Am J Transl Res 2020;12(10):6107-6121
www.ajtr.org /ISSN:1943-8141/AJTR0114979

Original Article

Porous fish collagen for cartilage tissue engineering

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Received May 25, 2020; Accepted September 12, 2020; Epub October 15, 2020; Published October 30, 2020

Abstract: Cartilage defects repair is still a challenge in clinical practice until now. Although many breakthroughs have been achieved in cartilage repair using tissue engineering technology, there are still no scaffolds available for large-scale clinical applications. Currently, fish collagen (FC) is a natural source that is considered as an alternative to mammal-derived collagen in engineering cartilage tissue due to its excellent biocompatibility, suitable biodegradability, lack of immunogenicity, rich sources, low cost and minimal risk of transmitting zoonoses, which implies great potential for use in cartilage regeneration. Herein, we successfully prepared three-dimensional porous FC scaffolds from three different concentrations of FC (0.5%, 1% and 2%) by freeze-drying technology. Our results indicated that increasing the FC concentration resulted in comparable levels of suitable biodegradability and good biocompatibility but lead to a concurrent decrease in pore size and porosity and a significant increase in water absorption capacity and mechanical properties; further, initial scaffold dimension was only sustained in the 2% FC concentration. Moreover, the *in vivo* immunological evaluation suggested that the FC scaffold evoke low immunogenicity. In addition, our results confirmed that the porous FC scaffold facilitated cartilage formation both *in vitro* and when placed subcutaneously in rabbits. The gross and autopsy outcomes at 12 weeks postoperation suggested that the porous FC scaffold achieved superior cartilage repair effect than what was observed in the empty group with no scaffold. Overall, our results demonstrated that porous FC scaffolds represent a promising prospective natural material for use in engineering cartilage for clinical applications.

Keywords: Fish collagen, natural biomaterials, porous scaffold, cartilage regeneration, tissue engineering

Introduction

After nearly thirty years of development, tissue engineering technology has witnessed the gradual breakthrough of key scientific and technical problems, and thus, cartilage defect repair strategies based on tissue engineering technology have entered the stage of clinical translation [1]. In cartilage tissue engineering, scaffolds play a vital role in promoting cells adherence, proliferation, differentiation, and cartilage extracellular matrix (ECM) formation [2]. At present, finding an ideal scaffold material is the key factor limiting the tissue-engineered cartilage for use in clinical application [3].

Synthetic scaffolds, including polyglycolide, polylactide, and poly (lactide-co-glycolide), have long been and most widely used synthetic polymers in cartilage regeneration [4]. However, the inherent defects of those scaffolds, such as poor biocompatibility and immune reactions result from its acidic degradation substances, seriously interfere with the stability of the *in vivo* engineered cartilage, which hinders its further clinical applications [5]. Compared with synthetic scaffolds, natural scaffolds from a wide range of sources exhibit many advantages, they are, easy to obtain and possess, exhibit biosafety and good biocompatibility, and have weak antigenicity. In addition, natural scaffolds contain unique amino acid sequences, that facilitate cell adhesion and differentiation [6]. Therefore, natural scaffolds represent ideal biomaterials for engineering cartilage for clinical applications.

Mammal-derived collagen (MC), mainly sourced from bovine tendon and porcine skin, is the most frequently-used natural scaffold to engineer cartilage tissue [7]. Although MC show several merits for use in cartilage tissue engineering, it is still limited by zoonosis, religious issues, immunogenicity and high cost [8]. Lately, fish collagen (FC) has been considered as an alternative source of MC due to its excellent biocompatibility, suitable biodegradability, lack of immunogenicity, rich sources, low cost and lack of transmission of zoonosis risk [9]. Previous studies have shown that FC has the capacity to act as an antioxidant in cells and promote chondrogenesis of stem cells [10-14]. Additionally, Kanchanit et al. reported that FC hydrolysates with diverse sizes evidently affected cartilage metabolism under both physiological and pathological conditions [10]. Collectively, these results demonstrated that FC is a promising natural scaffold for use in cartilage regeneration. Previous studies have fabricated FC into three-dimensional (3D) porous scaffolds and verified its satisfactory biocompatibility [15]; however, it is still unknown whether changes to the FC concentration wound affect physicochemical characteristics. In addition, the immunogenicity of the porous FC scaffold in animals remains unknown. Furthermore, no studies have yet been able to achieve substantial cartilage tissue regeneration, and whether the porous FC scaffold could be used to generate tangible cartilage tissue needs to be investigated. Moreover, no breakthrough has been made in cartilage repair *in situ* based on porous FC scaffolds. Only by determining these scientific issues will it be possible to use FC for clinical application in the future.

To address these challenges, we prepared 3D porous FC scaffolds from three different FC concentrations by freeze-drying technology to investigate the pore size, porosity, mechanical properties, and biodegradability of the scaffold. Afterwards, the biocompatibility and immunological reactivity were evaluated. Furthermore, chondrocytes were seeded into FC scaffolds, which was followed by *in vitro* culture and *in vivo* implantation to explore the feasibility of cartilage regeneration. Finally, we investigated the feasibility of the porous FC scaffolds in repairing articular cartilage *in situ* by rabbit model.

Methods and materials

Preparation of FC powder

Tilapia skin was purchased from Shanghai Fisheries Research Institute, and followed by washing, mincing, and stirring in NaOH fluid (0.1 M) for 24 hours and in acetic acid (1 M) for another 6 hours. Afterwards, the supernatant was digested with 0.8% pepsin under continuous stirring for 36 hours. Finally, the sediment was soaked in acetic acid (1 M), dialyzed and lyophilized to collect FC powder [9].

Porous FC scaffolds with different concentrations

The FC powder was suspended in deionized water to produce FC concentrations of 0.5%, 1%, and 2% weight/volume. Homogenized FC suspension was frozen at -40°C for 1 hours and followed by freeze-drying for 24 hours. Afterwards, the lyophilized FC scaffolds were crosslinked with a carbodiimide solution at 4°C for 24 hours and was lyophilized to form 3D porous scaffolds.

Characterizations of the porous FC scaffolds

Morphology observation: The macromorphology of three different concentrations of FC scaffolds, and samples after *in vitro* and *in vivo* culture were observed by a digital SLR camera (D750, Nikon, JP). The micromorphology of three different concentrations of FC scaffolds was evaluated via scanning electron microscopy (SEM, JEOL-6380LV, JP) at an accelerating voltage of 15 kV. ImageJ software was used to further analysis the average pore size of the scaffolds depends on the SEM images [16].

Porosity measurement: A liquid replacement method was used to determine the porosity of FC scaffolds. The original volume of ethanol was marked as V1, the volume after the scaffold was immersed in ethanol for 5 min was marked as V2, and the residual volume after taking out the wet scaffold was marked as V3. The porosity of FC scaffold was calculated by the formula: (V1-V3)/(V2-V3).

Area determination: After immersion in phosphate buffered saline (PBS) for 4 weeks, the FC scaffolds were photographed immediately after removed from wells for area determination. The

projected area of the FC scaffold was accessed by ImageJ according to the images.

Mechanical testing: The mechanical strength of the scaffolds was accessed using a mechanical testing machine (Instron-5542, Canton, MA) as previously reported [17]. Wafer-shaped scaffolds with 5 mm in diameter and 2 mm in height from the three groups were subjected to a compressive test. The Young's modulus was calculated according to the stress-strain curve.

Water absorption capacity: The initial dry weights of FC scaffolds with a size of 1 × 1 cm^2 and were determine as W_1. The scaffolds were then soaked in distilled water (15 mL). Scaffolds were taken out from the distilled water after 5 min and the wet weight were recorded as W_2. The water absorption ratio was determined by the formula: $(W_2 - W_1)/W_1 \times 100\%$ [6].

In vitro degradation: The dry scaffold was initial weighted as W_1, and then immersed in sterile PBS (pH = 7.4) at 37°C under continuously shaken. Scaffolds retrieved at 1, 2, 3, and 4 weeks were lyophilized and weighed as W_2, respectively. The degradation rate was reckoned by the following formula: $W_2/W_1 \times 100\%$ [18].

Biocompatibility of scaffold

Cell seeding efficiency: Chondrocytes at passage 2 from rabbit auricula were prepared according to previously established approaches [19]. A total of 200 μL chondrocytes suspension with a concentration of 75 × 10^6 cells/mL was evenly seeded into three different FC scaffolds. After 24 hours of incubation, the remaining chondrocytes were gathered and calculated, and then the cell seeding efficiency was determined as the following formula: (total cell number - remaining cell number)/total cell number × 100% [20].

Live & dead cell viability assay: Live & dead cell viability assay (Invitrogen, USA) was used to assessed the viability of the cells seeded on the scaffold for 1, 4, and 7 days. The cells were evaluated by confocal microscopy (Nikon, JP).

Cytotoxicity of scaffolds: FC scaffolds were soaked in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 72 hours. Chondrocytes at a density of 2 × 10^4 cells/mL were seeded in the scaffold extracts for 7 days [15]. Chondrocytes in the normal DMEM supplemented 10% FBS for 7 days was conducted as control group. Cell proliferation was assessed via a cell counting kit-8 (CCK-8; Dojindo, JP) base on the manufacturer's guidance.

In vivo immunological assessment: This study was complied with the Weifang Medical University Ethics Committee. Nine six-weeks aged Sprague Dawley mice were randomly divided into three groups, including 2% FC scaffold group, polyglycolic acid (PGA) scaffold group (positive control group), and no scaffold group (negative control group), to conduct an immunological assessment. The FC scaffold (2% concentration) and an equal weight (5 mg) of PGA were directly put into a dorsal subcutaneous pocket in mice. The negative control group was treated equally without scaffold placement. All mice survived within the predetermined study period and were euthanized after 14 days. The scaffolds with the surrounding tissue were retrieved for histological and immunohistochemical staining.

Cartilage formation in vitro and in vivo: Second-generation chondrocytes were prepared into a cell suspension of 1.0 × 10^8 cells/mL, uniformly inoculated on the 2% FC scaffold for 4 h at 37°C/5% CO_2 and then incubated in DMEM supplemented with 10% FBS. One half sample were cultured *in vitro* for 8 weeks and the other half sample were subcutaneously implanted into rabbits for 4 or 8 weeks.

Articular cartilage regeneration in rabbits: Rabbits (2.5 kg) were anesthetized with 10% chloral hydrate (4 mL/kg), and followed with their knee joints expose using medial parapatellar approach. Thereafter, the patellar groove was drilled using a stainless steel punch to create a cylindrical defect (4 mm in diameter and 4 mm depth). The defects were implanted with the 2% FC scaffolds in the experimental group, whereas the defects without scaffold implantation was served as the empty group [21]. The rabbits were sacrificed at 6 or 12 weeks after surgery and retrieved samples were evaluated via the International Cartilage Regeneration and Joint Preservation Society (ICRS) macroscopic and O'Driscoll histological assessment scores [22].

Histological staining: Sections derived from retrieved samples were stained with hematoxy-

lin & eosin (HE) for structural and inflammatory analysis, stained with safranin-O for glycosaminoglycan (GAG) components assessment, and stained with immunohistochemical type II collagen for cartilage-specific ECM evaluation [19]. In addition, safranin-O/Fast Green staining (Saf-O/FG) and toluidine blue staining were performed to discriminate cartilage and bone matrix in the rabbit joint samples. Furthermore, the proinflammatory cytokine tumor necrosis factor α (TNF-α) was immunohistochemically labeled to evaluate the inflammatory response using previously established methods [6].

Biochemical and biomechanical evaluation: The sulfated GAG content was analyzed via the Alcian Blue method [23]. The DNA content was quantified via a nucleic acid protein quantitation detector (Nanodrop 2000). The total collagen content was detected via a hydroxyproline assay base on previously described methods [24]. The Young's modulus of the engineered cartilage was detected and analyzed using the above described methods [19]. Each sample was repeated for three times.

Statistical analysis

All quantitative data are shown as mean ± standard deviation. One-way analysis of variance was used to evaluate the statistically significant differences between groups. Data were analyzed using SPSS13.0. $P < 0.05$ was considered statistically significant.

Results

Characteristic analysis of porous FC scaffolds

In the current study, porous FC scaffolds were produced form three concentrations (0.5%, 1%, and 2%) with freeze-drying. Gross images showed that all three groups presented a white cylinder-shaped appearance, and the samples became denser with increasing FC concentration (**Figure 1A1-C1**). The microstructure of the three FC scaffolds was observed via SEM (**Figure 1A2-C2**), from which the porous structure of the FC scaffolds can be clearly observed. Moreover, the pore size gradually decreased with the increased concentration, but the pores tended to be neat and uniform. Quantitative analyses revealed that the pore sizes at 0.5%, 1% and 2% concentrations were 278.4 ± 22.3 μm, 200.2 ± 15.8 μm, and 98.8 ± 17.1 μm, respectively, and the porosities were 93.2 ± 0.6%, 91.9 ± 0.7%, and 89.9 ± 0.5%, respectively. These data confirmed that scaffold pore size and porosity decreased with increasing FC concentration.

All FC scaffolds possessed the same original area and were immersed in PBS for 4 weeks to test their anti-contraction abilities. The retained area of FC scaffolds increased with increasing FC concentration, and only the 2% scaffold was able to sustain the initial dimensions (**Figure 2A**), the scaffolds with lower FC concentrations contracted during PBS immersion. The quantitative analyses of Young's modulus (**Figure 2B**) and water absorption capacity (**Figure 2C**) revealed increasing trends with increasing FC concentration, while the FC concentration had a minor effect on the degradation rate (**Figure 2D**).

Biocompatibility and adherence rate of porous FC scaffolds

Scaffolds with satisfactory cell compatibility and adherence rate are highly desirable for use in tissue engineering. The FC scaffold was colonized with chondrocytes to assess its potential of as a scaffold for cartilage engineering. The Live & dead staining assay showed that chondrocytes survived and proliferated well among three groups at 1-7 days post cell seeding (**Figure 3A**), which were further validated via the cell proliferation assay (**Figure 3C**). Additionally, all the three FC scaffolds showed comparable highly adherence rate (**Figure 3B**). All the three FC concentrations show comparable biocompatibility, biodegradability and adherence rates, while only the 2% concentration group exhibited superior mechanical properties, anti-contraction and water absorption capacity, thus, the FC scaffold at 2% concentration was selected for further immunological and cartilage regeneration tests.

Immunological assessment

Lack of immunogenicity is a prerequisite for a biomaterial to be used in clinical applications. We subcutaneously implanted the scaffold with a 2% concentration of FC scaffold in mice for immunological assessment (**Figure 4**). Our results indicated that both the negative control group and FC groups showed slight inflammation as evidenced by low levels of inflammatory

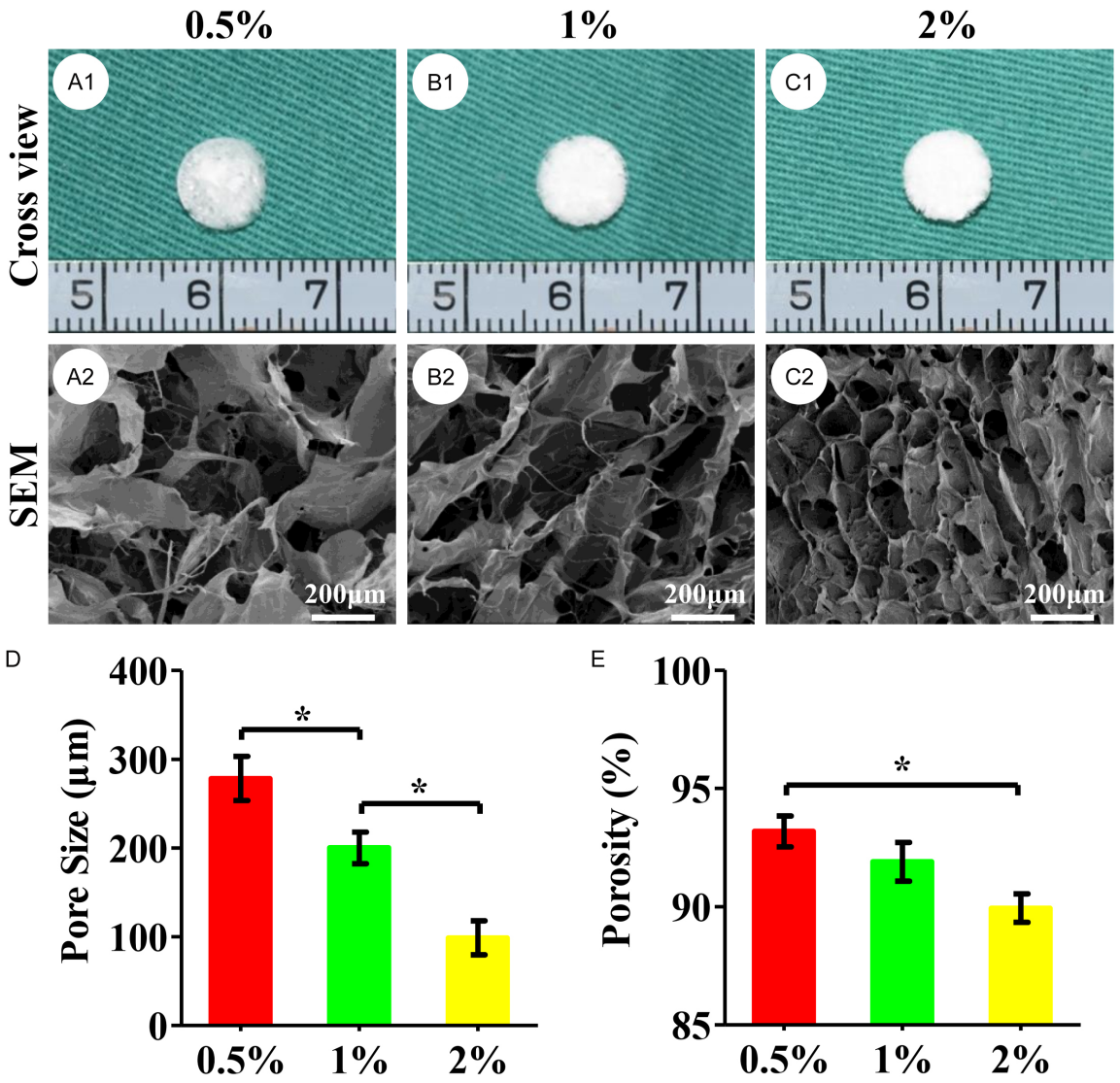

Figure 1. The porous structure of FC scaffolds produced from three different concentrations. The gross (A1-C1) and SEM (A2-C2) views of scaffolds produced from 0.5%, 1% and 2% concentrations of FC. Quantitative analyses of pore size (D) and porosity (E) scaffolds produced from 0.5%, 1% and 2% concentrations of FC. *$P < 0.05$.

cells and TNF-α, whereas the PGA group (positive control group) aroused severe inflammatory response as evidenced by high levels of inflammatory cells and TNF-α.

Cartilage formation in vitro and in vivo

The cartilage formation capacity of the FC scaffolds was validated *in vitro* and in rabbit with subcutaneous implantation. Our results demonstrated that the chondrocyte-FC scaffold samples could sustain their initial dimension (**Figure 5A**, **5B**). Furthermore, the gross views suggested gradually matured cartilage-like tissues with elongated *in vitro* culture time. Histological analysis revealed that preliminary cartilage-specific ECM deposition was formed in samples at 4 weeks (**Figure 5A1-A3**). Concerted with the gross views, matured lacunae structures and ample cartilage-specific ECM deposition were displayed in the neocartilage at 8 weeks (**Figure 5B1-B3**). Notably, gradual degradation of the FC scaffolds was obviously observed as the elongated in vivo cultivation time.

Cartilage regeneration in immunocompetent animals (rabbits) is highly desirable to predict the feasibility of future clinical application. Chondrocyte-FC scaffold constructs were subcutaneously incubated in rabbits. At 4 weeks, the engineered tissues already formed carti-

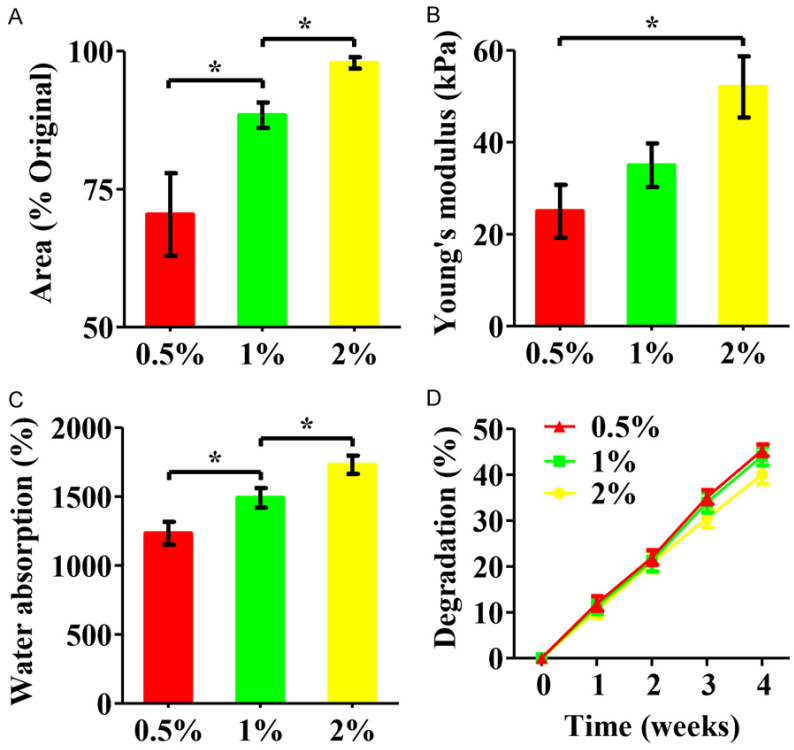

Figure 2. The mechanical properties, water absorption capacity and biodegradability of FC scaffolds produced from three different concentrations. The projected area of FC scaffolds after immersion in PBS for 4 weeks (A). Quantitative analyses of Young's modulus (B), water adsorption (C) and degradation rate (D) in all three FC scaffolds. *$P < 0.05$.

lage-like tissues (**Figure 6A**). Histological images revealed that the sample comprise mainly cartilage-specific ECM and partially undegraded FC scaffolds (**Figure 6A1-A3**). At 8 weeks, a more matured neocartilage with ivory-white appearance was displayed (**Figure 6B**). Histological images exhibited abundant homogeneous cartilage-specific ECM and typical lacuna structure with sparse undegraded materials (**Figure 6B1-B3**). Notably, no obvious inflammatory reaction was observed, predicting promising potential for clinical applications. Additionally, biomechanical and biochemical analysis revealed that Young's modulus, content of GAG, DNA and total collagen in *in vivo* engineered cartilage obviously increased over time, and samples at 8 weeks were even comparable to native articular cartilage (**Figure 6C-F**).

In situ cartilage repair in rabbit articular defect model

Cartilage repair *in situ* is the most direct evidence used to predict the potential of future clinical applications. In this study, rabbit articular cartilage defect was used to validate the *in situ* cartilage repair capacity of the FC scaffolds. At 6 weeks postoperation, the cartilage defects in the FC group was partially repaired and exhibited white cartilage-like tissue (**Figure 7B**), while the empty group was concave and showed a noticeable borderline between the normal tissue region and the defect region (**Figure 7A**). Histological images further confirmed that the defects in the empty group was invaded by a thin layer of fibrous tissue, as evidenced by HE staining and negative staining of Saf-O/FG, toluidine blue and type collagen II; these data reveal no cartilage-specific ECM deposition (**Figure 7A1-A4**). In stark contrast, the defect in the FC group was partially repaired by neocartilage tissue with positive cartilage-specific ECM staining (**Figure 7B1-B4**). At 12 weeks, gross views revealed complete repair of the defect with a flat surface in the FC group (**Figure 7D**) while a noticeable unrestored defect was displayed in the empty group (**Figure 7C**). Histologically, the defect in the FC group exhibited thick neocartilage tissue and was well fused with the surrounding normal cartilage tissue; further, there was positive cartilage-specific ECM staining (**Figure 7D1-D4**). However, the defect in the empty group predominantly filled by fibrous tissue with negative cartilage-specific ECM staining (**Figure 7C1-C4**). Conforming to the ICRS and O'Driscoll histological scores (**Figure 8A**, **8B**), the averages for the FC group were significantly superior to the empty group both at 6 and 12 weeks postoperation. Furthermore, the histological images revealed that no evident long-standing inflammatory reaction was observed in either the empty or FC groups, further confirming that the FC had no immunogenicity (**Figure 8C1**, **8C2** and **8D1**, **D2**). Collectively, these results demonstrated that the FC scaf-

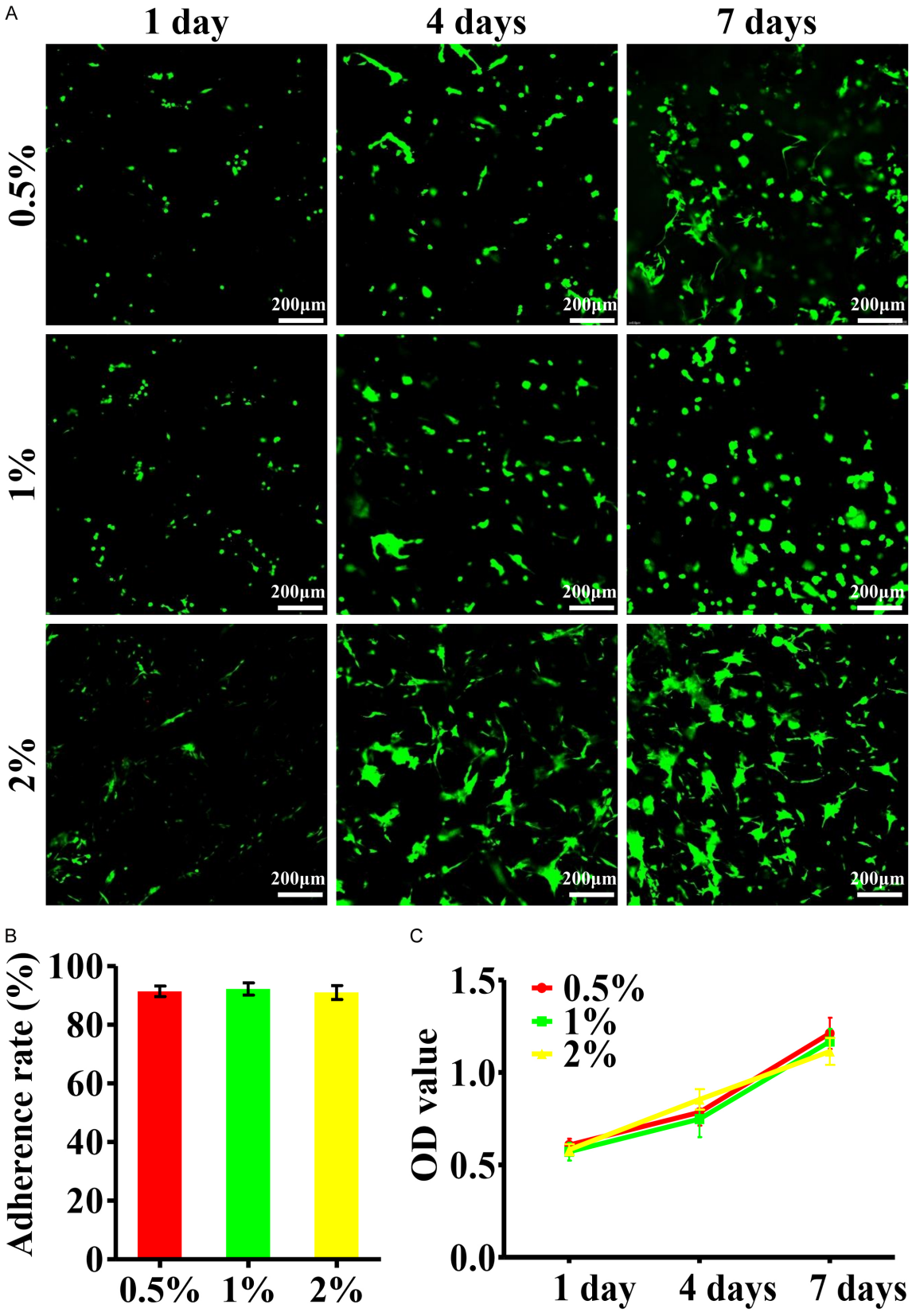

Figure 3. The biocompatibility evaluations of all three FC scaffolds. Live & Dead staining of scaffolds produced from three different concentrations of FC on days 1-7 (A). Cell adherence rate (B) and proliferation assay (C) in all three FC scaffolds.

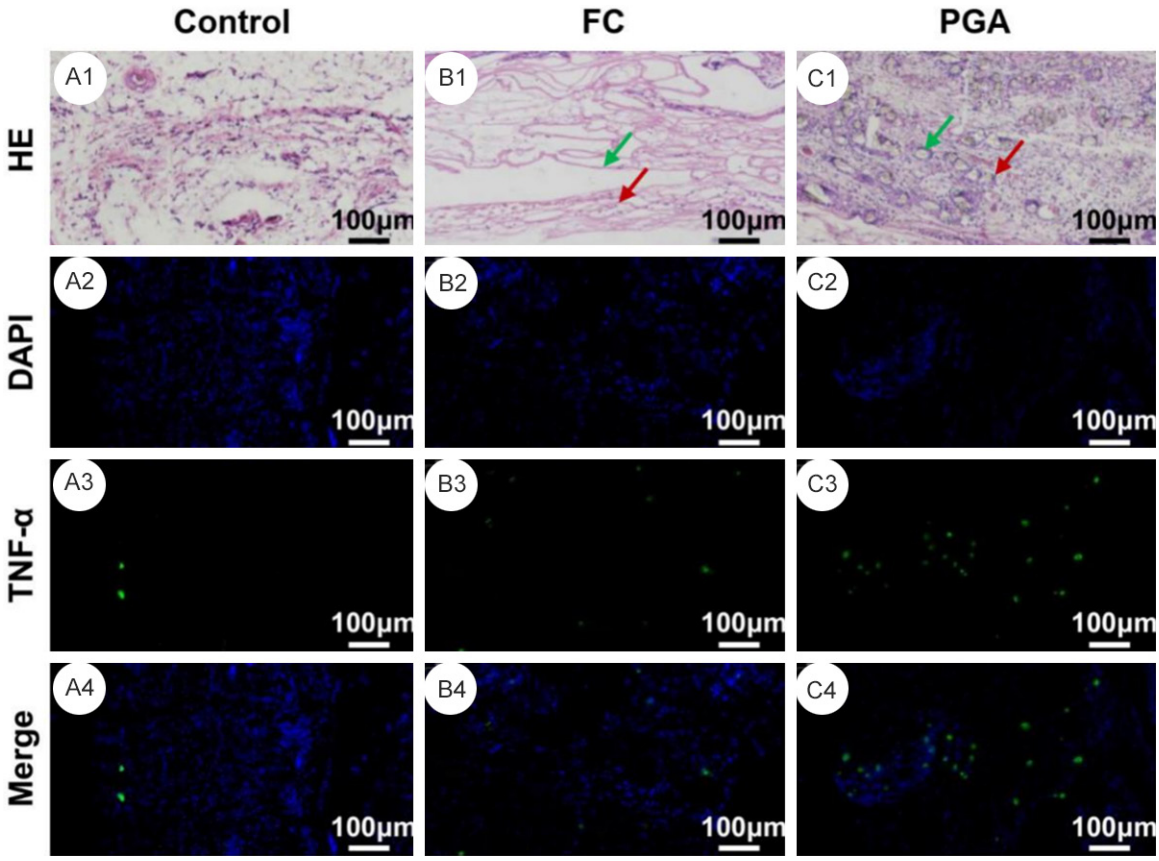

Figure 4. Immunological assessment after subcutaneous implantation in rabbits for 14 days. Images of HE (A1-C1) and immunofluorescence (A2-A4, B2-B4 and C2-C4) staining of the negative control group (without scaffold), 2% FC scaffold group and positive control group (PGA scaffold). Red arrows denote inflammatory cells. Green arrows denote residual scaffolds.

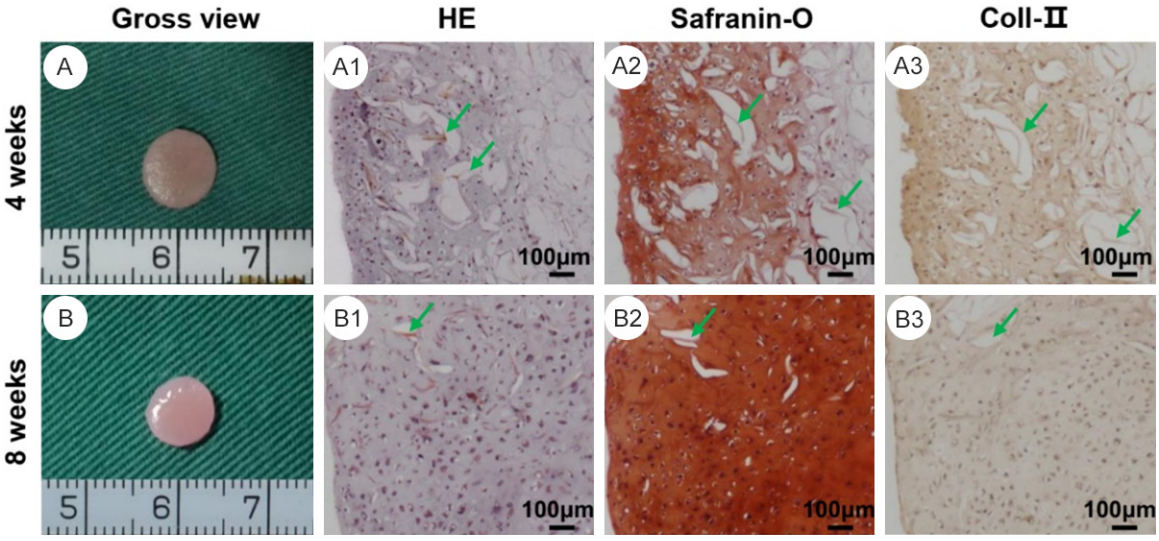

Figure 5. Cartilage regeneration *in vitro*. Gross view (A), HE (A1), safranin-O (A2) and type II collagen (A3) staining of chondrocyte-FC scaffold constructs *in vitro* cultured for 4 weeks. Gross view (B), HE (B1), safranin-O (B2) and type II collagen (B3) staining of chondrocyte-FC scaffold constructs *in vitro* cultured for 8 weeks. Green arrows indicate residual FC scaffolds.

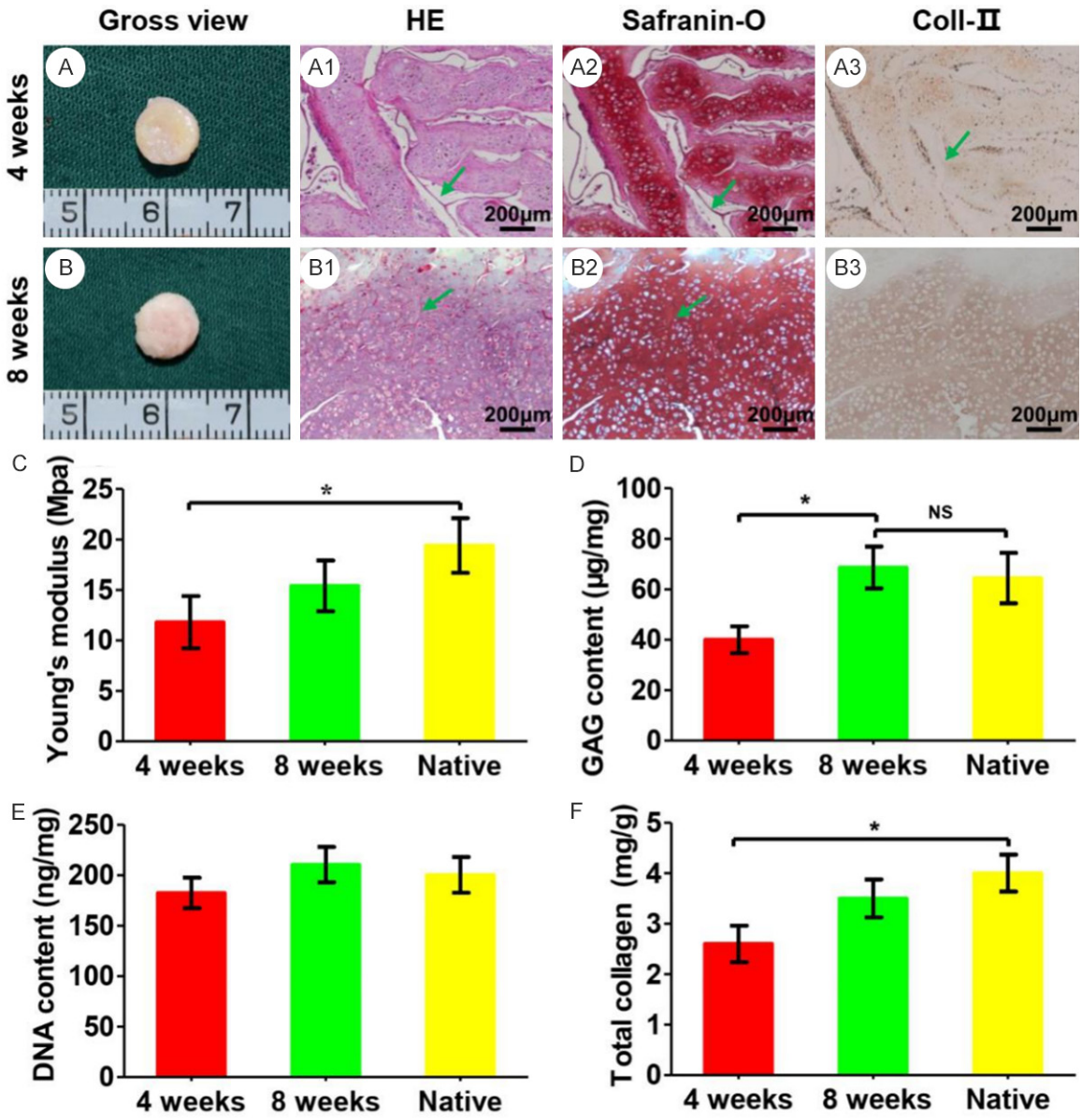

Figure 6. Cartilage regeneration subcutaneously in rabbits. Gross view (A), HE (A1), safranin-O (A2) and type II collagen (A3) staining of neocartilage subcutaneously implanted in rabbits for 4 weeks. Gross view (B), HE (B1), safranin-O (B2) and type II collagen (B3) staining of neocartilage subcutaneously implanted in rabbits for 8 weeks. Quantitative analyses, including Young's modulus (C), content of GAG (D) and DNA (E) as well as total collagen (F), of neocartilage subcutaneously implanted in rabbits for 4 and 8 weeks and native articular cartilage. Green arrows indicate residual FC scaffolds. *$P < 0.05$. NS denotes no significant difference.

fold could promote *in situ* cartilage repair in rabbits and is a potential alternative to MC for use in clinical applications.

Discussion

Compared with MC, FC not only shows good biocompatibility, but also possesses biosafety and economic benefits, which endow it with tremendous potential to repair cartilage defects in clinical applications in the future. However, no study to date has achieved substantial cartilage regeneration based on FC and investigated its feasibility to repair cartilage defects *in situ*. In this study, 3D porous FC scaffolds were successfully prepared by freeze-drying technology. Importantly, the scaffold with a 2% concentration of FC possessed suitable pore morphology, acceptable biodegradability, satisfac-

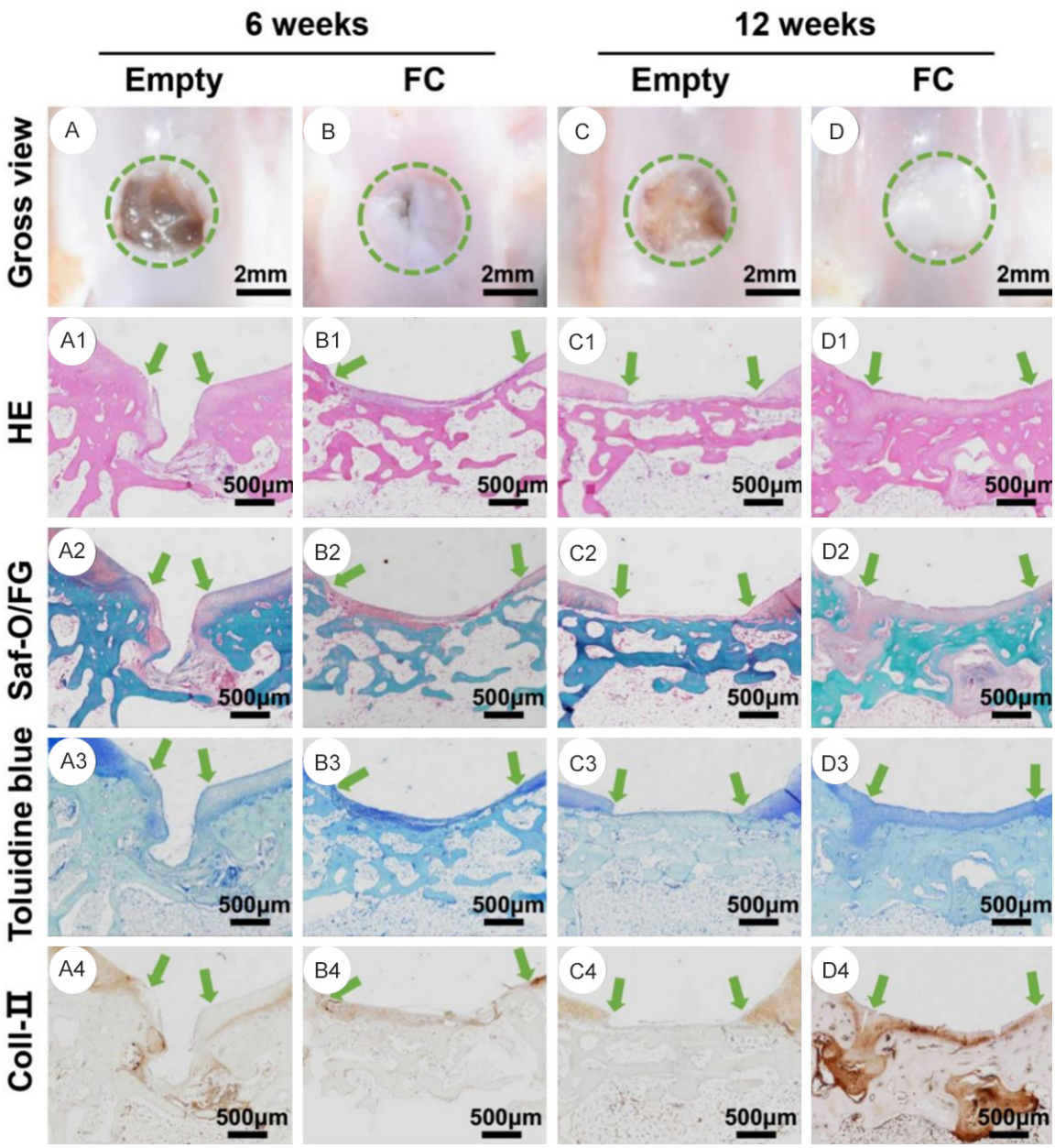

Figure 7. *In situ* cartilage repair in a rabbit articular defect model at 6 and 12 weeks postoperation. Gross images (A, B), and images of HE (A1, B1), Saf-O/FG (A2, B2), toluidine blue g (A3, B3), and type II collagen (A4, B4) staining of the repaired cartilage defects in the empty and FC groups at 6 weeks postoperation. Gross images(C, D), and images of HE (C1, D1), Saf-O/FG (C2, D2), toluidine blue (C3, D3), and type II collagen (C4, D4) staining of the repaired cartilage defects in the empty and FC groups at 12 weeks postoperation. Green arrows denote the border of native cartilage and defect areas.

tory biocompatibility, sound mechanical properties and low immunogenicity. In addition, the porous FC scaffold facilitated cartilage regeneration both *in vitro* and in rabbits following subcutaneous implantation. More importantly, the porous FC scaffolds could significantly promote cartilage repair *in situ* in a rabbit articular defect model. All of these results provide support for the porous FC scaffold as a clinical application for engineering cartilage.

MC has been widely applicated in biomedical owing to its perfect cell adherence, desirable biocompatibility, suitable biodegradability and self-assembly into various structures [25]. However, the applicability of MC is limited due

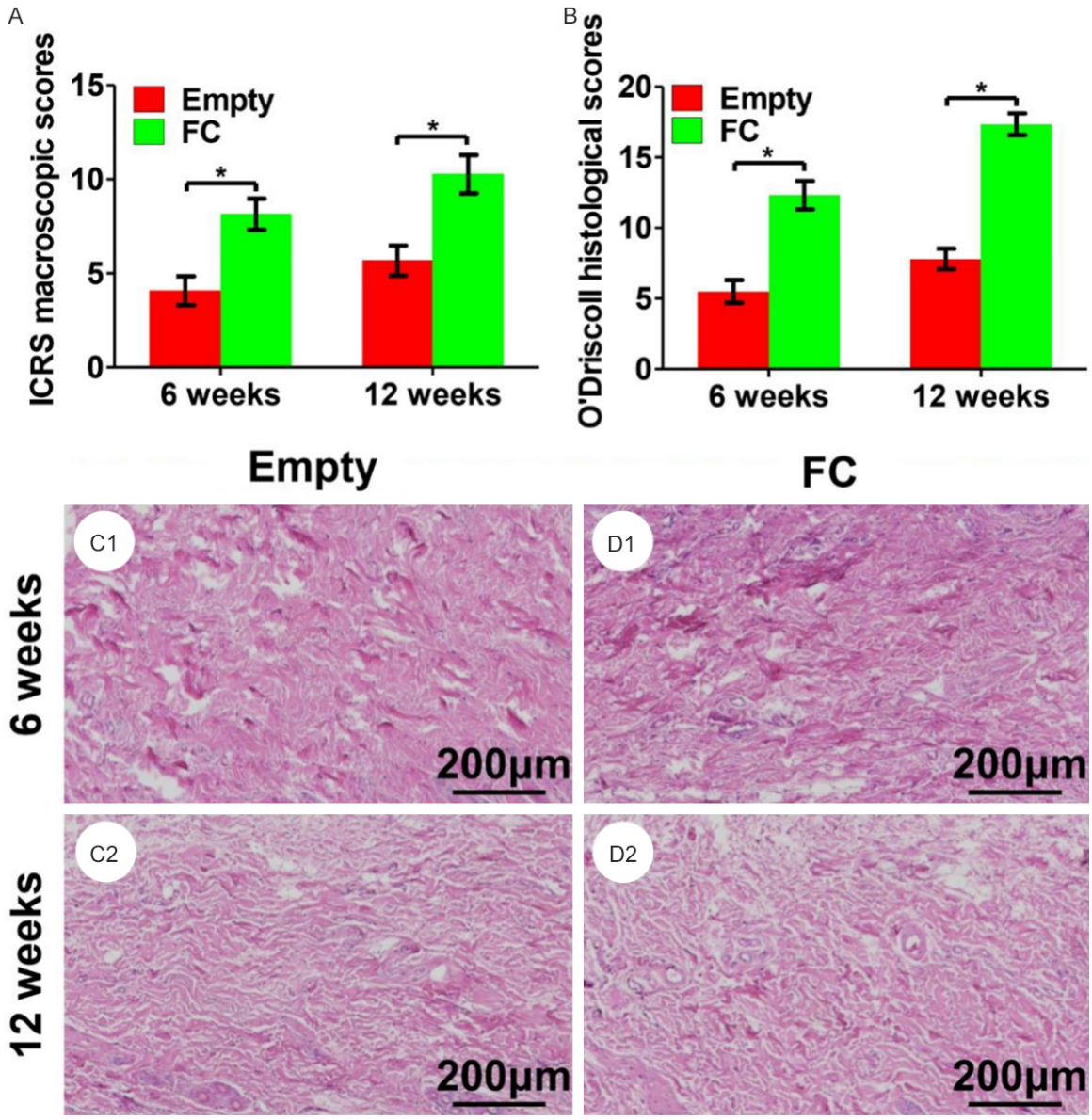

Figure 8. Macroscopic and histological evaluation of the repaired tissue and HE staining of synovial membrane. ICRS macroscopic assessment scores (A) and O'Driscoll histological assessment scores (B) of the repaired tissue. HE staining of the synovial membrane in the empty group at 6 weeks (C1) and 12 weeks (C2) postoperation. HE staining of the synovial membrane in the FC group at 6 weeks (D1) and 12 weeks (D2) postoperation.

to its high cost, religious issues, immunogenicity, and probability of disease transmission from its sources [8, 26]. As a consequence, increasing attention has been focused on finding an alternate natural scaffold to replace MC. Recently, FC was found to possess highly coincidental properties to that of MC, suggesting that it could be a potential alternative for use in cartilage tissue engineering [10, 12]. Previous study revealed that hydrolyzed FC solely potentially could induce and maintain chondrogenesis of adipose-derived stromal cell-derived [27]. In addition, some other studies demonstrated that combining FC and alginate could maximum mimic cartilage components and support chondrogenesis of human mesenchymal stem cells (hMSC) [12, 15]. Hence, FC is considered a desirable scaffold source for use in engineering cartilage tissue.

3D porous scaffolds with favorable interconnectivity and tremendous porosity are condu-

cive to cell attachment, distribution and proliferation, nutrient infiltration and ECM secretion. The use of controllable FC concentrations and freeze-drying mechanisms produced 3D porous scaffolds with diverse porosities and pore sizes, which present a decreasing trend with increasing FC concentration. A previous study indicated that a smaller pore size was conductive for cartilage regeneration [28], and a 50-100 μm size was recommended for good cell distribution and adhesion. Our results revealed that the pore size in the scaffold with a 2% concentration of FC was 98.8 ± 17.1 μm, thus, it was eligible for cartilage tissue engineering. In addition, changes in FC concentration showed different anti-contraction abilities. Our results showed that 2% FC can prevent contraction in PBS. Furthermore, scaffolds with sound mechanical properties are desirable for use in cartilage regeneration [29]. The mechanical properties of the FC scaffold increased with augmenting FC concentration. Additionally, the adsorption capacity of scaffolds may positively affect the nutrition retention of cell attachment [6]. In the current study, the absorption capacity of the FC scaffold was evaluated by immersion in distilled water. Our results indicated an increase in the water absorption capacity with increasing FC concentration. The scaffold with a 2% concentration of FC may have exhibited superior water absorption capacity because water is mainly absorbed within the scaffold and stored in the porous space of the scaffold [30]. One the one hand, the increased FC concentration enhance the water absorption; On the other hand, the 2% FC scaffold show anti-contraction ability to provide more space for water to be stored.

We then investigate the biocompatibility of the FC scaffold, including cell seeding efficiency, viability, and proliferation rate. As anticipated, our results confirmed that all three FC scaffolds showed desirable chondrocyte affinity, low toxicity, and excellent capacity to promote chondrocyte proliferation, validating our hypothesis that FC scaffold would be favorable for cartilage engineering. Similar noncytotoxic effects on human fibroblasts, smooth muscle cells and hMSC incubated with FC extracts were obtained by other researchers [15, 31]. Overall, considering the pore size, anti-contraction ability, mechanical strength, absorption capacity and biocompatibility, the FC scaffold with a 2% concentration was deemed as an appropriate scaffold for cartilage engineering.

No inflammatory reaction is highly desired for a scaffold to be used in cartilage tissue engineering. Previous studies reported that the immunogenicity in FC was comparable or even lower than those in MC and shows a minimal inflammatory response in mice [32]. In this study, the FC scaffold was subcutaneously implanted in mice for 14 days to assess their *in vivo* immunogenicity because there is a delay of 4-7 days before the initial adaptive immune response is triggered. The proinflammatory cytokine TNF-α is involved in host defense, and its overproduction can lead to chronic inflammatory diseases; it is produced mainly by macrophages in response to tissue damage or infection [33]. Both the FC scaffold group and injury without scaffold group (negative control group) showed slight inflammatory behavior with low levels of inflammatory cells and TNF-α. In addition, our results also suggest that scarcely inflammation was observed in subcutaneously and the synovial tissue around the knee joint, confirming the lack of immunogenicity of the FC scaffold.

Thus, whether porous FC scaffolds support cartilage regeneration is the major concern. Both the *in vitro* and *in vivo* engineered cartilage based on FC scaffolds matured with increasing culturing time, displayed homologous cell distribution, exhibited typical cartilage lacuna structure, and secreted a cartilage-specific ECM that stained positive for safranin-O and collagen type II. Notably, after 8 weeks of subcutaneously implantation, the FC scaffold was almost degraded, resulting in a “pure” neocartilage, and the neocartilage exhibited quantitative indexes, including Young’s modulus, content of DNA, GAG and total collagen, that were comparable to that of native articular cartilage. The underlying mechanism is that the interconnected 3D porous structure furnishes ideal space for chondrocytes to proliferate and secrete cartilage ECM and the subcutaneously niche acts as a bioreactor to facilitate nutrition infiltration for neocartilage.

The feasibility of repairing cartilage defects in situ is the most considerable indicator for the clinical application of 3D porous FC scaffold. In this study, the FC scaffold enhanced articular cartilage repair with well interface integration and a hyaline-like cartilage character after 12

weeks postoperation. Several reasonable speculation might be that 1) the excellent absorption capacity of FC scaffolds promotes the recruitment of host chondrocytes or bone marrow stem cells, and 2) the FC scaffold provide favorable niche for the recruited cells to survive and proliferation, and 3) the porous structure facilitates nutrient infiltration and enhances cartilage formation [16, 21]. Collectively, our results indicated that FC scaffolds holds tremendous clinical potential for cartilage repair.

Conclusion

In summary, we prepared and optimized a porous scaffold with FC at a 2% concentration, and it exhibited satisfactory physicochemical properties, including proper pore structure, acceptable degradation rate, good biocompatibility, sound mechanical strength and low immunogenicity. In addition, based on the FC scaffold, we achieved gratifying cartilage regeneration both *in vitro* and following subcutaneous implantation in rabbits, and we observed cartilage repair *in situ*. These results validate the potential clinical application of the porous FC scaffolds in future.

Acknowledgements

This research was financed by the National Key Research & Development Program of China (2018YFC1105800), the National Natural Science Foundation of China (81702257), the Hainan Provincial Natural Science Foundation of China (818QN313), the Project of Special Research on Battle Injury and Trauma of Changzheng Hospital (201709), and the Project of Special Research on Medical Protection of Second Military Medical University (WL-MS-09).

Disclosure of conflict of interest

None.

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