

Alberto Turri  
Christer Dahlin

## Comparative maxillary bone-defect healing by calcium-sulphate or deproteinized bovine bone particles and extra cellular matrix membranes in a guided bone regeneration setting: an experimental study in rabbits

### Authors' affiliations:

Alberto Turri, Christer Dahlin, Department of Biomaterials, BIOMATCELL VINN Excellence Center, Institute for Surgical Sciences, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

Alberto Turri, The Brånemark Clinic, Institute of Odontology, Public Dental Health Care, Gothenburg, Sweden

Christer Dahlin, Department of Oral, Maxillofacial Surgery and Research and Development, NU-Hospital Organisation, Trollhättan, Sweden

### Corresponding author:

Professor Christer Dahlin  
Department of Biomaterials, The Sahlgrenska Academy, University of Gothenburg, P.O Box 412, SE-405 30 Gothenburg, Sweden  
Tel.: +46 31 786 2969  
Fax: +46 31 786 2941  
e-mail: christer.dahlin@biomaterials.gu.se

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### Abstract

**Objectives:** The aim of this study was to histologically compare the dynamics of bone healing response between calcium sulphate (CaS) and deproteinized bovine bone mineral (DBBM) particles in guided bone regeneration utilizing an extracellular matrix membrane (ECM) as barrier.

**Materials and methods:** Eighteen rabbits were used in this study. 5 × 5 mm defects were created in the edentulous space between the incisors and molars in the maxilla. The CaS and DBBM particles were placed in the defects, with or without the placement of a membrane by means of random selection. Healing was evaluated at 2, 4 and 8 weeks by histology.

**Results:** A total resorption of the CaS material was seen already at 2 weeks. Only minor resorption could be seen of the DBBM particles. The CaS group showed significantly more bone regeneration at all three healing periods compared to the DBBM group. The addition of an ECM membrane demonstrated significant additional effect on bone regeneration. The CaS group showed significant increased amounts of blood vessels compared to the DBBM group.

**Conclusions:** This study showed that CaS in combination with an ECM membrane provided synergistic effects on bone regeneration, seemingly due to stimulating angiogenesis in the early healing process.

The use of dental implants has become a common treatment modality and an important component of modern dentistry. In many clinical situations, edentulous sites in the maxilla or mandible do not offer appropriate bone volume for implant placement. Possible causes for this lack of sufficient bone volume are various and include earlier bone atrophy, traumatic tooth extraction, bone resorption due to periodontal disease and physiological ridge contraction following tooth removal (Pietrokowski & Massler 1967; Araújo & Lindhe 2005).

For such defects, bone augmentation procedures are necessary prior to or in combination with the implant placement.

These procedures encompass the Guided Bone Regeneration (GBR) method, based on the use of barrier membranes sealing off an anatomical site for improved healing of bone tissue (Dahlin et al. 1988). Clinically, this

technique is an established treatment option for bone volume augmentation, and it is often applied in combination with a suitable bone filling material to achieve a more predictable and lasting regenerative outcome (Jung et al. 2013).

Historically, the gold standard for bone grafting has been autogenous bone from intra- or extraoral donor sites. However, harvesting autogenous bone has several downsides such as increasing patient morbidity, limited supply and clinician chair time (Raghoobar et al. 2001).

Theoretically, the ideal bone substitute should be used as intermediate phase scaffold. This means that it should maintain a three-dimensional biological support during bone healing (osteoconductive), possibly working as a guide to stimulate bone growth (osteoinductive) before being gradually replaced by the newly formed bone.

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Several materials, synthetically derived or processed from skeletal structures of other species (xenografts), have been developed to support bone formation.

Deproteinized bovine bone mineral (DBBM) is an inorganic bovine bone derivative that provides an osteoconductive scaffold and has a mineral content comparable to that of human bone allowing it to integrate with the host bone. It is by far the best documented bone substitute material used in combination with GBR (Retzepi & Donos 2010; Baldini et al. 2011). Although being described as a truly osteoconductive material, it has been suggested, in a few studies, that deproteinized bovine bone may also trigger osteoinductive mechanisms (Schwartz et al. 2000; Mladenović et al. 2013) and exert angiogenic activity (Lakey et al. 2000; Galindo-Moreno et al. 2014). Presently, there is a clear trend within biomaterials research to develop and explore ion-substituted biomaterials. Chemical composition, microstructure and crystallinity are considered as contributing factors determining the biological responses to these materials. In addition, it has been suggested that the incorporation of bioactive ions may have a favourable effect on the bone response during healing (Eppley & Pietrzak 2005; Cardemil et al. 2013; Dahlin et al. 2014). However, only scarce information can be found studying the possible interaction of GBR with these types of ion substituted filling materials. Calcium sulphate (CaS), with a rapid dissolution of Calcium ions, is a common bone substitute with the longest history of clinical use spanning more than 110 years. Medical grade calcium sulphate is highly biocompatible, osteoconductive, and it undergoes virtually complete resorption *in vivo* (Thomas & Puleo 2009). Several studies described osteogenic activity and a positive effect on angiogenesis when defects in a rabbit model were filled with calcium sulphate (Strocchi et al. 2002; Dasmah et al. 2011).

The aims of this study were to evaluate the biological response of a biphasic calcium sulphate representing biomaterials with active dissolution of active ions in comparison to deproteinized bovine bone in combination with guided bone regeneration in an experimental animal design.

## Material and methods

### Animals

The animal experiment was approved by the University of Gothenburg and Local Ethical Committee for Laboratory Animals.

A total of 18 adult New Zealand white rabbits of both sexes, weighing  $4.1 \pm 1$  kg (mean  $\pm$  SD), fed on a standard diet and water were used for the study.

During all surgical procedures, general anaesthesia was induced by intraperitoneal injections of diazepam (Kabi Pharmacia, Helsingborg, Sweden) at a dose of 1.5 mg/kg body weight and intramuscular injections of fluanisone and fentanyl (Hypnorm Vet, Janssen, Saunderton, UK) at a dose of 0.2 mg/kg body weight. In addition, approximately 2 ml of 2% lidocaine and epinephrine 12.5  $\mu$ g/ml (Xylocain with adrenalin, AstraZeneca, Södertälje, Sweden) was used as local anaesthesia. Each rabbit received analgesic (Temgesic 0.03 mg/kg; Reckitt & Coleman, Hull, Great Britain) subcutaneously prior to surgery and daily postoperatively.

In addition, antibiotics were given 1 day before and 2 days after surgery.

### Surgery

The bilateral edentulous areas between the incisors and the molars of the maxilla were used as experimental sites (Nannmark & Sennerby 2008). Full thickness mucoperiosteal flaps were reflected exposing the maxillary bone. After randomization, a  $5 \times 5$  mm defect on each side of the upper jaw was prepared with  $\varnothing$  2.3 mm burs with copious saline irrigation (Fig. 1). The defects were filled either with a biphasic calcium sulphate (CaS) (Bond-Bone<sup>®</sup>, MIS Implants Technologies Inc., Minden, Germany) or with DBBM (Bio-Oss<sup>®</sup>, Geistlich<sup>®</sup>, Wolhusen, Switzerland) (Fig. 2a–c). Half of the defects filled with biphasic CaS were then covered with an extracellular matrix membrane (DynaMatrix<sup>®</sup>, Keystone Dental, Burlington, MA, USA) while all DBBM filled defects were covered with a barrier membrane (positive control) according to the principles of GBR (Fig. 3a, b). The surgical sites were closed using resorbable sutures.

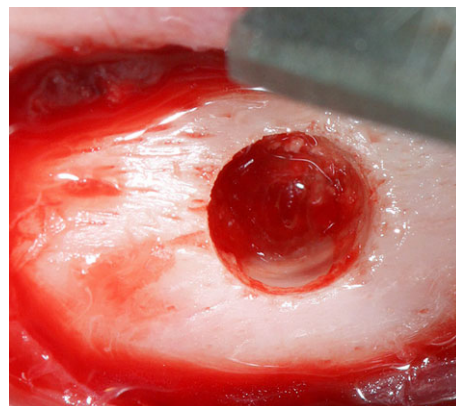


Fig. 1. Standardised test defect in the rabbit maxilla.

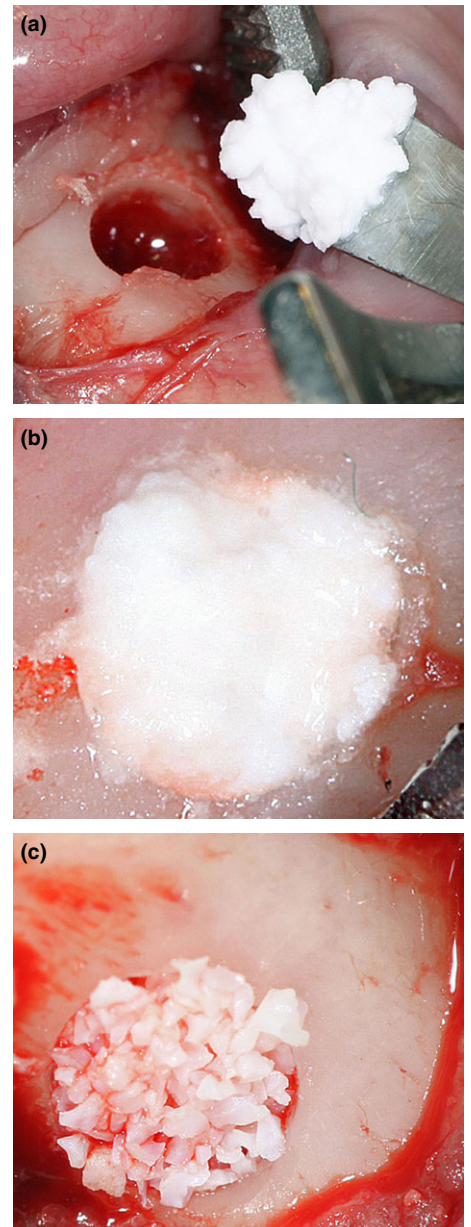


Fig. 2. (a) Calcium sulphate (CaS) material prior to insertion into defect. (b) The CaS material in position. (c) DBBM particles in position in defect.

### Healing periods

Eighteen rabbits were divided into three groups of six animals with the following healing periods: 2, 4 and 8 weeks.

At the end of each designated healing time, the animals were sacrificed by intravenous injection of sodium pentobarbital (60 mg/ml; ATL Apoteket Production & Laboratories, Stockholm, Sweden).

### Histological preparation and analysis

The specimens were removed *en block*, fixed using 4% formalin and then dehydrated in ascending series of ethanol and embedded in plastic (LR White; London Resin Co, Ltd,



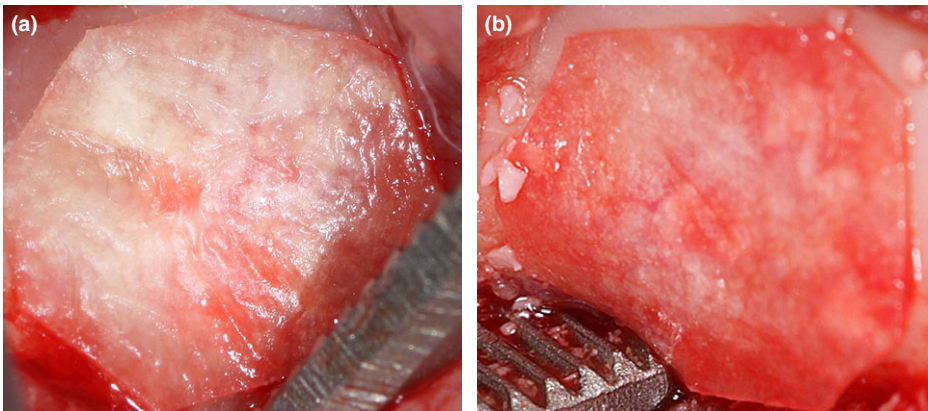


Fig. 3. (a) Extracellular matrix membrane in place covering the CaS graft material and the defect. (b) The extracellular matrix membranes (ECM) protecting a site filled with DBBM.

Berkshire, UK). From each site, one section was analyzed. Using a sawing and grinding technique (Exakt® Apparaturbau, Nordenstedt, Germany) 15–20 µm thick sections were produced, mounted on glass slides, and stained with 1% toluidine blue (Schenk et al. 1994).

The sections were viewed and analyzed using a light microscope (Nikon Eclipse E600; Tekno Optik, Stockholm, Sweden) connected to a personal computer with software for morphometric evaluation (Easy Image Measurements 2000; Tekno Optik) using a point counting procedure:

In the 2-, 4- and 8-week specimens, the following tissue elements were assessed and calculated as the relative volumes (%) occupied:

- newly formed bone
- residual grafting material
- blood vessels
- connective tissue/marrow space.

Using an ×10-magnification objective, a square grid (500 µm × 500 µm) was superimposed over different portions (apical, central, marginal) of the newly formed tissue in the defect. A total of 20 regions of interest (ROI) were calculated per section and expressed as mean values of the relative volumes (%).

#### Statistical analysis

A software package (SPSS 11.0; SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. Mean values (expressed as percentage) and standard deviations (SDs) were then calculated for each group and healing period. Differences between the means for the groups were assessed by performing Brunner Langer test (modified Wilcoxon nonparametric tests). Adjustments for multiple comparisons was performed using Dunnett-Hsu correction (Brunner 2010). A result was considered to be significant if  $P < 0.05$ .

## Results

All animals recovered well from surgery. One specimen from the 8 week group (Bond-Bone + membrane) was lost due to histological processing.

#### Histological evaluation

The histological orientation was performed in a bucco-palatal aspect. The defects areas were filled with either bone grafts, blood vessels, connective tissue and newly formed bone.

#### 2 weeks

The histological examination revealed a total resorption of the calcium-sulphate material (Table 1) (Fig. 4a, b). The corresponding amount of present DBBM particles was 32.7%. The percentage of blood vessels in the calcium sulphate treated defects was 11.3% (6.7% with membrane) in comparison to the DBBM group which showed 2.4% ( $P < 0.02$ ) (Table 2). In the calcium-sulphate groups, random new bone formation could be noted, always in conjunction with blood vessels (Fig. 4b). The amount of bone fill was 11.9%

for calcium sulphate alone and 22% when combined with a barrier membrane ( $P < 0.001$ ). In the DBBM filled defects traces of bone formation could be noted mostly in the bottom of the defects and to a lower degree (6.5%) (Table 3). The newly formed bone was mostly found deposited on the DBBM particles (Fig. 5a,b). The amount of connective tissue varied between 53 to 66% for the respective groups (See Table 4).

#### 4 weeks

After additional 2 weeks of healing, still no traces of calcium sulphate could be noted. The corresponding remaining volume of DBBM grafts was 30.3%. A steady growth of new bone towards the buccal aspect of the defect could be noted. The calcium-sulphate filled defects showed a significant higher density of bone (27.8%) and 32.8% with the presence of a membrane compared to DBBM + membrane (21.3%) ( $P < 0.001$ ) (Table 3). A significant increase in micro vessel density could also be noted at this time point (11.5 and 10.5%) in comparison to the DBBM group 2.2% ( $P < 0.001$ ) (Table 2).

#### 8 weeks

At 8 weeks of healing the DBBM specimen demonstrated a mean bone density of 18%, almost similar to calcium sulphate alone (19.9%) (Fig. 6). The combination of calcium sulphate and membrane showed higher density of bone at this time point (27.9%) (Table 3) although not significant ( $P < 0.061$ ). With regard to blood vessel density calcium sulphate alone showed higher values (14.1%) compared to DBBM + membrane (8.2%) and calcium sulphate + membrane (9.4%) respectively ( $P < n.s.$ ) (Table 2). The amount of remaining graft particles for the DBBM + membrane group was 17.8%. No presence of graft material in the calcium sulphate groups could be seen also at this time point (Table 1, Fig. 7).

**Table 1. Demonstrating the % residual graft material at 2, 4 and 8 weeks respectively**

DBBM + membrane% residual graft material	Mean	Range	SD
2 weeks	32.7	0–67.7	20.4
4 weeks	30.4	0–73.6	23.5
8 weeks	17.8	0–71.7	17
CaS% residual graft material	Mean	Range	SD
2 weeks	0	0–0	0
4 weeks	0	0–0	0
8 weeks	0	0–0	0
CaS + membrane% residual graft material	Mean	Range	SD
2 weeks	0	0–0	0
4 weeks	0	0–0	0
8 weeks	0	0–0	0

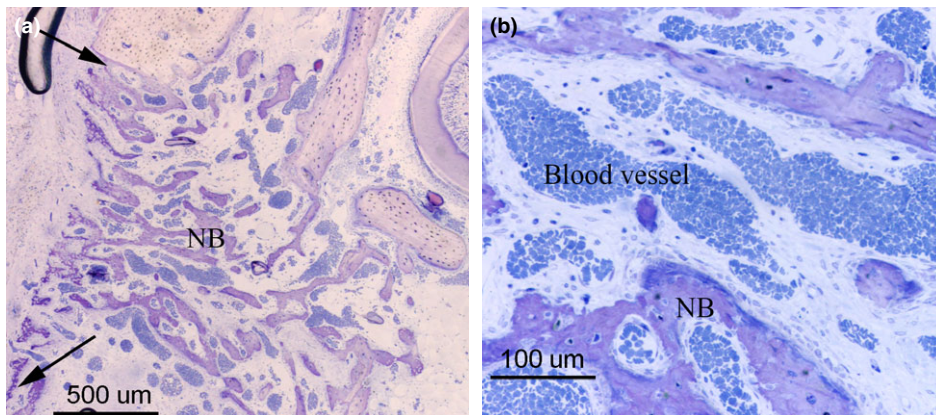


Fig. 4. (a, b) Histological sections of defects filled with CaS at 2 weeks of healing. Note the random bone formation and the massive amount of blood vessels. Original magnification  $\times 4$  and  $20\times$ . Toulidine blue stain.

**Table 2.** Showing the % of blood vessels within the defect area at 2, 4 and 8 weeks

DBBM + membrane% vessels	Mean	Range	SD
2 weeks	2.4	0–19.4	3.9
4 weeks	2.2	0–14.2	3.4
8 weeks	8.2	0–23.9	6.1

CaS% vessels	Mean	Range	SD
2 weeks	11.3	0–30.9	9.4
4 weeks	11.5	1–34.7	6.6
8 weeks	14.1	0.3–56.9	13

CaS + membrane% vessels	Mean	Range	SD
2 weeks	6.6	0–29	6.4
4 weeks	10.4	0.1–24.2	5.7
8 weeks	9.1	0.6–27.6	7.3

**Table 3.** Demonstrating the % bone formation at 2, 4 and 8 weeks

DBBM + membrane% bone formation	Mean	Range	SD
2 weeks	6.5	0–36.3	8.4
4 weeks	21.3	0–59.9	18.8
8 weeks	18.1	0–59	17.8

CaS% bone formation	Mean	Range	SD
2 weeks	11.9	0–32.9	10.2
4 weeks	27.8	0–85.6	15.6
8 weeks	19.9	0–65.9	13.9

CaS + membrane% bone formation	Mean	Range	SD
2 weeks	22	0–49.2	14.5
4 weeks	32.8	0–73.4	16.6
8 weeks	27.8	0–65.2	18.2

## Discussion

The present study was performed to study potential biological differences, in a GBR setting, when using a synthetic bone substitute material characterized by a high dissolution rate and hence release of high concentrations of ions in the wound area (CaS) compared to the use of an osteoconductive material

(DBBM) with a barrier membrane acting as control.

The histological examination revealed a total dissolution of the present calcium sulphate material already after 2 weeks of healing. This was regardless of the presence of a barrier membrane or not. Initially the amount of DBBM particles was approximately 30% which decreased to 18% at

8 weeks of healing. DBBM is claimed to be deproteinized and subsequently bone resorption by osteoclasts probably cannot occur (Schwartz et al. 2000). The reported reduction in area fraction of the DBBM may also depend on the surgical technique including pressure at application of the grafting material, the initial percentage of DBBM particles and the variation of particle size. There are also limitations in the histological preparation as well (Mordenfeld et al. 2010). A striking finding in particular at the 2 and 4 week time period was the different pattern of bone formation. In the DBBM group, a typical osteoconductive driven event was taking place with new bone formation growing from the bottom of the defect via deposition of bone on the DBBM particles towards the buccal portion of the cavity. The CaS group (with or without membrane placement) revealed a totally different picture with a significant increase in blood vessel density and an almost random formation of immature bone in the entire defect area. The presence of a vascular net is an important factor for bone neogenesis and maintenance and angiogenesis plays a crucial role in all regenerative processes. Angiogenesis is increased in surgical/injured sites due to the low oxygen tension and high metabolic activity present in the area (Carter et al. 2000). Hence, early establishment of the vascular system is essential in order to deliver oxygen and tissue nutrients and to clear away cellular debris (Lakey et al. 2000). The CaS material clearly stimulated angiogenesis in the present study. Strocchi et al. (2002) who in a rabbit study demonstrated more blood vessels in the sites treated with CaS demonstrated similar findings. Hence, vascular induction stimulated by CaS may increase the rate of bone neogenesis. Another possible mechanism could also be a direct effect on bone formation by means of stimulation via the calcium ions. According to Orsini et al. (2004) calcium powder acts as a direct source of calcium supply after resorption process that promotes osteogenic activity. This has also been proposed in a recent study by Dasmah et al. (2012) who evaluated histologically, CaS used for sinus floor augmentation in a clinical setting. They also reported a significant resorption of the CaS graft material with only 8.8% of remaining graft material after 4 months of healing. In their histological observations they noted histological signs of acellular substitution of CaS with bone-like tissue. We did not see any similar findings in the present study.



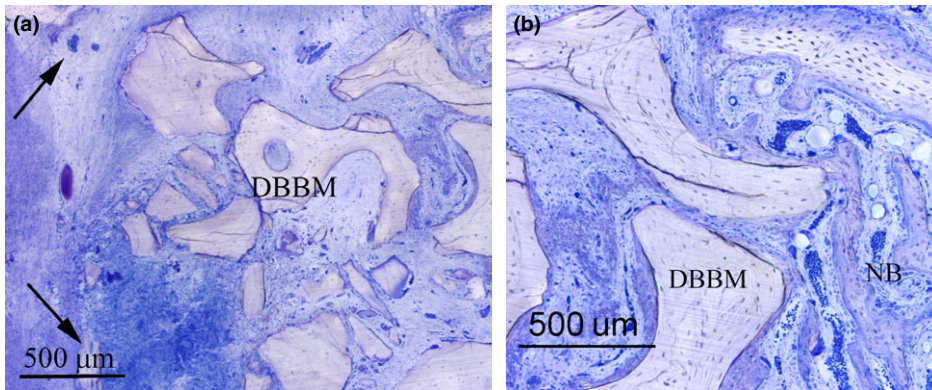


Fig. 5. (a, b) Histological sections of defects filled with DBBM at 2 weeks of healing. Note the more appositional growth of new bone from the bottom of the defect via the DBBM particles. Original magnification  $\times 4$  and  $20\times$ . Toulidine blue stain.

**Table 4. Showing the % connective tissue at 2, 4 and 8 weeks of healing**

DBBM + membrane% connective tissue	Mean	Range	SD
2 weeks	53.2	17.4–86.5	16
4 weeks	41.9	6.5–93.9	20.2
8 weeks	49.8	15.2–97	22.2

CaS% connective tissue	Mean	Range	SD
2 weeks	64.6	15.8–99.3	20.5
4 weeks	57.6	12.1–90.9	16.2
8 weeks	61.3	19.1–98	16.9

CaS + membrane% connective tissue	Mean	Range	SD
2 weeks	66.5	27.7–99.3	20.1
4 weeks	54	6.7–90.1	17.5
8 weeks	61.1	27.2–99	19.1

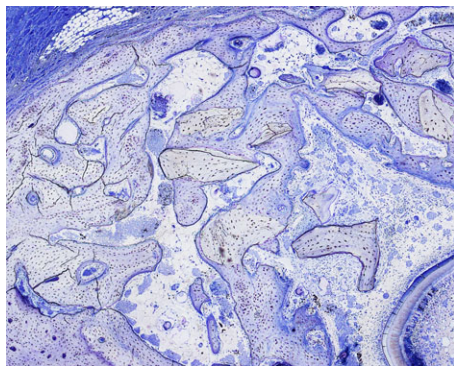


Fig. 6. Histological section of defect previously filled with DBBM at 8 weeks of healing. Note the apposition of bone around the DBBM particles throughout the defect hereby increasing the total bone volume in the defect. ( $\times 4$ , Toulidine blue stain).

The combination of a barrier membrane and various bone substitute materials has over the year proven, both in experimental and clinical settings, to be a synergistic concept to achieve predictable bone regeneration and successful long-term outcome in particular in conjunction with oral implant

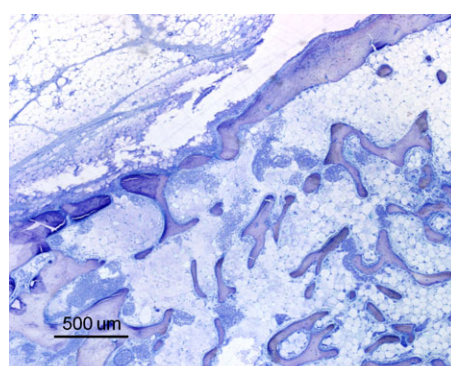


Fig. 7. Histological section of defect previously filled with CaS at 8 weeks of healing. Only scarce presence of bone trabeculae can be noted. The majority of the defect is filled with adipose tissue. ( $\times 4$ , Toulidine blue stain).

treatment (Dahlin et al. 1991; Hämmerle & Jung 2003; Stavropoulos et al. 2004; Simion et al. 2007; Donos et al. 2008; Esposito et al. 2009; Jung et al. 2013). However, most studies have been performed using osteoconductive materials such as DBBM. The combination of a barrier membrane and the

use of CaS as a filling material are not so well explored in the literature. Dasmah et al. (2011) studied the intramembranous bone tissue response towards CaS in the same animal model as in our study but without the placement of a barrier membrane. They also reported that the CaS-graft exhibited a fast resorption rate while stimulating angiogenesis. In the present study, the addition of a barrier membrane to the CaS graft demonstrated a significant less blood vessel density at 2 weeks in comparison to CaS alone indicating a slightly compromised wound healing situation during the initial phase of healing due to the placement of the barrier membranes. However at 4 and 8 weeks respectively not statistical significance could be noted. Overall both CaS alone or in combination with a bioactive membrane showed significant higher blood vessel density in comparison to when using DBBM as filling material at 2 and 4 weeks of healing. With regard to bone formation the combination of CaS and membrane demonstrated synergistic effect with significant higher values of % bone formation compared to DBBM and membrane as well as CaS alone. The high amount of bone density in the CaS and membrane group could also be due to the bioactive properties of the extracellular matrix membrane used in the present study. The material is claimed to include stimulatory factors essential for early angiogenesis (Hodde et al. 2005). In a recent study by Al-Asfour et al. (2013) the extracellular matrix membranes (ECM) were studied histologically in an identical animal model in the rabbit maxilla. They found ingrowth of micro vessels within the material already after 4 weeks of healing. Furthermore they also claimed that the material did not degrade, instead it was well integrated in the tissue. These findings were confirmed in the present study where the ECM membrane could be identified also at 8 weeks of healing.

There were obvious differences in the pattern of bone regeneration between the DBBM and the CaS sites in this study. The DBBM sites demonstrating a more osteoconductive pattern of bone formation on the bone substitute particles while the CaS treated sites showed total resorption of the graft material and a random bone formation combined with a high density of blood vessels in the sites. One has to consider that the time span of this study was relatively short and little is known what will happen over time with the CaS group. Furthermore, it could be stated

that the fact that a barrier membrane was present did not One could speculate upon that in future studies a combination of the two material groups offers an interesting alternative to maintain a predictable long-term result. Further studies in this direction are needed.

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## Conclusion

The present study showed that CaS in combination with an ECM membrane provides synergistic effects on bone regeneration, seemingly due to stimulating angiogenesis in the early healing process.

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