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Enhancement of chondrogenic differentiation of rabbit mesenchymal stem cells by oriented nanofiber yarn-collagen type I/hyaluronate hybrid

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ABSTRACT

Cartilage defects cause joint pain and loss of mobility. It is crucial to induce the chondrogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) by both biological and structural signals in cartilage tissue engineering. Sponge-like scaffolds fabricated using native cartilage extracellular matrix components can induce the BMSC differentiation by biological signals and limited structural signals. In this study, an oriented poly(L-lactic acid)-co-poly(ε-caprolactone) P(LLA-CL)/collagen type I (Col-I) nanofiber yarn mesh, fabricated by dynamic liquid electrospinning served as a skeleton for a freeze-dried Col-I/hyaluronate (HA) chondral phase (SPONGE) containing both structural and biological signals to guide BMSC chondrogenic differentiation. In vitro results show that the Yarn Col-I/HA hybrid scaffold (Yarn-CH) promotes orientation, adhesion and proliferation of BMSCs better than SPONGE. Furthermore, BMSCs seeded on the Yarn-CH scaffold demonstrated a large increase in the glycosaminoglycan content and expression of collagen type II following a 21-day culture.

1. Introduction

Cartilage defects cause joint pain and loss of mobility due to osteoarthritis, trauma, aging, and developmental disorders [1–3]. Nowadays, cell-based therapy for cartilage repair provides a promising means to restore joint functions [4,5]. In cartilage tissue engineering, bone marrow mesenchymal stem cells (BMSCs) have been commonly employed as a major source for seeding cells for tissue engineering application to solve the problem of the deficit of chondrocytes [6,7]. However, it is crucial to induce the differentiation of BMSCs into the cartilaginous phenotype for sufficient repair of cartilage.

The BMSCs' chondrogenesis can be influenced by both biological and structural signals from various scaffolds [5,8,9]. Sponge-like scaffolds, especially, fabricated using native cartilage extracellular matrix (ECM) components, have shown sound, promising repair results in regard to the biological signals that guide BMSC chondrogenic differentiation [10,11]. However, there is very polarized, dense organization of fibrils in native cartilage. It has been demonstrated that the oriented fibers (similar to native cartilage) can better regulate the distribution, alignment, and migration of BMSCs compared to non-oriented scaffolds [12–14]. Thereafter, incorporation of oriented fibers into the sponge-like scaffolds, fabricated using native cartilage ECM components, may provide a more physiological microenvironment containing both structural and biological signals to guide BMSC chondrogenic differentiation.

We previously reported our precedent setting use of a novel nanofiber yarn (Yarn) established by a dynamic, liquid electrospinning method, mainly created out of the blend of poly(L-lactic acid)-co-poly(ε-caprolactone) P(LLA-CL) and natural biomaterials [15]. This Yarn scaffold was biodegradable, biocompatible and highly porous in nature. Hyaluronate (HA) is a major component of ECM in cartilage tissue which can trigger a sophisticated signaling pathway to retain the phenotype of chondrocytes [16]. In this study, an oriented yarn mesh was fabricated by our previously established dynamic, liquid electrospinning method and subsequently used as the skeleton of the

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freeze-dried Collagen type I (Col-I)/HA scaffold to reproduce certain cartilaginous environmental cues, especially within the superficial zone. It was hypothesized that the Yarn Col-I/HA hybrid scaffold (Yarn-CH) could improve the chondrogenic differentiation of BMSCs. The BMSC chondrogenesis of the Yarn-CH was investigated.

2. Experimental

2.1. Materials

Poly(L-lactic acid)-co-poly(ε-caprolactone) (P(LLA-CL)) (Mw = 300 kDa; LA:CL = 75:25) was obtained from Nara Medical University, Japan. Collagen type I (Col-I) and hyaluronic acid (HA, sodium salt, Mw = 1.0 MDa) were purchased from Sichuan Ming-Rang Bio-Tech Co. Ltd, China and Sigma-Aldrich Chemical Co. (St. Louis, MO), respectively. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco (Grand Island, NY). All other reagents and media were of reagent grade or better and purchased from Invitrogen, unless otherwise indicated.

2.2. Fabrication of nanofiber yarn-Col-I/HA hybrid scaffold

The fabrication of the nanofiber yarn-collagen type I/hyaluronate hybrid scaffold (Yarn-CH) involved the processes of electrospinning combined with freeze-drying. Specifically, at the outset, a dynamic, liquid supporting system [17] for electrospinning was adopted to prepare the yarn mesh which served as a skeleton for the porous scaffold. A water vortex was created in the basin including a hole at the bottom with a diameter of 8 mm. The drained water was deposited in a tank and recycled to the basin using a pump to keep the water level constant. A blended solution of Col-I and P(LLA-CL) (8 wt.%, Col-I:P(LLA-CL) = 10:90) in 1,1,1,3,3,3-hexafluoro-2-propanol was loaded into a syringe, charged with a voltage of 15 kV and then fed at a feed rate of 1 mL/h. Electrospun nanofibers were continually deposited in the water vortex and twisted into yarn by flowing along with the water through the hole. The yarn was collected by a rotating mandrel (60 r/min), frozen at −80 °C for 6 h and freeze-dried overnight. Col-I and HA were dissolved in 0.05 M acetic acid under consistent stirring overnight to yield the blended solution with a concentration of 1% (Col-I:HA = 1:1).

For fabrication of Yarn-CH, the Col-I/HA solution was dripped onto the surface of the yarn until the yarn was fully immersed in the solution. Then, the yarn, suspended in the Col-I/HA solution was frozen at a target temperature of −80 °C for 6 h and freeze-dried overnight in preparation for the final scaffolding. Finally, the Yarn-CH was punched into dish scaffolds (5 mm in diameter) for further study. As a control, a Col-I/HA sponge (SPONGE) was fabricated via freeze-drying. The Col-I/HA solution was subsequently frozen at −80 °C for 6 h and then freeze-dried overnight to obtain the SPONGE.

2.3. Characteristics of the scaffolds

For scanning electron microscope (SEM) observation, all specimens were sputter coated with gold. The surface morphology and the pore size of the scaffolds were determined using a Hitachi TM 1000 SEM at an accelerated voltage of 10 kV.

Porosities of the scaffolds were determined using the liquid displacement method [18,19]. Briefly, the scaffold was immersed in a given volume (V1) of hexane in a measuring cylinder for 10 min and the total volume of the scaffold and hexane was recorded as V2. The resulting volume was recorded as V3, after the removal of the scaffold. The porosity (ρ) of each scaffold was calculated using the following formula: Porosity (%) = (V1 − V3) / (V2 − V3).

2.4. Isolation and culture of rabbit BMSCs

Animal experiments were carried out in accordance with the policies of Shanghai Jiao Tong University School of Medicine and the National Institutes of Health. Rabbit bone marrow mesenchymal stem cells (BMSCs) were obtained from 16-week-old New Zealand white rabbits as in our previous study [20]. Briefly, under anesthesia induced by intramuscular administration of ketamine hydrochloride (60 mg/kg) and xylazine (6 mg/kg), about 5 mL of marrow was extracted from the right iliac crest by a needle, flushed with 10 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%, w/v), penicillin (100 units/mL), and streptomycin (100 mg/mL) and then cultured at 37 °C, 5% CO_2 in a humidified incubator. The unattached cells were removed via the culture medium changing after 5 days of incubation and exchange the cell medium once every three days. After the primary 2-week culture, the cells were detached for subculture. Third-passage cells were used in the experiment. To seed cells onto the samples, cells were pipetted directly onto the specimens at a density of 1×10^4 cells/cm^2 (for proliferation and morphology assay) or 5×10^7 cells/mL (for chondrogenic differentiation assay) for different lengths of culture time. After sterilizing by immersion in 75% ethanol for 1.5 h, the electrospun matrices in a 96-well plate were washed repeatedly with phosphate-buffered saline (PBS, pH 7.4) to remove residual ethanol. A tissue culture plate was used as a control.

2.5. Confocal analysis for cell orientation

The cytoskeletal arrangements on different specimens were observed by the use of actin staining after 24 h. Briefly, the BMSCs on the different specimens were fixed in 4% paraformaldehyde for 10 min. After removing the fixative, the cells were permeabilized using 0.2% Triton X-100 (Sigma Aldrich) for 10 min and then rinsed three times in PBS. The cytoskeleton was stained with 20 μg/mL of phalloidin (Sigma) and the cell nuclei were stained by 1 μg/mL DAPI for 5 min before imaging under a confocal laser scanning microscope (Leica TCS SP2; Leica Microsystems, Heidelberg, Germany) [21].

2.6. Cell proliferation

Cells cultured on different specimens were analyzed for proliferation by 3-(4, 5-dimethyl-2-thia-zolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. After incubation for 1 and 4 days, MTT solution (5 mg/ml) (Sigma) was added to each sample (100 μL/well) for an additional 4 h

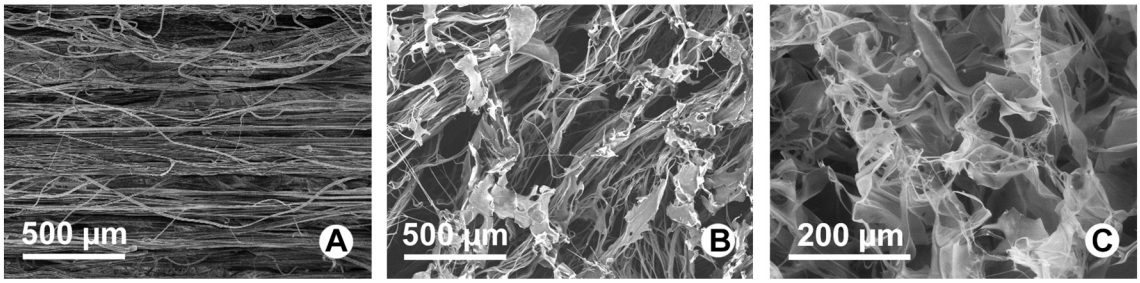

Fig. 1. SEM showing surface morphological features of Yarn (A), Yarn-CH (B) and SPONGE (C), respectively.

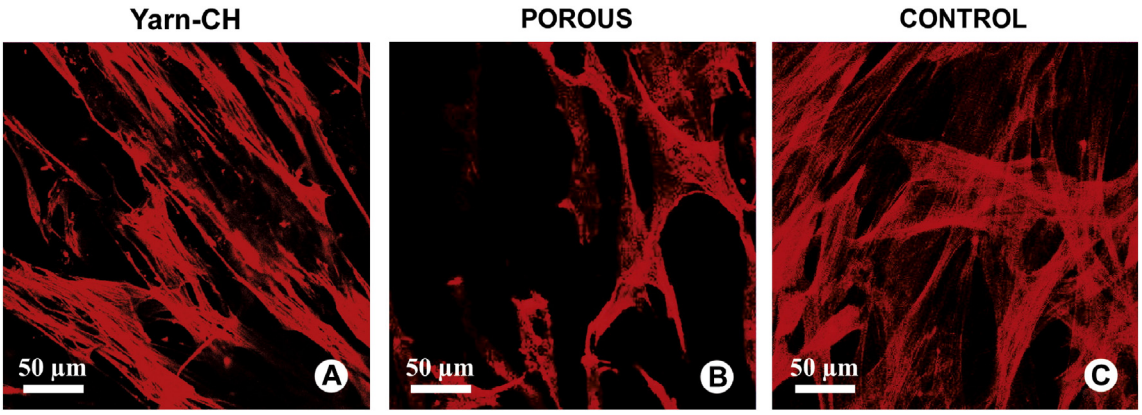

Fig. 2. Cytoskeletal arrangement of BMSCs on the surface of Yarn-CH (A), SPONGE (B) and culture tissue plate (control) (C).

at 37 °C. Then, the medium was removed, and the samples were washed with PBS. Dimethyl sulfoxide (1 mL) was used to dissolve the MTT formazan crystals on a shaking platform for 10 min, and 200 μL of the solution was transferred into a 96-well plate to determine the absorbance at 490 nm using a spectrophotometer (Synergy 2; BioTek, Winooski, VT).

2.7. Chondrogenic differentiation of BMSCs

The scaffolds were pre-immersed in culture media for 24 h to promote cell attachment. After cell seeding with a final seeding density of 3×10^6 cells/scaffold, the cell-scaffold constructs were incubated for 4 h to allow the cells to completely adhere to the scaffolds. Then the constructs were placed in a six-well plate and incubated in the normal medium described above or a chondrogenic medium, which contained 10 ng/mL TGF-β1 (R&D), 100 nmol/L dexamethasone, 50 μg/mL ascorbate-2-phosphate, 40 μg/mL proline, 100 μg/mL pyruvate (all from Sigma), and 1:100 diluted ITS Premix (Becton Dickinson). After incubation for 7 and 21 days, the samples were fixed in 4% paraformaldehyde and 5 μm cross sections were obtained after dehydration, clarification, infiltration and paraffin embedding. The sections were stained with safranin O (Sigma-Aldrich, USA) and images were obtained using a microscope (LEICA DM 4000 B).

2.8. Quantitative analysis of in vitro chondrogenesis

After 1 and 3 weeks of in vitro culture, glycosaminoglycan (GAG) content, and total collagen content were analyzed according to previously described methods to quantitatively evaluate the effects of oriented nanofibers on in vitro chondrogenesis [22]. Briefly, the cell–scaffold complex was rinsed with doubly-distilled H_2O, lyophilized for 12 h followed by adding 1 mL cold H_2O and incubated at 4 °C in a microcentrifuge tube overnight. After lysis with repeated freeze thawing and sonication cycles, samples were centrifuged at 10,000 rpm for 3 min. The supernatant was mixed with 9-dimethylmethylene blue chloride solution (Sigma). Then, the absorption was spectrophotometrically measured at 520 nm. Unknown sample concentrations were determined by a standard curve of known concentrations of chondroitin sulfate B.

2.9. Real-time PCR for chondrogenic gene expression

Using real-time PCR, the mRNA expression of collagen type II and aggrecan was analyzed from each sample. Total RNA was isolated from the samples using TRIZOL reagent according to the manufacturer's instructions. The concentration and integrity of RNA were estimated by evaluation of the A260/280 absorbance ratio. Subsequently, RNA (2 mg) was reversed transcribed into complementary DNA using the SuperScript III. After the reverse transcription reaction, real-time PCR was performed by an ABI 7500 system using SYBR® Premix Ex TaqTM (Takara, Dalian, China) according to the manufacturer's instructions. The conditions of real-time PCR were carried out at 40 cycles of 94 °C for 5 s and 60 °C for 34 s, followed by a melting curve analysis. The cartilage-specific gene primers used for this analysis were as follows: COL II: 5′-AAGAGCGGTGACT-3′ and 5′-TGCTGTCTCCATAGCTGAAGT-3′; aggrecan: 5′-AGGTCGTGGTGAAAGGTGTTG-3′ and 5′-GTAGGTTCTC ACGCCAGGGA-3′; GAPDH: 5′-TCACCATCTTCCAGGAGCGA-3′ and 5′-CACAATGCCGAAGTGGTCGT-3′.

2.10. Statistical analysis

Descriptive statistics were used to determine group means and standard deviations for numerical data, and analysis was performed using analysis of variance (ANOVA) for multiple comparisons. Statistical significance was defined as a P-value < 0.05.

3. Results

3.1. Scaffold characterization

SEM image (Fig. 1A) showed nanofiber yarn (Yarn) while obvious different microstructures can be detected in the Yarn-CH and SPONGE groups (Fig. 1B, C). It was observed that the Yarn-CH had porous surface structures composed of the aligned Yarn and the honeycomb-like matrix. Opened and interconnected pores bound by the Yarn and matrix could be found, implying that the Yarn-CH had a well-designed three-dimensional structure. In the non-oriented porous group, the SPONGE only presented a randomly-arranged porous structure without paralleled Yarn penetrating the matrix.

The interconnecting Yarn-CH had an almost negligible effect on the porous structure of the sponge-like scaffold; the average porosities were (84 ± 6)% and (89 ± 7)% in the Yarn-CH group and SPONGE

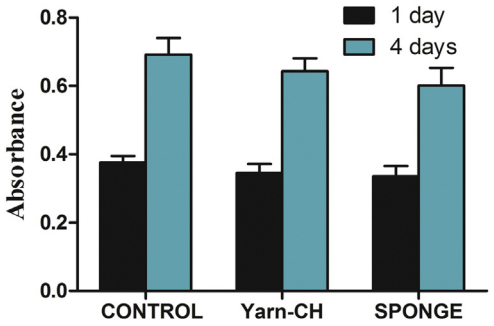

Fig. 3. MTT analysis of BMSCs on the surface of Control, Yarn-CH and SPONGE (each group, n = 3).

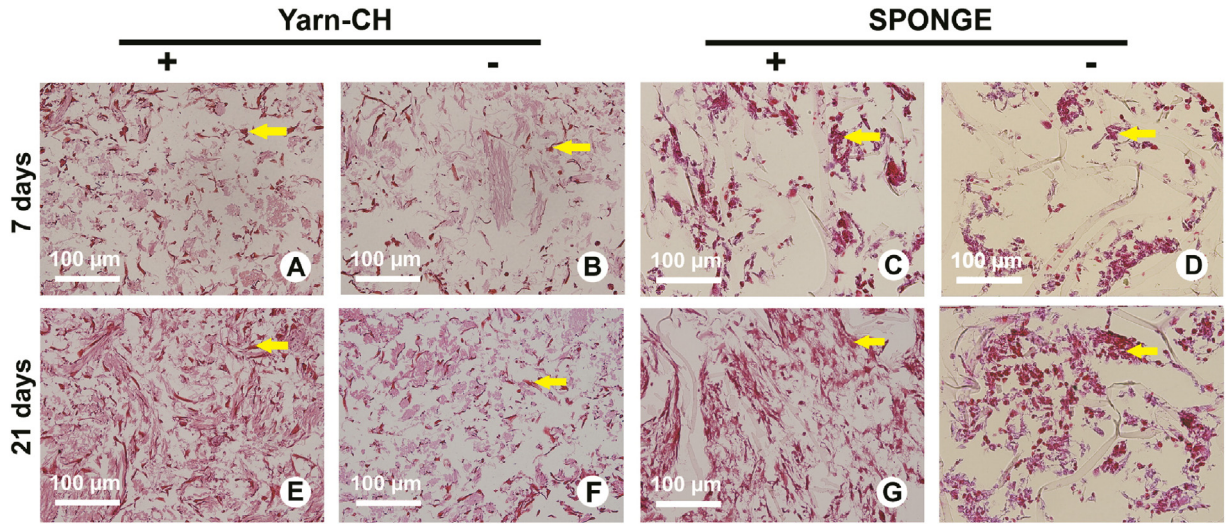

Fig. 4. Safranin O staining from the specimens cultured in chondrogenic differentiation medium for 7 days on YARN-CH (A) and SPONGE (C) and for 21 days on YARN-CH (E) and SPONGE (G). Images of safranin O staining cultured in the normal growth medium for 7 days on YARN-CH (B) and SPONGE (D) and for 21 days on YARN-CH (F) and SPONGE (H). The yellow arrows show the matrix. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

group, respectively. The Yarn-CH and SPONGE shared the pore size of (100 ± 30) μm and (110 ± 30) μm, respectively. The diameter of the yarn is 20 ± 10 μm. The Yarn-CH chondral phase had a tensile strength of 3.43 ± 0.15 MPa which was significantly higher compared to that of 1.07 ± 0.23 MPa in the SPONGE chondral phase ($P < 0.05$).

3.2. Cell orientation analysis

Four days after cell seeding (Fig. 2), it was revealed by a confocal laser scanning microscope that the BMSCs exhibited an elongated morphology on the surface of YARN-CH, SPONGE and tissue culture plate. The cells seeded on the Yarn-CH showed a preferential orientation along the direction of the fibers while no organized growth pattern on the SPONGE and tissue culture plate was found.

3.3. Cell proliferation

By comparing the proliferation of BMSCs on different surfaces after 1- and 4-day culture, the cell viability after both time points showed a similar trend; the cells were better proliferated on the surfaces of the Yarn-CH after 4 days compared to the SPONGE (Fig. 3). However, no statistic difference was detected.

3.4. Chondrogenic differentiation

After one and three weeks of in vitro culture, safranin O staining was carried out to determine the production of ECM (Fig. 4A–H). It was found that the staining was more intensive in the Yarn-CH group than that in the SPONGE group at both time points. The constructs in the Yarn-CH group formed a relatively homogeneous structure, more ECM was observed in both outer and inner regions of the Yarn group; contrarily less ECM and relatively loose structure were discovered in the SPONGE group. Notably, the addition of chondrogenic factors could promote matrix formation in both groups.

3.5. Quantitative analysis of in vitro chondrogenesis

Using GAG as a chondrogenic differentiation marker, the total GAG amounts synthesized at days 7 and 21 days were quantified, respectively (Fig. 5). In the chondrogenic differentiation media, no obvious difference in GAG production was found between the Yarn-CH and SPONGE groups at day 7; however, BMSCs seeded on the Yarn-CH scaffold demonstrated a large increase in the GAG content following a 21-day culture, approximately a 1.5-fold rise compared to the SPONGE group ($P < 0.05$).

3.6. Real-time PCR for chondrogenic gene expression

Quantitative real-time PCR was used to measure the chondrogenic gene expression. The expression of collagen type II was up-regulated in both groups at 21 days when compared with 7 days, especially in the Yarn-CH group, in which a nearly 22-fold increase was measured, whereas in the SPONGE group, only a 12-fold rise was found (Fig. 6A). Strikingly, the aggrecan gene expression was almost the same in the two groups, which was slightly higher in the Yarn-CH group but without significant difference (Fig. 6B).

4. Discussions

The techniques of dynamic liquid electrospinning and freeze-drying utilized in this study offer a new approach to the fabrication of cartilage scaffolds with maintained porosity and simultaneously advanced chondrogenic differentiation of BMSCs when compared with sponge-like scaffolds. Porous nanofiber yarn-collagen type I/hyaluronate hybrid

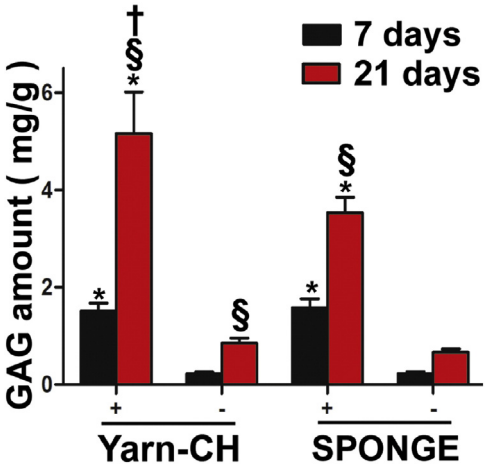

Fig. 5. The total GAG amounts at day 7 or 21 were quantified. *$P < 0.05$ compared with the same scaffold receiving undifferentiated culture at the same time point; §$P < 0.05$ compared with the same scaffold receiving the same treatment at 7 days; †$P < 0.05$ compared with SPONGE receiving the same treatment at the same time point.

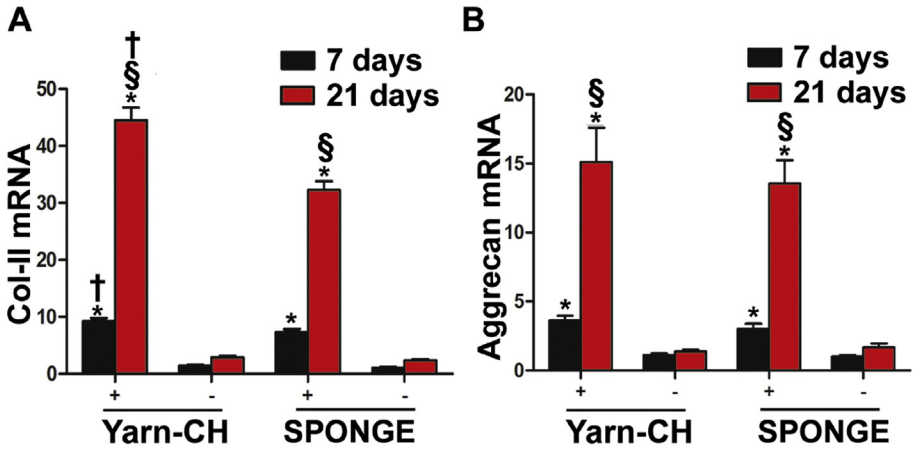

Fig. 6. The quantitative real-time PCR was used to measure the expression of collagen type II (A) and aggrecan (B). Data are expressed as mean ± SD (each group, n = 3). *P < 0.05 compared with the same scaffold receiving undifferentiated culture at the same time point; §P < 0.05 compared with the same scaffold receiving the same treatment at 7 days; †P < 0.05 compared with SPONGE receiving the same treatment at the same time point.

scaffold (Yarn-CH) inspired by both the fibrous structure and the components of native cartilage ECM can promote the proliferation, orientation and chondrogenesis differentiation of BMSCs.

The previous study has confirmed that the potency of the scaffold containing the ECM component efficiently induced chondrogenic differentiation of BMSCs and thus resulted in accumulation of GAG and collagen type II (Col-II) when compared with the pellet [23]. In particular, Hyaff, a HA based scaffold, was revealed to facilitate cartilage formation better in a fiber shape than in a sponge shape [24]. Thereafter, all these results suggest that the composition and structure of scaffolds were both biological and structural signals to support the formation of Col-II and proteoglycan from chondrocytes and BMSCs [25,26].

The scaffold design of a parallel-oriented structure mimicking the superficial zone of articular cartilage exhibits structural signals to guide BMSC chondrogenic differentiation [12–14]. Native articular cartilage is an avascular tissue consisting of four different zones. The superficial zone is crucial for the compressive strength of the cartilage and also may act as a seal to isolate cartilage from the immune system at the joint surface [27]. Furthermore, as the superficial zone of naturally functioning articular cartilage, a very polarized, dense organization of fibrils is oriented parallelly to the plane of the articular surface [18]. Thereafter, incorporation of oriented fibers into the sponge-like scaffolds may provide a more physiological microenvironment containing both structural and biological signals to guide BMSC chondrogenic differentiation.

The current study indicates that the majority of cells on the Yarn-CH was indeed oriented along a dominant direction, while the cells were randomly distributed and spread in all directions in the SPONGE (Fig. 3). The effect of Yarn-CH to support chondrogenic differentiation and promote cartilaginous matrix deposition was shown by safranin O staining, in that significantly more proteoglycan was found in the Yarn-CH group compared with the non-oriented porous group (Fig. 4A–H). Similar results were revealed by real time PCR, as higher expression of chondrogenic genes (Fig. 4J, K). In addition, the production of GAG content was much higher in the oriented Yarn-CH group (Fig. 4I). Other than the fact that Yarn-CH can imitate natural ECM matrix, Yarn-CH was likely to provide optimal conditions for cell attachment and further to affect cell proliferation. All these occurrences may be because of the combination of biological signal and structural factors for BMSC differentiation in Yarn-CH. Especially, oriented fibers may be a supplementary factor to enhance the formation of Col-II and GAG while mimicking the superficial zone of naturally functioning articular cartilage.

The deep zone with a longitudinally oriented structure takes the most important responsibility in distributing loads and resisting compression. Thereafter, further study about the scaffold design of longitudinally oriented structure should be performed. Furthermore, the fate of in vitro engineered cartilage based on oriented scaffolds in an animal model, especially the in vivo results of the engineered superficial zone of the articular cartilage, should be performed for further design and study. And, it is necessary to further investigate the effect of Yarn alone on the chondrogenic differentiation of BMSCs.

5. Conclusions

Electrospun P(LLA-CL) nanofiber yarn (Yarn) can be fabricated by a dynamic liquid supporting system for subsequent application into a freeze-dried collagen type I/hyaluronate sponge scaffold for chondral defect. Using Yarn as skeleton, the ideal porosity of the freeze-dried collagen type I (Col-I)/hyaluronate (HA) sponge scaffold (SPONGE) can be maintained as the SPONGE. Our in vitro results show that such Yarn Col-I/HA hybrid scaffold can promote the proliferation, orientation and chondrogenesis differentiation of BMSCs to reproduce some cartilaginous environmental cues, especially in the superficial zone, when compared to SPONGE.

Acknowledgments

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