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| Original Article |

The Effect of Platelet Rich Plasma on Osteogenesis in a Long Bone Segmental Defect : Is the Platelet Rich Plasma Effective for Bone Reconstruction?

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Abstract : The effect of platelet rich plasma (PRP) on osteogenesis in the defect of long bone is controversial. The purpose of this study was to investigate the effect of PRP on new bone formation in a segmental defect of rabbit ulna. Unilateral 15 mm mid-diaphyseal defect was created in the ulna of twenty skeletally mature New Zealand White rabbits. The defects in ten rabbits each were treated with autogenous PRP in a polycarprolactone scaffold (PRP treated group) and remain empty defect without any material (contol group). 12 weeks and 20 weeks after surgery, five rabbits in each group were sacrificed to assess new bone formation. New bone formation assessed by plain radiography, microCT scan and histology did not differ significantly between PRP treated group and control group. This study demonstrated that PRP alone had no evidence of promoting osteogenesis in a long bone segmental defect of rabbit ulna.

Key words: *platelet rich plasma, osteogenesis, new bone, defect, rabbit*

1. Introduction

The use of platelet rich plasam (PRP) in tissue engineering is a developing area for clinicians and researchers and has been employed in a various fields of surgery. Although the growth factors and mechanisms involved are still poorly understood, the easy application of PRP in clinical practice and lack of immunologic reactions hold promise.

A lot of studies have been conducted to investigated the effect of PRP on bone formation. However, the results are controversial. Marx et al[1] used PRP for the reconstruction of maxillofacial defects in human and found that PRP resulted in a quicker maturation of autogenous bone transplants and higher bone density. Another clinical study tested the efficacy of platelet-rich plasma in three bilateral sinus graft cases with grafts of an organic bovine bone that contained minimal or no autogenous bone.[2] Histomorphometric analysis indicated that the addition of platelet-rich plasma to the grafts did not make a significant difference either in vital bone production or in interfacial bone contact on the test implants. Experimental *in vivo* findings are also inconsistent. In a rat femur defects model, the addition of PRP to PCL-TCP scaffolds accelerated early vascular ingrowth and enhanced bone healing.[3] In a sheep tibia segmental defect model, PRP did not enhance new bone formation in a critical size defect with a low regenerative potential.[4] In a rabbit radius segmental defect model, PRP delivered in a calcium deficient hydroxyapatite scaffold improved bone healing.[5]

Because of the controversial results, there is still need for further research regarding the possible osteogenic potency of PRP particularly in diaphyseal bone. The purpose of this study was to investigate the effect of PRP on new bone formation in a segmental defect of long bone. For this purpose, PRP loaded polycarprolactone scaffolds were implanted into the segemental defect of rabbit ulna. New bone formation was evaluated by plain radiography, microCT scan and histology after 12 weeks and 20 weeks. Our hypothesis was that PRP would promote bone formation.

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2. Materials and Methods

2.1 Experimental Design

All animal experimental procedures were performed under the guidelines for animal scientific procedures approved by the insititution's ethical committee. Twenty skeletally mature male New Zealand white rabbits (age 14-16 weeks, body weight 3.0-3.5 kg) were used in this study. Unilateral 15mm segmental bone defect was surgically made on midshaft of rabbit ulna. The rabbits were randomly assigned to one of two groups; those untreated group (Control group, n=10) and those PRP treated group (PRP treated group, n=10). Five rabbits in each group were sacrificed to process microCT scan and histology at 12 weeks and 20 weeks after surgery.

2.2 Preparation of Scaffolds

Poly (ε-caprolactone) scaffolds (Osteopore International Pte Ltd, Singapore), were fabricated by fused deposition modeling (FDM). Each scaffold manifests a lay-down pattern of 0/60/120 degree with porosity of 70%, and average pore size of 0.515 mm as determined by micro-CT analysis. The scaffolds have a compressive modulus of 23.1±6.16 MPa and compressive strength of 6.38±0.82 MPa. The structure of PCL scaffold is a honeycomb array of interconnected equilateral triangles with regular porous morphology. For this study, scaffolds are specially designed to fill bone defect. It is a cylindrical shape with a 15 mm of length and 4 mm of diameter.

2.3 Preparation of Platelet-Rich Plasma

Under anesthesia, 50 ml of blood was aspirated from the aorta of rabbit using a heparinized syringe. 50 ml of blood was first centrifuged at 2000 rpm for 3 min to separate PRP and platelet poor plasma (PPP) portions from the red blood cell fraction. Then, the PRP and PPP portions were again centrifuged at 5000 rpm for 5 min to concentrate PRP (5 ml). A loading volume consisting of 20 ul human lyophilized thrombin, reconstituted in 10% $CaCl_2$ solution, and 120 ul of PRP mixture was sequentially pipette loaded onto each PCL-TCP scaffold placed in a 24 well plate. The loading volume was determined based on the volume of scaffold. PRP clotted upon contact with the thrombin-$CaCl_2$ solution in the scaffold after 30 min incubation at 37°C.

2.4 Surgical Procedure

The rabbits were anesthetized with an intramuscular injection of Ketamine 35 mg/kg and Xylazine 5 mg/kg, and left limbs were then shaved and prepared aseptically for surgery. A longitudinal skin incision was made over the ulna and underlying muscles were retracted, exposing the mid-diaphysis of the ulna. Periosteum of operative site was excised. Proximal osteotomy site was first marked using a 2.7 mm drill at 4 cm distal to the olecranon tip and then osteotomy was performed using a hand osteotome. The distal osteotomy site was marked at 15 mm proximal to the first one and then osteotmy was performed. The 15 mm ulna bone was removed to create segmental bone defect. The bone defect was lavaged with 0.9% normal saline. Twenty rabbits were randomly assigned to two groups. In control group 1 (N=10), segmental defect was left untreated without any materials. In PRP treated group, PCL-TCP scaffold prepared with PRP was implanted into the bone defect. After implantation of scaffold, the muscle layer and the skin were closed layer by layer. Postoperatively, the rabbits were moved freely without external support. Intramuscular antibiotics were administered twice a day for three days after surgery. The general health of the rabbits was monitored daily throughout the study.

2.5 Radiographic Examination

Anteroposterior and lateral radiographs were taken immediately after surgery and then monthly (0, 2, 4, 8, 12, 16 and 20 weeks after surgery). Radiographic evaluation was used to qualitatively estimate bone formation within the defect as well as the timing of callus formation, bridging of defect, corticalization, and recanalization of marrow. A quantitative radiological scoring system was used to stage the temporal sequence of defect-bridiging observed in this study. The average score for anteroposterior and lateral plain radiography for each specimen in each group was determined at 12 and 20 weeks. A score of 0 was assigned to an appearance equivalent to that of the initial defect. A score of 1 was assigned when bone formation was from both ends of the defect was first observed. A score of 2 was assigned when consolidation of new bone from these locations was observed, in addition to new bone formation within the defect. A score of 3 was assigned when new bone was seen to fill the defect completely. Cortical continuity was assigned to a score of 4. Cortical remodeling in combination with restoration of the medullary canal was assigned to a score of 5.

2.6 Microcomputed Tomography (Micro-CT) Examination

New bone formation was evaluated by the quantitative Micro-CT scan (Skyscan1072, SKYSCAN, Antwerpen, Belgium) at 12 or 20 weeks after surgery. The scanner was set at a voltage of 80 kVp and a current of 100 uA. Resolution was set to medium, which created a 1024×1024 pixel image matrix. Isotropic slice data were obtained by the system and reconstructed into 2D images (TomoNT™, SKYSCAN, Belgium). These slice images

were compiled and analyzed to render 3D images and obtain quantitative architectural parameters. The bone volume fraction (BVF), trabecular thickness (TTh), and trabecular separation (TSp) were recorded.

2.7 Histologic Examination

At 12 or 20 weeks after surgery, each specimen was fixed in 10% neutral-buffered formalin for 2 weeks and then processed for histology. Dehydration was accomplished using a graded series of ethyl alcohols (Harleco, USA) and clearing with xylene substitute (Thermo Electron Corporation, USA). Infiltration was performed using a graded series of methyl methacrylate solutions (ACROS Organics, USA). Embedding was done using a catalyzed mixture of Osteo-Bed resin solution containing 2.5 g of benzoyl peroxide (Sigma, USA) per 100 ml. Specimens were embedded in catalyzed resin mixture under vacuum for at least 48 h. After polymerization, specimens were placed in a freezer for 24 h. Specimens were trimmed of excess plastic (Isomet 1000, Buehler, USA), ground to expose relevant portions (Ecomed 3, Buehler, USA) and longitudinally sectioned (3 μm thick) using a microtome (Microm HM355S, Richard Allan Scientific, USA). Sections from each specimen were then stained with Hematoxylin and Eosin to evaluate new bone formation. Stained samples were examined under a light microscope.

2.8 Statistical Analysis

Differences in radiologic score, the bone volume fraction (BVF), trabecular thickness (TTh), and trabecular separation (TSp) between control group and PRP treated group were assessed using the Mann-Whitney's U-test. Statistical analyses were performed using the SPSS (SPSS for Windows Release 12.0, SPSS Inc, Chicago, IL). A p-value less than 0.05 was considered as statistically significant.

3. Results

3.1 Radiographic Findings

The qualitative radiologic scores indicated that there was no difference between control and scaffold groups in each time of follow up period (Table 1). At 4 weeks after surgery, new bone formation was identified from one or both ends of the defect in all specimens. However, we could not identify complete defect-bridging in all specimens of both groups during follow- up period (Fig 1).

3.2 Micro-CT Analysis

The bone volume fraction (BVF), trabecular thickness (Tth), and trabecular separation (Tsp) were shown in Table 2. At 12 weeks, there were no significant differences in BVF, Tth, Tsp between control and PRP treated group. At 20 weeks, there was no significant difference in BVF, Tth and Tsp between two groups. However, the BVF and Tth were increased significantly between 12 and 20 weeks in both groups ($p < 0.05$).

Table 1. Radiologic scores of Control and PRP treated group

	Radiologic scores
12 wk	
Control	1.5±0.7
PRP treated	1.1±0.7
p value	>0.05
20 wk	
Control	2±0.7
PRP treated	2.1±1.3
p value	>0.05

Values were reported as mean and standard deviations

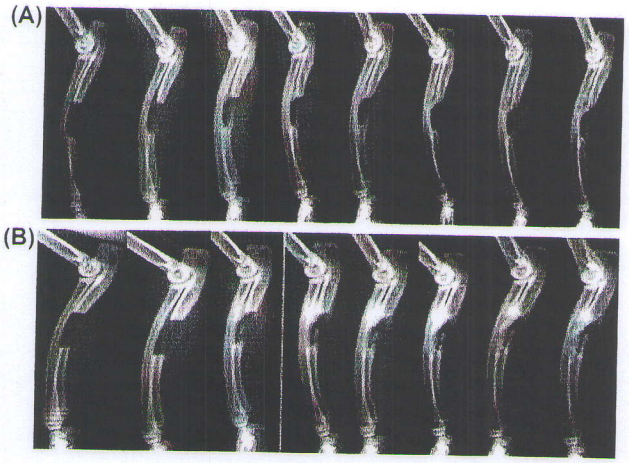

Figure 1. Plain lateral radiographs taken 0, 2, 4, 8, 12, 16, 20 weeks postoperatively. (A) Control group (B) PRP treated group. At 4 weeks after surgery, new bone formation was identified from one or both ends of the defect in all specimens. However, defect-bridging was not identified in all specimens of both groups during follow- up period.

3.3 Histological Findings

In general, the histological findings were in accordance with the results of radiography and microCT analysis in both groups. Newly formed bone was identified from one or both ends of defect, but little or no new bone was found in the central portion of empty defects and within the pores of scaffold (Fig 2). Fibrous connective tissues were observed within the defect. There was no significant difference of the amount of new bone

Table 2. New bone formation in microCT

	*BVF (%)	†Tth (mm)	§Tsp (mm)
12 wk			
Control	14.9±5.2	0.4±0.3	1.2±0.4
PRP treated	16.9±5.3	0.4±0.2	1.3±0.7
p value	> 0.05	> 0.05	> 0.05
20 wk			
Control	26.3±5.5	0.6±0.4	1.3±0.8
PRP treated	26.8±5.9	0.7±0.5	1.2±0.5
p value	> 0.05	> 0.05	> 0.05

Values were reported as mean and standard deviations.
*BVF : Bone volume fraction, †Tth : Trabecular thickness
§Tsp : Trabecular separation

tissue between control and PRP treated group at 20 weeks after surgery.

4. Discussion

The hypothesis for this study was that PRP delivered in a PCL scaffold promotes bone healing in a long bone segmental defect of rabbit ulna. The results of this experimental study demonstrated that PRP alone had no evidence of promoting osteogenesis in a long bone segmental defect of rabbit ulna model. The clinical use of PRP for a wide variety of applications has been described, particularly in periodontal, craniofacial, and spinal surgery.[1,6-9] These studies provide strong evidence to support the clinical use of PRP, but clinical and animal studies that found positive effects for PRP were mostly performed in well-vascularized cancellous bone defects where an abundant presence of precursor cells can be assumed.

The effect of PRP on bone cells may not be due to the action of a single growth factor but to the synergistic effects of the many such factors derived from platelets. However, the osteogenic

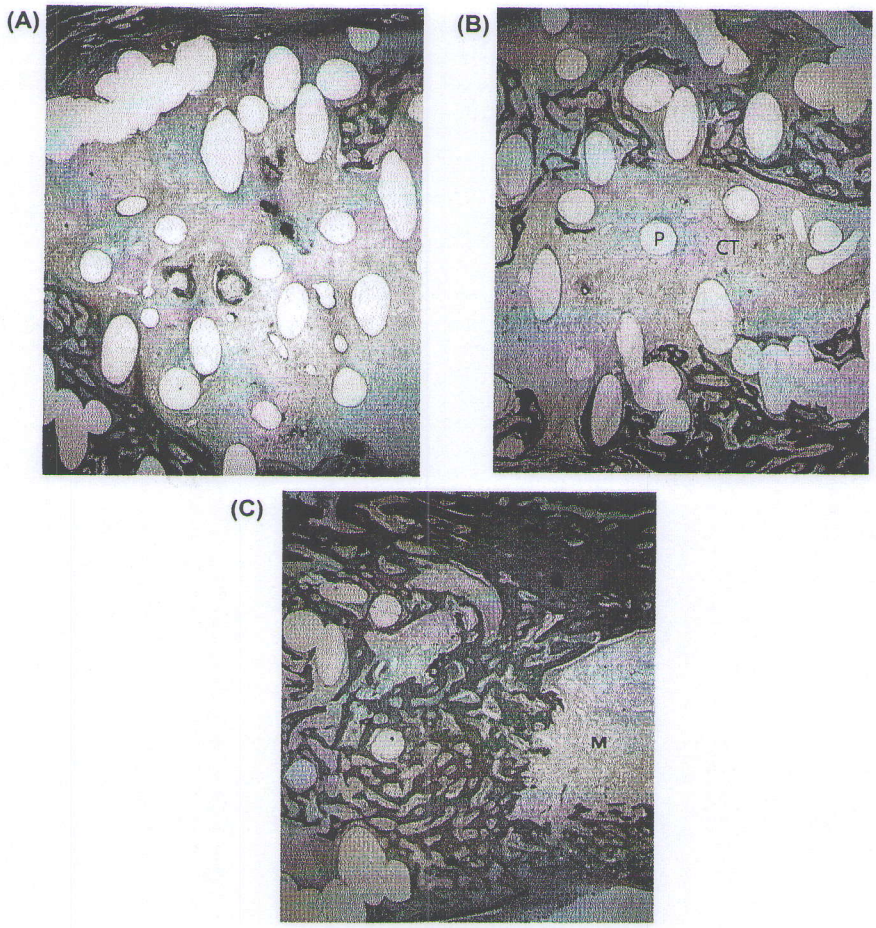

Figure 2. Hematoxylin and eosin (H&E) staining of 20 weeks specimen treated with PRP delivered in a PCL scaffold. (A) Distal end of segmental defect of ulna (B) Central part of segmental defect of ulna (C) Proximal end of segmental defect of ulna (×4). There was new bone formation from proximal end of segmental defect of ulna, but little bone formation was found in the central part of defect. P = pore of scaffold, M = medullary cavity of proximal ulna, CT = connective tissue.

capacity of PRP remains controversial. PRP does not contain bone morphogenic proteins, the most potent osteoinductive proteins, which promote stem cell differentiation into the osteoblastic lineage, it is therefore assumed that PRP has weak osteogenic properties. Several studies have reported that PRP is a potent mitogenic, but does not have strong osteogenic properties *in vitro* and *in vivo*. [5,10,11] One study showed that PRP alone does not enhance new bone formation in a critical size long bone defect of sheep tibia with a low regenerative potential.[4] The reason for the failure of PRP in their studies might be that the potency of the growth factors liberated by PRP is too weak to induce bone formation in defects with low regenerative capacity. Another studies [10,11] evaluated the effect of PRP on osteogenic differentiation and ectopic bone formation of human mesenchymal stem cells (MSC) in distinct resorbable calcium phosphate ceramics. In their studies, PRP improved proliferation of MSC on both calcium phosphate scaffolds, but had a weak influence on osteogenic properties. They reported that the addition of PRP to MSC did not improve osteogenesis in a critical sized radius defect of rabbit model.[5] Our study showed that the bone volume fraction, trabecular thickness, trabecular separation assessed by microCT scan did not differ from untreated control group. We could not find evidence of beneficial effect of PRP on osteogenesis.

The method of PRP preparation can affect the results of this study since this can significantly influence the concentrations of platelets and growth factors, and consequently their osteogenic capacity.[12] A review of literature reveals a rampant lack of standardization in the preparation of PRP. A lack of standardized protocol to produce and evaluate PRP in the literature can help explain the inconsistent clinical and experimental results. Grageda proposed that in addition to a uniform protocol, future studies should quantify growth factor concentration. However, several studies found no correlation between platelet concentration and the observed biological effects.[4,13,14] Only few authors have investigated concentration-dependent effects. Schlegel et al. found somewhat better results with higher (6.5-fold compared to normal blood) than with lower platelet concentrations (4.1-fold) on bone regeneration in scull defects of minipigs.[15,16] In the present study, the platelet concentration ranged from four to seven times to normal blood. Recently an *in vitro* study showed that the concentration of growth factors were species dependent, being highest in human PRP, followed by goat and rat PRP.[17]

One of factors that can influence osteogenesis is the scaffold.[18] The PCL scaffolds have been shown previously to support ectopic bone formation in rat model,[19] repair critical-sized calvarial defects in rabbit model,[20] orbital defects in a pig model,[21] and burr hole defects in patients with chronic subdural hematoma.[22] The efficacy of PCL scaffolds as delivery systems for PRP have been documented recently.[3,23] Rai et al demonstrated the beneficial effect of PRP on vascularization and bone ingrowth within 3D PCL-TCP scaffolds.[3] In the present study, the rate of bone formation was inferior in comparison to cancellous bone graft group. The bone volume fraction was 26.9% in PRP treated group after 12 weeks. Kasten et al [5] reported 92.2% of bone bolume fraction in the cancellous bone graft group. This might be due to the low osteogenesis effect of PRP or scaffold itself. However, the rate of new bone formation with PCL scaffold was similar with previous studies. Kasten et al[5] examined a calcium deficit hydroxyl apatite scaffold with PRP in a similar segmental defect of rabbit ulna. They found 24.8% of bone formation. Louisa et al [24] investigated a coral scaffold with MSC and reported 30% of bone formation. Geiger et al[25] found bone formation of 28% in coral scaffolds in a rabbit radius defect and an increase up to 60% with the addition of VEGF after 12 weeks. Therefore, the ability of PCL scaffold is not inferior to other scaffold. Future studies using other bioactive materials instead of PRP should be required.

Currently, it is common to combine the PRP with autograft, allograft, demineralized bone matrix or other graft material to fill bony defects in the mandible or cranium. Lowery, Kulkarni and Pennisi administered PRP and autogenous bone grafts during spinal fusion with good results, obtaining union in all their patients.[6] Kitoh et al[26] used PRP and bone marrow stem cells during osteogenesis distraction in three patients, and Acceleration of bone formation was observed. In this present study, other osteoinductive materials were not combined. A comparison would have been interesting to judge the osteogenic potential of PRP in combination of other osteoinductive materials. However, the purpose of this study was to investigate the effect of PRP alone and not to compare it to other kinds of growth factors. Several study groups are required and it is very hard to estimate the effect of PRP alone if more than two materials are combined. This should be addressed in future studies.

Several limitations of this study should be addressed. First, only one specific concentration of PRP was used. It would be better to see the effects of different concentrations of PRP since an optimal concentration of PRP has yet to be defined. Second, only one scaffold formulation was used. It is therefore impossible to know if this is the best formulation or if alternatives would produce superior outcomes. There could have been additional groups included with alternative PCL-TCP ratios and different geometries/porosity of scaffold to

determine the most favourable scaffold design. In addition, there was no study group with scaffold alone. Third, we did not quantify the growth factors concentrations. It is therefore impossible to know if optimal range of growth factors is released from platelets. Fourth, there were a small number of specimens. This study does not have enough statistical power to document a true statistical difference between controls and treatment groups. There is a need for well controlled, randomized studies that meet the requirements for properly powered studies. In addition, it may be beneficial to see some immunochemistry of osteogenic markers to confirm that there is no effect at the molecular level.

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