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ORIGINAL ARTICLE

Effects of Type I Collagen Concentration in Hydrogel on the Growth and Phenotypic Expression of Rat Chondrocytes

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Abstract It is controversial whether type I collagen itself can maintain and improve chondrogenic phenotype of chondrocytes in a three-dimensional (3D) environment. In this study, we examined the effect of type I collagen concentration in hydrogel (0.5, 1, and 2 mg/ml) on the growth and phenotype expression of rat chondrocytes *in vitro*. All collagen hydrogels showed substantial contractions during culture, in a concentration-dependent manner, which was due to the cell proliferation. The cell viability was shown to be the highest in 2 mg/ml collagen gel. The mRNA expression of chondrogenic phenotypes, including SOX9, type II collagen, and aggrecan, was significantly up-regulated, particularly in 1 mg/ml collagen gel. Furthermore, the production of type II collagen and glycosaminoglycan (GAG) content was also enhanced. The results suggest that type I collagen hydrogel is not detrimental to, but may be useful for, the chondrocyte culture for cartilage tissue engineering.

Keywords Type I collagen · Chondrocyte · Proliferation · Chondrogenic phenotype

1 Introduction

Articular cartilage is composed of sparse chondrocytes and a dense extracellular matrix (ECM). Type II collagen and glycosaminoglycan (GAG) are primary compositions of the ECM. In particular, the mechanical function of articular cartilage is dependent on the quantity of type II collagen [1]. Therefore, type II collagen has been widely studied for the cartilage repair and regeneration [2, 3].

Type I collagen is the most abundant collagen type, which is mostly found in bone, tendon, ligament and skin [4]. Hence, it is widely used to prepare scaffolds that mimic natural ECM of those tissues [5–7]. Even though type II collagen might be the most appropriate scaffold for cartilage tissue engineering, attempt has also been made to use the type I collagen for reconstruction of injured cartilage. Yasui et al. [8] previously observed that the chondrocytes proliferated and maintained their cartilage phenotype in type I collagen gel. Negri et al. [9] also demonstrated that human chondrocytes were capable of multiplying in type I collagen to present cartilage ECM. We fabricated previously alginate-hyaluronic acid based hydrogel with the incorporation of type I collagen [10]. The results showed that collagen proportion significantly accelerated chondrocytes proliferation and preserved chondrogenic phenotypes [10]. The type I collagen-assisted autologous chondrocyte transplantation (ACT) has also been applied to clinics. For instance, Nöth et al. [11] used type I collagen-based ACT for the treatment of three young patients. The results showed a good integration into the host articular

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cartilage tissue 12 months after surgery. Behrens et al. [12] reported also that the collagen I/III-assisted ACT for twenty-five patients improved the function of knee over a period of up to 5 years after operation.

In contrast, Chen et al. [13] revealed that type I collagen incorporated with dexamethasone and TGF-β1 reduced GAG production and mRNA level of SOX9 and aggrecan, with little expression of type II collagen gene in the cultured mesenchymal progenitor cells. Farjanel et al. [14] demonstrated that the chondrocytes exposed to type I collagen could lose their differentiated phenotype. In addition, the expansion of the chondrocytes in monolayer culture results in a rapid cell dedifferentiation. The collagen biosynthesis also switches from type II to type I collagen [15]. The type I collagen expression is indicative of the dedifferentiated status of chondrocytes.

Therefore, there are still some controversies as to the use of type I collagen in the culture of cells for cartilage tissue engineering. In this study we aim to investigate the effect of type I collagen in a hydrogel form on the growth and phenotype expression of the chondrocytes. Among the parameters, different concentrations of collagen gels were used. The cell-associated gel contraction behavior, and the proliferation and chondrocyte-related molecular expression (gene and protein levels) of cells were investigated, which will guide if the type I collagen 3D gel matrix can be useful for cartilage tissue engineering.

2 Materials and methods

2.1 Preparation of collagen hydrogels

The type I collagen solution (3.87 mg/ml, rat tail type I collagen, BD Biosciences, Bedford, MA, USA) was diluted with Dulbecco's Modified Eagle's Medium (DMEM; 10×; Gibco), 1% penicillin/streptomycin, and chondrocyte maintenance medium consisting of DMEM, 50 μg/ml ascorbic acid, 1% insulin-transferrin-selenium, 100 nM dexamethasone, 10% fetal bovine serum to yield final concentrations of 0.5, 1, and 2 mg/ml. The solutions were kept on ice less than a minute until neutralizing. A neutralization of the solution was carried out by adding 1 N NaOH aqueous solution: the volume of 1 N NaOH needed was equaled to 0.023 times of the volume of collagen solution, which was predetermined to adjust pH at 7.4.

For culture experiments, rat chondrocytes from articular cartilage of the knees were monolayer-expanded through four passages according to the procedures in a previous study [16], and thoroughly mixed with each collagen hydrogel to reach a final concentration of 3×10^5 cells/ml. The cell-seeded hydrogels were poured into bottomless polydimethylsiloxane molds with a dimension of 8 mm diameter and 2 mm thickness. The hydrogels were allowed to polymerize in a humidified incubator at 37 °C for 30 min. After gelation, the hydrogels were cultured with the chondrocyte maintenance medium or the chondrogenic medium for 14 days. The chondrogenic medium consists of the chondrocyte maintenance medium supplemented with 10 ng/ml transforming growth factor-β1 (PeproTech, USA). The media were changed twice every week.

2.2 Collagen gel contraction assay

At each culturing time, the diameter of collagen gels was measured with a ruler. The extent of contraction of the collagen gels was expressed as the percentage of initial area. Each experiment was performed in triplicate.

2.3 Cellular proliferation assay

The cell-seeded hydrogels were placed in 24-well plates and incubated in the chondrocyte maintenance medium for 1, 4 and 7 days, to evaluate the cell proliferation behavior. Cell proliferation was assessed using a CellTiter 96 aqueous one solution cell proliferation kit (MTS assay, Promega, USA). The culture medium was removed and a diluted MTS solution was added to each sample and allowed to react for 3 h at 37 °C. A 200 μl aliquot of the reaction sample was used for a colorimetric measurement at a wavelength of 490 nm using a microplate reader (Molecular Devices, USA). Three replicate samples were tested.

2.4 Cell viability

For the cell viability assay, the cell-seeded hydrogels were placed in 24-well plates and incubated in the chondrocyte maintenance medium for 1 and 7 days. At each culturing time, the cell viability in the gels was detected by the Live/Dead assay (Molecular Probes, Reduced Biohazard Viability/Cytotoxicity Kit, USA) according to manufacturer's instruction. The samples were incubated in Live/Dead assay stain solution for 30 min at room temperature, and subsequently observed under an inverted fluorescence microscope equipped with a DP-72 digital camera (DP2-BSW, Olympus Co., Tokyo, Japan). The viable cells were indicated with green fluorescence signals while dead cells were indicated with red fluorescence signals.

2.5 Quantitative real-time polymerase chain reaction (qPCR)

For qPCR assay, the cell-seeded hydrogels were incubated in the chondrogenic medium for 7 and 14 days. At each culturing time, the expression of cartilage-related genes

Table 1 Primer sequences of chondrogenic genes for qPCR

Gene	Forward sequence	Reverse sequence
SOX9	5′-CGTCAACGGCTCCAGCA-3′	5′-TGCGCCCACACCATGA-3′
Type II collagen	5′-GAGTGGAAGAGCGGAGACTACTG-3′	5′-CTCCATGTTGCAGAAGACTTTCA-3′
Aggrecan	5′-CTAGCTGCTTAGCAGGGATAACG-3′	5′-TGACCCGCAGAGTCACAAAG-3′
GAPDH	5′-TGAACGGGAAGCTCACTGG-3′	5′-TCCACCACCCTGTTGCTGTA-3′

was analyzed using a Rotor-Gene RG-3000A qPCR machine (Australia). The first strand cDNA was synthesized from the total RNA (1 μg) using a SuperScript first strand synthesis system for real-time PCR (Invitrogen, USA) according to the manufacturer's instruction. The reaction mixture was made up to 50 μl. Real-time PCR was conducted using SYBR GreenER qPCR SuperMix reagents (Invitrogen, USA). The relative transcript quantities were calculated using the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous reference gene amplified from the samples. The primer sequences of the genes are summarized in Table 1.

2.6 Immunofluorescence staining

To detect the expression of type II collagen, the samples were cultured in the chondrogenic medium for 14 days. Thereafter, the culture medium was removed and the harvested samples were fixed with 4% paraformaldehyde (PFA) for 20 min, incubated with 5% normal goat serum (Vector Laboratories, USA) in PBS for 30 min to suppress nonspecific staining, and then incubated with a primary antibody, anti-type II collagen (1:150 dilution, sc-52658; Santa Cruz Biotechnology, USA), for 24 h at 4 °C. The specimens were subsequently incubated with the FITC-conjugated antibody against mouse IgG (1:100 dilution, 115-095-003; Jackson Immunoresearch, USA) for 50 min at room temperature. The nuclei of the cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min. The samples were examined with an inverted fluorescence microscope equipped with a DP-72 digital camera (Olympus Co., Tokyo, Japan).

2.7 Histological analysis of *in vitro* samples

For the assessment of GAG content, the cell-seeded hydrogels were incubated in the chondrogenic medium. After 14 days, the samples were harvested and fixed with 4% PFA, and then stained with Safranin O (Sigma-Aldrich, USA) and Alcian blue (Sigma-Aldrich, USA). Images were observed under an optical microscope.

2.8 Statistical analysis

Data are shown as the mean ± one standard deviation. Statistical analysis was performed using one-way ANOVA followed by a post hoc LSD test. $p < 0.05$ was considered to be statistically significant.

3 Results

3.1 Contraction of hydrogels

Unfixed hydrogels were observed at each time point. 0.5 mg/ml collagen gel contracted most rapidly compared with other groups. 1.0 mg/ml gel contracted moderately, and 2.0 mg/ml gel contracted most slowly among three groups (Fig. 1). This observation confirmed that the gel contraction was in a collagen concentration-dependent manner.

3.2 Cellular growth behaviors

The cell-gel constructs were cultured using the chondrogenic medium for 14 days, and the cell morphology was observed under a phase contrast microscope, as shown in Fig. 2. In all groups, both round- and spindle-shaped cells were observed at day 1. Thereafter, the cells embedded in 2 mg/ml collagen gel presented a more elongated morphology than in 0.5 and 1 mg/ml collagen gels with time.

The Live/Dead assay was used to detect the viability of the cells in the gels (Fig. 3). The results showed that the chondrocytes were mostly alive and only few dead cells were observed in all groups at day 1. However, dead cells significantly increased in 0.5 and 1 mg/ml collagen gels after 7 days of culture. At this time point, the viability of cells remained high in 2 mg/ml collagen gel.

The cell growth level was examined by MTS cell proliferation assay, as shown in Fig. 4. All groups showed similar cell growth behaviors at day 1. Thereafter, the proliferation level of cells increased in a collagen concentration-dependent manner.

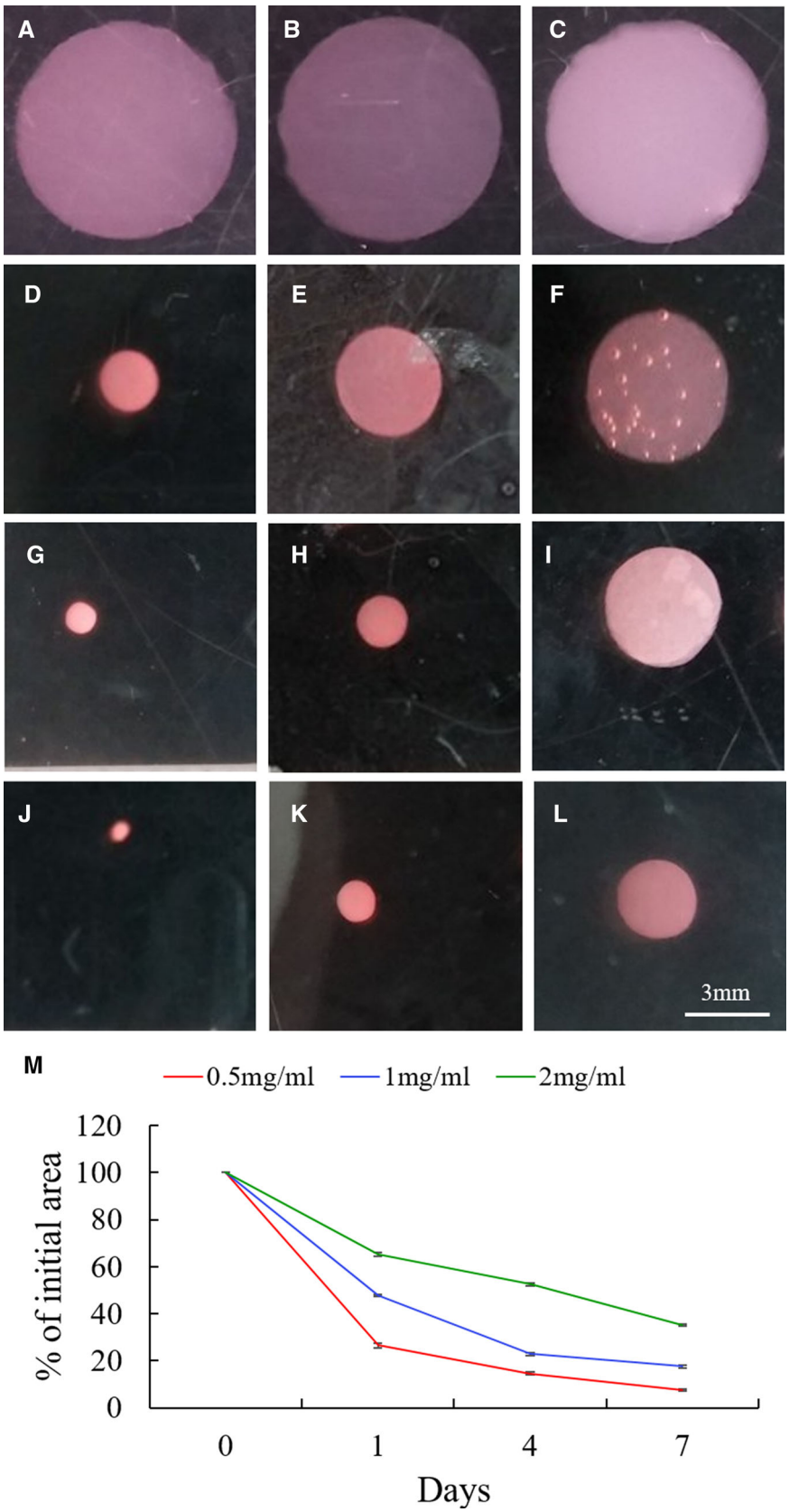

Fig. 1 Effect of collagen concentration on the gel contraction by rat chondrocytes. **A**–**L** Images of collagen gel contraction. **A**, **D**, **G**, **J** 0.5 mg/ml collagen gels; **B**, **E**, **H**, **K** 1 mg/ml collagen gels; **C**, **F**, **I**, **L** 2 mg/ml collagen gels. **A**–**C** culture day 0; **D**–**F** culture day 1; **G**–**I** culture day 4; **J**–**L** culture day 7. **M** Quantitative analysis of collagen gel contraction

3.3 Chondrogenic phenotype expressions

The expression of chondrocyte-related genes was analyzed at day 7 and 14 (Fig. 5). The SOX9, considered an early marker of chondrogenesis, increased in all groups with time. At day 7, the expression of SOX9 was higher in 2 mg/ml gel group than in other groups. At day 14, the gene expression was significantly higher in 1 and 2 mg/ml gel groups than in 0.5 mg/ml gel group. The type II collagen and aggrecan—relatively late stage markers of chondrogenesis—were expressed slightly higher in 2 mg/ml gel group than in other gel groups at day 7. These gene expressions significantly increased in 1 mg/ml gel group compared with those in 2 and 0.5 mg/ml gel groups at day 14.

After culturing for 14 days the production of type II collagen was further examined by the immunofluorescence staining. As shown in Fig. 6, the green fluorescence signals were found mainly in the peripheral area of the gels in 0.5 and 2 mg/ml groups. Interestingly, the signals were observed in both peripheral and center regions of the gels in 1 mg/ml group. More positive staining was found in 1 mg/ml gel than in 0.5 and 2 mg/ml gels. In order to support the immunofluorescence staining result, the production of cartilaginous matrix of the cell-gel constructs at 14 days was then investigated by histological staining. Safranin O and Alcian blue were widely used for the histochemical detection of cartilaginous matrices, glycosaminoglycan (GAG). As shown in Fig. 7, all gel groups were positive for Safranin O and Alcian blue stainings at day 14. Both stainings were observed to be more intense and homogeneous in 1 mg/ml gel group than in the other groups.

4 Discussion

Type I collagen, a major extracellular component of connective tissues, has proven to be one of the most promising biomaterials for tissue engineering because of its excellent biocompatibility, biodegradability, rich source and easy processing [17]. It has been widely applied to bone [6, 7], nerve [18, 19] and tendon/ligament tissue engineering

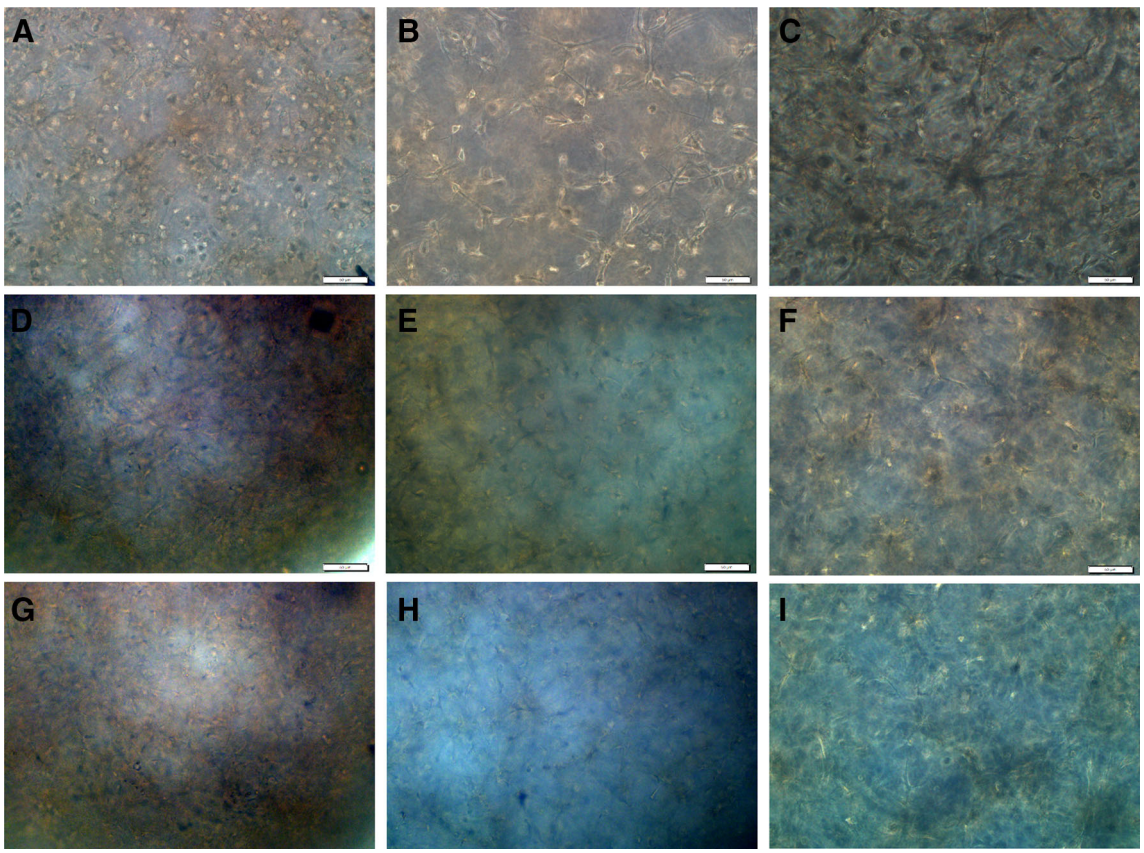

Fig. 2 Phase contrast images of the chondrocytes cultured in collagen gels at day 1, day 7, and day 14. **A**, **D**, **G** 0.5 mg/ml collagen gels; **B**, **E**, **H** 1 mg/ml collagen gels; **C**, **F**, **I** 2 mg/ml collagen gels. **A–C** culture day 1; **D–F** culture day 7; **G–I** culture day 14

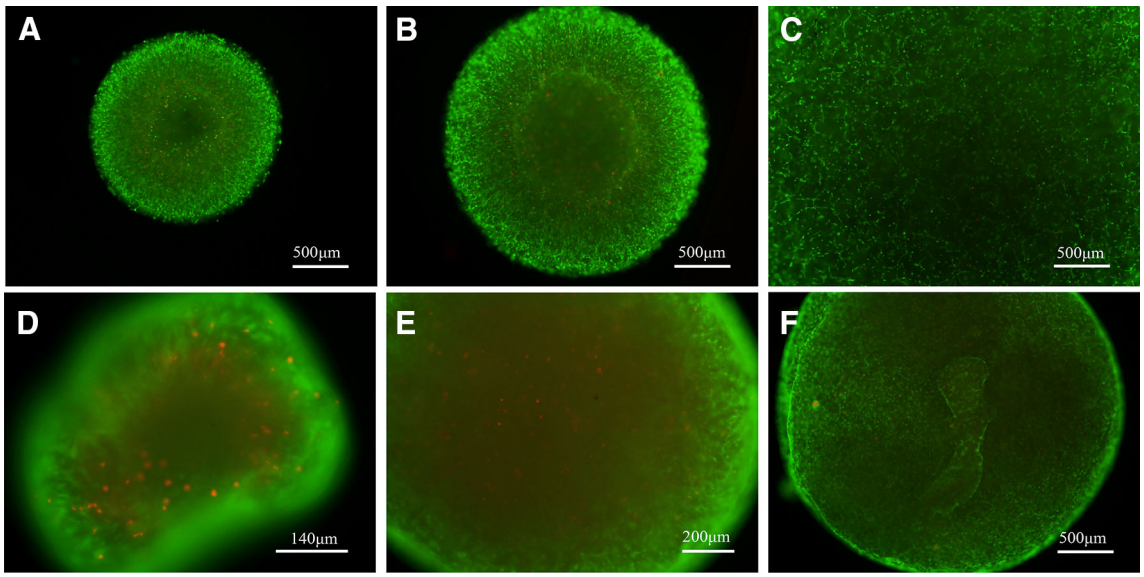

Fig. 3 Fluorescence image of cells cultured in collagen gels at day 1 and day 7 by the live/dead assay. Live cells marked with green-fluorescent calcein, and dead cells labeled with red-fluorescent ethidium homodimer-1. **A**, **D** 0.5 mg/ml collagen gels; **B**, **E** 1 mg/ml collagen gels; **C**, **F** 2 mg/ml collagen gels. **A–C** culture day 1; **D–F** culture day 7

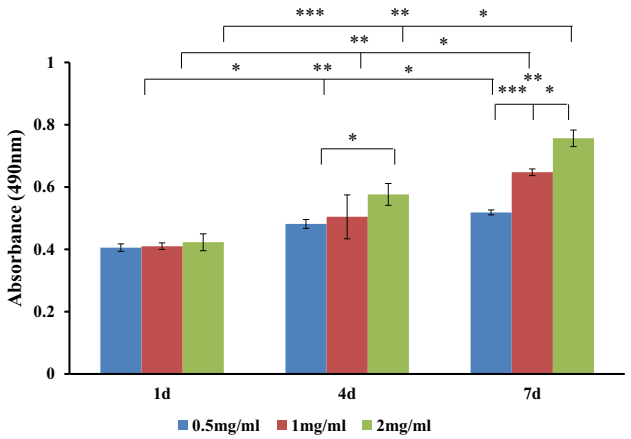

Fig. 4 MTS assay of the cell viability in collagen gels (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, n = 3)

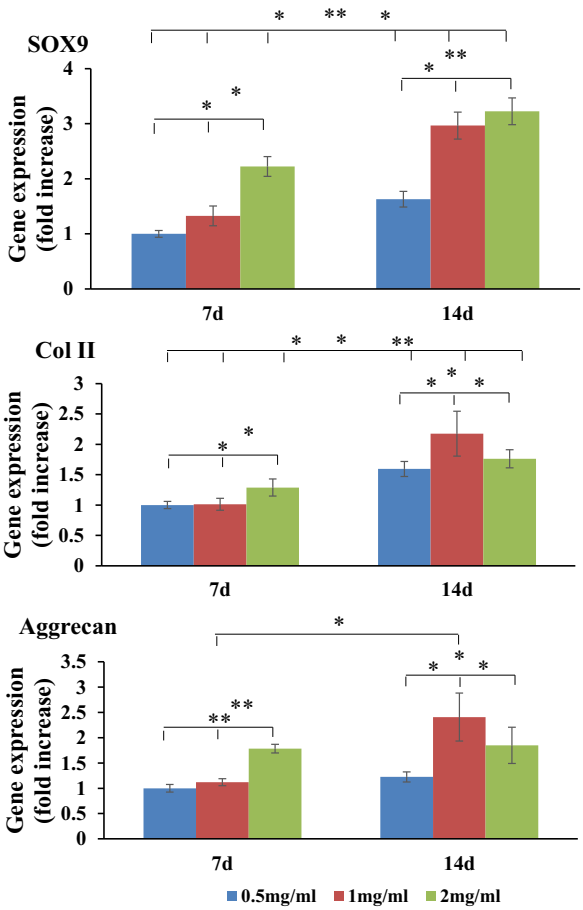

Fig. 5 Quantitative PCR chondrogenic gene expressions of the cells in collagen gels (*$p < 0.05$; **$p < 0.01$; n = 3)

[20, 21]. Although type II collagen is the main protein of a cartilage ECM, type I collagen has also been demonstrated to be beneficial for supporting cartilage regeneration [22, 23]. In this study, we observed that the gel contraction and cell growth were collagen concentration-dependent; while the collagen gel contracted the most in the lowest concentration (0.5 mg/ml), the chondrocytes proliferated the most rapidly in the highest concentration (2 mg/ml). In fact, type I collagen gel has been widely used to encapsulate cells of different origins, having proven the cell and tissue compatibility. Therefore, the slower cell growth in lower collagen concentration groups (0.5 and 1 mg/ml) compared with that in 2 mg/ml is not from the toxicity of

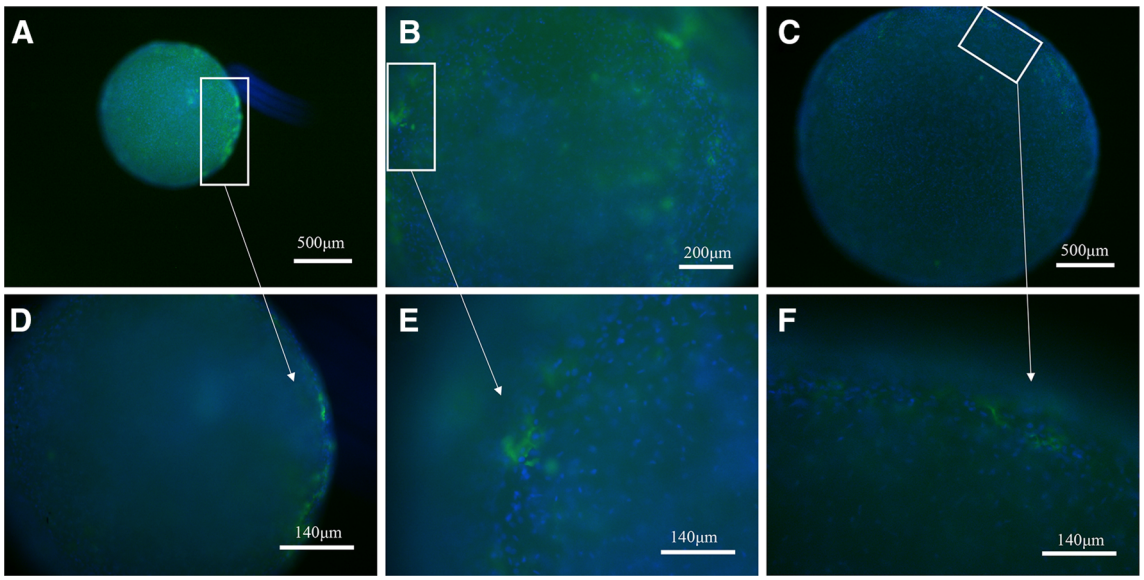

Fig. 6 Type II collagen immunofluorescence staining of cells in collagen gels at day 14. **A**, **D** 0.5 mg/ml collagen gels; **B**, **E** 1 mg/ml collagen gels; **C**, **F** 2 mg/ml collagen gels

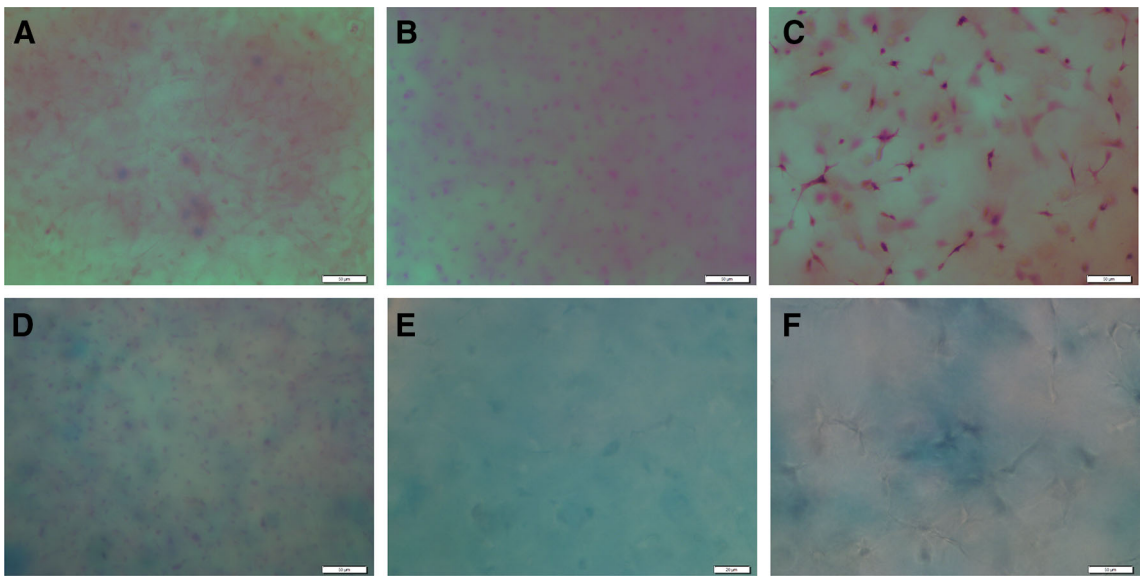

Fig. 7 Glycosaminoglycan (GAG) production of the cells in collagen gels visualized by Safranin O and Alcian blue staining at day 14. **A**, **D** 0.5 mg/ml collagen gels; **B**, **E** 1 mg/ml collagen gels; **C**, **F** 2 mg/ml collagen gels. **A**–**C** Safranin O staining; **D**–**F** Alcian blue staining

collagen, but maybe due to the low density of collagen fibrils that are actively engaged in the cellular adhesion, spreading and division.

The contraction of collagen gels during cultures has been reported by Zhou et al. [24]. They demonstrated that the gels with lower collagen concentrations contracted more than the gels with higher concentrations, in good agreement with our results (Fig. 1). The contraction of the collagen hydrogel is considered as a major obstacle for the applications in tissue engineering, and high cell density and low collagen concentration cause uncontrollable contraction of the gels [24, 25]. Therefore, many studies have been carried out to solve the collagen shrinkage issue. Visscher et al. [26] showed that collagen hydrogel contraction could be prevented by a 3D-printed polye-ε-caprolactone cage. Sheu et al. [27] revealed that cross-linking of collagen hydrogel using glutaraldehyde enhanced the strength and inhibited the contraction of the gel, which however,

showed substantial cytotoxicity. Fibrin was incorporated into the collagen matrix to improve the mechanical property and to resist the gel contraction [28].

Here we thus hypothesized that the collagen concentration might affect the hydrogel contraction level. Initially, the collagen concentrations of 0.5–3 mg/ml were tried, however, the 3 mg/ml collagen solution produced some bubbles due to a high viscosity during the mixing with cells; thus the range of 0.5–2 mg/ml collagen concentrations was selected for this study. The gel contraction result demonstrated well that increasing the collagen concentration reduced the gel contraction significantly, supporting the merit of 2 mg/ml group.

However, the chondrocyte phenotype expression was not dependent on the collagen concentration, which was different from the cell growth and gel contraction behaviors. The chondrogenic genes and the cartilage ECM appeared to be the most significantly up-regulated in 1 mg/ml collagen gel (Figs. 5, 6, 7). We consider that the cells may need to aggregate for the proper maintenance of chondrogenic phenotypes, thus the gel contraction to a certain level can help this cellular contacts and aggregation. In fact, low cell seeding density results in very little cartilaginous ECM [29], while high cell density can form clinically relevant level of cartilage tissue [30, 31]. Therefore, the collagen gel concentration and cell density are considered to be properly used in order to gain optimal cellular functions for cartilage.

Type I collagen has significant advantages for tissue engineering applications because of its ability to promote cell adhesion and proliferation through collagen-binding integrins [32, 33]. In particular, sufficient number of cells is required for cartilage repair, thus the use of type I collagen is biologically relevant to increase the cell number for cartilage tissue engineering. However, a long-term expansion of cells often leads to dedifferentiation of the chondrocytes. Thus the maintenance of cartilage phenotype is another important consideration in cartilage tissue engineering [34–36]. Within hydrogels composed of some natural materials including alginate and agarose, the chondrocytes were shown to maintain in part their chondrogenic phenotype, suggesting the importance of 3D gel matrix environment for chondrocytes culture [37–39].

In this study the chondrocytes cultured in 3D collagen gels exhibited a mixture of round and spindle-shaped cells. In fact, the round shape is indicative of chondrocyte phenotype maintenance, and the dedifferentiated phenotype is often characterized by a change from the round to the spindle-shaped cells [40], therefore, the chondrocytes are considered to preserve in part their chondrogenic phenotype within type I collagen gels. Our previous work also demonstrated that the combination of type I collagen with other natural polymers, such as alginate and hyaluronic acid, increased the cell proliferation and chondrogenic gene expression [10]; therefore, the role of type I collagen to be played in chondrocyte cultures is considered beneficial, suggesting its potential use as a 3D gel matrix for cartilage regeneration. In this case, the gel contraction and the cell seeding density need to be carefully considered to enable proper cell growth and cell–cell contact induced aggregation, and ultimately to preserve the chondrogenic phenotypes and cartilage formation *in vivo*, which remains as further study.

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Compliance with ethical standards

Conflict of interest The authors have no financial conflict of interest.

Ethical statement The chondrocyte isolation from Sprague–Dawley rats was according to the consent from Dankook University Institutional Animal Care and Use Committee (DKU-IRB-2014-039).

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