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Combination of chondrocytes and chondrons improves extracellular matrix production to promote the repairs of defective knee cartilage in rabbits



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ABSTRACT

Background: Chondrons are composed of chondrocytes and the surrounding pericellular matrix (PCM) and function to enhance chondrocyte-mediated cartilage tissue engineering. This study aimed at investigating the potential effect of combined chondrocytes with chondrons on the production of proteoglycan and collagen-II (Col-2) and the repair of defective knee cartilage in rabbits.

Methods: Chondrocytes and chondrons were isolated from the knee cartilage of rabbits, and cultured alone or co-cultured for varying periods in vitro. Their morphology was characterized by histology. The levels of aggrecan (AGG), Col-2 and glycosaminoglycan (GAG) expression were quantified by qRT-PCR, Alcian blue-based precipitation and ELISA. The effect of combined chondrocytes with chondrons in alginate spheres on the repair of defective knee cartilage was examined in rabbits.

Results: The isolated chondrocytes and chondrons displayed unique morphology and began to proliferate on day 3 and 6 post culture, respectively, accompanied by completely degenerated PCM on day 6 post culture. Evidently, chondrocytes had stronger proliferation capacity than chondrons. Longitudinal analyses indicated that culture of chondrons, but not chondrocytes, increased AGG mRNA transcripts and GAG levels with time and Col-2 mRNA transcripts only on day 3 post culture. Compared with chondrocytes or chondrons alone, co-culture of chondrocytes and chondrons significantly up-regulated AGG and Col-2 expression and GAG production, particularly at a ratio of 1:1. Implantation with chondrocytes and chondrons at 1:1 significantly promoted the repair of defective knee cartilage in rabbits, accompanied by reduced the Wakiteni scores with time.

Conclusion: Combined chondrons with chondrocytes promoted the production of extracellular matrix and the repair of defective knee cartilage in rabbits.

The translational potential of this article: This study explores that the combination of chondrons and chondrocytes may be new therapeutic strategy for cartilage tissue engineering and repair of defective cartilage.

1. Introduction

Articular cartilage is a unique connective tissue and functions to help the joint movement by transmitting the movement-related loads. Because it is prone to injury and difficult to repair, many peoples suffer from articular cartilage damage. Even in young and middle-aged patients, the trauma-related articular cartilage is usually difficult to be treated and can seriously affect their life quality. The advance in tissue engineering technology has led new therapeutic strategies to promote cartilage repair and regeneration. However, it is well known that chondrocytes are prone to dedifferentiation during the process of cartilage repair in vitro [1,2].

The dedifferentiated chondrocytes usually lose some properties, leading to a poor cartilage repair. This, together with degeneration of the repaired tissues, becomes a major problem to restrict the clinical application of tissue engineering [3,4]. In addition, which types of cells for implantation and how to protect from their degeneration have not been clarified.

Chondrocytes are commonly used as the seed cells for cartilage engineering and they usually need to be expanded in vitro before implantation due to the limited resource of cartilage tissues. However, chondrocytes cultured in vitro can lose their differentiated phenotypes and cellular mechanical properties with a reduced anabolic capacity [5].

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Chondrocytes are the unique type of cells in articular cartilage tissues and surrounded by the pericellular matrix (PCM). The PCM is specific extracellular matrix (ECM) and composed of type VI and IX collagens, perlecan, hyaluronan, aggrecan monomers, biglycan, and other small aggregates and other components [6]. These molecules, together with the enclosed chondrocytes, form the “chondron” [7]. Because the PCM is a circular matrix surrounding chondrocytes, it is theoretically an important “medium” for the functions of chondrocytes [8]. Previous works have shown that the PCM in the cell–matrix interface acts as a sensor of regulatory signals from the environment [9,10] and is crucial for the spatial organization and functions of superficial chondrocytes [11,12]. As a result, the PCM naturally forms a protective layer for the enclosed chondrocytes from physical and osmotic damages to modulate their biosynthetic responses [13,14]. Moreover, its interactions with Col-6 in the PCM surrounding the cells can support the survival of chondrocytes [15]. However, the roles of articular chondrons, especially for the PCM, in the functions of rabbit chondrocytes are not fully understood [16].

Our previous study and those of others have shown that chondrons with the intact PCM can be obtained from rabbit articular cartilage by sequentially enzymatic digestions using dispase and type II collagenase [17,18]. With a modified *in vitro* chondron microtubule suction mechanical analysis model, we found that freshly digested chondrons had high biomechanical properties, which were declined with aging [19], indicating that chondrons had better mechanical advantages in tissue engineering cartilage and repair [20]. It is notable that growth factors, such as osteogenic protein-1 (OP-1) [21], tumor growth factor beta 1 (TGF- β 1) [22], insulin-like growth factor-1 (IGF-1) [21] and others, and continual mechanical stimulation in the chondrocyte culture environment can promote the formation of the PCM [23]. The Col-6 component in the PCM generated by hydrogel-embedded chondrocytes in culture has potential implications for the success of tissue engineering [24]. Accordingly, we hypothesize that chondrons, including the wrapped chondrocytes as a whole, can have better biological properties to promote the repair of articular cartilage defects.

This study aimed to explore the effects of chondrocyte/chondron co-culture on the morphology, proliferation and matrix synthesis in chondrocytes *in vitro* and on the repair of articular cartilage defects *in vivo*.

2. Methods

2.1. Animals

New Zealand White rabbits (two-month-old, $n = 18$) were obtained from Shanxi Medical University Experimental Animal Center and housed in a specific pathogen-free room with normal rabbit chow and water. This study was approved by the Institutional Animal Care and Use Committee of Shanxi Medical University.

2.2. Isolation of chondrocytes and chondrons

Rabbit chondrocytes and chondrons were isolated, as a previous report [20]. In brief, the rabbits were anesthetized with xylazine (2 mg/kg body weight) and euthanized by decapitation. Their full-thickness articular cartilages were dissected from their femoral condyles and tibial plateau of the knee joints. The chondrocytes were isolated from cartilage tissues by sequential enzymatic digestions with 14 U/mL pronase (Sigma, St. Louis, Missouri, USA) in Dulbecco's Modified Eagle Medium-F12 (DMEM-F12, HyClone, Beijing, China) at 37 °C for 90 min and then with 31.25 U/mL collagenase-2 (Sigma) in DMEM-F12 at 37 °C overnight (about 10–12 h). Chondrons were isolated by enzymatic digestions using 30 U/mL dispase (Sigma) and 250 U/mL collagenase-2 (Sigma) in DMEM-F12 at 37 °C with shaking for 3 h. The digested tissue products were filtered through an 100- μ m nylon cell strainer (BD, Franklin Lakes, New Jersey, USA) and centrifuged. The isolated chondrocytes and chondrons were collected, respectively.

2.3. Culture of chondrocytes with chondrons and histology

The isolated chondrocytes and chondrons (2×10^5 cells/well) were cultured in 6-well plates in DMEM/F12 medium with 10% fetal bovine serum (FBS, HyClone) at 37 °C and 5% CO₂ for 1, 3, 6, and 9 days, respectively. The cultured monolayer chondrocytes and chondrons on glass slides were fixed in Acetone for 10 min, stained with hematoxylin (3 min) and eosin (3 min). The morphological characteristics of chondrocytes and chondrons were observed by phase-contrast microscopy. In addition, chondrocytes and chondrons (2×10^5 cells/well) were cultured alone or mixed at a ratio of 2:1, 1:1 and 1:2, respectively for 6 days.

2.4. Glycosaminoglycan (GAG)

The levels of GAG released from the cultured chondrocytes and/or chondrons ($n = 6$ per group) were determined by Alcian blue-based precipitation [25]. Briefly, the proteins in cell culture supernatants and control media (100 μ l each, in triplicate) were denatured with guanidine-HCl, sulfuric acid, Triton X-100 and the contained GAG was precipitated by Alcian blue solution (74,240, Chroma-Gesellschaft, K \ddot{o} ngen, Germany) at 4 °C for 1 h. The precipitants in individual tubes were high-centrifuged. After being washed, the pellets were solved with guanidine-HCl/propanol solution, which were measured for the absorbance at 600 nm in a microplate reader. The levels of GAG in individual samples were determined by a standard curve established using the different concentrations of GAG.

2.5. Enzyme-linked immunosorbent assays (ELISA)

The levels of Col-2 in the supernatants of cultured cells were quantified by ELISA using the Rabbit-collagen type II ELISA Kit (E10H2107, R&D System, USA), according to the manufacturer's protocol. Briefly, the supernatants of different groups ($n = 6$ per group) of cultured cells were tested simultaneously in duplicate. The levels of Col-2 in individual samples were quantified using a standard curve established with the different concentrations of Col-2 provided [26,27].

2.6. MTT assay

The viability and proliferation of chondrocytes and chondrons were examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [28]. Briefly, chondrocytes and chondrons (5×10^3 cells/well) were cultured in triplicate in 96-well plates in DMEM/F12 medium for 1, 3, 6, and 9 days. During the last 4-h culture, individual wells were added with MTT solution (M1020, Beijing Solarbio Science & Technology, China) and the generated formazan was dissolved in DMSO, followed by measuring their absorbance at 490 nm.

2.7. Quantitative real-time PCR (qRT-PCR)

The relative levels of aggrecan (AGG) and Col-2 to the control GAPDH mRNA transcripts were quantified by qRT-PCR [29]. Briefly, we extracted total RNAs from the different groups of cells using Trizol reagent (15, 596–026, Invitrogen) and reversely transcribed them into cDNA using the iScript™ cDNA Synthesis Kit (K1642, Fermentas, MD, USA). Subsequently, we quantified the relative levels of AGG and Col-2 mRNA transcripts using the QuantiTect SYBR Green PCR Kit (K0251, Fermentas) and specific primers. The primer sequences were AGG: Forward 5'-TCTACCGCTGTGAGGTGATGC-3' Reverse 5'-TTCACCACGAC-CTCCAAGG-3', Col-2: Forward 5'-ACACTGCCAACGTCCAGATG-3' Reverse 5'-GTGATGTTCTGGGAGCCCTC-3'; GAPDH: Forward 5'-GGTGAAGGTCGGAGTGAACG-3', Reverse 5'-AGTAAAAGCAG-CCCTGGTGA-3'. Data were analyzed by $2^{-\Delta\Delta Ct}$.

2.8. Cell encapsulation in alginate spheres

The cell encapsulation in alginate was prepared as described previously [30]. Briefly, chondrocytes, chondrons and chondrocytes/chondrons (1:1) were suspended in 1.2% alginate in 0.15 M NaCl at 5.8×10^6 cells/ml and dropped into a 102 mM CaCl₂ solution to form different spheres that were extensively washed.

2.9. A rabbit model of osteochondral defects

A rabbit model of osteochondral defects was generated, as described previously [31]. Briefly, New Zealand white rabbits (four-month-old, n = 30) were anesthetized with xylazine (2 mg/kg body weight) and maintained with 10% chloral hydrate (1.0 ml/kg). The animals were subjected to a medial parapatellar incision in the right knee joint, and their cartilages were drilled to form a hole (4.0 mm in diameter and 3 mm in depth) in the trochlear center of femurs. The hole in individual rabbits was filled with, or without, different groups of alginate spheres (n = 10 per group), followed by suturing its skin. The animals were injected intramuscularly with Penicillin daily for three consecutive days. At 6 and 12 weeks post operation, five rabbits from each group were euthanized and their femoral trochlear grooves were dissected for subsequent experiments.

2.10. Histologic evaluation

The femoral trochlear groove tissues were routinely fixed, decalcified for 4 weeks, and paraffin-embedded. The sagittal sections (5 µm) were routinely stained with haematoxylin and eosin or Safranin O. In addition, the sections were subjected to immunohistochemistry using anti-Col-2 (1:200). The tissue repairs in the defect areas of each rabbit were evaluated for cell morphology, matrix staining, surface regularity, cartilage thickness, and the donor integration in the recipients using the modified Wakitani grading system [32,33] with a score range of 0–14. The lower the score, the better the repair effect (Table 1). The scoring was performed in a blinded manner by two observers, and there was no significant interobserver difference.

2.11. Statistical analysis

Data are present as mean ± standard deviation (SD). The difference between the groups was analyzed by analysis of variance (ANOVA) using SPSS 13.0 software (SPSS, USA). Statistical significance level (α) was set at 0.05.

3. Results

3.1. Characterization of rabbit chondrocytes and chondrons

To understand the role of chondrons in the function of chondrocytes, rabbit chondrocytes and chondrons were isolated and cultured for varying periods. Following culture for one day, both chondrocytes and chondrons were small and rounded. Due to the PCM surrounding chondrocytes, the chondrons was still rounded at 3 days post culture, whereas the chondrocytes displayed like fibroblasts with sharp spindles on day 3 post culture. When the PCM is completely degraded, the chondrons also displayed like fibroblasts with sharp spindles on day 6 post culture. The number of adherent cells in the cultured chondrons decreased significantly, compared to chondrocyte on day 3 and 6 post culture (Fig. 1A).

MTT assays indicated that the chondrocytes began their proliferation on day 3 post culture and gradually increased with the prolonged culture time periods while the chondrons appeared to increase their OD values on day 6 post culture, which were further greater at a later time (Fig. 1B). As a result, the proliferation of chondrocytes was significantly stronger than that of chondrons on day 3, 6 or 9 post culture. Furthermore, qRT-PCR revealed that while there was a similar level of AGG mRNA

Table 1

The modified Wakitani histological scoring system for evaluation of cartilage repair.

Category	Points
Cell morphology	
Hyaline cartilage	0
Mostly hyaline cartilage	1
Mostly fibrocartilage	2
Mostly non-cartilage	3
Non-cartilage only	4
Matrix-staining intensity ^a	
Normal (compared with host adjacent cartilage)	0
Slightly reduces	1
Markedly reduced	2
No metachromatic stain	3
Surface regularity ^b	
Smooth (>3/4)	0
Moderate (1/2 to 3/4)	1
Irregular (1/4 to 1/2)	2
Severely irregular (<1/4)	3
Thickness of cartilage ^c	
>2/3	0
1/3 to 2/3	1
<1/3	2
Integration of donor with host adjacent cartilage	
Both edges integrated	0
One edge integrated	1
Neither edge integrated	2
Total maximum	14

^a Metachromasia matrixes including proteoglycan staining intensity by Safranin O and Col-2 immunohistochemistry staining compared with host adjacent cartilage

^b Total smooth area of the reparative cartilage compared with the entire area of the cartilage defect

^c Average thickness of the reparative cartilage compared with that of the surrounding cartilage.

transcripts in the cultured chondrocytes significantly higher levels of AGG mRNA transcripts were detected in the cultured chondrons on day 6 and 9 post culture (Fig. 2A). In contrast, we detected very low levels of Col-2 mRNA transcripts in the different groups of cells, except for a dramatically higher level of Col-2 mRNA transcripts in the chondrons on day 3 post culture (Fig. 2B). Moreover, Alcian blue-based precipitation detected gradually increased levels of GAG in the supernatants of cultured chondrocytes and chondrons beginning on day 3 post culture (Fig. 2C). ELISA detected similarly low levels of Col-2 in the supernatants of cultured chondrocytes and chondrons (Fig. 2D). Together, such data indicated both rabbit chondrocytes and chondrons exhibited their biological characteristics in vitro.

3.2. Co-culture of chondrocytes with chondrons enhances the extracellular matrix production in vitro

It is well known that chondrons can support the function of chondrocytes [34]. To determine the role of rabbit chondrons, we co-cultured chondrocytes with chondrons for 6 days. We found that while culture of chondrocytes or chondrons alone only promoted low levels of AGG mRNA transcription co-culture of chondrocytes with chondrons at a ratio of 2:1 or 1:1 significantly increased the relative levels of AGG mRNA transcripts (Fig. 3A). The highest levels of AGG mRNA transcripts were detected in the co-cultured cells at 1:1. A similar pattern of Col-2 mRNA transcripts was observed among these groups of cells (Fig. 3B). In comparison with that in the chondrocytes or chondrons alone, Alcian blue-based precipitation detected significantly higher levels of GAG in the supernatants of co-cultured chondrocytes and chondrons, particularly for those with a ratio of 1:1 (Fig. 3C). However, ELISA revealed that there was no significant difference in the levels of Col-2 in the supernatants of cultured cells, regardless of their culture alone or co-culture (Fig. 3D).

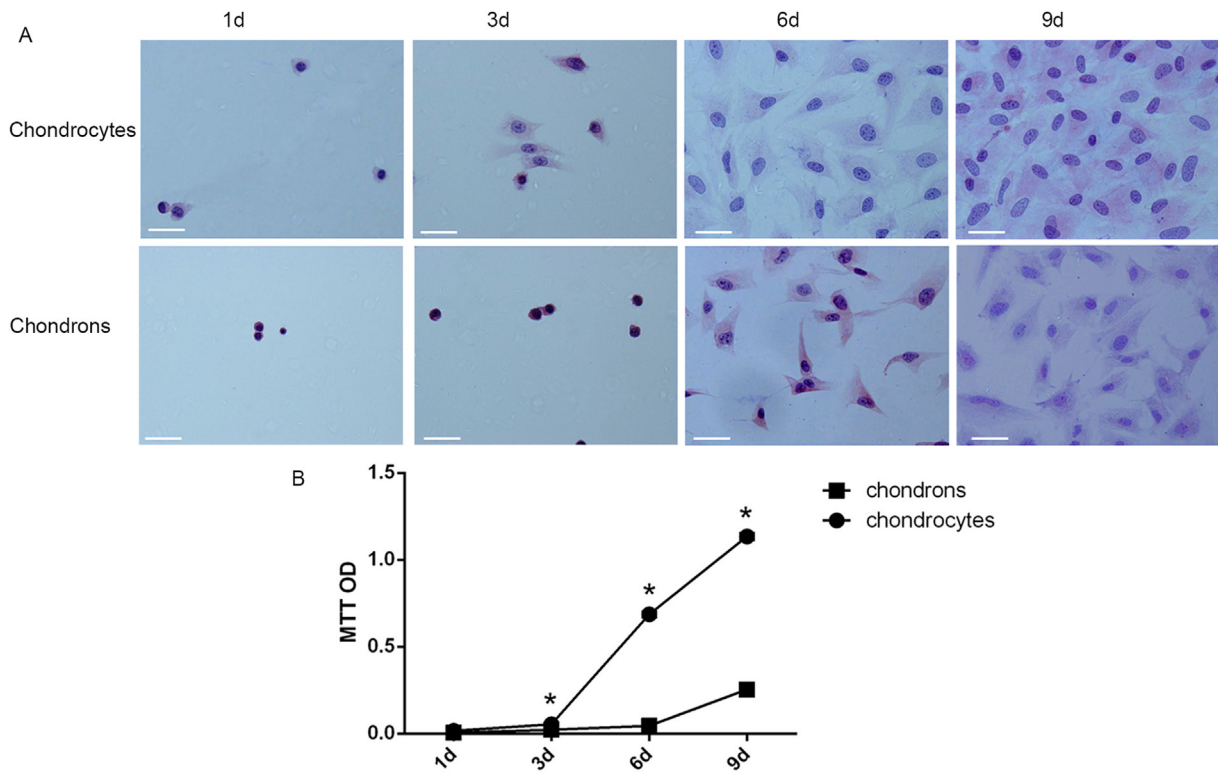


Figure 1. Morphological characterization of rabbit chondrocytes and chondrons. The isolated chondrocytes and chondrons were cultured for the indicated time periods and stained by H&E (A). The upper rows: Chondrocytes; The lower rows: Chondrons. Scale bar = 5µm (B) MTT analysis of the proliferation of chondrocytes and chondrons at days 1, 3, 6 and 9 post culture (n = 30). *P < 0.05 vs. the chondron group.

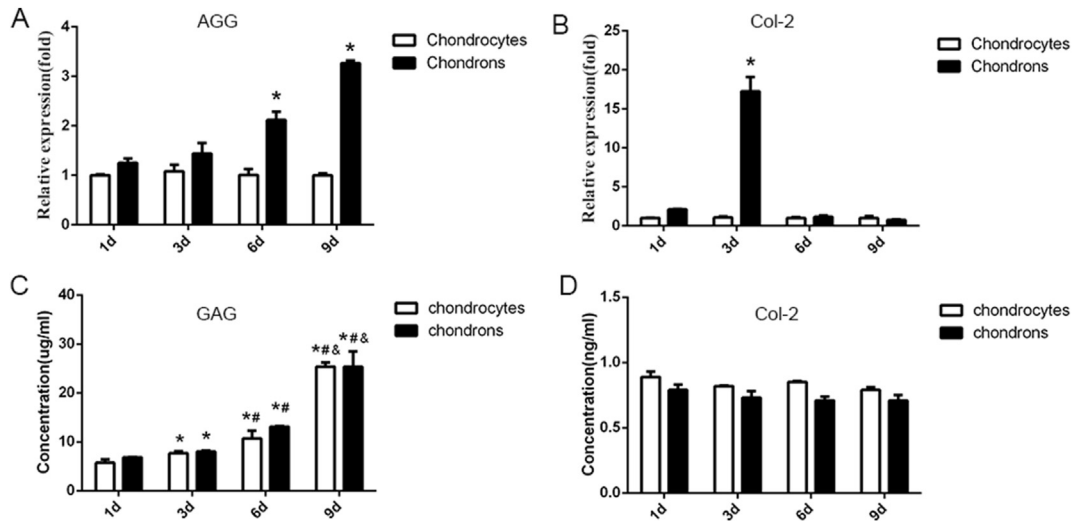


Figure 2. Analysis of mRNA transcripts, GAG and Col-2 production in rabbit chondrocytes and chondrons.

3.3. Implantation of both chondrocytes and chondrons significantly accelerates the repairs of cartilage defects in rabbits

To explore the role of chondrons and chondrocytes in the repairs of cartilage defects, we induced knee cartilage defects in rabbits and implanted with chondrocytes/alginate, chondrons/alginate or chondrocytes/chondrons/alginate spheres, respectively (Fig. 4A). Six weeks later, we examined the repairs of defective knee cartilage in individual rabbits by histology and immunohistochemistry. As shown in Fig. 4B, the defective areas in the chondrocytes/chondrons group were filled with cartilage matrix whereas those in the chondrocyte or chondron group

were filled with a little cartilage matrix formation, rather with fibrous tissues. At 12 weeks post implantation, the cartilage matrix regenerated to cover all defective areas in the chondrocytes/chondrons group. In contrast, there was a little cartilage regeneration in the defective areas of the chondrocyte or chondrons group, accompanied by high levels of Col-2 expression, particularly in the chondron group (Fig. 4C). Quantitative analysis of cartilage repairs by the Wakitani score system revealed that in comparison with the chondrocyte group at 6 weeks post implantation, significantly reduced Wakitani scores were detected in the chondron group and the scores further significantly decreased in the chondrocyte/chondron group (Fig. 4D). Furthermore, the Wakitani scores were also

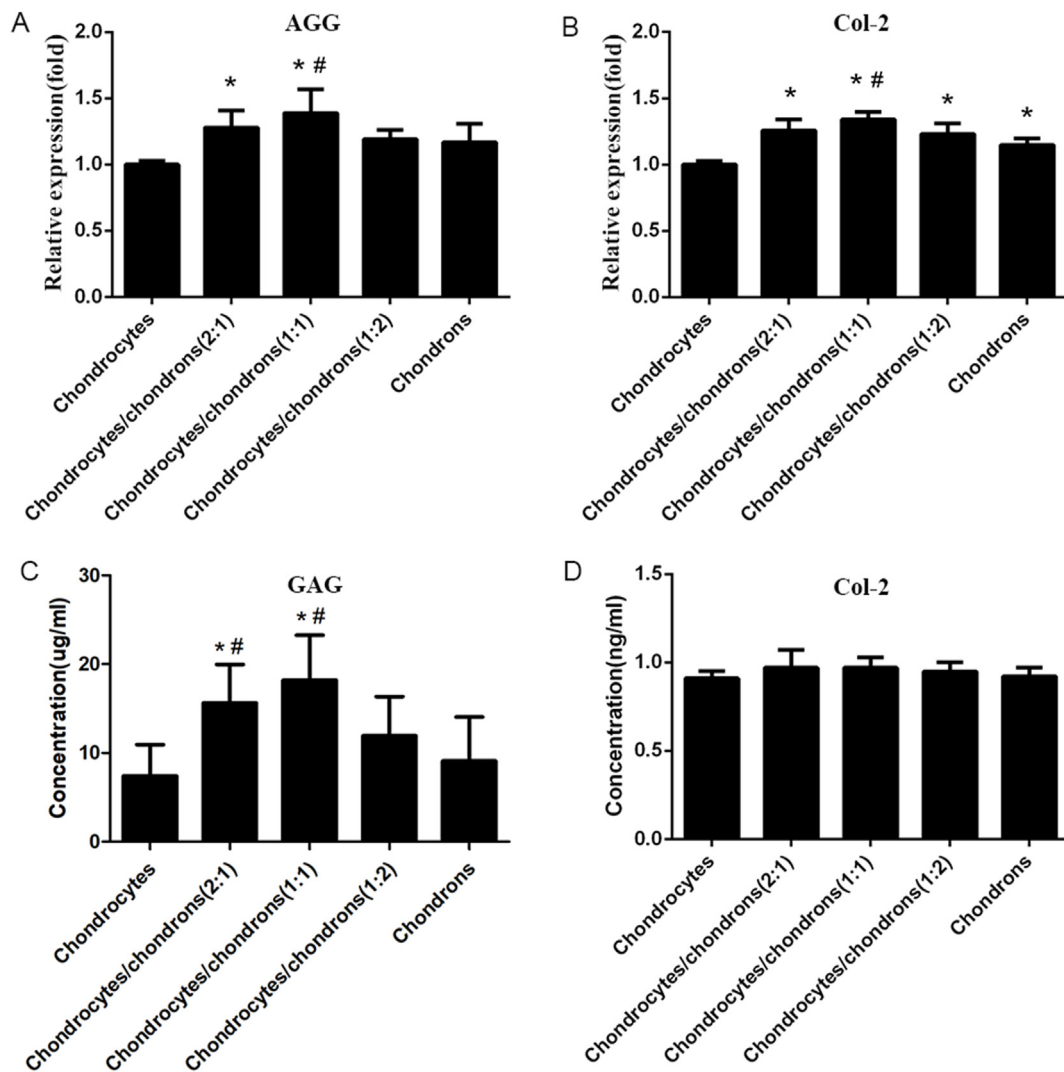


Figure 3. Analysis of mRNA transcripts, GAG and Col-2 production following co-culture of rabbit chondrocytes and chondrons in vitro.

significantly reduced in the chondrocyte group at 12 weeks post implantation, a hallmark of continual cartilage self-repair in the defective region of rabbits. Similarly, the Wakitani scores further significantly decreased in the chondron or chondrocytes/chondrons group, particularly in the combination group at 12 weeks post implantation. Collectively, such data indicated that implantation of both chondrocytes and chondrons accelerated the knee cartilage repair in rabbits.

4. Discussion

The cultured chondrocytes in vitro usually undergo degeneration by changing its Col-2, Col-11 and proteoglycan into Col-1, Col-3 and Col-5 expression, gradually becomes fibroblasts [35]. Chondrons are mainly composed of chondrocytes and surrounding PCM, and function to support the chondrocyte-related articular cartilage regeneration [36–39]. In this study, we isolated chondrocytes and chondrons from rabbit knee cartilage by sequential enzymatic digestions and we found that after culture, both chondrocytes and chondrons displayed their unique morphology and biological characteristics, consistent with our previous report [20].

The PCM is primarily composed of Col-6 surrounding chondrocytes and crucial for cartilage tissue engineering [40,41]. We found that the isolated chondrons displayed a round shape up to 3 days post culture, suggesting that the PCM surrounded the chondrocytes. In contrast, the

isolated chondrocytes were present as fibroblast-like morphology on day 3 post culture with obvious proliferation. The PCM in the cultured chondrons degraded on day 6 post culture, which led to the proliferation of the enclosed chondrocytes. Such data suggest that the chondron may be used as seeding cells, together with chondrocytes, for cartilage engineering.

The PCM in the chondrons provides a microenvironment for gene expression and metabolism in chondrocytes [42–44]. Actually, Vonk et al. [45] found that chondrons expressed higher levels of Col-2, but lower Col-1 than chondrocytes, which may stem from the inhibition of the PCM on lipid peroxidation on the cell membrane surface to reduce active oxidation, leading to increased Col-2 and Col-6 expression and metabolism in chondrocytes and attenuating their hypertrophy and dedifferentiation [46,47]. Although the numbers of chondrons were less than that of chondrocytes, we observed that the relative levels of AGG expression in the cultured chondrons were significantly higher than that in the chondrocytes. Furthermore, we detected similar levels of GAG and Col-2 in the supernatants of cultured chondrons and chondrocytes. Hence, chondrons may promote the gene expression of chondrocytes and delay their degeneration, benefitting matrix synthesis. When the composition of the PCM changes, the chondrons will degrade, causing the dedifferentiation of chondrocytes [48,49]. Thus, optimal culture of chondrocytes and chondrons to expand them may be valuable for cartilage engineering in vivo.

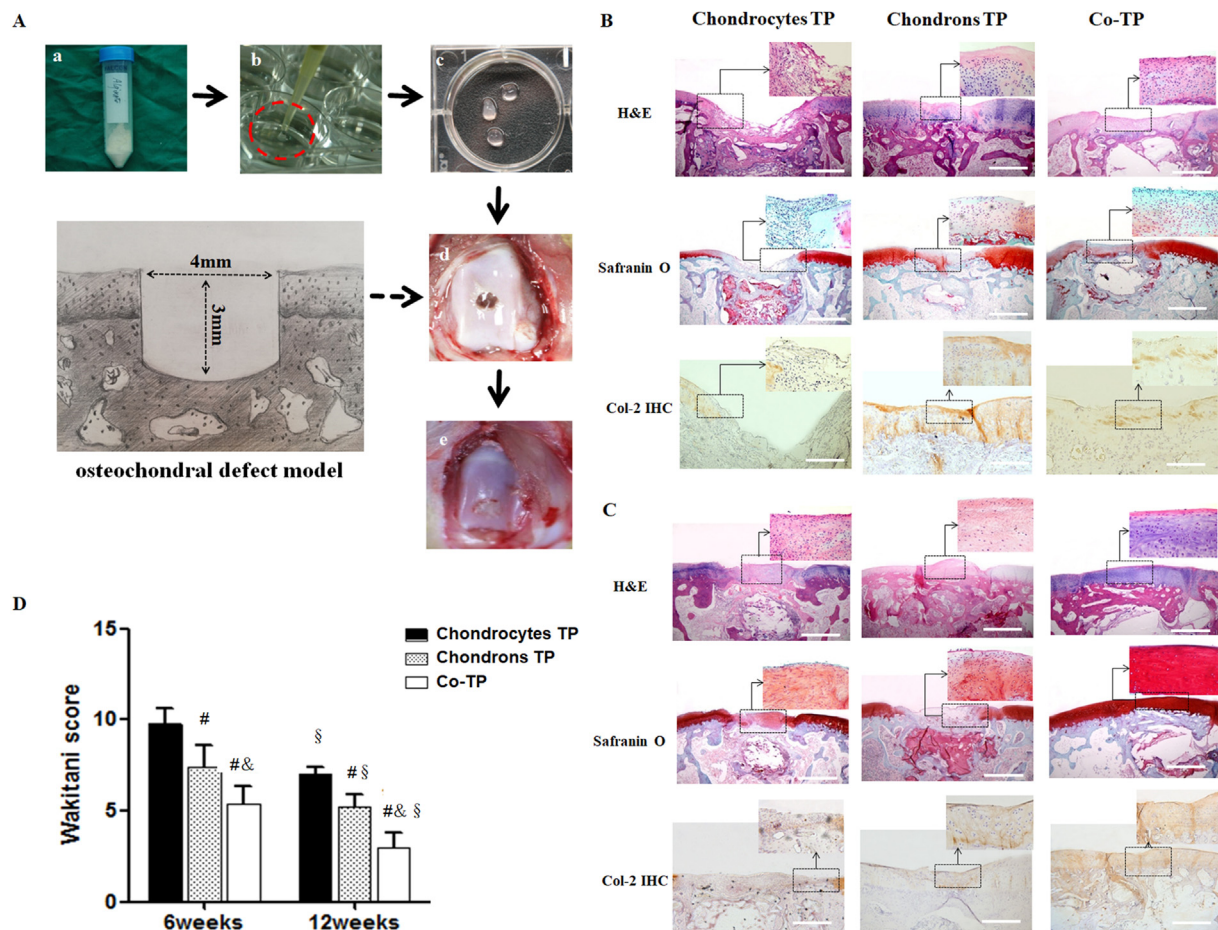


Figure 4. Histological and immunohistochemistry analysis of the repairs of defective knee cartilage in rabbits.

Recent works have shown that co-culture of human chondrons with MSCs produces more cartilage ECM than that in the co-cultured chondrocytes with MSCs [50,51]. In this study, we found that co-culture of chondrons with chondrocytes, particularly at a ratio of 1:1, significantly elevated the levels of AGG and Col-2 expression and GAG production in vitro, suggesting that co-culture of them at a good ratio may promote chondrogenesis. These results extended a previous observation that co-culture of MSCs with chondrons increases the deposition of ECM [41, 52].

Chondrocytes embedded in alginate exhibited a rounded morphology and a PCM feature [34]. This suggests that chondrocyte/alginate spheres even with genetically modified chondrocytes can be implanted into the defective regions of osteochondral articular cartilage to promote the repair of defective cartilage in vivo [30]. Recent studies have shown that co-administration of chondrons and adipose-derived stem cells or MSCs increases articular hyaline cartilage formation [53,54]. In this study, we found that implantation with chondrons/chondrocytes alginate spheres significantly accelerated the repairs of defective knee cartilage in rabbits by increasing the cartilage matrix and thicknesses, and reducing the Wakitani scores in a trend of time-dependence. Such data indicated that implantation of both chondrocytes and chondrons may be a new strategy for intervention of defective knee cartilage. We are interested in further investigating the molecular mechanisms underlying the action of this therapy in accelerating the repairs of defective knee cartilage.

5. Conclusions

This study indicated that rabbit chondrons expressed higher levels of proteoglycan and Col-2 than chondrocytes. Co-culture of chondrons with

chondrocytes, particularly at a ratio of 1:1, significantly elevated AGG, Col-2 and GAG production in vitro and implantation of chondrons/chondrocytes alginate spheres significantly accelerated the repairs of defective knee cartilage in rabbits by increasing the cartilage matrix and thicknesses, and reducing the Wakitani scores. Therefore, combination of chondrons and chondrocytes may be a new therapeutic strategy for cartilage tissue engineering and repair of defective cartilage.

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Declaration of competing interest

The authors have no conflicts of interest to disclose in relation of this article.

Following culture for varying periods, the relative levels of AGG (A, n = 4) and Col-2 (B, n = 4) mRNA transcripts were quantified by qRT-PCR and the levels of GAG (C, n = 6) and Col-2 (D, n = 6) in the supernatants of cultured chondrocytes or chondrons were measured by precipitation with Alcian Blue and ELISA respectively. Data are expressed as the means \pm SD of each group of cells from three separate experiments. *P < 0.05 vs the chondrocytes; #P < 0.05 vs. the chondrocytes on day 1 post culture; &P < 0.05 vs. the chondrocytes on day 3 post culture; §P < 0.05

vs. the chondrocytes on day 6 post culture.

Rabbit chondrocytes and chondrons were cultured alone or co-cultured at a ratio of 2:1, 1:1 or 1:2 for 6 days. The relative levels of AGG (A, n = 4) and Col-2 (B, n = 4) mRNA transcripts were quantified by qRT-PCR and the levels of GAG (C, n = 6) and Col-2 (D, n = 6) in the supernatants of cultured chondrocytes or chondrons were measured by precipitation with Alcian Blue and ELISA respectively. Data are expressed as the means \pm SD of each group of cells from three separate experiments. *P < 0.05, #P < 0.05 vs. the chondrocytes or chondrons alone, respectively.

A, Cell encapsulation in alginate spheres and implanted into the osteochondral defective knee in rabbits. B, HE, safranin O, and Col-2 immunohistochemical staining at 6 weeks post implantation. C, HE, safranin O, and Col-2 immunohistochemical staining at 12 weeks post implantation. Scale bar = 2 mm. D, Modified Wakitani scores among 3 groups at 6 and 12 weeks post implantation. #P < 0.05 vs. the chondrocyte group; & P < 0.05 vs. the chondron group; §P < 0.05 vs. the same group at 6 weeks post implantation. A total of five defect specimens in each group at each time point, and 3 sections of each sample were analyzed.

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