Efficacy of collagen and alginate hydrogels for the prevention of rat chondrocyte dedifferentiation

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Abstract

Dedifferentiation of chondrocytes remains a major problem in cartilage tissue engineering. The development of hydrogels that can preserve chondrogenic phenotype and prevent chondrocyte dedifferentiation is a meaningful strategy to solve dedifferentiation problem of chondrocytes. In the present study, three gels were prepared (alginate gel (Alg gel), type I collagen gel (Col gel), and their combination gel (Alg/Col gel)), and the in vitro efficacy of chondrocytes culture while preserving their phenotypes was investigated. While Col gel became substantially contracted with time, the cells encapsulated in Alg gel preserved the shape over the culture period of 14 days. The mechanical and cell-associated contraction behaviors of Alg/Col gel were similar to those of Alg. The cells in Alg and Alg/Col gels exhibited round morphology, whereas those in Col gel became elongated (i.e. fibroblast-like) during cultures. The cells proliferated with time in all gels with the highest proliferation being attained in Col gel. The expression of chondrogenic genes, including *SOX9*, type II collagen, and aggrecan, was significantly up-regulated in Alg/Col gel and Col gel, particularly in Col gel. However, the chondrocyte dedifferentiation markers, type I collagen and alkaline phosphatase (*ALP*), were also expressed at significant levels in Col gel, which being contrasted with the events in Alg and Alg/Col gels. The current results suggest the cells cultured in hydrogels can express chondrocyte dedifferentiation markers as well as chondrocyte markers, which draws attention to choose proper hydrogels for chondrocyte-based cartilage tissue engineering.

Introduction

Articular cartilage damage from sports injury and osteo-arthritis is almost intractable to regenerate due to the lack of vascularity.[1] While there have been numerous attempts to develop treatment techniques to repair cartilage defects, there remain significant challenges for the clinical application. Tissue engineering opens up an alternative therapeutic approach for articular cartilage repair.[2–6] However, one major problem of the techniques is that tissue engineering of cartilage grafts frequently causes osteogenic differentiation.[7]

Autologous chondrocyte implantation (ACI) is the treatment of choice for cartilage repair. However, acquiring sufficient cells before implantation needs a long expansion time and multiple passaging processes under monolayer cultures,

which often leads to dedifferentiation of the chondrocytes.[8,9] Dedifferentiation of the chondrocytes is a major contributor to osteogenic differentiation.[10] The chondrocyte dedifferentiation involves a gradual loss of the chondrocyte markers,

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which is characterized by RUNX2, type I collagen, alkaline phosphatase (*ALP*), and so on.[11–13] Therefore, it is becoming crucial to prevent chondrocyte dedifferentiation for clinical cartilage repair. Many efforts have been made to overcome the dedifferentiation of chondrocytes. Watt[14] found that high-seeding density of the chondrocytes resulted in less dedifferentiation than low-seeding density cultures due to high-seeding density can inhibit cell spreading with respect to low-seeding density. Meretoja et al.[15] found that cocultures of chondrocytes with mesenchymal stem cells (MSCs) promoted a stable chondrocyte phenotype and prevented their dedifferentiation due to the requirement of the low cell numbers and the low passages than the chondrocytes monolayer culture. Despite attempts to avoid dedifferentiation, the above methods still do not effectively maintain the chondrocyte phenotype. Thus, approaches to redifferentiation of dedifferentiated chondrocytes have been investigated using three-dimensional (3D) hydrogel culture systems. Grigolo et al.[16] noted that redifferentiation of dedifferentiated chondrocytes is largely conducted in hyaluronan hydrogel by increasing type II collagen and aggrecan expression levels. Benya et al.[12] found that the dedifferentiated chondrocytes could be redifferentiated in agarose gels via increased type II collagen and proteoglycan production. Another natural material, alginate hydrogel, has also been shown to support redifferentiation potential of dedifferentiated chondrocytes.[17,18] However, alginate inherently lacks the bioactive ligands necessary for cell anchoring and thereby possesses limited cell adhesion and proliferation.[19,20]

Previously, we showed that type I collagen hydrogel could not only support the chondrocytes growth and proliferation but also up-regulate the chondrogenic phenotypes.[21] The study of Van Susante et al.[20] noted that the chondrocytes proliferated become dedifferentiated in type I collagen gels although the cells grew slowly while expressing their typical chondrocyte phenotype. By considering those aspects and the merits of collagen and alginate gels, we hypothesized that their combination might provide proper hydrogel conditions to allow chondrocytes to grow and to prevent their dedifferentiation. In the present study, we examined the cell proliferation and shape change, and the expression of chondrogenic and dedifferentiation markers in alginate, collagen, or their combined gel.

Materials and methods

Hydrogels preparation

Three different hydrogels were prepared: a pure alginate (Alg), a pure collagen (Col), and a blend gel (Alg/Col). The sodium alginate (MW 32,000–250,000, Duksan Science, South Korea) was dissolved in a chondrocyte maintenance medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 50 μg/mL ascorbic acid, 1% insulin–transferrin–selenium, 100 nM dexamethasone, and 10% fetal bovine serum to form a 3.6 wt% stock solution

by magnetic force stirring for 8 h at 4°C. The type I collagen solution (3.87 mg/mL, rat tail type I collagen, BD Biosciences, Bedford, MA, USA) was diluted in the chondrocyte maintenance medium and neutralized to pH 7.4 by incorporating 1N NaOH to form a 2-mg/mL stock solution. The Alg solution and the Col solution were prepared by 1:1 dilution with the chondrocyte maintenance medium, respectively. To prepare the Alg/Col solution, the alginate and the collagen were mixed at a volume ratio 1:1 to obtain final concentration of 1.8 wt% Alg and 1 mg/mL Col.

Cells in hydrogels

For culture experiments, rat chondrocytes from articular cartilage of the knees were monolayer-expanded through two passages according to the procedures in a previous study.[22] The cell–hydrogel mixtures were prepared according to the procedures in a previous study.[21] The cells were thoroughly mixed with the above solutions to reach a final concentration of 4×10^5 cells/mL. The mixtures were poured into bottomless polydimethylsiloxane molds with dimensions of 8 mm diameter and 2 mm thickness. The Alg and the Alg/Col gels were immersed in 50 mM $CaCl_2$ for 8 min at room temperature to allow cross-linking, and the Col gel was allowed to polymerize in a humidified incubator at 37°C for 10 min. After gelation, the gels were cultured for 14 days in the chondrocyte maintenance medium. The media were changed every 2 days.

Dynamic mechanical analysis

Dynamic mechanical analysis was conducted to characterize the viscoelastic mechanical behaviors of the hydrogels (8 mm diameter, 4 mm height) using a dynamic mechanical analyzer (01dB-Metravib, Limonest Cedex, France). The storage moduli E′ and loss moduli E″ were measured as a function of time. The data were collected at 37°C.

Gel contraction assay

The cell-seeded hydrogels were placed in 48-well plates and incubated in the chondrocyte maintenance medium for 1, 7, and 14 days. At each culturing time, a diameter of each gel was measured with a ruler. The extent of contraction of the gels was expressed as the percentage of initial area. Each experiment was performed in triplicate.

Cell viability

For the cell viability assay, the cell-seeded hydrogels were placed in 48-well plates and incubated in the chondrocyte maintenance medium for 1 and 7 days. At each culturing time, the fluorescent cell viability in the gels was detected by the Live/Dead assay (Reduced Biohazard Viability/ Cytotoxicity Kit, Molecular Probes, Inc., Eugene, OR,

Table 1. Primer sequences of chondrogenic genes for qPCR.

Gene	Forward sequence	Reverse sequence
SOX9	5′-CTGAAGGGCTACGACTGGAC-3′	5′-TACTGGTCTGCCAGCTTCCT-3′
Type II collagen	5′-GAGTGGAAGAGCGGAGACTACTG-3′	5′-CTCCATGTTGCAGAAGACTTTCA-3′
Aggrecan	5′-CTAGCTGCTTAGCAGGGATAACG-3′	5′-TGACCCGCAGAGTCACAAAG-3′
Type I collagen	5′-CGTGACCAAAAACCAAAAGT-3′	5′-GGGGTGGAGAAAGGAACAGA-3′
ALP	5′-ACTGGTACTCGGACAATGAG-3′	5′-ATCGATGTCCTTGATGTTGT-3′
GAPDH	5′-TGAACGGGAAGCTCACTGG-3′	5′-TCCACCACCCTGTTGCTGTA-3′

qPCR: quantitative polymerase chain reaction; ALP: alkaline phosphatase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

USA) according to manufacturer's instruction. The gels 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, while dead cells were indicated with red fluorescence.

Cellular proliferation assay

The cell-seeded hydrogels were placed in 48-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, Madison, WI, USA). The culture medium was removed, and a diluted MTS solution was added to each sample and allowed to react for 4 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, Sunnyvale, CA, USA). Three replicate samples were tested.

Expression pattern of chondrogenic genes

For reverse transcription polymerase chain reaction (RT-PCR) and quantitative polymerase chain reaction (qPCR) assay, the cell-seeded hydrogels were incubated in the chondrocyte maintenance medium for 14 days. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) was used for cDNA synthesis with a Superscript kit (Invitrogen, Carlsbad, CA, USA) with random hexamers. RT-PCR reactions were conducted using 35 cycles at 95°C for 30 s, 55°C for 30 s, then 72°C for 60 s. PCR products were run on 1.5% agarose gel for 30 min at 100 V. qPCR was carried out using RealAmp SYBR qPCR master mix (GeneAll Biotechnology Co., LTD, Seoul, Korea) and a real-time PCR system (StepOnePlus, Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. The reaction mixture was made up to 50 μL. The relative transcript quantities were calculated using the 2-ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous reference gene amplified from the samples. The primer sequences used for RT-PCR and qPCR are summarized in Table 1. The reactions were run in triplicate in three independent experiments.

Immunofluorescence staining

To detect the expression of type II collagen, the samples were cultured in the chondrocyte maintenance medium for 14 days. Thereafter, the culture medium was removed, and the harvested gels were fixed with 4% paraformaldehyde (PFA) for 20 min, incubated with 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in phosphate buffered saline (PBS) for 30 min to suppress nonspecific staining, and then incubated with a primary antibody, anti-type II collagen (Santa Cruz Biotechnology, Dallas, Texas, USA), at a dilution of 1:150 overnight at 4°C. The samples were subsequently incubated with the fluorescein isothiocyanate (FITC)-conjugated antibody against mouse IgG at a dilution of 1:100 (Jackson Immunoresearch, West Grove, PA, 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.).

Glycosaminoglycan content measurement

The cell-seeded hydrogels were incubated in the chondrocyte maintenance medium. After 14 days, the samples were harvested and dried at 60°C for 5 h. Then, the dry weight of the samples was measured. For the assessment of glycosaminoglycan (GAG) content, Rheumera Proteoglycan Detection Kit (Cat# 8000, Astartebio Ltd., Bothell, WA, USA) was used. Briefly, the dry samples were digested with 300 μg/mL Papain in 20 mM PBS (pH 6.8) at 60°C for 1 h, and then 5 mL of 50 mM Tris/HCl (pH 8.0) and 10 mM iodoacetic acid were added. Dimethyl methylene blue (DMMB) assay was conducted according to the manufacturer's instructions. An aliquot of 0.1 mL sample was mixed with 0.1 mL of DMMB solution, and an
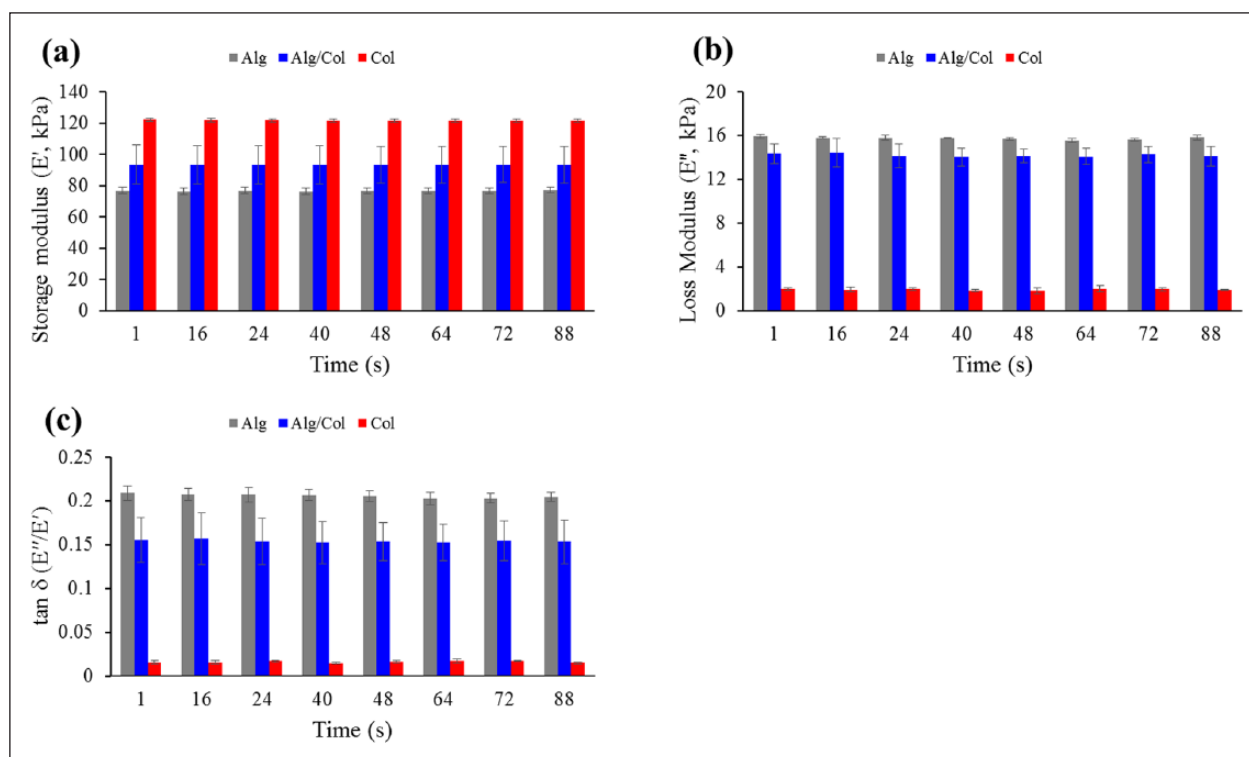
Figure 1. Time evolution of E′, E″, and tan δ (E″/E′) for each of the hydrogels at 37°C: (a) storage modulus (E′) measures the sample's elastic behavior, (b) loss modulus (E″) measures the sample's viscous response, and (c) tan δ is the ratio of the loss to the storage.

absorbance was read at 525 nm using a spectrophotometer. Chondroitin sulfate from bovine trachea (Astartebio Ltd., USA) was used to create a calibration curve to correlate the measured absorbance to known amount of GAG. The amount of GAG measured from each sample was normalized to dry weight for all samples.

Statistical analysis

Data are shown as the mean ± 1 standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by a post hoc least significant difference (LSD) test; $p < 0.05$ was considered to be statistically significant.

Results

Mechanical behaviors of hydrogels

The mechanical properties of the hydrogels were analyzed by a dynamic mechanical analyzer (Figure 1). Col gel had the highest E′ compared with other gels, Alg/Col gel had the moderate E′, and Alg gel had the lowest E′ among three groups, suggesting that Col gel had the highest elasticity. In contrast, Alg gel had the highest E″ among three groups, Alg/Col gel had the moderate E″, and Col gel had the lowest E″ compared with other groups, suggesting that Alg gel showed the highest viscous behavior. Meanwhile, the

E″/E′ (equivalent to tan δ) ratio of Col gel was relatively low, indicating a low viscosity and a high elasticity. Alg/Col gel had the moderate tan δ values. These data suggest that Alg/Col gel has viscoelastic behaviors more similar to the Alg gel than to the Col gel.

Contraction of hydrogels

Unfixed hydrogels were observed at each culturing time point. Col gel showed substantial contractions during cultures in a time-dependent manner. Both Alg and Alg/Col gels hardly contracted at the same time point (Figure 2). This observation confirmed that the gel contraction was prevented through the addition of alginate.

Cellular growth behaviors

The cell–gel constructs were cultured using the chondrocyte maintenance medium for 14 days, and the cell morphology was observed under a phase contrast microscope, as shown in Figure 3. In Alg gel and Alg/Col gel, the cells exhibited a round-shaped morphology at each time point. In Col gel, photomicrograph showed the presence of a mixture of round- and spindle-shaped cells at day 1. Thereafter, the cells became a more elongated shape with time.

The Live/Dead assay was used to detect the viability of the cells in the gels (Figure 4). The results showed that most
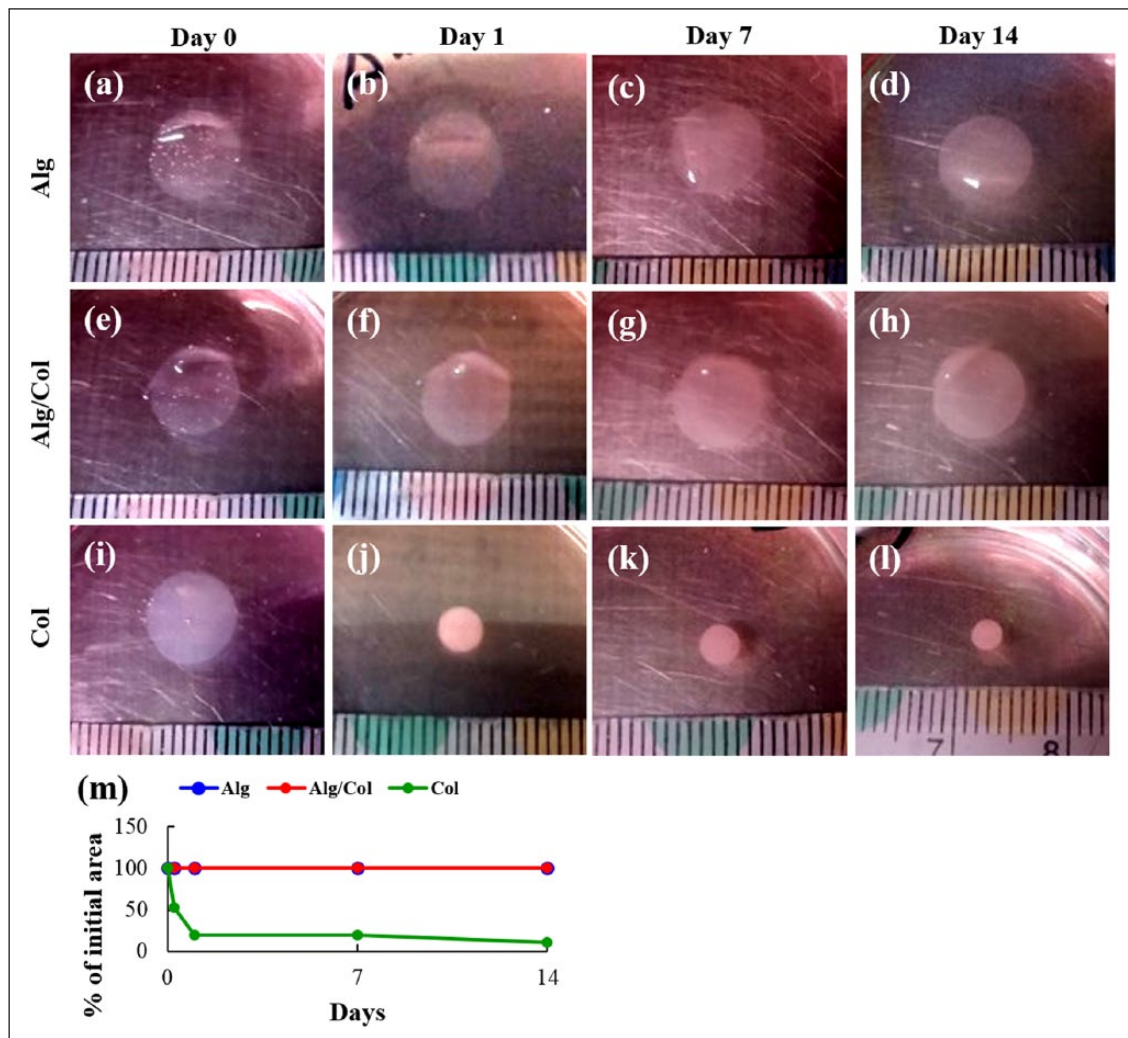
Figure 2. Gel contraction assay: (a–d) Alg gel, (e–h) Alg/Col gel, (i–l) Col gel; (a, e, i) day 0, (b, f, j) day 1, (c, g, k) day 7, (d, h, l) day 14; and (m) quantitative analysis of contractility of various gels.

of the chondrocytes were alive, and only few dead cells were observed in all groups at day 1. However, dead cells had significantly increased in Col gel after 7 days of culture. At this time point, the viability of cells remained also high in Col gel.

The cell growth level was examined by MTS cell proliferation assay, as shown in Figure 5. All gels showed a similar MTS level at day 1. Thereafter, the cells in Alg/Col gel and Col gel were shown to grow slowly. In particular, Col gel showed the highest cell proliferation.

Chondrogenic phenotype expressions

The expression of chondrocyte-related genes, *SOX9*, type II collagen, and aggrecan, was analyzed at day 14 (Figure 6). The genes increased with the addition of collagen in the Alg/Col gel and appeared to be the most significantly up-regulated in the Col gel. The genes of chondrocyte dedifferentiation, type I collagen and *ALP*, were only expressed in the

Col gel. In contrast, the genes were not detected in Alg gel and Alg/Col gel. The expression of type II collagen was further examined by the immunofluorescence staining (Figure 7). Positive signals for type II collagen were clearly observed in the three groups. Large cartilaginous nodules were observed in Alg/Col gel and small nodules in Alg gel. The chondrocytes remained mostly as single cells in the peripheral area of Col gel. The production of cartilaginous matrix of the cell–gel constructs at 14 days was further investigated by the DMMB assay (Figure 8). The results showed that the production of GAG was the highest in Col gel, moderate in Alg/Col gel, and the lowest in Alg gel.

Discussion and conclusion

This study investigated the efficacy of hydrogels based on natural polymers of type I collagen and alginate to provide a chondro-permissive microenvironment that can encourage cellular proliferation and preserve chondrogenic phenotype.
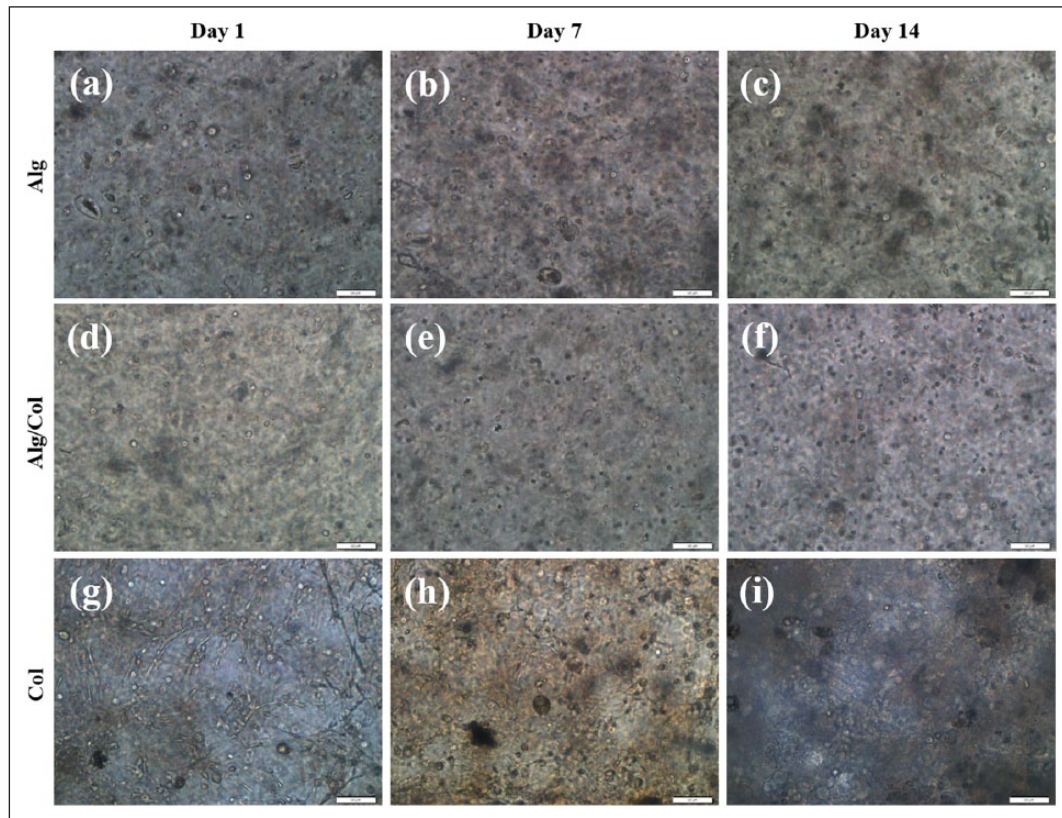
Figure 3. Phase contrast images of the chondrocytes cultured within various gels at day 1, day 7, and day 14: (a–c) Alg gel, (d–f) Alg/Col gel, (g–i) Col gel; (a, d, g) day 1, (b, e, h) day 7, (c, f, i) day 14. Scale bar: 50 μm.
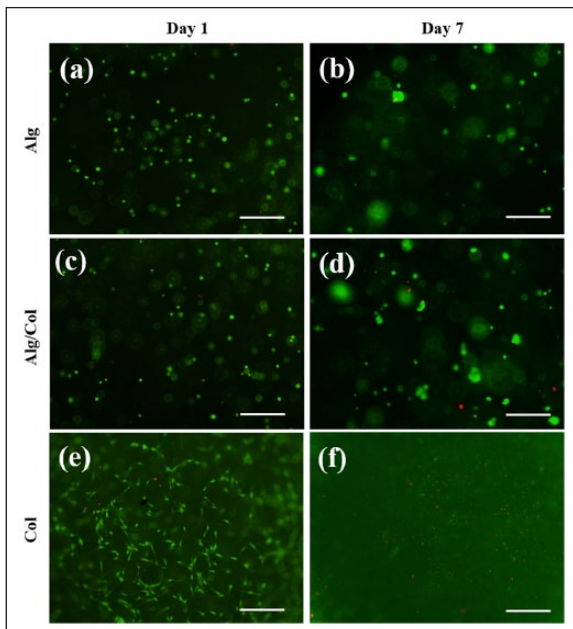

Figure 4. Fluorescence image of chondrocytes cultured in various gels at day 1 and day 7 by the Live/Dead assay. Live cells were marked with green-fluorescent calcein, and dead cells were labeled with red-fluorescent ethidium homodimer-1. (a, b) Alg gel, (c, d) Alg/Col gel, (e, f) Col gel; (a, c, e) day 1, (b, d, f) day 7. Scale bar: 240 μm.
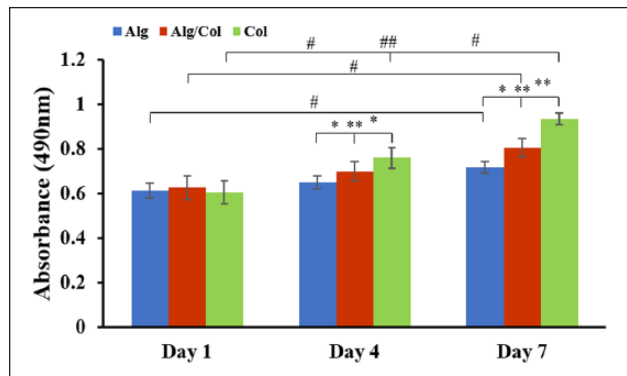

Figure 5. MTS assay for the cell viability in various gels at day 1, day 4, and day 7. Statistical analysis was performed using one-way ANOVA followed by a post hoc LSD test. Symbols above bars indicate statistically significant differences (#,*$p < 0.05$, ##,**$p < 0.01$, n = 3). # indicates differences with same gel group (different time-points), * indicates differences with different gel groups (same time-points).

Therapies for articular cartilage repair include microfracture and cell-based ACI. Despite promising results, these clinical techniques fail to generate tissues that adequately restore damaged cartilage.[23,24] Hydrogels are promising candidates for culture of cells in cartilage
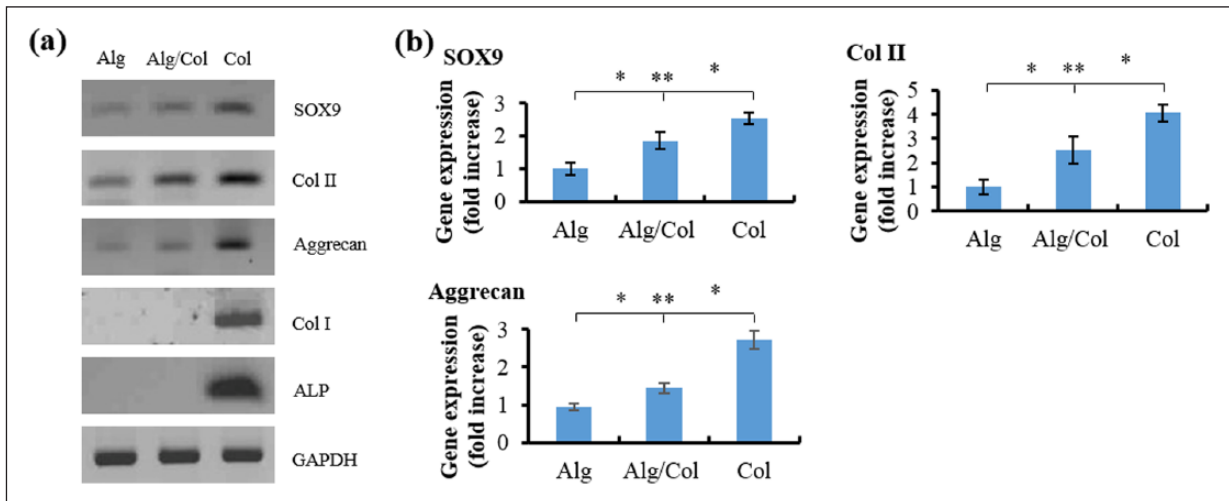
Figure 6. PCR assay for chondrogenic gene expressions in various gels at day 14: (a) RT-PCR and (b) qPCR. Statistical analysis was performed using one-way ANOVA followed by a post hoc LSD test. * indicates statistically significant differences with different gel groups (*p < 0.05,**p < 0.01, n = 3).
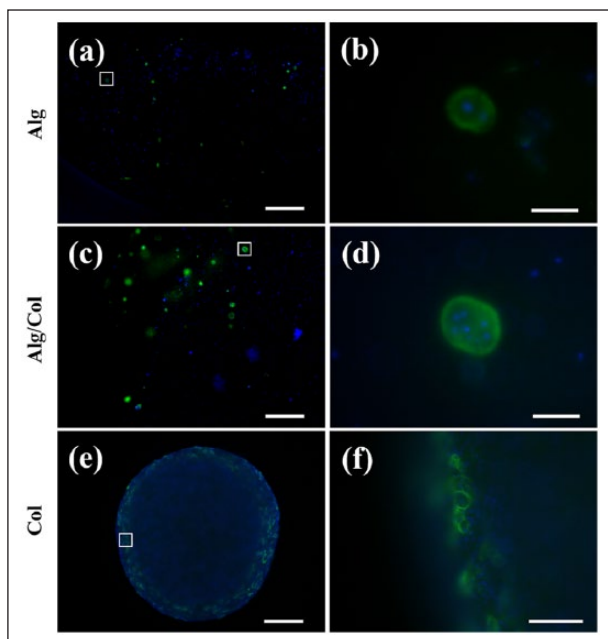

Figure 7. Type II collagen immunofluorescence images of the chondrocytes in various gels at day 14. (a) Alg gel, (c) Alg/Col gel, (e) Col gel; (b) enlargement of boxed area in (a), (d) enlargement of boxed area in (c), (f) enlargement of boxed area in (e). Scale bar: 500 μm (a, c, e), 60 μm (b, d), and 140 μm (f).
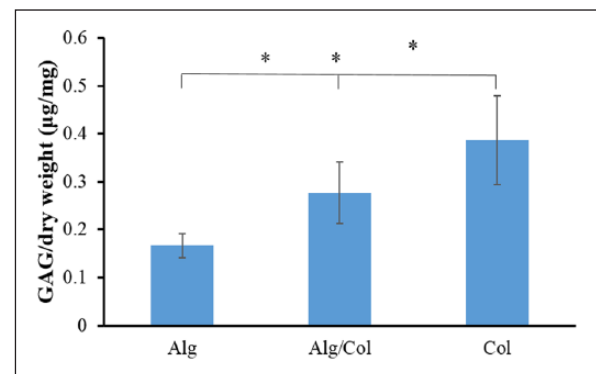

Figure 8. GAG production in various gels determined by DMMB assay at day 14. Statistical analysis was performed using one-way ANOVA followed by a post hoc LSD test. * indicates statistically significant differences with different gel groups (*p < 0.05, n = 3).

regeneration. They can mimic the native extracellular matrix with compositional and mechanical similarity to cartilage.[25,26] The rheological behavior of cell-free hydrogels, as examined by a dynamic mechanical analyzer, revealed that the moderate range of E′ of Alg/Col gel (90 kPa) being similar to that of tibia cartilage (30∼110 kPa)[27] may be favorable for cells to sense and secrete cartilaginous matrix.

When the chondrocytes are expanded in monolayer, they undergo dedifferentiation and hypertrophic differentiation. Chondrocyte hypertrophic differentiation is the gradual development process from chondrogenic differentiation to cartilage mineralization.[28,29] Therefore, it is possible that the expression of cartilage-related genes in Col gel is higher than that in other gels (Figure 6). Most importantly, the genes of chondrocyte dedifferentiation, type I collagen and *ALP*, were highly expressed in Col gel; the genes were not detected in Alg gel and Alg/Col gel (Figure 6). The results indicated that the chondrocytes in Col gel underwent dedifferentiation process and those in Alg gel and Alg/Col gel preserved chondrogenic phenotype. Van Susante et al.[20] noted that collagen gels promoted the proliferation and fibroblast-like dedifferentiation of chondrocytes, whereas alginate gel maintained

chondrocyte phenotype, which being in good agreement with our results.

Type I collagen displays a typical triple helix structure to form collagen fibers. The presence of the integrin-recognized sequence Arg-Gly-Asp (RGD) in the type I collagen can help the chondrocytes to attach and proliferate,[30] which can cause the cells to exhibit also fibroblastic-like morphology predominantly in Col gel. On the other hand, alginate inherently lacks the RGD sequence necessary for cell anchoring and thereby limiting cell adhesion and proliferation.[19,20] As a result, the cells in Alg gel cannot spread but form a round morphology, which is often favorable for the preservation of chondrocyte phenotype. The combination of alginate and collagen composition implemented in this study appeared to provide some optimized hydrogel conditions for chondrocytes to proliferate while preserving chondrogenic phenotype with suppressed dedifferentiation.

In fact, articular cartilage can withstand high compressive loads with little damage or degeneration due to the specific biological roles of chondrocytes under mechanical environments.[31] There are clear evidences for the important role of mechanical loading to facilitate articular cartilage regeneration. Most mechanical loading studies have been undertaken primarily under hydrostatic pressure and compression conditions. For example, hydrostatic pressure up-regulated type II collagen expression[32,33] and proteoglycan biosynthesis,[33–35] while down-regulating the expression of matrix metalloproteinase[36,37] and type I collagen[36] in chondrocyte cultures. It has also been established that compressive loading promotes chondrogenic differentiation,[38] increases cartilaginous matrix production,[39,40] and reduces the expression of hypertrophic markers[39] in MSC- and chondrocyte-seeded hydrogels. Those studies suggest that hydrostatic compression can positively affect the dedifferentiated chondrocytes and undifferentiated MSCs. Therefore, we speculate that the present 3D hydrogel culture system combined with stiffness-related biomechanical cues would prevent more effectively the chondrocyte dedifferentiation. However, more in-depth in vitro study is still needed to clarify the prevention mechanisms of chondrocyte dedifferentiation using our 3D hydrogel culture system, including the biological crosstalk between cells and hydrogels and potential signaling pathways of the preservation of chondrogenic phenotype.

In conclusion, the chondrocytes cultured in Alg/Col gel, when compared to those in Alg or Col gel, exhibited a level of proliferation and preserved chondrogenic phenotype with significantly suppressed dedifferentiation. Although cartilage-related genes were expressed at the highest levels in Col gel, the collagen gel contraction and the expression of dedifferentiated markers of chondrocytes could be prevented in the Alg/Col gel. The results suggest that the importance of gel matrix environment for the growth and the preservation of the chondrocyte phenotype with a simultaneous inhibition of dedifferentiation. These aspects may need special consideration to optimize hydrogel conditions for successful chondrocyte-based cartilage tissue engineering.

Declaration of conflicting interests

Funding

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