



Evaluating the Cartilage-Specific Genes Expression in Co-culture of Chondrocytes With 2 Different Mesenchymal Stem Cells: Adipose and Wharton's Jelly Derived

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Abstract

Objectives: Articular cartilage has a limited potential for self-repairing due to the scarce number of chondrocytes and avascularity. As regards the low proliferative potential of chondrocyte under in vitro conditions, nowadays mesenchymal stem cells are introduced for osteoarthritis (OA) treatment. Accordingly, the present study aimed to evaluate whether co-culture of chondrocytes with 2 different sources of mesenchymal stem cells, namely, adipose and Wharton's jelly derived can promote the expression of cartilage specific genes by chondrocytes.

Materials and Methods: Mesenchymal stem cells were isolated from both adipose tissue and Wharton's jelly. The obtained cells were co-cultured with chondrocytes or cultivated alone as control for 3 days. Then, cartilage specific genes including collagen II, Sox9, cartilage oligomeric matrix protein (COMP), and aggrecan were evaluated using the real time Reverse transcription polymerase chain reaction (RT-PCR).

Results: The expression of genes in chondrocytes alone was significantly higher compared to the co-culture of chondrocytes with both stem cell types. In addition, the results revealed that co-culture of Wharton jelly derived stem cells (WJSCs) with chondrocyte led to higher expression of genes compared to WJSCs alone. However, the co-culture of the adipose-derived stem cells (ASCs) demonstrated no significant change.

Conclusions: Generally, no up-regulation of cartilage specific genes was observed by co-cultivation of chondrocytes with ASCs or WJSCs compared to chondrocytes. Further, stem cells derived from Wharton's jelly expressed higher levels of the above mentioned genes compared to those of the ASCs.

Keywords: Adipose tissue, Cartilage, Stem cells, Osteoarthritis, Wharton's jelly.

Introduction

Hyaline cartilage is a precisely arranged type of connective tissue found in different parts of the body such as nose, xiphoid process, synovial joints, and the like (1). Articular cartilage, which covers the end of long bones in all the synovial joint, functions as a shock-absorber lubricating the joint surfaces and protecting the subchondral bone (2). In addition, this cartilage has limited intrinsic potency for self-repairing due to the lack of vessels, nerve supply, and lymph, and its untreated damages can gradually lead to osteoarthritis (OA) (3). Further, poor ability of articular chondrocytes for migrating to the lesion site and compensating the lost extracellular matrix (ECM) components is regarded as another obstacle in cartilage healing (4). The OA which affects more than 250 million people worldwide, is a "wear and tear" kind of disease that occurs due to an imbalance between the catabolic and

anabolic function in cartilage tissue (5). Conventional efforts for OA include surgical repairs such as mosaicplasty, microdrilling, and microfracture techniques that normally lead to the formation of fibrocartilage tissue with lower biomechanical properties (6).

During the past few years, regenerative therapies represented promising prospects for reconstructing the cartilage lesions (2). Cartilage tissue engineering is based on using the cells, scaffolds, and stimulating factors. Autologous chondrocytes obtained from a less loaded site were first introduced in 1987 (7,8). Despite the encouraging clinical outcomes, using chondrocyte has some drawbacks including donor site morbidity which limits intrinsic proliferative capacity and cellular dedifferentiation during monolayer expansion that results in losing the chondrocyte characteristic morphology and secreting fibrous tissue components by these 2 surgery steps (8-10).

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Therefore, recent studies shifted to use mesenchymal stem cells (MSCs) with superior differentiation ability and self-renewal characteristic (10,11).

These multipotent cells are identified by their ability to differentiate into adipocytes, osteoblasts, and chondrocytes (12). The MSCs are found in numerous tissues such as bone marrow, adipose tissue, umbilical cord blood, and Wharton's jelly (13). Adipose derived stem cells, among others, have gained attention due to their abundance through the body, high proliferative potency, and minimal morbidity (4). Based on the reports, ASCs have greater ability for performing cartilage function compared to the chondrocytes (14,15).

Despite the advantages of adipose-derived stem cells (ASCs) as a source for cartilage regeneration strategies, chondrogenic potential of these cells is less than bone marrow or synovial derived stem cells (16).

Furthermore, MSCs are present in placenta and fetal membranes such as Wharton's jelly (17). The fetal membrane is considered a promising source of cells known as Wharton jelly derived stem cells (WJ-SCs) because of its immunomodulatory properties (18), high proliferative role, and relatively similar composition of its ECM to hyaline cartilage (19).

It has been reported that WJSCs can secrete enriched glycosaminoglycan and collagen type II and form a cartilage-like tissue in pellet culture conditions. These investigations provide a rationale for attempting to use WJ-SCs in OA and rheumatoid arthritis (RA).

In spite of above-mentioned advantages of MSCs application, these cells can produce cartilage-like tissue only after considerable chondrogenic induction which can farther lead to hypertrophy and formation of calcified tissue (19).

Therefore, co-culture of articular chondrocytes and MSCs has recently been proposed in order to overcome this obstacle (20). Considering the controversial information on using different stem cell sources in cartilage tissue engineering and based on the described advantages of the co-culture system, this study sought to compare the combination of articular chondrocytes with ASCs or WJ-SCs in order to understand which one has the superior potency to optimize the chondrogenesis.

Materials and Methods

Chondrocyte Isolation and Expansion

Cartilage tissue samples were obtained from patients who underwent arthroplasty surgery for femoral neck fracture. Written consent was obtained from all the patients.

The cartilage samples were transferred to the cell culture lab under aseptic conditions, washed 3X with phosphate-buffered saline (PBS) containing 1% penicillin/streptomycin (P/S), and finally sliced into 1×1 mm pieces using sterile scalpel. Briefly, chondrocyte was isolated by incubation with pronase (1%) for 60 minutes in a shaking water bath followed by incubation with collagenase (0.2%)

for 4-6 hours. After digesting the main parts, enzyme activity was stopped by adding Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and then the isolated cells were passed through Nylon mesh (70 μ M size) in order to remove the undigested pieces. The obtained chondrocytes were centrifuged for 10 minutes at 1500 rpm and then counted. They were plated at a density of 5×10^5 /T75 flasks and incubated at 37°C/5% CO₂ until reaching 70% confluency of about 70% chondrocytes at second passage and using for the purpose of this study.

Isolation of Wharton's Jelly Derived Stem Cells

The umbilical cord samples were obtained from women undergoing the caesarean section (CS) surgery. The samples were transported to the lab while stored in the sterile PBS. After 3X washing, umbilical cord pieces were put in 70% ethanol. Then, a longitudinal incision was created using the sterile scissor and the vessels were removed carefully. Subsequently, the exposed Wharton's jelly tissue was divided into small pieces (1×2 mm) and placed in T25 culture flasks. Samples were incubated with DMEM culture medium containing 30% FBS in 37°C/5% CO₂ and checked every 3 days to observe the migrated cells. After reaching 70% confluency, the cells were trypsinized and expanded into T75 flasks. The WJSCs at fourth passage were used to perform this study.

Isolation and Expansion of Adipose-Derived Stem Cells

After the patients underwent laparotomy, the adipose tissue samples were collected in sterile PBS and then mechanically digested into small fragments using a sterile scalpel. Enzymatical digestion was conducted by incubating the adipose tissue samples in collagenase type I (0.2% in free DMEM) per gram while shaking in a water bath for 60 minutes. Then, the cells were centrifuged at 1500 rpm for 10 minutes. The obtained cells were counted employing trypan blue exclusion dye test and plated at a density of 3×10^5 / T75 flasks in 37°C/5% CO₂ until reaching confluency. The ASCs at fourth passage were used for evaluation.

Experimental Design

A co-culture system was designed to evaluate whether ASCs or WJSCs can promote chondrocyte ECM component synthesis. To this end, chondrocytes and WJSCs or chondrocyte and ASCs at a proportion of 50/50 were seeded in T75 culture flasks for 72 hours. Finally, the samples were obtained and prepared for further evaluation of gene expression using the real-time reverse transcriptase polymerase chain reaction (RT-PCR) technique.

Real-Time Reverse Transcriptase Polymerase Chain Reaction

The total cellular RNA was extracted to examine the cartilage-specific gene expression profile applying the

RNX-PLUS reagent kit. Based on the manufacturer's instruction, chloroform was added to the samples while they were placed on ice. After centrifuging at 12 000 rpm for 15 minutes, the isopropanol was added and centrifuged at the same rate for 5 minutes. Then, the supernatant was discarded and 75% ethanol was added to the samples. After centrifuging at 7500 rpm for 7 minutes, the pellet was dissolved in diethyl pyrocarbonate (DEPC) treated water. The total RNA was used to synthesize the cDNA using a reverse transcriptase kit. The RT-PCRs were performed employing the real-time PCR detection system with SYBR Green Master Mix under the condition of 2 seconds at 98°C and 5 seconds at the annealing temperature of 60°C. The primer sequences are listed in Table 1. Additionally, the levels of gene expression were determined by the $2^{-\Delta\Delta Ct}$ method and β -actin was used as the internal control. All experiments were performed in triplicate.

Statistical Analysis

It involved using the GraphPad data which were demonstrated as mean \pm standard deviation (SD). The statistical difference between different groups was evaluated by one-way ANOVA followed by *t* test. A $P < 0.05$ was considered statistically significant.

Results

The Effects of ASCs and Chondrocytes Co-culture on the Expression of Cartilage Specific Genes

The co-culturing of the cells (chondrocytes and ASCs) was performed in order to understand whether direct contact of ASCs with chondrocytes can enhance the expression of cartilage specific genes by these cells. As is displayed in Figure 1A, the expression of collagen type II, aggrecan, cartilage oligomeric matrix protein (COMP), and Sox-9 decreased significantly in co-cultivated cells when compared to that of the chondrocytes alone. Figure 1B illustrates the comparison of gene expression between the co-cultured cells and ASCs alone. Based on the findings, no significant difference was observed in expression of collagen II, Sox-9, and COMP between the 2 groups.

Table 1. Primer Sequences Used in Real Time RT-PCR and the Related Annealing Temperature

Gene	Primer	Annealing Temperature
Sox9-F	AGAGAGGACCAACCAGAATTC	57°C for 30 s
Sox9-R	TGGGTAATGCGCTTGGATAG	57°C for 30 s
Coll2-F	GGCAATAGCAGGTTCCAGTACA	59°C for 30 s
Coll2-R	CGATAACAGTCTTGCCCCACTT	59°C for 30 s
Comp-F	TGCAATGACACCATCCAG	56°C for 30 s
Comp-R	ACACACACTTTATTTGTCTCTC	56°C for 30 s
ACAN-F	CAACTACCCGGCCATCC	56°C for 30 s
ACAN-R	GATGGCTCTGTAATGGAACAC	56°C for 30 s
B actin-F	TCCTCCCTG GAG AAG AGC TA	58°C for 45 s
B actin-R	TCA GGAGGA GCA ATG ATC TTG	58°C for 45 s

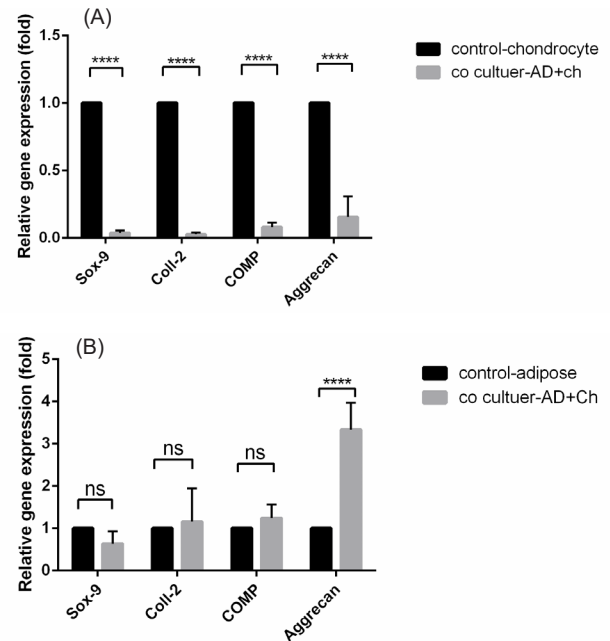


Figure 1. Comparison of cartilage specific gene expression between (A) Co-culture of chondrocytes/ASCs and chondrocytes alone, (B) Co-culture of chondrocytes/ASCs, (C) and ASCs alone. **** $P < 0.00001$; ns: Non-significant.

However, the expression of the aggrecan significantly increased in ASCs cultivated with chondrocytes compared to the ASCs alone ($P < 0.00001$).

The Effects of Chondrocytes and WJSCs Co-culture on Expression of Cartilage Specific Genes

To evaluate the probable role of WJSCs and chondrocyte co-cultivation on the gene expression profile, these 2 cell types were cultivated together and the results of their gene expression were compared to the cultured chondrocyte or WJSCs alone. As Figure 2A demonstrates, the expression of all genes including collagen II, Sox-9, aggrecan, and COMP were decreased significantly compared to the chondrocytes alone. However, the expression of Sox-9, aggrecan, and COMP significantly increased by the co-cultivated cells compared to the WJSCs alone (Figure 2B).

Discussion

Based on this experimental study, these findings were obtained. The expression of cartilage specific genes by chondrocytes alone was significantly higher when compared to the chondrocytes co-cultured with ASCs. In addition, co-culturing of chondrocytes with WJSCs cannot affect the expression of collagen II, Sox-9, aggrecan, and COMP genes compared to the chondrocytes alone.

OA affects different tissues in the synovial joints, but articular cartilage is the major target tissue in this disease (21). The only cell population in cartilage tissue is chondrocytes. Due to avascularity of this tissue, chondrocytes receive nutrients and low oxygen level through diffusion from synovial fluid and capillaries

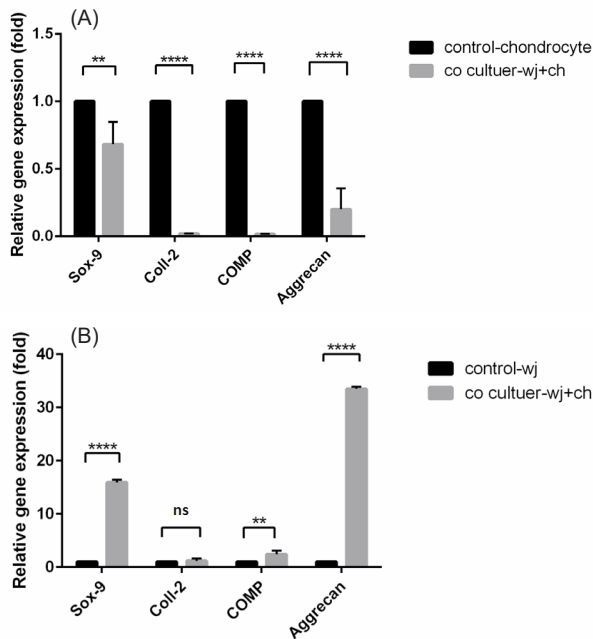


Figure 2. The Analysis of Cartilage Specific Gene Expression Between (A) Chondrocytes alone and chondrocytes co-cultivated with WJSCs, (B) WJSCs alone, and (C) Co-culture of chondrocyte and WJSCs. ** $P < 0.001$, **** $P < 0.00001$.

which are located in subchondral bone (22), therefore the regenerative capacity of cartilage tissue is limited. Further, chondrocytes which are responsible for maintaining the normal balance in cartilage ECM synthesis and degradation, have low proliferation rate both in cartilage matrix and under in vitro conditions (20). Recently, strategies for using the stem cells are introduced to promote the healing of damaged cartilage (23). MSCs are one of the encouraging cell sources for cartilage tissue engineering. These cells can be harvested from different tissues such as bone marrow, adipose tissue, umbilical cord, and the like (19). The ASCs, among these sources, acquired attention due to abundance, quickly expansion under in vitro systems, and differentiation capacity toward chondrocytes (4). These features can conquer the drawbacks of using chondrocytes in cartilage tissue engineering. Furthermore, application of stem cells alone has some difficulties such as using the inductive factors for differentiating these cells into chondrocyte which occasionally results in forming the calcified tissue rather than cartilage (24,25). Accordingly, mixing the stem cells and mature chondrocytes in a co-culture system is considered a promising approach in this field. The probable mechanism through which these cultivated cells can affect each other is possibly and partially related to the paracrine factors and signals produced by the cells (23).

Several studies were conducted regarding the co-culture systems and controversial results were obtained in this regard. For example, Tsuchiya reported that mixed pellet culture of human MSCs and bovine articular chondrocytes led to the upregulation of chondrocyte

proliferation and production of cartilaginous ECM (20). Moreover, Tsuchiya et al revealed that co-culture of ASCs with chondrocytes increased the level of cartilage ECM genes. However, at the level of proteins no significant changes were found. Additionally, Tsuchiya et al reported no chondrogenic effects of chondrocytes on MSCs in a co-culture pellet system, which is in line with the results of the current study (20-26).

Due to these contradictory results and the fact that previous studies have not yet revealed whether or not ASCs and chondrocytes co-culture can yield better outcomes, in the present study 2 different co-cultures including chondrocytes/ASCs and chondrocytes/WJSCs were compared. The results demonstrated that mixing the ASCs and chondrocytes in a co-culture system had no role in upregulating the cartilage specific genes compared to the chondrocytes alone. In addition, expression of these genes in chondrocytes alone was significantly higher compared to the co-culture of WJSCs and chondrocytes. However, the expression level of these genes in WJSCs/chondrocyte was significantly higher compared to that of ASCs and chondrocytes co-culture.

As previously explained, another source for obtaining stem cells is the human umbilical cord Wharton's jelly with superior properties compared to ASCs in terms of proliferation rate, stemness characteristics, and tolerance in allograft transplantation (27). These cells can be differentiated into chondrocytes when receiving chondrogenic inducers (28). In a previous study performed by our team, it was revealed that treating chondrocytes with conditioned medium of Wharton's jelly enhanced the expression of cartilage specific genes (23). Since controversial results were obtained with respect to the co-culturing of stem cells and chondrocyte for augmenting the chondrocyte proliferation and differentiating the stem cells into chondrocytes, the present study attempted to compare 2 co-culture conditions, namely, chondrocyte/ASCs and chondrocyte/WJSCs. The obtained results indicated that none of these co-culture systems led to the upregulation of cartilage specific genes compared to the chondrocytes alone. Further, it was found that the expression level of COMP, aggrecan, and Sox-9 genes in co-culture of WJSCs with chondrocytes significantly increased compared to the culture of WJSCs alone. However, in ASC and chondrocyte co-culture, only aggrecan gene expression increased significantly compared to the ASCs alone. As previously described, the MSCs derived from Wharton's jelly can indigenously express some cartilage specific genes such as collagen type II, Sox-9, hyaluronic acid, and aggrecan (22) In other words, higher expression level of cartilage specific genes can be interpreted by WJSCs compared to the ASCs which was found in this study. Furthermore, based on the results, the expression level of Sox-9 was up-regulated in the co-culture of WJSCs and chondrocytes compared to the WJSCs alone. The Sox-9 is

considered the major transcription factor during the early steps of chondrogenesis and its depletion causes severe skeletal malformation. Moreover, the presence of this gene is necessary for aggregation of MSCs before cartilage formation (29,30). Additionally, synthesizing ECM components of cartilage such as collagen II and Aggrecan is regulated by this transcription factor (23). Aggrecan is another essential molecule in cartilage ECM, which is important for maintaining the hydrating structure of the cartilage and gives the load-bearing properties to this tissue (31). Aggrecan and the other key component of the cartilage matrix, namely, collagen type II, form the major part of ECM (32). The COMP is a non-collagenous protein in cartilage which interacts with other components like collagens and aggrecan and keeps the integration of ECM molecules. In sum, the results revealed that the expression of the above-mentioned genes had no effect on increasing the chondrocytes co-cultured with ASCs or with WJSCs compared to the chondrocytes alone. Conversely, the expression of these key genes increased in WJSC combined with chondrocytes compared to the WJSCs alone.

Conclusions

Generally speaking, the results of the present study indicated that stem cells derived from Wharton's jelly have more potential for expressing the cartilage specific genes when co-cultured with chondrocytes compared to the ASCs. In addition, based on the findings, none of the ASCs or WJSCs can affect the expression of these genes by chondrocytes in co-culture systems compared to chondrocyte alone.

Conflict of Interests

Authors have no conflict of interests.

Ethical Issues

The study protocol was approved by the Medical Ethics Committee of Tabriz University of Medical Sciences under the ethical code of 5/104/512.

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