Histologic Evaluation of the Osteoinductive Property of Autogenous Demineralized Dentin Matrix on Surgical Bone Defects in Rabbit Skulls Using Human Amniotic Membrane for Guided Bone Regeneration

Mônica Fernandes Gomes, MD, DDS[1]/Mário James da Silva dos Anjos, MD, DDS[2]/
Terezinha de Oliveira Nogueira, MD, DDS[3]/Sérgio Augusto Catanzaro Guimarães, MD, DDS[4]

The aim of this investigation was to evaluate the osteoinductive property of autogenous demineralized dentin matrix (ADDM) on experimental surgical bone defects in the parietal bone of rabbits using the guided bone regeneration (GBR) technique incorporating human amniotic membrane (HAM). Thirty-six rabbits were divided into 2 groups, HAM and ADDM+HAM. It was possible to conclude that HAM did not interfere with bone repair and was resorbed. Slices of ADDM induced direct bone formation and were incorporated by the newly formed bone tissue and remodeled. The bone defects healed faster in the ADDM+HAM group than in the group with HAM only. (Int J Oral Maxillofac Implants 2001; 16:563–571)

Key words: *autogenous demineralized dentin matrix, biologic dressings, bone repair, guided tissue regeneration, human amniotic membrane*

Many studies have been performed attempting to accelerate bone repair using different osteoinductive materials, such as autogenous demineralized bone matrix,[1–3] autogenous demineralized dentin matrix (ADDM),[4–7] and bone growth factors.[3] The guided bone regeneration (GBR) technique promotes the increase of osteoblastic proliferation and synthesis of bone matrix. The whole process is controlled by complex molecular interactions that act upon mesenchymal cells, producing their proliferation and differentiation.[6]

Previous investigations have shown that proteins associated with bone matrix have chemotactic, mitogenic, and osteogenic potential.[3,5,8] These effects have been seen as well with dentin matrix.[5,6] The chemotactic and osteogenic potential of bone and dentin matrix are associated with bone morphogenetic proteins (BMPs).[5] Bone matrix is the greatest source of growth factors among mineralized tissues. Some of these factors are produced by osteoblasts, eg, insulin growth factor (IGF), transforming growth factor (TGF), fibroblastic growth factor (FGF), and platelet-derived growth factor (PDGF). However, additional factors are produced by other cells related to bone tissue, such as macrophages, which produce interleukin (IL) and tumor necrosis factor (TNF). Dentin matrix contains significant amounts of these growth factors.[3,5,7,9,10] The importance of ADDM as an osteoinductive implant material has been verified in the literature.[2,4,7]

[1]Professor, Department of Biopathology and Diagnosis, São José dos Campos Dental School, São Paulo State University, UNESP, São Paulo, Brazil.
[2]Professor, Department of Dentistry, Ceará Federal University, Ceará, Brazil.
[3]Associate Professor, Department of Biopathology and Diagnosis, São José dos Campos Dental School, São Paulo State University, UNESP, São Paulo, Brazil.
[4]Associate Professor, Department of Pathology, Bauru Dental School, São Paulo University; and Department of Pathology, School of Medicine, UNIMAR, Marília, São Paulo, Brazil.

Reprint requests: Dr Mônica Fernandes Gomes, Faculdade de Odontologia de São José dos Campos, UNESP, Depto. de Biopatologia e Diagnóstico, Av. Eng. Francisco José Longo, 777 / Cep: 12.245-000, São José dos Campos, São Paulo, Brasil. Fax: +55-12-3213620. E-mail: mfgomes@fosjc.unesp.br

Fig 1 The ADDM slices were immersed in a closed container containing ethyl alcohol 70°/gentamicin (5 mL/0.2 sol).

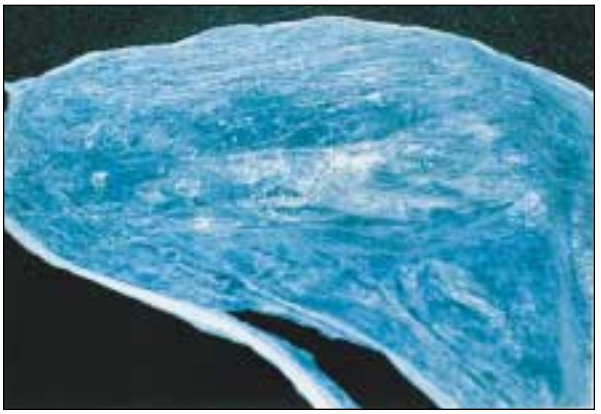


Fig 2 Macroscopic aspect of the lyophilized human amniotic membrane.

Clinical and experimental studies with GBR have shown positive results of the technique in different biologic models.[11,12] The technique is based on the theory that different types of cells adjacent to a wound area produce different types of tissues at different times.[9,13–15] Bone repair could be optimized by using a membrane as a barrier to obstruct the invasion of undesirable cells at the bone healing area. This technique permits the preferential repopulation of specific cells at specific places, which will then be able to regenerate the type of bone tissue lost in the area.[16]

Since the beginning of the 19th century, human amniotic membrane (HAM) has been used as a biologic dressing in wound areas.[17–19] Studies indicate that the use of this membrane is mainly associated with the early induction of the repair process, as well as the promotion of pain relief and hemostasis.[20,21] Other publications indicate the existence of angiogenic and growth factors in the amnion, which stimulate the repair process.[18,22] Antibacterial properties of this membrane have also been reported when it is applied to infected wounds.[23] It is believed that the antibacterial property of HAM is related to the presence of lysozyme present in amniotic fluid.[24] According to Needham[25] and Talmi and coworkers,[23] lysozyme is a powerful bactericidal enzyme in high concentration in HAM, and it acts against many gram-negative microorganisms. Some authors have suggested that HAM is relatively well tolerated when used as a self-graft.[26,27]

The purpose of the present investigation was to evaluate the osteoinductive activity of ADDM together with a GBR technique incorporating the use of HAM in experimental surgical bone defects in the parietal bone of rabbits.

MATERIALS AND METHODS

Thirty-six adult rabbits with an average weight of 3.5 kg were used and divided into 2 groups, experimental (ADDM+HAM) and control (HAM). The ADDM was obtained by extraction of the central incisor of the rabbits used in the experimental group and prepared in slices, according to the technique of Catanzaro-Guimarães and associates.[4] The extracted teeth were processed by the following techniques. The tooth pulps were extirpated by a retrograde technique and the residues of the periodontal ligament were removed by scaling of the root. The teeth were washed with sterile physiologic serum at 2°C and then immersed in a 0.6 N hydrochloric acid solution at 2°C until completely demineralized. The specimens were then washed in distilled water under constant agitation for 3 hours for total acid removal. After this process, the ADDM was cut into slices of approximately 8 µm thickness with the aid of freezing microtomy. These slices were immersed in a closed container containing ethyl alcohol 70°/gentamicin (5 mL/0.2 sol) and stored at 2°C until the time of implantation (Fig 1).

The HAM was obtained by cesarean section from selected human subjects. Acceptable conditions for utilization of the membrane were: absence of previous membrane rupture, no history of sexually transmitted diseases or infectious/contagious

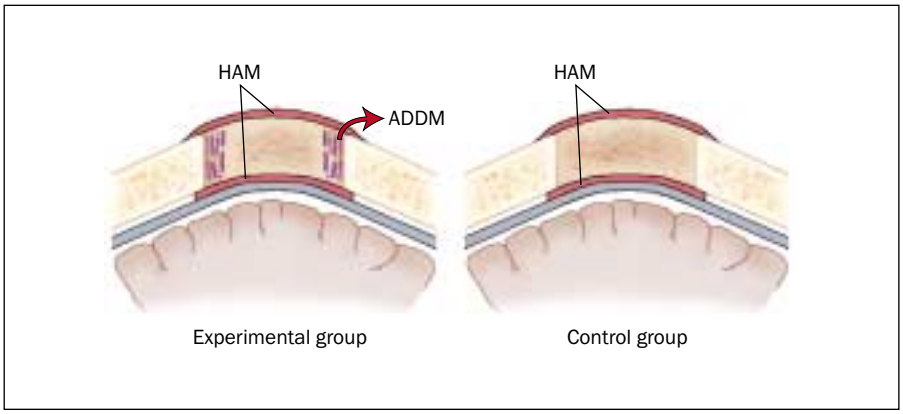


Fig 3 Transverse incisions of the defect in rabbit parietal bone. HAM = human amniotic membrane; ADDM = autogenous demineralized dentin matrix.

diseases, no pelvic inflammatory disease, no toxemia, and absence of abnormal amniotic fluid.[27,28] This membrane was lyophilized and sterilized with ethylene oxide (Fig 2).

The animals were anesthetized intramuscularly with Rompum (pre-anesthetic, Bayer SA, São Paulo, Brazil) and Ketamina (anesthetic, Holliday-Scott SA, São Paulo, Brazil). The pre-anesthetic agent was applied 5 minutes before administration of the anesthetic. An incision was made in the median sagittal plane, followed by muscular dissection, plane to plane, and incision of the periosteum. Subsequently, a surgical bone defect was created, with the aid of a 5.0-mm trephine activated by a surgical micromotor. The bone defect had an elliptic form (1.0×0.5 cm), with a depth equal to the thickness of the removed cortical bone.

In the experimental group, HAM was positioned on the floor of the bone defect and ADDM was placed in slices, internally and at the periphery of the bone defect. The superficial surface of the bone defect was completely recovered by HAM. Immediately afterward, the periosteum was sutured, as well as the skin. In the control group, HAM was placed on the defect floor and on the surface of the surgical bone defect and the defect was filled with blood clot. Then, the periosteum and the skin were sutured (Fig 3).

Thirty, 60, 90, and 120 days after the surgery, 3 animals in each group were sacrificed. The bone containing the created defect was removed en bloc, fixed in 10% formalin, and submitted to light microscopic analysis. The sections were obtained in longitudinal direction and showed about 8 µm of thickness. The sections were stained by hematoxylin-eosin, Mallory trichrome, and Schmorl.

RESULTS

Defects at 30 Days

In the experimental group (ADDM+HAM), the region of the bone defect showed immature bone tissue throughout the defect and, in some areas at the surface, the presence of osteogenic connective tissue could be observed. This tissue showed a discrete infiltration of mononuclear inflammatory cells, with a predominance of lymphocytes. Above the surgical bone defect, HAM and some mononuclear inflammatory cells were observed in direct contact with the HAM surface. In a panoramic view, a greater number of immature bone trabeculae were seen at the extremities of the surgical bone defect than in the central part, characterizing a centripetal growth pattern. The ADDM slices, implanted at the limits of the defect, were incorporated into the newly formed bone matrix (Fig 4). In some areas, osteoclastic cells and numerous osteoprogenitor cells were observed on the surface of the ADDM slices (Fig 5).

In the control group (HAM), the defects were filled with bone tissue and osteogenic connective tissue. The newly formed bone trabeculae were immature and extended from the limits of the defect to the central part of the surgical bone defect, characterizing centripetal bone growth.

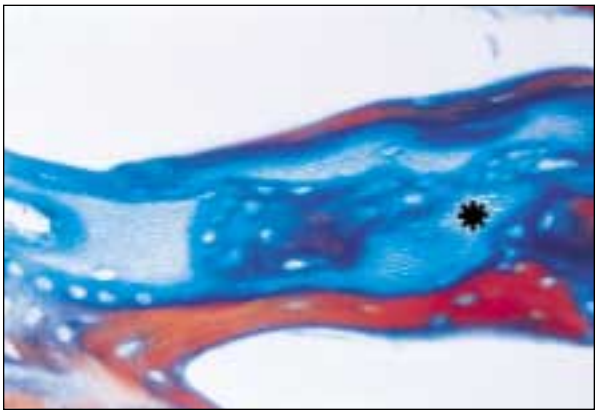


Fig 4 Experimental group at 30 days. Autogenous demineralized dentin matrix slices *(asterisk)* have been incorporated into the newly formed bone matrix (Mallory trichrome; magnification ×200).

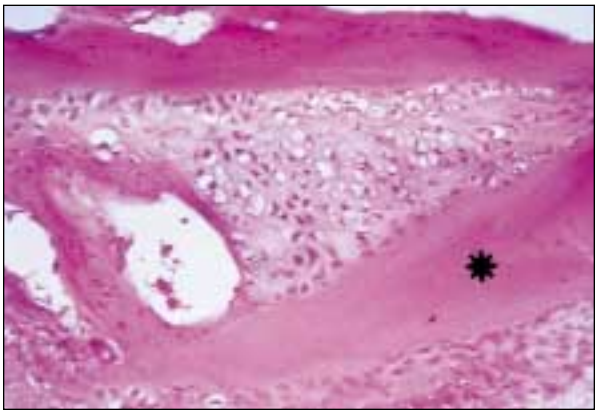


Fig 5 Experimental group at 30 days. Numerous osteoprogenitor cells can be seen on the surface of the autogenous demineralized dentin matrix slices *(asterisk)* (hematoxylin-eosin; magnification ×200).

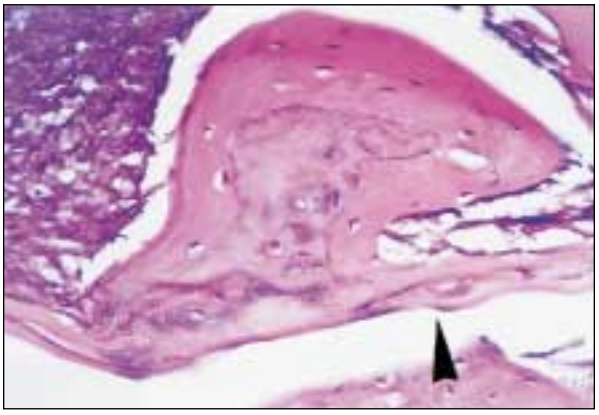


Fig 6 Control group at 30 days. Newly formed bone is in contact with human amniotic membrane *(arrow)* (hematoxylin-eosin; magnification ×200).

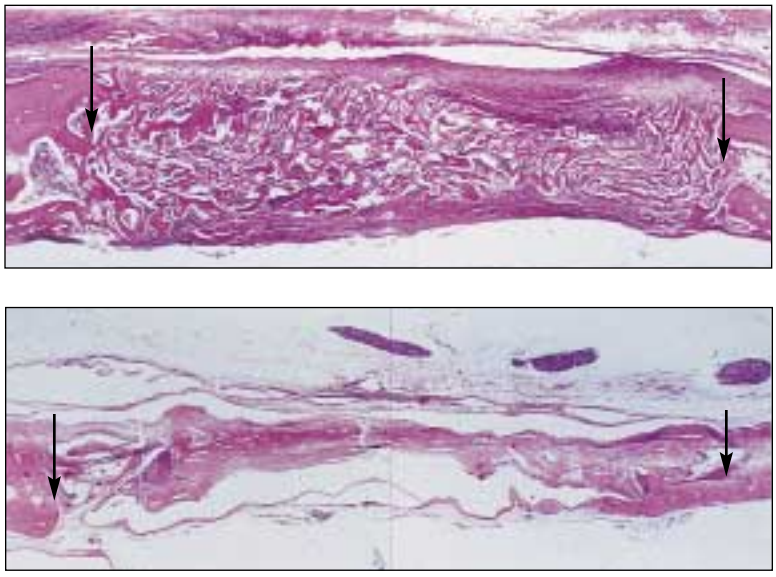
Newly formed bone and a discrete infiltrate of lymphocytic inflammatory cells were observed in contact with the HAM, along with intense osteoclastic activity, characterizing a resorption phenomenon (Fig 6). In a panoramic view, it was noted that the thickness of the newly formed bone trabeculae was thinner in comparison to the thickness of the original bone trabeculae. No evidence of HAM rejection was observed in either control or experimental sites (Figs 7a and 7b).

Defects at 60 Days

In the experimental group, the surgical bone defect was completely filled by mature bone tissue, with thick and well-defined bone trabeculae and areas of newly formed bone. At one of the defect extremities, the presence of an exophytic bone growth was verified, with numerous bone trabeculae and reduced medullary spaces. The absence of HAM was also observed, and ADDM slices incorporated in bone matrix were resorbing during the bone remodeling process (Fig 8). In the control group, the region of the surgical bone defect was completely filled with bone tissue. This tissue showed red and yellow bone marrow with wide medullary spaces. On the floor of the defect, the presence of HAM was still seen in contact with mononuclear inflammatory cells and immature bone trabeculae. No signs of the rejection of HAM were evident in either control or experimental sites (Figs 9a and 9b).

Defects at 90 Days

In the experimental group, the surgical bone defect was filled with bone tissue, with numerous bone trabeculae and wide medullary spaces present. The bone trabeculae were mature, thick, and well defined. However, there were areas of newly formed bone showing trabeculae with many osteoblasts. In a panoramic view, the newly formed bone tissue presented discrete variations in thickness in comparison to the original cortical bone level. In the control group, almost the entire bone defect was filled with mature bone tissue. At one of the lateral limits of a defect, the discrete presence of osteogenic connective tissue was observed. In this area, HAM remnants were still present in contact with this membrane, along with macrophages, characterizing a resorption phenomenon. Adjacent to the HAM, few newly formed bone trabeculae were seen. In the panoramic view, the area of the bone



Figs 7a and 7b Photomicrographs of the bone defect region after a period of 30 days: *(Top)* Autogenous demineralized dentin matrix with human amniotic membrane and *(bottom)* human amniotic membrane only. Limits of the surgical bone defect are indicated by arrows (hematoxylin-eosin; magnification ×25).

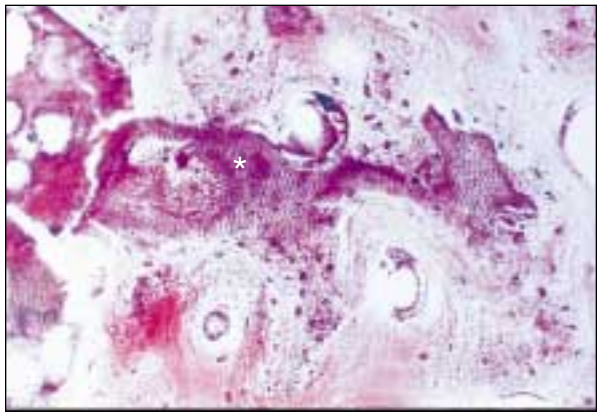
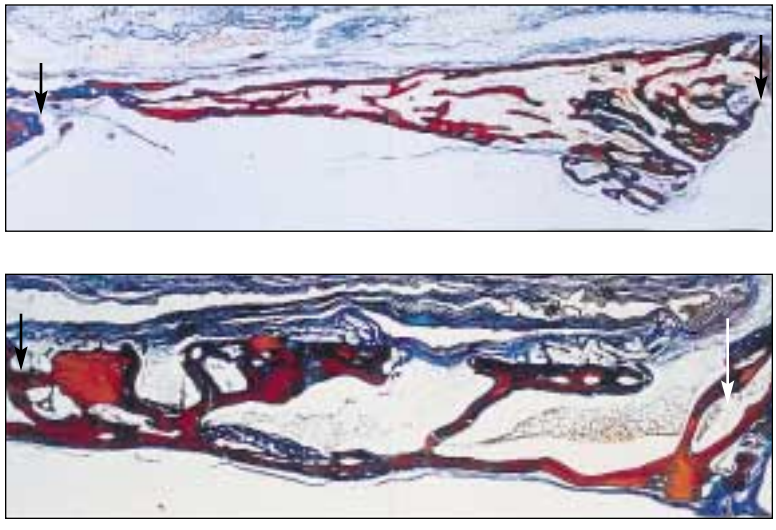
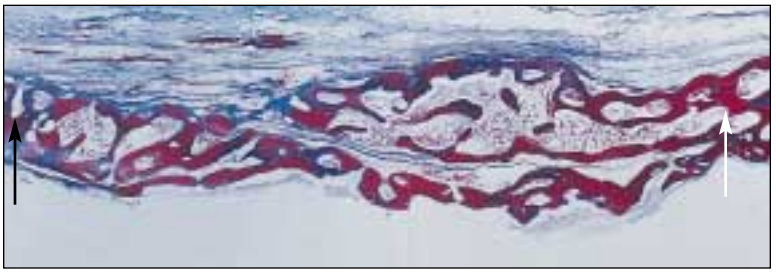
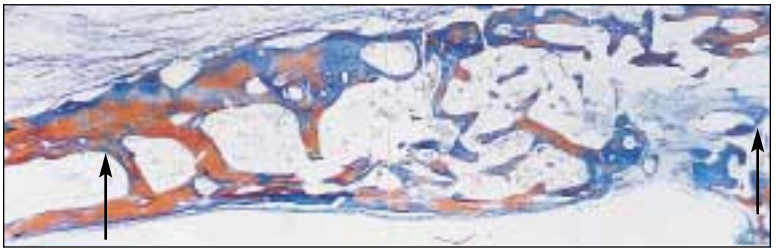


Fig 8 Experimental group, 60 days. Autogenous demineralized dentin matrix slice is incorporated in bone matrix *(asterisk)* and is being resorbed during the bone remodeling process (Schmorl; magnification ×200).

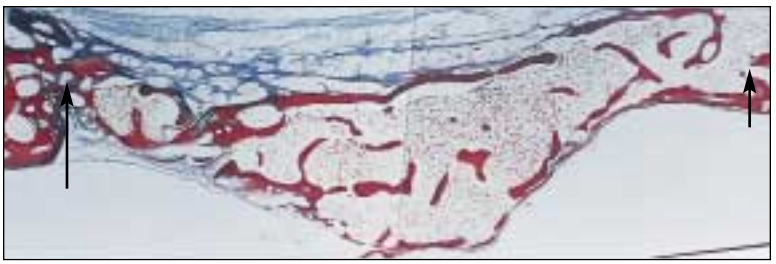


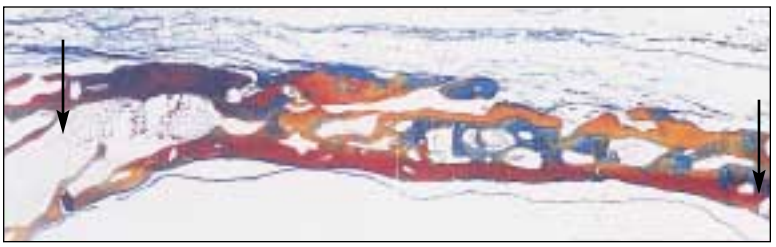
Figs 9a and 9b Photomicrographs of the bone defect region after a period of 60 days: *(Top)* Autogenous demineralized dentin matrix with human amniotic membrane and *(bottom)* human amniotic membrane only. Limits of the surgical bone defect region are indicated by arrows (hematoxylin-eosin and Mallory trichromic; magnification ×25).





Figs 10a and 10b Photomicrographs of the bone defect region after a period of 90 days: *(Top)* Autogenous demineralized dentin matrix with human amniotic membrane and *(bottom)* human amniotic membrane only. Limits of the surgical bone defect region are indicated by arrows (hematoxylin-eosin and Mallory trichromic; magnification ×25).





Figs 11a and 11b Photomicrographs of the region of the bone defect in the period of 120 days: *(Top)* Autogenous demineralized dentin matrix with human amniotic membrane and *(bottom)* human amniotic membrane only. Limits of the surgical bone defect region are indicated by arrows (hematoxylin-eosin; magnification ×25).

defect had increased in thickness in both control and experimental sites in comparison to the preexisting bone area (Figs 10a and 10b).

Defects at 120 Days

In the experimental group, the region of the surgical bone defect was totally filled with mature bone tissue. In a panoramic view, it could be seen that the newly formed bone was thicker in the central region than on the limits of the defect, demonstrating an exophytic bone growth. In the central region, the presence of a small amount of mature bone trabeculae with wide medullary spaces and yellow and red bone marrow was observed. In the control group, the region of the surgical bone defect was filled by mature bone tissue, with regular medullary spaces and yellow and red bone marrow. The newly formed cortical bone was well defined (Figs 11a and 11b). The healed parietal bone was thinner in the control group than in the experimental group.

DISCUSSION

The search for ideal characteristics of an osteoinducing implant material constitutes a continuous challenge in biomedical research. Biocompatibility, material storage without loss of viability, ease of obtaining

the material, cost/benefit relationship, and, mainly, osteoinductive potential, have been the parameters for various studies of osteoinductive biologic materials.[6] Osteoinduction has been observed using materials such as hematopoietic bone marrow, fresh spongious bone, compact fresh bone, bone matrix, demineralized dentin, hydroxyapatite, and others. These materials have been used in reconstructive surgery to replace the loss of large amounts of bone tissue in situations involving craniofacial deformities, multiple trauma, orthopedic and oncologic surgery, neurosurgery, and periodontal surgery.[2,4,6,7,10,30–39]

Some studies have shown the formation of bone tissue and cartilage after implantation of demineralized dentin in the intramuscular regions of experimental animal groups.[4,7] These phenomena occur because of the presence of an osteoinducing substratum—BMPs that have osteoinductive properties.[30,34,40,41] The process of preparation of the ADDM used in this study[4,40] probably did not jeopardize the activity of the existing BMP in the dentin, since a greater amount of bone tissue was seen in the experimental group than in the control group.

In recent years, researchers have used growth factors such as IGF, PDGF, BMPs, and others in combination with occlusive membranes to accelerate the bone repair process.[42,43] Some authors reported the presence of these growth factors in bone matrix and dentin matrix, explaining in this way the osteoinductive activity of ADDM.[3–6]

No membrane of embryonic origin such as HAM had been used previously with the GBR technique. Knowing its antibacterial properties,[23] the presence of angiogenic[17] and growth factors,[22] and the absence of rejection of HAM by the rabbits,[44,45] it was the authors' primary purpose to investigate the influence of its application in the repair of surgical bone defects. A second purpose was to observe its behavior in the host tissue when associated with the osteogenic material dentin matrix.

In this study, microscopic results showed that in the 30-day period, a greater amount of bone tissue was formed in the experimental group than in the control group. In a panoramic view, the control group showed a thinner layer of bone tissue in comparison to the experimental group, and the amniotic membrane had been phagocytosed by macrophage cells, characterizing a resorption phenomenon. This process was also seen in the studies of Gomes and associates.[45] The absence of bacterial colonies in both groups was also observed. This may have been the result of the antibacterial activity of HAM, in accordance with the findings of Needham,[25] Robson and coworkers,[28] Colocho and colleagues,[24] Matthews and associates,[20] and Talmi and coworkers.[23]

After 60 days, bone formation was more exuberant in the defects in which ADDM slices had been implanted. At this point, ADDM slices were no longer evident. In the control group, the presence of this membrane on the surface of surgical bone defects was still observed.

After 90 days, bone formation was also more exuberant in the experimental group than in the control group. In the experimental group, HAM and ADDM slices were no longer seen. In the control group, remnants of HAM were still observed on the surface of the surgical bone defect.

After 120 days, in the experimental group, the amount of mature bone trabeculae was greater in comparison to the control group. In the control group, the presence of HAM was no longer observed on the surface of the region where the surgical bone defect had been created.

The biocompatibility of HAM and ADDM evidenced in these results associated with the osteogenic effect of ADDM corroborate the results of Norris and associates,[46] Catanzaro-Guimarães and coworkers,[4] Fishman and colleagues,[47] Gage and associates,[19] Badawy and coworkers,[44] Catanzaro-Guimarães,[6] and Gonçalves.[7] According to the observations of Trelford and associates[48] and Catanzaro-Guimarães and coworkers,[4] this is a result of the low antigenicity of HAM and ADDM.

On the other hand, in this investigation, it was also verified that ADDM has chemotactic properties, attracting osteoprogenitor cells and osteoblasts toward its region, promoting acceleration of the bone repair process at the surgical bone defect. According to Urist and Strates,[8] Catanzaro-Guimarães and associates,[4] and Gonçalves,[7] these phenomena occur because of the release of growth factors from ADDM—more evidence of its osteoinductive and osteoconductive properties, which were also observed in these studies. From the results seen at 60 days, the ADDM was already in a complete resorption phase and the remodeling process occurred simultaneously with this phase.

In this investigation, there was no control group without HAM, since regular bone repair in surgical defects in the rabbit skull, without inclusion of any material or substances, has already been studied extensively.[11,12]

CONCLUSION

It was concluded that the HAM did not interfere with bone repair and bone resorption processes in the healing of parietal defects in rabbits. The ADDM slices stimulated new bone formation and

were completely incorporated into the newly formed bone tissue, having been resorbed during the bone remodeling process. Bone repair appeared to be faster in the experimental group (sites with ADDM slices) than in the control group (no ADDM slices).

ACKNOWLEDGMENT

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