

# Does oseltamivir protect human chondrocyte and nucleus pulposus cells from degeneration by inhibiting senescence and proinflammation mediated by the NLRP3 inflammasome and NF- $\kappa$ B?

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**Abstract.** – **OBJECTIVE:** Recent drug design studies suggest that inflammation is among the most important factors in the development of both intervertebral disc (IVD) degeneration (IVDD) and osteoarthritis (OA) due to cartilage damage. This study aimed to investigate whether the anti-inflammatory drug oseltamivir has a toxic effect on IVD and cartilage tissue cells. It assessed what effect oseltamivir has on hypoxia-inducible factor (HIF)-1 alpha (HIF1 $\alpha$ ), which plays an important role in anabolic pathways in IVD and cartilage tissue. In addition, the study analyzed whether oseltamivir could inhibit the release of inflammatory interleukin-1 beta (IL-1 $\beta$ ) via the nuclear factor kappa-B (NF- $\kappa$ B) signaling pathway by activating the nucleotide-binding oligomerization domain and leucine-rich repeat protein-3 (NLRP3) inflammasome.

**MATERIALS AND METHODS:** Human lumbar IVD (n = 8) tissues were isolated for annulus fibrosus (AF) and nucleus pulposus (NP) primary cell cultures, and human tibial and femoral cartilage tissues (n = 8) were isolated for primary chondrocyte cultures. Untreated groups served as the control and oseltamivir-treated groups as the study sample. Cell viability and cytotoxicity were evaluated at 0, 24, 48, and 72 h in all groups for changes in HIF-1 $\alpha$ , IL-1 $\beta$ , NF- $\kappa$ B, and the NLRP3-inflammasome protein expressions using Western blotting. The  $\alpha$  significance value was < 0.05.

**RESULTS:** In the oseltamivir-treated groups, cell proliferation decreased in both AF/NP cell and

chondrocyte cultures obtained from IVD cartilage tissues. After Western blotting analysis, changes were observed in the protein expressions of HIF-1 $\alpha$ , IL-1 $\beta$ , NF- $\kappa$ B, and the NLRP3 inflammasome in both AF/NP cells and chondrocytes. The results were statistically significant ( $p < 0.05$ ).

**CONCLUSIONS:** Oseltamivir treatment may be a promising regenerative strategy to manage IVDD and osteoarthritic cartilage tissues.

*Key Words:*

Degeneration, Inflammation, NF- $\kappa$ B, NLRP3 inflammasome, Oseltamivir.

## Abbreviations

AF: annulus fibrosus; HIF 1 $\alpha$ : hypoxia-inducible factor-1 alpha; IL-1 $\beta$ : interleukin-1 beta; IVD: intervertebral disc; IVDD: intervertebral disc degeneration; NF- $\kappa$ B: nuclear factor kappa-B; NLRP3: nucleotide-binding oligomerization domain, leucine-rich repeat (LRR)-containing proteins (NLR) family pyrin domain containing 3; NP: nucleus pulposus; OA: osteoarthritis.

## Introduction

Senescent cell accumulation increases with organism age<sup>1,2</sup>, which decreases cellular proliferation and can impair tissue regeneration<sup>3</sup>. Chon-

drocyte activity and the secretion of inflammatory mediators are also associated with cell senescence<sup>4</sup>. Inflammation may cause cartilage destruction, pain, functional loss, and deteriorated quality of life<sup>4,6</sup>. In spite of this connection, the relationship between cellular senescence and intervertebral disc (IVD) and cartilage tissue elements remains unclear.

Some studies<sup>7,8</sup> have suggested that examining the inflammation mechanism in the nucleus pulposus (NP) is key to understanding and preventing IVD degeneration (IVDD)<sup>7</sup>, as it is pivotal in this degeneration's development<sup>8</sup>. Within IVDD, both inflammation and poor chondrocyte viability increase the degradation of cartilage tissue, thereby progressing osteoarthritis (OA)<sup>9</sup>. OA is a disease associated with chondrocyte conditioning and extracellular matrix (ECM) catabolism, resulting in a chondrocyte imbalance due to ECM degradation and the release of inflammatory factors<sup>10</sup>.

Inflammatory molecules play a key role in anabolic and catabolic signaling pathways. They accelerate IVDD<sup>11</sup> by affecting the annulus fibrosus (AF) and NP cells, as well as OA by degrading chondrocytes<sup>12</sup>. The molecules behind both IVDD and OA are the proinflammatory cytokine proteins interleukin-1 beta (IL-1 $\beta$ ), transcription nuclear factor kappa-B (NF- $\kappa$ B), hypoxia-inducible factor (HIF)-1 alpha (HIF-1 $\alpha$ ), and the nucleotide-binding oligomerization domain and leucine-rich repeat protein-3 (NLRP3) inflammasome.

The activation of the NF- $\kappa$ B signaling pathway, which plays a key role in inflammation<sup>13</sup>, inhibits proteases that degrade the ECM and disintegrin and metalloproteinase with thrombospondin motifs, resulting in the induction of chondrocyte anabolism and expression<sup>10</sup>. NF- $\kappa$ B are well-recognized factors that cooperatively mediate the activation of the NLRP3 inflammasome. NLRP3-ASC interaction and the subsequent activation of procaspase 1 mediate the activation and maturation of proinflammatory cytokines, such as pro-IL-1 $\beta$  and pro-IL-18, which induce inflammatory responses, including NF- $\kappa$ B activation, and lead to a type of cell death called pyroptosis<sup>14</sup>. NF- $\kappa$ B activation in osteoarthritic chondrocytes then mediates the production of chemokines and catabolic proinflammatory cytokines, such as IL-1 $\beta$ .

These molecules decrease collagen synthesis and "augment NF- $\kappa$ B signaling through a positive feedback loop" as shown by Li et al<sup>10</sup>. Procytokines are further stimulated by the actions of the NLRP3 inflammasome and NF- $\kappa$ B, after which inflammation begins. IL-1 $\beta$  can accelerate IVDD by promoting the degradation of the ECM, inflammatory

cascade, angiogenesis and neo-innervation, and NP cell apoptosis<sup>7</sup>. One overriding aspect of IVD cell biology, however, is that NP and inner AF cells are removed from the blood supply<sup>15</sup>.

In turn, HIF-1 $\alpha$  expressions are components of a key pathway that supports chondrocyte and AF/NP cell survival during embryonic bone development<sup>16-19</sup>. HIF-1 $\alpha$  can thus be considered an important element in balancing anabolism and catabolism, as well as cell protection and death in cartilage and many other tissues<sup>3-12,16-19</sup>. Chondrocytes especially are dependent on the adaptive functions of HIF-1 $\alpha$  to maintain ATP levels and thereby ECM synthesis during OA<sup>20</sup>, as they must withstand considerable hypoxic conditions within avascular articular cartilage<sup>16,21</sup>. Indeed, the functional inhibition of HIF-1 $\alpha$  leads to increased articular chondrocyte apoptosis and murine OA<sup>22</sup>. In addition, in osteoarthritic cartilage, HIF-1 levels are significantly increased, and its activity correlates to the severity of degenerative cartilage changes<sup>17</sup>.

Oseltamivir, which is directed against viral neuraminidase enzymes, is a first-line antiviral drug that in recent years has been frequently prescribed to treat both influenza A virus (IAV) infections<sup>23</sup> and coronavirus disease 2019 (COVID-19), as caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection<sup>24-29</sup>. It reduces IAV replication and inflammatory symptoms by inhibiting NF- $\kappa$ B, as associated with decreased NLRP3 inflammatory activation<sup>28</sup>. It acts by activating IAV through the phosphorylation of I $\kappa$ B $\alpha$  by means of p65<sup>22</sup>. In this way, oseltamivir suppresses the NLRP3 inflammasome-mediated secretion of IL-1 $\beta$  and the production of free oxygen radicals<sup>23</sup>.

However, researchers often investigate oseltamivir for insight into its cytotoxicity<sup>28,29</sup>, as it has been reported to result in respiratory suppression as a result of central nervous system suppression, as well as hypothermia, hypoactivity, and sudden death<sup>23</sup>. Accordingly, no studies have investigated the drug's effects on AF/NP cells, ECM structure, or IVD.

In response, for this study oseltamivir was applied to human primary IVD cell and chondrocyte cultures to assess its cytotoxicity and effects on ECM structure, cell morphology, and the protein expressions of IL-1 $\beta$ , HIF-1 $\alpha$ , NF- $\kappa$ B, and the NLRP3 inflammasome. The study further examined whether oseltamivir could be a pharmacological agent that can heal degenerated chondrocytes and AF/NP cells toward regeneration by manipulating the inflammatory signaling pathway associ-

ated with HIF-1 $\alpha$ , IL-1 $\beta$ , NF- $\kappa$ B, and the NLRP3 inflammasome.

## Materials and Methods

### **Criteria for the Inclusion or Exclusion of Patient Tissues**

The tissues of patients with neutropenia, leukopenia, or thrombocytopenia, active tuberculosis, active hepatitis B or C, a history of liver or kidney dysfunction, and pregnant women were not used in the preparation of the primary cell cultures. The tissues of patients with an allergy or hypersensitivity to oseltamivir were also excluded from the study.

### **Surgical Resection of Tissues and Preparation of Primary Cell Cultures**

IVD tissues were obtained from patients (Pfirsman grading scale stage 4; 4 females, 4 males; mean age:  $42.45 \pm 2.58$  years) diagnosed with lumbar disc herniation but who did not respond to conservative medical treatment and underwent lumbar microdiscectomy. Chondrocyte cell culture tissues were obtained from patients at Kellgren-Lawrence radiological scale grade IV (4 females, 4 males; mean age:  $64.37 \pm 7.31$  years). The osteochondral tissues were obtained from the lateral condyles of the tibia, the medial condyles of the femur, and the ends of the tibial plateau during total knee arthroplasty<sup>21</sup>. The samples were separated from the chondral tissues with the aid of a scalpel, with the intact and healthy parts of the resected tissue used in the culture stage<sup>21</sup>. The tissues were then transferred to Falcon tubes containing a freshly prepared medium and transported to the laboratory at 4°C.

### **Drug Treatment of the Cell Cultures**

The Falcon tubes containing the tissue samples, 5% penicillin-streptomycin/amphotericin, and Dulbecco's Modified Eagle Medium (DMEM) were placed in a flow cabin. The tissues were cleared of red blood cells in three consecutive washes using sterile phosphate buffer saline. After the samples were mechanically de-integrated, they were transferred to new Falcon tubes containing *Clostridium histolyticum*-based collagenase type I and type II enzymes dissolved in a complete medium for the enzymatic degradation of IVD tissues. The samples were incubated overnight in an incubator set to 37.4°C at 5% CO<sub>2</sub>. The tissues were then centrifuged twice

consecutively at 4°C at 1,300 rpm for 10 min. Cell pellets at the bottom were resuspended using a prepared DMEM culture medium. In the preparation of the cartilage tissue cell cultures, enzymatic digestion tissues were incubated in a 200 units/mL collagenase type II enzyme mixture, dissolved in a complete medium 100 mL DMEM containing 1 mL of penicillin-streptomycin (10,000 U/mL), 2.50 mg/mL Amphotericin B, 1 mL L-glutamine (200 mM), 1 mL ITS premix, and 10% fetal bovine serum for 16 h in a CO<sub>2</sub> incubator<sup>21</sup>. Afterward, the tissue samples were centrifuged at 4°C at 1,200 rpm for 10 min to discard collagenase<sup>16</sup>. Sedimented cartilage cell pellets were resuspended in fresh complete medium and transferred to flasks to obtain the primary cultures<sup>21</sup>. Samples of the NP/AF cells and chondrocytes were transferred into wells and incubated for approximately 21 days<sup>18</sup>.

The confluent chondrocytes and AF/NP cells were both passaged and seeded at  $1.6 \times 10^4$  cells per well in 96-well plates for the analyses. After additional overnight incubation, oseltamivir solutions (oseltamivir diluted in DMEM) were added to samples that had over 90% confluency in the cell culture container and adhered to the surface ( $15 \mu\text{M/mL/well}$ )<sup>30</sup>. Because oseltamivir reduces the duration of shedding and the viral titer and can shorten the length of symptoms by 0.5-3 days<sup>31,32</sup>, all molecular analysis were performed within a maximum of 3 days.

### **Cell Viability, Toxicity, and Proliferation Analyses Using MTT and Enzyme-Linked Immunosorbent Assay**

The viability and proliferation of the AF/NP cells and chondrocytes were tested *via* Enzyme-Linked Immunosorbent Assay (ELISA) using a commercial kit with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT)<sup>33</sup>. Oseltamivir's cytotoxicity was also examined. To perform the analysis, the culture medium containing oseltamivir was discarded from the wells, and 100  $\mu\text{l}$  of an MTT stock solution (MTT dissolved in 1 ml sterile phosphate buffer saline at a pH of 7.4, 12 mM) was added to each well. After the primary cell culture samples were incubated for 2 h at 37°C without light, dimethyl sulfoxide was added to the samples to stop the reaction. Then, the wells were incubated for an additional 10 min at 37°C before undergoing photometric analysis at a wavelength of 540 nm. The viability of the control group before the addition of oseltamivir was accepted as 100%<sup>34</sup>.

### **Acridine Orange, Propidium Iodide, and Janus Green-B Staining**

Acridine orange (AO) and propidium iodide (PI) nucleic acid binding dyes were used to determine cell viability and confirm the MTT-ELISA results. As extant studies have established, AO produces green fluorescence by staining all nucleated cells, whether alive or dead, while PI penetrates only dead cells with poor membrane integrity and stains nucleated cells to produce red fluorescence<sup>35</sup>. When stained with both AO and PI, all cells with viable nuclei produce green fluorescence, and all cells with dead nuclei produce red fluorescence. In the preparation of the AO and PI dyes, 4 mg AO (dissolved in 2 mL 99% ETOH), 10 g sodium-ethylenediaminetetraacetic acid, 4 mg PI, and 50 mL FBS were mixed well. Sterile distilled water was added to this mixture until it reached a volume of 200 mL<sup>35</sup>. In addition, Janus Green-B dye (0.02% solution of JG-B, Sigma Chemical Co., St. Louis, MO, USA) prepared in saline (150 mM NaCl, 3 mM KCl, 4.9 mM MgCl, 1.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.6 mM NaHCO<sub>3</sub>, pH 7.4) was used for more detailed morphological examinations.

### **Protein Level Evaluation via Western Blotting**

Total protein amount was determined spectrophotometrically using the Bradford method in protein lysates obtained from the samples. To determine expression levels *via* immunoblotting, the proteins were separated with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Then, the polyvinylidene difluoride transfer membrane, which has a high binding capacity with proteins and nucleic acids and is thus an ideal membrane for protein transfer, was used. Immunoblotting for the target proteins was performed using a WesternBreeze™ Chemiluminescent kit (Catalog No: WB7104, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Primary antibodies specific to HIF-1 $\alpha$  (Catalog No: MA1516, Thermo Fisher Scientific, Waltham, MA, USA), STAT 3 (Cat. No: MA113042, Thermo Fisher Scientific, USA), phospho-STAT3 (ser727; Cat. no: MA515208, Thermo Fisher Scientific, Waltham, MA, USA), and the NLRP-3 inflammasome (Cat. No: MA516274, Thermo Fisher Scientific, Waltham, MA, USA) were used. The  $\beta$ -Actin protein (Catalog No: MA1-140, Thermo Fisher Scientific, Waltham, MA, USA) was used as an endogenous control in Western blotting. Af-

ter treatment with a primary antibody specific to each protein, sequential washes were performed. The membranes were incubated with an alkaline phosphatase-conjugated secondary antibody, and then, treated with a substrate solution after three washes. The protein bands were transferred to an X-ray film (Thermo Fisher Scientific, Cat#34090, Waltham, MA, USA) and analyzed using ImageJ software to determine the amount of protein in each sample<sup>36</sup>.

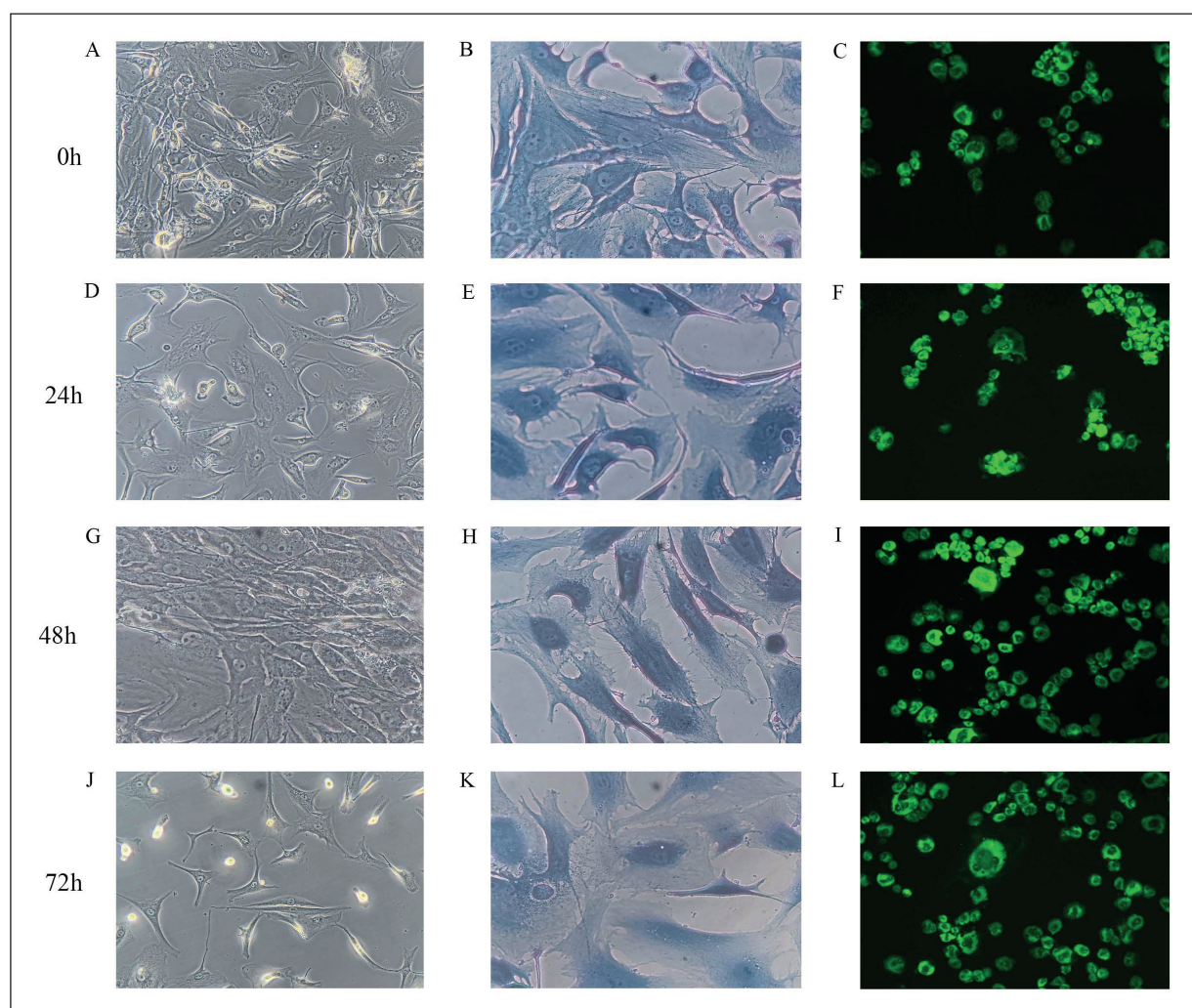
### **Statistical Analysis**

Statistical analysis was performed with Minitab version 22.0. The data were reported as percentages (%), mean  $\pm$  standard deviation, minimum (min), and maximum (max). After one-way analysis of variance (ANOVA), Tukey's test of significant difference (Tukey's HSD) was used to test for differences between sample means for significance. The confidence level was 95%, and the alpha significance value was assumed as  $< 0.05$ .

## **Results**

Upon evaluating the primary IVD tissue cultures, the administration of oseltamivir did not cause changes in cell morphology, nor did AO and PI staining reveal any acute cell death (Figure 1). Similarly, after evaluating the primary chondrocyte tissue cultures, drug administration did not cause any changes in cell morphology, and AO and PI staining revealed no acute cell death (Figure 2). However, cell viability decreased by 21.49%, 32.70%, and 53.72% at 24, 48, and 72 h, respectively in the oseltamivir-treated AF and NP samples compared to the control samples ( $p < 0.05$ ). Likewise, cell viability increased by 8.21% and 8.58% at 24 and 48 h but decreased by 34.63% at 72 h in the oseltamivir-treated chondrocyte samples compared to the control samples ( $p < 0.05$ ; Tables I and II, Figure 3).

NLRP-3 inflammasome, HIF-1 $\alpha$ , IL-1 $\beta$ , and NF- $\kappa$ B protein expressions were evaluated *via* Western blot in all untreated control and oseltamivir-treated groups at 24, 48, and 72 h. The chart included images of the analyses performed with protein samples obtained from the IVD cultures (Figure 4) and chondrocyte cultures (Figure 5). The bands in the Western blot charts were evaluated using ImageJ for protein fold expression changes in the samples.  $\beta$ -Actin protein expression in all groups was accepted as 1 (100%), and the data were normalized (Table III).



**Figure 1.** Morphological evaluation of IVD culture cells. The first column presents the inverted microscopy (20× magnification) results, the second column the Janus green-stained (40× magnification), and the third column the AO and PI-stained (10× magnification) samples. **A-C**, Control groups with no drug administered. **D-F**, The oseltamivir-treated culture groups after 24 h. **G-I**, Groups after 48 h. **K, L**, are the groups after 72 h.

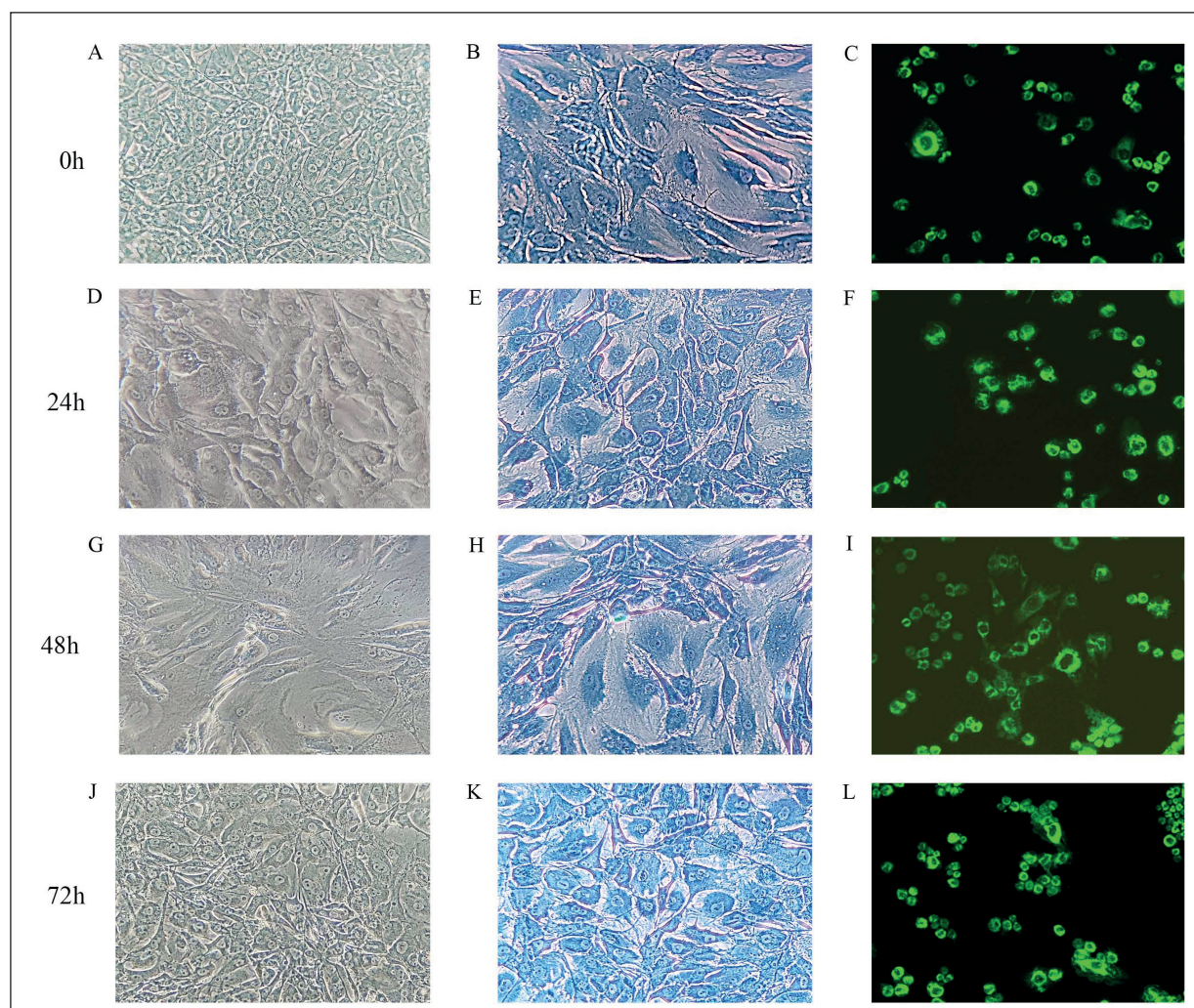
In the AF and NP cell cultures, NLRP3 inflammasome protein expression increased 2.06 times in the oseltamivir-treated samples compared to the control samples at 24 h but was normalized at 48 and 72 h. HIF- $\alpha$  expression increased 2.75, 1.52, and 1.36 times at 24, 48, and 72h, respectively in the oseltamivir-treated samples compared to the control samples. IL-1 $\beta$  expression increased 2.15-fold at 24 h, though it was normalized at 48 h ( $r = 0.96$ ) and decreased by 50% at 72 h. NF- $\kappa$ B expression increased 3.58 and 5.82 times at 24 and 48 h but was normalized at 72 h ( $r = 0.94$ ).

In the chondrocyte cultures, NLRP3 inflammasome protein expression was at the same level in both the study and control samples after 24 h ( $r = 1.02$ ), but slightly decreased at 48 and 72 h ( $r =$

0.79 and 0.84, respectively). HIF-1 $\alpha$  expression increased by 1.68 and 1.37 at 24 and 48 h in the oseltamivir-treated samples compared to the control samples. However, it decreased at a rate similar to that in the control group at 72 h ( $r = 1.10$ ). IL-1 $\beta$  expression increased 1.23 and 1.25 times at 24 and 48 h, though it decreased by 25% at 72 h. Lastly, NF- $\kappa$ B expression increased by 1.90 and 1.30 at 24 and 48 h in the oseltamivir-treated samples compared to the control samples, then, normalized at 72 h ( $r = 0.95$ ).

## Discussion

In the present study, the drug oseltamivir was applied to human primary IVD cell and chondro-



**Figure 2.** Morphological evaluation of chondrocyte culture cells. The first column displays the inverted microscopy (20× magnification) results, the second column the Janus green-stained (20× magnification) samples, and the third column the AO and PI-stained (10× magnification) samples. **A-C**, Control groups with no drug administered. **D-F**, The oseltamivir-treated culture groups after 24 h. **G-I**, The groups after 48 h. **J-L**, are the groups after 72 h.

cyte cultures. The study aimed to investigate the cytotoxicity of oseltamivir and any changes it caused in ECM structure, cell morphology, and the protein expressions of IL-1 $\beta$ , HIF-1 $\alpha$ , NF- $\kappa$ B, and the NLRP3 inflammasome. It also examined whether oseltamivir could be a pharmacological agent that can heal degenerated chondrocytes and AF/NP cells toward regeneration by manipulating the inflammatory signaling pathway associated with those proteins.

As extant research has shown, IVD and cartilage tissues are alymphatic<sup>21,34,35</sup>. Most drugs administered to the body orally or parenterally thus accumulate in the synovial fluid compartment. From there, they can diffuse first into the hyaluronan or synovial tissues and then into body fluids<sup>37</sup>.

Afterward, they reach cells by passing through the pores in the hyaline membrane located in the disc distance<sup>21,34,35</sup>.

With this accumulation in bodily tissues, drugs can affect some cell signaling pathways. Moreover, the investigation of pathways, such as the NLRP3 inflammasome and NF- $\kappa$ B, which are related to IVDD and IVD senescence, has gained popularity<sup>38,39</sup>. The NLRP3 inflammasome especially plays an important role in the regulation of NP inflammation, and activated NF- $\kappa$ B supports NLRP3 inflammasome activation and IL-1 $\beta$  release, which in turn support NP degeneration<sup>40</sup>. In the present study, in the AF/NP cultures, IL-1 $\beta$  expression increased 2.15 times at 24 h in the oseltamivir-treated samples compared to the control

**Table I.** Tukey HSD grouping of the proliferation data after descriptive analysis and ANOVA in the oseltamivir-treated samples.

Primary cell culture	Variable	Time (hours)	Mean±StDev	Tukey HSD Grouping*
AF and NP cells	Group 1 Absorbance (nm)	0	0.309±0.01315	E
		24	0.414±0.00089	C
		48	0.458±0.00258	B
		72	0.564±0.00089	A
	Group 2 Absorbance (nm)	0	0.309±0.01315	E
		24	0.325±0.00089	D
		48	0.308±0.00089	E
		72	0.261±0.00089	F
Chondrocytes	Group 1 Absorbance (nm)	0	0.249±0.00089	G
		24	0.292±0.00089	F
		48	0.326±0.00089	D
		72	0.563±0.00000	A
	Group 2 Absorbance (nm)	0	0.249±0.00089	G
		24	0.316±0.00089	E
		48	0.354±0.00089	D
		72	0.368±0.00089	B

\*A: Highest rate of cell viability and proliferation. G: Lowest rate of cell viability and proliferation. AF and NP cells; Group 1 untreated samples and Group 2 oseltamivir-treated samples. Chondrocytes; Group 1 untreated samples and Group 2 oseltamivir-treated samples.

samples. However, it was normalized at 48 h ( $r = 0.96$ ) and decreased by 50% at 72 h. In the chondrocyte cultures, IL-1 $\beta$  expression increased 1.23 and 1.25 times at 24 and 48 h, only to decrease by 25% at 72 h.

Apart from IVD inflammation, NLRP3 inflammasome activation is associated with pyroptosis, ECM degradation, and IVD cell apoptosis<sup>40,41</sup>. Accordingly, the detrimental effects of the NLRP3 inflammasome in IVDD have been extensively examined<sup>42</sup>. Increased IL-1 $\beta$  due to NLRP3 inflammasome activation increases the secretion of matrix metalloproteinases and causes NP degradation<sup>43</sup>. Pyroptosis can also lead to IL-1 $\beta$ , though distinct from apoptosis and simple cell necrosis; it is proinflammatory and mediated by the NLRP3 inflammasome<sup>44</sup>.

Similarly, the activation of the NF- $\kappa$ B signaling pathway promotes catabolism through the production of metalloproteases and enhances apoptosis

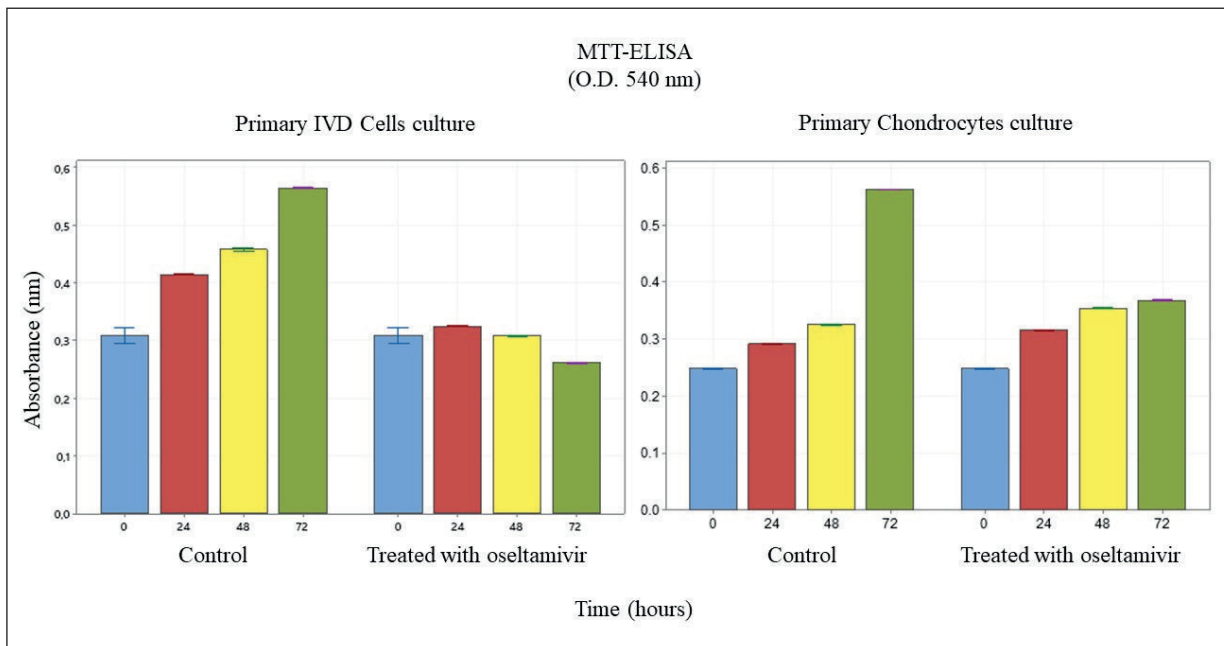
through the release of apoptotic biomarkers<sup>45</sup>. Indeed, in NF- $\kappa$ B-induced modulated chondrocytes, apoptosis and OA progression are regulated by the NLRP3 inflammasome<sup>46</sup>. In a study evaluating the relationship between the replicative senescence of chondrocytes and NF- $\kappa$ B target gene expression, the researchers emphasized that NF- $\kappa$ B proteins form a ubiquitously expressed transcription factor that plays an important role in apoptosis, cell senescence, cell inflammation, and immunity<sup>47</sup>. If NF- $\kappa$ B activation can be inhibited, then, lumbar IVDD can be slowed<sup>48</sup>.

In the present study, in the AF and NP cell cultures, NLRP3 protein expression increased 2.06 times in the oseltamivir-treated samples compared to the control samples at 24 h, but was normalized at 48 and 72 h. In the chondrocyte cultures, NLRP3 protein expression was at the same level for both the study and control samples at 24 h ( $r = 1.02$ ) and slightly decreased at 48 and 72 h ( $r =$

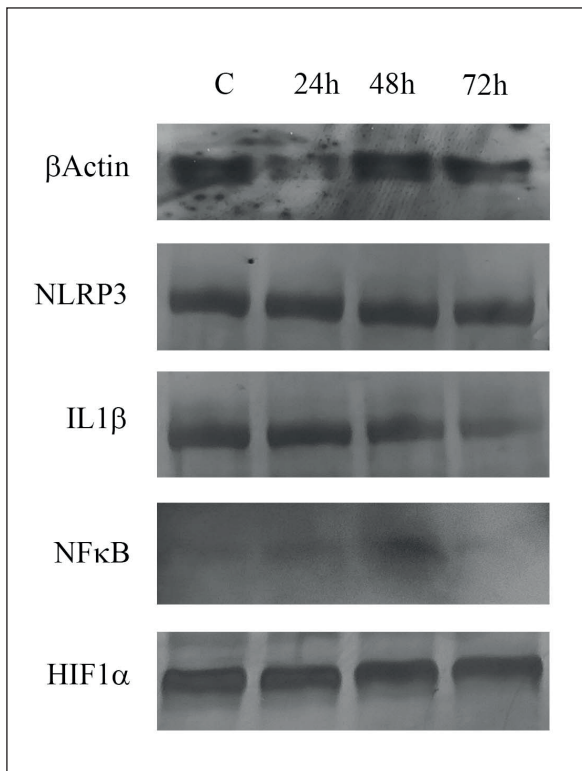
**Table II.** Assessment of the cell viability, toxicity, and proliferation of AF and NP cells and chondrocytes following oseltamivir treatment.

Primary cell culture	Source	Adj SS	Adj MS	F-value	p-value*
AF and NP cells	Groups	0.220052	0.220052	4941.29	0.000
	Time (hours)	0.068192	0.022731	510.42	0.000
	Groups vs. Time	0.146338	0.048779	1095.35	0.000
Chondrocytes	Groups	0.015337	0.015337	21909.64	0.000
	Time (hours)	0.303920	0.101307	144723.93	0.000
	Groups vs. Time	0.102818	0.034273	48961.07	0.000

\* $p < 0.05$  vs. group 1 and  $p < 0.05$  vs. group 2. Data was analyzed using a one-way analysis of variance followed by a post-hoc Turkey Pairwise Comparison test.



**Figure 3.** Comparison of the absorbance viability values in the oseltamivir-treated IVD and chondrocyte cultures with the control groups.



**Figure 4.** Western blotting analysis of HIF-1 $\alpha$ , IL-1 $\beta$ , the NLRP3 inflammasome, and NF- $\kappa$ B protein expression in human AF/NP cells with and without oseltamivir proteins in IVD tissues from the Pfirrmann scale grade IV case groups (n = 8). Data are presented as frequencies.

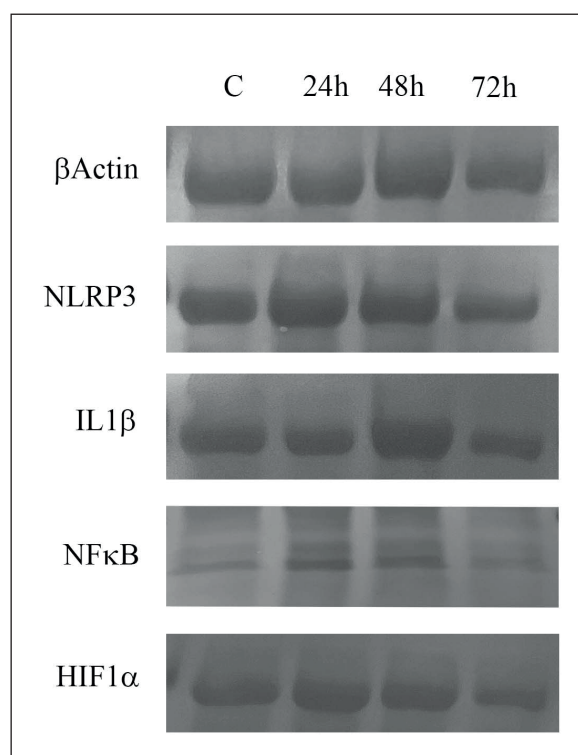
0.79 and 0.84, respectively). Additionally, NF- $\kappa$ B expression increased 3.58-fold and 5.82-fold at 24 and 48 h in the oseltamivir-treated samples compared to the control samples but was normalized at 72 h ( $r = 0.94$ ). In the chondrocyte cultures, NF- $\kappa$ B expression likewise increased 1.90 and 1.30 times at 24 and 48 h in the oseltamivir-treated samples compared to the control samples but was normalized at 72 h ( $r = 0.95$ ).

NP is a vascular tissue that is nourished by passive diffusion through pores in the hyaline membrane of adjacent endplates. Therefore, NP cells are physiologically hypoxic. While changes in oxygen levels are part of cellular adaptation, HIF-1 $\alpha$ , one of the key genes in this cellular adaptation process, can be activated in hypoxic conditions and adapt to tissue functions to lower oxygen levels by mediating transcription factors and enzymes<sup>49</sup>. HIF-1 $\alpha$  is also an activator of vascular endothelial growth factor and is critical in the angiogenic response to hypoxia<sup>50</sup>. Furthermore, HIF-1 $\alpha$  gene expression is an important component that supports chondrocyte survival during embryonic bone development<sup>21</sup>. It is a transcription factor that regulates the transcription of a wide variety of genes involved in cell survival. The hypoxic environment, hormones, and growth factors induce HIF-1 $\alpha$  translocation to the nucleus and regulate the expression of its target

genes<sup>21</sup>. Thus, HIF-1 $\alpha$  controls hypoxia-induced ECM synthesis in chondrocytes and is considered a positive regulator of cartilage regeneration<sup>21</sup>. In the present study on AF and NP cell cultures, HIF-1 $\alpha$  expression increased 2.75, 1.52, and 1.36 times at 24, 48, and 72 h, respectively in the oseltamivir-treated samples compared to the control samples. In the chondrocyte cultures, HIF-1 $\alpha$  expression increased 1.68 and 1.37 times at 24 and 48 h in the oseltamivir-treated samples compared to the control samples, though it decreased at a level similar to the control group at 72 h ( $r = 1.10$ ).

Many experimental studies examining the effects of oseltamivir generally have used commercial cell lines<sup>51</sup> or animal tissues<sup>52</sup>. Commercial cell lines contain only one cell type and lack complex coordination mechanisms in the cells' microenvironment<sup>53</sup>. They do not have the same genotypic and/or phenotypic characteristics as in the human body; therefore, results obtained from studies using commercial cell lines can be misleading<sup>53</sup>. The sensitivity of animal tissue differs from that of human tissue as well<sup>53</sup>. Therefore, the results obtained from studies using animal tissues and those using human tissues may also differ<sup>54</sup>. In the present study, primary cell cultures prepared from human degenerated IVD were used, which may enhance its value.

No morphological changes or cell deaths that could result from oseltamivir cytotoxicity were observed in the AF/NP and chondrocyte cells in this study's oseltamivir-treated culture samples. However, proliferation decreased in the AF and NP cell samples ( $p < 0.05$ ). In turn, proliferation increased slightly at 24 and 48 h in the oseltamivir-treated chondrocyte samples, though cell viability decreased, and proliferation was suppressed at 72 h ( $p < 0.05$ ). The protein expressions of HIF-1 $\alpha$ , IL-1 $\beta$ , NF- $\kappa$ B, and the NLRP3 inflammasome in both the AF/NP and chondrocyte cell cultures changed at certain time inter-



**Figure 5.** Western blotting analysis of HIF-1 $\alpha$ , IL-1 $\beta$ , the NLRP3 inflammasome, and NF- $\kappa$ B protein expression in human chondrocytes with and without oseltamivir proteins in cartilage tissues from the Kellgren-Lawrence radiological scale grade IV case groups ( $n = 8$ ). Data are presented as frequencies.

vals as well in the oseltamivir-treated samples compared to the control samples ( $p < 0.05$ ). In the AF and NP cell cultures, HIF- $\alpha$  increased, IL-1 $\beta$  decreased, and NF- $\kappa$ B and the NLRP3 inflammasome were normalized at 72 h in the oseltamivir-treated samples compared to the control group ( $p < 0.05$ ). In the chondrocyte tissue cells, IL-1 $\beta$  and the NLRP3 inflammasome decreased at 72 h, while HIF-1 $\alpha$  and NF- $\kappa$ B expressions were close to the control group samples at 72 h.

**Table III.** Protein expression fold change data obtained from Western blot analysis.

Primary Cell Culture	Protein	0h	24h	48h	72h
AF and NP Cells	$\beta$ -actin	1	1	1	1
	NLRP3	1	2.06	1.02	1.03
	HIF-1 $\alpha$	1	2.75	1.52	1.36
	IL-1 $\beta$	1	2.15	0.96	0.50
	NF- $\kappa$ B	1	3.58	5.82	0.94
Chondrocytes	$\beta$ -actin	1	1	1	1
	NLRP3	1	1.02	0.79	0.84
	HIF-1 $\alpha$	1	1.68	1.37	1.10
	IL-1 $\beta$	1	1.23	1.25	0.75
	NF- $\kappa$ B	1	1.90	1.30	0.95

All culture samples were prepared using tissues obtained from patients who belonged to the same ethnic group, generating the first limitation of this research. Regarding the study's second limitation, oseltamivir phosphate is an inactive prodrug that exerts pharmacologic activity when hydrolyzed *in vivo* to its active form, oseltamivir carboxylate<sup>31</sup>. However, any medication administered to the body that passes into the systemic circulation without undergoing chemical changes is defined as bioavailable. Orally administered medications also first pass through the liver and are extensively metabolized<sup>55</sup>. This research was carried out in an *in vitro* experimental setup, so the compensatory mechanisms in the body were disabled.

## Conclusions

Oseltamivir can inhibit viral neuraminidase enzymes through the regulation or increase of HIF-1 $\alpha$ , suppression of IL-1 $\beta$ , and normalization of the NLRP3 inflammasome with NF- $\kappa$ B, which mediate senescence and proinflammation. Accordingly, it may be used in the treatment of both cartilage and IVD tissue degeneration. As it is known, primary cultures obtained directly from the tissue, as used in our study, exhibit a heterogeneous structure containing cells belonging to the whole tissue and extracellular elements. Indeed, primary cultures are *in vitro* systems that reflect tissue integrity and physiology first-rate. For this reason, it describes best the response to drug administration or the effect in clinical practice after *in vivo* systems. The results of our study shed light on the clinical outcome.

## Conflict of Interest

The authors report no conflicts of interest.

## Availability of Data

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

## Ethical Approval

Informed consent forms were obtained from patients whose tissues were used to prepare primary cell cultures. Approval has been also obtained from the local ethics committee of the School of Medicine of Izmir Bakircay University (Date: 12/01/2022 Number: 485).

## Authors' Contributions

I.Y., is the principal author of this study, designed the study and the experiments. I.Y., and H.A., provided the dissolution of the drug in suitable solvent and applied oseltamivir to the cell cultures they prepared from the tissues. K.O., identified the cases to be included in the study and surgically resected the tissues from the cases to be used in the preparation of chondrocyte cultures. N.K., identified the cases to be included in the study and surgically resected the tissues from the cases to be used in the preparation of AF and NP cell cultures. I.Y., HA, N.K., and DYS worked on the experiments and the pharmaco-molecular analysis of the samples. I.Y., KO, and N.K. collected the data. I.Y., DYS, N.K., and H.O. carried out the statistical analysis. I.Y., H.A., K.O., N.K., D.Y.S., and H.O. read and approved the final manuscript.

## Acknowledgments

None.

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## References

- 1) Qiu HB, Bian WG, Zhang LJ, Mei N, Wu Y, Wei YQ, Han XZ. Inhibition of p53/p21 by TWIST alleviates TNF- $\alpha$  induced nucleus pulposus cell senescence *in vitro*. *Eur Rev Med Pharmacol Sci* 2020; 24: 12645-12654.
- 2) Shao JH, Fu QW, Li LX, Zhou R, Liu N, Peng JH, Chen Y. Prx II reduces oxidative stress and cell senescence in chondrocytes by activating the p16-CDK4/6-pRb-E2F signaling pathway. *Eur Rev Med Pharmacol Sci* 2020; 24: 3448-3458.
- 3) Coryell PR, Diekman BO, Loeser RF. Mechanisms and therapeutic implications of cellular senescence in osteoarthritis. *Nat Rev Rheumatol* 2021; 17: 47-57.
- 4) Rezus E, Cardoneanu A, Burlui A, Luca A, Codreanu C, Tamba BI, Stanciu GD, Dima N, Bădescu C, Rezuş C. The Link Between Inflammation and Degenerative Joint Diseases. *Int J Mol Sci* 2019; 20: 614.
- 5) Murphy MP, Koepke LS, Lopez MT, Tong X, Ambrosi TH, Gulati GS, Marecic O, Wang Y, Ransom RC, Hoover MY, Steininger H, Zhao L, Walkiewicz MP, Quarto N, Levi B, Wan DC, Weissman IL, Goodman SB, Yang F, Longaker MT, Chan CKF. Articular cartilage regeneration by activated skeletal stem cells. *Nat Med* 2020; 26: 1583-1592.
- 6) Shui XL, Lin W, Mao CW, Feng YZ, Kong JZ, Chen SM. Blockade of IL-17 alleviated inflammation in rat arthritis and MMP-13 expression. *Eur Rev Med Pharmacol Sci* 2017; 21: 2329-2337.

- 7) Sun Y, Leng P, Song M, Li D, Guo P, Xu X, Gao H, Li Z, Li C, Zhang H. Piezo1 activates the NLRP3 inflammasome in nucleus pulposus cell-mediated by Ca<sup>2+</sup>/NF- $\kappa$ B pathway. *Int Immunopharmacol* 2020; 85: 106681.
- 8) Guo Z, Qiu C, Mecca C, Zhang Y, Bian J, Wang Y, Wu X, Wang T, Su W, Li X, Zhang W, Chen B, Xiang H. Elevated lymphotoxin- $\alpha$  (TNF $\beta$ ) is associated with intervertebral disc degeneration. *BMC Musculoskelet Disord* 2021; 22: 77.
- 9) Wang C, Gao Y, Zhang Z, Chen C, Chi Q, Xu K, Yang L. Ursolic acid protects chondrocytes, exhibits anti-inflammatory properties via regulation of the NF- $\kappa$ B/NLRP3 inflammasome pathway and ameliorates osteoarthritis. *Biomed Pharmacother* 2020; 130: 110568.
- 10) Li Z, Huang Z, Zhang H, Lu J, Tian Y, Wei Y, Yang Y, Bai L. P2X7 Receptor Induces Pyroptotic Inflammation and Cartilage Degradation in Osteoarthritis via NF- $\kappa$ B/NLRP3 Crosstalk. *Oxid Med Cell Longev* 2021; 2021: 8868361.
- 11) Wang Y, Che M, Xin J, Zheng Z, Li J, Zhang S) The role of IL-1 $\beta$  and TNF- $\alpha$  in intervertebral disc degeneration. *Biomed Pharmacother* 2020; 131: 110660.
- 12) Zheng L, Zhang Z, Sheng P, Mobasheri A. The role of metabolism in chondrocyte dysfunction and the progression of osteoarthritis. *Ageing Res Rev* 2021; 66: 101249.
- 13) Vetuschi A, Pompili S, Gaudio E, Latella G, Sferra R. PPAR- $\gamma$  with its anti-inflammatory and anti-fibrotic action could be an effective therapeutic target in IBD. *Eur Rev Med Pharmacol Sci*. 2018; 22: 8839-8848.
- 14) Saberi M, Zhang X, Mobasheri A. Targeting mitochondrial dysfunction with small molecules in intervertebral disc aging and degeneration. *Geroscience* 2021; 43: 517-537.
- 15) Risbud MV, Schipani E, Shapiro IM. Hypoxic regulation of nucleus pulposus cell survival: from niche to notch. *Am J Pathol* 2010; 176: 1577-1583.
- 16) Gelse K, Pfander D, Obier S, Knaup KX, Wiesener M, Hennig FF, Swoboda B. Role of hypoxia-inducible factor 1 alpha in the integrity of articular cartilage in murine knee joints. *Arthritis Res Ther* 2008; 10: R111.
- 17) Kong P, Chen R, Zou FQ, Wang Y, Liu MC, Wang WG. HIF-1 $\alpha$  repairs degenerative chondrocyte glycolytic metabolism by the transcriptional regulation of Runx2. *Eur Rev Med Pharmacol Sci* 2021; 25: 1206-1214.
- 18) Karaarslan N, Yilmaz I, Ozbek H, Sirin DY, Kaplan N, Akyuva Y, Gonultas A, Ates O. Are Specific Gene Expressions of Extracellular Matrix and Nucleus Pulposus Affected by Primary Cell Cultures Prepared from Intact or Degenerative Intervertebral Disc Tissues? *Turk Neurosurg* 2019; 29: 43-52.
- 19) Sirin DY, Karaarslan N. Evaluation of the effects of pregabalin on chondrocyte proliferation and CHAD, HIF-1 $\alpha$ , and COL2A1 gene expression. *Arch Med Sci* 2018; 14: 1340-1347.
- 20) Pfander D, Cramer T, Swoboda B. Hypoxia and HIF-1alpha in osteoarthritis. *Int Orthop* 2005; 29: 6-9.
- 21) Kaplan N, Yilmaz I, Karaarslan N, Kaya YE, Sirin DY, Ozbek H. Does Nimodipine, a Selective Calcium Channel Blocker, Impair Chondrocyte Proliferation or Damage Extracellular Matrix Structures? *Curr Pharm Biotechnol* 2019; 20: 517-524.
- 22) Schipani E, Ryan HE, Didrickson S, Kobayashi T, Knight M, Johnson RS. Hypoxia in cartilage: HIF-1alpha is essential for chondrocyte growth arrest and survival. *Genes Dev* 2001; 15: 2865-2876.
- 23) Jia X, Liu B, Bao L, Lv Q, Li F, Li H, An Y, Zhang X, Cao B, Wang C. Delayed oseltamivir plus sirolimus treatment attenuates H1N1 virus-induced severe lung injury correlated with repressed NLRP3 inflammasome activation and inflammatory cell infiltration. *PLoS Pathog* 2018; 14: e1007428.
- 24) Bagheri Novir S, Aram MR. Quantum mechanical studies of the adsorption of Remdesivir, as an effective drug for treatment of COVID-19, on the surface of pristine, COOH-functionalized and S-, Si- and Al- doped carbon nanotubes. *Physica E Low Dimens Syst Nanostruct* 2021; 129: 114668.
- 25) Wu R, Wang L, Kuo HD, Shannar A, Peter R, Chou PJ, Li S, Hudlikar R, Liu X, Liu Z, Poiani GJ, Amorosa L, Brunetti L, Kong AN. An Update on Current Therapeutic Drugs Treating COVID-19. *Curr Pharmacol Rep* 2020; 6: 56-70.
- 26) Anedda L, Bianchini L, Cuzzolin L, Finco G, Fanos V, Marcialis MA. What if COVID-19 affects the child: which weapons and how to use them. *Eur Rev Med Pharmacol Sci* 2021; 25: 3325-3337.
- 27) Hussain M, Galvin HD, Haw TY, Nutsford AN, Husain M. Drug resistance in influenza A virus: the epidemiology and management. *Infect Drug Resist* 2017; 10: 121-134.
- 28) Wang W, Yin R, Zhang M, Yu R, Hao C, Zhang L, Jiang T. Boronic Acid Modifications Enhance the Anti-Influenza A Virus Activities of Novel Quindoline Derivatives. *J Med Chem*. 2017; 60: 2840-2852.
- 29) Tan Q, Duan L, Ma Y, Wu F, Huang Q, Mao K, Xiao W, Xia H, Zhang S, Zhou E, Ma P, Song S, Li Y, Zhao Z, Sun Y, Li Z, Geng W, Yin Z, Jin Y. Is oseltamivir suitable for fighting against COVID-19: In silico assessment, in vitro and retrospective study. *Bioorg Chem* 2020; 104: 104257.
- 30) Smeed DF, Hurst BL, Wong MH, Bailey KW, Morrey JD. Effects of double combinations of amantadine, oseltamivir, and ribavirin on influenza A (H5N1) virus infections in cell culture and in mice. *Antimicrob Agents Chemother* 2009; 53: 2120-2128.
- 31) Treanor JJ, Hayden FG, Vrooman PS, Barbarash R, Bettis R, Riff D, Singh S, Kinnersley N, Ward P, Mills RG. Efficacy and safety of the oral neuraminidase inhibitor oseltamivir in treating acute influenza: a randomized controlled trial. *US Oral Neuraminidase Study Group. JAMA* 2000; 283: 1016-1024.
- 32) Sur M, Lopez MJ, Baker MB. Oseltamivir. [Updated 2021 Oct 2]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK539909/> (accessed on: 05.04.2022).
- 33) Wang MC, Wang D, Lu YH, Li ZH, Jing HY. Protective effect of MAPK signaling pathway me-

- diated by ITGB3 gene silencing on myocardial ischemia-reperfusion injury in mice and its mechanism. *Eur Rev Med Pharmacol Sci* 2021; 25: 820-836.
- 34) Akgun FS, Sirin DY, Yilmaz I, Karaarslan N, Ozbek H, Simsek AT, Kaya YE, Kaplan N, Akyuva Y, Caliskan T, Ates O. Investigation of the effect of dipyrone on cells isolated from intervertebral disc tissue. *Exp Ther Med* 2019; 18: 216-224.
  - 35) Karaarslan N, Yilmaz I, Ozbek H, Yasar Sirin D, Kaplan N, Caliskan T, Ozdemir C, Akyuva Y, Ates O. Are radio-contrast agents commonly used in discography toxic to the intact intervertebral disc tissue cells? *Basic Clin Pharmacol Toxicol* 2019; 124: 181-189.
  - 36) Yilmaz I, Karaarslan N, Yasar Sirin D, Ozbek H. Pharmacological assessment of the effects of anandamide and its antagonists on hippocampal tissue in Wistar albino rats. *Eur Rev Med Pharmacol Sci* 2020; 24: 11871-11882.
  - 37) Zhu SB, Xu YQ, Gao H, Deng Y. NF- $\kappa$ B inhibitor QNZ protects human chondrocyte degeneration by promoting glucose uptake through Glut4 activation. *Eur Rev Med Pharmacol Sci* 2020; 24: 4642-4651.
  - 38) Xia C, Zeng Z, Fang B, Tao M, Gu C, Zheng L, Wang Y, Shi Y, Fang C, Mei S, Chen Q, Zhao J, Lin X, Fan S, Jin Y, Chen P. Mesenchymal stem cell-derived exosomes ameliorate intervertebral disc degeneration via anti-oxidant and anti-inflammatory effects. *Free Radic Biol Med* 2019; 143: 1-15.
  - 39) Sun PF, Kong WK, Liu L, Liu Y, Liu FM, Liu WJ, Yu H, Yang WL, Li GQ, Sun QR. Osteopontin accelerates chondrocyte proliferation in osteoarthritis rats through the NF- $\kappa$ B signaling pathway. *Eur Rev Med Pharmacol Sci* 2020; 24: 2836-2842.
  - 40) Zhao K, An R, Xiang Q, Li G, Wang K, Song Y, Liao Z, Li S, Hua W, Feng X, Wu X, Zhang Y, Das A, Yang C. Acid-sensing ion channels regulate nucleus pulposus cell inflammation and pyroptosis via the NLRP3 inflammasome in intervertebral disc degeneration. *Cell Prolif* 2021; 54: e12941.
  - 41) Chao-Yang G, Peng C, Hai-Hong Z. Roles of NLRP3 inflammasome in intervertebral disc degeneration. *Osteoarthritis Cartilage* 2021; 29: 793-801.
  - 42) Zhao Y, Qiu C, Wang W, Peng J, Cheng X, Shang-guan Y, Xu M, Li J, Qu R, Chen X, Jia S, Luo D, Liu L, Li P, Guo F, Vasilev K, Liu L, Hayball J, Dong S, Pan X, Li Y, Guo L, Cheng L, Li W. Cortistatin protects against intervertebral disc degeneration through targeting mitochondrial ROS-dependent NLRP3 inflammasome activation. *Theranostics* 2020; 10: 7015-7033.
  - 43) Mariotte A, De Cauwer A, Po C, Abou-Faycal C, Pichot A, Paul N, Aouadi I, Carapito R, Frisch B, Macquin C, Chatelus E, Sibilia J, Armspach JP, Bahram S, Georgel P. A mouse model of MSU-induced acute inflammation in vivo suggests imiquimod-dependent targeting of IL-1 $\beta$  as relevant therapy for gout patients. *Theranostics* 2020; 10: 2158-2171.
  - 44) Zu Y, Mu Y, Li Q, Zhang ST, Yan HJ. Icarin alleviates osteoarthritis by inhibiting NLRP3-mediated pyroptosis. *J Orthop Surg Res* 2019; 14: 307.
  - 45) Li Z, Zhang K, Li X, Pan H, Li S, Chen F, Zhang J, Zheng Z, Wang J, Liu H. Wnt5a suppresses inflammation-driven intervertebral disc degeneration via a TNF- $\alpha$ /NF- $\kappa$ B-Wnt5a negative-feedback loop. *Osteoarthritis Cartilage* 2018; 26: 966-977.
  - 46) Xu H, Zhang J, Shi X, Li X, Zheng C. NF- $\kappa$ B inducible miR-30b-5p aggravates joint pain and loss of articular cartilage via targeting SIRT1-FoxO3a-mediated NLRP3 inflammasome. *Aging (Albany NY)* 2021; 13: 20774-20792.
  - 47) Wu Y, Chen L, Wang Y, Li W, Lin Y, Yu D, Zhang L, Li F, Pan Z. Overexpression of Sirtuin 6 suppresses cellular senescence and NF- $\kappa$ B mediated inflammatory responses in osteoarthritis development. *Sci Rep* 2015; 5: 17602.
  - 48) Ma T, Guo CJ, Zhao X, Wu L, Sun SX, Jin QH. The effect of curcumin on NF- $\kappa$ B expression in rat with lumbar intervertebral disc degeneration. *Eur Rev Med Pharmacol Sci* 2015; 19: 1305-1314.
  - 49) Zhang B, Zhao Q, Li Y, Zhang J. Moxibustion alleviates intervertebral disc degeneration via activation of the HIF-1 $\alpha$ /VEGF pathway in a rat model. *Am J Transl Res* 2019; 11: 6221-6231.
  - 50) Zhu WJ, Chang BY, Wang XF, Zang YF, Zheng ZX, Zhao HJ, Cui QD. FBW7 regulates HIF-1 $\alpha$ /VEGF pathway in the IL-1 $\beta$  induced chondrocytes degeneration. *Eur Rev Med Pharmacol Sci* 2020; 24: 5914-5924.
  - 51) Xiong R, Zhang L, Li S, Sun Y, Ding M, Wang Y, Zhao Y, Wu Y, Shang W, Jiang X, Shan J, Shen Z, Tong Y, Xu L, Chen Y, Liu Y, Zou G, Lavillette D, Zhao Z, Wang R, Zhu L, Xiao G, Lan K, Li H, Xu K. Novel and potent inhibitors targeting DHODH are broad-spectrum antivirals against RNA viruses including newly-emerged coronavirus SARS-CoV-2. *Protein Cell* 2020; 11: 723-739.
  - 52) Sidwell RW, Smee DF. Peramivir (BCX-1812, RWJ-270201): potential new therapy for influenza. *Expert Opin Investig Drugs* 2002; 11: 859-869.
  - 53) Karaarslan N, Yilmaz I, Sirin DY. Toxicity of the acetyl-para-aminophenol group of medicines to intact intervertebral disc tissue cells. *Exp Ther Med* 2021; 21: 147.
  - 54) Yilmaz I, Karaarslan N, Ozbek H. Practical Performance of Hippocampal Tissue Resection in Rats in Pharmacomolecular Research. *Turk Neurosurg* 2021; 31: 112-118.
  - 55) Yilmaz I, Karaarslan N. Examining the effects of HMG-CoA reductase inhibitors on anabolic and catabolic signaling pathway proteins associated with degenerative disc disease. *Eur Rev Med Pharmacol Sci* 2022; 26: 2990-3000.