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In vivo regeneration functionalities of experimental organo-biomaterials containing water-soluble nacre extract

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Abstract

Background: Novel multifunctional biomaterials were recently designed to allow for an optimized tissue regeneration process.

Purpose: To comprehensively assess (photographic, radiographic and histological) the in vivo functionality of demineralized bovine bone matrix (DBM) associated with an experimental marine organic extract (MOE) from nacre in a sheep ectopic grafting model.

Materials and methods: Synthesis of MOE was based on mixing powdered nacre (0.05 g, particles average size <0.1 mm) with acetic acid (5 mL, pH 7) under constant stirring for 72 hours (25 °C). Polyethylene tubes (3/animal, n = 4, diameter: 5.0 mm × length: 10.0 mm) from the control (empty) or experimental groups (DBM or DBM + MOE) were then intramuscularly implanted into the lumbar regions of sheep (n = 8, 2-years old, ≈45 kg). Animals were euthanized at 3 and 6 months to allow for the collection of tissue samples. Tissue samples were fixed in formalin 10% (buffered, 7 days) in preparation for photographic,

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radiographic and histological assessments. Acquired images were then analyzed using digital image analysis software to quantify the amount of neoformed tissues, whereas radiographic and histological analyses were performed to determine radiopacity and classification of tissues deposited inside of the tubes.

Results: Photographic and radiographic analyses have shown that both pure (unaltered) and MOE-modified DBM were capable of depositing neoformed tissues (at 3 and 6 months), where higher levels of deposition and radiopacity were observed on groups treated with experimental materials. Histological results, however, demonstrated that tissues formed from both unaltered and MOE-modified DBM were only fibrous connective in origin.

Conclusions: As an ectopic grafting in sheep, the experimental organo-biomaterial association applied did not reveal any osteoinductive property but led to a fibrous tissue repair only.

Keywords: Dentistry, Biomedical engineering, Materials science

1. Introduction

Traditional regenerative techniques aim to guide the repair of bone defects using autografts (gold standard) [1], allografts or xenografts [2, 3]. However, despite their widespread use [4], significant and critical limitations such as limited tissue availability [4, 5], donor site morbidity [6], transmission of diseases [7] (HIV, bovine spongiform [8, 9] encephalitis or hepatitis) and graft-related issues (sequestration, infection and rejection) [10] have been previously reported in the literature, and therefore, represent an important and current challenge faced by oral surgeons worldwide. Such disadvantages have precipitated the development of novel biocompatible materials for bone substitution using natural or sintered hydroxyapatite (HA) [11], β-tricalcium phosphate (TCP), [12] and more recently, low-temperature apatites (LTA) [13]. These materials were designed to provide a 3-D matrix with structural (scaffolding), osteoconductive and osteoinductive functionalities that allow for a faster and optimized regenerative bone repair [14, 15, 16, 17, 18].

Novel biomaterials extracted from organic sources (herein designated as organo-biomaterials) have been considered as a cost-effective and promising [19] source of implantable graft materials with major industrial and scientific applications. Organo-biomaterials include demineralized bone matrix (DBM) [20, 21], bone morphogenic proteins (BMPs) [22, 23] and aragonite crystal tablets extracted from the nacre lining of pearl oysters (*Pincata maxima*) [24]. As previously reviewed by Gruskin et al. [17], DBM is a composite material containing collagens (predominantly type 1, with small amounts of collagen types IV and X), proteins, growth factors, calcium phosphates (up to 6%) and trace amounts of cellular debris. Its processing is based on bone removal, soft tissue debridement, antibiotic soaking

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(sterilization), reduction into fibers or particles, demineralization in chloridric acid (0.5−0.6 N) followed by one or more rounds of freeze-drying [17]. This process results in a hydrophilic and bio-resorbable organic matrix rich in signaling molecules and bone morphogenic proteins (BMPs) [17, 20] that has been demonstrated to have important scaffolding, tissue-support and low antigenicity and toxicity behaviors [18].

Likewise, nacre is a self-assembled nanocomposite consisting of organic (layered biopolymer, 5−20 nm thick/layer) and inorganic components (polygonal aragonite, 200−500 nm thick) with a unique and highly organized hierarchical structure that resembles the native structure of bone [25]. The present study's rationale for the selection of nacre-extracts as bone-growth promoters was based on the excellent biocompatibility, biodegradability and osteoinductive functionalities, that were previously shown to result in the production of highly-mineralized extracellular matrixes in shorter periods of time [26, 27, 28]. In addition, a recent report suggested that materials based on nacre may participate in highly dynamical biomineralization processes in mammals (e.g., sheep and humans) without triggering inflammatory reactions or fibrous formation [25].

More recently, a study investigating the impact of bone characteristics on the healing of soft tissues, indicated that bone mineral content, volume and support are considered as biomodulation factors that may directly affect the recovery process of soft tissues [29]. In this context, novel organo-biomaterials have been extensively investigated, both in vitro and in vivo (mice, rat, rabbit and sheep) [30, 31, 32, 33, 34, 35], in regards to their osteoconductive and osteoinductive properties, as well as their role in the regulation, formation, remodeling and regeneration of both hard and soft tissues surrounding dental implants and bone defects [36]. Recent approaches have combined the use of DBM with water-soluble nacre matrix to promote mineral content replenishment while providing an organic matrix that is capable of promoting the stimulation of living cells and enough mechanical support for an enhanced recovery of tissues [26]. Therefore, the aim of the present in vivo pilot study was to comprehensively assess (photographic, radiographic and histologically) the in vivo functionality of DBM associated with MOE from nacre in a sheep ectopic grafting model.

2. Materials and methods

2.1. Ethics statement

The present randomized and controlled in vivo pilot study was submitted and approved by the Ethics Committee on Animal Use of the Positivo University (CEUA 21/11).

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2.2. Marine organic extract preparation

The method used for the preparation of the experimental MOE was developed by our laboratory. In brief, whole and frozen brown mussels (*Perna perna*), a bivalve mollusk from the Mytilidae family, were obtained from a local seafood supplier in preparation for the MOE extraction. The shells of thawed mussels were separated, thoroughly washed in tap water (30 sec/each) and air dried for at least 24 hours. A bone pestle (Moedor de osso tipo pilão, Kopp, Paraná, Brazil) was then used to reduce the shells into large particulates that were further ground into a fine loose-powder (average size <0.1 mm) using a ball mill (AMEF, São Paulo, Brazil). A portion of the powdered nacre (0.05 g) was then mixed with acetic acid (5 mL, pH 7) at room temperature. The mixture was continuously stirred for a 72-hour period using a magnetic stir plate (Tongtuo, Hayward, USA). The experimental MOE sample was then centrifuged (4000 rpm, 15 min, MPW-350R, MPW Med. Instruments, Warsaw, Poland) to separate solid particles suspended from the liquid nacre extract.

2.3. Experimental design

Eight female sheep (2 years old, ≈ 45 kg) from the same herd were selected to participate in the present in vivo pilot study. The animals were then randomly assigned to each experimental time monitoring group (3 or 6 months) using a freeware available online [Research Randomizer (Version 4.0), Social Physcology Network, Pennsylvania, USA; www.randomizer.org]. Sterile polyethylene tubes (3/animal, diameter: 5.0 mm × length: 10.0 mm) as control (empty, *sham*) and containing DBM (GenOx Org, Baumer, São Paulo, Brazil) without (pure DBM) and with 40 μL of MOE (DBM + MOE) were then intramuscularly implanted into the lumbar region of each animal. Animals pertaining to each implantation period (either 3 or 6 months) have received a total of 3 tubes, as described in Table 1. The rationale for the implantation model selected was based on its ability to mimic unstable mechanical environments [1] and evaluate the biocompatibility and regenerative

Table 1. Experimental design.

Experimental Groups	Implantation time	
	3 months (4 animals)	6 months (4 animals)
Sham	S3	S6
DBM	D3	D6
DBM + MOE	DM3	DM6

A total of 8 animals participated in the present pilot study. Each animal received one empty tube (Sham, S), one tube with pure demineralized bovine bone matrix (DBM, GenOx Org™) and one tube with demineralized bovine bone matrix associated with the marine organic extract (MOE). S3 = group Sham of 3 months. S6 = group Sham of 6 months. D3 = group DBM of 3 months. D6 = group DBM of 6 months. DM3 = group DBM + MOE of 3 months. DM6 = group DBM + MOE of 6 months.

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capacity of biomaterials [37]. The same materials were also used in the mandible of the sheep in critical defects, but this research was assigned to a different paper.

2.4. Surgical procedures

The European Community guidelines for the handling, care and use of laboratory animals (DE 86/609/CEE) were followed in the present study [34]. Prior to surgery, the animals were deprived from solid food and water for a period of 24 and 8 hours, respectively. At the day of surgery, Acepromazine (0.55 mg/kg, Acepran 1%, Vetnil, São Paulo, Brazil) and Ketamine (20 mg/kg, Dopalen, Vetbrands, São Paulo, Brazil) were administered intramuscularly as a pre-anesthesia medication. Animals were then induced to anesthesia with Sodium Thiopental (5 mg/kg, Thiopentax, São Paulo, Brazil) that was intravenously administered and animals' sedation was maintained with oxygen vaporization (3 L/min, Biochimico, Rio de Janeiro, Brazil) with Isoflurane (oxygen flow of 3 L/min., Biochimico, Itatiaia, RJ, Brasil). Intraoperative procedures also included the intramuscularly administration of anti-inflammatory (Ketoprofen 10%, 3 mg/kg, Biofarm Quimica e Farmacêutica Ltda, São Paulo, Brazil) and antibiotic (Enrofloxacin 10%, 2.5 mg/kg, Chemitec agro Veterinária, São Paulo, Brazil) drugs.

After traditional trichotomy procedures, surgical sites were randomly assigned either to the right or to the left of vertebral column and were outlined 3 cm above from the sacral promontory. Surgical sites extended 20 cm anteriorly and 6 cm laterally from the vertebral column, and were disinfected with povidone-iodine (Rioquímica, São José do Rio Preto, Brazil). Three incisions ($\approx$ 3 cm/each, 4 cm apart) were made on the dermis to expose the muscular fascia (*Longissimus dorsi*) where one incision ($\approx$ 1 cm, parallel to column) per surgical site was then performed to allow individual intramuscularly implantation of sterile polyethylene tubes (control and experimental) among muscular fibers, as shown in Fig. 1.

A simple suture of the muscle and fascia was performed with a resorbable material (Vicryl 5-0, Ethicon, São Paulo, Brazil), whereas the continuous external dermis suture was performed with a non-resorbable material (Nylon 5-0, Shalon Fios Cirúrgicos Ltda., Goias, Brazil).

2.5. Euthanization procedures

Intramuscularly administration of anti-inflammatory (Ketoprofen 10%, 3 mg/kg, Biofarm Quimica e Farmacêutica Ltda, São Paulo, Brazil) and antibiotic (Enrofloxacin 10%, 2.5 mg/kg, Chemitec agro Veterinária, São Paulo, Brazil) drugs were performed for 3 and 5 days, respectively. Euthanize procedures (either at 3 or 6 months) were performed using Sodium Thiopental IV (8 mg/kg, Thiopentax, São Paulo, Brazil) to allow for the removal of tissue samples containing the tubes, with

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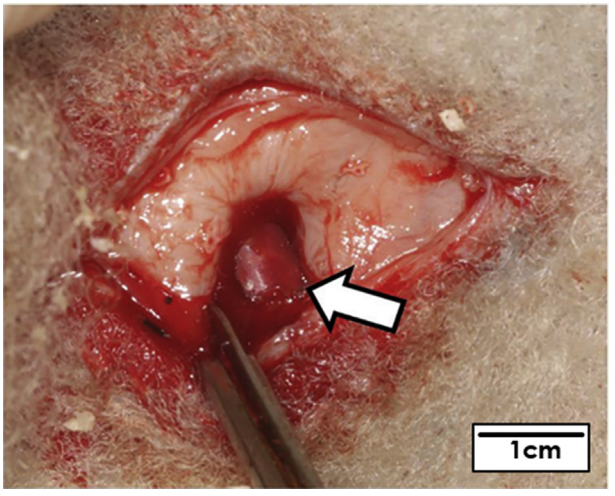

Fig. 1. Tube filled with biomaterial being inserted into the lumber muscle *longissimus dorsi* of the sheep (white arrow).

approximately 1 cm of surrounding tissues all around each tube. After removal, collected samples were immediately fixed in 10% neutral buffered formalin (Sigma-Aldrich, São Paulo, Brazil; 4 °C). Samples were kept immersed in the fixation solution for 7 days, in order to guarantee the preservation of fragile tissues inside the tubes.

2.6. Photographic analysis

After fixation, polyethylene tubes (control and experimental) were carefully removed from the excess surrounding muscle; tissues were removed from inside the tubes and photographed using a digital camera (Canon T3i, 105 mm macro lens, Oita Japan). Digital photographs were then exported into a computer where the diameter of the newly formed tissue (central region) was measured [3×, metric scale (mm)] on each sample (Fig. 2), using the ImageJ computer software (Freeware, available online at http://imagej.nih.gov/ij/).

2.7. Radiographic analysis

Each sample was then individually radiographed (0.005 mSv, 0.3 seconds) to determine the radiopacity levels achieved within each experimental group tested with a standard X-ray device (Spectro 70x, Dabi Atlante, São Paulo, Brazil) coupled with a digital sensor (RVG 5100, Kodak Company, New York, U.S.A.)

2.8. Histological analysis

Samples were subjected to decalcification in trichloroacetic acid (TCA) for 7 days. Decalcified samples were then embedded in paraffin blocks and serially sectioned

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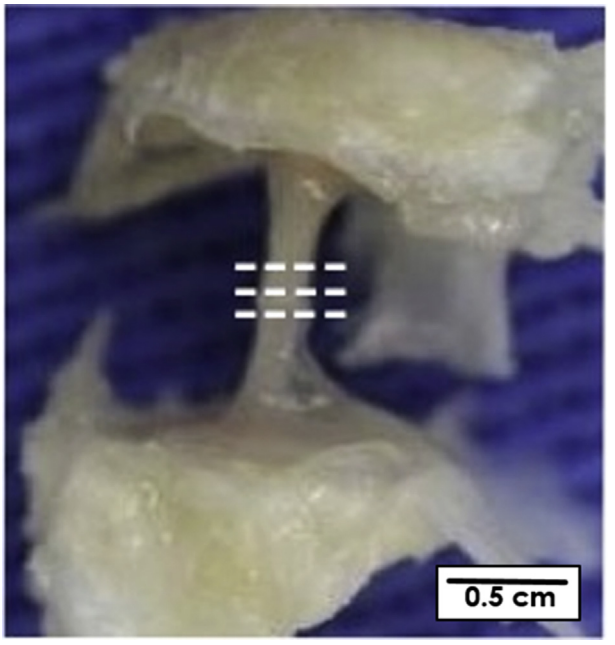

Fig. 2. Newly formed tissue within the tube has an hourglass shape. The white dotted lines represent the three regions used to determine the average thickness of the newly formed tissue inside the tube.

(6 μm/slice). Each slice was then stained with hematoxylin and eosin (HE) and Masson's trichrome (MT) in accordance with traditional histological techniques. Stained slices were imaged with an optical microscope (BX 41, Olympus Optical Company, Tokyo, Japan) coupled with a digital camera (Canon T3i, Oita Japan). Images were analyzed using Photoshop CS4 (Adobe Systems Inc., California, U.S.A.) and ImageJ (Freeware, available at http://imagej.nih.gov/ij/). The objective measurement ($pixels^2$) of the formation of collagenous matrix on MT-stained samples was performed at the central region of each sample (200× magnification), also using ImageJ.

2.9. Statistical analysis

Mean values from the photographic histological analyses were analyzed for normal distribution using the Shapiro-Wilk test, where the results were not normally distributed. Thus, Kruskal-Wallis was used to test samples' distribution and Fisher's least significant differences for treatment comparisons ($p < 0.05$).

3. Results

3.1. Photographic analysis

The results obtained from the image analysis, at three and six months, are presented in Figs. 3 and 4, respectively. All groups were capable of promoting the deposition

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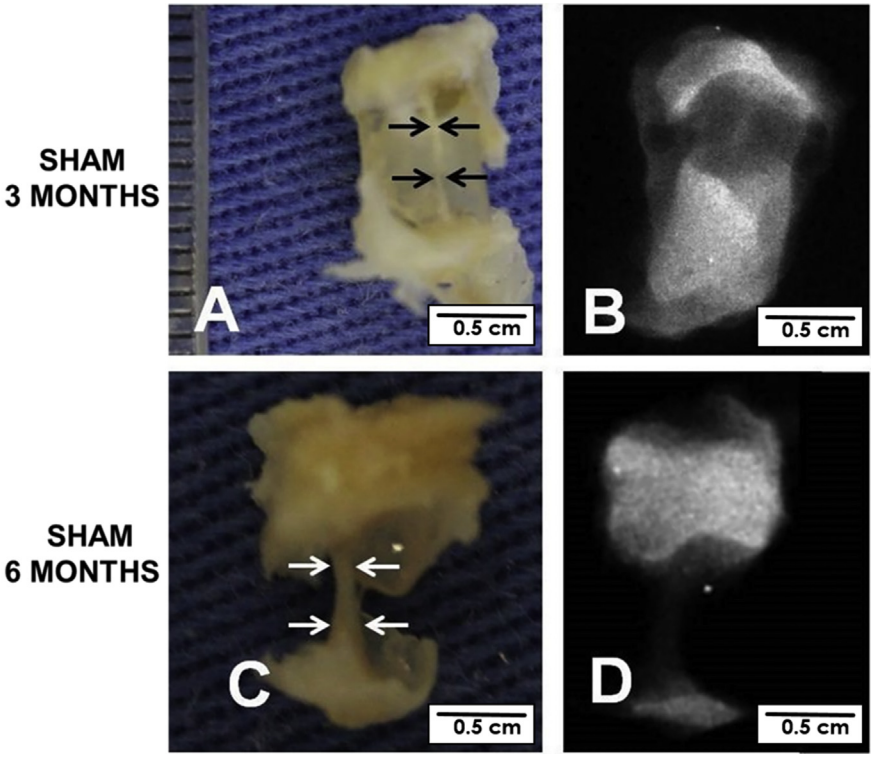

Fig. 3. Macroscopy and radiography of tissue formed in the presence of clot (sham). (A) 3-month sample; black arrows indicate the thickness of the newly formed tissue. (B) Radiograph of the sample in A. (C) 6-month sample; white arrows indicate the thickness of the newly formed tissue. (D) Radiograph of the sample in C.

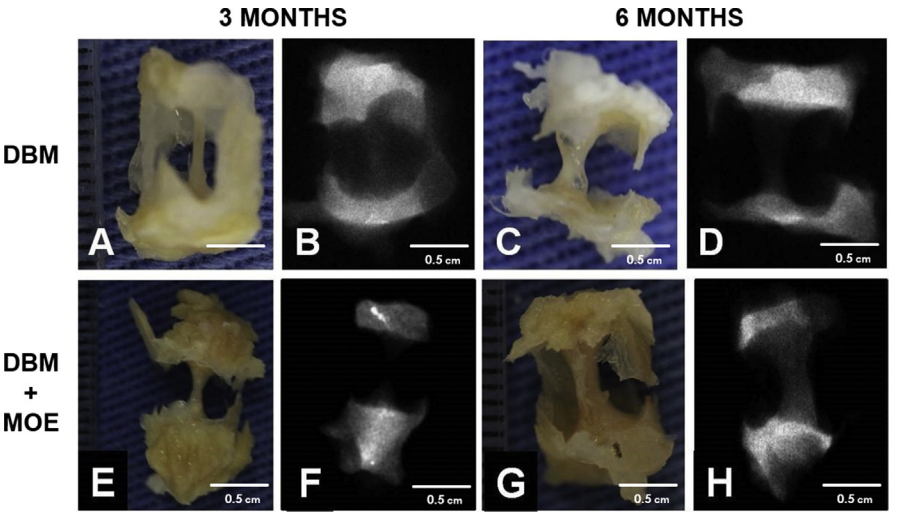

Fig. 4. Macroscopy and radiography of tissue formed inside the tubes in the presence of demineralized bovine bone matrix (DBM) and marine organic extract (MOE). (A) At 3 months. (B) Radiograph of the sample in A. (C) At 6 months. (D) Radiograph of the sample in C. (E) At 3 months with MOE. (F) Radiograph of the sample in E. (G) At 6 months with MOE. (H) Radiograph of the sample in G.

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of neoformed tissues. The levels of tissue formation observed at three months, have demonstrated that samples from the control group (sham) displayed the lowest levels of tissue formation, whereas experimental groups containing either DBM or DBM + MOE showed more tissue formation compared to the control. At six months, a similar trend of tissue formation was observed where samples from the experimental group treated with DBM + MOE displayed the highest levels of tissue formation among all groups investigated. The obtained results further demonstrated that the combined use of DBM and MOE led to a significant increase in the levels of newly formed tissue in the DM6 group (Table 2.)

3.2. Radiographic analysis

The visual examination of radiographs indicated that both the control and experimental groups seemed to promote the formation of mineral tissues that were radiopaque at radiographic assessment (Figs. 3 and 4), thereby suggesting that the control group could have promising osteoconductive and osteoinductive properties. This result was not confirmed by the histological analysis, and the observed radiopacity was explained by the tissue fixation technique used, with periods that were longer than normal. Such protocol was made necessary to preserve the structure and morphology of fragile tissues formed within the tubes at the time of euthanasia (3 and 6 months).

3.3. Histological analysis

The results from the histological assessment have clearly demonstrated that specimens from the control group (sham, Fig. 5, Table 3) did not promote any formation of mineralized tissues. In fact, what was microscopically observed, was the presence of a fibrous tissue of connective origin with organized collagenous bundles that were deposited in a parallel orientation to the long axis of the polyethylene tubes, nurtured by regular blood vessels presence − basically the same tissue characteristics at either 3 and 6 months (Fig. 5). A similar behavior was observed for samples from experimental groups treated with DBM or DBM + MOE, bundles of collagen fibers with

Table 2. Thickness of the center of the newly formed tissue.

Groups	Monitoring period	
	3 months	6 months
Sham	0.50 mm^{c}	0.59 mm^{c}
DBM	1.04 mm^{bc}	1.87 mm^{bc}
DBM + MOE	1.35 mm^{b}	3.40 mm^{a}

Sham = empty tube, negative control. DBM = Demineralized bovine bone matrix. MOE = marine organic extract. Values followed by the same superscript letters are statistically similar ($p < 0.05$).

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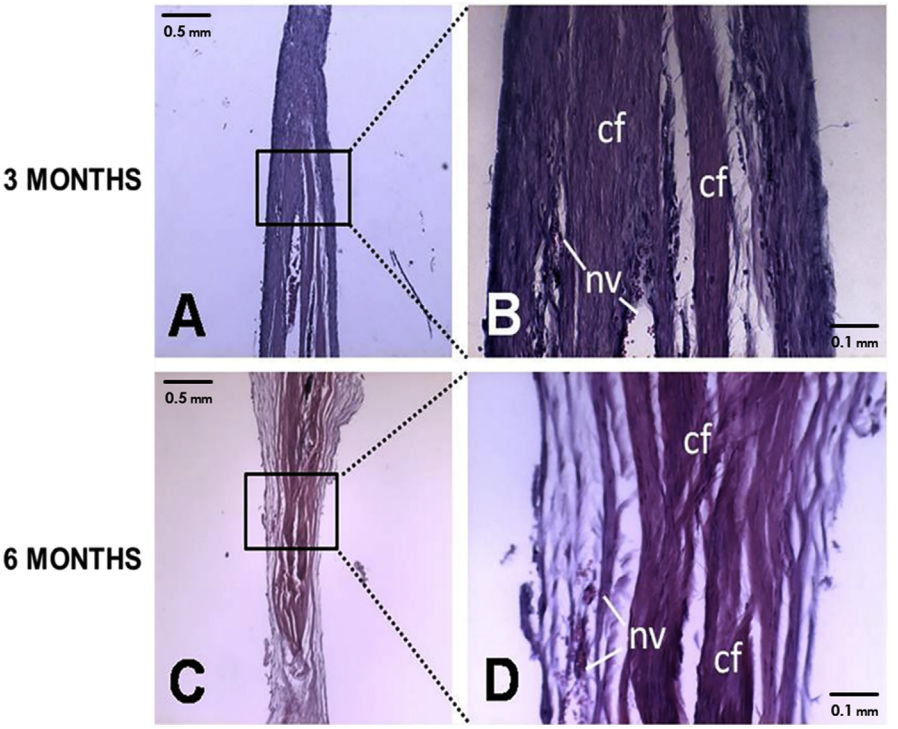

Fig. 5. Sham microscopy images. (A and B) At 3 months. (A) Original magnification ×40, HE (Hematoxylin and Eosin). (B) Original magnification ×200, HE. (C and D) At 6 months. (C) Original magnification ×40, HE. (D) Original magnification ×200, HE. nv = neovascularization; cf = connective fiber bundles.

blood vessels within. No giant cells of foreign-body, macrophages or other inflammatory cells were evident on these groups, neither at 3 or 6 months. At six months of observation, the use of these biomaterials led to the attainment of increased deposition of collagenous fibers with the presence of an interconnected fiber matrix, especially observed on DBM + MOE group (Fig. 6, Table 3).

4. Discussion

The present controlled and randomized pilot study aimed to investigate the in vivo utility of DBM modified or not by an experimental MOE extracted from nacre to

Table 3. Collagenous matrix area at 3 and 6 months.

Groups	Areas (pixels^2 × 10^5)	
	3 months	6 months
Sham	4.75^b	6.20^b
DBM	7.36^b	6.37^b
DBM + MOE	8.65^b	11.16^a

Sham = empty tube, negative control. DBM = Demineralized bovine bone matrix. MOE = marine organic extract. Values followed by the same superscript letters are statistically similar ($p < 0.05$).

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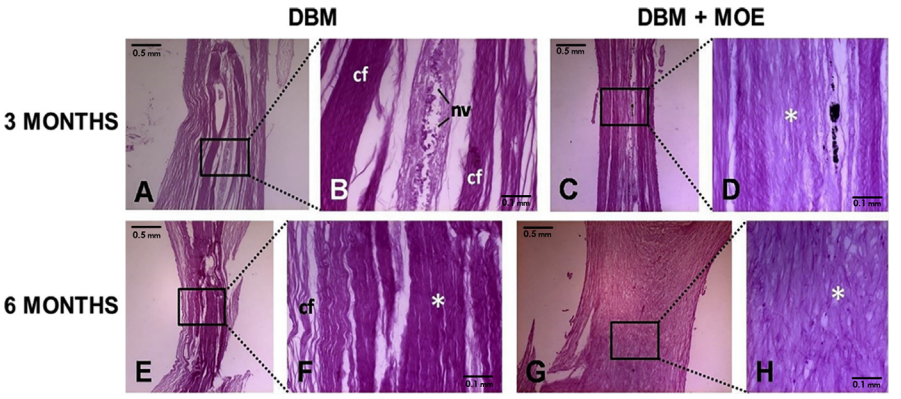

Fig. 6. Demineralized bone matrix (DBM) microscopy images. (A and B) At 3 months with pure DBM only. (C and D) At 3 months with the association of DBM and MOE. (E and F) 6 months of monitoring with pure DBM. (G and H) 6 months monitoring with DBM associated with MOE. A, C, E, and G = Original magnification ×40, HE (Hematoxylin and Eosin). B, D, F, and H = Original magnification ×200, HE. nv = neovascularization; cf = connective bundle fibers.

promote the guided deposition of tissues, perhaps bone. The rationale for the selection of the animal model used, was based on reports previously published in the literature who have demonstrated that sheep models (i) are sensitive, fast and efficient [34], (ii) provide testing conditions that are similar to those found in humans (both physiologically and biochemically) [38] and (iii) have an outstanding ability to screen foreign-body related reactions (such as the accumulation of macrophages, formation of giant cells and deposition of fibrous connective tissues) [39], that are typically triggered by implantable materials such as the ones herein investigated (e.g., polyethylene tubes, DBM and DBM + MOE).

Polyethylene tubes were selected as non-reactive carriers for the organo-biomaterials investigated based on the results reported in a previous publication from our group [40], where the intramuscularly implantation of tubes, of similar compositions, did not result in allergic or inflammatory reactions in a sheep model. Shah et al. [41], while investigating the expression of wound healing cellular biomarkers, have demonstrated that polyethylene tubes tested under comparable conditions (both experimental and surgical), did not result in the observation of either acute or chronic infiltrates, thereby demonstrating the high biocompatibility levels of polyethylene tubes and further corroborating the present study's rationale for the selection of polyethylene carriers.

Implantable biomaterials and bone substitutes, from both natural or synthetic origins, are being currently used in many areas of dentistry and medicine to guide the repair of critical bone defects (>10 mm) [42, 43], where the lack of support [44], may result in longer than usual healing periods and esthetic outcomes below patients' expectations and needs [45, 46]. In this direction, graft materials from organic sources (e.g., DBM) may be used as scaffolds to promote the deposition of undifferentiated

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mesenchymal cells, upregulate the adhesion and expression of osteoblasts and, ultimately, lead to the optimized formation of bony tissues [18, 20, 21]. Nacre was selected as a bone-growth promoter because of its biocompatibility, low antigenicity properties and growth factor concentrations (e.g. BMP-like) in levels that have been previously considered adequate for use in humans [19, 23]. The latter factor is of particular importance during the engineering step of novel implantable organo-biomaterials, because previous studies have demonstrated that undesired heterotopic ossification at the implantation site, exostosis, adverse life-threatening and inflammatory reactions (spinal surgery and off-label use) have been correlated with the presence of materials containing high concentrations of bone growth factors (BMP, BMP-2 or rhBMP-2) in a dose-dependent manner [47, 48, 49, 50].

Even though the organo-biomaterials investigated in the present study (DBM and MOE) were previously demonstrated to have BMPs in their compositions [17, 23, 51], the histological findings herein reported, have suggested that BMP concentrations present in the experimental organo-biomaterials investigated did not result in the formation of ectopic bone. Our results have also demonstrated that the experimental organo-biomaterials used in this work were resorbed at 3 and 6 months, and were completely replaced by newly formed tissue (fibrous connective only) in quantities that completely filled the implanted polyethylene tubes, which in turn, may demonstrate the potential application to guide the formation of tissues. Such relevant property may suggest at least that experimental materials investigated may be considered an interesting alternative to current biomaterials that are capable of maintaining the volume of tissues but are rarely resorbed [15].

Although the radiopaque aspect seen in the radiographic analysis was not due to mineralization but to the tissue fixation periods that were longer than normal [52], the results reported from the photographic and radiographic analyses, at 3 and 6 months, have indicated high-levels of deposition of neoformed tissues, where implants pertaining to the control group (sham) displayed the lowest levels of tissue formation among the groups. However, the radiopaque aspect herein found means that fixation methods can interfere with the results of x-ray imaging analytical methods used in the detection of mineralization levels of neoformed tissues (e.g. radiography, micro computed tomography).

Wu et al. [1] have demonstrated that subcutaneous implantation of native deproteinized bovine bone (DBB) resulted in the formation of soft connective tissues associated with significant numbers of foreign-body giant cells (inflammatory reaction) in a rat model. In the present study, probably due to the time of monitoring, no residual DBM particles or giant cells were detected.

According to previous studies, intramuscular and subcutaneous models are routinely used to evaluate the osteoconductivity [53] and biocompatibility [54] in the search of functionalities of biomaterials. However, the lack of mechanical stability that is

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commonly associated with these types of implantation sites, may translate into deleterious movements of implanted materials [1], subsequent encapsulation (dense fibrous connective tissues) and overall reduction of engineered osteogenic functionalities [55]. In this context, the use of biomaterial or association with high remodeling capacity may be problematic because these materials could accelerate the resorption process and result in tissues of low or none mineralization levels – some authors suggest the incorporation of bioceramic nanoparticles to counteract this effect [56]. That might be the main reason and limitation for the findings of the present work. This laboratory is part of a research group currently working with the development of nanostructured bioceramic particles.

5. Conclusions

The present controlled and randomized study has reported a protocol for the fabrication of MOE obtained from nacre and its in vivo functionality in ectopic grafting when associated or not with DBM. This experimental organo-biomaterial association did not reveal any osteoinductive property but led to a fibrous tissue repair only. Other studies are made necessary to improve nacre's osteoinductive, osteogenic characteristics and to further investigate the in vivo utility of these novel organo-biomaterials.[56]

Declarations

Author contribution statement

João C Zielak: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

David G Neto, Makeli A C Zielak: Performed the experiments; Analyzed and interpreted the data.

Leonardo Brunet Savaris: Analyzed and interpreted the data; Wrote the paper.

Fernando L E Florez: Analyzed and interpreted the data; Wrote the paper.

Tatiana Miranda Deliberador: Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

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Additional information

No additional information is available for this paper.

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