J Mater Sci: Mater Med (2011) 22:997–1004
DOI 10.1007/s10856-011-4278-7

Biological monitoring of a xenomaterial for grafting: an evaluation in critical-size calvarial defects

Thais Accorsi-Mendonça · Willian Fernando Zambuzzi · Clóvis Monteiro Bramante · Tânia Mari Cestari · Rumio Taga · Márcia Sader · Glória Dulce de Almeida Soares · José Mauro Granjeiro

Received: 13 October 2010 / Accepted: 28 February 2011 / Published online: 20 March 2011

Abstract Our purpose was to evaluate the osteoconduction potential of mixed bovine bone (MBB) xenografts as an alternative for bone grafting of critical-size defects in the calvaria of rats. After surgery, in the time intervals of 1, 3, 6, and 9 months, rats were killed and their skulls collected, radiographed and histologically prepared for analysis. The data obtained from histological analysis reported that the particles of MBB did not promote an intense immunological response, evidencing its biocompatibility in rats. Our results clearly showed the interesting evidence that MBB was not completely reabsorbed at 9 months while a small amount of newly formed bone was deposited by osteoprogenitor cells bordering the defect. However, this discrete bone-forming stimulation was unable to regenerate the bone defect. Overall, our results suggest that the properties of MBB are not suitable for stimulating intense bone regeneration in critical bone defects in rats.

T. Accorsi-Mendonça
Department of Endodontics, University of Grande Rio, Duque de Caxias, RJ 25071-202, Brazil

C. M. Bramante
Department of Endodontics, Bauru Dental School, University of Sao Paulo, Sao Paulo, Brazil

W. F. Zambuzzi · J. M. Granjeiro
Projects Division, The National Institute of Metrology, Standardization and Industrial Quality (INMETRO), Rio de Janeiro, Brazil

T. M. Cestari · R. Taga
Department of Biological Sciences, Bauru Dental School, University of Sao Paulo, Sao Paulo, Brazil

M. Sader · G. D. de Almeida Soares
Metallurgy and Materials Engineering, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

J. M. Granjeiro (✉)
Department of Cell and Molecular Biology, Institute of Biology, Fluminense Federal University, UFF, Outeiro de São João Baptista, s/n, Campus do Valonguinho, Centro/Niteroi, RJ 24020-150, Brazil
e-mail: jmgranjeiro@vm.uff.br

W. F. Zambuzzi · J. M. Granjeiro
Cell therapy center, University Hospital Antonio Pedro, UFF, Niterói, RJ, Brazil

1 Introduction

Over the last decades an increase of interest has been noticed in relation to the development and production of novel biomedical materials which are able to conduct bone regeneration in lesions. Although autogenous bone grafts present excellent biological properties (such as osteogenic and osteo-induction potentials), they also present a number of inconveniences which have lead researchers to search for an ideal substitute material.

Recent studies have demonstrated that the organic or anorganic matrix derived from bovine bone is biocompatible [1, 2]. This source of materials is convenient because it is easily available and cheap to purchase. On the other hand, an adequate preparation of this material needs to be performed in order to avoid risks such as the transmission of zoonosis [3]. The effectiveness of various bone processing techniques has made it possible to use such biomaterials, of a variety of species, for medical applications [4–6]. It is important to point out that hydroxyapatite and collagen structures are very similar among mammal's species [7].

Bovine materials present both biocompatibility and osteoconductivity. These important biological properties

allow an apposition of newly formed bone from osteoprogenitor cells located at the border of the host tissue. It is very important to mention that mixed bovine bone, particularly its anorganic phase promotes a support of calcium ions and phosphate moieties while its organic phase guarantees an adequate source of collagenic proteins, which are very important for guiding cell migration from host tissue. Hence, both phases supported by bovine bone grafts are essentials for stimulating the neoformation of bone [8]. It is known that materials exclusively constituted of organic matrix present poor mechanical resistance, which does not allow a block implantation followed by immediate function of the receptor site, or even implant fixation with screws. Therefore, the main goal is to develop a material that allows the use of immediate load, maintaining the characteristics of biocompatibility, osteoconduction, and osteoinduction [9, 10].

Seeking to fulfill these expectations bovine bone is processed using hydrogen peroxide, organic solvents and caotropic agents that promote the removal of cells, proteins and lipids. The resulting material contains both the organic (type I collagen and other non-collagenic proteins) and anorganic (biological apatite) structures that are suitable for the treatment of bone loss. This protocol to produce mixed materials has been widely used by biomedical companies, such as those which produce Tutoplast® (Mentor) and Kiel Bone® (Kiel) [11].

Despite the wide range of available biomaterials for bone replacement, an ideal biomaterial with both suitable mechanical and biological properties has still not been developed. Still, the xenomaterial evaluated in this work is obtained from the cancellous region of bovine long bones, treated with solvents, caotrophic and peroxide agents, without removing the mineral matrix or structural framework of collagen fibers in order to preserve its mechanical and resistance properties. This study aimed to evaluate the biological response of this material when implanted in a living system. Also, the experimental model in bone size defects allowed the prediction of osteconductivity of the tested material. In order to eliminate the migration of fibroblastic cells surrounding materials immediately after implantation, we used an absorbable membrane such as suggested by Oliveira et al. 2006 [12].

2 Materials and methods

This study was approved by the Ethics Committee of Animal research of the School of Dentistry of Bauru, University of São Paulo. A total of 40 rats (*Rattus norvegicus*), male adults (5 months old and average weight of 350 g) were randomly divided in two groups according to the treatment: 1) clot (control group) and 2) MBB implant (test group). The experimental periods tested were 1, 3, 6 and 9 months.

2.1 Materials

Mixed bovine bone (MBB) was produced from the cancellous portion of bovine long bone, after submitted to mechanical crushing and chemical etch with hydrogen peroxide, organic solvents, alkalis and chaotropic agents. After biomaterial treatment with saline phosphate buffer, it was lyophilized and sterilized by gamma radiation (25 kGy). The membrane used in this study is from cortical demineralized bone and is commercially available in Brazil (Gen-Derm®, Baumer S.A., Brazil). The topography of MBB and absorbable membrane were analyzed using a scanning electron microscope—SEM (JEOL, JSM-6460LV, Japan), working at 15 kV, with energy dispersive X-ray spectrometry—EDX (Thermo, Noran System Six model 200). The samples were sputter-coated with gold (Emitech, K550, USA) and secondary electrons (SE) and back scattered electrons (BSE) images were employed, while elemental composition was evaluated by EDX.

2.2 Surgical procedures

Trichotomy and incision were performed at the frontal-parietal region of each rat under general intramuscular anesthesia with xylazine hydrochloride (Anasedan, Vetbrands) and ketamine hydrochloride (Dopalen, Vetbrands, Brasil), in a 1:1 proportion. A perforation throughout the whole thickness of the diploe was performed with a 9 mm external diameter surgical trephine (Dentoflex®, Brazil) and continuous irrigation with saline solution. The critical size defect in the control group received only a blood clot while the test group defect was filled with the biomaterial mixed with blood. In both groups the bone defect was covered by the absorbable membrane.

2.3 Collection of specimens and histotechnical processing

For all experimental periods, rats were killed with an anesthetic overdose and the skulls were removed and immediately fixed in 10% buffered formaldehyde during 1 week. The skulls were then radiographed using optic plates with the following parameters: 70kVp, 10mAs, 40 cm film-focus distance and 0.26 s exposition time. The images were evaluated using the Digora system (Sordex Orion Corporation, Helsink, Finland) to follow the positioning of the material inside the bone defect.

Specimens were demineralized in a solution of EDTA (Merck, Germany) pH 7.2 for a period of approximately

2 months with weekly solution changes. After demineralization, the specimens were dehydrated in ethanol, cleared in xylol and embedded in Histosec (Merck, Germany), following the classical steps of histological procedure. Semi-serial sections of 5 μm thickness in a lateral-lateral direction were obtained and stained with the Hematoxylin-Eosin [13].

2.4 Histological analysis

An Axioskop 2 binocular microscope (Carl Zeiss, North America) was used to examine and evaluate the histological cuts according to bone neo-formation in the border of the defect and around biomaterial particles, presence and quality of the inflammatory infiltrate, intensity of the angiogenesis and occurrence of fibrosis between particles and/or in the defect space. The photomicrographs were obtained in an Axioskop (Carl Zeiss, North America) photomicroscope containing a photographic camera and a MC200 software chip. The osteoconductive capacity of the biomaterial was evaluated verifying new bone formation directly in the surface of particles.

3 Results

The morphology of the porous structure of MBB materials and particles size was evaluated by SEM (Fig. 1). The particles showed a bimodal distribution with pores in the range of 10–20 μm and smaller ones around 0.1–1.0 μm. Micrographs showed particles sizes in the range of 50–100 μm without the existence of concavities. The

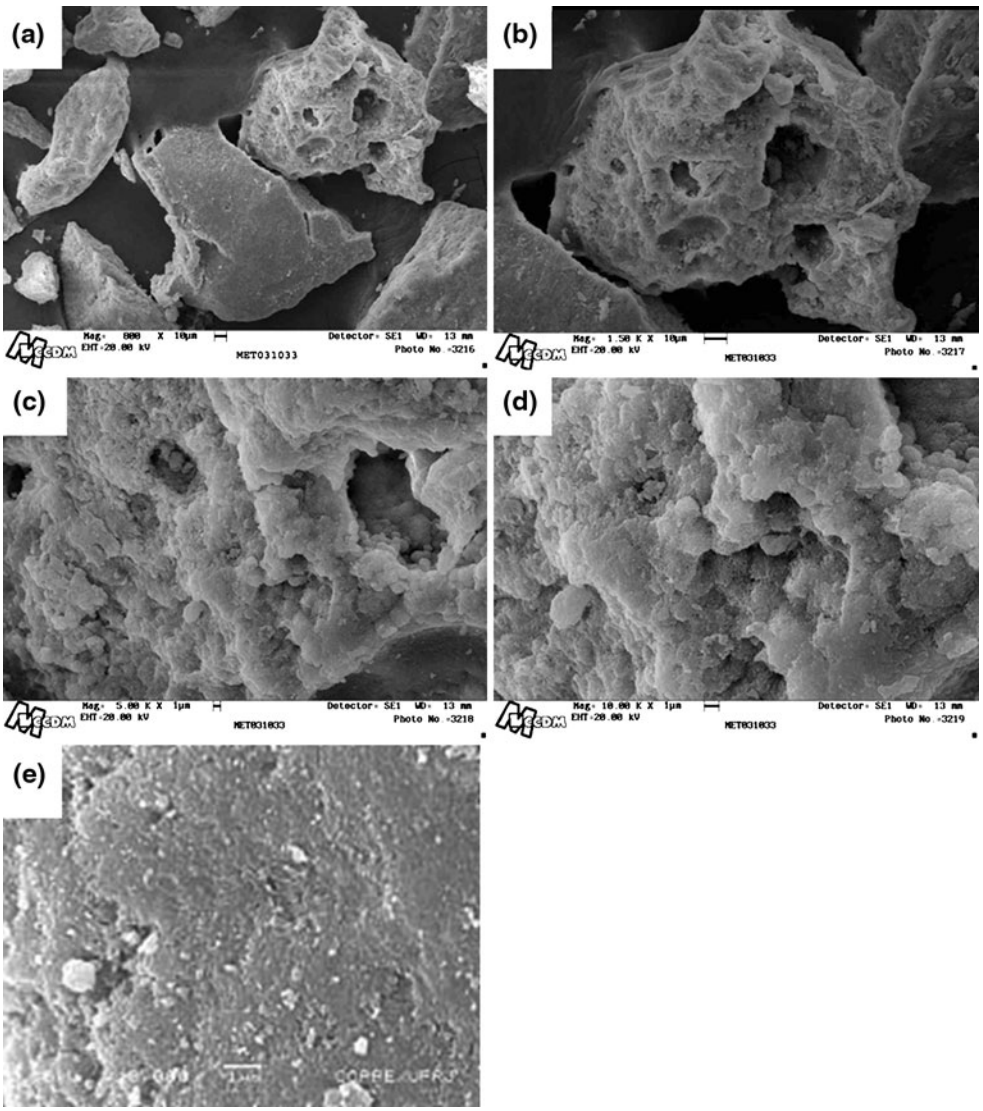

Fig. 1 Scanning electron microscopy of Mixed Bovine Bone. **a** Note particle size variation, 800×; **b** presence of concavities and micropores, 1500×; **c** 5000×; **d** irregular topography presenting pores in their surface, 10000×; **e** presence of nanoparticles on MBB surface, 10000×

absorbable membrane presented a uniform surface in SEM with pores sizes compatible with Harvers and Volkman′s channels (Fig. 2a). The backscattering images (Fig. 2a–e), showed some white particles with diameter of 1 μm on the membrane surface. On the dot mapping image it was noticed that these particles are rich in calcium (Fig. 2d), which was confirmed in the EDX spectrum (Fig. 2g) while on the matrix the spectrum shows only the presence of carbon (Fig. 2f).

Radiographic data showed that the control group was widely radiolucent for all periods and small radiopaque areas in defect border, which suggests mineralized tissue (Fig. 3a; arrows); while in the test group it was possible to identify some radiopaque areas representing the implanted

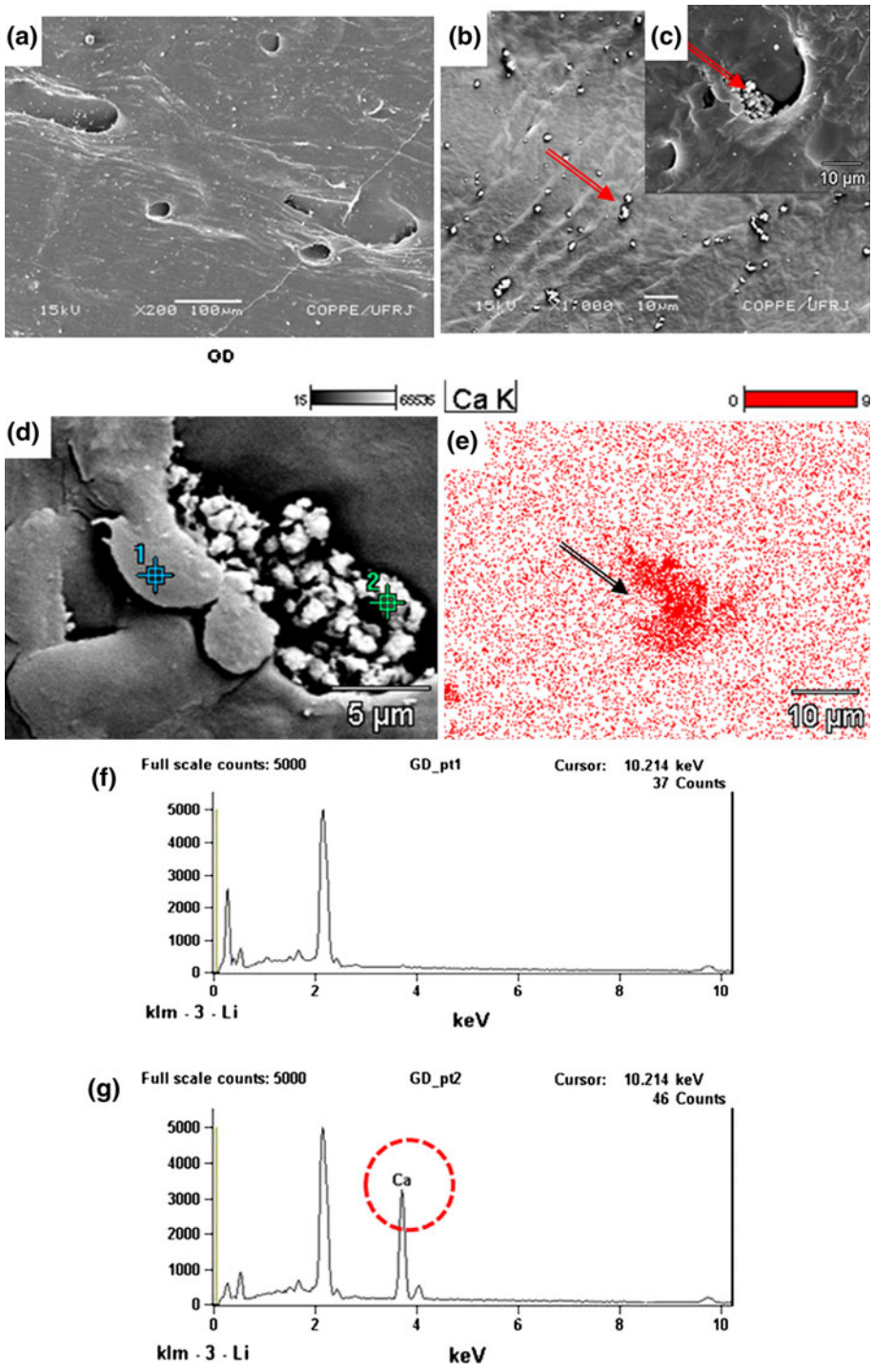

Fig. 2 Analysis of Gen-Derm® surface with EDX probe associated to SEM. **a** Scanning electron microscopy (SEM); **b–c** SEM with backscattered electrons; **d** calcium dot mapping; **e** SEM with backscattered electrons; **f** EDX espectroscopy for E1 region; **g** EDX espectroscopy for E2 region Voltage: 15.00; Magnification: 6000×

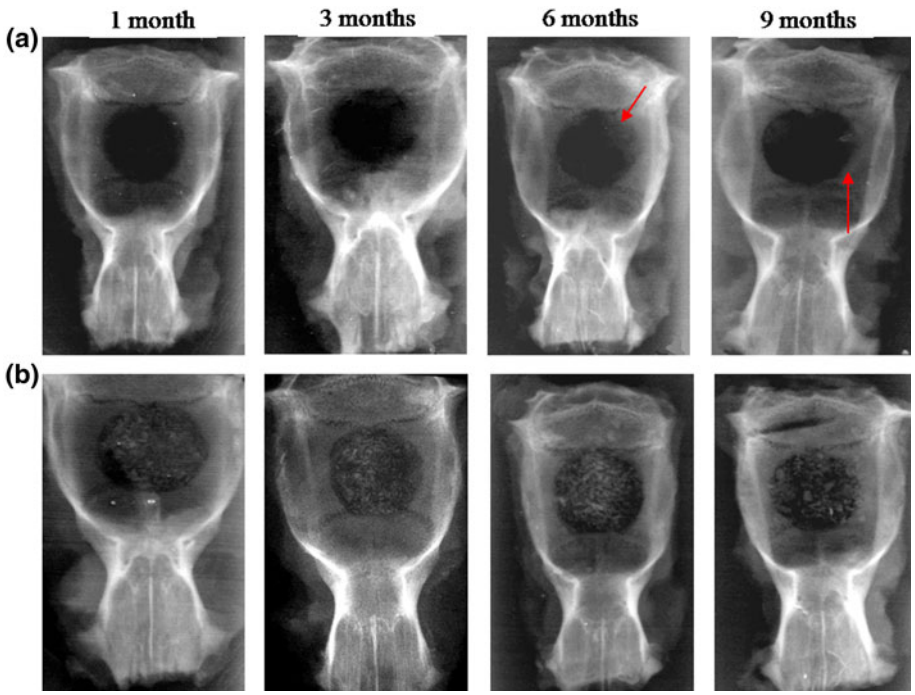

Fig. 3 Digital radiographic images. Control group **a** Blood Clot, *arrows* show areas that suggest presence of mineralized tissue at defect border; MBB **b** radiopaque formations in the middle of the defect with variable shapes and sizes

particles (Fig. 3b). Overall, data from histological analysis revealed that the defects in both groups were not completely filled by bone tissue in any of evaluated periods, while absorbable membrane was absent in all specimens up to 1 month after the surgery.

In the control group (Fig. 4a) the defect was filled by fibrous connective tissue with different degrees of cellularity and fibrosis and few areas of bone neoformation (Fig. 4b–d; nb—new bone). At the early periods a conspicuous cells diversity and high number of blood vessels was observed (Fig. 4d; arrow), while in the later periods the fibrous tissue was predominant and no mineralized tissue in the center of the lesion was observed (Fig. 4e–f; arrows).

On the other hand, in the test group (Fig. 5a), the implanted particles were distributed into bone defect space (Fig. 5c; points) surrounded by fibrous connective tissue. It was clear to observe that bone neoformation was present only in the borders of defects (Fig. 5; nb—new bone). In general, the particles were surrounded by neoformed bone or fibrous tissue (Fig. 5, biomaterial: point; fibrous tissue: FT). At the 9 months, it was possible to identify distinct points of bone neoformation on the central region of some defects (Fig. 5e). Despite the presence of some multinucleated giant cells, few reabsorbtion areas were observed up to 9 months.

4 Discussion

This study extends what is already known regarding to the potential use of bovine xenografts for osseous tissue repair and regeneration. As previously described, the purpose of this study was to evaluate the bone regenerative effects of mixed bovine bone (called MBB) in a critical-size rat calvarial defect model. As we have mentioned, most xenograft materials sold in the market are predominantly inorganic and organic-free that are also treated under high temperatures (>700°C) which culminate in low mechanical properties. MBB is a mixture of inorganic/organic components of bovine bone that are properly treated to remove cellular debris and fat tissue using chemical methods to avoiding heating. It is clear that the organic portion (mainly collagen) is the component associated with better mechanical properties, such as elasticity, tensile strength, and resistance.

In order to monitor the biological events in response to MBB, we decided to use a very classical bone critical size defect on rat's skulls. Thus, 8-mm-diameter defects were created, where either MBB (test group) or blood clots (control group) were implanted. The biological monitoring was carried out until up to 9 months after surgery. From our point of view, the critical-size rat calvarial defect used in this study was a very convenient model for evaluating bone regenerative effects of MBB since this model is relatively accessible, inexpensive, simple, and reproducible once spontaneous healing does not occur in the control specimen [14]. It has also been shown to be effective for evaluating bone formation potential [15].

In order to guide bone healing, the MBB or blood clot were covered by an absorbable membrane to reduce the risk of fibroblastic proliferation and fibrous tissue growth on the area of the bone defect, and to stop the migration of

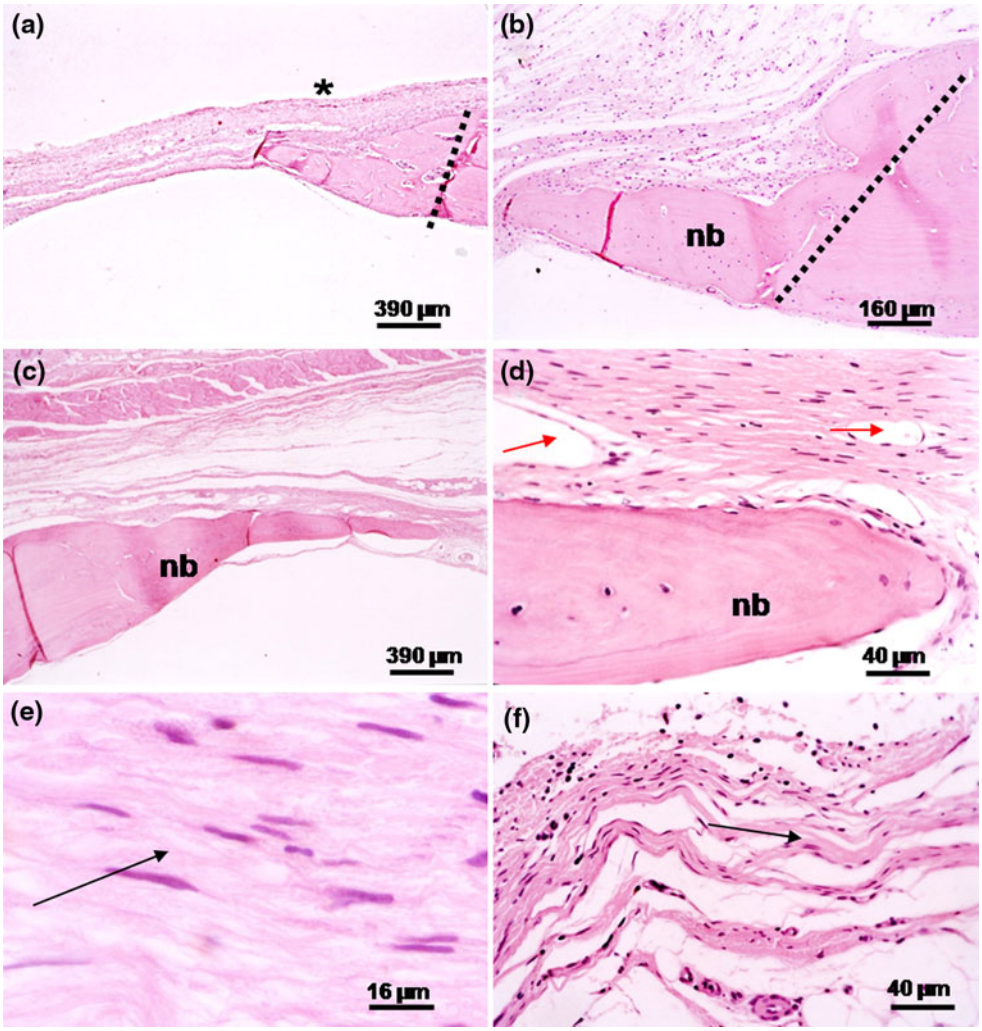

Fig. 4 Photomicrographies of the control group. **a** 1 month: lesion border (*dotted line*) reveals discreet bone formation and fibrous connective tissue in the defect space. Note membrane absence (*asterisk*), 4×; **b** 3 months: edges of the lesion (*dotted line*) and discreet bone neoformation (*nb* new bone), 10×; **c** 6 months: bone neoformation at the edges of the lesion, 4×); **d** 6 months: covered with dense connective tissue rich in cells and blood vessels—*arrow*, 40×; **e** 6 months: however, fewer number of cells and densely fibrous tissue (*arrow*) are seen when distant from bone edges, 100×; **f** 9 months: intense fibrosis (*arrow*) at central area. Magnification: 40×, Hematoxylin-Eosin

osteoprogenitor cells from the edges of the lesion [16]. As expected, radiographic and histological results obtained in the control group, showed absence of complete repair of the defect by bone tissue throughout all experiment periods. In fact, bone formation was observed close to the border of the lesions, which was promoted by viable osteoprogenitor cells residents in the physiologically active bone tissue, while other regions were filled by fibrous connective tissue. These results are in accordance with other authors who used the same biological model in rats [17].

Defects filled by MBB were not completely healed, probably due to fibroblastic activity/proliferation and partial fibrosis. The connective tissue growth surrounding particles compromises some chemo-attractive effects (if any) of MBB, impairing the recruitment of osteoprogenitor cells. This can be explained by the early degradation of the membrane, which allowed connective tissue growth into the defect and inhibited new bone formation. We were supported by previous studies performed by our group, where we have shown that a very similar membrane remained up to 6 months after surgery [18]. However, the time expected for membranes to degrade is a controversial issue in literature [18, 19]. The divergence present in literature might be due to variation of membrane thickness and animal model used. Moreover, we have identified some mineral particles on the membrane surfaces, and this can contribute to faster absorption, as they are capable to attract resorption cells. Also, the presence of calcium can stimulate an inflammatory reaction contributing to the release of

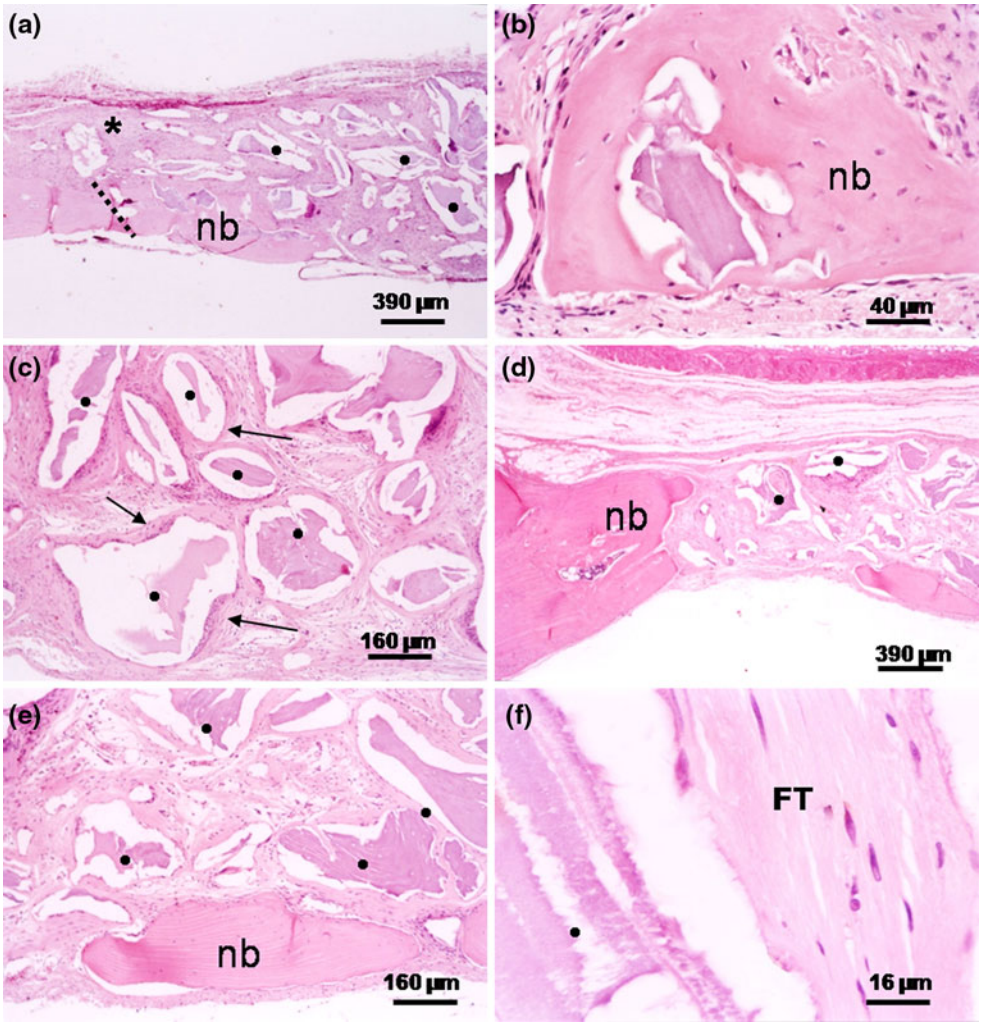

Fig. 5 Photomicrograph of group treated with MBB. **a** 1 month: lesion borders (*dotted line*) show new bone formation (*nb*) from the edges of the lesion, the biomaterial particles (*point*) and membrane absence (*asterisk*), 4×; **b** 3 months: biomaterial particle (*point*) near the edge surrounded with new bone (*nb*), 40×; **c** 6 months: particles in the central region surrounded with fibrous connective tissue (*arrows*), 10×; **d** 9 months: discrete new bone formation (*nb*) at the edge of the defect, 4×; **e** 9 months: new bone (*nb*) in central area, 10×; **f** 9 months: particle (*point*) surrounded with fibrous connective tissue (*FT*). Magnification: 100×. Hematoxylin-Eosin

cytokines and proteases [20], which contribute to organic molecules degradation. Also, very recently we have proposed that the transformations that occur on the surface of hydroxyapatite are fundamental for providing an ideal environment for osteoblast adhesion [21, 22].

Other important point was the lack of bone growth around MBB particles. All defects filled with MBB, particles were surrounded by fibrous connective tissue. Also, to control osteoblast adhesion and proliferation [23–26], the surface of biomaterials seem to modulate the inflammatory reaction [27]. The presence of irregular particles seems to enhance the inflammatory reaction in response to MBB. In fact, among the characteristics of biomaterials, the shape and the size of the particles are very important in relation to the modulation of inflammatory response. Needle shaped particles induced a larger production of TNF-alpha, IL-6 and IL-10 by cells. In a lower degree, small particles induced an increase of the expression and production of the cytokines studied (TNF-alpha, IL-6 and IL-10) [28, 29]. In the future, new studies must be developed to address the relationship between size/shape and biological response. These results will impact the rational use of granulate biomaterial for clinicians and with mean as advance in regenerative medicine and cell therapy.

5 Conclusion

Based on biological monitoring of MBB, we can conclude that these particles were not able to stimulate intense bone

regeneration in critical defects of rats, reinforcing the need of the rational use of adequate membrane barriers to allow the growth of newly formed bone.

Acknowledgments The authors would like to thank Dr. Flávio Monteiro Amado and Miss Daniele Santi Ceolin for exceptional technical support. This work was supported by CAPES (CAPES Foundation), FAPESP (The State of São Paulo Research Foundation—99/10655-5, 01/10707-7, and 04/08095-1), CNPq (National Council for Scientific and Technological Development—350084/03-3, 475721/2003-9, 505350/04-1 and 504.808/04-4).

References

1. Zambuzzi WF, Oliveira RC, Pereira FL, Cestari TM, Taga R, Granjeiro JM. Rat subcutaneous tissue response to macrogranular porous anorganic bovine bone graft. Braz Dent J. 2006;17(4):274–8.
2. Molly L, Vandromme H, Quirynen M, Schepers E, Adams JL, Van Steenberghe D. Bone formation following implantation of bone biomaterials into extraction sites. J Periodontol. 2008;79(6):1108–15.
3. Wenz B, Oesch B, Horst M. Analysis of the risk of transmitting bovine spongiform encephalopathy through bone grafts derived from bovine bone. Biomaterials. 2001;22(12):1599–606.
4. Anderson A, Mucalo MR, Johnson GS, Lorier MA. The processing and characterization of animal-derived bone to yield materials with biomedical applications. Part III: material and mechanical properties of fresh and processed bovine cancellous bone. J Mater Sci Mater Med. 2000;11(11):743–9.
5. Johnson GS, Mucalo MR, Lorier MA. The processing and characterization of animal-derived bone to yield materials with biomedical applications: part 1: modifiable porous implants from bovine condyle cancellous bone and characterization of bone materials as a function of processing. J Mater Sci Mater Med. 2000;11(7):427–41.
6. Meyer S, Floerkemeier T, Windhagen H. Histological osseointegration of Tutobone: first results in human. Arch Orthop Trauma Surg. 2008;128(6):539–44.
7. Le Geros RZ. Calcium phosphates in oral biology and medicine. Monographs in Oral Science. Karger. New York, USA, 1991.
8. Sciadini MF, Dawson JM, Johnson KD. Evaluation of bovine-derived bone protein with a natural coral carrier as a bone-graft substitute in a canine segmental defect model. J Orthop Res. 1997;15(6):844–57.
9. Jensen SS, Aaboe M, Pinholt EM, Hjorting-Hansen E, Melsen F, Ruyter IE. Tissue reactions and material characteristics of four bone substitutes. Int J Oral Maxillof Impl. 1996;11(1):55–66.
10. Cornelini R, Cangini F, Martuscelli G, Wennström J. Deproteinized bovine bone and biodegradable barrier membranes to support healing following immediate placement of transmucosal implants: a short-term controlled clinical trial. Int J Period Rest Dent. 2004;24(6):555–63.
11. Tadic D, Epple M. A thorough physicochemical characterisation of 14 calcium phosphate-based bone substitution materials in comparison to natural bone. Biomaterials. 2004;25(6):987–96.
12. de Oliveira RC, Carneiro E, Cestari TM, Taga R, Granjeiro JM. Dynamics of subcutaneous tissue response to the implantation of tetracycline-treated or untreated membrane of demineralized bovine cortical bone in rats. J Biomater Appl. 2006;21(2):167–78.
13. Luna LG. Manual of histologic staining methods of the armed forces institute of pathology. New York, USA: McGraw-Hill Book Company; 1968.
14. Frame JW. A convenient animal model for testing bone substitute materials. J Oral Surg. 1980;38(3):176–80.
15. Selvig KA. Discussion: animal models in reconstructive therapy. J Periodontol. 1994;65(12):1169–72.
16. Wang HL, Caroll WJ. Guided bone regeneration using bone grafts and collagen membranes. Quint Int. 2001;3(7):504–15.
17. Bosch C, Melsen B, Vargervik K. Importance of the critical-size bone defect in testing bone-regenerating materials. J Craniofac Surg. 1998;9(4):310–6.
18. Taga ML, Granjeiro JM, Cestari TM, Taga R. Healing of critical-size cranial defects in guinea pigs using a bovine bone-derived resorbable membrane. Int J Oral Maxillofac Impl. 2008;23(3):427–36.
19. Herculano MA, Tella OI Jr, Prandini MN, Alves MTS. Inhibition of peridural fibrosis after laminectomy using biological sheet in rat model. Arq Neuropsiquiatr. 2006;64(2A):259–63.
20. Zambuzzi WF, Paiva KB, Menezes R, Oliveira RC, Taga R, Granjeiro JM. MMP-9 and CD68(+) cells are required for tissue remodeling in response to natural hydroxyapatite. J Mol Histol. 2009;40(4):301–9.
21. Bertazzo S, Zambuzzi WF, Campos DD, Ferreira CV, Bertran CA. A simple method for enhancing cell adhesion to hydroxyapatite surface. Clin Oral Implants Res. 2010;21(12):1411–3.
22. Bertazzo S, Zambuzzi WF, Campos DD, Ogeda TL, Ferreira CV, Bertran CA. Hydroxyapatite surface solubility and effect on cell adhesion. Colloids Surf B Biointerfaces. 2010;78(2):177–84.
23. Bertazzo S, Zambuzzi WF, da Silva HA, Ferreira CV, Bertran CA. Bioactivation of alumina by surface modification: a possibility for improving the applicability of alumina in bone, oral repair. Clin Oral Implants Res. 2009;20(3):288–93.
24. Zambuzzi WF, Bruni-Cardoso A, Granjeiro JM, Peppelenbosch MP, de Carvalho HF, Aoyama H, Ferreira CV. On the road to understanding of the osteoblast adhesion: cytoskeleton organization is rearranged by distinct signaling pathways. J Cell Biochem. 2009;108(1):134–44.
25. Zambuzzi WF, Milani R, Teti A. Expanding the role of Src and protein-tyrosine phosphatases balance in modulating osteoblast metabolism: lessons from mice. Biochimie. 2010;92(4):327–32.
26. Milani R, Ferreira CV, Granjeiro JM, Paredes-Gamero EJ, Silva RA, Justo GZ, Nader HB, Galembeck E, Peppelenbosch MP, Aoyama H, Zambuzzi WF. Phosphoproteome reveals an atlas of protein signaling networks during osteoblast adhesion. J Cell Biochem. 2010;109(5):957–66.
27. Laquerriere P, Grandjean-Laquerriere A, Addadi-Rebbah S, Jallot E, Laurent-Maquin D, Frayssinet P, Guenounou M. MMP-2, MMP-9 and their inhibitors TIMP-2 and TIMP-1 production by human monocytes in vitro in the presence of different forms of hydroxyapatite particles. Biomaterials. 2004;25(13):2515–24.
28. Laquerriere P, Grandjean-Laquerriere A, Jallot E, Balossier G, Frayssinet P, Guenounou M. Importance of hydroxyapatite particles characteristics on cytokines production by human monocytes in vitro. Biomaterials. 2003;24(16):2739–47.
29. Nadra I, Boccaccini AR, Philippidis P, Whelan LC, McCarthy GM, Haskard DO, Landis RC. Effect of particle size on hydroxyapatite crystal-induced tumor necrosis factor alpha secretion by macrophages. Atherosclerosis. 2008;196(1):98–105.