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ORIGINAL ARTICLE

Application of Synovial Fluid Mesenchymal Stem Cells: Platelet-rich Plasma Hydrogel for Focal Cartilage Defect

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Purpose: To evaluate the effectiveness of synovial fluid mesenchymal stem cells (SFMSCs) mixed with platelet-rich plasma (PRP) and thermosensitive hydrogel in the management of porcine articular cartilage defects *in vitro* and *in vivo*.

Methods: The *in vitro* experiment was designed to evaluate the differential potential of cell-laden PRP composite hydrogels, i.e., porcine SFMSCs plus PRP with thermosensitive hydrogel. The chondrogenic-related gene expressions were evaluated for 3 weeks. For the *in vivo* experiment, an osteochondral defect was first created in the medial condyle of the porcine femurs, which was filled with PRP composite hydrogel, i.e., PRP with thermosensitive hydrogel, in the right femur. The left femur was embedded with cell-laden PRP composite hydrogel. The gross morphology, microscopic histology, and immunohistological staining were evaluated at 4 weeks and 8 weeks after implantation.

Results: The *in vitro* study demonstrated that the cell-laden PRP composite hydrogel appeared to have profound chondrogenic potential. The *in vivo* study also revealed that the cell-laden PRP composite hydrogels had better therapeutic effects with increased cell growth and maturation of chondrocyte than that of the PRP composite hydrogel only group.

Conclusion: The SFMSCs with PRP and thermosensitive hydrogel have a great capability for chondrocyte regeneration and maturation.

1. Introduction

Articular cartilage damage can be caused by different types of diseases and injuries.[1] In some cases, this severe damage and injury may lead to disability in walking, resulting in poor patient ambulatory status.[2] Because the healing potential of articular cartilage is poor, it is difficult to provide a good therapeutic management dealing with the articular defect. Currently, several therapeutic methods for improving articular cartilage repair has been applied including physical therapy, joint debridement, and autologous chondrocyte or cartilage transplantation.[3] Nevertheless, the replaced cartilage during the healing process is usually fibrocartilage, instead of hyaline–cartilage, which is the form of cartilage in composite joints and supports the connections between bones. Indeed, there are no well-established therapeutic methods or programs to repair articular cartilage injury functionally.[4]

In recent years, platelet-rich plasma (PRP) has attracted more attention as an important therapeutic for various forms of sport-related injuries.[5] PRP has been considered as a new therapeutic candidate and applied to accelerate the healing process in orthopedic-related disorders such as chondrogenesis, chondral defects, chronic elbow tendinosis, Achilles tendinopathy, and rotator cuff repair.[6] In 1998, the application of PRP in accelerating bone wound healing was first introduced by Dr Robert E. Marx and colleagues[7] who proposed that PRP can promote osteoid production, collagen synthesis, and cell proliferation, contributing to speeding up the healing process in the initial phases. It had been previously reported that several growth factors are found in PRP, including basic fibroblast growth factor, insulin-like growth factor-I, platelet-derived growth factor, transforming growth factor-β1 (TGF-β1), and vascular endothelial growth factor, suggesting that those factors may increase cell proliferation and extracellular matrix depositions and lead to acceleration of healing in the injured articular cartilage.

Controlled drug-delivery systems are designed and aimed to deliver the drugs to the specific targeted sites at desirable times to achieve the best therapeutic efficacy.[8] Over the past decades,

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temperature-responsive polymers have attracted great attention as superior carriers for injectable drug delivery systems, which are potentially useful in minimally invasive surgery.[9] Compared to the other biodegradable polymers, the injectable thermogelling polymers possess several additional advantages,[10] including easy preparation, high encapsulation efficiency of drugs or bioactive molecules, and freedom from harmful organic solvents in the formulation process. An ideal thermogelling polymer for drug delivery should exhibit a suitable sol–gel transition behavior; in other words, the polymer is in a state of solution under room temperature while turning into gel-like state at body temperature.

Mesenchymal stem cells (MSCs) isolated and purified from different types of mesenchymal tissue such as bone marrow, blood, periosteum, fat, and other tissues display characteristics of self-renewal and differentiation.[11] Recently, it has been suggested that synovial fluid (SF) in the joint capsule is an MSC-rich source. In general, more MSCs derived from SF (SFMSCs) can be obtained from patients with anterior cruciate ligament injury or osteoarthristis than from healthy volunteers. The number of SFMSCs is also increased after meniscus injury, leading to the promotion of spontaneous meniscus healing.[12] It has been suggested that the synovial fluid may serve as an MSCs-rich reservoir. The SFMSCs will proliferate, differentiate, and recruit to the injury site for tissue repairing post–intra-articular tissue injury. Previous *in vivo* and *in vitro* research suggests that the SFMSCs display a great potential in chondrogenesis.[13] Furthermore, Gullo and De Bari[14] found that the subpopulations of human SFMSCs with the combined markers of CD39 and CD73 displayed greater chondro-osteogenic potency.

The purpose of this study was to evaluate the therapeutic potential using a new methodology to repair the injured articular cartilage *in vitro* and *in vivo* (Figure 1). This combined therapeutic candidate consists of PRP coupled with thermohydrogel and then embedded into cartilage defects. In order to compare the cartilage repair potential of SFMSCs, we separated SFMSCs into different experiment groups for further characterization.

2. Methods

2.1. Isolation of autologous SFMSCs from pigs

All porcine procedures were conducted according to the Guide for the Care and Use of Laboratory Animals and approved by the Committee of Experimental Animal Sciences of Taipei Medical University, Taipei, Taiwan. Female pigs (weight about 25–35 kg) were anesthetized with an intramuscular injection of a mixture of 1 mL xylazine and 10 mL ketamine. Ten mL of saline was injected into the knee joint with an 18-gauge needle and 20 mL syringe, left for 30 seconds, and then taken out by the same route. Under sterilized conditions, the synovial fluid saline solution was centrifuged at 300*g* for 5 minutes to remove the supernatant, and the pelleted cells were resuspended in Dulbecco modified Eagle's medium (Invitrogen, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (HyClone, Logan, UT, USA) and 1% antibiotics/antimycotics (10,000 U/mL penicillin, 10 mg/mL streptomycin, and 25 μg/mL amphotericin B; Biological Industries, Kibbutz Beit-Haemek, Israel). Nucleated cells were plated at 6×10^3 cells/cm^2 in 75 cm^2 flasks and incubated at 37°C with 5% humidified CO_2 for 10 days. The culture medium was replenished twice a week. The cell morphology was observed by microscopy.

2.2. Autologous PRP preparation

PRP was collected by drawing the whole blood of pigs via neck vessel penetration. Using a syringe with an 18-gauge needle, 9.8 mL blood was aspirated and mixed with sterilized 0.2 mL sodium citrate (55 mM; Sigma–Aldrich, St Louis, MO, USA) for anticoagulation. The whole blood was then centrifuged at 300*g* (Eppendorf, Hamburg, Germany) for 10 minutes, and the superstratum formed the PRP. The platelets in the PRP and in whole blood were counted using a hemocytometer.

2.3. Preparation of PRP composite hydrogel

To prepare composite hydrogel, the thermohydrogel was first synthesized as previously described.[15] Briefly, the monomethoxypoly (ethylene glycol)-co-poly (lactic-co-glycolic acid) (mPEG-PLGA) diblock copolymer was synthesized by the ring-opening polymerization of monomers and mPEG in the presence of stannous 2-ethylhexanoate. After this, diblock copolymer was mixed with 10% PRP (v/v) and 2% alginate solution (v/v) as composite hydrogel for SFMSC cultivation.

2.4. In vitro evaluation

The SFMSCs were harvested by trypsin (Invitrogen) for the experiments and resuspended in fresh medium. Cell suspension (around

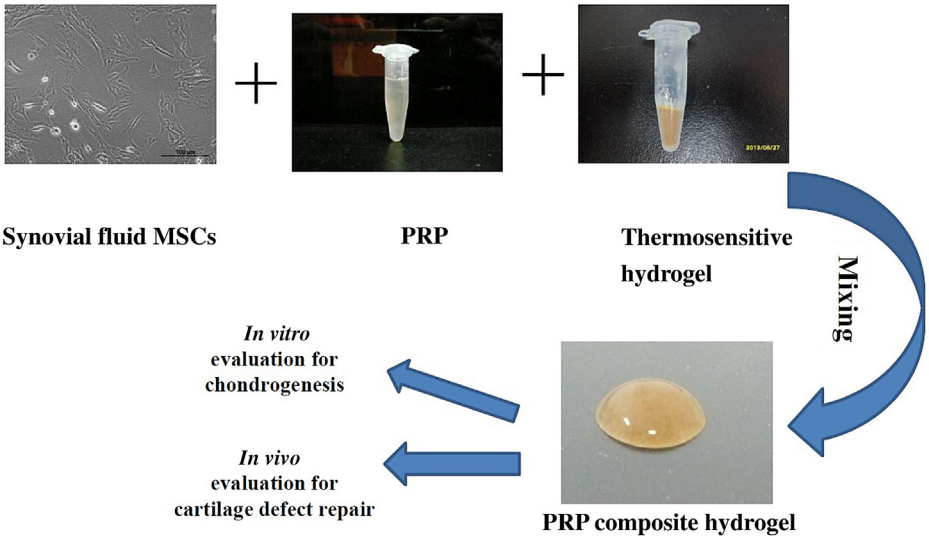

Figure 1 The schematic experimental designation for this study. MSC = mesenchymal stem cells; PRP = platelet-rich plasma.

1.2×10^7 cells) was injected to 2.4 mL of the PRP composite hydrogels to mix homogeneously. A 200 μL aliquot of cell-laden PRP composite hydrogels was put into a 24-well culture dish at 4°C, 0.5% calcium chloride was added. Finally, cell-laden PRP composite hydrogels were incubated at 37°C in an atmosphere of 5% CO_2 and 95% humidity. Medium changes were made at 2–3-day intervals and scaffolds were examined at time points up to 28 days. Cell distribution and chondrogenic marker genes expression were examined.

2.5. In vivo transplantation

The research protocol of this experiment was reviewed and approved by the Ethics Committee of the university and the pigs were cared for following international rules.

The PRP composite hydrogels (with or without SFMSCs) were pre-prepared for *in vivo* transplantation. Twelve female pigs (weight 25–35 kg) were anesthetized with an intramuscular injection of a mixture of 1 mL xylazine and 10 mL ketamine. The cartilage defects of pigs were made by the following steps (Figure 2). Briefly, the patella was dislocated laterally, and the patellar groove exposed. The articular cartilage defect (diameter about 4.5 mm and 3 mm depth) was created through the articular cartilage and into the subchondral bone of patellar groove of the knee with a 4.5 mm diameter dermal punch. In the left knee, the cartilage defects were filled with the PRP composite hydrogels seeded with SFMSCs (2×10^6 cells each hydrogel; cell-composite hydrogel, CH). In the other (right) knee, the cartilage defects was filled with an empty PRP composite hydrogels without cells using the same protocols (empty cell-composite hydrogel, ECH). After surgery, all pigs were allowed to unrestricted cage activity without immobilization until sacrificed 4 weeks or 8 weeks later.

2.6. Gene expression by real-time polymerase chain reaction

The cultured cells in the PRP composite hydrogel with fibrogenic medium were analyzed by real-time polymerase chain reaction (PCR) to investigate temporal mRNA expression changes of type II collagen and aggrecan. Total RNA was extracted from the cultured cells inside the PRP composite hydrogels. The hydrogels were incubated with TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Subsequent steps followed the manufacturers' instructions. Total RNA of each sample was measured by a spectrophotometer (Beckman, Fullerton, CA, USA) and then was reverse transcribed with oligo (dT) primer using Moloney murine leukemia virus reverse transcriptase by First Strand synthesis kit (Fermentas, Glen Burnie, MD, USA) for cDNA synthesis. The resultant solution (2.0 μL) was amplified in triplicate by real-time PCR (Applied Biosystems, Foster, CA, USA) with SyBr Green Master Mix reagent (Applied Biosystems). Glyceraldehyde phosphate dehydrogenase was also amplified under the same conditions as a normal control. The primers for the specific genes were designed according to the published sequences available in GenBank using Primer Express 2.0 (Applied Biosystems) as shown in Table 1. The comparative Ct method was used for gene expression quantification.

Table 1 Sequences of primers used in real-time polymerase chain reaction.

Gene symbol	Primer sequences (5′→3′)	GenBank accession no.
Aggrecan	GCTACGGAGACAAGGATGAGTTC CGTAAAAGACCTCACCCTCCAT	L38480
Type II collagen	CCTGTGCGACGACATAATCTGT GGTCCTTTAGGTCCTACGATATCCT	AF027122
Glyceraldehyde phosphate dehydrogenase	GGAGAAAGCTGCTAA ACGACCTGGTCCTCGGTGTA	L23961

2.7. Histology staining

The cartilage defect specimens were harvested 4 weeks and 8 week after transplantation. The cartilage defect specimen were washed three times in phosphate-buffered saline, fixed for 12 hours in 10% phosphate buffered formalin (Sigma–Aldrich) at room temperature, dehydrated in serial ethanol solutions followed by xylene, and embedded in paraffin. The embedded samples were sectioned at 5 μm thickness, deparaffinized in xylene, rehydrated, and stained with hematoxylin and eosin or alcian blue according to the manufacturers' protocols. For immunohistochemistry staining, the slide was first blocked with normal goat serum for 45 minutes. Mouse anti-human monoclonal primary antibodies of type II collagen diluted 1:100 (Abcam, Cambridge, UK) diluted 1:100, were applied at 4° for 24 hours, followed by incubation with Horseradish Peroxidase (HRP) conjugated anti-mouse secondary antibody diluted 1:250. Diaminobenzidine (Sigma–Aldrich) was used as the substrate, which presents the brown color of the immunopositive cells. Counterstaining was done with hematoxylin. The slides were dehydrated prior to being coverslipped.

2.8. Statistical analysis

The gene expression results are expressed as mean ± standard deviation for three experiments for each test. The control and

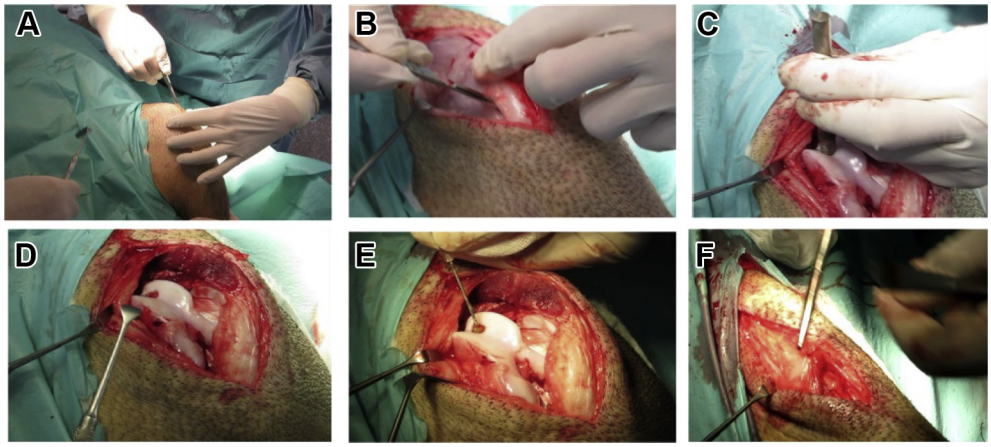

Figure 2 The animal protocol for cartilage focal defect: (A) skin open; (B) muscle cutting; (C) defect creation; (D) defect appearance; (E) platelet-rich plasma composite hydrogel injection; and (F) skin suture.

experimental groups of the *in vitro* study were compared with each other by *t* test. The *in vivo* data collected in this study were based on six tests. The control and experimental groups of *in vivo* evaluation were compared with each other and with unoperated tissue.

3. Results

3.1. In vitro study

To ensure the successful preparation of PRPs, the number of platelets was confirmed and counted. The results showed that the number of platelets in whole blood was $4.5 \pm 0.8 \times 10^8$ platelets/mL, and that in PRP was $1.9 \pm 0.6 \times 10^9$ platelets/mL (422% more in PRP than in whole blood).

To check the cell distribution, the immunofluorescence staining was used to identify the location of the cells within the PRP composite hydrogels prior to transplantation (Figure 3). SFMSCs were distributed homogenously in the PRP composite hydrogels before transplantation and displayed a great characteristic in proliferation.

To evaluate the potential of SFMSCs cultivated in PRP composite hydrogel for differentiation, the chondrigenic-related gene expressions were measured and compared by real-time PCR. According to previous reports, MSCs could differentiate into chondrocytes via stimulation of growth factors derived from the TGF-β family.[16] As shown in Figure 4, the chondrigenic-related gene expressions of collagen type II and aggrecan was significantly increased after 3-weeks of cultivation. The experimental results also indicate that PRP composite hydrogel may play a central role in accelerating chondrogenesis.

3.2. In vivo study

The PRP composite hydrogels with SFMSCs (CH) or without SFMSCs (ECH) were compared. The pigs were sacrificed 4 weeks or 8 weeks after transplantation and the cartilage was examined.

Generally, the defects in both knees were all completely filled by the repair tissue at both 4 weeks and 8 weeks after transplantation (Figure 5). In the ECH group (right knee), the repair tissue 8 weeks after transplantation turned to mostly white from the deep brown color seen in the repair tissue 4 weeks after transplantation. These experimental results indicate that the thermosensitive hydrogel was degraded gradually within the 8 weeks. In the CH group (left knee), the repair tissue 8 weeks after transplantation showed only the white color of cartilage-like tissue, whereas the repair tissue 4 week after transplantation appeared light brown in color, indicating that the CH group might not only degrade the thermosensitive hydrogel, but also promote the SFMSCs proliferation and differentiation.

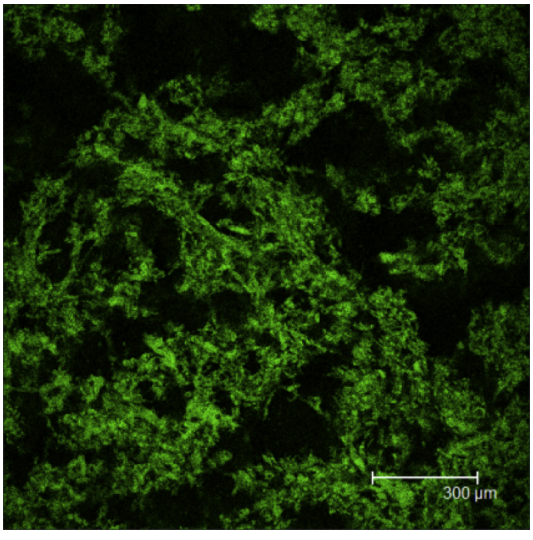

Figure 3 Synovial mesenchymal stem cells cultured on platelet-rich plasma composite hydrogels.

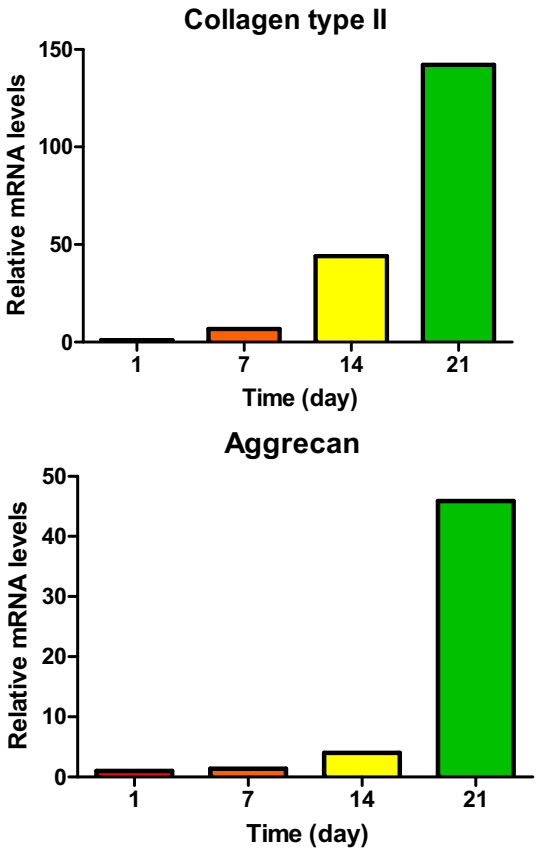

Figure 4 The chondrogenic-related gene expression of synovial mesenchymal stem cells cultivated on platelet-rich plasma composite hydrogels *in vitro*.

For further confirmation, a closer examination of histology of CH or ECH is shown in Figure 6. As shown in the ECH group, the repair tissue showed more extracellular matrix deposition at 8 weeks than at 4 weeks after transplantation. In addition, as shown in the CH group, the repair tissue showed more cells with lacuna structure after 8-week transplantation than after 4-week transplantation (Figure 6). Furthermore, cell clusters of typical cartilage characteristics were also observed (Figure 6). These observations indicate that the SFMSCs cultivated on the PRP composite hydrogel facilitate chondrocyte-like cell formation and regenerate cartilage-like tissue in the repair sites in the CH group, whereas animals in the ECH group failed to form cartilage tissue in the repair sites.

As shown in Figure 7, the glycosaminoglycan (GAG) secretion in the CH group showed a little more alcian blue staining than the ECH group 4 weeks after transplantation. Furthermore, the increased GAG secretion was observed in the CH group than in the ECH group 8 weeks after transplantation. These observations indicate that the CH group seemed to induce more GAG secretion than the ECH group. In addition, this indicates that PRP composite hydrogel in the ECH group could also trigger increased GAG secretion. In immunohistological staining for type II collagen, positive results were also shown in both the CH and ECH groups 8 weeks after transplantation (Figure 8). Like the alcian blue staining results, the CH group obviously showed more type II collagen secretion than the

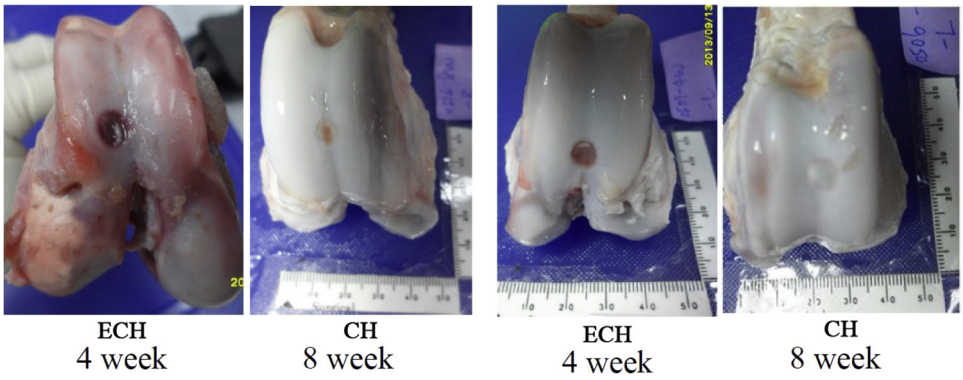

Figure 5 The gross morphology of rabbit after 4-week and 8-week implantation. CH = cell-laden hydrogel; ECH = empty cell-laden hydrogel.

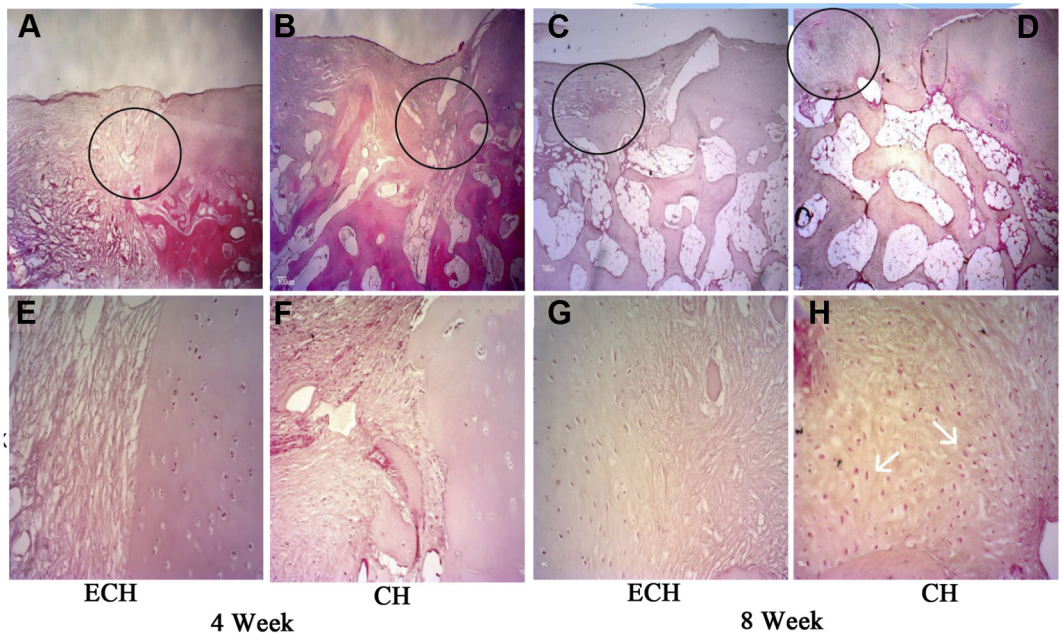

Figure 6 (A–H) Histology examination with hematoxylin and eosin stain of defects after 4 weeks and 8 weeks. The stain shows the full vision of repair site (A, C) empty cell-laden hydrogel and (B, D) cell-laden hydrogel; (E–H) are zoomed in from the gating circle in A–D, respectively (the arrows indicate the cell location).

ECH group at both 4 weeks and 8 weeks after transplantation. Our results also indicate that the SFMSCs in the PRP composite hydrogel facilitate the formation of cartilage tissue. This finding also indicates that the SFMSCs could be induced into functional chondrocytes within the PRP composite hydrogel under physiological environment at the defect site.

4. Discussion

The *in vitro* and *in vivo* studies reveal chondrogenesis by SFMSCs with the mixture of PRP and thermohydrogel. The use of SFMSCs in the management of cartilage defects is rarely mentioned in the literature. Pei et al[17] used allogeneic synovium-derived stem cells to repair rabbit femoral condyle cartilage defect. The cartilage defect was filled with hyaline cartilage with integration to the surrounding cartilage 6 months later. Lee et al[16] compared synovium-derived mesenchymal cells with 100% PRP gel to 100% PRP alone gel to manage rabbit cartilage defects. The PRP alone group showed fibrous tissue in the cartilage defect. The synovium-derived mesenchymal cell group showed greater hyaline cartilage formation with no cartilage defect. The PRP with thermosensitive hydrogel was used in this study instead of 100% PRP gel because executing this technique with arthroscopic instrumentation might be performed in the future. During arthroscopic surgery, the knee usually fills with a large amount of fluid, so the 100% PRP gel would be washed out quickly during surgery. The thermosensitive hydrogel has better affinity to PRP and prevents PRP from being washed out.

MSCs differentiate into mesenchymal lineages such as cartilage and bone under certain environment condition.[18] SFMSCs have several advantages for clinical use. The synovium is an easily available tissue either during arthroscopic or open surgery. The good quantity and quality of SFMSCs are both revealed in *in vivo* and *in vitro* studies. The SFMSCs had less donor site mobility than bone marrow mesenchymal cells. Jones and Pei[19] also showed that the SFMSCs display superiority in cartilage formation compared to other sources of mesenchymal cells. Our study also revealed that SFMSCs with PRP and thermohydrogel had better chondrogenicity than PRP and thermohydrogel.

PRP is prepared by centrifuging analogous, anticoagulated whole blood, and contains several times the concentration of platelets compared to whole blood. PRP has high levels of growth factors to stimulate bone and cartilage growth in the local environment. Nowadays, there are many different kinds of PRP

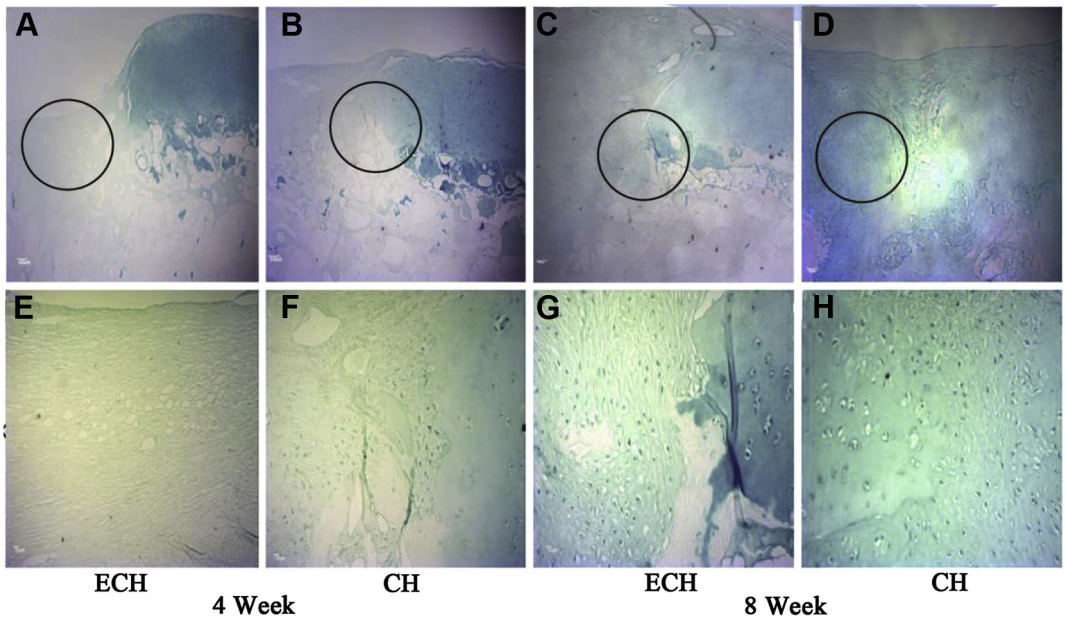

Figure 7 (A–H) Histology examination with alcian blue of defects after 4 weeks and 8 weeks. The stain shows the full vision of repair site (A, C) empty cell-laden hydrogel and (B, D) cell-laden hydrogel; (E–H) are zoomed in from the gating circle in A–D, respectively. CH = cell-laden hydrogel; ECH = empty cell-laden hydrogel.

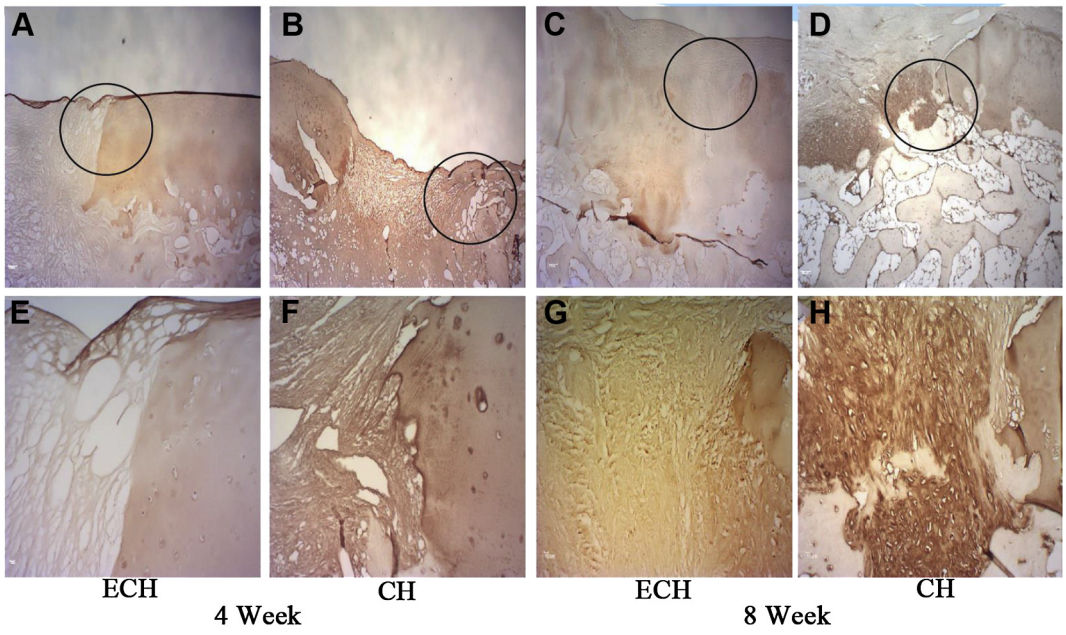

Figure 8 (A–H) Immunohistology with type I collagen of defects after 4 weeks and 8 weeks. The stain shows the full vision of repair site (A, C) empty cell-laden hydrogel and (B, D) cell-laden hydrogel; (E–H) are zoomed in from the gating circle in A–D, respectively. CH = cell-laden hydrogel; ECH = empty cell-laden hydrogel.

preparation, so the real PRP composition is different for each manufacturer's kit. Delong et al[20] proposed a Platelets Activation White blood cells (PAW) classification for PRP based on platelet concentration, activation method, and white blood cell counts. The classification for PRP in this study used the PAW system. The PAW classification provided an easy and effective method to document the PRP component and activation method. It could allow physicians to understand each different PRP faster and make further research more convenient.

Many articles mention the relation between PRP and chondrocytes.[21,22] Smyth et al[21] wrote a systematic review article about PRP in the pathologic processes of cartilage. PRP improves chondrocyte viability and promotes chondrocyte proliferation *in vitro*. PRP also increases gene expression including SOX9, aggrecan, bone morphogenetic protein, and TGF-β *in vitro*.[22] More than half of *in vivo* studies reveal that PRP improves gross and histologic appearance. The GAG secretion and type II collage contents of cartilage were increased *in vivo*. Our *in vitro* results also concur with the report.

Zhu et al[23] reviewed the application of PRP for cartilage defects and osteoarthritis. In an animal study, PRP may promote cartilage proliferation and stimulate hyaline cartilage formation. In the management of knee osteoarthritis, PRP have better and longer symptom relief compared to hyaluronic acid. PRP is a safe and effective

treatment for knee osteoarthritis regardless of patient age. The use of PRP in human cartilage defects also reveals promising results. PRP with hydrogel would create a better local environment for cartilage regeneration after microfracture procedure during an operation.

PEG-PLGA hydrogel has been well studied in recent literature. The biodegradation and hydrophilicity of PEG-PLGA copolymers can be managed by choosing the ratio of its hydrophilic and hydrophobic constituents.[24] The specific thermosensitive transformation makes PEG-PLGA hydrogel useful in the delivery of special medication into the human body. The sol-to-gel transition occurs at temperatures <37°C so that the special drug could be encapsulated within the structure.[25] The PEG-PLGA hydrogel is used in many medical fields including antitumor and anti-TB medication.[26] The PEG-PLGA hydrogel also showed a good result in the orthopedic field. Ni et al[27] used PEG-PLGA hydrogel in corporation with an acellular bone matrix. The biocompability of this composite to the rats was confirmed with macroscopic and microscopic examination. Fu et al[28] found that PEGP-poly(epsilon-caprolactone)-PEG and hydroxyapatite had good biocompatibility and biodegradability, and can guide bone regeneration processes. The incorporation of PRP and PEG-PLGA hydrogel has not been studied previously. Our research reveals that this composite would promote chondrogenesis *in vivo* and *in vitro*. Moreover, the better result of the CH group may indicate that this composite would not interrupt SFMSC transformation and proliferation. It may also indicate that SFMSCs could survive in this PRP-PEG-PLGA composite. This would be a promising result for further research focusing on the PEG-PLGA and PRP composite.

This study had several limitations. First, PRP preparation is different and diverse. Our promising result may not the same for other brands of PRP kit. Second, it is difficult to control the PEG-PLGA thermosensitive transformation in the same body temperature for each pig. The PRP of each porcine was different. So, the true PRP-PEG-PLGA composite would show some differences in each pig. Third, the quantity of SFMSCs was not controlled in our study. This meant that each CH group may have had different numbers of SFMSCs. Although we did not control the quantity of many factors, the result was still promising. We believe that the SFMSC with PRP-PEG-PLGA composite has potency for chondrogenesity.

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