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Original

Freeze Dried Bone Matrix on Rat Critical Size Defect Regeneration

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Abstract: Bone allografts are commonly used for bone regeneration. The aim of this study was evaluate the efficacy of a freeze dried bone matrix (FDBM) in critical size defect (CZD) rat calvaria. Eighteen Wistar female rats (body weight 150 ± 50 g) with CZD (5mm) were divided in two groups: group 1, using freeze dried bone matrix; and group 2, only with coagulum. All samples were evaluated on the 1st, 3rd and 6th weeks post-surgery by soft X-ray, histological and histometric studies. Soft X-ray results showed a radiolucent image with many irregular radiopaque areas. Histologically, bone regeneration was initiated from the 3rd week, when a thin layer of new woven bone could be seen adjacent to the matrix. At the 6th week, lamellar bone covered over half (61.8 %) of the defect area. The lack of FDBM resorption allowed its incorporation to the new regenerated bone. This behavior is important in circumstances where it is necessary not only to stimulate bone regeneration but also increase the volume in affected areas, such as during the placement of dental implants. The results obtained in this research are encouraging for the use of freeze dried bone matrix as a bone graft material.

Key words: Freeze dried bone, Critical-sized defect, Bone regeneration

Introduction

Angiogenesis, osteogenesis and chronic wounds healing are all natural processes that occur in the body. Bone is a mineralized connective tissue capable of regeneration. This tightly coordinated event requires the synchronized activities of osteoblasts, osteoclasts, osteocytes and lining cells, arranged in an anatomic structure known as Basic Multicellular Unit (BMU)[1]. When bone is affected by certain pathologies compromising its microarchitecture and causing extensive damage to the extracellular matrix, regenerative capacity diminished or lost, forming a critical size defect (CZD)[2].

Actually there are different strategies to solve this problem. Autogenous bone graft is still considered to be the "gold standard" in bone regeneration because its osteogenic activity[3]. In this process, bone morphogenetic protein components stimulate undifferentiated mesenchymal stem cells to become osteoprogenitor cells. Also, autogenous bone does not cause immune responses in the host, as well as, avoiding the risk of disease transmission. However, the amount of bone needed, sometimes its higher than it could be extracted from the patients; it may cause significant morbidity and cannot be storage[4]. We currently have human bone, available in banks, called allograft. According to the Pacific Coast Tissue Bank, bone bank preserves bone morphogenetic protein, giving osteoinductive properties[5]. And it has a very low immunological response in the host[6].

Human allografts can be classified into cortical, cancellous and cortico-cancellous according to the source and there are no differences on bone regeneration effects. They can also be classified into freeze dried bone allografts (FDBA) and decalcified freeze dried bone allografts (DFDBA), also known as demineralized bone matrix (DBM), according to their decalcification process[7]. Aghaloo et al. in 2005 evaluated a human bone allograft with and without demineralization, frozen and lyophilized, alone or in combination with platelet rich plasma, and the results showed no significant differences between groups[8]. Intini et al. in 2008, described a limited ability to promote bone regeneration when they evaluated demineralized, frozen and lyophilized human bone in rat calvarial defects[9].

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Table 1. New Bone Volume

Week	Bone Volume	
	Control Group	FDBM Group
1st	0.14 % ± 0.1	0.70 % ± 0.02
3rd	0.65 % ± 0.2	37 % ± 0.4 ^{y*}
6th	1.06 % ± 0.2	61. 8 % ± 0.5 ^{y*}

Mean and Standard Deviation Values at 1st, 3rd, and 6th weeks from Control and FDBM Groups. New bone volume was expressed in % *p < 0.05, Mann-Whitney test.

In a pilot study of our laboratory, we have observed that a freeze dried bone matrix (FDBM) increased bone volume in a non critical post extraction sockets model in rats. Based on these encouraging results we evaluated the bone matrix in a critical size bone defect model in rat calvaria.

Material and Methods

Animals

Eighteen female Rattus norvegicus var. Wistar rats (150 ± 50 g), approximately 9 wks old, were obtained from the Animal Research Center (Medical School, Tucumán University, Argentina). Animals were housed in pairs, in a specific pathogen-free environment, with a temperature of 22.4 ºC to 23.8 ºC, relative humidity of 45 % to 62 %, and a 12-hour light-dark cycle. A standard commercial diet and tap water were available ad libitum. During the study, animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals, 8th Edition (NRC, 2001) from the National Academic Press (Washington DC, USA).

Experimental Design

The research protocol was approved by the local Ethical Committee for Animal Research (Tucumán National University & CONICET). Critical size defects were created in calvaria as previously described[10-12]. Briefly, defects were created manually with 5-mm-diameter ad hoc punch with smooth edges. Animals were randomly assigned between two groups and the created defects were filled with: Group 1: freeze dried bone matrix (FDBM), (Laboratorio de Hemoderivados, Universidad Nacional de Córdoba, Argentina); Group 2: maintained only with coagulum. After 1, 3, or 6 weeks post-surgery, animals were killed. Bone samples were obtained and fixed in 20% buffered formalin phosphate for 24 hours.

Soft X ray - High-resolution Films

All samples were subjected to soft X-ray with high resolution Kodak film (mammography type, 18 x 23 cm; GBA Mamograf HF Digital brand equipment, Buenos Aires, Argentina). Exposure time was 0.8 sec, at 27.5 kV and 7.0 mA[13].

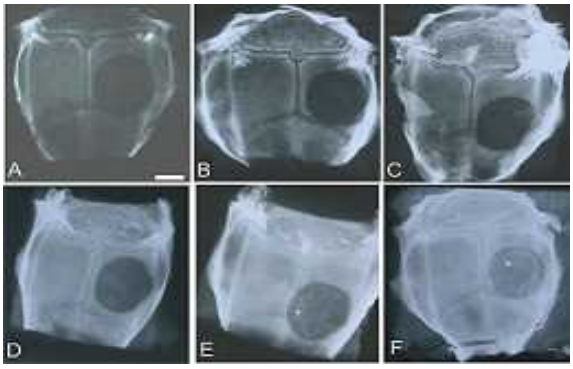

Figure 1. Images of soft X-ray in high resolution film at 1st, 3rd and 6th week showing 5 mm CZD, with rounded lytic shadows. Inside, amorphous opacities can be seen on defect area (*). Control (A - C) and FDBM (D-F) groups. bar = 1mm.

Histopathology

The specimens were decalcified with modified Morse solution (Okayama University Dental School) and embedded in paraffin in a routine manner. Two serial 4 ìm central section per defect were selected and stained with Hematoxylin & Eosin (H&E) and Masson's Trichrome. They were examined by light microscopy. A single pathologist evaluated all tissues. Subsequently, another pathologist (certified by the Argentina Health Ministry Nº 31455) performed an independent review to verify microscopic observations. The reported results reflect the mutually-agreed-upon diagnoses by both pathologists.

Histometric Studies

Photomicrographs were taken from slides of each specimen by means of a Sony digital camera adapted to an Olympus CH30 microscope. The photos obtained by Soft Pinnacle Studio 9.4 with 116.7X magnification were evaluated by Image Pro Plus analysis system (Media Cybernetics, Silver Spring, MD, USA Version 4.5.0.29 for Windows 1998/NT/2000). New bone formation was quantified at the CZD area and expressed in percentage.

Statistical Analysis

Data are presented as mean ± standard deviation, as indicated in Table 1. Morphometric results were analysed by the Mann Whitney test ($p < 0.05$).

Results

Soft X ray – High resolution Films

In the untreated group, a radiolucent area corresponding to the defect site was observed in the three periods (Fig. 1A- 1C). On treated groups at the 1st week post-surgery, soft X-ray images of CZD showed slightly radiopaque areas. It was covering almost the entire defect, which corresponds to the implanted membrane presence. During the 3rd and 6th weeks, intense radiopaque spots were observed inside the CZD area (Fig. 1D – 1F).

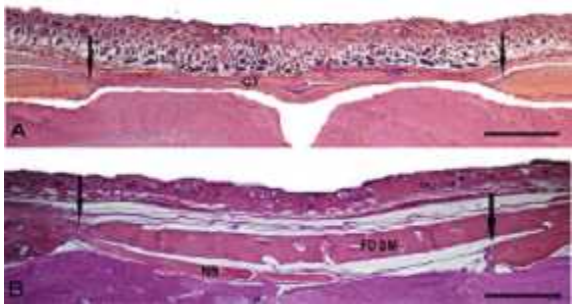

Figure 2. Histological analysis of CZD at 6th week at low magnification (A) CZD maintained only with coagulum (control group). TG: granulation tissue. (B) FDBM group. FDBM: freeze dried bone matrix. NB: new bone covering over half of the defect area. The defect margins are depicted by black arrows. H&E, bar = 2 mm.

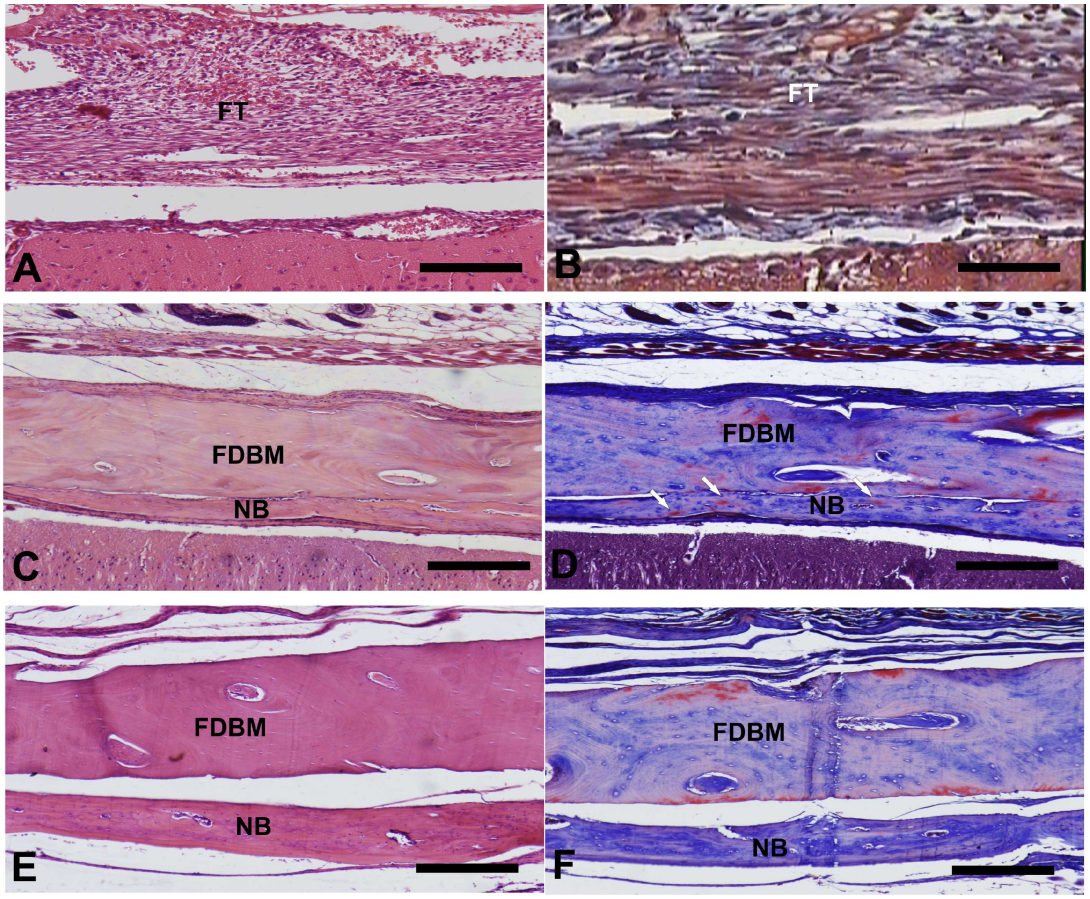

Figure 3. Histological analysis of CZD at high-magnification (A) H&E and (B) Masson's Trichrome stained sections of FT (Fibrous Connective Tissue) in CZD area at control group 6 th week (C) H&E stained sections of FDBM group, showing a thin layer of NB (new woven bone adjacent to the matrix). (D) Masson's Trichrome stained sections of FDBM group, new bone formation with some foci of mineralization (arrows) 3rd week. (E) H&E and (F) Masson's Trichrome stained sections of lamellar bone bellow the FDBM 6th week, bar = 200μm.

Histopathology and Histometric Observations

All animals survived the surgical procedures and were available for evaluation. No signs of infection were registered during the healing period or at the time of retrieval.

Figures 2A and B shows at low magnification critical-sized defect at 6 wks in control and FDBM groups.

At the control group, during the 1st week post-surgery in the defect area granulation tissue and vessels congestion were found in the superior sagittal sinus. In the 3rd and 6th weeks, the CZD area showed fibrous connective tissue healing. In all periods, there was almost no regenerated bone in the defects except in the immediate vicinity of the surgical margins (Fig. 3A, 3B).

In the FDBM group, during the 1st week, the CZD area showed granulation tissue and the matrix appeared associated with fibrino hemorrhagic exudates. In the 3rd week, there was a fibroblastic tissue and the implanted matrix was thicker by new woven bone. The new bone formed showed some foci of mineralization (Fig. 3C, 3D). During the 6th week lamellar bone

ossicles were observed below the matrix and covering over half of the defect area. Bone matrix resorption did not occur and it was incorporated into the new bone tissue (Fig. 3E, 3F).

The Table 1 shows the results of histometric analysis. The new bone formed with the FDBM at 1st week (0.70 % ± 0.02) compared with that formed in the CG (0.14 % ± 0.1) showing no significant statistical differences. At 3rd and at 6th weeks FDBM showed significant bone formed (37 % ± 0.4 and 61. 8 % ±0.5 respectively) and compared with the CG (0.65 % ±0.2 and 1.06 % ± 0.2 respectively) (p = 0.03 at 3rd week and 0.02 at 6th week).

Discussion

Freeze dried bone matrix was able to stimulate critical size bone regeneration on rats. CZD is an established model for the evaluation of bone replacement materials; defined as the minimal dimension of a bi-cortical bone lesion that does not heal spontaneously during the life time of an animal, and which requires an additional treatment to complete bone reparation. In our experience a CZD of 5 mm of diameter repair by a fibrous tissue after six weeks[11-13].

In the present study we evaluated a FDBM at 1st, 3rd and 6th weeks after surgery. The defect regeneration was initiated from the 3 rd week; where a thin film of new woven bone could be seen adjacent to the matrix. At 6th week, lamellar bone covered over half of the defect area (61.8 %). These results allowed us to suggest that the matrix had an osteoconductive but also an osteoinductive effect because it stimulated the differentiation of osteoblastic precursors from the subcutaneous tissue and the duramatter14). In accordance with our research, Borie et al. observed central bone formation in a rabbit CSD, induced by freeze dried bone allograft after 15 days[15]. However, there are other authors that mentioned that in critical defects bone formation occurred mainly from the defect margins and poorly in the center[16].

Another interesting finding in our results was the presence of foci of mineralization of the new bone at 3rd week. This showed that the material stimulated mineral precipitation from early stages. Borie *et al*. compare an allograft with auto graft in CZD regeneration, and bone mineralization was higher and earlier when an allograft was implanted[15]. The most recent review shows that there is no reason to prefer autogenous bone over bone substitutes[17]. However, most authors argue that autologous bone graft is currently the gold standard[3].

The persistence of the FDBM at the 6th week, also revealed its osteoconductive effect, acting as a scaffold for cell proliferation. Moreover, the lack of resorption of the matrix allowed its incorporation to the new regenerated bone. This behavior is important in circumstances where it is necessary not only to stimulate the regeneration of the defect but also increase the volume in the affected area, such as during the placement of dental implants. In a preliminary study from our laboratory, we have observed an increase in the volume of new bone in non-critical models of post extraction sockets rats when placed the FDBM, compared with those where only induced to coagulum formation. Intini et al. also describe the allograft persisted, however, there was no difference in the amount of new bone formation between the control and treated groups.

The bone matrix had an osteoinductive and osteoconductive effect in CZD regeneration. Furthermore it was highly biocompatible and did not generate adverse inflammatory reactions showing excellent tolerance by the body. The results obtained in this and preliminary study, encourage the use of it as a bone graft material.

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