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TISSUE ENGINEERING CONSTRUCTS AND CELL SUBSTRATES

Original Research

Analysis of bone formation and membrane resorption in guided bone regeneration using deproteinized bovine bone mineral versus calcium sulfate

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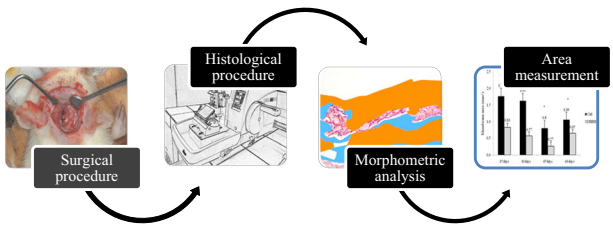
Abstract

Guided Bone Regeneration (GBR) is a technique based on the use of a physical barrier that isolates the region of bone regeneration from adjacent tissues. The objective of this study was to compare GBR, adopting a critical-size defect model in rat calvaria and using collagen membrane separately combined with two filling materials, each having different resorption rates. A circular defect 8 mm in diameter was made in the calvaria of Wistar rats. The defects were then filled with calcium sulfate (CaS group) or deproteinized bovine bone mineral (DBBM group) and covered by resorbable collagen membrane. The animals were killed 15, 30, 45 and 60 days after the surgical procedure. Samples were collected, fixed in 4% paraformaldehyde and processed for paraffin embedding. The resultant sections were stained with H&E for histological and histomorphometric study. For the histomorphometric study, the area of membrane was quantified along with the amount of bone formed in the region of the membrane. Calcium sulfate was reabsorbed more rapidly compared to DBBM. The CaS group had the highest percentages of remaining membrane at 15, 30, 45 and 60 days, compared to the DBBM group. The DBBM group had the highest amount of new bone at 45 and 60 days compared to the CaS group. Based on these results, it was concluded that the type of filling material may influence both the resorption of collagen membrane and amount of bone formed.

Graphical Abstract



1 Introduction

Guided Bone Regeneration (GBR) may be used in several fields of dentistry and medicine to correct or reestablish adequate levels of bone tissue in bone defect areas. It is one of the most commonly used techniques for promoting bone regeneration, and is most employed in the areas of periodontics and implant dentistry to preserve the dimensions of the tooth socket [1], increase the alveolar ridge vertically or

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horizontally, raise the maxillary sinus and fill bone defects. In the case of implants, GBR can provide the time and space required for the migration of regenerative cells to the repair site [2].

To enhance bone tissue healing, the GBR technique employs a membrane that acts as a physical barrier over the defect to isolate it from non-osteogenic tissues with a higher proliferation rate, such as connective tissue, and hinder the development of scar tissue [3, 4].

The first membranes used in GBR were of the non-resorbable type, obtained from expanded polytetrafluoroethylene (e-PTFE), which provide biocompatibility and structural stability during the healing period. The main disadvantages of use of this material is the need for a second surgical intervention to remove it [5], and the fact that this results in a higher rate of postoperative complications [6], including early exposure of the membrane [7]. To circumvent these problems, other materials that are resorbable, obtained naturally or synthetically, have been used [8].

Membranes derived from collagen can substitute non-resorbable materials effectively [8] as they have osteoconductive properties which encourage the regeneration process [4] and have low immunogenic activity, since collagen is part of the structure of bone tissue [9]. The main disadvantage of collagen membranes is their low structural strength, which leads to the need for an additional supporting material to maintain its three-dimensional shape and prevent collapse into the defect [10, 11].

Suitable options for filling material that can be combined with membranes include autogenous bone, allogeneic bone, xenografts, alloplastic materials and combinations of these four [12]. Xenogeneic materials and alloplastic grafts have assumed prominence in dentistry due to limitations of the use of autogenous and allogenic bone [6]. One of the most studied graft materials is deproteinized bovine bone mineral (DBBM), which exhibits osteoconductive properties [13, 14] and has fewer drawbacks compared with autogenous grafts [15].

Calcium sulfate (CaS) can also be used as a filling material for it is biocompatible, osteoconductive [16, 17] and bioactive in vitro [18]. In addition, CaS has osteogenic and angiogenic potential [19, 20] and the ability to osteointegrate [21]. While both materials possess favorable properties for bone regeneration, bovine bone and calcium sulfate have different resorption rates in the body, which can influence the rate of bone regeneration during GBR. Bovine bone has a lower resorption rate [22] than calcium sulfate, with the latter being rapidly and completely resorbed in vivo [16]. The hypothesis of the present study was that GBR can be modulated by the influence of the type of filling material used. To test this hypothesis, the objective of the study was to compare GBR in a critical-defect model in rat calvaria, employing histological and histomorphometric analysis, and using collagen membrane separately combined with two different filling materials, deproteinized bovine bone mineral and calcium sulfate, each having different resorption rates.

2 Materials and methods

All procedures involving the use of animals were approved (under permit number 047/2013) by the Ethics Committee on the Use of Animals (CEUA) of the Maringa State University, constituted under the terms of article 8 of Federal Law 11794/2008.

2.1 Experimental procedures in animals

For this study, 40 male rats (*Rattus norvegicus albinus*; Wistar) weighing between 180 and 200 g were used. Following an intramuscular injection at the proportion of 1:1 of a solution ($1.0\,mL.Kg^{-1}$) of ketamine (Dopalen 10%, Sespo, Paulinia, Brazil) and xylazine (Rompun 2%, Bayer, São Paulo, Brazil), a linear 15 mm incision was made in the calvarial region of the animals, following a transversal imaginary line connecting the base of the ears. The bone tissue was then exposed by the total reflection of the skin-muscle-periosteal tissue and a full-thickness 8 mm circular critical-size bone defect [23] was made using a trephine bur (Neodent, Curitiba, Brazil) with sterile saline irrigation (LBS Laborasa, Sao Paulo, Brazil). The defects were filled with calcium sulfate (CaS group) or deproteinized bovine bone mineral (DBBM group).

In the CaS group, the defects were filled with calcium sulfate hemihydrate (Asfer, Sao Caetano do Sul, Brazil). Briefly, the powder was sterilized by autoclaving (121 °C for 20 min). Subsequently, 40 g of the powder was mixed with 100 mL of sterile saline, according to the manufacturer's instructions, resulting in a paste preparation used to fill the defect. In the DBBM group, the defects were filled using inorganic bovine bone Bio-Oss (Geistlich Pharma AG, Wolhusen, Switzerland). Both defects were covered with a square (10×10 mm) demineralized cortical-derived resorbable membrane (GenDerm, Baumer, Mogi Mirim, Brazil) that fully covered the defect [24]. The periosteum was than repositioned and the skin sutured. The area was disinfected with iodine 2% solution. The animals remained under observation at a controlled temperature until complete recovery from the surgery. The rats were housed in temperature-controlled rooms (23 ± 1 °C) and received water and food (Nuvilab/Nuvital, Sogorb, Sao Paulo, Brazil) ad libitum, under a light/dark cycle of 12 h.

The animals were killed 15, 30, 45 and 60 days after surgery ($n = 5$ animals/time of observation/group). They were euthanized by anesthetic overdose ($3.0\,mL.Kg^{-1}$).

Subsequently, samples of bone with periosteum containing the defects (with safety margin) were removed, fixed in 4% paraformaldehyde for 24–48 h and decalcified in Morse's modified solution for 2–3 weeks. After decalcification, the samples were cut transversely in the central region of the defect and processed for paraffin embedding. Semi-serial 7 μm-thick sections were cut in a center-margin direction and stained with hematoxylin and eosin to allow an overall evaluation of the bone regeneration process, including the occurrence of inflammatory response, development of fibrosis and osteogenesis.

2.2 Histomorphometric evaluation

The histomorphometric study was performed to quantify the rate of resorption of the membrane and the amount of bone formed in the region of the membrane (in the surrounding region and/or within the membrane itself).

One hundred randomly chosen images of the slides were taken for each group (total 200 images) using a high-resolution camera (Nikon, DS-Fi1c, Shimjuku, Japan) coupled to an optical microscope (Nikon, Eclipse 80i, Shimjuku, Japan) with 2X objective. The following parameters were measured for each image: total area of membrane (mm^2) and total area of bone formation associated with the membrane (mm^2), i.e. the bone formed within the membrane or immediately adjacent to it. The images obtained were analyzed and the areas quantified using an image analysis program (ImageJ®, ScionCorp., Frederick, MD, USA). For the periods of 15 and 30 days, because the size of the defect and membrane was larger than the visual field obtained with the 2X objective, two images were required to measure the area.

2.3 Statistical analysis

The Kolmogorov–Smirnov normality test was applied to the data. The measurements of the membrane area, expressed as median and standard deviation, were then analyzed using the Mann–Whitney non-parametric test to compare the test group (CaS) and the control group (DBBM) at each of the observation timepoints. In addition to the analysis of the remaining membrane area, the area of formed bone associated with the membrane was analyzed by comparing the CaS and DBBM groups for all observational periods. Results were expressed as median and standard deviation for the two groups. The observation time of 15 days was not considered for this variable due to the small amount of bone formed in this period. Due to the different variability between the data, the non-parametric test was used. For the calculation of percentages of the remaining membrane and bone formed, an area of 2 mm^2, corresponding to the area of the original membrane before being implanted into the animal, was considered equivalent to 100% [25, 26]. An alpha level of 5% was adopted for all statistical analyses.

3 Results

3.1 Histological evaluation of bone regeneration

Fifteen days: at this timepoint, most of the calcium sulfate had been absorbed. The remnants were granulated and sparsely distributed within the interior and at the margins of the defects filled by acellularized and vascularized connective tissue from the periphery. A larger number of multinucleated giant cells were adjacent to these granules. Fibroblasts, isolated macrophages and neutrophils were also identified, characterized by mild and diffuse inflammation in some areas.

Infiltration of cells with strongly basophilic condensed nuclei into the collagen membrane was observed. Neutrophils were identified among the cell types.

In terms of bone formation, there was growth from the margins of the defect and appositional growth on the top and bottom surfaces of the remaining bone plate. In some animals, there was development of small bony formations in the connective tissue within the central area of the defect (Fig. 1a–c).

In the DBBM group, there was some deposition of bone tissue replacing the collagen membrane, which appeared to be more extensively resorbed in this period compared with the CaS group. The formation of connective tissue around the bovine bone granules, the presence of discrete inflammatory infiltrate and formation of bone tissue from the margins of the defect and around the biomaterial particles were also observed (Fig. 1d).

There were no signs of fibrosis in any of the groups.

Thirty days: this period was notable for greater amounts of bone tissue replacing the inner surface of the collagen membrane. Several bone formations, independent from the remaining bone margins, were also observed. The central-most region of the defect was filled with a small amount of calcium sulfate interspersed with loose connective tissue. Giant cells were still sparsely located in this tissue (Fig. 2a–c).

The animals from the DBBM group exhibited a collagen membrane showing a high degree of resorption. In this group, there was also bone deposition on the collagen membrane. Some of the Bio-Oss® granules had been resorbed while some were surrounded by macrophages (Fig. 2d).

Complete regeneration of the defect had not occurred in any of the animals.

Forty-five days: the collagen membrane of the animals from the calcium sulfate group was more extensively

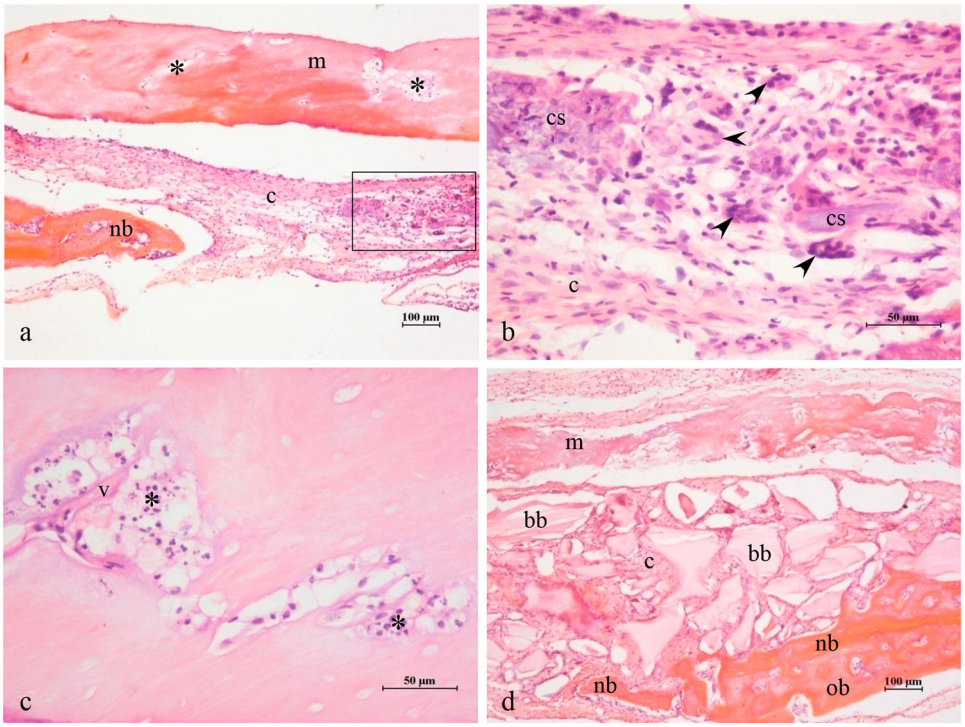

Fig. 1 Photomicrograph of calvarial bone defects filled with calcium sulfate **a, b** and **c** or deproteinized bovine bone mineral **d** 15 days after graft. In **a**, note the new bone (nb) growing from margin of defect, and the presence of collagen membrane (m) with small reabsorption areas (*). Under the membrane, the defect was filled by vascularized connective tissue (c) and remaining calcium sulfate (delimited area, detailed in **b**). Photomicrograph **b** shows details from delimited area in **a**. Note the presence of multinucleated giant cells (arrowhead) around fragments of calcium sulfate (cs). In **c**, detail showing resorption area (*) of collagen membrane filled with vascularized (v) soft tissue and neutrophils. In **d**, bone defect filled with granules of deproteinized bovine bone mineral (bb). Note new bone (nb) growing over old bone (ob) (appositional growth). The spaces between granules are filled by connective tissue (c). H&E stain

resorbed compared to the previous period. Bone formation on the membrane, however, had not evolved. In the DBBM group, the membrane had been almost totally replaced by bone tissue.

For this period, the greatest growth from the margins of the defect was observed in the DBBM group. There was a reduction of cellularity in both groups (Fig. 3a, b).

Sixty days: the calcium sulfate was almost completely resorbed. The remaining particles were enveloped in phagocytic cells. The defect was filled by loose connective tissue, most of which had some fibrotic areas. This group showed little or no bone formation beyond that observed at 45 days. In animals for which osteogenesis had occurred, the tissue developed within and/or on the collagen membrane. In this period, there appeared to be less bone formation compared to the defects filled with lyophilized bovine bone. None of the defects were completely filled by bone tissue (Fig. 3c).

Compared with the defects filled by calcium sulfate, there was greater bone growth into the defect in the DBBM group from the defect margin and osteoconduction toward Bio-Oss® granules. Most of the collagen membrane had been replaced by bone and, to a lesser extent, by loose connective tissue. The defect was not completely regenerated (Fig. 3d).

3.2 Histomorphometric evaluation

The collagen membrane was partially replaced by bone tissue and partially resorbed. Therefore, the results of the analysis of the membrane area represent the remnants of the membrane at each observation timepoint. Figure 4 depicts the median area (mm^2) of the collagen membrane. In the DBBM group, the remaining membrane percentage was 40.0, 28.5%, 13.5% and 31.0% at 15, 30, 45 and 60 days, respectively. In the CaS group, the percentages were 88.5, 81.5, 40.0% and 53.0%, respectively.

The Fig. 5 shows the median area (mm^2) of bone formed in the region of the membrane. Analysis at 15 days of observation could not be performed due to the small amount of bone tissue formed. In the DBBM group, the percentage of bone formed was 0.8, 12.5% and 53.0%, at 30, 45 and 60 days, respectively. In the CaS group, the percentages were 9.0%, 1.0% and 32.5%.

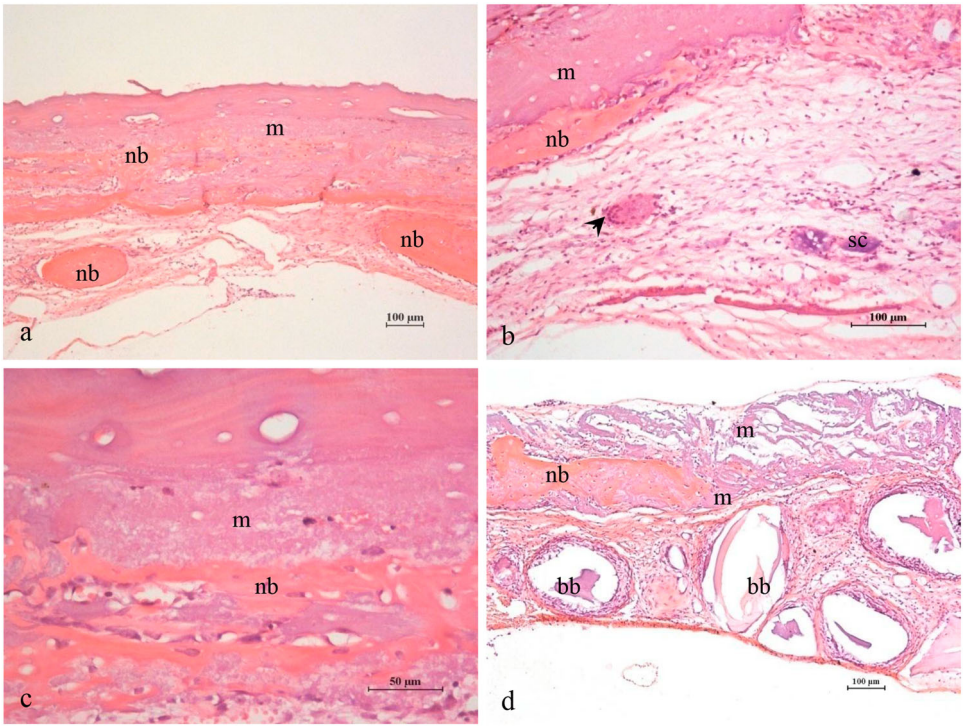

Fig. 2 Photomicrograph of calvarial bone defects filled with calcium sulfate **a**, **b** and **c** or deproteinized bovine bone mineral **d** 30 days after graft. For this period, note **a** the greater formation of bone tissue (nb), mainly in the collagen membrane (m), compared to the previous period. In the connective tissue under the membrane, note new bone (nb) islets. **b** For this period, few multinucleated giant cells (arrowhead) and fragments of calcium sulfate (cs) were observed. In **c**, detail showing bone formation (nb) in the center of the collagen membrane (m). In defects filled with deproteinized bovine bone mineral **d**, part of the membrane (m) is being resorbed and part is being replaced by bone tissue (nb). The fragments of deproteinized bovine bone mineral (bb) are enveloped by connective tissue. H&E stain

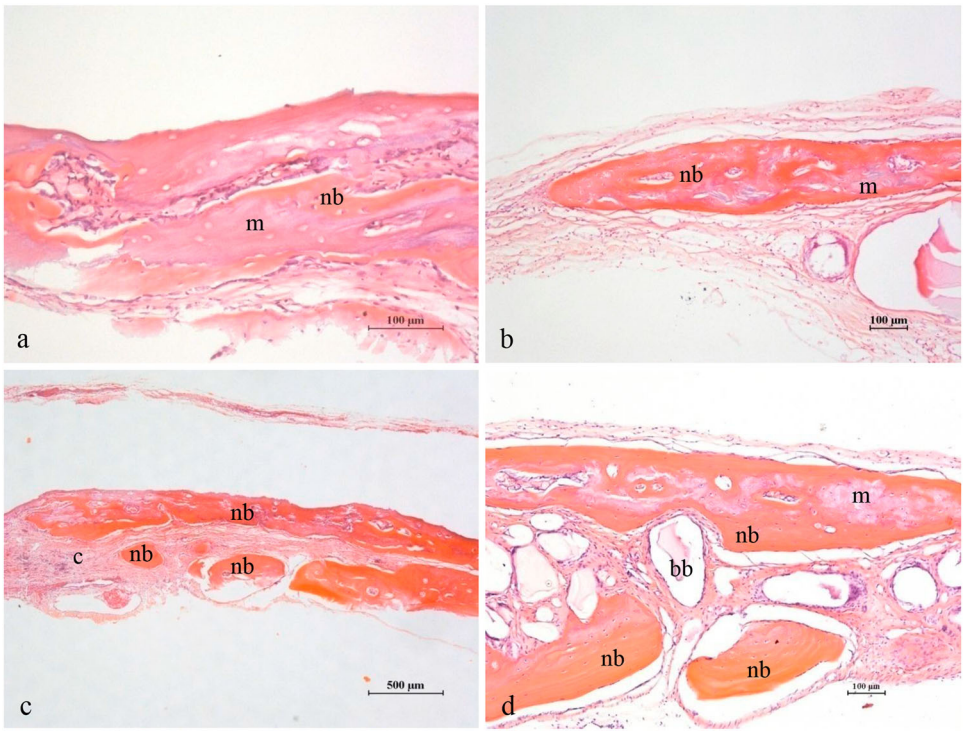

Fig. 3 Photomicrograph of calvarial bone defects filled with calcium sulfate **a** and **c** or deproteinized bovine bone mineral (b and d). **a**, **b** represent samples obtained 45 days after graft; **c** and **d**, 60 days after graft. All images show new bone (nb) replacing the membrane (m). After 60 days, **c** the site of calcium sulfate grafting was occupied by vascularized connective tissue (c) and small bone islets, and at **d** the site of grafting with bovine bone mineral, some fragments of deproteinized bovine bone mineral (bb) were replaced by bone (nb). H&E stain

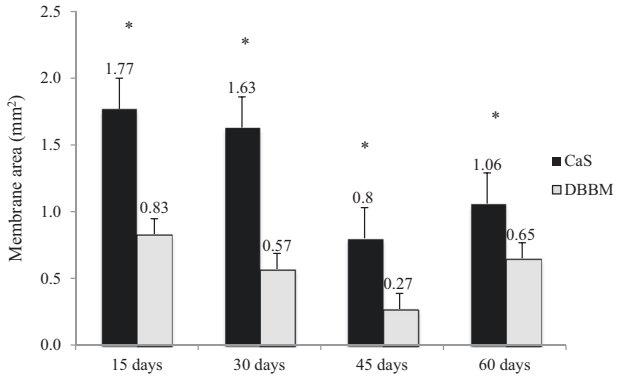

Fig. 4 Graph of collagen membrane area after implantation onto critical-size defect in rat calvaria. The defects were filled with calcium sulfate (CaS group) or deproteinized bovine bone mineral (DBBM group). The samples were collected at 15, 30, 45 and 60 days after implantation. Results expressed as median ($n = 10$). (*) represents statistical difference. Mann–Whitney test

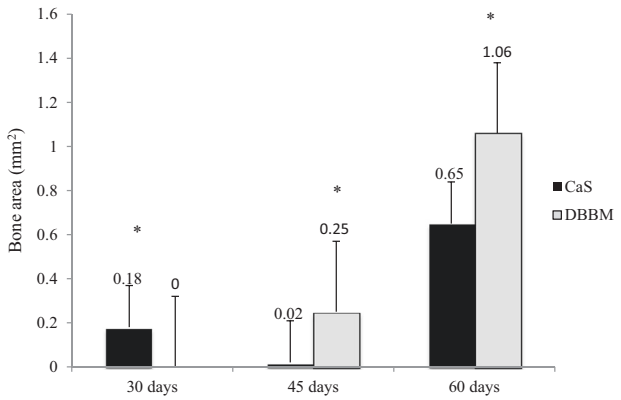

Fig. 5 Graph of bone formation area in collagen membrane after implantation onto critical-size defect in rat calvaria. The defects were filled with calcium sulfate (CaS group) or deproteinized bovine bone mineral (DBBM group). The samples were collected at 15, 30, 45 and 60 days after implantation. However, due to the small amount of bone formed at 15 days of observation in both groups, quantification was not possible. Results expressed as median ($n = 10$). (*) represents statistical difference. Mann–Whitney test

4 Discussion

The effectiveness of the application of resorbable membranes for the treatment of critically-sized defects has been described for different species, including rats [26–29], rabbits [30, 31] and dogs [32]. In the present study, the membrane was used as a cover to isolate the site of the bone defect and prevent non-osteogenic tissues from interfering with bone regeneration. Due to the fact that biodegradable membranes are obtained from less rigid materials, a filler material is required to prevent the collapse of the membrane into the region of the defect and to allow room for clot formation and the evolution of bone regeneration [33, 34]. Bio-Oss® and calcium sulfate were used to determine whether guided bone regeneration could be modulated, because both materials are biocompatible, possess osteoconductive and osteogenic properties and induce insignificant inflammatory response [35]. These fillers do, however, differ significantly in terms of the speed at which they are resorbed at the sites of bone regeneration.

Bio-Oss® can be completely incorporated through the mechanism of osteoconduction into the bone tissue, up to 11 years after graft surgery [22], while calcium sulfate can be resorbed within a few weeks [36, 37]. The calcium sulfate resorption process occurs by a combination of cellular activity and dissolution in body fluid [37]. This causes an increase in the local concentration of calcium ions which, when in contact with body fluids, leads to the local precipitation of calcium phosphate, the deposits of which are used by the body to form bone tissue at the regeneration sites [38–41].

Although the use of the collagen membrane is a well-established procedure, there is no consensus regarding the minimum length of time the resorbable membrane should remain in the body. Premature resorption of the membrane may lead to a loss of graft material and compromise the quantity and quality of the bone tissue formed in this region [42]. Some aspects that should be considered are the size of the bone defect, the animal model used, the intensity of the inflammatory reaction induced by the membrane [43], as well as the internal structure of the membrane and amount of collagen present [44, 45]. According to Kozlovsky et al. [46], minimum membrane permanence time should be six months or longer to allow adequate bone formation. It is generally accepted that resorbable membranes should remain at the regeneration site for at least four weeks to exercise the physical barrier function in regenerative procedures [2]. In the present study, the membrane had not been completely resorbed in either group by the maximum observation period of 60 days. However, the remaining area of the membrane at this timepoint was greater in the CaS group (53%) than in the DBBM group (31%). After four weeks of observation (30 days), the percentage of remaining membrane in the CaS group was approximately 81.5% vs. 28.5% in the DBBM group. This difference in percentages suggests that the filling material influences the amount of resorption of this type of collagen membrane and may interfere with its clinical outcome.

It is important to note that the membrane was resorbed and also replaced by bone. The presence of neutrophilic infiltrate denoted membrane degradation in some areas, alternating with the deposition of bone matrix in others. In the DBBM group at 60 days, 98.3% of the remaining membrane corresponded to bone while 1.6% was membrane residues. In the CaS group, 32.7% corresponded to formed bone tissue and 67.3% represented membrane remnants, showing that the process of bone formation on the

membrane was more effective in the DBBM group than in the CaS group, suggesting that the filling material may have influenced both bone formation and membrane resorption.

The presence of calcium particles on the surface of collagen membranes [47], and the fact that membranes can act as a scaffold for osteogenic cells [46, 48], are factors that may favor the formation of bone tissue combined with the membrane. In alveolar ridge defects, the membrane fibers were incorporated into the matrix of the new bone which, in turn, was integrated into the ridge bone [46, 48]. These characteristics reinforce the importance of the use of membranes. Bone formation associated with collagen membrane has been described by other authors using different study models [46, 49].

5 Conclusions

We concluded that the filling materials, deproteinized bovine bone mineral and calcium sulfate, can influence both the resorption of collagen membrane and the dynamics of the formation of bone tissue associated with collagen membrane during the guided bone regeneration procedure.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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