Research Article

Characterization of Human Chondrocytes from Less- vs. Severely-Affected Osteoarthritic Cartilage and Evaluation of their Ability to Develop into In Vitro 3D Models

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Abstract: Osteoarthritis (OA) is a joint disease involving cartilage degeneration. This study aimed to compare properties of chondrocytes from less-affected (LA-Cartilage) and severely-affected (SA-Cartilage) of human OA articular cartilage. Based on Dougados classification, OA cartilage was classified into two groups; less-affected (Grade 0-1) and severely-affected (Grade 2-3). Chondrocytes from each group were cultured until passage (P) 4. Growth, migration, stem cell properties and chondrogenic properties under normal and inflammatory conditions, and the formation of in vitro 3D cartilage tissues were compared between groups. The growth and migratory properties of LA-chondrocytes and SA-chondrocytes were similar, except that the migration rate of SA-chondrocytes was significantly higher at P0 compared to LA-chondrocytes. Both LA-chondrocytes and SA-chondrocytes expressed mesenchymal stem cell markers and tri-lineage differentiation, but the expression of stem cell markers decreased significantly with increasing passage number. Exposure to inflammatory conditions induced distinct morphological changes and significant increases in expression of SOX9 at P4 and MMP3 at P1 for LA-chondrocytes. LA-chondrocytes and SA-chondrocytes able to develop into in vitro 3D constructs, but SA-chondrocytes exhibited superior cartilage-like properties. Chondrocytes from both less- and severely-affected regions are suitable to be used in clinical applications, however, chondrocytes from severely-affected regions could be a more favorable cell source.

Keywords: 3D models; Cartilage; Chondrocytes; Osteoarthritis (OA)

1. Introduction

Osteoarthritis (OA) is a pathological process involving damage to the articular cartilage matrix, leading to pain and immobility. It is a leading cause of disability, especially in older patients, and adversely affects patient quality of life in terms of serious physical suffering, reduced work-related productivity, and high healthcare and non-healthcare related costs [1,2]. Due to the progression of cartilage degradation in OA patients, urgent interventions to repair and restore articular cartilage functions are needed. There are three treatment interventions for OA: non-pharmacological, pharmacological and surgical [3]. In many patients, these treatments are combined and chosen based on individual needs and risk factors. Given the increasing numbers of people with OA worldwide,

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extensive research is being conducted to study and understand the mechanism behind OA and the most effective medical approach [2].

One of the most favorable treatments for OA nowadays is joint arthroplasty, which is the replacement of the diseased joint with an implant [4]. However, increased cases of long-term prosthesis failure limit its effectiveness for individuals below 50 years old [5]. In younger people, the most suitable treatment for cartilage damage is resurfacing of the joint with a functional replacement tissue [1].

An alternative to standard treatment, cell therapy and implantation of tissue-engineered cartilage constructs have gained tremendous attention recently. Cells are the main components of cell-based cartilage repair strategy, especially for OA. Several cell types have been tested for the treatment of OA, including autologous chondrocytes [6] and undifferentiated or differentiated mesenchymal stem cells (MSCs) [7,8]. Autologous chondrocyte implantation (ACI) has been tested in the clinical setting worldwide and is considered the most advanced articular cartilage repair technique. Chondrocytes for ACI are harvested from the non-load bearing area of the knee, which creates a new lesion in the knee [9]. Thus, chondrocytes harvested at the periphery of the OA load bearing area, which is severely-affected, are worth investigating to study their potential as a cell source for ACI.

The key quality that determines whether a cell is suitable for use in OA treatment is based on their ability to produce and extracellular matrix (ECM) that is high in collagen type 2, i.e. hyaline cartilage, and low in collagen type 1 to avoid the production of fibrocartilage. Moreover, the adaptation of chondrocytes injected into an inflamed knee joint and their ability to regenerate cartilage tissue also play vital roles in the success of ACI. In this study, chondrocytes collected from two OA cartilage regions based on the severity of OA, i.e., the lateral femoral condyle and medial femoral condyle of the varus knee, designated as less-affected chondrocytes (LA-chondrocytes) and severely-affected chondrocytes (SA-chondrocytes), respectively. LA-chondrocytes and SA-chondrocytes were cultured and studied regarding their similarities and differences in terms of proliferation, migration, stem cell properties, gene expression, protein production and their properties in the presence of an inflammatory environment. These chondrocytes were also studied under 3D conditions to evaluate their ability to produce cartilage tissue.

2. Results

2.1 Histological analysis of cartilage tissue

Intact articular cartilage was graded according to the Dougados classification scoring system and classified into two groups, i.e. less-affected (Grade 0-1) and severely-affected (Grade 2-3) cartilage (Figure 1A). Histological analysis was performed to verify the grading accuracy of intact articular cartilage. As shown in Figure 1B, in less-affected cartilage tissue, the thickness of the superficial zone was maintained or decreased minimally, and the surface integrity was occasionally broken as softening and swelling occurred. Severely-affected cartilage showed a much thinner superficial layer with small and profound branched fissures extending from the superficial zone to the middle zone. In some parts, the superficial zone of cartilage was lost, and the formation of cell clusters was observed with a reduction in ECM.

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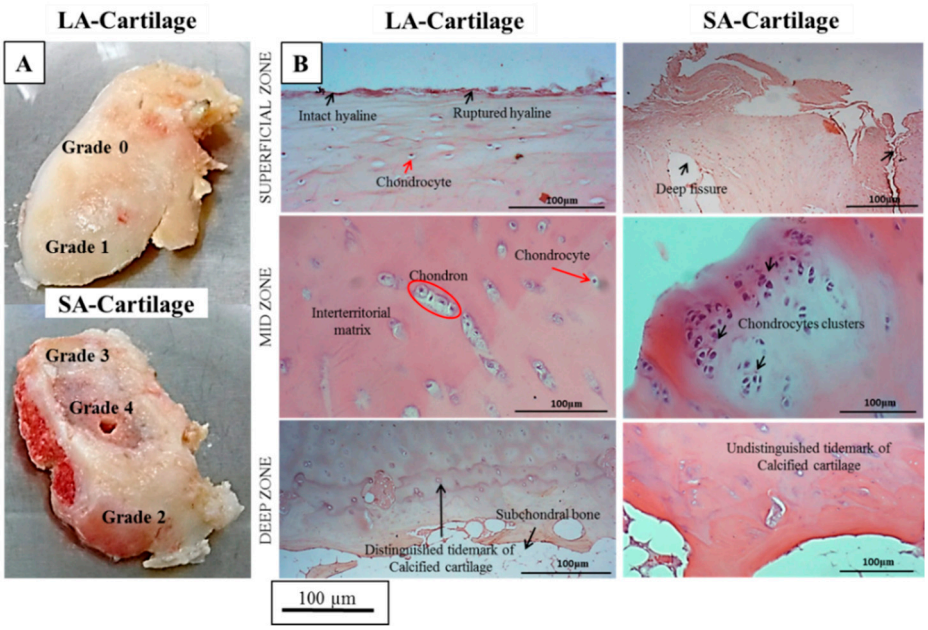

Figure 1. (A) Gross tissue grading based on the Dougados classification system. (B) H&E staining of less-affected osteoarthritic cartilage and severely-affected osteoarthritic cartilage

2.2 Proliferation and migration of chondrocytes

It was found that LA-chondrocytes proliferated slightly faster than SA-chondrocytes at all passages except for P0. However, no significant difference was found. In contrast, SA-chondrocytes migrated significantly faster at P0 (0.27±0.07 h-1) than LA-chondrocytes. At subsequent passages, the migration rate of SA-chondrocytes was higher than that of LA-chondrocytes, but this was not statistically significant (Figure 2).

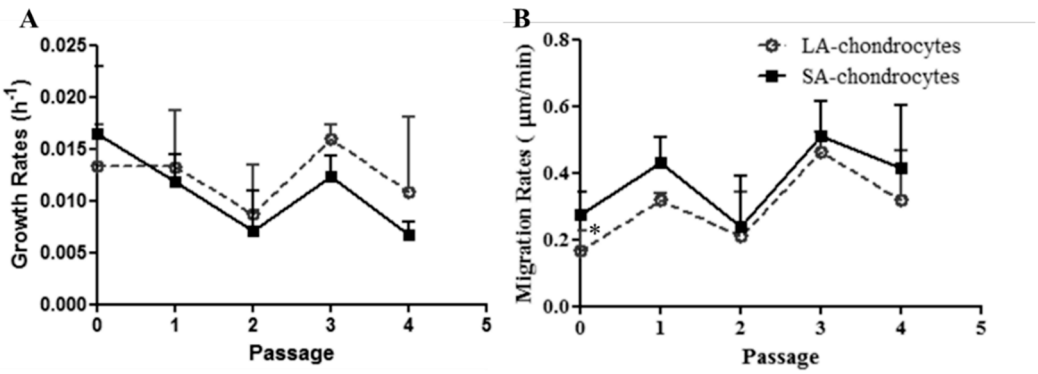

Figure 2. (A) Chondrocyte growth rate from P0 to P4. (B) Chondrocyte migration rate from P0 to P4. *indicates a significant difference ($p<0.05$).

2.3 Stem cell properties of chondrocytes

The expression of mesenchymal stem cell (MSC) markers such as CD13, CD44 and CD105 and hematopoietic stem cell (HSC) markers such as CD14, CD34, CD45 and HLA-DR was evaluated in LA-chondrocytes and SA-chondrocytes. It was found that LA-chondrocytes and SA-chondrocytes expressed all MSC markers at P1 and P4. However, both LA-chondrocytes and SA-chondrocytes showed a significantly decreased level of CD13 and CD105 at P4 than that at P1. In the case of LA-chondrocytes, the expression of CD13 decreased from 83% at P1 to 59% at P4, and the expression of

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CD105 decreased from 94% at P1 to 50% at P4. The level of expression of CD13 by SA-chondrocytes decreased from 95% at P1 to 47% at P4, and the expression of CD105 decreased from 97% at P1 to 32% at P4. In contrast, the level of CD44 expression remained unchanged. On the contrary, the expression of HSC markers was less than 10% for LA-chondrocytes and SA-chondrocytes (Figure 3).

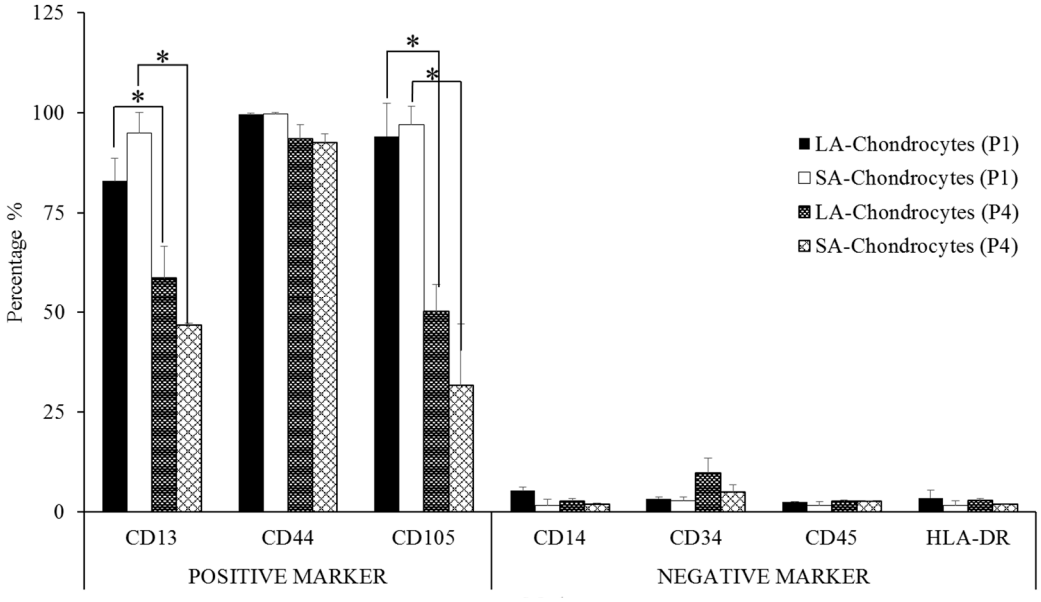

Figure 3. Bar graph showing the percentage of cells positive for MSC and HSC markers (n=3). * indicates a significant difference ($p<0.05$).

LA-chondrocytes and SA-chondrocytes were also tested for their capacity to differentiate toward the adipogenic, osteogenic and chondrogenic lineages at P1 and P4 (Figure 4). It was found that LA-chondrocytes and SA-chondrocytes demonstrated multilineage potential, positive staining for toluidine blue, indicating that the cells were of chondrogenic origin, regardless of their passage number, upon induction to a specific lineage. Uninduced chondrocytes also showed whereas no positive staining for the adipogenic and osteogenic markers was detected for the uninduced cells. However, a significant difference was observed for SA-chondrocytes, which shows the aggregated formation of the cells after exposure to chondrogenic induction medium compared with the control that only been cultured with F12:DMEM medium containing 10% FBS.

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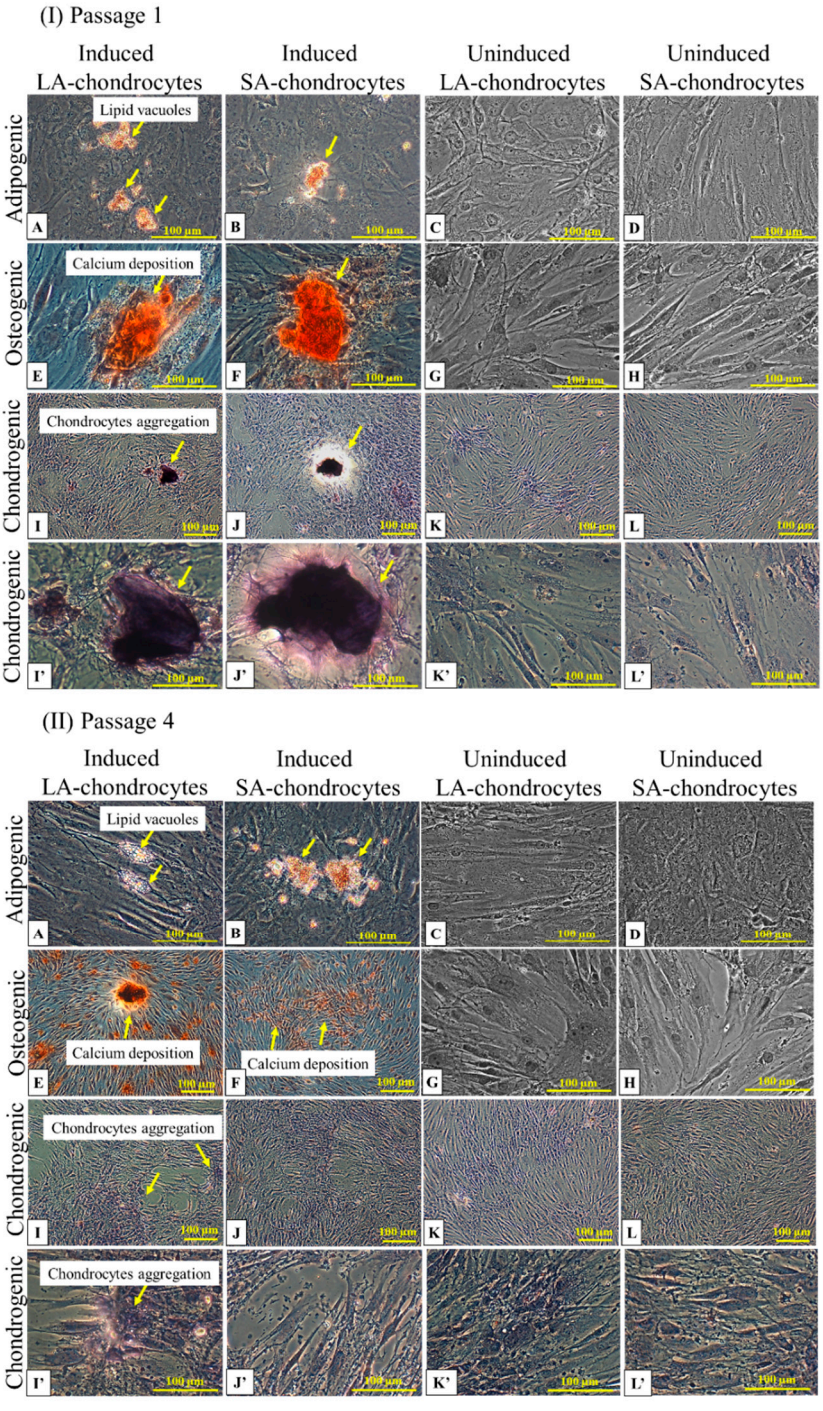

Figure 4. Oil red O, alizarin red and toluidine blue staining for the adipogenic, osteogenic and chondrogenic lineages, respectively, at P1 (I) and P4 (II) for chondrocytes from LA-chondrocytes (A, E, I) and SA-chondrocytes (B, F, J) three weeks after induction. Uninduced LA-chondrocytes and SA-chondrocytes represent chondrocytes cultured in F12 + 10% FBS without any induction; LA-C (C, G, K) and SA-chondrocytes (D, H, L). Magnified images of I, J, K, and L are labeled as I', J,' K' and L' respectively.

2.4 Effect of inflammatory cytokines on chondrocytes

LA-chondrocytes and SA-chondrocytes were exposed to an inflammatory medium that contained IL-1β (1 ng/mL) and TNF-α (10 ng/mL) in serum-free culture medium. After 3 days of exposure, both LA-chondrocytes and SA-chondrocytes showed a distressed cell morphology, a prominently pronounced nucleus and the presence of cellular protrusions as compared to non-

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exposed chondrocytes (Figure 5). LA-chondrocytes and SA-chondrocytes were stained for COL1, COL2 and SOX9, and demonstrated that both LA-chondrocytes and SA-chondrocytes produced COL1, COL2 and SOX9 both at P1 and P4 (Figure 6).

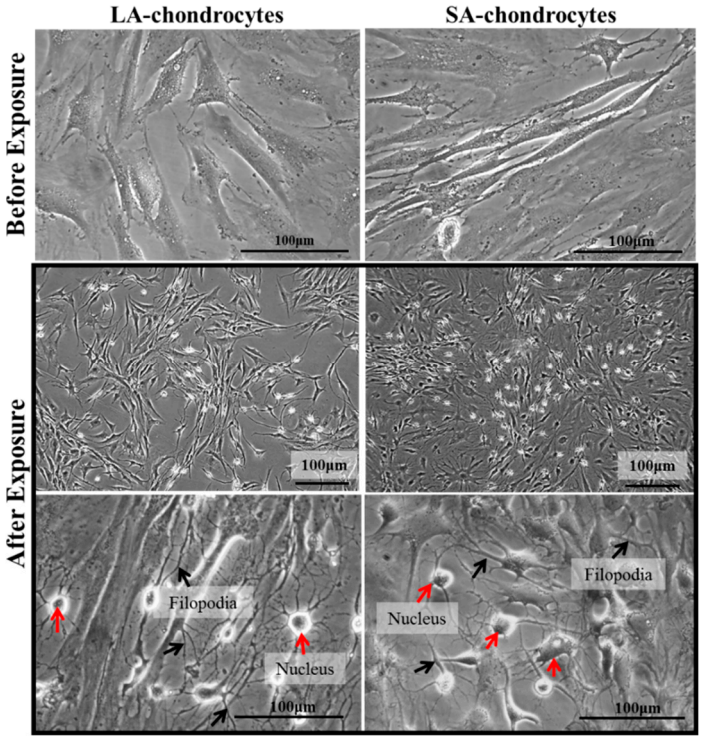

Figure 5. The morphology of chondrocytes before and after 3 days of exposure to inflammatory conditions. Prominent nuclei (red arrow) and cellular protrusions (black arrow) were observed in chondrocytes from LA-chondrocytes and SA-chondrocytes.

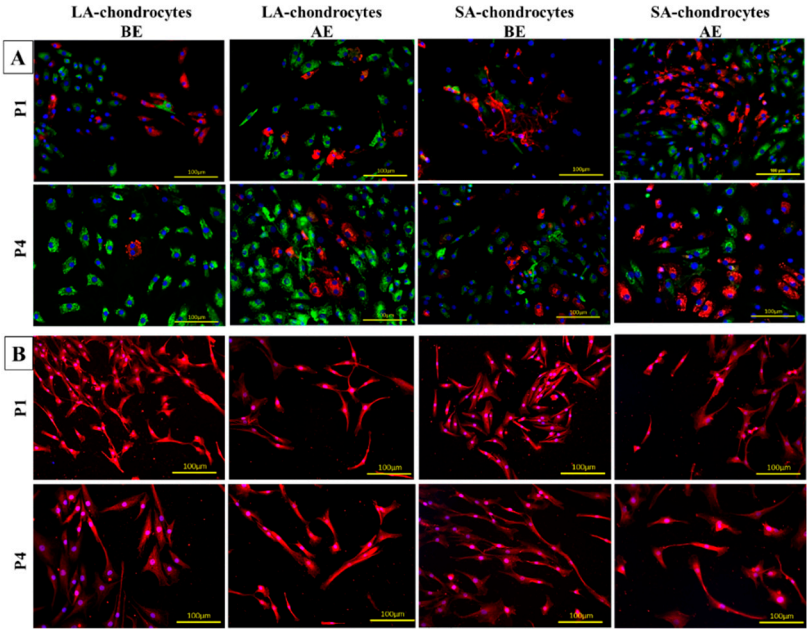

Figure 6. Immunocytochemical analysis of LA-chondrocytes and SA-chondrocytes before exposure (BE) and after exposure (AE) to inflammatory conditions at P1 and P4. (A) COL1 (green) and COL2 (red). (B) SOX9 (red). Nuclei were counterstained with DAPI (blue).

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For the quantitative evaluation, the relative expression of catabolic and anabolic genes was compared between LA-chondrocytes and SA-chondrocytes before and after exposure to inflammatory conditions at P1 and P4. LA-chondrocytes at P4 showed significantly higher SOX 9 expression after exposure to inflammatory conditions. The expression of COL2 was also increased after exposure. However, LA-chondrocytes and SA-chondrocytes at P1 and P4 showed overall increasing expression patterns of the catabolic markers IL-1, MMP13 and MMP3 after exposure to inflammatory conditions. LA-chondrocytes showed significantly higher expression of MMP3 at P1 (Figure 7).

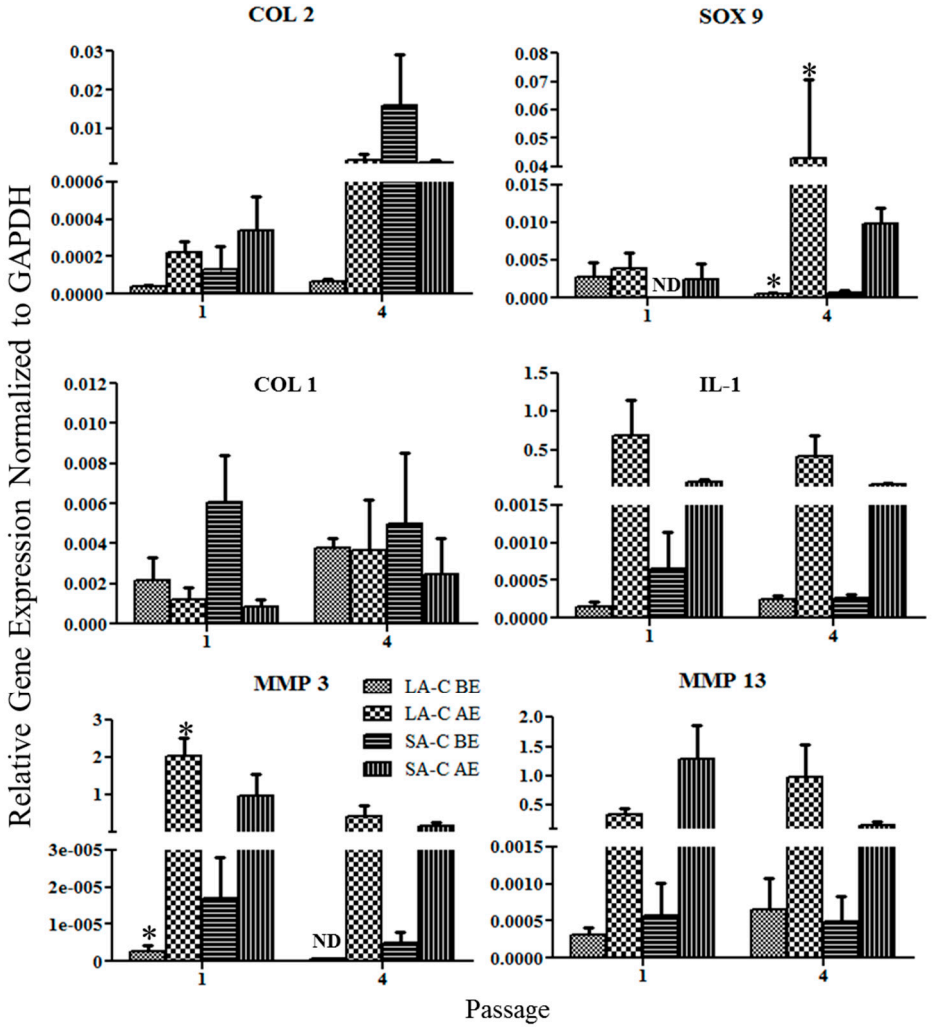

Figure 7. Quantitative expression of anabolic and catabolic genes for chondrocytes from LA-chondrocytes and SA-chondrocytes at P1 and P4. ND = Not detected. * indicates a statistically significant difference between LA-chondrocytes before exposure (BE) and after exposure (AE) at P1 and P4, respectively ($p<0.05$).

2.5 3D cartilage constructs

The morphology of 3D constructs varied depending on the culture period and type of chondrocytes. The construct with LA-chondrocytes remained attached to the mould until 28 days of culture. However, the SA-chondrocyte construct started to detach from the mould without any physical manipulation as early as day 3 of culture and freely floated inside the culture medium for 28 days. The 3D constructs were harvested at 14, 21 and 28 days. It was found that the SA-chondrocyte constructs were smaller in size compared to those composed of LA-chondrocytes, but this was not statistically significant (Figure 8A). After 21 days of culture, the LA-chondrocyte construct started to

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show a cartilage-like appearance, which was firm and maintained its shape when palpated. However, at day 28, the constructs lost their glossy cartilage-like appearance, and became very fragile. Conversely, the SA-chondrocyte constructs started to look like cartilage tissue by 14 days and maintained this structure until 28 days of culture (Figure 8B). The construct was firm when palpated and was able to resist compression.

Histological analysis of the 3D constructs demonstrated that the SA-chondrocyte constructs had greater ECM production compared to the constructs composed of LA-chondrocytes. Especially at day 21, the ECM of the SA-chondrocyte constructs was dense and stained much more intensely compared to that of the LA-chondrocytes. At day 28, the ECM was thicker and denser in the SA-chondrocyte construct, while the ECM of the LA-chondrocyte constructs was almost transparent, possibly responsible for the fragility of the constructs (Figure 8C,D).

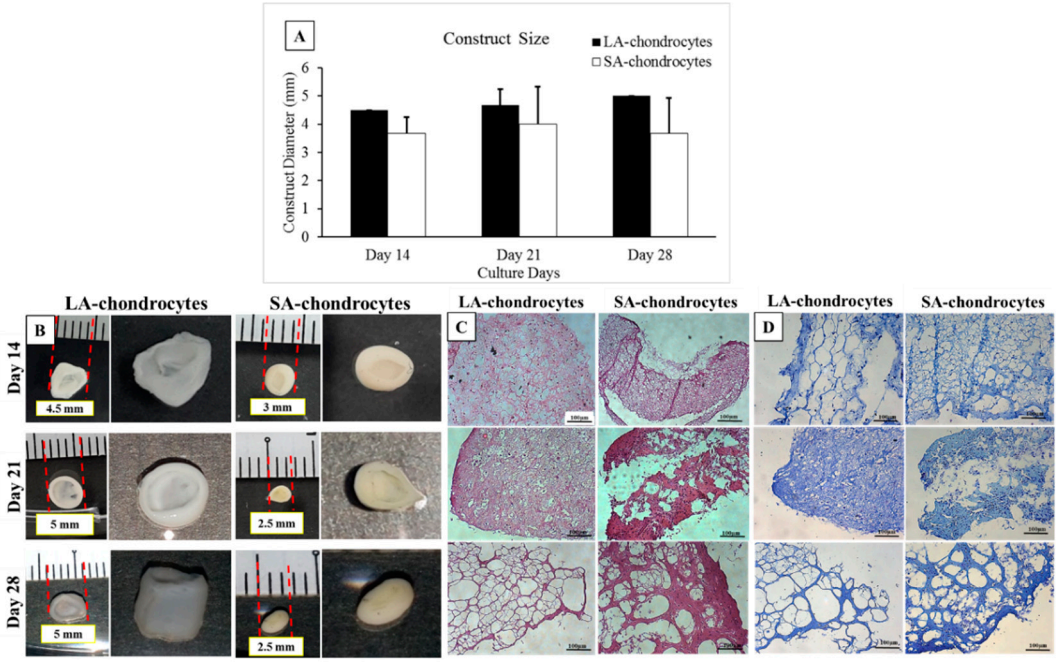

Figure 8. The diameter of less and severely-affected chondrocytes in the 3D construct (A) at days 14, 21 and 28. Representative images of the constructs (B), photomicrographs of the 3D chondrocyte cultures stained with haematoxylin and eosin (C) and toluidine blue (D). ECM produced by the construct was stained red-orange by eosin and purplish blue by toluidine blue.

3. Discussion

In a synovial joint, articular cartilage, which consists of chondrocytes, is directly affected by the progression of osteoarthritis (OA). It has long been known that articular cartilage has a poor ability to self-repair due to its avascularity [10]. The repair process is only initiated once the defect reaches the osteochondral layer, which leads to infiltration of undifferentiated MSCs from the bone marrow [11]. However, chondrocytes from articular cartilage can be isolated from its surrounding matrix via enzymatic digestion and cultured in vitro. Cultured chondrocytes have been used for clinical applications [12], but the quality of the cells needs to be evaluated. In this study, chondrocytes isolated from less and severely-affected regions of an OA knee were compared to study their potential for use as a cell source for chondrocyte implantation.

The intact cartilage tissue preserved the morphological structure of the tissue with no sign of degradation; the tissue was rich in ECM and GAG proteins. Less-affected cartilage from an OA knee showed structural alterations that included a reduction in cartilage thickness of the superficial and the middle zones. The collagen network was damaged and dominated by collagen type 1 fibers. In contrast, severely-affected cartilage showed deep surface clefts, a non-intact tidemark and fibrillation.

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These findings are in line with the observations made by Musumeci et al. [13]. Although the structural features of cartilage tissue are distinctly different in less versus severely-affected regions of the OA knee, the isolated chondrocytes demonstrated similar growth and migratory properties. In our previous study [14], we demonstrated confluent LA-chondrocytes tend to form aggregates while SA-chondrocytes grow in a monolayer.

Chondrocytes obtained from OA joints are thought to be affected by disease conditions, and not suitable for therapeutic purposes [15]. Instead, it is believed that MSCs should be employed for interventions. Macroscopically, intact OA cartilage contains migratory cells with a chondroprogenitor phenotype; these cells demonstrate a similar surface marker profile compared to bone marrow-derived MSCs [16,17]. In this study, we showed that LA-chondrocytes and SA-chondrocytes express MSC markers such as CD13, CD44 and CD105 and are negative for hematopoietic markers such as CD14, CD34, CD45 and HLA-DR.

CD13, also known as aminopeptidase N, is a metalloprotease gene that is significantly increased in OA cartilage [18]. In this study, both LA-chondrocytes and SA-chondrocytes were positive for CD13, which was approximately 80% at P1, and decreased to around 50% at P4. The percentage was significantly higher for SA-chondrocytes at P1 compared to P4. In contrast, both LA-chondrocytes and SA-chondrocytes retained their expression of CD44 from passage 1 to passage 4. CD44, also known as a homing cell adhesion molecule (HCAM), is a hyaluronan receptor that allows chondrocytes to sense and respond to changes in the ECM [19]. It has been reported that CD44 has multiple functions besides acting as an HA receptor. CD44 also serves as a regulator of cell proliferation and migration [16]. Previously, Chow et al. [20] showed that exposure to IL-1 upregulates CD44 in chondrocytes from bovine and human articular cartilage. In this study, CD105 expression was high in LA- and SA-chondrocytes cultured at passage 1 and was significantly reduced at later passages. This indicates that cells isolated from LA-chondrocytes and SA-chondrocytes demonstrated a mixed MSC and chondrocyte phenotype, but the MSC properties diminished when cells were cultured on a plastic surface, while the chondrogenic phenotype persisted.

The differentiation potential of LA-chondrocytes and SA-chondrocytes towards the adipogenic, osteogenic and chondrogenic lineages was also evaluated to confirm their stem cell properties. It was shown that, upon induction, LA-chondrocytes and SA-chondrocytes (both at P1 and P4) were able to differentiate into the adipogenic and osteogenic lineages, which was detected by the deposition of lipid droplets and calcium, respectively. However, the uninduced group (control) did not show any sign of differentiation toward the adipogenic and osteogenic lineages. In contrast, both LA-chondrocytes and SA-chondrocytes demonstrated chondrogenic properties with or without induction, indicating that the cells are of chondrocyte origin. The combined results on cell surface marker expression and multilineage differentiation shows that chondrocytes harvested from OA patients have properties similar to those of MSCs. The results of this study also demonstrate that there was no distinct difference in the stem cell properties of LA-chondrocytes and SA-chondrocytes. A previous study by Alsalameh et al. [21] also demonstrated that cartilage tissue contains an MSC subpopulation, and that this population is significantly higher in OA cartilage compared to normal cartilage. However, it contributes to a small fraction of total cells, and it is expected that most of the cells are of chondrogenic origin.

It is well-documented that OA is accompanied by the presence of inflammatory mediators in synovial fluid. Inflammatory cytokines such as IL-1β and TNF-α are known to be upregulated during OA progression [22]. IL-1β has been described to cause inflammation and promote cartilage catabolism and at the same time increase the expression of matrix-degrading enzymes in the cartilage [12,23-25]. Previous studies have demonstrated that chondrocytes exposed to inflammatory conditions in vivo behave normally when cultured in vitro in a favourable environment. However, considering the clinical applications, it is important to understand their cellular properties when they are exposed to inflammatory conditions after implantation in an OA knee. In this study, LA-chondrocytes and SA-chondrocytes were exposed to IL-1β and TNF-α, and the morphological and chondrogenic properties of LA-chondrocytes and SA-chondrocytes before and after exposure were assessed. It was found that, upon exposure to inflammatory cytokines, both LA-chondrocytes and

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SA-chondrocytes showed a distressed cell morphology, a prominently pronounced nucleus and the presence of cellular protrusions, as compared to cells before exposure. Moreover, irrespective of passage number, the expression of all tested catabolic and anabolic markers demonstrated increasing trends in both LA-chondrocytes and SA-chondrocytes after exposure to the inflammatory environment, except for COL1. Significantly higher expression of SOX9 and MMP3 was observed for LA-chondrocytes at P4 and P1, respectively. The transcription factor SOX9 has been shown to be the central factor related to COL2 expression, i.e. the major anabolic product of chondrocytes. A study by Aigner et al. [26] found that normal adult human articular chondrocytes in vivo contain high SOX9 levels, which are decreased in OA cartilage. This result shows that chondrocytes exposed to inflammatory conditions undergo dedifferentiation, expressing higher levels of anabolic markers and a reduced probability of forming fibrocartilage. A previous study by Guilak, & Weinberg [27] showed that the presence of relevant pathophysiological levels of IL-1 resulted in limited matrix accumulation in chondrocytes. However, prolonged exposure to IL-1 protects the cells from the apoptotic effects of this cytokine.

Chondrocytes need a 3D growth surface to retain their original phenotype and functions. When cultured on a plastic surface, chondrocytes tend to differentiate into the fibrocartilage lineage [28]. After several passages, the cells start to lose their phenotypic characteristics and normal functions. However, after subsequent culture in a 3D environment, culture expanded chondrocytes regain their natural phenotype and function. In this study, we tested the ability of LA-chondrocytes and SA-chondrocytes to form 3D cartilage tissue. It was found that both LA-chondrocytes and SA-chondrocytes can develop 3D constructs when cultured with fibrin. However, SA-chondrocyte constructs had a smaller diameter and had a cartilage-like appearance, which was firm and able to return to its shape when palpated. Moreover, SA-chondrocyte constructs demonstrated greater ECM production, especially at day 21, compared to LA-chondrocyte constructs.

4. Materials and Methods

4.1 Sample collection and cell culture

This study was approved by the Universiti Kebangsaan Malaysia Research and Ethics Committee (FF-2014-215). Human articular cartilage tissue was obtained from consenting OA patients undergoing total knee replacement surgery (n = 23; 43-75 years old; mean age 62.1±8.08 years). Articular cartilage was graded according to the Dougados classification for gross tissue and the Osteoarthritis Research Society International (OARSI) guidelines for stained tissue. All specimens were processed within 2 to 6 hours after surgery. Based on the Dougados classification assessment, cartilage tissues were broadly classified into two groups; less-affected (Grade 0-1) and severely-affected (Grade 2-3). Less-affected cartilage was collected from an unaffected lateral part of OA patient's varus knee, whereas severely-affected cartilage was collected from an affected medial part of the varus knee.

Cartilage was cut and separated from the subchondral bone and washed with Dulbecco's phosphate buffered saline (DPBS; Invitrogen, UK). The cartilage was diced finely using a sharp scalpel blade and digested for 4 hours at 37°C using 0.6% collagenase type II (Worthington, USA) under continuous agitation. The suspension containing chondrocytes was centrifuged, and the cell pellet was washed twice with DPBS, followed by re-suspension in Ham's F12 nutrient mixture: Dulbecco's modified Eagle's medium (F12:DMEM; Gibco, USA) with 10% foetal bovine serum (FBS; Gibco), denoted as FD. The cells were then seeded into 6-well culture plates (Greiner Bio-one) and incubated at 37°C in a 5% CO2 incubator with the culture medium being replaced every 2-3 days. Upon reaching 80%-90% confluence, the medium was discarded, and cells were detached using trypsin-EDTA (Gibco). The cell suspension was then centrifuged, and the pellet was suspended in FD. Chondrocytes were seeded in 6-well, 12-well and 24-well plates (Greiner Bio-one) for RNA extraction, proliferation and migration analysis, and immunocytochemical analysis, respectively.

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4.2 Growth rate and migration rate

For the growth and migration rate evaluation (29), cells were observed via live imaging at 20 min intervals for 72 h using a Nikon A1 microscope (Nikon, Japan). The growth rate was evaluated by counting cells from 24 h to 96 h using Image J software (National Institutes of Health, USA). The migration rate was assessed by measuring the X and Y (center) coordinate of a cell every 20 min for 1 h using NIS-element software (Nikon). The growth and migration rates were calculated using the formulas below:

$$\text{Growth rate } (h^{-1}) = \frac{\ln(\text{Cell count at 24 h} / \text{Cell count at 96 h})}{72 \text{ h}}$$

$$\text{Migration rate} = \frac{\sqrt{\frac{(x_2 - x_1)^2 + (y_2 - y_1)^2}{20 \text{ min}}} + \sqrt{\frac{(x_3 - x_2)^2 + (y_3 - y_2)^2}{20 \text{ min}}} + \sqrt{\frac{(x_4 - x_3)^2 + (y_4 - y_3)^2}{20 \text{ min}}}}{3}$$

x1, x2, x3, x4 = x coordinates at 0 min, 20 min, 40 min, and 60 min, respectively
y1, y2, y3, y4 = y coordinates at 0 min, 20 min, 40 min, and 60 min, respectively

4.3 Flow cytometry

Flow cytometry was performed to evaluate the stem cell properties of LA-chondrocytes and SA-chondrocytes. Cells were trypsinised and divided into 1×104 cells/tube. Then, the cells were incubated with primary antibodies against CD13, CD44, CD105, CD14, CD34, CD45 and HLA-DR (Invitrogen, UK), then subsequently stained with secondary antibodies (Gibco). Cell analysis was performed using a FACSCalibur apparatus (Biosciences, USA) and BD CellQuest Pro software (Biosciences).

4.4 Multilineage differentiation of LA-chondrocytes and SA-chondrocytes

The multilineage differentiation potential of LA-chondrocytes and SA-chondrocytes was performed between passage 1 and 4 by inducing the cells into the adipocyte, osteocyte and chondrocyte lineages as described elsewhere (30). Induction of LA-chondrocytes and SA-chondrocytes into the adipogenic lineage was performed in FD supplemented with 3% FBS (Gibco, Invitrogen, USA), 0.25 mmol/L 3-isobutyl-1-methylxanthine, 100 nmol/mL dexamethasone and 100 nmol/L human recombinant insulin (all reagents purchased from Sigma-Aldrich, USA). After 21 days of induction, cells were fixed with 10% formalin, and the cells were stained with 0.36% Oil Red O (Sigma-Aldrich) for 50 minutes. Osteogenic differentiation of LA-chondrocytes and SA-chondrocytes was carried out in α-MEM supplemented with 10% FBS (Gibco, Invitrogen),

0.1 μM dexamethasone, 10 μM β-glycerol phosphate and 0.2 μM ascorbic acid-2-phosphate (all reagents purchased from Sigma-Aldrich unless stated otherwise) for 21 days. Differentiation was assessed by staining with Alizarin red (Sigma-Aldrich) for 1 hour. Excess stain was washed off using DPBS. Chondrogenic differentiation of LA-chondrocytes and SA-chondrocytes was performed by culturing cells in FD supplemented with 10 ng/mL of transforming growth factor-beta-3 (TGF-β) (Invitrogen, UK). Briefly, 5×105 LA-chondrocytes and SA-chondrocytes were pelleted in a polypropylene tube and cultured in chondrogenic medium for 21 days. The resulting loose tissue was smeared onto a glass slide and dried. The slides then stained with 0.04% toluidine blue (Gainland, UK). The slides were then rinsed with tap water and dried for approximately 10 minutes at room temperature before being dipped in xylene (VWR International LTD, USA). The same procedure was used to stain induced chondrocytes cultured on a 2D plastic surface but omitting the smearing step. Each of the groups was cultured in medium without induction factors as a control. Controls and differentiated cultures were then evaluated using a bright field microscope.

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4.5 Evaluation of chondrocyte properties in an inflammatory environment

LA-chondrocytes and SA-chondrocytes were cultured until confluence, and cells were washed three times with DPBS. Then, inflammatory medium, i.e., F12-DMEM medium containing 1 ng/mL human interleukin-1 beta (IL-1 ; Abcam, USA) and 10 ng/mL human tumor necrosis factor-alpha (TNF- ; Abcam) was added to the culture. Cells were incubated for three days with the medium changed every day. Subsequently, cells were observed under an inverted microscope before lysis to collect RNA for RT-PCR analysis.

4.6 Quantitative RT-PCR analysis

Total RNA was extracted from 1.0×106 chondrocytes at passages 1 and 4. The iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad, Hercules, CA) was used to perform the RT-PCR analysis. Human GAPDH was used as the housekeeping gene. The quantitative RT-PCR protocol was performed using a Bio-Rad iCycler (Bio-Rad) set for 40 cycles for each run. The data were analysed using Bio-Rad iCycler software (Bio-Rad). For gene expression quantification, the comparative Ct method was used. The Ct values of the gene of interest were normalised to GAPDH. The Ct value was calculated when the fluorescence of the sample exceeded a threshold level. Table I shows the primer sequence used for RT-PCR.

4.7 Immunocytochemistry

LA-chondrocytes and SA-chondrocytes were also subjected to immunocytochemical analysis. The cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), followed by permeabilisation with 0.5% Triton X (Sigma-Aldrich) solution and blocking with 10% goat serum (Sigma-Aldrich). The cells were incubated together with mouse anti-collagen type 1 antibody (Origene, USA) and rabbit anti-collagen type 2 antibody (Abcam) overnight at 4°C. On the following day, the cells were incubated with Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen) and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) for 1 hour at 37°C in the dark. Then, the nuclei was counterstained with 4',6-diamidino-2-phenylindole (DAPI; Life Technologies, USA). The cells were then observed and images were captured using a Nikon Eclipse Ti fluorescence microscope (Nikon, Japan).

4.8 Preparation of 3D constructs

LA-chondrocytes and SA-chondrocytes at passage 4 were cultured separately until 90% confluent. The cells were then trypsinised and mixed with fibrin-rich plasma collected from the blood of a human donor. Fibrin-rich plasma was collected by withdrawing venous blood from a human donor, which was then centrifuged at 5000 rpm for 5 minutes at 4°C. The mixture of cells and 50 µL of fibrin was polymerised with 5 µL of CaCl2 (Invitrogen) and seeded at a density of 5x105 cells/construct in a 5 mm diameter mould, prepared using 8% agarose (Invitrogen). The 3D construct was analysed at 14 days, 21 days and 28 days for ECM production using toluidine blue and haematoxylin and eosin staining, as described earlier.

4.9 Statistical analysis

All parameters were analysed using six experimental replicates (samples) with three technical replicates for each sample, unless stated otherwise. Data are expressed as a mean ± standard error of the mean and analysed using Student's t-test. $P<0.05$ was considered significantly different.

5. Conclusions

In this study, it was shown that chondrocytes harvested from cartilage tissue with variable osteoarthritic severity have similar properties regarding their proliferation and migration rate, expression of stem cell markers and multilineage potential. However, upon exposure to

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inflammatory conditions, LA-chondrocytes demonstrated significant upregulation of SOX9 and MMP3 at P4 and P1, respectively, whereas no significant differences in catabolic and anabolic markerd were observed for SA-chondrocytes after exposure to inflammatory cytokines, indicating the SA-chondrocytes are more resistant to inflammatory conditions. In addition, SA-chondrocytes were found to produce a mature cartilage-like 3D construct and expressed more matrix proteins compared to LA-chondrocyte constructs. Thus, considering the clinical perspective, chondrocytes from severely-affected regions could be a viable cell source for the treatment of OA. These results may also help developing an in vitro cartilage model, which is vital to research and the pharmaceutical industry due to the limited availability of OA animal models.

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Abbreviations

2D	Two Dimensional
3D	Three Dimensional
ACI	Autologous Chondrocytes Implantation
AE	After Exposure
BE	Before Exposure
$CaCl_2$	Calcium Chloride
CD	Cluster of Differentiation
CO_2	Carbon dioxide
COL1	Collagen type 1
COL2	Collagen type 2
DAPI	4′, 6-diamidino-2-phenylindole
DPBS	Dulbecco's Phosphate Buffered Saline
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
et al.	(et alia); and others
FBS	Fetal Bovine Serum
FD	Dulbecco's Modified Eagle Medium: Nutrient Mixture
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H&E	Hematoxylin & Eosin
HCAM	Homing Cell Adhesion Molecule
HLA-DR	Human Leukocyte Antigen-antigen D Related
HSC	Hematopoietic Stem Cell
IgG	Immunoglobulin G
IL-1	Interleukin-1

IL-1β	Interleukin-1 Beta
LA-Cartilage	Less Affected Cartilage
LA-Chondrocytes	Less Affected Chondrocytes
MMP	Matrix metalloproteinases
MSC	Mesenchymal Stem Cell
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
P	Passage
P0	Passage 0
P1	Passage 1
P4	Passage 4
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SA-Cartilage	Severely Affected Cartilage
SA-Chondrocytes	Severely Affected Chondrocytes
TGF-β	Transforming Growth Factor Beta
TNF-α	Tumor Necrosis Factor Alpha
UK	United Kingdom
UKM	Universiti Kebangsaan Malaysia
UKMMC	Universiti Kebangsaan Malaysia Medical Centre
USA	United States of America
α-MEM	Minimum Essential Medium Eagle-alpha modification

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