

Effect of Platelet Rich Plasma Incorporated to Autologous Bone Graft on Collagen Production in vivo

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Abstract

Platelet-rich plasma (PRP) has become a valuable adjunct to several procedures in oral and maxillofacial surgery as it can promote bone tissue regeneration without the risk of toxicity and immunogenic reactions. Moreover, collagen plays an important role in wound healing.

To investigate the effect of PRP incorporated to nonvascularized autogenous bone grafts on collagen production in vivo using an *Ovis aries* critical-sized mandibular bone defect model.

The extracted PRP was mixed with autogenous bone mills and was augmented to the mandibular bone defect model. The volume of collagen produced at 3 and 6 weeks was evaluated. Masson's trichrome was used for histological examination, and the volume of collagen produced was quantified using ImageJ.

The PRP group had a significantly higher collagen volume at 3 and 6 weeks than the control group, and the volume of collagen produced at 6 weeks was higher than that produced at 3 weeks in the PRP group. However, the results did not significantly differ.

Incorporating PRP to autogenous bone grafts increases collagen production at 3 and 6 weeks, which can facilitate mandibular bone healing in sheep.

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Introduction

Bony defect reconstruction after tooth extraction, abscess, cyst, and neoplastic tissue resection, and trauma and congenital deformity reconstruction remain a challenge in oral and maxillofacial surgery.¹ Unlike allografts, xenografts, and biomaterial-based bone grafts, autologous bone grafts (autografts) offer biocompatibility without the risk of rejection from the host.² Thus, autograft is the gold standard and the most commonly used bone substitutes (about 83% of all bone augmentation cases) in clinical settings, even though it has disadvantages including donor site morbidities and high cost.³ In critical-sized defects measuring more than 6 cm, vascularized bone

grafts are normally indicated for successful bone reconstruction.⁴ Compared with nonvascularized bone grafts, vascularized bone grafts only offer a limited amount of tissues to be harvested; pose greater risks including donor site morbidities and postoperative infection and bone resorption; and require higher cost, longer surgical time and hospitalization period, and highly skilled operators with more complex armamentarium.⁵ Interestingly, Saleem et al. (2018) reported that incorporating platelet-rich plasma (PRP) to nonvascularized autografts successfully induced bone regeneration in critical-sized defects.⁶

PRP is an autologous concentrate of platelets suspended in a small volume of plasma rich with platelet-derived growth factors.⁷ It is extracted by centrifugating blood, which is then mixed with bone granules. Thus, it is a simple and affordable yet effective strategy for improving vascularization and bone regeneration with additional antimicrobial properties.^{8,9} Oley et al. (2018) and Gonzalez-Ocasio et al. (2017) demonstrated that incorporating PRP to autografts successfully improved bone regeneration in mandibular bone defects.^{10,11}

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Collagen is a well-known marker of osteogenesis. Moreover, it is the most abundant protein found in the human body and is the main organic component of the bone matrix.¹² Hence, it plays an important role in wound healing processes, including bone formation.^{13,14} We hypothesized that incorporating PRP to nonvascularized autografts has a positive effect on bone regeneration based on a high collagen production in an *Ovis aries* critical-sized mandibular bone defect model. Therefore, this study aimed to assess this hypothesis.

Materials and methods

Sample preparation

The current study used 24 sheep, and it was approved by the Veterinary Ethical Commission, Faculty of Veterinary Medicine, Bogor Agricultural University (IPB) (approval no: 005/KEH/SKE/III/2020). The animals were divided into four groups (n=6) (Table 1).

	PRP groups	Control groups
At 3 months	Group I	Group II
At 6 months	Group III	Group IV

Table 1. Experiment and control groups.

In groups I and III, the autografts were mixed with PRP and augmented to the defect. Then, the samples were necropsied at 3 and 6 weeks post-surgery. Groups II and IV (control groups) were augmented with autografts without PRP. Then, samples were necropsied at 3 and 6 weeks post-surgery.

Prior to surgery, all samples were evaluated by a veterinarian and were placed in the acclimatization phase for 2 weeks. The surgery was performed under general anesthesia (premedication: atropine sulfate [0.4 mg/kg body weight] for 20 min, followed by xylazine tranquilizer [0.22 mg/kg body weight] and atropine and ketamine [11 mg/kg body weight] for 10 min).

The sheep hair was removed from the surgical site and disinfected with povidone iodine. Next, 20 mL of blood sample was collected from the jugular vein and placed in a tube (BD Vacutainer®) containing 4.5 mL of sodium citrate, which is an anticoagulant. Then, it was centrifuged at 720 g (2500 rpm) for 5 min. Then, with a 5-mL syringe, one-third of the middle layer of the plasma (approximately 1.5 mL) was

obtained just above the red blood cells, leaving the top plasma layer and red blood cell deposits. Using five tubes, approximately 8 mL of plasma was collected. Next, the plasma was transferred to 2 × 4-mL vacuum tubes without additives and was centrifuged again at 720 × g (2500 rpm) for 5 min. Then, PRP (approximately 2 mL) was obtained by taking 25% plasma from the bottom of the tube.

Surgery

On the right jaw via an extraoral incision, resection (2×1, 5 cm) was performed on the buccal plate (about three teeth from the canine to the premolar) with a fissure bur. The harvested bones were milled manually by pounding and sieving with a mesh (diameter: 1.5×1.5 mm). The milled bone granules were mixed with PRP and CaCl₂ (bone:PRP:CaCl₂=1 g:1.67 mL:0.083 mL) and were stirred for about 3 min until a tender consistency was achieved. In the control groups, the bone grafts were mixed with NaCl (bone:NaCl=1 g:1.67 mL) and were stirred for 3 min. The mixtures were placed on the defect and covered with 3×2-cm Batan® membrane. Then, the sheep was reared in the research facility of Veterinary Medicine Faculty, IPB University, until the designated period.

Postsurgical evaluation

All sheep healed well after surgery. At 3 and 6 weeks, the sheep (n=6) were euthanized and necropsied to prepare the mandibles for histological examinations. The mandible segment from each sheep, including the graft area, was fixed with 10% formalin buffer, dehydrated with alcohol (100%, 95%, and 70%), stained with Masson's trichrome (Weigert's iron hematoxylin, Biebrich scarlet-acid fuchsin, and aniline blue solutions), and then dehydrated again with 95% ethyl alcohol. Histological examinations were conducted with a microscope equipped with a camera/optilab at a magnification of 100× in five fields of view. The collagen appeared blue, and the nuclei was black. Meanwhile, the muscle, cytoplasm, and keratin were red. To quantify the percentage of collagen density, images were processed using ImageJ.¹⁵

Statistical analysis

Normality tests were conducted using the Shapiro–Wilk test, and the Lavene's test was used to assess for homogeneity. For normally distributed data, one-way analysis of variance (p-value of < 0.05) was used. However, if the data were not normally distributed, the Kruskal–Wallis

test and the Mann–Whitney U test were utilized to assess differences among the experimental groups (p value < 0.05). Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS) v.22 software.

Results

One sheep in group I had bone overgrowth in the lingual side. One sheep in group II presented with intraoral pus discharge, and another sheep in group IV had a pathological fracture.

Histological examinations revealed an increased percentage of collagen area in the PRP groups compared with the control groups. The PRP 6-month group (group III) had a higher collagen production than the PRP 3-month group (group II). Figure 1 and Table 2 show the volume of collagen produced among all groups.

In Figure 1, the collagen area (blue staining) looked fibrillar, and group (a) I had a denser collagen area than (b) group II. However, group (c) III had a less dense collagen area. Meanwhile, the collagen area of group (c) III was denser than that of group (d) IV. However, the results did not significantly differ between groups (b) II and (d) IV. These observations were confirmed via quantification using ImageJ, as shown in Table 2.

Groups	Collagen density (%)	Significant values		
		II	III	IV
I. PRP at 3 weeks	20.25	* $P<.0001$	* $P=.002$	-
II. Control at 3 weeks	11.68	-	-	$P=.051$
III. PRP at 6 weeks	28.78	-	-	* $P=.002$
IV. Control at 6 weeks	18.35	-	-	-

Table 2. Collagen density (%) after bone grafting calculated via a histological image analysis with ImageJ: (a) Group I: autograft + PRP at 3 weeks, (b) group II: autograft + PRP at 6 weeks, (c) control at 3 weeks, and (d) control at 6 weeks, along with their significance level (Kruskal–Wallis test and Mann–Whitney U test) (* $P<.05$ indicates significant differences between two groups) (n=6).

Discussion

In oral and maxillofacial surgery, blood derivatives, including platelet-rich plasma (PRP), have been widely used. PRP is rich in growth factors, including transforming growth factor- β (TGF- β 1 and TGF- β 2), vascular endothelial

growth factors, platelet-derived growth factors (PDGF- $\alpha\alpha$, PDGF- $\beta\beta$, and PDGF- $\beta\alpha$), and endothelial growth factors.¹⁶ Growth factors stimulate chemotaxis, mitogenesis, collagen matrix synthesis, and tissue healing when applied to bone defects.¹⁷

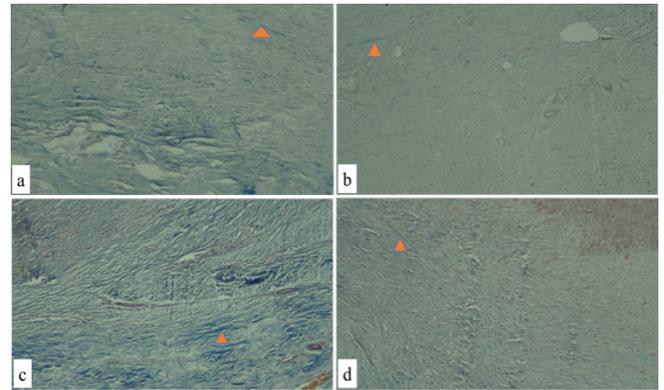


Figure 1. Representative histological images of the mandibular bone sections of *Ovis aries* stained with Masson's Trichrome: (a) Group I: autograft + PRP at 3 weeks, (b) group II: autograft + PRP at 6 weeks, (c) control at 3 weeks, and (d) control at 6 weeks. Collagen was indicated by blue color (red arrow).

To assess bone regeneration, quantification was performed using collagen, a well-known biomarker of osteogenesis. Immediately after the injury, macrophages produce inflammatory mediators, such as cytokines and growth factors, which stimulate angiogenesis and new extracellular matrix (ECM) synthesis. This indicates a higher supply of oxygen and nutrients for cell survival, proliferation, and growth.¹⁸ In this study, the percentage of collagen produced at 6 weeks (group III) was higher than that at 3 weeks (group I). The formation of collagen starts from day 7 and increases over time. When the period is longer, the healing time is prolonged. Thus, thicker matrix strands of collagen areas were formed, and the 6-week samples produced a higher volume of collagen area than the 3-week samples.

Therefore, the incorporation of PRP enhances bone healing (Figure 1 and Table 2). Moreover, Daradka et al. (2019)¹⁹, Oley et al. (2018)¹⁰, and Gawai et al. (2015)²⁰ have shown that PRP incorporation in bone graft enhances bone regeneration. However, the efficacy of PRP in improving bone regeneration remains

debatable. Despite several reports concluding the positive effect of PRP on bone healing, some studies showed no significant improvement on bone regeneration.²¹⁻²³ These differences can be attributed to different factors including variations in blood content from each sample and lack of standardized protocols in the experimental variables. Furthermore, in clinical application, wound healing process may also be influenced by numerous intrinsic and extrinsic factors, such as mechanism of injury, blood supply, age, nutrition, smoking, infection, jaw mobilization, oral hygiene, and comorbidities.²⁴

In vivo PRP studies should use large animals, such as rabbits, minipigs, sheep, and dogs because a large volume of blood is required. The sheep (*Ovis aries*) model was chosen because the mandibular shape of sheep is similar to that of humans.²⁵ Since sheep are ruminant animals, the results could be biased due to mastication activity. Bone mills were chosen instead of block graft as the particulate form offers larger total surface area than the block graft to optimize the incorporation of growth factors from PRP. Cautious handling is required in milling the bone as the processing may damage bone cells, or activate osteoclasts, which, in turn, may cause bone resorption.²⁶ Furthermore, although the sterilization of the operating area has been minimized by shaving the hair and keeping the cage clean, the operative site was at risk for infection. In this study, there was a sample in the 3-week control group that presented with pus discharge in the lingual part, and another sample in the 6-week control group had a pathological fracture. The absence of such occurrence in PRP samples might be attributed to the possible antibacterial properties of PRP.⁹ Nevertheless, further antimicrobial studies must be conducted to validate these results.

Conclusions

This *in vivo* study showed that incorporating PRP to the autogenous bone graft increases collagen production at 3 and 6 weeks, which can facilitate mandibular bone healing in sheep. Therefore, incorporating PRP to nonvascularized autografts can be a minimally invasive alternative to vascularized bone grafts in critical-sized mandibular bone defect reconstruction in oral and maxillofacial surgery.

However, further clinical studies should be performed to validate this hypothesis.

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Declaration of Interest

The authors report no conflict of interest.

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