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Original Article

Title: Standardized *in vivo* evaluation of biocompatibility and bioresorption of a new synthetic membrane for guided bone regeneration

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ABSTRACT

This study aimed to evaluate the physico-chemical properties, biocompatibility and bioabsorption of 3 different new membranes for bone guided regeneration (PLGA associated with hydroxyapatite and β-TCP) with three thicknesses (200, 500 and 700 µm) implanted in mice subcutaneously.

Scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and the quantification of Carbon, Hidrogen and Nitrogen were used to characterize the physico-chemical properties. One hundred Balb-C mice were divided into 5 experimental groups: Group 1 - Sham (without implantation); Group 2 - 200 µm; Group 3 - 500 µm; Group 4 - 700 µm; and Group 5 - Pratix®. Each group was subdivided into four experimental periods (7, 30, 60 and 90 days). Samples were collected and processed for histological and histomorphometrical evaluation.

The membranes showed no moderate or severe tissue reactions in the experimental periods studied. The 500 µm membrane did not show tissue reaction for any experimental periods studied. The 200 µm membrane membranes began to exhibit fragmentation after 30- day, while the 500 and 700 µm membranes started the fragmentation at 90-day.

All membranes studied were biocompatible, and the 500µm membrane showed the best results being a promissory membrane for bone guided regeneration.

KEY WORDS: Membranes; Polymer; Biocompatibility; Inflammatory cells; Calcium Phosphate.

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1. INTRODUCTION

The bone is a specialized connective tissue capable of regenerating completely. Therefore, regenerative procedures are used for gaining bone tissue, allowing professionals the possibility of rehabilitating a patient both aesthetically and restoring function. However, bone tissue's growth pattern requires a longer time for its formation compared to non-mineralized connective tissue, since the fibroblasts proliferate and produce matrices of collagen more quickly than the osteoblasts that form the bone tissue [1].

Guided Bone Regeneration treatment (GBR), in which the regeneration of defects is predictably achieved by the application of occlusive membranes, was developed based on the Guided Tissue Regeneration (GTR) concept. GBR involves mechanically excluding the population of non-osteogenic cells from the surrounding soft tissues and allowing the population of osteogenic cells originating from native bone to inhabit the defect, thus preventing the growth of unwanted soft tissue in the bone defect [2]. Experimental studies have provided significant evidence that bone regeneration is significantly increased when the invasion of soft tissues into bone defects is mechanically prevented [3,4].

In the last 40 years, different membranes have been developed to act as physical barriers in the treatment of bone defects. Today, resorbable collagen and aliphatic polyester membranes are the most commonly used materials to eliminate the need for a second surgery for membrane removal [5,6]. However, collagen undergoes rapid degradation due to the action of inflammatory cells in the region, which resultd in poor mechanical resistance leading to collapse [7].

Among the polymers used, the most applicable in medicine are those that, when degraded, generate soluble and non-toxic products that are compatible with

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the organism. The aliphatic polyesters derived from lactic acid and glycolic acid undergo hydrolytic degradation. However, despite the excellent mechanical properties of the PLA, its biocompatibility, biodegradability, hydrophobicity, brittleness, acidic degradation products and high cost of PLA restrict its application [8]. The PLA polymer shows a slower hydrolysis rate compared to the PGA polymer in the human body, so for a suitable degradation of the polymer, the PLA polymer has been combined with the PGA polymer as a copolymer, improving its characteristics [9]. The insertion of calcium phosphate (hydroxyapatite and β-TCP) prevents collapses by improving mechanical properties [10].

The aim of this study was to evaluate the physico-chemical properties, biocompatibility and bioabsorption of 3 different thicknesses of PLGA membranes associated with calcium phosphate (PLGA + hydroxyapatite + β-TCP) implanted in mice subcutaneously, following ISO 10993-6: 2016. This study used mice as an experimental model due to their rapid prolificity, easy handling, known biology and genome and not very expensive maintenance, as well as similarity of the results with humans.

2. MATERIALS AND METHODS

Control membrane

A commercially available PLGA dental membrane (Pratix®, Baumer, São Paulo, SP, Brazil) was purchased for comparison of the biocompatibility and bioabsorption rates in the subcutaneous tissue of rats.

Physico-chemical characterization of experimental membranes

The crystalline mineral phases present in the membranes, their crystallinity and the proportion of the hydroxyapatite and β-tricalcium phosphate phases were

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examined by Rietveld XRD Quantification (XRD). The XRD patterns were obtained with a Empyrean - Panalytical diffractometer operating at 45 kV and 40mA, with CuKα radiation (Cu - 1,540598 Å), with temperature of 25ºC and relative humidity of the air about 55%. The data were collected in the 2θ range of 10°–70° with a step of 0.02° point per second.

The contents of the hydroxyapatite and β-tricalcium phosphate phases in the samples were evaluated by the relative intensities of specific peaks of βTCP and HA XRD patterns in the sample as described by Balmain et al. [11]. Vibrational modes of phosphate and hydroxyl groups in samples were analyzed by Fourier transform infrared spectroscopy. The spectra were obtained in a Thermo Scientific, Nicolet iS50, operating in transmission mode from 650 to 4000 cm^{-1}. Scans number: 8, Resolution: 4 cm^{-1}.

The microstructure of the membrane was investigated by using a scanning electron microscope (FEG - ZEISS® - mod. SUPRA 55VP) of the surface and the transversal section with 300, 1000, 3000, 10000 and 20000 X. The carbon, hydrogen and nitrogen contents were quantified in duplicate with the organic elemental analyzer (CHN SO PE 2400 series II, PerkinElmer – Table 1).

	Carbon %	Hydrogen %	Nitrogen %
1	39.5	4.7	0.1
2	39.2	4.3	0.1
Mean	39.4	4.5	0.1

Tabela 1 - CHN SO PE 2400 series II, PerkinElmer

Animals Caracterization and Experimentals Group

This study was carried out in compliance with the guidelines of the 3Rs Program (Reduction, Refinement and Replacement), whose objective is to reduce the number of animals used during experimentation, to minimize pain and

discomfort and avoid euthanasia at the end of experimentation (NC3Rs 2010), and reported according to the ARRIVE guidelines regarding relevant items[11]. The Ethical Committee of Universidade Federal Fluminense approved the study and the protocol no. is CEUA/UFF: 869. One hundred Balb-C mice, male and female, weighing approximately 30g, were provided by the Laboratory Animals Center at Fluminense Federal University (Niterói, Rio de Janeiro, Brazil). The animals were divided into 5 experimental groups: Group 1—Sham (without membrane implantation); Group 2—PLGA membrane + HA + β-TCP (200 μm); Group 3—PLGA membrane + HA + β-TCP (500 μm); Group 4—PLGA membrane + HA + β-TCP (700 μm); and Group 5—Pratix® membrane implantation. The materials were supplied by FGM Materiais Odontológicos LTDA. All experimental groups were subdivided into 4 experimental periods (7, 30, 60 and 90 days) with 5 animals in each group/experimental period. Before and after the study, all animals were kept in isolators with a maximum of 5 animals in each and fed with pelleted feed and water *ad libitum*.

Surgical Procedure and Production of Samples

After a 24-hour fast, all animals were submitted to general anestesia by the intraperitoneal route, following Fluminense Federal University protocol, with a 0.6 ml injection of anesthetic solution prepared with 1.0 ml of 10% Ketamine (Dopalen®-100mg / ml), 0.5 ml of 2% xylazine (Anasedan® 20mg / ml) and 8.5 ml saline sterile (KabiPac®).

Three minutes later, degermation and trichotomy were performed with chlorhexidine degermant and chlorhexidine alcoholic 2% solutions (Riohex Scrub®, Rioquimica; São José do Rio Preto, São Paulo, Brazil). An approximately 1-cm-long incision was made in the epithelium of the dorsal region of the animal,

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followed by divulsion of the muscular fascia skin with the aid of scalpel and blunt-tipped scissors, exposing the subcutaneous tissue for insertion of the membrane (1 cm) into the subcutaneous region. This was followed by a 5-0 nylon suture (Ethicon®, Johnson & Johnson, Brazil) and antisepsis with gauze and alcoholic chlorhexidine solution (Figure 1).

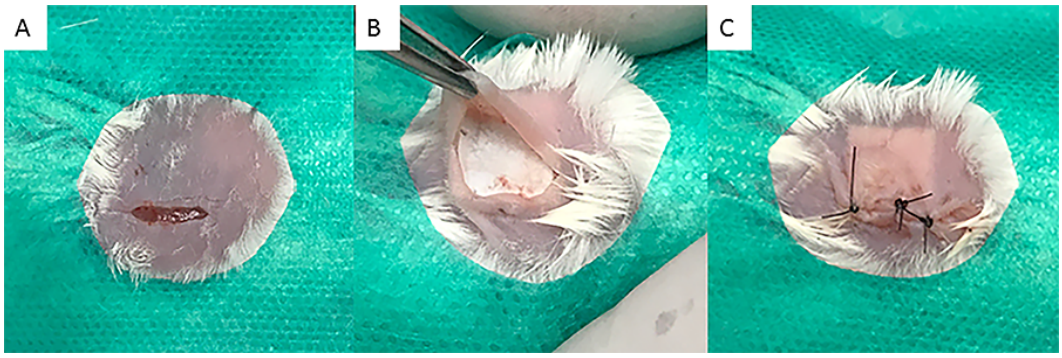

Figure 1 - Sequence of surgical procedures performed. **(A)**. Trichotomy, skin antisepsis of the back region of the animals and incision of approximately 1cm in length on the back region of the animals, according to ISO 10993-6 / 2016; (**B).** After divulsion of the cutaneous tissue, a fragment of 1cm membrane was implanted and tested according to the experimental groups and (**C).** Replacement of cutaneous tissue over the implanted membrane and suture with 5-0 mononylon (Ethicon®, Johnson & Johnson, Brazil).

In the postoperative period, the animals were kept in the Animal Experimentation Laboratory (AEL/UFF) and divided in isolaters based on their experimental groups, where they received food and water at will. On the day of surgery and on each of the following two days, 5 mg/kg of Meloxicam (Eurofarma Laboratórios LTDA, São Paulo, SP, Brazil) was administered subcutaneously every 24 hours.

The mice were euthanized after the respective experimental periods with lethal doses of anesthetic solution, and the samples and adjacent subcutaneous

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tissue (± 5 mm with safety margins) were collected, fixed, decalcified, dehydrated, clarified and included in paraffin to obtain 5 µm thickness. The slices were stained with Hematoxylin and Eosin (HE) and observed using a light field microscope at 40X magnification (Nikon Eclipse E400, Tokyo, Japan). These images were captured by a high resolution digital camera (Sony® HD DSC HX9V 16.2 Mega Pixels) using a 10x acroplan objective lens at the Laboratory of Applied Biotechnology – UFF (LABA) for descriptive and semiquantitative histological evaluation for the presence of inflammatory infiltrate, vascular neoformation, extension and type of necrosis, presence of fatty infiltrate and fibrosis and membrane bioabsorption.

3. RESULTS

3.1. Structure and characteristics of the PLGA membrane

The synthetic PLGA membrane is shown in Figure 2. The membrane exhibited a micro-fibrous aspect to favor guide bone regeneration. The difference in the morphology between the different membranes thickness is clearly visible on the 20.000X magnification observed by SEM.

The micro-fibrous surface exhibited a highly porous structure with interlaced non-woven fibres. The diameters of the fibres ranged from 0.2 to 2 µm (Figure 2). The XRD evaluation showed two crystallines mineral phases present in the membranes (HA and β-TCP), and the proportion of the hydroxyapatite (54.2%) and β-tricalcium phosphate phases (45.8%) (Figure 3). The carbon, hydrogen and nitrogen contents were quantified according the Table 1. It was observed the mean value for Carbon (39.4%), Hydrogen (4.5%) and Nitrogen (0.1%). The FTIR showed that samples showed predominant absorptions of poly (lactic acid-co-glycolic acid) (PLGA) (Figure 4).

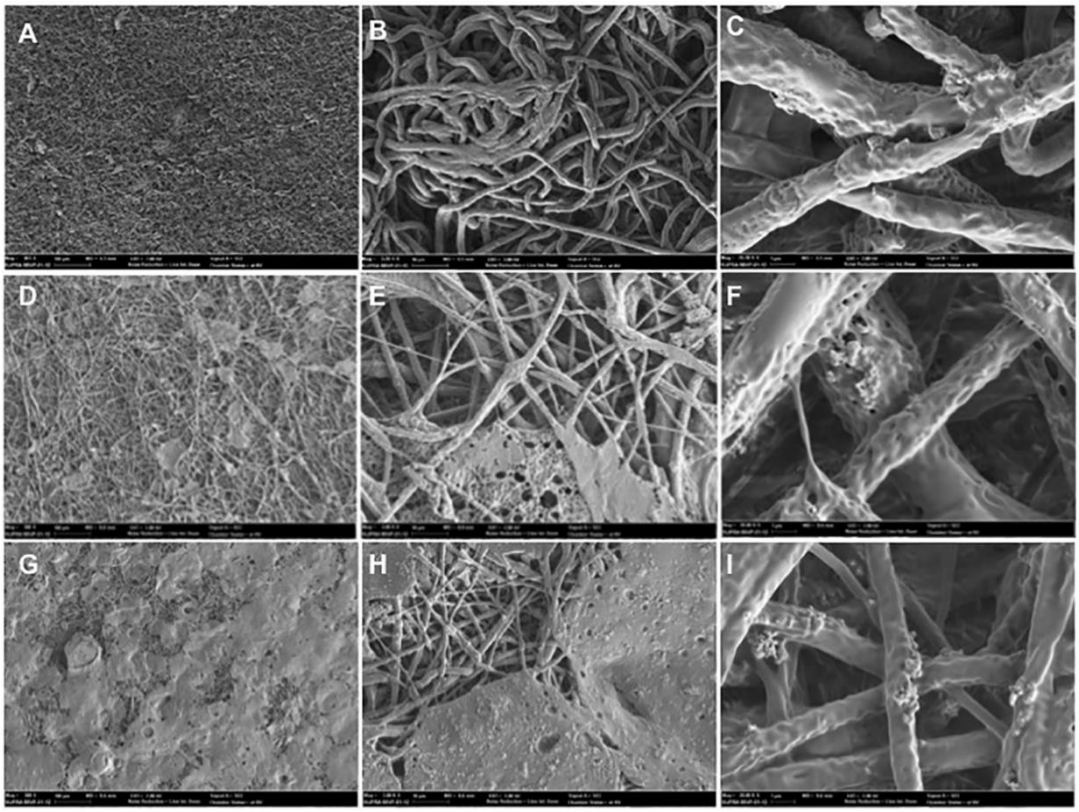

Figure 2 - Scanning electronmicroscopy (SEM) micrographs of the membrane surface at 300 X magnification, scale bar=100 µm (**A, D** and **G**), 3000 X magnification, scale bar = 10 µm (**B, E** and **H**), and 20000 X magnification, scale bar=1 µm (**C, F** and **I**). **A, B** and **C** (200 µm membrane), **D, E** and **F** (500 µm membrane), and **G, H** and **I** (700 µm membrane).

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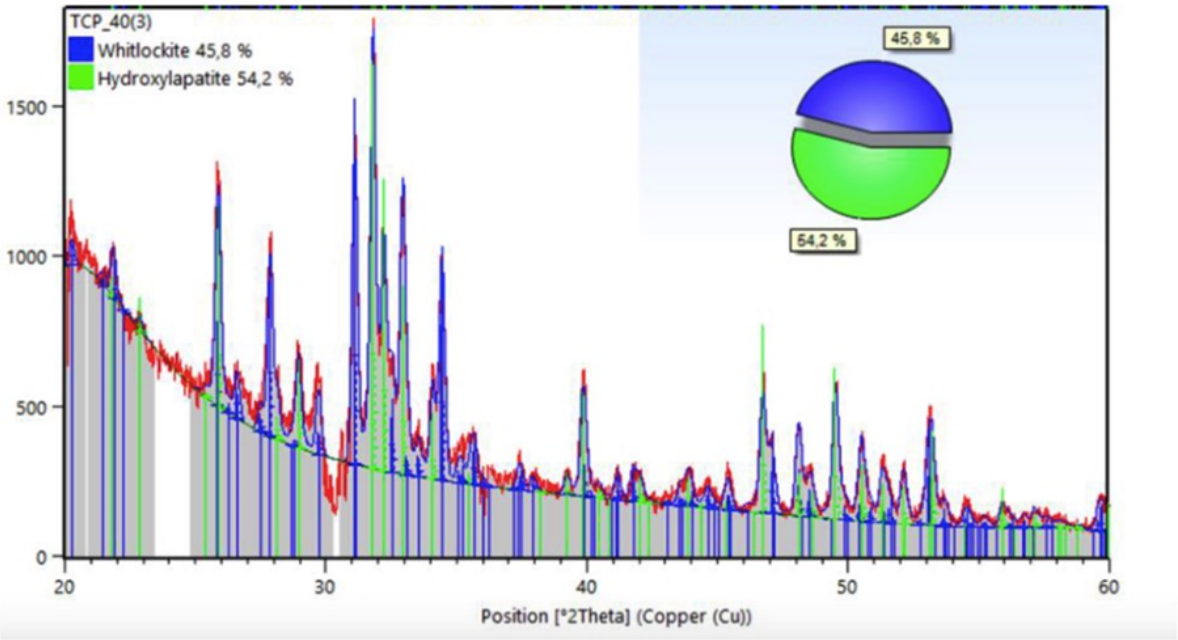

Figure 3 - X-ray diffraction pattern (XRD) of membrane sample. Observe the peak representing hydroxyapatite and β-TCP. The quantitative analysis showed the presence of two phases: 45.8% β-TCP and 54.2% HA.

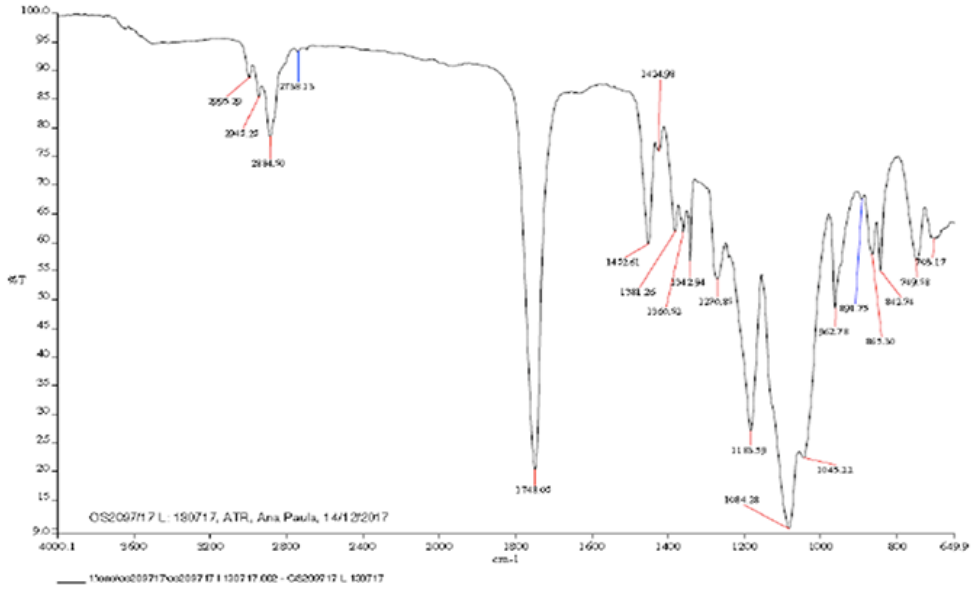

Figure 4 - Fourier transformed infrared (FTIR) spectra of the membrane sample. Observe the polymerics peaks and phosphate peaks.

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3.2. In vivo implantation

Overall, the animals tolerated well the anesthesia, pre-surgery and post-surgery, without complications or setbacks. Biological effects after the implantation of the different experimental membranes were evaluated according to the criteria established by ISO 10993-6: 2016 / Part 6 / Annex E and the descriptive analysis of the tissue response to the membranes was evaluated as a function of tissue disposition in the different membranes: bioabsorption, presence of inflammatory cells, vascular neoformation and presence of fibrosis. The degree of inflammation was evaluated and quantified manually according to the number and distribution of inflammatory cells present at the membrane-tissue interface, that is, polymorphonuclear cells, lymphocytes, plasma cells, macrophages and giant cells. In addition, the degree of degeneration (debris) was determined by morphological alterations necrosis extension.

For the 7-day experimental period, small vascular neoformation with minimal capillary proliferation (presence of 1 to 3 bulbs per examined field) was observed in Group Sham. Also found were a cicatricial process with an absence of multinucleated giant cells and macrophages and an abundance of lymphocytes and plasmocytes, in addition to the moderate presence of polymorphonuclear cells. In Group 2 (200μm), a moderate infiltration of lymphocytes, macrophages and multinucleated giant cells was observed, along with a slight presence of polymorphonuclear cells. Moderate vascular neoformation with capillary proliferation was also present (presence of a wide range of capillaries per examined field).

In Group 3 (500 μm), a moderate infiltration of lymphocytes and macrophages and a discrete infiltration of plasmacytes and multinucleated giant

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cells were observed, as were mild vascular neoformation with proliferation of capillaries. Group 4 (700 µm) presented integral membranes with a moderate presence of lymphocytes, plasma cells and macrophages and a discrete polymorphonuclear infiltration. Local neovascularization with small capillary proliferation was also observed in this group. The control Group (Pratix ®) did not visualize the membrane in any cuts performed; therefore, the cellular response tissue of the periphery membrane was evaluated. There was a moderate presence of lymphocytes, macrophages and polymorphonuclear infiltration. Also observed in this group was moderate local neovascularization with small capillary proliferation (Figure 5).

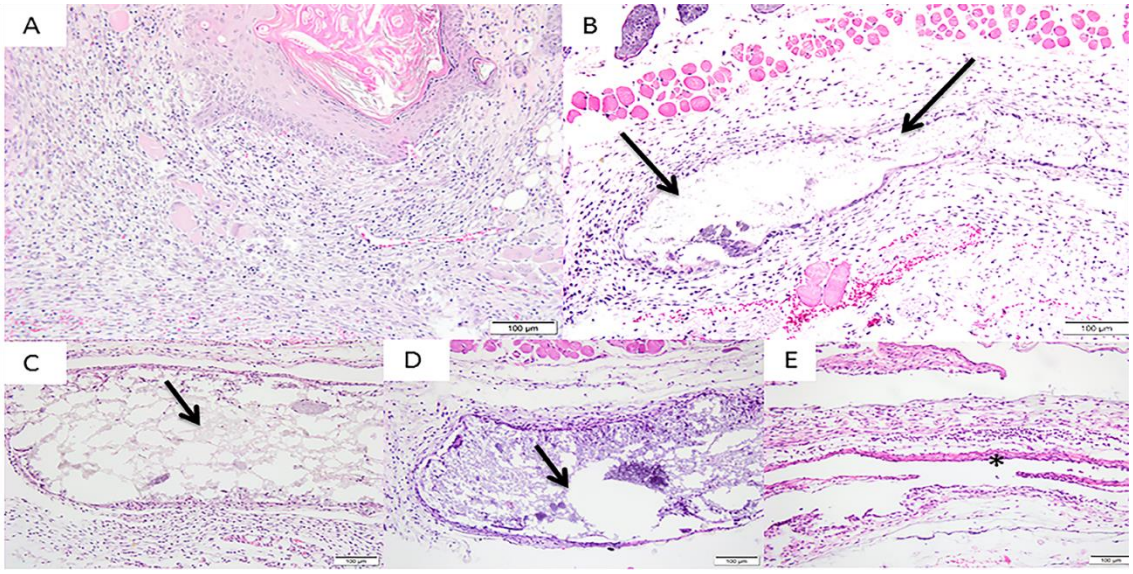

Figure 5 - Photomicrographs of the experimental groups after 7 days of implantation. Group 1 (Sham) – (**A**); Group 2 (200 µm) - (**B**); Group 3 (500 µm) - (**C**); Group 4 (700 µm) – (**D**); and Group 5 (Pratix®) – (**E**). Observe the presence of the fragment-free membrane in all groups. → Membranes; * Pratix® membrane site; Magnification: 10 X and 20X, coloring: Hematoxylin and Eosin.

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In the 30-day experimental period, a discrete presence of lymphocytes and plasma cells and a moderate presence of macrophages were observed in Group Sham. Regarding tissue response, a discrete presence of vascular neoformation with few vascular proliferations characterized this sample. In Group 2 (200 μm), membranes began to exhibit fragmentation with tissue invasion. There was a moderate presence of multinucleated giant cells and macrophages, a discrete presence of lymphocytes with an absence of polymorphonuclear cells and mild local neovascularization.

The membranes of the 500 μm and 700 μm Groups showed no changes in their integrity after 30 days. The discrete presence of lymphocytes, plasma cells, macrophages and a few multinucleated giant cells, with a moderate tissue response of local neovascularization, was observed in Group 3. Group 4 had a discrete presence of lymphocytes and plasma cells, a moderate presence of multinucleated giant cells and few polymorphonuclear cells. Regarding tissue response, there was moderate vascular neoformation with proliferation of more than 7 shoots per field. The Pratix® membranes used as a control group did not adhere to the subcutaneous tissue, so during the removal of the samples they were detached from the tissue and the tissue adjacent to the implantation site of this membrane was observed, revealing a discrete lymphoplasmocytic infiltration and a moderate infiltration of macrophages (Figure 6).

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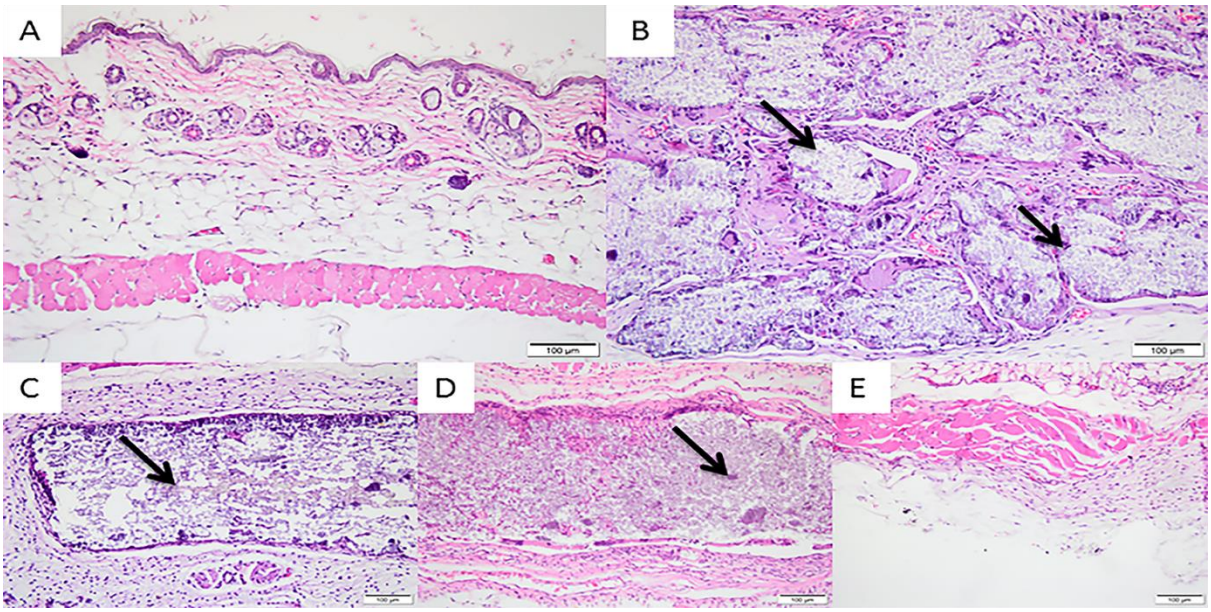

Figure 6 - Photomicrographs of the experimental groups after 30 days of implantation. Group 1 (Sham) – (**A**); Group 2 (200 µm) – (**B**); Group 3 (500 µm) – (**C**); Group 4 (700 µm) – (**D**); and Group 5 (Pratix®) – (**E**). Observe the presence in the 200 µm membrane of fragmented and non-fragmented aspects in the 500 µm and 700 µm groups. ⟶ Membranes; * Pratix® membrane site; Magnification: 10 X and 20X, coloring: Hematoxylin and Eosin.

After 60 days, a discrete presence of lymphocytes, plasma cells and macrophages was observed in the Sham Group. There were no multinucleated giant cells or polymorphonuclear cells with a slight presence of vascular neoformation shoots. In Group 2, there was a moderate presence of lymphocytes, macrophages and multinucleated giant cells, a discrete presence of plasma cells and an absence of polymorphonuclear cells. Groups 3 and 4 presented a discrete / moderate presence of lymphocytes, macrophages, multinucleated giant cells and plasma cells. However, while Group 3 presented mild vascular neoformation, Groups 2, 4 and 5 showed moderate neovascularization. Group 5 was

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macroscopically detached from the tissue, without connection to the subcutaneous tissue. The presence of multinucleated and polymorphonuclear giant cells was not observed and there was only a moderate presence of lymphocytes and macrophages (Figure 7).

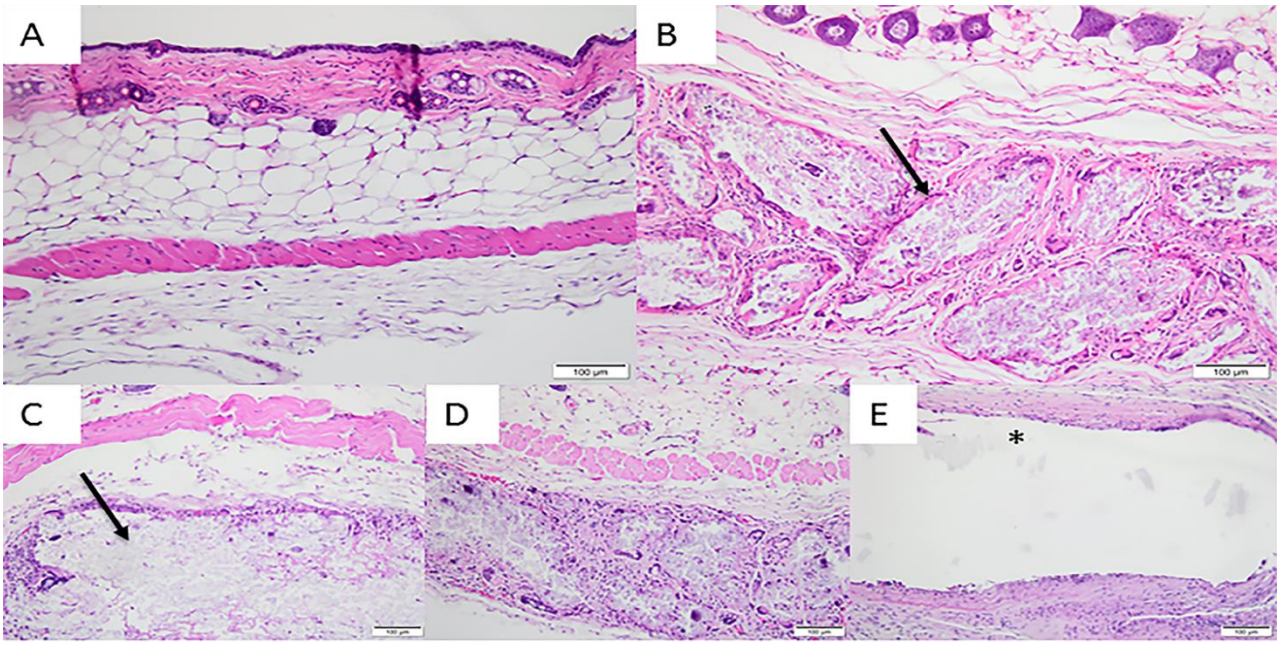

Figure 7 - Photomicrographs of the experimental groups after 60 days of implantation. Group 1 (Sham) – (**A**); Group 2 (200 µm) – (**B**); Group 3 (500 µm) – (**C**); Group 4 (700 µm) – (**D**); and Group 5 (Pratix®) – (**E**). ⟶ Membranes; * Pratix® membrane site; Magnification: 10 X and 20X, coloring: Hematoxylin and Eosin.

After the 90-day period, Group 1 showed discrete lymphocyte and macrophages infiltration, and an absence of multinucleated giant cells and polymorphonuclear cells. The 200 µm group presented a moderate presence of macrophages, lymphocytes and multinucleated giant cells and a discrete presence of plasma cells. In the 500 µm and 700 µm groups, there was no presence of polymorphonuclear cells, plasmocytes and moderate lymphocytes,

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multinucleated giant cells and macrophages. The control group had a discrete presence of polymorphonuclear cells and a moderate presence of macrophages, multinucleated giant cells, lymphocytes and plasma cells. All presented moderate vascular neoformation, with the exception of Group 1, which had discrete neoformation (Figure 8).

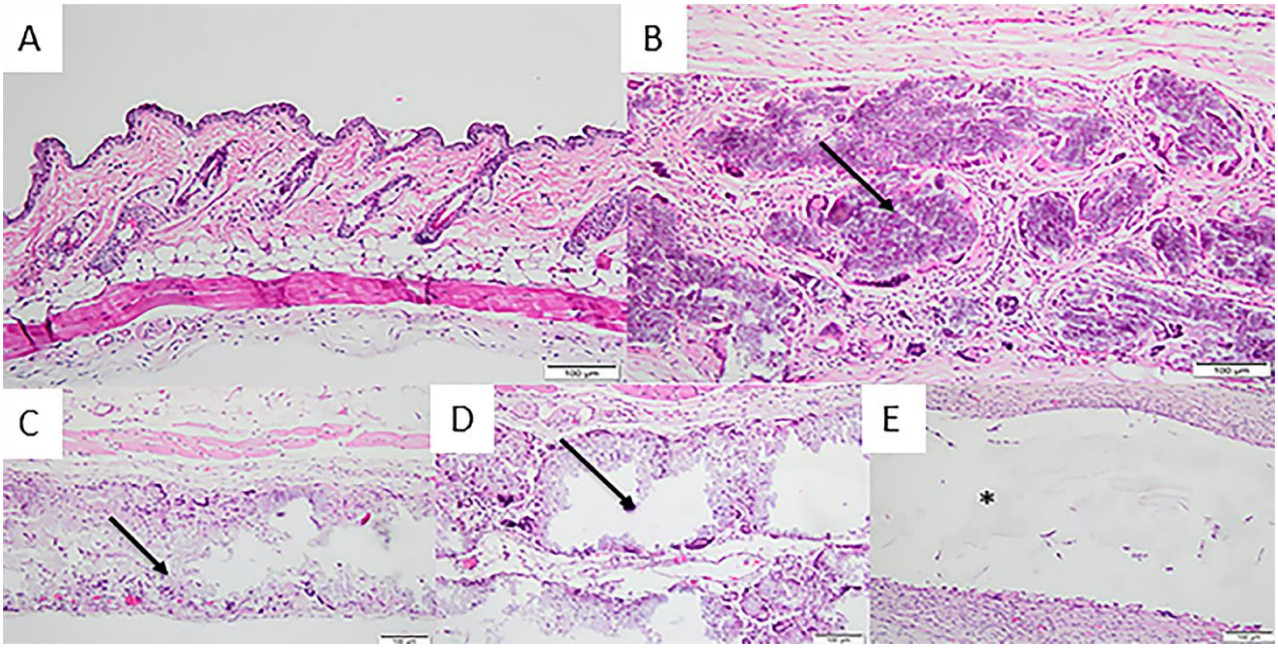

Figure 8 - Photomicrographs of the experimental groups after 90 days of implantation. Group 1 (Sham) – (**A**) ; Group 2 (200 µm) – (**B**); Group 3 (500 µm) – (**C**); Group 4 (700 µm) – (**D**); and Group 5 (Pratix®) – (**E**). → Membranes; * Pratix® membrane site; Magnification: 10 X and 20X, coloring: Hematoxylin and Eosin.

No areas of necrosis, fibrosis or fatty infiltration were present for all experimental periods.

According to ISO, at the end of the evaluation test samples may present scores ranging from 0.0 to 2.9 (absence of tissue reaction), 3.0 to 8.9 (discrete tissue reaction), 9.0 to 15 (moderate tissue reaction) and above 15.1 (severe

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tissue reaction). Thus, after evaluation of each cell type and tissue within the conditions of this study, following ISO 10993-6/2016, it was observed that the test sample of 200 µm showed a slight tissue reaction (4) compared to the control sample after 7 days of implantation, whereas the test samples of 500 µm (2,6) and 700 µm (0) showed no tissue reactions after 7 days of deployment. After 30 days, the tested membrane of 700 µm showed a slight tissue reaction (4.4) when compared to the control sample, whereas the 200 µm (1,2) and 500 µm (1,8) samples did not present a tissue reaction. After 60 days, the 200 µm membrane presented a slight tissue reaction (7) when compared to the control group. There was an absence of tissue reaction for the membranes of 500 µm (2,6) and 700 µm (3,2). After 90 days of implantation, the membrane of 700 µm showed tissue reaction (3.8), while the membrane of 200 µm obtained a score of 0.6 and the membrane of 500 µm obtained a score of 0, characterizing an absence of tissue reaction.

4. DISCUSSION

For membranes to be used effectively in the GTR process, they must have biocompatibility characteristics, occlusive properties, capacity to maintain space, tissue integration and have easy clinical manageability [12,13]. The desirable characteristics of the membrane barriers used for guide bone regeneration therapy include biocompatibility to allow integration into host tissues without creating an inflammatory response, a degradation profile accompanying tissue neoformation, mechanical and physical properties sufficient to allow membrane installation and sufficient sustained force to not collapse and to perform the proper barrier role [14].

Studies demonstrate that membranes containing PLGA + HA and β-TCP can contribute mainly due to their mechanical properties, thus improving structural integrity and flexibility [15,16]. In addition, the release of calcium and phosphorus ions during the degradation of HA and β-TCP may be involved in bone metabolism and promote the formation of new bone [17]. Sanaei-Rad et al. [18] demonstrated that HA improved osteogenic properties, strength and structural stability. Seyedjafari et al. [19] evaluated the incorporation of nanohydroxyapatite on the surface of an electrospun poly (l-lactide)-PLLA associated with human cord blood-derived unrestricted somatic stem cells, *in vitro* under osteogenic induction and *in vivo* after subcutaneous implantation, by demonstrating adequate mechanical properties and improving the osteogenic differentiation of somatic stem cells.

The membranes tested did not present moderate or severe tissue reactions in the experimental periods studied, not differing from the patterns presented by the control group. Therefore, they are biocompatible. The present results demonstrate that all membranes were intact after 7 days of implantation, but after 30 days the 200 μm membrane was partially fragmented and lower mechanical stability was observed, while the membranes of 500 μm and 700 μm started the process of fragmentation only after 90 days of implantation. The 200 μm membrane showed a slight tissue reaction after 7 and 60 days, while the membrane of 700 μm showed a slight tissue reaction after 30 and 90 days. The 500 μm membrane did not show tissue reaction in any experimental periods studied. To play its role as a barrier, absorbable membranes should remain for at least three to four weeks [20].

Therefore, we verified that all thicknesses of membranes are biocompatible to subcutaneous connective tissue of mice; however, the membrane of 200 µm presented a faster absorption when compared to those of another thickness and to the control group. The membrane of 500 µm only started fragmentation after 90 days, an ideal time to exclude mechanically non-osteogenic and non tissue response cell populations, thus making it a good thickness for this type of membrane.

Particles of thermally deproteinized inorganic bone (hydroxyapatite) have been demonstrated to induce a chronic inflammatory reaction with the presence of giant cells and fibrosis of the particles in the subcutaneous tissue of rats[21]. Therefore, it is reasonable to suggest that the recruitment of giant cells may be related to the persistence of remnants of the mineral phase on the membrane.

5. CONCLUSION

With the limitations of this preclinical study, it is possible to conclude that the tested membranes are well tolerated by the tissues, are not completely resorbed at 90 days. Absorbable membranes derived from PLGA + HA + β-TCP could be used as barriers to promote tissue regeneration in surgical techniques of guided tissue regeneration, but further *in vivo* (bone repair model) and clinical studies should be conducted to determine the clinical efficacy of these membranes.

AUTHOR CONTRIBUTIONS

Conceptualization - Lívia da Costa Pereira, Mônica Diuana Calasans-Maia and Rafael Seabra Louro.

Methodology – Lívia da Costa Pereira, Rodrigo Figueiredo de Brito Resende and Marcelo José Pinheiro Guedes de Uzeda.

Histomorphological analysis - Adriana Terezinha Neves Novellino Alves.

Writing - original draft preparation – Lívia da Costa Pereira.

Supervision – Rafael Seabra Louro and Monica Diuana Calasans-Maia.

Writing - review and editing - Carlos Fernando de Almeida Barros Mourão.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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