The Effect of Autologous Platelet-Rich Plasma on Bone Regeneration by Autologous Mesenchymal Stem Cells Loaded onto Allogeneic Cancellous Bone Granules

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Keywords

Bone regeneration · Mesenchymal stem cell transplantation · Allografts · Bone transplantation · Platelet-rich plasma · Tissue engineering

Abstract

To develop a clinically effective bone regeneration strategy, we compared bone regeneration using allogeneic cancellous bone granule scaffolds loaded with autologous bone marrow-derived mesenchymal stem cells (BM-MSC) with or without autologous platelet-rich plasma (PRP). Critical-sized segmental bone defects were made at the mid-shaft of both radiuses in 41 New Zealand White rabbits. Small-sized allogeneic cancellous bone granules (300–700 µm in diameter) loaded with BM-MSC were implanted on one side, and PRP was added. On the other side, autologous BM-MSC loaded onto allogeneic cancellous granules were grafted as a control. Bone regeneration was assessed by radiographic evaluation at 4, 8, and 16 weeks postimplantation and by micro-computed tomography (micro-CT) and histological evaluation of the retrieved specimens at 8 and 16 weeks. The experimental group did not show significantly higher bone quantity indices than the control group at any time point.

Micro-CT analysis revealed that both groups had similar mean total volumes, surface areas, and other parameters at 8 and 16 weeks. Histological evaluation of 8- and 16-week specimens also showed a similar progression of new bone formation and maturation. In this experiment using a contralateral control group in the same individual, an initial single addition of PRP in allogeneic cancellous bone granules loaded with BM-MSC for critical-sized bone defects in the weight-bearing area did not induce a consequent difference in bone healing. Further research into the optimal preparation and application of PRP is necessary. Furthermore, studies involving a greater number of subjects and larger experimental animals could determine the clinical relevance of PRP treatment.

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Introduction

Autologous bone grafting is frequently used to facilitate healing of massive bone defects resulting from high-energy trauma, tumor resection, osteomyelitis, delayed union, nonunion, osteotomy, and arthrodesis [Van Heest and Swiontkowski, 1999], and it remains the gold stan-

Abbreviations used in this paper

A	allogenic cancellous bone + mesenchymal stem cells
B	allogenic cancellous bone + mesenchymal stem cells + platelet-rich plasma
BM	bone marrow
BM-MSc	autologous bone marrow-derived mesenchymal stem cells
BQI	bone quantity index
D	density
micro-CT	micro-computed tomography
MSC	mesenchymal stem cells
NWR	New Zealand White rabbits
PRP	platelet-rich plasma
TGF	transforming growth factor
V	volume

dard to which other modalities are compared. Autologous bone grafting promotes osteogenesis through osteoinductive, osteoconductive, and osteogenic mechanisms, diminishes the risk of infectious disease transmission, and provokes no immune response [Gazdag et al., 1995]; however, this procedure is limited by donor site morbidity and the amount of bone that can be retrieved [Younger and Chapman, 1989; Gazdag et al., 1995; Rubin and Yaremchuk, 1997]. Therefore, bone regeneration by means of tissue engineering has attracted increasing interest. Tissue engineering involves the use of combinations of scaffolds, cells, and growth factors [Kasten et al., 2008]. The resulting bone regeneration shows promise for the management of massive bone defects [Lee et al., 2014].

Among various bone substitutes used as scaffolds in tissue engineering, lyophilized allogeneic cancellous bone is frequently employed to promote osteogenesis due to its established osteoconductivity [Pruss et al., 2002]. Allogeneic cancellous bone is almost ideal in terms of its porosity and biocompatibility; it has a rough surface and numerous pores to enable cell adhesion, spreading, proliferation, and intercellular contact [Albrektsson and Johansson, 2001; Pruss et al., 2002; Kim et al., 2012].

As the cellular component in bone engineering, the use of autologous bone marrow-derived mesenchymal stem cells (BM-MSc) is an alternative method for bone regeneration. Mesenchymal stem cells (MSC) are multipotent cells, which can be easily isolated from bone marrow (BM) aspirate; they also proliferate rapidly into large populations *in vitro* and differentiate into osteogenic cells. A strong osteogenic capacity has been demonstrated in animal models and clinical trials [Pittenger et al., 1999;

Quarto et al., 2001; Chao et al., 2007]. We have demonstrated the osteogenic potential of autologous MSC loaded onto allogeneic cancellous bone granules compared to allogeneic bone granules lacking cells [Lee et al., 2010].

Growth factors influence chemotaxis induction, differentiation and proliferation of osteoblasts and their precursors, and ultimately the synthetic activity of bone cells to regulate fracture healing, bone regeneration, and physiological remodeling [Bostrom et al., 1999; Weibrich et al., 2002; Kasten et al., 2008]. The alpha granules of platelets contain numerous growth factors, such as transforming growth factor (TGF)- β_1 , TGF- β_2 , fibroblast growth factor-2, platelet-derived growth factors, insulin-like growth factor-1, epidermal growth factor, hepatocyte growth factor, and vascular endothelial growth factor, which exert beneficial effects on the healing process [Bostrom et al., 1999; Weibrich et al., 2003]. Platelet-rich plasma (PRP) is a volume of plasma that has a platelet concentration above the baseline value [Tözüm and Demiralp, 2003]. Therefore, PRP is expected to stimulate bone healing and be an easy and more physiological means of applying growth factors to bone defects [Bostrom et al., 1999; Weibrich et al., 2002]. Because it is prepared by intraoperative centrifugation from whole blood on the day of surgery, and poses no risk of disease transmission, PRP has gained considerable attention over the past decade [Kasten et al., 2008]. However, there have been conflicting reports about the effect of PRP on bone regeneration [Kassolis et al., 2000; Choi et al., 2004; Fennis et al., 2004; Li et al., 2004; Roldán et al., 2004; Schlegel et al., 2004; Yamada et al., 2004; Raghoobar et al., 2005; Thorwarth et al., 2005; Plachokova et al., 2007; Kasten et al., 2008; Batista et al., 2011; Ohba et al., 2012a; Parizi et al., 2012; Shafiei-Sarvestani et al., 2012; El Backly et al., 2013; Lin et al., 2013]. In the present study, we hypothesized that additional application of autologous PRP may promote bone regeneration in transplantation of allogeneic cancellous bone granules loaded with autologous BM-MSc.

Materials and Methods

Experimental Animals

Forty-one male New Zealand White rabbits (NWR), each weighing 2.5 kg, were housed individually in rabbit cages under identical feeding and management conditions. The animals were acclimatized for 2 weeks until reaching a weight >3 kg prior to the experiments. A critical-sized segmental bone defect [Bolander and Balian, 1986], 15 mm in length, was made in the mid-portion of both radiuses. The defect in one radius was filled with small allogeneic cancellous bone granules containing cultured autologous

Fig. 1. Scanning electron micrographs of allogeneic cancellous bone granules loaded with bone marrow-derived mesenchymal stem cells. **a** Porous structure and rough surface. **b** Mesenchymal stem cells attached to the surface of bone granules in a higher-magnification image.

BM-MSc and PRP (experimental group), whereas allogeneic cancellous bone granules with autologous BM-MSc without PRP were grafted into the other radius of the same rabbit as a control (control group). For result analysis, we planned to sacrifice 20 rabbits 8 weeks after the operation and 21 rabbits 16 weeks after the operation. All animal experiments were approved by the Institutional Animal Care and Use Committee at the College of Medicine of The Catholic University of Korea.

Isolation and Culture of BM-MSc

The entire process was performed as in previous studies from our lab [Lee et al., 2010, 2014; Kim et al., 2012; Kang et al., 2014]. In brief, rabbits were anesthetized, and 10 mL BM were aspirated by penetrating the cortex of the posterior iliac crest of each animal using an 18-gauge needle attached to a 50-mL syringe containing 0.6 mL heparinized saline solution (3,000 U heparin). After processing with the Ficoll-Paque PREMIUM gradient (density 1.077 g/mL; GE Healthcare Bio-Sciences, Uppsala, Sweden) and centrifugation, adherent nucleated cells were harvested from the aspirated BM specimen. Dispersed cells were plated at a density of 1×10^6 /mL and cultured in Minimum Essential Medium, Alpha Modification, with 15% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% antibiotic/antimycotic in a humidified atmosphere of 5% CO₂ at 37°C. Cells were subcultured upon reaching 80–90% confluence, and the culture medium was changed every 3 days. Only cells at the third passage were used in this study.

Fabrication of MSC/Allograft Composites

This process was also performed as in previous studies from our lab [Lee et al., 2010, 2014; Kim et al., 2012; Kang et al., 2014]. Allogeneic cancellous bone was harvested from iliac crests, proximal and distal femurs, and the proximal tibias of NWR weighing 2.5 kg. All soft tissues were removed, and the bones were segmented into small granules. Optimally sized bone granules (300–700 μm in diameter) were obtained by sieving with defined meshes. The bone granules were rinsed sequentially with high-pressured sterile water, 95% ethanol, and diethyl ether to remove remaining marrow and blood components, followed by air-drying under a laminar flow hood. After removal of the remaining lipids and immunogenic components with 0.5% hydrogen peroxide, the bone graft granules were lyophilized in a vacuum chamber at –20°C for

48 h. The packed granules were sterilized by 25 kGy gamma irradiation. BM-derived proliferating autologous MSC (2×10^6 cells/mL) were seeded onto 0.3-mL allogeneic cancellous bone granules, and the composites were cultured in an incubator for 48 h before implantation in Minimum Essential Medium, Alpha Modification, with 15% fetal bovine serum and 1% antibiotic/antimycotic in a humidified chamber with 5% CO₂ at 37°C.

Scanning Electron Microscopic Observation

Impregnations of MSC into the allograft were confirmed using scanning electron microscopy (Fig. 1). For tissue preparation, the specimens were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer overnight. After washing in 0.1 M phosphate buffer, they were postfixed with 1% osmium tetroxide in the same buffer for 1 h, dehydrated in ascending concentrations of acetone from 50 to 100% for 12 h at each concentration, and then transferred to hexamethyldisilazane and allowed to air-dry. All samples were coated with gold using a sputter coater and examined using a scanning electron microscope (JSM-5410LV; JEOL, Tokyo, Japan) operated with an accelerating voltage of 15 kV.

PRP Preparation

Rabbits were anesthetized as described for isolation of the MSC. Immediately before surgery and transplantation, whole blood of male NWR was withdrawn from the auricular vein. Using a syringe rinsed with heparin and a 24-gauge needle, 10 mL blood was aspirated. While an assistant centrifuged the blood using a PRP kit and an ABS brake system centrifuge (Pro-PRP 20; Good-morning Bio, Seoul, Korea) at 2,800 rpm for 5 min, an operator started the surgical procedure. One milliliter of supernatant including the buffy coat was collected as PRP using a 2.5-mL syringe. To identify platelet concentrations at least indirectly, we obtained 11 mL whole blood at 2-day intervals 3 times from 3 rabbits, which were not used in subsequent experiments. Platelet counts were measured using 1 mL out of 11 mL whole blood and 1 mL PRP extracted from the remaining 10 mL. With the exception of one unmeasurable case due to platelet aggregation, the mean platelet count in whole blood was $373.75 \pm 181.64 \times 10^3$ (68×10^3 to 590×10^3)/μL, and that in PRP was $1,015.88 \pm 968.36 \times 10^3$ (168×10^3 to $2,836 \times 10^3$)/μL; therefore, the mean count was 271.80% higher in PRP than in whole blood.

Fig. 2. Serial radiographs of a representative subject in the experimental and control groups demonstrate progression of bone regeneration and remodeling. Both groups show a similar bone healing process. MSC, mesenchymal stem cells; PRP, platelet-rich plasma.

Table 1. Radiographic assessment criteria for bone volume and bone density

Parameter	Score
<i>Bone volume</i>	
Complete or overfilling of the defect site	5
75–99% filling of the defect	4
50–74% filling of the defect	3
25–49% filling of the defect	2
Less than 24% filling of defect	1
<i>Bone density</i>	
Same as the normal cortical bone density	5
Higher than the mid-density between cortical and cancellous bone	4
Lower than the mid-density between cortical and cancellous bone	3
Same as the normal cancellous bone density	2
Less than the cancellous bone density	1

Surgery and Transplantation Procedure

Rabbits were placed in the supine position and the forearms were shaved, prepped, and draped for aseptic surgery. A 3.5-cm, longitudinal, dorsoradial incision was made, and the tissue overlying the mid-shaft of the radius was meticulously dissected. A 1.5-cm segment of the radius and overlying periosteum was resected using an electrical saw, and a sheet of dialysis membrane was placed on the exposed ulna to prevent bone growth from the ulnar surface. A 0.3-mL aliquot of allogeneic cancellous bone granules and autologous BM-MSC were implanted in the radial defect, and 1 mL PRP was added. The time from preparation to application of PRP was less than 10 min in every case. A prepared composite of allogeneic cancellous bone granules with autologous BM-MSC was grafted without PRP in the contralateral radius. The grafted composites were completely packed within the defect. The surrounding muscles and fascia were repaired precisely using absorbable 3-0 polyglactin sutures (Vicryl Plus; Ethicon, Cincinnati, OH, USA) to prevent grafts from migrating out of the defect upon movement of the animals. The skin was closed with continuous running 4-0 nylon sutures. Postoperatively, rabbits were injected intramuscularly with gentamicin (40 mg/day) and they were allowed to walk freely in their cages.

Fig. 3. Bone quantity indices (BQI). **a** The experimental group with platelet-rich plasma (PRP) shows higher BQI than the control group at 1, 4, and 8 weeks after implantation in all 28 rabbits. **b** In 12 rabbits in the 16-week survival group, however, the control group without PRP showed a higher BQI at 4 weeks postimplantation. Differences in BQI are not statistically significant. MSC, mesenchymal stem cells.

Radiologic Evaluation

Anteroposterior radiographs of the rabbit forearms were obtained using 50 kV and a current-exposure time product value of 2 mAs at 1, 4, 8, and 16 weeks postimplantation and saved as digital images. The source-to-image distance and field of view were 120 cm and $20 \times 13 \text{ cm}^2$. Three independent examiners assessed the plain radiograph images in terms of the volume (V) and density (D) of regenerated bone using our 5 criteria (Table 1), 3 times, respectively, at 3-week intervals. The quantity of regenerated bone (bone quantity index; BQI) was calculated using the following equation: $\text{BQI}^2 = V \times D$, where V denotes the assessed mean bone volume and D denotes the assessed mean bone density [Lee et al., 2010, 2014; Kim et al., 2012; Kang et al., 2014].

Micro-Computed Tomography Scanning

The rabbits were humanely euthanized 8 or 16 weeks postimplantation, and the regenerated bone segments with ~2 mm of adjacent normal bone at both ends were harvested. The specimens were immersed in 10% neutral-buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for tissue fixation for 72 h and preserved in absolute alcohol until use. At each time point, harvested, alcohol-preserved specimens were scanned using a micro-computed tomography (micro-CT) scanner (SkyScan-1173; Bruker microCT, Kontich, Belgium). The specimens were scanned as 0.012-mm-thick sections and digital micro-radiographic images were acquired. The scanned images were reconstructed using NRecon software (version 1.6.8.0; Bruker microCT) and remodeled using realistic 3-D visualization (Bruker microCT), and 2-D tomogram images were obtained using SkyScan 1173 control software. Bone volume, bone surface area, trabecular number, thickness, and sep-

aration of regenerated bone were measured on 45 transverse sections at 20-pixel intervals for each specimen using the provided CT analyzer software (version 1.14.4.1; Bruker microCT), and the means of the measured values were used for comparison.

Histological Examination

After micro-CT, the collected specimens were decalcified in a 10% neutral buffered formalin solution for 14 days, followed by dehydration and paraffin embedding. Transverse sections of 1-mm thickness were cut through the central level of the implantation and they were used to cut longitudinal sections in a direction parallel to the long axis of the bone. The longitudinal sections were stained with Masson's trichrome and H&E. Histological evaluation for resorption of the grafted bone granule, the degree of new bone formation, the maturation pattern including evidence of early remodeling and any adverse inflammatory response was performed using an Olympus BX51 microscope (Olympus, Tokyo, Japan).

Statistical Analyses

Twenty-eight animals with complete data were subjected to the final analysis, after excluding 5 deaths associated with anesthetic problems, 4 fractures, 2 wound problems due to self-injury, and 2 inadequate graft placements (8-week survival group, $n = 16$; 16-week survival group, $n = 12$). All data in the present study are shown as means $\pm$ SD. Statistical analysis was performed using SPSS 18.0 statistical software for Windows (SPSS Inc., Chicago, IL, USA). According to the normality of the distribution of the data, the Mann-Whitney test or Student's *t* test was applied to determine the significance of differences between the two groups. $p < 0.05$ was considered statistically significant.

Fig. 4. Micro-computed tomography analysis. **a** Projection images were acquired using micro-computed tomography (SkyScan1173; Bruker microCT). Scanning conditions were 100 kV, 60 μ A, rotation step 0.4°, and image pixel resolution 19.89 μ m. **b** Three-dimensional images were obtained with CTVoX software (version

2.0; Bruker microCT). **c** Axial images were reconstructed with NRecon (version 1.6.8.0; Bruker microCT). **d** The region of interest was considered the new bone area. **e** Finally, a segmented binary image for the region of interest was used for morphometric analysis with CT-analyzer software (version 1.14.4.1; Bruker-CT).

Results

Radiologic Evaluation of Plain Radiographs

Serial radiographs revealed progression of grafted bone granule resorption, bone regeneration, and evidence of early-phase remodeling such as recanalization at the grafted sites in both the experimental and control groups in a time-dependent manner (Fig. 2). The experimental group showed higher mean BQI at 4 and 8 weeks postimplantation in all NWR and at 16 weeks postimplantation in the 12 rabbits of the 16-week survival group. However, no significant difference was detected at any time point (Fig. 3).

Micro-Computed Tomography

Representative sections and the 3-D micro-CT images showed similar bone regeneration in the experimental and control groups (Fig. 4). The 16-week survival group showed an increased mineral density compared to the 8-week survival group. The experimental group had similar mean total bone volume, surface area, trabecular thickness, number, and separation values compared to the control groups at 8 and 16 weeks (Fig. 5; Table 2).

Histological Analysis

Histological evaluation of the 8-week specimens revealed newly formed bone with immature osteoids sur-

Fig. 5. Micro-computed tomography assessments. There were no significant differences in bone volume (**a**) or surface area (**b**) between the experimental and control groups. A, allogenic cancellous bone + mesenchymal stem cells; B, allogenic cancellous bone + mesenchymal stem cells + platelet-rich plasma.

Table 2. Micro-computed tomography assessment

Survival time, weeks	Parameter	Experimental group	Control group	<i>p</i> value
8	Volume, mm ³	133.34±35.67	135.16±41.85	0.895
	Surface, mm ²	1,611.4±417.9	1,608.4±635.8	0.498
	Trabecular thickness, mm	0.308±0.043	0.302±0.041	0.851
	Trabecular number/mm	0.637±0.053	0.717±0.276	0.474
	Trabecular separation, mm	1.681±0.525	1.628±0.592	0.794
16	Volume, mm ³	97.69±32.96	109.47±27.28	0.350
	Surface, mm ²	992.2±437.7	1,164.0±504.2	0.382
	Trabecular thickness, mm	0.447±0.067	0.454±0.082	0.808
	Trabecular number/mm	0.343±0.111	0.375±0.132	0.644
	Trabecular separation, mm	1.821±0.352	1.589±0.342	0.115

rounded by osteoblasts and nucleated viable osteocytes, and remnant grafted bone granules with vacant lacunae in the experimental and control groups. The 16-week specimens showed more newly formed bone, more mineralization, and greater medullary space formation than the 8-week specimens; however, there was no clear difference between the 2 groups. No evidence of an immune reaction suggesting an adverse response to the grafted components, such as inflammatory cell/macrophage infiltration or bone trabecular atrophy in or around the newly regenerated bones, was observed in either group (Fig. 6).

Discussion

Preclinical studies have reported positive effects of PRP on bone regeneration in weight-bearing areas (Table 3). However, those studies included numerous different factors that could have influenced the results and most did not include a contralateral control group in the same experimental animal, which complicated evaluation of the effect of PRP. Various species, e.g., NWR, Japanese white rabbits [Ohba et al., 2012a], Mongrel dogs [Choi et al., 2004], and Danish Landrace pigs [Li et al., 2004], have been used as experimental animals. The age, sex, and

Fig. 6. Histological evaluations. There were no differences between the experimental (**a, b**) and control groups (**c, d**) in terms of new bone formation and mineralization, bone maturation pattern, or bone remodeling in the 8-week specimens. There was no difference between the experimental (**e, f**) and control groups (**g, h**) in the 16-week specimens. **a, c, e, g** H&E staining. $\times 40$. **b, d, f, h** Masson's trichrome staining. $\times 40$.

weight of the NWR have also differed [Kasten et al., 2008; Batista et al., 2011; Parizi et al., 2012; El Backly et al., 2013; Lin et al., 2013]. Baseline platelet counts vary markedly within and among species [Table 6 in Kahn et al., 2006], and the difference in the metabolism of small and large animals may influence the rate of bone healing [Mooren et al., 2010]. A critical defect is more proper for evaluating the bone regeneration potential of PRP and the eventual clinical application of preclinical results, particularly in weight-bearing bones. Few studies have assessed weight-bearing long-bone defects [Kasten et al., 2008], and the

defect size and location differ among the studies [Parizi et al., 2012; Shafiei-Sarvestani et al., 2012; El Backly et al., 2013]. In addition, various materials such as coral species [Guillemin et al., 1987; Parizi et al., 2012], deproteinized bovine bone material [Hämmerle et al., 1997], nanohydroxyapatite/poly(ester urethane) [El Backly et al., 2013], and β -tricalcium phosphate [Li et al., 2004] have been used as bone regeneration scaffolds. MSC were used in a few preclinical studies on the effect of PRP on bone regeneration [Yamada et al., 2004; Kasten et al., 2008; El Backly et al., 2013; Lin et al., 2013].

Table 3. Previous preclinical studies on the effect of PRP on bone regeneration in a weight-bearing area

Reference	Species	Defect	Scaffold	Cell	PRP	Contralateral control group	Implication for effect
Batista et al., 2011	NWR	Tibia NC	β -Tricalcium phosphate		Autologous	–	+
Ohba et al., 2012a	JWR	Femur NC	Hydroxyapatite Electrically polarized hydroxyapatite		Autologous	+ +	+ +
Ohba et al., 2012b	JWR	Femur, tibia NC	Hydroxyapatite Electrically polarized hydroxyapatite		Autologous	– –	– +
Parizi et al., 2012	NWR	Radius C	Coral		Human	–	+
Shafiei-Sarvestani, 2012	NWR	Radius C	Coral Hydroxyapatite		Human	– –	+ +
El Backly, 2013	NWR	Ulna C	Nanohydroxyapatite/polyester urethane	BMSC	Autologous	–	+
Lin et al., 2013	NWR	Femur NC	Nanohydroxyapatite-type I collagen beads	BMSC	Autologous	–	+

NWR, New Zealand white rabbit; JWR, Japanese white rabbit; NC, noncritical; C, critical; BMSC, bone marrow-derived mesenchymal stem cells; PRP, platelet-rich plasma.

Allogeneic, pooled, or xenogenic PRP has been used instead of autologous PRP in some preclinical studies [Ohba et al., 2012a, b; Parizi et al., 2012; Shafiei-Sarvestani et al., 2012; El Backly et al., 2013], and human PRP has been reported to be more potent than animal-derived PRP for bone regeneration [Mooren et al., 2010]. An *in vitro* study demonstrated that the growth factor concentration is highest in human PRP, followed by goat and rat PRP [van den Dolder et al., 2006]. Another study reported that human PRP does not contain bone morphogenic protein, the most potent osteoinductive protein and unique growth factor, that promotes stem cell differentiation into the osteoblastic lineage [Cook, 1999]. These factors could result in the different findings between clinical and preclinical studies; therefore, these factors must be taken into consideration.

PRP has been applied to bone defects after gel preparation in many studies [Kassolis et al., 2000; Choi et al., 2004; Yamada et al., 2004; Plachokova et al., 2007; Ohba et al., 2012a; El Backly et al., 2013; Lin et al., 2013]. Because platelet gel can act as a biologic adhesive and barrier, and function as a sealant [Kassolis et al., 2000], it can maintain the PRP concentration at the defect site, further promoting bone regeneration even more. However, PRP with gel preparation can be inserted only during surgery. Therefore, research using PRP without gel preparation is

warranted. While the effective period of PRP is unclear [Fennis et al., 2004], some literature has reported that the mitogenic properties of cytokines in PRP work during the first 1–2 days, the platelet life span is <5 days [Plachokova et al., 2007; Mooren et al., 2010], and the majority of bone regeneration after PRP application occurs within the first month [Fennis et al., 2004; Raghoobar et al., 2005; Plachokova et al., 2007]. Therefore, repeated percutaneous implantation of PRP without gel preparation might facilitate bone regeneration. In a previous rabbit study, PRP was injected percutaneously 4 days after scaffold implantation [Shafiei-Sarvestani et al., 2012].

The efficacy of PRP is dependent on the preparation method because this exerts a critical influence on platelet and growth factor concentrations [Weibrich et al., 2003]. Differences in the preparation method, such as the source and volume of blood for extraction, instruments, and rotation frequency and duration of centrifugation, have been used; indeed, some reports did not provide details of the preparation process. One study in rabbits reported that 0.15 mL PRP with a mean of 807.564 ± 211.490 platelets $\times$ mm^{–3} from 4 mL blood with a mean of 320.133 ± 42.323 platelets $\times$ mm^{–3} was extracted using a 2-step technique of centrifugation at 2,400 rpm for 8 min and at 2,400 rpm for 5 min [Ohba et al., 2012a]. Another study presented that a mean PRP of $104.53 \pm 47.67 \times 10^7$ plate-

lets/mL from 9.8 mL blood of a mean of $28.03 \pm 13.34 \times 10^7$ platelets/mL was collected by 1-step centrifugation at 1,200 rpm for 10 min [Lin et al., 2013]. In addition, a PRP of $3 \times 10^6/\mu\text{L}$ was obtained using 3-step centrifugation twice at 209 g for 16 min, and at 1,500 g for 12 min [El Backly et al., 2013]. A human study reported that the numbers of platelets, white blood cells, and growth factors obtained varied according to the separation method as well as among subjects and with repetitive blood draws [Mazzocca et al., 2012]. In recent human studies, all separation techniques resulted in a significant increase in platelet counts, and 2-step procedures did not result in concentrations significantly higher than those achieved with 1-step procedures [Foster et al., 2009; Pauly et al., 2010; Mazzocca et al., 2012]. One-step centrifugation achieved a mean 2.7-fold increase in platelet concentration in this study.

The optimal platelet concentration in PRP for bone regeneration is controversial [Kassolis et al., 2000; Roldán et al., 2004]. Some studies have indicated that high concentrations of platelets would result in superior and more rapid bone formation [Fennis et al., 2004; Schlegel et al., 2004; Thorwarth et al., 2005; Parizi et al., 2012], whereas others have suggested that the platelet concentration might not be correlated with the biological effect [Aghaloo et al., 2002; Kim et al., 2002], or that a higher platelet concentration may be biologically unfavorable [Nikolidakis and Jansen, 2008; Foster et al., 2009; Mazzucco et al., 2009]. Thus, information on the dose-effect relationship and the optimum quantity of PRP is lacking [Kassolis et al., 2000; Fennis et al., 2004]. PRP should be considered a medicine, and its pharmacological effects should be investigated. Therefore, further studies using different quantities and doses are warranted.

The BQI in the radiographic assessment increased gradually for the first 4 weeks, decreased thereafter, and was lowest at 8 weeks in all rabbits and at 16 weeks in 12 rabbits of the 16-week survival group after implantation in both groups. The difference in BQI was not significant at any time point. These results may be due to variation in the quantity of PRP or an insufficient enhancing effect of PRP on bone regeneration in this setting (Fig. 3).

None of the micro-CT evaluation parameters of bone regeneration and maturation were different (Table 2; Fig. 5). These findings suggest that PRP does not promote bone regeneration when allogeneic cancellous bone granules loaded with autologous MSC are transplanted into critical long-bone defects. Micro-CT analysis is a more objective measure than radiological assessment; one study demonstrated that only 3-D models based on 3-D

micro-CT can visualize the final spatial distribution of newly formed bone over the total defect area [Mooren et al., 2010].

The histologic findings were consistent with those of micro-CT. We found no evidence of an adverse immune response in the experimental group or control group, suggesting that the implants had very little or no immunogenicity and they were biocompatible (Fig. 6).

This study had several limitations. First, the statistical power was limited by the relatively small number of rabbits used. Second, as the platelet concentration could not be measured in all rabbits, it was unclear whether a consistent quantity of PRP was applied. Third, withdrawal of 10 mL whole blood to extract PRP may have affected the small rabbits and this may have impacted the restoration ability. Although this rabbit model facilitates investigation of bone regeneration, its blood volume might be insufficient to maintain PRP quality and a healthy perioperative status after blood aspiration.

In conclusion, an initial single addition of PRP in transplantation of allogeneic cancellous bone granules loaded with autologous BM-MSC for critical-sized bone defects in the weight-bearing area did not induce a significant difference in bone healing in this experiment with a contralateral control in the same animal. Further research into the optimal preparation method, concentration, and application frequency of PRP is necessary. Furthermore, studies involving a greater number of subjects and larger animals should be performed to evaluate the clinical relevance of PRP.

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Disclosure Statement

The authors declare no conflict of interests.

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