Joint Diseases and Related Surgery

Jt Dis Relat Surg
2023;34(3):628-639

ORIGINAL ARTICLE

The effect of oral hydroxychloroquine on chondral defect: An experimental study

Erdal Uzun, MD[1], Semih Güvercin, MD[1], Ali Eray Günay, MD[2], İbrahim Halil Kafadar, MD[1], Demet Bolat, MD[3], Arzu Hanım Yay, MD[3,4], Ahmet Güney, MD[1]

[1]Department of Orthopedics and Traumatology, Medicine Faculty of Erciyes University, Kayseri, Türkiye
[2]Department of Orthopedics and Traumatology, Kayseri City Hospital, Kayseri, Türkiye
[3]Department of Histology and Embryology, Medicine Faculty of Erciyes University, Kayseri, Türkiye
[4]Erciyes University, Genome and Stem Cell Center, Kayseri, Türkiye

ABSTRACT

Objectives: This study aims to evaluate the time- and dose-dependent effects of oral hydroxychloroquine (HCQ) on focal full-thickness knee chondral defect healing in a rabbit model.

Materials and methods: Cartilage defects of 4×4 mm^2 were created on both medial femoral condyles of 24 New Zealand rabbits. The rabbits were divided into six groups (A-F) according to HCQ administration and sacrifice time: A (three-week control) and B (six-week control) received no additional interventions; C (20 mg/kg HCQ, three weeks); D (20 mg/kg HCQ, six weeks); E (40 mg/kg HCQ, three weeks); and F (40 mg/kg HCQ, six weeks). Osteochondral specimens were evaluated macroscopically, histologically, and immunohistochemically. The terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method was used to detect apoptotic cells.

Results: The International Cartilage Repair Society (ICRS) scores were significantly higher in the experimental groups than in the controls ($p<0.001$). The Wakitani scores in Group D showed a significant improvement compared to those in Group B ($p<0.01$). The 20 mg/kg HCQ treatment groups showed better recovery than the controls ($p<0.01$). High-dose HCQ (40 mg/kg) treatment significantly reduced the intensity of collagen type 2 immunoreactivity compared to that in the groups receiving 20 mg/kg of HCQ ($p<0.01$). Collagen type 2 expression in Group F was significantly lower than that in Group D ($p<0.01$). There were more TUNEL-positive cells in the repair sites of Groups E and F than in the lower-dose experimental groups and untreated experimental groups ($p<0.001$).

Conclusion: A low dose of HCQ improved cartilage repair, while higher doses of HCQ exerted a negative effect on cartilage regeneration in rabbits. In the presence of defective cartilage, the use of HCQ at an appropriate dose and time is important for cartilage health.

Keywords: Cartilage defect, hydroxychloroquine, oral, rabbit knee, treatment.

Articular cartilage plays an important role in joint movement and has an essential function of load-bearing in the structural organization of the normal joint.[1] Articular cartilage injuries are one of the most common orthopedic problems of the knee joint;[2] however, their treatment has always been challenging for clinicians, due to the lack of blood supply and limited regeneration capacity.[1,3-5] Injured articular cartilage is usually repaired with fibrous tissue, although this lacks the strength and mechanics of normal hyaline cartilage.[1] The resultant repaired tissue, with its poor quality biochemical and biomechanical properties, may eventually fail, leading to the development of osteoarthritis (OA) or degenerative joint disease, causing joint swelling, loss of function, pain, and impaired quality of life.[1,3,4,6] Therefore, protection of the knee joint cartilage and treatment injuries to it have gained importance.[1,3,7,8] The effects of many pharmacological and biological agents on cartilage healing and injury have been evaluated.[9-14]

Received: March 21, 2023
Accepted: June 01, 2023
Published online: August 21, 2023

Correspondence: Erdal Uzun, MD. Erciyes Üniversitesi Tıp Fakültesi Ortopedi ve Travmatoloji Anabilim Dalı, 38030 Melikgazi, Kayseri.

E-mail: nuzuladre@gmail.com

Doi: 10.52312/jdrs.2023.1114

Citation: Uzun E, Güvercin S, Günay AE, Kafadar İH, Bolat D, Hanım Yay A, et al. The effect of oral hydroxychloroquine on chondral defect: An experimental study. Jt Dis Relat Surg 2023;34(3):628-639. doi: 10.52312/jdrs.2023.1114.

Hydroxychloroquine (HCQ) is a hydroxylated analog of chloroquine (CQ) which has been widely used in the treatment of malaria since 1940s,[15] as well as in rheumatoid arthritis (RA) and other chronic inflammatory conditions, as a disease-modifying anti-rheumatic drug (DMARD) therapy.[16] It has also been used in the treatment of novel coronavirus disease 2019 (COVID-19), although this application has been controversial.[17,18] Several clinical studies have demonstrated the beneficial effects of HCQ in hand OA.[19,20] Numerous adverse effects of HCQ have also been identified in clinical use, such as pruritus, nausea, vomiting, headache, abdominal pain, anorexia, blurred vision, and urticaria.[21,22]

Previously, Volastro et al.[23] evaluated the protective or destructive effects of CQ in a rabbit cartilage damage model. They observed local toxic (destructive) effects when applied intra-articularly, while curative effects were seen when applied systemically (intraperitoneally). Moreover, in an *in vitro* study, Li et al.[24] showed that the application of HCQ in cell culture reduced collagen type 2 production and increased the level of matrix metalloproteinases (MMP-13), which have the potential to damage the cartilage. However, the effects of HCQ on defective cartilage have not yet been evaluated *in vivo*.

In the present study, we hypothesized that oral HCQ could affect cartilage formation and regeneration in a rabbit chondral defect model in a dose- and time-dependent manner. We, therefore, aimed to evaluate the effects of oral HCQ, which is widely used over an extended period in various diseases, on a cartilage defect model using histological, macroscopical, and immunohistochemical (IHC) analyses.

MATERIALS AND METHODS

A total of 24 mature (three-month old) male New Zealand white rabbits weighing between 2,500 and 3,500 g were used in this study. All animal experiments were conducted in accordance with the guidelines provided by the animal committee of the local government and international guidelines. The animals were randomly divided into six groups of four (eight knees). Group A received only cartilage defects (no additional interventions -three-week negative control); Group B received only cartilage defects (no additional interventions -six-week negative control); Group C received cartilage defects with oral HCQ (20 mg/kg) and three-week follow-up (FU); Group D received cartilage defects with oral HCQ (20 mg/kg) and six-week FU; Group E received cartilage defects with oral HCQ (40 mg/kg) and three-week FU; and Group F received cartilage defects with oral HCQ (40 mg/kg) and six-week FU.

Surgical technique

All rabbits were operated under general anesthesia using an intramuscular injection of 2.5 to 10 mg/kg of xylazine (Rompun®; Bayer, Leverkusen, Germany) and 20 to 40 mg/kg of ketamine (Ketalar®; Zentiva, Kirklareli, Türkiye). Cefazolin sodium (50 mg/kg; Sefazol®; Mustafa Nevzat Pharmaceuticals, Istanbul, Türkiye) was administered to all rabbits as antibiotic prophylaxis. Both knees were shaved, and the surgical areas were cleaned with povidone-iodine and prepared with sterile coverages. After the rabbits were placed in the supine position, an anterior midline incision was made, a medial parapatellar arthrotomy was performed, the patella was dislocated laterally, and the femoral condyle was exposed. A total of 48 full-thickness cartilage defects were created in the weight-bearing area of the medial femoral condyles on both knees of each animal (diameter: 4 mm, thickness: 4 mm) using a sharp, round, cylindrically shaped, hollow-edged tool (Figure 1). All calcified cartilage was carefully removed with a curette and debrided, and the underlying subchondral bone plate was exposed. The knee capsule was, then, tightly closed with simple sharp 2-0 Vicryl sutures, and the skin was closed with 3/0 PDS sutures. The incision was dressed with povidone-iodine. The rabbits were kept in separate environments during the recovery period after anesthesia and placed in their pre-operative locations after recovery. Knee joint movement and weight-bearing were not restricted, and the animals were followed until sacrifice.

Hydroxychloroquine administration

After HCQ was supplied in powder form, measurements were made on a precision balance in the facilities of the Histology Department of Erciyes University, according to the weights of the subjects (2,500 to 3,500 g). The application of HCQ according to the groups was as follows: control groups (Groups A and B) were administered 5 mL of physiological saline (PS) by oral gavage, starting on the first day after surgery. The experimental groups (Groups C, D, E, and F) were administered two different doses of HCQ once per day, and in Groups C and E, 20 mg/kg was mixed with 5 mL PS and administered to the subjects via orogastric lavage. In Groups D and F, 40 mg/kg was mixed with 5 mL of PS and administered to the subjects via orogastric lavage.

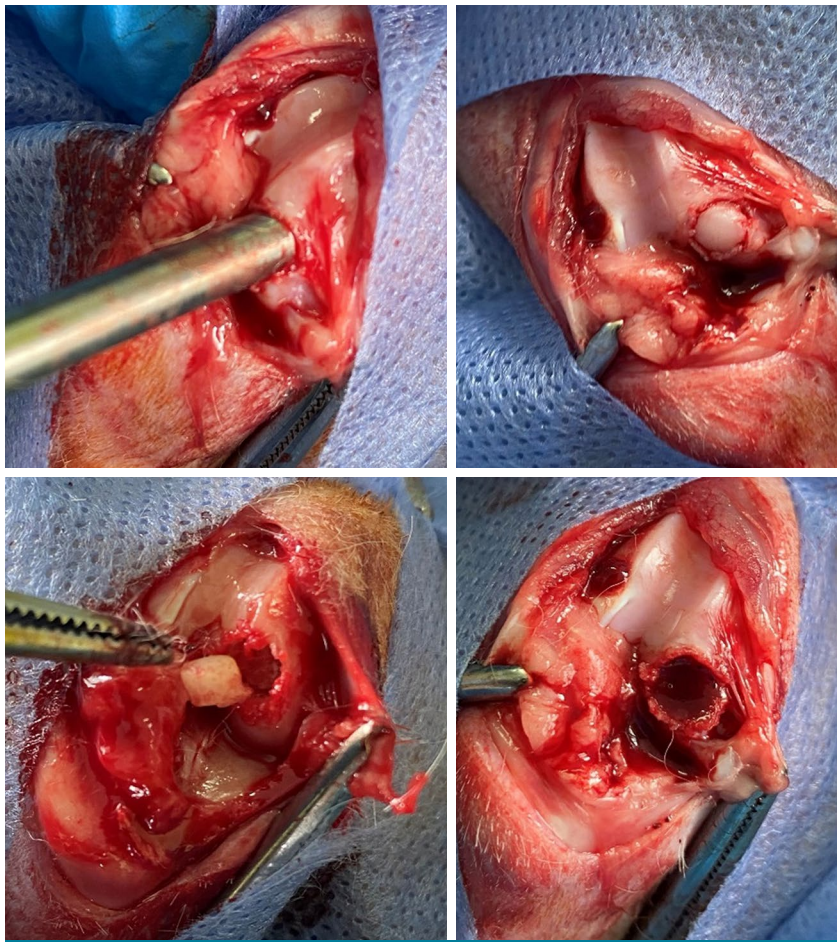

FIGURE 1. Full-thickness cartilage defects were created in the weight-bearing area of the medial femoral condyles on both knees of the animals (diameter: 4 mm, thickness: 4 mm) using a sharp round cylindrical shaped and hollow edged tool.

Postoperatively, the animals were sacrificed with an intracardiac injection of potassium chloride at two separate times. While Groups A, C, and E were sacrificed at the third week, Groups B, D, and F were sacrificed at the sixth week, and the medial femoral condyles were harvested.

Macroscopic examination

The regenerated tissues of the defect sites were photographed and blindly evaluated by two investigators macroscopically, using the International Cartilage Repair Society (ICRS) scores.[25]

Histopathological examination

After macroscopic evaluation, specimens of the distal medial femoral condyles were embedded in 10% formalin for 72 h and decalcified in 10% ethylenediaminetetraacetic acid (EDTA)-buffered saline solution. The samples were, then, serially dehydrated in ethanol and embedded in paraffin blocks. Subsequently, 5-µm sections were taken perpendicular to the articular surface from the paraffin blocks prepared for light microscopic examination using a microtome (Leica RM 2155; Leica Microsystems Nussloch GmbH, Germany). The 5-µm thick sections obtained from joint tissues were stored in an oven at 60°C for one night. The sections were, then, initially treated with hematoxylin and eosin (H&E) and Masson's trichrome (MT) staining to assess histoarchitectural changes. Two histologists blindly graded the histological preparations using a computer-assisted light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan) and Wakitani histology scoring systems.[26] There were no significant differences in the histological scores between the observers.

Immunohistochemical procedure

The IHC staining method is based on the binding of a marker with a specific antigenic detection feature on the cell surface. In this study, the expression of collagen type 2 (bs-0709R, Bioss) in defective cartilage tissues was demonstrated in the IHC examination. For IHC staining, avidin-biotin peroxidase was applied using Lab Vision™ UltraVision™ Large Volume Detection System: anti-Polyvalent, HRP (ready-to-use) (Thermo Scientific, TA-125-HL; Thermo Fisher Scientific Inc., MA, USA) IHC staining kit. To avoid tissue spillage, 5-µm sections from the paraffin-embedded blocks were prepared with routine FU after 10% formalin fixation and decalcification on poly lysine slides. The sections were rehydrated by first passing them through xylene and a graded alcohol series and, then, washed three times for 5 min with phosphate-buffered saline (PBS). For antigen recovery, the sections were boiled in a microwave oven at 600 Watts for 5 min with 10% citrate buffer, washed twice for 5 min with PBS (pH 7.4), and treated with 3% hydrogen peroxide (H_2O_2) for 12 min to inhibit endogenous peroxidase activity. The remaining procedures were carried out in a chamber to prevent the tissues from drying out. Each solution was applied to ensure that it covered the tissue completely. Block serum was applied to the sections, which were washed with PBS for 20 min at room temperature to ensure that the regions outside the antigenic areas were covered. Collagen type 2 primary antibody was dropped and incubated overnight at +4ºC on the tissues in the preparations. After washing, the sections were incubated with biotin-secondary antibodies for 30 min to ensure binding to the primary antibody, and the washing process was repeated. The sections were, then, treated with streptavidin peroxidase for 30 min and washed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Lab vision, UltraVision detection system, Large volume DAB substrate system, TA-125) peroxidase substrate kit for 1 to 10 min to make the immunoreactivities visible, and washed with deionized H_2O for 5 min. Subsequently, the tissues, which were placed in increasing alcohol concentrations for 5 min each, were placed in xylene for 15 min, and closed with a closure solution (Entellan™; Merck KGaA, Darmstadt, Germany). The sections treated using the IHC staining method were examined under a light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan)), and microscopic photographs were taken from 10 different areas at 40× magnification for each tissue. Collagen type 2 immunoreactivity intensities were calculated from the photographs taken using ImageJ software, and the results were recorded.[25,27]

TUNEL method

The terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method was used to detect apoptotic cells in the articular cartilage sections. Paraffin-embedded cross-sections of knee joint tissues with a thickness of 5 µm were deparaffinized, rehydrated in graded alcohol (pure alcohol, 96%, 80%, 70%, 60%, and 50%), and washed three times with PBS at room temperature. The cross-sections were, then, incubated with the TUNEL kit (in situ TUNEL reaction using ApopTag® Fluorescein In Situ Apoptosis Detection Kit; EMD Millipore Corp., Darmstadt, Germany) for apoptotic cell detection in a humid and dark environment and, then, rinsed with PBS. Deparaffinized sections were transferred to water and passed through a decreasing alcohol series. After 5 min of equilibration buffer treatment at room temperature, the reaction buffer and TdT enzyme mixture were prepared, dripped onto the sections, and the preparations were incubated at 37°C for 1 h. After stopping the reaction with stop buffer and treating for 30 min with the fluorescein marker anti-digoxigenin antibody, nuclei were stained with the 4,6-diamidino-2-phenylindole (DAPI) chromogen. The prepared samples were evaluated using a fluorescence microscope (Olympus BX51; Olympus Corp., Tokyo, Japan). To assess the number of TUNEL+apoptotic cells, photographs were taken from at least 10 different areas of each tissue using a 40× magnification. In the tissues obtained after immunofluorescence staining, positively stained apoptotic cells were carefully counted using the ImageJ software program, and statistical analysis was performed.[25]

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 8.0 (GraphPad Inc., San Diego, CA, USA). Descriptive data were expressed in mean ± standard deviation (SD), median (min-max) or number and frequency, where applicable. The normality of distributions was evaluated using the Shapiro-Wilk test. The comparison of two groups in more than one group was performed using the Bonferroni analysis and one-way analysis of variance (ANOVA). The Kruskal-Wallis test was used for comparisons between more than two groups. The Dunn's test was used for multiple comparisons.

A p value of <0.05 was considered statistically significant.

RESULTS

Macroscopic evaluation

All the rabbits completed the study. No complications or adverse effects related to the surgery or HCQ administration were observed. In all groups, the defect areas were filled with varying proportions of repaired tissues macroscopically. In the HCQ 20 and 40 mg/kg groups, the surface of the newly formed cartilage appeared to be more regular than that in the controls, and ICRS scores were significantly higher in the third- and sixth-week study groups than those in the controls (p<0.001), indicating no significant difference among the study groups (p>0.05) (Table I). The macroscopic samples of the groups are shown in Figure 2.

Histopathological evaluation

Histological images of the tissues belonging to each group were analyzed by H&E and MT staining. In the degenerating articular cartilage, decreased cell population ratios were seen in all samples. Three weeks after its formation, cell distribution was irregular at the histological level in the defect area, and granulation tissue was observed in the subchondral bone. The repaired tissue histologically consisted of fibrous-like tissue with fibroblastic cell morphology, with no signs of cartilage regeneration (Figure 3a).

The untreated group (B) mostly had irregular surfaces with a hyaline matrix mixed with fibrocartilage. However, there was a significantly more regenerated cartilaginous tissue in Group B than in Group A. The joint damage in Group B was characterized by a lack of superficial and intermediate regions and a decrease in the thickness of the cartilage layer (Figure 3b). In Groups C and D, the repaired tissue was not sufficiently integrated with the adjacent cartilage and subchondral bone. However, the cartilage layers could be distinguished more clearly in Group D than in Group C, except for in the superficial region (SR) (Figures 3c, d). In Group E, the surface of the repaired tissue was irregular and thinner than that of the adjacent cartilage. An irregular cartilage surface with predominantly irregular distribution of cells was observed in this group. In Group F, the articular cartilage surface was smoother than that in Group E. Cell distribution was irregular in Groups E and F; chondrocyte-like cells appeared in clusters or scattered (Figures 3e, f). At the end of the study period (six weeks after chondral

TABLE I
ICRS scores, Wakitani scores, collagen type 2 immunoreactivity intensities, and TUNEL positive apoptotic cell counts of experimental groups

	Groups														
	A			B			C			D			E			F			
	Mean±SD	Median	Q1-Q3	Mean±SD	Median	Q1-Q3	Mean±SD	Median	Q1-Q3	Mean±SD	Median	Q1-Q3	Mean±SD	Median	Q1-Q3	Mean±SD	Median	Q1-Q3	p
ICRS		1.0[a]	0.5-1.0		2.0[ab]	1.0-3.0		2.5[ab]	2.0-3.0		3.0[b]	3.0-3.0		3.0[b]	2.5-3.0		3.5[b]	3.0-4.0	<0.001
Wakitani score		8.0[a]	7.0-8.0		5.0[ab]	4.75-6.25		5.5[ab]	5.0-6.0		5.0[b]	4.0-5.0		7.0[ac]	6.0-7.0		5.0[abc]	5.0-6.0	<0.01
Collagen II	77.83±5.70[a]			86.49±19.50[ac]			87.90±12.43[ac]			92.32±5.78[ac]			85.08±9.56[ac]			78.20±10.59[a]			<0.01
TUNEL		2.0[a]	0.0-3.0		2.0[ab]	1.0-5.0		4.0[ab]	0.0-7.0		5.0[cb]	3.0-8.0		9.0[d]	9.0-11.0		9.0[cd]	6.0-10.0	<0.001

ICRS: International Cartilage Repair Society; TUNEL: Terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick end labeling; SD: Standard deviation; Q: Quartile; The same letters on the same line indicate the similarity between the groups, and different letters indicate the difference between the groups.

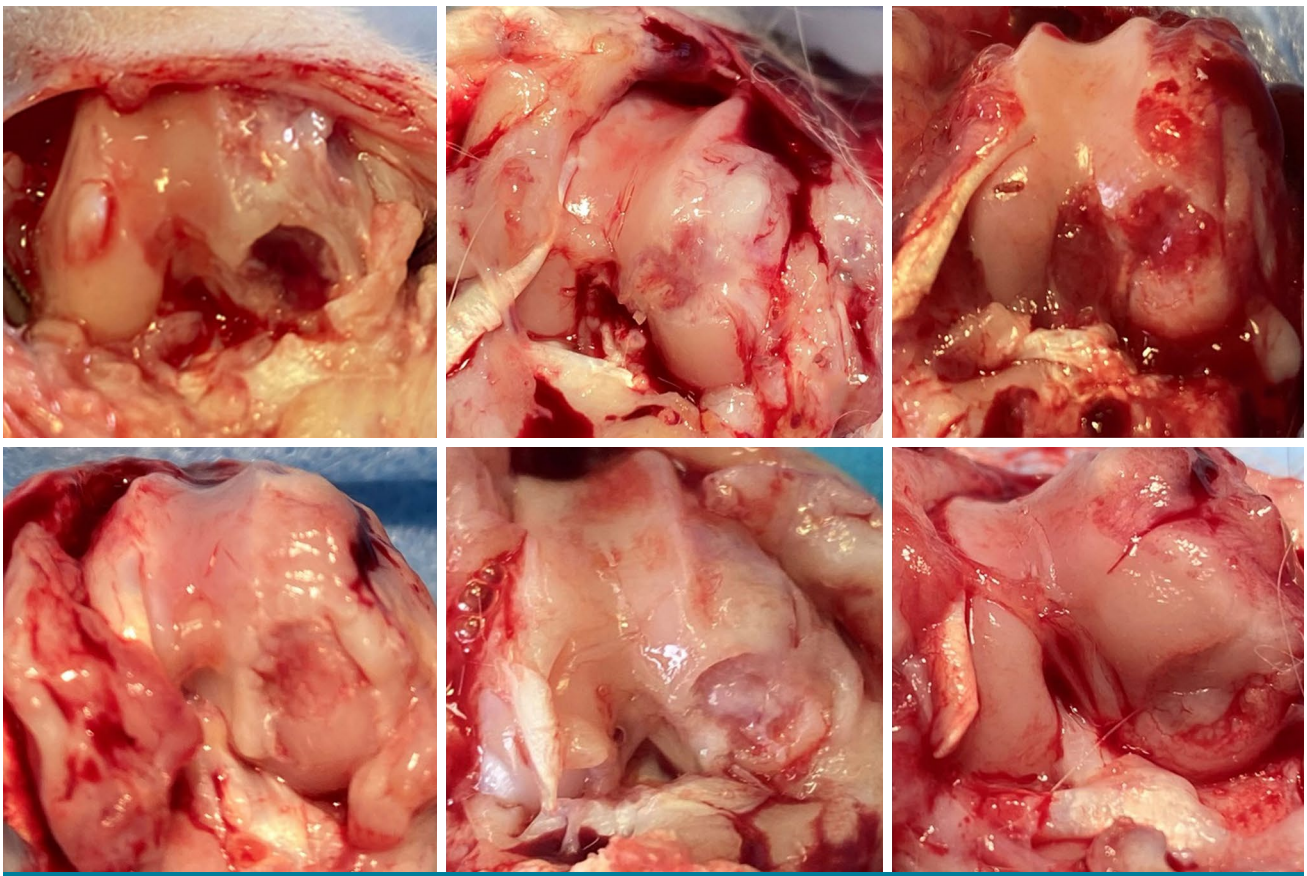

FIGURE 2. Macroscopic samples of the groups.

defect formation), no inflammation was observed in either experimental group.

Regenerated cartilage was scored according to the Wakitani Visual Histological Assessment Scale using H&E-stained sections (Table I). In this study, regenerated tissue was not clearly observed in the defect areas of Group A. There was more repaired tissue filling the defect in the HCQ treatment groups. According to quantitative analysis with Wakitani histological scoring, after three weeks of HCQ treatment, there was no significant difference between Groups C and E, which were treated with 20 mg/kg and 40 mg/kg of HCQ, respectively. The total histological scores of Groups C and E were improved compared to that of Group A, although it did not reach statistical significance ($p>0.05$). While the improvement in Group D's sixth-week scores was statistically significant compared to that in Group B ($p<0.01$), the difference in osteochondral repair scores between Groups D and F was not significant ($p>0.05$). The results of Groups C and D indicated a greater recovery in the 20 mg/kg of HCQ treatment groups compared to the controls, consistent with the H&E findings. The findings from the experimental groups showed that HCQ administered at an appropriate dose could improve cartilage repair after oral administration.

In the preparations stained with MT, collagen fibers in the experimental groups receiving HCQ treatment showed a dense blue color, whereas pale stained areas were observed in degenerated surface cartilage areas. These areas were less prominent in Groups C and D than in Groups E and F (Figure 4).

Immunohistochemical evaluation of collagen type 2

Collagen type 2 was present in the deeper parts of the repair tissue stained with IHC in Groups C and D, and collagen type 2 expression was not observed in the superficial layer in the experimental groups receiving HCQ treatment (Figure 5). The repaired tissue exhibited a mixture of fibroblastic and hyaline-like cartilage cell morphologies. Tissue in the experimental groups

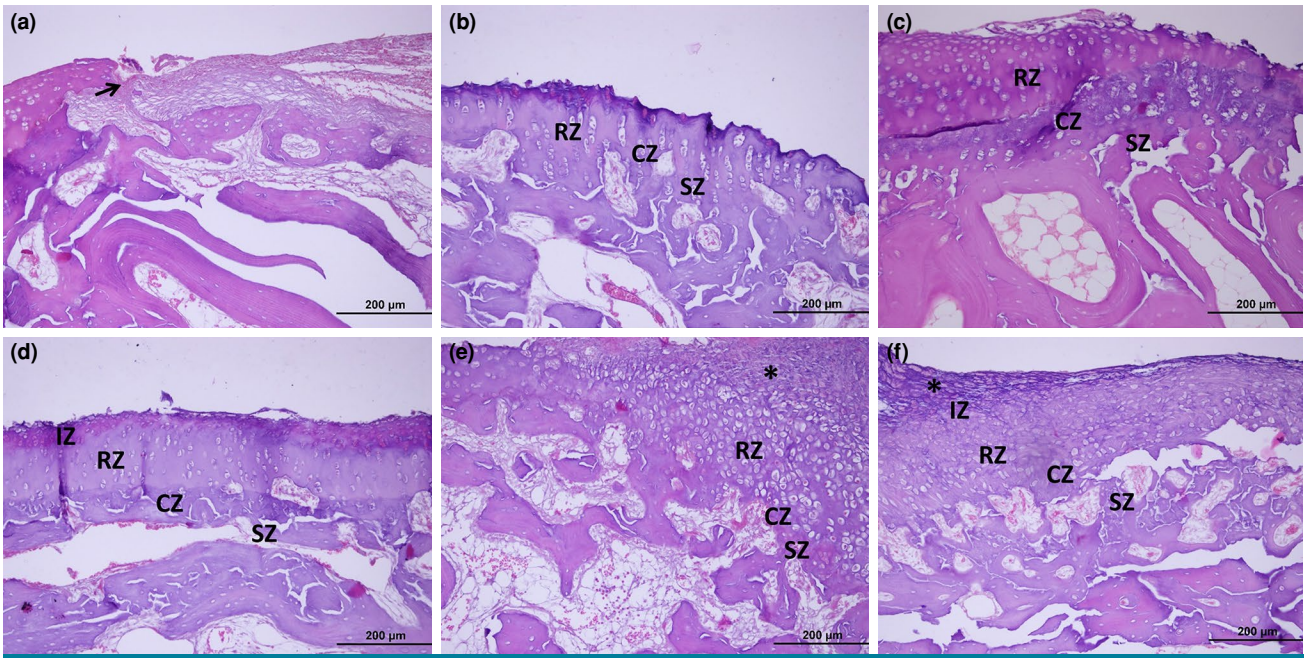

FIGURE 3. (a) Week 3: the superficial zone, the intermediate zone, the radial zone of the cartilage layer, and the defect region where the ossification zone cannot be distinguished (arrow). **(b)** Week 6: the cartilage layer was thin, as there was no superficial and intermediate zone. **(c)** Treatment group with 20 mg/kg of HCQ, Week 3: layers of radial zone, calcified zone, and subchondral zone over the cartilage defect. **(d)** Treatment group with 20 mg/kg of HCQ at Week 6: the articular surface is irregular, but the osteochondral zone is intact. **(e)** Treatment group with 40 mg/kg of HCQ at Week 3. **(f)** Treatment group with 40 mg/kg of HCQ at Week 6.

RZ: Radial zone; CZ: Calcified zone; SZ: Superficial zone; IZ: Intermediate zone; HCQ: Hydroxychloroquine; SB: Subchondral bone; * Decreased cell population; (H&E, ×20).

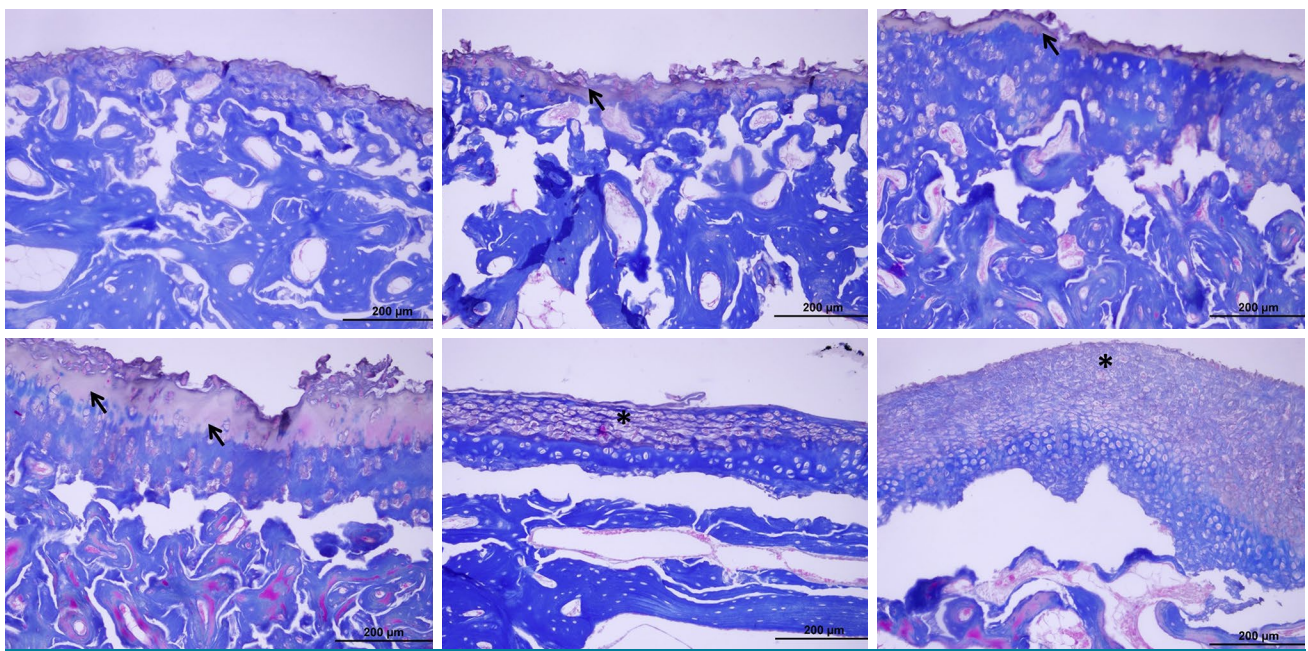

FIGURE 4. Matrix stained pale blue due to collagen fiber reduction (arrow). Decreased cell population (*) (×20, Masson's trichrome).

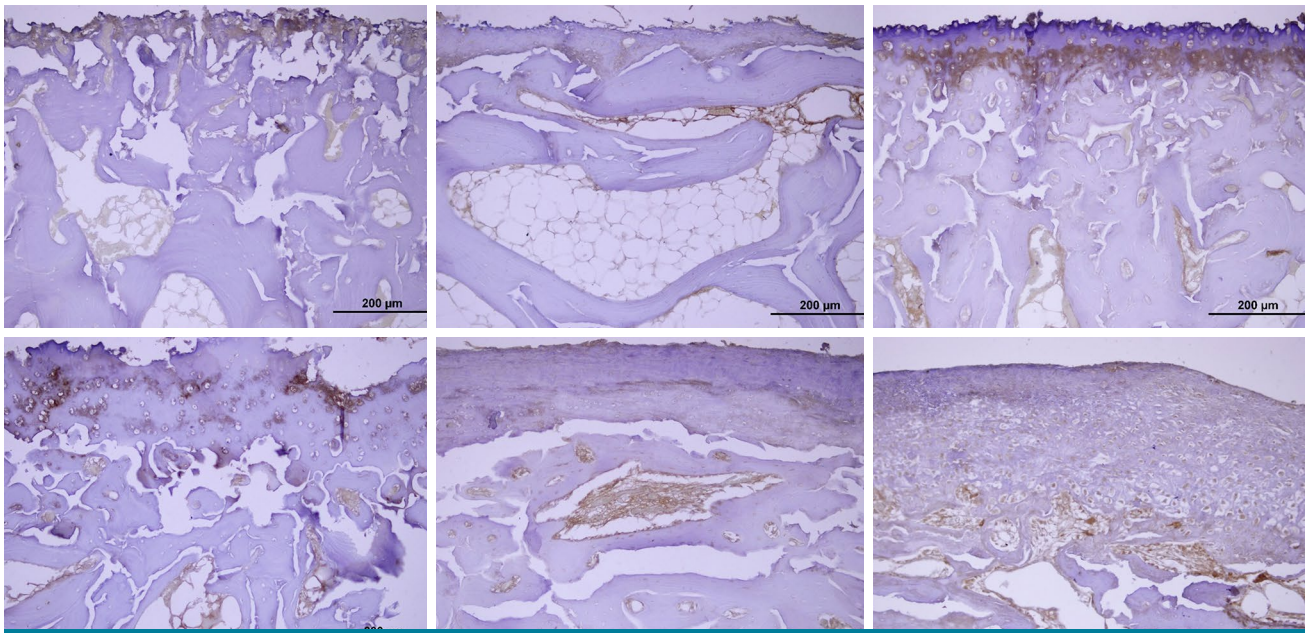

FIGURE 5. Immunohistochemically stained collagen type 2 image. The repaired tissue in groups E and F consisted of fibrous-like tissue with histologically fibroblastic cell morphology and showed weak collagen type 2 staining.

receiving high-dose HCQ (40 mg/kg) treatment (Groups E and F) exhibited a significantly reduced intensity of collagen type 2 immunoreactivity compared to the experimental groups receiving 20 mg/kg of HCQ treatment (Groups C and D). The repaired tissue in Group D was thicker than that in Group C, and showed high IHC staining intensity for collagen type 2. At six weeks, collagen type 2 expression in Group F was significantly lower than that in Group D ($p<0.01$).

TUNEL evaluation

To determine the effect of oral HCQ on apoptosis of chondral defect repair tissue, all sections were stained with the TUNEL kit, as described above. In osteochondral specimens, administration of high doses of HCQ (Groups E and F) affected the entire repair tissue layer. When osteochondral specimens treated with HCQ were evaluated suing the TUNEL test, there was an increase in TUNEL at the repair sites (E and F) of the experimental groups administered high-dose HCQ, compared to the untreated (A and B) experimental groups and the experimental groups (C and D) treated with 20 mg/kg of HCQ. The number of apoptotic cells was remarkable in Groups E and F (Figure 6). The results showed that 40 mg/kg of HCQ treatment significantly increased apoptosis ($p<0.01$) (Table I).

DISCUSSION

In this *in vivo* study, we, for the first time, evaluated the dose- and time-dependent effects of oral HCQ, which is widely used over an extended period in various diseases, on a rabbit cartilage defect model using histological, macroscopical, and IHC analyses. The main findings were that a lower dose of HCQ could improve cartilage repair after oral administration and although oral HCQ had a positive effect on the filling of cartilage defects macroscopically and histopathologically, IHC analysis revealed that, particularly especially with long-term and high-dose usage, it had a negative effect on cartilage proliferation and increased apoptosis.

The safe therapeutic dose of HCQ for routine use in humans is approximately 6 to 6.5 mg/kg/day (average 200 to 400 mg per day), particularly in long-term treatments.[28] Studies of CQ in various animal models have shown that it is approximately 40% more toxic than HCQ when administrated orally.[29] The acute lethal and tolerated doses for chronic use of CQ in rabbits were reported to be 85 mg/kg and 25 mg/kg, respectively, for 42 to 72 days.[30,31] The acute lethal dose or tolerated value of HCQ in rabbits is unknown. However, in some experimental animals, various tolerated doses have been reported: in dogs, 20 mg/kg/day; in rats, >80 mg/kg/day

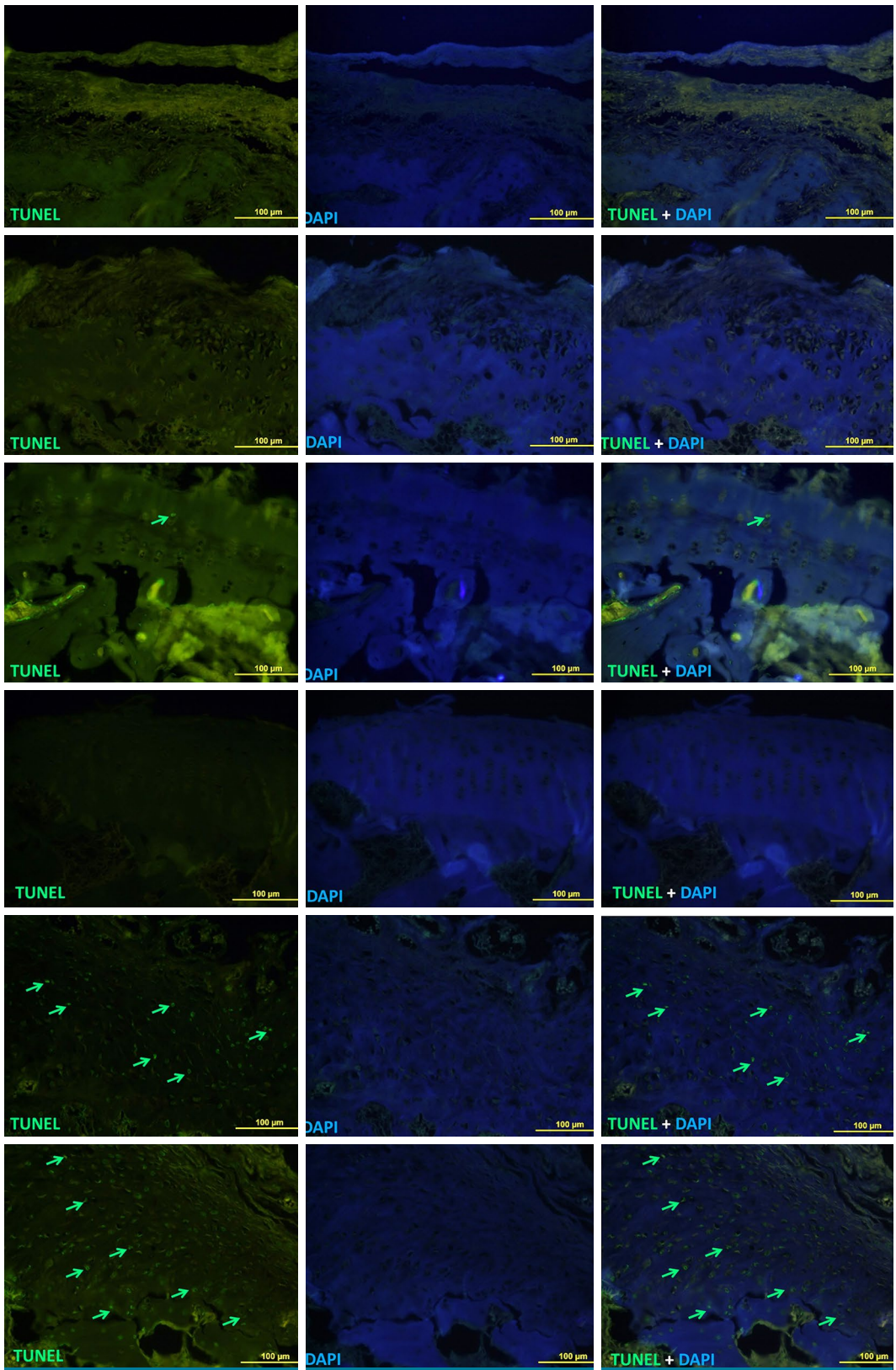

FIGURE 6. Apoptotic cells in the joint tissues of the experimental groups were determined by the TUNEL method.

TUNEL: Terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick end labeling; DAPI: 4,6-diamidino-2-phenylindole; TUNEL + apoptotic cell (×40; green arrow).

and >250 mg/kg/day to <400 mg/kg/day; and monkeys, >60 mg/kg/day in a 10-month test.[29] The daily tolerable doses of CQ for rabbits and monkeys are similar (rabbit, >25 mg/kg/day *vs.* monkey, >25 mg/kg/day to <50 mg/kg/day). Thus, 40 mg/kg/day of HCQ administration in rabbits is considered tolerable and above the therapeutic dose in rabbits, compared to >60 mg/kg/day over 10 months for monkeys. The approximate calculation of HCQ doses applied in our study was designed to be compatible with previous studies, and to be non-toxic and therapeutic for the experimental animals (20 mg/kg/day *vs.* 40 mg/kg/day).

In experimental studies on the treatment of cartilage injuries, the main goal is to provide healing with hyaline cartilage. To address this issue, very long FUs of ≥6 months are usually required.[32] However, these long-term FUs are not always possible in terms of the workforce and cost of research. Moreover, loss of participants increases during FU, which makes it difficult to evaluate the results. In the literature, when the positive or negative effects of various substances or drugs on cartilage defects or repair processes were evaluated, evaluations were made at different time points, ranging from two to 24 weeks.[23,26,32-34] In this study, histopathological, IHC, and morphological changes in healing tissue were evaluated at two different time periods: at Weeks 3 and 6.

Chloroquine and HCQ, its hydroxylated analog, are 4-aminoquinoline compounds. They have been widely used in the treatment of malaria since 1940s.[15] The latter is an approved therapy for several autoimmune diseases, such as RA and systemic lupus erythematosus, owing to its anti-inflammatory properties.[16,21] It is usually preferred over CQ, due to of its lower toxicity under rheumatic conditions.[29] Its potential efficacy in a wide variety of conditions has also been highlighted, including endocrine diseases, infectious diseases, and coagulopathies.[35] The true mechanism of action of HCQ in controlling these diseases has not been fully understood yet. The HCQ is thought to act by inhibiting antigen presentation, interfering with lysosomal activity and toll-like receptor signaling, and terminating circulating immune complexes.[36] Although it is controversial, it has been used in the treatment of COVID-19 in recent years.[17,18] In addition, several clinical studies have demonstrated beneficial effects of HCQ in hand OA.[19,20] Vuolteenaho et al.[37] concluded that it suppresses nitric oxide production induced by interleukin (IL)-1β in osteoarthritic cartilage, and that it could be useful in the treatment of OA. In the present study, we observed that a lower dose of HCQ (20 mg/kg) improved cartilage repair after oral administration; however, a higher dose of HCQ (40 mg/kg) had a negative effect on cartilage regeneration and increased apoptosis. Cartilage proliferation and healing occurred in the control group at six weeks; however, the same effect was achieved by the third week with the use of 20 mg/kg of HCQ. Therefore, the use of appropriate doses of HCQ in cartilage injuries may accelerate healing.

Furthermore, HCQ is only available in oral dosage forms and should be consumed with food to reduce the risk of adverse gastrointestinal reactions. Almost all of the drug is rapidly absorbed by the gastrointestinal tract, and HCQ reaches its maximum plasma concentration in approximately 3 h and is rapidly distributed to tissues. It has a large volume of distribution of 100 to 1,000 L/kg and is slowly released from tissues and metabolized. Its metabolites are excreted in the urine. The initial half-life is three to five days; however, the terminal elimination half-life is one to two months.[21] In humans, particularly in individuals of African descent, its main adverse effects include pruritus, nausea, vomiting, headache, abdominal pain, loss of appetite, blurred vision, and urticaria. Rarely observed reactions include psychosis, seizures, agranulocytosis, exfoliative dermatitis, graying of hair, alopecia, electrocardiographic changes (QRS widening, T-wave abnormalities), hypotension, and hemolysis in individuals with glucose-6-phosphate dehydrogenase enzyme deficiency.[21,22] Moreover, in this study, we observed a negative effect of oral HCQ on cartilage regeneration, as it increased apoptosis in damaged cartilage tissue at higher doses. To fully determine its cartilage toxicity, further studies using different doses and time points are needed.

In their study, Volastro et al.[23] evaluated the protective and destructive effects of CQ in a rabbit cartilage damage model. They examined its dose-dependent effects via systemic (intraperitoneal) and local (intra-articular) administration. The authors observed local toxic (destructive) effects in intra-articular application (twice a week, five-week FU), while they histologically demonstrated that it could be curative when applied systemically (intraperitoneal). They emphasized the importance of investigating the appropriate dose and duration of CQ and suggested avoiding local application in the presence of severe cartilage degeneration, and being careful while implementing long-term systemic application. Moreover, in an *in vitro* study, Li et al.[24] showed that the application of HCQ in cell culture

reduced collagen type 2 production and increased the level of MMP-13, which have the potential to damage the cartilage. Consistent with the findings of this study, we observed that the use of appropriate doses of HCQ was beneficial for cartilage regeneration and proliferation histopathologically and IHC, while HCQ could damage cartilage by increasing apoptosis in the presence of cartilage defects, particularly in long-term and high-dose use.

Hydroxychloroquine, which has been used for many years and has advantages such as low cost and easy accessibility, has also been clinically shown to be effective for treating several diseases, and its side effects in various tissues have been reported. However, its effects on cartilage have not been clearly elucidated yet. This *in vivo* study is valuable, as it is the first report in the literature to demonstrate the effects of orally administered HCQ on injured cartilage in an experimental model.

Nonetheless, this study has several limitations. First, we evaluated only two doses at only time points (relatively early period); evaluation of applications at different doses and longer time intervals would be useful in revealing the real beneficial and toxic value effects on cartilage. Second, we were unable to record mechanical and biochemical data of the repaired tissues. Mechanical function is known to be critical for mechanical loading, and the mechanical character of the repair tissue is an outcome that should be incorporated into treatment protocols. Therefore, further studies are required to evaluate the mechanical and biochemical properties of the repaired cartilage tissue.

In conclusion, our study results indicate that the use of oral HCQ in focal cartilage defects is safe and contributes to cartilage healing and proliferation when administered at an appropriate dose in rabbits. Higher doses of HCQ have a negative effect on cartilage regeneration and increase apoptosis. Further studies are needed to evaluate the effects of HCQ at different doses and time points.

Ethics Committee Approval: The study protocol was approved by the Erciyes University Animal Experiments Local Ethics Committee (date: 02.12.2020, no: 20/164). The study was conducted in the facilities of the Orthopedics and Traumatology, Histology, and Experimental Animal Production and Research Center.

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: E.U., A.E.G., A.G.; Design: E.U., İ.H.K., D.B., A.H.Y.; Control/supervision: A.G., A.H.Y.; Data collection and/or processing: E.U., S.G., D.B.; Analysis and/or interpretation: E.U., İ.H.K., A.G.; Literature review: E.U., A.E.G., S.G., D.B.; Writing the article: E.U., A.H.Y., A.G.; Critical review: A.G., A.H.Y.

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

Funding: TUBITAK 1002 program supported this study.

REFERENCES

1. Messent EA, Ward RJ, Tonkin CJ, Buckland-Wright C. Osteophytes, juxta-articular radiolucencies and cancellous bone changes in the proximal tibia of patients with knee osteoarthritis. Osteoarthritis Cartilage 2007;15:179-86. doi: 10.1016/j.joca.2006.06.020.
2. Hangody LR, Gal T, Vasarhelyi G, Hangody G, Bukhari Syed RI, Hangody L. Results of ultra-fresh osteochondral allograft transplantation for large cartilage defects in the knee joint. Jt Dis Relat Surg 2022;33:521-30. doi: 10.52312/jdrs.2022.836.
3. Ahern BJ, Parvizi J, Boston R, Schaer TP. Preclinical animal models in single site cartilage defect testing: A systematic review. Osteoarthritis Cartilage 2009;17:705-13. doi: 10.1016/j.joca.2008.11.008.
4. Sun Y, Feng Y, Zhang CQ, Chen SB, Cheng XG. The regenerative effect of platelet-rich plasma on healing in large osteochondral defects. Int Orthop 2010;34:589-97. doi: 10.1007/s00264-009-0793-2.
5. Atik OŞ, Hangody LR, Turan S. Total versus unicompartmental knee arthroplasty. Jt Dis Relat Surg 2023;34:235-6. doi: 10.52312/jdrs.2023.57913.
6. Haberal B, Şimşek EK, Baysan Çebi HP, Tuç Ö, Verdi H, Ataç FB. Lack of association between MMP13 (rs3819089), ADAM12 (rs3740199-rs1871054) and ADAMTS14 (rs4747096) genotypes and advanced-stage knee osteoarthritis. Jt Dis Relat Surg 2021;32:299-305. doi: 10.52312/jdrs.2021.64.
7. Chubinskaya S, Hurtig M, Rueger DC. OP-1/BMP-7 in cartilage repair. Int Orthop 2007;31:773-81. doi: 10.1007/s00264-007-0423-9.
8. Akeda K, An HS, Okuma M, Attawia M, Miyamoto K, Thonar EJ, et al. Platelet-rich plasma stimulates porcine articular chondrocyte proliferation and matrix biosynthesis. Osteoarthritis Cartilage 2006;14:1272-80. doi: 10.1016/j.joca.2006.05.008.
9. Singh JA, Noorbaloochi S, MacDonald R, Maxwell LJ. Chondroitin for osteoarthritis. Cochrane Database Syst Rev 2015;1:CD005614. doi: 10.1002/14651858.CD005614.pub2.
10. Raynauld JP, Buckland-Wright C, Ward R, Choquette D, Haraoui B, Martel-Pelletier J, et al. Safety and efficacy of long-term intraarticular steroid injections in osteoarthritis of the knee: A randomized, double-blind, placebo-controlled trial. Arthritis Rheum 2003;48:370-7. doi: 10.1002/art.10777.
11. Strauss EJ, Hart JA, Miller MD, Altman RD, Rosen JE. Hyaluronic acid viscosupplementation and osteoarthritis: Current uses and future directions. Am J Sports Med 2009;37:1636-44. doi: 10.1177/0363546508326984.
12. Venosa M, Calafiore F, Mazzoleni M, Romanini E, Cerciello S, Calvisi V. Platelet-rich plasma and adipose-derived mesenchymal stem cells in association with arthroscopic microfracture of knee articular cartilage defects: A pilot randomized controlled trial. Adv Orthop 2022;2022:6048477. doi: 10.1155/2022/6048477.

13. Gök S, Ozan F, Akay E, Yamak K, Kayalı C, Altay T. Effect of boric acid on cartilage formation of osteochondral defects in rabbit knee: An experimental study. Ulus Travma Acil Cerrahi Derg 2021;27:504-9. doi: 10.14744/tjtes.2020.17748.
14. Yildiz E, Ersen A, Yener E, Comunoglu N, Sen C. The effect of intraarticular insulin on chondral defect repair. Cartilage 2021;13(2_suppl):684S-691S. doi: 10.1177/1947603520938462.
15. Lawrenson AS, Cooper DL, O'Neill PM, Berry NG. Study of the antimalarial activity of 4-aminoquinoline compounds against chloroquine-sensitive and chloroquine-resistant parasite strains. J Mol Model 2018;24:237. doi: 10.1007/s00894-018-3755-z.
16. Kedor C, Detert J, Rau R, Wassenberg S, Listing J, Klaus P, et al. Hydroxychloroquine in patients with inflammatory and erosive osteoarthritis of the hands: Results of the OA-TREAT study-a randomised, double-blind, placebo-controlled, multicentre, investigator-initiated trial. RMD Open 2021;7:e001660. doi: 10.1136/rmdopen-2021-001660.
17. Qaseem A, Yost J, Etxeandia-Ikobaltzeta I, Miller MC, Abraham GM, Obley AJ, et al. Should clinicians use chloroquine or hydroxychloroquine alone or in combination with azithromycin for the prophylaxis or treatment of COVID-19? Living practice points from the American College of Physicians (Version 1). Ann Intern Med 2020;173:137-42. doi: 10.7326/M20-1998.
18. Prodromos C, Rumschlag T. Hydroxychloroquine is effective, and consistently so when provided early, for COVID-19: A systematic review. New Microbes New Infect 2020;38:100776. doi: 10.1016/j.nmni.2020.100776.
19. Kingsbury SR, Tharmanathan P, Keding A, Ronaldson SJ, Grainger A, Wakefield RJ, et al. Hydroxychloroquine effectiveness in reducing symptoms of hand osteoarthritis: A randomized trial. Ann Intern Med 2018;168:385-95. doi: 10.7326/M17-1430.
20. Lee W, Ruijgrok L, Boxma-de Klerk B, Kok MR, Kloppenburg M, Gerards A, et al. Efficacy of hydroxychloroquine in hand osteoarthritis: A randomized, double-blind, placebo-controlled trial. Arthritis Care Res (Hoboken) 2018;70:1320-5. doi: 10.1002/acr.23471.
21. PLAQUENIL® (hydroxychloroquine sulfate) [package insert]. Barbados: Concordia Pharmaceuticals Inc.; 2018.
22. Wong AO, Gurung B, Wong WS, Mak SY, Tse WW, Li CM, et al. Adverse effects of hydroxychloroquine and azithromycin on contractility and arrhythmogenicity revealed by human engineered cardiac tissues. J Mol Cell Cardiol 2021;153:106-10. doi: 10.1016/j.yjmcc.2020.12.014.
23. Volastro PS, Malawista SE, Chrisman OD. Chloroquine: Protective and destructive effects on injured rabbit cartilage in vivo. Clin Orthop Relat Res 1973;(91):243-8. doi: 10.1097/00003086-197303000-00033.
24. Li T, Shi HY, Hua YX, Gao C, Xia Q, Yang G, et al. Hydroxychloroquine induces inhibition of collagen type II and oligomeric matrix protein COMP expression in chondrocytes. Bangladesh J Pharmacol [Internet] 2016;11:372-7. doi: 10.3329/bjp.v11i2.24882.
25. van den Borne MP, Raijmakers N J, Vanlauwe J, Victor J, de Jong SN, Bellemans J, et al. International Cartilage Repair Society (ICRS) and Oswestry macroscopic cartilage evaluation scores validated for use in Autologous Chondrocyte Implantation (ACI) and microfracture. Osteoarthritis Cartilage 2007;15(:1397-402. doi: 10.1016/j.joca.2007.05.005.
26. Wakitani S, Goto T, Pineda SJ, Young RG, Mansour JM, Caplan AI, et al. Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. J Bone Joint Surg [Am] 1994;76:579-92. doi: 10.2106/00004623-199404000-00013.
27. Yay A, Onses MS, Sahmetlioglu E, Ceyhan A, Pekdemir S, Onder GO, et al. Raman spectroscopy: A novel experimental approach to evaluating cisplatin induced tissue damage. Talanta 2020;207:120343. doi: 10.1016/j.talanta.2019.120343.
28. Browning DJ. Pharmacology of chloroquine and hydroxychloroquine. Hydroxychloroquine Chloroquine Retin 2014:35-63. doi: 10.1007/978-1-4939-0597-3_2.
29. McChesney EW. Animal toxicity and pharmacokinetics of hydroxychloroquine sulfate. Am J Med 1983;75:11-8. doi: 10.1016/0002-9343(83)91265-2.
30. Aguayo AJ, Hudgson P. Observations on the short-term effects of chloroquine on skeletal muscle. An experimental study in the rabbit. J Neurol Sci 1970;11:301-25. doi: 10.1016/0022-510x(70)90080-8.
31. Smith B, O'Grady F. Experimental chloroquine myopathy. J Neurol Neurosurg Psychiatry 1966;29:255-8. doi: 10.1136/jnnp.29.3.255.
32. Rudert M, Wilms U, Hoberg M, Wirth CJ. Cell-based treatment of osteochondral defects in the rabbit knee with natural and synthetic matrices: Cellular seeding determines the outcome. Arch Orthop Trauma Surg 2005;125:598-608. doi: 10.1007/s00402-005-0008-2.
33. Tuncay I, Erkocak OF, Acar MA, Toy H. The effect of hyaluronan combined with microfracture on the treatment of chondral defects: An experimental study in a rabbit model. Eur J Orthop Surg Traumatol 2013;23:753-8. doi: 10.1007/s00590-012-1080-2.
34. Tuncay IC, Ozdemir BH, Demirörs H, Karaeminogullari O, Tandogan NR. Pedunculated synovium grafts in articular cartilage defects in rabbits. J Invest Surg 2005;18:115-22. doi: 10.1080/08941930590956101.
35. Olsen NJ, Schleich MA, Karp DR. Multifaceted effects of hydroxychloroquine in human disease. Semin Arthritis Rheum 2013;43:264-72. doi: 10.1016/j.semarthrit.2013.01.001.
36. Wallace DJ, Gudsoorkar VS, Weisman MH, Venuturupalli SR. New insights into mechanisms of therapeutic effects of antimalarial agents in SLE. Nat Rev Rheumatol 2012;8:522-33. doi: 10.1038/nrrheum.2012.106.
37. Vuolteenaho K, Kujala P, Moilanen T, Moilanen E. Aurothiomalate and hydroxychloroquine inhibit nitric oxide production in chondrocytes and in human osteoarthritic cartilage. Scand J Rheumatol 2005;34:475-9. doi: 10.1080/03009740510026797.