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Effects of Choukroun's platelet-rich fibrin on bone regeneration in combination with deproteinized bovine bone mineral in maxillary sinus augmentation: A histological and histomorphometric study

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ABSTRACT

Purpose: The potential effect of Choukroun's platelet-rich fibrin (PRF) in combination with allograft on promoting bone regeneration has been discussed in previous publications. This study aims to evaluate an influence of PRF on bone regeneration in sinus augmentation in combination with a xenograft, deproteinised bovine bone.

Materials and methods: Eleven sinuses from 10 patients with posterior maxillary bone atrophy were selected for the study. As a test group, six sinus floor elevations were grafted with a Bio-Oss and PRF mixture, and as control group, five sinuses were treated with Bio-Oss alone. Clinical and radiographic examinations were performed pre- and postoperatively. After 6 months of sinus augmentation, bone biopsies were obtained from the grafted posterior maxilla, and un-decalcified ground sections were prepared. Bone characteristics were evaluated using histological observation and histomorphometric analyses.

Results: No adverse effect was observed in any case within the follow-up period of 6 months after sinus augmentation. Histological observation showed similar morphological characteristics for both the PRF and control groups. The percentage of new bone formation in the PRF group was about 1.4 times of that in control (18.35% ± 5.62% vs. 12.95% ± 5.33%), while the percentage of residual bone substitute in the control group was about 1.5 times higher as that in the PRF group (28.54% ± 12.01% vs. 19.16% ± 6.89%). The percentage of contact length between newly formed bone and bone substitute in the PRF group was 21.45% ± 14.57% vs. 18.57% ± 5.39% in the control. No significant statistical differences between the two groups were found in these observed parameters.

Conclusions: Our preliminary result demonstrated neither an advantage nor disadvantage of the application of PRF in combination with deproteinised bovine bone mineral in sinus augmentation after a healing period of 6 months.

1. Introduction

Platelet-rich plasma (PRP) has been increasingly investigated as a potential bioactive substance for improving bone regeneration, not only because it is an easily available autologous material, but also because it contains a high concentration of bioactive proteins (Marx et al., 1998; Zimmermann et al., 2001; Weibrich et al., 2002), which are able to stimulate cell proliferation, angiogenesis, matrix remodelling, and intrinsic bone regeneration in alveolar bone defects (Nevins et al., 2005; Ridgway et al., 2008; Simion et al., 2008). Combinations of growth factors, which are present in PRR, seem to have a synergistic effect on healing processes and tissue regeneration (Lynch et al., 1989; Greenhalgh et al., 1993).

PRP has been shown to promote cell proliferation and expression of osteogenic markers in human osteoblasts in vitro (Kanno et al., 2005; Clausen et al., 2006; Uggeri et al., 2007). However, the effect of PRP on bone regeneration in in vivo studies is contradictory. New bone formation was detected in a PRP grafted canine model in the first 2 months (Gerard et al., 2006; Gerard et al., 2007). In contrast, PRP gel does not enhance the bone healing process in mandibular defects in rabbits (Kazakos et al., 2011). The results of human studies are also inconsistent (Marx et al., 1998; Wiltfang et al., 2003; Raghoebar et al., 2005, Marukawa et al., 2010). Dispute has been focused on therapeutic platelet level (Marx, 2004), intrinsic

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osteoinductive property (Schilephake, 2002), and mostly short-term effects, because of the quickly fading level of bioactive proteins (Schmitz and Hollinger, 2001; Marx, 2004). Because the early effect of PRP on bone regeneration is generally supported (Schlegel et al., 2004; Butterfield et al., 2005; Kasten et al., 2008), new approaches have focused on prolonging the effect of PRP by using different activators (Anitua, 1999; Lacoste et al., 2003; Tsay et al., 2005) and release carriers (Hokugo et al., 2007).

Recently, an autologous platelet- and leucocyte-enriched fibrin matrix called Choukroun's platelet-rich fibrin (PRF) was introduced as a second-generation platelet concentrate (Dohan et al., 2006a). Centrifugation of freshly collected blood without adding any anti-coagulant or thrombin results in the natural formation of a leuko-cyte- and platelet-rich fibrin clot with bioactive proteins trapped inside, which represents a system for slow release of growth factors. In addition, fibrin formation supports cell migration (Dohan

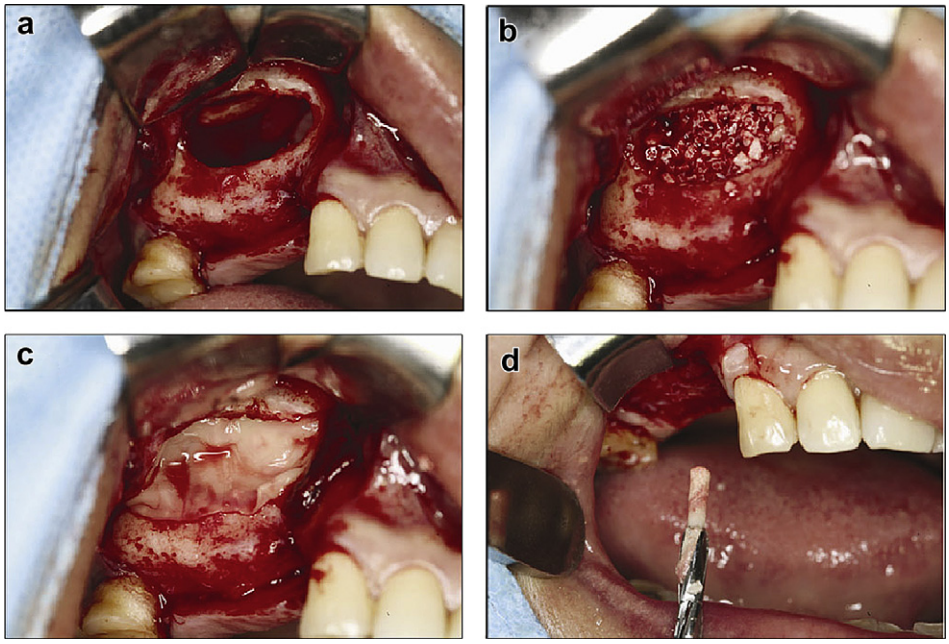

Fig. 1. Surgical procedures of sinus augmentation and removal of biopsy. (a) Osteotomy of lateral wall of sinus and elevated Schneiderian membrane. (b) Mixture of Bio-Oss and PRF was packed into the augmentation area. (c) The osteotomy window was covered with PRF membrane. (d) Biopsy was taken after 6 months.

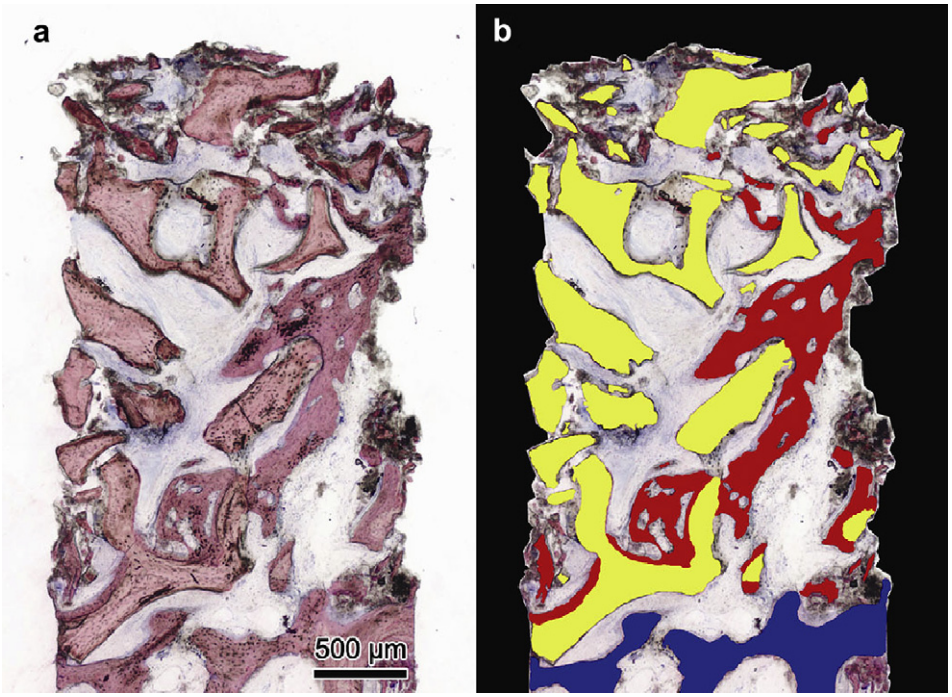

Fig. 2. Histology and histomorphometric evaluation of the biopsy section. (a) Un-decalcified ground section stained with Levai-Laczko stain. (b) Interactive colouring of different structures: local host bone (blue), Bio-Oss (yellow) and newly formed bone (red).

Ehrenfest et al., 2009a). In a human sinus augmentation study (Choukroun et al., 2006), PRF accelerated bone production induced by the application of freeze-dried bone allograft (FDBA). The results implied that a bone substitute in combination with PRF is a promising approach to the acceleration of bone regeneration.

To date, with the exception of FDBA, no study has been done on the adjuvant effect of bone substitutes in combination with PRF. Bio-Oss®, which is deproteinised bovine bone mineral, is a well-documented bone substitute used in dentistry (Piattelli et al., 1999; Valentini et al., 2000; Traini et al., 2007) and one of the most widely used bone substitutes, used both alone and in mixtures for sinus augmentation (Valentini et al., 1998; Yildirim et al., 2000; Hallman et al., 2001; Yildirim et al., 2001). It has been shown to be biocompatible, osteoconductive, and is considered to be slowly resorbable (Artzi et al., 2001; Simion et al., 2007). In the present study, we applied PRF in combination with Bio-Oss in sinus augmentation to evaluate the potential effect of PRF on bone regeneration.

2. Materials and methods

2.1. Patients

For this study, 10 patients (two female and eight male) were selected, each having been diagnosed radiographically to exhibit maxillary bone atrophy with a residual crest height of less than 5 mm. The sinuses were randomly assigned either to the experimental group consisting of six sinuses from six patients (mean age, 43.5 years; range, 30–49), or the control group containing five sinuses of five patients (mean age, 46.2 years; range, 37–53). The study was conducted in accordance with the standards of the Declaration of Helsinki (2002) and was approved by the Ethical Board of Peking University (Nr. IRB00001052-0719). Patients were informed about the study protocols and given written consent. Exclusion criteria included blood platelet disorders, medication with aspirin before surgery, infectious and metabolic diseases, and radiotherapy, as well as acute and chronic maxillary sinus inflammation.

2.2. PRF preparation

PRF was produced using an established technique (Choukroun et al., 2006) and the patients' peripheral blood samples were taken at the beginning of the operation. Immediately after drawing blood, the vacutainers (BD, Franklin Lakes, NJ, USA) were centrifuged at about 300*g* for 10 min (Labofuge 300, Kendro Laboratory Products GmbH, Osterrode, Germany). After coagulation, each PRF clot was prepared in fragment or membrane forms.

2.3. Sinus augmentation

All surgery was performed with local anaesthesia, and the sinus augmentation followed the lateral wall protocol. In brief, after a buccal mucoperiosteal flap was raised, an osteotomy was prepared in the lateral wall of the maxillary sinus. After the Schneiderian membrane was carefully elevated, the grafting material was packed into the space between the sinus floor and the Schneiderian membrane. A mixture of deproteinised bovine bone mineral (Bio-Oss®, Geistlich Pharma AG, Wolhusen, Switzerland) and PRF preparation was inserted in sinuses of the test group. The PRF membrane was covered on the access window before flap closure (Fig. 1). In the control group the sinuses were grafted with Bio-Oss alone and covered with mucosa just as in the test group. Dental implants (Replace, Nobel Biocare) were placed 6 months after sinus augmentation. To standardize procedures all operations were performed by one surgeon with a longstanding experience with the technique.

During the second stage of implant placement, biopsies were taken from the alveolar crest using a trephine bur 2 mm in diameter and 13 mm in length (Fig. 1d). Altogether, six samples of the PRF group and five samples of the control group were obtained. Core biopsy samples included parts of pristine alveolar crest bone as well as grafted areas of the implant site. Implants were then inserted at the sites where the biopsies had been taken. The samples were fixed immediately in buffered 4% formalin at 4 °C for histological preparation.

2.4. Clinical and radiographic evaluation

Clinically, all patients were followed up postoperatively after the first week, the first month, 3 months, and 6 months. The clinical evaluation included assessment of complications during surgery and postoperative healing (inflammation, wound dehiscence, and loss of grafted bone particles). Orthopantomograms (OPT) or dental computed tomography scans were taken immediately after operation as well as postoperatively after 3 and 6 months. Radiographic evaluation focused on the amount and density of mineralized tissue present in the sinus augmentation area.

2.5. Histology and histomorphometry

Biopsy samples were treated using Donath's technique (Donath, 1988) for the preparation of thin ground sections. Samples were dehydrated in ascending grades of alcohol and embedded in a light-curing resin (Technovit® 7200 VLC + BPO; Kulzer & Co., Wehrheim, Germany). Histological ground sections with a thickness of 20–30 μm were produced with the Exakt Cutting and Grinding Equipment (Exakt Apparatebau,

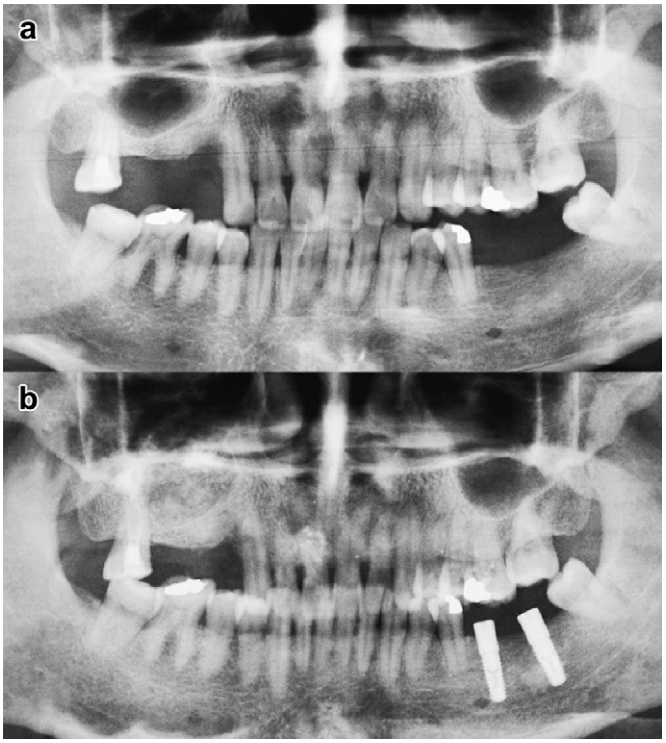

Fig. 3. Orthopantomogram examination of a case with PRF/Bio-Oss mixture grafted sinus (a) before operation and (b) 6 months after operation.

Norderstedt, Germany) and stained with the Levai-Laczko stain. All sections were analysed with a light microscope to describe the structure, quality, and composition of bone and the bone substitute. In addition, dynamic processes like resorption and new bone formation were evaluated.

Histomorphometric analyses were performed as described previously (Jakse et al., 2007). The ground sections were photographed with a digital camera (Nikon DXM 1200; Nikon Corp., Tokyo, Japan) mounted on a microscope (Nikon FXA; Nikon Corp.). The images depicted the entire surface of the biopsy at a resolution of 1.12 μm/pixel (i.e., 1 mm is equivalent to 890 pixels). The region of interest (ROI) was defined as the augmented area formed by the sinus elevation procedure, upward from the sinus floor. Mature bone of the alveolar crest present before the surgical intervention was excluded. The histological structures of interest, i.e., Bio-Oss and the newly formed bone, were interactively retraced in different colours with Adobe Photoshop CS2 (Adobe, San Jose, CA, USA) (Fig. 2). The areas of these structures (in mm^2) and contact length between Bio-Oss and newly formed bone (in mm) were measured with the morphometry program Definiens XD Version 1.1.1; Build 1199 (Definiens, Munich, Germany). From these primary measurements, the percentage of newly formed bone in the region of interest (BV/TV), the percentage of remaining bone substitute material (BSV/TV), and the percentage of the contour length of the bone substitute material in contact with new bone (bone-to-bone substitute contact) were calculated.

2.6. Statistics

All the parameters have been presented as a mean and standard deviation. Significant differences between the PRF and control groups were evaluated by Student's *t*-test.

3. Results

3.1. Clinical and radiographic observation

Healing processes under routine clinical examination during the first 6 months were uneventful for all patients and locations. Postoperative radiographic evaluation revealed the presence of mineralized tissue (bone and bone substitute) adequate in amount and density in all cases. There were no obvious signs of resorption (Fig. 3).

3.2. Histology

Biopsies of the PRF and control groups showed a very similar composition and distribution of histologic structures (Fig. 4). In both groups, no significant signs of an inflammatory reaction could be detected. The Bio-Oss particles were distributed homogenously within the augmented area.

The formation of new bone bridging the gaps between Bio-Oss particles was observed in samples of both groups. The newly formed bone was characterized as woven bone in contrast to the

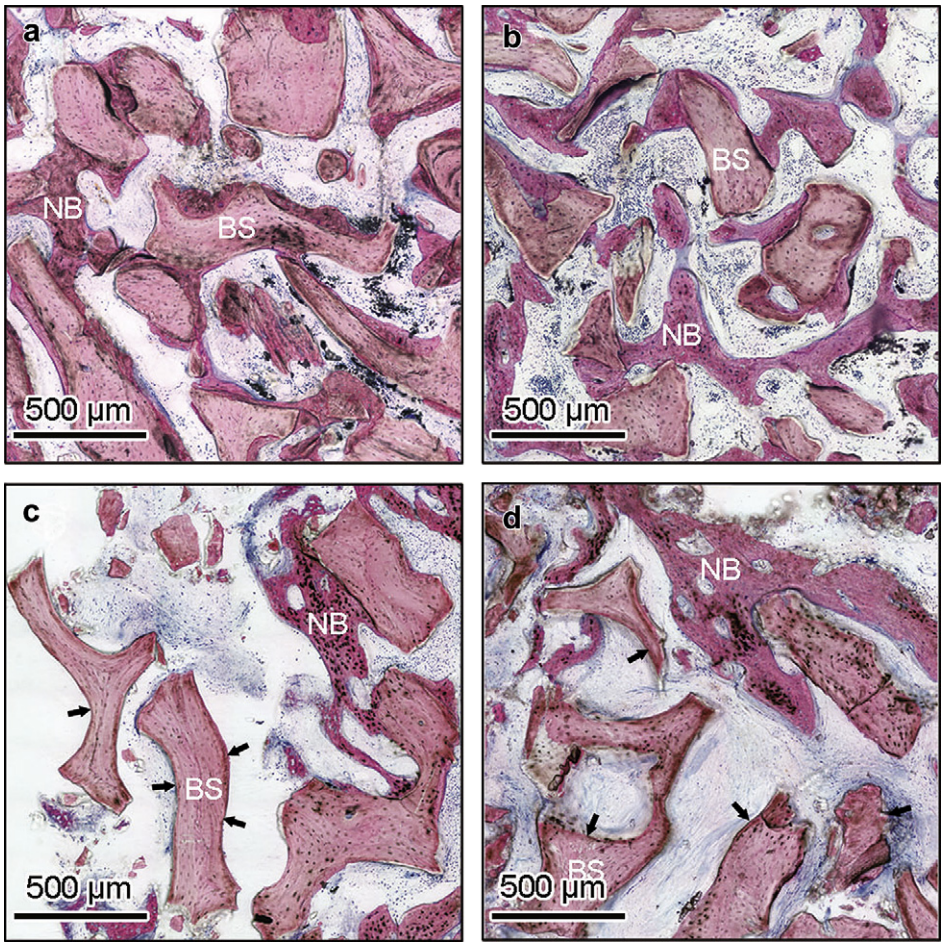

Fig. 4. Histologic images of control (a, c) and PRF (b, d) specimens. New bone can be observed on the surface of Bio-Oss. Bio-Oss showed very good contact with new bone in some areas (a, b) and very poor contact (arrows) in others (c, d). This phenomenon was present in the control and the PRF group. Levai-Laczko stain. NB: newly formed bone; BS: Bio-Oss.

mature skeletal tissue of the alveolar crest, consisting of lamellar bone. There was still on-going formation of woven bone (Fig. 5c) as well as resorption of mineralized tissue, especially of Bio-Oss particles. There were also more blood vessels distributed in regions with large amounts of newly formed bone. In some areas, Bio-Oss particles were intact with woven bone showing signs of slight resorption (clear seams and osteoclasts; Fig. 5c–e). In areas with poor bone formation, mostly situated at a greater distance from the sinus floor (Fig. 4c and d), the predominant histological structure was fibrous connective tissue, while blood vessels were scarce. In such regions, most of the Bio-Oss particles were covered and surrounded by connective tissue fibres (Fig. 5a and b). No obvious resorption and osteoclasts were found on the surface of these particles.

3.3. Histomorphometry

The effect of PRF on new bone formation was evaluated by measuring the amount of newly formed bone and residual bone substitute (Bio-Oss) in the region of interest (Figs. 6 and 7). The percentage of newly formed bone in PRF group was about 1.4 times that of the control group (18.35% ± 5.62% vs. 12.95% ± 5.33%). The percentage of residual bone substitute (Bio-Oss) in the PRF group was 19.16% ± 6.89%, while in the control group it was about 1.5 times that of PRF (28.54% ± 12.01%). No statistically significant difference between groups was found in either of the observed parameters ($P = 0.138$, $P = 0.141$). There was also no significant difference between the two groups concerning the percentage of the contour length of the bone substitute material in contact with new bone. Bone-to-bone substitute contact was 21.45% ± 14.57% in the PRF group and 18.57% ± 5.39% in the control group (Fig. 8).

4. Discussion

PRF is regarded as a promising biomaterial that ensures the slow release of growth factors and contains a strong fibrin matrix (Dohan Ehrenfest et al., 2009a). For instance, Dohan Ehrenfest et al. (2009b) showed a significant slow release of growth factors such as transforming growth factor (TGF-β1), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF)-AB from PRF membrane for 7 days. Considering the remarkable difference in ratios between slowly released and immediately extracted growth

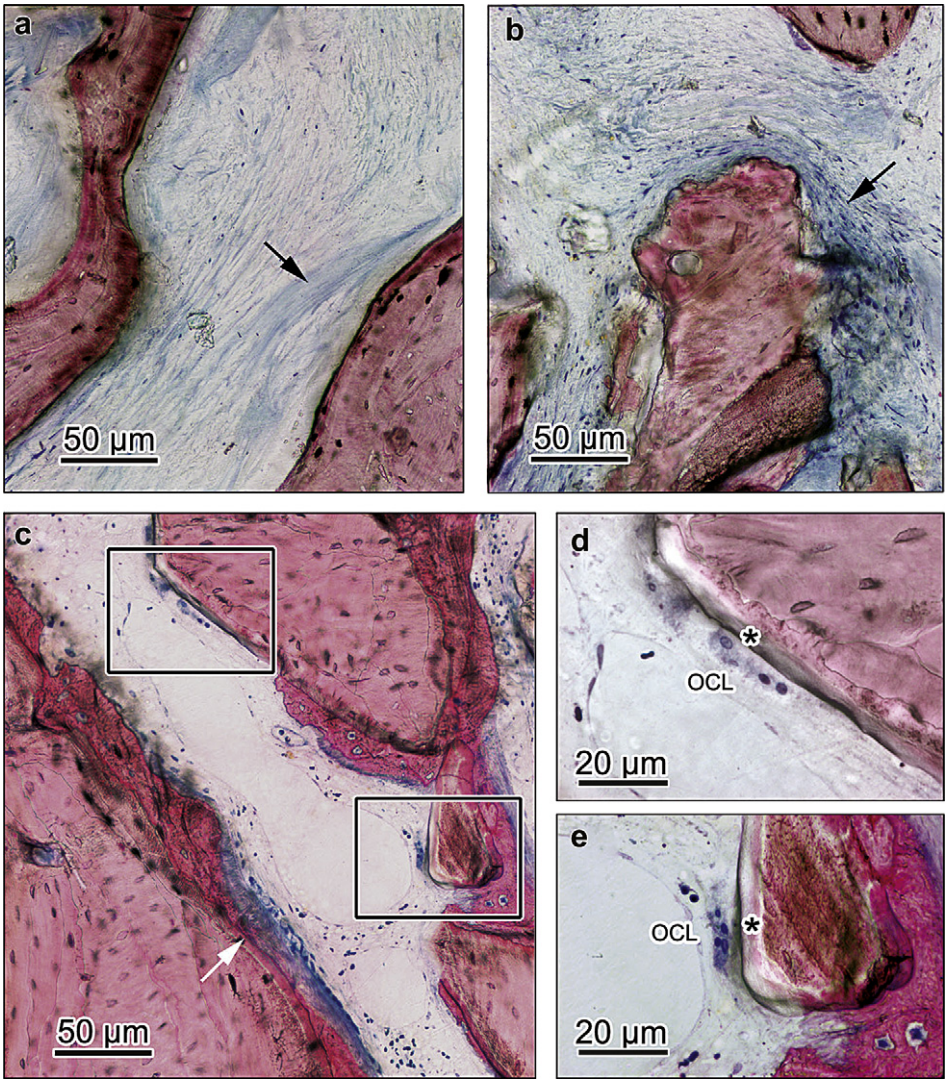

Fig. 5. Histologic details of osseointegration, bone formation and resorption of bone substitute. In areas (a, b) where no contact between Bio-Oss and newly formed bone developed, surfaces were covered with fibrous tissue (black arrows). Large vessels with very thin walls were present in regions with bone formation (white arrow) (c) and bone substitute resorption (d, e). Osteoclasts (OCL) attached to the surface of bone substitute. Clear seams (*) are a sign of resorption. Levai-Laczko stain.

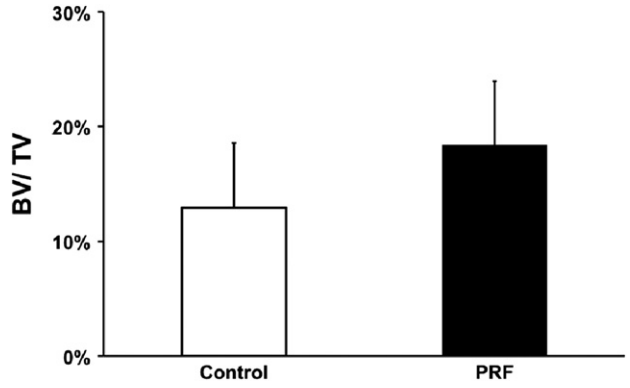

Fig. 6. Percentage of bone tissue in the augmented area (BV/TV).

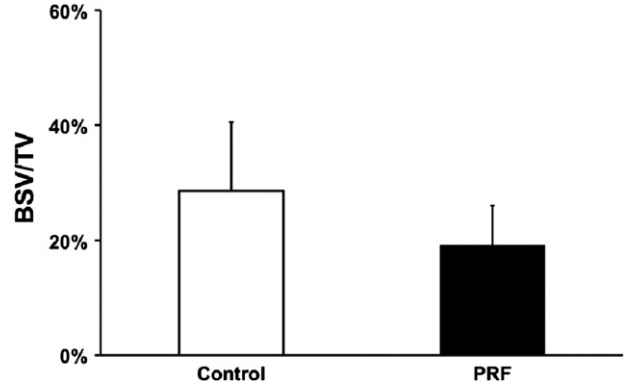

Fig. 7. Percentage of bone substitute in the augmented area (BSV/TV).

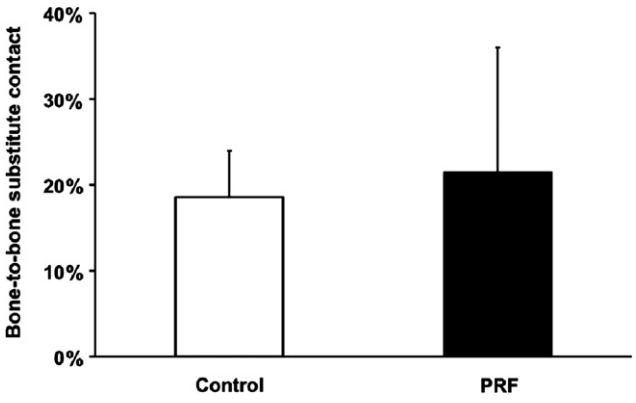

Fig. 8. Percentage of the bone substitute contour in contact with newly formed bone (Bone-to-bone substitute contact).

factors, leucocytes were considered to play a key role in the slow release of TGF-β1 and VEGF. Compared to PRP, the amounts of TGF-β1 and PDGF-BB measured in supernatant and exudates from PRF were significantly lower (Dohan et al., 2006b), which indicates that PRF platelet cytokines were trapped in the fibrin meshes and would be released during matrix remodelling. Fibrin was applied as a delivery system of growth factors in tissue engineering. It has also been reported that fibrin prolonged the release of BMP-2 as its carrier (Schmoekel et al., 2004). A recent study in which the degradation of growth factors in wounds was mimicked by trypsin treatment showed that the protection of fibrin might result in limited proteolysis of TGF-β1 and PDGF-AB (Lundquist et al., 2008). Moreover, fibrin has a significant effect on collagen synthesis of osteoblast-like cells (Kawase et al., 2003). It was emphasized that the synergetic activities between cytokines and fibrin matrix were much more important than the cytokine release pattern alone (Dohan and Choukroun, 2007).

In vitro, PRF significantly improved proliferation of human osteoblasts in a dose-dependent manner, and the expression of alkaline phosphatase activity was enhanced in a time-dependent manner with PRF (Dohan Ehrenfest et al., 2009c). The authors supposed that the growth factors and fibrin matrix structure might be responsible for those biological events. Nevertheless, in contrast to in vitro studies, only limited data from clinical investigations about the effect of PRF on bone regeneration are available (Choukroun et al., 2006). To our knowledge, the synergistic effect of PRF and xenograft in sinus augmentation has not been studied. In the present study, we focused on the effect of PRF in combination with Bio-Oss in sinus augmentation 6 months after the operation. The percentage of newly formed bone and residual bone substitute in the control group (12.95% and 28.54%, Figs. 6 and 7) matched with previous data (14.7% and 29.7%) in a similar model (Yildirim et al., 2000). In the PRF group, the percentage of newly formed bone (18.35%) is slightly higher than that in control (12.95%), while the percentage of residual bone substitute appeared lower in the experimental group (19.16%) compared with the control (28.54%). The percentage of contact length between newly formed bone and bone substitute in both groups (PRF: 21.45%, control: 18.57%) in the present study seems to be lower compared to that in the previous study (29.1%) (Yildirim et al., 2000). However, no statistically significant difference was determined in any of the parameters observed in the present study.

Histologically, Bio-Oss showed good contact as well as poor contact with newly formed bone in both groups. In regions where good contact between newly formed bone and Bio-Oss developed, well-vascularized vital bone marrow was present. In areas with poor contact, Bio-Oss particles were surrounded by fibrous tissue containing only a small number of blood vessels. The resorption of Bio-Oss in both groups appeared to be a similar process with the presence of "clear seam" on the surface, as reported by Valentini et al. (2000).

In clinical studies, Choukroun et al. (2006) demonstrated a potentially synergistic effect of PRF adjunction to FDBA, increasing the graft volume without changing the maturation quality in new bone. However, our study showed that PRF addition to Bio-Oss has no significantly synergistic effect on neither new bone formation nor on the graft volume. These different results could be due to different bioactive properties of these two graft materials. A different study indicated that new bone formation declined with increasing distance from the host bone (Busenlechner et al., 2009). In the present study, poor bone formation was mostly situated at a greater distance from the sinus floor, which is in line with these data. It implies that the sinus floor plays an important role in bone regeneration as the source of precursor cells. PRF mixed with Bio-Oss might stimulate migration of precursor cells to situ by a lesser extent than PRF mixed with FDBA. The absence of precursor cells could be a reason of the lacking effect of PRF mixed with Bio-Oss on the bone formation. This hypothesis is supported by a finding that bone regeneration in the rat is accelerated after transplantation of bone marrow cells (Qi et al., 2006). Additionally, Bio-Oss is a slowly resorbable bone substitute (Artzi et al., 2001; Simion et al., 2007). Probably the slow resorption property retards the replacement of new bone formation. The resorption of Bio-Oss appeared to be a similar process with the presence of a "clear seam" on the surface, as reported by Valentini et al. (2000). Histologically, Bio-Oss particles were mostly intact, only partly replaced by newly formed bone. FDBA and Bio-Oss are derived from different sources and as carrier substances they may provide different structural foundations for further cellular and molecular events in bone regeneration. Therefore, to support our

hypothesis, further study is necessary to compare the synergistic effects of PRF addition to different grafts in vitro and in vivo.

5. Conclusion

In summary, our preliminary results have demonstrated neither advantage nor disadvantage in the application of PRF in combination with deproteinised bovine bone mineral in sinus augmentation, when evaluated after a healing period of 6 months.

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