

# Effects of different concentrations of type-I collagen hydrogel on the growth and differentiation of chondrocytes

DESHAN HU and XIULI SHAN

Department of Stomatology, The Fifth People's Hospital of Jinan, Jinan, Shandong 250022, P.R. China

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**Abstract.** The objective of this study was to analyze the effects of type-I collagen hydrogel of different concentrations on the growth and differentiation of rabbit chondrocytes. Articular cartilage from New Zealand white rabbits was harvested and cultured. Second-generation chondrocytes were collected for *in vitro* culture with 10, 7, and 5 mg/ml type-I collagen hydrogel, respectively (denoted as groups A, B, and C). After *in vitro* culture for 1 day, chondrocytes were stained with fluorescein diacetate (FDA)/propidium iodide (PI), and cell viability was observed by laser confocal microscopy. After *in vitro* culture for 14 days, the histological patterns were observed by H&E and toluidine blue staining. The expression of chondrocyte-related genes were measured by real-time quantitative RT-PCR. After *in vitro* culture for 1 day, FDA/PI staining showed that the cell density of group A was significantly higher than that of group B and C. After *in vitro* culture for 14 days, H&E staining showed that chondrocytes showed obvious aggregation in group A, partial proliferation and aggregation in group B, and uniform distribution in group C. Toluidine blue staining showed that chondrocytes in group A had aggregation areas and some were stained purple-red, fewer chondrocytes were aggregated with different staining around them in group B, and the aggregation of chondrocytes was not obvious. However, the distribution of chondrocytes was uniform with different staining in group C. After *in vitro* culture for 2 weeks, the levels of polymerized proteoglycan and type-II collagen mRNA were not significantly different between the three groups ( $P>0.05$ ). The levels of type-I collagen, type-X collagen, and Sox9 mRNA in group A were significantly higher than those in group B and C ( $P<0.05$ ). In conclusion, high concentration type-I collagen hydrogel can promote chondrocyte fibrosis and upregulation of type-I collagen, type-X collagen, and Sox9 mRNA.

## Introduction

Articular cartilage injury is a common clinical orthopedic disease. Self-repair of articular cartilage is difficult at pathogenic sites, and there are no specific methods to repair articular cartilage injury in clinical practice (1). In recent years, with rapid developments in modern biology and advanced materials, the application of cartilage tissue engineering has promoted the emergence of new methods to repair articular cartilage injury (2). Articular cartilage is rich in type-II collagen, and the synthesis and secretion of type-II collagen are specific indicators of articular chondrocytes to maintain their differentiated phenotype. In addition, they are essential bases for the evaluation of cartilage tissue engineering (3). In cartilage tissue engineering, commonly used accelerants include growth factors, such as exogenous growth factors, which are expensive and can easily biodegrade. Moreover, they may have immunogenicity and other problems, limiting their clinical application (4). Therefore, it is necessary to actively explore more effective accelerants with improved bio-performance. Type-I collagen is widely distributed. Studies have confirmed that (5) type-I collagen has good biocompatibility and biodegradability, and has been used in hemostasis, and in artificial skin and corneal repair. Previous studies showed that (6) type-I collagen scaffolds have good prospects in cartilage tissue engineering and cartilage repair. However, there are few studies on the effects of type-I collagen hydrogel of different concentrations on chondrocyte growth and differentiation. To further optimize the concentration of type-I collagen in collagen hydrogel, its effects on articular chondrocytes from New Zealand white rabbits were analyzed.

## Materials and methods

**Experimental animals.** Three newborn New Zealand white rabbits (male or female) aged 1 week were selected. The average weight was 0.17 kg. The rabbits were provided by the animal center of the Fifth People's Hospital of Jinan. The study was approved by Ethics Committee of the the Fifth People's Hospital of Jinan.

**Reagents.** Type-I collagen was extracted from calf skin, and prepared by the pathology laboratory of the Fifth People's Hospital of Jinan;  $\alpha$ -MEM medium (Shanghai Huiying Biotechnology Co., Ltd.); trypsin (Beijing Geyuan Tianrun

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*Correspondence to:* Dr Deshan Hu, Department of Stomatology, The Fifth People's Hospital of Jinan, 24297 Jingshi Road, Jinan, Shandong 250022, P.R. China  
E-mail: drdeshanhu@163.com

**Key words:** type-I collagen hydrogel, chondrocyte, Sox9

Biotechnology Co., Ltd.); type-II collagenase (Shanghai Shize Biotechnology Co., Ltd.); fluorescein diacetate (FDA)/propidium iodide (PI) dye (Shanghai Hualan Chemical Technology Co., Ltd.); DNA extraction kit (Shenzhen Baoankang Biology Co., Ltd.); ReactTra Ace Qpcr RT kit [Toyobo (Shanghai) Biotechnology Co., Ltd.]; acetic acid, NaOH, formaldehyde, and other chemical reagents were from Sinopharm Group Chemical Reagent Co., Ltd.

**Preparation of collagen hydrogel.** Appropriate amounts of type-I collagen and 0.5 mol/l acetic acid aqueous solution were weighed and mixed. The pH of the solution was adjusted to approximately 7.5 using 2 mol/l NaOH solution at 4°C, and the final concentration of type-I collagen was 10 mg/ml. The solution was then diluted to 7 and 5 mg/ml, respectively. Approximately 0.5 ml of collagen solutions of different concentrations were placed in a water bath at constant temperature (37°C) for 30 min to prepare the collagen hydrogel with type-I collagen concentrations of 10, 7, and 5 mg/ml.

**Chondrocyte extraction and inoculation.** Articular cartilages from three New Zealand white rabbits were harvested, and the chondrocytes were separated by enzyme digestion, and cultured. Chondrocytes were then collected, and appropriate amounts of  $\alpha$ -MEM medium were added to prepare cell suspensions. Cell suspensions were seeded in culture dishes at the density of  $1 \times 10^5$  cells/ml, and then cultured in an illumination incubator. After cells were confluent, 0.25% trypsin was used for digestion, followed by subculture. The morphology of second-generation chondrocytes was observed under an inverted fluorescence phase-contrast microscope.

Second-generation chondrocytes were collected and cultured *in vitro* with 10, 7, and 5 mg/ml type-I collagen hydrogel, respectively. The above three sterile collagen solutions were mixed and the suspensions had a concentration of  $5 \times 10^6$  cells/ml. The cell suspensions were then cultured in an incubator and added to the 300  $\mu$ l collagen hydrogel-chondrocyte complex after the collagen solution produced hydrogel, denoted as groups A, B, and C with 10, 7, and 5 mg/ml type-I collagen hydrogel, respectively. The complex continued to be cultured in an incubator for 2 weeks, and the solution was replaced every 2 days.

**Observation of cell viability.** The collagen hydrogel-chondrocyte complexes (one in each group) were selected after *in vitro* culture for 1 day, and stained with fluorescein diacetate (FDA)/propidium iodide (PI). Cell viability was observed under a laser confocal microscope.

**Histological observation.** The collagen hydrogel-chondrocyte complexes were selected after *in vitro* culture for 2 weeks and fixed using 10% neutral formaldehyde, followed by paraffin embedding and sectioning at 5  $\mu$ m. Sections were then stained with H&E and toluidine blue to observe the cell distribution.

**Real-time quantitative RT-PCR.** The collagen hydrogel-chondrocyte complexes were selected after *in vitro* culture for 2 weeks. Real-time quantitative RT-PCR was used to measure the mRNA levels of type-II collagen, polymerized proteoglycan, type-I collagen, type-X collagen, Sox9, and other

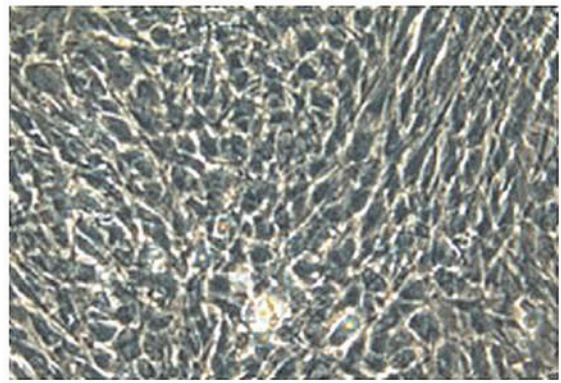


Figure 1. Morphological observation of second-generation chondrocytes after *in vitro* culture for 1 day. Inverted fluorescence phase-contrast microscopy showed that the cells were mostly polygonal with good growth status (x200).

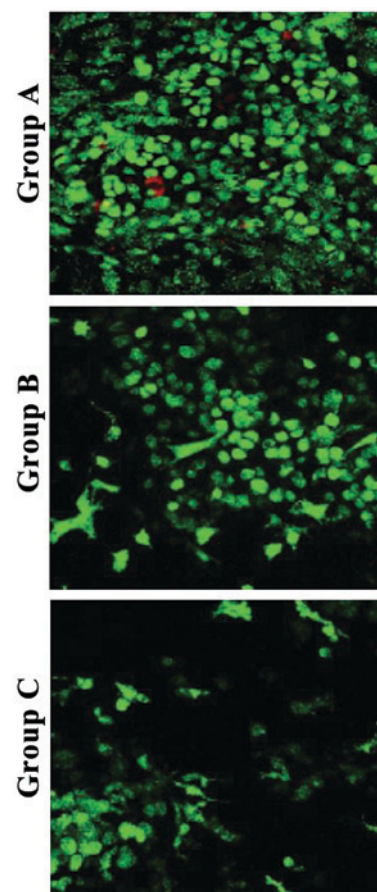


Figure 2. FDA/PI staining of collagen hydrogel-chondrocyte complexes after *in vitro* culture for 1 day in the three groups. Most of the chondrocytes in collagen hydrogels of groups A, B, and C were stained green, while a few were stained red (x200).

chondrocyte-related genes. The housekeeping gene, GAPDH, was used to normalize the expression of each gene. mRNA was extracted from samples using a specific extraction kit, followed by reverse transcription into cDNA using a ReverTra Ace Qpcr RT kit. The cDNA was then amplified using SsoFast™ EvaGreen® Supermix reagent (Beijing Changlitong Technology Co., Ltd.) in a CFX960 real-time fluorescence quantitative PCR instrument. The expression of each gene was analyzed, and each sample was measured three times. Related

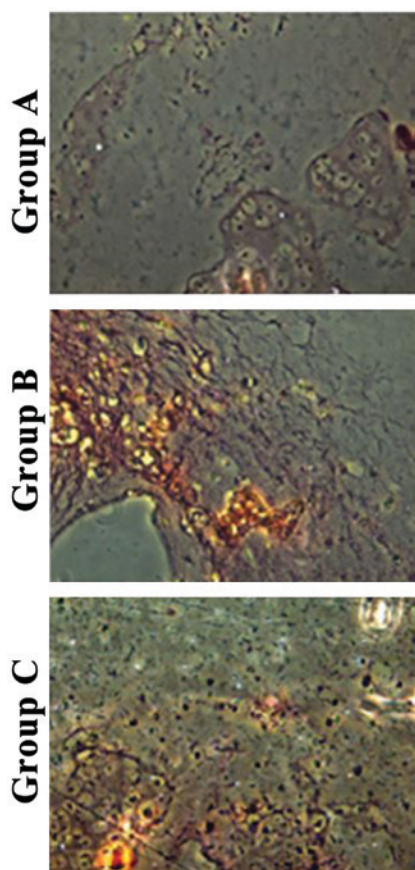


Figure 3. H&E staining and observation of collagen hydrogel-chondrocyte complexes after *in vitro* culture for 2 weeks in the three groups. Chondrocytes were aggregated significantly in group A, some chondrocytes showed proliferation and aggregation in group B, and the distribution of chondrocytes was relatively uniform in group C (x200).

primers were designed and synthesized by Shanghai Gongda Co., Ltd.

**Statistical analysis.** SPSS 20.0 (IBM, New York, NY, USA) statistical software was used for data analysis. Numerical data are presented as mean  $\pm$  SD, and compared using the paired samples t-test. The Chi-square test was used for comparisons of numerical data.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

### *Chondrocyte inoculation and observation of viability.*

Observation by inverted fluorescence phase-contrast microscopy showed that the second-generation chondrocytes were polygonal with diameter of approximately 8-10  $\mu\text{m}$ , and had good growth status (Fig. 1). After collagen hydrogel-chondrocyte complexes in groups A, B, and C were cultured *in vitro* for 1 day, and stained with FDA/PI, laser confocal microscopy showed that the distribution of chondrocytes was uniform. Most were spherical, while some were polygonal in shape, with a tendency to proliferate and aggregate. Most of the chondrocytes in collagen hydrogels of groups A, B, and C were stained green, while a few were stained red. The cell density of group A was significantly higher than that of group B and C (Fig. 2).

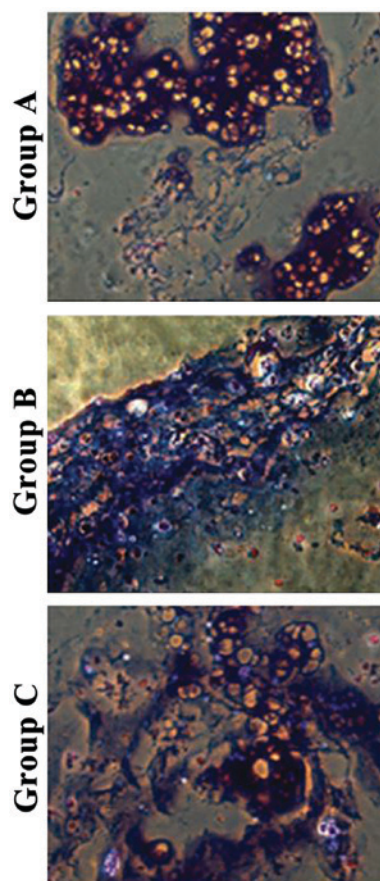


Figure 4. Toluidine blue staining and observation of collagen hydrogel-chondrocyte complexes after *in vitro* culture for 2 weeks in the three groups. Chondrocytes had areas of aggregation, and some were stained purple-red in group A. Fewer chondrocytes were aggregated with different staining around them in group B, and the aggregation of chondrocytes was not obvious. However, the distribution of chondrocytes was uniform with different staining in group C (x200).

**Histological observation.** H&E staining showed that chondrocytes showed obvious aggregation in group A, partial proliferation and aggregation in group B, and uniform distribution in group C (Fig. 3).

Toluidine blue staining showed that chondrocytes in group A had aggregation areas and some were stained purple-red. In group B, fewer chondrocytes were aggregated and had different staining color and intensity around them, and aggregation was not obvious. In group C, the distribution of chondrocytes was uniform with different staining (Fig. 4).

**Comparison of the relative levels of polymerized proteoglycan mRNA in each group.** The relative levels of polymerized proteoglycan mRNA were  $0.936 \pm 0.178$  in group A,  $0.925 \pm 0.136$  in group B, and  $0.917 \pm 0.135$  in group C. There were no significant differences in the relative levels of polymerized proteoglycan mRNA between the three groups ( $P > 0.05$ ) (Fig. 5).

**Comparison of the relative levels of type-II collagen mRNA in each group.** The relative levels of type-II collagen mRNA were  $0.772 \pm 0.201$  in group A,  $0.741 \pm 0.183$  in group B, and  $0.752 \pm 0.165$  in group C. There were no significant differences in the relative levels of type-II collagen mRNA between the three groups ( $P > 0.05$ ) (Fig. 6).

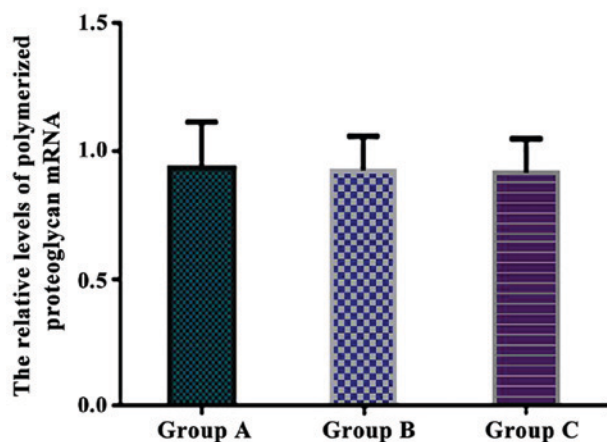


Figure 5. Comparison of the relative levels of polymerized proteoglycan mRNA in each group. Real-time quantitative RT-PCR showed that there were no significant differences in the relative levels of polymerized proteoglycan mRNA between the three groups.

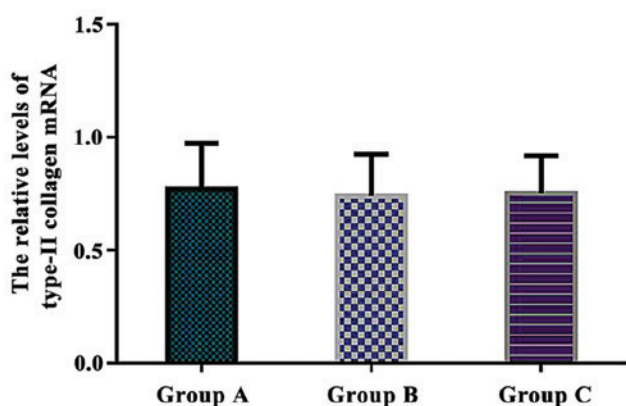


Figure 6. Comparison of the relative levels of type-II collagen mRNA in each group. Real-time quantitative RT-PCR showed that there were no significant differences in the relative levels of type-II collagen mRNA between the three groups ( $P > 0.05$ ).

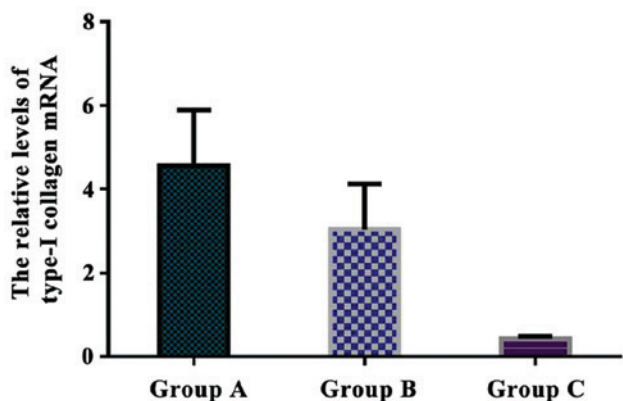


Figure 7. Comparison of the relative levels of type-I collagen mRNA in each group. Real-time quantitative RT-PCR showed that the relative expression of type-I collagen mRNA in group A was significantly higher than that in group B and C ( $P < 0.05$ ). There was a significant difference in type-I collagen mRNA expression between group B and C ( $P < 0.05$ ).

*Comparison of the relative levels of type-I collagen mRNA in each group.* The relative levels of type-I collagen mRNA

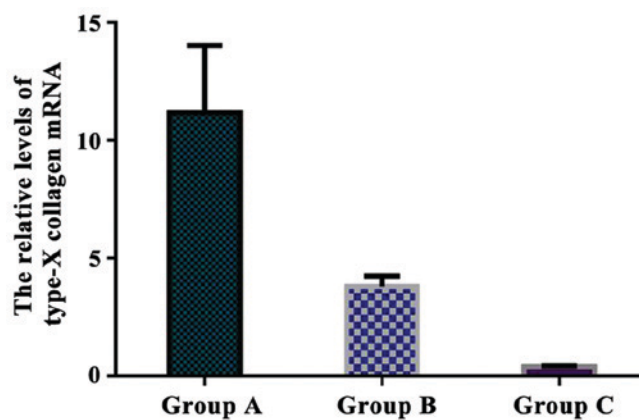


Figure 8. Comparison of the relative levels of type-X collagen mRNA in each group. Real-time quantitative RT-PCR showed that the relative expression of type-X collagen mRNA in group A was significantly higher than that in group B and C ( $P < 0.05$ ). There was a significant difference in type-X collagen mRNA expression between group B and C ( $P < 0.05$ ).

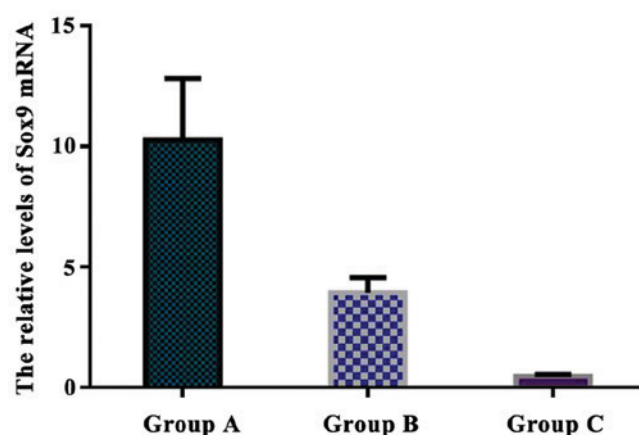


Figure 9. Comparison of the relative levels of Sox9 mRNA in each group. Real-time quantitative RT-PCR showed that the relative expression of Sox9 mRNA in group A was significantly higher than that in group B and C ( $P < 0.05$ ). There was a significant difference in Sox9 mRNA expression between group B and C ( $P < 0.05$ ).

were  $4.561 \pm 1.327$  in group A,  $3.046 \pm 1.075$  in group B, and  $0.432 \pm 0.058$  in group C. The relative expression of type-I collagen mRNA in group A was significantly higher than that in group B and C ( $P < 0.05$ ). Additionally, there was a significant difference in type-I collagen mRNA expression between group B and C ( $P < 0.05$ ) (Fig. 7).

*Comparison of the relative levels of type-X collagen mRNA in each group.* The relative levels of type-X collagen mRNA were  $11.172 \pm 2.845$  in group A,  $3.801 \pm 0.446$  in group B, and  $0.403 \pm 0.027$  in group C. The relative expression of type-X collagen mRNA in group A was significantly higher than that in group B and C ( $P < 0.05$ ). There was a significant difference in type-X collagen expression between group B and C ( $P < 0.05$ ) (Fig. 8).

*Comparison of the relative levels of Sox9 mRNA in each group.* The relative levels of Sox9 mRNA were  $10.263 \pm 2.551$  in group A,  $3.942 \pm 0.628$  in group B, and  $0.477 \pm 0.085$  in

group C. The relative expression of Sox9 mRNA in group A was significantly higher than that in group B and C ( $P < 0.05$ ). There was a significant difference in Sox9 mRNA expression between group B and C ( $P < 0.05$ ) (Fig. 9).

## Discussion

Collagen hydrogel is a form of hydrogel that is sensitive to temperature. It is a form of collagen fiber formed by continuous aggregation of collagen molecules of a neutral collagen solution under heating conditions or at 37°C (7-9). Related studies showed that (10) collagen concentration is positively correlated with collagen fiber density, which is an important factor for several physical and chemical indexes, such as the mechanical properties and water absorption of collagen gel. Related data showed that (11-13) collagen molecules have biological activity, and can actively participate in signal transduction and cellular behavior at the molecular level. Collagen concentration in the collagen hydrogel therefore plays an important role in its biological function. The biological functions of collagen hydrogels containing different concentrations of collagen may be different (12,14,15). Herein, we aimed to investigate the effects of type-I collagen hydrogel of different concentrations on the growth and differentiation of rabbit chondrocytes.

Related data showed that (16) collagen phenotype plays a highly important role in maintaining the normal structure of cartilage. At different stages of chondrocyte differentiation, the expression of collagen phenotype is different. In the process of differentiation, articular cartilage and epiphyseal cartilage mainly synthesize type-II, VI, IX, and XI collagen, whereas hypertrophic chondrocytes specifically synthesize type-X and II collagen. Type-X and I collagen are synthesized in the process from chondrocyte hypertrophy to differentiation into osteoblast-like cells (17). Type-II collagen and Aggrecan are markers of chondrocytes (18,19). The type-I collagen gene is expressed by fibrous tissues, and can be used to determine whether chondrocytes are differentiated into fibrous tissues (20-22). Sox9 also belongs to the group of chondrocyte fibrosis-related genes, and the type-X collagen gene is an important marker of hypertrophic chondrocytes (23,24). This study showed that the levels of Aggrecan and type-II collagen mRNA in groups A, B, and C were not significantly different after *in vitro* culture for 2 weeks ( $P > 0.05$ ). The levels of type-I collagen, type-X collagen, and Sox9 mRNA in group A were significantly higher than those in group B and C ( $P < 0.05$ ). The levels of type-I collagen, type-X collagen, and Sox9 mRNA were upregulated with increasing concentration of type-I collagen, suggesting that the increase of type-I collagen concentration may induce chondrocyte fibrosis and hypertrophy, thus affecting the formation of normal cartilage tissue. H&E staining showed that chondrocytes were aggregated significantly in group A, some chondrocytes showed proliferation and aggregation in group B, and the distribution of chondrocytes was relatively uniform in group C, indicating that cell differentiation had occurred.

In conclusion, high concentration of type-I collagen hydrogel can promote chondrocyte fibrosis and upregulation of the expression of type-I collagen, type-X collagen, and Sox9 mRNA. High concentration type-I collagen hydrogel can result in hypertrophy of articular chondrocytes in a premature

manner, which may promote the development of articular cartilage to osteoarthritis.

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