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ORIGINAL ARTICLE

The effects of thymoquinone on steroid-induced femoral head osteonecrosis: An experimental study in rats

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Steroid-induced osteonecrosis (ON) of the femoral head represents one of the most significant complications of long-term high-dose steroid use.[1] Steroids are commonly used in the treatment of chronic autoimmune diseases such as systemic lupus erythematosus, nephrotic syndrome, asthma, after the organ transplantation, and in the patients hospitalized due to novel coronavirus disease-2019 (COVID-19).[2] The early diagnosis of ON of the femoral head (ONFH) is critical, since it mainly influences the young and middle-aged population, and progression of the disease to collapse of the femoral head occurs in most patients, if untreated.

Although it has been argued that steroid-induced ON develops due to an ischemia in the bone,

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ABSTRACT

Objectives: In this study, we aimed to evaluate the antioxidant, anti-apoptotic, osteoblastic and hypolipidemic effects of thymoquinone (TQ) treatment on the steroid-induced osteonecrosis of femoral head (ONFH) model in rats.

Materials and methods: A total of 24 rats were randomly divided into four groups: the control group administered saline; the TQ group administered 10 mg/kg/day TQ orally; lipopolysaccharide/methylprednisolone (LPS/MPS) group administered 20 µg/kg intraperitoneally LPS and 40 mg/kg intramuscularly MPS to establish ONFH model; and the LPS/MPS+TQ group administered both LPS/MPS and, then, TQ once daily for four weeks. All rats were sacrificed after intracardiac blood collection and their right femurs were removed.

Results: Micro-computed tomography showed a higher bone mineral density and lower porosity, Tr.Sp and Tr.Sep data were detected in the LPS/MPS+TQ group. In histopathology, osteonecrosis increased significantly in the LPS/MPS group and osteonecrosis decreased in the LPS/MPS+TQ group compared to the LPS/MPS group ($p=0.0077$). Histomorphometric examination revealed that the percentage of BV/TV in the LPS/MPS group was significantly lower compared to control and other groups ($p<0.01$ and $p<0.05$, respectively), while it reached normal rates in the LPS/MPS+TQ group. Immunohistochemically, antioxidant, anti-apoptotic, and angiogenesis indicators (8-hydroxy-20-deoxyguanosine [8-OHdG], malondialdehyde [MDA], B-cell lymphoma [Bcl-2], caspase-3, vascular endothelial growth factor [VEGF]) were significantly improved in tissue and serum with TQ. Furthermore, TQ significantly reduced low- and high-density lipoprotein cholesterol ratio and carboxy-terminal type 1 collagen crosslink (CTX) in serum.

Conclusion: Vascular and hematopoietic cell damages that occur due to steroid-induced deoxyribonucleic acid (DNA) oxidative and lipid peroxidative damages in an ONFH model can be successfully ameliorated by TQ administration. This antioxidant and anti-apoptotic effects of TQ may be a promising treatment option for early stage of osteonecrosis.

Keywords: Lipopolysaccharide, methylprednisolone, osteonecrosis of femoral head, thymoquinone.

the detailed mechanism of corticosteroid use in the development of ON still remains unclear. Several studies have suggested the thrombotic and fibrinolytic disorders predisposing both hypercoagulation and hypofibrinolysis as the possible etiologic causes of steroid-induced ON. The decrease of autophagy level induced by dexamethasone may be one of the causes of steroid-induced ON.[3] Vascular endothelial dysfunction, which is capable of increasing hypercoagulation, has also played a role in the pathophysiology of steroid-induced ON. Recent studies have reported that *in vivo* oxidative stress plays an essential role in the pathogenesis of steroid-induced ON.[4]

Thymoquinone (TQ) is an active compound isolated from the Nigella sativa plant. As a naturally derived substance, TQ has recently received particular attention and has been comprehensively studied for its therapeutic properties.[5] It has been demonstrated that TQ has antioxidant and anti-inflammatory properties, positive impacts on bone metabolism, bone formation, and bone healing. Thymoquinone has various biological activities, including protective effects against articular diseases and hypolipidemic effects, as well as reducing the number of osteoclasts and increasing osteoblastic activity.[5]

The primary objective of the current research on ONFH is to prevent the development of ON after the use of steroid drugs for any reason. In this context, different treatments with anticoagulant agents, antilipidemic agents, and antioxidant agents have been suggested in early-stage ONFH. In the present study, we aimed to evaluate the effectiveness of TQ treatment in preventing ONFH in a model of steroid-induced ONFH in young adult male rats using its antioxidant, anti-osteoclastic, osteoblastic, and hypolipidemic effects.

MATERIALS AND METHODS

Animals

The study was conducted with 24 young adult male Sprague-Dawley rats aged 12 weeks and with an average weight of 356 (range, 330 to 375) g. All rats were kept daily in 22°C conditions with 12 h of light/dark cycle. Standardized rodent feed and tap water *(ad libitum)* were given unlimitedly, regardless of the period before and after drug administration. All rats were observed and followed for seven days in the animal care laboratory before the experiment to exclude the possibility of occurrence of underlying disease.

Experimental design

A methylprednisolone (MPS) and lipopolysaccharide (LPS)-induced ONFH model was established according to the literature.[6,7] Twenty-four rats were randomly allocated and divided into four groups with six rats in each group: *(i)* Control group, *(ii)* TQ group, *(iii)* LPS/MPS group, and *(iv)* LPS/MPS+TQ group. To establish a ONFH model, a total of 12 rats in LPS/MPS and LPS/MPS+TQ group were intraperitoneally administered two doses of 20 μg/kg LPS (*Escherichia coli* O55:B5, Sigma-Aldrich, St. Louis, MO, USA) on Days 1 and 2 with a 24-h interval, and the same rats were intramuscularly administered by three doses of 40 mg/kg MPS sodium succinate (Mustafa Nevzat İlaç Sanayi AŞ, İstanbul, Türkiye) with a 24-h interval (Figure 1).

Saline solution was administered to the control group by orogastric lavage once a day starting from the first day of protocol. A total of 10 mg/kg/day TQ (Sigma-Aldrich Chemistry, St. Louis, MO, USA) was administered to the TQ group via orogastric gavage once a day starting from the first day. To establish the ONFH model, LPS/MPS group was administered saline by orogastric gavage once a day starting from the first day. All rats in the LPS/MPS+TQ group were administered 10 mg/kg/day TQ (Sigma-Aldrich Chemistry, St. Louis, MO, USA) by orogastric gavage once a day starting from the first day.

At the end of fourth week, intracardiac blood samples were collected for biochemical analysis. All rats were, then, sacrificed under general anesthesia and their right femurs were surgically removed. All bones were preserved in 10% formaldehyde for histological, immunohistochemical, and radiological evaluations.

Radiological examination

All the excised femur bones were prepared for three-dimensional (3D) scanning and analysis by micro-computed tomography (CT) (SkyScan 1174v2, Kontich-Belgium). Each sample was scanned 360 degrees with 0.90-degree rotation in about 58 min. Four hundred raw images in the TIFF format obtained after scanning were reconstructed using the NRecon (v. 1.6.10.2) program, and approximately 780 horizontal sections in the BMP format were obtained. Calibrations for bone mineral density (BMD) were first applied to dataset. All BMDs were analyzed using calcium densities 0.25 g/mm^3 and 0.75 g/mm^3 CaHA calibration bar (phantom).

The images were, then, transferred to CTAn (v. 1.16.4.1+) program, which allows densitometric

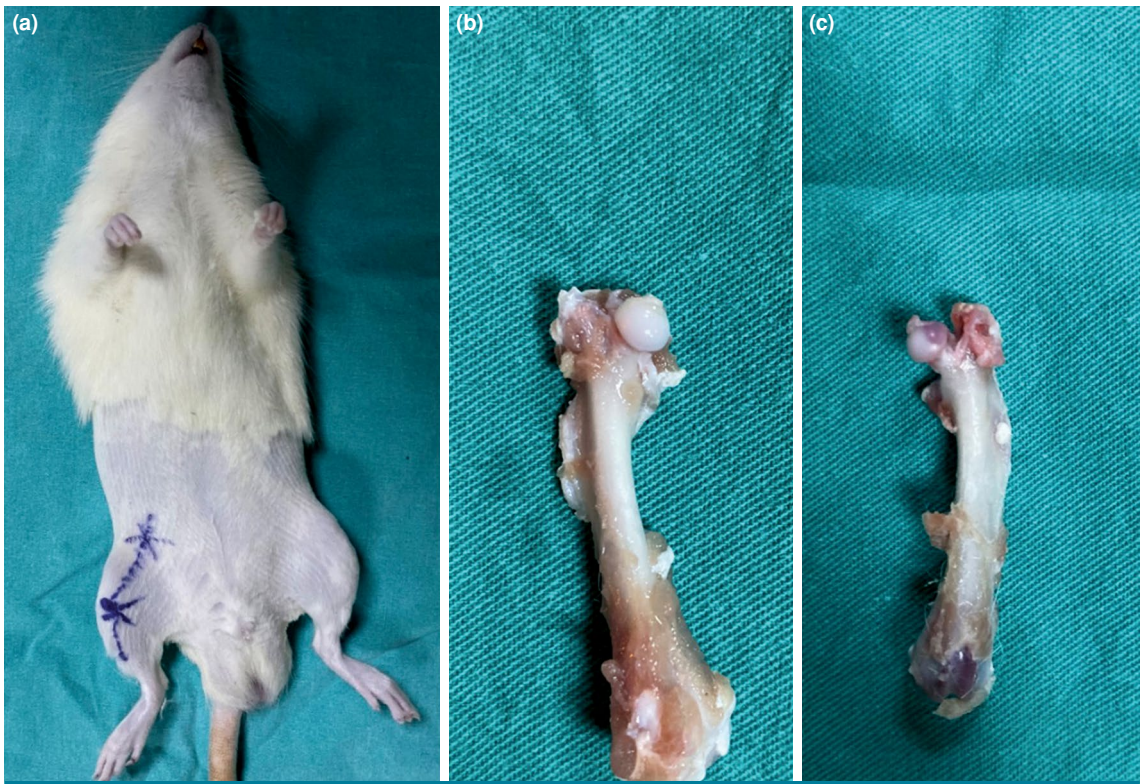

FIGURE 1. (a) Pre-surgical view of the femur after rat sacrification. **(b)** Post-removal view of a rat femur of the control group. **(c)** Osteonecrosis induced femoral head in a rat femur.

and morphometric measurements to create quantitative parameters and visual models. To determine the boundaries of area to be measured, a circular region of interest (ROI) was drawn semi-automatically on the horizontal plane on sections. Volumetric ratios were calculated separately with ROIs and threshold data. The data of samples were transferred to CTVol (v. 2.3.2.0) program, and 3D modeling images were obtained (Figure 2).

Using the ROI created in the femoral head, BMD in g/cm^3, bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), porosity, trabecular distance (Tr.Sp.), trabecular separation (Tr.Sep), trabecular bone model factor (Tb.Pf.), and bone surface area/bone volume (BS/BV) were calculated using CTAn software (v. 1.16.4.1+).

Histopathological and histomorphometric examinations

The sections were fixed in 10% buffered formaldehyde solution and decalcified in a 10% formic acid solution for three days. After a routine tissue tracking and tissues were embedded in paraffin blocks. A total of 3 to 4-µm longitudinal sections were prepared and stained with hematoxylin-eosin (H&E). The histopathological examination was performed under light microscope (Zeiss Axio microscope with Zen 2.3 lite program) and photographed by an attached camera (AxioCam IC).

The histomorphometric measurements were performed by AutoCAD 2017 (Autodesk) program in accordance with the literature and standard nomenclature.[8,9] The following parameters were calculated: percentage of BV/TV, trabecular bone circumference/total volume (BS/TV, mm^2/mm^3), BS/BV (mm^2/mm^3), Tb.Th (µm), Tb.N (mm^{-1}).

Immunohistochemical examination

Monoclonal and polyclonal antibodies against 8-hydroxy-20-deoxyguanosine (8-OHdG), malondialdehyde (MDA), anti-apoptotic protein B-cell lymphoma (Bcl-2) and proapoptotic protein caspase-3, and platelet/endothelial cell adhesion molecule-1 (PECAM/CD31) and vascular endothelial growth factor (VEGF) were

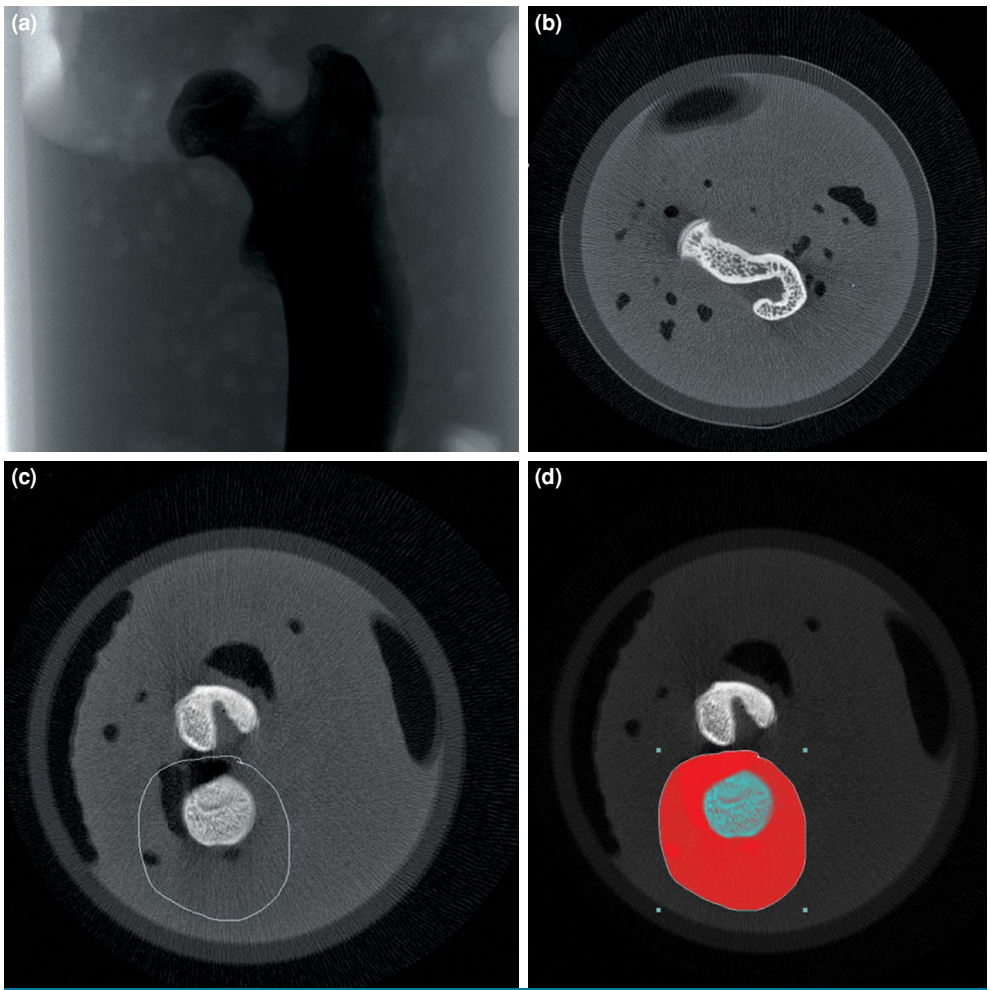

FIGURE 2. Three-dimensional visualizations of micro-CT imaging of femoral heads in avascular necrosis model. **(a)** Raw image **(b)** Horizontal section **(c)** A circular region of interest drawn semi-automatically on the horizontal plane **(d)** The designated threshold data.
CT: Computed tomography.

used for immunohistochemical staining with the streptavidin-biotin-peroxidase method as described in the literature.[8] Positive immunolabeling was analyzed blindly with a semi-quantitatively modified H-SCORE between 0 and 300.[10]

Biochemical examination

Intracardiac blood was drawn immediately after sacrification, and a hematological evaluation was carried out. Samples were centrifuged at 3,000 rpm for 10 min and stored at -80 °C until analysis. 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 cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were measured with double antibody sandwich technology enzyme-linked immunosorbent assay (ELISA) (MBS268427, MBS774706, MBS774291, MBS774451, MBS775098, MBS775451, MBS775433, MBS774773, MBS774933 MyBioSource, Inc., San Diego, CA, USA) kits.

Statistical analysis

Statistical analysis was performed using the GraphPad Instat version 3.06 software (GraphPad Inc., CA, USA). Continuous data were expressed in mean ± standard deviation (SD), while categorical variables were expressed in number and frequency. The distribution of the variables was tested using the Kolmogorov-Smirnov test. Categorical variables were compared using the chi-square test for independence. Normally distributed numerical variables were compared by one-way analysis of variance (ANOVA)

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

Groups	Control	TQ	LPS/MPS	LPS/MPS+TQ	
Parameter	Mean±SD	Mean±SD	Mean±SD	Mean±SD	*p*
Bone mineral density (g/cm^3)	0.84±0.07	0.74±0.09	0.71±0.03*	0.79±0.04	**0.032**
BV/TV (%)	34.97±4.56	34.14±11.0	26.02±5.94	26.52±2.89	0.072
Trabecular thickness (mm)	0.55±0.19	0.50±0.06	0.39±0.04	0.43±0.10	0.150
Trabecular number (mm^{-1})	61.17±30.5	56.83±24.5	28.93±30.5	35.17±18.9	0.135
Porosity (%)	66.47±1.89	71.46±1.58	76.30±3.73**	72.99±5.11*	**0.0045**
Trabecular space (mm^{-1})	0.64±0.15	0.66±0.06	1.57±0.35***	0.86±0.16	**<0.0001**
Trabecular separation (mm)	0.87±0.16	1.07±0.38	1.58±0.33**,†	1.14±0.15	**0.0027**
Trabecular bone model factor (mm^{-1})	-15.11±2.35	-15.06±3.19	-14.63±2.49	-16.15±1.46	0.739
BS/BV (mm^{-1})	6.57±1.65	5.41±2.48	4.79±0.74	4.84±0.87	0.213

SD: Standard deviation; TQ: Thymoquinone; LPS: Lipopolysaccharide; MPS: Methylprednisolone; BV: Bone volume; TV: Total volume; BS: Bone surface area; BV: Bone volume; * p<0.05; ** p<0.01 *vs.* control group; *** p<0.001 *vs.* all groups; † p<0.05 *vs.* TQ group.

test, and Tukey-Kramer multiple comparison test as a *post-hoc* test. Non-normally distributed numerical variables were compared by the Kruskal-Wallis (non-parametric ANOVA) test, and Dunn's multiple comparisons test as a *post-hoc* test. *P* values of <0.05, <0.01, and <0.001 were considered statistically significant.

RESULTS

Radiological findings

The mean BMD decreased significantly in the LPS/MPS group compared to the control group (p<0.05), but increased in the LPS/MPS+TQ group and were comparable with the control levels (Table I). The mean porosity increased significantly in both LPS/MPS and LPS/MPS+TQ groups compared to the control group (p<0.01 and p<0.05, respectively), but the increase was higher in the LPS/MPS group. The mean Tr.Sp and Tr.Sep increased significantly in the LPS/MPS group compared to the control group (p<0.001 and p<0.01, respectively), but decreased in the LPS/MPS+TQ group and were comparable with the control levels.

Histopathological findings

Figure 3 represents light micrographs. Osteonecrosis was defined by the presence of empty lacunae or osteocytes with pyknotic nuclei in bone trabeculae and, additionally, the necrosis in the bone marrow accompanying to the diffuse distribution of osteocytes.

Table II shows the distributions of histopathological findings in the ONFH model. Osteonecrosis increased significantly in the LPS/MPS group, while it decreased significantly in the LPS/MPS+TQ group compared to the LPS/MPS group (p=0.0077). Femoral head abnormality was observed in two animals of LPS/MPS group, and no statistically significant difference was observed between the groups (p=0.242).

The number of animals with irregular, thin, spaced and broken trabeculae was significantly higher in the LPS/MPS group, while it was lower in the LPS/MPS+TQ group (p<0.05) compared to the control group. The number of animals with regular trabeculae was significantly higher in control and TQ groups compared to other groups (p=0.0148). While the number of animals with trabeculae containing prominent osteocytes was significantly lower in the LPS/MPS group (p=0.046), those with trabeculae containing sparse osteocytes was also low compared to the other groups (p=0.0017) (Table II).

The number of animals with increased hematopoietic cell count was significantly higher in the TQ group than in other groups (p=0.0024). Normal morphology of bone marrow was not observed in any of the animals in the LPS/MPS group, and hemorrhage and necrosis were detected in most of them. Additionally, the majority had bone marrow with decreased hematopoietic cell count, but these data did not significantly differ between the groups (Table II).

The rates of pycnotic cells, empty lacunae, and thrombosed vessels in trabeculae were significantly higher in the LPS/MPS group than those in the control group (p=0.0167, p=0.0158, and p=0.0308, respectively), while these rates decreased in the LPS/MPS+TQ group and were comparable with the control group (Table II).

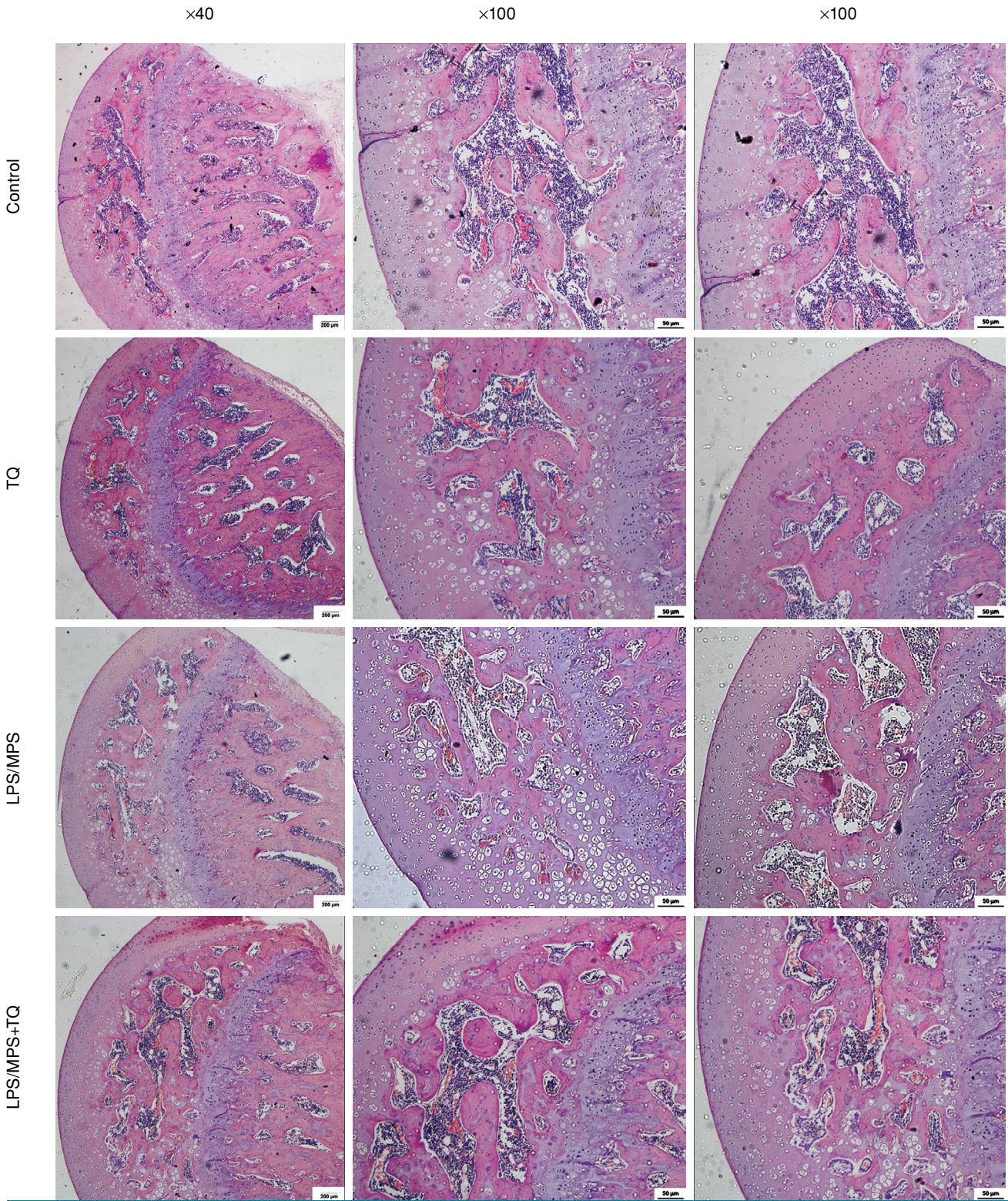

FIGURE 3. Light microscopic images of femoral head sections in avascular necrosis model. H&E, ×40 and ×100.
LPS: Lipopolysaccharide; MPS: Methylprednisolone; TQ: Thymoquinone.

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

Groups	Control	TQ	LPS/MPS	LPS/MPS+TQ	
Parameter			X/n		*p*
Osteonecrosis	0/6	1/6	5/6	1/6	**0.0077**
Femur head anomaly	0/6	0/6	2/6	1/6	0.242
Bone trabeculae					
Regular	6/6	5/6	1/6	4/6	**0.0148**
Partially regular	0/6	1/6	0/6	2/6	0.242
Irregular	0/6	0/6	5/6	0/6	**0.003**
Thin	0/6	1/6	5/6	0/6	**0.0017**
Intermittent/space	1/6	1/6	5/6	0/6	**0.0077**
Fractured	0/6	0/6	5/6	0/6	**0.0003**
Granulation	0/6	1/6	3/6	3/6	0.142
Fibrosis	0/6	1/6	1/6	2/6	0.494
Osteocytes					
Marked	6/6	5/6	2/6	5/6	**0.046**
Sparse	0/6	1/6	5/6	0/6	**0.0017**
Bone marrow					
Normal	4/6	2/6	0/6	2/6	0.112
Increased adipocyte	4/6	5/6	1/6	4/6	0.104
Large adipocytes	2/6	4/6	0/6	3/6	0.101
Hemorrhage	4/6	5/6	5/6	4/6	0.828
Thrombus	1/6	1/6	2/6	2/6	0.828
Macrophage	4/6	2/6	2/6	2/6	0.561
Increased HC	0/6	4/6	0/6	0/6	**0.0024**
Decreased HC	2/6	1/6	5/6	2/6	0.104
HC necrosis	2/6	4/6	5/6	4/6	0.337
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	*p*
Pyknotic cell (%)	8.02±5.57	16.91±13.04	46.28±17.97*	24.05±15.75	**0.0167**
Empty lacunae (%)	12.4±7.27	16.81±7.09	24.16±6.42*	20.33±8.89	**0.0158**
Vascular thrombosis (%)	9.0±12.45	35.0±27.53	53.33±32.06*	22.22±27.22	**0.0308**

TQ: Thymoquinone; LPS: Lipopolysaccharide; MPS: Methylprednisolone; X/n: Number of animal with pathology/total number of animals; HC: Hematopoietic cells; SD: Standard deviation; * p<0.05 *vs.* control group.

Histomorphometric findings

The mean BV/TV was significantly lower in the LPS/MPS group than those in control and other groups (p<0.01 and p<0.05, respectively), while it reached normal rates in the LPS/MPS+TQ group. The mean ratio of BS/TV and BS/BV did not differ significantly between the groups (p=0.342 and p=0.339, respectively). The mean Tb.Th and Tb.N did not differ significantly between the control and experimental groups (p=0.366 and p=0.326, respectively).

Immunohistochemical findings

The 8-OHdG immunoreactivities in the vessels, adipocytes, bone marrow, and trabeculae increased significantly in the LPS/MPS group compared to the other groups (p<0.001) (Figure 4), but regressed to normal levels, particularly in vessels, adipocytes, and bone marrow in the LPS/MPS+TQ group. No significant difference was detected between the control group and TQ and LPS/MPS+TQ groups (Figure 5).

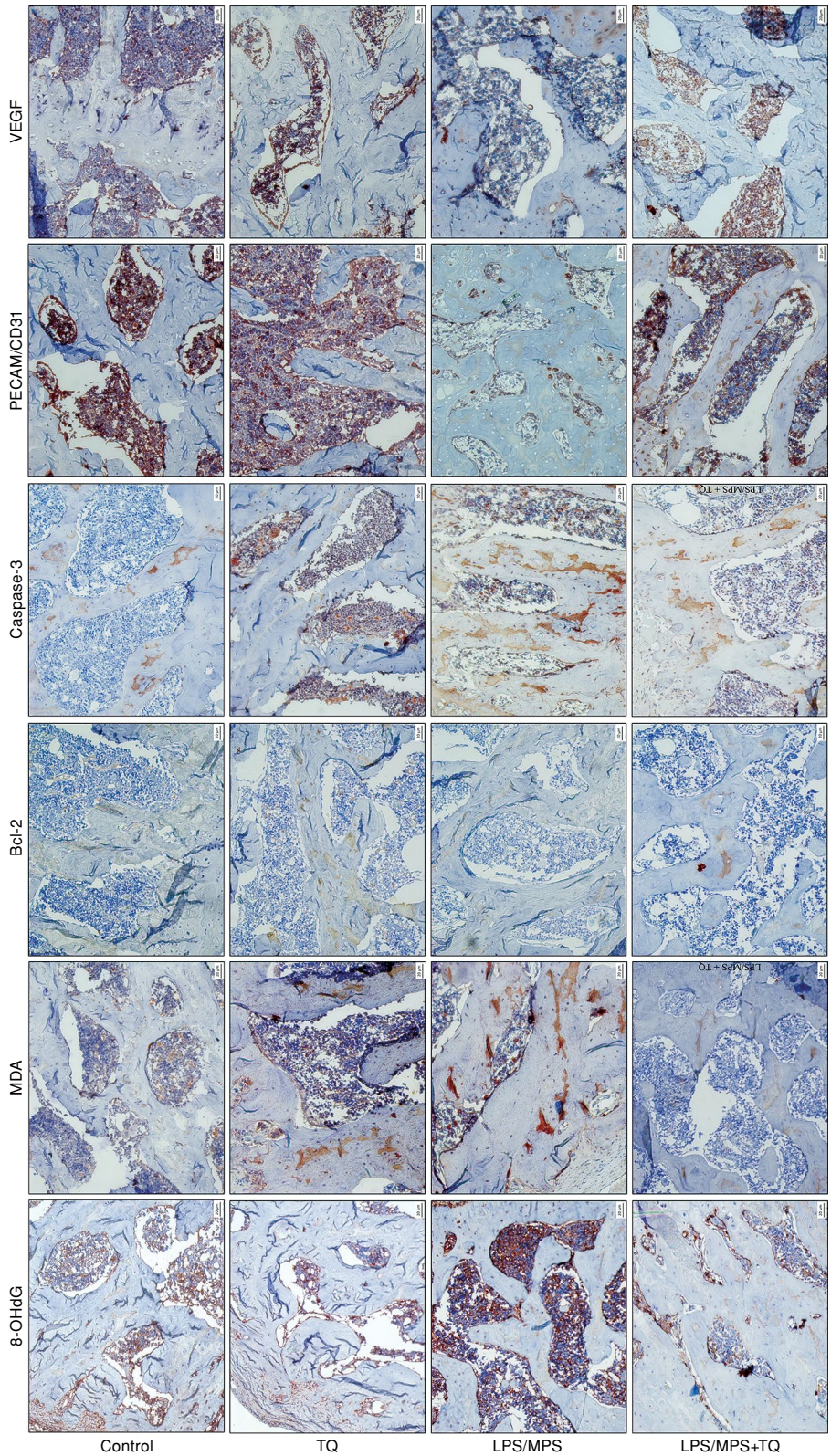

FIGURE 4. Light microscopic images of immunohistochemistry analysis of femoral head sections in avascular necrosis model. H&E, ×200.

TQ: Thymoquinone; LPS/MPS: Lipopolysaccharide/methylprednisolone; 8-OHdG: 8-hydroxy-20-deoxyguanosine; MDA: Malondialdehyde; PECAM/CD31: Platelet/endothelial cell adhesion molecule 1; VEGF: Vascular endothelial growth factor.

The MDA levels increased significantly in vessels and adipocytes of the LPS/MPS group compared to other groups ($p<0.001$ and $p<0.01$), but did not change in bone marrow. Although the mean MDA level in vessels regressed significantly in the LPS/MPS+TQ group compared to the LPS/MPS group, there was a 10-fold increase in level of the LPS/MPS+TQ group compared to the control group ($p<0.05$). While considerable decreases were observed in MDA levels of the adipocytes in LPS/MPS+TQ group, no significant change was observed in the bone marrow (Figure 5).

No Bcl-2 immunoreactivity was observed in the adipocytes of the LPS/MPS group and no significant difference between the other groups ($p<0.01$). The Bcl-2 level was relatively low in the bone marrow of the LPS/MPS group and exhibited a significant difference with the other groups ($p<0.01$). Despite a high Bcl-2 level in the bone marrow of the LPS/MPS+TQ group compared to the LPS/MPS group, it was significantly lower than those of the control group ($p<0.05$). Significantly lower levels of Bcl-2 in trabeculae were detected in all experimental groups compared to the control group ($p<0.05$) (Figure 5).

No statistically significant difference was found in caspase-3 levels of the adipocytes between the groups (Figure 4), but significantly increased caspase-3 levels were identified in bone marrow and trabeculae of the LPS/MPS group compared to the other groups ($p<0.01$). Caspase-3 levels regressed to control levels in LPS/MPS+TQ group. However, the levels in the bone marrow of this group increased by 10 times compared to the control group ($p<0.05$) (Figure 5).

The PECAM/CD31 and VEGF immunoreactivities were examined only in vessels to determine vascular damage (Figure 4). The mean PECAM/CD31 levels decreased in the LPS/MPS group; however, there was no statistically significant difference between the groups ($p=0.068$). On the other hand, the mean VEGF levels decreased significantly in the LPS/MPS group compared to the other groups ($p=0.0012$), while reached almost normal levels in the LPS/MPS+TQ group (Figure 5).

Biochemical findings

Serum MDA levels increased significantly in the LPS/MPS group compared to all groups ($p<0.001$). Despite increases in MDA levels in both groups treated with TQ compared to the control group, TQ treatment could significantly reduce the MDA levels,

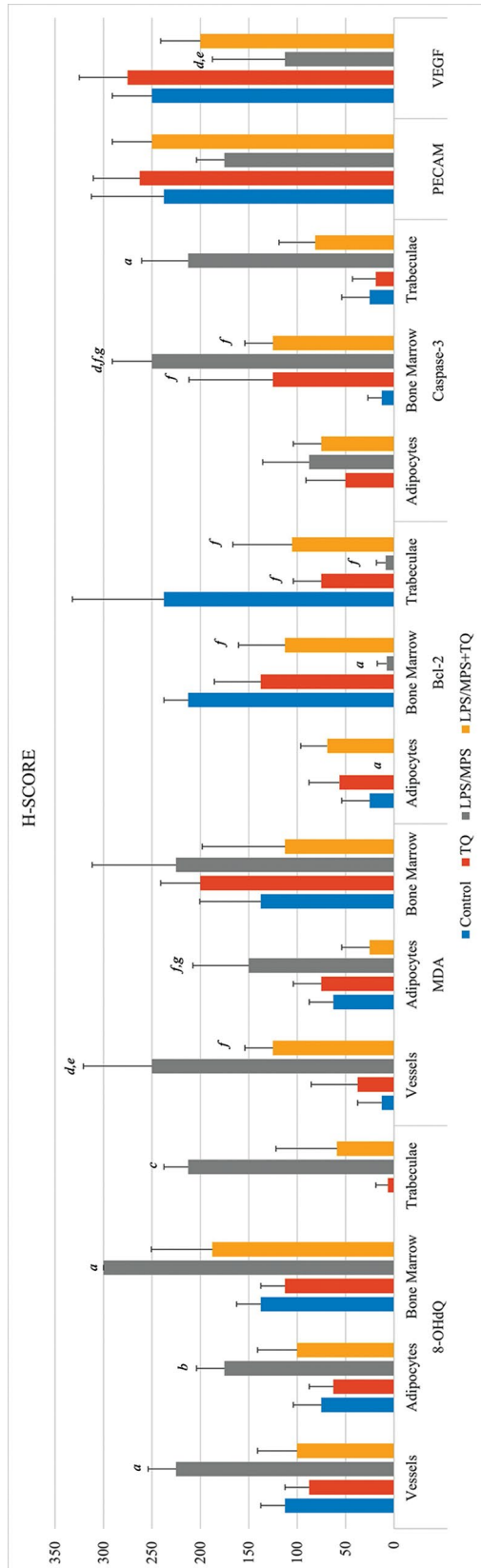

FIGURE 5. Graphical presentation of immunoreactivities of femoral head sections in avascular necrosis model and a statistical comparison according to the groups. All values are given as mean and error bars are standard deviations. TQ: Thymoquinone; LPS: Lipopolysaccharide; MPS: Methylprednisolone; 8-OHdG: 8-hydroxy-20-deoxyguanosine; MDA: Malondialdehyde; PECAM/CD31: Platelet/endothelial cell adhesion molecule 1; VEGF: Vascular endothelial growth factor; a: $p<0.01$; b: $p<0.05$; c: $p<0.001$ *vs.* other groups; d: $p<0.05$ *vs.* control group and TQ group; e: $p<0.05$; g: $p<0.01$ *vs.* LPS/MPS+TQ group; f: $p<0.05$ *vs.* control group.

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

Groups	Control	TQ	LPS/MPS	LPS/MPS+TQ	
Parameter	Mean±SD	Mean±SD	Mean±SD	Mean±SD	*p*
Oxidative stress					
MDA (nmol/mL)	3.74±0.24	5.22±0.26[b,f]	7.17±0.77[c]	5.45±0.58[b,f]	**<0.0001**
GSH (mmol/L)	109.20±10.18	78.33±5.65[b,e]	46.83±21.37[c]	76.83±4.62[b,e]	**<0.0001**
Osteoprotection					
BALP (ng/mL)	54.33±3.56	52.33±5.61	63.00±13.51	61.00±5.14	0.0922
CTX-1 (ng/mL)	27.00±6.51	24.83±3.25[e]	37.80±7.22[a]	27.50±4.04[d]	**0.0036**
OPG (ng/mL)	2.97±0.77	2.62±0.59	2.06±0.44	2.40±0.40	0.0942
Lipid profile					
TG (mmol/L)	2.58±0.38	4.30±0.43[c,f,g]	5.62±0.63[c]	5.08±0.42c	**<0.0001**
TC (µmol/L)	19.00±2.24	20.17±1.60	23.83±4.26	21.83±2.86	0.0718
HDL-C (µmol/L)	163.33±34.16	178.00±9.08[f,g]	99.00±15.77[b]	117.50±34.75[a]	**0.0002**
LDL-C (mmol/L)	4.06±0.36	4.73±0.22[e]	5.70±0.56[c]	5.30±0.45[c]	**<0.0001**

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

particularly in the LPS/MPS+TQ group (p<0.01), compared to those of LPS/MPS group (Table III).

The GSH levels decreased significantly in LPS/MPS group compared to all groups (p<0.001). The levels in the LPS/MPS+TQ group were significantly higher than those in the LPS/MPS group (p<0.01) (Table III).

The mean CTX level in the LPS/MPS group was significantly higher than those in the control, TQ and LPS/MPS+TQ groups (p<0.05, p<0.05, and p<0.01, respectively), but regressed to the control level in the LPS/MPS+TQ group. Although serum BALP level increased in the LPS/MPS group, no statistically significant difference was observed between the groups (p=0.0922). Despite the low mean OPG level in the LPS/MPS group and an increase in the TQ applied group, no statistically significant difference was found between the groups (p=0.0942) (Table III).

Furthermore, TG increased significantly in the LPS/MPS group compared to the control group, whereas a similar increase was observed in the LPS/MPS+TQ group (p<0.001). Although the increase in the TQ group was higher than those in the control group (p<0.001), it was significantly lower than those in the LPS/MPS group (p<0.001 and p<0.05, respectively). There was no statistically significant difference in TC levels between the groups, despite slight increases in the LPS/MPS and LPS/MPS+TQ groups compared to the other groups (p=0.0718) (Table III).

The mean HDL-C levels decreased significantly in the LPS/MPS and LPS/MPS+TQ groups compared to the control group (p<0.01 and p<0.05, respectively), while they increased significantly in the TQ group compared to the rats with ONFH (p<0.001 and p<0.05, respectively). While the mean LDL-C level increased significantly in the LPS/MPS and LPS/MPS+TQ groups compared to the control group (p<0.001), the mean value was significantly lower in rats treated with only TQ compared to the LPS/MPS group (p<0.01) (Table III).

DISCUSSION

Osteonecrosis is a pathological process which mainly impairs the femoral head and gradually progresses to the fracture of the subchondral bone, the collapse of the femoral head surface, and the destruction of the hip joint. Although the etiology of ON is attributed to a number of factors, steroid administrations are the most common predisposing causal factors associated with the development of ON.[11] In the present study, we conducted radiological, histopathological, histomorphometric, immunohistochemical, and biochemical analyses to evaluate the efficacy of TQ, an important active chemical component, and its systemic effects in osteonecrotic areas using its antioxidant,

antiosteoclastic, osteoblastic, and hypolipidemic effects in the steroid-induced ONFH model in rats.

Various pharmacological interventions were used to reduce ON-induced bone loss and ONFH. The recommended treatments included vasodilators, statins, bisphosphonates, and anticoagulants to revascularize the femoral head. Karakaplan et al.[12] showed that platelet-rich plasma therapy had positive effects on ONFH. Despite various options available for pharmacological treatment, there is currently no strong consensus on the efficacy of any of these agents over the other. Thus, there is still a need for a safe and effective treatments to prevent ONFH in steroid-treated subjects.

The injury on the vascular endothelium in ON may make an important initial contribution to local hypercoagulation capacity and intravascular thrombus formation.[13] Vascular endothelial cells are susceptible to injury due to oxidative stress.[14] Accordingly, it has also been shown that hematopoietic cell damage occurs in the early phase of steroid-induced ON.[15] In an animal study, Li et al.[16] demonstrated that the incidence of ON decreased with edaravone therapy following steroid administration, and the reduction of small-sized perfused vessels stopped, and the increase in intravascular thrombus formation was significantly improved. This beneficial effect was explained in two ways: first, as the preservation of the structural function of the intraosseous vascular system and secondly, as the protection of hematopoietic cells from oxidative stress. Our study revealed that the bone marrow lost its normal morphology and hematopoietic cell count decreased in rats with ONFH. While necrosis and hemorrhage were found to accompany these, thrombus was identified in some of them. The bone marrow preserved its normal morphology and hematopoietic cell count increased in many animals of the TQ group. Nevertheless, hematopoietic necrosis was also observed in two animals of this group. These findings suggest that TQ has significantly beneficial effects in preventing the development of ON by preventing oxidative damage to hematopoietic cells and the vascular structure in the bone marrow.

In vitro and *in vivo* data have indicated that steroid-associated oxidative damage plays an essential role in the development of ON. Shortly after steroid administration, protective antioxidative mechanisms are weakened, leading to tissue peroxidation and protein changes in bone tissues.[17] Many researchers have observed oxidative stress in steroid-induced ONFH sites or have reported that an antioxidant agent effectively suppresses the development of ON.[4,18-21] In the present study, we also established a steroid-induced ONFH model and investigated the antioxidant effect of TQ. Examining 8-OHdG immunoreactivity identified an increased damage in ONFH model and that TQ almost completely ameliorated this damage in all parts of tissue. The levels of MDA, main form of aldehyde resulting from tissue lipid peroxidation and widely used as biomarker of oxidative stress, increased significantly in the vessels and adipocytes of rats with ONFH, both in tissues and in serum, and MDA levels in vessels and adipocytes regressed significantly with TQ application, but did not reach to the control levels.

As an important biochemical marker of the antioxidant mechanism, GSH has been shown to be a scavenger of a wide variety of reactive species, including reactive oxygen species (ROS).[22] Elevated MDA levels, together with decreased GSH levels, are important indicators of oxidative damage. Li et al.[16] found a significant decrease in GSH levels shortly after steroid administration in ON model of rabbits. In our study, we revealed that serum levels of GSH decreased significantly in ONFH model and increased significantly with TQ treatment. These data suggest that the treatment effect of TQ on LPS and steroid-induced ONFH may be due to inducing the antioxidant mechanism by inhibiting lipid peroxidation and related oxidative stress. This is an expected result, since TQ has previously been shown to inhibit oxidative damage in various oxidation-associated diseases.[23,24]

The beneficial effects of TQ on osteoporosis and bone healing have been reported.[25,26] Kirui et al.[27] showed that TQ improved bone healing with little or no side effects on rat femurs. Changes in the bone tissue trabecular architecture in ONFH can be easily calculated morphometrically by micro-CT. Huang et al.[28] examined bone morphological changes with micro-CT in the steroid-induced ONFH model and found that BMD decreased significantly in the ONFH group compared to the other groups. Zheng et al.[29] used micro-CT to study perfusion, decalcification, vascular structures, and trabecular architecture in the femoral heads of rats in LPS- and MPS-induced ONFH model and recorded that the BMD, BV/TV, Tb. N values decreased significantly in ONFH group compared to control group and the mean Tb.Sp value increased. In the present study, we monitored the trabecular changes in femoral head by micro-CT and identified that the mean BMD, similar to previous studies in the literature, decreased significantly in LPS/MPS-induced ONFH, but

increased in rats treated with TQ and were comparable with the control levels. Furthermore, the mean porosity, Tr.Sp and Tr.Sep increased significantly in LPS/MPS group compared to the control group, but decreased in the TQ group. Although the histomorphometric measurements showed TQ increased BV/TV ratio in treatment group compared to the LPS/MPS group, micro-CT findings showed no significant difference in BV/TV and BS/BV ratios between the groups.

Bone alkaline phosphatase and OPG are bone formation markers, and CTX is a bone resorption marker. The alkaline phosphatase (ALP) content is an important indicator of differentiation of mesenchymal stem cells into osteoblasts during osteogenesis. Measuring ALP level is used to determine the presence and differentiation degree of osteoblasts. The ALP activity was shown to increase significantly in serum at the end of the fourth week in steroid-induced ONFH model in rats,[30] and there are also studies revealing that it is downregulated in bone tissue and decreases in serum until the 12th week.[28] Osteoprotegerin is a glycoprotein secreted by osteoblasts in a differentiation-dependent manner and acts as a "decoy receptor" which regulates osteoclast functions and lifespan, by regulating the receptor activator of nuclear factor kappa-B ligand (RANKL). It has been demonstrated that steroid administration increases CTX levels, one of the indicators of bone remodeling in femoral head, while it reduces OPG levels and consequently causes the disruption in osteoblast functions.[30] In our study, although serum BALP level increased and OPG levels decreased in rats with ON, and TQ reversed these effects, no significant difference was observed between the groups. However, the serum CTX level of LPS/MPS rats was significantly higher than those of other groups and regressed significantly to the control level in the TQ and LPS/MPS+TQ groups, suggesting that TQ may reverse the bone resorption by mediating the CTX in ON.

Vascular endothelial growth factor produced by osteoblasts is a significant regulator of bone formation and repair. Its expression decreases with the increase in apoptosis of osteoblast and osteocyte induced by steroid administration.[31] Moreover, decreased VEGF expression was shown to impair angiogenesis and bone regeneration in the femoral head.[32] In this study, we indicated that vascular thrombosis rates increased with steroid administration in femoral head, while these rates decreased in TQ-treated rats and were even comparable with the control group. The mean PECAM/CD31 levels decreased in the LPS/MPS group and increased in the LPS/MPS+TQ group, but there was no statistically significant difference between the groups. Furthermore, VEGF immunoreactivities decreased in LPS/MPS group, but reached normal levels in the LPS/MPS+TQ group. These findings indicate that TQ is successful in ameliorating vascular damage in ONFH, thereby suggesting a therapeutic effect on local vascular supply.

Decreased vascularity and bone ischemia represent the traditional etiological background of ON. Recently, osteocyte apoptosis is considered a mechanism in the pathogenesis of ON caused by corticosteroid use.[11,15,33,34] Apoptotic osteocytes were detected in the pathological examination of the femoral heads in patients who underwent total hip arthroplasty due to steroid-induced ONFH, and these findings were not observed in trauma or alcohol-induced ONFH.[35] It is thought that apoptotic cells accumulate, leading to the disruption of the osteocyte-lacunar-canalicular system and vascular spaces and to the collapse of femoral head. In the present study, the apoptotic pathways in the femoral head by examining the caspase-3, a proapoptotic protein in the bone marrow, and anti-apoptotic protein Bcl-2 levels. We detected very low Bcl-2 levels in the adipocytes, bone marrow, and trabeculae of LPS/MPS-induced ON. In addition, the TQ treatment increased Bcl-2 levels, particularly in the adipocytes and bone marrow. We demonstrated that immunoreactivities of caspase-3 increased significantly in trabeculae of the LPS/MPS group and regressed almost to the control levels in TQ group. Therefore, we suggest that TQ treatment may be an effective approach to prevent steroid-induced ON in the femoral head by suppressing the apoptosis of osteoblasts and osteocytes.

Hyperlipidemia has been also implicated in the pathogenesis of ONFH.[36] Many studies have shown that statin group drugs, which are lipid-lowering agents, significantly reduce the incidence of steroid-induced ON.[37,38] On the contrary, hyperlipidemia leads to the formation of fat emboli in peripheral blood, causes bone microvascular occlusions, increases intraosseous pressure, and worsens the dysfunction of bone microcirculation.[39] In the current study, we examined the lipid profile in the steroid-induced ONFH model and found that serum TG and TC levels increased significantly in the ONFH group, but TQ treatment could not reduce these levels. On the other hand, TQ treatment reversed the reduction of HDL-C and elevation of LDL-C in LPS/MPS-induced model.

The LDL/HDL ratio has been reported to be a risk factor for the development of ON, and an increase in this ratio may also increase the size of bone marrow fat cells.[40] This explains the decrease in HDL-C and increase in LDL-C in the ONFH model revealed by our findings. Furthermore, the fact that TQ reversed these changes suggests that it may be used to treat ONFH by modulating the cholesterol levels of lipid metabolism.

Various animal species, such as rabbits, dogs, birds, and sheep, have been used as experimental models of steroid-induced ONFH. However, there are few studies using rats in the ONFH model, although our study has certain limitations. First, we used a few number of animals and performed biochemical analyses in only one blood sample. To comply with 3R rule (replacement, reduction, and refinement) accepted in scientific studies carried out on animals, less stimulation was done for fewer animals and appropriate conditions. Intraperitoneal and intramuscular injections in the first week of study and oral gavage application throughout the study may cause a moderate stress in rats. Therefore, a biochemical analysis could be performed at the end of the study. Another limitation of our study is the administration of a single dosage for TQ. The effect of different doses needs to be further investigated to determine the optimal dose in reducing the damage of steroid-induced ON. Moreover, a four-week observation period design used in our study can be shortened to determine the short-term efficacy of TQ treatment.

In conclusion, our study results demonstrated that the oxidative stress significantly contributed to the development of ONFH and TQ, a powerful antioxidant and might significantly reduce ON damage and reduce serum LDL/HDL ratio. Moreover, positive effects of TQ on bone turnover were demonstrated by micro-CT and biochemically. Many mechanisms are known in the pathogenesis of steroid-induced ONFH, and the present preliminary study, conducted for the first time in the literature, assessed all possible mechanisms.[41] Yet, more research and further studies are required to support the findings and clarify the mechanisms of beneficial impacts of TQ on steroid-induced ONFH.

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