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ORIGINAL ARTICLE

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# Porous Microcarrier-Enabled Three-Dimensional Culture of Chondrocytes for Cartilage Engineering: A Feasibility Study

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Cartilage repair is substantially intractable due to poor self-healing ability. Porous microspheres can be a fascinating three-dimensional matrix for cell culture and injectable carrier in cartilage engineering. In this study, we assessed the feasible use of porous biopolymer microspheres for chondrocyte carriers. When seeded onto the blended biopolymer microspheres and followed by a dynamic spinner flask culture, the chondrocytes showed robust growth behaviors during the culture period. The gene expressions of *SOX9*, type II collagen, and aggrecan were significantly upregulated after 2-week of culture. Furthermore, immunolocalization of type II collagen and secretion of glycosaminolglycan became prominent. The results suggest the feasible usefulness of the porous microspheres as the cell culture matrix and the subsequent delivery into cartilage defects.
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**Key Words:** Porous microspheres; Blend biopolymer; Chondrocyte; Cartilage engineering

## INTRODUCTION

Once articular cartilage is damaged in adult, it is almost intractable to regenerate due to the limited capacity for self-repair [1]. The long-term outcome of current clinical treatments has often been unsatisfactory. On the other hands, tissue engineering approach, i.e., utilizing cells and matrices together to regain the biological functions of damaged tissues, has shown some promise for the cartilage repair [2]. Among the cell sources introduced for cartilage engineering, chondrocytes, mesenchymal stem cells, and pluripotent stem cells have been used [3]; on the while, as for the biomatrices, synthetic and natural polymers, including poly(lactic acid), poly(glycolic acid), their copolymer, alginate, chitosan, and hyaluronan have often been used [4-7].

Scaffolds provide the three-dimensional (3D) environment onto which cells can grow and produce extracellular matrix of cartilage. While different types have been developed, including hydrogels [8], sponges [9], and fibrous meshes [10], injectable form has some merits of minimal incision during the transplantation [11,12]. One typical form of injectable scaffold is the microspheres that can be used to expand and scale up cells through 3D culture methods and then fill large defect spaces [12,13].

Previously, we showed that blended poly(D, L-lactide) (PLDLA)/poly(caprolactone) (PCL) porous microspheres significantly accelerated osteoblasts expansion and differentiation under proper dynamic culture conditions [13]. Based on this, we seek to utilize the blended microspheres for cartilage engineering. As a first step toward this, here we evaluate the chondrocyte growth and maintenance of chondrogenic phenotypes upon the porous microspheres under dynamic culture conditions, which can provide information on the feasible usefulness of the porous microspheres for future cartilage tissue engineering.

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## MATERIALS AND METHODS

### Microsphere preparation

PCL (80 kDa, Sigma-Aldrich, USA) blended with PLDLA (L-lactide: D, L-lactide=70:30, Sigma-Aldrich, USA) was fabricated into porous microspheres as described in our previous report [13]. In brief, the PCL and PLDLA were dissolved separately in chloroform at 10%. To generate pores, camphene was

 

added at 60% in the solvent. The PLDLA and PCL solutions were mixed at 1:3 by volume with the camphene solution. The solution was dropped into ice-cooled 2% poly(vinyl alcohol) solution, while gentle stirring at 450 rpm. After the pore generation, the microspheres were filtered through Millipore filter paper, washed with ice-cooled distilled water, and freeze-dried for further uses. The microsphere morphology was observed by scanning electron microscopy (SEM, Hitachi S-3000H, Japan) and optical microscopy. The size distribution of microspheres was determined based on the images.

### Cell seeding and culture

Rat primary articular chondrocytes used in this study were harvested from articular cartilage of the knees of Sprague Dawley rats. Briefly, the collected cartilage slices were minced into about 1 $mm^3$ pieces and incubated in 0.2% type II collagenase solution for 18 h. The cell suspension was filtrated through a 40 μm cell strainer and centrifuged at 300×g at room temperature for 10 min. The cell pellets were washed twice with phosphate buffer saline (PBS). The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, 4.5 g/L glucose; Gibco-BRL, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator containing 5% $CO_2$ at 37°C. The cells were used after four passages for the following experiments.

Before seeding cells, microspheres were sterilized with 70% ethanol for 2 h and washed with PBS solution three times. One milliliter of $1\times10^6$ suspended cells were added to the pre-wetted microspheres of 30 mg with the culture medium for 12 h, and cell-microsphere constructs were incubated under shaking with a sway of 45° at 3 rpm for 6 h using MyLab SLRM-3 Intelli-Mixer (SLRM-3, SeouLin Bioscience, Korea).

Thereafter, the cell-microsphere constructs were divided into experimental group and control group, and then a spinner flask culture system (S-flask 4500-1L, TAITEC, Japan) was employed. The control group was cultured in normal medium (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin); whereas, the experimental group was cultured in chondrogenic medium consisting of the normal medium supplemented with 50 μg/mL ascorbic acid, 1% ITS premix (Sigma, USA), 100 nM dexamethasone (Sigma, USA), and 10 ng/mL transforming growth factor-β1 (PeproTech, USA). The stirring speed of the spinner flask was set at 30 rpm. The experiments were carried out up to 14 days. The media were changed twice each week.

### Cell behavior observation in 2D and 3D environment

After 14 days, the cell-microsphere constructs were moved onto a 35-mm culture dish or embedded into 3D type I collagen hydrogels. The collagen hydrogels were prepared as described in our previous study [14]. The final concentration of collagen was 2 mg/mL. The constructs of both groups were cultured using the same normal medium at each time point. An inverted optical microscope (Olympus, Japan) was used to observe the cell behaviors.

### Quantitative real-time polymerase chain reaction

Quantitative analysis of the chondrocyte-related genes expressed at day 14 was conducted using a Rotor-Gene RG-3000A qPCR machine (Australia). The first strand complementary DNA 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 instructions. 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 ΔΔCt method with β-Actin as the endogenous reference gene amplified from the samples. The primer sequences of the genes are summarized in Table 1.

### Immunofluorescence staining

Immunostaining of the harvested samples at day 14 was performed to detect the expression of type II collagen. The samples were 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 30 min at room temperature. The nuclei of the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. The

**Table 1.** Primer sequences of chondrogenic genes for quantitative real-time polymerase chain reaction

| 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' |
| β-actin | 5'-ACGTTGACATCCGTAAAGAC-3' | 5'-TAATCTCCTTCTGCATCCTG-3' |

slides were examined with an inverted fluorescence microscope equipped with a DP-72 digital camera (Olympus Co., USA).

### Dimethyl methylene blue assay for GAG content

To measure GAG content, Rheumera Proteoglycan Detection Kit (Cat# 8000, Astartebio Ltd., USA) was used. Briefly, the 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 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.

### Statistics

Data were analyzed by the Student's t-test. All data shown are expressed as the mean±one standard deviation, and the statistical significance is considered at $p<0.05$, $p<0.01$, or $p<0.001$.

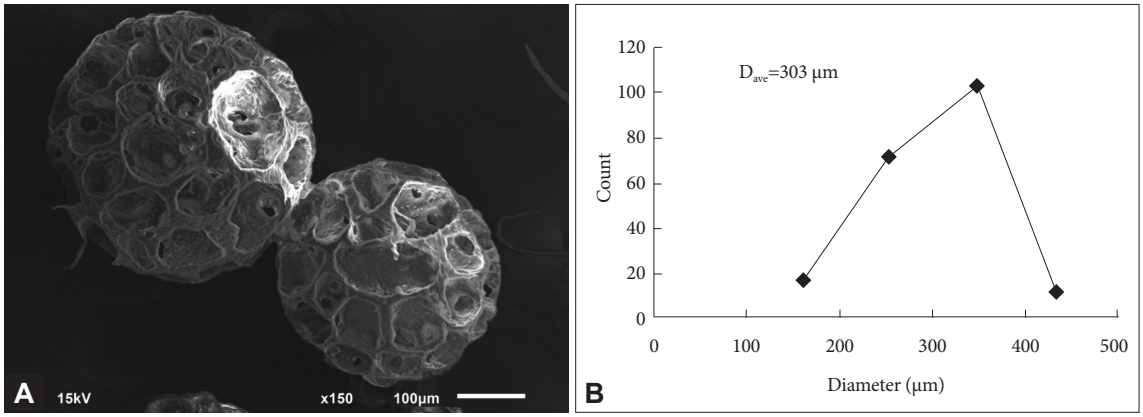


**Figure 1.** Porous microsphere characteristics. (A) SEM morphology appearance. (B) Size distribution of the microspheres. Average diameter of the microspheres is 303 μm (from a measurement of 202 microspheres). Scale bar indicates 100 μm. SEM: scanning electron microscopy.

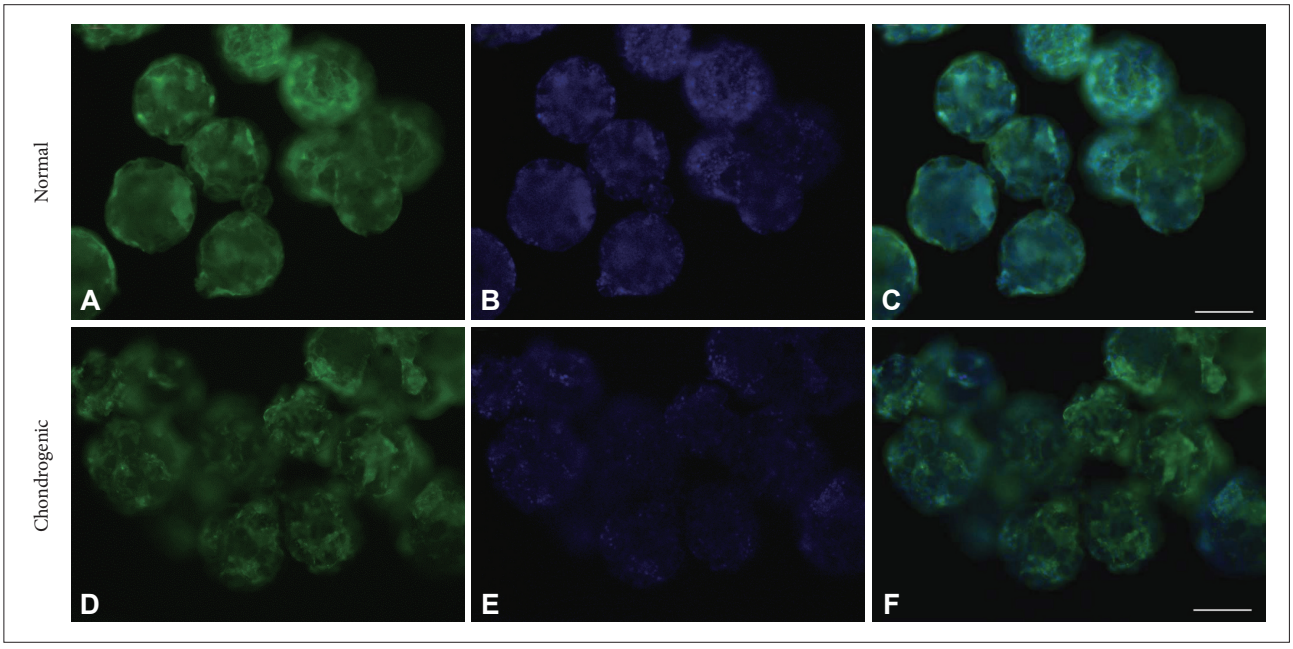


**Figure 2.** Fluorescence images of cell growth on the microspheres recorded for 14 days. Green, actin cytoskeletons stained with Alexa Fluor 488-conjugated phalloidin; blue, nuclei counterstained with DAPI. (A, B, and C) Control group, (D, E, and F) Experimental group. Scale bars indicate 200 μm. DAPI: 4',6- diamidino-2-phenylindole.

## RESULTS

### Porous microspheres

The blended PCL/PLDLA microspheres, as characterized by SEM, demonstrate well the highly porous morphology (Fig. 1A). The microspheres show a relatively broad size distribution; however, we choose those in the range of 150−450 μm (average diameter=303 μm, as shown in Fig. 1B) for the cell culture studies. The pores are highly open-channeled throughout the sphere and the sizes of pores are approximately 50−100 μm.

### Cell growth and migration behaviors

After 14 days of culture under dynamic spinner flask conditions, similar growth and distribution of the chondrocytes on the microspheres were observed in both the experimental and control groups as visualized by F-actin staining (Fig. 2). The stained images demonstrate robust growth of cells and the well-constructed cells upon/within the porous microspheres under both culture conditions. The number of cells adhered initially to the microspheres was approximately $1.81\times10^5$, corresponding to 18.1% of the initial cells seeded.

The cell-microsphere constructs were then moved onto a 35-mm culture dish or embedded within a collagen hydrogel to examine the cell migration toward surroundings. When transferred to 2D plastic dish, the chondrocytes cultured without the chondrogenic medium (control group) were shown to substantially migrate from the microspheres. Moreover, the cells actively proliferate and spread on the 2D dish with a spindle shape, showing the behaviors similar to fibroblastic cells (Fig. 3A). However, the cells cultured with chondrogenic cues (experimental group) rarely migrate to grow on the plastic substrate (Fig. 3B). When the cell-microsphere constructs were cultured within the 3D collagen gel, both culture conditions (differentiated and undifferentiated) enable the outgrowth of cells (the chon-

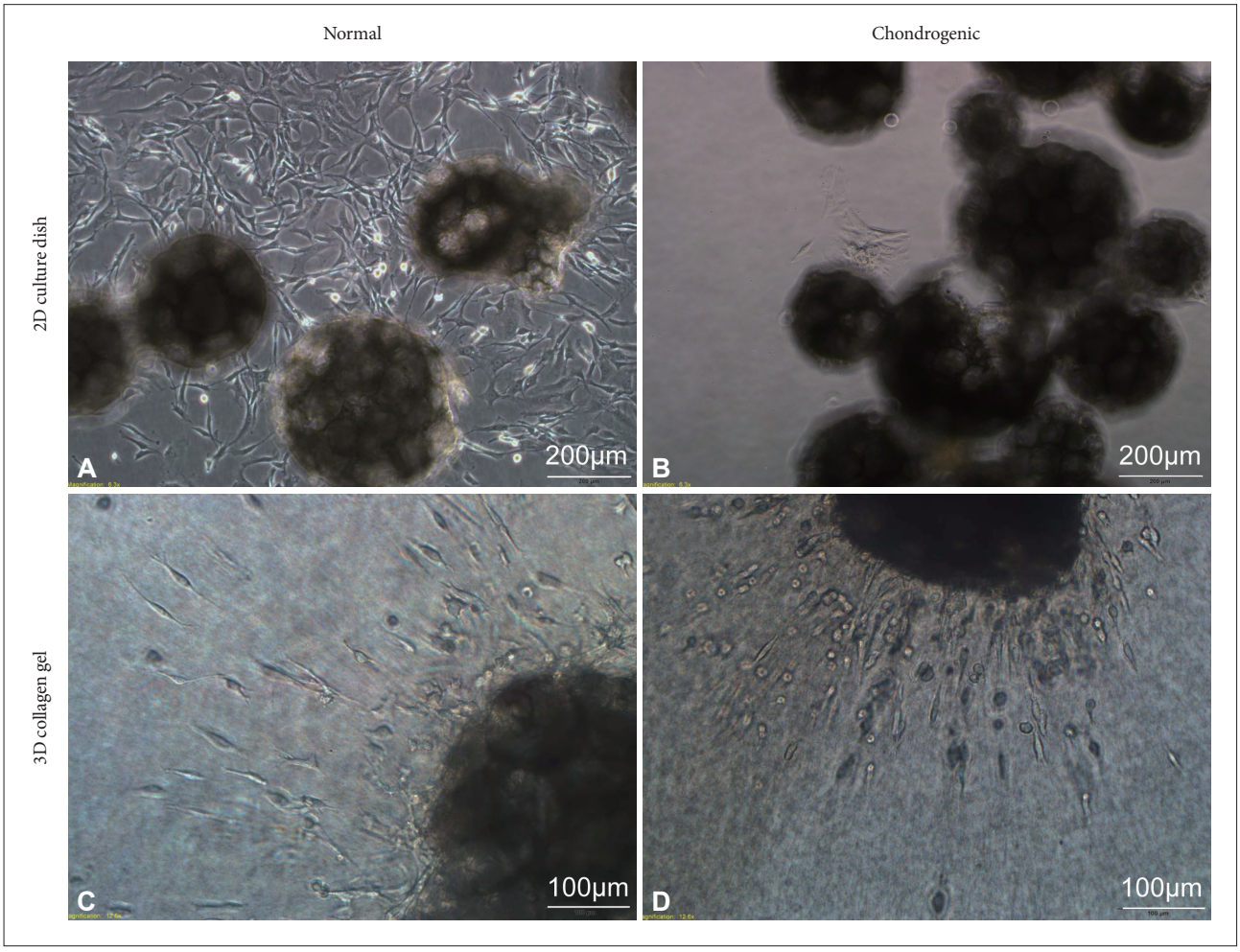


**Figure 3.** Cell migration behaviors from the cell-microsphere constructs, in 2D and 3D conditions. (A and C) Control group, (B and D) Experimental group. (A and B) The cell-microsphere constructs were cultured on 35-mm 2D plastic dish for 7 days, (C and D) The constructs were cultured within 3D collagen gels for 3 days. 2D: two-dimensional, 3D: three-dimensional.

drocytes toward gel matrix) although the cell shapes are slightly different. The cells in the control group move actively toward collagen gel and form a spindle shape in radial direction (Fig. 3C). On the other hand, the cells differentiated also migrate toward collagen gel, which though, in a bit limited way, i.e., the sprouting process is not so progressive as the case in control cells, rather, a major fraction of cells are round-shaped (as arrowed), preserving a typical morphology of chondrocytes (Fig. 3D). This is attributed to the chondrocyte maturation in the chondrogenic medium.

### Chondrogenic phenotype expressions

The expression of chondrocyte-related genes was then analyzed at 14 days (Fig. 4). The qPCR analyses demonstrate that the experimental group shows significantly higher expressions of *SOX9*, type II collagen, and aggrecan than the control group, by a difference of 2.5-, 4.2-, and 3-fold, respectively. The specific expression of type II collagen was further examined by the immunofluorescence staining (Fig. 5). The result shows that the experimental group has more positive signals with respect to the control group. The DMMB assay shows that the production of GAG is much higher in the experimental group than the control group (Fig. 6), indicating the cells substantially secrete mature extracellular matrix molecules under the culture conditions.

## DISCUSSION

The blended PCL/PLDLA microspheres used herein were those applied in our previous work [14]. This porous structure

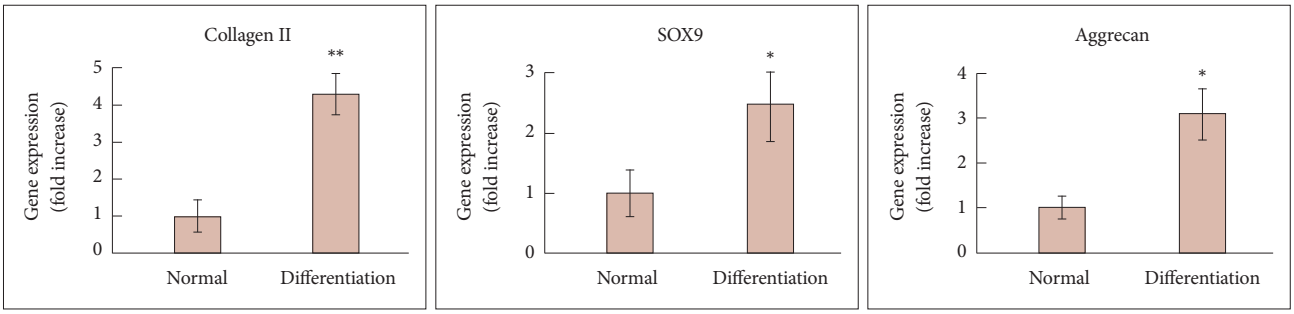


**Figure 4.** Quantitative PCR analyses of chondrogenic gene expression at 14 days of chondrocytes cultured on the microspheres under the chondrogenic medium and the normal medium. A significant difference between groups was noted at *$p$<0.05, **$p$<0.01. n=3. PCR: polymerase chain reaction.

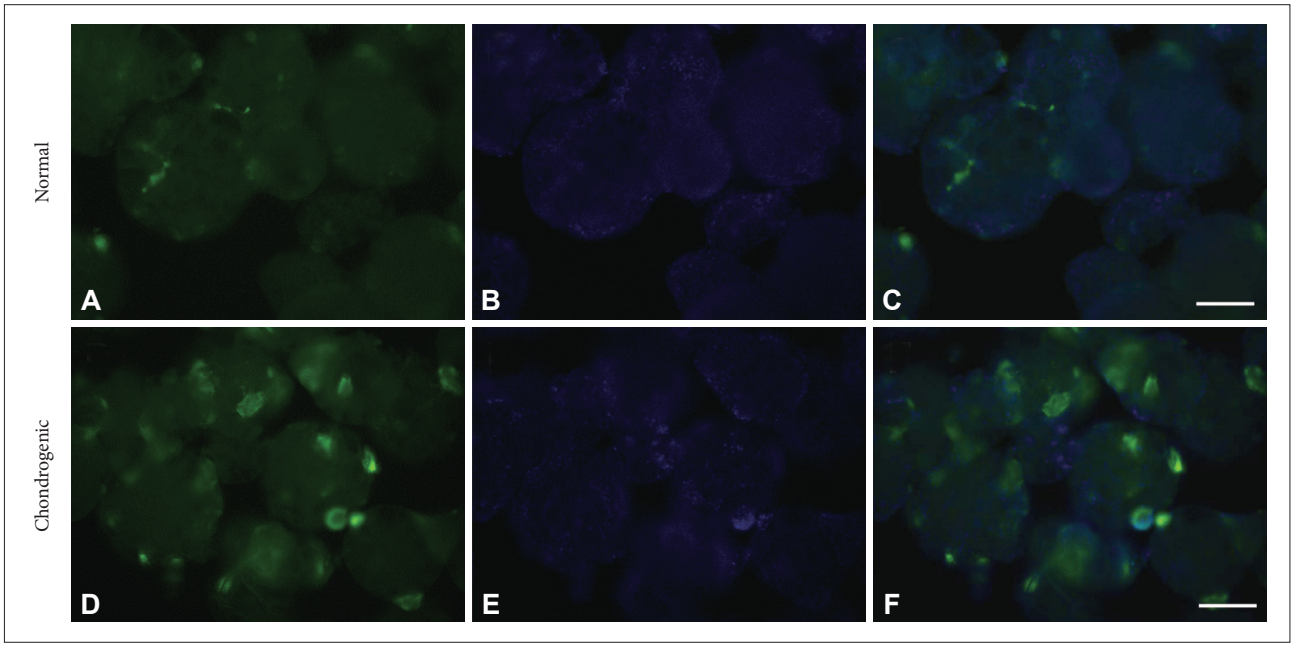


**Figure 5.** Immunofluorescence images of the cell-microsphere constructs cultured for 14 days. (A, B, and C) Control group, (D, E, and F) Experimental group. (A and D) FITC-conjugated type II collagen (green), (B and E) DAPI-stained nuclei of cells (blue), and (C and F) merged images. Scale bars indicate 200 μm. FITC: fluorescein isothiocyanate, DAPI: 4',6-diamidino-2-phenylindole.

is required not only to achieve sufficient cell seeding density (loading capacity) within the scaffold, but also to facilitate in- and out-transport of nutrients and oxygen for subsequent cell survival and proliferation [5]. Furthermore, this unique form of porous microspheres can be potentially considered as an injectable carrier of cells for cartilage tissue engineering, enabling minimally invasive surgery of joint space under arthroscopy; the injectable delivery of cells can minimize patient suffering and operating time, and fill various sizes and shapes of defects easily [17]. Even so, for the successful application of the microcarriers in cartilage repair, several issues related with the microcarrier design and injection, i.e., the microsphere size and pore structure as well as the possible shear-induced cell damage and syringe blocking, should be carefully considered which need further studies.

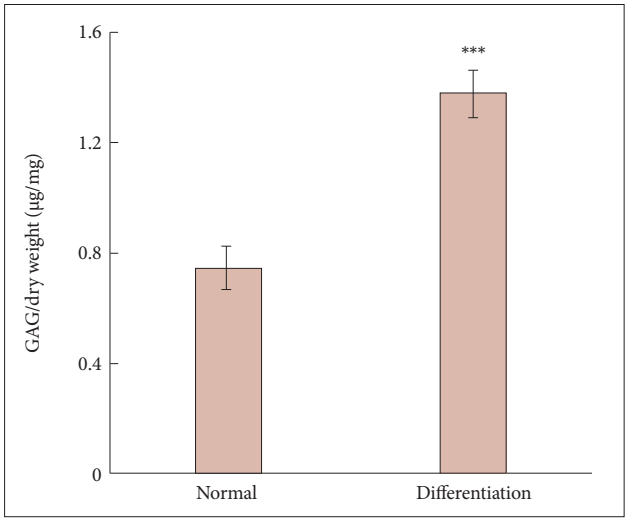


**Figure 6.** GAG content of the cell-microsphere constructs after 14 days of culture, as determined by DMMB assay. GAG content was normalized to dry weight for all samples. A significant difference between groups was noted at ***$p$<0.001, n=3. GAG: glycosaminoglycan, DMMB: dimethyl methylene blue.

Cell migration in 3D environments also occurs when the cell-microsphere constructs are injected to a cartilage defect wherein cells are interactive with surrounding tissue environment. One interesting point in the results is that the chondrogenic committed cells appeared to favor the collagen gel matrix as they migrate toward the gel, which however, was not readily observed in the 2D plastic dish. It is thought that the soft gel matrix condition, not the stiff dish substrate, might be attractive for chondrocyte to recognize and populate upon, and regarding this further study may be needed to elucidate the underlying mechanisms.

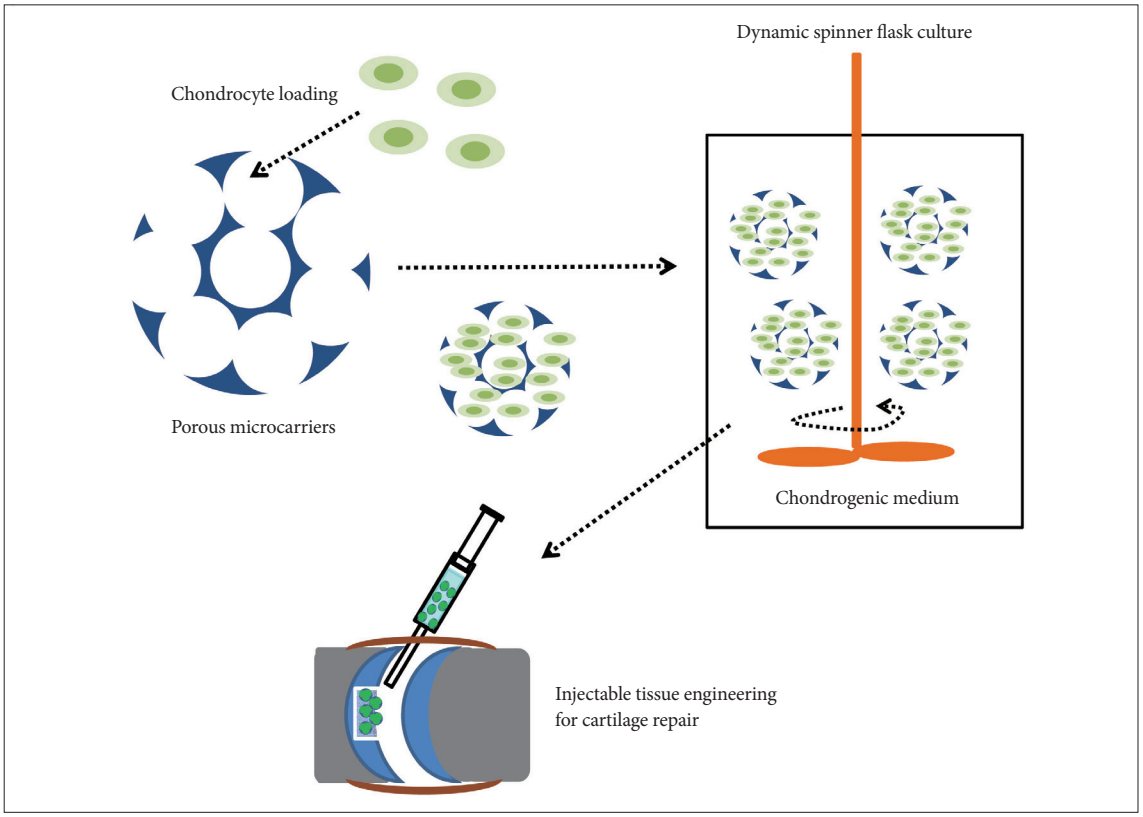


**Figure 7.** Schematic illustration showing the future cartilage engineering approach that can utilize the construct of chondrocytes/porous microspheres enabled by the dynamic and chondrogenic induction culture.

Our results demonstrate that chondrogenic environment allows the chondrocytes to secret a substantial level of cartilage extracellular matrix molecules, and further imply the porous microspheres provide appropriate 3D matrix conditions for the primary chondrocytes to engage in matrix synthesis and maturation process when supplied with proper biochemical signals, which is ultimately useful for the cartilage regeneration.

The current experiments, although considered as a priori study, explain the feasible use of the microcarrier system for chondrocyte culture in cartilage engineering. In particular, the loading of cells onto biodegradable porous microspheres followed by the dynamic spinner flask culture method sufficiently populates and expands primary chondrocytes. Upon the chondrogenic biochemical cues, the cells are primed to express substantial chondrogenic markers. Further study remains as to confirm the in vivo efficacy of the cell-microcarrier constructs for their applications in cartilage regeneration. Figure 7 illustrates the future cartilage engineering approach that can utilize a part of this study, where the chondrocyte/porous microsphere constructs are generated by the dynamic and chondrogenic-induction culture.

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## Conflicts of Interest

The authors have no financial conflicts of interest.

## Ethical Statement

There are no animal experiments carried out for this article.

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