Joint Diseases and Related Surgery

Jt Dis Relat Surg
2024;35(1):i-xiii

ORIGINAL ARTICLE

The protective role of vitamins C and E in steroid-induced femoral head osteonecrosis: An experimental study in rats

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ABSTRACT

Objectives: This study aimed to determine whether vitamin C (VC) and vitamin E (VE) can effectively protect the femoral head and reduce the risk of developing osteonecrosis in rats that have been treated with steroids.

Materials and methods: The study was conducted on 30 young adult male Sprague-Dawley rats (mean weight: 356±18 g; range, 330 to 375 g), which were randomly assigned to one of five groups. The control group received saline solution, while the other groups were given lipopolysaccharide/methylprednisolone (LPS/MPS) to induce osteonecrosis. Three groups in which osteonecrosis was induced were also intraperitoneally administered either VC, VE, or both once a day for four weeks. Intracardiac blood samples were taken at the end of the fourth week for biochemical examination, and the rats were then sacrificed under general anesthesia. After sacrification, right femurs were removed for histopathological, immunohistochemical, and radiologic examinations.

Results: The results showed that the mean trabecular number increased significantly in the VC+VE group. There was a substantial decrease observed in the mean trabecular separation within the LPS/MPS group compared to the control group, although trabecular separation decreased in all three vitamin groups compared to the LPS/MPS group. The surface area/bone volume was significantly increased in the VC+VE group compared to the LPS/MPS group. Histological, immunohistochemical, and radiological examinations showed that the administration of VC and VE significantly reduced oxidative stress, inflammation, and microvascular dysfunction in rats with steroid-induced femoral head osteonecrosis.

Conclusion: This study suggests that VC, VE, and particularly VC+VE have a protective effect on the femoral head in rats with steroid-induced femoral head osteonecrosis. These findings may lead to new treatment options for patients.

Keywords: Lipopolysaccharide, methylprednisolone, osteonecrosis of femoral head, vitamin C, vitamin E.

The illness known as steroid-induced osteonecrosis of the femoral head (ONFH) is an incapacitating ailment that afflicts a considerable population of individuals who have had extended steroid therapy. This condition is characterized by the death of bone tissue in the femoral head, which can lead to hip joint arthritis and even the need for total joint replacement. The development of osteonecrosis is a complex process that is influenced by a variety of factors, including oxidative stress, inflammation, and microvascular dysfunction.[1,2]

Dexamethasone and other glucocorticoid steroids are frequently used in chronic autoimmune diseases, asthma, organ transplantation, nephrotic syndrome, and hospitalized patients with COVID-19 (coronavirus disease 2019).[3-5] Osteonecrosis of the femoral head is a condition that accounts for around 2 to 10% of all hip arthroplasty procedures conducted in the USA and Europe.[6]

Received: August 28, 2023
Accepted: September 26, 2023
Published online: November 02, 2023

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Doi: 10.52312/jdrs.2023.1405

Citation: Beytemur O, Dasci MF, Gök Yurttaş A, Yaprak Bayrak B, Alagöz E. The protective role of vitamins C and E in steroid-induced femoral head osteonecrosis: An experimental study in rats. Jt Dis Relat Surg 2024;35(1):i-xiii. doi: 10.52312/jdrs.2023.1405.

Despite claims that ischemia in the bone causes osteonecrosis to develop, the precise pathogenesis of steroid-induced osteonecrosis is unknown. According to recent research, oxidative stress plays a significant role in the pathogenesis of steroid-induced osteonecrosis *in vivo*.[2]

Reactive oxygen species (ROS) are a byproduct of metabolic processes such as mitochondrial ATP production and inflammation,[7] disrupting cellular function by damaging macromolecules, including DNA (deoxyribonucleic acid), proteins, and lipids. Peroxidation of fatty acids in low-density lipoprotein (LDL) by ROS is an early pathogenic step in atherosclerosis.[3]

Vitamin C (VC) and vitamin E (VE) are powerful dietary antioxidants.[8] Vitamin C is a potent antioxidant that has been shown to protect against oxidative stress and improve microvascular function. Vitamin E has been shown to have anti-inflammatory properties and to enhance the function of other antioxidants in the body. Vitamin C is a more powerful antioxidant than VE, and VC has positive effects on bone development and maintenance through genes that produce bone matrix in osteoblasts.[9]

The primary objective of ongoing research on ONFH is to prevent the occurrence of this condition following the use of steroid drugs, regardless of the underlying etiology. A number of therapeutic approaches using anticoagulant, antilipidemic, and antioxidant agents have been suggested for the treatment of early-stage ONFH to achieve this objective. This study aimed to provide valuable information about the therapeutic potential of VC and VE in treating steroid-induced ONFH and contribute to our understanding of the underlying mechanisms of osteonecrosis, which may lead to the discovery of new targets for therapeutic intervention.

MATERIALS AND METHODS

The experiment was carried out using 30 young adult male Sprague-Dawley rats (mean weight: 356±18 g; range, 330 to 375 g). All rats were housed in a constant 22°C environment with a 12-h cycle of light and darkness. Irrespective of the temporal sequence preceding and following the medication delivery, unlimited quantities of standardized mouse chow and tap water were provided ad libitum. Prior to the commencement of the experiment, a comprehensive monitoring and follow-up process was conducted on all rats for a duration of seven days at the animal care laboratory. This rigorous procedure aimed to exclude the possibility of any preexisting health conditions among the subjects.

Thirty rats were randomly assigned to one of five groups, each with six rats: *(i)* control group, *(ii)* lipopolysaccharide (LPS)/methylprednisolone (MPS) group, *(iii)* VC group, *(iv)* VE group, and *(v)* VC+VE group. The sole therapy given to the control group was a saline solution. A total of 24 rats were assigned to different groups to establish an ONFH model. These rats were administered two intraperitoneal doses of 20 g/kg LPS derived from *Escherichia coli* O55:B5 (Sigma-Aldrich, St. Louis, MO, USA) on days 1 and 2, with a 24-h interval between doses. Additionally, the same rats received three intramuscular doses of 40 mg/kg MPS sodium succinate (Mustafa Nevzat İlaç Sanayi A.Ş., İstanbul, Türkiye).

As stated in the literature, from the first day of the study, 30 mg/kg VC (L-ascorbic acid; Sigma-Aldrich, St. Louis, MO, USA) to the VC group, 15 mg/kg VE (DL-Alpha-Tocopherol; Sigma-Aldrich, St. Louis, MO, USA) to the VE group, and both drugs with same doses to the VC+VE group were intraperitoneally administered once a day for four weeks.[10]

Biochemical analysis was conducted on intracardiac blood samples obtained at the conclusion of the fourth week. Subsequently, the rats were subjected to euthanasia while under the influence of general anesthesia, followed by the surgical removal of their right femur. To facilitate histological, immunohistochemical, and radiological studies, all bones were preserved in a 10% formaldehyde solution.

Radiological examination

The femur bones that were extracted were subjected to preparation for the purpose of three-dimensional scanning and micro-computed tomography (CT) evaluation using the SkyScan 1174v2 system (SkyScan 1174v2; Bruker-microCT, Kontich, Belgium). The dataset underwent an initial calibration process for the purpose of measuring bone mineral density (BMD). The BMD was calculated using calcium densities of 0.25 g/mm^3 and 0.75 g/mm^3 for the calcium hydroxyapatite (CaHA) calibration bars, which served as phantoms.

Images were uploaded to the CTAn program version 1.16.4.1+ (Bruker micro-CT, Belgium) to enable densitometry and morphometry quantitative parameters and visualization. On the cross-section of the section, a semiautomated circular region of interest (ROI) was generated to indicate the limits of the measurement area. The computation

of volume ratios was conducted in a manner that was independent of both the ROI and threshold information. Upon inputting the sample data into CTVol version 2.3.2.0 (Bruker micro-CT, Belgium), a series of three-dimensional simulated pictures were produced.

The CTAn software was employed to compute various parameters related to bone density and structure. These parameters include BMD measured in grams per cubic centimeter (g/cm^3), bone volume fraction (BV/TV), porosity, trabecular distance (Tr. Sp.), trabecular separation (Tr. Sep.), trabecular bone model factor (Tb. Pf.), and bone surface area to bone volume ratio (BS/BV). The ROI used for analysis was generated based on the femoral head.

Histopathological and histomorphometric examinations

The femoral heads were immersed in a solution of 10% buffered formaldehyde for fixation and then subjected to decalcification in a 10% formic acid solution for a duration of three days. The femoral heads were placed in paraffin blocks following a standard tissue tracking procedure. Hematoxylin-eosin was used to stain 3- to 4-m longitudinal sections. The consecutive sections were checked under microscope after staining for the observation of whole parts of cartilage. A light microscope (Zeiss Axio microscope with the Zen 2.3 lite program; Carl Zeiss AG [Global Corporate Headquarter], Oberkochen, Germany) was used for the histopathological analysis, and an attached camera (AxioCam IC; Carl Zeiss AG [Global Corporate Headquarter], Oberkochen, Germany) was used to take pictures.

The AutoCAD 2017 (Autodesk Inc., San Francisco, CA, USA) was used to take the histomorphometric measurements in accordance with the literature and accepted terminology.[11,12] The percentage of BV/TV, trabecular bone circumference/total volume (BS/TV, mm^2/mm^3), BS/BV (mm^2/mm^3), trabecular thickness (Tb. Th, μm), and the mean trabecular number (Tb. N, mm^{-1}) were determined.

Immunohistochemical examination

Monoclonal and polyclonal antibodies were utilized to target specific biomarkers, including 8-hydroxy-20-deoxyguanosine (8-OHdG), malondialdehyde (MDA), antiapoptotic protein Bcl-2, proapoptotic protein caspase-3, platelet/endothelial cell adhesion molecule 1 (PECAM/CD31), and vascular endothelial growth factor (VEGF). These antibodies were employed in an immunohistochemical staining procedure, following the streptavidin-biotin-peroxidase technique as described in the relevant literature.[12] For this process, 3 to 4 μm-thick longitudinal sections taken from paraffin blocks prepared on poly-L-lysine coated slides for use in light microscopic examinations were deparaffinized in an oven at 58°C for 1one night and then rehydrated with decreasing grade alcohols. After the antigen exposure by heat or microwave irradiation, the sections were kept in 0.3% H_2O_2 solution to block the endogenous peroxidase activity. All paraffin sections were soaked in a blocking solution in accordance with the procedure of the kit used for immunostaining. Afterward, the blocking solution was removed, and the sections were incubated overnight at +4°C in a humid environment using the above-mentioned primary antibodies in optimal dilution. Consequently, the sections were washed with 0.01 M phosphate-buffered saline (PBS) and incubated for 15 min, incubated in the secondary antibody, then washed with PBS and incubated for 15 min. The sections were treated with a horseradish peroxidase substrate with a streptavidin complex. In the staining, DAB (3,3′-diaminobenzidine) was used as the chromogen. After washing with PBS, the rehydrated sections were covered with an aqueous sealing solution.

The presence of specific immune labeling was detected under the microscope in comparison with negative and positive control staining. In the tissue sample selected for the negative control, the entire procedure was applied in the same way, but the antibody diluent was used instead of the primary antibody. The staining intensity of five locations exhibiting positive immunological labeling with their respective antigens was assessed using a semiquantitative modified H-score. Two researchers assigned scores ranging from 0 to 300 to five different locations and subsequently computed the mean values for each region.[13] Hence, the localization of the proteins in the femoral head was established, and the alterations in protein expression, as well as the regional disparities, were assessed using a semiquantitative approach.

Biochemical examination

Upon euthanasia, intracardiac blood samples were instantly collected and afterward submitted to hematological analysis. The samples underwent centrifugation at a speed of 3,000 revolutions/min for a duration of 10 min. After undergoing centrifugation, the samples were stored at a temperature of −80 °C until they were subjected to analysis. The concentrations of serum MDA and glutathione (GSH), bone alkaline phosphatase

(BALP), carboxy-terminal type 1 collagen crosslink (CTX), osteoprotegerin (OPG), triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, and LDL cholesterol were measured using enzyme-linked immunosorbent assay (ELISA) kits that utilized double antibody sandwich technology, in accordance with the manufacturer's instructions (MyBioSource, Inc., San Diego, CA, USA; catalog number: MBS268427 for MDA; MBS774706 for GSH; MBS774291 for BALP; MBS774451 for CTX; MBS775098 for OPG; MBS775451 for TG; MBS775433 for TC; MBS774773 for HDL; MBS774933 for LDL).

The assay was based on competitive binding of antibodies. The ELISA kits consisted of a number of plates with 96 microtiter wells on each plate and standard solutions to compare the findings. All of the microtiter wells were coated with antibodies directed toward an antigenic site on the target molecule. Standard measurement range (curve range) for MDA was 0.156-12 nmol/mL (minimum detectable: 0.05 nmol/mL); for GSH was 10-400 mmol/L (sensitivity: 1.0 mmol/Lt); for BALP was 2-100 ng/mL (sensitivity is 0.1 ng/mL); for CTX was 1-48 ng/mL (sensitivity is 1.0 ng/mL); for OPG was 1-16 ng/mL (sensitivity is 0.1 ng/mL); for TG was 0.5-12 mmol/L (sensitivity: 0.01 mmol/L); for TC was 1-120 μmol/L (sensitivity: 1.0 μmol/L); for HDL was 50-1600 μmol/L (sensitivity: 10 μmol/L); for LDL was 0.2-8 mmol/L (sensitivity: 0.1 mmol/L). Intra-assay sensitivity for MDA was ≤8%, whereas it was <10% for GSH, BALP, CTX, TG, TC, HDL, and LDL and <100% for OPG. Interassay sensitivity for MDA was ≤12%, while it was <15% for GSH, BALP, CTX, OPG, TG, TC, HDL, and LDL.

Statistical analysis

Data were analyzed using the GraphPad InStat version 3.06 (GraphPad Software Inc., Le Jolla, CA, USA). All numerical data were recorded as mean±standard deviation (SD), and all categorical variables were recorded as number of animals present/total number of animals. The distribution of the variables was assessed using the Kolmogorov-Smirnov test. The chi-square test for independence was employed to assess the relationship between categorical variables. A one-way analysis of variance test was employed to compare numerical variables that adhere to a normal distribution. Subsequently, a post hoc analysis was undertaken using the Tukey-Kramer multiple comparison test. The Kruskal-Wallis test, which is a nonparametric method for conducting an analysis of variance, was employed to compare numerical variables that had nonnormal distributions. The post hoc test used in this study was Dunn's multiple comparisons test. Values of $p<0.05$, $p<0.01$, and $p<0.001$ were accepted as statistically significant for different groups.

RESULTS

Radiological findings

There was no statistically significant difference between all groups in BMD results (Table I). The mean trabecular number (Tb. N) increased significantly in the VC+VE group compared to the control group ($p<0.05$), the LPS/MPS group ($p<0.01$), and the VE group ($p<0.05$). The mean Tr. Sep. decreased significantly in the LPS/MPS group compared to the control group ($p<0.05$). Although Tr. Sep. decreased in all three vitamin groups compared to the LPS/MPS group, no

TABLE I
Comparison of radiological findings of femoral head sections according to the groups (n=6)

	Control	LPS/MPS	Vitamin C	Vitamin E	Vitamin C+E	
Parameter	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	*p*
BMD (g/cm^3)	0.88±0.07	0.81±0.04	0.85±0.03	0.84±0.06	0.86±0.05	0.135
BV/TV (%)	26.86±3.42	20.38±6.19	21.96±2.86	21.86±5.36	21.12±5.13	0.097
Tb. Th (mm)	0.46±0.06	0.39±0.04	0.42±0.08	0.42±0.05	0.43±0.05	0.497
Tb. N (mm^{-1})	62.71±28.2	53.2±39.8	82.0±45.8	70.7±42.4	145.5±49.9^{a,b,c}	0.0064
Porosity (%)	73.14±3.42	79.62±6.19	78.04±2.86	78.13±4.89	78.88±5.13	0.086
Tb. Sp (mm^{-1})	0.50±0.12	0.63±0.07	0.55±0.04	0.54±0.14	0.55±0.15	0.352
Tb. Sep (mm)	1.15±0.16	1.78±0.54^{a}	1.42±0.14	1.46±0.36	1.54±0.44	0.044
Tb. Pf. (mm^{-1})	-15.21±2.0	-16.25±2.06	-16.95±3.63	-13.91±3.8	-15.56±2.05	0.411
BS/BV (mm^{-1})	6.91±0.76	5.68±0.67	6.53±1.98	5.95±0.78	7.84±1.09b	0.0305

SD: Standard deviation; BMD: Bone mineral density; BV/TV: Bone volume/total volume; Tb. Th: Trabecular thickness; Tb. N.: Trabecular number; Tb. Sp: Trabecular space; Tb. Sep.: Trabecular separation; Tb. Pf.: Trabecular bone model factor; BS/BV: Bone surface area/bone volume; a $p<0.05$ *vs.* control group; b $p<0.01$ *vs.* LPS/MPS group; c $p<0.05$ *vs.* Vitamin E group.

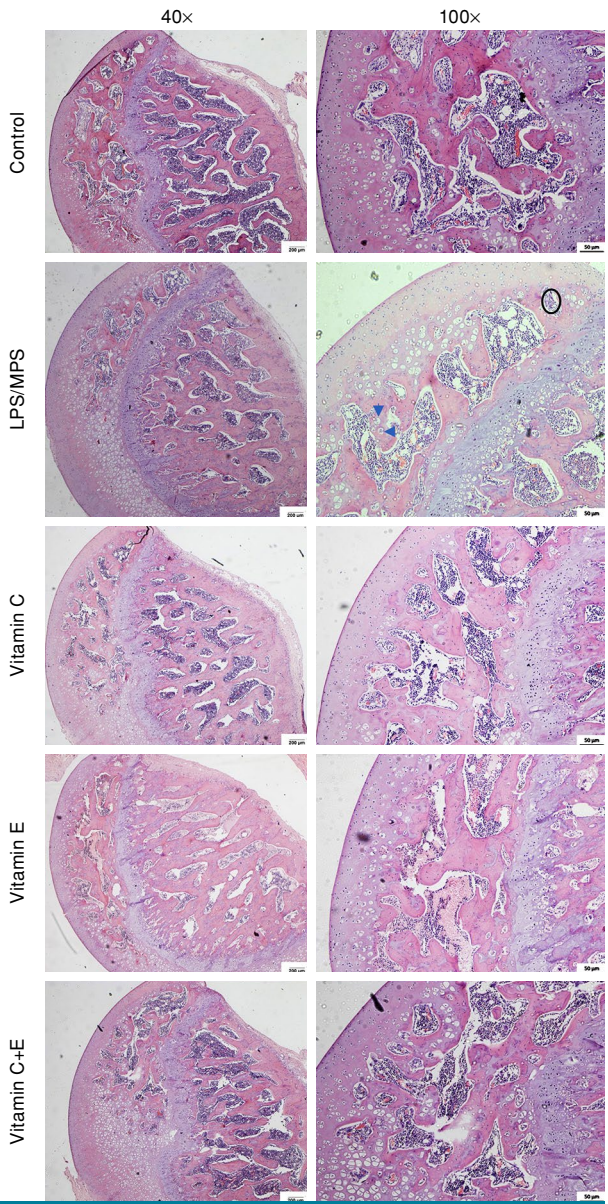

FIGURE 1. Light microscopic images of femoral head sections in the avascular necrosis model. The osteonecrosis was defined by the presence of empty lacunae or osteocytes with pyknotic nuclei (blue arrowhead) in bone trabeculae and the necrosis in the bone marrow (black circle) accompanying the diffuse distribution of osteocytes in the ONFH model (H&E, ×40 and ×100).
LPS/MPS: Lipopolysaccharide/Methylprednisolone.

statistically significant difference was observed. While there was no significant difference in the mean BS/BV in the VC and VE groups compared to the LPS/MPS group, it was significantly increased in the VC+VE group compared to the LPS/MPS group ($p<0.01$).

Histopathological findings

Figure 1 depicts light micrographs illustrating the identification of osteonecrosis. Osteonecrosis is characterized by the presence of empty lacunae or osteocytes with pyknotic nuclei within bone trabeculae. Furthermore, the necrosis in the bone marrow is observed with the widespread distribution of osteocytes in the ONFH model.

The incidence of osteonecrosis exhibited a substantial rise in all rats belonging to the LPS/MPS group, whereas it showed a significant decrease in the VC, VE, and VC+VE groups in comparison to the LPS/MPS group ($p=0.0003$, (Table II). An anomaly in the femoral head, characterized by a pale anterolateral area, was found in all animals belonging to the LPS/MPS group. This observation revealed a statistically significant difference between the groups ($p=0.0015$).

The prevalence of animals exhibiting partly regular, thin, spaced, and fibrotic trabeculae was found to be considerably greater in the LPS/MPS group, whereas it was shown to be reduced in the groups that received vitamin supplementation in comparison to the LPS/MPS group ($p<0.05$). The presence of regular trabeculae was mostly detected in the control group in comparison to the other groups ($p<0.0001$). There was no statistically significant difference between the groups regarding granulation. The LPS/MPS group exhibited a substantial decrease in the number of animals with trabeculae containing prominent osteocytes, whereas the number of animals with trabeculae containing sparse osteocytes was increased compared to the control group (Table II, $p=0.0026$).

The number of animals with decreased cellularity of bone marrow was higher in the LPS/MPS group than those in other groups ($p=0.042$). Normal morphology of bone marrow was only observed in one of the rats of the LPS/MPS group. Hemorrhage and vascular congestion in the bone marrow were detected in all of the LPS/MPS group ($p<0.0001$ and $p<0.0239$, respectively; Table II).

The rates of pyknotic cells and empty lacunae in trabeculae were significantly higher in the LPS/MPS group than those in the control group ($p=0.0158$ and $p=0.017$, respectively), while these rates decreased in the vitamin-administered groups, which were comparable with the rate of the control group. The vascular thrombosis did not show any significant difference between groups ($p=0.419$, Table II).

TABLE II
Comparison of histopathological findings of femoral head sections according to the groups (n=6)

Parameters	Control X/N	LPS/MPS X/N	Vitamin C X/N	Vitamin E X/N	Vitamin C+E X/N	*p*
Osteonecrosis	0/6	6/6	1/6	1/6	0/6	0.0003
Pale anterolateral region	0/6	6/6	1/6	1/6	4/6	0.0015
Bone Trabeculae						
Regular	6/6	0/6	1/6	0/6	0/6	**<0.0001**
Partially regular	0/6	5/6	4/6	3/6	6/6	**0.0053**
Irregular	0/6	1/6	1/6	3/6	0/6	0.125
Thin	0/6	6/6	5/6	4/6	6/6	**0.0006**
Intermittent/Space	0/6	6/6	1/6	4/6	2/6	**0.0034**
Granulation	0/6	0/6	0/6	0/6	1/6	0.388
Fibrosis	0/6	6/6	0/6	0/6	0/6	**<0.0001**
Osteocytes						
Marked	6/6	0/6	3/6	5/6	5/6	**0.0026**
Sparse	0/6	6/6	3/6	1/6	1/6	**0.0026**
Bone marrow						
Normal	6/6	1/6	3/6	3/6	4/6	0.0621
Decreased cellularity	0/6	5/6	3/6	4/6	4/6	**0.042**
Increased adipocyte %	0/6	5/6	3/6	4/6	3/6	0.053
Hemorrhage	0/6	6/6	4/6	4/6	5/6	**0.0049**
Vascular congestion	0/6	6/6	6/6	6/6	6/6	**<0.0001**
Megakaryocyte	4/6	3/6	0/6	0/6	1/6	**0.0239**
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	*p*
Pyknotic cell (%)	2.36±2.21	14.12± 2.19[a]	13.8±6.65	10.76±5.28	13.26±7.74	**0.0158**
Empty lacuna (%)	2.17±0.94	12.87±2.88[a]	12.63±6.61[a]	14.33±11.43	10.38±4.52	**0.017**
Vascular thrombosis (%)	5.56±13.61	27.78±34.83	19.44±22.15	22.22±19.48	7.86±11.4	0.419

X/N: Number of animal with pathology / Total number of animals; HC: Hematopoietic cells; SD: Standard deviation; [a] p<0.05 *vs.* control group

TABLE III
Comparison of histomorphometric findings of femoral head sections according to the groups (n=6)

Parameters	Control Mean±SD	LPS/MPS Mean±SD	Vitamin C Mean±SD	Vitamin E Mean±SD	Vitamin C+E Mean±SD	*p*
BV/TV (%)	67.41±2.01	58.13±8.54	57.78±2.47	69.69±5.96	57.17±0.76	0.0057
BS/TV (mm^2/mm^3)	99.35±16.11	94.01±16.29	105.13±27.79	98.03±11.28	98.12±24.01	0.9347
BS/BV (mm^2/mm^3)	14759.2±2529.0	16373.2±3239.8	18124.7±4233.1	14160.9±2079.7	17187.9±4340.9	**0.4173**
Tb.Th (µm)	138.57±23.91	125.86±22.84	114.28±22.58	144.85±21.37	121.14±28.58	**0.4747**
Tb.N (mm^{-1})	0.050±0.008	0.047±0.008	0.053±0.014	0.049±0.005	0.049±0.012	**0.9122**

SD: Standard deviation; BV/TV: Bone Volume/Total Volume; BS/TV: Bone circumference/total volume; BS/BV: Bone surface area/bone volume; Tb.Th: Trabecular thickness; Tb.N: Trabecular number.

Histomorphometric findings

The histomorphometric findings are presented in Table III. The mean BV/TV was considerably lower in the LPS/MPS (58.13±8.54%), VC (57.78±2.47%), and VC+VE (57.17±0.76%) groups compared to the control (67.41±2.01%) and VE (69.69±5.96%) groups (p=0.0057). There was no significant difference in the mean ratio of BS/TV and BS/BV between the groups (p=0.9347 and p=0.4173, respectively). There were no significant differences seen in the mean values of Tb. Th and Tb. N between

the control and experimental groups (p=0.4747 and p=0.9122, respectively).

Immunohistochemical findings

Figures 2 and 3 represent the immunohistochemical findings of ONFH model at tissue level. Immunoreactivity of 8-OHdG in the chondrocytes and trabeculae increased significantly in the LPS/MPS group compared to other groups (p=0.016, Figures 2, 3) but decreased in the chondrocytes and trabeculae of vitamin-administered groups (p<0.05). No significant difference was detected in MDA and Bcl-2 levels among the groups (Figure 3). A significant decrease was found in caspase-3 level of the bone marrow of the VC+VE group compared to the LPS/MPS group (p=0.0029), but no difference was detected in caspase-3 levels of chondrocytes and trabeculae between groups (Figure 3).

The immunoreactivity of PECAM/CD31 and VEGF was only assessed in vascular structures to evaluate the extent of vascular injury (Figures 2, 3). No significant difference was found for both immunoreactivities among all groups (Figure 3).

Biochemical findings

Biochemical analysis showed that the serum MDA levels increased significantly in the LPS/MPS and VE groups compared to other groups (p=0.0006) but decreased in the VC+VE group (Table IV). The mean GSH levels decreased in the LPS/MPS group compared to the control group but significantly increased in the vitamin-administered groups compared to the LPS/MPS group (p=0.0002, Table IV). No statistically significant difference was found in the serum BALP, CTX, and OPG levels between groups (p>0.05, Table IV).

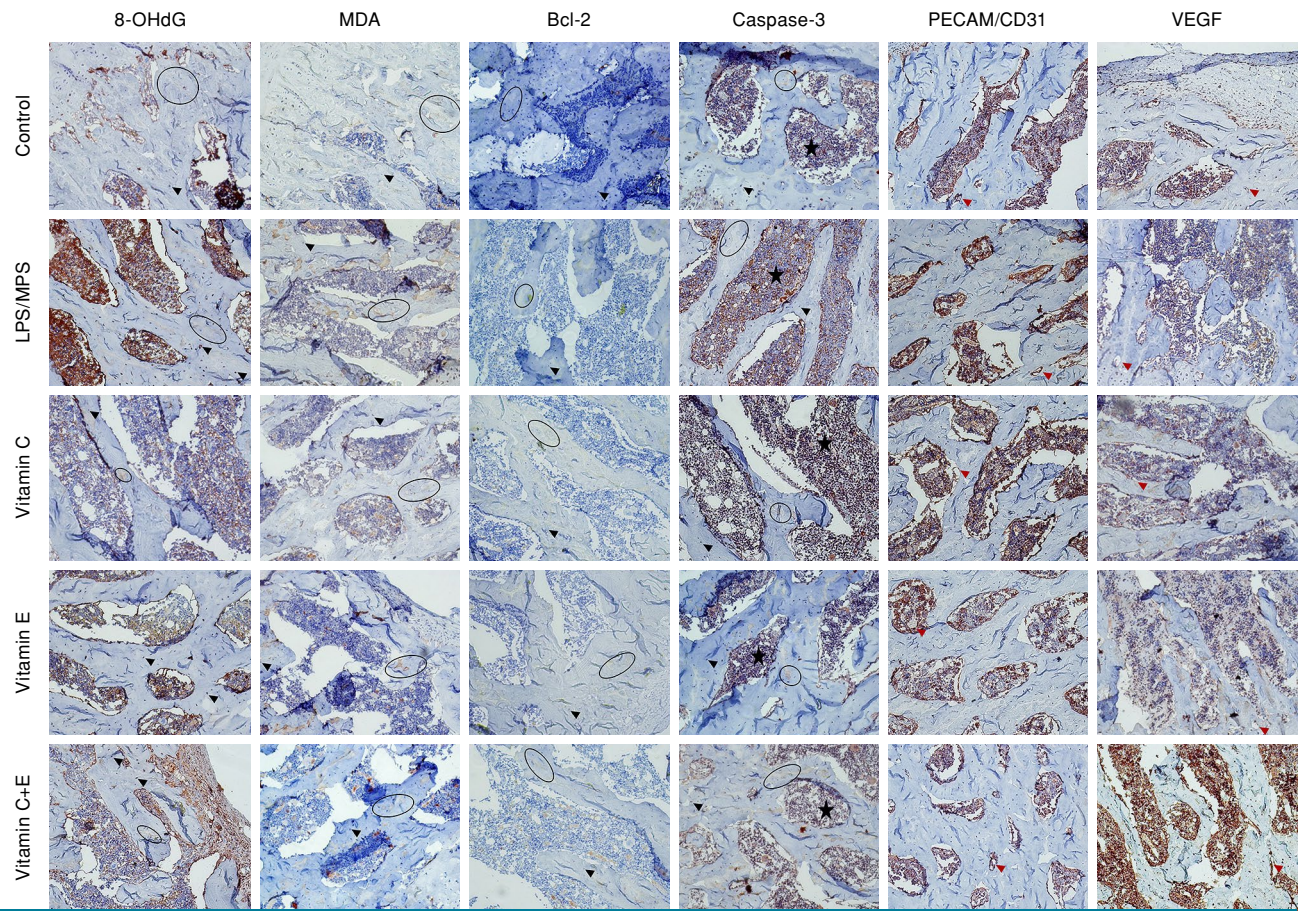

FIGURE 2. Light microscopic images of immunohistochemical analysis of femoral head sections in the avascular necrosis model. Chondrocytes are indicated by black arrowhead, trabeculae by black circles, bone marrow by black star, and vessels by red arrow.

8-OHdG: 8-hydroxy-20-deoxyguanosine; MDA: Malondialdehyde; Bcl-2: B-cell lymphoma 2; PECAM/CD31: Platelet/endothelial cell adhesion molecule 1; VEGF: Vascular endothelial growth factor; LPS/MPS: Lipopolysaccharide/methylprednisolone (H&E, ×200).

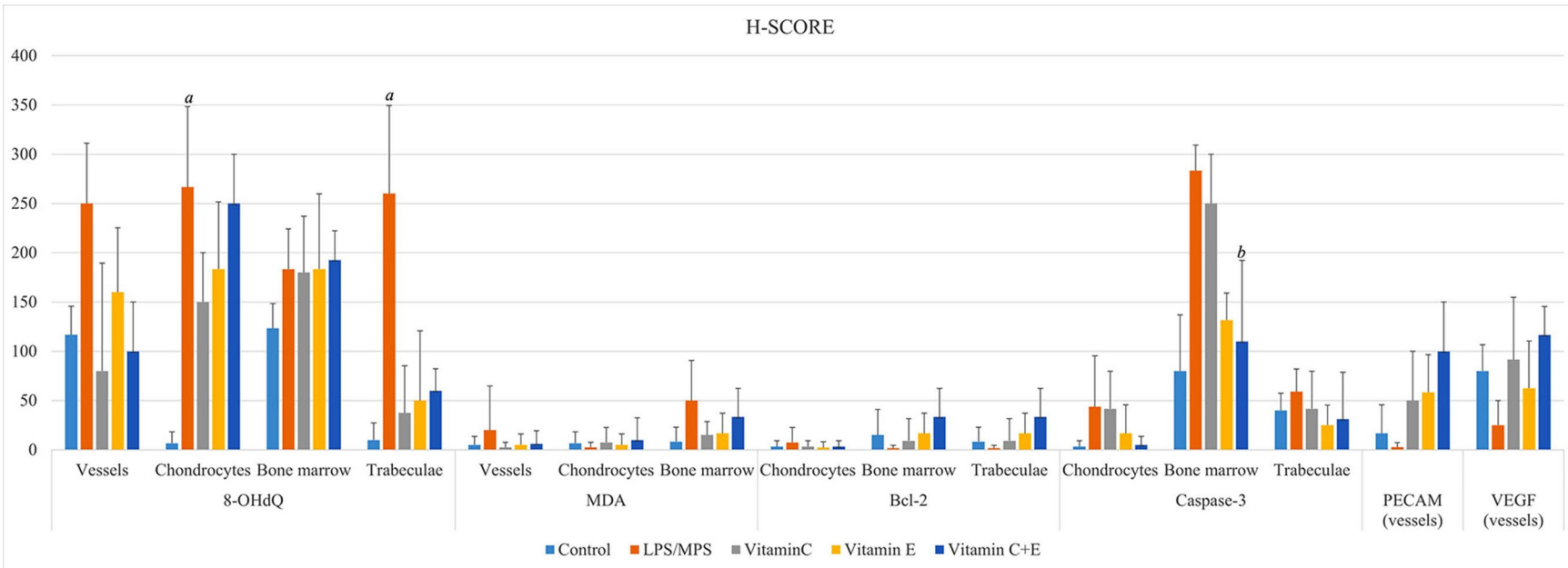

FIGURE 3. Graphical presentation of immunoreactivities of femoral head sections in the avascular necrosis model and statistical comparison of immunoreactivities according to the groups.

8-OHdG: 8-hydroxy-20-deoxyguanosine; MDA: Malondialdehyde; Bcl-2: B-cell lymphoma 2; PECAM/CD31: Platelet/endothelial cell adhesion molecule 1; VEGF: Vascular endothelial growth factor; LPS/MPS: Lipopolysaccharide/methylprednisolone; a: $p<0.05$ *vs.* control group; b: $p<0.01$ *vs.* LPS/MPS group. All values are given as mean and error bars are standard deviations.

TABLE IV
Comparison of biochemical findings of femoral head sections according to groups (n=6) with the avascular necrosis model

Parameters	Control Mean±SD	LPS/MPS Mean±SD	Vitamin C Mean±SD	Vitamin E Mean±SD	Vitamin C+E Mean±SD	*p*
Oxidative stress						
MDA (nmol/mL)	3.54±0.40	7.75±0.93[a]	6.35±0.68	7.17±0.77[a]	4.15±0.31	**0.0006**
GSH (mmol/L)	91.33±9.85	50.5±19.3	111.2± 16.87[b]	125±17.8[c]	135±11.64[d]	**0.0002**
Osteoprotection						
BALP (ng/mL)	51.38±6.39	54.5±11.2	55.17±6.37	50.75±2.99	61.2±4.76	0.108
CTX-1 (ng/mL)	29.88±7.66	34.67±5.79	37.67±5.76	36.25±5.25	32±6.04	0.212
OPG (ng/mL)	3.19±0.79	2.8±0.49	2.85±0.24	2.98±0.39	3.06±0.77	0.875
Lipid profile						
TG (mmol/L)	2.84±0.61	6.05±0.37[e]	4.67±0.53	4.35±0.58	5.28±0.41	**0.0003**
TC (µmol/L)	29.88±11.80	65.5±7.71[e]	60.0±9.14[f]	49.75±10.2	56.0±4.36	**0.0009**
HDL (µmol/L)	103.2±13.37	69.0±9.74	94.67±9.20	143.75±41.9[c]	149±30.9[c]	**0.0006**
LDL (mmol/L)	4.42±0.29	5.81±0.55	3.9±0.33c	4.13±0.34	3.66±0.49[d]	**0.0004**

LPS/MPS: Lipopolysaccharide/methylprednisolone; SD: Standard deviation; MDA: Malondialdehyde; GSH: Glutathione; BALP: Bone alkaline phosphatase; CTX-1: Carboxy-terminal type 1 collagen crosslink; OPG: Osteoprotegerin; TG: Triglyceride; TC: Total cholesterol; HDL: High density lipoprotein; LDL: Low density lipoprotein; a: p<0.01, e: p<0.001, f: p<0.05 *vs.* control group; c: p<0.01, d: p<0.001 *vs.* LPS/MPS group.

The mean TG and TC concentrations increased significantly in the LPS/MPS group compared to the control group (p<0.001), whereas all vitamin-administered rats showed decreased concentrations of TG and TC compared to the LPS/MPS group (p=0.0003 and p=0.0009, respectively), except for the TC concentration in the VC group did not reduce to the control levels (Table IV).

The mean HDL concentrations increased significantly in the VE and VC+VE groups compared to the LPS/MPS group (p=0.0006), while mean LDL concentrations decreased significantly in the VC and VC+VE groups compared to LPS/MPS group (p=0.0004, Table IV).

DISCUSSION

Osteonecrosis is predominantly characterized by the pathological progression of femoral head impairment, leading to the sequential occurrence of subchondral bone fracture, collapse of the femoral head surface, and eventual destruction of the hip joint. We found that the VC+VE combination group had significantly better results than the other groups regarding Tb. N, BS/BV, some histopathological findings, MDA, GSH, the lipid profile, and caspase-3.

Steroid consumption is the predominant etiological factor for nontraumatic osteonecrosis.[14] Acute lymphoblastic leukemia, rheumatoid arthritis, systemic lupus erythematosus, and other disorders are all treated with steroids as indispensable medications. However, one of the most dangerous conditions for orthopedists is steroid-induced ONFH, and the only alternative for therapy at the terminal stage is a hip replacement.[15] Studies on epidemiology in East Asia reveal that steroids were directly related to 47.4% of all cases classified as nontraumatic ONFH.[16] Finding novel treatments is crucial since ONFH has major effects and incurs expensive costs.[17] Further investigation is required to ascertain the exact mechanism behind ONFH generated by steroid use. Oxidative stress disorders, however, may be one of the most prevalent risk factors among all those mentioned, and they may also benefit the most from intervention.

Pharmacological interventions for steroid-induced ONFH, such as vasodilators, statins, bisphosphonates, and anticoagulants, exert their therapeutic effects via several mechanisms; nevertheless, no single intervention has demonstrated superior efficacy compared to the others.[18] The study conducted by Karakaplan et al.[19] demonstrated that the utilization of platelet-rich plasma therapy yields beneficial effects on ONFH. Despite the wide range of therapy options available, there is still no significant agreement on which of these treatment is more effective than the others. Therefore, a safe and effective therapy to stop ONFH in steroid-treated patients is still required.

The morphometric assessment of trabecular architectural alterations in ONFH may be conveniently performed by micro-CT analysis. Jiang

et al.[20] showed that tetramethylpyrazine treatment protected rats from steroid-induced effects on the levels of the above microstructural parameters such as Tr. N and BS/BV. Additionally, another study showed that salvianolic acid B treatment significantly reversed in micro-CT the steroid-induced changes in bone volume, Tb. Pf., Tb. Th, and Tb. N in rats.[21] Zhou et al.[22] showed that cartilage oligomeric matrix protein-angiopoietin 1 treatment in femoral head necrosis in rats resulted in significantly improved trabecular number and thickness and BV/TV in micro-CT compared to the other groups. In parallel with these findings in the literature, Tr. N and BS/BV were found to be significantly higher than other groups in our study.

In recent times, there has been a growing recognition of osteocyte apoptosis as a potential mechanism behind the development of osteonecrosis resulting from the use of corticosteroids.[23,24] Apoptotic osteocytes were observed during the pathological examination of femoral heads in patients who had undergone total hip arthroplasty due to steroid-induced ONFH. These findings have not been documented in instances of ONFH that arise from traumatic events or excessive alcohol use.[25] In their study investigating the impact of erythropoietin on glucocorticoid-induced osteonecrosis in rats, Chen et al.[14] observed that the administration of steroids resulted in an elevation of the apoptotic index in osteoblasts and osteocytes, as well as an increase in the immunoreactivity of caspase-3, a protein associated with promoting apoptosis. However, the application of erythropoietin effectively mitigated these effects.[14] Similar to these findings, a significant decrease was found in the caspase-3 level of the bone marrow of the VC+VE group compared to the LPS/MPS group in our study.

Several studies have indicated that disruption of lipid metabolism may serve as a key factor behind the development of obesity-related hyperlipidemia.[26,27] Hyperlipidemia has the potential to have detrimental effects on vascular endothelial cells, resulting in the development of prothrombotic circumstances. Additionally, it can contribute to the occurrence of fat embolism within the peripheral blood, leading to the obstruction of bone microvessels. Consequently, this process elevates intraosseous pressure and exacerbates the impairment of bone microcirculation.[28,29] The association between the LDL/HDL ratio and the development of osteonecrosis has been established, and an elevation in this ratio has been found to potentially contribute to an enlargement of adipocytes inside the bone marrow.[30] In our study, the VC+VE combination significantly reduced the mean TG and TC concentrations and increased the mean HDL concentrations compared to the LPS/MPS group.

Osteonecrosis of the femoral head is a pathological disorder affecting the musculoskeletal system, defined by the progressive degeneration of the femoral head, which eventually results in its complete collapse during the later stages of the disease. The cellular and molecular mechanisms responsible for steroid-induced osteonecrosis are still a matter of debate and disagreement in the academic community. According to recent study findings, the increased quantity and enhanced activity of osteoclasts have been identified as the underlying cause of bone loss in the femoral head, ultimately resulting in its failure.[31-33] A study examined whether changes in ROS level or osteoclasts contributed to the pathogenesis of ONFH following steroid use by promoting osteoclast formation and resorption. As a result, ROS-bound enzymes and osteoclasts were detected in ONFH samples of both patients and rodent models mimicking ONFH.[34] The existence of oxidation inside bone has been shown by the identification of anti-8-OHdG antibody expression, which serves as an indicator of oxidative stress. This expression has been seen in arterioles located in the medial vascular muscle of femoral heads that were surgically removed from individuals diagnosed with steroid-induced osteonecrosis.[35] In our study, 8-OHdG immunoreactivity in the chondrocytes and trabeculae increased significantly in the LPS/MPS group and decreased in the vitamin-administered groups.

Antioxidant vitamins possess a diverse range of biological functions. Vitamin C and VE are well-known antioxidants. Vitamin E was thought to be an important chain-breaking antioxidant. Vitamin C is another important water-soluble antioxidant.[10] Vitamin E is also the most important lipophilic antioxidant and is found mainly in cell membranes and helps maintain membrane stability. Vitamin C is the most important free radical scavenger in extracellular fluids and protects biomembranes from peroxidative damage.[10] Our study focused on the effects of VC and VE given alone or in combination on oxidative stress parameters in rats with steroid-induced ONFH.

Further investigation has shown that antioxidants possess the capability to efficiently eradicate free radicals or sustain the equilibrium of free radicals, consequently playing a significant role in upholding regular cellular metabolism and averting DNA damage.[36] Vitamin E is a powerful free radical

scavenger that interacts with active free radicals and converts lipid peroxide into hydroxyl lipids.[37] It may also inhibit VE oxidative enzymes and activate the antioxidase system.[38] Hence, the administration of VE can enhance the antioxidant capacity and safeguard DNA from damage caused by free radicals. Elevated levels of MDA, in conjunction with lower levels of GSH in serum, serve as significant indications of oxidative damage.[39] In our study, increasing MDA levels and decreasing GSH levels in serum with LPS/MPS application indicate increased oxidative stress, and in the group administered VC+VE combination, these rates were reversed, showing that antioxidant mechanisms were working strongly.

In one study, VE statistically significantly reduced the incidence of osteonecrosis in a rabbit model with corticosteroid-induced osteonecrosis.[40] This finding suggests that VE intake may prevent corticosteroid-induced osteonecrosis. However, there were still rabbits that developed osteonecrosis. Vitamin E did not reduce the number of sites of development of osteonecrosis in individual rabbits that have developed osteonecrosis. They found no effect of VE on the severity of osteonecrosis. In conclusion, it shows that the cause of osteonecrosis is multifactorial.[24,40] There may be a threshold in the development of osteonecrosis, which may mean the beginning of the development of osteonecrosis, even if prophylactic treatment is prescribed once it is reached.[41]

Two commonly used inductive methodologies were frequently employed to prove the relevance of osteonecrosis in relation to steroid use in animals. The prevalence was as low as 43% while using a single injection of high-dose MPS (20 mg/kg). The alternative was to inject three high-dose MPS injections (20 mg/kg) after two high-dose LPS (100 μg/kg) injections, which resulted in a greater osteonecrosis incidence (85%). We used the technique that was described in the literature, and histopathological examination revealed that it had an acceptably high rate of site-specific femoral head necrosis.[42,43] The diagnosis of osteonecrosis was established in histopathology by seeing empty lacunae or pyknotic osteocyte nuclei within the bone trabeculae, accompanied by necrosis of surrounding bone marrow cells. In our study, the rate of osteonecrosis in the LPS/MPS group was 100% (6/6), and no rat died during the period, proving that this model is secure and reliable.

The comprehensive elucidation of the exact pathophysiological process underlying the onset of steroid-induced ONFH has to be fully established. However, among the several risk factors that have been discovered, oxidative stress disorders appear to be particularly prevalent. Interventions targeting oxidative stress disorders may thus provide significant benefits.[44] Strong dietary antioxidants, VC, and VE directly decrease oxidative stress by contributing their electrons to antioxidant processes to stop the oxidation of other molecules. Two *in vitro* studies have demonstrated the antioxidant effects of VC and VE.[45,46]

Experimental studies have yielded data suggesting that oxidative stress plays a substantial role in the etiology of steroid-induced ONFH. Multiple studies have provided evidence indicating that locations of steroid-induced ONFH display oxidative stress, whereas the progression of osteonecrosis can be notably inhibited by the use of antioxidants.[47-49] In an animal model, oxidative damage occurred in the bone marrow after steroid administration and before the development of ONFH.[50] The study conducted by Ichiseki et al.[2] revealed that the mitigation of oxidative stress has the potential to decrease the occurrence of ONFH in both rabbits and rats,[2,51] but Kuribayashi et al.[41] indicated that VE with no impact on lipid metabolism might prevent ONFH in rabbits with an antioxidant effect. In this study, we also constructed a steroid-induced ONFH model and explored the antioxidant effects of VC, VE, and their combination.

The findings suggest that VC, VE, and a combination of both may have a protective effect against the progression of osteonecrosis in the rat model, possibly by decreasing oxidative stress and apoptosis. The lack of significant difference in BMD between the groups indicates that further studies with longer treatment periods may be necessary to see the impact of vitamins on BMD in this model. Additionally, the study is limited by the use of only a single rat model, and the results may not be directly applicable to humans. Further studies with larger sample sizes and different animal models, as well as studies in humans, are necessary to fully understand the potential benefits and limitations of vitamins in the treatment of osteonecrosis.

In conclusion, the VC+VE combination therapy had a significant positive effect on Tb. N, BS/BV, some histopathological findings, MDA, GSH, the lipid profile, and caspase-3 in rats with steroid-induced ONFH. The results of this study may lead to new treatment options for patients with this condition and help reduce the need for total joint replacement. Additionally, the study will contribute

to our understanding of the underlying mechanisms of osteonecrosis and may lead to the discovery of new targets for therapeutic intervention.

Acknowledgments: We would like to thank the Istanbul Bağcılar Experimental Research and Skill Development Center for their kind contributions and support. We also appreciate the assistance of Göker Külüslü from the Istanbul Technical University Biomedical Engineering Department for his help during the micro-CT scan analyses.

Ethics Committee Approval: The study protocol was approved by the Istanbul Bağcılar Training and Research Hospital Animal Experiments Local Ethics Committee (date: 31.07.2018, no: 2018-90). All animal experiments were conducted in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (revised, 1985).

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: Writing, editing, analysis: O.B.; Writing, data collection, statistics, proofreading: M.F.D.; Data collection, analysis, proofreading: A.G.Y.; Data collection, proofreading, editing: B.Y.B.; Supervision, proofreading, editing: E.A.; All authors contributed to the study conception and design. All authors read and approved the final manuscript.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding: The authors received no financial support for the research and/or authorship of this article.

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