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Evaluation of the Effects of HDAC Activity in Hydroxychloroquine Applied Human Primary Chondrocyte and Nucleus Pulposus Cultures

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ABSTRACT

AIM: To evaluate the in vitro effects of hydroxychloroquine (HCQ) on histone deacetylase (HDAC) enzyme activity and interleukin (IL)-6, IL-10, and tumor necrosis factor-alpha (TNF-α) expressions.

MATERIAL and METHODS: Primary cell cultures were prepared. Samples that did not receive any medication constituted the control group, while culture samples treated with HCQ served as the study group. The surface morphology of cells and the extracellular matrix (ECM) were evaluated by Giemsa staining and inverted light microscopy. Cell viability, proliferation, and cytotoxicity were determined by 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium-bromide (MTT) analysis. The cultures were simultaneously stained with acridine orange (AO)/propidium iodide (PI) and viewed under fluorescence microscopy. HDAC enzyme activity and IL-6, IL-10, and TNF-α expression were evaluated using commercial enzyme-linked immunosorbent assay kits. The obtained data were analyzed using statistical methods. The alpha significance level was accepted as $p<0.05$.

RESULTS: HCQ applied to cell cultures at the tested doses and durations showed cytotoxic effects on cell viability, proliferation, and cell or ECM morphology. It increased HDAC activity in chondrocytes and caused a proinflammatory response, indicated by an increase in TNF-α in the cells ($p<0.05$).

CONCLUSION: The results of this study emphasized that the cytotoxic effect of HCQ increased HDAC activity; therefore, this proinflammatory response should be taken into consideration in the clinical use of HCQ.

KEYWORDS: Histone deacetylase, Hydroxychloroquine, Interleukin, Tnf-A, Valproic acid

INTRODUCTION

Hydroxychloroquine (HCQ) is an antiprotozoal drug, but its anti-inflammatory and analgesic properties have led to its pharmaceutical use in the treatment of connective tissue diseases such as systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, and antiphospholipid syndrome (16). HCQ was also used in the treatment of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), also called COVID-19, during the epidemic that rapidly affected the world and caused great public health concerns in 2019 (16).

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HCQ diffuses into synovial tissue and is particularly preferred for the treatment of rheumatoid arthritis because it can act on synovial tissue (14). A neuronal cartilage tissue like the synovium is alymphatic (9), but most medications administered to the body in any form, orally or parenterally, are known to accumulate in the synovial fluid compartment. Medications or nutrients diffuse in significant quantities, first into hyaluronan or synovial tissues and then into body fluids. They then reach cells by passing through the pores in the hyaline membrane in cartilage tissue (9). Drugs taken into the body first diffuse into the hyaluronan or synovial tissues and then reach intervertebral disc (IVD) cells by passing through the pores in the hyaline membranes in the IVD cavity (34). The subsequent effects of HCQ in the synovial cells are not fully understood, but they potentially involve modifications of chromatin and DNA that control gene expression at the transcriptional level.

Many biological events, including proliferation, are controlled by modifications in the chromatin DNA of associated genes *via* histone acetylation (19). The electrostatic charge of histones changes with acetylation, thereby facilitating the binding of transcription factors to DNA and decreasing the affinity of histones to DNA due to transcription activation. The acetylation state of histones is determined by a reversible equilibrium that forms between the histone acetyltransferase and histone deacetylase (HDAC) enzyme families. HDAC enzymes have been the target of numerous medications by reason of their effects on key biological events, including cell cycle regulation, cell proliferation, survival, differentiation, metabolism, and DNA repair. The HDAC enzyme family is a promising medication target because of its association with these cellular processes. For this reason, HDAC enzymes have become a popular research topic. Consequently, compounds that can inhibit these enzymes have been identified and now have a substantial place in treatment protocols.

A study has reported that HDAC enzymes regulate the stability and activity of the hypoxia-induced factor-1 alpha (HIF-1α) protein in annulus fibrosus (AF)/nucleus pulposus (NP) cells (17); however, their roles in AF/NP cells and in chondrocytes have not been fully elucidated. Furthermore, no study has yet evaluated the effect of HCQ treatment on AF/NP cells and on chondrocyte proliferation or HDAC enzyme activity in degenerative joint diseases. Many cytokines' signaling pathways have been associated with cartilage degeneration or disease (11). Interleukin (IL)-6 is a proinflammatory cytokine involved in the pathophysiology of several age-related diseases, including osteoporosis (13). IL-6 can induce proinflammatory mediators (33), and extracellular matrix (ECM)–degrading enzymes that cause cartilage degeneration. Experimental osteoarthritis (OA) models have shown that blockade of IL-6 can protect against cartilage degradation (28). Similarly, increased expression of tumor necrosis factor-alpha (TNF-α), a proinflammatory cytokine, is observed in osteoporosis patients and causes death in IVD cells (30) and cartilage cells (32). IL-10 is an anti-inflammatory cytokine involved in the suppression of inflammation of the synovial membrane; it slows the progression of OA and promotes cartilage turnover (21). Histone acetylation or deacetylation (i.e., HDAC activity) is known to have an effect on inflammatory responses activated in immune reactions, aging, and cell stress.

The main aim of this study was to determine the potential toxicity of HCQ as regards IVD tissue, cartilage tissue cells, and ECM structure. Its second aim was to evaluate IL-6, IL-10, and TNF-α expression in two cultures and to evaluate whether HCQ could induce inflammatory responses and change in HDAC enzyme activity.

MATERIAL and METHODS

Approval was obtained from the ethics committee of the School of Medicine of Izmir Bakircay University (date: 24/01/2024, no: 1443/1423). The experiments were repeated at least three times to minimize errors. Tissues used in the preparation of primary cultures were resected by the same surgeons. The researchers who performed the molecular and histopathological analyses in the laboratory were blinded to the medication status and medication type in each cell culture and to the research purpose. All experiments were performed simultaneously at 0, 24, 48, and 72 hours (h) after drug administration.

Inclusion or Exclusion Criteria for Tissues Used in the Preparation of Primary Cell Cultures

Tissues from patients with hypersensitivity to 4-aminoquinoline group medications and patients with glucose-6-phosphate dehydrogenase deficiency were not used for cell cultures in this study. Tissues belonging to cases who used gentamicin, neomycin, tobramycin, or moxifloxacin antibiotics or cimetidine, neostigmine, pyridostigmine, amiodarone, or antiepileptic medications that could interact with HCQ were also excluded from the study.

Dissection of Tissues by Surgical Resection and Preparation of Primary Cell Cultures

The intact and healthy parts of the resective tissues were used in the culture stage. Primary AF/NP cell cultures were prepared from the tissues of patients (n=4) with lumbar disc herniation, Pfirrmann grade IV, who were unresponsive to conservative management and medical treatment. The IVD tissues were obtained through microdiscectomy.

Tissues used in the preparation of primary chondrocyte cell cultures were obtained from patients who did not respond to conservative medical treatment and who had undergone total knee arthroplasty operation due to gonarthrosis with Kellgren-Lawrence radiological scale grade IV (n=4). Osteochondral tissue was prepared from tissues taken from the lateral condyles of the tibia, the medial condyles of the femur, and the ends of the tibial plateau in total knee arthroplasty operations. A scalpel was used to separate the osteochondral tissue from the chondral tissue.

All the tissues were transferred to Falcon tubes containing medium and 5% antibiotics (PS, 10,000 U/mL, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and transferred to the laboratory at 4 °C under aseptic conditions (34). The tissue was degraded in separate petri dishes, first mechanically with the help of a rongeur, and then enzymatically using 200 units/mL *Clostridium histolycum* (1 mg/mL) dissolved in Hank's Balanced Salt Solution (Thermo Fisher) (34). The samples were

kept overnight in an incubator (Nüve Laboratory & Sterilisation, cat. no. 06750, Ankara, Turkey) in a 5% CO_2 environment. The next morning, the tissue was centrifuged at 4 °C, 900 g, twice consecutively for 10 min. The supernatant was discarded, and the pellets were resuspended in freshly prepared cell culture medium and transferred to individual flasks. Cells that were viable and adhered to the surface of the flasks were counted on a Neubauer slide after staining with Trypan blue (cat. no. T8154; SigmaAldrich; Merck KGaA, Darmstadt, Germany).

The cells were seeded at 1×10^4 cells/well in 96-well plates for 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium-bromide (MTT) analyses and acridine orange (AO)/propidium iodide (PI) staining, and at 2×10^5 cells/well in 24-well plates for HDAC activity assays and IL-6, IL-10, and TNF-α enzyme-linked immunosorbent assay (ELISA) experiments. These cultures were incubated for 24 h at 37 °C in an incubator containing 5% CO_2 before the administration of medication.

Application of Medications to Primary Cultures

HCQ is both metabolized and inhibited by the liver's CYP3A4 enzyme *via* N-dealkylation reaction. The plasma half-life of HCQ is almost 40 days (16). In clinical practice, HCQ is administered orally for a total of 5 days, with a 2 × 400 mg/day loading dose, followed by maintenance doses of 2 × 200 mg/4 days (16).

Doses that mimic the clinical doses in cell culture studies have been reported in the literature (2,3). The final concentration of HCQ was reported to be 5 μg/mL (2) in *in vitro* studies and 0.5 mM for valproic acid (VPA) (5). In this study, the cell cultures were treated with 5 μg/mL HCQ (Plaquenil® 200 mg tablet; Sanofi, Istanbul, Turkey) and 0.5 mM VPA (Convulex® 150 mg capsule, Liba Laboratories, G.L. Pharma, Austria). The samples were divided into three groups: control (no applied drug); VPA-treated (0.5 nM VPA), and HCQ-treated (100 μM HCQ).

Histopathological Evaluations by Inverted Light Microscopy with Fluorescence Illumination

The surface morphologies of the cells and ECM were evaluated using inverted light microscopy (Olympus microscope, cat. no. CKX41; Olympus Soft Imaging Solutions GmbH, Münster, Germany). Giemsa staining (GS500; Sigma-Aldrich; Merck; 37°C for 10 min) was used to observe morphological changes, such as cytoplasmic borders, ECM structure, chromatin condensation, and cell condensation. The cells were viewed at magnifications from ×4 to ×40. Membrane permeability tests were performed while viewing with fluorescence illumination under similar magnifications. Live and dead cells were counted after staining with acridine orange (AO)/propidium iodide (PI) dye, which causes live cells to glow green and the nuclei of dead cells to glow red (35).

Evaluations of Cell Viability, Toxicity, and Proliferation

Cell proliferation was determined using a commercial kit based on the principle of the color change resulting from the production of formazan thiazole blue dye from tetrazolium due to the increasing dehydrogenase enzyme activity of proliferating cells (Vybrant MTT Cell Proliferation Assay, Cat#V13154, Thermo Fisher). Cell viability was determined using MTT and measured at 540 nm (chondrocytes and AF/NP cells), using the viability of the control cells as 100%. Proliferation was calculated with the "*Test optical density (OD)/Control ODX100"* formula, and proliferation inhibition was calculated with the "*1-Test OD/Control OD*" formula (9).

HDAC Activity/Inhibition Assay

A Biovision HDAC Activity Colorimetric Assay Kit (catalogue no: K331-100, Milpitas, CA 95035, USA) was used to evaluate the effects of HCQ treatment on HDAC activity. After cell treatment for the specified time, the culture supernatant was collected and stored at –20°C for later determination of IL-6, IL-10, and TNF-α content. The cells attached to the culture dish were washed once with 1X PBS. After complete removal of the medium, the cells were lysed in nuclear and cytoplasmic extraction buffer containing 5 mM M EDTA, pH 8.0; 50 mM Tris-HCl, pH 8.0; 0.5% NP; and protease inhibitor cocktail (Abcam, ab65621, Cambridge, United Kingdom). Cell debris was precipitated by centrifugation and discarded. The protein in the supernatant containing the nuclear extract was determined by the Bradford method, and all samples were diluted with ddH2O to contain 300 mg/mL protein. An 85 μL volume of each sample was loaded into the wells of the ELISA plate, followed by 10 μL of 10X HDAC assay buffer and 5 μL of HDAC colorimetric substrate, and the plates were incubated at 37 °C for 1 h. The reaction was then stopped by adding 10 μL of lysine developer and incubating the plate at 37 °C for a further 30 min. The OD values at 400 nm absorbance were then obtained using an ELISA microplate reader (an ELISA reader was obtained from Mindray BioMedical Electronics Co., Ltd., MR 96A, Shenzhen, China). In this experiment, the HeLa nuclear extract provided with the kit was used as a positive control to confirm the presence of HDAC activity, while the negative control consisted of trichostatin (TSA), an HDAC inhibitor included in the kit.

Boster commercial kits (IL-6: catalogue no: EK0410, IL-10: catalogue no: EK0416, and TNF-α: catalogue no: EK0525, Boster Biological Technology, CA 94566, USA) were used to determine the amounts of IL-6, IL-10, and TNF-α, and the values were expressed on a protein basis. For IL-6, IL-10, and TNF-α, standard curves were created using the standards included in the kit, and the amounts of IL-6, IL-10, and TNF-α in the samples were calculated as a percentage of the total protein. The following steps were used for IL-6, IL-10, and TNF-α ELISA assays. A sample (100 mL) was added to the kit microplate strips and incubated at 37°C for 90 min. The plate was emptied, and 100 μL of 1X biotinylated antihuman IL-6, IL-10, or TNF-α was added and incubated at 37°C for 60 min. The plate was then washed thrice with 1X wash buffer, and 100 mL of 1X avidin biotin peroxidase complex was added and incubated at 37°C for 30 min. After five washes, 90 μL of color-developing reagent was added, and the samples were incubated for 25 min at 37°C. Finally, 100 μL of stop solution was added to each well, and the OD values were obtained using an ELISA microplate reader.

Statistical Analyses

The Minitab (Version 22.0) statistical package program was used to evaluate the data. Variables are presented as mean ± standard deviation and frequency (%). The Pearson correlation coefficient (r) was used to measure the strength of the relationship between two variables and their relationships with each other. Tukey's honest significant difference test (HSD) was used after a one-way analysis of variance (ANOVA) for comparison of data, with $p<0.05$ accepted as statistically significant.

RESULTS

Demographic Data

The mean age of the gonarthrosis cases (*n*=4; 2 male, 2 female) whose tissue was used in the preparation of primary cartilage tissue cell cultures was 59.28 ± 1.85 years. The mean age of the lumbar disc herniation cases (*n*=4; 2 male, 2 female) whose tissue was used in the preparation of primary AF/NP cell cultures was 48.62 ± 4.27 years.

Microscopic Evaluations

At 0, 24, 48, and 72 h, the chondrocytes morphology was evaluated using an inverted microscope after Giemsa staining (Figure 1).

Figures 1A and 1B show unstained and Giemsa-stained samples, respectively, from the 0 h control group. Figures 1D and 1E show the unstained and stained cells after 24 h treatment with VPA; Figures 1J and 1K show the unstained and stained cells after 48 h VPA treatment; and Figures 1P and 1Q show the unstained and stained cells after 72 h VPA treatment. Figures 1G and 1H show the unstained and stained cells after 24 h HCQ treatment; Figures 1M and 1N show the unstained and stained cells after 48 h HCQ treatment; and Figures 1S and 1T show the unstained and stained cells after 72 h HCQ treatment.

The images confirmed that the cell morphology was significantly destroyed by the HCQ treatment, especially at 72 h. By contrast, the VPA treatment was less damaging to the cell morphology at the applied doses, and the cells continued to proliferate.

Figures 1C, 1F, 1L, and 1R show the images of AO/PI-stained, VPA-treated cultures after 0, 24, 48, and 72 h, respectively, while Figures 1I, 1O, and 1U are images of AO/PI-stained, HCQ-treated cultures after 24, 48, and 72 h, respectively. No cell death was evident in any of the cultures, in agreement with the MTT analysis results.

Figure 2A (unstained AF/NP cells), 2C (AO/PI-stained AF/NP cells), and 2E (Giemsa-stained AF/NP cells) show the images of control groups, HCQ-treated cultures after 72 h, respectively, while Figures 2B (unstained AF/NP cells), 2D (AO/PI-stained AF/NP cells), and 2E (Giemsa-stained AF/NP cells) are the images of HCQ-treated cultures after 72 h, respectively. Unlike chondrocyte cultures, cell death was observed in the MTT analysis results of AF/NP cell cultures (Figure 2D red areas).

Evaluation of Proliferation

The viability of the control group was accepted as 100%, and the viability of the experimental groups was calculated as a percentage of the control value. The percentage viability in HCQ-treated cultures was 180% at 24 h, 169.3% at 48 h, and 98.8% at 72 h (Figure 3). The viability of the VPA-treated chondrocytes was 92% at 24 h, 121% at 48 h, and 200% at 72 h ($p<0.05$). These data confirmed that the administration of HCQ or VPA did not affect cell viability at the applied doses, and that the chondrocyte continued to proliferate. However, as a result of HCQ application, it was observed that proliferation decreased by 60% to 80% and that cell deaths occurred in AF/NP cell culture samples compared to the cell samples in the control group (Figure 2, Figure 4).

Evaluation of HDAC Enzyme Activation/Inhibition

HDAC activity was suppressed by 95.5% when **TSA** was added to the lysates obtained from the 0 h control group (HDAC activity 100%). Similarly, VPA suppressed HDAC activity by 93.1% when applied to the lysates obtained from the 0 h control group ($p<0.05$). These two assays constituted the technical negative control. The HDAC activity was 100% for the HeLa nuclear extract included in the kit, and this was used as our technical positive control. VPA treatment suppressed HDAC activity in the cell cultures by 95.62% after 24 h, by 94.68% after 48 h, and by 94.68% after 72 h ($p<0.05$). By contrast, HCQ increased HDAC activity by 49.7% at 24 h, by 19.7% at 48 h, and by 2.7% at 72 h (Figure 5).

At the end of the 72nd h, since the viability and cell proliferation in AF/NP cell cultures decreased by 60%*80% compared to the control group samples, HDAC activity analyzes could not be performed in AF/NP cell cultures, unlike chondrocyte cultures.

IL-6, IL-10, and TNF-α ELISA Results

IL-6, IL-10, and TNF-α amounts were accepted as 100% in the 0 h control group, and the percentages were calculated for all three proteins in all experimental groups. IL-6 expression was 99.90% in VPA-treated cultures after 24 h, 71.15% after 48 h, and 53.92% after 72 h ($p<0.05$). IL-10 expression in VPA-treated cultures was 124.88% after 24 h, 90.78% after 48 h, and 79.8% after 72 h ($p<0.05$). TNF-α expression in VPA-treated cultures was 141.79% after 24 h, 162.97% after 48 h, and 80.81% after 72 h ($p<0.05$) (Figure 5).

IL-6 expression in HCQ-treated cultures was 40.83% after 24 h, 41.38% after 48 h, and 106.14% after 72 h ($p<0.05$). IL-10 expression in HCQ-treated cultures was 64.81% after 24 h, 72.46% after 48 h, and 79.37% after 72 h ($p<0.05$). TNF-α expression in HCQ-treated cultures was 63.49% after 24 h, 149.07% after 48 h, and 40.82% after 72 h (Figure 6).

All data obtained were also analyzed statistically. After one-way ANOVA (Table I), data from the Tukey HSD results were gathered to determine in which groups differences were significant (Table II).

The analyses showed that while there is a strong negative relationship between IL-6 and TNF-α, there is a moderate

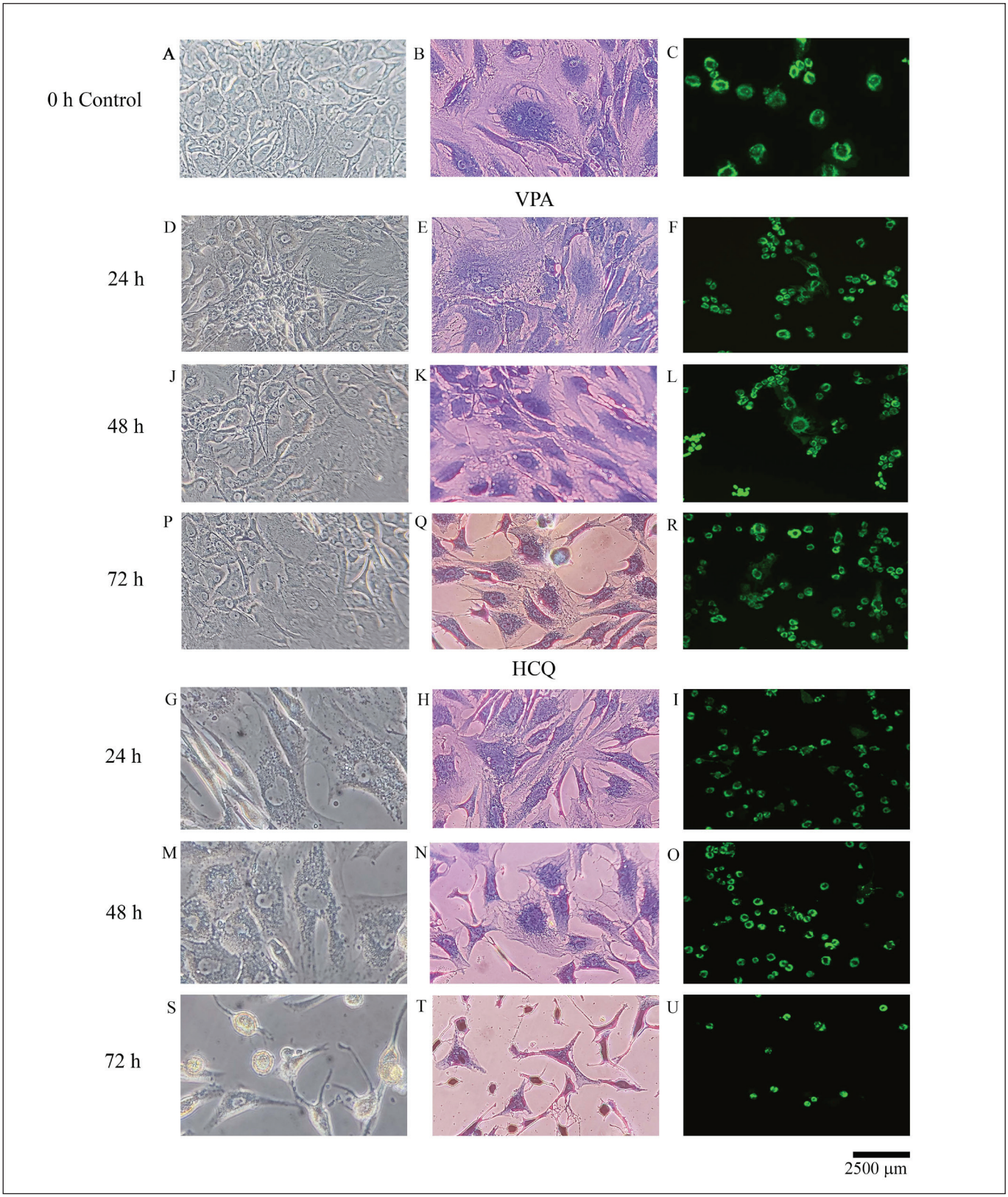

Figure 1: Morphological evaluation of chondrocyte cultures under invert light microscope (2500 µm magnification).

positive relationship between IL-10 and TNF-α. There is a strong positive relationship between IL-6 and IL-10, there is a weak negative relationship between IL-6 and HDAC activity (%), and there is a negative relationship between IL-10 and HDAC activity (%), but this time it is moderately negative. While it is understood that there is a moderately strong negative relationship between HDAC activity (%) and TNF-α, it is observed that there is a moderately strong negative relationship between viability (%) and TNF-α. In addition, a weakly negative relationship is observed between viability (%) and IL-6 or IL-10 (Figure 7).

As in the HDAC enzyme activity measurements, at the end of the 72nd h, AF/NP cell proliferation and viability decreased by 60% to 80% compared to the control group samples. IL-6, -10, and TNF-α values tested in chondrocyte culture samples could not be tested by ELISA analyses.

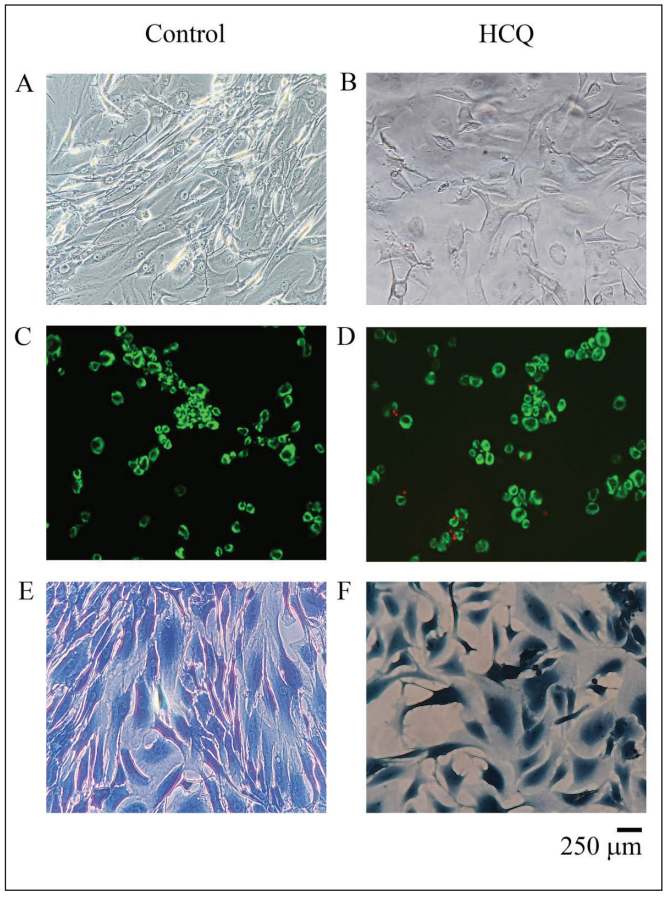

Figure 2: Morphological evaluation of AF/NP cell cultures under invert light microscope (250 µm magnification).

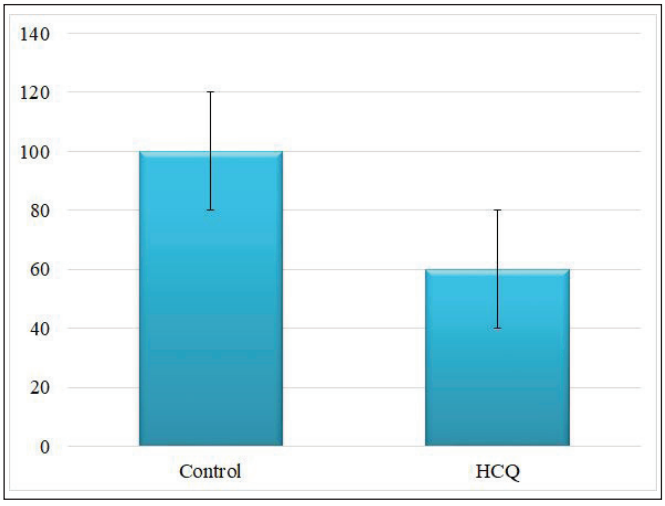

Figure 4: HDAC activity (%): 0 h control group (HDAC activity 100%) *vs*. HCQ- or VPA-treated cultures.

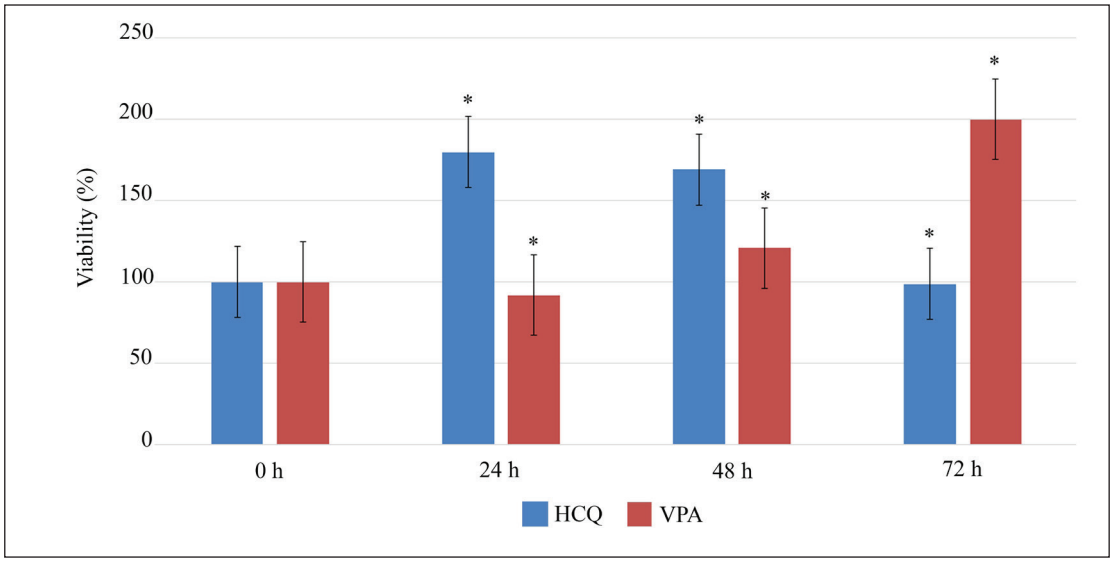

Figure 3: Evaluation of proliferation: percentage of viability calculated in HCQ- and VPA-treated cultures.

Table I: Results of Statistical Analysis Performed After Drug Application to Chondrocyte Cultures

Source (Application)	Adj SS	Adj MS	Model Summary [(R-sq(pred)]	F-value	p-value
TNF-α	39780.0	6629.99	99.99%	46385.97	**0.000**
IL-6	14988.4	2498.07	100.00%	28260789.12	**0.000**
IL-10	7319.97	1219.99	99.94%	8451.89	**0.000**
HDAC activity (%)	70469.6	11744.9	100.00%	3941432.89	**0.000**
Viability (%)	35909.7	5984.95	99.95%	10421.55	**0.000**

Adj SS: *Adjusted sum of squares,* ***Adj MS:*** *Adjusted mean square.*

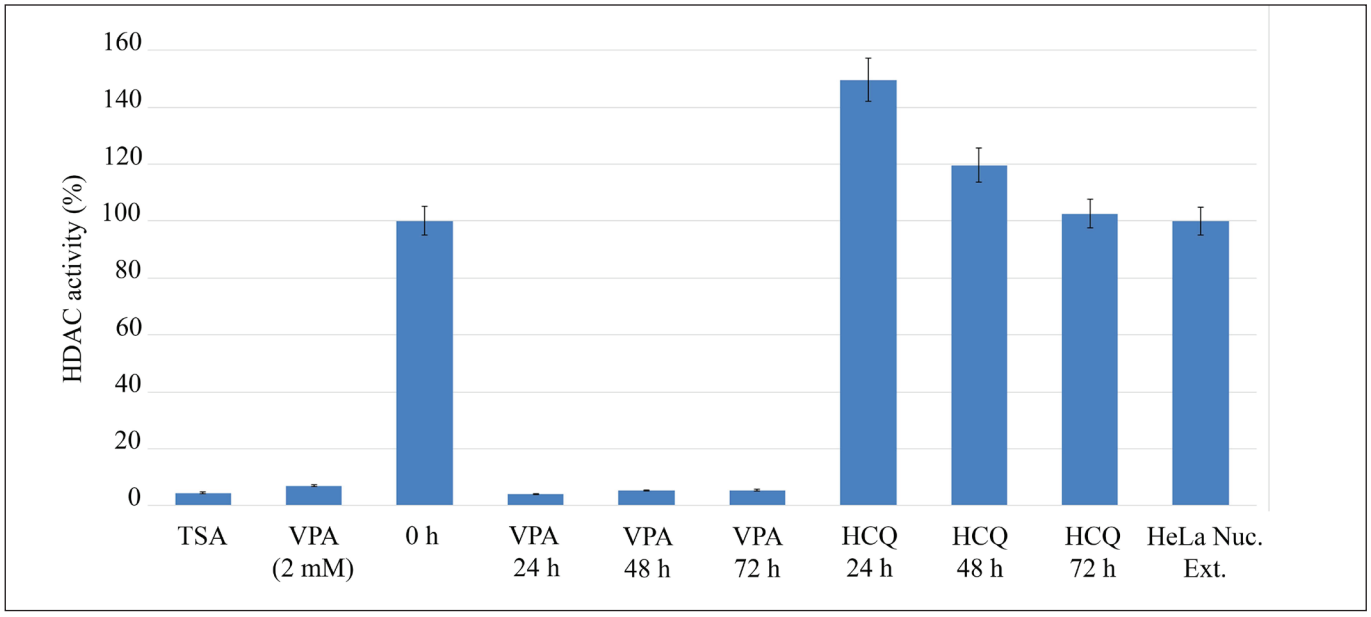

Figure 5: IL-6, IL-10, and TNF-α ELISA: percentage of IL-6, IL-10, and TNF-α amounts; 0 h control group (100%) *vs*. HCQ- or VPA-treated cultures.

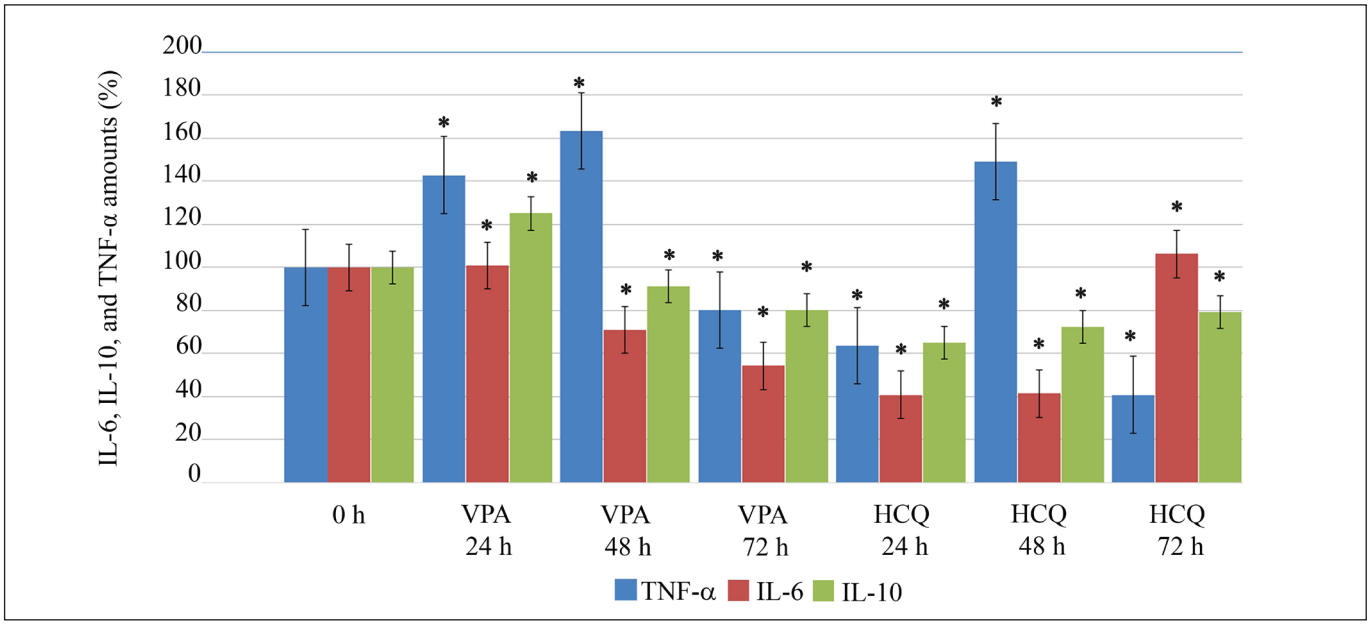

Figure 6: IL-6, IL-10, and TNF-α ELISA results.

Table II: Tukey Pairwise Comparisons Performed After ANOVA on Chondrocyte Cell Cultures (Grouping Information Using the Tukey's HSD Method and 95% Confidence Interval)

Application		Mean	Grouping
TNF-α	VPA 48 h	163.268	A
	HCQ 48 h	149.069	B
	VPA 24 h	142.859	C
	Control	100.000	D
	VPA 72 h	80.000	E
	HCQ 24 h	63.491	F
	HCQ 72 h	40.819	G
IL-6	HCQ 72 h	106.140	A
	VPA 24 h	100.901	B
	Control	100.000	C
	VPA 48 h	70.852	D
	VPA 72 h	54.219	E
	HCQ 48 h	41.381	F
	HCQ 24 h	40.830	G
Il-10	VPA 24 h	125.000	A
	Control	100.000	B
	VPA 48 h	91.270	C
	VPA 72 h	80.000	D
	HCQ 72 h	79.368	D
	HCQ 48 h	72.461	E
	HCQ 24 h	64.812	F
HDAC activitiy (%)	HCQ 24 h	149.690	A
	HCQ 48 h	119.701	B
	HCQ 72 h	102.688	C
	Control	100.062	D
	VPA 72 h	5.411	E
	VPA 48 h	5.320	E
	VPA 24 h	4.098	F
Viability (%)	VPA 72 h	200.0	A
	HCQ 24 h	180.0	B
	HCQ 48 h	169.3	C
	VPA 48 h	121.0	D
	Control	100.0	E
	HCQ 72 h	98.8	E
	VPA 24 h	92.0	F

DISCUSSION

HCQ inhibits the glycosylation of angiotensin converting enzyme-2 in the host cell, thereby preventing virus entry into the cell (15). It is a medication with weakly alkaline properties that promote an inhibitory effect by increasing the endosome pH and preventing acidification (15). After oral ingestion, HCQ is rapidly absorbed, reaches a high tissue concentration, and can accumulate in tissues (4). HCQ has been used for years because it provides anti-inflammatory and analgesia in antirheumatic diseases (1), but it has also been shown to be effective *in vitro* against SARS-COV-1 and MERS-CoV viruses. It also showed strong inhibition of SARS-CoV 2, a virus belonging to the same family, in Vero E6 cells, with an EC50 value of 1.13 μM at 48 h (20). This observation suggests its potential use as an antiviral prophylaxis based on its inhibition of virus entry, but the clinical studies and statistical data are presently insufficient to support this recommendation, and the clinical use of HCQ in this way remains controversial (10).

The blood concentration of HCQ peaks shortly after the absorption phase and then falls relatively quickly due to rapid partitioning into organs (23). Specifically, many studies have reported that the absorption rate constant reaches almost complete absorption within an average of 3 h (4).

HCQ, which has good oral absorption in humans, has been scientifically confirmed to reach a mean maximum blood concentration (Cmax) of 129.6 ng/mL after 3.26 (tmax) h, even after a single oral administration of 200 mg in healthy individuals. HCQ also has a reported bioavailability between 74% and 87%, and this drug and its metabolites can accumulate rapidly in tissues when administered at chronic dosages. However, the plasma half-life increases proportionally with dosage increase (4,23).

Many studies on HCQ are available in the literature (16,20), but none have investigated the effect of this drug on HDAC activity including AF/NP cells and chondrocytes. Moreover, the cytotoxicity of HCQ in both cartilage and IVD tissue cell cultures has not been examined. More importantly, no research has provided information about the relationship between HCQ and the inflammation observed in cartilage and IVD tissues due to possible effects on HDAC activation or inhibition. Therefore, the data presented in this study make a significant contribution to the existing literature.

The present research evaluated whether HCQ administration had a cytotoxic effect on both chondrocytes and AF/NP cells. HCQ administration at the doses and times used, according to the MTT analysis, did not cause any acute cytotoxicity; the chondrocytes remained viable and continued to proliferate. However, as a result of HCQ application, it was observed that, unlike chondrocytes, proliferation decreased significantly and cell death occurred in AF/NP cell culture samples.

AO/PI staining also supported the MTT findings, as no cell death was evident after AO/PI staining (Figure 1). However, the morphological evaluations revealed a different picture, as the inverted microscopy images of the unstained and Giemsa-stained cultures revealed that HCQ treatment caused deterioration of the cell morphology and that losses of cytoplasmic contents of the cells had decreased, especially in cultures exposed for 72 h (Figure 1S and 1T). The morphological degeneration was not as clear at 24 and 48 h as at 72 h, but cytoplasmic contents and granulation were also decreased in cultures treated for 24 and 48 h (Figure 1G and 1M).

The question of whether HCQ administration causes inflammation in chondrocytes was also addressed here, including

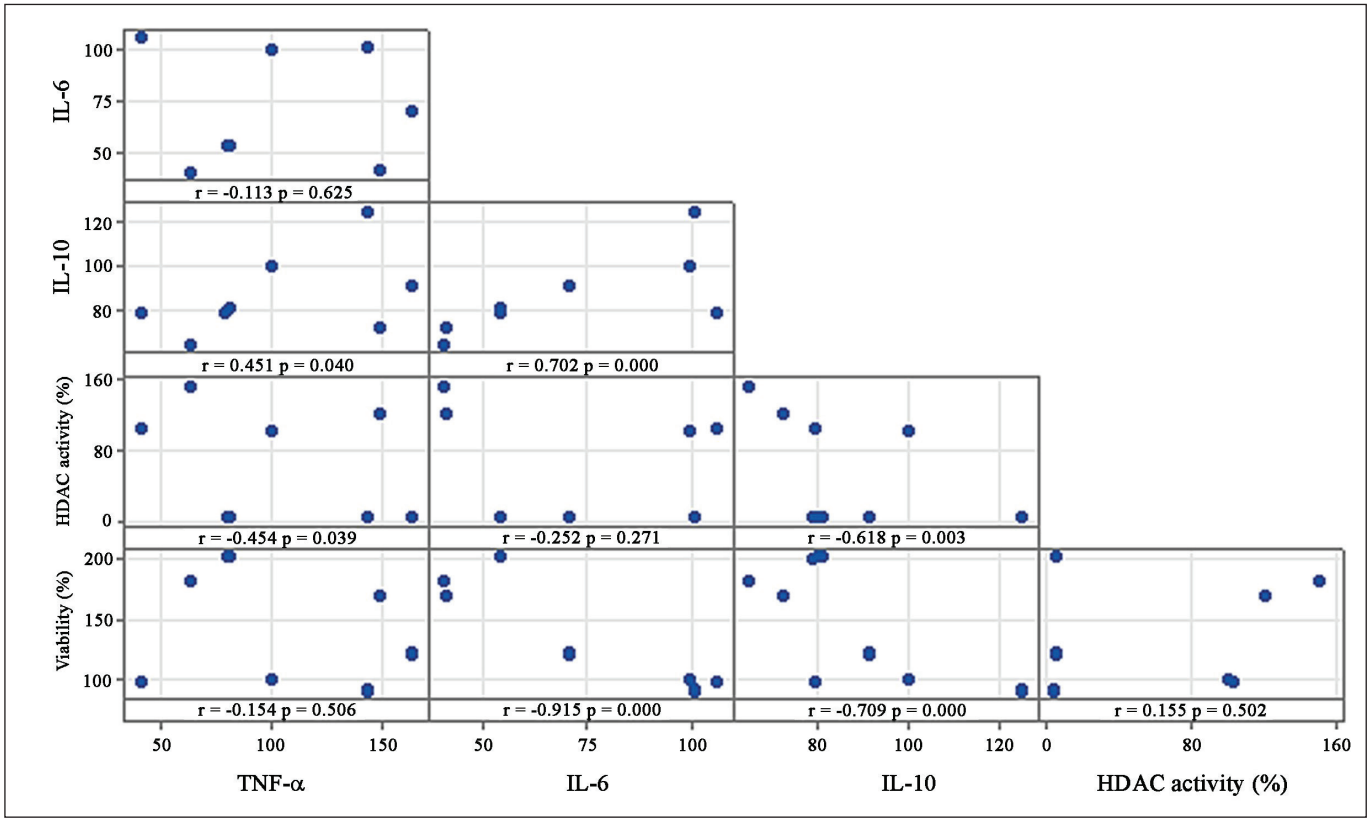

Figure 7: Matrix plot of TNF-**α**, IL-6, IL-10; HDAC activity (%); viability (%). ***ρ:*** *pairwise Pearson correlation.*

the potential involvement of an inflammation mechanism related to HDAC activity. Histone–DNA and histone–histone relationships are altered by HDAC activity, which therefore can control many biological events, including gene expression and DNA packaging, replication, and repair (31).

Currently, HDACs are classified into four families (class I, II, III, and IV) according to their function, structure, distribution, and expression pattern. Various roles for different HDAC family members have been reported (31). For example, HDAC1 activity is decreased in human mesenchymal stem cells during chondrogenesis, while HDAC1 inhibition elevates cartilage-specific gene expression and promotes human mesenchymal stem cell chondrogenesis (18). Conversely, HDAC6 inhibition by TubA maintains chondrocyte survival and prevents ECM degradation (22).

Schoepflin et al. reported HDAC6 associated with and deacetylated HSP90, an important cofactor for HIF-1 functions in NP cells, and HDAC6 inhibition decreased p300 transactivation in NP cells (17). Taken together, these results suggest that although multiple class I and class IIa HDACs control HIF-1 stability, HDAC6, a class IIb HDAC, is a novel mediator of HIF-1 activity in NP cells possibly through promoting the action of critical HIF-1 cofactors (17).

HDAC inhibitor has recently been shown to inhibit synovial proliferation (26), and HDAC enzymes related to the NF-KB and NOTCH signaling pathways play a key role in osteogenesis (12). HDAC enzymes also play important roles in chondrogenesis (25), and the epigenetic deactivation of genes, such as HDAC genes, regulates chondrocyte resistance to apoptosis (29). Many studies have also reported that HDAC regulates HIF-1α protein stability and activity in chondrocytes.

Complex roles have been suggested for many pathways, including those involving HDACs, in the pathogenesis of cartilage-like tissue (6). Therefore, our study did not target a specific HDAC family; instead, total HDAC activity was evaluated in primary chondrocyte cultures. The application of VPA, an HDAC inhibitor, suppressed HDAC activity in the cultures, whereas the cultures treated with HCQ showed increased HDAC activity (Figure 5).

Cartilage tissue pathogenesis can be triggered by a number of stress-related stimuli, including proinflammatory cytokines such as IL-6 and TNF-α (27). Morphological changes observed in OA include cartilage erosion as well as varying degrees of synovial inflammation. Current studies link these OA changes to a complex network of biochemical factors, including the activation of proteolytic enzymes that degrade cartilage macromolecules. Cytokines such as IL-6 and TNF-α, which are produced by activated synoviocytes, mononuclear cells, or the articular cartilage itself, significantly increase the expression of metalloproteinase genes. Cytokines also significantly suppress the regenerative pathways required to restore the integrity of degraded ECM (7).

Human chondrocytes synthesize IL-10 and express IL-10R on their surfaces. Since IL-10 inhibits the expression of IL-1

and TNF-α, an increase in IL-10 in OA chondrocytes could protect cartilage tissue against the catabolic effects of these inflammatory cytokines. However, the functions of IL-10 in cartilage may extend beyond the activities generated in the immunological environment. For example, the high levels of IL-10 and IL-10R in fetal cartilage, an actively growing tissue, suggest that IL-10 may control chondrocyte metabolism under physiological conditions. IL-10 has been reported to limit cartilage damage caused by blood present in the environment, especially as a result of trauma or surgery (8).

In our study, the amount of TNF-α increased in cultures treated with VPA, while total HDAC activity was suppressed by approximately 95%. However, the amount of TNF-α decreased by 20% at 72 h compared with the control. The amount of IL-6 at 24 h was the same as in the control, and it decreased at 48 h and 72 h by 28.95% and 46.68%, respectively. IL-10 level increased by 25.8% at 24 h but decreased by 9% at 48 h and 21% at 72 h compared with the control. These results could be interpreted as a rapid response of chondrocyte cells to the increased TNF-α levels in the first 48 h due to HDAC inhibition, with continued viability and proliferation due to the increased amount of IL-10.

HDAC activity increased in chondrocyte cultures treated with HCQ, and the cells showed greater viability and proliferation compared with VPA-treated cultures; however, the morphology of the HCQ-treated cells changed significantly. HCQ treatment decreased TNF-α expression by 36.51% at 24 h but increased it by 49.07% at 48 h. At 72 h, the amount of TNF-α was 58.18% less than in the control. The amount of IL-10 in the HCQ-treated cultures decreased by 35.19% at 24 h, by 27.54% at 48 h, and by 20.63% at 72 h. By contrast, VPA treatment increased IL-10 expression, presumably due to the VPA-induced increases in the amount of TNF-α.

IL-10 overexpression is known to modulate some of the catabolic properties of TNF-α in chondrocytes. Our data showed that cartilage cells treated with VPA to suppress HDAC activity could escape the catabolism that would occur in response to TNF-α by increasing IL-10 expression. However, HDAC activity continued or was even increased in cells treated with HCQ; therefore, no modulation of TNF-α catabolism occurred. The identity of the actual HDAC enzyme responsible for this effect should be investigated, and other possible pathways should definitely be evaluated.

The failure to modulate TNF-α could have played a role in the morphological degeneration observed in the HCQ-treated cells; however, the small number of cases examined here limits any generalization. Tissue was obtained from only eight cases for the preparation of human primary chondrocyte cell cultures; therefore, repeating the experiments would help alleviate this limitation. Another limitation of ours is that, as a result of HCQ application, proliferation in AF/NP cell culture samples decreased almost to the point of stopping, so HDAC enzyme activity and IL-6, IL-10, and TNF-α levels could not be measured. Additionally, the lack of information regarding the bioavailability of the drugs tested in this study is a limitation. The part of any medication administered to the body that passes into systemic circulation without undergoing chemical changes is defined as its bioavailability. Orally administered medications first pass through the liver, where they undergo extensive metabolism. Molecules of the medications absorbed from the gastrointestinal mucosa enter the portal circulation through the capillaries. Some of the absorbed medication may reach systemic circulation *via* the inferior vena cava, or they may be converted to their inactive metabolites by enzymes in the mucous epithelium or liver cells. The unchanged form of medication entering the enterohepatic cycle and/or its metabolic products are excreted into the bile. This research was carried out in an *in vitro* experimental setup, with no access to the compensatory mechanisms in the body. The HCQ used in the study did not undergo any significant first pass metabolism (24). The present findings, which were derived using *in vitro* conditions and primary cultures, should be supported by clinical studies involving more cases.

CONCLUSION

This research was an *in vitro* experimental study, so its findings may not reflect clinical results and responses. In this study, HCQ, applied at the dosage and times used here, caused cytotoxic effects that appeared as reduced cell viability and proliferation and disrupted cell and ECM morphology in primary cell cultures. HCQ treatment increased the HDAC activity recovered from the culture medium and caused a proinflammatory response, indicated by an increase in TNF-α in chondrocytes. For this reason, duration and dosage should be considered in the clinical use of HCQ.

Declarations

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Availability of data and materials: The data and materials generated/analyzed in the present study are available from the corresponding author upon request.

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AUTHORSHIP CONTRIBUTION

Study conception and design: YEK, IY, TT, SO
Data collection: YEK, IY, TT, SO, DYS, NK
Analysis and interpretation of results: IY, DYS, NK
Draft manuscript preparation: IY, TT, DYS, NK
Critical revision of the article: IY, NK, DYS, HO
Other (study supervision, fundings, materials, etc...): IY, NK, DYS, HO
All authors (YEK, NK, IY, TT, SO, DYS, HO) reviewed the results and approved the final version of the manuscript.

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