Evaluation of Platelet-Rich Plasma in Combination with Deproteinized Bovine Bone Mineral in the Rabbit Cranium

Yadollah Soleymani Shayesteh, Mohammad Amin Shafiee Ardestani, Afshin Khorsand, Pouria Motahhary, Mohammad Dehghan

Introduction: Reconstruction methods are an essential prerequisite for functional rehabilitation of the stomatognathic system. Platelet-rich plasma (PRP) offers a new and potentially useful adjunct to bone substitute materials in oral and maxillofacial reconstructive surgery. This animal study investigated the influence of PRP on the regeneration of bony defects, treated with deproteinized bovine bone mineral (DBBM).
Materials and Methods: Twenty New Zealand white rabbits were included in this randomised, blinded study. Three equal 3 x 6 mm cranial bone defects were created and immediately grafted with DBBM, PRP + DBBM, or no treatment as control. The defects were evaluated with histological and histomorphometric analysis performed at 2, 4, 8 and 12 weeks.
Results: The results showed a significant increase in histomorphometric bone area and trabecular maturity in both DBBM and DBBM + PRP samples as compared with the control after 4, 8 and 12 weeks. A significant increase in bone formation was seen with the addition of PRP to DBBM after 2, 4 and 8 weeks. At 12 weeks, the level of bone formation had adjusted similarly between the two groups. There was also a significant increase in the rate of biodegradation of DBBM particles after the addition of PRP throughout the study period. Neither foreign body reaction nor severe inflammation was seen in any of the specimens evaluated.
Conclusions: Under the limitations of this study, PRP added additional benefits when DBBM was used in rabbit cranial defects concerning the speed of bone regeneration and the rate of biomaterial degradation.

Key words: animal study, bone grafting, deproteinized bovine bone mineral, histomorphometry, platelet-rich plasma

INTRODUCTION

Autogenous bone grafts from intra- and extra-oral donor sites, regarded as gold standard, are used especially to regenerate bony defects in the craniofacial region (Nkenke et al, 2001). The disadvantage of donor morbidity, using autogenous bone, can be avoided by using bone substitutes. A variety of degradable or permanent, mainly osteoconductive bone substitutes like tricalcium phosphate or xenogenic hydroxyapatite ceramics are available (Lynch et al, 1999).
The use of platelet-rich plasma (PRP) offers a potentially useful adjunct to autogenous, allogenic, and xenogenic graft materials in oral and maxillofacial bone and implant reconstructive surgery (Marx, 2004). Some authors also suggest that the addition of PRP to osteoconductive grafting materials can potentiate osteoinduction (Kim et al, 2002). Clinical trials suggest that the combination of auto-

genous bone graft (Marx et al, 1998) and bone graft substitutes (Kassolis et al, 2000) with growth factors such as cytokines contained in PRP may be suitable to enhance bone density. More particularly, Marx et al's study (1998) showed that combining PRP with autogenous bone in mandibular continuity defects resulted in significantly faster radiographic maturation and a histomorphometrically denser bony regenerate.

Platelets are very important in the wound-healing process. They arrive quickly at the wound site and begin the coagulation process. They release multiple wound-healing growth factors and cytokines, including platelet-derived growth factor (PDGF), transforming growth factors (TGF-β1 and TGF-β2), vascular endothelial growth factor (VEGF), epithelial cell growth factor (EGF), basic fibroblast growth factor (bFGF), and platelet activating factor-4 (PAF-4) (Lynch et, 1999; Weibrich et al, 2003b). These growth factors are thought to contribute to bone regeneration and increased vascularity, and play an important role in the process of wound healing (Marx, 2004). Questions exist as to whether PRP can be used with alloplasts, xenografts or allograft materials without the incorporation of autogenous donor bone to create a bone graft that is comparable to autogenous bone concerning bone regenerative capacity. Results of studies attempting to answer this question are conflicting or equivocal (Anitua, 1999; Shanaman et al, 2001).

The present study was undertaken to use an animal model to evaluate the effectiveness of PRP on bone healing utilising deproteinized bovine bone mineral (DBBM) in rabbit non-critical-sized calvarial defects.

STUDY DESIGN

Animal surgical procedure

Twenty New Zealand white male rabbits between 2.5 and 3 kg mass were included in this randomised, blinded prospective study. The study protocol was first approved by Tehran University of Medical Sciences ethics committee.

Each rabbit was anaesthetised with ketamine 10% (40 mg/kg) and xylazin 2% (5 mg/kg) (Alafason, Woeden-Holland), administered intra-muscularly. The fur was shaved over the cranium, scrubbed with 7.5% povidine iodine and draped in a sterile fashion. Surgical procedure followed a coronal–sagittal approach, the periosteum was dissected and three identical full thickness bony defects (terminated over the Dura mater) were created with a round bur (3 x 6 mm) in the frontal and parietal bones distanced approximately 2 mm from the sagittal and coronal sutures. The defects were randomly filled with DBBM (Bio-Oss®, Geistlich and Sons, Wolhusen, Switzerland), DBBM + PRP, and one defect was left unfilled to serve as control defect. A volume of 0.5 ml PRP was available for each defect and was mixed with approximately 30 mg of DBBM particles (particle size 0.25–1 mm), in a sterile dish. The wound was closed with resorbable 4/0 sutures (Vicryl, Johnson & Johnson, Somerville, NJ, USA) for periosteal closure and non resorbable 4/0 suture (SURGIPRO™, Polypropylene Monofilament, Richmond, VA, USA) for the calvarial skin. The rabbits recovered from anaesthesia without complications. They were given postoperative narcotic pain medication (ketoprofen 0.1 mg/day) for 3 days and antibiotic (Enrofloxacin 0.6 mg/day) for 1 week subcutaneously.

PRP preparation

PRP samples were prepared using a modification of Curasan technique (Weibrich et al, 2003b). The 6 ml of autologous blood drawn from each rabbit was combined with 0.5 ml of anticoagulant citrate dextrose phosphate (ACDA) to prevent coagulation. The blood was centrifuged at 1200 rpm (160 *g*) for 20 minutes to separate the plasma containing the platelets from the red cells (Clements 2000 centrifuge, Sydney, Australia).

The plasma was drawn off the top, and centrifuged for an additional 15 minutes at 2000 rpm (400 *g*) to separate the platelets. The platelet-poor plasma was separated from the PRP along with the buffy coat. The buffy coat and PRP, approximately 0.5 ml, was re-suspended and used within minutes to add to the grafting materials. Platelet counts were performed on each sample, including a peripheral blood count and PRP count.

Sample preparation

Rabbits were euthanised using pentobarbital, 100 mg/kg intravenously at 2, 4, 8 and 12 weeks. There were 5 rabbits in each group.

The entire cranium was removed with a reciprocating saw, without encroaching on the grafted areas. Specimens were treated with 20% formic acid decalcifying solution for three days. They were then dehydrated with alcohols and embedded in paraf-

Fig 1 40x magnified H&E-stained photomicrographs of the experimental groups at 2, 4, 8 and 12 weeks; Fig 1 (i) illustrates the control defect at the end of the study period.

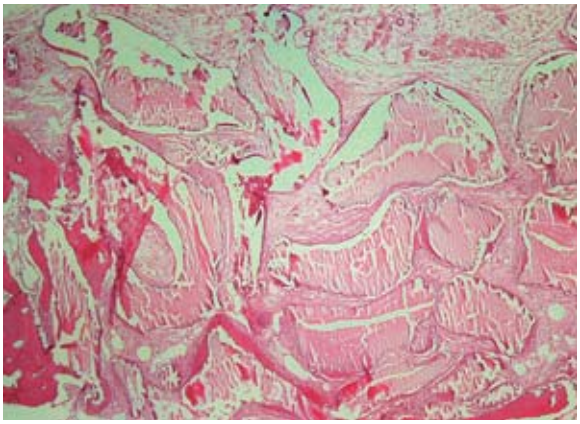

Fig 1a DBBM at 2 weeks.

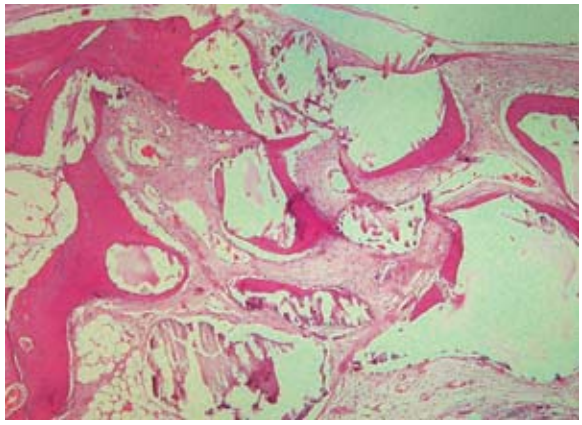

Fig 1b PRP + DBBM at 2 weeks.

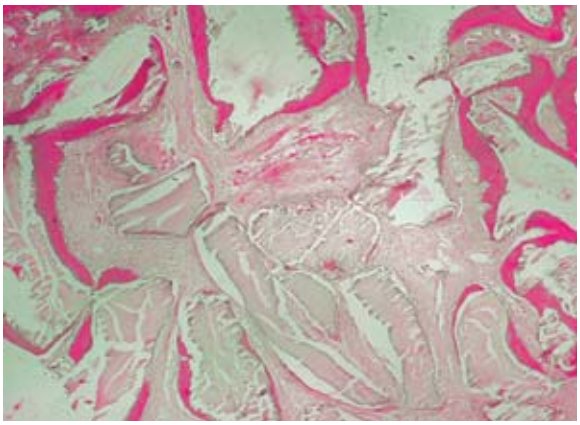

Fig 1c DBBM at 4 weeks.

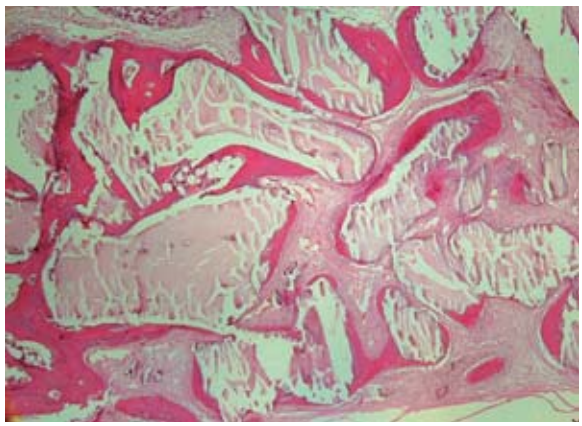

Fig 1d PRP + DBBM at 4 weeks.

fin. Histological sections (5 µm thickness) were prepared perpendicular to the long axis of each defect with an anterior to posterior direction. Finally, 20 sections of each defect (350 µm distance between two succeeding sections) were provided. The specimens were stained with haemotoxylin and eosin (H&E). Histological evaluation was performed at 40x, 100x and 400x magnification. The 40x magnification was used for histomorphometric analysis.

Sample evaluation

Magnified micrographs (400x) (Olympus BX 51, Olympus Co., Tokyo, Japan) were used for assessment of foreign body reaction as defined by giant cells and concomitant granulomatous reaction. The same magnification was also used to assess the interface between bone and biomaterial particles. Polarised light microscopy was used to determine the proportion of lamellar and woven trabeculaes in each specimen. Concentrically aligned collagen bundles in the bony trabeculaes were interpreted as lamellar bone, whereas irregularly oriented collagen fibres in the trabeculaes were documented as woven bone.

Magnified photomicrographs (40x) (Olympus DP12 digital camera, Olympus Co., Tokyo, Japan) were used (Fig 1) for computing the histomorphometric bone and biomaterial areas using graphics

Fig 1 40x magnified H&E-stained photomicrographs of the experimental groups at 2, 4, 8 and 12 weeks; Fig 1 (i) illustrates the control defect at the end of the study period.

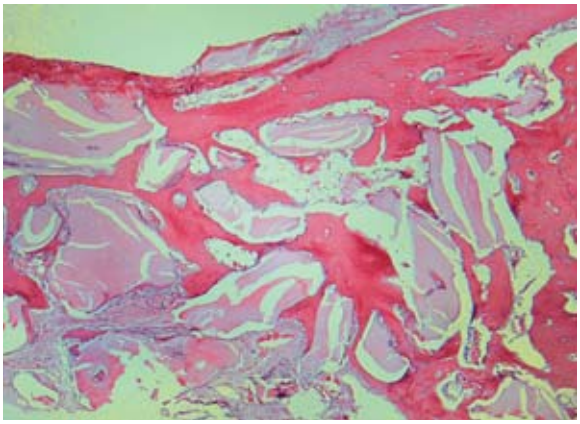

Fig 1e DBBM at 8 weeks.

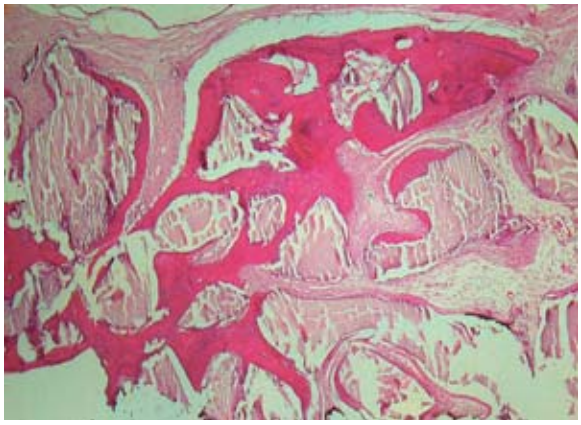

Fig 1f PRP + DBBM at 8 weeks.

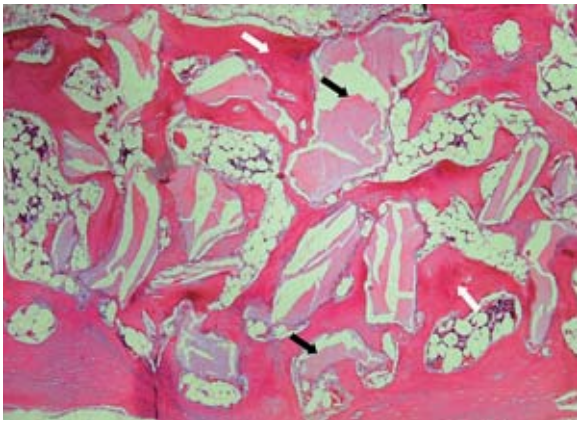

Fig 1g DBBM at 12 weeks; new bone trabeculae (white arrows) and remaining DBBM particles (black arrows).

Fig 1h PRP + DBBM at 12 weeks.

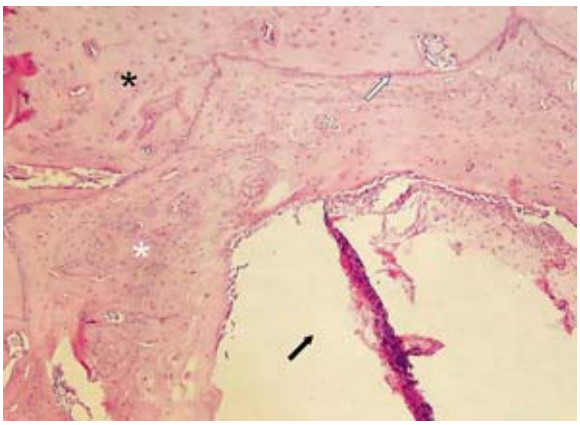

Fig 1i Control at 12 weeks; original calvarial bone (black asterisk), reversal line (white arrow), new bone trabeculae (white asterisk) and original defect area (black arrow).

software (Photoshop 8 CS, Adobe Photoshop CS). Areas including newly regenerated bone were selected according to their similar colour properties. The pixel counts of these areas were calculated (Photoshop 8 CS) and divided by the total number of pixels of each photomicrograph. The same procedure was used for calculating the histomorphometric area of remaining biomaterials. Evaluators were blinded with regard to the use of PRP and also time period for each sample.

Statistical evaluation

Statistical analysis included Kruskal-Wallis and Dunn procedure for qualitative variables and ANOVA (repeated measure) with Post Hoc for quantitative variables using SPSS software (SPSS for Windows version 11.5).

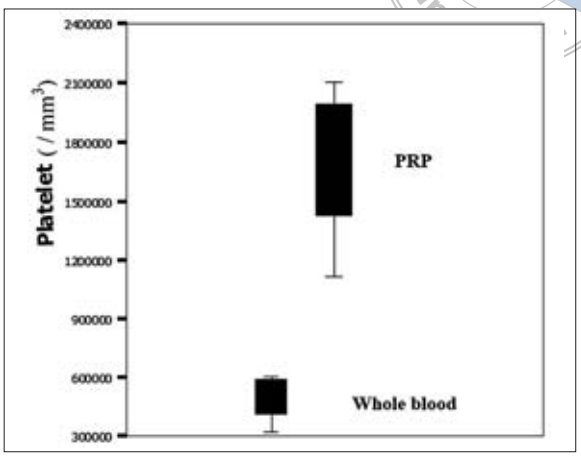

Fig 2 Changes in platelet count in the process of PRP preparation.

RESULTS

Platelet counts confirmed that the PRP preparation technique used in this study produced a source of highly concentrated platelets with the collection efficiency (Weibrich et al, 2003a) of 34.96% ± 4.06 [collection efficiency = (volume PRP x platelet count PRP)/(volume whole blood x platelet count whole blood) x 100]. The average peripheral blood platelet count was 473274 ± 49067/mm^3. The average PRP platelet count was 1670064 ± 345548/mm^3 (Fig 2).

Histological evaluation

Neither foreign body reaction nor severe inflammation was seen in any of the specimens evaluated. Also direct bone–biomaterial contact without intervening connective tissue was seen whenever new bone formation occurred in DBBM (+PRP) groups. Polarised light microscopy showed statistically significant differences between DBBM + PRP and DBBM with control group concerning the proportion of lamellar trabeculaes at all intervals in favour of using DBBM particles. Adding PRP caused a slight tendency towards more frequent lamellar trabeculaes (Table 1), although the differences were not statistically significant (Table 3).

Histomorphometric evaluation

The descriptive statistics of histomorphometric data from all the three evaluated groups are shown in Table 2.

Fig 3 shows the histomorphometric bone area as a function of time for each of the study groups. DBBM and DBBM + PRP showed statistically significant

Table 1 Type of regenerated bone in each study group at different time intervals (described as percentages of newly regenerated trabeculaes).

Study group	12 weeks			8 weeks			4 weeks			2 weeks		
	III	II	I	III	II	I	III	II	I	III	II	I
PRP + DBBM	0	37.5	62.5	0	60	40	0	77.5	22.5	77.5	22.5	0
DBBM	0	40	60	0	62.5	37.5	0	82.5	17.5	82.5	17.5	0
Control	0	70	30	20	80	0	45	55	0	100	0	0

I, lamellar bone; II, lamellar and woven bone; III, woven bone

Table 2 Mean and standard deviation of the percentages of newly regenerated bone and remaining biomaterial particles in each study group at different time intervals.

Time	Study group	Regenerated bone	Remaining DBBM particles
2 weeks	PRP + DBBM	9.60 ± 3.74	62.71 ± 4.98
	DBBM	7.86 ± 1.42	67.27± 4.17
	Control	7.97 ± 2.58	
4 weeks	PRP + DBBM	24.72 ± 4.18	38.82 ± 3.53
	DBBM	15.30 ± 2.65	49.05 ± 4.27
	Control	11.47 ± 2.87	
8 weeks	PRP + DBBM	37.18 ± 4.43	30.06 ± 4.02
	DBBM	33.35 ± 4.25	33.04 ± 4.24
	Control	19.27 ± 2.58	
12 weeks	PRP + DBBM	58.92 ± 5.10	19.69 ± 4.19
	DBBM	57.70 ± 4.74	22.79 ± 4.45
	Control	26.89 ± 3.68	

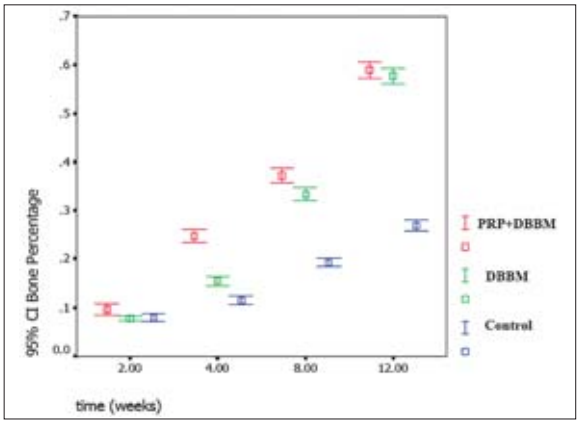

Fig 3 Histomorphometric evaluation of bone area over the 12-week study period.

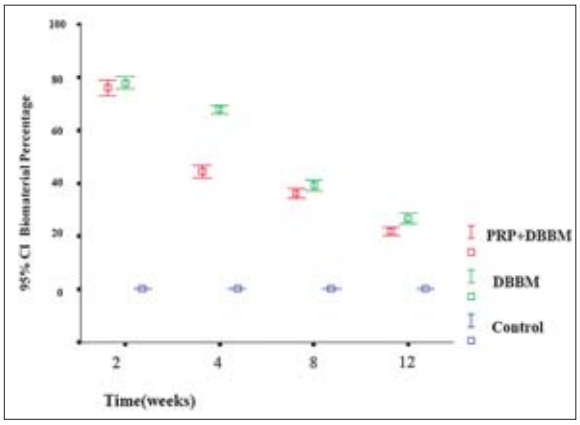

Fig 4 Histomorphometric evaluation of biomaterial area over the 12-week study period.

increases in the amount of regenerated bone over control sites at 4, 8 and 12 weeks ($p < 0.001$). Adding PRP also significantly increased the amount of bone area compared with DBBM alone in all intervals except for 12 weeks. Fig 4 shows the histomorphometric percentage of the remaining biomaterial area as a function of time for each of the experimental groups. DBBM + PRP constantly showed a greater rate of biomaterial degradation in comparison with DBBM alone. The differences were also statistically significant (Table 3).

Table 3 Comparison of the type and amount of newly regenerated bone and remaining biomaterial particles between different study groups at different time intervals.

Time	Variable	Biomaterial percentage	Bone percentage	Type of bone
2 weeks	PRP+DBBM/DBBM	*	*	
	DBBM/control			*
	PRP+DBBM/control			*
4 weeks	PRP+DBBM/DBBM	*	*	
	DBBM/control		*	*
	PRP+DBBM/control		*	*
8 weeks	PRP+DBBM/DBBM	*	*	
	DBBM/control		*	*
	PRP+DBBM/control		*	*
12 weeks	PRP+DBBM/DBBM	*		
	DBBM/control		*	*
	PRP+DBBM/control		*	*

** Statistical significance in 95% confidence interval.*

DISCUSSION

Researchers in oral and maxillofacial surgery continuously strive to improve on current bone-grafting techniques and provide a faster and denser bony regenerate. Growth factors are a realistic way to improve and expedite both soft-tissue and bony wound healing. Platelets contain angiogenic, mitogenic, and vascular growth factors in their granules (Lynch et al, 1999; Weibrich et al, 2003b). Platelets have a physiological store of VEGF, which can be released when platelets are activated, secreted, or aggregated by collagen. TGF-β1 and TGF-β2 have been shown to inhibit bone resorption, as well as to trigger rapid maturation of collagen in early wounds (Maloney et al, 1998). PDGF increases the population of wound-healing cells and recruits other angiogenic growth factors to the wound site (Lynch et al, 1999). It is therefore a reasonable hypothesis that increasing the concentration of platelets in a bone defect may lead to improved, faster healing.

In the present study, the combined effects of using PRP and a xenograft (DBBM) in treating non-critical-sized rabbit cranial defects were evaluated. Histological assessment revealed that DBBM is a biocompatible, osteoconductive grafting material with no sign of foreign body reaction and/or severe inflammation in conjunction with its application. Adding PRP to this material did not affect its biocompatibility. These results are in accordance with Donos et al (2005), Slotte and Lundgren (1999) and Berglundh and Lindhe (1997).

Microscopic assessment with polarised light showed that with increasing time, trabecular bone maturation (from woven bone to lamellar bone) occurs in all experimental groups (including controls), but the amount of lamellar bone was significantly greater in grafted areas compared with controls in all intervals. Philippart et al (2003), Artzi et al (2000) and Maiorana et al (2000) also showed that lamellar bone formation occurs in the areas where PRP and/or DBBM were used.

In the present study, PRP did not significantly increase the proportion of lamellar trabeculaes throughout the study period. In contrast, Marx reported that 4 months after using autogenous bone graft, in combination with PRP, or autogenous bone graft alone, the proportion of mature bony trabeculaes (85% versus 30%) and trabecular bone density (80% versus 59%) were significantly different between the two groups, in favour of using PRP (Marx, 2004). Fundamental differences between autogenous bone and xenogenic bone substitutes, smaller sample size in the present study and also different study periods could be the reason for such a discrepancy between the results.

Histomorphometric evaluation of the amount of newly regenerated bone and remaining biomaterial particles showed that adding PRP to DBBM caused a significant increase in bone formation at 2, 4 and 8 weeks, but the level of bone regeneration had adjusted in PRP (+) and PRP (-) groups at 12 weeks. These findings are in accordance with Aghaloo et al (2004), Wiltfang et al (2003) and Kim et al (2002), but differ from the results of Wiltfang et al (2004), Fuerst et al (2003) and Froum et al (2002). Differences in the study design (animal/human), collection efficiency of the PRP kit, methods of evaluation (radiography/histology) and shape, size and configuration of the defects evaluated have been implicated for such a discrepancy in the results of PRP studies.

Another interesting finding was the ability of xenogenic biomaterial (DBBM) to contribute to physiological bone remodelling as manifested by continuous reduction in the amount of histomorphometric area of DBBM particles between succeeding intervals; a similar finding was reported by Berglundh and Lindhe (1997) and Merkx et al (2000). Additionally, PRP significantly increased the rate of degradation of the biomaterial (DBBM) in all time intervals. Replacement of bovine bone mineral with natural bone appeared to be a slow process (Berglundh and Lindhe, 1997; Skoglund et al, 1997); therefore the later finding of the present study (i.e. acceleration of the process of DBBM degradation by addition of PRP) could be important.

As Marx (a pioneer in the field of PRP) stated, PRP works via the degranulation of α-granules in platelets, which contain the synthesised and prepackaged growth factors. The secreted growth factors bind to the external surface of cell membranes of cells in the graft, flap or wound via transmembrane receptors. Mesenchymal stem cells and osteoblasts are major brands of cells that express these receptors. Using PRP in conjunction with various grafting materials accelerates recruitment of osteoblasts, and perhaps, more realistically, mesenchymal cells to the grafted area, which leads to accelerated cellular proliferation, matrix formation, osteoid and collagen production (Marx, 2004).

It should be mentioned that according to the concept of 'coupling' (inter-dependency of osteoblasts and osteoclasts in bone remodelling), osteoblasts promote formation and activation of osteoclasts (via production of cytokines like IL-1, IL-6 and leukemia inhibiting factor). On the other hand, osteoclasts facilitate osteoblast differentiation (via exposure of osteogenic substrates) (Newman et al, 2002).

Accordingly, it can be postulated that PRP causes an accelerated remodelling process, in which osteoclasts arrive at the grafted area more quickly and invade DBBM particles more effectively (as part of the wound-cleansing process). This phenomenon could be expressed as continuous and accelerated reduction in the histomorphometric area of remaining biomaterial particles, which was seen in the present study (Fig 4).

The sample size was relatively small, consisting of 5 rabbits in each time period of 2, 4, 8, and 12 weeks. On the other hand, a true critical-sized cranial defect in the rabbit model is 15 mm (Vikjaer et al, 1997). Therefore, three critical-sized defects cannot be created in the rabbit cranium due to the small size of the cranium. We chose a non-critical-sized defect to evaluate the early healing, and the potential ability of PRP to improve this early healing when it was added to DBBM particles.

CONCLUSIONS

Within the limitations of this study, it can be concluded that PRP provides additional benefits when xenogenic bone substitute (DBBM with the trade name of Bio-Oss®) was used for bone regeneration in rabbit cranial defects, concerning the speed of bone regeneration (until the eighth week of healing) and the rate of biomaterial degradation throughout the study period.

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