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ORIGINAL ARTICLE

TERM

Alginate-Hyaluronic Acid-Collagen Composite Hydrogel Favorable for the Culture of Chondrocytes and Their Phenotype Maintenance

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Articular cartilage has limited regeneration capacity, thus significant challenge has been made to restore the functions. The development of hydrogels that can encapsulate and multiply cells, and then effectively maintain the chondrocyte phenotype is a meaningful strategy to this cartilage repair. In this study, we prepared alginate-hyaluronic acid based hydrogel with type I collagen being incorporated, namely Alg-HA-Col composite hydrogel. The incorporation of Col enhanced the chemical interaction of molecules, and the thermal stability and dynamic mechanical properties of the resultant hydrogels. The primary chondrocytes isolated from rat cartilage were cultured within the composite hydrogel and the cell viability recorded revealed active proliferation over a period of 21 days. The mRNA levels of chondrocyte phenotypes, including SOX9, collagen type II, and aggrecan, were significantly up-regulated when the cells were cultured within the Alg-HA-Col gel than those cultured within the Alg-HA. Furthermore, the secretion of sulphated glycosaminoglycan, a cartilage-specific matrix molecule, was recorded higher in the collagen-added composite hydrogel. Although more in-depth studies are required such as the *in vivo* functions, the currently-prepared Alg-HA-Col composite hydrogel is considered to provide favorable 3-dimensional matrix conditions for the cultivation of chondrocytes. Moreover, the cell-cultured constructs may be useful for the cartilage repair and tissue engineering.

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Key Words: Chondrocytes; Alginate; Hyaluronic acid; Collagen type I; Cartilage regeneration

INTRODUCTION

Articular cartilage is an avascular connective tissue populated by chondrocytes and has poor intrinsic regeneration capability [1-3]. For the cartilage repair and regeneration, cell transplant is considered as one of the most promising treatments [4]. In autologous chondrocyte implantation, chondrocytes are used to restore damaged cartilage, relieving the pain while improving the joint function [5,6]. However, there are still hurdles to use cells as a therapeutic medicine for cartilage tissue regeneration. Chondrocytes are easily dedifferentiated in *in vitro* cultures, and it is difficult to obtain enough population of cells required. To address this limitation well-designed culture systems or 3D scaffolds for chondrocytes should be established [7-10].

Extra cellular matrix (ECM) supports the chondrocytes during culture, maintains their characteristics or recovers phenotypes [11-13]. Hydrogels are widely studied for this purpose to provide an *in vivo* mimic niche for chondrocytes [14]. In fact, chondrocytes easily dedifferentiate into fibroblastic cells on 2D culture conditions, whereas 3D cultures using hydrogels restrain this dedifferentiation [15]. Moreover, the hydrogel matrix properties can alter the cell survivability and maintenance of cellular phenotype [16]. Therefore, finding a suitable matrix component, i.e., chondrocyte-favorable ECM, is an important issue in cartilage regeneration [17].

Natural polysaccharides including alginate (Alg) and hyaluronic acid (HA) are frequently used as a hydrogel matrix due to their water-soluble, biocompatible, biodegradable, and non-immunogenic characteristics [18,19]. Alg is a suitable material to encapsulate and deliver cells under mild cross-linking conditions [20]. HA is prevalent in the cartilage and the synovial fluid of joints, and has a special role in the retention of proteogly-

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cans and water that is important in regenerating cartilage tissue [21-24]. Furthermore, HA regulates cellular processes by binding to cell surface receptors like CD44 [25]. The combination of Alg and HA components allowed the hydrogel formation easily, and produced cartilaginous ECM molecules in the *in vitro* culture of chondrocytes [26-30]. However, Alg has a low bioactivity for the chondrocyte proliferation [31-33] and HA is rapidly degraded during cell cultures, thus requiring more optimized physio-chemical properties for cartilage regeneration [34].

Here, we add collagen type I (Col) to the Alg-HA composite gel. Col is frequently used in the hydrogel synthesis in concert with other biopolymer components to provide structure and mechanical stability and/or bioactive and cell stimulating motifs [35]. Col is the adhesion molecule enabling many different types of cells to recognize the matrix through integrins, like α1β1, α2β1, α10β1 and α11β1 [36,37]. Another aspect of Col, particularly in relation with Alg or HA molecules, is the possible chemical interactions, i.e., Col amine groups can interact with carboxyl groups of Alg or HA, resulting in the improvement of stability of the hydrogel. We hypothesize that these characteristics of Col and its relation with other components may provide the biophysical/chemical environment favorable for the culture of chondrocytes and cartilage engineering. In fact some studies have also reported the beneficial role of Col in the cell maintenance and chondrogenesis [38-40].

The aim of this study is thus to establish an effective 3D matrix based on Alg-HA-Col components for the culture, maintenance, and expansion of chondrocytes without losing their phonotype. The Alg-HA-Col hydrogel was engineered to provide the chondrocytes with tissue ECM-like conditions, thus to preserve therapeutic requirement of phenotypically stable chondrocytes for cartilage tissue engineering.

MATERIALS AND METHODS

Materials

Sodium Alg (MW 32000−250000) was purchased from Duksan Science Co. Ltd., (Ansan, Korea), type I collagen solution (Col) from rat tail (4 mg/mL) was from BD Biosciences (USA), and sodium hyaluronate (HA, MW 4800 kDa) from Genewel Co. Ltd. (Hwaseong, Korea).

Hydrogels fabrication

The sodium Alg and the sodium hyaluronate (HA) were dissolved in Dulbecco's modified Eagle medium (DMEM) by magnetic stirring for 24 h at 4°C. The Alg-HA solution was mixed with Col solution at an equivalent volume, and the final ratio of Alg:HA:Col was 10:2:1 by weight. The pH of the mixture solution was adjusted to pH 7.4 by the addition of 1 M NaOH aqueous solution. This ratio was based on our preliminary works that could generate a hydrogel with proper physical stability and suitability for cell culture inside the gel. The composite was crosslinked by the addition of 50 mM $CaCl_2$, and the gelation was made by incubating at 37°C for 15 min in 5% CO_2 incubator.

Characterizations of physico-chemical properties

The chemical bond structure of the samples was analyzed by Fourier transform infrared spectrometer (FT-IR; Varian 640-IR, Varian Australia Pty Ltd., Mulgrave, Australia) in the wavelength range of 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1}. For the measurement, the samples were lyophilized (Freeze dryer, Ilshin Lab. Co. Ltd., Korea). Dynamic mechanical analysis was conducted to characterize the viscoelastic mechanical behaviors of the hydrogels (6 mm diameter, 4 mm height), ranging in the frequency from 0.5 to 10 Hz with a constant amplitude displacement of 0.1 mm using a dynamic mechanical analyzer (01dB-Metravib, Limonest Cedex, France). All experiments were performed at room temperature and the average values for storage (E') modulus (E'') were obtained. Differential scanning calorimetry (DSC) was carried out by using a thermal analysis system (Setaram Instrumentation, Caluire, France) calibrated and operated in the temperature range of 30−400°C. The hydrated samples (5 mg) was heated at a rate of 10°C min^{-1} in an aluminum pan sealed by a lid, under a nitrogen atmosphere using an empty pan as the reference.

Chondrocyte preparation

Chondrocytes were isolated from the resting zone of costochondral cartilage growth plate and rib region of adult Sprague-Dawley rats, and a detail protocol was described elsewhere [41]. Briefly, dissected ribs were cut into small pieces and digested with 10 mL Dulbecco's Phosphate-Buffered (DPBS) containing 0.25% EDTA and incubated for 20 min at 37°C, and then followed by an incubation in 0.25% trypsin (Gibco, Invitrogen, Carlsbad, CA, USA) for 1 h and 0.2% collagenase type 2 for 3 h. The digested tissues were centrifuged at 2000 rpm and then filtrated with sterile 45-μm sieve filter (BD Bioscience, Franklin Lakes, NJ, USA). Chondrocytes were plated at a density of 25000 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium (DMEM)-low glucose containing 10% fetal bovine serum, 1% penicillin/streptomycin, 50 μg/mL ascorbic acid, 1% ITS premix (Sigma-Aldrich Korea Ltd., Korea), in an atmosphere of 5% CO_2 and 95% humidity at 37°C. For the *in vitro* cell test, at passage 3, the confluent cells were prepared for encapsulating into the hydrogels.

In vitro cell tests

The samples were divided into two experimental groups; Alg/

HA/Col and Alg/HA. 3×10^5 cells were encapsulated in each hydrogel of 1 mL. During the chondrocytes culture, the culture medium was changed every three days until harvest.

After culture of 14 days, the viability of cells in the hydrogel was examined by a Live/Dead assay (Invitrogen Corporation, Carlsbad, CA, USA). In order to evaluate the cell distribution inside the hydrogels, the fluorescence images were taken from sliced hydrogels at 3 days. The live/dead cell analysis of the constructs was made by washing with DMEM medium three times, followed by an incubation at 37°C for 30 min in a solution containing 4 μM EthD-1 (ethidium homodimer1) and 2 M calcein AM made in sterile PBS. The constructs were observed using a fluorescence microscope (Olympus, lX 71, Japan) with excitation filters of 450−490 nm [green, Calcein-acetoxymethyl (AM)] and 510−560 nm [red, ethidium homodimer-1 (EthD-1)], LIVE/DEAD® Kit (Invitrogen, Carlsbad, CA, USA).

DNA quantification assay

The dsDNA content was determined by a fluorescence image with excitation at 480 nm and emission at 520 nm in 96 well plates using a microplate reader (SpectraMax plus, Microplate reader, USA). The binding of Pico Green dye (Invitrogen, Carlsbad, CA, USA) was used as against standard for calf thymus DNA, according to the manufacturer's protocol.

Quantitative real-time polymerase chain reaction

After culture for 7 and 21 days, the expression of the cartilage-associated genes, including SOX-9, collagen type II (Col II), and Aggrecan (Agg) was confirmed by qPCR. The first strand complementary DNA was synthesized from the total RNA (1 μg) using a superscript first strand synthesis system for RT-PCR (Bioneer Ltd., Daejeon, Korea) 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, Carlsbad, CA, USA). The relative transcript quantities were calculated using the $2^{-\Delta\Delta Ct}$ method, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous reference amplified from the samples. The primer sequences of cartilage-associated genes (Bioneer Ltd., Daejeon, Korea) used for the real-time PCR are shown in Table 1.

Histological evaluation

For general histological staining, cryocut slides were prepared by fixing hydrogels in 4% paraformaldehyde followed by freezing the samples in O.C.T compound (Tissue-Tek, Sakura, Japan) at -80°C and sectioning at thickness 10 μm by a cryocut microtome (leica CM3050S, GmbH). For Safranin 'O' staining analysis, cryosections were stained with fast green for 5 min, quickly rinsed in 1% acetic acid solution, stained with 0.1% Safranin O solution for 5 min, and dehydrated in 100% ethanol. The slides were mounted by putting cover slip with resin mounting medium.

To assess immunofluorescence staining for collagen type II, cryocut slides as mentioned were rinsed with DPBS to remove embedding compound followed by a permeabilization with 0.1% Triton X-100 for 10 min. The samples were then blocked with 1% BSA, after which they were probed with primary antibody (collagen type 2A1, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) with incubation overnight at 4°C, and then washed with DPBS three times. After this, samples were incubated with TRITC labeled secondary rabbit anti-mouse antibody (dilution 1:500) (Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature, and mounted with VECTASHEILD (H-1000, Victor Lab, Burlingame, CA, USA) for antifade. Images were captured using the fluorescence microscope (Olympus lX 71, Olympus Corp., Tokyo, Japan).

GAG content measurement

At harvest, dissolution buffer (55 mM sodium citrate, 30 mM EDTA, 0.15 M NaCl) were used to isolate cells followed by a digestion in papain digestion buffer containing 1.0 mL of 20 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA, 2 mM dithiothreitol and 300 g Papain for 40 min at 60°C. GAG content was determined spectrophotometrically at 525 nm followed by the reaction with 1, 9-dimethylmethylene blue dye (DMMB) (Proteoglycan detection kit, Astarte Biologics, Redmond, WA, USA) using chondroitin sulfate (Bovine Trachea, Astarte Biologic, Redmond, WA, USA) standard 10 μg.

Statistical analysis

All results are presented as means±one standard deviations from four replicate samples (n=4). Statistical analysis was performed by Student's t-test, and $p<0.05$ or $p<0.01$ were considered to be significant for the statistical analyses.

Table 1. Primer sequence for chondrogenic specific genes

Gene		Sequences
GAPDH	Forward	5'-CTGGAAGATGGTGATGG-3'
	Reverse	5'-GATTTGGTCGTATTGGGCG-3'
Aggrecan	Forward	5'-TCGCAAGTCCCTTCCACATC-3'
	Reverse	5'-TCAAGGCGTCCTGAAGTGTC-3'
Collagen type II	Forward	5'-GCTGGTGCACAAGGTCCTAT-3'
	Reverse	5'-AGGGCCAGAAGTACCCTGAT-3'
SOX9	Forward	5'-CCAGCAAGAACAAGCCACAC-3'
	Reverse	5'-CTTGCCCAGAGTCTTGCTGA-3'

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

RESULTS

Characteristics of produced hydrogels

The possible chemical interactions between components were characterized by FT-IR (Fig. 1A). In general, pure Col has amide peaks at characteristic wavelengths (amide I at ~1610 cm^{-1}, and amide II at ~1400 cm^{-1}) [42]. A closer examination of these amide peaks in the Alg-HA-Col composite gel showed some changes; a significant decrease in the symmetric stretching amide I with a strong amide II peak remained in the composite hydrogel. Furthermore, the amide I band appeared to shift toward higher wavenumber (i.e., blue shift, from 1403 cm^{-1} to 1408 cm^{-1}), which was attributed to the interactions between collagen and other components (primarily HA). When the Col molecules form chemical interactions with other molecules incorporated, this blue shift in amide band has often been observed [43,44]. The DSC thermal analysis of the composite samples revealed that the glass transition temperature (T_g) of the composites was higher in Alg-HA-Col than in Alg-HA (from 65°C to 72°C) (Fig. 1B), and the result also suggests the possible additional chemical interactions between Col and Alg-HA because the increase in Tg is implicated in strengthening of the chemical bond structures of polymer networks. The mechanical properties of the hydrogels were analyzed by a dynamic mechanical analyzer. The storage modulus change in a frequency sweep of 0.5 Hz to 10 Hz showed a typical hydrogel behavior under a dynamic compression and relaxation mode (Fig. 1C). The modulus values of both hydrogels were in the ranges of ~50−80 kPa, and the Alg-HA-Col hydrogel revealed higher values than the Alg-HA. This modulus range of hydrogels is considered to provide matrix stiffness similar to the cartilage tissue, which is favorable for cells to sense and se-

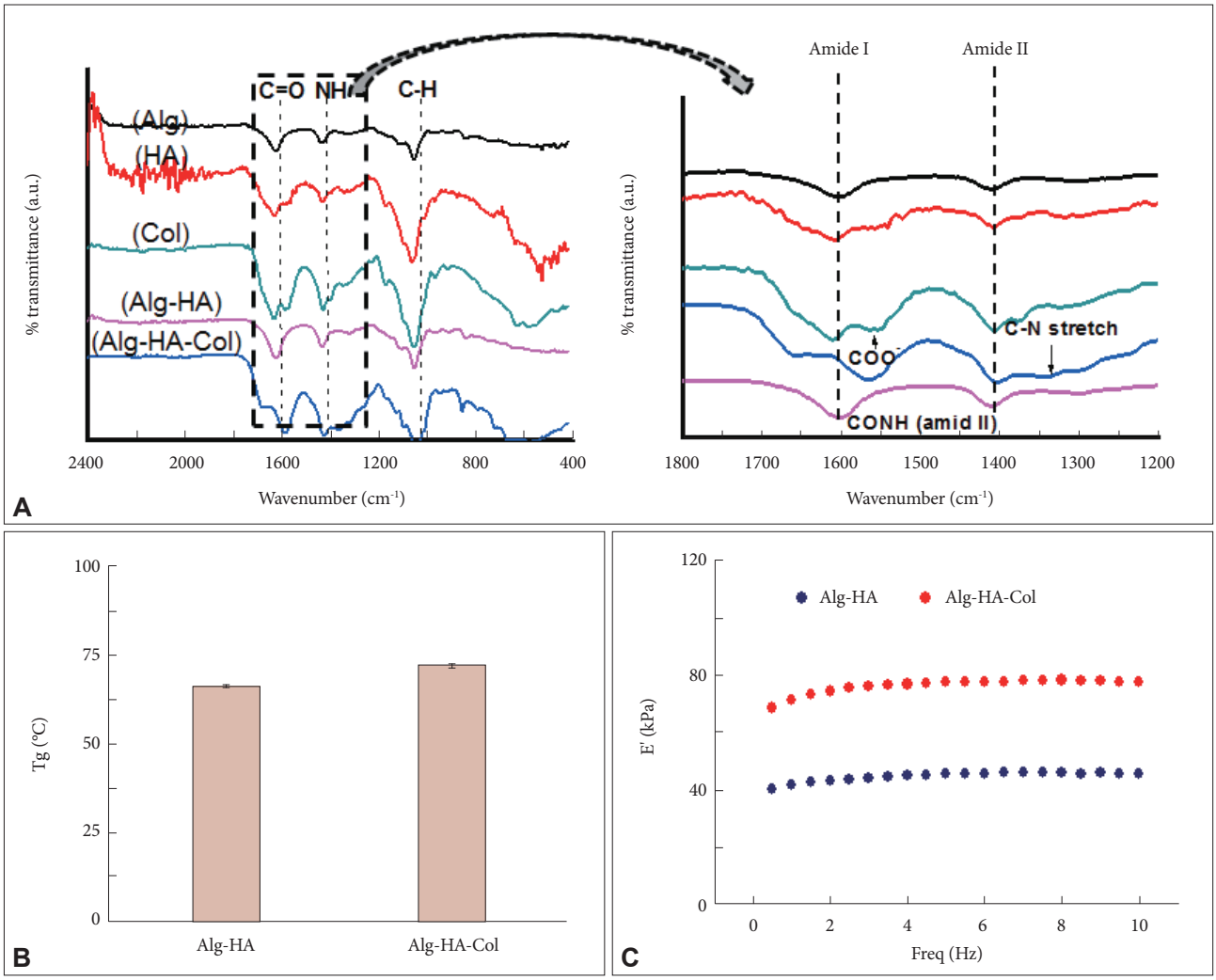

Figure 1. Characterizations of composite hydrogels: (A) FT-IR spectra (enlarged spectrum also included), (B) differential scanning calorimetry, and (C) dynamic mechanical elastic behavior of samples. FT-IR: fourier transform infrared spectrometer.

crete cartilaginous matrix [31].

Cellular growth behaviors in hydrogels

Chondrocytes were cultured within the two types of hydrogels for up to 21 days, and the cell morphology was examined under a phase contrast microscope, as shown in Figure 2. Cells were observed to distribute at low density in both hydrogels at day 3, and some cellular aggregates were developed in the hydrogels at day 7. In particular, the aggregates were larger in Alg-HA-Col than in Alg-HA. From days 7 to 21 the cell population was substantially enhanced, and spherical aggregates of chondrocytes were profoundly observed in Alg-HA-Col hydrogel. The cellular behaviors of this spherical aggregate formation (round cell shape not elongated) are indicative of a chondrogenic maintenance of chondrocytes, and the volumetric increase of the aggregates is considered to represent the maturation of cells [45,46]. Even the chondrocytes, once deprived of their phenotypes in 2D conventional cultures, can regain their de-differentiated phenotype when cultured within the properly-controlled 3D matrices, with the sign of aggregated and spherical morphology of cells rather than flattened morphology [47]. Thus the morphological data of cell aggregates suggests that the Alg-HA-Col composite hydrogel can be considered to provide a matrix condition suitable for chondrocytes to multiply the number and to maintain phenotype, which is ultimately helpful for the production of cartilage ECMs.

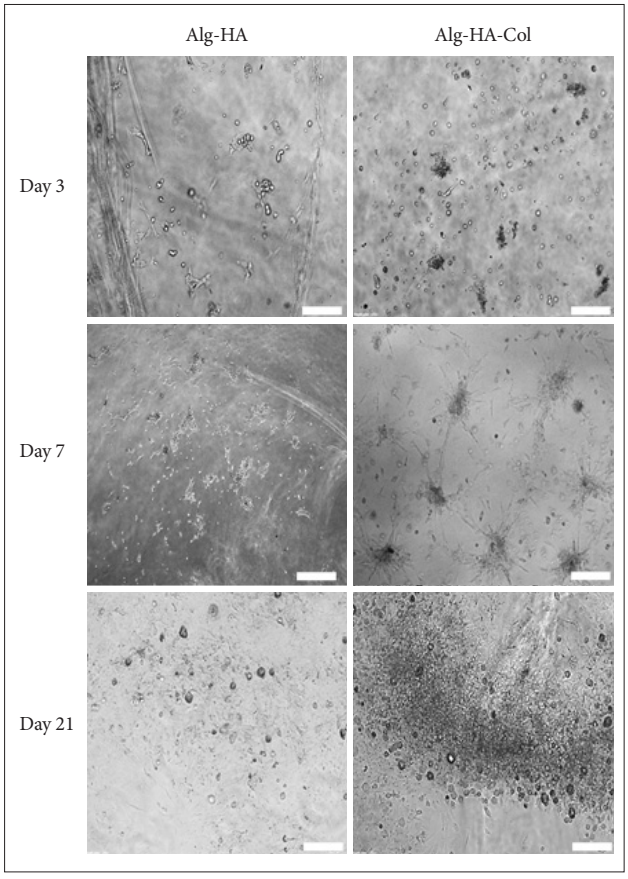

Figure 2. Phase contrast images of the chondrocytes cultured within Alg-HA and Alg-HA-Col hydrogels for 3, 7, and 21 days. Scale bar: 500 μm.

The live/dead assay of cells revealed a major cell population was viable in both hydrogels (Fig. 3A). The DNA content of cells grown in the hydrogels showed significantly higher level in Alg-HA-Col than in Alg-HA, particularly at day 21, suggesting the chondrocytes proliferated more actively within the Alg-HA-Col gel during this culture period (Fig. 3B). The reason for this improvement in chondrocyte proliferation is that the collagen type I can also present essential binding motifs for chondrocytes to recognize through integrins, including α1β1, α2β1, α10β1, and α11β1 (mainly those with β1 subset) [48-50]. Therefore, the addition of collagen to the composite gel is considered to favor the cellular recognition of the gel matrix conditions. Considering that a sufficient cell number is generally required for the cell therapy in cartilage engineering, this high proliferative nature of chondrocytes supports the merited aspect of the Alg-HA-Col hydrogels.

Production of chondrogenic markers

Next we analyzed the chondrogenic gene expressions of the cells within the hydrogels at 7 and 21 days, as shown in Figure 4. *Sox9* is expressed at an early stage of a chondrogenic differentiation, showing higher levels at day 7 than at day 21 for both gels. In particular, the gene level was significantly higher when cultured within Alg-HA-Col than within Alg-HA. Other chondrogenic markers, including collagen type II and aggrecan, were also analyzed. Cells cultured within Alg-HA-Col expressed higher gene levels than those within Alg-HA. In particular, the gene expression levels increased with time (from day 7 to 21) for both gels, implying these two genes are late chondrogenic markers.

Next, the safranin-O stain of cells was performed at day 21 to evaluate the production of GAG molecules (visualized in orange color). The results showed that the stain in Alg-HA-Col gel was higher than that in Alg-HA gel (Fig. 5A). Furthermore, the immunofluorescence staining for collagen type II revealed more positive stain of cells in Alg-HA-Col gel than in Alg-HA gel (Fig. 5B). The DMMB assay showed that the amount of GAG content gradually increased from day 7 to day 21, and the GAG content was significantly higher in Alg-HA-Col gel than in Alg-HA gel, in good agreement with the results of immunohistochemistry (Fig. 5C).

DISCUSSION

The aim of this study is to evaluate the potential of composite Alg-HA-Col 3D gel in the culture of chondrocytes and the maintenance of chondrogenic phenotypes. The three components (alginate, HA, and collagen type I) used herein were shown to have chemical interactions as deduced from IR spectrum and Tg value; and the modulus values also justify proper

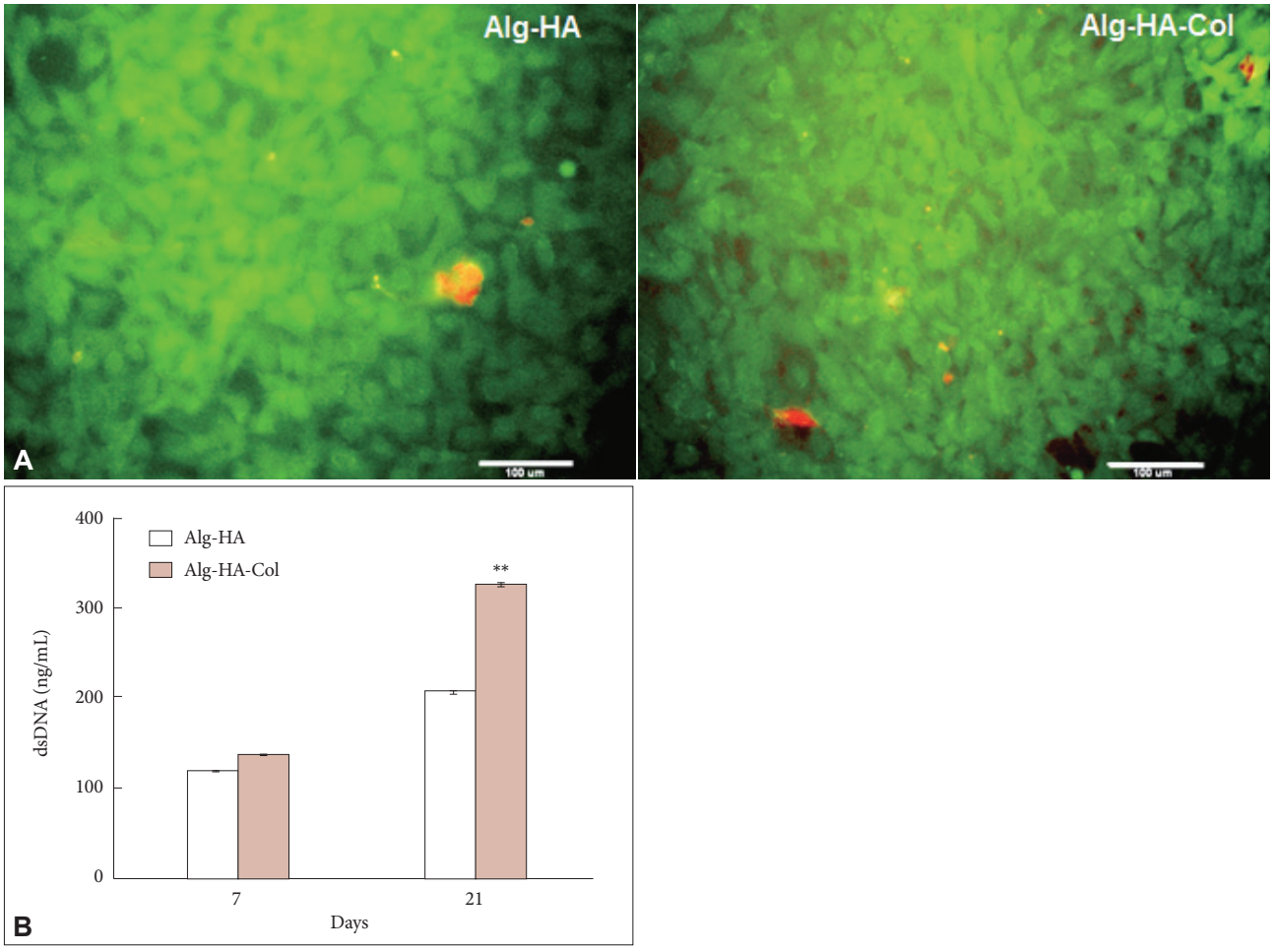

Figure 3. (A) Live (green)/dead (red) cell fluorescence images. (B) Quantification of total DNA content. *p<0.05 statistical significant between two groups at each period (n=3, Student's t-test). Scale bar in (A): 100 μm.

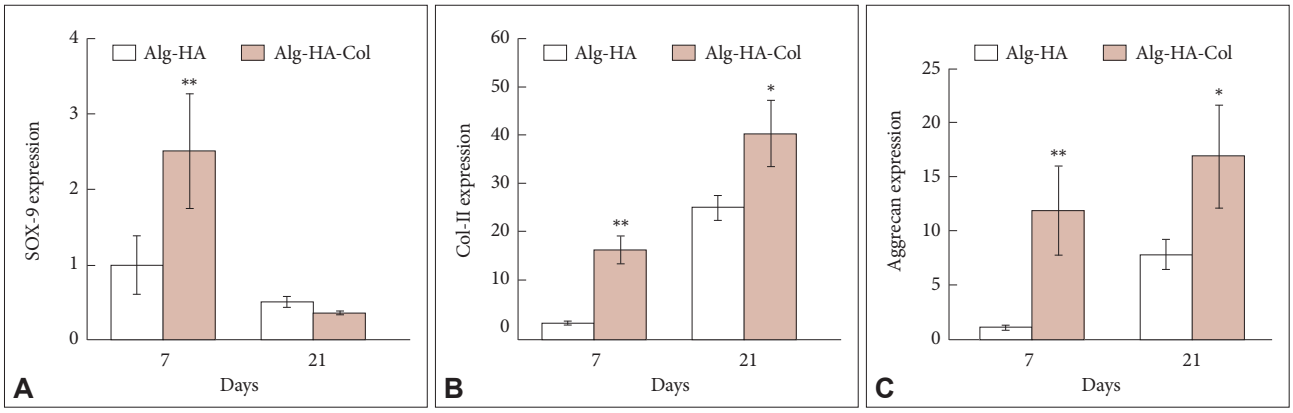

Figure 4. Q-PCR mRNA expression of (A) SOX9, (B) collagen type II, and (C) aggrecan, as quantified by $2^{-\Delta\Delta Ct}$ at 7 and 21 days. *p<0.05 and **p<0.01 statistical significant between two groups at each period (n=3, Student's t-test).

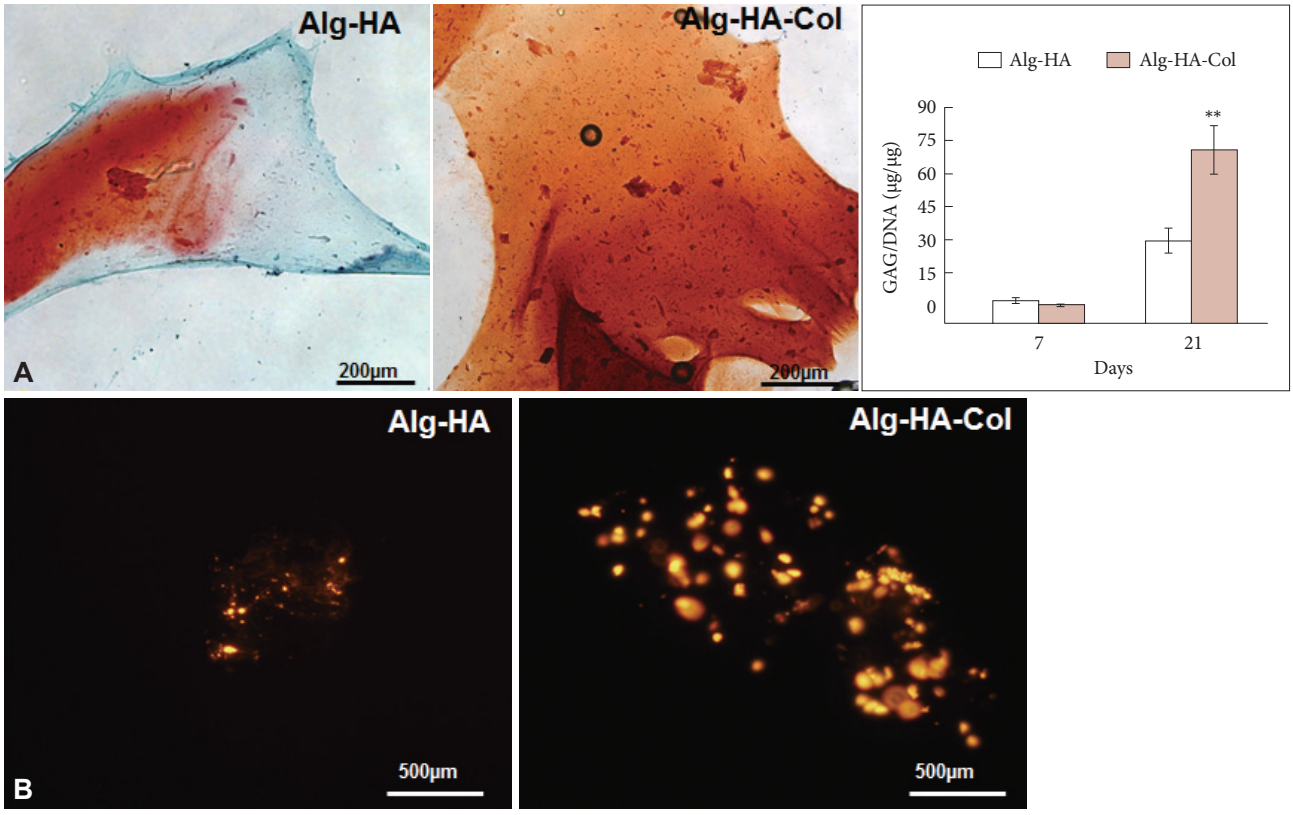

Figure 5. Observation of ECM production in chondrocytes within the hydrogels: (A) Safranin 'O' staining for GAG, and GAG quantification by DMMB assay, showing total sulphated proteoglycan. (B) Immunostaining for collagen type II. $^{**}p<0.01$ statistical significant between two groups at each period (n=3, Student's t-test).

conditions for chondrocyte culture and cartilage engineering. In particular, the presence of RGD domains in collagen and CD44 ligand in HA can synergistically help the chondrocyte attachment and proliferation. These aspects of the gels engineered herein may provide 3D matrix conditions favorable for the culture of chondrocytes and their phenotype maintenance.

The spherical aggregate formation (round cell shape not elongated) of cells in the composite gel is indicative of a chondrogenic maintenance, and the volumetric increase of the aggregates is considered to represent the maturation of cells [45,46]. Even the chondrocytes, once deprived of their phenotypes in 2D conventional cultures, can regain their de-differentiated phenotype when cultured within the properly-controlled 3D matrices, with the sign of aggregated and spherical morphology of cells rather than flattened morphology [47]. Thus the morphological data of cell aggregates suggests that the Alg-HA-Col composite hydrogel can be considered to provide a matrix condition suitable for chondrocytes to multiply the number and to maintain phenotype, which is ultimately helpful for the production of cartilage ECMs.

Cells were shown to have higher proliferation potential in collagen-containing gel. The reason might be that the collagen type I also presents essential binding motifs for chondrocytes to recognize through integrins, including α1β1, α2β1, α10β1, and α11β1 (mainly those with β1 subset) [48-50]. Therefore, the addition of collagen to the composite gel is considered to favor the cellular recognition of the gel matrix conditions. Considering that a sufficient cell number is generally required for the cell therapy in cartilage engineering, this high proliferative nature of chondrocytes supports the merited aspect of the Alg-HA-Col hydrogels.

The gene expression results demonstrated the essential chondrogenic phenotypes were highly stimulated in proper time courses when cultured within the Alg-HA-Col gel matrix, confirming that the cells substantially proliferated and aggregated during the period became highly functional with significant expressions of chondrogenic markers. Moreover, the quantification assays to confirm the collagen type II and GAG contents demonstrated the cellular aggregates were highly functional in producing major ECM molecules in cartilage, suggesting the Alg-HA-Col gel could provide biophysical (mechanical and stability) and biochemical (adhesion motifs) environment for chondrocytes to develop cartilage-like matrix during the long-term *in vitro* cultures. Another aspect in the later stage is to

overcome the hypertrophy as this is critical for the chondrocyte maturation and deregulation of ossification, which must be addressed in future study.

Conclusively, the incorporation of Col to Alg-HA gel enhanced the physico-chemical stability through chemical interactions and provided a binding motif for chondrocytes, which resulted in a higher proliferation capacity. The chondrogenic phenotypes were well preserved in the composite gels over the culture of 3 weeks, demonstrating the expression of a series of chondrogenic genes and the production of cartilage ECMs. The findings suggest that the Alg-HA-Col composite gel may provide 3D matrix conditions mimicking native cartilage ECM, and thus find a suitable matrix for future cartilage tissue engineering.

Conflicts of Interest

The authors have no financial conflicts 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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