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Cytoprotective Role of Chondrocyte Coculturing for Enhanced Cartilage Regeneration

(Peranan Sitopelindung Pengkulturan bersama Kondrosit untuk Penjanaan Semula Rawan Dipertingkat)

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ABSTRACT

Osteoarthritis is characterized by the progressive deterioration of articular cartilage, leading to joint pain and functional impairments. Current treatment options are limited in their ability to stimulate cartilage regeneration. Evidence suggests that the co-culture technique, involving the interplay of multiple cell types, may effectively restore damaged cartilage. This investigation assessed the regenerative impact of co-culture on osteoarthritis-affected rat knee joints. Gene expression profiling validated phenotypic and biochemical expression analysis. Compared to the control group, the co-cultivated cohorts showed elevated levels of cartilage-specific markers, such as collagen and aggrecan. Notably, the group with stress and co-cultured with normal and osteoarthritic chondrocytes (H_2O_2+N+OA) demonstrated significant results, including lower LDH release (15.06 ± 1.461), decreased glycosaminoglycan levels (1.551 ± 0.1487), and reduced cell death percentage (17.50 ± 3.536) compared to the H_2O_2 control. Safranin-O staining retention also increased (28.89 ± 2.846), indicating enhanced cartilage matrix retention. Enhanced expression of survival markers such as Bcl (0.3974 ± 0.02241) and decreased apoptotic markers like Bax (0.2961 ± 0.01199) were observed, confirming the stimulation of critical genes involved in cartilage development and matrix synthesis. These findings support the potential of co-culture technology to accelerate cartilage regeneration and offer an innovative strategy to impede osteoarthritis progression.

Keywords: Cartilage regeneration; cellular survival; co-culturing; osteoarthritis

ABSTRAK

Osteoartitis dicirikan oleh kemerosotan progresif rawan artikul yang membawa kepada sakit sendi dan gangguan fungsi. Pilihan rawatan semasa adalah terhad dalam keupayaannya untuk merangsang pertumbuhan semula rawan. Bukti menunjukkan bahawa teknik pengkulturan bersama yang melibatkan interaksi pelbagai jenis sel boleh memulihkan rawan yang rosak dengan berkesan. Penyelidikan ini menilai kesan penjanaan semula pengkulturan bersama pada sendi lutut tikus yang terjejas oleh osteoartitis. Pemprofilan pengekspresan gen mengesahkan analisis pengekspresan fenotip dan biokimia. Berbanding dengan kumpulan kawalan, kohort yang diusahakan bersama menunjukkan peningkatan tahap penanda khusus rawan, seperti kolagen dan aggrecan. Terutamanya, kumpulan dengan tekanan dan pengkulturan bersama dengan kondrosit normal dan kondrosit osteoartitis (H_2O_2+N+OA) menunjukkan hasil yang ketara, termasuk pelepasan LDH yang lebih rendah (15.06 ± 1.461), penurunan paras glikosaminoglikan (1.551 ± 0.1487) dan pengurangan peratusan kematian sel (17.50 ± 3.536) berbanding kawalan H_2O_2. Pengekalan pewarnaan Safranin-O juga meningkat (28.89 ± 2.846), menunjukkan pengekalan matriks rawan dipertingkat. Pengekspresan penanda kelangsungan hidup yang dipertingkatkan seperti Bcl (0.3974 ± 0.02241) dan penurunan penanda apoptosis seperti Bax (0.2961 ± 0.01199) telah diperhatikan, mengesahkan rangsangan gen kritikal yang terlibat dalam pembangunan rawan dan sintesis matriks. Penemuan ini menyokong potensi teknologi pengkulturan bersama untuk mempercepatkan pertumbuhan semula rawan dan menawarkan strategi inovatif untuk menghalang perkembangan osteoartitis.

Kata kunci: Kelangsungan hidup sel; osteoartitis; pengkulturan bersama; penjanaan semula rawan

INTRODUCTION

Osteoarthritis is a degenerative disease of joints that mostly affects the articular cartilage, joint capsule, synovial membrane, and subchondral bone (Silva et al. 2019). Knee osteoarthritis is the most frequent type, accounting for twice as many cases as hip or hand osteoarthritis (Driban et al. 2020). It is the biggest cause of disability in adults, affecting approximately 300 million individuals globally. Articular cartilage, a connective tissue found within joints, degrades in osteoarthritis, resulting in a loss of its smooth, low-friction surface (Tschaikowsky et al. 2022). Adult cartilage is mostly made up of extracellular matrix, with a low number of chondrocytes. The water content of cartilage, which accounts for 65-80% of its weight, increases in osteoarthritis due to increased permeability and matrix instability. As a result, as the modulus of elasticity of the cartilage declines, so does its ability to sustain human weight (Mieloch et al. 2019).

The extracellular matrix of articular cartilage is developed, maintained, and repaired exclusively by specialized, metabolically active cells called chondrocytes (Yang et al. 2020). The extracellular matrix (ECM) is the primary target of cartilage degradation in osteoarthritis. However, repairing injured cartilage is challenging due to the lack of cartilage's neural, vascular, and lymphatic nature (Hu et al. 2021). Osteoarthritis can be caused by oxidative stress due to the induction of reactive oxygen species. Increased levels of ROS have been observed in OA patients. Oxidative stress can disturb the anabolic activities of chondrocytes and lead to cartilage degradation by causing apoptosis and senescence of chondrocytes (Liu et al. 2022). Intraarticular injection of a number of substances, including enzymes (papain, collagenase, trypsin, hyaluronidases), cytokines (interleukin (IL)-1, transforming growth factor (TGF), and chemicals (monosodium iodoacetate), induces pathologic changes (Xu et al. 2020). *In vitro* studies have shown that mimicking OA can be achieved by inducing oxidative stress through hydrogen peroxide (Liu et al. 2021).

Cartilage defects can be addressed either conservatively or surgically. NSAIDs, or nonsteroidal anti-inflammatory drugs, are routinely used in conventional pain therapy (Wolff et al. 2021). Although their long-term usefulness is unknown, chondroitin sulphate, glucosamine, and hyaluronic acid food supplements may also lessen discomfort. Use of a brace, physical therapy, and rehabilitation may offer momentary pain relief (Nguyen et al. 2016). There are several surgical methods available to repair articular cartilage, but they are not renewable and produce more fibrous cartilage than hyaline cartilage. Tissue engineering methods have been developed to treat articular cartilage damage based on improved understanding of the molecular, cellular, and microenvironmental characteristics of cartilage (Del Bakhshayesh et al. 2020). Autologous chondrocytes implantation (ACI) and implantation of MSCs are two alternative clinical trials for the regeneration of defected articular cartilage (Niemeyer et al. 2016), but they have limitations in accessibility of healthy chondrocytes, non-homologous regeneration of cartilage, and dedifferentiation during the expansion of culture (Ogura et al. 2020).

Co-culture is a method of cultivating different populations of cells in contact with each other and has been used for studying cell interactions (Tan et al. 2019). Chondrocytes were co-cultured to investigate issues related to mesenchymal stem cells (MSCs) transplantation (Cao et al. 2019). Chondrocytes have been shown to release factors that promote chondrogenesis in MSCs, prevent the terminal differentiation of chondrocytes, and inhibit hypertrophy of MSCs during co-culture (Marchan et al. 2022).

This study aimed to examine the growth traits of osteoarthritic and normal chondrocytes in female Wistar rats. The study also used a co-culture system to investigate the impact of paracrine factors secreted by healthy, OA, and Normal + OA chondrocytes on hydrogen peroxide-induced damage. The findings showed that in H_2O_2-induced injury, normal and Normal +OA chondrocytes enhanced proteoglycan content and cell survival while decreasing apoptosis and cell damage. These results imply that chondrocytes, both healthy and OA, have therapeutic potential for the treatment of osteoarthritis.

MATERIALS AND METHODS

ANIMALS

Healthy female Wistar rats aged 4-6 months were procured from the Animal House of the National Centre of Excellence in Molecular Biology (CEMB), Lahore, Pakistan, for chondrocyte culture. The experiments conducted received approval from the Institutional Animal Ethics Committee at the National Centre of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan as recommended by the American Veterinary Medical Association (AVMA) and approves the said research work.

PREPARATION OF RAT MODELS

A group of 20 rats aged 4-6 months and weighing 150-200 g were randomly divided into two groups: An osteoarthritis group and a normal group, with 10 rats in each group. Anesthesia was induced in all rats with an optimized dose of ketamine (88 mg/kg BW) and xylazine (14 mg/kg BW), Collagenase-B injections (Roche. Cat no. 11 088 823 103) were given in the knee of each rat on day 1, followed by two additional doses on the 3rd and 6th day after the first injection.

SAMPLE COLLECTION

Normal and one osteoarthritis rat were selected. Rats were anesthetized and blood was collected directly from hearts of normal and osteoarthritic rats and preserved. The

rats were then sacrificed using chloroform and their right knees were obtained and placed in a petri plate containing sterile 1X PBS (Gibco Cat No.70011) with penicillin and streptomycin.

SERUM ISOLATION

The clotted blood of the normal and osteoarthritis rat was centrifuged at 1200 rpm for 10 min, resulting in the separation of the serum-containing upper layer. The serum was then preserved in a 1.5 mL centrifuge tube at a temperature of -20 °C.

ISOLATION OF CHONDROCYTES FROM RAT'S KNEES

The knee joints were placed in a petri plate with sterile 1X PBS (100 U/mL penicillin and 100 g/mL streptomycin). Soft tissues and cartilage fragments were taken from the articular heads. The cartilage was then incubated for 2 h at 37 °C and 5% CO_2 in Collagenase D (Gibco, cat no 17104-019) solution (3 mg/mL). Under the same conditions, the bones were removed from the joint and incubated overnight in Collagenase IV solution (0.5 mg/mL). The resultant cell suspension was filtered, centrifuged, rinsed with PBS, and resuspended in low glucose DMEM medium (Sigma Cat No. D5523) supplemented with 20% fetal bovine serum (Sigma Cat No. F9665). The cells were seeded in six-well plates and cultured at 37 °C and 5% CO_2 for three days, with new medium replenished every third day, until they reached 70-80% confluence and sub cultured to next passage. Cells at passage 3 (P3) were used for further experiment.

SULFATED GLYCOSAMINOGLYCAN ASSAY

A reagent blank with 100 µL of deionized water was prepared. A calibration curve was created using standards and the reagent blank. Test samples, initially 90 µL, were adjusted to 100 µL with deionized water. Blyscan dye reagent was added to each tube and incubated on a mechanical shaker for 30 min. Dissociation reagent was added, capped, and vortexed to release the bound dye. After centrifuging at 12000 rpm for 5 min to remove foam, 200 µL of each sample was transferred to a 96-well plate, and the absorbance was measured at 656 nm.

BIOCHEMICAL ASSAYS

Serum from normal and OA groups (n=6) was screened for levels of alkaline phosphatase (DiaSys Diagnostics Systems, Germany) and LDH assay (Sigma Aldrich, USA) to assess acute knee injury (cartilage).

ALKALINE PHOSPHATASE ASSAY

To make the working reagent, R1 and R2 reagents from the DiaSys kit (Germany) were mixed thoroughly. Then, 20 µL of serum was added to 1000 µL of working reagent, and the absorbance was measured at 405 nm after 1, 2, and 3 min. The calculations were performed following the instructions provided by the manufacturer.

LDH ASSAY

Chondrocytes from passages one to seven were analyzed for cytotoxicity using the LDH assay. Media (100 µL) from each group was added to a 96-well plate and the LDH concentration was determined using a kit from Sigma Aldrich (SWUSA) following the manufacturer's protocol. Using a Spectramax PLUS 384 spectrophotometer absorbance was measured at 490 nm with a reference value taken at 690 nm.

INDUCTION OF OXIDATIVE STRESS IN NORMAL CHONDROCYTES AND CO-CULTURE EXPERIMENT

Normal chondrocytes were randomly separated into five groups and planted in a 6-well plate (Becton Dickinson, USA) at a density of 1×10^4 cells per well. The chondrocytes were injured by being exposed to 200 µM H_2O_2 for 3 h (Merck, USA). After the 3-h H_2O_2 exposure, inserts were put into the wells, and on the membranes of the inserts, equal numbers of freshly trypsinized normal chondrocytes, OA chondrocytes, or a mixture of normal + OA chondrocytes were implanted. Although they shared the same DMEM media, the cells above the inserts and the chondrocytes below were not in close proximity to one another. The regular chondrocytes were used for further investigation after co-culturing for 36 h.

TRYPAN BLUE EXCLUSION ASSAY

The Trypan Blue staining protocol for microscopy involved preparing a cell suspension with an equal volume of Trypan Blue dye (0.2-0.4 %), incubating the mixture at 25 °C for 2 min, and spreading it onto a microscope slide. A coverslip was placed over the cell suspension, and the slide was observed under a microscope. Live cells appeared translucent, while dead cells were stained blue. The number of live and dead cells was counted and analyzed.

SAFRANIN-O STAINING

The proteoglycan content of the experimental groups was measured by safranin-O staining. The cells were fixed with 4% PFA and then incubated in 1.5% Safranin-O (ICN Biomedicals, Germany) for 30 min at room temperature. After staining, the cells were washed three times with 1X PBS, followed by 0. 02% fast green staining for 2 min. One percent freshly prepared acetic acid was added for 10 s, and the cells were washed with 1X PBS three times. The stained cells were observed under an Olympus BX61 microscope for further analysis.

IMMUNOCYTOCHEMISTRY

For immunocytochemistry characterization, 5×10^4 cells were plated in a 12-well plate. The cells were washed, fixed with 4% PFA, blocked with 5% BSA. Primary antibodies (Col1a1 and Aggrecan) were applied and incubated with the cells. After washing, a fluorochrome-conjugated secondary antibody was added. Rhodamine-conjugated Donkey anti-goat secondary antibody was used. The cells were stained with DAPI, mounted, and imaged using an Olympus BX61 microscope.

RNA EXTRACTION

The TRIzol Reagent was used to extract RNA from cells in according to the manufacturer's instructions (TRIzol™ LS Reagen-10296010), the pellet was washed, dried, and dissolved in RNAse-free water. The RNA was quantified using a Nanodrop ND 1000 spectrophotometer.

STATISTICAL ANALYSIS

In order to find meaningful differences between the groups, one-way ANOVA with Bonferroni's test was used in the statistical analysis. Statistical significance was defined as a p-value less than 0.05. Results were presented as mean ± SD. GraphPad Prism software (version 8.00) for Windows, was used to create the graphs.

RESULTS & DISCUSSION

MORPHOLOGICAL AND X-RAY ANALYSIS OF THE RAT'S KNEE

X-ray analysis was performed for the confirmation of OA rat model. There was difference between right and left knee of the rat apparently. Loss of cartilage and narrowing of joint space was observed in OA knee as compared to normal knee (Figure 1(A)(b). Similarly, the morphology of isolated knee was observed which showed significant inflammation in the OA rat knee as compared to the normal knee (Figure 1(B)) (Nirmal et al. 2017). X-ray analysis confirmed the development of OA in the injected knees, characterized by cartilage loss and joint space narrowing as shown in Figure 1. Similar osteoarthritis models were prepared in mice models to study the effect of 17β-estradiol on bone marrow (BM) cell differentiation *in vivo* (Ganova, Belenska-Todorova & Ivanovska 2023).

MICROSCOPICAL ANALYSIS OF INJURED CHONDROCYTES

Normal chondrocytes in culture are rounded or polygonal with smooth surfaces and good adherence. After 3 h of H_2O_2 exposure, chondrocytes became swollen, detached, and irregularly shaped due to oxidative stress, showing decreased viability and signs of apoptosis, indicating significant cellular damage as shown in Figure 2.

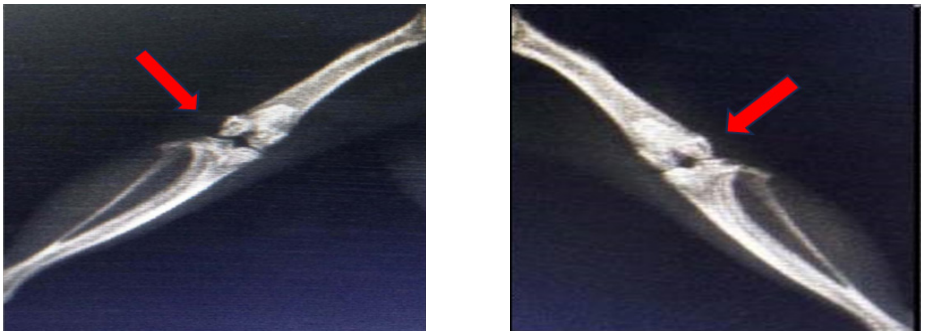
CO-CULTURING EFFECTED CELLS MORPHOLOGY AND CONFLUENCY

It was found that the co-cultured cells displayed greater confluence in comparison to the H_2O_2 treated cells. The H_2O_2 treated cells in the Normal (N) and Normal+ OA (N+OA) co-culture groups supported the cellular attachment along with marked cell growth when compared to the OA group as shown in Figure 3. Co-culture techniques have emerged as valuable tools for studying cell interactions, particularly in the context of OA and injured chondrocytes (Borciani et al. 2020). Previous research has shown promising results in co-culturing human mesenchymal stem cells (MSCs) and chondrocytes to address cartilage issues (Chen et al. 2020). In this study, chondrocytes from normal, OA, and a combination of both were co-cultured with normal chondrocytes subjected to hydrogen peroxide-induced stress.

CO-CULTURING DECREASED NECROSIS ACTIVITY IN STRESSED CHONDROCYTES

High LDH levels have been reported to be a sign of either acute or ongoing cell injury (Wen et al. 2016). The necrotic activity in co-cultured groups was observed by the increased level of LDH released in H_2O_2 control cells as compared to the normal cells. H_2O_2 control cells released a significant higher amount of LDH when compared to the normal cells (N) and co-cultured groups. The H_2O_2+N (14.60 ± 1.132) and H_2O_2+N+OA (15.06 ± 1.461) groups exhibited a significantly lower LDH release in comparison to the H_2O_2 control group. However, the H_2O_2+OA (19.19 ± 1.267) group did not display any significant decrease in LDH levels when compared to the H_2O_2 control group (21.01 ± 1.455) as shown in Figure 4(A). It has been reported that culturing of chondrocytes minimizes the necrotic activity in osteoarthritic knee and improves the ECM formation (Song et al. 2018). Our study suggests that co-culture groups significantly reduce the necrotic activity by ameliorating the oxidative stress and improving the ECM.

Alkaline phosphatase is a marker of bone health, it breaks down proteins by removing phosphate group. An increase in level of alkaline phosphatase can be indication of bone disease (Sekaran, Vimalraj & Thangavelu 2021). Similarly, H_2O_2 control group (32.74 ± 3.800) exhibited a higher level of ALP in comparison to the normal (11.78 ± 1.475) and co-cultured groups. The H_2O_2+OA group (28.25 ± 1.577) displayed higher concentration of ALP when compared to the H_2O_2+N (20.28 ± 1.189) and H_2O_2+N+OA (21.62 ± 2.122) co-cultured groups. The H_2O_2+N (20.28 ± 1.189) and H_2O_2+N+OA (21.62 ± 2.122) co-cultured groups had a similar concentration of ALP, which was lower than that of the H_2O_2 control (32.74 ± 3.800) and H_2O_2+OA groups (28.25 ± 1.577) as shown in Figure 4(B). Similar results were reported in co-culturing of osteoclasts/osteoblasts for bone regeneration (Mandatori et al. 2021).



(a) (b)

FIGURE 1.A. X-ray analysis. (a) Normal left knee of rat (b) OA right knee of rat

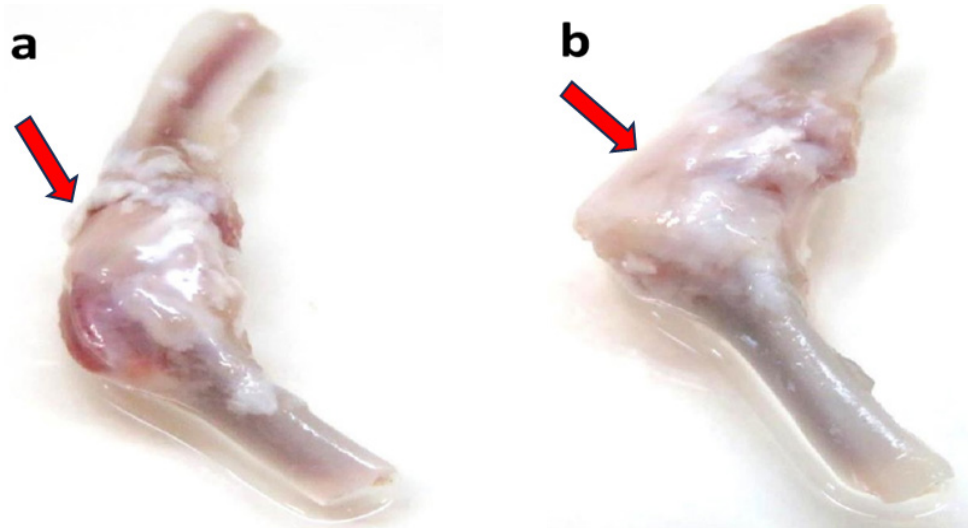


FIGURE 1.B: The gross morphological aspects of the osteoarthritic knee model showing hard, yellow fibrotic tissue (b) Normal knee joint showing soft and smooth cartilage surface

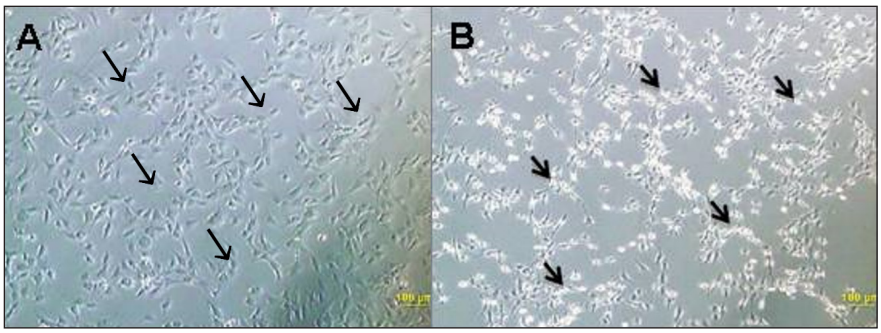


FIGURE 2. Exposure to H_2O_2 (A) Arrows show the normal chondrocytes before H_2O_2 exposure and (B) Chondrocytes after 3 h of H_2O_2 exposure

CO-CULTURING REDUCED THE GLYCOSAMINOGLYCANS RELEASE

Glycosaminoglycans (GAGs) are located primarily on the surface of cells and in the extracellular matrix (Gulati & Poluri 2016). In this study, there was significant decrease in GAGs content in degraded cartilage as compared to normal cartilage due to loss of tissue integrity and ECM. It was observed that the H_2O_2 control group (2.372 ± 0.1436) had a significantly higher level of glycosaminoglycans released when compared to the other co-cultured groups as shown in Figure 5. The H_2O_2+N (1.694 ± 0.1162) and H_2O_2+N+OA (1.551 ± 0.1487) groups exhibited a significant decreased level of glycosaminoglycans when compared to the H_2O_2 control group (2.372 ± 0.1436). Comparing our results to a previously reported similar study using Rabbit as *in vivo* osteoarthritis model, similar results were exhibited by the co-culture group (chondrocytes/chondrons in 1:1 ratio) as compared to the normal and control group (Duan et al. 2021).

CO-CULTURING MITIGATED THE CELLULAR DEATH

The percentage viability was calculated to compare the normal chondrocytes to those treated with H_2O_2, it was found that normal chondrocytes had higher viability (4.000 ± 1.414). The H_2O_2 control group showed a higher percentage of dead cells (78.00 ± 2.828) when compared to co-culture groups. Specifically, H_2O_2+N (13.50 ± 2.121) and H_2O_2+N+OA (17.50 ± 3.536) had significantly lower percentages of dead cells than the H_2O_2 control group (78.00 ± 2.828) and H_2O_2+OA cells (17.50 ± 3.536) as depicted in Figure 6. In a reported study cell viability was assessed using the trypan blue exclusion assay (Deszcz et al. 2020), where normal chondrocytes showed higher viability compared to H_2O_2-treated chondrocytes. Osteoarthritic chondrocytes retained more trypan blue dye than normal chondrocytes. In current study, normal chondrocytes showed more viability as compared to H_2O_2 treated chondrocytes.

CO-CULTURING IMPROVED THE PROTEOGLYCAN CONTENT

The stability of matrix of articular cartilage is maintained by proteoglycans and collagen (Peng et al. 2021). On the other hand, loss of proteoglycans and collagen is associated with cartilage damage associated with osteoarthritis (Gauci et al. 2017). In this study, normal cells appeared darkly stained and retained more Safranin-O color, while other groups retained less stain. The H_2O_2-treated cells (10.79 ± 2.051) were significantly less stained than the normal (76.86 ± 3.564) and co-cultured groups. There was a statistically significant increase in the percentage of Safranin-O stain in the H_2O_2+N (27.36 ± 2.318) and H_2O_2+N+OA (28.89 ± 2.846) groups compared to the H_2O_2 control group as shown in Figure 7. This data is in accordance with previous study showing increased proteoglycan contents in combination of ADMSCs and chondrocytes co-cultures (Cho, Kim & Kim 2018).

CO-CULTURING ENHANCED THE AGGRECAN AND COLLAGEN EXPRESSION

The extracellular matrix in chondrocytes contains components like aggrecan that are essential to the structural integrity and functionality of cartilage (Wang et al. 2020). In the present study, aggrecan expression was high in normal cells as compared with the H_2O_2 treated cells as the cellular matrix components were degraded due to oxidative stress produced by H_2O_2. Aggrecan's expression was highly exhibited normal cells (33.23 ± 1.426) compared to H_2O_2 cells (6.906 ± 1.153), where its expression decreased. The positive expression for aggrecan were observed in normal cells (33.23 ± 1.426), H_2O_2+N (17.25 ± 1.707) & H_2O_2+N+OA (15.53 ± 0.9970) cells, while its expression decreased in H_2O_2 (6.906 ± 1.153) and H_2O_2+OA cells (10.05 ± 1.355) as shown in Figure 8.

Similarly, the expression of collagen, a matrix protein found in the extracellular space of connective tissues was found to be significantly higher in normal chondrocytes compared to injured cells. Immunostaining results showed decreased expression of collagen in H_2O_2 cells (7.146 ± 0.8563) and H_2O_2 cells co-cultured with OA chondrocytes (8.798 ± 0.6739) as compared to normal cells (27.18 ± 1.194) and H_2O_2 cells co-cultured with normal (17.05 ± 0.5798) and Normal+ OA cells (15.57 ± 0.7856). The H_2O_2+N and H_2O_2+N+OA groups showed statistically significant increased expression of collagen (17.05 ± 0.5798 and 15.57 ± 0.7856, respectively) compared to the H_2O_2 control group as shown in Figure 9. In a similar study using naturally derived antioxidant compounds such as allicin, sulforaphane, and lycopene which enhanced antioxidase and chondrogenic matrix synthesis in H_2O_2-stimulated osteoarthritis in *in vitro* experiments (Yang et al. 2020).

GENE EXPRESSION ANALYSIS

Gene expression analysis of caspase and Bax key players in apoptotic cell death (Orning & Lien 2021), showed higher expression levels in the hydrogen peroxide (H_2O_2) treated cells, consistent with a previous study (Li et al. 2023). Gene expression analysis of apoptotic markers Bax and caspase 3 indicated higher expression levels of both markers in the H_2O_2 group (Bax, 0.5152 ± 0.03251; Caspase, 0.7097 ± 0.02828) compared to the H_2O_2+N (Bax, 0.2747 ± 0.009835; Caspase, 0.4492 ± 0.03536) and H_2O_2+N+OA groups (Bax, 0.2961 ± 0.01199; Caspase, 0.5266 ± 0.02121). Similar results for Caspase were seen when a former research study conducted trials on minimizing the inflammatory response in osteoarthritic knee of female mice by transplanting primary chondrocytes culture (Lohan et al. 2016). Similarly the results of Bax correspond to previous research to lookout the rat bone

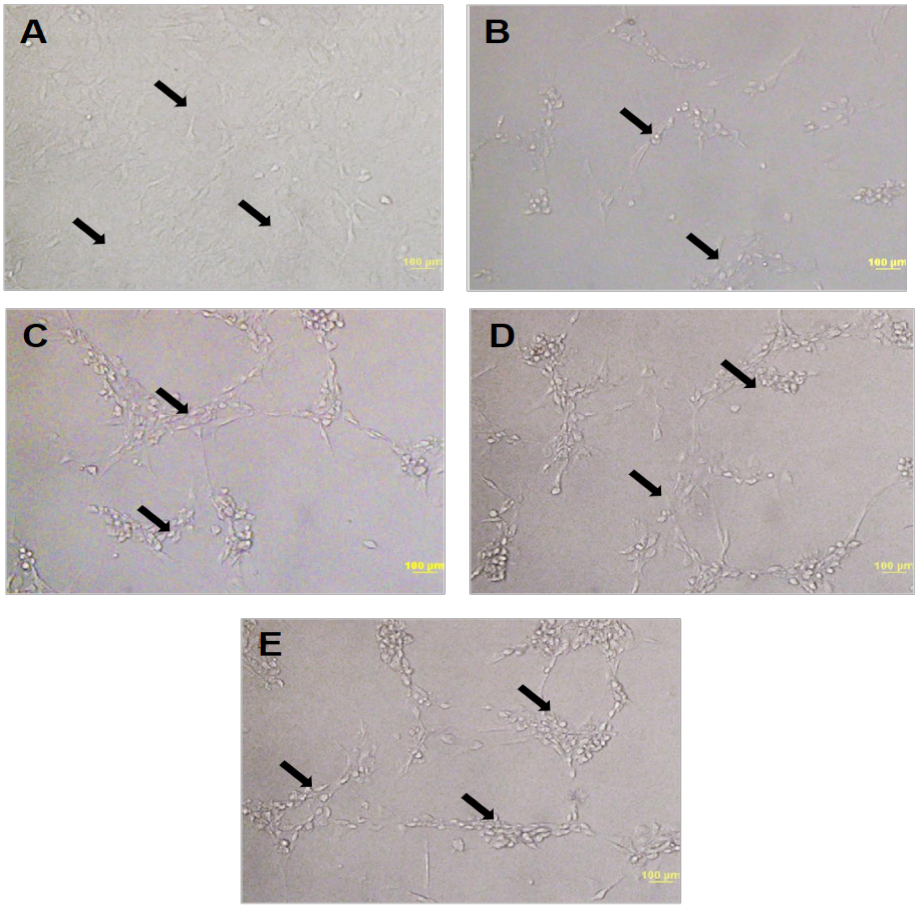


FIGURE 3: (A) Normal cells (B) H_2O_2 treated cells detached from their surface due to oxidative stress (C) H_2O_2 treated cells co-cultured with normal cells (D) H_2O_2 treated cells co-cultured with OA cells (E) H_2O_2 treated cells co-cultured with mixture of normal and OA cells.

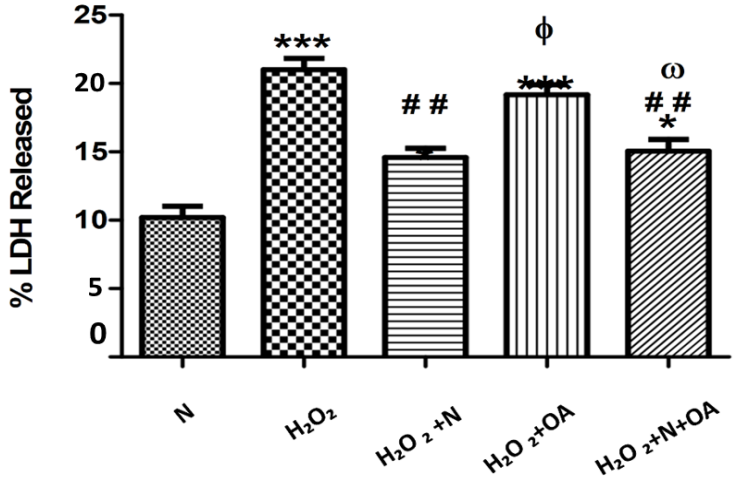
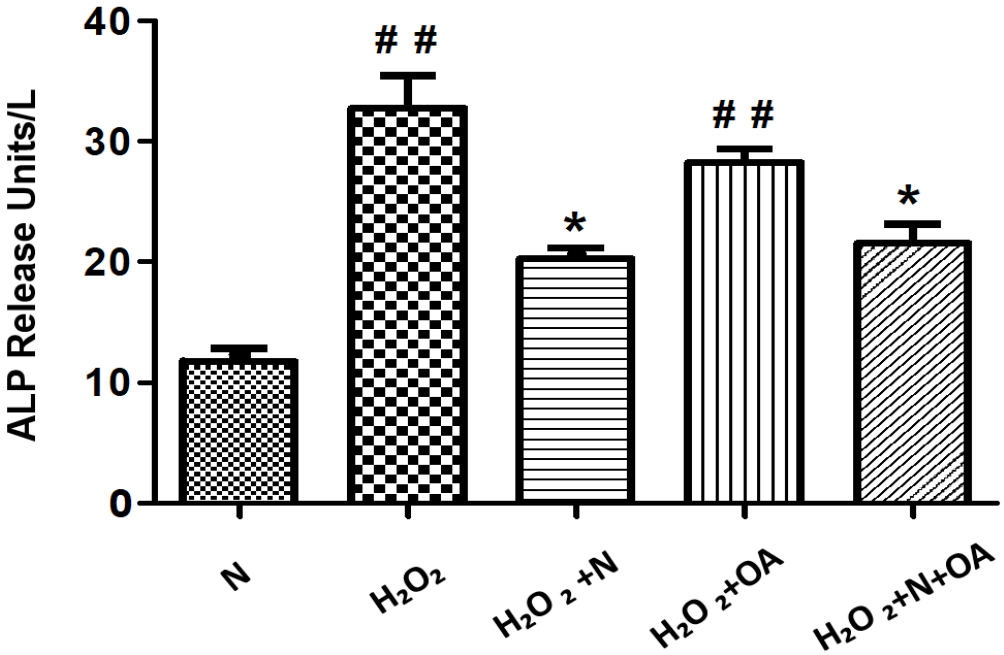
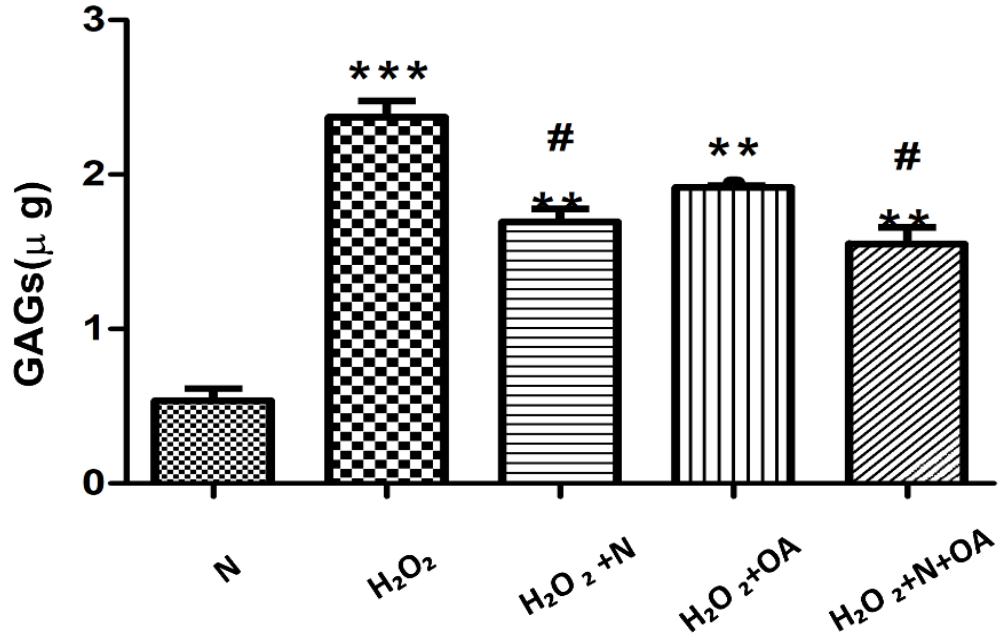


FIGURE 4 (A): Graphical representation of LDH released in co-culture groups: Data are represented as Mean ± SD. ##p < 0.01 for H_2O_2 vs. H_2O_2+N and H_2O_2+N+OA, *p < 0.05 for N vs. H_2O_2+N and H_2O_2+N+OA, ***p < 0.001 for N vs. H_2O_2 and H_2O_2+OA. ϕp < 0.05 for H_2O_2+N vs. H_2O_2+OA, ωp < 0.05 for H_2O_2+OA vs. H_2O_2+N+OA



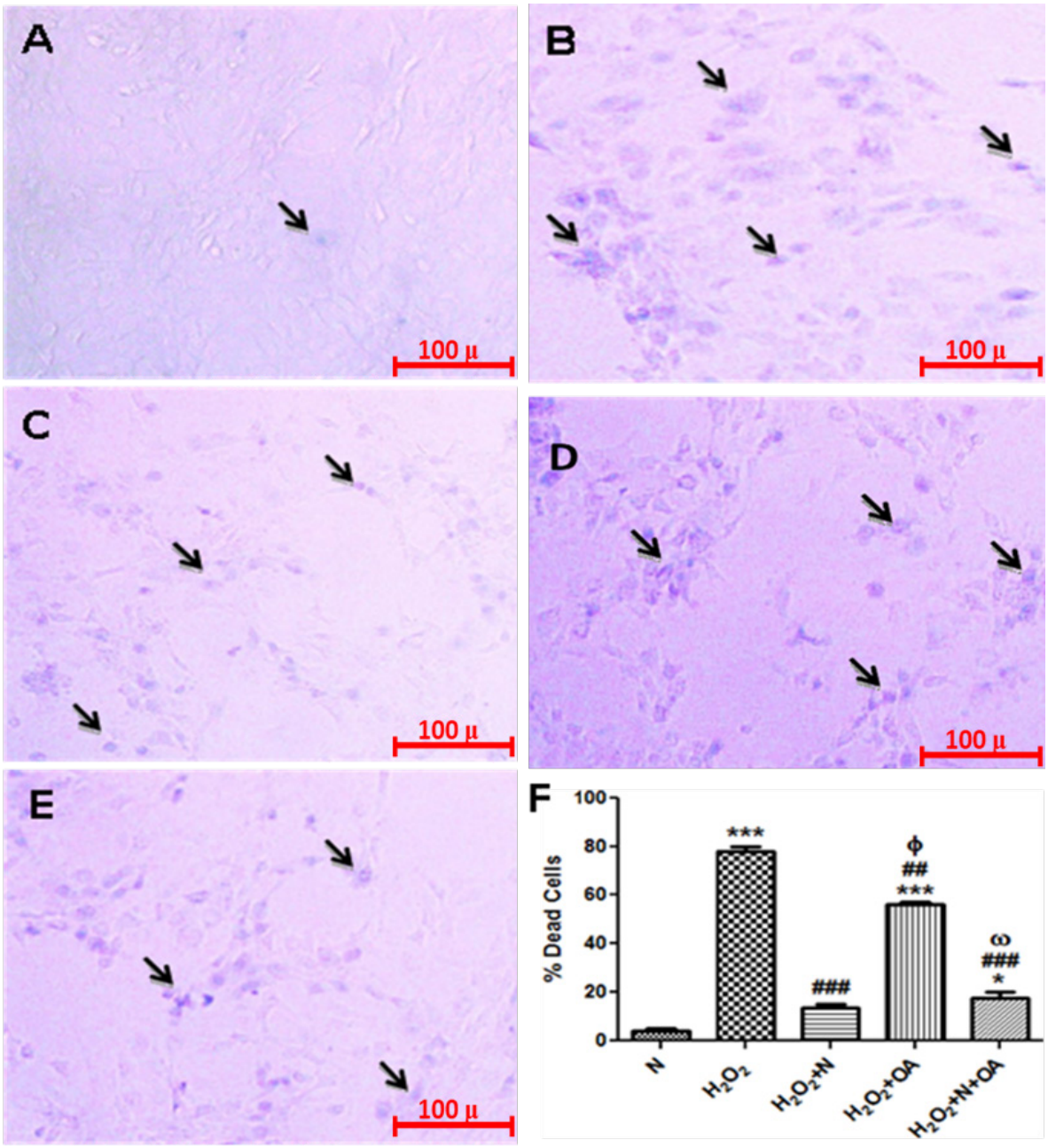
Data are represented as Mean ± SD. *p < 0.05 for H_2O_2 vs. H_2O_2+Normal and H_2O_2 vs. H_2O_2+Normal+ OA; ##p < 0.01 for Normal vs. H_2O_2 and H_2O_2+OA

FIGURE 4(B). Graphical representation of ALP released in co-culture groups



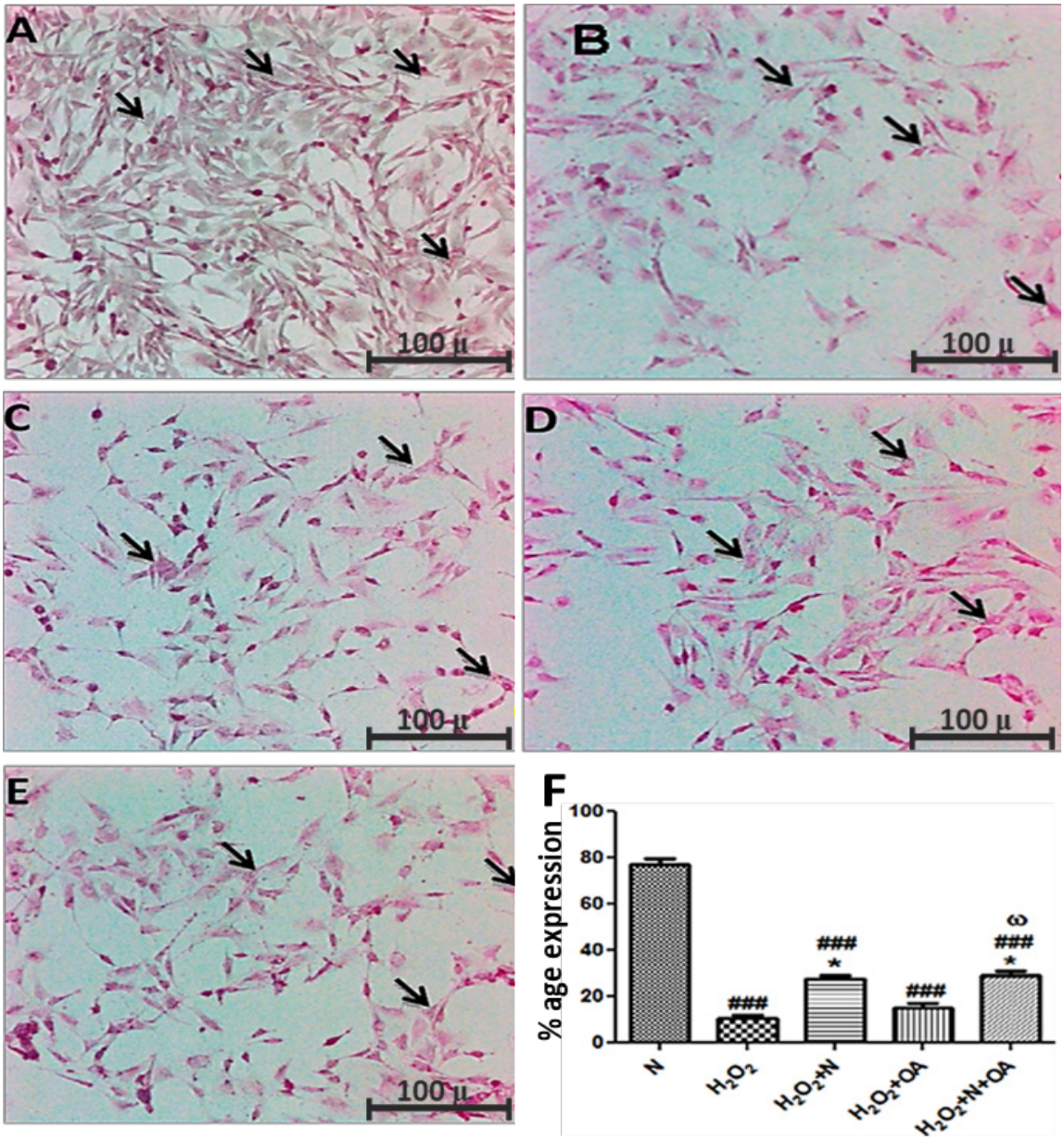
Data are represented as Mean ± SD. **p < 0.01 for Normal vs. H_2O_2+Normal, Normal vs. H_2O_2+OA, and Normal vs. H_2O_2+Normal+ OA; ***p < 0.001 for Normal vs. H_2O_2; #p < 0.05 for H_2O_2 vs. H_2O_2+Normal and H_2O_2 vs. H_2O_2+Normal+ OA

FIGURE 5. Graphical representation of glycosaminoglycans released in co-culture groups



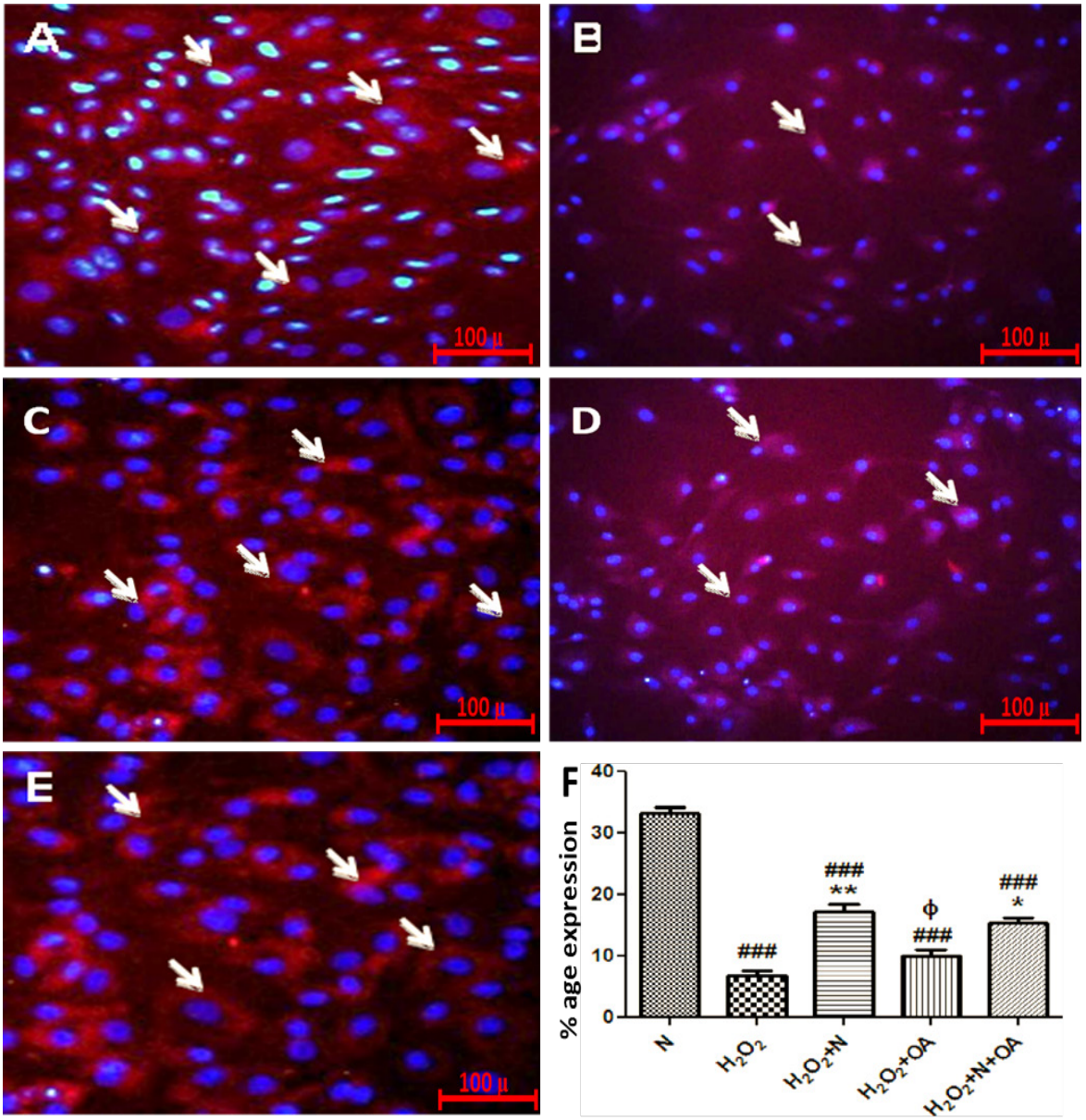
Data are represented as Mean ± SD. **p < 0.01 for Normal vs. H_2O_2+Normal, Normal vs. H_2O_2+OA, and Normal vs. H_2O_2+Normal+ OA; ***p < 0.001 for Normal vs. H_2O_2; #p < 0.05 for H_2O_2 vs. H_2O_2+Normal and H_2O_2 vs. H_2O_2+Normal+ OA

FIGURE 6. Trypan Blue Assay: (A) Normal cells, (B) H_2O_2 treated cells, (C) H_2O_2 treated cells co- cultured with normal cells, (D) H_2O_2 treated cells co-cultured with OA cells, (E) H_2O_2 treated cells co-cultured with Normal+ OA cells, and (F) Graphical representation of comparison of trypan blue assay in co- culture groups



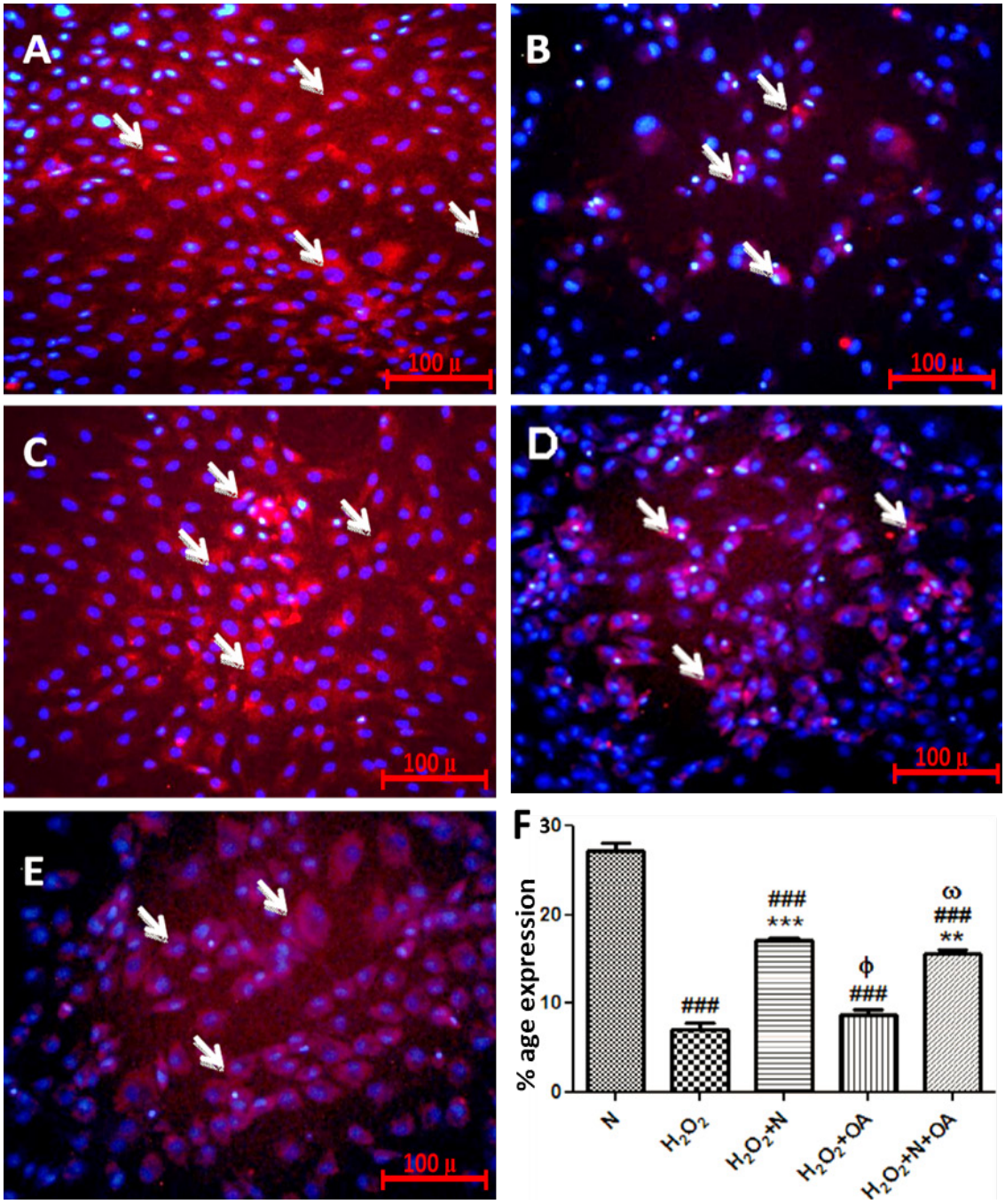
Data are represented as Mean ± SD. **p < 0.01 for Normal vs. H_2O_2+Normal, Normal vs. H_2O_2+OA, and Normal vs. H_2O_2+Normal+ OA; ***p < 0.001 for Normal vs. H_2O_2; #p < 0.05 for H_2O_2 vs. H_2O_2+Normal and H_2O_2 vs. H_2O_2+Normal+ OA

FIGURE 7. Safranin O Staining: (A) Normal cells, (B) H_2O_2 treated cells, (C) H_2O_2 treated cells co- cultured with normal cells, (D) H_2O_2 treated cells co-cultured with OA cells, (E) H_2O_2 treated cells co- cultured with Normal+ OA cells, and (F) Quantitative analysis of safranin staining in experimental groups through Image J



Data are represented as Mean ± SD. **p < 0.01 for Normal vs. H_2O_2+Normal, Normal vs. H_2O_2+OA, and Normal vs. H_2O_2+Normal+ OA; ***p < 0.001 for Normal vs. H_2O_2; #p < 0.05 for H_2O_2 vs. H_2O_2+Normal and H_2O_2 vs. H_2O_2+Normal+ OA

FIGURE 8. Immunostaining for Aggrecan: (A) Normal cells (B) H_2O_2 treated cells (C) H_2O_2 treated cells co-cultured with normal cells (D) H_2O_2 treated cells co-cultured with OA cells (E) H_2O_2 treated cells co-cultures with Normal+ OA cells (F) Quantitative analysis of aggrecan expression in experimental groups through Image J



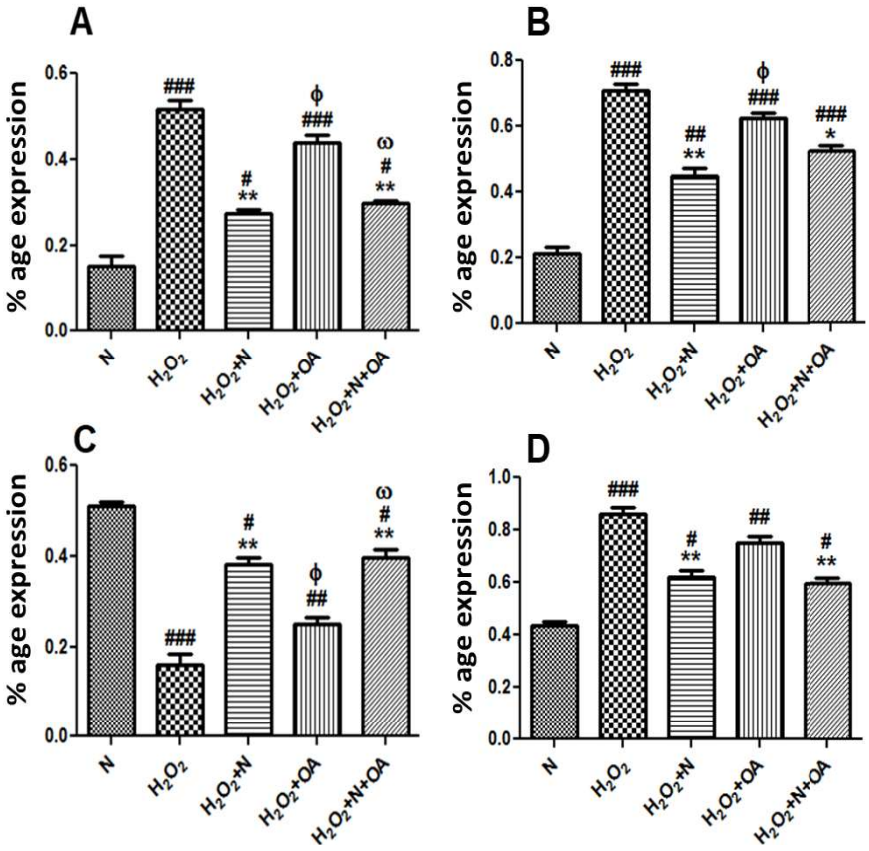
Data are represented as Mean ± SD. **p < 0.01 for Normal vs. H_2O_2+Normal, Normal vs. H_2O_2+OA, and Normal vs. H_2O_2+Normal+ OA; ***p < 0.001 for Normal vs. H_2O_2; #p < 0.05 for H_2O_2 vs. H_2O_2+Normal and H_2O_2 vs. H_2O_2+Normal+ OA

FIGURE 9. Immunostaining for Collagen: (A) Normal cells, (B) H_2O_2 treated cells, (C) H_2O_2 treated cells co-cultured with normal cells, (D) H_2O_2 treated cells co-cultured with OA cells, (E) H_2O_2 treated cells co-cultured with Normal+ OA cells, and (F) Quantitative analysis of collagen expression in experimental groups through Image J

marrow derived mesenchymal stem cell's potential in intervertebral disc regeneration (Ekram et al. 2021).

In contrast, the survival marker Bcl, showed higher expression in H_2O_2+N (0.3819 ± 0.02195) and H_2O_2+N+OA groups (0.3974 ± 0.02241) but lower expression in the H_2O_2 (0.1586 ± 0.03519) and H_2O_2+OA groups (0.2508 ± 0.02052). H_2O_2+N and H_2O_2+N+OA groups showed significant increases in Bcl expression due to cell proliferation. The ALP marker, important in bone mineralization, was also analyzed and results were in line with other degradative markers (0.8592 ± 0.03528) and H_2O_2+OA groups (0.7498 ± 0.02757), while expression levels were decreased in the H_2O_2+N (0.6225 ± 0.03487) and H_2O_2+N+OA groups (0.5959 ± 0.02757) as shown in Figure 10(A)-10(D). In this study, the expression of Bcl and ALP was higher in H_2O_2+N and H_2O_2+N+OA groups. However, the expression of Bcl was lower in H_2O_2 control and H_2O_2+OA groups. H_2O_2+N and H_2O_2+N+OA showed significant results due to cell proliferation.

Taken together, these findings demonstrate that co-culturing normal and Normal+ OA chondrocytes can mitigate the damaging effects induced by hydrogen peroxide. The co-culture technique promoted increased production of proteoglycans, collagen, aggrecan, and Bcl, while simultaneously reducing the expression of caspase, annexin, Bax, and ALP. Moreover, the co-cultured groups exhibited reduced levels of senescent cells, LDH, ALP, glycosaminoglycans, and glucose, along with improved cell viability. These results highlight the potential of the co-culture approach for *in vitro* and *in-vivo* cartilage repair in osteoarthritis models. To better understand the molecular processes by which stressed chondrocytes are kept intact by normal and normal + OA chondrocyte co-cultures, future studies might investigate pre-treating chondrocytes with hydrogen peroxide prior to transplanting in animal models. Such research would increase our understanding of the possible therapeutic uses of co-culturing methods in cartilage regeneration and repair.



Data are represented as Mean ± SD. **p < 0.01 for Normal vs. H_2O_2+Normal, Normal vs. H_2O_2+OA, and Normal vs. H_2O_2+ Normal+ OA; ***p < 0.001 for Normal vs. H_2O_2; #p < 0.05 for H_2O_2 vs. H_2O_2+Normal and H_2O_2 vs. H_2O_2+ Normal+ OA

FIGURE 10. Quantitative Gene expression analysis: Quantitative analysis of gene expression in experimental groups through Image J. One way ANOVA was applied to check the significance of the data. (A) Bax, (B) Caspase 3, (C) Bcl, and (D) ALP

CONCLUSION

In conclusion, co-culture may be a viable treatment for osteoarthritis, especially in rat knee joints. The co-cultured group showed improved chondrocyte phenotype, backed by increased cartilage-specific marker expression. This was confirmed by gene expression profiling, which showed the activation of cartilage development and matrix synthesis genes. In addition, H_2O_2-induced injury boosted proteoglycan levels, cell survival, and decreased apoptosis and cell damage. Healthy and osteoarthritic chondrocytes in co-culture improved cartilage repair and provided a unique way to halt osteoarthritis progression.

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REFERENCES

Borciani, G., Montalbano, G., Baldini, N., Cerqueni, G., Vitale-Brovarone, C. & Ciapetti, G. 2020. Co–culture systems of osteoblasts and osteoclasts: Simulating *in vitro* bone remodeling in regenerative approaches. *Acta Biomaterialia* 108: 22-45.

Cao, X., Luo, P., Huang, J., Liang, C., He, J., Wang, Z., Shan, D., Peng, C. & Wu, S. 2019. Intraarticular senescent chondrocytes impair the cartilage regeneration capacity of mesenchymal stem cells. *Stem Cell Research & Therapy* 10: 86.

Chen, Y., Ouyang, X., Wu, Y., Guo, S., Xie, Y. & Wang, G. 2020. Co-culture and mechanical stimulation on mesenchymal stem cells and chondrocytes for cartilage tissue engineering. *Current Stem Cell Research & Therapy* 15(1): 54-60.

Cho, H., Kim, D. & Kim, K. 2018. Engineered co-culture strategies using stem cells for facilitated chondrogenic differentiation and cartilage repair. *Biotechnology and Bioprocess Engineering* 23: 261-270.

Del Bakhshayesh, A.R., Babaie, S., Nasrabadi, H.T., Asadi, N., Akbarzadeh, A. & Abedelahi, A. 2020. An overview of various treatment strategies, especially tissue engineering for damaged articular cartilage. *Artificial Cells Nanomedicine, and Biotechnology* 48(1): 1089-1104.

Deszcz, I., Lis-Nawara, A., Grelewski, P., Dragan, S. & Bar, J. 2020. Utility of direct 3D co-culture model for chondrogenic differentiation of mesenchymal stem cells on hyaluronan scaffold (Hyaff-11). *Regenerative Biomaterials* 7(6): 543-552.

Driban, J.B., Harkey, M.S., Barbe, M.F., Ward, R.J., MacKay, J.W., Davis, J.E., Lu, B., Price, L.L., Eaton, C.B., Lo, G.H. & McAlindon, T.E. 2020. Risk factors and the natural history of accelerated knee osteoarthritis: A narrative review. *BMC Musculoskeletal Disorders* 21: 332.

Duan, W., Zhao, Y., Ren, X., Zhao, R., Li, Q., Sun, Z., Song, W., Yang, Y., Li, P. & Wei, X. 2021. Combination of chondrocytes and chondrons improves extracellular matrix production to promote the repairs of defective knee cartilage in rabbits. *Journal of Orthopaedic Translation* 28: 47-54.

Ekram, S., Khalid, S., Bashir, I., Salim, A. & Khan, I. 2021. Human umbilical cord-derived mesenchymal stem cells and their chondroprogenitor derivatives reduced pain and inflammation signaling and promote regeneration in a rat intervertebral disc degeneration model. *Molecular and Cellular Biochemistry* 476: 3191-3205.

Ganova, P., Belenska-Todorova, L. & Ivanovska, N. 2023. Effect of estradiol on chondrocytes in the active stage of collagenase-induced osteoarthritis. *Current Bioactive Compounds* 19(1): 3-10.

Gauci, S.J., Stanton, H., Little, C.B. & Fosang, A.J. 2017. Proteoglycan and collagen degradation in osteoarthritis. In *Cartilage: Volume 2: Pathophysiology*, edited by Grässel, S. & Aszódi, A. Springer, Cham. pp. 41-61. https://doi.org/10.1007/978-3-319-45803-8_3

Gulati, K. & Poluri, K.M. 2016. Mechanistic and therapeutic overview of glycosaminoglycans: the unsung heroes of biomolecular signaling. *Glycoconjugate Journal* 33(1): 1-17.

Hu, H., Liu, W., Sun, C., Wang, Q., Yang, W., Zhang, Z., Xia, Z., Shao, Z. & Wang, B. 2021. Endogenous repair and regeneration of injured articular cartilage: A challenging but promising therapeutic strategy. *Aging and Disease* 12(3): 886.

Li, W., Yin, X., Yan, Y., Liu, C. & Li, G. 2023. Kurarinone attenuates hydrogen peroxide-induced oxidative stress and apoptosis through activating the PI3K/Akt signaling by upregulating IGF1 expression in human ovarian granulosa cells. *Environmental Toxicology* 38(1): 28-38.

Liu, G., Liu, Q., Yan, B., Zhu, Z. & Xu, Y. 2021. USP7 inhibition alleviates H_2O_2-induced injury in chondrocytes via inhibiting NOX_4/$NLRP_3$ pathway. *Frontiers in Pharmacology* 11: 617270.

Liu, L., Luo, P., Yang, M., Wang, J., Hou, W. & Xu, P. 2022. The role of oxidative stress in the development of knee osteoarthritis: A comprehensive research review. *Frontiers in Molecular Biosciences* 9: 1001212.

Lohan, P., Treacy, O., Lynch, K., Barry, F., Murphy, M., Griffin, M.D., Ritter, T. & Ryan, A.E. 2016. Culture expanded primary chondrocytes have potent immunomodulatory properties and do not induce an allogeneic immune response. *Osteoarthritis and Cartilage* 24(3): 521-533.

Mandatori, D., Penolazzi, L., Pelusi, L., Lambertini, E., Michelucci, F., Porreca, A., Cerritelli, P., Pipino, C., Di Iorio, A. & Bruni, D. 2021. Three-dimensional co-culture system of human osteoblasts and osteoclast precursors from osteoporotic patients as an innovative model to study the role of nutrients: Focus on vitamin K2. *Nutrients* 13(8): 2823.

Marchan, J., Wittig, O., Diaz-Solano, D., Gomez, M. & Cardier, J.E. 2022. Enhanced chondrogenesis from chondrocytes co-cultured on mesenchymal stromal cells: Implication for cartilage repair. *Injury* 53(2): 399-407.

Mieloch, A.A., Richter, M., Trzeciak, T., Giersig, M. & Rybka, J.D. 2019. Osteoarthritis severely decreases the elasticity and hardness of knee joint cartilage: A nanoindentation study. *Journal of Clinical Medicine* 8(11): 1865.

Nguyen, C., Lefèvre-Colau, M-M., Poiraudeau, S. & Rannou, F. 2016. Rehabilitation (exercise and strength training) and osteoarthritis: A critical narrative review. *Annals of Physical and Rehabilitation Medicine* 59(3): 190-195.

Niemeyer, P., Albrecht, D., Andereya, S., Angele, P., Ateschrang, A., Aurich, M., Baumann, M., Bosch, U., Erggelet, C. & Fickert, S. 2016. Autologous chondrocyte implantation (ACI) for cartilage defects of the knee: A guideline by the working group "Clinical Tissue Regeneration" of the German Society of Orthopaedics and Trauma (DGOU). *The Knee* 23(3): 426-435.

Nirmal, P.S., Jagtap, S.D., Narkhede, A.N., Nagarkar, B.E. & Harsulkar, A.M. 2017. New herbal composition (OA-F2) protects cartilage degeneration in a rat model of collagenase induced osteoarthritis. *BMC Complementary and Alternative Medicine* 17: 6.

Ogura, T., Ackermann, J., Mestriner, A.B., Merkely, G. & Gomoll, A.H. 2020. Minimal clinically important differences and substantial clinical benefit in patient-reported outcome measures after autologous chondrocyte implantation. *Cartilage* 11(4): 412-422.

Orning, P. & Lien, E. 2021. Multiple roles of caspase-8 in cell death, inflammation, and innate immunity. *Journal of Leucocyte Biology* 109(1): 121-141.

Peng, Z., Sun, H., Bunpetch, V., Koh, Y., Wen, Y., Wu, D. & Ouyang, H. 2021. The regulation of cartilage extracellular matrix homeostasis in joint cartilage degeneration and regeneration. *Biomaterials* 268: 120555.

Sekaran, S., Vimalraj, S. & Thangavelu, L. 2021. The physiological and pathological role of tissue nonspecific alkaline phosphatase beyond mineralization. *Biomolecules* 11(11): 1564.

Silva, J.C., Carvalho, M.S., Han, X., Xia, K., Mikael, P.E., Cabral, J.M.S., Ferreira, F.C. & Linhardt, R.J. 2019. Compositional and structural analysis of glycosaminoglycans in cell-derived extracellular matrices. *Glycoconjugate Journal* 36: 141-154.

Song, E.K., Jeon, J., Jang, D.G., Kim, H.E., Sim, H.J., Kwon, K.Y., Medina-Ruiz, S., Jang, H-J., Lee, A.R. & Rho, J.G. 2018. ITGBL1 modulates integrin activity to promote cartilage formation and protect against arthritis. *Science Translational Medicine* 10(462): eaam7486.

Tan, Z.Q., Leow, H.Y., Lee, D.C.W., Karisnan, K., Song, A.A.L., Mai, C.W., Yap, W.S., Lim, S.H.E. & Lai, K.S. 2019. Co-culture systems for the production of secondary metabolites: Current and future prospects. *The Open Biotechnology Journal* 13: 18-26.

Tschaikowsky, M., Brander, S., Barth, V., Thomann, R., Rolauffs, B., Balzer, B.N. & Hugel, T. 2022. The articular cartilage surface is impaired by a loss of thick collagen fibers and formation of type I collagen in early osteoarthritis. *Acta Biomaterialia* 146: 274-283.

Wang, C., Brisson, B.K., Terajima, M., Li, Q., Han, B., Goldberg, A.M., Sherry Liu, X., Marcolongo, M.S., Enomoto-Iwamoto, M. & Yamauchi, M. 2020. Type III collagen is a key regulator of the collagen fibrillar structure and biomechanics of articular cartilage and meniscus. *Matrix Biology* 85: 47-67.

Wen, Z., Lei, Z., Yao, L., Jiang, P., Gu, T., Ren, F., Liu, Y., Gou, C., Li, X. & Wen, T. 2016. Circulating histones are major mediators of systemic inflammation and cellular injury in patients with acute liver failure. *Cell Death & Disease* 7(9): e2391.

Wolff, D.G., Christophersen, C., Brown, S.M. & Mulcahey, M.K. 2021. Topical nonsteroidal anti-inflammatory drugs in the treatment of knee osteoarthritis: A systematic review and meta-analysis. *The Physician and Sportsmedicine* 49(4): 381-391.

Xu, J., Yan, L., Yan, B., Zhou, L., Tong, P. & Shan, L. 2020. Osteoarthritis pain model induced by intra-articular injection of mono-iodoacetate in rats. *Journal of Visualized Experiments* 159: e60649.

Yang, J., Song, X., Feng, Y., Liu, N., Fu, Z., Wu, J., Li, T., Chen, H., Chen, J. & Chen, C. 2020. Natural ingredients-derived antioxidants attenuate H_2O_2-induced oxidative stress and have chondroprotective effects on human osteoarthritic chondrocytes via Keap1/Nrf2 pathway. *Free Radical Biology and Medicine* 152: 854-864.

Yang, Z., Li, H., Yuan, Z., Fu, L., Jiang, S., Gao, C., Wang, F., Zha, K., Tian, G. & Sun, Z. 2020. Endogenous cell recruitment strategy for articular cartilage regeneration. *Acta Biomaterialia* 114: 31-52.

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