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The Effect of Thrombin Activation of Platelet-Rich Plasma on Demineralized Bone Matrix Osteoinductivity

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Background: Demineralized bone matrix is an osteoinductive and osteoconductive material that is often used in orthopaedic surgery to induce bone formation. Autologous platelet-rich plasma, which contains proliferative and chemoattractant growth factors, has been used as a demineralized bone matrix adjuvant with mixed results. One variable during clinical use appears to be whether the platelet-rich plasma is activated with thrombin or is implanted in a liquid form with intact platelets. The objective of the present study was to determine if platelet-rich plasma can increase the osteoinductivity of demineralized bone matrix when used without thrombin activation.

Methods: The bioactivity of the demineralized bone matrix was evaluated in vitro by determining alkaline phosphatase production by C2C12 myoblast cells. The effect of thrombin activation on platelet-rich plasma was studied in vitro by evaluating osteosarcoma and bone marrow stromal cells for cell number and transforming growth factor-β1 activation. Demineralized bone matrices supplemented with platelet-rich plasma, with or without thrombin activation, were implanted intramuscularly in athymic rats and were examined at fourteen, twenty-eight, and fifty-six days. Histological samples were analyzed for osteogenesis and chondrogenesis. Osteogenesis was further characterized on the basis of alkaline phosphatase activity.

Results: In vitro, thrombin triggered an immediate release of growth factors from the platelet-rich plasma, and the platelet-rich plasma increased the number of both osteosarcoma and stromal cells in a dose-dependent manner. Thrombin activation of the platelet-rich plasma eliminated such stimulatory effects. In vivo, the platelet-rich plasma stimulated chondrogenesis on Day 14 and osteogenesis on Days 28 and 56, whereas thrombin-activated platelet-rich plasma acted as an inhibitor of such events. In addition, inflammatory cells were detected in demineralized bone matrix samples that were mixed with thrombin-activated platelet-rich plasma. These cells were not present in matrix mixed with platelet-rich plasma alone.

Conclusions: Platelet-rich plasma significantly increased in vivo demineralized bone matrix osteoinductivity only when used without thrombin activation.

Clinical Relevance: Platelet-rich plasma has the potential to increase the osteoinductivity of demineralized bone matrix in clinical applications. On the other hand, its activation by thrombin immediately prior to implantation significantly inhibits this activity.

Demineralized bone matrix (DBM) is used as an adjuvant in orthopaedic surgery because it contains a variety of osteoinductive proteins[1]. These osteoinductive bone morphogenetic proteins, specifically BMP-2, BMP-4, and BMP-7, initiate stem cell differentiation, which leads to new bone formation[2]. Platelet-rich plasma has been used as an autologous source of chemoattractant and mitogenic growth factors that are able to enhance the biologic activity of de-

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mineralized bone matrix[3]. Growth factors released from platelets include platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), platelet-derived epidermal growth factor (PDEGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived angiogenesis factor (PDAF), and insulin-like growth factor-I (IGF-I)[4-7]. The growth factors contained in platelet-rich plasma are believed to enhance important steps of the bone-healing cascade such as stem cell recruitment, angiogenesis, extracellular matrix production, and remodeling[8].

The use of platelet-rich plasma to enhance bone regeneration and soft-tissue maturation has increased in many surgical fields over the last decade. However, controversy exists regarding its added benefit. While some authors have reported a stimulatory effect in association with platelet-rich plasma[9-11], others did not observe any improvement[12,13] or even found inhibitory effects[14,15].

Platelet-rich plasma is typically implanted in a gel form enhanced with the addition of a clinical dose of thrombin (1000 U/mL in 10% $CaCl_2$)[16]. The solution that is used to rehydrate the thrombin contains calcium in order to neutralize the anticoagulant effects associated with the chelating reaction. Thrombin not only catalyzes the conversion of plasma fibrinogen into fibrin to create a platelet-rich plasma gel that has improved handling properties as compared with liquid platelet-rich plasma but also triggers platelet aggregation and degranulation[17]. Thus, thrombin causes the platelets to release their growth factor content when applied to the wound site. While the typical application of platelet-rich plasma includes activation with thrombin, clinical benefits of platelet-rich plasma have been documented without the use of thrombin activation[18].

The purpose of the present study was to determine whether platelet-rich plasma could augment the osteoinductivity of demineralized bone matrix and whether this effect was dependent on thrombin activation. We hypothesized that the addition of thrombin to platelet-rich plasma not only changes the physical property of platelet-rich plasma but may also alter the osteoinductive activity of demineralized bone matrix.

Materials and Methods

Platelet-Rich Plasma Preparation

Fifty-four milliliters of blood from a healthy, thirty-five-year-old male donor was collected into each of two 60-mL syringes that had been prefilled with 6 mL of anticoagulant citrate dextrose A (ACD-A). Platelet-rich plasma was prepared with the GPS II system (Biomet Biologics, Warsaw, Indiana). After centrifugation at 3200 revolutions per minute for fifteen minutes, three basic components (red blood cells, platelet-rich plasma, and platelet-poor plasma) were separated. This platelet-rich plasma preparation system produces 6 mL of platelet-rich plasma with an eightfold increase in platelet concentration over baseline and 30 mL of platelet-poor plasma[5,19]. Freshly prepared platelet-rich plasma was used immediately for in vivo implantation.

Aliquots of platelet-rich plasma, platelet-poor plasma, and whole blood were also saved for in vitro studies. In the vial with 2 mL of platelet-rich plasma, 200 μL of thrombin (Jones Pharma, Bristol, Tennessee; bovine origin, 1000 U/mL in 10% $CaCl_2$) was added, followed by vortexing. After incubation for twenty minutes at room temperature, the clotted platelet-rich plasma was centrifuged at 8000 revolutions per minute for ten minutes. This process was repeated with platelet-poor plasma and whole blood. The resulting supernatants were removed to new vials and were used for in vitro studies within two hours (for cell culture) or were stored at −80°C (for ELISA [enzyme-linked immunosorbent assay] tests).

TABLE I In Vitro Characterization of Demineralized Bone Matrix*

Demineralized Bone Matrix	C2C12 Alkaline Phosphatase Activity† *(μmol/mg protein/min)*	Bioactive Index†
Lot 1	0.285 ± 0.043	0.94
Lot 2	0.198 ± 0.023	0.52
Lot 3	0.132 ± 0.020	0.20

*N = 6. All three groups were significantly different with regard to both alkaline phosphatase activity and the bioactive index ($p < 0.05$). †The values are given as the mean and the standard deviation. ‡The bioactive index was calculated as $(ALP_{test} - ALP_{neg})/(ALP_{pos} - ALP_{neg})$. Demineralized bone matrix activity was classified according to this index as low (<0.20), moderate (0.20 to 0.79), or high (≥0.80). The values are given as the mean.

Platelet-Rich Plasma Growth Factor Profiles

The levels of TGF-β1 (Human TGF-β1 Quantikine ELISA Kit, DB100B; R&D Systems, Minneapolis, Minnesota), VEGF (Human VEGF Quantikine ELISA Kit, DVE00; R&D Systems), and PDGF-BB (Human PDGF-BB Quantikine ELISA Kit, DBB00; R&D Systems) in the platelet-rich plasma, platelet-poor plasma, and whole blood supernatants were measured with use of commercially available ELISA kits. The platelet-rich plasma and whole blood samples were activated with thrombin as described above. The TGF-β1 samples were additionally activated with 1N HCl in accordance with the manufacturer's instructions for the ELISA. The optical densities of the microplate wells were measured with a microplate reader (SpectraMax Plus[384]; Molecular Devices, Sunnyvale, California) at 405 nm. The data were analyzed with SoftMax Pro 4.6 (Molecular Devices). Sample concentrations from duplicate measurements were obtained by interpolating from the standard curve.

TGF-β was selected as a representative growth factor in platelet-rich plasma, and its activity was tested before and after thrombin activation. TGF-β exists in both a latent and an active form. The latent form has a C-terminal pro-region that must be cleaved in order for the protein to interact with a TGF-β receptor[20]. TGF-β activity in the platelet-rich plasma with and without thrombin activation was assayed with use of mink lung cells transfected with plasminogen activator inhibitor-1 (PAI-1) promoter-luciferase (Luc) reporter[21]. The cells were provided as a generous gift by Dr. D.B. Rifkin (New York University Medical Center)[22]. Briefly, PAI-1-Luc-transformed

TABLE II Growth Factor Profiles of Whole Blood, Platelet-Poor Plasma, and Platelet-Rich Plasma*

	PDGF-BB† (ng/mL)	VEGF† (pg/mL)	TGF-β1§ (ng/mL)
Whole blood	4.6 ± 0.2	44.3 ± 6.5	14 ± 0.4
Platelet-poor plasma	0.5 ± 0.02#	17.6 ± 1.1	3.8 ± 0.1#
Platelet-rich plasma	18 ± 0.9#	245 ± 16#	100 ± 1.6#

*The values are given as the mean and the standard deviation. †PDGF = platelet-derived growth factor. ‡VEGF = vascular endothelial growth factor. §TGF-β1 = transforming growth factor-β1. #Different from whole blood ($p < 0.05$).

mink lung cells were plated in a forty-eight-well culture plate at a density of 90,000 cells/well in 10% fetal bovine serum (FBS; HyClone, Logan, Utah)/DMEM (Dulbecco modified Eagle medium; Mediatech, Manassas, Virginia) containing 200 μg/mL geneticin (G418; Clontech Laboratories, Mountain View, California) and were allowed to attach for 5.0 hours at 37°C. After washing twice with DMEM, 10μL of 1:2 serial-diluted platelet-rich plasma was added in a 0.5% FBS/DMEM test medium. After seventeen hours, cells were rinsed twice with ice-cold PBS (phosphate-buffered saline solution) before testing for luciferase activity with use of a luciferase assay kit (Promega, Madison, Wisconsin). Only active TGF-β in platelet-rich plasma contributes to the PAI-1-linked luciferase activity.

Effect of Platelet-Rich Plasma on Osteoblast-Like Cell and Stem Cell Number

Saos-2 cells derived from a human osteosarcoma (HTB-85; ATCC, Manassas, Virginia) and rat bone marrow-derived stromal cells, prepared according to the method described by Hall et al.[23], were used for the cell number study. The cell assay was based on the ability of mitochondrial dehydrogenases to oxidize thiazolyl blue (MTT) (Promega). In each ninety-six-well plate, 15,000 cells/well were plated in 10% FBS/DMEM for a twenty-four-hour adhesion period. Right after the growth medium was exchanged to serum-free medium, 10 μL of PBS-serial-diluted platelet-rich plasma or platelet-poor plasma, prepared as described above with and without thrombin activation, was added to each well. Following incubation for forty-five hours, 20 μL of MTT solution (5 mg/mL) was added to the cells. After an additional three hours of incubation, the medium was aspirated and the cells were washed twice with phosphate-buffered saline solution. Cells were lysed with 120 μL of dimethyl sulfoxide (DMSO) for five minutes. The plates were read on a microplate reader (Molecular Devices) with use of test and reference wavelengths of 570 and 620 nm, respectively. The absorbance value is proportional to the number of cells in the dish.

Demineralized Bone Matrix Implant Preparation and Implantation

Three lots of human demineralized bone matrix were acquired from an American Association of Tissue Banks-accredited tissue bank. The biologic activity of each lot was determined in vitro by means of C2C12 rat myoblast cell line (CRL-1772; ATCC) pro-

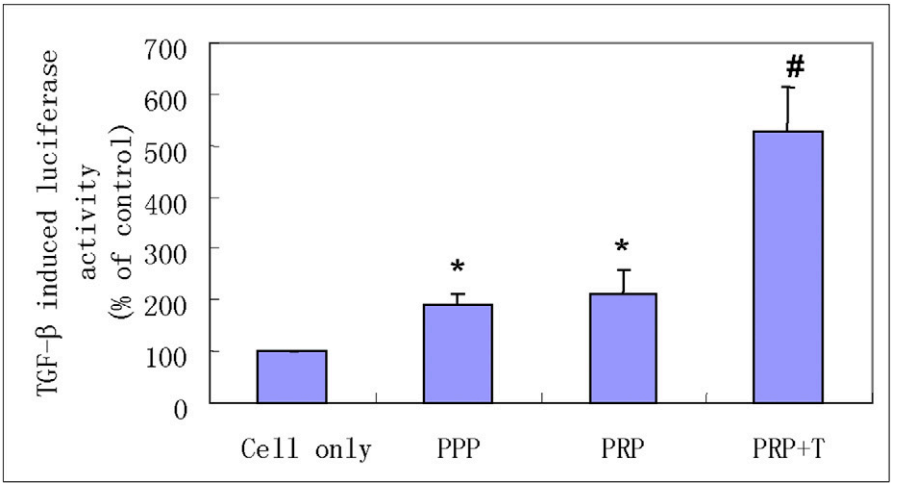

Fig. 1

Bar graph illustrating TGF-β activity in platelet-rich plasma before and after activation by thrombin. Freshly prepared platelet-rich plasma activated by thrombin (PRP + T) together with platelet-poor plasma (PPP) and platelet-rich plasma without activation (PRP) were tested with use of a mink lung epithelial cell culture method. Luciferase activity was assayed after seventeen hours of incubation. The results are expressed as the mean and the standard deviation ($n = 6$). *$p < 0.05$ as compared with the thrombin-treated platelet-rich plasma group. #$p < 0.05$ as compared with the control group.

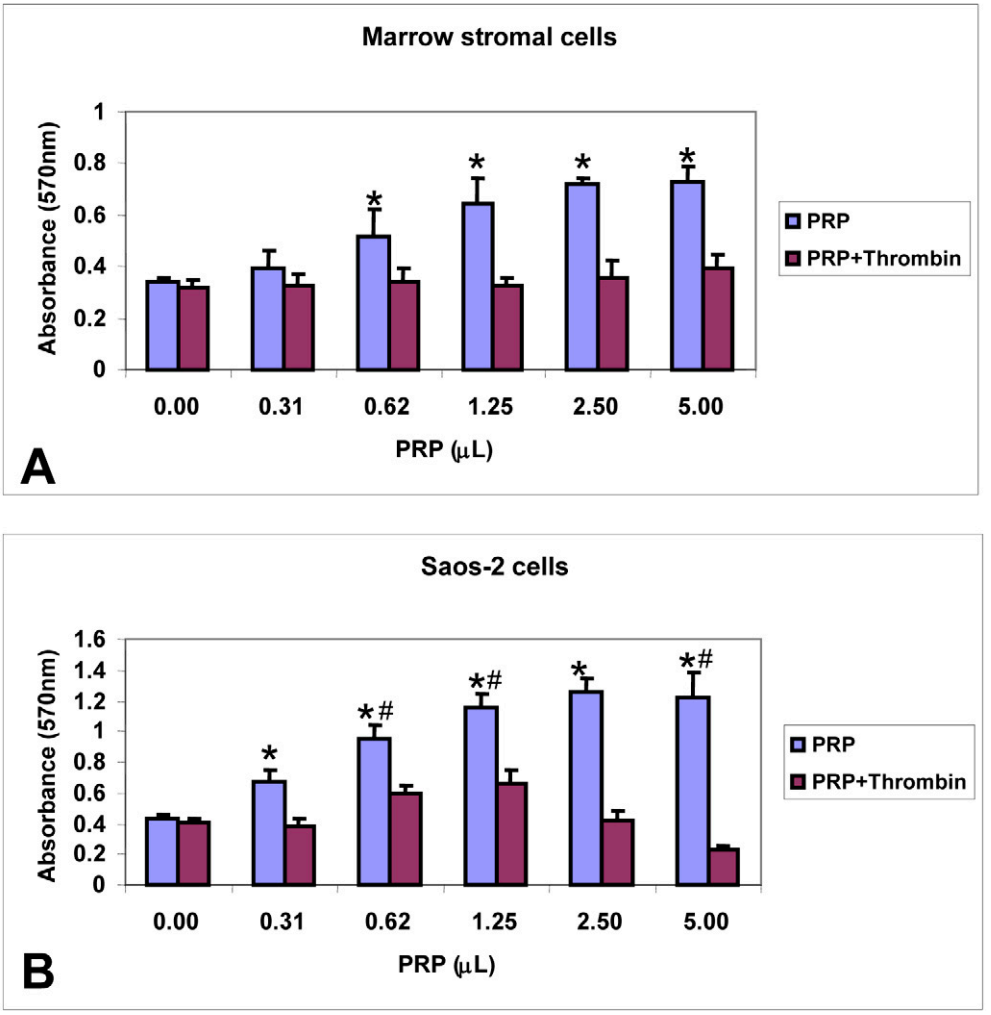

Fig. 2

Bar graphs illustrating the effect of platelet-rich plasma on the number of bone marrow stromal cells (A) and Saos-2 cells (B). Platelet-rich plasma (PRP) or thrombin-activated platelet-rich plasma (PRP + Thrombin) was added into cell cultures for forty-eight hours. Viable cells were assayed with the MTT assay (shown as the mean and the standard deviation based on five specimens). *$p < 0.05$ (platelet-rich plasma as compared with control [0 μL]). #$p < 0.05$ (platelet-rich plasma as compared with thrombin-activated platelet-rich plasma).

duction of alkaline phosphatase (ALP) and in vivo following intramuscular implantation in an athymic rat[24]. The activity of the demineralized bone matrix was characterized with a bioactive index (BI) calculated from the in vitro assay with use of a known active lot of demineralized bone matrix for the positive control and no demineralized bone matrix for the negative control ($[ALP_{test} - ALP_{negative}]/[ALP_{positive} - ALP_{negative}]$). On the basis of the BI value, demineralized bone matrix activity was classified as low (<0.20), moderate (0.20 to 0.79), or high (≥0.80) (Table I). This BI value is based on an in vitro experiment and is only a predictor of in vivo activity.

Twenty-seven male athymic rats weighing 150 to 175 g (Harlan Laboratories, Indianapolis, Indiana) were used in the study, which was approved by the University of Southern California Institutional Animal Care and Use Committee. Six pouches were created in the abdominal muscles, three on each side, by means of blunt dissection. Three implantation types were studied: (1) demineralized bone matrix with unactivated platelet-rich plasma, (2) demineralized bone matrix with thrombin-activated platelet-rich plasma, and (3) demineralized bone matrix alone. In each case, 50 mg of demineralized bone matrix was implanted in each site. Types 1 and 2 included 200 μL of platelet-rich plasma. Type 2 also received 20 μL of thrombin (1000 U/mL) reconstituted in 10% $CaCl_2$. Each animal received two implants of each type. The implantation sites with regard to placement in the rat were randomized. Explants were retrieved after fourteen, twenty-eight, and fifty-six days. One-half of each explant underwent histological staining, and the second half was assayed for alkaline phosphatase activity with use of previously described methods[24]. The histological scoring system and in vivo alkaline phosphatase determination have been found to correlate with the

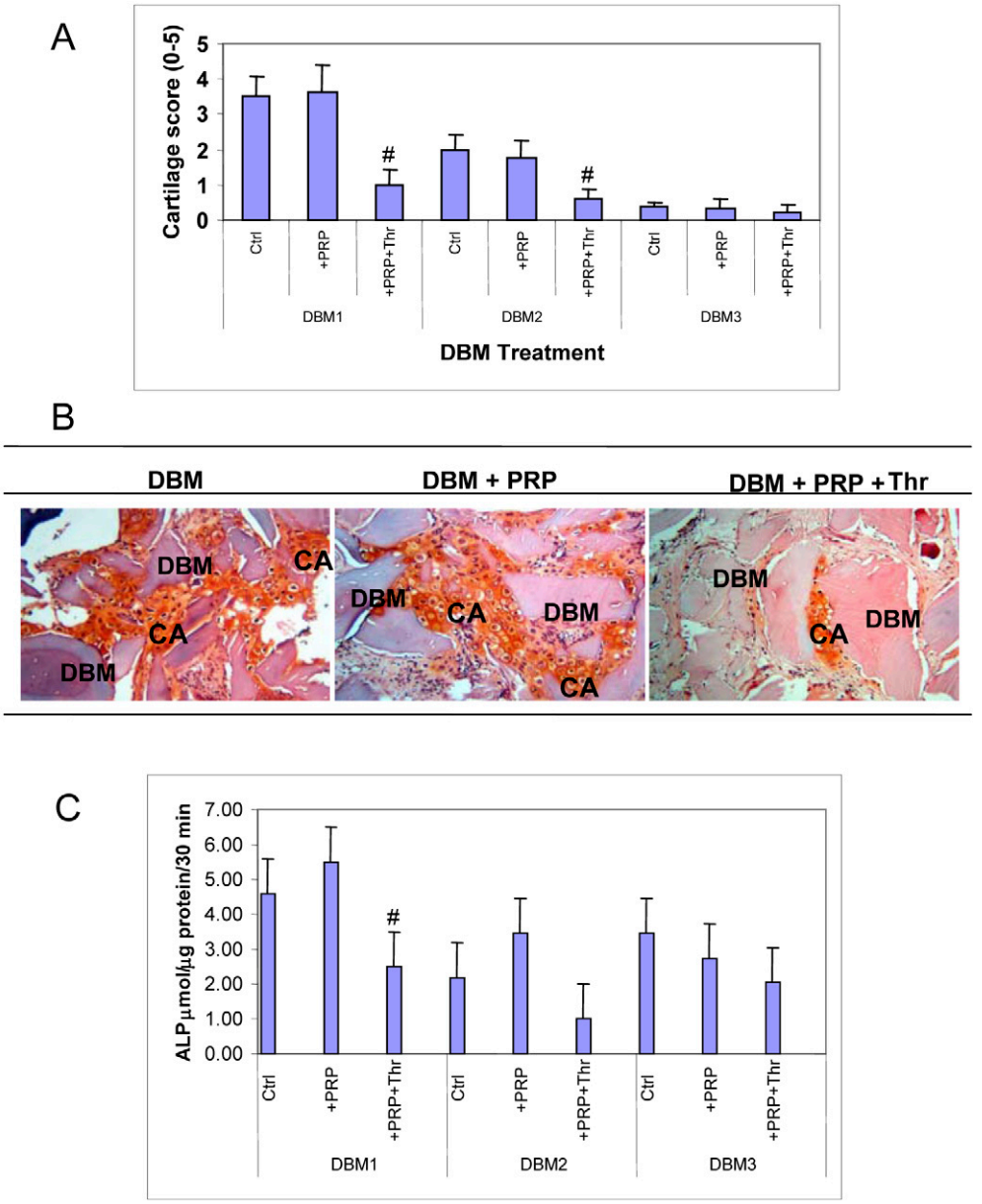

Fig. 3

Effect of platelet-rich plasma on demineralized bone matrix chondrogenesis at fourteen days after intramuscular implantation. PRP = platelet-rich plasma, and Thr = Thrombin. DBM1, DBM2, and DBM3 refer to the three lots of demineralized bone matrix. #$p < 0.05$ as compared with control (Ctrl). *A:* Bar graph illustrating the cartilage scores. *B:* Photomicrograph showing the induction of new cartilage in response to demineralized bone matrix (lot 1) (safranin O, ×100). CA = cartilage. *C:* Bar graph illustrating the alkaline phosphatase (ALP) activity from the explants (shown as the mean and the standard deviation based on nine specimens).

osteoinductive activity of demineralized bone matrix following twenty-eight days of implantation[24].

Alkaline Phosphatase Evaluation

One-half of each explant was homogenized in extraction buffer (1% Triton X-100). Alkaline phosphatase activity was assayed with 50 µL of this solution with use of 150 µL of 0.3-mM pNPP (Sigma-Aldrich, St. Louis, Missouri) as a substrate[25]. Absorbance was detected at 410 nm after thirty minutes of incubation. The alkaline phosphatase activity was normalized to the total protein content of the extract (BCA Protein Assay; Pierce, Rockford, Illinois)[24].

Histological Evaluation

Explants were fixed in 10% neutral buffered formalin, decalcified in 5% formic acid, embedded in paraffin, sectioned, and

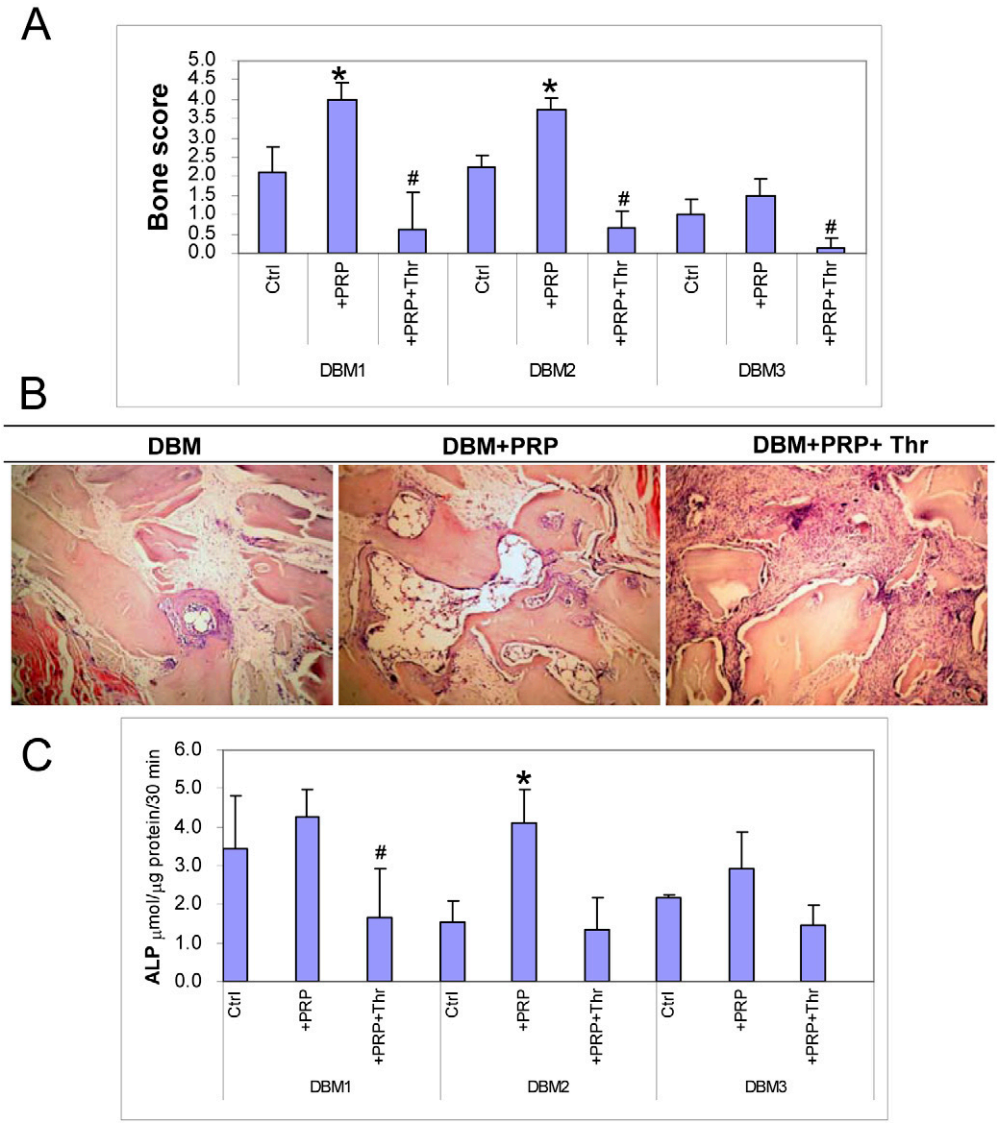

Fig. 4

Effect of platelet-rich plasma on demineralized bone matrix osteogenesis at twenty-eight days after intramuscular implantation. PRP = platelet-rich plasma, and Thr = thrombin. DBM1, DBM2, and DBM3 refer to the three lots of demineralized bone matrix. * or #: $p < 0.05$ as compared with control (Ctrl). *A:* Bar graph illustrating the bone formation scores. *B:* Photomicrograph showing the induction of new bone in response to demineralized bone matrix (lot 1) (hematoxylin and eosin, ×100). *C:* Bar graph illustrating the alkaline phosphatase (ALP) activity from the explants (shown as the mean and the standard deviation based on nine specimens).

stained with either hematoxylin or eosin or safranin-O. Three consecutive cross-sectional cuts were made at each of three different levels of the explant to visualize any induced cartilage and/or bone formation. Each section was evaluated for evidence of chondrogenesis, osteoinduction, and/or inflammation with use of a modified qualitative scoring system as described previously[24]. Briefly, de novo bone formation was scored on a 5-point scale as 1 (fibrous tissue only), 2 (cartilage only), 3 (new bone and bone marrow involving 10% of the area), 4 (new bone and bone marrow involving 20% of the area), or 5 (new bone and bone marrow involving ≥40% of the area). All slides were viewed at 100× magnification, and each section was evaluated with the qualitative scoring system by two blinded, independent examiners (B.H. and Z.Y.).

Statistical Analysis

All of the results were expressed as the mean and the standard deviation. Significant differences were determined with use of a one-way analysis of variance followed by a Student-Newman-Keuls test ($\alpha = 0.05$). Additionally, the 95% confidence interval for the difference in the means was calculated for the histological observations.

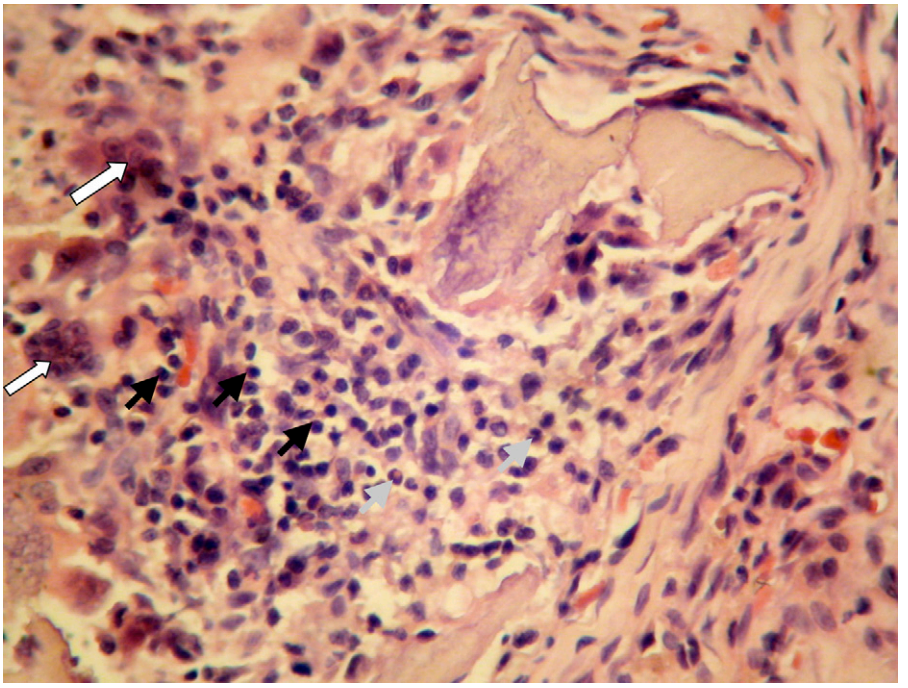

Fig. 5

Photomicrograph, made at twenty-eight days after intramuscular implantation, showing inflammation surrounding demineralized bone matrix particles implanted with platelet-rich plasma activated with thrombin (hematoxylin and eosin, ×200). Black arrows = lymphocytes, gray arrows = neutrophils, and white arrows = foreign-body giant cells.

Source of Funding

Financial support for this study was provided by Biomet (Warsaw, Indiana) and the Wright Foundation (University of Southern California, Los Angeles, California). The funding was used to support the salary of a research associate who performed cell culture and biochemical analysis. In addition, the funding was utilized for the purchase of materials and supplies.

Results

Growth Factors in Platelet-Rich Plasma

Aliquots of thrombin-activated platelet-rich plasma, unactivated platelet-poor plasma, and thrombin-activated whole blood were tested for growth factor concentrations with ELISA kits. There was a fourfold to sevenfold increase in growth factor concentration in the platelet-rich plasma samples as compared with the baseline whole blood (Table II). These values are consistent with the growth factor content seen in clinically derived platelet-rich plasma with use of the same platelet-rich plasma preparation system[5].

Thrombin Activation of Platelet-Rich Plasma

The extent of TGF-β activation in platelet-rich plasma was measured with a specific and sensitive cell-culture method for luciferase activity with use of mink lung epithelial cells transfected with PAI-1 promoter-luciferase reporter. Only active TGF-β can contribute to luciferase activity in platelet-rich plasma preparations. Before thrombin activation, TGF-β in platelet-rich plasma was in a latent form. The thrombin-activated platelet-rich plasma preparations significantly increased luciferase activity. However, when freshly prepared platelet-rich plasma was not activated with thrombin, the TGF-β activity was as low as that in platelet-poor plasma in the cell culture assay (Fig. 1). This is in contrast to the ELISA preparations, in which TGF-β in both the thrombin-activated and unactivated platelet-rich plasma samples was converted to the active form by means of 1N hydrochloric acid treatment; thus, differences in activity were not detected. With an increase in storage time, the platelet-rich plasma began to exhibit spontaneous activation and consequently increased activity (data not shown). These results suggest that exogenous addition of thrombin activates TGF-β from the latent to the active form immediately, as tested in the present study and in others[14].

Effect of Platelet-Rich Plasma on Cell Number

When platelet-rich plasma was tested for its biologic effect in an in vitro cell culture system, it demonstrated a dose-dependent increase in the number of marrow stromal cells at forty-eight hours. However, when thrombin-activated platelet-rich plasma was evaluated, no increase in cell number was seen (Fig. 2, *A*).

When the biologic potential of platelet-rich plasma was assessed on osteosarcoma cells (Saos-2), a dose-dependent increase in cell number was seen at forty-eight hours. When the supernatant of thrombin-activated platelet-rich plasma

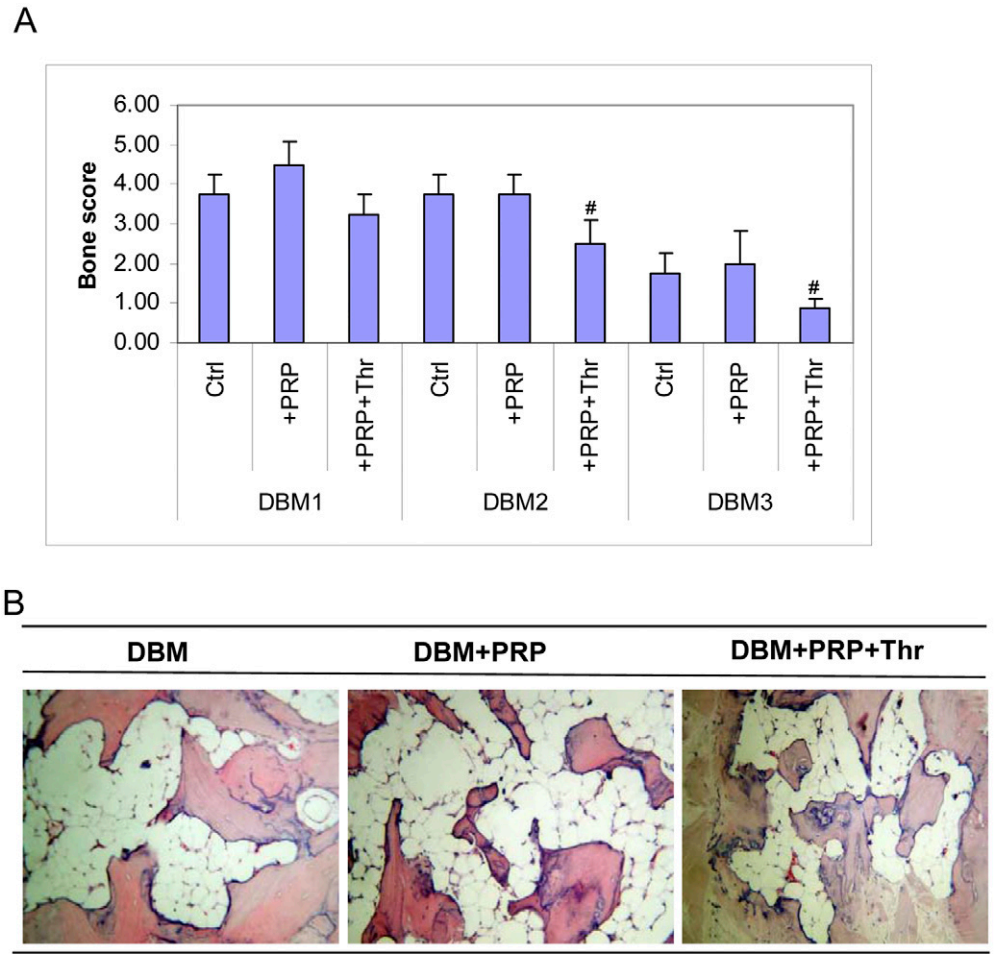

Fig. 6

Effect of platelet-rich plasma on demineralized bone matrix osteogenesis at fifty-six days after intramuscular implantation. PRP = platelet-rich plasma, and Thr = Thrombin. DBM1, DBM2, and DBM3 refer to the three lots of demineralized bone matrix. *A:* Bar graph showing the bone formation scores (shown as the mean and the standard deviation based on nine specimens). #$p < 0.05$ as compared with control (Ctrl). *B:* Photomicrographs showing the induction of new bone in response to demineralized bone matrix (lot 1) (hematoxylin and eosin, ×100).

was added at low doses (0 to 125μL), a positive effect was observed, although the effect was significantly less than that noted in association with platelet-rich plasma without thrombin activation. Furthermore, high doses of thrombin-activated platelet-rich plasma decreased Saos-2 cell number (Fig. 2, *B*). These results suggest that the growth factors released from the platelet-rich plasma have different dose-dependent effects on different cell types; thrombin present in the activated samples also may have deleterious effects on the viability and/or growth of the cells.

In Vivo Chondrogenic and Osteogenic Effects of Platelet-Rich Plasma

Chondrogenesis and Osteogenesis on Day 14

Ectopic bone formation induced by demineralized bone matrix with the addition of platelet-rich plasma or thrombin-activated platelet-rich plasma was tested in an athymic rat intramuscular model. After fourteen days, no new bone formation could be observed and only cartilage was seen. The addition of platelet-rich plasma to demineralized bone matrix did not show a significant effect on cartilage scores at fourteen days in comparison with demineralized bone matrix alone (Fig. 3, *A*). However, there was a significant decrease in cartilage formation in the thrombin-activated platelet-rich plasma-demineralized bone matrix group when compared with either the demineralized bone matrix-alone group or the demineralized bone matrix with platelet-rich plasma group ($p < 0.05$). Cartilage exhibiting positive safranin-O staining of proteoglycans was found within and around the demineralized bone matrix particles (Fig. 3, *B*). In both the demineralized bone matrix group and the platelet-rich plasma-demineralized bone matrix group, the quantity of cartilage and the morphology of

chondrocytes were similar, although the cartilage looked more hypertrophic in the platelet-rich plasma group. In contrast, new cartilage was found only in very small and local areas when thrombin-activated platelet-rich plasma was added. Moreover, more inflammatory cells, including macrophages and monocytes, were found between demineralized bone matrix particles in the thrombin-activated platelet-rich plasma group (data not shown). This trend held for every lot of demineralized bone matrix combined with thrombin-activated platelet-rich plasma.

Platelet-rich plasma increased alkaline phosphatase activity in comparison with corresponding demineralized bone matrix controls (lot 1 and lot 2), whereas thrombin-activated platelet-rich plasma actually decreased alkaline phosphatase activity. The decrease, however, was only significant for demineralized bone matrix lot 1 (Fig. 3, *C*). These results, combined with the cartilage scores, suggest that the activation of platelet-rich plasma in the presence of demineralized bone matrix either inhibits chondrogenesis or stimulates the removal of calcified cartilage at Day 14. The increased inflammation seen in association with the thrombin-activated platelet-rich plasma may have contributed to the decrease in cartilage seen at Day 14.

Bone Formation Potential of Demineralized Bone Matrix on Days 28 and 56

In the athymic rat, most cartilage is replaced by osteoblasts, osteocytes, and bone marrow by twenty-eight days following the intramuscular implantation of demineralized bone matrix[26]. In the present study, platelet-rich plasma enhanced demineralized bone matrix bone formation as determined histologically and on the basis of alkaline phosphatase activity ($p < 0.05$) (Fig. 4). As an example, in the experiments involving demineralized bone matrix lot 1, the 95% confidence interval for the difference in means was $0.296 \leq 1.875 \leq 3.454$ for the comparison between demineralized bone matrix alone and demineralized bone matrix with platelet-rich plasma and $-0.317 \leq 1.5 \leq 3.317$ for the comparison between demineralized bone matrix alone and demineralized bone matrix with platelet-rich plasma and thrombin. Histologically, demineralized bone matrix alone did exhibit osteoinductivity with islands of bone marrow and new bone forming between the residual demineralized bone matrix particles. In the platelet-rich plasma group, much more bone marrow and new bone was evident. However, thrombin activation of the platelet-rich plasma dramatically decreased bone formation as determined histologically and on the basis of alkaline phosphatase activity ($p < 0.05$). No bone marrow or new bone was seen, and the residual demineralized bone matrix particles were surrounded by fibrous tissue. Subchronic inflammation, which was rated as medium to severe, was again persistent in the thrombin-activated groups (Fig. 5).

Results at the fifty-six-day harvest period were similar to those at the twenty-eight-day time point. By fifty-six days, the results for the demineralized bone matrix-alone group appeared to be similar to those for the unactivated platelet-rich plasma-demineralized bone matrix group. The unactivated platelet-rich plasma-demineralized bone matrix group showed the highest new bone formation score, whereas the thrombin-activated platelet-rich plasma-demineralized bone matrix group continued to have the lowest score (Fig. 6). As an example, in the experiments involving demineralized bone matrix lot 1, the 95% confidence interval for the difference in the means was $-0.787 \leq 0.75 \leq 2.287$ for the comparison between demineralized bone matrix alone and demineralized bone matrix and platelet-rich plasma and $-0.998 \leq 0.5 \leq 1.998$ for the comparison between demineralized bone matrix alone and demineralized bone matrix with platelet-rich plasma and thrombin.

Discussion

Platelet-rich plasma has been proposed as an autologous source of growth factors that can be used clinically as an adjuvant to enhance the quantity, quality, and rate of bone regeneration induced by demineralized bone matrix[6,7]. However, controversies persist regarding its benefits for bone regeneration. Specifically, Ranly et al. demonstrated that platelet-rich plasma decreased the osteoinductivity of demineralized bone matrix in an athymic mouse model[14]. In that study, the platelet-rich plasma was activated with bovine thrombin (1000 U/mL) and then was mixed with demineralized bone matrix for implantation into the gastrocnemius muscles of nude mice. Osteoinductivity was evaluated after fifty-six days of implantation.

However, there are some design differences between the present study and the study by Ranly et al. Two different athymic species were used, with one study involving the use of the thigh muscle in mice and the other study involving the use of the abdominal muscle in rats. The two species could have different metabolic rates, and different implantation sites can have different levels of demineralized bone matrix activity[27,28]. These factors could affect bone-forming ability and the time course of bone formation. Finally, two separate platelet-rich plasma preparation devices were used, which provided different platelet concentrations. Despite differences in design, both studies demonstrated that human platelet-rich plasma activated with 1000 U/mL of bovine thrombin reduces the osteoinductivity of demineralized bone matrix[14]. However, unlike the previously published study, the current study demonstrates that platelet-rich plasma does increase the osteoinductivity of demineralized bone matrix, but only when it has not been activated by thrombin.

Other studies have indicated that thrombin activation of platelet-rich plasma diminishes bone formation. In a goat study, platelet-rich plasma significantly increased the bone contact around a calcium phosphate-coated implant as compared with controls whereas platelet-rich plasma activated with thrombin (300 U/mL in 10% $CaCl_2$) did not[29]. In a mini-pig model, thrombin-activated platelet-rich plasma (0.5 mL Tissucol thrombin-fibrin solution) failed to show any significant effects on peri-implant bone formation after a six-week healing period[30]. Even though clinically improved bone fusion

has been achieved with the augmentation of graft material with platelet-rich plasma[31,32], adverse effects on bone graft consolidation also have been reported[15]. Unfortunately, the use of thrombin was not discussed in those clinical studies, so it is impossible to discern the effects of thrombin activation.

Platelet-rich plasma can be considered as a biologic adjuvant for demineralized bone matrix because of its mitogenic, chemoattractant, and angiogenic growth factor profile. The platelet-rich plasma used in the present study had a growth factor profile that was similar to those that have been previously reported[5,33]. The platelet-rich plasma demonstrated a dose-dependent increase in the number of both marrow stromal stem cells and osteoblast-like cells in vitro. Both in vitro and in vivo, thrombin-activated platelet-rich plasma either had no stimulatory effect or had an inhibitory effect. The thrombin-activated platelet-rich plasma samples demonstrated an immediate release of growth factors and consequently supplied higher doses of growth factors as compared with the platelet-rich plasma without thrombin activation. The unactivated platelet-rich plasma also likely releases growth factors, but at a much slower rate, providing an overall sustained lower dose to the cells.

In contrast, other investigators have shown that the use of autologous human thrombin to activate platelet-rich plasma enhanced the proliferation of mesenchymal stem cells[34]. Autologous preparations typically provide thrombin doses much lower than bovine thrombin (typically around 50 U/mL[35]). The potential effects of such differences need to be determined.

The dose of thrombin appears to play a role, at least in vitro. Low doses of thrombin-activated platelet-rich plasma have been shown to increase both the migration and the number of bone marrow-derived mesenchymal progenitor cells and endothelial cells[6,7,33,36], whereas high concentrations have demonstrated limited effects on the proliferation of osteoblast and alveolar bone cells[37]. A similar dose-dependent biphasic response of TGF-β on osteoblast proliferation and differentiation has been shown previously[38]. It is not surprising that the biologic effect of growth factors and enzymes is dose-dependent. Therefore, the dose of thrombin used in published studies must also be considered when interpreting results with platelet-rich plasma.

The source of the thrombin also must be considered in clinical applications. Bovine thrombin has been documented to react with endogenous Factor V, resulting in bleeding complications[39,40]. There also have been clinical reports of bovine thrombin generating anaphylactic shock[41-43]. In addition, bovine thrombin can increase the risk of transmission of bovine spongiform encephalopathy. For these reasons, only autologous thrombin has been allowed to be used clinically in Europe. Furthermore, in the present study, the in vivo thrombin-activated samples caused increased inflammation in comparison with the unactivated platelet-rich plasma and demineralized bone matrix samples. While athymic rats cannot demonstrate an immune reaction per se, this result is still interesting. It is unclear at this time why this inflammation occurred, but this result cannot be discounted as a factor in decreasing the osteoinductivity of the demineralized bone matrix.

Thrombin also has biologic activities beyond serving as a serine protease to catalyze the conversion of fibrinogen to fibrin and binding to platelets to initiate aggregation and activation[44]. It can elicit a range of responses from a wide variety of cells[45,46]. Specifically, thrombin (0.1 to 10 U/mL) has been shown to exhibit regulatory effects on osteoblast cell function, including cell proliferation and the secretion of endogenous cytokines[47].

In the present study, a demineralized bone matrix heterotopic bone formation model was used to assess the supplementary effects of platelet-rich plasma on demineralized bone matrix osteoinductivity. Demineralized bone matrix, an osteoinductive material, has a spectrum of intrinsic growth factors that induce endochondral bone formation[26]. To test the potential of platelet-rich plasma as an adjuvant in different bone formation circumstances, three human demineralized bone matrix lots were chosen with a high, medium, and low activity index[24]. The demineralized bone matrix was implanted in the abdominal muscle instead of the thigh muscle of the rat. This implant location, being more remote from the skeleton, may help to eliminate any growth factor or cell influence from neighboring bone. With use of this more challenging model design, platelet-rich plasma enhanced both chondrogenesis and osteogenesis. These effects were greater when the demineralized bone matrix had higher osteoinductivity as compared with lots of demineralized bone matrix with low osteoinductivity.

As others have noted[14], we found that thrombin activation allows for an immediate release of growth factors from the platelets[44] and converts TGF-β to an active form so that it can interact with the cell surface receptors. These results indicate that the platelets must become activated in some way before TGF-β can be available for biologic use. Without thrombin, other activators of platelets, such as the collagen present in demineralized bone matrix[48], could stimulate this phenomenon over an extended period of time. This protracted activation rate could result in sustained release of the growth factors and may reduce the inhibitory effects associated with higher doses (biphasic activity)[38,49,50].

The use of platelet-rich plasma as an adjuvant to bone-grafting, especially in combination with demineralized bone matrix, is controversial. The clinical relevance of the present study is its demonstration that, if properly administered, platelet-rich plasma can enhance the osteoinductivity of demineralized bone matrix preparations. On the other hand, because bovine-derived thrombin-activated platelet-rich plasma (1000 U/mL) inhibited this effect, bovine-derived thrombin-activated platelet-rich plasma is not recommended to be used in combination with demineralized bone matrix. However, it can be concluded from these studies that platelet-rich plasma continues to be an attractive adjuvant to demineralized bone matrix but that prior activation should be discouraged. ■

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