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ORIGINAL ARTICLE

The effects of hydroxychloroquine-induced oxidative stress on fracture healing in an experimental rat model

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Hydroxychloroquine is a derivative of 4-aminoquinoline and is known as a lysosomotropic agent. Hydroxychloroquine was first used to prevent or cure malaria. While red blood cells do not contain organelles, malaria-infected erythrocytes can accumulate HCQ in the acidic digestive vacuoles of the parasite according to the pH gradient, thus preventing hemoglobin polymerization.[1] In addition, various viral, rheumatologic, dermatologic, and immunologic diseases have been treated with HCQ. Due to its lysosomotropic, immunomodulatory, anti-inflammatory, anti-infective, antithrombotic, antitumor, and beneficial metabolic effects, HCQ is a versatile drug.[2] In the context of the COVID-19 (coronavirus disease 2019) pandemic, it was included in treatment protocols in many countries.[3]

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ABSTRACT

Objectives: The purpose of this study was to investigate whether hydroxychloroquine (HCQ) sulfate causes oxidative stress (OS) and its effect on fracture healing in an experimental rat model.

Materials and methods: In this experimental study, open diaphyseal femur fractures were induced in 24 eight-week-old male rats (mean weight: 225±25 g; range, 200 to 250 g) and then fixed with K-wire. The rats were divided into four groups: HCQ-2, control-2 (C-2), HCQ-4, and control-4 (C-4). During the study period, rats in the HCQ groups received an HCQ solution (160 mg/kg/day), whereas rats in the control groups received saline. The HCQ-2 and C-2 groups were sacrificed on the 14th day, and the HCQ-4 and C-4 groups were sacrificed on the 28th day. After sacrifice, malondialdehyde levels induced by OS were calculated for each rat, and fracture healing was evaluated radiographically, histomorphometrically, histopathologically, and immunohistochemically.

Results: Malondialdehyde levels were higher in the HCQ groups than in the control groups ($p<0.05$). Hydroxychloroquine caused OS in rats. The ratio of total callus diameter to femur bone diameter was lower in HCQ groups compared to control groups ($p<0.05$). No differences were observed when comparing radiological and histological healing results between the control and HCQ groups. Alkaline phosphatase levels were lower in the HCQ-4 group than the C-4 group at week four ($p<0.05$), although osteocalcin and osteopontin levels did not differ between groups ($p>0.05$). Oxidative stress had no adverse effects on histologic healing outcomes and osteoblast functions. Cathepsin K and tartrate-resistant acid phosphatase-5b levels were higher in the HCQ-4 group than in the C-4 group ($p<0.05$). While the number and function of osteoclasts increased due to OS in callus tissue, a decrease in the number of chondrocytes was observed.

Conclusion: Hydroxychloroquine-induced OS increases the number and function of osteoclasts and decreases the number of hypertrophic chondrocytes and endochondral ossification but has no significant effect on mid-late osteoblast products and histological fracture healing scores.

Keywords: Femur, fracture healing, hydroxychloroquine sulfate, oxidative stress, rat.

According to a study by Both et al.,[4] HCQ decreased bone resorption by decreasing cathepsin K (Cat-K) and increased tartrate-resistant acid phosphatase-5b (TRAP5b) by increasing the number of osteoclast mononuclear cells (OCLs) *in vitro*. In the same study, they also examined serum bone resorption marker beta-crosslaps (β-CTx) in patients with rheumatoid arthritis. At the end of six months of HCQ treatment, serum β-CTx was found to be lower than baseline. In addition, Both et al.[5] showed that HCQ reduced the development and mineralization of osteoblasts produced from human mesenchymal stem cells *in vitro*. While *in vitro* studies failed to show that chloroquine (CQ)/HCQ treatment causes oxidative stress (OS), these results were confirmed by *in vivo* studies. Animal studies demonstrate that CQ treatment has systemic OS effects.[6,7] Studies have shown that OS is caused by its uptake into the human body, including its use in the treatment of COVID-19 (coronavirus disease 2019).[8] The aim of our study was to investigate how HCQ-induced OS affects fracture healing using an animal model.

MATERIALS AND METHODS

The experimental study was conducted on 24 eight-week-old male Wistar albino rats (mean weight: 225±25 g; range, 200 to 250 g). The rats were blindly randomized and divided into four groups, with six rats in each group: the HCQ-2 group, which was administered 160 mg/kg/day of the HCQ sulfate solution by gastric lavage in the two weeks after surgery;[9] the HCQ-4 group, which was administered 160 mg/kg/day of the HCQ sulfate solution by gastric lavage in the four weeks after surgery;[9] the control-2 (C-2) group, which was administered the same amount of saline every day in the two weeks after surgery; the control-4 (C-4) group, which was administered the same amount of saline every day in the four weeks after surgery. The rats had access to unlimited tap water and normal pellet chow throughout the experiment. Rats were housed in cycles of 12 hours of light and darkness at a temperature of 24±2°C.

Surgical procedure

All rats were anesthetized by intraperitoneal 5 mg/kg xylazine hydrochloride (Rompun®; Bayer, Istanbul, Türkiye) and 80 mg/kg ketamine hydrochloride (Ketalar®; Pfizer Inc., Istanbul, Türkiye). After disinfection, the right legs were shaved. An open osteotomy model was used.[10] Transverse osteotomy of the right femur at the mid-diaphysis was performed using an electric saw with a 0.38 mm blade (ConMed Linvatec PowerPro Oscillator 6125; CONMED Corp., Utica, NY, USA), and the fracture site was stabilized intramedullary by inserting a sterilized K-wire (Figure 1). No immobilization technique was used after the surgical procedure. At the end of the study period, the rats were sacrificed by the decapitation method.

Drug preparation

Hydroxychloroquine sulfate in powder form was dissolved daily in water at 40 mg/mL in an open glass petri dish. The solution was homogenized using a vortex mixer. Approximately 1 mL of solution daily was sufficient for each experimental rat.

Oxidative stress evaluation

The level of OS was determined by collecting 5 mL of blood from each rat after sacrification. The amount of malondialdehyde in the blood samples was determined according to the method described in the literature, and the results were expressed in nmol/mg protein.[11]

Radiological analysis

After sacrifice, all operated femurs were removed without damaging the callus tissue, and radiological assessment was performed. Anteroposterior digital radiographs of each femur were obtained at 100% magnification (Figure 2). Two orthopedic surgeons evaluated the radiographs in a blinded fashion according to the Lane–Sandhu classification (Table I).[12] Intraclass correlation coefficients (ICCs) were used to examine agreement and disagreement between measurements within and between observers. Intra- and inter-observer reliability was assessed based on radiographic measurements of all subjects repeated twice at one-month intervals by two orthopedic surgeons.

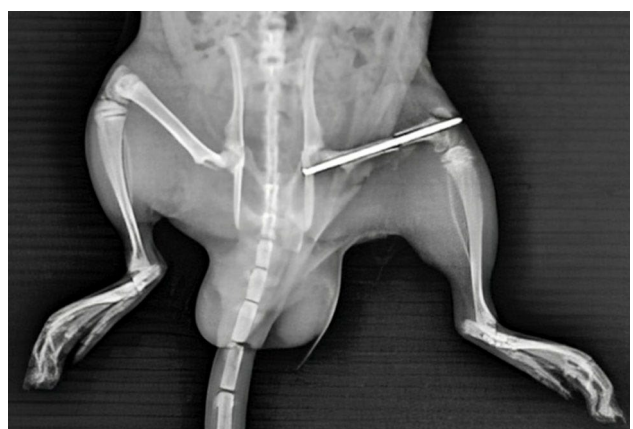

FIGURE 1. Early postoperative radiograph taken after the surgical procedure.

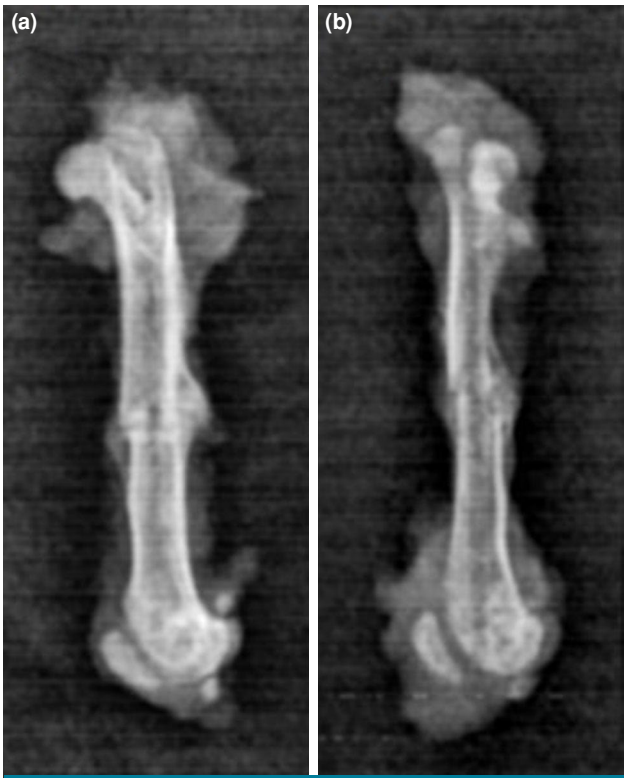

FIGURE 2. Radiograph samples taken after sacrification for radiological evaluation of fracture healing. **(a)** from the Control-2 group, **(b)** from the HCQ-2 group.
HCQ: Hydroxychloroquine.

TABLE I
Radiological scoring system of fracture healing

0	No callus
1	Callus formation present
2	Beginning of bone healing
3	No apparent fracture line
4	Complete bone healing

TABLE II
Histological scoring system of fracture healing

1	Fibrous tissue
2	Predominant fibrous tissue with minimal cartilage tissue
3	Cartilage tissue and fibrous tissue in a uniform manner
4	Predominant cartilage tissue with minimal fibrous tissue
5	Cartilage tissue
6	Predominant cartilage tissue with minimal immature bone
7	Immature bone and cartilage tissue in a uniform manner
8	Predominant immature bone with minimal cartilage tissue
9	Bone healing with immature bone
10	Bone healing with matured bone

Histomorphometric analysis

The Olympus DP72 image analysis software (Olympus, Tokyo, Japan) was used to quantitatively analyze serial sections of the fracture site for the histomorphometric analysis. According to the literature, the ratio of total callus diameter to femoral bone diameter (TCD/FBD) was calculated as a percentage.[13]

Histopathological analysis

Tissue samples were decalcified and preserved in 10% buffered formalin solution for histopathologic examination. Normal tissue examination was followed by paraffin blocking. Longitudinal sections 3 to 4 μm in thickness were stained with hematoxylin-eosin and Masson's trichrome. Sections were examined under ×20 and ×40 magnification with a light microscope (BX61; Olympus, Tokyo, Japan) and photographed (DP72; Olympus, Tokyo, Japan). Five or more randomly selected sections were evaluated using the histologic healing grading system of Huo et al.[14] (Table II). The sections were assessed by a histologist in a blinded fashion.

Immunohistochemical analysis

After four weeks, immunohistochemical staining was performed using the streptavidin-biotin-peroxidase technique. Monoclonal/polyclonal antibodies were used to label alkaline phosphatase (ALP), osteocalcin (OC), osteopontin (OPN), Cat-K, and TRAP5b.[15-17] Five regions that showed positive immunoreactivity were analyzed for staining intensity using a modified histoscore (H- SCORE), ranging from 0 to 300 points, according to the literature.[18] Sections were assessed in a blinded fashion by the same histologist. Intraobserver reliability was assessed by histopathologic and immunohistochemical measurements of all subjects repeated twice at one-month intervals by the same histologist.

Statistical analysis

The number of rats used in each group was determined using power analysis with G*Power version 3.1.9.4 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany), with an alpha of 0.05, beta of 0.30, and effect size of 0.95. It was determined that at least six rats were required

for each experimental or control group. Statistical analysis was performed using GraphPad Instat version 3.06 (GraphPad Software Inc., La Jolla, San Diego, CA, USA). A t-test was used to compare the means of two independent groups, and the Tukey-Kramer multiple comparison test and the Kruskal-Wallis comparison test were used to analyze the differences between groups. A *p*-value <0.05 was considered statistically significant.

RESULTS

Malondialdehyde levels were statistically higher in the HCQ-2 group than in the C-2 group and higher in the HCQ-4 group than in the C-4 group (p<0.05). Malondialdehyde values were statistically higher in the HCQ-4 group than in the HCQ-2 group and higher in the C-4 group than in the C-2 group (p<0.05, Table III).

There was no statistical difference in histological healing scores between the C-2 and HCQ-2 groups, as well as the C-4 and HCQ-4 groups (p>0.05). Histological healing scores were higher in the HCQ-4 group than in the HCQ-2 group and higher in the C-4 group than in the C-2 group, but there was no statistical difference (p>0.05, Table IV). The histological sections showed that the cartilage callus tissue almost disappeared, and the areas of immature bone tissue relatively increased in the fracture sections of HCQ groups (Figure 3, 4).

At week four, the ALP value was statistically lower in the HCQ-4 group than in the C-4 group

TABLE III
The levels of MDA in blood samples taken from rats

Oxidative stress parameter	Control 2nd week Mean±SD	Control 4th week Mean±SD	HCQ 2nd week Mean±SD	HCQ 4th week Mean±SD	*p*	
Malondialdehyde (nmoL/mg protein)	16.0±2.4	27.2±4.0	24.0±3.6	46.2±7.0	C-2 *vs.* HCQ-2	0.002
					HCQ-2 *vs.* HCQ-4	<0.001
					C-2 *vs.* C-4	0.001
					C-4 *vs.* HCQ-4	0.001

MDA: Malondialdehyde; SD: Standard deviation; HCQ: Hydroxychloroquine; C-2: Control-2; C-4: Control-4.

TABLE IV
Comparison of histological healing scores and immunoreactivities

	Control 2nd week Mean±SD	Control 4th week Mean±SD	HCQ 2nd week Mean±SD	HCQ 4th week Mean±SD	*p**	
Histological healing scores	4.3±1.2	6.1±0.9	4.7±1.0	6.8±0.9	C-2 *vs.* HCQ-2	0.267
					HCQ-2 *vs.* HCQ-4	0.051
					C-2 *vs.* C-4	0.059
					C-4 *vs.* HCQ-4	0.124
ALP (H-score) (0-300 points)	-	123.6±34.7	-	51.8±31.9	C-4 *vs.* HCQ-4	**0.003**
OC (H-score) (0-300 points)	-	176.6±65.5	-	185±58.2	C-4 *vs.* HCQ-4	0.821
OPN (H-score) (0-300 points)	-	261.6±22.2	-	251±29.6	C-4 *vs.* HCQ-4	0.497
Cat-K (H-score) (0-300 points)	-	64.1±38.7	-	124.6±31.7	C-4 *vs.* HCQ-4	**0.014**
TRAP5b (H-score) (0-300 points)	-	63.5±55.1	-	160.1±31.0	C-4 *vs.* HCQ-4	**0.007**

SD: Standard deviation; ALP: Alkaline phosphatase; OC: Osteocalcin; OPN: Osteopontin; Cat-K: Cathepsin-K; TRAP5b: Tartrate resistant acid phosphatase 5b; C-2: Control-2; C-4: Control-4; Intra-observer reliability was found to be excellent (ICC=0.986-0.996). * T test, Tukey-Kramer and Kruskal Wallis tests was used to compare the groups.

(p<0.05). No statistical difference in the values of OC and OPN was observed between the C-4 and HCQ-4 groups (p>0.05). Cathepsin K and TRAP5b scores were statistically higher in the HCQ-4 group than in the C-4 group (p<0.05, Figure 5, Table IV).

Regarding radiological scores, there was no statistical difference between the C-2 and HCQ-2 groups, as well as the C-4 and HCQ-4 groups (p>0.05). Radiological scores were statistically higher in the HCQ-4 group than in the HCQ-2 group and higher in the C-4 group than in the C-2 group (p<0.05, Table V).

Regarding histomorphometric analysis, the TCD/FBD ratio was statistically lower in the HCQ-2 group than in the C-2 group and lower in the HCQ-4 group than in the C-4 group (p<0.05). The TCD/FBD ratio was statistically lower in the HCQ-4 group than in the HCQ-2 group and lower in the C-4 group than in the C-2 group (p<0.05, Table VI).

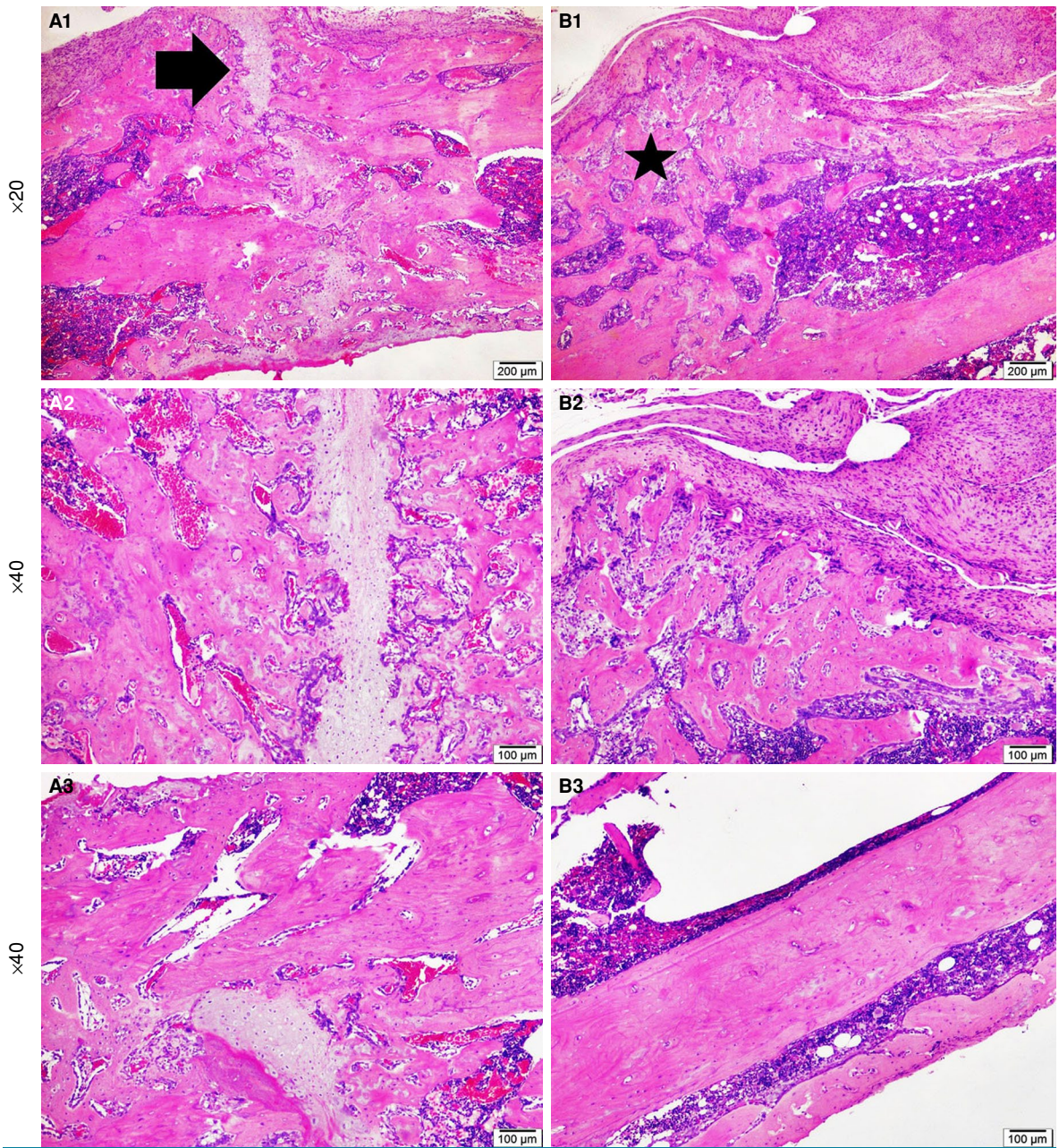

FIGURE 3. A1, A2, A3 from the Control-4 group, shows large amount of cartilage callus tissue (arrow), **B1, B2, B3** from the HCQ-4 group, shows relatively increased immature bone tissue (star) (H&E, ×20 and ×40).
HCQ: Hydroxychloroquine.

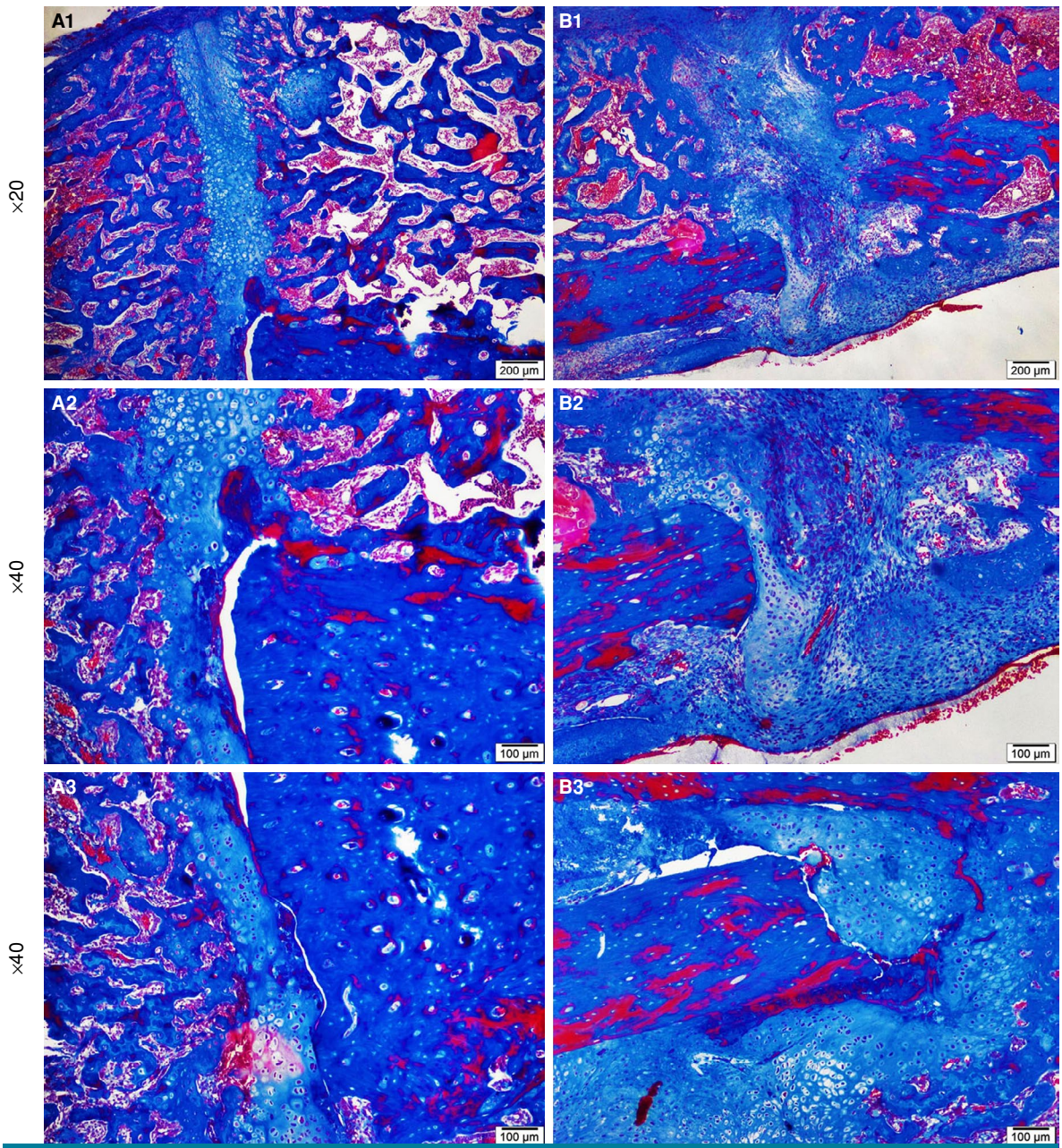

FIGURE 4. A1, A2, A3 from the Control-4 group, **B1, B2, B3** from the HCQ-4 group (Masson trichrome, ×20 and ×40).
HCQ: Hydroxychloroquine.

DISCUSSION

In the initial phase of fracture healing, inflammation and ischemia produce reactive oxygen species. An increase in reactive oxygen species during fracture healing has also been demonstrated in animal studies.[19] Reactive oxygen species and reactive nitric oxide species are overproduced during OS, resulting in redox imbalance and physiological damage. Apoptosis of chondrocytes, osteoblasts, and osteocytes can be induced by an excess of OS, and OCLs can be activated to begin the resorption of bone.[20] Malondialdehyde, is an oxidative metabolite and an indicator of lipid peroxidation.[9] As expected from the fracture healing process, malondialdehyde levels were higher in the HCQ-4 group than in the HCQ-2 group and higher in the C-4 group than in the C-2 group. Malondialdehyde levels were higher in the HCQ-2 group than in the C-2 group and higher in

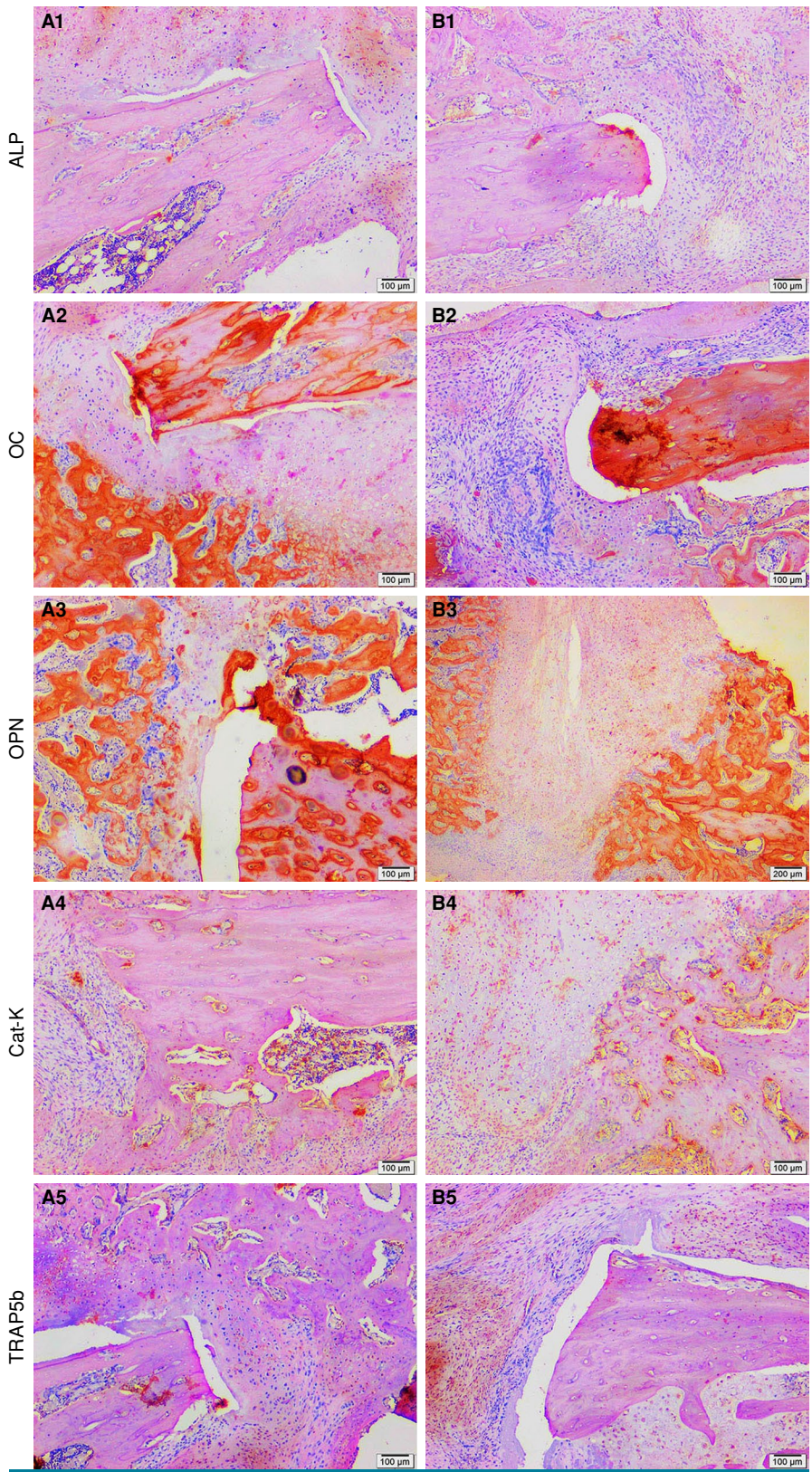

FIGURE 5. ALP, OC, OPN, Cat-K and TRAP5b immunoreactivities, total magnification ×40. **A1, A2, A3, A4, A5** from the Control-4 group, **B1, B2, B3, B4, B5** from the HCQ-4 group.

ALP: Alkaline phosphatase; OC: Osteocalcin; OPN: Osteopontin; Cat-K: Cathepsin K; TRAP5b: Tartrate-resistant acid phosphatase-5b.

TABLE V
Comparison of radiological scores

Lane and Sandhu radiological scores	Control group Mean±SD		HCQ group Mean±SD		*p*	
2nd week	1±0.2		1.15±0.3		C-2 *vs.* HCQ-2	0.392
4th week	2±0.4		2.33±0.7		C-4 *vs.* HCQ-4	0.785
p value	C-2 *vs.* C-4	0.008	HCQ-2 *vs.* HCQ-4	0.007		

HCQ: Hydroxychloroquine; SD: Standard deviation; C-2: Control-2; C-4: Control-4; Intra- and inter-observer reliability were found to be excellent (ICC=0.986-0.996).

TABLE VI
Comparison of histomorphometric results

Total callus diameter/femoral bone diameter ratio (%)	Control group Mean±SD		HCQ group Mean±SD		*p*	
2nd week	65.6±7.5		34.6±18.8		C-2 *vs.* HCQ-2	0.041
4th week	16.4±1.8		8.6±4.7		C-4 *vs.* HCQ-4	0.018
p value	C-2 *vs.* C-4	0.020	HCQ-2 *vs.* HCQ-4	0.009		

HCQ: Hydroxychloroquine; SD: Standard deviation; C-2: Control-2; C-4: Control-4; Intraobserver reliability was found to be excellent (ICC=0.986-0.996).

the HCQ-4 group than in the C-4 group. This means that the HCQ groups were exposed to OS.

In our study, the histological sections showed that the cartilage callus tissue almost completely disappeared, and the areas of immature bone tissue relatively increased in the fracture sections of the HCQ groups. In correlation with these results, the histological healing results of the HCQ groups were higher than those of the control groups. However, there was no difference between the HCQ and control groups. In an experimental HCQ study, there was no difference between the control and experimental groups in histological healing outcomes at three and six weeks.[21] In a clinical trial, the use of methotrexate, sulfasalazine, HCQ, or combination treatment was not associated with the incidence of fractures in patients with rheumatoid arthritis.[22]

The ALP enzyme phenotype belongs to hypertrophic chondrocytes, preosteoblasts, and odontoblasts.[23] While ALP is known as an early osteoblastic marker, OC is considered a mid to late osteoblastic marker.[24] Osteopontin is an extracellular matrix protein produced by osteoblasts. It has been shown that OPN expression is low in fibroblast-like cells and high in transchondral ossification regions during distraction osteogenesis.[25] In our study, the ALP level was lower in the HCQ-4 group than in the C-4 group, but no difference was observed between the groups in terms of OC and OPN levels. This means that the OS caused by HCQ had no direct effect on the mid-late products (OC and OPN) of osteoblasts, whereas the decreased levels of ALP were associated with a decreased number of hypertrophic chondrocytes and endochondral ossification. The absence of change in histological fracture healing scores despite decreased endochondral ossification can be attributed to compensatory intramembranous ossification. Cathepsin K and TRAP5b levels were higher in the HCQ-4 group than in the C-4 group. This means that OS caused by HCQ increased osteoclastogenesis.

According to Lee et al.,[26] the disease-modifying antirheumatic drugs methotrexate, sulfasalazine, and infliximab suppressed the production of OCLs in human bone marrow cell cultures, whereas HCQ had no effect on osteoclast formation.

In a periodontitis model in rats, He et al.[27] showed that topical application of CQ reduced inflammation, osteoclastogenesis, and alveolar bone resorption by suppressing autophagy. In an osteoporosis model in mice, therapy with CQ had no effect on bone resorption and bone mass, did not alter the activities of osteoblasts but inhibited OCL development *in vitro*, bone resorption induced by parathyroid hormone *in vivo*, and ovariectomy *in vivo*.[28]

When the above-mentioned studies were analysed, HCQ reduced bone destruction through autophagy and lysosome inhibition. The main cells

that HCQ is expected to act on are OCLs with high lysosomal activity. Considering fracture healing, HCQ is expected to affect predominantly the remodeling phase of bone metabolism. In our study, we observed that HCQ increased OS parameters in rats and increased OCL number and functions but did not affect the mid-late products of osteoblasts. Although we observed a decrease in hypertrophic chondrocyte count and endochondral ossification, there was no significant difference between the control and HCQ groups in terms of histological fracture healing scores.

In the previously mentioned experimental HCQ study,[21] radiographic callus formation and the callus/diaphysis ratio were used to compare fracture healing. It was found that callus formation and callus/diaphysis ratio were lower in the experimental groups than in the control group at both the third and sixth weeks. In our study, no difference was found between the control and HCQ groups in terms of radiological scores. Since radiographic evaluation was not sufficient to assess callus volume, histomorphometric evaluation was used.[29] The TCD/FBD ratio was lower in the HCQ-2 group than in the C-2 group and lower in the HCQ-4 group than in the C-4 group, which means that the total callus diameter decreased in the HCQ groups. This can be explained by the smaller cartilage callus tissue detected in the histological sections.

There are some limitations to this study. Staining is limited because of the high price of immunohistochemical dyes. Considering ethical concerns, the number of experimental animals was kept as low as possible.

In conclusion, HCQ-induced OS increases the number and function of osteoclasts and decreases the number of hypertrophic chondrocytes and endochondral ossification but has no significant effect on mid-late osteoblast products and histological fracture healing scores.

Ethics Committee Approval: Approval from the local animal experimentation ethics committee was granted by Bağcılar Training and Research Hospital (date: 10.05.2020; no: 2020/27). The Declaration of Helsinki on the Guide for the Care and Use of Experimental Animals was followed in the conduct of this study.

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

Author Contributions: Idea/Concept/Writing the Article: Y.Ö.; Control/Supervision: O.B.; Data Collection and/or Processing, literature review: Y.G., O.B.; Analysis and/or interpretation: Y.Ö., E.Y.S.; Critical review: M.A.G.

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