|--------------------------|----------------|----------------|----------------|---------|
| Age (years) | 61.6±9.3 | 64.7±6.8 | 60.4±8.1 | 0.060 |
| HT (n, %) | 38/60 (63.3) | 24/42 (57.1) | 27/44 (61.4) | 0.821 |
| DM (n, %) | 21/60 (35) | 18/42 (42.9) | 12/44 (27.3) | 0.317 |
| HL (n, %) | 6/60 (10) | 4/42 (9.5) | 6/44 (13.6) | 0.791 |
| Smoking (n, %) | 31/60 (51.7) | 28/42 (66.7) | 12/44 (27.3) | 0.001 |
| LVEF, mm | 60.2±3.7 | 58.6±4.1 | 60.1±3.4 | 0.096 |
| SYNTAX score | 11.1±5.2 | 27.1±4.2 | | <0.001 |
| | Median (IQR) | Median (IQR) | Median (IQR) | |
| LAD, mm | 36.5 (6) | 35.5 (5) | 36 (5) | 0.445 |
| LVEDd, mm | 47.5 (7) | 49 (7) | 46 (7) | 0.313 |

Values are expressed as mean±SD or median (IQR: Interquartile range) HT: Hypertension, DM: Diabetes Mellitus, HL: Hyperlipidemia, EF: Left ventricular ejection fraction, LAD: Left atrium diameter, LVEDd: Left ventricular end-diastolic diameter.

Table 2. Electrocardiographic measurements of the study groups.

| Variables | Group 1 (n=66) | Group 2 (n=50) | Group 3 (n=44) | P value |
|--------------------------|----------------|----------------|----------------|---------|
| Heart rate (bpm) | 69.4±12.8 | 71.5±13.2 | 69.8±11.2 | 0.759 |
| QT max. (ms) | 364.4±28.3 | 359.4±28.8 | 360.3±22.4 | 0.600 |
| QT min. (ms) | 346.2±28.4 | 342.1±29.3 | 345.2±23.4 | 0.756 |
| QT mean (ms) | 355.3±27.9 | 350.9±28.2 | 352.7±22.7 | 0.716 |
| QTc (ms) | 378.5±27.6 | 378.7±28.9 | 377.9±22.1 | 0.990 |
| Tp-e/JT | 0.326±0.04 | 0.325±0.04 | 0.309±0.04 | 0.118 |
| Tp-e/JTc | 0.306±0.04 | 0.300±0.05 | 0.287±0.4 | 0.118 |
| Tp-e/QT | 0.238±0.02 | 0.230±0.02 | 0.227±0.02 | 0.131 |
| Tp-e/QTc | 0.224±0.03 | 0.213±0.03 | 0.212±0.2 | 0.097 |
| | Median (IQR) | Median (IQR) | Median (IQR) | |
| Tp-e (ms) | 85 (16) | 80 (14) | 81 (13) | 0.072 |
| QRS duration (ms) | 94.5 (16) | 95 (13) | 92 (12) | 0.131 |
| JT (ms) | 264 (38) | 259.7 (38) | 261.9 (34) | 0.141 |
| JTc (ms) | 281 (36.7) | 262 (49.2) | 283 (36.5) | 0.392 |
| QTd (ms) | 18 (12.2) | 19.5 (14) | 14 (8) | 0.062 |
| cQTd | 21 (13) | 21.5 (15.2) | 16 (7.5) | 0.065 |

Values are expressed as mean ±SD or median (IQR: Interquartile range). QTc: Corrected QT, QTd: QT dispersion, bpm: Beat per minute, ms: Millisecond, Tp-e: T peak and end interval, cQTd: corrected QT dispersion.

Results

The participants mean age was 62.4 ± 9.1 years. There were 51 female and 109 male patients. There were no major variations between the groups in the prevalence of hypertension, diabetes mellitus and hyperlipidemia. In Group-2, smoking was substantially higher in comparison to other groups. Additionally, left atrium diameter, and ejection fraction and end-diastolic diameter of left ventricle (LV) did not vary significantly between groups (Table 1). QRS duration, HR, and QT, QTd and JT values were similar between groups. Furthermore, relatively recent repolarization indices such as values of cQTd and Tp-e, and ratios of Tp-e/JT, Tp-e/JTc, Tp-e/QT and Tp-e/QTc were not substantially different between groups (Table 2).

Discussion

In the present analysis, we observed that the length of QRS, HR, QTd, JT, Tp-e values and Tp-e/JT and Tp-e/QT ratios did not vary significantly between high-SYNTAX and low-SYNTAX and normal coronary anatomy groups.

SCD is typically triggered by ventricular arrhythmias, especially ventricular fibrillation [2,3]. Myocardial ischemia and infarction are among the most common underlying mechanisms for these life-threatening ventricular arrhythmias [15,16]. There is a 'border zone' between normal and hypoxic/ischemic tissues that can promote the occurrence of arrhythmias through several mechanisms such as automatic action during myocardial ischemia [17,18]. Shortened refractoriness and slowed conduction are the most pronounced electrophysiological impacts of myocardial ischemia [19].

The interval between the QRS complex and the end of the T wave in ECG is the product of

ventricular depolarization and repolarization. Cardiac electrical changes during this phase, especially during VR, can lead to fatal ventricular arrhythmias [20]. The time from the onset of ventricular depolarization to the end of repolarization is the QT interval. The JT interval is a mere VR time component and has been suggested to be more VR specific than the QT [21,22].

It has recently been suggested that the VR dispersion corresponds to the Tp-e interval [23,24]. The ratio of Tp-e/QT has been hypothesized as a potential arrhythmogenesis index independent of the length of the QT interval. This relatively new VR index stay to be fixed despite alterations in HR [8,9].

It has been shown that QT, QTc and QTd values predict life-threatening ventricular arrhythmic events and SCD in CAD. Corrected QTd was increased in patients with healthy CAD and was linked to the degree and severity of coronary atherosclerosis [25]. Panikkath et al. [26] compared ECGs of SCD cases ($n=353$; mean age, 66.6 years) with living controls having CAD ($n=342$; mean age, 64.7 years) using a case-control design. The mean Tp-e value, and QRS and QTc durations were significantly prolonged and the Tp-e/QT ratio was higher in SCD cases. After adjusting for gender, age, QRS duration, value of QTc and LV function, Tp-e remained a significant predictor of SCD. In subjects with normal QTc, Tp-e remained significantly correlated with SCD [27]. Helmy et al. [28] observed a strong positive association between QTc dispersion and SS among patients with STEMI during the past six months. Bektas el al. [29] examined 172 patients who underwent coronary angiography for SAP retrospectively. Patients were grouped as patients with a low and high SS (< 22 and > 22). Although the QTc and QT values were identical, the Tp-e in the high SYNTAX group

was significantly higher ($p=0.045$). Furthermore, both the Tp-e/QTc and Tp-e/QT ratios in the high SYNTAX group were higher ($p<0.001$). However, in our study we found that QT duration, Tp-e interval and Tp-e/QTc and Tp-e/QT were similar between high and low SYNTAX groups and also were similar to normal coronary artery group as well. The lack of clinical value of such electrocardiographic parameters may be due to scarce of very severe or subtotal stenosis that may cause significant ischemia and ischemia induced arrhythmias. The number of patients presenting with arrhythmia in stable angina pectoris not having necrosis or severe ischemia itself may be so rare and it may not be possible to detect significant ECG changes in such a small study group.

Ipek et al. [30] stated that intervals of Tp-e and cTp-e, and ratio of Tp-e/QT were significantly higher among SAP patients with a high SS. The intervals of Tp-e and cTp-e, and ratio of Tp-e/QT were associated prominently and moderately with SS ($r=0.435/p=0.005$, $r=0.395/p=0.004$ and $r=0.348/p=0.011$, respectively). On the other hand, they have not handled intervals of JT and JTc, and Tp-e/JT or Tp-e/JTc. Contrary to their results, in our study there were no substantial differences between high SS, low SS and normal coronary artery groups regarding Tp-e and JT intervals, and Tp-e/QTc, Tp-e/JTc and Tp-e/JT.

One of the most major limitations of this analysis is the relatively small sample size. Additionally, another constraint is the manual calculation of non-computer-aided ECG measurements. In addition, classifying patients with methods such as myocardial perfusion scintigraphy or fractional flow reserve according to evidence of ischemia could reveal more reliable results. The study lacks follow up for the detection of arrhythmias and their association with ECG parameters.

Conclusion

Several surface ECG predictors of ventricular arrhythmias, including QTd, JT and Tp-e values and their ratios to JT and QT, are not significantly correlated with SYNTAX score-assessed CAD severity.

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Platelet to lymphocyte ratio in differentiation of benign and malignant thyroid nodules

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ABSTRACT

Aim: Differentiation of thyroid nodules, either as benign or malignant, is a real diagnostic challenge. Inflammation has an important role in development of the malignancy. Therefore, inflammatory markers are associated with malignant thyroid nodules. Platelet /lymphocyte ratio (PLR) is also one of the novel inflammatory indices derived from hemogram tests. We hypothesized whether PLR was associated with malignant thyroid nodules. For this purpose, we compared PLR levels of the patients with benign thyroid nodules to the PLR of the subjects with malignant nodules.

Methods: The subjects who visited outpatient internal medicine clinics of our institution with a diagnosis of thyroid nodule were enrolled to the present retrospective study. According to the examination of the fine needle aspiration cytology (FNAC) specimen of the nodules, patients grouped into benign or malignant nodule groups. PLR of groups were compared.

Results: Median PLR values of the benign and malignant thyroid nodule groups were 106 (48-432) % and 119 (48-365) %, respectively ($p=0.001$). PLR value higher than 106% has 69% sensitivity and 51% specificity in detecting malignant nodules (AUC: 0.59, $p=0.001$, 95% CI: 0.54-0.65). PLR was positively correlated with TSH level ($r=0.10$, $p=0.34$).

Conclusion: We suggest that elevated PLR could be an additional tool to differentiate malignant thyroid nodules from benign ones in supportive of sonography, scintigraphy and cytology.

Keywords: Platelet lymphocyte ratio, thyroid nodule, malign, benign, inflammation.

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Introduction

Differentiation of thyroid nodules, either as benign or malignant, is a real diagnostic challenge. Since a considerable amount of thyroid nodules are malignant, differentiation of these nodules is crucial. Nodules may develop in thyroid gland especially in iodine

deficient areas of the world and its prevalence in women may reach up to 5% [1]. Detection rate of thyroid nodules by ultrasound scanning is a bit higher as 20% [2].

Inflammation has an important role in development of the malignancy [3]. Therefore, inflammatory markers are associated with malignant thyroid nodules. For instance, c-reactive protein (CRP) and novel inflammatory markers, such as, mean platelet volume, red cell distribution width and neutrophil/lymphocyte ratio are elevated in malignant thyroid nodules [4-7].

Platelet /lymphocyte ratio (PLR) is also one of the novel inflammatory indices derived from hemogram tests. It has substantial diagnostic and prognostic value in certain conditions. PLR has been correlated with glycated hemoglobin (HbA1c) levels in subjects with type 2 diabetes mellitus [8]. Moreover, authors suggested PLR as a prognostic factor of survival in colorectal cancer patients [9].

As other cancer types and type 2 diabetes mellitus, malignant thyroid nodules are associated with inflammation. Therefore, we hypothesized whether PLR was associated with malignant thyroid nodules. For this purpose, we compared PLR levels of the patients with benign thyroid nodules to the PLR of the subjects with malignant nodules.

Materials and methods

The subjects who visited outpatient internal medicine clinics of our institution between May 2018 and December 2020 with a diagnosis of thyroid nodule were enrolled to the present retrospective study. Ethical approval obtained from institutional ethics committee (approval number: 2021-26). The study was conducted in accordance with the principles of the Declaration of Helsinki. All patients and the institution were informed about the study and their written consents were obtained. According to the cytological examination of the fine needle aspiration biopsy (FNAB) specimen of the nodules, patients grouped into benign or malignant nodule groups.

Age, sex, thyroid stimulating hormone (TSH), white blood cell count (WBC), hemoglobin (Hb), hematocrit (Htc), lymphocyte count (lym), platelet count (PLT) were obtained from the laboratory tests of the participants that held before FNAB. Exclusion criteria were as follows; inflammatory conditions including thyroiditis, recent infection, type 2 diabetes

mellitus, cancer, hematologic conditions that may affect platelet count, use of medicines that may interfere with hematopoiesis or thrombocyte functions (i.e. corticosteroids, acetyl salicylate). Hemogram tests were held with Abbott Cell-Dyn 3700 automatic analyzer device (Abbott Laboratories, Abbott Park, IL, USA). TSH assay was done with original kits of the manufacturer in Abbott Architect i2000SR device (Abbott Laboratories, Abbott Park, IL, USA).

Statistical analysis conducted by SPSS software (SPSS15.0; SPSS Inc., Chicago, IL, USA). Fitness of the measurable variables to normal distribution was evaluated by Kolmogorov-Smirnov test. Variables with normal distribution were compared with student t test and expressed as mean \pm Standard Deviation. Variables without normal distribution were compared with Mann Whitney u test and expressed as median (minimum-maximum). Comparison of the frequency data was held with X^2 test and these data were expressed as percentage. Significance level is considered as a p value lower than 0.05 in all statistical tests.

Results

After application of exclusion criteria, remaining 443 patients were enrolled to the study. Benign thyroid nodule group was consisted of 207, while malignant thyroid nodule group was consisted of 236 subjects.

Mean ages of the benign and malignant thyroid nodule groups were 43.1 ± 7.4 years and 44.6 ± 11.6 years, respectively ($p=0.12$).

Forty nine (24%) of the subjects were male and 158 (76%) were female in benign thyroid nodule group while 55 (23%) were male and 181 (77%) were female in malignant thyroid nodule group. Gender was not statistically different among study groups, either ($p=0.93$).

The Hb ($p=0.49$), Htc ($p=0.07$), WBC ($p=0.08$), Plt ($p=0.76$) and TSH ($p=0.16$) of the benign and malignant thyroid nodule groups were not statistically different.

Median PLR values of the benign and malignant thyroid nodule groups were 106 (48-432) % and 119 (48-365) %, respectively. PLR value of the malignant thyroid nodule group was significantly higher than that of the benign thyroid nodule group ($p=0.001$).

A ROC analysis revealed that a PLR value higher than 106% has 69% sensitivity and 51% specificity in detecting malignant nodules ($AUC: 0.59$, $p=0.001$, 95% CI: 0.54-0.65). Figure 1 shows the ROC curve of PLR in detecting malignant thyroid nodules.

In Pearson's correlation analysis, PLR was positively correlated with TSH level ($r=0.10$, $p=0.34$). Figure 2 shows the correlation between PLR and TSH.

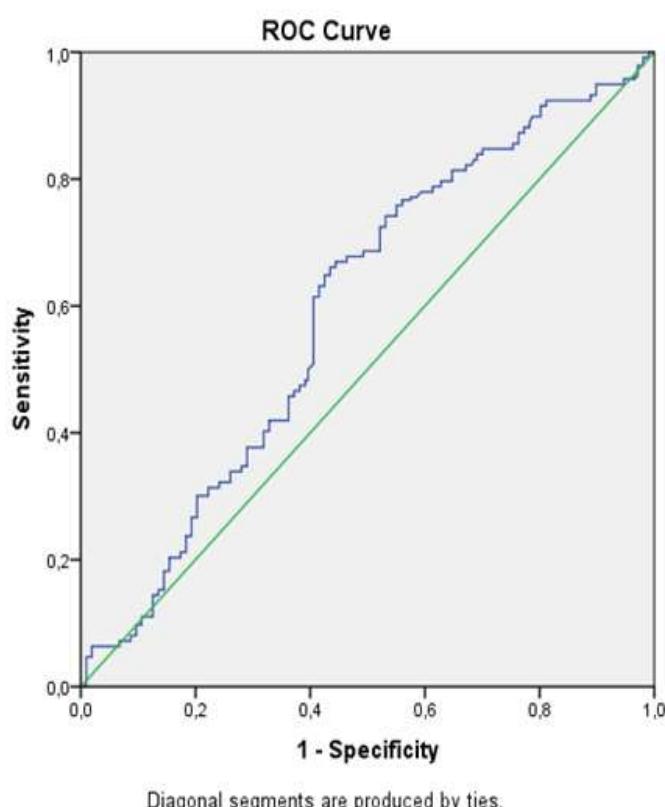


Figure 1. The ROC curve of PLR in detecting malignant nodules in thyroid gland.

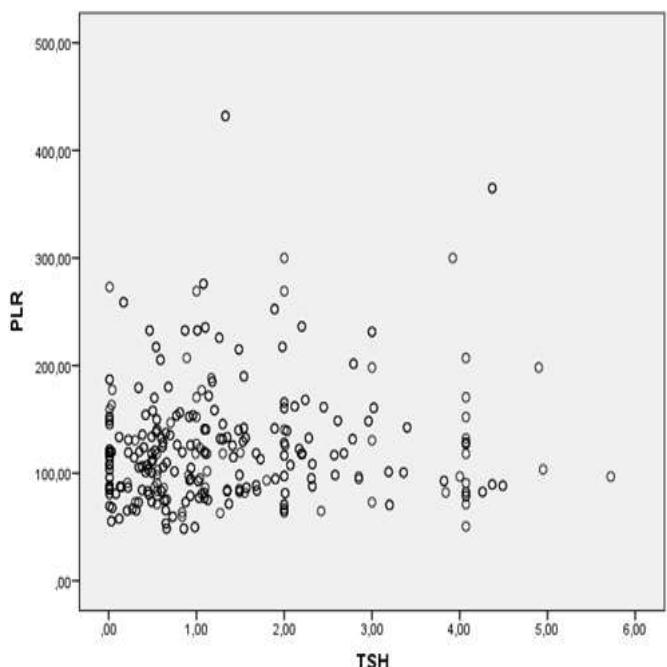


Figure 2. Correlation between TSH and PLR.

Discussion

Present study demonstrated that PLR of the subjects with malignant thyroid nodule is significantly elevated compared to the PLR of the subjects with benign thyroid nodule. Another important outcome of present study is moderate sensitivity and specificity of the PLR in detecting malignant nodules in thyroid gland. Finally, significant positive correlation between TSH and PLR was revealed by present work. Recent studies pointed out the diagnostic and prognostic role of PLR in various inflammatory conditions. Atak et al reported that PLR was associated with type 2 diabetes mellitus and correlated with HbA1c [8]. PLR levels of the subjects with lower extremity deep venous thrombosis were significantly higher than the PLR levels of the subjects without thrombosis [10]. Moreover, patients with Covid-19 have increased PLR levels compared to the healthy controls [11]. In addition, elevated PLR was suggested to be a predictor of early mechanical ventilation requirement in subjects with Covid-

19 [12]. Both, type 2 diabetes mellitus, venous thrombosis and Covid-19 infection are inflammatory conditions as malignant thyroid nodules. Therefore, the findings of present study, which revealed elevated PLR levels in patients with malignant thyroid nodules, are consisted with literature.

The role of inflammation in cancer development is pivotal [13,14]. PLR is a novel inflammatory marker. Therefore, cancer is also associated with increased PLR values. A study from Poland reported that PLR was correlated with survival in patients with endometrium cancer [15]. High PLR levels were correlated with worse overall survival in oral squamous cell carcinoma patients [16]. Preoperative PLR level has been proposed as an independent prognostic factor of survival in rectal cancer [17]. Authors reported that PLR level in pretreatment period could be a prognostic index in patients with non-small cell lung cancer [18]. The PLR of the patients with recurrent ovarian cancer are higher than the PLR of healthy subjects [19]. Malignancy attracts inflammatory cells and causes an increase in circulating inflammatory mediators. Malignant thyroid nodules are also causing such inflammatory burden. As shown in present study, PLR levels of these subjects are increased compared to the PLR levels of the patients with benign thyroid nodules.

Thyroid nodules are associated with inflammation. Authors found that the prevalence of thyroid nodules is higher in subjects with hyperhomocysteinemia, another condition that precedes inflammation [20]. Sit et al reported that mean platelet volume and neutrophil/lymphocyte ratios were predictors of malignancy in thyroid nodules [6,7]. Moreover, erythrocyte distribution width has been introduced as a predictor of malignant thyroid nodules in patients with nodular goiter [5].

Both, mean platelet volume, neutrophil/lymphocyte ratio and erythrocyte distribution width are inflammatory markers of hemogram tests, as the PLR. Therefore, similar elevation in PLR noticed in malignant nodules compared to benign nodules in present study. Sonographic characteristics for suspicious malignancy in thyroid nodules include speculated edges, vertical shape, microcalcification and hypoechoogenicity, which all have diagnostic sensitivity less than 50% [21]. Another tool for differentiating malignant and benign nodules is scintigraphy. Cold nodules in thyroid scintigraphy images are more likely to be malignant. However, nearly 3 of every 4 cold nodules is not malignant and malignancy detected in cytology of the 6% of hot nodules [22]. Gold standard method of the diagnosis of thyroid nodules as benign or malignant is fine needle aspiration cytology (FNAC), however, false negative and false positive results of FNAC have been reported around 10% [23]. Therefore, elevated PLR in malignant thyroid nodules compared to benign nodules could be a useful adjunctive test in discrimination of these nodules.

Relatively small study population and retrospective design of the work are two main limitations of our study. However, to the best of our knowledge, this is the first study in literature reported elevated PLR levels in patients with malignant thyroid nodules.

In conclusion, we suggest that elevated PLR could be an additional tool to differentiate malignant thyroid nodules from benign ones in supportive of sonography, scintigraphy and cytology.

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An overview of vitamin B12 and iron deficiencies as a risk factors in children with febrile seizure etiology

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ABSTRACT

Aim: To determine serum iron, ferritin, folate, and vitamin B12 deficiency and associated hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), and red cell distribution width (RDW) values in children undergoing febrile seizure, and thus to reveal their potential etiological role.

Method: The serum iron, folate, vitamin B12, and ferritin, and Hb, Hct, MCV, and RDW values of 98 patients undergoing FS and presenting to the pediatric neurology department and of 64 control patients were retrieved retrospectively and compared. Patient group data were also compared within the group.

Results: Serum iron, ferritin, and vitamin B12 values were significantly lower in the patient group than in the control group. Intragroup comparison revealed higher RDW values in patients with more than three FS and in those with complicated seizures.

Conclusion: This is the first study to investigate the relationship between vitamin B12, folate, and iron deficiency and FS. It should be remembered that deficiencies in these micronutrients, which are not routinely investigated in patients presenting with FS, may play a role in the etiology, and that the frequency can decrease with treatment. It should also not be forgotten that FS patients with high RDW values may be at risk of frequent seizure recurrence.

Key words: Febrile seizure, nutrition, risk factors, vitamin B12, iron deficiencies, children, outcome.

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Introduction

Febrile seizure (FS) is an acute, symptomatic type of seizure most frequently seen in the first five years of childhood and affecting approximately 2-5% of children [1,2]. FS has been defined as seizures induced by fever in children older than one month, with no previous history of afebrile seizure, in the absence of any central nervous system infection, metabolic or

electrolyte disorder, intoxication or trauma [3]. The American Academy of Pediatrics defines the age range at which FS is seen as 6-60 months [4]. The great majority of FS (65-70%) present as simple FS [1]. Simple FS has good prognosis and is not linked to mortality, hemiplegia, or intellectual disability [5]. Generalized FS less than 15 min in duration and not repeated within 24 h are defined as simple FS [1,6]. However, although they are described as ‘simple,’ such seizures are nevertheless causes of grave anxiety for families [7].

Iron serves as a cofactor for various enzymes in the body and is an important micronutrient involved in the synthesis of deoxyribonucleic

acid (DNA) and in the production of neurotransmitters and hormones. It is also an essential component of enzymes involved in a range of neurochemical reactions, such as myelin formation, brain energy metabolism, and neurotransmitter metabolism [8]. Numerous studies have shown that iron deficiency is associated with FS development in children [9,10]. Infants with iron deficiency are known to exhibit later cognitive, motor, social, emotional, and neurophysiological development than their peers, and studies have emphasized the importance of protecting the developing brain against iron deficiency [11]. This deficiency has been reported to cause poor physical performance through its role in muscle contraction and energy metabolism, and to lead to immune dysfunction due to its impact on the immune system [12]. This function impairment in the immune system can contribute to frequent fevers as a cause of increasing infections, and thus to more frequent FS.

Vitamin B₁₂ cannot be synthesized in the human body, but together with folic acid it plays a role in DNA synthesis, and cell division and proliferation. Deficiency is generally associated with insufficient intake in diet. It causes a wide spectrum of symptoms by affecting various systems.

Described symptoms include growth and development delay, gastrointestinal motility disorders, hyperpigmentation, stomatitis, glossitis, orthostatic hypotension, tachycardia, irritability, lethargy, decreased activity, hypotonia, ataxia, hyporeflexia, tremor, seizure, movement disorders, vision disorders, loss of taste and smell, altered consciousness and memory, regression in acquired motor skills, paresthesia, vibration and proprioception disorders, psychiatric disorders, and coma [13,14]. Although neurological

disorders and seizures have been described with serum vitamin B₁₂ and folic acid deficiency [15-17], the numbers of studies showing the relationship with FS are limited.

Addressing treatable causes that may be involved in the etiology of FS can successfully prevent the disease. The purpose of the present study was to determine serum iron, ferritin, folate, and vitamin B₁₂ deficiency and associated hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), and red cell distribution width (RDW) values in children undergoing FS, and thus to reveal their potential etiological role.

Materials and methods

The study was conducted at the Duzce University Faculty of Medicine Department of Pediatrics Division of Child Neurology, Turkey, between March 2019 and December 2020. It was approved by the local ethics committee (2020/248). The study was conducted in accordance with the principles of the Declaration of Helsinki. All patients and the institution were informed about the study and their written consents were obtained.

Ninety-eight patients and 64 healthy individuals as the control group identified retrospectively by scanning the hospital automation system were included in the study. Patients aged 1-60 months, with no previous history of afebrile seizure, with no history of trauma capable of resulting in seizure, in whom central nervous system infection and metabolic or electrolyte disorder were excluded, and with seizure induced only by fever were diagnosed with FS [3,4]. Generalized FS less than 15 min in duration and not recurring with 24 h was defined as simple FS [1,6]. Patients with FS attending the pediatric neurology clinic for routine controls at least two weeks after seizure

and at a time when no disease was present, and whose Hb, Hct, MCV, RDW, serum vitamin B12, folate, iron, and ferritin levels were investigated were included in the study. FS patients other than these 98 individuals, with insufficient available data, with histories of chronic disease or using vitamins were excluded from the study. The control group consisted of 64 children aged 1-60 months, attending the pediatric clinic for routine healthy child examination, whose Hb, Hct, MCV, RDW, serum vitamin B12, folate, iron, and ferritin levels were investigated, with no history of disease or malnutrition, and not receiving additional vitamin supplementation. FS group age and gender, history of FS and epilepsy in the family, parental consanguinity, type of delivery, birth week, history of hospitalization in the infant, degree of fever at time of seizure, presence of complicated or simple FS, body weight, electroencephalogram (EEG) results, antiseizure treatment requirements, neurological examination findings at time of seizure, and cranial magnetic resonance imaging (CMRI) results were recorded. The patient and control groups were compared in terms of age, gender, and Hb, Hct, MCV, RDW, serum vitamin B12, folate, iron, and ferritin levels. The patient group was subdivided into children with three or fewer simple/complicated seizures and those with more than three seizures, and the same parameters were compared between these subgroups.

Statistical analysis

Continuous data were expressed as mean \pm SD (min-max), and categorical variables as frequency and percentage for each group. A range of statistical tests were applied, depending on normality of data distribution. The Kolmogorov-Smirnov test was used to

determine the normality of distribution of the variables. The independent samples t-test was applied to normally distributed variables, while Pearson's chi-square test and Fisher's exact test were applied to categorical variables. The results were assessed within a 95% reliance and at a significance level of $p < 0.05$. Analyses were performed on Statistical Package for Social Sciences 25.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Results

The mean age of the patient group was 21 \pm 12 months. Sixty-eight percent were boys and 32% girls. No family history of FS or epilepsy was present in 44% of the patients. A history of FS was present in 44.3% of families, of both FS and epilepsy in 6.2%, and of epilepsy in 5.2%. The parental consanguinity rate was 9.3%. Additionally, 79.2% of patients were born on term, 21.8% preterm, 64.9% by cesarean section, and 35.1% by the normal vaginal pathway. A history of hospitalization in the neonatal period was present in 17.5% of patients. In terms of body temperature, 35.1% of patients experienced seizures at 37-37.9°C, 36.1% at 38-38.9°C, and 6.2% at more than 39°C. Body temperature was unknown in 22.7% of cases. FS was simple in 72.2% of patients, complex in 25.8%, and febrile status epilepticus in 2.1%. In addition, 83.5% patients were normal weight, 8.2% were overweight, and 8.2% were underweight. EEG was not performed on 29.9% of patients, while EEG results were normal in 44.3% and abnormal in 25.8% (18.6% generalized, 7.2% focal abnormality). Results were normal in 21 of the 22 patients on whom CMRI was performed, while dysgenesis of the corpus callosum splenium was determined in one. NO FS prophylaxis was being used by 52.6% of patients, 1.7% received intermittent

prophylaxis, and 29.9% were on continuous prophylaxis. Neurological examinations at time of seizure were normal in 97.6% of patients. The distribution of all patient group categories is shown in Table 1.

The mean age of the control group was 26 ± 14 months, 33 (51.6%) were boys and 31 (49.4%) were girls. Age and gender distributions were similar between the two groups. Vitamin B12, iron and ferritin levels were significantly lower in the patient group compared to the control group. No difference was observed between folate, Hb, Hct, MCV, or RDW values (Table 2).

When the patient group was subdivided into individuals with three or fewer seizures (80 patients) and those with more than three seizures, the only difference in the study parameters was observed in significantly higher RDW values among patients undergoing more than three seizures (Table 3). When the patient group was subdivided into simple and complicated FS, no significant difference was observed between the parameters, although RDW values were higher in the complicated FS group, but the difference was not statistically significant ($p=0.08$) (Table 4).

Table 1. Demographic and clinical data of patients with febrile seizures.

| Variables | N (%) or Mean \pm SD | Variables | N (%) or Mean \pm SD |
|------------------------------|------------------------|---------------------------------|------------------------|
| Age (month) | 21 \pm 12 | FS classification | |
| Gender | | Basic FS | 70 (72.2) |
| Female | 66 (%68) | Complex FS | 25 (25.8) |
| Male | 31 (%32) | FSE | 2 (2.1) |
| Family history | | Neurological examination | |
| Absent | 43 (44.3) | Normal | 93 (97.9) |
| FC | 43 (44.3) | Abnormal | 4 (2.1) |
| Epilepsy | 5 (5.2) | | |
| Epilepsy and FC | 6 (6.2) | Weight | |
| | | >97p | 8 (8.2) |
| Consanguinity | | <3p | 8 (8.2) |
| Absent | 88 (90.7) | Normal | 81 (83.5) |
| Present | 9 (9.3) | | |
| Delivery type | | EEG result | |
| C/S | 63 (64.9) | Normal | 43 (44.3) |
| Normal | 34 (35.1) | Generalized abnormality | 18 (18.6) |
| Gestation week | | Focal abnormality | 7 (7.2) |
| Preterm | 21 (21.8) | n.a | 29 (29.9) |
| Term | 76 (79.2) | | |
| NICU history | | CMRI result | |
| Present | 17 (17.5) | Normal | 20 (20.6%) |
| Absent | 80 (82.5) | Abnormal | 2 (2.06%) |
| Body temperature (°C) | | n.a. Treatment | 75 (77.3%) |
| Missing data | 22 (22.7) | Absent | 51 (52.6) |
| 37-38 | 34 (35.1) | Intermittent prophylaxis | 17 (17.5) |
| 38-39 | 35 (36.1) | Continuous prophylaxis | 29 (29.9) |
| 39-40 | 6 (6.2) | | |

FS: febrile seizure, C/S: cesarean section, NICU: newborn intensive care unit, EEG: electroencephalogram, CMRI: cranial magnetic resonance imaging, FSE: Febrile status epilepticus, n.a: not available.

Table 2. A comparison of demographic characteristics and laboratory parameters between patients with febrile seizures and the control group.

| Variables | Patient (N=97) | Control (N=64) | <i>p</i> |
|--------------------|-------------------|-------------------|------------------------------|
| Age(months) | 21±12 | 26±14 | 0.018^a |
| Gender | | | 0.047^b |
| Male | 66(%68) | 33(%51.6) | |
| Female | 31(%32) | 31(%48.4) | |
| Vitamin B12 | 373±176 | 527±259 | <0.001^a |
| Iron | 49±26 | 65±22 | <0.001^a |
| Ferritin | 28±32 | 65±22 | 0.001^a |
| Folate | 13.1±5.2 | 13.04±4.9 | 0.8^a |
| Hb | 11.7±1.1 | 11.9±0.7 | 0.3^a |
| Hct | 35±4 | 34±4 | 0.6^a |
| MCV | 83±69 | 75±10 | 0.3^a |
| RDW | 15.1±4.1 | 14.5±1.6 | 0.2^a |

Hb: hemoglobin, *Hct:* hematocrit, *MCV:* mean corpuscular volume, *RDW:* red cell distribution width. Data expressed as n (%). Bold *p* values indicate statistical significance at $\alpha=0.05$.

^a Independent samples t-test, ^b Pearson's chi-square test.

Table 3. The relationship between seizure frequency and laboratory findings.

| Variables | Seizure frequency ≤3(n=80) | Seizure frequency >3 (n=17) | <i>p</i> |
|--------------------|-------------------------------|--------------------------------|------------------------------|
| Age(months) | 21.9±12.6 | 16.6±11 | 0.1^a |
| Gender | | | 0.1^b |
| Male | 52(65%) | 14(82.6%) | |
| Female | 28(35%) | 3(17.4%) | |
| Vitamin B12 | 362±162 | 427±226 | 0.16^a |
| Iron | 26±23 | 47±28 | 0.8^a |
| Ferritin | 26±23 | 38±57 | 0.14^a |
| Folate | 13.1±5.2 | 11.04±4.9 | 0.13^a |
| Hb | 11.7±1.1 | 11.6±1.7 | 0.5^a |
| Hct | 35.1±4.3 | 35.7±3.6 | 0.6^a |
| MCV | 85±75 | 75±4 | 0.5^a |
| RDW | 14±1 | 18±9 | <0.001^a |

Hb: hemoglobin, *Hct:* hematocrit, *MCV:* mean corpuscular volume, *RDW:* red cell distribution width. Data expressed as n (%). Bold *p* values indicate statistical significance at $\alpha=0.05$.

^a Independent samples t-test, ^b Pearson's chi-square test.

Table 4. Relationships between type of febrile seizure and laboratory findings.

| Variables | Basic FC (n=70) | Complex FC (n=25) | <i>p</i> |
|--------------------|--------------------|----------------------|-------------------------|
| Age(month) | 22.1±12 | 18.6±9.8 | 0.23^a |
| Gender | | | 0.1^b |
| Male | 52(74.3%) | 13(52%) | |
| Female | 18(25.7%) | 12(48%) | |
| Vitamin B12 | 370±180 | 370±176 | 0.96^a |
| Iron | 49.5±30 | 49.7±30.5 | 0.9^a |
| Ferritin | 22±16 | 41±54 | 0.01^a |
| Folate | 12.1±4.2 | 13.9±6.4 | 0.3^a |
| Hb | 11.7±1.2 | 11.6±0.9 | 0.6^a |
| Hct | 35.2±4.4 | 35±3.6 | 0.8^a |
| MCV | 86±81 | 77±5 | 0.5^a |
| RDW | 14±1 | 16±7.8 | 0.08^a |

Hb: hemoglobin, *Hct:* hematocrit, *MCV:* mean corpuscular volume, *RDW:* red cell distribution width, *FS:* febrile seizure. Data expressed as n (%). Bold *p* values indicate statistical significance.

Discussion

FS is the most common form of childhood seizure [1]. Despite its high prevalence, the etiopathogenesis is complex and has not yet been fully explained. Numerous factors, including age, gender, infections, immunizations, cytokines, ethnicity, low calcium, sodium, iron, and zinc levels, microcytic anemia, progression to nursery school, and a history of hospitalization exceeding 28 days in the neonatal period have all been implicated in the development of FS [1,18-23].

Iron and ferritin levels in this research were significantly lower in the patient group than in the control group. Studies performed over many years have emphasized the association between low iron levels and iron deficiency anemia (IDA) and FS. In 1996, Pisacane et al. determined higher IDA in patients with FS compared to both a hospital group and the

normal population [10]. A case-control study from 2017 compared Hb, Hct, RDW, MCV, mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) values between a 30-member FS group and a non-febrile control group enrolled at the same time. All values in the case group were significantly in favor of anemia [24]. No difference was observed between our patient and control groups in terms of Hb, Hct, MCV, and RDW values.

In their meta-analysis of 17 studies, Kwak et al. determined a high level of correlation between IDA and FS (*OR*, 1.98; 95% *CI*, 1.26–3.13; *p*=0.003). While MCV (*OR*, 2.08; 95% *CI*, 1.36–3.17; *p* = 0.001) and plasma ferritin (*OR*, 3.78; 95% *CI*, 1.80–7.94; *p* < 0.001) exhibited moderate correlation with FS in some comparisons, no relationship was determined in two studies involving serum iron (*OR*, 0.57; 95% *CI*, 0.24–1.37; *p* = 0.210) [9].

A rat study reported a low seizure threshold in mice exposed to iron deficiency in the postnatal period, and showed that those mice were more susceptible to some types of seizure [25]. The mechanisms by which iron deficiency leads to seizure by causing excitatory-inhibitor imbalance are not yet fully understood.

A recent prospective study comparing 63 FS patients and a control group of 65 patients with nonconvulsive febrile illness reported significantly low serum iron, plasma ferritin, and transferrin saturation in the FS group. Consistent with the present study, iron deficiency was reported to increase the risk if FS even in the absence of IDA [26].

However, there are also studies reporting either the opposite or else no relationship. Kobrinsky et al. reported that iron deficiency can play a protective role in the context of FS [27]. Soheilipoor et al. compared 158 children with FS and 149 febrile children without seizures in

terms of Hb, Hct, RBC count, MCV, MCH, MCHC, values and weight for age. Greater IDA was observed in febrile children without seizures, while no association was found between growth delay and FS [28]. Since no low-weight control group was included in the present study, no comparison was performed with growth delay. We observed no association between our patients' anemia-related values and those of the control group. In their meta-analysis, Habibian et al. observed an increased prevalence of anemia in patients with FS. The authors determined a moderately increased risk of FS with IDA in societies in which anemia is less common, compared to those in which it is only seen at high levels [29].

Serum vitamin B12 levels were significantly lower in the FS group than in the control group in the present study. However, there was no difference in serum folate levels between the two groups. The number of studies examining the relationship between serum vitamin B12 and folate levels and FS is limited. Vitamin B12 and folic acid serve on the methionine-homocysteine pathway responsible for the provision of methyl groups essential for DNA and protein synthesis. Vitamin B12 catalyzed the formation of methionine from homocysteine and the conversion of methylmalonyl coenzyme A (MMA) to succinyl coenzyme A [30,31]. Although the exact role of vitamin B12 in epileptogenesis is unclear, various mechanisms have been proposed in the mechanism, including MMA accumulation due to deficiency leading to impairment in myelin synthesis [30], increased homocysteine contributing to neurological dysfunction by way of oxidative stress [30-32], a decreased S-adenosylmethionine/ S adenosylhomocysteine ratio compromising the methylation pathway [33], and the protective effect of S-adenosylmethionine against N-

methyl aspartate receptor (NMDA)-mediated glutamate neurotoxicity [34].

Folate and cobalamin levels were investigated in both serum and cerebrospinal fluid (CSF) in 40 febrile children (18 FS, 22 non-FS) and in serum in 20 healthy children. Serum folate levels were significantly higher in all patients compared to the healthy group, the highest values being determined in FS patients. No difference was found in CSF levels between the FS and non-seizure febrile patients. This was interpreted as indicating that even if folate levels are elevated in serum, passage to the brain is prevented by the blood-brain barrier, and it therefore exhibits no seizure-triggering. Patients' serum cobalamin levels were lower than in the control group, while no difference was observed between the FS patients and the febrile non-seizure patients, and no association with FS could be established [35].

Similarly to the present study, Özkale et al. determined lower vitamin B12 levels in an FS group compared to a seizure-free group, while no difference was observed between the two groups in terms of serum folate, homocysteine, Hb, MCV, macrocytosis, or anemia. Folic acid levels were also lower among patients in the FS group with more than three seizures and in those experiencing seizures at a temperature range of 37.5-39°. The authors associated vitamin B12 deficiency with an increased disposition to FS, and folic acid deficiency with an increased frequency of FS [36].

When patients with FS frequencies of three or fewer were compared with those with frequencies of more than three, RDW values were significantly higher in the latter group. When our FS patients were divided into simple and complex seizures, no difference was determined between any parameters. RDW values were higher in the complicated group, but not significantly so. Considering that RDW

values will naturally rise in case of iron, vitamin B12 and folate deficiency, elevation in RDW values in children undergoing frequent seizures may be useful in predicting vitamin B12, folate and iron deficiencies. In addition, the absence of significance among other parameters at intergroup comparison among the FS patients may be due to the unequal numbers in patient distribution.

The principal limitation of this study is its retrospective nature. We were therefore unable to evaluate parameters such as homocysteine, methylmalonic acid, transferrin saturation, and iron binding capacity. Our case number may also be regarded as insufficient in the light of the incidence of FS in the general community. However, anemia evaluation and serum iron, ferritin, folic acid, and vitamin B12 measurement are not routinely recommended in children presenting with FS. Considering that patients in which all parameters were fully examined were included in the study, the number is adequate.

Conclusions

The present study is the first study which investigates the relationship between Hb, Hct, MCV, RDW parameters and iron, vitamin B12, folate deficiencies with FS. Our findings show that although low serum ferritin, iron, and vitamin B12 levels do not cause anemia, they can increase the tendency to FS. We recommend that iron and vitamin B12 be examined in FS patients with RDW elevation due to the possibility of frequent seizure recurrence. Since the etiology of FS is multifactorial, the treatment of iron and vitamin B12 deficiencies may be effective in reducing the development of FS. Further prospective studies involving large case numbers are now needed in order to identify the mechanisms involved in FS development.

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Ethical statement: The study was approved by the Local Ethics Committee of Düzce University (Date and decision no: 2020/248).

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