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COMPARATIVE STUDY OF β-TRICALCIUM PHOSPHATE MIXED WITH PLATELET-RICH PLASMA VERSUS β-TRICALCIUM PHOSPHATE, A BONE SUBSTITUTE MATERIAL IN DENTISTRY

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Animal experiments were carried out with osteoconductive bone substitute β-tricalcium phosphate (β-TCP), with the aim of assessing the effects of the growth factors synthesised by thrombocytes on the speed of β-TCP incorporation and on the quality of newly formed bone. The question to be answered was the extent to which platelet-rich plasma (PRP) accelerated the resorption of β-TCP and the formation of new bone. Two teeth were removed symmetrically from each side of the mandible of 12 Beagle dogs; the resulting cavities were filled on one side with β-TCP alone, and on the other side with a mixture of β-TCP + PRP (obtained from autologous blood). The quality of the newly formed bone and the effects of this PRP were studied by histological and histomorphometric methods. In week 6, bone formation was already more effective when PRP was applied in comparison with β-TCP alone, and in week 12 the growth was significantly greater. The results demonstrate that the use of PRP accelerates the remodelling of new bone created by β-TCP.

Key words: Bone substitution, platelet-rich plasma, β-tricalcium phosphate, remodelling, histomorphometry

During the past decade, the development of oral and maxillofacial surgery has increased the need for the substitution of lost bone (Gera et al., 2002). Various substances of human or animal origin or synthetic materials may be used to fill bone defects (Sonis et al., 1985).

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However, none of the methods employed at present are perfect. The best results can be achieved with autologous bone grafts from the aspect of bone transformation. The procedure called 'bone-grafting' by clinicians is in fact bone marrow transplantation. The aim of this procedure is to obtain osteocomponent cells from the bone donor. These cells are fundamentally endosteal osteoblasts and medullary substance cells (Caplan, 1987). The graft contains these osteocomponent cells and mineralised network-substance islets, together with fibrin from the coagulated blood and platelets. The endosteal osteoblasts and medullary substance cells survive the first 3–5 days because of their surface location, and because they are able to take up nutrients from the recipient tissue via simple diffusion. The osteocytes of the mineralised network substance die: when they are embedded in the mineral material, they are shut off from the nutrients. The use of autologous bone has the great disadvantages of the donor area and the stress on the patient, with the possibilities of complications (Stanford, 1987).

Bio-Oss, an organic bone-substitute of bovine origin, has proved to be a very popular product (Benke et al., 2001). Histological examinations have confirmed that similar processes of incorporation occur like in autologous bone. It has been stated that particles of Bio-Oss undergo only a very slow transformation and still can be detected 7 years after implantation (Schlegel, 1996), whereas other authors have described a perfect transformation following the passage of a longer time (Berglund and Lindhe, 1997). No sensitivity to bone substitutes of bovine origin has been reported yet. In consequence of the fear of bovine spongiform encephalopathy, however, nowadays there is a reluctance to utilise materials of bovine origin (Hönig et al., 1999). This may also be the reason why interest in synthetic bone substitutes has increased so significantly in recent years (Frame et al., 1981). Animal experiments and clinical results have revealed that β-tricalcium phosphate (β-TCP) has the advantages that neither β-TCP itself nor its breakdown products are toxic, and it does not contain viruses, prions, or other organic agents (Velich and Szabó, 2002). The new bone tissue grows into its continuous porous structure.

It breaks down synchronously with the bone transformation, and it is converted quantitatively to bone (Foitzek and Stamm, 1997). Its resorption is connected to non-cellular elements by chemical dissolution. It is tissue-friendly and its transformation is not accompanied by inflammatory symptoms. The question arises whether the incorporation (remodelling) of β-TCP can be enhanced by the addition of growth factors that promote ossification. Growth factors permit faster bone regeneration and the development of more solid, denser bone (Carlson, 2000). Investigations and observations relating to the addition of platelet-rich plasma (PRP) to bone grafts have demonstrated the early solidification of the graft, a doubling of the mineralisation rate, and a 15–30% improvement in trabecular bone density (Marx, 1999). PRP is an autologous platelet concentrate prepared by centrifugation. Fibrin network of gel form can be created from PRP af-

ter thrombin and calcium administration, providing more effective usage of PRP. There is excessive release of different growth factors from the platelets of PRP. Recently platelet-derived growth factor (PDGF) and transforming growth factors β_1 and β_2 (TGF-β) are thought to be the most important factors released from these thrombocytes (Ross et al., 1986). The growth factors produced by the platelets are the first factors that appear in the wound to stimulate bone regeneration. The most important specific effects of PDGF is the activation of mitogenesis, angiogenesis and macrophages (Antoniades and Williams, 1983). 10^6 platelets contain approximately 0.06 ng PDGF, i.e. ca. 1200 molecules per platelet (Sonnleitner et al., 2000). This large amount of PDGF much more induces the activity of osteogenic cells than the graft and the coagulum (Ross et al., 1986). Additionally, it is supposed that the dense fibrin network produced by the PRP promotes osteoconduction via solidification of the graft.

TGF-β is a member of the bone morphogenic protein (BMP) family of growth and differentiation factors (Celeste et al., 1990). The proteins (TGF-β_1 and TGF-β_2) are general growth factors participating in general connective tissue repair and in bone generation (Roberts and Spron, 1993). These TGF-β proteins are parts of a system that maintains a long-term healing and bone-regenerating mechanism and they have an important role in the regulation of bone remodelling. The most important functions of TGF-β_1 and TGF-β_2 are the chemotaxis and mitogenesis of osteoblast precursors, and enhancement of collagen matrix deposition during wound or bone healing. Furthermore, they inhibit osteoclast formation and bone resorption, therefore they promote bone formation rather than resorption. Since PRP is an autologous preparation, this excludes disease transmission or allergic reactions which can possibly occur with allogenic or endogenous preparations.

Materials and methods

In the present experiment, β-TCP, a bone substitute material with osteoconductive effect used for bone replacement was compared with a mixture of β-TCP and PRP (a thrombocyte concentrate obtained from autologous blood) from the aspect of effectiveness for bone augmentation in Beagle dogs. The formation of new bone, i.e. the remodelling of these bone substitutes, was evaluated by histological and histomorphometric methods (Szabó et al., 2001). Ten adult Beagle dogs, aged 2–3 years, were involved in the study. They were obtained from a special breeding unit, where they had undergone regular immunisation and deworming. They were housed in individual cages with a floor area of 1 m × 2 m in an isolated animal house. Following the study, all the dogs were adopted. As prophylaxis, dental calculus was removed and a state of excellent oral hygiene was achieved: a daily dose of 0.2 ml/kg body weight of Baytril /enrofloxacin 5% (Bayer) was administered for 7 days. Prior to surgery, the dogs were anaesthe-

tised intramuscularly with a combination of 20–30 μg/kg Domitor/medetomidine hydrochloride (Pfizer), the dose depending on the behaviour of the animal, and 0.2 mg/kg Butomidor/Butorphanol (Richter-Pharma AG). Since the operation and the immediate postoperative period involved pain, a daily dose of 0.6 mg/kg Butorphanol was administered intramuscularly, for 7 days postoperatively.

Surgery

Before the intervention, 5 ml of venous blood was obtained with a Curasan separation kit for the preparation of PRP. The blood anticoagulated with Na-EDTA was centrifuged at 2400 rpm for 10 min at room temperature (Hettich EBA 8S). The supernatant (plasma containing platelets) was removed and re-centrifuged at 4000 rpm for 10 min. Following removal of the supernatant, the thrombocyte pellet that remained in the tube was suspended in 0.3 ml plasma with the aid of a test tube shaker (Vortex Genie).

Under appropriate depth of narcosis the animal was fixed in lateral position, and premolars 2 and 3 were extracted on both sides. After the extraction, a bed drill 3 mm in diameter was used to prepare the final bone defect 4 mm deep. Physiological saline solution was utilised for cooling. The cavity on the right side in the mandible was filled with β-TCP (Cerasorb, Curasan, 500–1000 micron particle size), while the left side was filled with a mixture of the same β-TCP and PRP. The wound was closed in two layers with resorbing sutures (Vycril, Braun). The dogs were fitted with a collar and the conditions for undisturbed healing were provided: the animals received presoaked canine maintenance feed (Hills).

Clinical follow-up

Following the intervention 12 during weeks, daily the general condition of the dogs and the augmented areas were inspected in order to establish whether the presence of the material implanted could still be recognised.

Preparation of histological samples

In the 6th and 12th weeks following the intervention, histological samples were taken from the dogs. Under anaesthesia, a cylinder of bone 3 mm long was removed from each of the augmented areas with a bone trephine 4 mm in diameter (Meisinger). The tissue sample was fixed in 10% neutral formalin and embedded in Durcupan resin, and sections were then prepared with a Diatome. The sections were stained with toluidine blue, haematoxylin-eosin and Goldner trichrome stain. These stained sections were examined under the microscope (Olympus 2000) at magnifications of 10×, 20× and 40×. Subsequently, a histomorphometric evaluation was performed: the areas of mineralised matrix, the bony tissue and the soft tissue in the region of the augmentation were expressed as percentages of the total area. The data of tissue samples from both sides in the

individual animals, and the changes in them, were compared. Statistical evaluation of the results was carried out with one-side, two-sample paired Student's *t*-test.

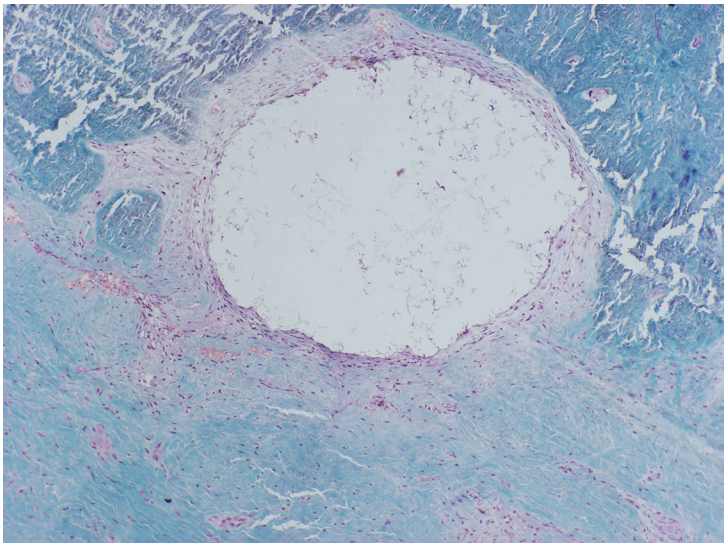

Fig. 1. Six weeks after β-TCP implantation. Rounded Cerasorb granule surrounded by a fibrous encapsulation. No peripheral or internal osteoid bridges can be seen. Granules are partly dissolved by the acidic staining. Goldner's trichrome staining, original magnification × 10

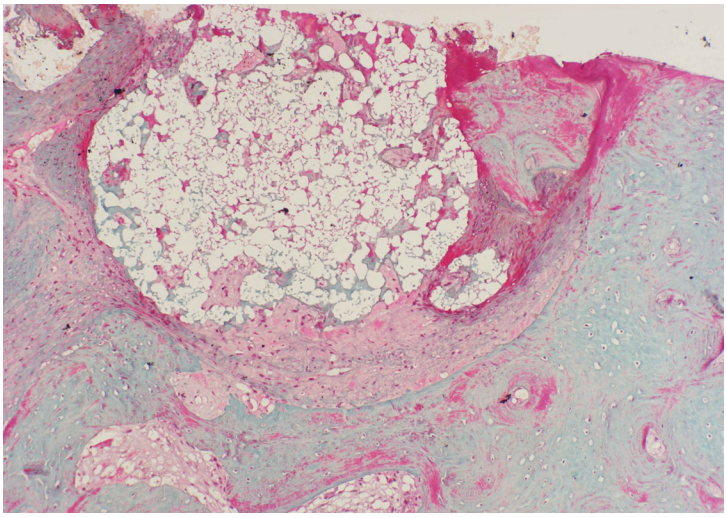

Fig. 2. Six weeks after combination of β-TCP and Platelet Rich Plasma implantation. β-TCP granule is surrounded by cell-rich mesenchymal tissue. Connective tissue islands and bridging is characteristic in the pores of the granule. Goldner's trichrome staining, original magnification × 10

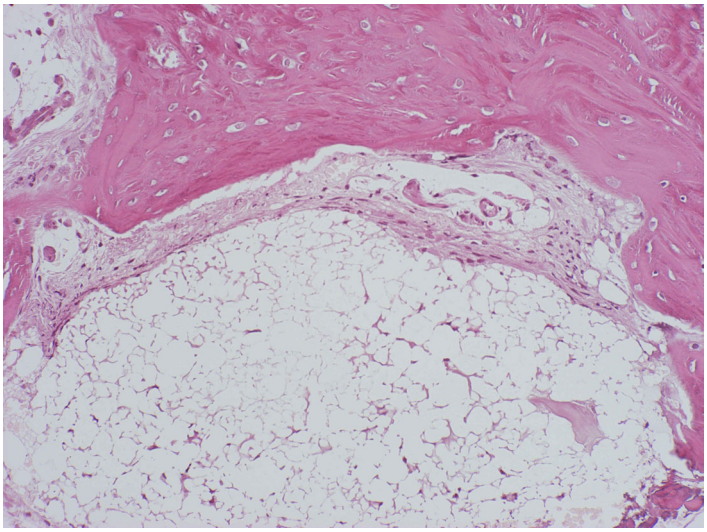

Fig. 3. Twelve weeks after β-TCP implantation. Osteogenic mesenchyma and newly formed bone with osteocytes in the vicinity of the β-TCP granule. Haematoxylin and eosin staining, original magnification × 40

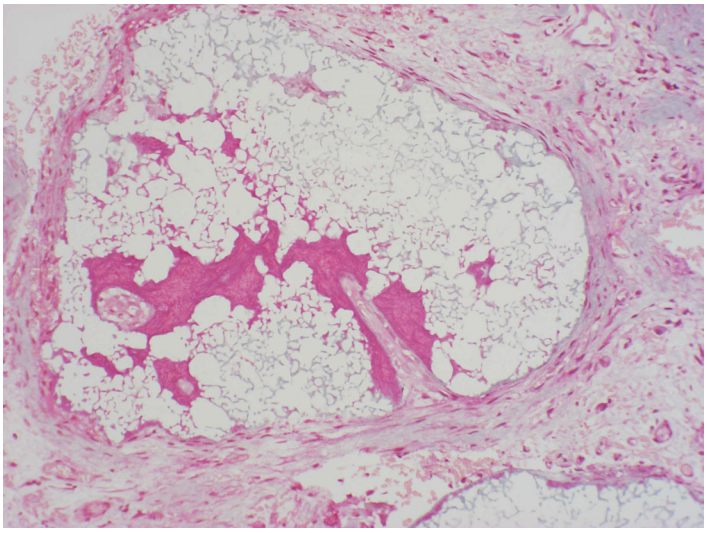

Fig. 4. Twelve weeks after combination of β-TCP and Platelet Rich Plasma implantation. Intra-granular budding of the richly capillarised osteogenic mesenchyma and osteoid bridges in the pores of the granule. Goldner's trichrome staining, original magnification × 20

Results

Clinical evaluation

After a 6-week healing period, numerous β-TCP granules could be clearly recognised with the naked eye under the mucosa-covered area. At week 12, only scattered particles were visible.

Examination with a dental probe showed the formation of new hard bone tissue in every defect after a healing time of 12 weeks. After removal of the mucosa and the periosteum, no obvious difference could be discerned with the naked eye between the cavities filled with β-TCP or with mixture of β-TCP+PRP. The sites of the filled defects could be identified only by measurement of the distance from the cuspid.

Evaluation of histological preparations

When only β-TCP was implanted, the granules were rounded and had a porous structure. In their neighbourhood, osteoid tissue in close connection with the granules could be seen. This confirms the biocompatibility and osteoconductive nature of β-TCP (Figs 1 and 2). In case of β-TCP+PRP implantation, red-staining tissue bridges with a homogeneous structure corresponding to osteoid, without cellular activity, could be seen inside in the granules. Bone with trabecular structure was observed in the adjacent areas, which was indicative of the formation of matured new bone. There was no detectable osteoclast activity in the area of the implant, showing that β-TCP granules were eliminated by physical resorption (Figs 3 and 4).

Histomorphometric evaluation

The histomorphometric procedure was applied to quantify the new bone formation of the various individual areas in the histological sections. The results are shown in Tables 1 and 2. At 6 weeks, there was no significant difference between the samples gained from the two sides (44.7 vs. 52.97; $p = 0.137$). At 12 weeks, however, the difference was statistically significant (51.09 vs. 65.06; $p = 0.033$). At week 6, the ratio of hard tissue area/soft tissue area in the case of the β-TCP+PRP graft was 52.97/40.27, while in the case of single β-TCP graft it was 44.77/52.13, showing the higher ratio of hard tissue in the PRP-containing sample. There was further increase in the ratio of hard tissue in the PRP-containing graft but not in the β-TCP graft (65.06/30.45 vs. 51.09/42.61, respectively), at week 12.

Table 1

The ratio of hard tissue area and soft tissue area at week 6

No.	β-TCP side		β-TCP+PRP side	
	Bone area %	Soft tissue %	Bone area %	Soft tissue %
1	41.63	58.37	31.68	68.27
2	43.91	56.08	38.75	53.39
3	17.41	78.48	27.65	62.50
4	42.64	57.35	53.57	37.42
5	67.94	32.06	76.75	7.47
6	17.73	75.33	81.41	18.58
7	26.08	63.26	32.17	61.76
8	56.05	35.59	35.03	45.94
9	64.16	35.84	72.50	27.51
10	70.15	28.99	80.15	19.85
Mean	44.77	52.13	52.97	40.27

Table 2

The ratio of hard tissue area and soft tissue area at week 12

No.	β-TCP side		β-TCP+PRP side	
	Bone area %	Soft tissue %	Bone area %	Soft tissue %
1	38.96	46.68	15.52	84.49
2	37.22	53.81	40.72	42.86
3	48.55	51.45	64.69	28.84
4	21.93	54.64	41.32	53.47
5	57.92	28.95	95.83	4.17
6	78.00	21.00	58.15	33.07
7	39.82	60.18	64.87	35.13
8	60.22	34.64	87.10	4.80
9	67.71	35.36	90.53	9.47
10	60.54	39.41	91.84	8.16
Mean	51.09	42.61	65.06	30.45

Discussion

These animal experiments were planned to study the effects of PRP-containing numerous factors belonging to the family of BMP, on the incorporation of a bone substitute material with osteoconductive effect. We compared the speed of the bone remodelling process and bone transformational tendency of β-TCP after artificial bone substitution with β-TCP alone and in the presence of PRP.

The examined bone substitute was applied with good results in human oral surgery for sinus elevation and to fill up mandibular and maxillary bone defects that developed due to atrophy cyst or trauma (Foitzek and Stamm, 1997; Szabó et al., 2001).

The chosen experimental animal was the Beagle dog, although it has a different regeneration ability from that of humans (Sonis et al., 1985). The results can form the basis of further human research in the future. This bone substitution material is very rarely used recently in small animal dentistry, but its excellent properties may make it popular soon among veterinary surgeons.

The clinical examination showed that the bone defects were completely filled up with newly formed bone both in β-TCP and β-TCP+PRP grafts after a healing time of 12 weeks. In every sample, β-TCP particles could be recognised, but the quality of the new tissue proved to be good. In every defect, histological and histomorphometric examinations demonstrated a picture corresponding fundamentally to bone tissue. In cases of β-TCP+PRP grafts, the β-TCP particles in the sections exhibited a greater degree of transformation than without PRP.

The present findings permit the conclusions that PRP accelerates the bone transformation of β-TCP more than β-TCP does when applied alone. Further research is necessary, however, to establish what changes occur after a healing period longer than 12 weeks. These results suggest that the application of bone substitute material in animal dentistry can help to save the teeth of animals which would be lost due to periodontitis, cyst formation or trauma without treatment. Furthermore, the application of PRP in these treatments can increase the efficiency of new bone formation. It is hoped that the use of this excellent synthetic bone substitute will become increasingly widespread in the treatment of animals, too.

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