Effect of Autologous Platelet-Rich Plasma in Combination With a Biphasic Synthetic Graft Material on Bone Healing in Critical-Size Cranial Defects

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Purpose: The aim of the study was to investigate the effect of autologous platelet-rich plasma (PRP) on the osteogenic potential of a biphasic synthetic graft material composed of hydroxyapatite and beta-tricalcium phosphate (HA/β-TCP) in critical-size cranial defects in rabbits.

Materials and Methods: Three circular bicortical critical-size cranial defects were created in each of 18 rabbits. The first of the defects was grafted with autologous PRP and HA/β-TCP, the second was grafted with HA/β-TCP without PRP, and the third was left unfilled as a negative control. Animals were euthanized at 2, 4, and 6 weeks after surgery. Harvested tissue specimens were evaluated histologically and histomorphometrically. Several parameters associated with osteoclastic and osteoblastic activities were measured and calculated. The results were statistically analyzed using the 1-way analysis of variance statistical method.

Results: Histologic analysis of the samples showed bone tissue formation at all experimental sites including untreated control defects. A statistically significant difference in new bone formation between the defects treated with HA/β-TCP + PRP and defects treated with HA/β-TCP alone was not observed. Control untreated defects showed the greatest bone regeneration.

Conclusions: In this animal model, autologous PRP had no effect on bone healing in addition to a biphasic HA/β-TCP synthetic graft material after 2, 4, and 6 weeks of implantation.

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In contemporary oral and maxillofacial surgery, the need to restore bone defects is imperative to avoid functional, aesthetic, and psychologic problems in patients. Osseous tissue is able to regenerate itself by obtaining the same structure and texture and thus to restore functionality after an injury or loss. It should however be noted that the main factor affecting the bone healing–restorative process is the size of the defect. The "critical size" of a bone defect is defined as the smallest bone defect for a type of body and bone, which cannot be fully restored through the normal healing process during life, as the largest area of the defect will be covered by dense fibrous connective tissue.[1]

To avoid this unfavorable outcome in bone regeneration, bone grafts are used to support the process. It is commonly accepted that bone autografts are the ideal solution in such cases because they have osteogenetic, osteoinductive, and osteoconductive properties; they do not transmit diseases; and they do not cause immune reactions while they are gradually absorbed and replaced by newly formed osseous tissue. However, the limited quantity of bone autografts that can be received and the postoperative complications at the donor site (postoperative pain, development of hematomas, risk of infection, injury of the innate anatomic structures, deformities, increased intervention, and nursing time, and cost of additional intervention) are drawbacks that limit their scope of application.[2]

Thus, in an effort to support the healing process, different biologic (human or animal) or synthetic grafts and various bone-regenerative factors such as growth factors, morphogenetic proteins, bioactive polypeptides, tissue-engineering techniques, and pluripotent stem cells have been used for the past 2 decades.[3–5]

Alloplasts are synthetic osteoconductive bone substitutes that are readily available in virtually unlimited supply and also eliminate the need for a patient donor site. The advantages of alloplastic materials include biocompatibility and lack of immune response and the ability of radiographic assessment after their implantation. These biomaterials should be sufficiently porous and interconnective for tissues to grow into and around the graft.[6,7] Among them, calcium phosphates such as hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP) are of great interest with high potential for bioapplications. Today, biphasic calcium phosphates made of HA and β-TCP are widely used for bone grafting because they combine the excellent bioactivity and stable phase of HA with the good resorbability of β-TCP.[8] The resorbability of these bone grafts depends on their HA/β-TCP ratios; the lower the ratio, the greater the resorbability of the bone graft at the recipient site.[9]

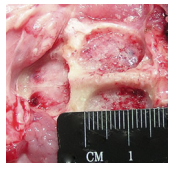

FIGURE 1. Intraoperative view of the three 15-mm critical-size cranial defects.

Platelet-rich plasma (PRP) is an autologous concentration of platelets in a small volume of plasma.[10,11] Because it is a concentration of platelets, it is also a concentration of the 7 protein growth factors proved to be actively secreted by platelets to initiate all wound healing. These growth factors include the 3 isomers of the platelet-derived growth factor (PDGF-AA, PDGF-BB, and PDGF-AB), the 2 isomers of the transforming growth factor-β (TGF-β_1 and TGF-β_2), the vascular endothelial growth factor, and the epidermal growth factor.[10] Moreover, PRP contains adhesive proteins such as vitronectin (in the α-granules of platelets), fibrin (in plasma), and fibronectin (in plasma).[12] 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,[13] while the handling of the particulate bone grafts is significantly improved.[14]

The purpose of this study was to investigate the effect of autologous PRP on the osteogenic potential of a biphasic synthetic graft material composed of HA and β-TCP in critical-size cranial defects in rabbits.

MATERIALS AND METHODS

The experimental procedures of this study were submitted and approved by the Veterinary Directorate of the Prefecture of Athens.

PRP Preparation

Platelet-rich plasma was prepared using a particular commercial kit (Curasan PRP kit; Curasan AG, Kleinostheim, Germany) according to the manufacturer's protocol. In each of the 18 experimental animals, 8 mL of blood was drawn from the central auricular artery in a monovette containing 1.1 mL of citrate phosphate dextrose anticoagulant. Each blood sample was then centrifuged at 2400 rotations/min for 10 minutes, at room temperature, to separate platelet-containing plasma from the red and white blood cells. This plasma was drawn into a new monovette and centrifuged again at 3600 rotations/min for 15 minutes, at room temperature. Through this second centrifugation, platelet-poor plasma was separated from PRP. After the removal of platelet-poor plasma, 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. Weibrich and Kleis[15] found that the platelet count in human PRP produced by the Curasan PRP kit is 1,140,500/μL, the amount of TGF-β_1 is 79.7 ng/mL, and the amount of PDGF-AB is 314.1 ng/mL. Given that the rabbit's hematologic profile is similar to that of humans, we hypothesized that the autologous rabbit PRP that we prepared for our experiment contained similar levels of platelets, TGF-β_1, and PDGF-AB.[16]

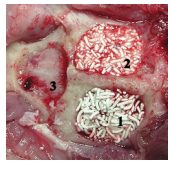

FIGURE 2. Site 1 filled with HA/β-TCP, site 2 filled with HA/β-TCP + PRP, and site 3 left unfilled (control).

TABLE 1. Histomorphometric Parameters Measured

Parameter	Abbreviation	Units of Measurement
Bone area	BAr	mm^2
Osteoid perimeter	OPm	mm
Osteoblast perimeter	ObPm	mm
Osteoclast perimeter	OcPm	mm
No. osteoblasts	NOb	Cardinal number
No. osteoclasts	NOc	Cardinal number

Surgical Procedure

Under general anesthesia, a semicircular incision was made in the skin over the top of the cranial vault and a cutaneous–periosteal flap was raised and reflected laterally to expose the parietal and frontal bones. Three identical 15-mm diameter bicortical cranial defects were created in each animal, and they were defined as critical-size defects (Fig. 1). A calvarial wound model has many similarities to the maxillofacial region. Morphologically and embryologically, the calvaria develops from a membrane precursor and thus resembles the membranous bones of the face. Anatomically, the calvaria consists of 2 cortical plates with regions of intervening cancellous bone similar to the mandible. The first of the defects was grafted with autologous PRP and HA/β-TCP (BONITmatrix, DOT GmbH, Rostock, Germany); the second was grafted with HA/β-TCP without PRP (experimental sites). The third defect was left unfilled (control site) (Fig. 2). BONITmatrix is a synthetic bone graft substitute for reconstruction of bone defects. It consists of a mixture of HA and β-TCP in a ratio of 60:40. In contrast to conventional HA- and β-TCP–based ceramics and bio-glasses, BONITmatrix is manufactured in a sol–gel process without sintering (patented sol–gel procedure DE 100 03 824). In this process, nanocrystalline calcium phosphates are embedded in a biologic active silicon dioxide matrix. This special low temperature process leads to a high interconnecting porosity inside the granules. The pore sizes are in the nanometer and micrometer ranges ensuring the products' very large internal surface of approximately 90 m^2/g. The interconnecting pore system with its high capillarity and the adsorptive capacity of the surface allow for the diffusion of biologic fluids. The surface binding of important growth factors present in blood supports osteogenesis. The material is osteoconductive and acts as a scaffold during osteogenesis. It has been shown that incorporation of silicate in calcium phosphate ceramics is followed by an increase of

TABLE 2. Histomorphometric Parameters Calculated

Parameter	Abbreviation
Bone volume/tissue volume	BV/TV
Osteoid volume/bone volume	OV/BV
Trabecular thickness	TbTh
No. osteoblasts/bone perimeter	NOb/BPm
No. osteoclasts/bone perimeter	NOc/BPm
No. osteoclasts/osteoclast perimeter	NOc/OcPm
Trabecular separation	TrSp
Trabecular perforation area	TrPfAr
Trabecular perforation perimeter	TrPfPm
No. osteoblasts	NOb
No. osteoclasts	NOc

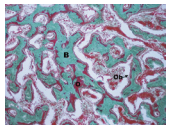

FIGURE 3. Representative histologic microphotograph of a control site at 2 weeks. Goldner trichrome staining showing newly formed mineralized bone (B), osteoid (O), and ream of osteoblasts (Ob) (original magnification ×120).

osteoblast attachment and proliferation.[17] Furthermore, in vivo and in vitro studies have demonstrated the biocompatibility and the bone regeneration potential of BONITmatrix.[18–21]

Each laboratory animal received antibiotics (Zinadol 30 mg/kg/24 h; GlaxoWellcome, Athens, Greece) and analgesics (Depon 15 mg/kg; Bristol-Myers Squibb, Athens, Greece) for 2 days postoperatively. The postoperative course of all animals was uneventful.

Histologic Preparation

Six animals were killed at 2, 4, and 6 weeks postsurgery. Right after their removal, specimens were fixed in 10% neutral buffered formalin. After this, they were placed in alcohol and methyl methacrylate and plasticized using hot polymerization. Finally, nondecalcified sections were obtained and stained with Goldner trichrome.

Histomorphometric Analysis

Slides were placed in a semiautomated histomorphometric measurement system. Histologic images were digitized, and histomorphometric parameters were measured on a computer using a special software (Osteomeasure, interactive measurement system for bone histomorphometry; OsteoMetrics, Atlanta, GA). The measurements are presented and explained in Tables 1 and 2.

Statistical Analysis

Histomorphometric results were statistically analyzed using the 1-way analysis of variance statistical method.

RESULTS

All subjects had an uneventful recovery.

Histologic Analysis

All sections were examined using an optical microscope under "blind" conditions. Analysis of Goldner-stained sections at 2, 4, and 6 weeks after implantation revealed the presence of osteoblasts, osteoid, and newly formed mineralized bone at all groups (Figs. 3–5). At experimental groups remaining graft granules appeared as amorphous masses incorporated in the newly formed tissues.

Histomorphometric Analysis

Histomorphometric analysis of parameters associated with osteoblastic and osteoclastic activities revealed that there was no statistically significant differences in new bone formation between

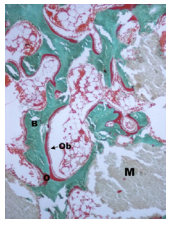

FIGURE 4. Representative histologic microphotograph of defect filled with HA/β-TCP at 4 weeks. Goldner trichrome staining showing newly formed mineralized bone (B), osteoid (O), ream of osteoblasts (Ob), and remaining graft particles (M) (original magnification ×120).

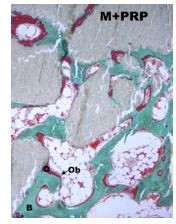

FIGURE 5. Representative histologic microphotograph of defect filled with HA/β-TCP + PRP at 6 weeks. Goldner trichrome staining showing newly formed mineralized bone (B), osteoid (O), ream of osteoblasts (Ob), and remaining graft particles (M) (original magnification ×120).

the defects filled with HA/β-TCP + PRP and defects filled with HA/β-TCP alone at 2, 4, and 6 weeks after surgery. In addition, the bone defects which were left unfilled (control group) displayed more pronounced bone regeneration (Tables 3–5).

In particular, at 2 weeks, there are no statistically significant differences between histomorphometric parameters that express new bone formation (bone volume/tissue volume [BV/TV], osteoid volume [OV]/BV) between the 3 groups. Statistically significant differences between specific parameters express a higher osteoclastic activity at the control group compared with the 2 experimental groups at this time point of observation (Table 3). At 4 weeks, there are no statistically significant differences between parameters that express new bone formation (BV/TV, OV/BV) between the 3 groups. Statistically significant differences between specific parameters express a higher osteoclastic activity at the control group compared with the experimental groups. The sites that were grafted only with HA/β-TCP express a higher osteoclastic activity compared with the sites that were filled with graft + PRP at this time point of observation (Table 4). At 6 weeks, the control group expresses significantly higher new bone formation and osteoblastic activity compared with the experimental groups. At this time point, there are no statistically significant differences between the 2 experimental groups

TABLE 3. Comparison of Histomorphometric Parameters Between the 3 Groups at 2 Weeks

Parameter	Group	n	Mean	SD	*P*
BV/TV	Control	6	43.3192	17.59582	NS
	Graft+ PRP	6	30.8762	17.22248	
	Graft	6	33.0611	13.51261	
OV/BV	Control	6	7.3801	3.01248	NS
	Graft + PRP	6	9.0197	4.09143	
	Graft	6	11.9532	4.19525	
OcS/BS	Control	6	8.1839	2.05900	0.030
	Graft + PRP	6	5.4705	2.54976	
	Graft	6	5.1050	.81468	
NOc/BPm	Control	6	.7227	.18777	0.022
	Graft + PRP	6	.5532	.16913	
	Graft	6	.4549	.05157	
OcPm	Control	6	8.2040	2.31850	0.05
	Graft + PRP	6	5.6310	2.19920	
	Graft	6	5.4447	1.61238	
NOb	Control	6	530.5000	88.42341	0.039
	Graft + PRP	6	681.0000	117.30644	
	Graft	6	655.1667	84.58704	
NOc	Control	6	72.5000	21.07842	0.044
	Graft + PRP	6	51.0000	18.14387	
	Graft	6	45.3333	13.41144	

n indicates number of samples; NS: no statistically significant difference.

TABLE 4. Comparison of Histomorphometric Parameters Between the 3 Groups at 4 Weeks

Parameter	Group	n	Mean	SD	*P*
BV/TV	Control	6	44.2686	24.98329	NS
	Graft +PRP	6	25.3490	4.78476	
	Graft	6	26.3885	8.28006	
OV/BV	Control	6	6.3793	2.76670	NS
	Graft +PRP	6	3.6294	2.63752	
	Graft	6	5.1085	4.04828	
OcS/BS	Control	6	9.1719	4.25005	0.006
	Graft +PRP	6	2.8575	1.30210	
	Graft	6	5.4733	2.18704	
TbTh	Control	6	248.9580	95.31720	0.025
	Graft +PRP	6	155.0931	29.23862	
	Graft	6	150.7014	40.77639	
NOb/OPmLm	Control	6	14.7099	7.50257	0.010
	Graft +PRP	6	35.0625	18.07377	
	Graft	6	43.0204	14.71553	
NOc/BPm	Control	6	.4815	.17863	0.040
	Graft +PRP	6	.2331	.11818	
	Graft	6	.3982	.16000	
NOc/OcPm	Control	6	6.9082	.70206	0.019
	Graft +PRP	6	8.0358	.50476	
	Graft	6	7.2299	.64857	
TbSp	Control	6	275.7838	165.67085	0.05
	Graft +PRP	6	463.2010	83.30334	
	Graft	6	440.8640	139.65547	
Bar	Control	6	11.3251	3.32650	0.002
	Graft +PRP	6	6.2573	1.25201	
	Graft	6	5.9240	2.06903	
TbPfAr	Control	6	12.4592	3.43298	0.001
	Graft +PRP	6	6.4536	1.33313	
	Graft	6	6.1397	2.17872	
VdAr	Control	6	1.1341	.44207	0.0005
	Graft +PRP	6	.1963	.10404	
	Graft	6	.1157	.04537	
Oar	Control	6	.6985	.39036	0.049
	Graft +PRP	6	.2377	.18752	
	Graft	6	.3516	.30387	
OcPm	Control	6	4.1570	2.08379	0.05
	Graft +PRP	6	1.9079	1.11246	
	Graft	6	3.1526	.80758	
NOc	Control	6	31.0000	10.41153	0.009
	Graft +PRP	6	14.0000	7.21110	
	Graft	6	22.8333	6.04704	

regarding parameters that showed new bone formation and osteoblastic and osteoclastic activities (Table 5).

DISCUSSION

So far, the ideal bone graft has not been found. Autogenous bone is still considered the "gold standard" among bone grafts because it is the only graft material that fulfills all 3 components of the bone regeneration triad (osteogenesis–osteoinduction–osteoconduction). However, it is not always easy to harvest, amounts may be insufficient, there are complications in the donor site, and in most cases, a second operation is necessary.[22]

For these disadvantages, research has focused for many years on the development of synthetic bone grafts with characteristics similar to those of autologous bone grafts. Intense research in regenerative medicine is toward accelerating the healing process using various biologic agents because growth factors may improve the biologic activity of bone graft substitutes.[22] Platelet-rich plasma could be promising in this effort.

Several studies have attempted to unravel the mechanism of action and efficacy of PRP, both in humans and in animal models.[23–26] Experimental studies showed that PRP does not offer additional benefits on bone regeneration when used in combination with bone substitutes. On the contrary, according to their results, when autografts are used, particularly in combination with PRP, bone regeneration is intense.[27–33] Moreover, experimental research is showing that PRP may inhibit bone regeneration.[34–39]

Apart from the neutral or negative findings of the aforementioned works on the contribution of PRP to bone regeneration, there are several experimental studies supporting that local application of PRP in bone defects alone or in combination with β-TCP enhances bone regeneration and remodeling of newly formed bone.[40–44]

The aim of our study was to investigate the effect of autologous PRP on bone regeneration in combination with HA/β-TCP, using critical-size cranial defects in rabbits. Studying in detail the histomorphometric parameters as measured at 2, 4, and 6 weeks after implantation of grafts, no significant difference in new bone formation between the experimental groups was observed with respect to osteoblastic and osteoclastic activities, osteoid deposition, and bone deposition.

Our findings are in agreement with those reported by Plachokova et al.[23,24] In their studies, the authors report that after 1, 2, and 4 weeks of implantation of a biphasic HA/β-TCP graft mixed with PRP in rat skull defects, no significant difference in bone formation between the PRP and non-PRP groups was observed neither histologically nor by microcomputed tomographic analysis.

TABLE 5. Comparison of Histomorphometric Parameters Between the 3 Groups at 6 Weeks

Parameter	Group	n	Mean	SD	*P*
BV/TV	Control	6	53.0566	17.80956	0.009
	Graft +PRP	6	26.6300	6.49956	
	Graft	6	30.9294	13.69448	
OV/BV	Control	6	5.0275	1.50356	NS
	Graft +PRP	6	4.6792	1.75009	
	Graft	6	3.3518	1.36974	
ObS/BS	Control	6	19.6900	1.48449	0.019
	Graft +PRP	6	13.6791	6.65619	
	Graft	6	10.7896	4.96134	
TbTh	Control	6	348.0371	74.32738	<0.0005
	Graft +PRP	6	169.7565	49.88563	
	Graft	6	168.2417	45.01883	
NOb/BPm	Control	6	5.4092	.36135	0.032
	Graft +PRP	6	3.9035	1.76438	
	Graft	6	3.2150	1.40511	
NOb/OPmLm	Control	6	13.5982	7.45198	0.029
	Graft +PRP	6	17.9626	3.90905	
	Graft	6	23.7597	5.68109	
Bar	Control	6	12.7336	4.27430	0.007
	Graft +PRP	6	6.3822	1.66838	
	Graft	6	7.293	3.05586	
TbPfPm	Control	6	42.6015	10.44587	0.014
	Graft +PRP	6	51.9410	8.78247	
	Graft	6	60.2359	7.45598	
TbPfAr	Control	6	13.2285	4.74930	0.016
	Graft +PRP	6	7.0007	2.21989	
	Graft	6	7.7851	3.16335	

In addition, the findings of our study do not support the hypothesis that the positive effects of PRP on bone grafts are seen early in the healing process.[45–49] Although platelets and growth factors are known to act in the early stage during bone regeneration because the life-span of platelets and the direct effect of growth factors last less than 5 days, our results showed no positive effect of PRP on new bone formation at 2 weeks.

The failure of the autologous PRP to enhance the osteoconductive potential of the biphasic HA/β-TCP graft in our study cannot be ascribed to poor preparation of the PRP. Autologous PRP was prepared using a certified commercially available system, and we followed the technique recommended by the manufacturer (Curasan PRP kit, Curasan, B. Brown Melsungen AG).

Calvarial defects from the control group where no graft was placed were characterized by a more pronounced bone regeneration, compared with the experimental sites. This outcome is significant and raises several concerns about the appropriateness of the rabbit as an experimental model in the research of bone graft healing. This experimental model has been criticized because bone formation in rabbits is more likely to be induced when compared with other animal models or in vivo clinical studies.[50] It seems that the cranial defects that we prepared turned out to be of noncritical size, although we assumed based on the literature[51] that in rabbits, a 15-mm cranial defect would be a critical-size defect for an implantation period raging from 2 to 6 weeks.

In theory, autologous PRP could improve the osteoconductive potential of a biphasic HA/β-TCP synthetic graft through the growth factors contained in it; this however was not verified in the current study. Thus, the efficacy of PRP in bone regeneration remains questionable because studies reach different conclusions. A recent study by van den Dolder et al[52] attempts to explain this issue, arguing that the concentrations of growth factors contained in PRP differ depending on the species and between individuals of the same species. Of course, other factors, such as the method of PRP preparation, the number of animals included in the study, the size of the defect, and sometimes the subjective analysis of the results also play a role.

It seems that our results reinforce the opinion by Marx[10] that PRP is an effective adjunct only to autologous bone grafts, which contain living osteogenetic cells, and not to bone substitutes.

CONCLUSIONS

On the basis of our results, we conclude that autologous PRP had no effect on new bone formation when added to a biphasic HA/β-TCP graft in critical-size cranial defects in rabbits at 2, 4, or 6 weeks after implantation. Our histologic and histomorphometric results confirmed the limited osteoconductive potential of the synthetic graft used in this study. Further investigations are necessary to elucidate the effect of PRP on osseous regeneration using biphasic HA/β-TCP grafts.

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