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Hydroxychloroquine induces oxidative stress and impairs fracture healing in rats

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ABSTRACT

Objectives: The aim of this study was to investigate whether hydroxychloroquine sulfate (HCQS) induced oxidative stress and how it affected the union of bone fractures in an experimental rat model.

Materials and methods: A total of 48 Wistar albino male rats were used. The rats were divided into six groups. To investigate the effects of oral administration of HCQS at varying doses between the third and sixth weeks, fracture healing processes were evaluated using radiography, histopathology, biochemistry, and dual-energy X-ray absorptiometry. The activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA) were measured to analyze the relationship between HCQS and oxidative stress.

Results: Radiographic scores, alkaline phosphatase levels, callus/diaphysis ratio, callus development, and bone mineral density were significantly lower in rats given HCQS at three and six weeks compared to the control group ($p<0.005$). When oxidative stress parameters were compared among the groups, all antioxidant parameters were statistically significant, indicating that antioxidant systems played a role in peripheral blood, when HCQS was used ($p<0.005$).

Conclusion: Oral HCQS intake impairs the fracture healing process by causing oxidative stress in rats. However, further biomolecular researches are needed to understand the underlying mechanism of these effects.

Keywords: Bone, fracture, healing, hydroxychloroquine sulfate, oxidative stress.

After the novel coronavirus disease 2019 (COVID-19) pandemic that occurred globally in 2020, most of the world took infectious disease measures to reduce the effects of the pandemic. In the treatment of patients infected with this viral pathogen that primarily affects the respiratory tract, besides antiviral agents, hydroxychloroquine sulfate (HCQS) was also found in the guidelines for a while, but was removed over time.[1-3]

The HCQS is a pharmacologically formulated 4-aminoquinoline derivative as the sulfate salt for oral use. It has been used as a first-line drug for both the chemoprophylaxis and treatment of malaria for more than 70 years. However, in the 2020 COVID-19 pandemic, it was used to create a cytokine storm against the virus in the metabolism of the patient, just as it is used for remission in severe rheumatoid arthritis (RA) attacks.[2,3]

The number of experimental studies on the bone metabolism of chloroquine (CQ) is quite limited.

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The main and one-of-a-kind mechanistic study was performed by Xiu et al.[4] In their study, although no effect of CQ on baseline bone resorption was found, the authors inhibited osteoclast genesis by inhibition of parathormone in ovariectomized rats. In the study by He et al.,[5] which can be a reference for other experimental but not clinical applications, CQ inhibited autophagy, playing a vital role in the pharmacological inflammation and bone resorption phase, thereby reducing osteoclast genesis and bone resorption. In the literature, there are studies investigating the effects of different agents on fracture healing.[6]

In the present study, we aimed to examine whether HCQS caused oxidative stress and how it affected the union of bone fractures in an experimental rat model.

MATERIALS AND METHODS

A total of 48 Wistar albino male rats (age 8 to 10 months and weight 300 to 320 g) were used. The rats were kept in individual cages. They were fed with a light-dark cycle and standard pellet feed and tap water for 14/10 h at a constant mean temperature of 23±2°C.

The number of rats involved was calculated based on power analysis with G*Power version 3.1.9.4 software (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) with α=0.05, β=0.10, effect size=0.80; minimum seven rats were required for each study and/or control group. Considering possible dropouts, each group consisted of eight rats. Accordingly, the subjects were randomly assigned to six groups of eight rats each: two control groups in terms of time intervals (Group 1-3rd w: control group sacrificed at three weeks; Group 1-6th w: control group sacrificed at six weeks) and four study groups regarding the time interval and dosage of HCQS (Group 2-3rd w: in solution formulation by gastric lavage, HCQS 2×3 mg loading dosage for one day, 1×0.9 mg maintenance dosage for four days, followed by sacrification at three weeks after the surgical procedure; Group 2-6th w: in solution formulation by gastric lavage, HCQS 2×3 mg loading dosage for one day, 1×0.9 mg maintenance dosage for four days, followed by sacrification at six weeks after the surgical procedure; Group 3-3rd w: in solution formulation by gastric lavage, HCQS 2×1.5 mg loading dosage for one day, 1×0.45 mg maintenance dosage for four days, followed by sacrification at three weeks after the surgical procedure; Group 3-6th w: in solution formulation by gastric lavage, HCQS 2×1.5 mg loading dosage for one day, 1×0.45 mg maintenance dosage for four days, followed by sacrification at six weeks after the surgical procedure).

A 1.2 mL sample of blood was taken before the femur was fractured for alkaline phosphatase (ALP) analysis. Before the rats were euthanized, anteroposterior and lateral femoral radiographies were obtained, whole femoral bone mineral density (BMD) was assessed, and 3 mL of blood was withdrawn to evaluate oxidative stress. After the rats were sacrificed, the entire right femurs were removed and preserved in a 10% formaldehyde solution for histopathological examination.

Radiographic measurements from all subjects were repeated twice with a one-month interval by a single orthopedic and a radiology specialist for radiological evaluation in a double-blind manner with 10 years of professional experience to assess intra- and inter-observer reliability. Moreover, two pathologists in a double-blind manner with five years of experience for histopathological evaluation also performed the same procedure, respectively. To assess agreement and disagreement between intra- and inter-observer measurements, a two-way mixed effects model and intraclass correlation coefficients (ICCs) were used.

Procedures on the preparation of HCQS oral solution formulation and administration to the subjects

The HCQS treatment posology in the information on drugs that can be used in the treatment of COVID-19 (severe acute respiratory syndrome-coronavirus 2 [SARS-CoV2] infection) was the last updated by the Republic of Türkiye, Ministry of Health on March 25th, 2020[7] as 10 mg/kg (max. 600 mg) PO×2 (loading dose), then 3 mg/kg PO×1 (maintenance dose) t.i.d. (max: 200 mg) four days total treatment duration of five days. Accordingly, the subjects were given via gastric lavage in suspension formulation. The average weight of the rats was calculated as 300 mg. A maximum of 0.4 mL was only given in gastric lavage in each session of an average of 300 mg rat.[8] In addition, the treatment posology was in a standard amount of active substance in each 0.4 mL volume to avoid confusion between the groups in gastric lavage application: if the loading dosage of 10 mg in 1,000 g is 2×1; if the loading posology of 3 mg in 300 mg, and the maintenance dose of 3 mg in 1,000 g is 1×1; calculated as 0.9 mg loading dose at 300 mg.

Stock formulation

For 120 mL oral suspension of HCQS25 mg/mL preparation, HCQS 200 mg film-coated 15 tablets,

ORA-Plus® 60 mL (Perrigo®, Perrigo Co., Minneapolis, MN, USA, NDC#0574-0303-16), which is a suspending vehicle used to simplify the process of extemporaneous compounding of oral suspensions, and 120 mL sterile water for irrigation according to the United States Pharmacopeia version 27 (USP 27) were used.

Materials used

Mortar and pestle, conical measuring tape, stirring stick, 70% isopropyl alcohol, amber colored glass bottle, and small plastic dosing cup were used.

Method of preparation in order:

i. Remove the film coating from the tablet surface using 70% isopropyl alcohol. The tablets are placed in a small dosing cup and 70% isopropyl alcohol is added only to remove the film coating on the tablet surface. Let stand for 10 sec. Never wait for more than 10 sec. Then, put the tablets on a paper towel. Gently rub the tablets to remove the coating.
ii. The tablets are taken in a pestle and crushed with a pestle, until they become fine powder.
iii. The powder is wetted by adding minimum amount of Ora-Plus® and turned into a smooth paste.
iv. While adding Ora-Plus®, geometrically mixing is performed after each addition.
v. This mixture is transferred to the conical measuring tape.
vi. The mortar and pestle hand are washed with some irrigation water and the remaining dose is transferred to the measuring tape.
vii. The mixture is mixed thoroughly and completed with up to 120 mL of water for irrigation.
viii. Amber is put into the bottle and shaken well.
ix. Labeled as α, β, δ, and ε.

Storage period and conditions

Information on storage at room temperature is not available in the literature. It can be stored in the refrigerator for 30 days. It must be shaken well before use.

Oral suspension of HCQS 25 mg/mL administration

Oral gastric lavage procedures were performed with 14 to 16-gauge gastric needles (Kent Scientific Corp., CT, USA; FNC 14-3-2 and 16-3-2) by the same researcher.

Group 2 (3rd w & 6th w): Loading dose 2×0.4 mL (3 mg) of 20 mL suspension labeled as α, on day one, and maintenance dose 1x0.4 mL (0.9 mg) of 40 mL suspension labeled as β on Days 2, 3, 4, and 5 was given.

Group 3 (3rd w & 6th w): Loading dose 2×0.4 mL (1.5 mg) of 20 mL suspension labeled as β, on day one, and maintenance dose 1x0.4 mL (0.45 mg) of 40 mL suspension labeled as ε on Days 2, 3, 4, and 5 was given.

Surgical procedure

All surgical procedures were performed in spontaneously breathing animals anesthetized with 6 to 8 mg/kg xylazine (Rompun® 2% solution, 50 mL vial, Bayer-Turk Pharmaceuticals Ltd., Istanbul, Türkiye) and 60 to 80 mg/kg ketamine (Ketalar® 50 mg/mL 10 mL vial, Pfizer Pharmaceuticals Ltd., Istanbul, Türkiye). Before any surgical procedure, a single dose of prophylactic antibiotic was given.

An arthrotomy was performed in all rats with a standard knee midline incision extended proximally. After the skin-subcutaneous tissue was passed, the knee joint was entered with a median parapatellar incision. After revealing the mid-distal parts of the right femur of all rats, it was osteotomized from this region with the help of an electric saw (Tri-Mic™, Triton Micro Technologies Inc., CA, USA; bone micro-saw sagittal, rotational and oscillating), and the femurs of the rats were retrogradely intramedullary rimier using a micro-drill. It was fixed with 1.5-mm Kirschner wires (K-wires). After fixation, care was paid to keep the K-wires in the femur from proximal and distal. Following fixation, the fracture line and the incision area were washed with 30 mL of 0.09% sodium chloride (NaCl). The patellar tendon and skin were, then, closed with 3/0 sharp needle Vicryl (Johnson & Johnson©, Brussels, Belgium), respectively. No other fixation was made after the wound dressing was done.

Biochemical evaluation

The ALP activity can be used to predict the course and rate of bone healing following sustained fractures.[9] Accordingly, blood ALP levels of the rats were measured simultaneously in our study using the ARCHITECT™ cSystem (Abbott Laboratories, Abbott Park, IL, USA).

Radiological evaluation

A previously described five-point scoring system modified by Warden et al.[10] was used to evaluate fracture healing for each group after sacrification at three and six weeks. The callus/diaphysis ratio

and volume of new callus formation at the femoral fracture site were also measured on plain X-rays three and six weeks after surgery.

Histopathological evaluation

A 10-point scale was used for the histological evaluation of fracture healing.[11]

BMD evaluation

The BMD of the femurs was determined using dual-energy X-ray absorptiometry on a Hologic QDR 4500 ELITE Acclaim Series (Hologic Inc., MA, USA). High resolution measurements of area, bone mineral content, and BMD data were obtained from a region of interest approximately 1.45×1.08 mm over the region of osteotomy in the left limb as well as the osteotomy-equivalent site in the right intact limb.

Oxidative stress evaluation

Sample preparation

To separate plasma, blood samples were centrifuged at 4,400 rpm for 5 min. The plasma samples were kept at -80°C, until the oxidative stress parameters were determined. A spectrophotometer was used to determine the antioxidant enzymes in the samples (UV-1800, Shimadzu Co., Kyoto, Japan). Protein content in samples was determined using the Lowry et al.'s[12] method.

Catalase (CAT) enzyme activity

The Aebi method was used to measure CAT activity in plasma samples at 25ºC. For 30 sec, the decomposition rate of the substrate hydrogen peroxide (H_2O_2) was measured spectrophotometrically at 240 nm. The CAT was calculated as U/g protein.[13]

Superoxide dismutase (SOD) enzyme activity

The SOD activity was measured as described by Fitzgerald et al.[14] Enzyme activity was calculated as U/g protein.

Malondialdehyde (MDA) levels

Ohkawa et al.[15] described a method for measuring MDA in samples to estimate lipid peroxidation. The results were given in nmoL/mg protein.

Glutathione peroxidase (GPx) enzyme activity

The GPx activity was measured as previously described by Pleban et al.[16] The results were given in U/mg protein.

Statistical analysis

Statistical analysis was performed using the IBM SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). The Kolmogorov-Smirnov test was used to check all variables in all six groups for normal distribution. Descriptive data were presented in mean ± standard deviation (SD), median (min-max) or number and frequency. The Mann-Whitney U test was used to assess differences among all six groups for variables with non-normal distribution. The Pearson chi-square test was used for univariate analysis of dependent and independent variables. One-way analysis of variance (ANOVA) was used to compare the results of different groups, followed by the Tukey multiple comparisons test. The Cox regression analysis was used to determine the factors influencing the score values. Subject scores were used as the dependent variable in Cox regression analysis, with follow-up time as the independent

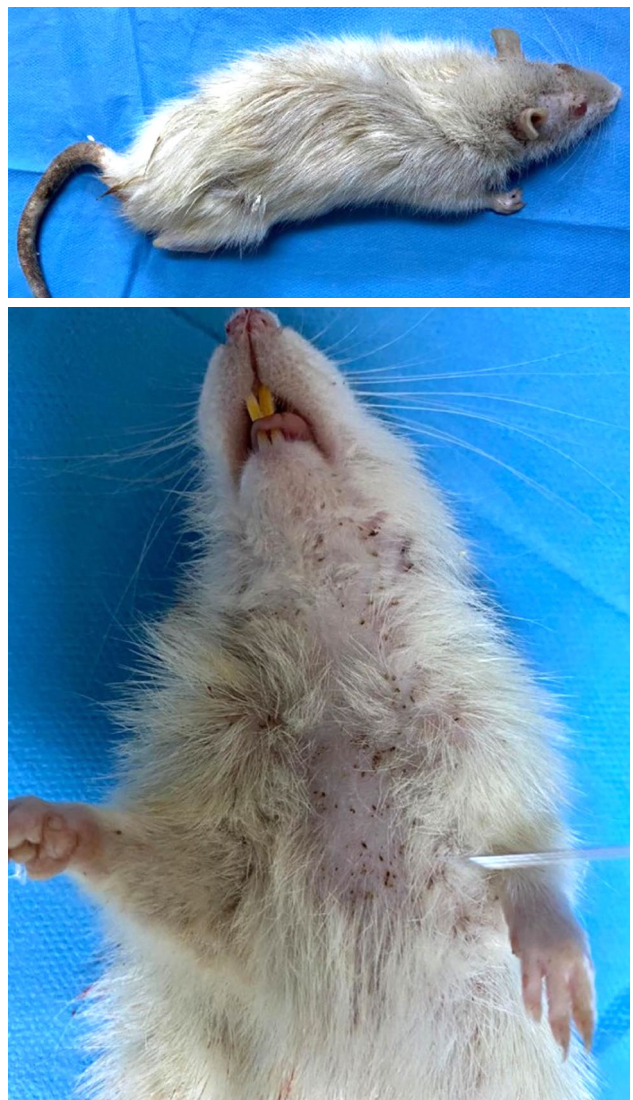

FIGURE 1. Clinical view of hydroxychloroquine-related hyperpigmentation.

TABLE I
Radiological scores for all groups

Groups (n=8 for each)	0 Point		1 Points		2 Points		3 points		*p* for overall difference
	n	%	n	%	n	%	n	%	
Group 1-3rd w: Control group sacrificed at third week	0	0	0	0	3	37.5	5	62.5	
Group 1-6th w: Control group sacrificed at sixth week	0	0	0	0	1	12.5	7	87.5	
Group 2-3rd w: Hydroxychloroquine Sulfate 2×3 mg loading dosage for 1 day, 1×0.9 mg maintenance dosage for 4 days and sacrificed at third week	0	0	3	37.5	4	50	1	12.5	
Group 2-6th w: Hydroxychloroquine Sulfate 2×3 mg loading dosage for 1 day, 1×0.9 mg maintenance dosage for 4 days and sacrificed at sixth week	0	0	4	50	3	37.5	1	12.5	0.00138
Group 3-3rd w: Hydroxychloroquine Sulfate 2×1.5 mg loading dosage for 1 day, 1×0.45 mg maintenance dosage for 4 days and sacrificed at third week	1	12.5	2	25	4	50	1	12.5	
Group 3-6th w: Hydroxychloroquine Sulfate 2×1.5 mg loading dosage for 1 day, 1×0.45 mg maintenance dosage for 4 days and sacrificed at sixth week	1	12.5	2	25	3	37.5	2	25	

w: Week.* Intra- and inter-observer reliability were evaluated based on radiographic measurements from all subjects repeated twice with an interval of one month by one orthopedics and traumatology and one radiology specialist with 10 years of professional experience. A two-way mixed effects model and intraclass correlation coefficients (ICC) were used to evaluate agreement and differences between intra- and inter-observer measurements. Intra- and inter-observer reliability were found to be excellent (ICC=0.986-0.996) and good (ICC=0.873-0.991), respectively.

variable. All statistical analyses were carried out using a backward conditional logistic regression model. A p value of <0.05 was considered statistically significant.

RESULTS

Almost all HCQS-treated subjects exhibited hydroxychloroquine-related hyperpigmentation, usually indicating superficial dermal deposition of

TABLE II
Comparison of callus/diaphysis ratio, callus formation, and serum alkaline phosphatase level between the scarification weeks

Groups (n=16 for each)	Callus/diaphysis ratio		Callus formation (mm)		Alkaline phosphatase level (U/mL)		
	3rd Week	6th Week	3rd Week	6th Week	Preoperative	3rd Week	6th Week
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Control groups: Group 1-3rd w and Group 1-6th w	3.3±0.2	4.1±0.5	4.5±0.4	5.2±0.4	230±29.1	367±51.4	421±48.5
Hydroxychloroquine Sulfate 2×3 mg loading dosage for 1 day, 1×0.9 mg maintenance dosage for 4 days groups: Group 2-3rd w and Group 2-6th w	1.4±0.3	2.6±0.6	0.3±0.1	1.0±0.3	242±18.2	128±37.7	241±23.4
Hydroxychloroquine Sulfate 2×1.5 mg loading dosage for 1 day, 1×0.45 mg maintenance dosage for 4 days groups: Group 3-3rd w and Group 3-6th w	2.9±0.1	3.2±0.2	0.7±0.3	2.1±0.3	228±11.4	259±31.5	297±19.4
Significance of time-dependent intergroup callus/diaphyseal change (p<0.005)	F=44.423 p<0.001		F=57.247 p<0.001		F=113.343 p<0.001		
One-way analysis of variance in repeated analysis	*		*		**		

SD: Standard deviation; w: Week; * Th post-hoc Tukey test showed a significant difference between the groups; ** In the post-hoc Tukey test, there was a significant difference between preoperative alkaline phosphatase and third- and sixth- week values. The difference between the Group 2-3rd w & Group 2-6th w and Group 3-3rd w & Group 3-6th w was significant.

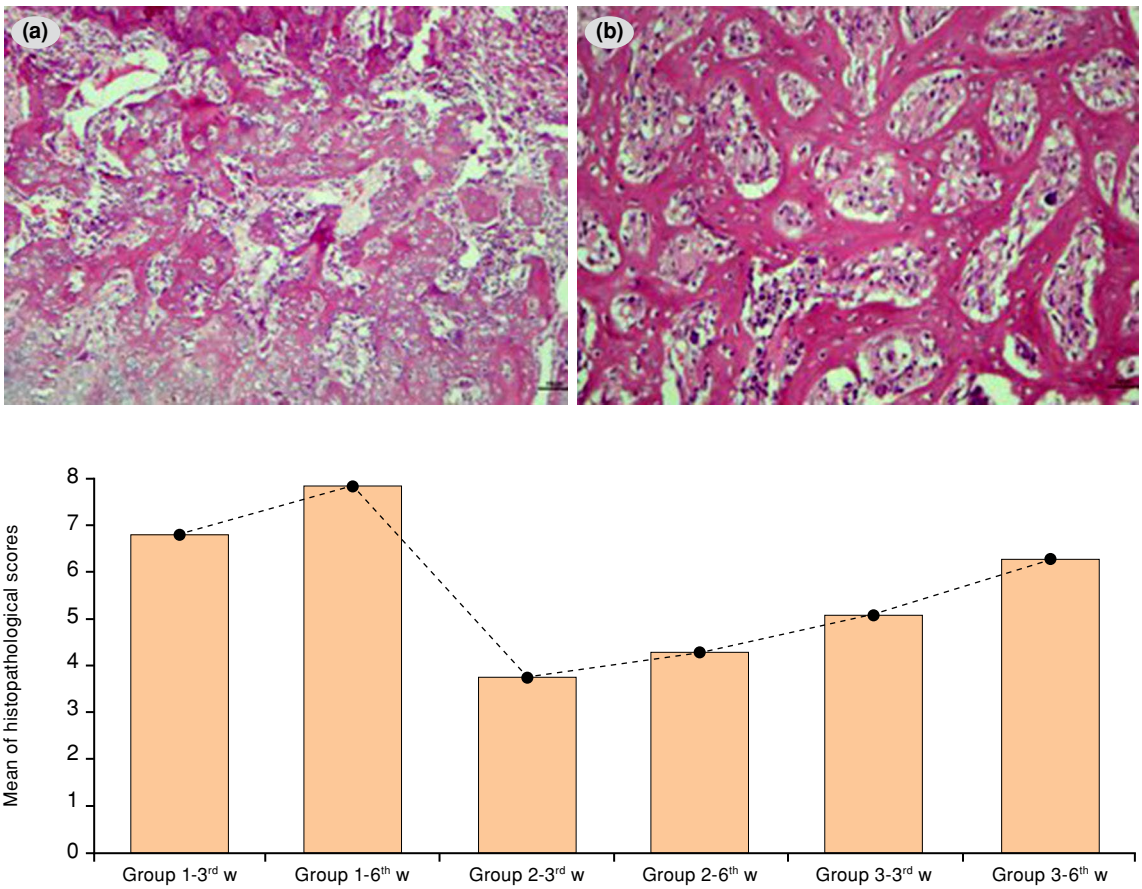

FIGURE 2. Histopathological samples and mean of scores. **(a)** Dominant proliferation of cartilage tissue at the fracture healing zone (H&E, ×100) in Group 1-3rd week. **(b)** Immature bone-rich proliferation at the fracture healing zone (H&E, ×200) in Group 1-6th week.
w: Week.

brown-pigmented granules of iron or melanin, or both (Figure 1).

Radiological scores were significantly lower in Groups 2 and 3 compared to the controls at both three and six weeks ($p<0.05$) (Table I). Group 1-6th w had the highest radiological score at six weeks among both the control and study groups ($p<0.001$).

In this study, we measured the callus/diaphysis ratio, callus tissue volume, and serum ALP levels, which are the most commonly used indicators of fracture union in radiological and biochemical follow ups (Table II). Radiological score intra- and inter-observer reliability were determined to be excellent (ICC=0.986-0.996) and good (ICC=0.873-0.991), respectively.

Between Weeks 3 and 6, the increase in callus masses in the control groups (Group 1-3rd w and Group 1-6th w) was significantly different from Group 2 and Group 3 ($p<0.05$). Although the decrease in Groups 2 and 3 was different from the control group at the end of six weeks, there was no significant difference between the groups, regardless of time interval ($p>0.05$). At preoperative, three, and six weeks, serum ALP levels were significantly different among the control, Group 2, and Group 3 groups ($p<0.05$). Although the increase in serum ALP levels was significantly different in Groups 2 and 3 compared to the control group at three weeks, there was no significant difference between Groups 2 and 3 ($p>0.05$).

Histopathological scores in all three groups were improved at six weeks, although there was no significant difference between the three and six weeks (Figure 2). For both inter-observer pathologists, the intra-observer and inter-observer reliability of histopathological evaluation scores was excellent (ICC=0.945–0.991).

TABLE III
Bone mineral density values for all groups

Groups (n=8 for each group)	Mean±SD (g/cm^2)	*p* for overall difference
Group 1-3rd w: Control group sacrificed at third week	0.145±0.007	<0.001
Group 1-6th w: Control group sacrificed at sixth week	0.178±0.003	
Group 2-3rd w: Hydroxychloroquine Sulfate 2×3 mg loading dosage for 1 day, 1×0.9 mg maintenance dosage for 4 days and sacrificed at third week	0.042±00.001	
Group 2-6th w: Hydroxychloroquine Sulfate 2×3 mg loading dosage for 1 day, 1×0.9 mg maintenance dosage for 4 days and sacrificed at sixth week	0.052±0.004	
Group 3-3rd w: Hydroxychloroquine Sulfate 2×1.5 mg loading dosage for 1 day, 1×0.45 mg maintenance dosage for 4 days and sacrificed at third week	0.072±0.002	
Group 3-6th w: Hydroxychloroquine Sulfate 2×1.5 mg loading dosage for 1 day, 1×0.45 mg maintenance dosage for 4 days and sacrificed at sixth week	0.092±0.006	

SD: Standard deviation; w: Week.

TABLE IV
Comparison of oxidative stress parameters in each group

	Group 1-3rd week	Group 2-3rd week	Group 3-3rd week	Group 1-6th week	Group 2-6th week	Group 3-6th week	
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	*p*
CAT (U/g protein)	1.5±0.6	4.6±0.4	2.7±0.7	1.3±0.8	3.5±1.0	1.8±0.4	0.001
SOD (U/g protein)	8.9±2.2	14.7±3.3	10.5±4.2	10.8±4.8	9.0±3.3	9.7±3.1	0.001
MDA (nmol/mg protein)	1.0±0.2	3.5±1.1	2.0±0.8	2.1±0.7	3.2±0.7	1.1±0.1	0.001
GPx (U/mg protein)	0.8±0.4	2.7±0.7	1.3±0.7	1.4±0.4	1.9±0.4	0.7±0.3	<0.001

SD: Standard deviation; CAT: Catalase; SOD: Superoxide dismutase; MDA: Malondialdehyde; GPx: Glutathione peroxidase.

In all three groups, BMD was found to be lower at three weeks than six weeks (Table III). There was no significant difference in the intra-group analysis results between the three and six weeks. Group 1-6th w had the highest BMD values ($p<0.05$).

The values of CAT, SOD, MDA, and GPx studied in plasma samples as oxidative stress parameters are shown in Table IV. In the comparison of oxidative stress parameters in each group, all antioxidant enzyme activities and lipid peroxidation levels were statistically significant in each group, suggesting that the use of HCQS caused lipid peroxidation and the antioxidant defense system played a role in peripheral blood (Table IV).

DISCUSSION

The results of this experimental study showed that radiological scores, ALP levels, callus/diaphysis ratio, callus formation and BMDs were significantly lower in the experimental femoral fracture model in rats given HCQS at both three and six weeks compared to the control group. To the best of our knowledge, this is the first study to show that HCQS negatively affects fracture healing by causing lipid peroxidation and changes in the antioxidant defense system.[17]

Another important result of the study is that although ALP level, callus formation, callus\diaphysis ratio, and BMD values were lower in Group 2 given dose-equivalent HCQS at three and six weeks, there was no statistically significant difference in Group 3 at both three and six weeks. In other words, HCQS adversely affects fracture healing in an experimental fracture model independent of time and dose.

When CQ is added to the cell culture, the osteoclast effect area decreases depending on the dose-dependent decrease in the number of osteoclasts; however, when the CQ concentration in the cell culture medium is increased, the total number of osteoclasts remains unchanged, although the osteoclast domain decreases.[4] It has been demonstrated that CQ

inhibits osteoclast formation by preventing tumor necrosis factor receptor-associated receptor 3 (TRAF3) lysosomal degradation *in vitro* and *in vivo* and does not inhibit receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast formation in TRAF3-deficient cells.[4] In the same study, CQ treatment showed no effect on baseline bone resorption and bone mass, did not affect osteoblast functions, but inhibited osteoclast formation *in vitro* and bone resorption induced by parathormone and ovariectomy *in vivo*.[4] Although this mechanism has proposed that CQ may prevent bone resorption in humans, the current study demonstrated that it impaired fracture healing, indicating the need for more detailed molecular studies on bone metabolism and fracture healing associated with HCQS.

In the experimental model of periodontitis, which is an inflammation characterized by alveolar bone resorption resulting from an imbalance in bone hemostasis, it has been shown that the number of osteoclasts in the high-dose CQ group was the least and CQ reduced inflammation and alveolar bone resorption.[5] In addition, bone resorption induced by inflammatory cells and osteoclasts was clearly dependent on autophagy. As a result of the study, the authors concluded that CQ could reduce inflammation, osteoclast genesis and bone resorption by inhibiting autophagy, which plays a vital role in the inflammation and bone resorption phase. Considering the results of the aforementioned study, it is likely that inhibition of autophagy and reduction in osteoclast number may affect the remodeling phase of fracture healing. Naturally, this effect may adversely affect fracture healing, but again more detailed molecular studies on bone metabolism and fracture are warranted.

In another mechanistic study on hydroxychloroquine, it inhibits K+ flux by blocking Ca+2-activated K+ channels in macrophages, resulting in impaired inflammatory activity and proinflammatory cytokine release.[18] It is possible that the negative effects of HCQS on fracture healing may have also occurred through this mechanism, as the ion channels, particularly the Ca^{+2}-activated K^{+} channels, contributed to the mechanosensory and mechanotransducive processes of the bone.[19]

Oxidative stress is the deterioration of the balance between oxidants, which occur as a natural result of the aerobic mechanism, and the antioxidant defense, attempting to limit them by enzymatic or non-enzymatic processes in favor of oxidants.[20] Excessive oxidative stress has the potential to cause apoptosis in chondrocytes, osteoblasts, and osteocytes, as well as to activate osteoclasts in bone resorption.[21] Due to inflammatory and ischemic conditions, reactive oxygen species (ROS) are produced during the early stages of fracture healing.[22,23] The ROS affect bone hemostasis by inhibiting anabolic cell differentiation, such as osteoblasts, osteocytes, and chondrocytes, and activating catabolic cells.[24-28] Oxidative stress is induced by both external and internal injuries of the body, and a more severe injury indicates a more severe oxidative stress.[29] Evidence suggests that HCQ may mediate oxidative stress and influence antioxidant enzymes.[30] As they are free radical scavengers, the SOD, CAT, and GPx enzymes serve as important antioxidant defense mechanisms, protecting against the deleterious effects of toxicants. Increased MDA levels indicate increased lipid peroxidation, which causes cellular damage and failure of the antioxidant defense mechanism to prevent excessive free radical production.[29] In the current study, we found that the groups given high-dose HCQS had higher MDA levels and CAT activity at the end of the third and sixth weeks compared to the control group. However, the SOD and GPX activities increased in the high-dose groups at the end of the third week, but no significant difference was observed at the end of the sixth week. In the light of these results, the oxidative stress caused by HCQS may have contributed to the negative effect of fracture healing. Uzar et al.[31] reported that HCQ caused oxidative stress in rat sciatic nerve and muscle tissues. In their study, MDA levels, as well as SOD and GPx activity, were significantly higher in the HCQ group than the control group, while CAT activity was not statistically different between the two groups. Farombi et al.[32] also measured oxidative stress parameters in malaria patients treated with CQ. The CQ-treated groups had significantly lower CAT and GPX activities in erythrocyte compared to the control groups, while SOD activity and MDA levels were increased. Ogunbayo et al.[33] reported that a single dose (10 mg/kg of body weight) of CQ administration changed the enzymatic antioxidant defense systems and increased MDA levels in the blood of rabbits within 6 h.

In another study, Muljacić et al.[9] showed that plasma ALP and bone-specific ALP levels measured at different time points in patients with long bone fractures were similar to each other and that monitoring the differences in these enzyme levels could allow early detection of fracture healing. A small increase in ALP and bone-specific ALP values during the first two weeks indicated successful fracture fixation, rapid bone healing, and minimal

or insignificant callus formation, whereas a large increase in ALP and bone-specific ALP activity during the first two weeks indicated inadequate fracture fixation, delayed bone healing, and visible and significant callus formation. In our study, the highest ALP levels were found in the control groups and the lowest in those receiving high-dose HCS. This result is consistent with the radiological scores, callus formation and callus/diaphysis ratio of the groups. The fact that the study is compatible with ALP, the biochemical marker of fracture union of the bones included in the radiological evaluation suggests that the study is self-consistent.

Although there is no study showing the clinical effect of HCQS on fracture healing in the literature, there is also no study evaluating the results of fracture treatment in malaria patients, where it is most commonly used. In the literature, there are studies investigating fracture healing only in RA cases. It has been reported that femoral intertrochanteric fractures in RA patients receiving HCQS have a higher rate of avascular necrosis and nonunion than expected in the general population.[34] In another study, when the patients with RA were compared with the healthy control group, there was no statistically difference between the two groups in terms of femoral intertrochanteric fractures, but in femoral shaft fractures in patients with RA, the rate of union was longer and delayed union was significantly higher.[30] In another study, the incidence of atypical femur fractures was higher in HCQS-treated RA patients; however, 81% of the cases had a history of glucocorticoid use and 45% of them had a history of non-steroidal anti-inflammatory drug use, while the use of HCQS was not specified.[35]

Nonetheless, there are several limitations to this study. First, micro-computed tomography investigations were unable to determine the callus size of the fracture sites due to technical limitations. Second, the study sample size is small, and the experiments could not be repeated due to technical constraints. Third, the use of a closed fracture model in rats may allow for the preservation of typical fractures and thermal effect of the saw on bone. Finally, biomechanical evaluation should be studied separately in further experimental studies.

In conclusion, oral HCQS intake impairs the fracture healing process by causing oxidative stress at the fracture site. Further biomolecular studies are required to highlight the underlying mechanism of these effects. In addition, the effects of the HCQS on bone metabolism in *in vitro* settings are warranted.

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

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