

The application of autologous platelet-rich plasma gel in cartilage regeneration

AIGUO XIE¹, LANJUN NIE¹, GAN SHEN¹, ZIWEI CUI², PENG XU², HUAQIANG GE² and QIAN TAN²

¹Department of Plastic Surgery, Second Affiliated Hospital of Nanjing Medical University;

²Department of Plastic Surgery, Drum Tower Clinical Medical College, Nanjing Medical University, Nanjing, Jiangsu 210008, P.R. China

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Abstract. Cartilage defect caused by disease or trauma remains a challenge for surgeons, owing to the limited healing capacity of cartilage tissues. Cartilage tissue engineering provides a novel approach to address this issue, and appears promising for patients with cartilage defects. The cell scaffold, as one of the three key elements of tissue engineering, plays an important role in cartilage tissue engineering. Platelet-rich plasma (PRP), which is a fraction of the plasma containing multiple growth factors, has become a major research focus in the context of its use as a bioactive scaffold for tissue engineering. Therefore, we investigated the value of using PRP scaffolds combined with chondrocytes in cartilage tissue engineering. In this study, we examined the levels of growth factors in PRP, and the effects of PRP on cell proliferation and matrix synthesis in rabbit chondrocytes cultured in PRP. Short-term *in vitro* culture followed by long-term *in vivo* implantation was performed to evaluate the chondrogenesis of neocartilage *in vivo*. The results show that PRP may provide a suitable environment for the proliferation and maturation of chondrocytes, and can be used as a promising bioactive scaffold for cartilage regeneration.

Introduction

The management of various cartilage lesions remains a challenge for surgeons, due to the limited regenerative capacity of cartilage tissues. A number of techniques have been developed for the treatment of cartilage defects during the past decade, aiming to address this complex issue. These techniques, including autologous cartilage implantation and autologous

chondrocyte implantation, have been used to repair cartilage defects. More recently, tissue engineering has been reported as a promising approach to repair cartilage defects. This technique involves harvesting a high number of cells from the human body and seeding them on a biodegradable scaffold, and then implanting these scaffolds to cover the defective areas. Therefore, the emergence of cartilage tissue engineering techniques provides potential solutions for the clinical repair of cartilage defects. In addition, seeding of chondrocytes on biodegradable scaffolds in order to allow formation of a three-dimensional cartilage tissue for implantation is a primary approach in cartilage tissue engineering aiming to repair cartilage defects. Cao *et al* (1) used a chondrocyte-polymer composite to produce tissue-engineered cartilage in the shape of a human ear. Luo *et al* (2) reconstructed a segmental tracheal defect by pedicled tissue-engineered trachea in rabbits.

A variety of biodegradable materials has been explored for eventual use as scaffolds in cartilage tissue engineering, including collagen gels and sponges, hyaluronic acid matrices, and poly(α -hydroxyesters), such as polyglycolic acid (PGA), polylactic acid (PLA) and their copolymers. Wu *et al* (3) were able to form mature cartilage tissue by using poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV), and improved the PHBV scaffold with bioglass to produce cartilage tissue with improved biomechanical and biochemical properties. Xue *et al* (4) reported that chondrogenesis of bone marrow-derived stromal cells seeded on PGA promotes regeneration of cartilage. However, the natural and artificial synthetic material used in these studies are still exogenous, often causing inflammatory and immune reactions. Therefore, research on biodegradable scaffolds produced by autologous material is receiving increasing attention. Platelet-rich plasma (PRP) is ideal for use as a biomedical material for tissue engineering due to its reduced immunogenicity and improved biocompatibility, in addition to its high content in growth factors.

Numerous studies have used PRP as a scaffold for bone tissue engineering. El Backly *et al* (5) used platelet-rich plasma to enhance the osteoconductive properties of a hydroxyapatite- β -tricalcium phosphate scaffold for late healing of critical-size rabbit calvarial defects. Jiang *et al* (6) repaired calvarial defects in rabbits using platelet-rich plasma as the scaffold, which carried bone marrow stromal cells.

Correspondence to: Dr Qian Tan, Department of Plastic Surgery, Drum Tower Clinical Medical College, Nanjing Medical University, 321 Zhong Shan Road, Nanjing, Jiangsu 210008, P.R. China
E-mail: smmutanqian@sina.com

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However, up to now, few studies have investigated the utility of autologous platelet-rich plasma gel (APG) in cartilage tissue engineering. Therefore, in this study, APG was prepared and studied. The chondrocytes were seeded on the scaffolds and cultured *in vitro* for one week, followed by *in vivo* implantation for six weeks, to observe the growth and proliferation of the chondrocytes and the engineered cartilage tissue in PRP. The level of growth factors, cell proliferation in the scaffold, extracellular matrix production, as well as biomechanical properties of the neocartilage were examined, to evaluate the value of the PRP scaffold in cartilage regeneration.

Materials and methods

Ethics statement. All experimental procedures conducted on animals in this study were approved by the Ethics Committee of the Nanjing Medical University.

Blood collection and cell isolation. New Zealand white rabbits (2-3 months old), obtained from the Animal Center of Nanjing Medical University (Nanjing, China) were used to isolate PRP and articular cartilage. Animals were first anesthetized by intramuscular injection of sodium pentobarbital (30 mg/kg) and ketamine hydrochloride (30 mg/kg). Blood (50 ml) was collected from the posterior auricular vein with a 14-G needle using a 50-ml syringe treated with an anti-coagulant citrate dextrose solution (Sigma-Aldrich, St. Louis, MO, USA). Following collection of peripheral blood, the animals were sacrificed by intravenously injecting supersaturated pentobarbital.

The articular cartilages were removed from the knees and hip joints of the rabbits and were cut into small pieces as described in (3). Chondrocytes were released from the cartilage slices by digesting with collagenase II (0.2% w/v; Invitrogen Life Technologies, Carlsbad, CA, USA). The isolated cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Irvine, UK). Cells were incubated at 37°C in a 5% CO₂ incubator, and the medium was changed every 3 days.

Preparation of PRP. PRP was isolated from fresh rabbit blood (~50 ml) as previously described (7). Briefly, the blood was treated with anti-coagulant and was separated into the plasma and the hemocyte (erythrocytes and leukocytes) fractions, and then separated into PRP by continuous two-step sedimentation (Fig. 1A). PRP was clotted by adding a 10% (v/v) thrombin solution in CaCl₂ (1,000 U/ml thrombin in 100 mM CaCl₂) to yield a final thrombin concentration of 100 U/ml. Soluble PRP was released from the clotted preparations by centrifugation at 1,500 g for 5 min.

Quantification of growth factors. The level of transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)-AB in whole blood, PRP and activated PRP (aPRP) was determined using corresponding enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN) as in (8).

Cell cultures with different concentrations of PRP and cell proliferation assay. Cell cultures in DMEM fetal calf serum-free medium (SFM) containing different concentrations of PRP (0, 5, 10, 20 and 30%) were used to test the effect of PRP on the proliferation of chondrocytes. Briefly, the chondrocytes at passage 2 were precultured in DMEM SFM for 24 h. The chondrocytes were then cultured under five different conditions: 0% PRP (SFM without PRP), 5% PRP (5% PRP in SFM), 10% PRP (10% PRP in SFM), 20% PRP (20% PRP in SFM), and 30% PRP (30% PRP in SFM). The proliferation of the chondrocytes was assessed with a cell counting kit (CCK)-based colorimetric assay as in (9), following the manufacturer's instructions (CCK-8 kit; Rockville, MD, USA). The optical density (OD) of the samples was measured on a DU800 spectrophotometer (Beckman Coulter, Brea, CA, USA).

Formation of chondrocyte/PRP composites. After subculturing twice, the chondrocyte suspension at passage 2 was collected by trypsin digestion and centrifuged at 352 x g for 5 min. The chondrocytes were washed 2 times with phosphate-buffered saline (PBS) to remove the residual serum, and cells were counted and resuspended in PRP, at a density of 5.0x10⁷ cells/ml. To activate PRP, 0.15 ml of a CaCl₂ and Gibco® bovine thrombin mixture (1,000 U/ml in 100 mg/ml CaCl₂; Thermo Fisher Scientific, Waltham, MA, USA) was added to the cell/PRP suspension. Seven days later, the composites were subcutaneously implanted into BALB-c-nu nude mice (4 weeks old, 20 g), obtained from the Animal Center of Nanjing Medical University. The composites were harvested at 6 weeks post-implantation.

Scanning electron microscopy (SEM) of in vitro-engineered tissue. The attachment, proliferation and matrix production of the cells on the scaffolds were examined using light microscopy (Eclipse TS100 microscope; Nikon, Tokyo, Japan) and SEM (XL-30; Philips, Amsterdam, The Netherlands).

Evaluation of in vivo-engineered tissue

Gross observation of in vivo-engineered tissue. The composites were harvested at 6 weeks after implantation and were recorded by image analysis.

Histological evaluation. Following implantation for 6 weeks, representative *in vivo*-formed cartilaginous tissue was fixed in neutral-buffered formalin, embedded in paraffin and sectioned (5-µm thick sections). The cross-sections were stained with hematoxylin and eosin (H&E) and safranin O (Sigma-Aldrich, St. Louis, MO, USA).

Five-micrometer cryosections were used for immunostaining with the collagen type II monoclonal mouse anti-rabbit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The samples were immersed in PBS containing 1% goat serum at 4°C overnight, in order to block the non-specific reactions. Subsequently, the sections were incubated in PBS containing 1% bovine serum albumin (BSA) and anti-collagen type II antibody (1:100 working dilution) at 25°C for 4 h. After washing with PBS three times, the samples were incubated in PBS containing 3% BSA. Finally, the samples were incubated in PBS containing 1% BSA and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:150 working dilution; Santa Cruz Biotechnology, Inc.) at 25°C for 4 h, followed by color

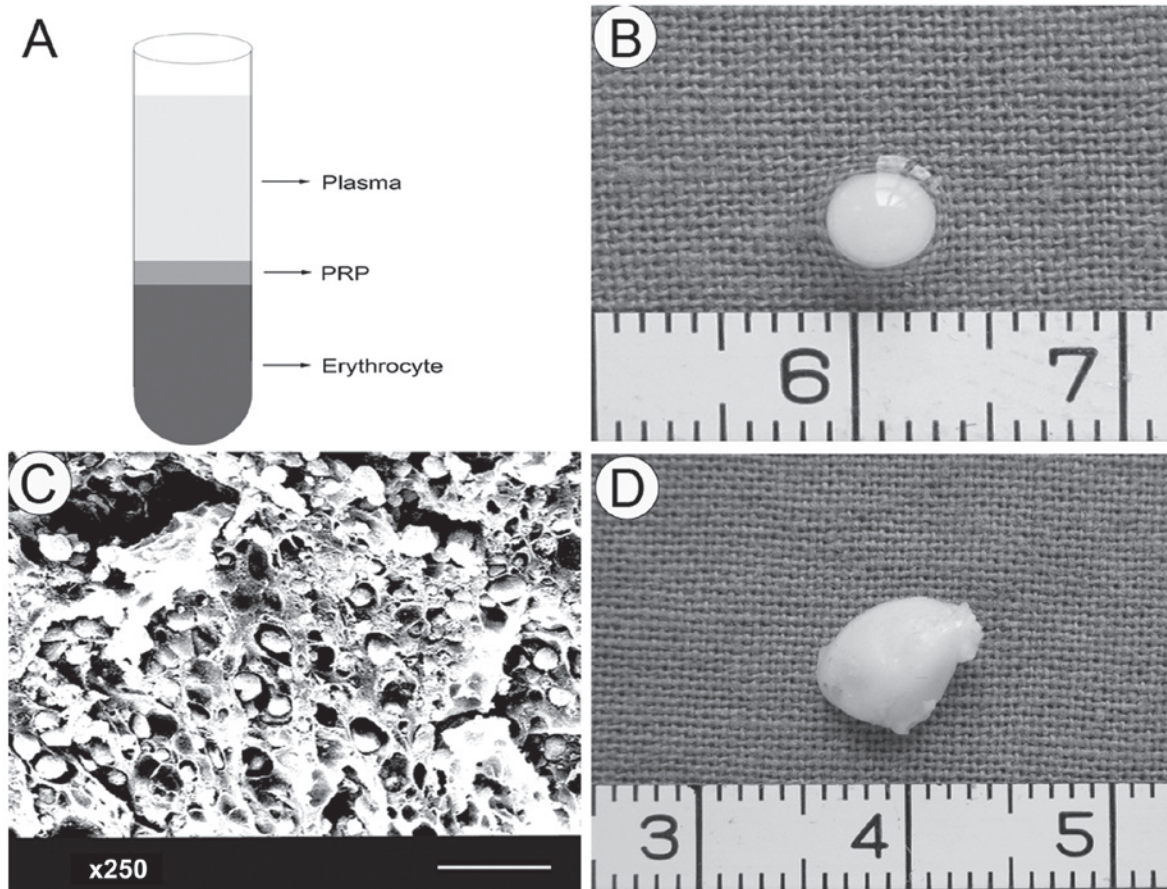


Figure 1. The optical and scanning electron microscopy (SEM) micrographs of the chondrocyte-autologous platelet-rich plasma gel (APG) composite. (A) Different fractions of the blood during platelet-rich plasma (PRP) preparation. (B) In the composite, the tissue is ivory-white and cartilage-like after 7-day culture *in vitro*. (C) SEM shows that scaffolds have a macroporous structure with open pores, and that chondrocytes grow well in APG after 3 days of *in vitro* culture. Scale bar, 100 μm . (D) Gross view of the composite after 6 weeks culture *in vivo*.

development with diaminobenzidine tetrahydrochloride (DAB; Santa Cruz Biotechnology, Inc), as in (3).

Quantitative analysis of *in vivo* cartilage formation. After 6 weeks of *in vivo* culture, the glycosaminoglycan (GAG) content (10,11) and the total collagen content were analyzed according to previously described methods (12). Briefly, the protein solutions of the specimens were prepared. The specific binding of Alcian Blue and polysulfated molecules of GAGs in cartilage were achieved by adding a series of reagents. All GAGs were precipitated specifically in guanidine-HCl by using a low pH in combination with detergent and high salt concentration. The precipitate was dissolved in a mixture of guanidine-HCl and propanol. The optical density (OD) of the samples was measured, and a linear standard curve between 0.5 and 20 mg was generated by adding known amounts of proteoglycans.

Biomechanical analysis. A biomechanical analyzer (Instron, Norwood, MA, USA) was used for the biomechanical tests. As previously described (3), a constant compressive strain rate of 1 mm/min was applied, until a maximal force of 100 N was achieved, and thus a force-displacement curve was obtained. The compressive modulus of the tested tissue was calculated based on the force-displacement curve.

Statistical analysis. All data were expressed as means \pm standard deviation (SD), with $n=6$. The differences were analyzed with a one-way analysis of variance (ANOVA), using SPSS

for Windows software, version 13.0 (SPSS, Inc., Chicago, IL, USA). A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Cell cultures and chondrocyte-APG composite formation. After 3 days of primary culture, cells reached 85-90% confluence. Following two additional subculturing rounds, the cells maintained their morphology, and chondrocytes at passage 2 were used for cartilage engineering. The chondrocyte-APG composite had an ivory-whitish appearance (Fig. 1B and D). When observed by SEM, the chondrocytes grew well in the APG scaffold, and the scaffold exhibited a macroporous structure with interconnected open pores, of size varying from 30 to 300 μm (Fig. 1C).

Quantification of growth factors. The results of ELISA assays showed that the level of TGF- β 1 increased from 3.10 ± 0.43 ng/ml in whole blood to 5.9 ± 0.67 ng/ml in PRP and 13.55 ± 1.53 ng/ml in aPRP (Fig. 2A). The concentration of IGF showed an increase from 14.50 ± 1.63 ng/ml in whole-blood to 45.03 ± 4.67 ng/ml in PRP and 110.06 ± 12.53 ng/ml in aPRP (Fig. 2B). The concentration of PDGF-AB showed an increase from 145.02 ± 16.3 pg/ml in whole blood to 263.00 ± 26.71 pg/ml in PRP and 503.00 ± 53.00 pg/ml in

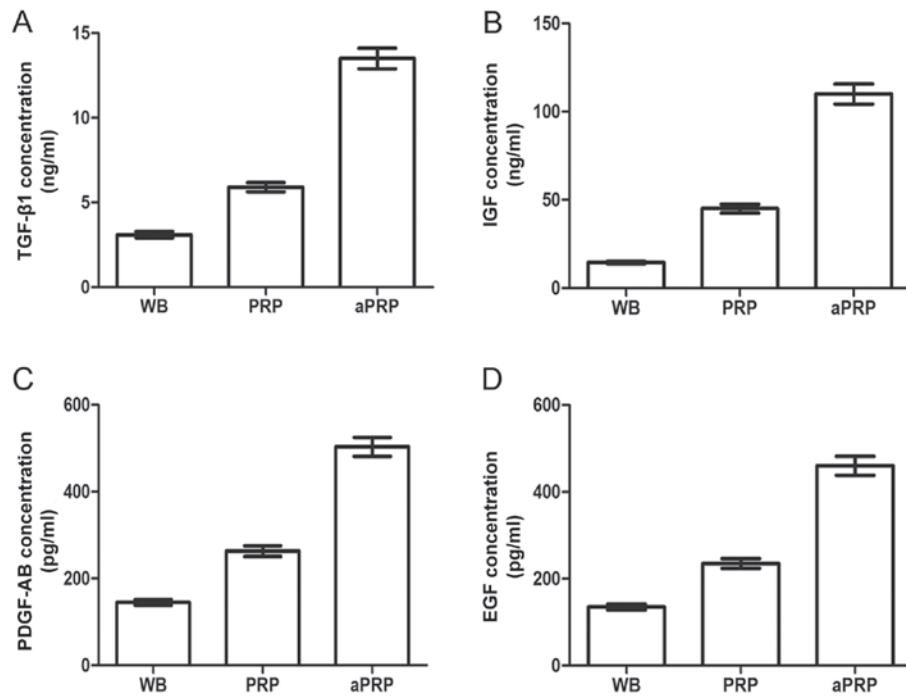


Figure 2. Quantification of growth factors. The concentration of transforming growth factor-β (TGF-β), insulin-like growth factor (IGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)-AB is increased in whole blood (WB), platelet-rich plasma (PRP), and activated PRP (aPRP).

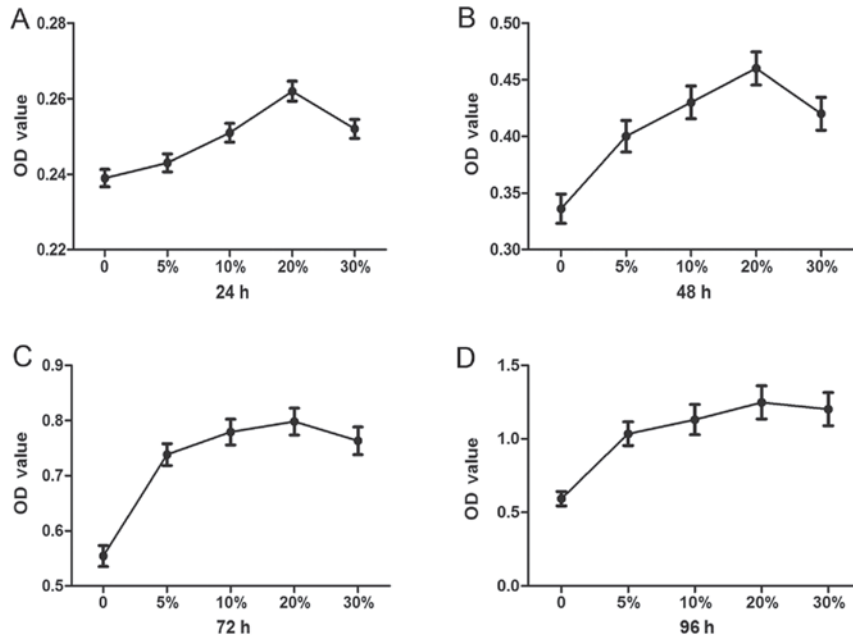


Figure 3. Cell proliferation in the presence of different concentrations of platelet-rich plasma (PRP). Cell proliferation increases at different concentrations of PRP, and reaches a peak at 20% PRP. OD, optical density.

aPRP (Fig. 2C). The concentration of EGF showed an increase from 135.12 ± 16.3 pg/ml in whole blood to 235.30 ± 25.67 pg/ml in PRP and 460.23 ± 50.98 pg/ml in aPRP (Fig. 2D).

The effect of different concentrations of PRP on cell proliferation. The result of the CCK assay showed that different concentrations of PRP have different effects on cell proliferation. Cell proliferation in the presence of different

concentrations of PRP (0, 5, 10, 20 and 30%) gradually increased, and reached a peak at 20% PRP (Fig. 3).

Evaluation of in vivo-engineered tissue

Gross evaluation of the in vivo-engineered composites. The chondrocyte-APG composites maintained their original size and presented a cartilage-like appearance following *in vivo* implantation for 6 weeks.

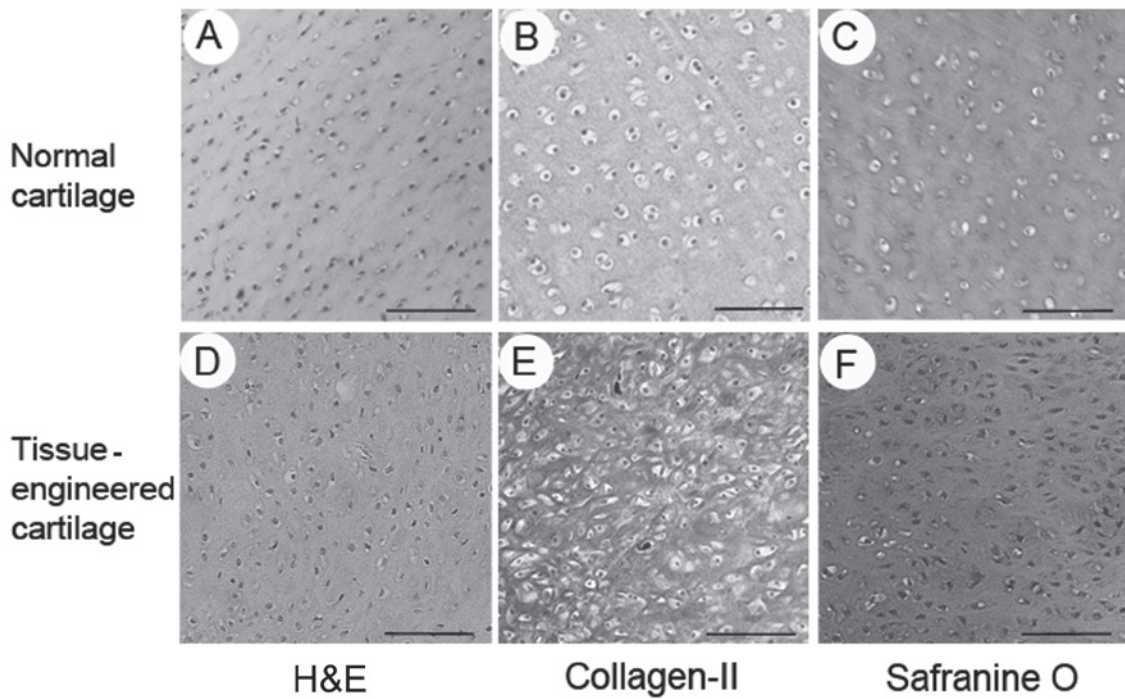


Figure 4. Histological and immunohistochemical examination of *in vivo*-engineered cartilage. (A-C) Normal cartilage and (D-F) tissue-engineered cartilage, (A and D) stained with H&E, (B and E) immunostained with collagen type II antibody, and (C and F) stained with safranin O. The chondrocyte-APG composite forms cartilage-like tissue. Lacuna-like structures, positive safranin O staining and type II collagen immunostaining are observed in the tissue-engineered cartilage, similar to the normal cartilage. Scale bar, 100 μ m. H&E, hematoxylin and eosin; APG, autologous platelet-rich plasma gel.

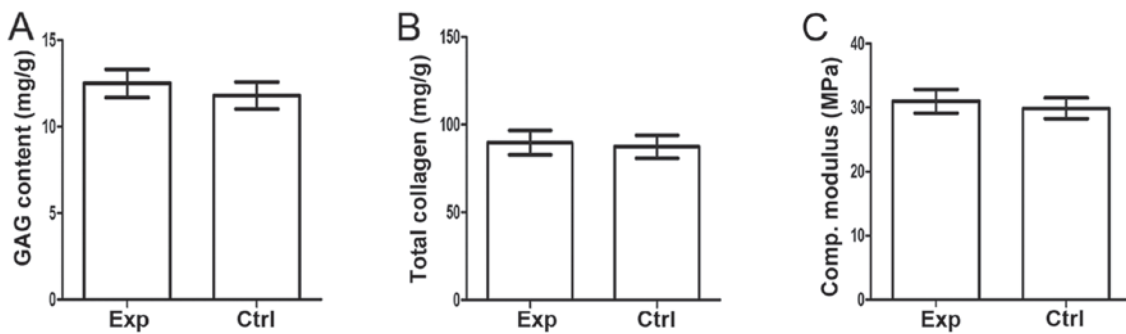


Figure 5. (A) Collagen content, (B) glycosaminoglycan (GAG) content and (C) compressive modulus in the tissue-engineered experimental group (Exp) and the normal cartilage control group (Ctrl). There is no significant difference in the collagen and GAG content, as well as in the compressive modulus between tissue-engineered and normal cartilage ($P > 0.05$).

Histology and immunohistochemistry. Histology and immunohistochemistry revealed the formation of tissue-engineered cartilage. Formation of the cartilage-like tissue was observed, with an obvious lacuna structure and positive staining for safranin O and type II collagen (Fig. 4).

Collagen and GAG content, and compressive modulus. Quantitative analysis of the collagen and GAG content demonstrated that there is no significant difference ($P > 0.05$) on extracellular matrix (ECM) contents between normal and tissue-engineered cartilage (Fig. 5A and B).

The mechanical properties of the cartilage are of importance, and the collagen and GAG content only partly reflect these. Analysis of the compressive modulus further indicated that there is no significant difference ($P > 0.05$) between tissue-engineered and normal cartilage (Fig. 5C).

Discussion

Cartilage defects have been a challenge for surgeons. However, developments in cartilage tissue engineering in the past few years have provided a means to repair cartilage defects (13-15). Certain cases of tissue-engineered cartilage implantation have been reported in the clinic (e.g., 16). The scaffold, which acts as a cell carrier, plays a significant role in the regeneration of the cartilage. Numerous natural or synthetic materials have been exploited as scaffolds during the past decade for cartilage tissue engineering. However, an optimal biodegradable scaffold for cartilage tissue engineering would ideally be less immunogenic than the currently used ones, with improved biocompatibility and an adequate degradation rate; in addition, the scaffold

is expected to promote attachment, proliferation and the activity of cells (17).

Autologous platelet-rich plasma gel (APG) is a fraction of the plasma containing multiple growth factors, such as TGF- β 1, PDGF, EGF and IGF-1, and which can induce biological changes in cell proliferation and matrix metabolism in a variety of connective tissues (18,19). APG can be produced by centrifugal separation of autologous whole-blood samples (20). Moreover, APG has an ideal three-dimensional structure, reduced immunogenicity and satisfactory biocompatibility. Scanning electron microscope analysis in this study revealed that APG has a three-dimensional nanostructure, and that cells grew well in APG. Due to the above advantages, APG appears promising as a novel biodegradable scaffold to be used in tissue engineering.

Despite the development of various natural or synthetic biomaterials for cartilage tissue engineering, the biological safety of scaffolds and the long-term biocompatibility between chondrocytes and scaffolds, or between tissue-engineered cartilage and acceptor tissue, still needs to be further investigated. Most of the currently used biodegradable material has been reported to cause certain inflammatory reactions, and in some cases, it is even rejected following implantation via immune reactions (21,22). Compared to other materials, APG is an autologous material with reduced immunogenicity and enhanced biocompatibility, and has been used in certain clinical cases. Torrero *et al* (23) treated 30 patients (18-65 years old, diagnosed with Outerbridge grade I-III chondropathy in the knee, pain for more than 3 months following conservative treatment, and no bone axial defects) affected by chondropathy of the knee with a single intrarticular injection of PRP, and statistical analysis with ANOVA showed significantly better ($P < 0.05$) results in these patients in terms of KOOS and VAS scores at 1, 3 and 6 months, in comparison to the pre-injection values. Redaelli *et al* (24) revitalized face and neck with PRP injection and also obtained satisfactory results.

In addition, PRP provides a high level of growth factors such as TGF- β 1, FGF etc. In the present study, we measured the concentration of growth factors in the whole blood, PRP and aPRP. The results showed higher concentrations of growth factors in PRP than that in the whole blood. Moreover, the activated PRP in APG showed the highest concentrations of growth factors among the three groups. The reason for that is that the platelets collected in PRP are activated by the addition of thrombin and calcium chloride, which induces the release of these factors from the α granules (25,26).

It was reported that most growth factors found in PRP can enhance cell proliferation and/or matrix production (18). TGF- β plays an important role in tissue regeneration, cell differentiation and embryonic development, and the application of TGF- β on the chondrocytes induces the synthesis of type I relative to type II collagen (27,28). Both IGF-I and IGF-II stimulate chondrocyte proliferation, and maintain the chondrocyte phenotype (29). We showed in this study that PRP, a highly concentrated natural combination of growth factors, has the ability to stimulate cell proliferation. Furthermore, we studied the effect of different concentrations of PRP on cell proliferation, and the result showed that cell proliferation is dose- and time-dependently increased by PRP, with the highest levels of cell proliferation observed at a dose of 20%

PRP. In addition to proliferation, the growth factors present in PRP are known to accelerate epithelial regeneration, promote angiogenesis, and ultimately improve wound healing in skin and periodontal tissue (30).

In conclusion, PRP provides a good environment for cell growth and proliferation, by supplementing the chondrocytes with all the essential nutrients. In this study, the chondrocyte-APG composite was formed on an ivory-whitish, cartilage-like tissue *in vitro* and *in vivo*. Histological and immunohistochemical examination of the engineered cartilage showed that the majority of the cells formed lacuna-like structures, with positive staining for safranin O and type II collagen.

In addition, there was no significant difference observed in the compressive modulus between the tissue-engineered and the normal cartilage groups. The mechanical properties of neocartilage tissue are determined by the content of the ECM (31). Previous studies reported that the ECM content (GAG and total collagen) in cartilage tissue contributes to the improvement of mechanical strength (e.g., 32). Our study also found that the GAG and total collagen content in tissue-engineered cartilage is similar to that in the normal cartilage tissue. Thus, the APG scaffold supported the growth of cells, which maintained their activity, fully expressed their phenotype, produced the extracellular matrix, and preserved the characteristic spherical morphology associated with the synthesis of type II collagen and cartilage-specific proteoglycans.

In summary, the APG scaffolds provide a high level of growth factors, which can efficiently promote chondrocyte proliferation. The chondrocyte-scaffold composites formed cartilage-like tissue of homogeneous structure, showing strong mechanical properties and high cartilage matrix content. Overall, these results suggest that the APG scaffolds may constitute a suitable biodegradable scaffolds for cartilage tissue engineering.

Acknowledgements

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