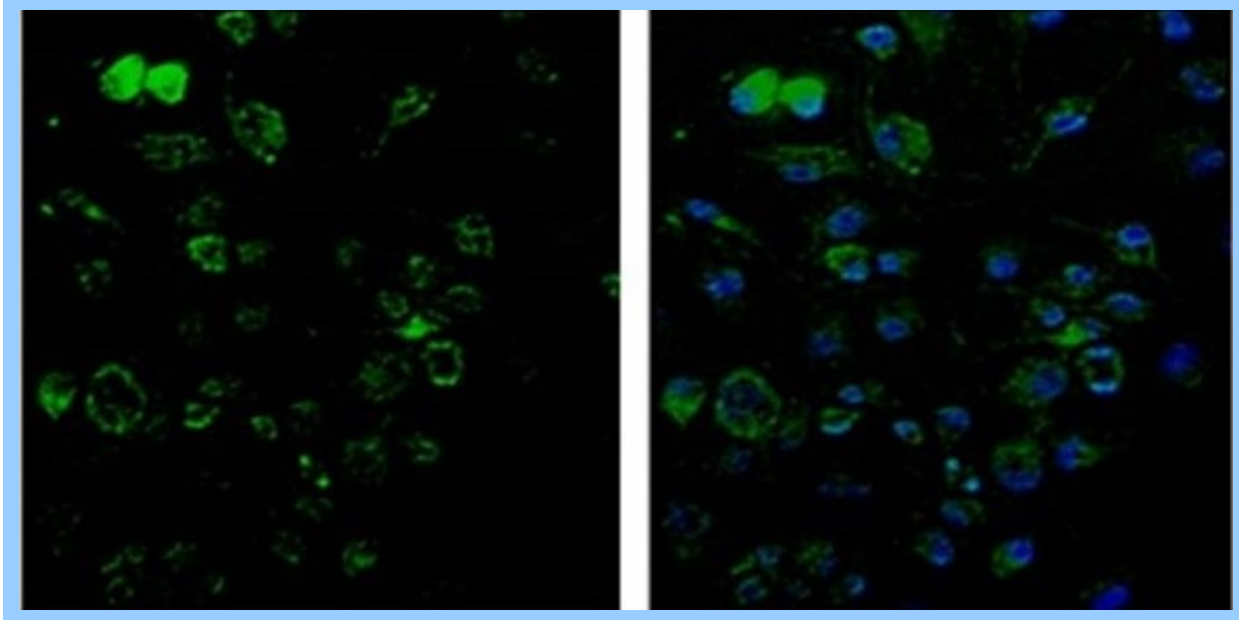

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Hydroxychloroquine induces inhibition of collagen type II and oligomeric matrix protein COMP expression in chondrocytes

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Abstract

The aim of this study was to investigate the effect of hydroxychloroquine on the level of collagen type II and oligomeric matrix protein COMP expre-ssion in chondrocytes of knee osteoarthritis. The rate of growth in cartilage cells was analyzed using MTT assay whereas the Col-2 and COMP expression levels were detected by RT-PCR and Western blotting analyses. For the determination of MMP-13 expression, ELISA test was used. The results revealed no significant change in the rate of cartilage cell proliferation in hydroxychloroquine-treated compared to untreated cells. Hydroxychloroquine treatment exhibited concentration- and time-dependent effect on the inhibition of collagen type II and COMP expression in chondrocytes. However, its treatment caused a significant enhancement in the expression levels of MMP-13 compared to the untreated cells. Therefore, hydroxychloroquine promotes expression of MMP-13 and reduces collagen type II and COMP expression levels in chondrocytes without any significant change in the growth of cells.

Introduction

Osteoarthritis is the disease of joints which results in articular cartilage degradation and remodeling of bones accompanied by joint pain and stiffness (Wattanachai et al., 2009: Gentili et al., 2009). In adult articular cartilage, the non-proliferating chondrocytes produce a large amount of extracellular matrix which mainly consists of two types of macromolecules, collagens (types II, IX and XI) and proteoglycans. Chondrocytes are responsible for the synthesis of sufficient quantity of the extracellular matrix molecules for establishing the cartilage homeostasis (Goldring, 2000; Roughley, 2001). The maintenance of equilibrium between the rate of extracellular matrix formation and its degeneration is performed by chondrocytes (Brondello et al., 2010). Cartilage extracellular matrix molecules such as type II collagen and sulfated proteogly can play a crucial role in regulating chondrocyte functions by mediating interaction between cell and matrix (Eyre, 2002). Degeneration of articular cartilage by inhibition of chondrocyte function is the major cause of cartilage diseases like osteoarthritis and rheumatoid arthritis (Cawston et al., 1999; Kim et al., 1999). Therefore, promotion of chondrocyte proliferation can exhibit an important effect on the management of cell functions.

Chloroquine and its synthetic derivatives have shown promising results for the treatment of disorders including prion disease (Korth et al., 2001), hepatitis C virus (Ashfaq et al., 2011) and various types of cancers (Vasquez-Martin et al., 2011; Mahoney et al., 2013). The

mechanism underlying the action of chloroquine and its derivatives is not fully understood yet. However, various mechanisms have been put forward from time to time. It is observed that use of chloroquine for long-term induces toxicity which hinders its application. However, the synthetic analogs of chloroquine like hydroxychloroquine have shown promising results for the treatment of systemic lupus erythematosus (Costedoat-Chalumeau et al., 2010) and rheumatoid polyarthritis (Suarez-Almazor et al., 2010) disorders without developing any severe toxicity.

The present study demonstrates the effect of hydroxychloroquine on the chondrocytes of knee osteoarthritis. It was observed that hydroxychloroquine treatment exhibited concentration- and time-dependent effect on the inhibition of collagen type II and COMP expression in chondrocytes.

Materials and Methods

Animals

The 6 week old male Sprague-Dawley rats were purchased from Beijing Vital River Experimental Animal Technology Co., Ltd.

Isolation and culture of cartilage cells

The articular cartilage from the rat knee joints was washed thrice with phosphate buffer solution and DMEM and then cut into thin 1 mm^3 sections. The sections were digested using type II collagenase, transferred to a 37°C incubator for the isolation of cartilage cells. The supernatant was centrifuged for 30 min for obtaining cell pellet from which cells were filtered using 200 mesh filters. The cells were distributed at a density of 2 x 10^6 cells per mL onto 6-well plates in DMEM supplemented with 10% FBS and incubated in a 5% CO_2 incubator. The inverted microscope was used for the observation of the cell cultures.

Identification of the cartilage cells

The cells of the second generation were distributed onto the cover slips and cultured for 72 hours. The cells were washed three times with phosphate buffer solution, fixed in 4% formalin for 45 min and incubated with goat serum for 1 hour at 37°C. After phosphate buffer solution washing, the cells were incubated with antibodies overnight at 4°C and the treated with FITC antibodies following phosphate buffer solution washing. The cells were incubated for 2 hours stained with DAPI and examined using fluorescence microscope.

RNA extraction and RT-PCR analysis

The cells were distributed at a density of 2.5 x 10^6 per well onto 6-well plates in DMEM medium and treated with various concentrations of hydroxychloroquine for the indicated time. TRIzol reagent (Invitrogen Life Sciences, Carlsbad, CA, USA) was used to isolate the total RNA from cells. The RNA (1 µg) samples were reverse transcribed into cDNA which was then used to determine the mRNA levels using GAPDH as the internal control. The sequences of the primers used for amplification of the PCR primers were as follows: Col-2F: 5′-TGCCCAGAAAATGAAAAAGG-3′, R: 5′-GTGT-ATGTGGCAATGCGTTC-3′; COMP F: 5′-GAGAACTTT-GCCGTTGAAGC-3′, R: 5′-GCTTCCTGTAGGTGGCA-ATC-3′. The 1.5% agarose gel electrophoresis was used for the detection and digital gel imaging system viewing the images.

Western blot analysis

The cells were distributed in culture flasks followed by treatment with hydroxychloroquine for indicated time periods at 37°C. After incubation, the cells were scraped, washed three times with PBS and then treated with Western blotting lysis buffer. The bicinchoninic acid protein assay was used for the determination of the concentration of proteins. Proteins were separated by electrophoresis on 12% SDS-polyacrylamide gels and the blots were transferred to PVDF membranes blocked with 5% skimmed milk in TBST solution. The membranes were incubated with the primary antibodies overnight at 4°C followed by TBST washing and incubation with secondary antibodies. ECL plus Western Blotting detection reagents (Molecular Imager Chemi-Doc XRS System; Bio-Rad, Hercules, CA, USA) was used to develop the membranes.

MMP13 detection with ELISA

For the determination of MMP13 expression level commercially available ELISA kits (Cayman Chemicals, Ann Arbor, MI) was used according to the manufactures instructions. The cell supernatant was put into the antibody coated 96-well plates followed by incubation for 3 hours at 37°C in 95% CO_2 atmosphere. The plates after incubation were washed and then incubated with chromogenic reaction liquid for 45 min at 37°C. The microplate ELISA reader (EL x 800™; BioTek Instruments, Inc., USA) was used to measure the absorbance at the wavelength of 455 nm.

Analysis of cell proliferation

MTT assay

The MTT colorimetric assay was used for the analysis of the cell viability. The cells were distributed onto 96-well plates at a density of 2.0 x 10^5 cells per well in DMEM culture medium supplemented with 10% fetal bovine serum. The cells were then exposed to various concentrations of hydroxychloroquine for 12, 24, 36 and 48 hours. After incubation, the medium was removed and 20 µL MTT solution was added to each well. Following incubation for 4 hours at 37°C dimethyl sulfoxide was added to dissolve purple-blue MTT formazan precipitate formed. ELISA reader (EL x 800™;

BioTek Instruments, Inc., USA) was used to measure the absorbance at 490 nm.

Bromodeoxyuridine incorporation assay

The chondrocytes were distributed at a density of 2 × 10^5 cells per well onto 96-well plates progressed to G0 phase using 0.4% FCS for 3 days. The cells were then treated with bromodeoxyuridine (3 µg/L) and incubated for 1 hour at 37°C in an atmosphere with 5% CO_2. The cells were washed thrice with phosphate buffer solution followed by fixing using methanol/acetic acid for 25 min. The cultures were treated with H_2O_2 for 45 min to quench the endogenous oxidases. Formamide was added to the cultures for denaturation of nucleic acids at a temperature of 100°C. The cells were treated with the anti-bromodeoxyuridine antibody followed by the measurement of optical density values the wavelength of 490 nm.

Statistical analysis

The data presented are the mean ± standard deviation and were analyzed using a two-tailed Student's t-test and two-sample assuming unequal variance. MS Excel 2007 software package (Microsoft Corp., Redmond, WA, USA) was used for the analysis of the data. The values were considered statistically significant at $p<0.05$.

Results

Identification of chondrocytes

The chondrocytes were identified using immunofluorescence staining. The cytoplasm of the chondrocytes showed positive staining on immunofluorescence staining (Figure 1).

Analysis of chondrocyte cell survival

Examination of the effect of hydroxychloroquine on the growth of chondrocytes using trypan blue staining showed no significant effect on the cell growth after various time points of the treatment (Table I).

Table I

Effect of HCQ on the rate of cartilage cell survival

Group	12 hours	24 hours	36 hours	48 hours
Control	98.4 ± 3.5	97. 7 ± 2.8	98.2 ± 2.9	96.8 ± 4.2
HCQ-treated*	97.6 ± 4.2	98.1 ± 3.3	96.5 ± 4.3	97.6 ± 3.5

*The cells were treated with 15 mg/mL of HCQ (hydroxychloroquine)

Effect on the expression of collagen type II and COMP

We used RT-PCR analysis to investigate the effect of hydroxychloroquine on the expression of collagen type II and COMP in the chondrocytes. The results showed that hydroxychloroquine treatment inhibited the expression of both collagen type II and COMP in chondrocytes after 36 hours (Table II). Compared to untreated cells the expression of collagen type II and COMP was significantly lower in the hydroxychloroquine-treated cells after 36 hours.

The results from Western blot analysis also revealed that hydroxychloroquine treatment inhibited the expression of collagen type II and COMP proteins in a dose-

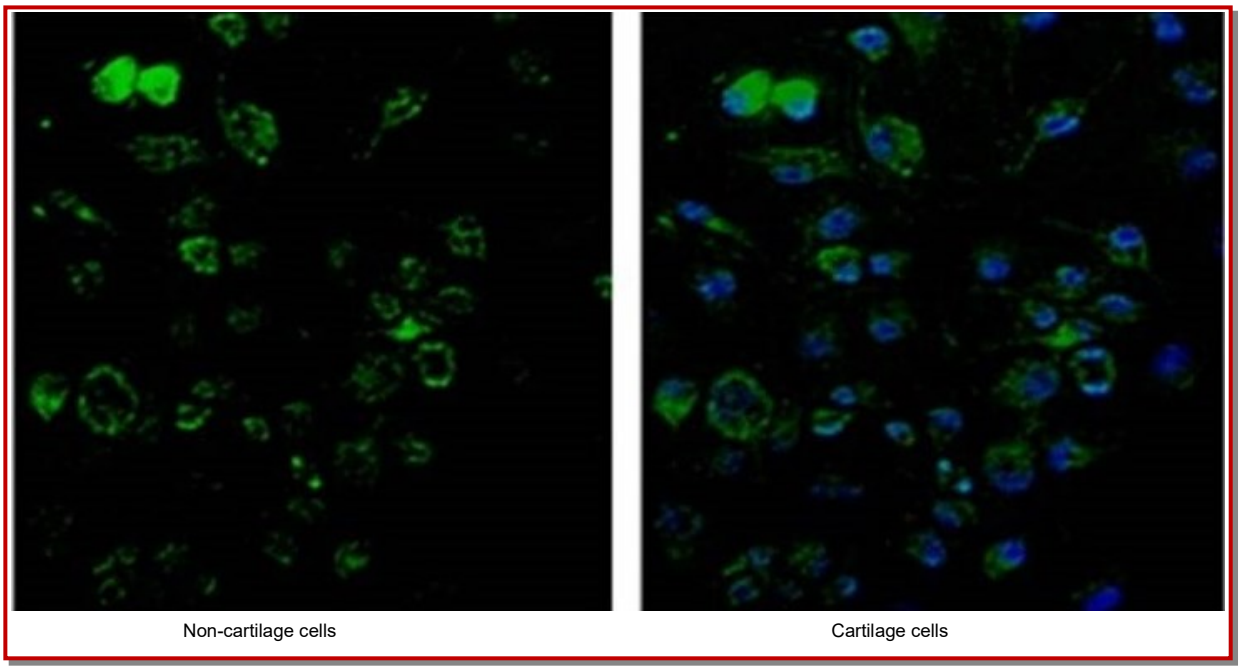

Figure 1: Cartilage cells showing positive results for immunofluorescence staining

Table II

Effect of HCQ on the level of Col-2 and COMP expression in the cartilage cells

	Group	12 hours	24 hours	36 hours	48 hours
Col-II	Control	11.3 ± 1.2	12.1 ± 1.1	15.5 ± 1.6	15.8 ± 1.8
	HCQ-treated	14.4 ± 2.0	18.5 ± 2.3	54.3 ± 3.6	56.5 ± 4.3
COMP	Control	9.6 ± 1.2	10.3 ± 1.4	12.5 ±1.6	12.4 ±1.8
	HCQ-treated	17.4 ± 1.7	42.5 ± 3.1	62.8 ± 3.3	63.4 ± 3.5

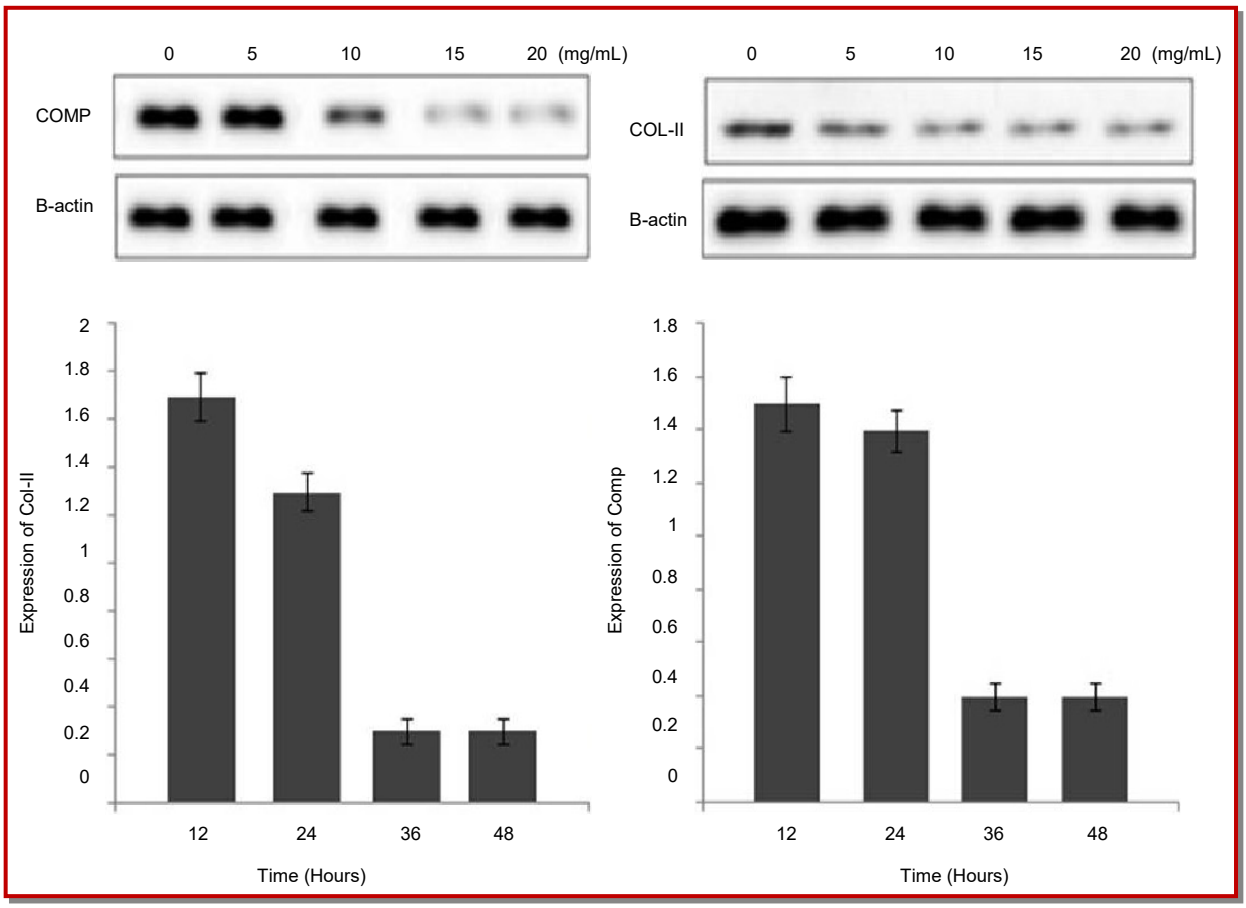

Figure 2: Effect of hydroxychloroquine on the expression of collagen type II and COMP using Western-blotting analyses

and time-dependent manner. Exposure of the chondrocytes to hydroxychloroquine for 36 hours resulted marked decrease in the expression of collagen type II and COMP proteins at concentration of 15 mg/mL (Figure 2).

Effect on MMP-13 expression

Hydroxychloroquine treatment exhibited a concentration-dependent effect on the expression level of the MMP-13. The concentration of hydroxychloroquine at which the expression level of MMP-13 was significantly higher compared to untreated cells was found to be 15 mg/mL (Table III).

Effect on the chondrocyte proliferation

The results from MTT assay revealed that hydroxychloroquine exhibited a concentration- and time-dependent effect on the rate of cell proliferation in chondrocytes. The effect of hydroxychloroquine on the chondrocyte cell proliferation was studied using 5, 10, 15 and 20

Table III

Effect of HCQ on the expression of MMP-13 in the chondrocytes at various time points after treatment

Group	12 hours	24 hours	36 hours	48 hours
Control	82.4 ± 4.6	81.3 ± 4.2	83.5 ± 5.3	84.9 ± 5.6
HCQ-treated	97.5 ± 6.3	98.6 ± 6.7	109.2 ± 7.6	110.1 ± 7.8

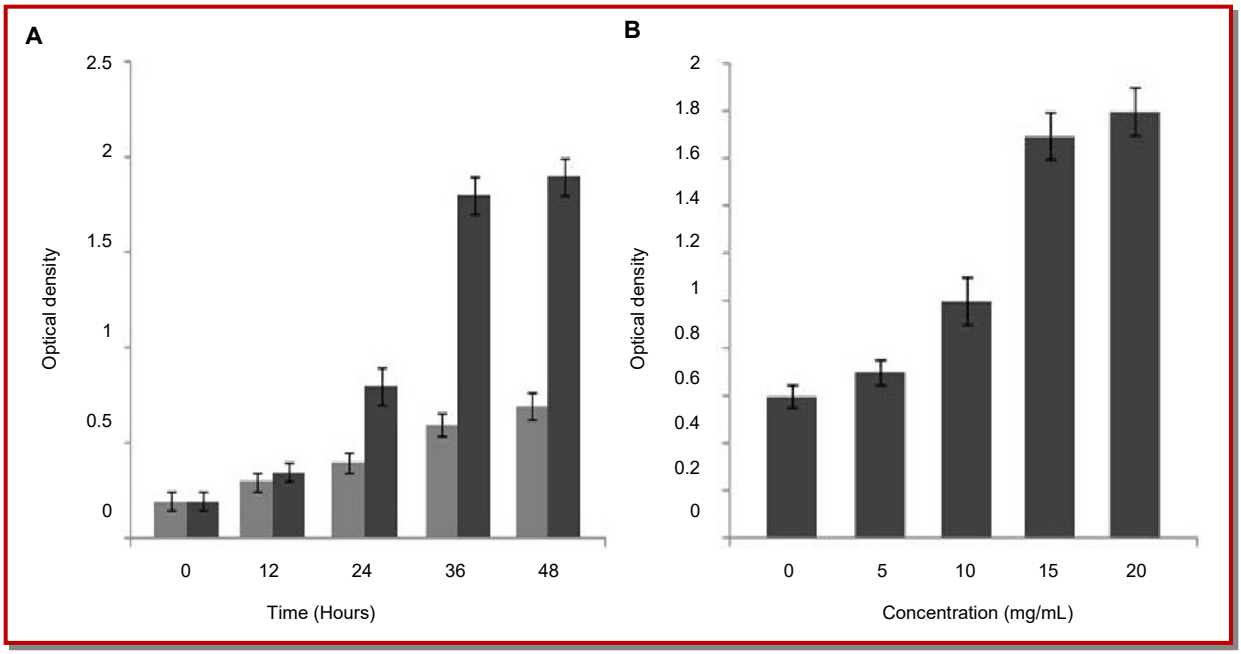

Figure 3: Effect of hydroxychloroquine on the proliferation of cartilage cells using MTT results (A) and bromodeoxyuridine incorporation assay (B)

mg/mL doses. Among the various doses of hydroxychloroquine tested the proliferation rate was significant at concentration of 15 mg/mL (Figure 3A). The significant effect on the proliferation rate using 15 mg/mL of hydroxychloroquine was observed at 36 hours after the treatment (Figure 3B). Therefore, the rate of chondrocyte proliferation was significantly higher at 15 mg/mL after 36 hours compared to the untreated cells. The proliferation rate in the hydroxychloroquine-treated chondrocytes was 37.5% higher compared to untreated cells.

Discussion

The present study demonstrates the effect of hydroxychloroquine on the chondrocytes of knee osteoarthritis. Chondrocytes possess reduced ability to undergo the process of cell proliferation and differentiation and the repaired fibrous cartilage formed shows least mechanical properties (Xu et al., 2013; Lo et al., 2013). Hydroxychloroquine treatment caused a significant increase in the rate of chondrocyte proliferation compared to the untreated cells. The enhancement in the proliferation of chondrocytes was found to be dose and time-dependent.

Chondrocytes and the extracellular matrix together comprise the articular cartilage however, the tendency of chondrocytes to express collagen type II and secrete cartilage matrix decreases with the passage of time (Lo et al., 2013). The collagen type II functions to facilitate the adherence of cells together along with the maintenance of cell and extracellular matrix interactions (Turajane et al., 2014). The process of cell signal transfer, protein activation and expression of genes depend on the interaction of collagen type II binding and its ligand (Guzman-Morales et al., 2014). The results from the present study demonstrated that the rate of proliferation of chondrocytes was markedly increased on exposure to hydroxychloroquine. The increased chondrocyte count was found to enhance the production of cartilage matrix and expression of collagen type II. For the treatment of knee osteoarthritis maintenance of osteoclasts plays an important role and it has been observed that COMP is associated with the regulation of osteoclasts. In our study, hydroxychloroquine treatment exhibited concentration- and time-dependent effects on the expression of COMP in chondrocytes. Hydroxychloroquine treatment significantly inhibited the expression of COMP in the cartilage cells. Our results also showed that hydroxychloroquine treatment enhanced the expression of MMP-13 level which in turn may be involved in the process of decreasing the expression of collagen type II.

Conclusion

Hydroxychloroquine treatment enhances the proliferation of chondrocytes, decreases the expression of collagen type II and COMP and enhances the expression level of MMP-13.

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Ethical Issue

All the experimental procedures involving the animals were performed according to the Guidance Suggestions for the Care and Use of Laboratory Animals 2006 administered by the Ministry of Science and Technology of the People's Republic of China.

Conflict of Interest

Authors declare no conflict of interest

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