ORIGINAL RESEARCH

International Journal of Surgery 12 (2014) 378–383

Contents lists available at ScienceDirect

International Journal of Surgery

journal homepage: www.journal-surgery.net

Original research

Augmentation of intramembranous bone in rabbit calvaria using an occlusive barrier in combination with demineralized bone matrix (DBM): A pilot study

Víctor Beltrán [a,b,*], Wilfried Engelke [a], Ruth Prieto [c], Iván Valdivia-Gandur [d], Pablo Navarro [e], María Cristina Manzanares [f], Eduardo Borie [b,g], Ramón Fuentes [b]

[a] Department of Maxillofacial Surgery, University of Göttingen, Germany
[b] Departamento Integral del Adulto, Facultad de Odontología, Universidad de La Frontera, Chile
[c] Facultad de Medicina, Universidad de La Frontera, Chile
[d] Unidad de Anatomía Humana, Departamento Biomédico, Universidad de Antofagasta, Chile
[e] Departamento de Matemáticas y Estadística, Facultad de Ingeniería, Universidad de La Frontera, Chile
[f] Unidad de Anatomía y Embriología Humana, Health University of Barcelona campus-Bellvitge, Universidad de Barcelona, Spain
[g] Ribeirão Preto Dental School, University of São Paulo, Ribeirão Preto, Brazil

ARTICLE INFO

Article history:
Received 17 November 2013
Received in revised form
1 February 2014
Accepted 13 March 2014
Available online 18 March 2014

Keywords:
Bone regeneration
Biomaterials
DBM
Barriers
Bone

ABSTRACT

The aim of this study was to histologically evaluate the performance of demineralized bone matrix (DBM) when compared with a blood clot in addition to an occlusive barrier in the bone regeneration process for bone defects in a rabbit model. Prefabricated metallic capsules with 4.5 mm and 3.5 mm dimensions were placed in five adult rabbit skulls. At the right side, the capsule was filled with DBM, and the clot was located on the left side. The barriers were supplied with a 0.5 mm horizontal peripheral flap and a vertical edge, fitting tightly into a circular slit prepared by a trephine in the skull. After a healing period of three months, the animals were sacrificed, and the samples were prepared for histological and histomorphometric analyses after capsule removal. Trabecular and medullar bone percentages were calculated from the different areas of the newly formed bone inside the metallic barriers, and non-parametric statistical analysis was used to describe the findings. The results showed a complete filling of newly formed bone inside the capsules of both groups. Less mature bone tissue was observed in the upper third of all samples, and a higher trabecular area was observed in the samples with DBM. The use of barriers resulted in the augmentation of newly formed bone in a three-month period. However, a higher trabecular area was observed in the barriers filled with DBM.

1. Introduction

Bone-graft substitutes are useful for increasing the healing of bony defects caused by traumatic injury, tumor removal, abnormal skeletal development, cyst removal, and prosthetic loosening. The common sources of bone graft materials include autogenous bone, vascularized fibular grafts, allogeneic bone, synthetic calcium phosphate bone-graft substitutes, and a variety of osteoinductive agents [1]. One of the most commonly used biomaterials for bone augmentation is demineralized bone matrix (DBM), which has osteoconductive and osteoinductive properties [2,3]. In this context, osteoconductivity is defined as the ability to provide a 3D configuration for the in-growth of host capillaries, perivascular tissue, and osteoprogenitor cells into the graft, and osteoinduction is defined as the ability to encourage the host to synthesize new bone [1]. Osteoinductivity can also be explained as the ability to stimulate the formation of bone in heterotopic sites, leading to the isolation of several osteoinductive factors, including bone morphogenetic proteins (BMPs), from extracts obtained from DBM [4].

However, the stabilization and retention of the graft material within the defect site is of paramount importance for bone regeneration. In this context, several studies have demonstrated the possibility of regenerating or generating bone prior to or in conjunction with implant treatment, and the effects of an occlusive titanium cap on bone generation beyond the skeletal envelope have

* Corresponding author. Dpto. Integral del Adulto, Dental School, Universiidad de La Frontera, Claro Solar N° 115 – Of. 420, 4781176 Temuco, Chile.
E-mail address: victor.beltran@ufrontera.cl (V. Beltrán).

http://dx.doi.org/10.1016/j.ijsu.2014.03.010
1743-9191/

also been evaluated [4–7]. In humans, vertical augmentation of the alveolar crest using membranes has not yet been established as a routine procedure, and a number of unresolved questions remain. The space-making function of membranes in particular has been underestimated until now [8]. A technique with occlusive barriers may influence augmentative procedures in various alveolar sites in the future because the concept is based on well-known surgical principles using a flapless approach and a secure space-making device [9].

Some authors have used rigid barriers in rabbit models and have observed favorable results with bone regeneration. In humans, similar devices have been placed in transverse maxillary bone defects, with results that depend mainly on the barrier size, the time of placement in the donor bone, and the associated use of biomaterials [8–11]. Engelke et al. [9] used small diameter barriers in combination with osteoconductive bone biomaterials, reporting favorable results for up to eight months after insertion in humans. However, the success of the methods, related to the characteristics of the stimulated bone receptor and the type of biomaterial used, has not been clarified in humans. Additionally, the presence of an osteoinductive commercial biomaterial and its behavior over the bone-regeneration process has not been reported in the literature.

Thus, the aim of this study was to histologically evaluate the performance of demineralized bone matrix when compared with a blood clot in addition to an occlusive barrier, in the bone regeneration process for bone defects in a rabbit model.

2. Materials and methods

2.1. Study design

The following study was experimental with a non-randomized sample. Five rabbits (*Oryctolagus cuniculus*) were selected for the study, with a mean weight of 3.1 $\pm$ 0.7 kg to minimize the growth effect. Two 4.5 mm defects were created in each rabbit skull in five randomly chosen rabbits. Each defect was created to apply an occlusive barrier with biomaterial or blood clot inside and to keep it separated from periosteal influence.

The research was conducted in accordance with the international ethical principles for biomedical research involving animals and was approved by the University de la Frontera Ethical Committee (protocol N° 06/008). The animals were confined and maintained with ad libitum feeding in separated closed cages with controlled temperature.

2.2. Surgical technique

Anesthesia was administered with ketamine 30 mg/kg and xylazine 5 mg/kg supplemented with intraoperative analgesia with buprenorphine 0.3 ml/kg at 30-min after the first application. In addition, diazepam 5 mg/mL, with a dose of 1 mg/kg i.m. was used to maintain neuroleptanalgesia levels and this was complemented with Hypnorm 0.1 ml/kg i.m. at intervals of 30 min during surgery. In addition, as a local anesthetic, 0.4 ml of lidocaine 2% was applied with a dose of 1:100,000 of epinephrine (Octocaine-100, Novocol Pharmaceutical, Ontario, Canada).

The rabbit's skin was shaved and treated for surgery with 70° ethanol and povidone-iodine to enable performance of the surgical stages under a sterile environment. A median lineal incision was made from the frontal to the occipital region, separating the skin and periosteum 3–4 cm laterally. Subsequently, two osteotomies were performed with a 4.5-mm trephine bur and continuous irrigation. After this, multiple perforations were created in the external cortical trephined area of the calvaria with low-speed round burs, 0.5 mm in diameter with safety stops to prevent intracranial perforation. This caused profuse bleeding from the blood vessels of the bone (Fig. 1A). Therefore, at the left side (clot group), a capsule was filled with blood from the spontaneous bleeding that occurred through small holes in the calvaria bone of the same rabbit (Fig. 1B). The other capsule, at the right side, was filled with the demineralized bone matrix (DBM, Dynagraft® putty-D, Keystone Dental, Burlington, Massachusetts, USA). The capsules were inserted on each side of the sagittal suture in the same specimen without contact (DBM group). Each capsule was 4.5 mm in diameter (3.7 mm internal diameter) and 3.5 mm in height, with internal and external smooth surface walls. Following surgery, the rabbits were medicated during the three first days with one daily dose of Oxytetracycline 200 mg/kg i.m and Meloxicam 0.2 mg/kg s.c.

2.3. Specimens processing

After three months, the animals were sacrificed with 1 ml of Hypnorm (i.m.) and 10 ml of sodium pentobarbital (i.v.). Biopsies were sampled from each rabbit calvaria that comprised the two previously inserted barriers. These were fixed by immersion in 4% tamponed formaldehyde for 48 h and refrigerated at 5 °C (41°F). Subsequently, the capsules were cut with a circular saw (0.2 mm thick) in the antero-posterior direction. The first cut was made to eliminate the lateral metal wall, the second was made at the center of the sample, and the third was made to eliminate the contralateral metal wall (each cut was parallel to the one above). In this way, two sections of the samples were obtained. Then, the metal part was eliminated carefully, and the samples were decalcified for conventional histology. Only the medial faces of the samples were used for histological analysis.

For histological analysis, Hematoxylin-eosin and Masson trichrome techniques were used. Trabecular and medullar bone percentages were calculated from the different areas of the newly formed bone inside the metallic barriers. For this purpose, the newly formed bone tissue was divided in thirds from the base cortical bone to the uppermost region of the newly formed bone at the clot (Fig. 2) and the DBM (Fig. 3).

In the histomorphometric analysis, the samples were analyzed in detail at all segments of the newly formed bone tissue. First, the basal bone of the cranium and the lower third of the newly formed bone tissue were differentiated. Serial parasagittal cuts were used to determine the amount and comparative distribution of trabecular bone, and the relationship with the trabeculae number of each third. Histomorphometric analysis was performed with a camera (NIKON DS-Fi1 (Sigth) and a Stemi 2000-C stereomicroscope) with 1280 $\times$ 960 pixels. The software Scion Image was used to measure the medullar and trabecular bone areas.

2.4. Statistical analysis

The Shapiro–Wilk test was applied to determine the normality of the data. After this, the Kruskal–Wallis test was used to determine the behavior of the population medians for all of the investigated thirds between the newly formed callus and the intramembranous or basal bone ($p \leq 0.05$).

3. Results

3.1. Animal behavior

No signs of discomfort or suffering were observed in the animals. Feeding was normal, and the weight was considered to be within normal parameters for all of the animals. The surgical site showed normal healing and the suture was reabsorbed 16–22 days post-surgical intervention without signs of infection.

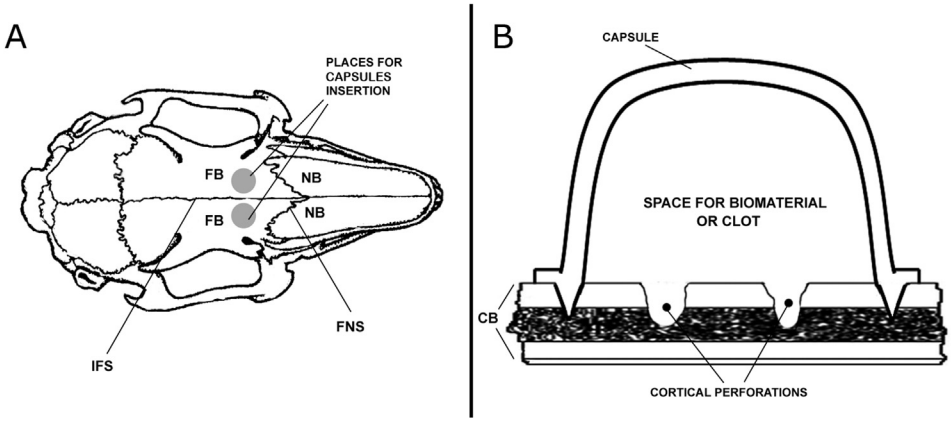

Fig. 1. A. Schematic of rabbit-skull, showing the locations for occlusive-barrier insertion. FB: Frontal Bone; NB: Nasal Bone; IFS: Interfrontal Suture; FNS: Frontonasal suture; B. Schematic showing a barrier inserted in the cortical area of the frontal bone. CB: cortical bone.

3.2. Histological and histomorphometric analyses

Newly formed bone tissue with slender trabeculae was observed in both treatment groups. Additionally, two areas showed similar distributions as the initial two-thirds of the newly formed bone tissue; the upper third showed apparently less mature bone tissue (Fig. 2A–D and Fig. 3A–D). Some DBM particles were observed within the trabecular bone in the capsules.

A tendency in favor of DBM was observed in relation to the amount of trabecular bone within the capsules for the sectioned thirds. This was more evident for the middle third and mild for the upper third (Fig. 4). A greater trabecular area was identified in the middle and lower thirds of the DBM samples, which was different from the upper third where only a minimal difference was observed between the two tissues.

Trabeculae numbers was clearly higher for DBM samples when compared with clots, mainly for the upper and middle thirds. However, the lower third of the newly formed bone showed a greater amount of trabeculae in the clot group but a greater area of trabecular bone in the DBM samples (Table 1). No significant difference was observed between the total trabecular areas of the samples with the DBM and the clot ($P \leq 0.05$). However, individual analysis of the thirds showed a statistical difference in favor of the DBM samples in the middle third (Table 1 and Fig. 4). In general, the trabecular bone area of the DBM specimens was the highest for the newly formed bone inside the barrier.

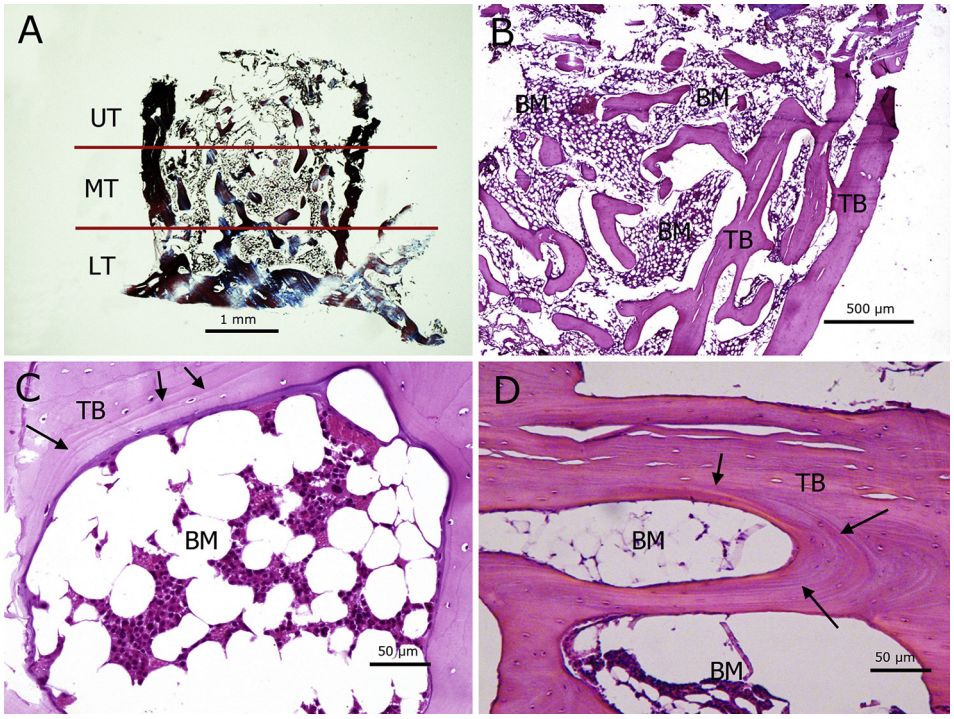

Fig. 2. Histological analysis of bone tissue obtained from the barrier with blood clots obtained from bleeding from the cortical bone perforation. A. Histological image of the frontal cut of bone tissue formed inside the capsule. UT: upper third; MT: middle third; LT: lower third; B, C and D show histological detail from the lower, middle and upper thirds, respectively. It is possible to observe trabecular bone (TB) and bone marrow (BM) in all thirds. Black arrows in image C and D indicate the lamellar organization of bone. A. Masson trichrome stain; B, C and D. Hematoxylin – eosin stain.

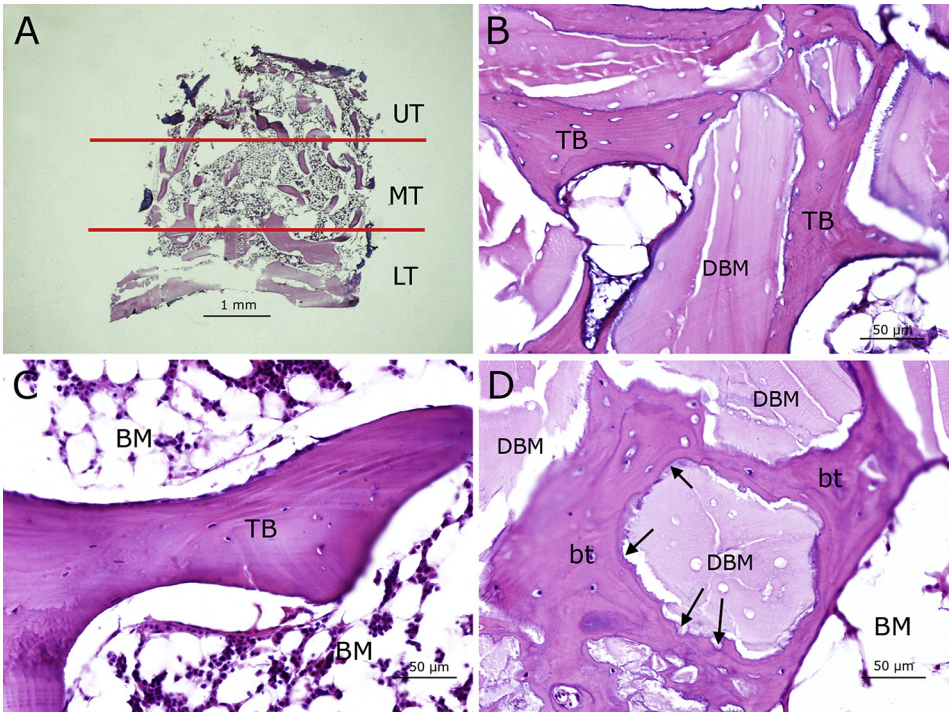

Fig. 3. Histological analysis of bone tissue obtained from the barrier with demineralized bone matrix (DBM) and blood clots obtained from bleeding from the cortical bone perforation; A. Histological image of the frontal cut of bone tissue formed inside the capsule. UT: upper third; MT: middle third; LT: lower third; B, C and D show histological detail from the lower, middle and upper thirds, respectively; B. Trabecular bone formed among DBM particles; C. Shows that mature trabecular bone formed in the middle third with bone marrow surrounding it (BM). D. Upper third shows immature bone tissue (bt), which was less calcified and similar to woven bone. The black arrows indicate Howship's lacunae, a typical sign of bone remodeling.

4. Discussion

To increase bone volume, a subperiosteal barrier has been used to allow the blood clot to develop bone tissue. This technique is called guided bone augmentation. Lundgren et al. [12] stated that the best method for guided bone augmentation is to use a stiff occlusive barrier. The stiffness of the barrier used in this study allowed shape maintenance and the creation of a space for graft placement, preventing collapse of the defect space and achieving the required volume. Another important feature is the occlusivity of

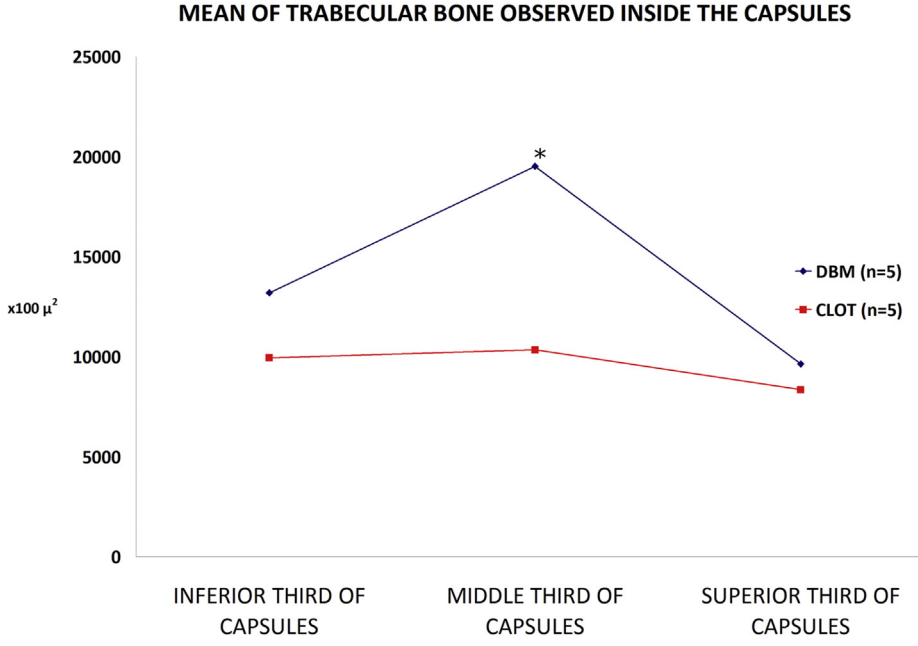

Fig. 4. Mean quantification of trabecular bone observed inside capsules. In general, the capsules with DBM showed a greater amount of trabecular bone compared with capsules with clots. However, only the middle third showed a statistically significant difference based on the analysis of nonparametric samples (95% confidence using the median).

Table 1
Relationship between the mean numbers of bone trabeculae observed and mean measurements made in different thirds of the capsules with DBM and clots. Although the means of the measurements show a tendency toward greater trabecular bone formation in samples with DBM, the analysis of nonparametric samples indicates that no statistically significant differences were present in this parameter between capsules with DBM and with clots. The mean numbers of trabeculae were approximated without decimals.

	Inferior third of capsules		Middle third of capsules		Superior third capsules		Total area of capsules	
	Mean of trabeculae observed	Mean of trabecular bone area (×100 ì2)	Mean of trabeculae observed	Mean of trabecular bone area (×100 ì2)	Mean of trabeculae observed	Mean of trabecular bone area (×100 ì2)	Mean of trabeculae observed	Mean of trabecular bone area (×100 ì2)
DBM ($n = 5$)	9	13,188.03	20	19,523.96	18	9628.79	47	42,340.78
CLOT ($n = 5$)	15	9940.12	12	10,337.43	12	8361.92	39	28,639.47

this barrier, which is necessary to avoid fibrous tissue formation [13]. The main disadvantage of these barriers is the tendency to become exposed during the healing period. However, the use of other non-resorbable devices such as non-rigid membranes is not recommended. This is because such membranes can be deformed or flattened during the augmentation procedure by tension from the adjacent soft tissue [14].

The capsule used in this study had a smooth surface because as stated by Lundgren et al. [4], the surface roughness does not seem to influence the amount of bone formation beyond the skeletal envelope.

This study made sure that the barriers that were used enabled bone-graft and blood-clot stabilization as well as space maintenance, as described in the literature [15,16]. However, it is also necessary to check some of the additional factors that are required for a successful outcome: barrier stability, peripheral sealing between barrier and bone, blood supply and access to bone-forming cells, among others [5]. If these factors are not controlled, the barrier's principal aims of keeping the biomaterial or the blood clot inside, and separate from periosteal influence, would not be possible.

Tamura et al. [17] used three-dimensional images constructed from computed tomography images after one or three months in rabbits, and observed bone regeneration, with newly formed bone along the cylinder wall and a central crater, with the same shape as the dome. These agree with the clinical observations of the present study. Here, it was demonstrated that it is possible to increase the skull bone thickness beyond the skeletal envelope, which agrees with other studies [5,18,19]. Additionally, the capsules were completely filled with newly formed bone which agrees with the results of Lundgren et al. [18]. However, the bone quality, mainly in the upper third, appeared to be poor. Some studies with barriers [17,20,21] found that the upper surface may present incomplete maturation, indicating that this process may require more than 90 days of bone healing. This may be the result of poor blood supply to the upper third in combination with a higher contact area with the barrier when compared with the middle and the lower thirds. All of the histological samples showed slender trabeculae, which is in accordance with some reports [5,10], where screwed cylinders with only clot inside were tested. It is important to note that these findings should not be rare because the original diploe of the rabbit skull exhibits slender trabeculae and a low degree of mineralization. Furthermore, Van Steenberghe et al. [10] proposed that the configuration and low mineralization degree of the newly formed bone inside the barrier may be related to the fact that it was not subjected to any functional stimuli.

Molly et al. [8] mention that although bone formation can be obtained under a barrier with just a clot, it is necessary to insert a bone graft to augment the bone volume. This statement is not in accordance with the results of this study because both groups exhibited bone augmentation with the barriers, despite the dissimilarity in the trabecular bone areas in the different thirds of the two groups. The same authors [8] reported that the behavior of some allografts and xenografts could be different when these grafts are placed under a stiff occlusive barrier.

Newly formed bone was identified inside both capsules. However, the trabecular bone area (for the newly formed bone inside the barrier) was the highest for the DBM specimens. Similar results were described by Slotte and Lundgren [6], who reported that domes filled with other bone-graft (cancellous deproteinized bovine bone) material revealed a higher amount of augmented tissue. Despite the great quantity of bone graft alternatives that are available in the market, DBM was used as the bone graft inside the capsule, mainly because of its osteoinductive capacity in combination with the successful reports in the literature [22,23]. Fuentes et al. [23,24] tested DBM and freeze-dried bone allograft (FDBA) in human alveolar sockets, and observed a more mature and mineralized bone tissue in the samples treated with DBM after a six-month healing period.

The use of the barrier seems to help with bone formation. However, the use of an osteoinductive graft material such as DBM seems to further increase the trabecular bone area. Nevertheless, further studies with a larger sample and other histological techniques could lead to better interpretation of these preliminary results.

5. Conclusions

The use of barriers resulted in the augmentation of newly formed bone in rabbit skulls in a three-month period. However, higher trabecular areas were found when the barriers were filled with DBM as bone grafts.

Ethical approval

Approved by the ethical committee, Universidad de La Frontera, Temuco, Chile (N° 06/008).

Conflicts of interest

No conflict of interest.

Sources of funding

The study was financed with self-funds.

References

[1] S.T. Moore, J.M. Katz, R.M. Zhukauskas, et al., Osteoconductivity and osteoinductivity of Puros(R) DBM putty, Journal of Biomaterials Applications 26 (2011) 151–171.

[2] W.R. Sassard, D.K. Eidman, P.M. Gray, et al., Augmenting local bone with Grafton demineralized bone matrix for posterolateral lumbar spine fusion: avoiding second site autologous bone harvest, Orthopedics 23 (2000) 1059–1064.

[3] G. Zimmermann, A. Moghaddam, Allograft bone matrix versus synthetic bone graft substitutes, Injury 42 (2011) S16–S21.
[4] A.K. Lundgren, D. Lundgren, A. Wennerberg, et al., Influence of surface roughness of barrier walls on guided bone augmentation: experimental study in rabbits, Clin Implant Dentistry and Related Research 1 (1999) 41–48.
[5] A.K. Lundgren, D. Lundgren, C. Hämmerle, et al., Influence of decortications of the donor bone on guided bone augmentation. An experimental study in the rabbit skull bone, Clinical Oral Implants Research 11 (2000) 99–106.
[6] C. Slotte, D. Lundgren, Augmentation of cavarial tissue using non-permeable silicone domes and bovine bone mineral. An experimental study in the rat, Clinical Oral Implants Research 10 (1999) 468–476.
[7] Y. Yamada, K. Nanba, K. Ito, Effects of occlusiveness of a titanium cap on bone generation beyond the skeletal envelope in the rabbit calvarium, Clinical Oral Implants Research 14 (2003) 455–463.
[8] L. Molly, M. Quirynen, K. Michiels, et al., Comparison between jaw bone augmentation by means of a stiff occlusive titanium membrane or an autologous hip graft: a retrospective clinical assessment, Clinical Oral Implants Research 17 (2006) 481–487.
[9] W. Engelke, O. Decco, M.J. Rau, Minimalinvasive augmentation des unterkiefer mit einem mikrofixierten starren formkörper, ZWR 113 (2004) 399–402.
[10] D. van Steenberghe, C. Johansson, M. Quirynen, et al., Bone augmentation by means of a stiff occlusive titanium barrier. A study in rabbits and humans, Clinical Oral Implants Research 14 (2003) 63–71.
[11] V. Beltrán, A. Matthijs, E. Borie, et al., Bone healing in transverse maxillary defects with different surgical procedures using anorganic bovine bone in humans, International Journal of Morphology 31 (2013) 75–81.
[12] A. Lundgren, D. Lundgren, A. Taylor, Influence of barrier occlusiveness on guided bone augmentation. An experimental study in the rat, Clinical Oral Implants Research 9 (1998) 251–260.
[13] Y.D. Rakhmatia, Y. Ayukawa, A. Furuhashi, et al., Current barrier membranes: titanium mesh and other membranes for guided bone regeneration in dental applications, Journal of Prosthodontic Research 57 (2013) 3–14.
[14] P.J. Louis, Vertical ridge augmentation using titanium mesh, Oral and Maxillofacial Surgery Clinics of North America 22 (2010) 353–368.
[15] H. Ozdemir, S. Ezirganli, M. Isa Kara, et al., Effects of platelet rich fibrin alone used with rigid titanium barrier, Archives of Oral Biology 58 (2013) 537–544.
[16] F. Watzinger, J. Luksch, W. Millesi, et al., Guided bone regeneration with titanium membranes: a clinical study, British Journal of Oral & Maxillofacial Surgery 38 (2000) 312–315.
[17] T. Tamura, Y. Fukase, E. Goke, et al., Three-dimensional evaluation for augmented bone using guided bone regeneration, Journal of Periodontal Research 40 (2005) 269–276.
[18] D. Lundgren, A.K. Lundgren, L. Sennerby, et al., Augmentation of intramembranous bone beyond the skeletal envelope using an occlusive titanium barrier. An experimental study in the rabbit, Clinical Oral Implants Research 6 (1995) 67–68.
[19] J. Schmid, C.H.F. Hämmerle, A.J. Olah, et al., Membrane permeability is unnecessary for guided generation of new bone. An experimental study in the rabbit, Clin Oral Impl Res 5 (1994) 125–130.
[20] A. Wennerberg, C.H. Hämmerle, S. Nyman, Influence of superface roughness of barrier walls on guided bone augmentation: experimental study in rabbits, Clinical Oral Implants Research 1 (2007) 41–48.
[21] M. Seiko, S. Sato, M. Saito, et al., Micro-computerized tomography analysis: dynamics of bone augmentation within a titanium cap in rabbit calvarium, Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics 106 (2008) 892–895.
[22] E.S. El-Chaar, Demineralized bone matrix in extraction sockets: a clinical and histologic case series, Implant Dentistry 22 (2013) 120–126.
[23] R. Fuentes, J.P.M. Issa, M.M. Iyomasa, et al., The behavior of demineralized bone matrix (DBM) in post-extraction sockets, International Journal of Morphology 30 (2012) 394–398.
[24] R. Fuentes, G. Oporto, S. Olate, et al., Histological evaluation of bone regeneration means freeze dried bone allograft (FDBA) in post exodontia sockets, International Journal of Morphology 29 (2011) 286–292.