HISTOLOGICAL AND MORPHOMETRIC ASPECTS OF RIDGE PRESERVATION WITH A MOLDABLE, *IN SITU* HARDENING BONE GRAFT SUBSTITUTE

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Abstract - Biphasic calcium phosphates (BCP) are widely used in alveolar ridge regeneration as a porous scaffold for new bone formation. The aim of this case series was to evaluate the regenerative effect of the combination of BCP and polylactide-co-glycolide (PLGA) which can serve as a barrier membrane during bone regeneration. The study included five patients. Four months into the healing period, bone samples were collected for histological and morphometric analyses. The results of morphometric analysis showed that newly formed bone represented 32.2 ± 6.8% of the tissue, 31.9 ± 8.9% was occupied by residual graft and 35.9 ± 13.5% by soft tissue. Active osteogenesis was seen around the particles of the graft. The particles were occupied mostly by immature woven bone and connective tissue. The quality and quantity of newly formed bone, after the use of BCP/PLGA for ridge preservation, can be adequate for successful implant therapy after tooth extraction.

Key words: Ridge preservation, BCP/PLGA, histology, morphometry, bone

INTRODUCTION

Most of the dimensional alterations of the alveolar ridge occur in the first three to six months after tooth extraction. During this healing period, the alveolar bone undergoes a marked horizontal (29-63% of bone loss) and vertical bone reduction (11-22% of bone loss) (Moya-Villaescusa et al., 2009; Tan et al., 2012). The level of these dimensional changes is reflected not only in the reduced bone volume, but it also affects the process of bone modeling inside the socket defect. The quality of new bone formation that occurs in an early phase of socket healing (examined in animal histological materials and human biopsies) showed a decrease in osteogenic activity eight weeks after tooth extraction with pre-

dominantly formed immature tissue (Chen et al., 2004; Araújo and Lindhe, 2005; Van der Weijden et al., 2009). The clinical consequences of these physiological changes are important for establishing subsequent treatment procedures for implant supported prosthetic restorations.

In order to preserve the original ridge dimensions following tooth extraction, ridge preservation procedures with various bone substitutes have been commonly used at the extraction sites (Irinakis, 2007; Araújo and Lindhe, 2010; Vignoletti et al., 2011; Hammerle et al., 2012). Synthetic calcium phosphate bone substitutes have similar chemical composition to the mineral component of bone (Wagoner Johnson et al., 2011; Bose and Tarafder,

2012), and in the alveolar ridge preservation method, ß-tricalcium phosphate (ß-TCP) and hydroxyapatite (HA) are the most commonly used calcium phosphates, with a composition of 40% ß-TCP and 60% HA (Kesmes et al., 2010; Mardas et al., 2010). Histological studies in experimental animal models and humans showed that the evidence of new bone formation was closely related to the pattern of material resorption (Artzi et al., 2008; De Coster et al., 2011). Namely, ß-TCP is a highly porous material with relatively fast biodegradation that is replaced with newly mineralized bone within three to nine months of healing (Habibovic et al., 2008). On the other hand, HA features a very low solubility, which may compensate for the fast resorption of ß-TCP and maintain the volume of the alveolar ridge (Anderegg et al., 1999; Fellah et al., 2010). Concerning this, it has been shown that HA remains integrated in the newly formed bone longer than ß-TCP (Barrere et al., 2006; Humber et al., 2010).

Most procedures for ridge preservation include, besides filling the socket, the application of a barrier membrane and a primary tissue closure for guided bone regeneration (GBR). However, barrier membrane exposure during healing has an important negative effect on GBR since wound dehiscence may lead to infection and disintegration of the membrane followed by loss of bone at the grafted area (Malchiodi et al., 1998; Machtei, 2001; Artzi et al., 2003). Several studies describe procedures for ridge preservation that do not rely on the use of membranes and primary tissue closure (Nair et al., 2006; Thoma et al., 2006; Aimetti et al., 2009; Brkovic et al., 2012). On the other hand, when the membrane is excluded from the treatment protocol, the bone graft substitutes that are used for such procedures will be exposed to the oral environment and potentially the risk of material loss and post-surgical infection may be increased.

It is suggested that the used bone graft substitutes have to be present as a single graft body retained in the socket. Materials that fulfill this condition and that have been used for ridge preservation without primary wound closure include *in situ* hardening

materials for bone regeneration (Brkovic et al., 2012) coated with a polylactide, which, owing to its composition, can assume the function of a barrier membrane (Gacić et al., 2009; Koković and Todorović, 2011).

The aim of the present case series was to evaluate moldable *in situ* hardening biphasic calcium phosphate/polylactide-co-glycolide as a bone graft substitute for ridge preservation, including its histological and histomorphometric analysis.

MATERIALS AND METHODS

Patient selection and surgical treatment

The study was designed as an interim study with one treatment group that included a series of five patients. All patients signed a written consent for participation in the study. The inclusion criterion for patients enrolled in the study was extraction of an upper one-root tooth subsequently restored with implant therapy. Patients were healthy nonsmokers without local intraoral infections. The extraction sockets in this study required all four walls. In addition, patients were selected based on their good general health (ASA I physical status).

Preoperatively, all patients underwent a rigorous oral hygiene regime seven days prior to the tooth extraction, including the use of 0.12% chlorhexidine mouth-rinse solution twice a day for 30 s and periodontal treatment when indicated. After a traumatic tooth extraction under local infiltration anesthesia (2 ml of 2% lidocaine + 1:100,000 epinephrine), removal of residual periodontal or periapical granulation tissue was performed. All extractions were done to prevent any damage to socket walls and interproximal papillae.

The post-extraction sockets were filled with a bone graft substitute (easy-graftCRYSTAL®, Degradable Solutions AG, Schlieren, Switzerland) consisting of granules and a liquid component (BioLinker™). The granules were composed of a microporous, biphasic calcium phosphate (BCP) compound

of 60% HA and 40% ß-TCP. Each granule was coated with a micrometer-sized film of polylactide-co-glycolide (PLGA), a resorbable polymer. Prior to application into the defect, the coated granules were mixed with the liquid component, which consisted of N-methyl-2-pyrrolidone (NMP) and water. NMP penetrated the coating, rendering it soft. The granules adhered to each other and formed a moldable mass, which was applied directly from the syringe into the prepared extraction sockets. Blood from the surrounding bone penetrated the porous graft, extracting the NMP from the material. Consequently, the moldable mass hardened into a scaffold of interconnected granules. No membrane was applied.

After the surgery, detailed instructions were given to all patients for postoperative care and a 7-day course of amoxicillin (Amoksicilin® 500 mg, Panfarma, Serbia) was prescribed. The regular follow-up for detection of possible complications and side effects was done at 3rd, 7th and 14th day and once monthly following the placement of a bone substitute.

The preserved alveolar sites were exposed after four months for implant placement (Bränemark® Implant System, Sweden). During implant placement, tissue samples were harvested with trephine drills (3 mm: diameter, 6 mm: length). The trephine cores for histological and morphometric analysis were taken from the center of the previously preserved sockets by navigation using a surgical template guide.

Histology and morphometry

The biopsy specimens were immediately fixed in 4% formaldehyde in 0.1 M phosphate-buffered saline pH 7.2. The samples were decalcified in EDTA and dehydrated in increasing concentrations of alcohol. After embedding in paraplast, longitudinal 5 μm-thick sections of the trephine drill cores were cut with a rotary microtome. The sections were placed on silica-coated glass slides and stained by Goldner's trichrome method (Romeis, 1989).

Image acquisition and morphometric assessment were performed using a microscope (Olympus, BX-51, Olympus, Japan) equipped with a microcator (Heidenhain MT1201, Heidenhain, USA) to control movements in the *z*-direction (0.2 μm accuracy), a motorized stage (Prior, Prior Scientific Inc., USA) for stepwise displacement in the *x–y* direction (1 μm accuracy), and a CCD video camera (PixeLink, PixeLINK, Canada) connected to a 19" computer monitor. Image acquisition and stage movement were controlled by the newCAST stereological software package (VIS – Visiopharm Integrator System, ver. 2.12.1.0; Visiopharm; Denmark) running on a personal computer.

Volume density estimation was used to determine the percentage of bone (mineralized and non-mineralized), residual graft material and soft tissue components (connective tissue and/or bone marrow) in the sample. Four central sections were analyzed per sample, with a spacing of 50 μm between sections. Morphometric assessment was performed using a final magnification of 490 x. The counting area was defined using a mask tool. An interactive test grid with uniformly spaced test points for histomorphometric assessment was provided by the newCAST software.

Test points hitting the unmineralized and mineralized bone matrix, residual graft materials, and soft tissue components were determined. Volume densities (V_V) were calculated as the ratio of the number of points hitting each tissue component divided by the number of points hitting the reference space, i.e. analyzed section:

$$V_V \, (\%) = P_p / P_t \times 100.$$

P_p, counted points hitting the tissue component, P_t, total of points of the test system_hitting reference space.

Volume density was calculated for each tissue component per analyzed section. Then, the average value for four sections was calculated (for each com-

Table 1. Patients' characteristics.

N	Gender	Age	Extracted tooth [1]	Reason for extraction	ASA Classification [2]
1.	male	1975	Implant region 22	Root fracture	ASA I
2.	male	1961	Implant region 22	Periodontal disease	ASA I
3.	female	1987	Implant region 12	Failed endodontic treatment	ASA I
4.	male	1969	Implant region 22	Failed endodontic treatment	ASA I
5.	female	1958	Implant region 25	Failed endodontic treatment	ASA I

[1] FDI tooth code
[2] ASA: American Society of Anesthesiologists Physical Status Classification System

Table 2. Volume densities of mineralized bone (MB), non-mineralized bone (NB) and bone blood vessels (BBV), bone graft substitute, connective tissue (CT) and connective tissue blood vessels (CTBV) in biopsy specimens.

N	MB [%]	NB [%]	BBV [%]	Total bone tissue [%]	Bone graft substitute [%]	CT [%]	CTBV [%]	Total connective tissue [%]
1.	10.1	21.2	0.2	31.6	20.7	45.0	2.6	47.7
2.	12.1	22.5	1.0	35.6	41.7	21.9	0.8	22.7
3.	8.8	22.5	2.4	33.7	27.3	35.9	3.0	38.9
4.	5.8	15.2	0.0	21.0	29.5	46.6	2.8	49.5
5.	11.5	26.5	1.0	39.0	40.1	18.8	2.1	20.9
	9.7 ± 2.5	21.6 ± 4.1	0.9 ± 0.9	32.2 ± 6.8	31.9 ± 8.9	33.6 ± 12.8	2.3 ± 0.9	35.9 ± 13.5

Values represent means ± SD.

ponent separately) representing the volume density of a sample i.e. a patient.

RESULTS

Patients and clinical outcome

The investigation involved five patients, three males and two females, mean age 38 ± 5 years, with a total of five extraction sockets. Reasons for extraction were classified into three diagnoses of root fracture, periodontal disease and unsuccessful endodontic treatment (Table 1). All treated sites healed uneventfully with spontaneous healing of the socket opening. There were no local complications and infections or patient discomfort during the observation period of four months. On the day of implant placement, there were clinically evident residual particles at the grafted areas and solid new bone formation.
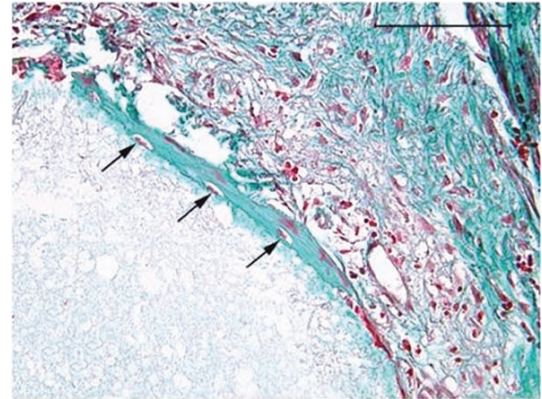
Histological analysis

All biopsies contained newly formed mineralized tissue. No residual bone was present in the samples. No evidence of inflammatory infiltrates, necrosis, foreign body reaction and no other signs of adverse reactions were detected.
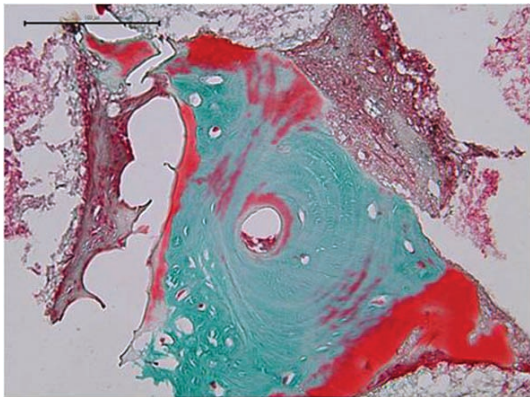
In all analyzed samples, bone tissue formed predominantly in the apical part of the extraction socket. Bone graft substitute particles, which are identified by their round shape, were mainly detected in the coronal portion of the samples (Fig. 1). The graft particles were partially surrounded by trabecular woven bone. Haversian canals surrounded by mature, lamellar bone were noted occasionally, suggesting that the formation of osteon-like structures had already started (Fig. 2). Active osteoblasts lined the osteoid surface (Fig. 3) and produced an osteoid layer (Figs. 3, 4) that soon after became mineralized, as seen in
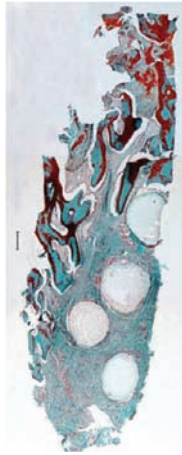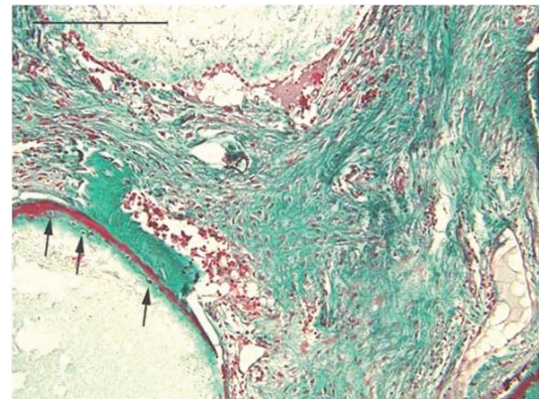
Fig. 1. Graft remnants are visible in the coronal region and islands of the new bone formation located throughout the central and apical portion. (Goldner's staining method, bar - 400µm).
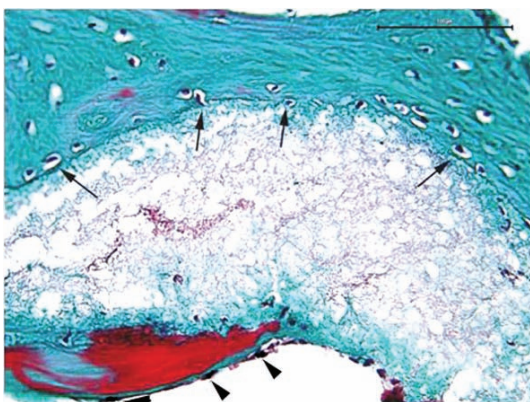

Fig. 2. Mature lamellar bone and woven bone surrounded by graft particles. (Goldner, bar - 200µm).

Fig. 3. Active osteoblasts (arrowhead) produce an osteoid layer (green) that soon after becomes mineralized (red). Graft particles connected (bridged) by zones of immature bone (arrows). (Goldner, bar - 100µm).

Fig. 4. Newly formed bone with osteocytes in lacunae (arrows) and ongoing bone formation. (Goldner, bar-100µm).

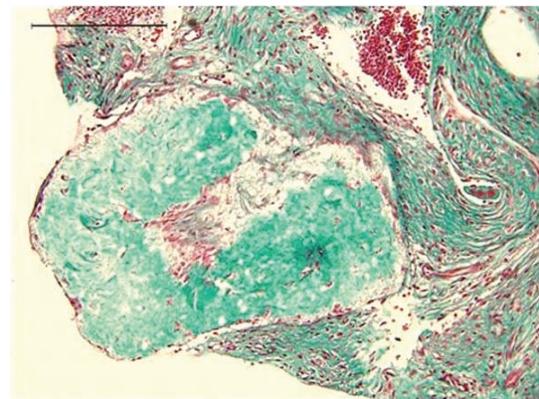**Fig. 5.** Formed bone tissue is in close contact with the surface of a graft particle (arrows). (Goldner, bar - 200µm).

Fig. 6. Graft particle, surrounded by connective tissue, in which the process of active degradation takes place. This connective tissue had the features of a provisional matrix (rich in mesenchymal cells, fibers and vessels). No acute or chronic cell infiltrate was present. (Goldner, bar - 200µm).
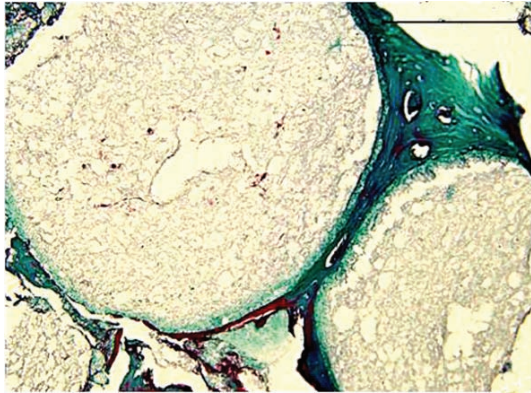
Fig. 7. Mineral particles with signs of degradation surrounded by newly formed bone. (Goldner, bar - 100μm).

Fig. 3 (red). Many trabeculae demonstrated very active bone remodeling, with a thick layer of osteoid surface at one side, which is indicative of new bone formation. In many areas, wide osteocyte lacunae with osteocytes were present.

In all specimens, graft material particles surrounded by the connective tissue with blood vessel, fibroblasts/fibrocytes, and collagen fibers, intermixed with newly formed bone tissue (Fig. 5) could be recognized. The presence of collagen fibers and cells inside the grafted material is an indication that resorption of the grafted material has started (Fig. 6). Together with bone formation around the grafted particles, this could represent a sign of material integration (Fig. 7). No osteoclastic activity was detected.

Morphometry

Details for the volume density estimation are given in Table 2 for individual samples. Bone formation, connective tissue as well as residual bone substitute were presented in similar percentages in the drill core samples. Total bone tissue represented 32.2 ± 6.8% of tissue, 31.9 ± 8.9% was occupied by residual graft and 35.9 ± 13.5 % by soft tissue.

DISCUSSION

This case series was conducted to produce a prelimi-

nary report of an *in situ* hardening, synthetic bone graft associated with PLGA, serving as a barrier membrane for ridge preservation. The present observational case series aimed to characterize the newly formed tissue in extraction sockets at the time point of implant placement.

Histomorphometric evaluation of single sections, which are often randomly chosen, may likely not represent the true tissue composition of the entire sample. Application of modern stereological method for volume density estimation of the analyzed components of the sample provides accurate and unbiased quantification of result (Gundersen and Jensen, 1987; Dorph-Petersen et al., 2001). The samples were serially sectioned and four longitudinal sections from the central portion of the sample spacing 50 μm were analyzed.

The absence of inflammatory infiltrates and foreign body reactions confirm the previously reported good biocompatibility of the *in situ* hardening bone graft substitute (Nair et al., 2006; Gacić et al., 2009; Koković and Todorović, 2011; Brkovic et al., 2012). Most of the grafted biomaterial particles were surrounded by newly formed bone, and rare gaps or connective, fibrous tissues were found at the biomaterial-bone interface. The present case series and other clinical studies (Trombelli et al., 2002; Pearce et al., 2007; Brkovic et al., 2012) indicate that bone formation starts from existing bone surfaces in the most apical part of the extraction defect, propagating along the surface of the bone graft substitute particles. This osteoconductive process leads to the formation of bridges of woven bone between the bone graft substitute particles, connecting them into a mass of mineralized tissue. Bone formation is followed by the remodeling and replacement of woven by mature lamellar bone. In the present study, bone formation was still ongoing after four months, as indicated by the rather large content of non-mineralized bone tissue in all samples and the relatively low number of osteon-like structures. The bone graft substitute particles seemed to act as local bone growth centers throughout the grafted area, according to the osteoblasts that were lying on the surface of the grafted

material. In addition, because of osteoblasts activity, bone formation started around grafted particles with the production of non-mineralized bone matrix proceeding to its mineralization. Generally, the amount of bone formed at the former defect site after ridge preservation varies between studies, presumably due to differences in study populations, defect anatomies, observation time points and material characteristics. Although beta-TCP is expected to degrade three times faster than HA, the predictability of BCP degradation in humans remains poor. It has been recently documented that non-resorbable particles of beta-TCP well incorporated inside a new bone formation were detected nine months after ridge preservation (Brkovic et al., 2008; Brkovic et al., 2012). Furthermore, *in vivo* experiments using rabbit critical-sized defects showed less bone formation in BCP-treated animal compared with autogenous bone grafts (Humber et al., 2010; Jan et al., 2010). After four months of healing, Kesmas et al. (2010) reported that the mean percentage of new bone formation, connective tissue, and residual graft particles in six biopsies after BCP treatment of post-extraction sockets were 28.00 ± 36.75%, 65.50 ± 25.85%, and 15.85 ± 8.70%, respectively. The differences in obtained results, especially in the percentage of residual graft particles, could be explained by the fact that in our cases all particles were coated with PLGA in comparison with the resorbable collagen dressing used in the study of Kesmas et al. (2010). It is also interesting to note that the study published by Mardas et al. (2010) showed that a new bone formation was observed in the apical part of the biopsies that was in direct contact with BCP, while the coronal parts of biopsies were occupied by a dense fibrous connective tissue surrounding BCP, eight months after material placement and the use of a barrier collagen membrane. These findings are in agreement with our results, which demonstrated that bone formation was not observed in the coronal thirds of the extraction sockets when PLGA was used instead of a collagen membrane. Considering this, it seems reasonable that the choice of materials used for the membrane function may have an influence on the quantity of new bone formation concerning the time-dependent degradation of the selected material.

It is obvious from the preliminary findings of our investigation that additional studies based on larger patient samples are necessary in order to identify the BCP/PLGA effect on bone formation before the alveolar ridge preservation procedure. Although all implants placed in previously preserved sites had clinically successful initial stability, it is important to address the biological mechanisms that may influence the implant-integration process and high survival rates after the preservation of extraction sockets with BCP/PLGA. Having in mind these study limitations, we can conclude that BCP/PLGA could be a useful *in situ* hardening grafting material in implant dentistry due to the quantity and quality of new bone formation ensured for ridge preservation procedures.

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