Experimental Study of the Effect of Autologous Platelet-Rich Plasma on the Early Phases of Osteoinduction by Allogenic Demineralized Bone Matrix

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Purpose: *To evaluate the effect of autologous platelet-rich plasma (PRP) on the early phases of osteoinduction by allogenic demineralized bone matrix (DBM) in rabbit intramuscular positions.*

Materials and Methods: *Allogenic DBM was produced from bones of 3 healthy rabbits. In each of 6 experimental animals, 0.3 mL autologous PRP was prepared and 2 muscle pouches were created, where 250 mg DBM + PRP (experimental sites) and 250 mg DBM without PRP (control sites) were randomly implanted. Animals were euthanized 3 weeks postoperatively.*

Results: *Histologic examination revealed uneventful healing in all cases, whereas remineralization of the periphery of the bone graft particles was a constant finding. In both control and experimental sites, fibroblasts and other mesenchymal cells (probably osteoprogenitor cells and preosteoblasts) were observed. The main histological difference was the recolonization of the empty lacunae of the bone graft particles by osteocytes at the control sites. The degradation of the graft at the control sites was statistically significantly quicker, although a statistically significant difference regarding the amount of the newly formed fibrous connective tissue was not observed.*

Conclusion: *The present study demonstrated that in this experimental model, the addition of PRP to DBM had a negative effect on the early phases of osteoinduction at 3 weeks of observation. (Implant Dent 2012;21:399–405)*

Key Words: PRP, DBM, osteoinduction, bone grafts, surgery

In contemporary oral surgery, autogenous bone is still considered as the gold standard for bone grafting materials. Autogenous bone possesses osteogenetic, osteoconductive, and osteoinductive properties, although it is nonallergenic and fully resorbable, eventually replaced by the patient's own bone.[1]

However, to avoid the additional surgery, which increases surgical time, cost, and also morbidity (donor site pain and possible infection), as well as the patient's inconvenience, in many cases the use of bone grafting substitutes derived from human, bovine, and synthetic sources constitutes an alternative solution. Bone allografts of human origin, obtained from cadaveric or living donors (femoral head), are also widely used. This category includes fresh-frozen bone, freeze-dried bone (FDB) and demineralized FDB (DFDB). In contemporary clinical practice, the use of allogenic DFDB, also known as demineralized bone matrix (DBM), is widespread.[2]

Demineralization by hydrochloric acid (HCl) removes the mineral phase of the bone graft material, leaving a matrix of demineralized bone consisting of collagen and noncollagen proteins. Among the noncollagen proteins, bone morphogenetic proteins (BMPs) are potent osteoinductive growth factors. Thus, the demineralization of the DBM allografts maximizes the

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bioavailability of BMPs, rendering the graft osteoinductive.[3,4]

Platelet-rich plasma (PRP) is formed by an autologous concentration of platelets in a small volume of plasma.[5,6] Although a normal blood clot formed in a wound or bone defect contains 94% red blood cells, 6% platelets, and <1% white blood cells, PRP includes 94% platelets, 5% red blood cells, and 1% white blood cells. The high concentration of platelets in PRP results, in turn, in a high concentration of growth factors released by platelets, although the ratio of cells that do not induce healing (ie, red blood cells) is minimized in the PRP clot. Seven growth factors have been identified in the α-granules of platelets: 3 isomers of platelet-derived growth factor (PDGF-aa, PDGF-bb, and PDGF-ab), 2 isomers of transforming growth factor-β (TGF-β_1 and TGF-β_2), vascular endothelial growth factor, and epidermal growth factor. Moreover, PRP contains vitronectin (in the α-granules of platelets), fibrin (in plasma), and fibronectin (in plasma), which are adhesive proteins.[7]

By applying PRP alone or in combination with a bone graft, the concentration of the growth factors and cell adhesion molecules will increase locally in the area of the bone defect, which seems to lead to faster and more effective bone regeneration,[8] although the handling of the particulate bone grafts is significantly improved.[9]

In 1998, Marx et al[10] published a pioneering clinical study that essentially introduced the use of PRP into everyday clinical practice. Since then, numerous experimental and clinical studies have examined the effects resulting from the use of PRP alone[9–13] or in combination with autografts,[11,14–17] lyophilized bone allografts,[18–21] synthetic grafts,[22,23] and inorganic bovine bone[24–26] with various positive and negative results.

It seems that there are still many questions to be answered about the physiology and activity of PRP, as well as its effect on the osteoinductive potential of DBM allografts. To draw reliable conclusions on whether the addition of PRP can affect the osteoinductive processes, triggered by the morphogenetic proteins of a DBM allograft, it is important to make experimental observations in extraskeletal sites, which has not yet been studied in detail so far.

The present experimental research is aimed at presenting a comparative evaluation of the osteoinductive activity in extraskeletal (intramuscular) sites in rabbits between (1) a DBM allograft and (2) a composite bone graft including the same allograft mixed with autologous PRP, at 3 weeks after implantation.

MATERIALS AND METHODS

Animals

Nine adult male New Zealand white rabbits, each weighing 3 kg (±250g), were used in this study with the approval of the Institutional Animal Care and Use Committee of the Veterinary Department of Athens Prefecture. The animals were fed a balanced rabbit diet and caged individually in a standard manner at the animal research facility "N. S. Christeas," Medical School, University of Athens. All animals were allowed 7 days from their arrival to the facility to be acclimatized to their new environment.

Experiment Design

Three New Zealand white rabbits were randomly selected and euthanized to prepare allogenic DBM. The remaining 6 New Zealand white rabbits served as the experimental animals. In each of the 6 experimental animals, 0.3 mL autologous PRP was prepared, and subsequently, 2 muscle pouches were surgically created, where 250 mg DBM + PRP (experimental sites) and 250 mg DBM without PRP (control sites) were randomly implanted. Experimental animals were euthanized 3 weeks postoperatively.

Graft Preparation

At the beginning of the study, 3 healthy rabbits were euthanized to prepare allogenic DBM. First, the diaphyses of the long bones were obtained under aseptic conditions. Then, based on the protocol of Reddi and Huggins,[27] these bones were dissected free of soft tissues and subsequently placed in a H_2O_2 solution (30%) and processed at high temperature for 15 minutes to remove the remaining blood, fat, and hydrosoluble proteins. Demineralization involved the processing of the bone particles in a 0.5N HCl solution in 3 cycles, lasting 1 hour each. Demineralized bones were then rinsed with distilled water and processed in absolute ethanol and anhydrous diethyl ether for 30 minutes. Dehydration was achieved by lyophilization for 24 hours (Terruzzi lyophilizing machine; Terruzzi, Spirano, Italy), until moisture in the final content of the bone particles would not exceed 3%. Demineralized lyophilized bones were segmented and pulverized, and the final DBM allograft was produced in powder form, using grain sieves. Particles of a size of 250 to 710 μm in diameter were collected. Subsequently, the DBM grafts from all 3 donor rabbits were mixed up to produce a graft, which would be homogenous in terms of donor origin. The bone graft and gelatin capsules (used to transfer and implant the graft) were sterilized with ultraviolet light for 24 hours. Based on the experimental protocol, each capsule was filled with 250 mg of DBM allograft.

PRP Preparation

In each of the 6 experimental animals, autologous PRP was prepared before the surgery. Eight milliliters of blood was drawn from the central auricular artery in a monovette containing 1.1 mL of citrate phosphate dextrose anticoagulant. PRP was prepared with the use of a particular commercial kit (Curasan PRP kit; Curasan AG, Kleinostheim, Germany) according to the manufacturer's protocol.

Each blood sample was then centrifuged at 2400 rotations/minute for 10 minutes, at room temperature, to separate platelet-containing plasma from red and white blood cells. This plasma was drawn into a new monovette and centrifuged again at 3600 rotations/minute for 15 minutes at room temperature. Through this second centrifugation, platelet-poor plasma (PPP) was separated from PRP. After the removal of PPP, the volume of PRP that remained in the monovette was approximately 0.3 mL. Subsequently, PRP was stirred for 20 seconds using a vibrating device and drawn into a syringe to be ready for use.

Surgical Protocol

Experimental animals received general anesthesia by orotracheal intubation. A 3-cm-long skin incision was made over the gluteus maximus muscle bilaterally, running parallel to the striated

muscle fibbers. After the muscle fascia had been exposed and prepared, blunt dissection was performed to the muscle to create an intramuscular pouch where the sterilized gelatin capsule containing the graft material was placed. Based on the experimental protocol, a capsule containing 250 mg of DBM was inserted into the pouch on the one side of each test animal (control sites), while another capsule containing 250 mg of DBM mixed with 0.3 mL of autologous PRP was implanted on the other side (experimental sites). According to the PRP manufacturer's instructions, to activate the PRP with thrombin, several drops of fresh blood from the laboratory animal were added to the capsule material (DBM + PRP) right before its intramuscular implantation. The muscle pouch was then sutured using nonabsorbable suture (silk 4-0; Ethicon, Johnson & Johnson Hellas, Athens, Greece), so that the implantation site would be easily identified after killing the animal. The surgical wound was then sutured by layers.

Each experimental animal received antibiotics (30 mg/kg of Zinadol every 24 hours [GlaxoWellcome, Athens, Greece]) and analgesics (15 mg/kg of Depon; Bristol-Myers Squibb, Athens, Greece) for 2 days postoperatively. The postoperative course of all animals was uneventful. All experimental animals were euthanized at 3 weeks postoperatively with an intravenous injection of sodium thiopental (100 mg/kg of Pentothal; Abbott Hellas, Athens, Greece).

Histological and Histomorphometric Evaluation

At killing, a part of the muscle containing the implant was removed from each gluteus muscle under aseptic conditions. Right after their removal, specimens were fixed in 10% neutral buffered formalin. After that, they were placed in alcohol and methyl methacrylate and plasticized by hot polymerisation. Finally, nondecalcified sections were obtained and stained with Goldner trichrome stain. Sections were first examined histologically using an optical microscope, under blind conditions. The slides were then placed in a semiautomated histomorphometric measurement system. Histological images were digitized and histomorphometric parameters regarding the percentage of newformed interstitial connective tissue volume, the percentage of remaining DBM volume, and the remaining DBM area were measured and quantified on a computer by means of specialized software (Osteomeasure, interactive measurement system for bone histomorphometry; Osteometrics; Atlanta, GA).

Statistical Analysis

The comparison of variables between the 2 groups (DBM vs DBM + PRP) was made using the independent samples *t* test. In case of nonnormal data distribution, the Mann-Whitney test was used. *P* values of ≤ 0.05 were set as the level of statistical significant difference.

RESULTS

Histological Findings

All sections were examined using an optical microscope under "blind" conditions. In all cases, a normal course of the healing process was reported. In all specimens, the graft appeared as noncellular bone particles containing empty bone lacunae. Remineralization was observed in the periphery of the bone graft particles, although their central part remained locally demineralized.

In control sites where only DBM was implanted, bone graft particles were densely distributed and enclosed in newly formed interstitial connective tissue (provisional matrix) containing numerous mesenchymal cells (probably osteoprogenitor and preosteoblasts like cells). Vascularization among the bone graft particles was poor. A rich phagocytic activity on the implant particles was observed, involving multinuclear giant cells (matrixclasts), although cells had recolonized the bone lacunae of a few bone graft particles (Figs. 1–3).

In experimental sites where DBM + PRP had been implanted, the bone graft particles were relatively densely distributed and enclosed in newly formed interstitial connective tissue, which appeared to be looser. Vascularization among the bone graft particles was also poor. The number of matrixclasts was markedly smaller, eliciting

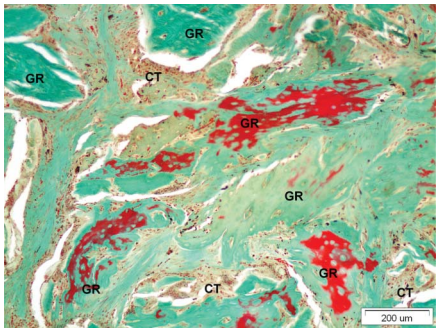

Fig. 1. Histopathological specimen (graft without PRP). The graft (GR) appears as noncellular bone particles containing empty bone lacunae, densely distributed and surrounded by connective tissue (CT) containing numerous mesenchymal cells. The remineralized peripheral part of the bone graft particles appears in green, while their central part remains demineralized and appears in red (Goldner trichrome stain: original magnification, ×10).

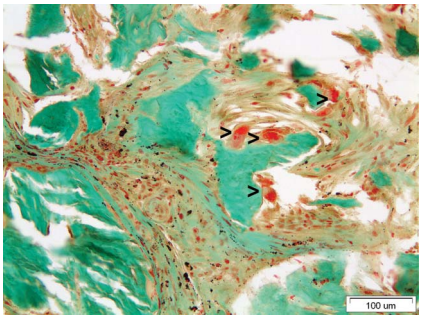

Fig. 2. Histopathological specimen (graft without PRP). A rich phagocytic activity on the implant particles is observed, involving multinuclear giant cells (arrows) (Goldner trichrome stain: original magnification, ×20).

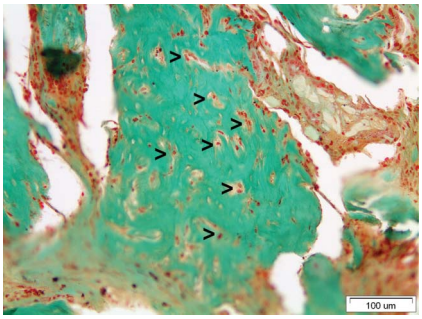

Fig. 3. Histopathological specimen (graft without PRP). Bone cells (arrows) recolonized the bone lacunae of a few bone graft particles (Goldner trichrome stain: original magnification, ×20).

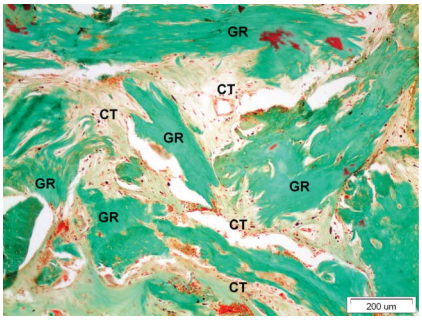

Fig. 4. Histopathological specimen (graft + PRP). Bone graft particles (GR) are relatively densely distributed and enclosed in newly formed loose interstitial connective tissue (CT) (Goldner trichrome stain: original magnification, ×10).

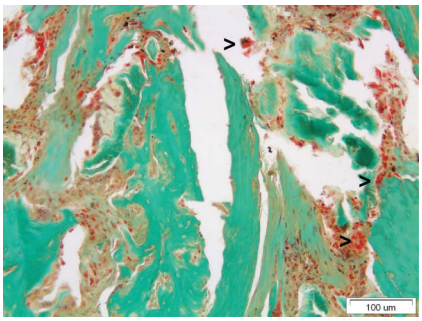

Fig. 5. Histopathological specimen (graft + PRP). Multinuclear giant cells (arrows) resorbing the bone graft particles (Goldner trichrome stain: original magnification, ×20).

a weaker phagocytosis of the implant particles (Figs. 4 and 5).

Histomorphometric and Statistical Analysis

The control sites presented a 14.552 ± 3.673 mean percentage of newly formed interstitial connective tissue volume, a 40.252 ± 6.403 mean percentage of remaining DBM volume, and a 1.206 ± 0.295 mean remaining DBM area.

The experimental sites presented an 11.970 ± 4.354 mean percentage of newly formed interstitial connective tissue volume, a 48.210 ± 5.877 mean percentage of remaining DBM volume, and a 1.555 ± 0.190 mean remaining DBM area.

Based on the above-mentioned findings, it can be concluded that at 3 weeks of observation, the amount of the newly formed interstitial connective tissue surrounding the graft particles was greater at control sites where only DBM was implanted. However, this effect was not statistically significant ($P > 0.05$). The addition of PRP reduced the phagocytosis of the bone graft particles as the remaining DBM area in the experimental sites was grater in a statistically significant manner ($P = 0.041$), and the mean percentage of remaining DBM volume in this group was also greater in a marginally statistically significant manner ($P = 0.060$).

DISCUSSION

Recent experimental and clinical studies have attempted to assess the effectiveness of DBM allografts in bone surgery. Both positive[28–30] and negative results[31–33] have been reported. The above-mentioned studies question the reliability and clinical effectiveness of commercially available DBM formulations in terms of providing a dependable osteoinductive effect, and it can be concluded that the results arising from the use of demineralized bone allografts are usually nonpredictable.

Applying the recent developments in tissue engineering, it has been suggested to combine a DBM graft with autologous PRP. The philosophy that the addition of PRP would enhance the osteoinductive potential of DBM is based on reasonable assumptions. Although the growth factors contained in PRP alone do not induce osteogenesis, they promote neoangiogenesis, thus improving the vascularization of the graft,[34] promote the chemotaxis, proliferation, differentiation, and metabolism of mesenchymal cells,[35] and interact *in situ* with the BMPs of the graft, thus enhancing their activity.[36,37] Therefore, PRP growth factors are, at least theoretically, expected to enhance the osteoinductive activity of the BMPs of the graft, create an environment containing more multipotent mesenchymal cells, which could be differentiated into osteogenetic cells by the BMPs of the graft, and promote the proliferation and function of the osteogenetic cells, which would be differentiated in the healing site of the graft. As a result, the addition of PRP to a DBM graft leads to the expectation that the osteoinductive potential of the graft will be enhanced.

Previous investigators examined the possible synergism between demineralized bone grafts and PRP with various results. Kim et al[19] placed osseointegrated implants in the iliac crest of dogs and created crestal bone defects around the implants, which were then covered with DFDB in powder form, with or without the addition of PRP. These defects were filled with newly formed tissue at 6 and 12 weeks. Histomorphometric analysis revealed a higher degree of direct bone-to-graft contact in the PRP group, and the authors report that bone defects around implants can be effectively treated with the use of DFDB and that the addition of PRP can improve bone regeneration. Aghaloo et al[20] used the experimental model of cranial defects in rabbits, to examine the effect of PRP on the healing of DFDB and FDB, but it did not confirm the hypothesis that bone regeneration can be enhanced by adding PRP to FDB grafts. Shanaman et al[18] used DFDB mixed with PRP for the augmentation of resorbed alveolar ridges in a series of patients. These authors concluded that the addition of PRP does not seem to improve the quality and quantity of newly formed bone. In a clinical study involving 29 periodontal patients, Markou et al[38] reported that the use of PRP alone or in combination with allogenic DFDB resulted in a significant clinical and radiological improvement in intraosseous periodontal defects during a 6-month follow-up period.

It must be stated that the above studies examine the effect of PRP on the osteoinductive activity of DBM in orthotopic sites. However, osteoinduction can be proved only in an environment deprived from differentiated osteogenetic cells. This means that any attempts to draw reliable conclusions on whether the addition of PRP can affect osteoinduction, triggered by the BMPs of the DBM graft, must be done in extraskeletal sites.

Our results showed that autologous PRP inhibits the specific osteoinductive potential of this allogenic DBM at 3 weeks after intramuscular implantation. The biological sequences of osteoinduction depend on the resorption of the DBM graft through its phagocytosis by so-called matrixclasts.[39]

Resorption releases additional BMPs from the matrix of the demineralized bone graft, which is an index of the graft's active osteoinductive performance.[40,41] Our data suggest that PRP inhibited this activity. Moreover, the addition of PRP had no effect on the production of the newly formed interstitial cellular connective tissue at the early phases of osteoinduction. The provisional connective tissue consists of mesenchymal cells (fibroblasts, osteoprogenitor cells, and preosteoblasts) embedded into a fibrous substrate. Therefore, this tissue is considered capable of developing into bone tissue and its presence is an index of the graft's osteoinductive activity.[42,43] Furthermore, the remineralization of the periphery of the bone graft particles, a constant finding in all histological sections, is suggested to be a consequence of osteoinduction and precedes new bone formation.[44–46]

In our study, animals were euthanized at 3 weeks to evaluate the effect of PRP at the early stages of osteoinduction, as there is a growing body of literature indicating that the positive effects of PRP on bone grafts are seen only early in the healing process.[16,47,48] Platelets and growth factors are known to act in the early stage during bone regeneration because life span of platelets and the direct effect of growth factors last less than 5 days.[10,49]

In 2007, Ranly et al[41] evaluated human PRP as an adjunct to human DBM implanted in intramuscular sites in nude mice. The authors concluded that PRP decreased the osteoinductivity of DBM at 8 weeks after implantation, and activities of both DBM and PRP were donor depended. However, in clinical practice, PRP is exclusively autologous and DBM grafts are allogenic,[5,39,40] and the use of xenogeneic growth factors and graft could be a confounding factor and affect the experimental results.

In the present study, allogenic DBM was produced according to the protocol proposed by Reddi and Huggins.[27] To avoid any variations in the osteoinductive potential of the DBM,[50] the DBM grafts from all 3 donor rabbits were mixed up to produce a graft homogenous in terms of donor origin. Moreover, young adult healthy rabbits were chosen as bone donors because it appears that young donors produce DBM with a higher osteoinductive potential.[51] The graft was prepared in powder form, with a particle size of 250 to 710 μm, as larger or smaller particle sizes seem to have a negative effect on the osteoinductive potential.[42,52] Finally, the DBM graft was not mixed with any excipients, which could improve its handling properties, but affect its behavior in an unexpected way.[53]

The failure of the autologous PRP to augment the specific osteoinductive activity of this allogenic DBM graft in our study cannot be ascribed to poor preparation of the PRP. Autologous PRP was prepared using a certified, commercially available system. In our experiment, we did not perform such measurements neither in the total amount of the rabbits' blood nor in autologous PRP, in order not to deviate from our primary experimental goal. However, according to independent studies by Weibrich and Kleis,[54] and Zhang et al,[23] this specific technique ensures an adequate concentration of platelets and TGF-β_1 and PDGF-ab growth factors in the produced PRP. Moreover, given that the rabbit hematological profile is similar to that of humans,[55] we assume that a drastic autologous PRP was prepared for our experiment.

Theoretically, PRP could improve the osteoinductive potential of a DBM graft through the growth factors contained in it; however, this was not verified in the present study. Although undifferentiated multipotent mesenchymal cells, fibroblasts, osteoblasts, and their precursors have receptors and respond positively to stimuli by platelet growth factors, at least *in vitro* cell cultures, this was not observed in our *in vivo* study. It seems that the interaction of PRP growth factors with each other, with the BMPs of graft and with the various cells in the multipotent environment of tissue healing, which is constantly changing and varies biochemically, is not totally clear. This reinforces the opinion by Marx[5] that PRP is an effective adjunct only to autologous bone grafts, which contain living osteogenetic cells, and not to bone substitutes such as DBM, which contains morphogenetic BMPs but has no living osteogenetic cells.

CONCLUSION

In conclusion, the present study demonstrated that autologous PRP has a negative effect on the early phases of osteoinduction by allogenic DBM implanted in intramuscular sites in rabbits.

DISCLOSURE

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