EVALUATION OF THE OSTEOINDUCTIVE POTENTIAL OF FREEZE-DRIED HUMAN AMNIOTIC MEMBRANE (EXPERIMENTAL STUDY)

Aliaa O. Kamal[1] *BDS*, Sherif S. Ayad[2] *PhD*, Ragab S. Hassan[2] *PhD*, Samir R. Nouh[3] *PhD*, Dina A. Nagy[4] *PhD*

ABSTRACT

INTRODUCTION: Bone regeneration represents an important challenge in oral surgery. Several means have been employed to improve bone healing, each having several advantages and disadvantages. The search for new materials and methods is an ongoing process.
OBJECTIVES: The aim of the present study was to evaluate the bone inductive effect of the freeze-dried human amniotic membrane (FDAM) as a novel biomaterial for bone regeneration.
MATERIALS AND METHODS: Twelve adult male dogs were involved in this study. Bilateral critical-sized mandibular defects were created in each dog. One defect was left uncovered as a control. The other defect was covered with a double layered sterile freeze-dried human amniotic membrane (FDAM) to serve as a study. At each of three time points - 4, 8, and 12 weeks - four dogs were euthanized and their mandibles were harvested en bloc and osteotomy sites were submitted for histochemical examination to evaluate bone healing.
RESULTS: The tissue samples were obtained after 4, 8, and 12 weeks for histochemical examination. The FDAM was found to enhance the blood supply to the defect area in the study group and gave rise to bone induction ($P < 0.001$).
CONCLUSIONS: Our study findings indicate that the FDAM has the potential for the enhancement of bone healing and bone induction.
KEYWORDS: Amniotic membrane, freeze-dried, bone regeneration, critical-sized bone defect.

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1. *BDS, Faculty of Dentistry, Alexandria University.*
2. *Professor of Oral and Maxillofacial Surgery, Faculty of Dentistry, Alexandria University.*
3. *Professor of Surgery, Vice Dean, Faculty of Veterinary Medicine, Alexandria University.*
4. *Lecturer of Oral Biology, Faculty of Dentistry, Alexandria University.*

INTRODUCTION

The repair of bone defects has always represented a huge challenge in the field of dentistry, as many injuries result in functional or esthetic impairment that remarkably reduces the quality of life of the individuals affected (1). Bone loss in the maxillofacial area may arise as a result of infections, trauma, cysts, neoplasia or congenital defects (2). These defects can range in size from small defects that can heal spontaneously to large critical-sized defects (CSD) that will neither heal spontaneously (3) nor regenerate more than 10 % of the lost bone during the patient's lifetime (4).

Ideal mandibular reconstruction should restore form, function, sensation, mastication efficiency and esthetics (5). Autogenous bone grafting has always been the gold standard in reconstructing critical-sized bone defects; however, it has shown multiple disadvantages, such as increased patient morbidity, the limited volume of transported bone and the need for a second operative site (6).

These disadvantages led to the emergence of the concept of Tissue Engineering (TE). TE is the application of scientific principles to the design, fabrication, modification, and growth of living tissues using biomaterials, cells and growth factors. The aim of bone tissue engineering is to regenerate lost bone via the use of growth factors and/or cells (5).

In bone TE, an appropriate carrier can deliver osteogenic growth factors to a defect site. Typically, these carriers are three-dimensional scaffolds that facilitate cell attachment and proliferation, and can themselves be used as a vehicle for the delivery of growth factors. That is why they are called "Growth Factor-Based Bone Grafts" (7).

The Human Amniotic Membrane (HAM) is the innermost layer of the fetal membranes. It can be harvested, sterilized and preserved in order to be available for use in different medical and dental fields. Several processing and preservation techniques have been developed, each having its advantages and disadvantages. One rising method is freeze-drying, which provides an inexpensive product that can easily be transported and stored at room temperature (8).

The HAM has many beneficial properties, such as; its antimicrobial activity, anti-angiogenic properties, inflammation suppression, scarring inhibition, wound healing and epithelialization (9-12), anti-tumorigenic properties (13), plus no immunogenicity and pain relief (12). Besides these characteristics, the HAM acts as an anatomical and vapor barrier (9), being able to preserve a moist atmosphere that promotes healing (14). That is why it is currently used as a biological membrane for the management of burns and skin ulcers (15). Furthermore, it has been identified as a suitable membrane for vestibuloplasty surgery (16).

Moreover, the HAM was found to contain a wide variety of enzymes, such as prostaglandin synthase (17); growth factors such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and transforming growth factors (TGF-α, and TGF-β) (18) and cytokines such as IL-6 and IL-8 (19). This is in addition to its content of collagen,

laminin, and fibronectin, all of which provide an appropriate substrate for bone induction (20).

The aforementioned unique combination of characteristics of the HAM is not found in any other natural or synthetic biocompatible material (9). That is why the aim of our experimental study was to explore the substantial benefits of freeze-dried HAM as a growth factor-based bone graft in the process of bone regeneration.

MATERIALS AND METHODS

I- Experimental Animals

This study was conducted on 12 healthy adult male dogs weighing 15-20 kilograms. Animals were kept under the same nutritional and environmental conditions in the animal house at the Department of Surgery, Faculty of Veterinary Medicine, Alexandria University, after gaining the approval of the Research Ethics Committee of the faculty. The study followed a split-mouth design creating two groups: Group A, in the right intraoral side, which served as the control group; and Group B, in the left intraoral side, which served as the study group. Each group included 12 defects.

II- Materials

Sterile freeze-dried human amniotic membrane (processed by the National Center for Radiation Research and Technology, Nasr City, Cairo, Egypt, and distributed by Badr Pharmaceutical Company) was used in this study.

This amniotic membrane was collected after caesarean section from women with a social and clinical history compatible with a healthy donor after serological testing to exclude viral infections. The membrane was then washed in saline with antibiotics (penicillin, 10,000 U/ml; streptomycin, 10,000 µg/ml; and amphotericin B, 25 µg/ml) and cut into 5 × 5 cm pieces. The samples were treated with 0.02 % ethylene diamine tetra acetic acid (EDTA) at 37 °C for 2 h with the purpose of removing epithelial cells. At this point the samples were freeze-dried and packed in vacuum to be radiation-sterilized with 25 Gy (21).

III- Surgical Procedures

All operating procedures were performed under the effect of sedation and general anesthesia. Sedation was performed by intramuscular (IM) injection of Xylazine HCl (Xyla-Ject, Adwia Pharmaceuticals, 10th of Ramadan City Industrial Area, Egypt) 1mg/kg, followed by induction of anesthesia using Ketamine HCl (Ketamine, Sigma Pharmaceutical Industries, Nasr City, Cairo, Egypt) 10mg/kg IM. Anesthesia was maintained by IM injections of Xylazine HCl 1mg/kg and Ketamine HCl 5mg/kg.

The surgical procedure was performed under aseptic conditions for all animals. Each animal received cefotaxime broad spectrum antibiotic (Cefotax, EPICO Pharmaceuticals, 10th of Ramadan City, Egypt) (1gm IM injection) as a prophylactic measure. The animals were placed in lateral recumbent position. Then, the surgical sites were painted with Povidone-iodine solution u.s.p 10% w/v (Betadine, NILE Company for Pharmaceutical and Chemical Industries, Cairo, Egypt.), followed by the administration of local anesthesia [Mepivacaine hydrochloride U.S.P. 20 mg (2%) + Levonordefrin hydrochloride 0.06 mg (Mepecaine – L, Alexandria Co. for Pharmaceuticals, Egypt)], for the purpose of hemostasis.

Both sides of the mandible were operated on; the right side for the control group and the left side for the study group.

An intraoral partial thickness trapezoidal flap was made in the premolar-molar area of the body of the mandible in each dog bilaterally using a Bard Parker scalpel handle no.3 and a disposable blade no.15. The extent of this flap was from the mesial side of the second premolar to the distal side of the second molar. The flap was then reflected through careful sharp dissection then the periosteum was completely removed to expose the bone.

Using a motor and a Primado 2 Micro Bone Saw (manufactured by NSK Global, Tokyo, Japan) along with constant irrigation with 0.9% saline solution for cooling, a bone defect with rectangular configuration and dimensions of 2cm mesiodistally X 1cm apicocoronally - measured by a ruler and marked by methylene blue prior to cutting - was created. The depth of the defect was equal to the full thickness of the cortical bone and it was completely away from the roots of the teeth in the area.

In the control group (right side) the bone defect was left to heal spontaneously, while in the study group (left side) the defect was covered with a double-layered sterile freeze-dried human amniotic membrane (Fig. 1).

Flap closure was achieved by interrupted sutures using vicryl 3-0 sutures.

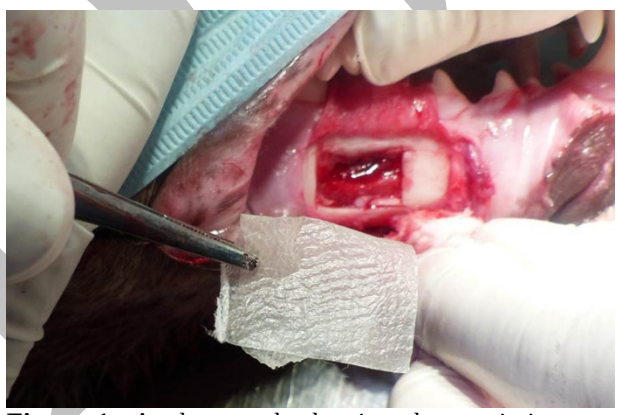

Figure 1: A photograph showing the amniotic membrane placement in the study group.

IV- Postoperative Care

All the dogs received the same course of antibiotic and anti-inflammatory drugs for 3 days in the form of broad spectrum antibiotic Cefotaxime (Cefotax, EPICO Pharmaceuticals, 10th of Ramadan city, Egypt) (1gm IM injection, twice daily) and Piroxicam 20mg/ml (Feldene, Pfizer Egypt, Dokki, Giza, Egypt) (1ml IM injection, three times per day). They were kept on a soft diet during the first postoperative week then returned to their normal food. The animals were kept under close daily observation during the first postoperative week to assess the presence or absence of any sign of infection or wound dehiscence.

Dogs were euthanized at 4, 8 and 12 weeks postoperatively, four dogs at a time, with an intravenous injection of one-shot thiopental sodium. Segments containing the defect areas with a part of the adjacent bone were retrieved to be prepared for histochemical examination. They were immediately fixed in 10% neutral buffered formalin for two weeks (22).

Preparation of the Histochemical Sections

Following fixation, the specimens were decalcified for 6-8 weeks in 8% trichloroacetic acid, then washed under running water and dehydrated in ascending grades of ethyl alcohol.

Specimens were then infiltrated by melted paraffin wax then embedded in a block of paraffin wax to be sectioned in a mesiodistal direction. Then, sections were cut at 4 microns thickness using rotatory microtome and stained with Mallory's trichrome stain, which uses three stains that are: aniline blue, acid fuchsin, and orange gelb (23), in order to examine the amount of mature and immature bone formed at the surgical site.

Histomorphometric Evaluation

Morphometric evaluation of the percentage of surface area of the newly-formed bone was assessed for each specimen using the "Image J 1.46" program. From each specimen, three slides of tissue from different standardized depths were made and used for quantification. Measurements were obtained from each slide then the mean values were calculated. The total surface area of the formed bone was measured using an objective lens of magnification 10 i.e.; the total magnification was 100.

Statistical Analysis

The collected data of the area percentage of the formed bone was statistically analyzed by means of the IBM SPSS software (Armonk, New York: IBM Corporation) using the t-test and then graphically illustrated.

RESULTS

I-Clinical Results

All the animals survived the experimental protocol of the present study very well. No signs of inflammation or infection were noted. The whole experimental period passed without complications.

II- Histochemical Results

Upon examination of the histochemical sections, the points of interest were: blood supply, collagen deposition, and new bone formation.

Blood supply was markedly increased in both groups by 8 weeks, with more blood vessels clearly noted in the study group.

Upon investigating the deposition of collagen fibers in both groups, it was noted that collagen deposition increased gradually in both the control and study groups throughout the experimental period, but it was denser and better organized in the study group.

Concerning new bone formation, minimal bone induction was noted in both groups at 4 weeks. At 8 weeks, signs of bone induction were noted in the study group (Fig. 2) more than the control group (Fig. 3) though the amount of new bone formed was still scarce. However, at 12 weeks, we could observe clear islands of newly-formed bone under the light microscope in the study group (Fig. 4), with a higher density and better organization of collagen fibers than the control group (Fig. 5). No complete filling of the defect occurred in any of the two groups.

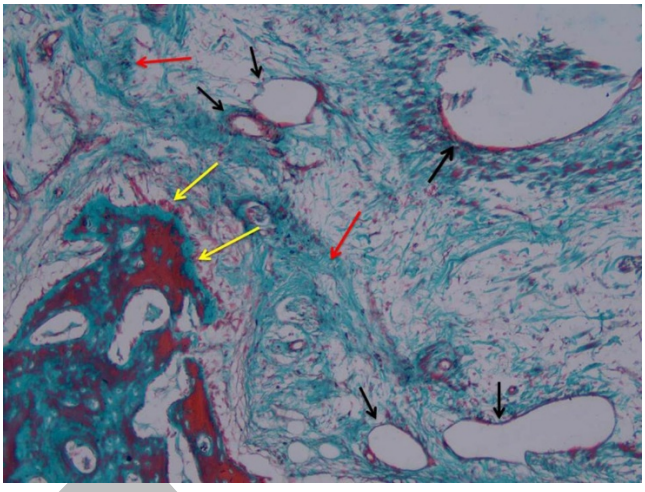

Figure 2: Light Micrograph of the study group at 8 weeks showing a remarkable increase in vascularity (black arrows) along with dense collagen fiber deposition (red arrows). Note the arrangement of osteoblasts along the borders of old bone (yellow arrows). (Trichrome stain, 100x).

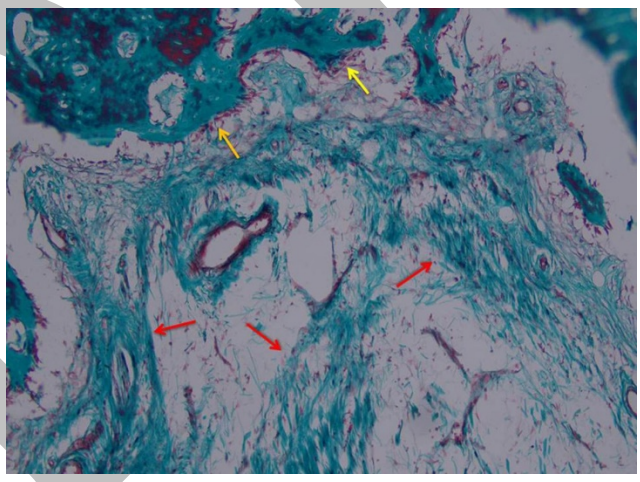

Figure 3: Light Micrograph of the control group at 8 weeks showing dense collagen fiber deposition (red arrows) and new blood vessel formation. Note the arrangement of osteoblasts along the borders of old bone (yellow arrows). (Trichrome stain, 100x).

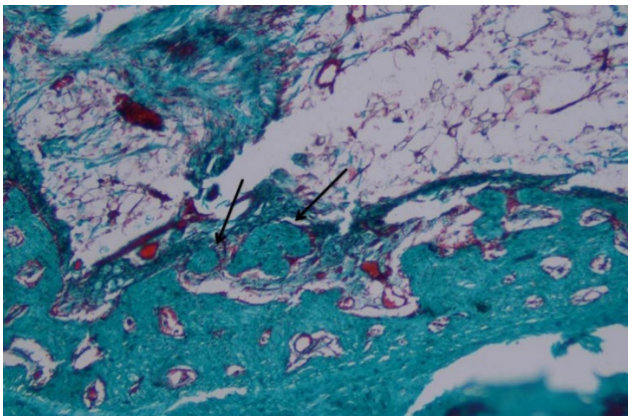

Figure 4: Light Micrograph of the study group at 12 weeks showing islands of newly-formed bone (black arrows) along with dense well-organized collagen fibers and increased vascularity with blood vessels appearing engorged with RBCs. (Trichrome stain, 100x).

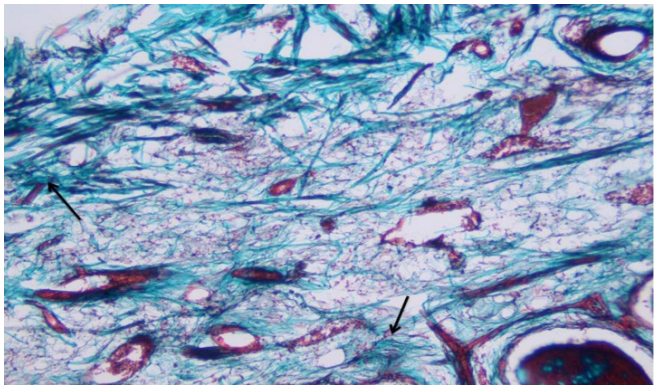

Figure 5: Light Micrograph of the control group at 12 weeks showing dense collagen bundles aggregation in a disorganized pattern and increased vascularity with blood vessels appearing engorged with RBCs. Note the bony spicules present between the collagen bundles (black arrows). (Trichrome stain 100x).

III- Histomorphometric and Statistical Results

The mean area percent of newly-formed bone in the two groups at different time points is summarized by mean and standard deviation in Table (1) and graphically illustrated in (Fig. 6). Minimal bone induction was noted in both groups at 4 weeks, with no significant difference between the two groups (P= 0.477). At 8 weeks, signs of bone induction were noted in the study group more than the control group (P< 0.003), where the mean area percent of new bone formation in the study group was found to be 5.65 ± 0.44, while that of the control group was 4.69 ± 0.38. At 12 weeks, the calculated mean area percent of the newly-formed bone in the study group was 11.8±1.07, which was significantly higher (P<0.001) than that in the control group which was 6.32±0.62

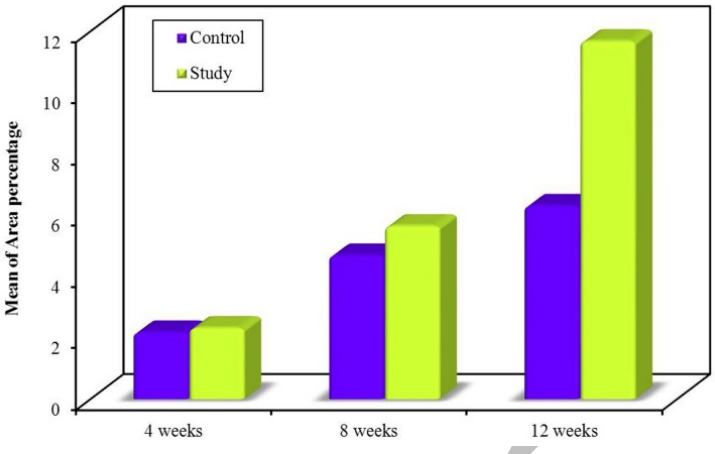

Figure 6: Comparison between the two studied groups according to area percentage.

Table (1): Comparison between the two studied groups according to area percentage.

Area percentage	Control (n= 6)	Study (n= 6)	t	P
4 weeks				
Min. – Max.	1.79 – 2.56	1.90 – 2.84	0.738	0.477
Mean ± SD.	2.18 ± 0.28	2.32 ± 0.37		
Median	2.11	2.27		
8 weeks				
Min. – Max.	4.04 – 5.19	4.83 – 6.07	4.002*	0.003*
Mean ± SD.	4.69 ± 0.38	5.65 ± 0.44		
Median	4.73	5.71		
12 weeks				
Min. – Max.	5.75 – 7.10	10.65 – 12.74	10.676*	<0.001*
Mean ± SD.	6.31 ± 0.62	11.68 ± 1.07		
Median	6.19	11.65		

t, p: t and p values for **Student t-test** for comparing between the two groups

*: Statistically significant at $p \leq 0.05$

DISCUSSION

In the present study, FDAM was used in order to evaluate its osteogenic potential and its possible contribution to the process of bony healing. FDAM is commercially available, quite inexpensive and can be preserved at room temperature; that is why it might represent a very economical form of growth factor based bone grafts (24). Although the work of Lim et al. (25) has shown significant disruption in the structure of the HAM and decrease in its growth factor content upon freeze drying in comparison to other preservation techniques, they themselves in addition to many others like Libera et al (26), Rodríguez-Ares et al (24) and Nakamura et al (21,27) revealed that these changes do not at all compromise the proposed clinical outcome, and that the FDAM brings about excellent clinical results that are comparable to the results obtained by using fresh or cryopreserved HAM.

The canine model was chosen due to the similarity of canine bone structure and architecture to human bone (28). Moreover, there is a considerable amount of literature discussing the usefulness of canine models as study models in research related to bone healing (29).This canine model was also used in a study by Samandari et al. (20). In contrast, Ríos et al. (30) used New Zealand Rabbits and Starecki et al. (31) used Sprague Dawley rats in their studies on the effects of the HAM in bone repair.

The present study was conducted on twelve healthy dogs. In each dog, two defects were induced; one on the right intraoral side (Group A) and the other on the left intraoral side (Group B). In group A, the defects were induced and then the mucosa was directly repositioned and sutured. In group B, the defects were covered with FDAM before flap

repositioning. The membrane did not require any stabilization other than flap re-positioning owing to the fact that the FDAM is wafer-like and becomes soft and flexible upon hydration, thus can be adapted well into the bone defect. The dogs were then euthanized at 4, 8 and 12 weeks postoperatively for histochemical evaluation of the healing process.

Only male dogs were used in our study to exclude any effect of female sex hormones on the structure, calcification and healing process of the bone.

In the present study, the healing of critical-sized bone defects was investigated due to the fact that they are quite challenging and resistant to healing (3). A critical-sized defect (CSD) is a bone defect that will not heal spontaneously during the animal's lifetime (3). The size of this defect varies from one animal species to another. These defects actually represent a suitable model for evaluating the usefulness of treatments, because bone regeneration in critical-sized defects only occurs in the presence of osteogenic materials (32). Experimentally, it was found that the size of the CSD for the dog was 15 mm without periosteum (4, 5, 32).

This type of defect was also investigated by Starecki et al. (31) in his study on the effect of commercially-available human amniotic membrane biomaterial on the healing of rat critical-sized femoral gap , while others like Sanz et al. (33) and Sanz-Martin et al. (34) chose to study smaller defects that are not necessarily of critical-sized.

The present study lasted for 12 weeks and was divided into three time intervals 4 weeks each in order to closely monitor all the stages of the regeneration process. After each interval, 4 dogs were euthanized and the defect sites were processed and submitted for histolchemical examination. Similarly, Samandari et al. (20) extended his study of the bone inductive capacity of the HAM in vestibuloplasty technique in dogs for the same period of time. Other researchers like Schenk et al. (35) in their study of bone regeneration in the canine mandible chose a longer period of time of 16 weeks divided into 2 intervals; while Park et al. (36) extended their study for only 8 weeks.

In the present study, the observations form the first experimental periods in both groups revealed no inflammatory reaction. This might be attributed to the fact that our first experimental period was after 4 weeks, a time when the regenerative potential of the tissue biology itself is able to limit and control the spread and continuation of inflammation.

In 1997, Guler et al. (37) studied the blood supply to FDAM when used as a graft in vestibuloplasty techniques and found that the FDAM indeed has an angiogenic effect. The results of the present study conformed to this finding, where histochemical examination revealed an increase in vasculature in the bone defects covered by the FDAM compared to control defects, being most noted at the 8-weeks period.

Of course, an increased blood supply to the healing area plays a very important role in accelerating and potentiating the healing process, through providing nourishment for the migration of bone marrow stem cells into the defect area and their subsequent differentiation and activation, eventually leading to the proposed result of bone regeneration.

Collagen fiber deposition was noted in both groups starting from the first postoperative month and remarkably increasing until the end of the experimental period. Better organization and condensation of those fibers were detected in the study group.

In the present study, very little bone deposition was noted in both the control and study groups by 4 weeks. By the 8th week, however, though the amount of bone was still scarce, a significant difference could be recorded in the rate of bone induction between the two groups ($P<0.003$). By the end of the experimental period, a significantly higher rate of bone deposition could be noted in the study group ($P<0.001$), where islands of newly-formed bone could be seen under the light microscope.

This finding was in accordance with the work of Samandari et al. (20) who found a significant increase in bone deposition in vestibuloplasty cases treated with HAM. Similarly, Ríos et al. in 2014 (31) found an increase in the density of the newly formed bone in rabbit femur defects treated with FDAM in comparison to control defects where FDAM was not used.

However, in the present study, no complete filling of the critical-sized bone defect occurred in any of the two groups. This suggests that, although the FDAM is potentially osteoinductive, it would be better to use it in conjunction with other bone grafting materials in order to achieve better results when it comes to reconstruction of significantly large defects.

CONCLUSION

The freeze-dried human amniotic membrane is potentially osteoinductive; however, it does not possess enough capability to be used as a sole method of bone regeneration in large bone defects.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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