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Repair of Osteochondral Defects in a Rabbit Model Using Bilayer Poly(Lactide-co-Glycolide) Scaffolds Loaded with Autologous Platelet-Rich Plasma

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: To examine the effects of the addition of autologous platelet-rich plasma (PRP) into bilayer poly(lactide-co-glycolide) (PLGA) scaffolds on the reconstruction of osteochondral defects in a rabbit model.





Material/Methods: Porous PLGA scaffolds were prepared in a bilayered manner to reflect the structure of chondral and subchondral bone. Bone defects, measuring 4 mm in diameter and 4 mm in thickness, were created in both knee joints in 18 healthy New Zealand white rabbits, aged between 120–180 days old. Rabbits were randomly divided into three groups: rabbits with bone defects implanted with bilayer PLGA scaffolds (PLGA group) (N=6); or with bilayer PLGA and autologous PRP (PLGA/PRP group) (N=6); and the untreated group (control group) (N=6). The gross morphology, histology, and immunohistochemistry for the expression of collagen type II and aggrecan were observed at 12 weeks after surgery and compared using a scoring system. Micro-computed tomography (CT) imaging and relative expression of specific genes were also assessed.

Results: The platelet concentrations in the PRP samples were found to be 4.9 times greater than that of whole blood samples. The total score on gross appearance and histology was greatest in the PLGA/PRP group, as was the expression of collagen II and aggrecan of the neo-tissue. Micro-CT imaging showed that more subchondral bone was formed in the PLGA/PRP group.

Conclusions: Bilayer PLGA scaffolds loaded with autologous PRP improve the reconstruction of osteochondral defects in the rabbit model.

MeSH Keywords: **Osteochondrosis • Platelet Activation • Polymethacrylic Acids • Tissue Scaffolds**

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Background

An important area orthopedic research is to develop reliable approaches for the repair and regeneration of damaged articular cartilage [1–6]. Although recent progress has been made in the field of cartilage repair, current clinical therapies including hyaluronan injection, subchondral drilling, autologous chondrocyte implantation, and mosaicplasty are still subject to practical difficulties [7,8]. Because articular cartilage and subchondral bone have specific anatomical levels and structures, cartilage damage is often accompanied by bone damage.

A suggested improvement in the approach to repair of defects in articular cartilage and bone has been to construct a tissue-engineered osteochondral composite. Previously reported studies had demonstrated the use of transforming growth factor (TGF)- β as an important requirement for chondrogenesis [9,10]. Tissue-engineered osteochondral composites have previously been prepared for repair of osteochondral defects. A construct of poly(lactide-co-glycolide) (PLGA) porous scaffold and chondrocytes sutured to a construct of tricalcium phosphate (TCP) and osteoblasts has been used [3]. A dual-layered composite of PLGA and hydroxyapatite-TCP or a bilayer system of PLGA scaffolds containing TGF- β 1 and bone morphogenetic protein-2 (BMP-2) in each layer have also been used for the repair of osteochondral defects [11–13]. In these studies, the implanted bilayer osteochondral composite was found to promote both cartilage and bone regeneration [11–13]. However, it is likely that biological growth factors act in a highly coordinated manner during osteochondral tissue development, and the use of a single factor to stimulate and regulate the process of chondrogenic differentiation may be a limited approach to a complex physiological process.

However, previous studies have shown that platelet-rich plasma (PRP) can release platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), TGF- β , insulin growth factor (IGF), and basic fibroblast growth factor (b-FGF) [14,15]. Recently PRP has gained wide acceptance in orthopedic surgery as a rich source of growth factors [16,17]. PRP has been widely used in the repair and regeneration of cartilage and bone, both as a scaffold and as part of a composite [18,19].

In this study, a bilayer strategy was employed using a porous scaffold composed of the biodegradable polymer PLGA, with different pore sizes, loaded with autologous PRP. PLGA has been widely used in clinical studies of ligament, tendon, cartilage, and bone regeneration [20–24]. Therefore, the effect of the addition of PRP into a PLGA scaffold is the aim of this preliminary study.

PRP has a high level of biological safety when used for tissue regeneration [25]. Potentially, autologous PRP has a wide range of potential applications in the field of regenerative medicine

and medical research. PRP has been shown to increase the proliferation rate of mesenchymal stem cells (MSC) and to cause chondrogenic differentiation of MSCs [18]. PRP may also enhance chondrocyte migration and stimulate chondrogenic differentiation in human subchondral progenitor cells [26]. The single-layer PLGA scaffold loaded with PRP has been shown to promote bone and cartilage regeneration in the patellar groove in the rabbit model [27]. However, to our knowledge, there have been no previous reports of the use of bilayer scaffolds loaded with PRP to repair both cartilage and bone simultaneously. For these reasons, the aim of this study was to prepare bilayer PLGA scaffolds loaded with PRP to include two layers of the same porosity but with different pore sizes, adhered together by a thin PLGA film. The porous scaffolds were implanted into defects in the medial femoral condyles of rabbits.

Material and Methods

Animals studied

Eighteen New Zealand white rabbits, 120–180 days old, weighing 2.8–3.2 kg were studied. All animals were healthy. The study protocol was approved by the Institutional Animal Care and Use Committee of the Southern Medical University.

Rabbits were randomly divided into three groups: rabbits with bone defects implanted with bilayer poly(lactide-co-glycolide) (PLGA) scaffolds (PLGA group) (N=6); or with bilayer PLGA and autologous platelet-rich plasma (PRP) (PLGA/PRP group) (N=6); and the untreated group (control group) (N=6) (Figure 1).

Manufacture of porous PLGA bilayer scaffolds

The room temperature compression molding and particulate leaching technique was used to generate a porous PLGA scaffold, as previously described [28–32]. Briefly, PLGA with a copolymer ratio of 85: 15 (lactic acid: glycolic acid) from Purac Co. Netherlands, was dissolved in dichloromethane and then mixed with salt particles to form a paste-like mixture. The mixture was pressed into a mold and kept under pressure for 24 h. After the mold was released, the shaped cylinder was produced. We also pre-prepared a PLGA thin film. The PLGA solution was cast on a petri dish. After air-drying for 48 h, PLGA film with a thickness of 300 μ m was produced.

To produce the bilayer scaffolds, the cylinders with different pore sizes and the PLGA film were adhered with dichloromethane under pressure and then cut into pieces measuring 4 mm in diameter and 4 mm in thickness, with the following characteristics: cartilage layer: 92% porosity, 50–100 μ m pore size, 300 μ m thickness; the adhesive layer: PLGA film with 300 μ m thickness; the subchondral layer: 92% porosity, 300–450 μ m

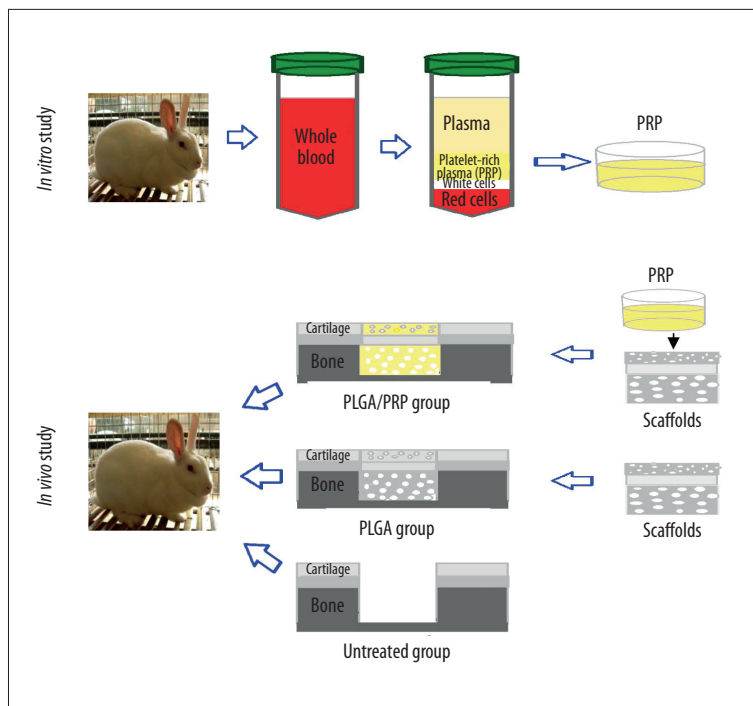


Figure 1. Study design. *In vitro* study:

Autologous platelet-rich plasma (PRP) was harvested from rabbit whole blood. *In vivo* study: 1) Bilayer poly(lactide-co-glycolide) (PLGA) scaffolds loaded with PRP were implanted into the osteochondral defects in the knee joints in the PLGA/PRP group. 2) Plain PLGA scaffolds were implanted into the osteochondral defects in the PLGA group. 3) The osteochondral defects were left untreated in the control group.

pore size, 3.4 mm thickness. After washing with water, we obtained the bilayer PLGA scaffold. The microstructure of the bilayer PLGA scaffolds were observed by scanning electron microscopy (SEM) (S-3000N, HITACHI, Japan) (Figure 2).

Preparation of autologous PRP

Blood samples were obtained from New Zealand rabbits in the experimental group. Autologous PRP was prepared using two centrifugation techniques, as previously described [33]. Briefly, rabbits were anesthetized and 9 ml of whole blood was drawn, from the central auricular artery of each rabbit, into sterile tubes each containing 1ml of acid citrate dextrose-A solution as an anticoagulant. The tubes were then spun at 1,496 rpm for 15 min in a centrifuge at room temperature. Blood was separated into three phases: platelet-poor plasma (top), platelet-rich plasma (middle), and erythrocytes (bottom). The top and middle layers were transferred to new tubes and centrifuged again at 2,115 rpm for 10 min. The supernatant was discarded and the remaining 0.8 ml of plasma containing the precipitated platelets was blended, as autologous PRP. The PRP was prepared before surgery. The platelets in the PRP and whole blood samples were counted manually.

Loading of the PRP onto the bilayer PLGA scaffolds

Twelve sterile bilayer PLGA scaffolds were carefully placed into a 6-well plate. The PRP and 10% calcium chloride (0.05 ml/ml of PRP) were added to the plate and 0.8 ml of PRP was absorbed into the two PLGA scaffolds in the 6-well plates, as shown in Figure 1.

Surgical implantation

An osteochondral defect, measuring 4 mm in diameter and 4 mm in thickness, was created through the articular cartilage and subchondral bone of each medial femoral condyle in the two knees of the 18 rabbits using a drill equipped with a 4 mm diameter drill bit. Twelve PLGA/PRP composites were implanted into the osteochondral defect in each knee of the same rabbit in the PLGA/PRP group. Twelve sterile PLGA scaffolds were implanted into the defects in the PLGA group, and osteochondral defects were left untreated in the control group (Figure 1). The rabbits were returned to their cages and allowed to move freely without joint immobilization. The rabbits (6 knees/group) were euthanized at four weeks and 12 weeks after surgery with an intravenous injection of pentobarbital (120 mg/kg).

Gross examination

Harvested samples of the rabbit knee joints were initially examined macroscopically. The gross appearance of the defects was assessed at four weeks and 12 weeks after surgery using a previously developed scale [34].

Histological evaluation

The distal femoral component of the rabbit knee joint was fixed and decalcified with 10% EDTA solution. Tissue blocks were prepared for processing using coronal sectioning of the center of the defect. Samples were then dehydrated, embedded,

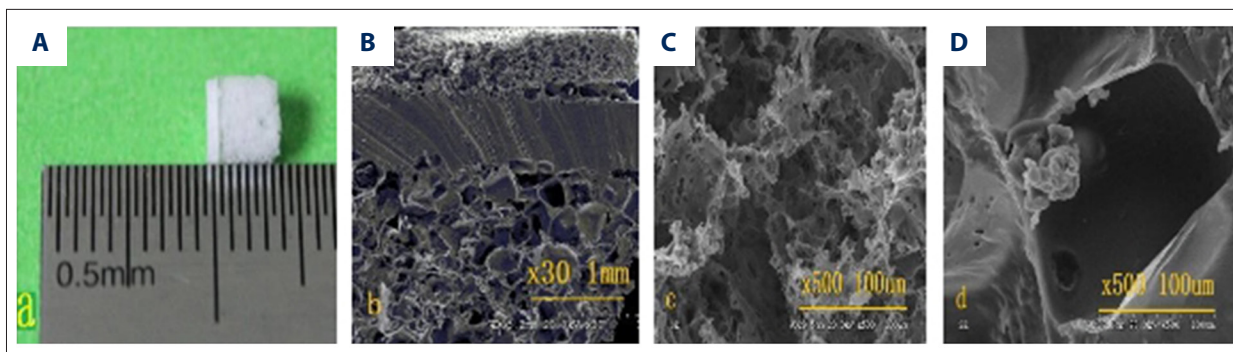


Figure 2. Imaging the poly(lactide-co-glycolide) (PLGA) scaffolds. (A) The gross appearance of the bilayer PLGA scaffolds prepared in this study. (B–D) Scanning electronmicroscopy (SEM) images (magnification, $\times 30$ and $\times 500$) of the sections of the scaffold.

cut, and stained with toluidine blue (Amresco, America) and Safranin-O/Fast Green staining for cartilage (Sigma, America). Sections were examined in a blinded manner and independently scored by three investigators according to the O'Driscoll histological grading scale, as described previous studies [27].

Immunohistochemical (IHC) evaluation

The expression of collagen type II was assessed by IHC evaluation, using a two-step IHC Detection Kit with an antibody (Merck, Germany) against type II collagen according to the manufacturer's instructions.

Quantitative polymerase chain reaction (qPCR)

At 12 weeks after the operation, a sharp biopsy punch with an inner diameter of 4 mm was used to cut a circle centered on the repaired area and tissue was collected by scraping, prior to the rabbits being euthanized. Pieces of cartilage from nine knees were immediately frozen. Total RNA was prepared using the Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The qPCR reaction was performed for aggrecan, collagen type I, collagen type II, and collagen type X, with mRNA detection performed using an ABI 7500 real-time PCR system and a SYBR Green PCR Kit (TaKaRa Japan). Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as an endogenous reference, and each sample was normalized to its GADPH content. All experiments were performed in duplicate.

Micro-computed tomography (CT) imaging

To observe the newly formed subchondral bone within the neo-tissue, a high-resolution microtomography scanner (Scanco μ -80) was used to visualize the medial femoral condyle of the rabbits in the three groups at 12 weeks.

Statistical analysis

Data were expressed as mean \pm standard deviation (SD). Statistical analysis was performed using SPSS 13.0 software for Windows. Statistically significant values were defined as $P < 0.05$ based on one-way analysis of variance (ANOVA).

Results

Characterization of the bilayer PLGA scaffolds

The gross appearance and the scanning electronmicroscopy (SEM) images of the poly(lactide-co-glycolide) (PLGA) scaffold are shown in Figure 2. The scaffold average pore sizes were 50–100 μ m in the cartilage layer and 300–450 μ m in the subchondral layer (Figure 2C, 2D). The porosities in both layers were approximately 92%. The adhesive layer was a solid PLGA film with thickness of 300 μ m (Figure 2B).

Assessment of autologous platelet-rich plasma (PRP)

The mean platelet number was $333.50 \pm 18.10 \times 10^3/\mu$ l in whole blood samples and $1633.26 \pm 89.64 \times 10^3/\mu$ l in PRP samples. Therefore, the platelet concentrations in the PRP samples was 4.9-fold that of the whole blood samples.

Gross examination

There were no signs of major infection in any rabbit and no rabbit died during the experimental procedures. The defects in the PLGA/PRP and the PLGA groups were quite similar in gross appearance at four weeks after implantation (Figure 3B, 3C). However, the central area of the defects in the untreated group remained depressed in appearance (Figure 3A). At 12 weeks, the regenerated tissue in the PLGA/PRP group integrated well with the adjacent cartilage; no obvious margins could be distinguished, and the neo-cartilage resembled hyaline cartilage (Figure 3F). In contrast, margins were obvious in the PLGA

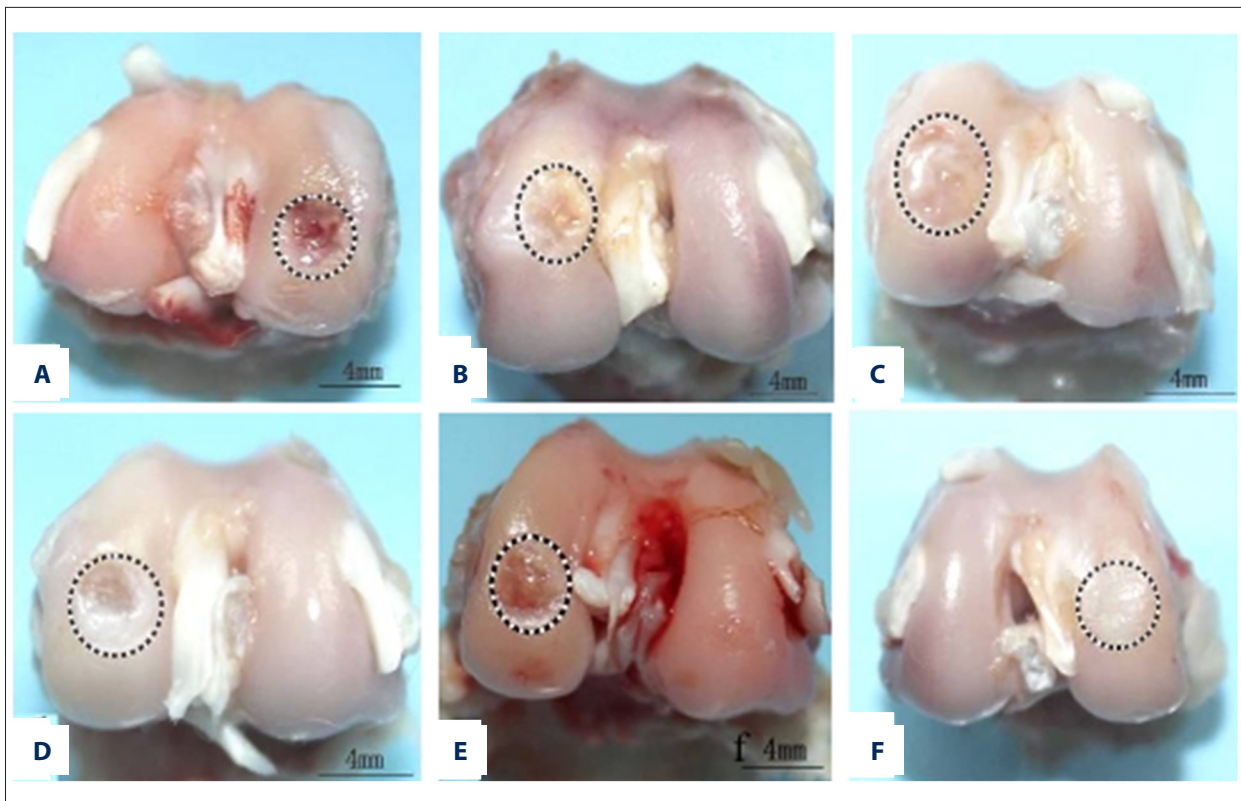


Figure 3. Gross appearance of the articular joint defects at four weeks and 12 weeks after surgery. (A, D) Untreated group. (B, E) Poly(lactide-co-glycolide) (PLGA) group. (C, F) PLGA/platelet-rich plasma (PRP) group. At (A–C) four weeks and at (D–F) 12 weeks. The circles indicate the sites of new tissue filling in the osteochondral defects.

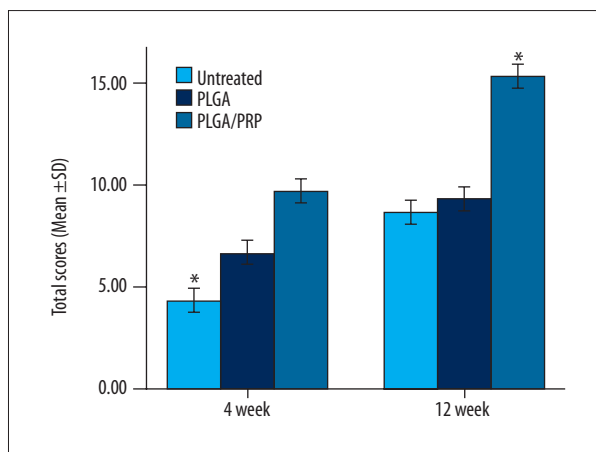


Figure 4. Total scores of the gross appearance of the articular joints at four weeks and 12 weeks after surgery. N=3. P<0.05, untreated group versus the poly(lactide-co-glycolide) (PLGA) group and the PLGA/platelet-rich plasma (PRP) group at four weeks.

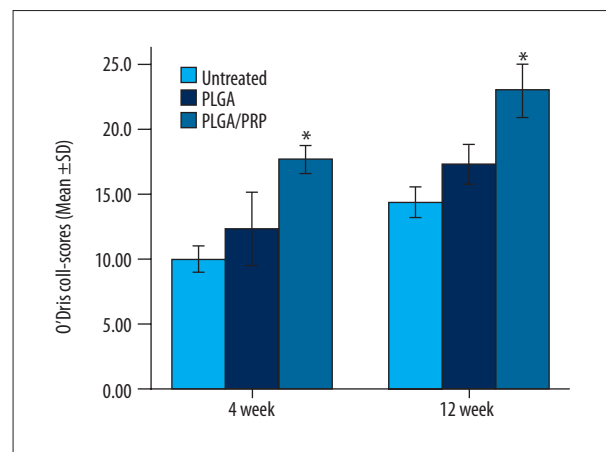


Figure 5. The O'Driscoll score at four weeks and 12 weeks after surgery. All values are mean ±SD of three experiments. The total score was greater in the poly(lactide-co-glycolide) (PLGA)/platelet-rich plasma (PRP) group than the PLGA and untreated groups at four weeks and 12 weeks. * P<0.05. PLGA/PRP group versus the PLGA group and untreated group.

group (Figure 3E) and the untreated group (Figure 3D), and the surfaces of the defects in these groups were less smooth than in the PLGA/PRP group.

The total scores for the gross appearance in all three groups at four weeks and 12 weeks are shown in Figure 4. At four weeks, the score in the PLGA/PRP group was higher than the PLGA group ($P=0.01$) and untreated group ($P<0.001$). At 12 weeks, the score in the PLGA/PRP group was significantly greater than in the PLGA group ($P<0.001$) and the untreated group ($P<0.001$).

Histological examination

Total scores of the O'Driscoll histological grading for each group are shown in Figure 5. The PLGA/PRP group scored significantly higher than the PLGA group ($P=0.013$) and untreated group ($P=0.003$) at four weeks after surgery. At 12 weeks after surgery, the PLGA/PRP group scored significantly higher than the PLGA group ($P=0.005$) and untreated group ($P=0.001$). The mean histological grade for the PLGA group was greater than the untreated group at four weeks and 12 weeks after surgery, even though this difference was not statistically significant.

At four weeks, the osteoarticular defects were partially filled with new tissue. The extracellular matrix was identified by Safranin-O/Fast Green and toluidine blue. The PLGA/PRP group demonstrated neo-cartilage that had migrated from the edge of the defect to the central region (Figures 6F, 7F). At 12 weeks, there was significantly more cartilage in the extracellular matrix and chondrocyte-like cells in the lacunae in the PLGA/PRP group (Figures 6L, 7L). The surfaces of defects were less smooth and fewer lacunae were found in the PLGA and untreated groups (Figures 6J, 6K, 7J, 7K).

Immunohistochemical evaluation

Positive immunohistochemical staining for collagen II was seen at four weeks and 12 weeks (Figure 8). The expression of collagen II was greatest in the PLGA/PRP group.

Analysis of gene expression

The relative expression level of the cartilage-related genes was analyzed by qPCR, as shown in Figure 9. The relative expression level of collagen type II and aggrecan in the PLGA/PRP group was significantly greater than in the PLGA group ($P<0.001$) and the untreated group ($P<0.001$). Similarly, the relative expression level of aggrecan in the PLGA/PRP group was also significantly greater than in the PLGA group ($P=0.002$) and the untreated group ($P=0.001$). The expression level of collagen type I in the PLGA/PRP group was not significantly different between the PLGA group ($P=0.165$) and the untreated group ($P=0.077$). The expression level of collagen type X was also not significantly

difference between the PLGA group ($P=0.654$) and the untreated group ($P=0.583$).

Micro-CT imaging

Subchondral bone regeneration within the defects was detected in all three groups. More mineralized bone was found in the PLGA/PRP group than in the untreated group, as shown in in Figure 10.

Discussion

The findings of this study showed that the addition of autologous platelet-rich plasma (PRP) into a bilayer poly(lactide-co-glycolide) (PLGA) scaffold had a positive effect on the repair of osteochondral defects in a rabbit model. In this study, the platelet concentration in PRP samples was found to be increased 4.9-fold when compared with whole blood samples, which may represent a form of 'therapeutic' PRP [25]. This finding may also be explained because a double centrifugation protocol was used that has previously been shown to induce higher platelet concentrations when compared with single centrifugation protocols [35].

A previously published study by Getgood and colleagues has shown that no exogenous activation of platelets with thrombin is required for the release of growth factors if PRP is combined with a polylactide osteochondral scaffold [24,36]. In this study, we loaded PRP into the bilayer PLGA scaffolds, and our findings showed that the new tissue formation in the PLGA/PRP group was greatest for all three groups studies. The possible explanation of this is PRP may enhance chondrocyte migration and stimulate chondrogenic differentiation of subchondral progenitor cells [26].

Furthermore, bilayer PLGA scaffolds with different pore diameters on the top and lower layer were used in the present study. Scaffolds with small pore size might facilitate the cartilage formation, as pore sizes $>300\ \mu\text{m}$ have previously been shown to promote vascularization and new bone formation [37,38]. For this study, we chose pore sizes of $50\text{--}100\ \mu\text{m}$ in the cartilage layer and $300\text{--}450\ \mu\text{m}$ in the subchondral layer. A thin solid PLGA film acted as the adhesive between the cartilage layer and subchondral layer to avoid blood vessel growth into the cartilage layer. Autologous PRP was loaded onto the scaffold and then implanted into the osteoarticular bone defects.

The results from this study in the rabbit model showed that the PLGA/PRP group developed more abundant cartilaginous extracellular matrix (ECM) than the PLGA group and the untreated group (Figures 6, 7). The surfaces of the osteoarticular bone defects in the untreated group were less smooth than in

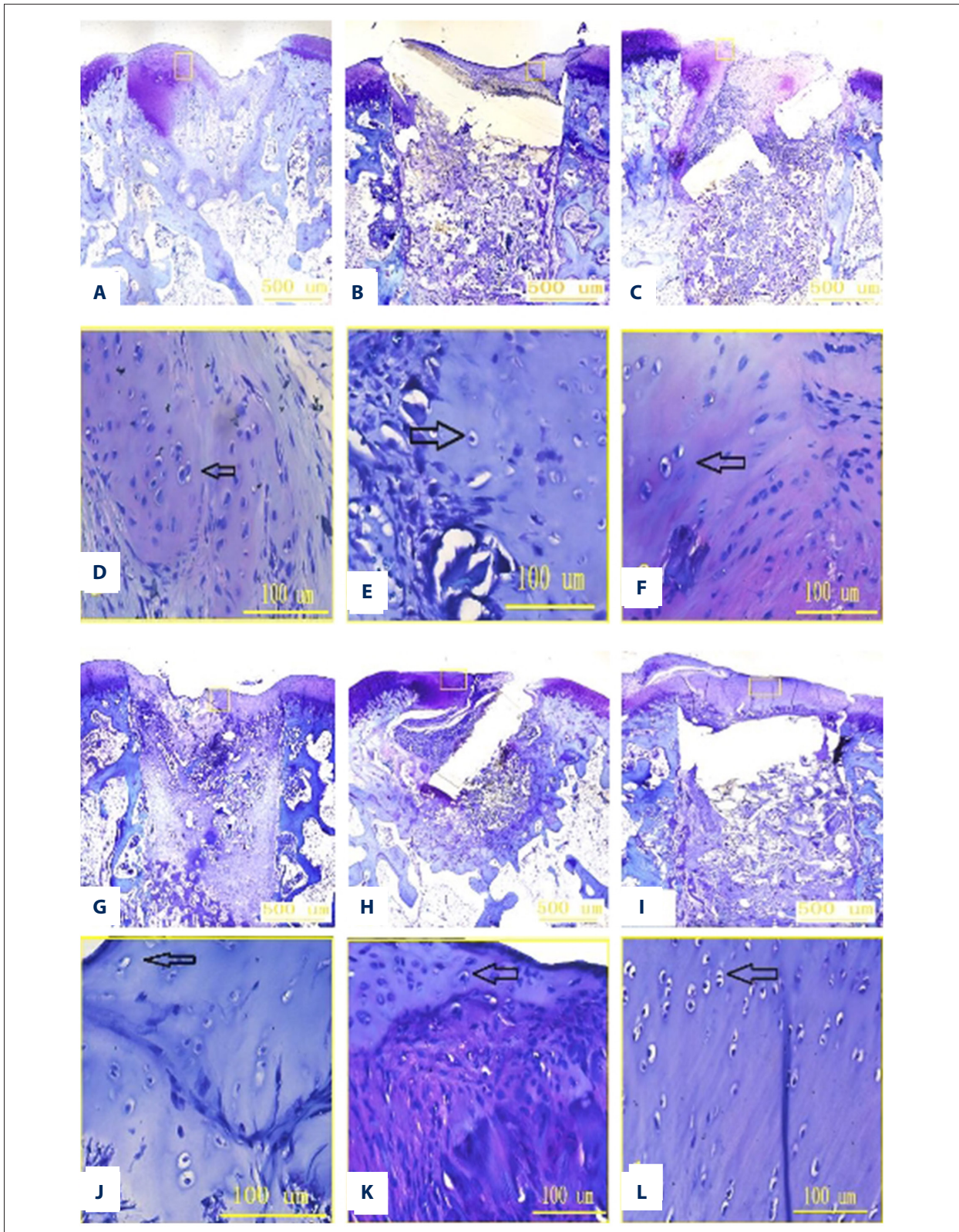


Figure 6. Toluidine blue staining of the osteoarticular joint defects. Toluidine blue staining at four weeks (A-F) and at 12 weeks (G-L) in: (A, D, G, J) the untreated group, (B, E, H, K) the poly(lactide-co-glycolide) (PLGA) group, and (C, F, I, L) PLGA/platelet-rich plasma (PRP) group. Arrows indicate cells within the lacunae.

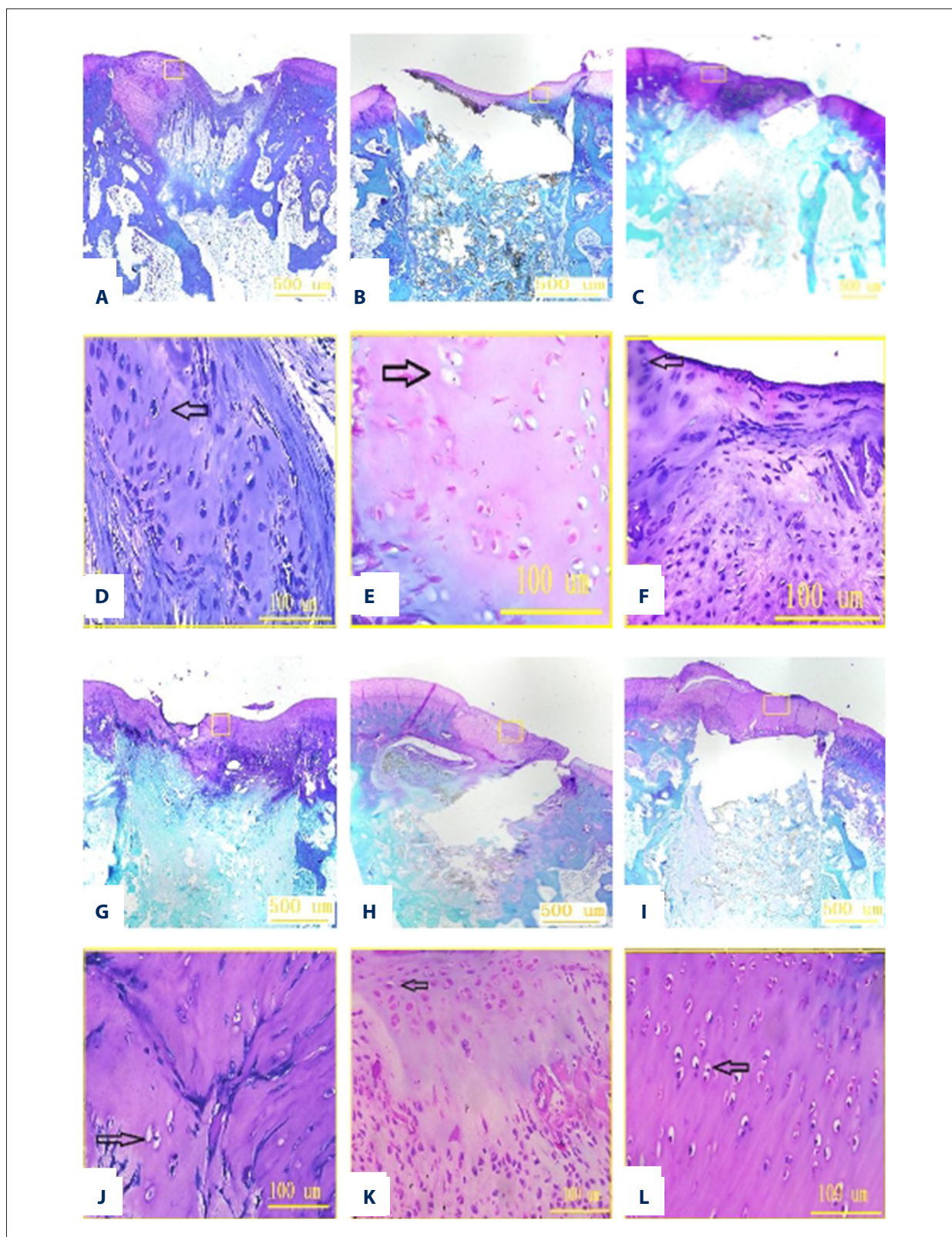


Figure 7. Safranin-O/Fast Green staining of the osteoarticular joint defects. Safranin-O/Fast Green staining for cartilage at four weeks (A–F) and at 12 weeks (G–L) in: (A, D, G, J) the untreated group, (B, E, H, K) the poly(lactide-co-glycolide) (PLGA) group, and (C, F, I, L) PLGA/platelet-rich plasma (PRP) group. Arrows indicate cells within the lacunae.

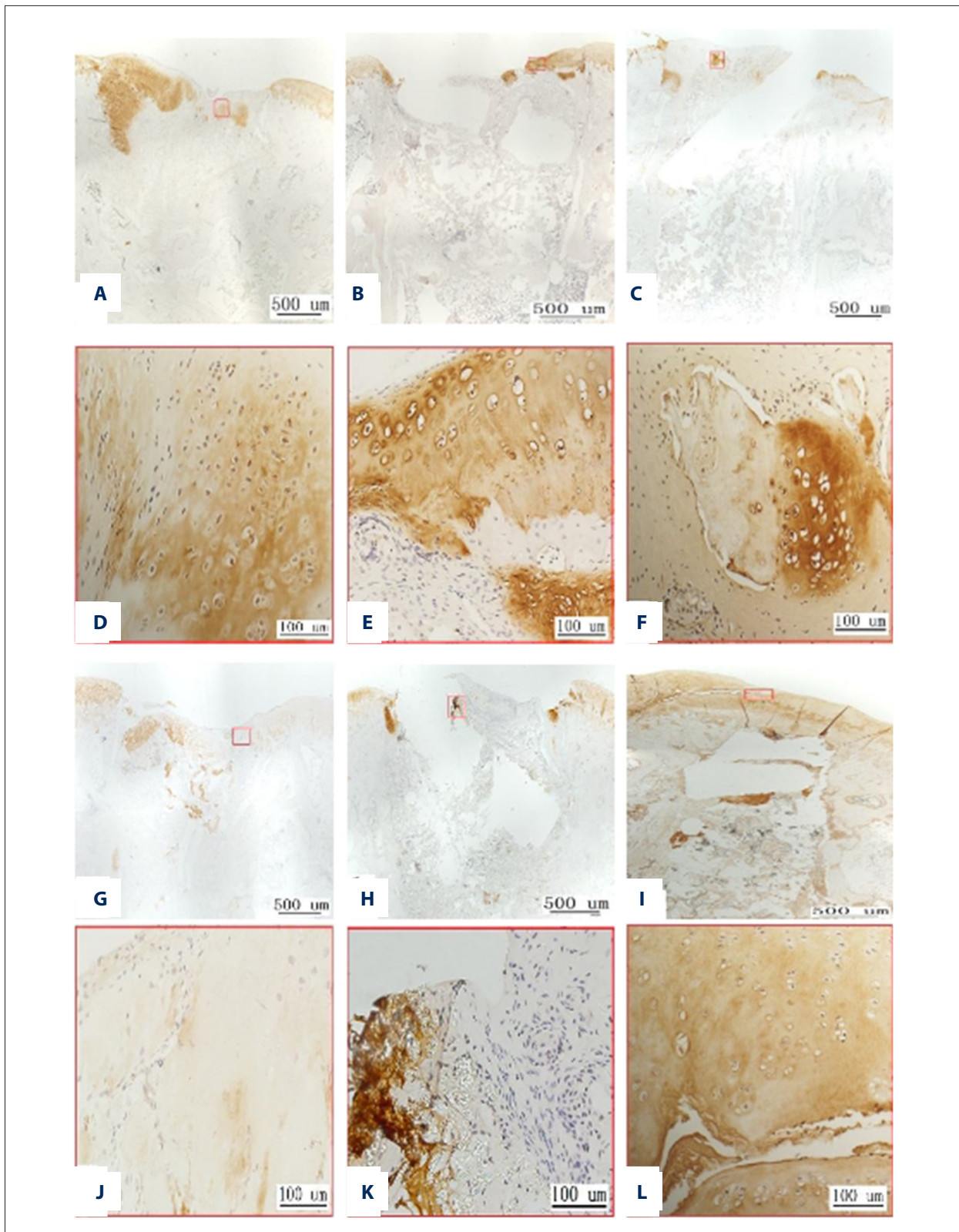


Figure 8. Positive type II collagen staining. At four weeks (A–F) and at 12 weeks (G–L) in the (A, D, G, J) untreated group, (B, E, H, K) the poly(lactide-co-glycolide) (PLGA) group, and (C, F, I, L) PLGA/platelet-rich plasma (PRP) group.

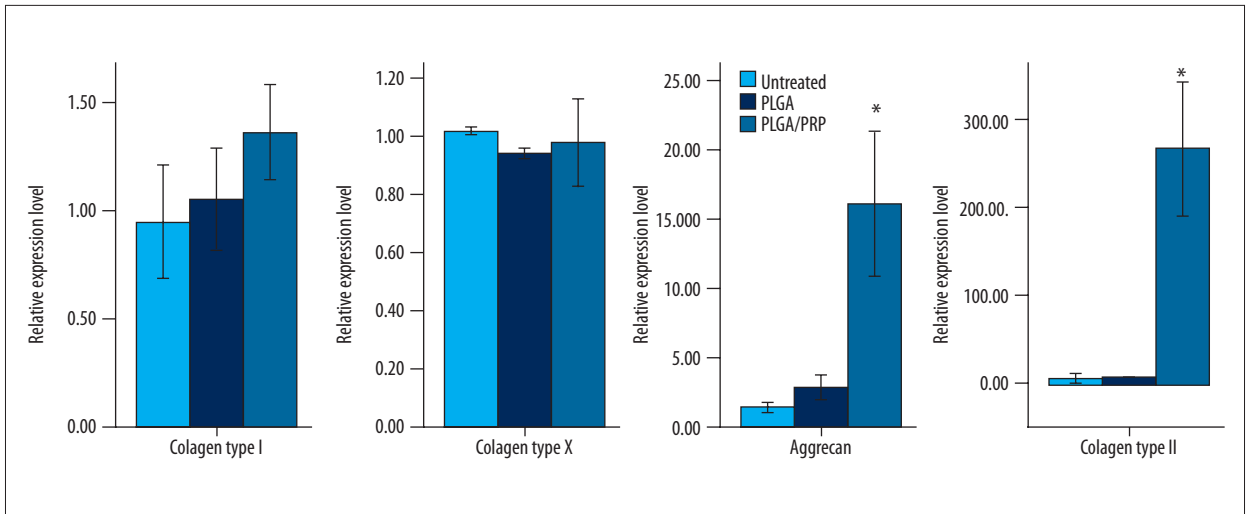


Figure 9. Relative gene expression levels of collagen type I, collagen type II, collagen type X, and aggrecan at 12 weeks. The bars show the mean (N=3) and the SD. The expression level of aggrecan and collagen type II was significantly greater in the poly(lactide-co-glycolide) (PLGA)/platelet-rich plasma (PRP) group than in the PLGA group and untreated groups (* $P < 0.05$).

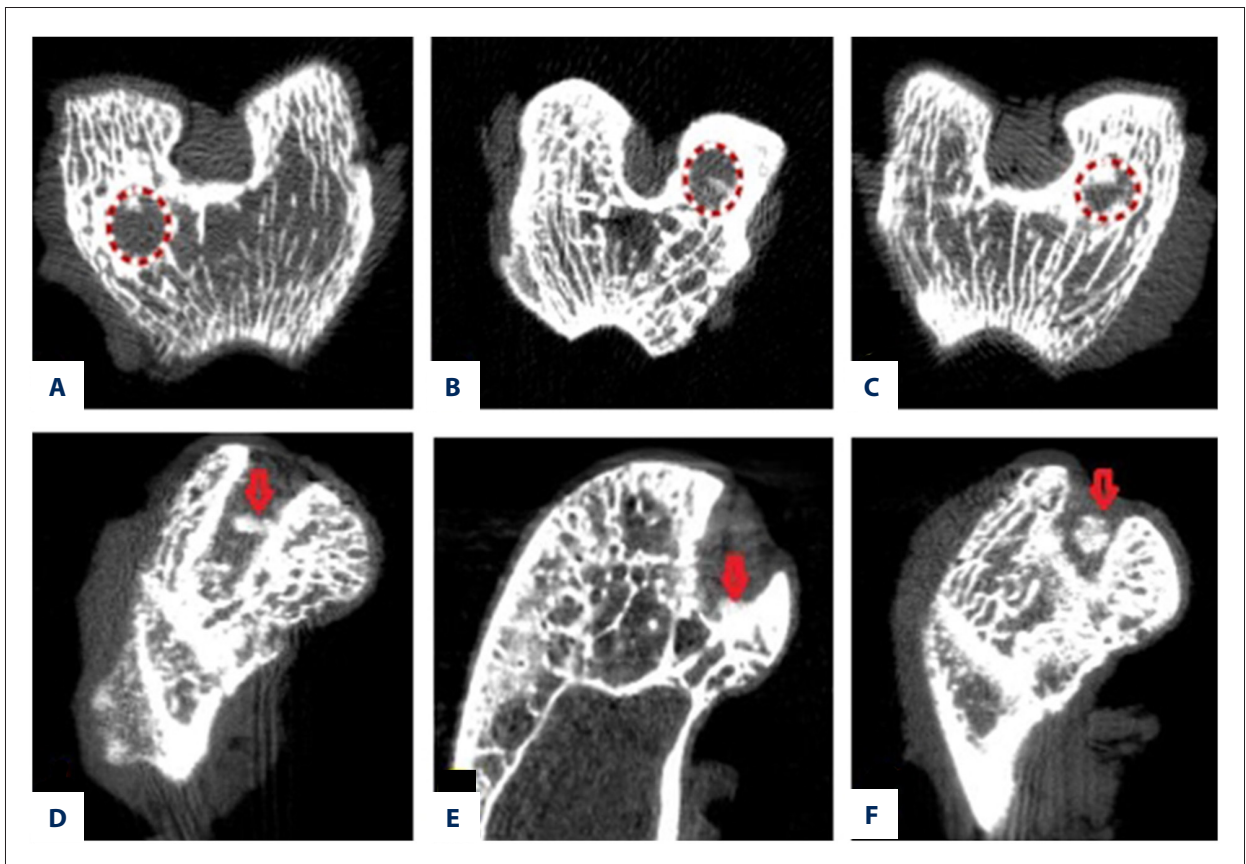


Figure 10. Micro-computed tomography (CT) images of newly formed bone at 12 weeks (A, D) nontreated group, (B, E) the poly(lactide-co-glycolide) (PLGA) group, and (C, F) the PLGA/platelet-rich plasma (PRP) group. The circles indicate the sites of new tissue filling in the defects. The arrow indicates the new bone.

the PLGA-treated group. Therefore, it may be assumed that the implantation of a bilayer PLGA scaffold facilitated chondrocyte migration to the defect and that this was enhanced when autologous PRP was loaded into the scaffold. One possible explanation for this finding is that chondrocytes derived from the surrounding normal cartilage may be activated by PRP and migrate into the composite within the defects. This explanation is supported by the findings from a previously published study [27].

Collagen type II is one of the most important components of hyaline cartilage. In this study, the relative expression level of collagen type II was significantly increased in the PLGA/PRP group when compared with the other groups, and positive immunohistochemical staining for collagen type II was also observed. Also, the expression of aggrecan was increased in the PLGA/PRP when compared with the PLGA group and the untreated group (Figure 9). This finding was demonstrated by the increased histochemical staining for ECM using Safranin-O/Fast Green staining for cartilage, and toluidine blue (Figures 6, 7). These results indicated that the neo-cartilage in the PLGA/PRP study group has the characteristics of hyaline cartilage. It seems that chondrocytes derived from the normal cartilage were activated and migrated into the scaffolds, with PRP stimulating articular chondrocyte proliferation and biosynthesis [39].

The regeneration of subchondral bone is important to the reconstruction of osteochondral defects, as the implantation of either PRP alone or the composite of PRP and bone marrow stem cells (BMSCs) could promote the bone regeneration [40,41]. Other studies have also shown PRP to have favorable effects on the regeneration of cartilage [42,43]. In the present study, the greatest volume of mineralized bone within the defects appeared in the PLGA/PRP group, according to the micro-CT imaging. This observation supports previous findings that PRP may enhance the formation of bone [39]. Previous studies have indicated that bone and cartilage regeneration is mainly related to cells originating from the bone marrow and that PRP can promote the differentiation and proliferation of these cells to form new cartilage and bone [41,43,44]. The present study in an *in vivo* animal model has shown that PRP may improve the proliferative activity of autologous chondrocytes, which is supported by previous *in vitro* studies [45].

It is now believed that the implantation of a scaffold loaded with a large number of cells is necessary for the reconstruction of osteochondral defects. However, in 2013, Hansen and colleagues confirmed that there was no positive effect on cartilage repair by increasing cell seeding density [46]. The results

of the present study indicate that the composite of the autologous PRP and PLGA, without any seeded cells, can promote the regeneration of cartilage and subchondral bone in the rabbit model. The findings of this study are consistent with those previously described and demonstrated the feasibility that cells originating from the bone marrow can differentiate into hyaline-like cartilage if a composite of bilayer PLGA scaffolds and autologous PRP are used simultaneously [27].

In 2011, a clinical study of the use of autologous matrix-induced chondrogenesis combined with platelet-rich plasma gel in five patients was reported [47]. Autologous PRP prepared before surgery has several advantages over allogeneic PRP and products produced in other species, including reduced cost when compared with the use of recombinant proteins. Although it would seem that the use of PRP could be recommended for widespread clinical use, the specific underlying mechanisms of action are still unclear, as is the ideal dose of PRP. Because the bilayer PLGA scaffolds do not degrade, long-term *in vivo* studies are required in the future. Also, cell-material interactions also remain another challenge [48–53]. However, the findings of this preliminary study in the rabbit animal model have afforded a potential future strategy to reconstruct osteochondral defects by implantation of bilayer PLGA scaffolds loaded with autologous PRP.

Conclusions

Bilayer autologous platelet-rich plasma (PRP) and poly(lactide-co-glycolide) (PLGA) scaffolds significantly improve the reconstruction of osteochondral defects in the rabbit model *in vivo*. Future long-term observational preclinical and clinical studies are required following the beneficial findings from this preliminary study.

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Conflict of interest

None declared.

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