Histological evaluation at different times after augmentation of extraction sites grafted with a magnesium-enriched hydroxyapatite: double-blinded randomized controlled trial

Today, teeth replacement with implant-supported prostheses is a predictable option of therapy. However, nowadays, esthetic demand requires a prosthetically driven approach. Thus, there is a high interest in minimizing tissue resorption after tooth extraction and maintain the contour of the alveolar crest. In fact, it is well known that the alveolar crest reduces in volume after tooth extraction, especially during the first 6 months and mostly on its buccal wall (Schropp et al. 2003).

It has been demonstrated that augmentation material does not prevent, it only reduces resorption process especially at the buccal wall (Caneva et al. 2011). Using post-extraction ridge preservation procedure, 85% of the initial ridge dimensions could be preserved, allowing a correct implant placement (Cornelini et al. 2004; Chen et al. 2007). On the other hand, immediate implant placement without bone grafting was shown not to prevent this process (Caneva et al. 2011).

New generation of hydroxyapatites (HAs), so-called biomimetic scaffolds, simulates bone structure not only from a chemical point of view but also microscopically, reconstructing micropores and their interconnections. Investigation of the healing of a nanocrystalline synthetic HA demonstrated sprouting vessels from pre-existent host vessels enter the granules. Later, they form an intergranular network that probably transports osteoblastic precursor cells into the granules (Götze et al. 2008). This may be...
relevant for osteoconductive and probably also osteoinductive features leading to early osteogenesis and subsequent remodeling of the newly formed bone (Götz et al. 2010).

Within the graft material category of HAs, partial substitution of HA with magnesium (Mg-enriched) results in a non-stoichiometric structure. Because this kind of material is chemically and stoichiometrically similar to bone mineral matrix and the process of sintering is missing in production process, Mg-enriched HA is completely resorbed after 6–12 months (Trombelli et al. 2010).

It was shown that autogenous bone biopsies provided higher vital over comparable total bone levels than Mg-enriched HA after 5 months of healing, but Mg-enriched HA grafts revealed higher expression of certain specific markers of osteoblast differentiation and bone formation, associated with a lower osteoclastogenic potential (Crespi et al. 2009c). Further, it was proven for socket preservation procedure that, 3 months after placement, Mg-enriched HA could reduce bone resorption more than calcium sulfate and control group. Coincidently, Mg-enriched HA revealed clearly more residual material and less vital bone than calcium sulfate and control groups (Crespi et al. 2009b). After 2–3 months, implants have been inserted, and thereafter, bone resorption was neglectable up to 24 months (Crespi et al. 2009a; Sisti et al. 2012).

No study has been leaded so far regarding the transition from 3-month results with histologically residual material in less vital bone with clinically reduced resorption and long-term histologically complete resorption with clinically proven stability of augmented areas. Investigations on immunohistochemistry of Mg-enriched HA osteogenesis are missing as well. This gave reason to focus on the angiogenesis–osteogenesis interplay.

Early angiogenesis and osteogenesis are determinants for establishment of healthy natural bone formation in augmented regions. Angiogenesis, in fact, means the ingrowth of new vessels from pre-existent ones in the local bone and is caused by activation of endothelial and vessel wall stem cells (Adams & Alitalo 2007). It is considered to be the most important process for healing and osteogenesis of implanted substitutes (Santos & Reis 2010; Götz et al. 2011). Only thereafter, the new bone takes part in vital biological remodeling process with surrounding earlier bone to ensure long-term bone stability (Chiapasco et al. 2006).

In the present trial, traditional histologic stainings and immunohistologic antibodies were adopted for evaluation of vessels and bone formation. Additionally, caveolin-1 antibody was added, for the first time in a study on bone regeneration in dental field. The protein caveolin-1 is a principal component of caveolae, which are subcompartments of plasma membrane in vessels and capillaries (Rothberg et al. 1992). For these reasons, caveolin-1 antibody was demonstrated to be ideal as a marker for quantity and quality of vessels (Sawada et al. 2007; Hada et al. 2012). Moreover, caveolin-1 has been identified to interact with variety of cellular proteins, and it plays a role in different metabolism and disease processes (Tamaskar & Zhou 2008; Le Lay et al. 2009). Furthermore, it takes function in bone metabolism (Sawada et al. 2007; Hada et al. 2012), and its indirect analysis allows for identification of osteoclasts and osteoblasts. (Gerger et al. 2004).

Aim of this study was to histologically and immunohistochemically analyze the early vascularization and the osteogenesis–angiogenesis interplay of the post-extraction sites after augmentation, using a magnesium-enriched HA (SINTlife®, Finceraica, Faenza, Italy).

This nano-structured hydroxyapatite presents in fact chemical and morphologic properties close to natural bone. Its porosity was demonstrated to reach 90% of the volume, with macro-pores ranging between 2 and 5 μm and pores of interconnection ranging between 0.8 and 0.2 μm (Fig. 1a,b). Additionally, partial substitution of HA with magnesium (Mg) results in a non-stoichiometric structure, and because it is more similar to bone mineral matrix, it was demonstrated to be completely resorbed after 6–12 months (Trombelli 2010).

Materials and methods

This study was performed following the principles outlined in the Declaration of Helsinki in 2008 on experimentation involving human subjects and was approved by the ethical committee of the University of Greifswald (Reg-Nr.: BB 13/11a). The design of this study was a randomized controlled prospective clinical trial.

Patient selection

Any patients requiring at least one tooth extraction in the maxillary premolar area who were at least 18 years old and able to sign an informed consent form were eligible for inclusion in this study.

They were enrolled in the study according to specific exclusion and inclusion criteria (Table 1). Ten patients were selected for this study that included a 4-month follow-up after extraction. All were asked to sign a written informed consent form. Preliminary screening was performed on the basis of clinical examination and intraoral radiography. Then, according to examinations, patients underwent scaling, root planning, oral hygiene instruction or any periodontal treatment necessary to provide an oral environment more favorable to wound healing. All patients received prophylactic antibiotic therapy (amoxicillin and clavulanic acid 2 g 1 h before tooth extraction and 1 g twice a day for 4 days postoperatively). Patients were informed about the procedure but were blinded regarding the group they belonged.

Ten patients (six women and four men) were recruited. The mean age was 58.4 ± 13.6 years.

Treatment

The whole treatment followed standard protocols for ridge preservation (Sisti et al. 2012).
was performed every week. Patients’ follow-up and rinsing twice daily with 0.12% chlorhexidine limited to soft brushing for the first 2 weeks. Oral hygiene at the surgical site was made provisional fixed partial denture (FPD) was removed and a re-entry process took into account the follow-up variables: patient gender, age, biotype, extraction sockets underwent reopening at 2 months after surgery. Ten to 14 days later, the sutures were removed. Two to 3 months after implant placement, definitive restorations were seated.

Histological processing
The specimens taken from the test sites were immersed in 4% buffered formalin for 1 week at room temperature and then decalcified for 1 week in ethylenediaminetetraacetic acid (EDTA). After decalcification, bone cores were rinsed with running water for 24 h; routinely dehydrated in a series of increasing concentrations of ethanol (50%, 70%, 80%, 90%, 96%, and 100%); placed in xylol for 12 h; and then embedded in paraffin. Serial longitudinal sections of about 5 μm were stained with hematoxylin/eosin (H.E., to recognize different tissue types) and Masson-Goldner trichrome (to differentiate collagen, graft and bone tissues). Additionally, to identify osteoclast-like cells, selected tissue sections were stained to demonstrate TRAP as described by Gerber et al. (2006).

A blind histological evaluation of all sections stained was undertaken by two independent investigators. The slices were observed and photographed under a Nikon light microscope (Eclipse E600) equipped with a calibrated digital camera (DXM1200; Nikon, Tokyo, Japan). Histomorphometric measurements were performed on images at a magnification of 10× using image analysis software (Image J, NIH, v. 1.61, http://rsb.info.nih.gov/ij/image/). The percentage of mineralized tissue, connective tissue, and residual graft material was calculated for all sections.

**Table 1. Patient and study site inclusion and exclusion criteria**

<table>
<thead>
<tr>
<th>Patients inclusion criteria</th>
<th>Patients exclusion criteria</th>
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<tr>
<td>Patients with need for tooth extraction and following implant placement. Furthermore, patients with missing teeth and lack of hard and/or soft tissues and desire for implant prosthodontic treatment</td>
<td>All absolute contraindication for implant treatment such as pregnancy or severe poor general health, for example Severe renal or liver disease History of a radiotherapy in the head region Chemotherapy at the time of surgical procedure Non-compensated diabetes mellitus symptoms of a maxillary sinus disease Active periodontal disease Poor oral hygiene</td>
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**Randomization procedure**
According to the randomization, augmented extraction sockets underwent reopening at 2 (Group 1) or 4 months (Group 2). Sites were assigned to groups according to predefined randomization tables. A balanced random permuted block approach, ensuring that, at any point in a trial, roughly equal numbers of participants are allocated to all groups, was used to prepare the randomization tables to prevent an unequal balance between the two treatments. To reduce the chance of unfavorable splits between the two groups in terms of key prognostic factors, the randomization process took into account the following variables: patient gender, age, biotype, and site location in dental arch. Assignment was performed using a sealed envelope.

**Biopsy**
According to the randomization (either 2 or 4 months after augmentation), the provisional FPD was removed and a re-entry procedure was performed. In both groups, proepreventive antibiotic prophylaxis was carried out, and after anesthesia, a full-thickness flap was raised. Using a small trephine (internal diameter 2.0 mm, external diameter 2.8; Hu-Friedy, Rockwell Chicago, IL, USA), a bone specimen was collected before implant placement. A 13 mm in length and 4.25 mm in diameter implant (Premium; Sweden & Martina, Due Carrare, Padua, Italy) was inserted. Single interrupted 5.0 monofilament sutures were used for flap adaptation in both groups. Ten to 14 days later, the sutures were removed. Two to 3 months after implant placement, definitive restorations were seated.

**Histological processing**
The specimens taken from the test sites were immersed in 4% buffered formalin for 1 week at room temperature and then decalcified for 1 week in ethylenediaminetetraacetic acid (EDTA). After decalcification, bone cores were rinsed with running water for 24 h; routinely dehydrated in a series of increasing concentrations of ethanol (50%, 70%, 80%, 90%, 96%, and 100%); placed in xylol for 12 h; and then embedded in paraffin. Serial longitudinal sections of about 5 μm were stained with hematoxylin/eosin (H.E., to recognize different tissue types) and Masson-Goldner trichrome (to differentiate collagen, graft and bone tissues). Additionally, to identify osteoclast-like cells, selected tissue sections were stained to demonstrate TRAP as described by Gerber et al. (2006).

**Immunohistochemistry**
Selected sections from the middle parts of the series were deparaffinized, rehydrated, and rinsed for 10 min in Tris-buffered saline (TBS). Once rinsed, sections were pretreated with PBS containing 1% bovine serum albumin for 20 min at RT and digested with 0.4% pepsin (Roche, Mannheim, Germany) for 10 min at 37°C. Following further rinsing, sections were incubated with the primary antibodies in a humid chamber. Staining was performed following the instructions for the ABC-systems (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA, USA). Bound antibodies were visualized using a New Fuchsine alkaline phosphatase substrate protocol or diaminobenzidine. Sections were counter stained in hematoxylin and then cover-slipped. As control for the specificity of the caveolin-1 antibody, antigen-pre-incubated antibodies were used as shown recently (Kunert-Keil et al. 2011). For negative controls, the primary antibody was replaced by PBS and PBS used in the same way. Details and incubation protocols are available for consultation in Table 2.

**Quantitative and qualitative analysis of capillary-like structures**
A simultaneous blind test was conducted in identical conditions (researchers, equipment, and chemicals) to determine the level of antigen. From each section, at random, five 100 serial longitudinal sections were immunohistochemically stained. The percentage of stained area was measured using a calibrated digital camera (FX2000; Nikon, Tokyo, Japan) equipped with a Nikon Light microscope (Eclipse E600). The slides were scanned using a calibrated digital camera (DXM1200; Nikon, Tokyo, Japan). Histomorphometric measurements were performed on images at a magnification of 10× using image analysis software (Image J, NIH, v. 1.61, http://rsb.info.nih.gov/ij/image/). The percentage of mineralized tissue, connective tissue, and residual graft material was calculated for all sections.

**Table 2. Details and incubation protocols of the used antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isotype</th>
<th>Producer</th>
<th>Incubation protocol</th>
<th>For staining of</th>
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<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>Rabbit polyclonal</td>
<td>Quartett (Berlin, Germany)</td>
<td>Ready to use, on, 4°C</td>
<td>Osteoblast cells</td>
</tr>
<tr>
<td>Caveolin-1</td>
<td>Rabbit polyclonal</td>
<td>DianoVa (Hamburg, Germany)</td>
<td>1 : 10000, on, 4°C</td>
<td>Blood vessels, osteoblasts, osteoclasts</td>
</tr>
<tr>
<td>CD34</td>
<td>Rabbit monoclonal</td>
<td>Epitomics (Burlingame, CA, USA)</td>
<td>1 : 100, 30 min, RT</td>
<td>Endothelial cells</td>
</tr>
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On, overnight; RT, room temperature.
digital pictures of different parts of the tissue were taken (200× magnification, 3CCD color camera, Hitachi HV-C20M; Hitachi Denshi Ltd, Tokyo, Japan, and Axiolab, Carl Zeiss, Gottingen, Germany). For standardization of the measurement, the optical density of white background color was attuned to 250 in each picture. For all sections, mean optical densities and the quantity of capillary-like structures were assessed using KSRun software (imaging system KS400, release 3.0; Zeiss, Vision GmbH, Munich, Germany) (Gerger et al. 2004; Kunert-Keil et al. 2011). The criteria for a capillary-like structure were central lumina surrounded by caveolin-1 or CD34 positively stained cells.

**Statistical analysis**

Mean values and standard deviations were calculated for each outcome variable. For each group, obtained values were compared using nonparametric Mann–Whitney U-test. P < 0.05 was considered statistically significant.

Statistical analysis was performed using the SPSS version 20 software (IBM Corporation, Somers, NY, USA).

**Results**

At the end of the study, no dropout occurred. According to the randomization, five biopsies were grafted at 2 months (Group 1), the other five at 4 months (Group 2).

All surgical interventions and postoperative healing period were without any serious complication or side effect for all patients. In the first postoperative day, some patients showed a moderate swelling without experiencing pain. After 1 week, no inflammation symptom was detectable.

**Histology**

According to the decalcification process of the bone graft material, sections showed Mg-enriched HA granules as amorphous or granular bodies of different sizes. In all biopsies, histological results demonstrated no inflammatory cell or foreign body reaction after 2 and 4 months of healing, respectively. The bone formation and remodeling could be categorized into four different osteogenious transformation stages (I–IV), as described formerly by Gerber et al. (2006) and Götz et al. (2008).

Stage 1: Two of five cases in Group 1 were classified in the first stage of healing. They presented non-degraded granules with smooth surface, mostly covered by a dense connective tissue and capillaries (Fig. 2a,b).

Stage 2: Three of five cases in Group 1 were classified in the second stage of healing. Samples presented well-vascularized connective tissue filling the spaces between the grafted material residues. Furthermore, osteoid protrusions extended into the bone graft material. These protrusions appeared green with Masson-Goldner staining and indicate the occurrence of a collagenous matrix. Some of the woven bone trabeculae were interconnected with each other (Fig. 3a,b).

Stage 3: Two of five cases in Group 2 were classified in the third stage of healing. Samples presented increased bone graft material degradation and remodeling of woven into lamellar bone. Large areas of newly formed bone appeared mineralized (Fig. 4a,b).

Stage 4: Three of five cases in Group 2 were classified in the fourth stage of healing. Samples presented residuals of woven bone within lamellar bone. Two of five biopsy specimens appeared to be nearly completely ossified. Small material residues were sometimes visible (Fig. 5a,b).

**Alkaline phosphatase**

Stage 1: Strong staining of alkaline phosphatase (AP) was found in fibroblasts of the connective tissue as well as in the peripheral area of granules (Fig. 2c).

Stage 3: Two of five cases in Group 2 were classified in the third stage of healing. Samples presented increased bone graft material degradation and remodeling of woven into lamellar bone. Large areas of newly formed bone appeared mineralized (Fig. 4a,b).

Stage 4: Three of five cases in Group 2 were classified in the fourth stage of healing. Samples presented residuals of woven bone within lamellar bone. Two of five biopsy specimens appeared to be nearly completely ossified. Small material residues were sometimes visible (Fig. 5a,b).
Stage 3: AP staining was detected in the peripheral area of granules, osteoblast cells at the surface of woven bone, fibroblast cells and in osteocytes of the newly formed bone (Fig. 4c).

Stage 4: AP-positive cells were still visible in the connective tissue and around blood vessels. However, the staining intensity decreased (Fig. 5c).

TRAP staining
Stage 1: Osteoclast-like cells as well as mononuclear cells were TRAP stained. These TRAP-positive cells appeared at the surface of the granules and were also visible in the connective tissue, mainly around vessels (Fig. 2d).

Stage 2: TRAP-positive osteoclast-like cells appeared at the surface of the granules. In addition, mononuclear cells stained with TRAP were visible in the connective tissue (Fig. 3d).

Stage 3: The amount of TRAP-positive mono- and multinuclear cells decreased. However, on the surfaces of newly formed bone and around residual material, TRAP-stained cells were still detectable (Fig. 4d).

Stage 4: The number of TRAP-positive cells was even more reduced as in stage 3 (Fig. 5d).

Caveolin-1 and CD34
Stage 1: Strong caveolin-1 staining was found in endothelial cells as well as the whole vessel wall, including smooth muscle cells. Furthermore, osteoblast and osteoclast cells showed strong expression of caveolin-1, whereas fibroblast cells of the connective tissue were mainly unstained (Fig. 2e). Only walls of vessel-like structures resulted strongly positive to CD34 antibodies (Fig. 2f).

Stage 2: As shown in stage 1, strong caveolin-1 and CD34 expression was found in endothelial cells as well as in the whole vessel wall [Fig. 3e,f]. In contrast, osteoblast and osteoclast cells showed weak expression of caveolin-1 [Fig. 3e].

Stage 3: No change in caveolin-1 and CD34 expressing cells was found at these stages compared to stages 1 and 2, whereas the staining intensity of the cells decreased [Fig. 4e,f].

Stage 4: As shown for stage 3, caveolin-1 and CD34 expressing cells were detectable. However, the staining intensity of caveolin-1 seemed to be increased in osteoblast cells on the surface of woven bone [Fig. 5e]. The expression level of CD34 was even more reduced as in stage 3 (Fig. 5f).

Histomorphometry
Histomorphometric analysis, as shown in Table 3, presented a regenerated bone mean value of 15.0% after 2 months of healing. After 4 months of healing, a 5.1-fold increased bone mean value was observed (77.4%; \( P = 0.016 \)). At the same time, 21.7% and 11.6% of graft material was still visible on average respectively in Groups 1 and 2. On average, connective tissue/marrow spaces occupied 63.3% of the bi-optical test sections after 2 months of healing. This area was significantly reduced to 11% (\( P = 0.016 \)) after 4 months (Table 3).

Computerized analysis of the staining intensity (optical density) of caveolin-1 in capillary-like structures is shown in Fig. 6a. A significant decrease in caveolin-1 protein expression was found comparing Group 1 and 2 even not considering osteogenous transformation stages (2 vs. 4 months: 645 ± 66 vs. 256 ± 188; \( P = 0.029 \)). Quantitation of the CD34 expression confirmed these results. The CD34 expression was also significantly reduced 4 months after material insertion compared to 2 months of bone healing (2 vs. 4 months, 301 ± 190 vs. 88 ± 54, \( P = 0.016 \), Fig. 6b and Table 3).

Discussion
From a theoretical point of view, there are several reasons to consider preservation of the alveolar ridge immediately following tooth extraction. The first is to stabilize the...
coagulum within the socket and minimize possible shrinkage of the hard tissues. The second is to provide a scaffold for the in-growth of cellular and vascular components for new bone regeneration.

Several studies were conducted to test techniques and materials able to contrast physiologic resorption process in post-extraction sites. Available data provide controversial outcomes; however, literature confirmed that sites grafted with nano-structured HAs showed new bone regeneration even in the early stage (after 3 months). Histomorphometric data after 4 months from the present study were confirmed by recently published study on preservation technique of crestal bone after extraction grafted with the same hydroxyapatite (Crespi et al. 2011). However, similar results in terms of bone formation, presence of graft particles and connective tissue were found in the same study also using porcine bone substitute. The similar biologic behavior might highlight once again the importance of the scaffold properties performed by graft materials. In sinus lift procedure, the same Mg-enriched HA as in the present study showed after 5 months 29.7% vital bone vs. 78.4% vital bone for autogenous bone graft ($P < 0.05$), while overall bone volume was nearly the same [Crespi et al. 2009a,b,c].

Coincidentally, Mg-enriched HA revealed clearly more residual material and less vital bone than calcium sulfate (Crespi et al. 2009b). This dissimilarity can be explained by the different vertical height where the biopsy was taken. In fact, in case of calcium sulfate, almost 2.5 mm of radiographic vertical bone resorption was demonstrated. On the other hand, Mg-enriched HAP showed <0.5 mm of radiographic vertical bone resorption.

In the present randomized controlled clinical trial, changes of the extraction sites at different times (2 and 4 months) after augmentation using a Mg-enriched HA as in the present study showed after 5 months 29.7% vital bone vs. 78.4% vital bone for autogenous bone graft ($P < 0.05$), while overall bone volume was nearly the same [Crespi et al. 2009a,b,c].

In fact, according to Jung et al. [2004], graft materials seem not able to modify resorption pattern of post-extraction socket. Furthermore, some grafts (bovine bone matrix) are supposed to lessen healing process and bone neo-formation (Becker et al. 1998; Araújo & Lindhe 2009; Araújo et al. 2009). On the other hand, in post-extraction sockets with buccal bone wall defects, the same graft covered by collagen membrane seemed to be effective from a quantitative point of view even if histologic data showed ongoing healing process after 7 months [Carmagnola et al. 2003].

In the present randomized controlled clinical trial, changes of the extraction sites at different times (2 and 4 months) after augmentation using a Mg-enriched HA were histologically analyzed. In Group 1, 8 weeks after tooth extraction, bone biopsies were composed of a low-density trabecular bone with few interspersed graft particles. After 16 weeks of healing (Group 2), newly formed bone appeared to have filled any defect: hydroxyapatite particles became well incorporated into new bone formation creating a dense cancellous network.

At the end of the study, the histomorphometric analysis points out the rare bone regeneration after two and impressing ossification in comparison after 4 months.

To explain the histologic outcomes of Group 2 compared to Group 1, osteoconductive properties of new generation HAs might be advocated. In fact, these properties were recently demonstrated in animal [Ripamonti et al. 2008, 2009] and human studies [Götz et al. 2008]. The hydroxyapatite nano-porosity was demonstrated to allow bone matrix proteins to adhere and promote differentiation of osteoblast precursor cells [Götz et al. 2008].

These data seem to be in accordance with results reported by Götz et al. [2008] and Canullo & Dellavia [2009]. Although in sinus lift and GBR procedures, it was demonstrated that sites grafted with nano-structured HAs showed new bone regeneration even in the early stage (after 3 months).

Histomorphometric data after 4 months from the present study were confirmed by recently published study on preservation technique of crestal bone after extraction grafted with the same hydroxyapatite (Crespi et al. 2011). However, similar results in terms of bone formation, presence of graft particles and connective tissue were found in the same study also using porcine bone substitute. The similar biologic behavior might highlight once again the importance of the scaffold properties performed by graft materials. In sinus lift procedure, the same Mg-enriched HA as in the present study showed after 5 months 29.7% vital bone vs. 78.4% vital bone for autogenous bone graft ($P < 0.05$), while overall bone volume was nearly the same [Crespi et al. 2009a,b,c].

In the present study, together with traditional histology and histomorphometry, Mg-enriched HAP effectiveness in post-extraction procedures was measured analyzing the angiogenesis–osteogenesis interplay.

In fact, experimental studies investigating the properties of some synthetic substitute...
materials have already demonstrated the importance of angiogenesis from the host tissue that is closely related to new bone formation (Kakudo et al. 2006).

Using indirect immunohistochemistry and enzymatic analysis, in fact, high expression of alkaline phosphatase (AP), TRAP, and CD34 (vessel density) was found in early stages of osteogenous transformation. On the other hand, the amount of vessel-like structures was significantly reduced 4 months after grafting material insertion. Furthermore, a decrease in the expression of AP and TRAP was also detectable. The reduction of AP expression is based on the progressive osteogenesis with subsequent reduction of pre-osteoblasts, osteoblasts, and young osteocytes. These data allow to speculate that Mg-enriched HA is a suitable material for post-extraction ridge preservation, ensuring early angiogenesis followed by early osteogenesis.

These results are definitely substantiated by caveolin-1 evaluation. As reported in literature (Frank et al. 2003), caveolin-1 could be detected in endothelial cells as well as in the whole vessel wall, including smooth muscle cells. Furthermore, osteoblast and osteoclast cells showed strong expression of caveolin-1, whereas fibroblast cells of the connective tissue were mainly unstained (Solomon et al. 2000; Lofthouse et al. 2001; Sawada et al. 2007; Hada et al. 2012). Particularly, caveolin-1 is strongly abundant in endothelial cells regulating functions as angiogenesis, vascular permeability, and transcytosis (Frank et al. 2003). In a recent study on caveolin-1-deficient mice, angiogenesis was found to be markedly reduced in comparison with control mice (Woodman et al. 2003). Furthermore, caveolin-1 knockout mice have increased bone size and stiffness, because caveolin-1 deficiency leads to increased osteoblast differentiation as shown by cell studies from the same group (Rubin et al. 2007). Thus, the caveolin-1 antibody can be used for the analysis of osteoblasts, osteoclasts as well as blood vessels.
In the present study, focusing on the angio-
genesis–osteogenesis interplay, strong expres-
sion of caveolin-1 was found in pre-
osteoblasts, osteoblasts, and osteoclasts: while staining density showed 645 ± 65 after 2 months for blood vessels, the expres-
sion of caveolin-1 in capillary-like structures decreased to 256 ± 188 after 4 months. Results of angiogenesis–osteogenesis inter-
play and analysis of AP and CD34 as well as TRAP staining allow to conclude that, from an histologic point of view, blood vessel den-
sity developed in opposite to osteogenesis and that high vascularization after 2 months could provide a highly accelerated ossifica-
tion of the augmented region, confirming tra-
ditional histology and histomorphometry.

From a clinical point of view, it may be concluded that Mg-enriched HA is a suitable mate-
rial for ridge preservation and ensures early angiogenesis and osteogenesis, suggesting that implant placement could be appro-
piate even after 2 months. However, at this early stage, immediate loading could be risky. On the other hand, at 4 months, the regen-
erated bone could bear loading stress.

Limitation of the study was represented by the small patient sample size, which was antagonized by a strict randomization proto-
col. However, more data are needed, and especially, influence of local soft tissue and genetic terms [healing bone pattern] has to be investigated.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** CONSORT checklist.

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