Improved Bone Healing by Angiogenic Factor-Enriched Platelet-Rich Plasma and Its Synergistic Enhancement by Bone Morphogenetic Protein-2

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Purpose: *The purpose of this study was to modify the method of platelet-rich plasma (PRP) preparation for obtaining optimal angiogenic potential and accelerate bone healing. Also, the potential synergistic effect of a suboptimal concentration of bone morphogenic protein-2 (BMP-2) and modified PRP (mPRP) on bone healing was evaluated in vivo.* ***Materials and Methods:*** *The angiogenic factor-enriched PRP, which included peripheral blood mononuclear cells (mostly lymphocytes and monocytes, excluding polymorphonuclear leukocytes [PMNs], was achieved by lowering concentrations of thrombin and $CaCl_2$, after pre-activation with shear stress using a table-top vortex machine and collagen. In vitro, endothelial cell migration activity in the mPRP group was compared to conventional PRP preparation using a modified Boyden chamber assay. In an animal study, PGA scaffold, PGA scaffold + mPRP, PGA scaffold + mPRP + rhBMP-2, and PGA scaffold + rhBMP-2 were applied to critical-sized calvarial defects in 28 nude rats. At 2 weeks, periosteal blood flow was measured using laser Doppler perfusion imaging, and bone formation was evaluated at 8 weeks by histology, dual energy x-ray absorptiometry, and micro-computed tomography.* ***Results:*** *mPRP induced faster migration of cord blood–derived outgrowth endothelial-like cells. In vivo, the group with mPRP with a low dose of rhBMP-2 showed significantly increased numbers of blood vessels at 2 weeks and notable synergistic effect on bone healing at 8 weeks as evaluated with histology, bone mineral density and bone mineral content, and μCT.* ***Conclusion:*** *The mPRP used in this study improved vascular perfusion around the defect and resulted in enhanced bone healing. Also, combining mPRP with a suboptimal dosage of rhBMP-2 improved bone formation and enhanced bone density.* Int J Oral Maxillofac Implants 2008;23:818–826

Key words: *angiogenic potential, athymic rat, calvarial defect, rhBMP-2*

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Fast and predictable healing of bone grafts would provide great benefits for implant dentistry. One of the recent strategies to accelerate bone graft healing includes the use of platelet-rich plasma (PRP). Since Balk reported in 1971 that growth factors may be preserved in platelets,[1] platelets have been identified as containing factors such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF)-like protein, and connective tissue activating peptide-III (CTAP-III).[2,3] Most of these factors are involved in angiogenesis and osteogenesis. Moreover, Sipe et al[4] reported evidence for the presence of bone morphogenetic proteins (BMPs)-2, 4, and 6, potent osteogenic inducers, within megakaryocytes and platelets. With this composition, autogenous PRP could be an attractive and potent material for bone graft procedures, as it has additional advantages, including hydrogel forma-

tion, suitable for cellular migration and proliferation while being immunologically inactive. Also, recent studies[5,6] indicate that combinations of pure factors seem to work better than single factors, supporting the promise of PRP.

The genuine effect of PRP on osteogenesis is, however, very controversial, and PRP has failed to show advantages in promoting healing of bone grafts in some recent animal and human applications.[7,8] Possible causes for such unsatisfactory results include an underestimation of the importance of the various factors involved in PRP activation and function. For example, thrombin and calcium, potent platelet activators, could induce immediate growth factor release from platelets in a dose-dependent manner. If their concentration is too high, however, vascular endothelial proliferation could be inhibited[9] and the fate of stem cells altered.[10] Also, PRP normally exerts its effects very early in the healing period, as the lifespan of a platelet in a wound and the period of direct influence of its growth factors are less than 5 days.[11] Therefore, the mismatch between the long-term process of bone healing and the mode of action of PRP suggests that the angiogenesis effects of PRP may be more relevant than its direct effects on osteogenesis. Angiogenesis, a process of new vessel formation, plays a critical role in bone development. Reconstruction of the circulatory system is one of the earliest events during bone healing. The early establishment of a functionally intact vascular network appears not only to precede the event of bone formation[12] but also to have a substantial influence on the quality of the resulting bone.[13]

In the present study, the method of PRP activation and preparation was modified to maximize its angiogenic potential, and its bone healing effect was evaluated in vivo. For the present study, it was hypothesized that incorporation of a suboptimal concentration of BMP-2 into the angiogenic factor-enriched PRP would show a synergistic effect on bone healing. To evaluate the osteoconductive effect of angiogenic factor-enriched PRP, fibronectin-coated woven polyglycolic acid (PGA) scaffolds were used as a carrier in a critical-size rat calvarial defect model.

MATERIALS AND METHODS

Preparation of the Angiogenic Factor-Enriched PRP

Fresh human adult whole blood was purchased from AllCells (Emeryville, CA). Peripheral blood mononuclear cells (PBMNCs) and platelets were separately isolated by a density gradient centrifugation (Histopaque-1077; Sigma, St. Louis, MO) according to the manufacturer's instructions. PBMNCs and platelet concentrate were collected by additional centrifugation (200*g* and 1,400*g*, respectively). Degranulation from platelets was induced with 10 U/mL of thrombin (Sigma) and 0.2 mg/mL $CaCl_2 \cdot 2H_2O$ after preactivation with shear stress and 20 μg/mL of collagen using a table-top vortex machine. The rotation rate was 100 rpm for the first 15 seconds and then 3,500 rpm for 5 minutes. Angiogenic factor-enriched PRP was prepared by incorporation of PBMNCs into the PRP.

Endothelial Migration into Fibrin Gel

Vascular endothelial cell migration activity was measured using a modified Boyden chamber assay described by Osusky et al.[14] Cord blood–derived outgrowth endothelial-like cells (CBOECs) having progenitor characteristics were cultured in the laboratory and used for the experiment. At 80% confluence in culture dishes, cells were starved in EBM-2 medium for 24 hours. 2.0×10^5 CBOECs in 1 mL of serum-free starvation medium were placed into the transwell fibrin gel-coated insert (24-mm diameter, 3.0 μm pore size, Corning Incorporated Costar, Corning, NY). One hundred microliters of fibrinogen solution (4 mg/mL, Sigma) were applied to the insert and polymerized with 2 kinds of thrombin and calcium solution (10 U/mL of thrombin and 0.2 mg/mL $CaCl_2 \cdot 2H_2O$ and 142.8 U/ml of thrombin and 14.3 mg/mL $CaCl_2 \cdot 2H_2O$) for 30 minutes at 37°C. Fresh EBM-2 medium (3 mL) with the modified PRP or the conventional PRP (300 μL) was added to the lower chamber and incubated overnight. The insert chamber membrane was fixed with 100% cold methanol and was cut off around the edge and stained with 0.2% of DAPI (4′-6-Diamidino-2-phenylindole) for 5 minutes. The lower surface of the membrane, which contained the migrated cells, was placed face-up on a histologic slide and covered with a cover slide mounted with phosphate-buffered saline (PBS)/glycerol solution. The migrated cells were quantified in 5 random fields for each membrane using fluorescence microscopy. Assays were performed in triplicate.

Implantation of Fibronectin-Coated PGA Scaffolds Loaded with PRP or rhBMP-2

Harvard University and National Institutes of Health (NIH) animal care guidelines were followed in all procedures. Anesthesia and pain control were used according to the recommended routines for the species. Twenty-eight NIH nude rats were anesthetized using isoflurane inhalation anesthesia (E-Z Anesthesia; Euthanex, Palmer, PA). Buprenorphine HCl, 0.02 to 0.03 mg/kg, was administered presurgically. After a midline skin incision, a critical-size cal-

varial through-and-through defect, 8.0 mm in diameter, was trephined into the central portion of the cranium using a dental handpiece and trephine bur (Ace Surgical Supply Co, Brockton, MA) under constant irrigation with sterile saline. Using an aseptic technique, 50 μL of angiogenic factor-enriched PRP (1×10^7 PBMNCs + 1×10^9 activated platelets) was soaked into the fibronectin-coated PGA scaffold. Ready-made PGA mesh 1.2 mm in thickness (Synthecon, Houston, TX) was cut into pieces 7.5 mm in diameter and then the circular scaffolds were soaked in 100 μg/mL fibronectin solution (Sigma) for 30 minutes and dried overnight in a clean bench under an ultraviolet lamp for sterilization. The cell-loaded scaffolds were kept at 37°C during site preparation to maintain cell viability in the experimental group. Certain angiogenic factor-enriched PRP/BMP-2 scaffolds also contained 200 ng of recombinant human BMP-2 (rhBMP-2; R&D Systems, Minneapolis, MN). The constructs were inserted into the defects and the margins of the wound were closed using nylon sutures. The animals were monitored until they had recovered from anesthesia. In addition, the same scaffolds without PRP or additional factors (blanks) and scaffolds containing either PRP or rhBMP-2 only were applied in the same manner.

Laser Doppler Analysis and Immunohistochemistry

At 2 weeks, periosteal blood flow over the cranial defect was measured using laser Doppler perfusion imaging (LDPI; PeriScan; Perimed, Stockholm, Sweden) in 3 animals per group. After a skin incision and meticulous supraperiosteal dissection under inhalation anesthesia, consecutive perfusion measurements were obtained by scanning the size of the region of interest; 10×10 mm^2 above the defects. After LDPI measurements, the animals were euthanized and the cranial bones, including the defects were retrieved, fixed in 10% zinc-buffered formalin, and decalcified. Paraffin tissue sections were immunostained for von Willebrand factor (vWF) using a commercial vessel staining kit (Chemicon, Temecula, CA) and imaged by means of a Nikon Eclipse E800 light microscope and a Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI). Blood vessels, marked by vWF staining, were counted manually at 100× magnification in randomly selected sites of the defect and normalized to the tissue area with the use of NIH Image J Software.

New Bone Formation

At 8 weeks, 4 animals from each group were euthanized, and the implants were retrieved and fixed in 10% zinc-buffered formalin. The implants were scanned for bone mineral content (g) and bone mineral density (g/cm^2) in the region of interest (ROI) sized 8×8 mm^2 using dual energy x-ray absorptiometry (DEXA, ODR2000+, Hologic, Waltham, MA). The specimens were also imaged 2-dimensionally with microradiography (Faxitron; Hewlett Packard, McMinnville, OR) and 3-dimensionally with microcomputerized tomography (×CT40, ScanoMedical, Bassersdorf, Switzerland). The samples were then decalcified, embedded with paraffin, and sectioned from the center of the defects. Sections were stained with hematoxylin-eosin.

Statistical Analysis

The Wilcoxon rank sum test was applied to compare the conventional method to the modified method, and also compare experimental groups (PRP only, rhBMP-2 only, and PRP + rhBMP-2) to the control group. The level of significance was set at 5%.

RESULTS

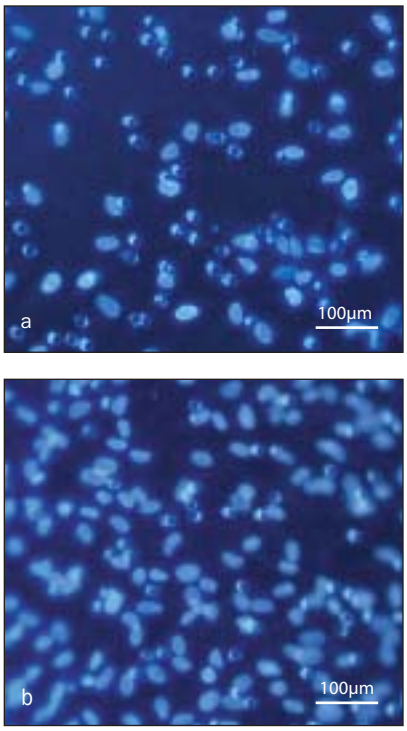
Angiogenic Effects

Angiogenic factor-enriched PRP induced faster migration of CBOECs in an in vitro model (Fig 1).

The blood vessel density within defects was analyzed 2 weeks postimplantation. Compared to the control group (42 ± 8 vessels/cm^2, Fig 2), PRP (66 ± 12 vessels/cm^2), rhBMP-2 (72 ± 16 vessels/cm^2) and PRP + rhBMP-2 (92 ± 13 vessels/cm^2) showed significantly increased numbers of blood vessels ($P < .05$). Supraperiosteal blood perfusions was also improved in the defects grafted with PRP, PRP + rhBMP-2, and rhBMP-2 compared to the control group, but there was no statistically significant difference except for the PRP + rhBMP-2 condition ($P < .05$; Fig 3).

New Bone Formation

All groups showed favorable tissue response with no significant inflammatory reaction (Fig 4). In microradiographic images, the control group showed minimal bone formation in the defect, while the angiogenic factor-enriched PRP and rhBMP-2 group showed increased bone healing around the defect margins. The healing of the calvarial defects was improved by angiogenic factor-enriched PRP. This was also confirmed by microCT images (Figs 5 and 6). Interestingly, synergistic effects on bone healing with coadministration of PRP and rhBMP-2 were noted. Human angiogenic factor-enriched PRP demonstrated significantly increased bone mineral content (0.017 ± 0.003 g) and bone mineral density (0.046 ± 0.0006 g/cm^2) versus control defects (bone mineral content of 0.011 ± 0.003 and bone mineral density of 0.037 ± 0.002; $P < .05$; Fig 7). The rhBMP-2 condition



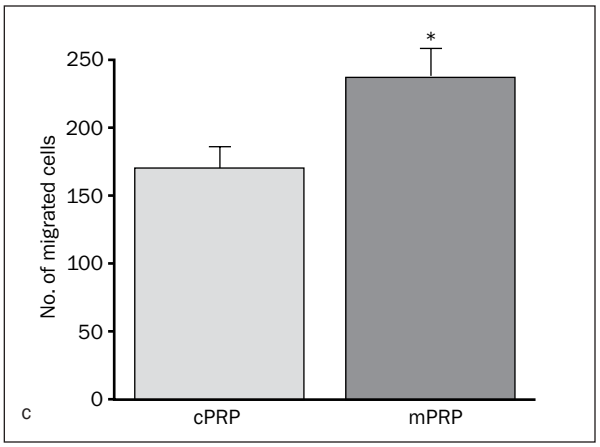
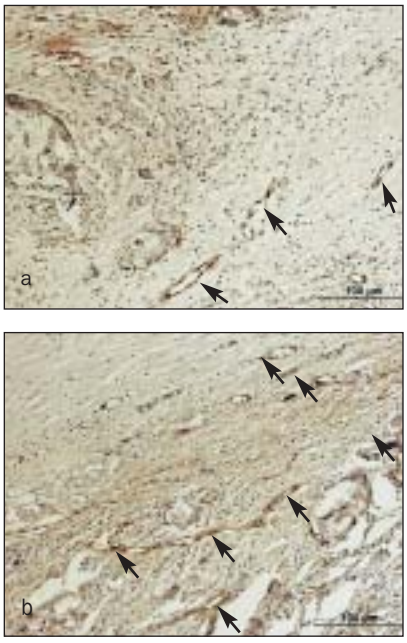


Fig 1 In vitro fibrin gel migration of CBOECs after 48 hours. *(a)* Fibrin gel formed with PRP activated by 142.8 U/mL thrombin and 4.3 mg/mL $CaCl_2$ (conventional PRP) and *(b)* fibrin gel formed with PRP activated by 10 U/mL thrombin and 0.2 mg/mL $CaCl_2$ (modified PRP). *(c)* Quantification of the number of cells that migrated in response to the conventional PRP preparation (cPRP) and modified PRP (mPRP). *Indicates statistically significant difference ($P < .05$). Values represent means and standard deviations.



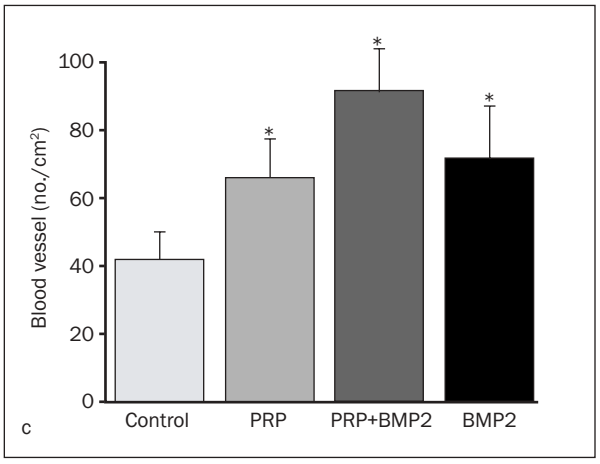


Fig 2 Staining for vWF in histologic sections from critical-size (8 mm) rat craniotomy defects following implantation of *(a)* fibronectin-coated PGA scaffold only and *(b)* PGA scaffold + angiogenic factors-enriched PRP at 2 weeks. Blood vessels are denoted with arrows. *(c)* Quantitative analysis of the number of blood vessels in all groups (*Indicates statistically significant difference compared to control; $P < .05$). Values represent mean and standard deviation.

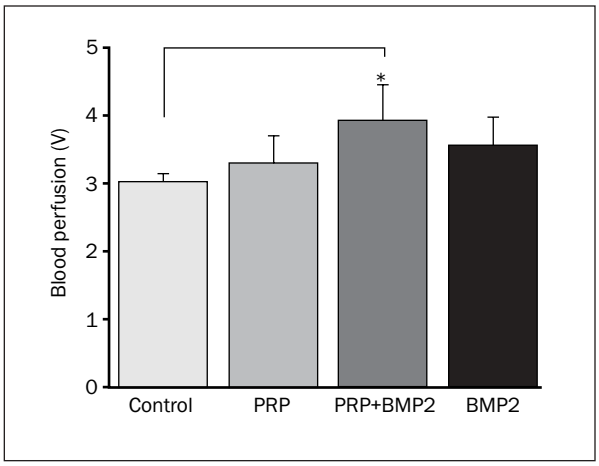


Fig 3 Supraperiosteal blood perfusion after implantation of fibronectin-coated PGA scaffolds containing angiogenic factor-enriched PRP and rhBMP-2 at 2 weeks. Laser Doppler perfusion imaging values represent mean and standard deviations. *Indicates statistically significant difference compared to control ($P < .05$).

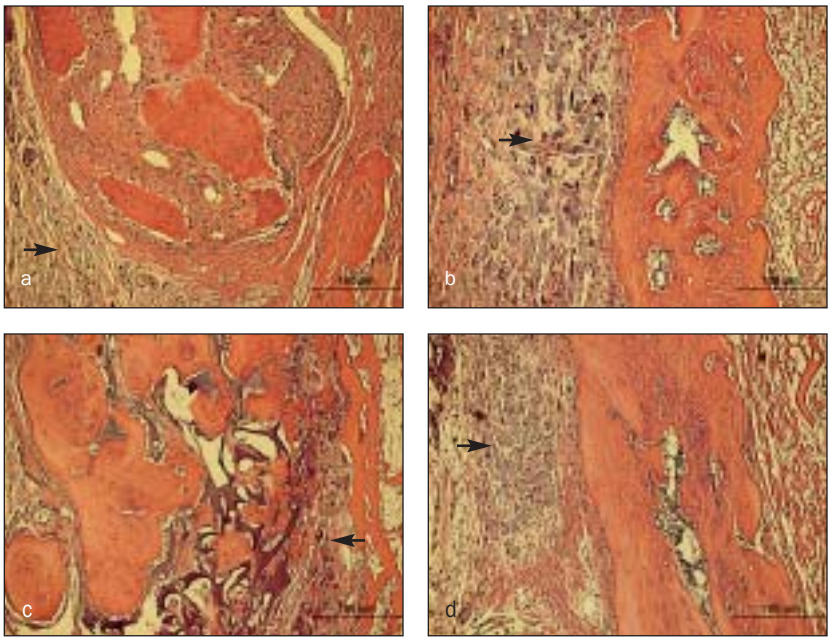


Fig 4 Photomicrographs of histologic sections of critical-size (8 mm) rat craniotomy defects following implantation of PGA scaffold, angiogenic factor-enriched PRP, and rhBMP-2 (8 weeks). *(a)* blank scaffold only, *(b)* PGA scaffold + PRP, *(c)* PGA scaffold + PRP + rhBMP-2 (200 ng), and *(d)* PGA scaffold + rhBMP-2 (200 ng). Compared to the blank scaffold *(a)*, PRP/PGA *(b)*, and rhBMP-2/PGA *(d)* scaffolds *(black arrow)* are replaced with more new bone. With the PRP + rhBMP-2/PGA scaffold *(c)*, new bone is thicker and fewer scaffold remnants remain *(black arrows)*.

also induced significant bone healing (bone mineral content, 0.024 ± 0.003; bone mineral density, 0.046 ± 0.001), and PRP combined with rhBMP-2 showed the greatest effects (bone mineral content, 0.034 ± 0.002; bone mineral density, 0.066 ± 0.011).

DISCUSSION

These results demonstrate that angiogenic factor-enriched PRP, optimized for early angiogenesis, enhances bone healing in the critical-size rat calvarial defect. More importantly, these findings show that this modified PRP and BMP-2 act synergistically to enhance both bone formation and bone healing. Two factors must likely be considered to overcome the reported inconsistency of the PRP effect in bone healing. One is the short life span of platelets and their effects, and the other is the insufficiency of the cellular component in some autogenous cortical bone graft and alloplastic materials. Since the life span of platelets is not more than 1 week, it can hardly be expected that their effect be retained during matrix formation by osteoblasts. In some studies, unsatisfactory effects of bone healing with PRP have been attributed to this short effective time.[15] It is well-known that angiogenesis plays a critical role in bone formation and healing, and microenvironmental alterations stemming from a lack of proper blood supply and ensuing tissue hypoxia may also directly hinder osteoblast differentiation.[16] Iliac cancellous marrow contains abundant cellular components that activate angiogenesis and migration of osteoprogenitors, maintain their survival, and promote their differentiation into osteoblasts. In particulate cortical bone or alloplastic bone substitutes, however, proper

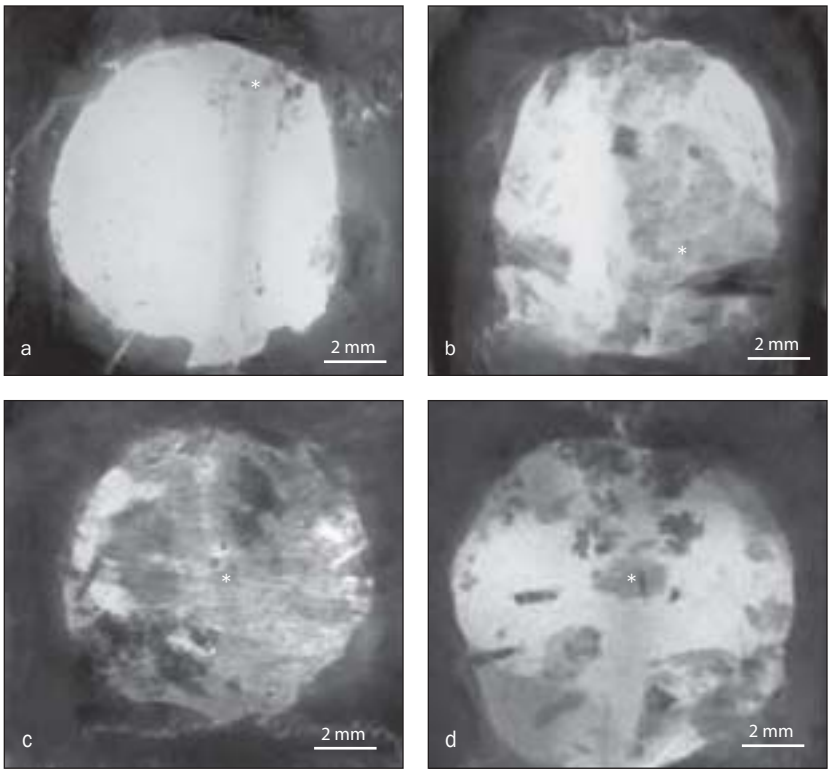


Fig 5 Radiographic images of the craniotomy defects following implantation of fibronectin-coated PGA nonwoven fabric scaffolds with or without angiogenic factors-enriched PRP and rhBMP-2 (8 weeks). *(a)* PGA scaffold only, *(b)* PGA scaffold + PRP, *(c)* PGA scaffold + PRP + rhBMP-2 (200 ng), and *(d)* PGA scaffold + rhBMP-2 (200 ng). PRP/PGA *(b)* and rhBMP-2/PGA *(d)* scaffolds show more sporadic new bone formations *(white asterisk)* than the blank scaffold *(a)*. In PRP + rhBMP-2/PGA scaffold, new bone formation is distributed in the whole defect.

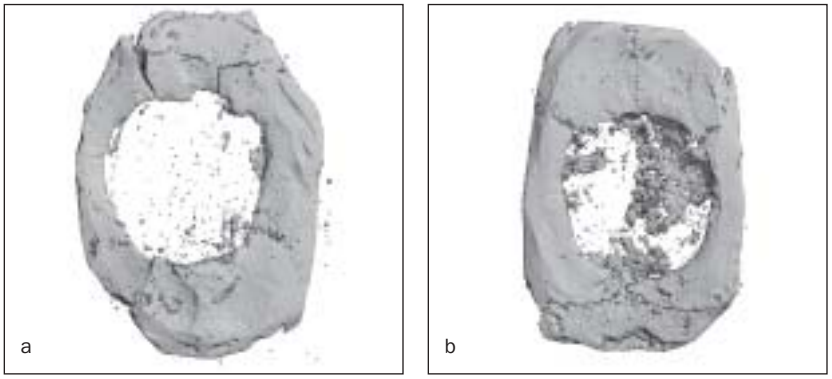


Fig 6 Three-dimensional reconstruction of μCT images of the craniotomy defects (8 weeks). *(a)* blank PGA scaffold only; *(b)* PGA scaffold + PRP.

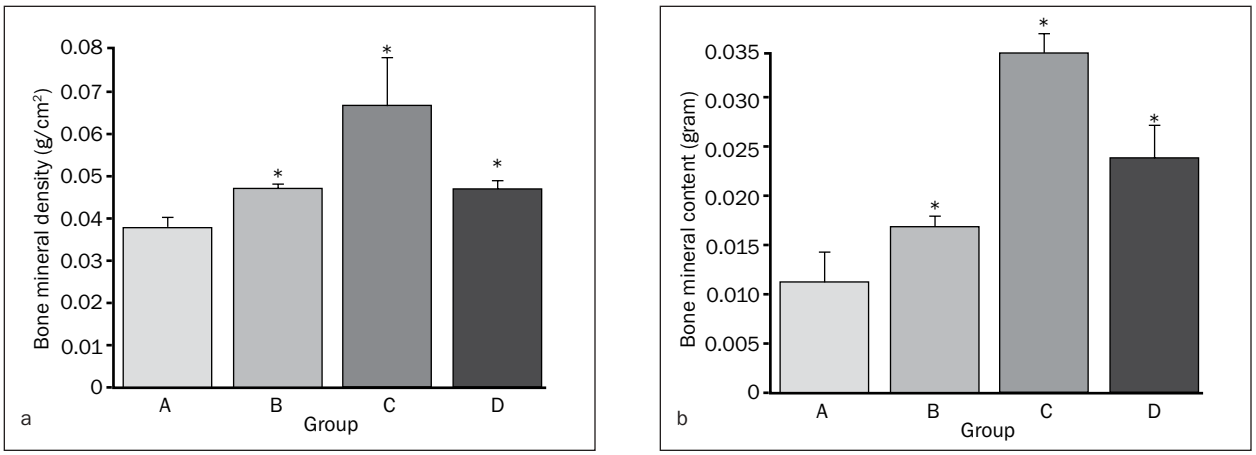


Fig 7 *(a)* Bone mineral density and *(b)* bone mineral content. Group A = PGA scaffold only; group B = PGA scaffold and angiogenic factors-enriched PRP; group C = PGA scaffold, angiogenic factors-enriched PRP, and rhBMP-2 (200 ng); and group D = PGA scaffold and rhBMP-2 (200 ng). Values represent means and standard deviations. *Indicates statistically significant difference as compared to group A; $P = .05$.

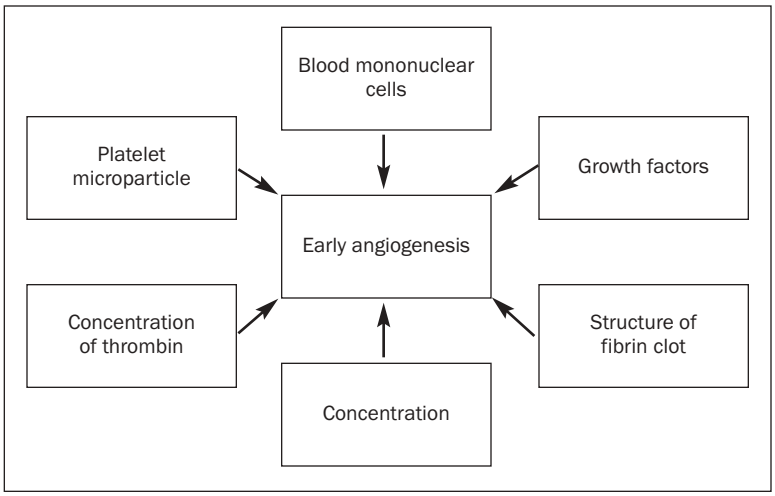


Fig 8 Factors to be considered for preparation of PRP to improve new bone formation through enhanced early angiogenesis.

osteoblastic differentiation or proliferation might be delayed until the hypoxic condition is improved. This may be a reason for the conflicting results with PRP in bone healing. The importance of the cellular component in PRP-induced bone healing has been supported by studies using a combined graft of stromal stem cells, PRP, and allogeneic bone in sheep long-bone critical-size defects.[17]

Through literature review, 5 factors in PRP preparation that likely influence angiogenesis can be identified. They are the concentrations of thrombin and calcium, initial release of VEGF, morphology of the fibrin clot, production of platelet microparticles (PMPs), and peripheral blood mononuclear cells (PBMNCs). In a pilot study, it was found that a low dose of thrombin (10 U/mL per 1×10^9 platelets) with 0.2 mg/mL $CaCl_2$ could enhance proliferation of vascular endothelial cells and initial VEGF release (data not shown). These results correspond with previous studies. Borrelli et al[9] reported that 0.1 U/mL of thrombin is the maximum concentration to increase endothelial cell proliferation done with 4×10^4 cell number, and Maloney et al[18] found that over 1 U/mL of thrombin per 3×10^8 platelets approximately decreased the initial release of VEGF. Also, thrombin is known to increase the migration of osteogenic cells (up to 1 U/mL).[19] One potential limitation, though, is that low amounts of thrombin decrease the activation of platelets, resulting in diminished production of PMPs. Microparticles (0.05 to 1 μm) are small vesicles that are released from all cell types subjected to activation, and their composition reflects the state of the membrane of the originating cell. Although the exact functions of microparticles are uncertain, microparticles generated from activated platelets (PMPs) are known to stimulate vascular endothelial cell proliferation, angiogenesis, and vascular hyperactivity, resulting in increased cellular migration, hematopoietic cell proliferation, and their engraftment.[20] It also has been reported that the effect of 5×10^5/mL PMPs appears to be at least as potent as that of 50 ng/mL VEGF in vitro. Therefore, sufficient PMP production is another strategy to enhance rapid angiogenesis.[21] Decreased PMP production could be compensated with application of shear stress and preactivation with a low dose of collagen (20 μg/mL), according to a previous report.[22] This type of preparation led to faster migration of endothelial progenitors through a fibrin clot than conventional PRP (high dose thrombin and calcium), and it was suggested that the fibrin configuration influenced cell behavior in addition to the increased angiogenic factors. In previous studies, it was shown that high thrombin concentrations make fibrin highly branched and reduce cell migration into bone defects.[9] Further studies are needed to clarify the correlation between fibrin morphology and cell migration.

In conventional PRP, the whole leukocyte fraction usually has been excluded because of an adverse inflammatory reaction. But it has been suggested that peripheral blood mononuclear cells, one component of the leukocyte cell population, have beneficial effects in angiogenesis. Some of these cells enhance arterial formation and collateral arterial growth,[23] and their effects can be augmented by incorporation of platelets. An advantage of inclusion of the peripheral blood mononuclear cell fraction in PRP is that the angiogenic effect can be maintained up to 1 month, and this complements the short-term effect of the growth factors in PRP.[24] The present study demonstrates that these integrated approaches in PRP preparation improve vascular perfusion around the defect (periosteum) and new vessel formation inside the defect and result in enhanced bone healing (Fig 8). The angiogenic factor-enriched PRP improved bone healing and increased bone density.

Another interesting feature of these studies is that PRP and BMP-2 acted synergistically in bone formation to enhance bone density. A major challenge to the successful application of BMP in the clinical situation is the significant amounts of the protein required for bone formation.[25] Combining anabolic agents that have a synergistic effect is an attractive solution to this problem. Although there is some evidence of BMPs in platelets, PRP is known to have a strong mitogenic effect without osteogenic differentiation. The present study shows that angiogenic factor-enriched PRP supports the function of a reduced concentration of BMP. This could be advantageous, as lower BMP doses may diminish adverse reactions to exogenous, genetically modified synthetic proteins and also reduce the cost of the therapy. This result is supported by previous findings of synergy in bone formation by combined VEGF and BMP-4 application and coadministration of a low dose of fibroblast growth factor and BMP-2 in vivo.[5,26,27] But it is unclear whether this synergistic effect comes from an enhanced osteogenic function of BMP-2 by PRP or augmented angiogenesis of PRP by the BMP-2. BMP-2 is an important protein in vascular development, and in the present study, vascular perfusion and vessel formation were improved with a small amount of BMP-2 (200 ng per animal). However, this finding contradicts previous reports, which indicated that the osteogenic differentiation induced by BMPs can be suppressed by platelet-released supernatant in vitro.[28] Such conflicting results may be attributable to the differences between in vivo and in vitro studies. Additional experiments are needed to clarify the exact mechanism and the optimal ratio between angiogenic factor-enriched PRP and BMP-2.

In summary, the modified PRP used in this study plays a favorable role in bone regeneration, and its effects appear to be mediated by improved angiogenesis. The findings also suggest a reduced effective dose of osteogenic protein (BMP-2) when used with this PRP preparation. Bone healing is a multifactorial process, and PRP contains many components whose interactions may be altered by the preparation method. Therefore, all these factors should be carefully considered and controlled to generate beneficial effects and prevent detrimental reactions.

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