

Review

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Review

Immunohistochemical, Ultrastructural, and Histological Analysis of Two Type 1 Collagen Membranes Used for Tissue Regeneration in the Subepithelial Region

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Abstract: Collagen membranes for tissue regeneration have been widely used in medicine and dentistry. Collagen membranes have a high biocompatibility and adequate healing time. The objective of the present work was to develop the combination of the use of type 1 collagen membranes in association with synthetic nanohydroxyapatite grafts. This study used 32 adult male Wistar rats weighing 250 g at 12 weeks of age. Critical defects of 8 mm were made in the temporoparietal region. The rats were divided into 5 groups of 8 rats each: sham group (only bone defects were made without being filled with biomaterial); Bio-Oss Group - the defect was filled with xenogeneic hydroxyapatite (Bio-Oss® - Geistlich); Bio-Oss + Membrane - the defect was filled with xenogeneic hydroxyapatite (Bio-Oss® - Geistlich) associated with collagen membrane (Mucrograft® - Geistlich); Blue-Bone® Group - the defect was filled with synthetic hydroxyapatite (Blue-Bone® - Regener); and Blue-Bone® + Green® Membrane Group - the defect was filled with synthetic hydroxyapatite (Blue-Bone® - Regener) associated with the type 1 collagen membrane (Green® Membrane Perio - Regener). The immunohistomic analysis showed that type 1 collagen membrane did not have contaminants. The ultrastructure analysis showed the type 1 collagen membrane has thin collagen fibers. The histology results showed it was possible through Goldner's Trichrome to measure the amount of collagen in each group analyzed. In conclusion, the type 1 collagen membrane, called Green Membrane Perio, has fibers organically organized, can be indicated for tissue regeneration, and the application is considered safe and biocompatible.

Keywords: biomaterials; membrane; biocompatibility; bone; collagen type 1

Introduction

Tissue regeneration using membrane barriers and bone grafts in maxillofacial surgery has been used for many years. In these surgeries, grafts are used in conjunction with membrane barriers. Barriers are often used to maintain the stability of the grafts, mainly when biomaterial granules are used. With this procedure, the success rate increases, and the formation of new bone improves. The collagen membranes act as a barrier against aggressive agents or assist in bone reconstruction.

However, research in this area is essential for better developing new materials and improving regenerative processes, [1].

During the development of new biomaterials, several complementary tests are necessary to evaluate the product's performance. Among the tests, the immunohistochemistry test confirms the material's biocompatibility, [2]. Ultrastructure analysis using SEM and TEM provides extremely reliable images. Several characteristics of collagen can be identified, such as the layers' shape and arrangements of the collagen fibers. Based on electron microscope images, it is possible to estimate the performance of the biomaterial, the organic structures, and the presence of foreign bodies on the surface, [3–5].

Histology is also an excellent tool for diagnosing specific structures. Through it, it is possible to score the perception of the matrix, different cells, proteins, and structures. Collagen is used for various types of regeneration, including bone and tissue. Collagen is used in different stages of any regeneration, [6,7].

The homogeneous roughness of the collagen membrane is one of the main characteristics that it must present. Irregularities on the surface generate points of weakness. The irregularities modify the adhered cells' shape and the extracellular matrix's structure. These changes modify the reabsorption time of the membrane and do not fulfill its function. These changes impair the process of regeneration involved in the tissues, [8–11].

This study evaluated the performance of collagen membranes used in maxillofacial surgeries. The membranes were used to maintain the mechanical stability of grafts placed in rats' temporal cavities. The tests determined the percentage of collagen in the subepithelial region of the rat calvaria. Several analyses were used, including SEM, TEM, histology, roughness, and immunohistochemistry.

Materials and Methodology

Animal Groups

In the present work, an animal model was used. The experimental protocol was approved by the local Animal Ethics Committee (nº 001/2019). Thirty-two adult male Wistar rats were used, with a body weight of 35 g, aged 12 weeks, provided by Roberto Alcântara from the Biology Institute of the State University of Rio de Janeiro. The animals were kept in cages with ad libitum access to food and water. The light/dark cycle (lights on at 7:00 a.m. and off at 7:00 p.m.), and temperature (22 °C) were kept constant.

Surgery

Rats were anesthetized with Ketamine and Xylazine intraperitoneally. Trichotomy was initiated (A), then a triangular incision was made (BC), and critical bone defects of 8 mm in diameter were created in the temporoparietal region using a trephine and the membrane

The critical defects were filled with bone substitute particles. Two bone substitutes were used: Geistlich Bio-Oss® and Blue-Bone®. The Geistlich Bio-Oss® is a xenogenic bone substitute used in regenerative dentistry. The Blue-Bone® is a synthetic hydroxyapatite made by Regener Co, Brazil.

The rats were distributed into five groups (n=8). Only bone defects were created in the Sham group without placing any biomaterial. In the four groups, the defects were filled with bone substitute using or not a membrane, as follows:

- Sham group: only bone defects were made without being filled with biomaterial
- Bio-Oss Group: the critical defects were filled with hydroxyapatite xenogeneic biomaterial (Bio-Oss® - Geistlich)
- Bio-Oss® + Mucograft® Membrane Group (Geistlich): the critical defects were filled with xenogeneic hydroxyapatite (Bio-Oss® - Geistlich), and collagen membrane (Mucograft® - Geistlich).

- Blue-Bone Group: the critical defects were filled with synthetic hydroxyapatite (Blue-Bone® - Regener)
- Blue-Bone + Green Membrane Perio® Group Perio: The critical defects were filled with synthetic hydroxyapatite (Blue-Bone® - Regener Co), and a collagen membrane (Green Membrane Perio® - Regener Co).

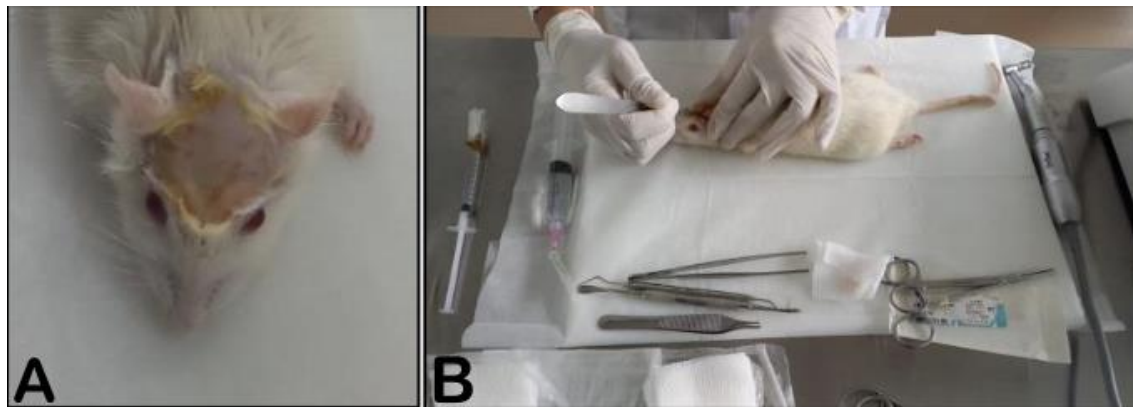


Figure 1. Surgery procedure. Detail of the trichotomy (A) and the beginning of the incision in the rat's calvaria (B).

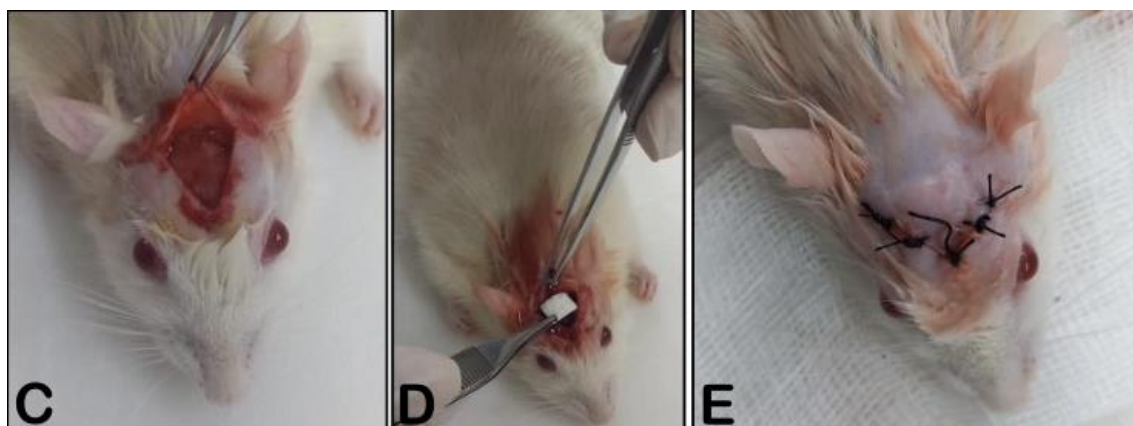


Figure 2. Surgery procedure. Start of the triangular incision (C), placement of the membrane (D), and suture (E).

Euthanasia

Twelve weeks after surgery, the animals were sacrificed with ketamine hydrochloride/xylazine solution (1/1, 0.3 mg/kg, ip) under anesthesia. The animals were decapitated, and the heads were used in the histological procedures (Figure 3).

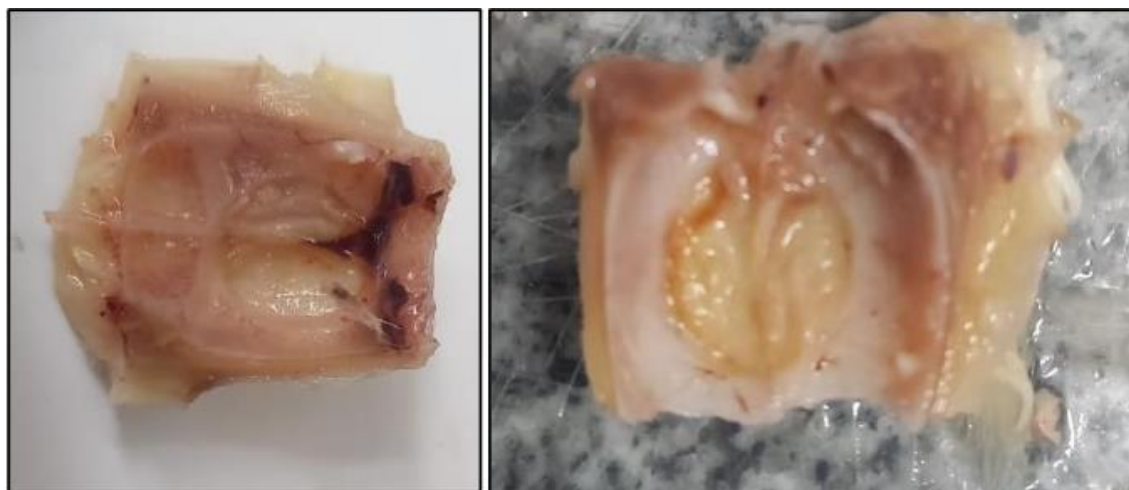


Figure 3. Animal after euthanasia. Two sections of the parietal region of the calvaria prepared for analysis.

Morphological Analysis Protocol

Animal heads were cut and decalcified in EDTA (7.0%) in phosphate-buffered saline (PBS) (0.1 M, pH 7.4) for 40 days. Specimens were washed in distilled water, dehydrated in alcohol (70, 95, 100%), cleared in dimethyl benzene, and embedded in ParaplastTM (Sigma—Aldrich, St. Louis, MO, USA) at 65 °C. Serial 7 µm sections were cut with a microtome (LEICA, Nussloch, Germany) and collected on silanized slides.

Goldner's Trichrome Staining Protocol

The slides were deparaffinized and then rehydrated with alcohol (100, 95, 70%), rinsed in distilled water, and immersed in Weigert's iron hematoxylin (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes. After rinsing in distilled water, the slides were immersed in Biebrich's fuchsin solution (Sigma-Aldrich) for 15 minutes, rinsed again in distilled water, and differentiated in phosphomolybdovanic acid solution for 10 minutes. Then, the slides were immersed in an aniline blue solution for 5 minutes, rinsed with distilled water, dehydrated, and covered with coverslips.

Image Acquisition and Histomorphometry

Three randomized Goldner trichrome, PAS-stained, and immune-labeled slides were photographed with an optical microscope (Carl Zeiss-JVC TK-1270 color video camera, Oberkochen, Germany) at 400 magnification. Images were quantified using GraphPad Prism Version 8.0 (GraphPad, San Diego, CA, USA).

Green-stained areas in Goldner trichrome representing newly formed collagen type 1 (Figure 4).

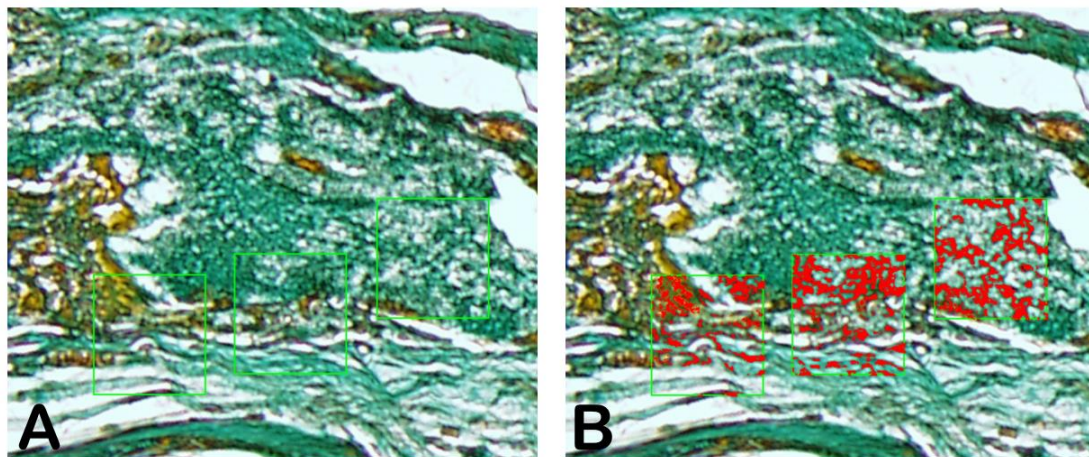


Figure 4. Histomorphometric analysis performed on a Goldner trichrome stained slide. Representative scheme performed by GraphPad Prism Version 8.0 (GraphPad, San Diego, CA, USA), the areas of delimitation chosen (A), to measure the percentage of green coloration representing newly formed collagen type 1 (B).

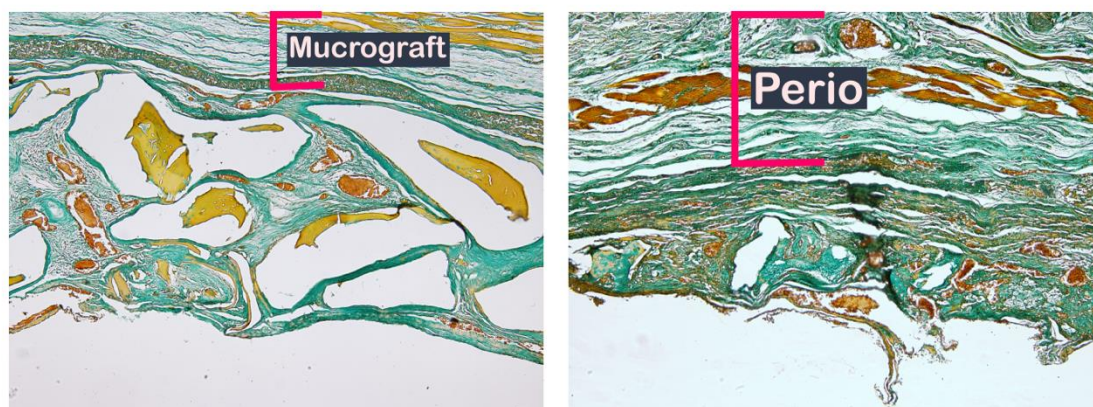


Figure 5. Photomicrograph of an animal calvaria sample 8 weeks after surgery. The mucrograft Area and Perio Area.

Histomorphometric analysis was performed on a Goldner trichrome-stained slide. In the representative scheme performed by GraphPad Prism Version 8.0 (GraphPad, San Diego, CA, USA), we can observe the areas of delimitation chosen (A) to measure the percentage of green coloration representing newly formed collagen type 1 (B).

Statistical Analysis

Data were analyzed using one-way ANOVA followed by a Wilcoxon Matched-Pairs test ($p < 0.05$). All analyzes were conducted with specific software (GraphPad Prism Version 8.0 and BioEstat 5.0).

Figure: Photomicrograph of an animal calvaria sample 8 weeks after surgery; 1–25× magnification: Mucrograft Area and Perio Area.

Scanning Electron Microscopy

Transmission Electron Microscope

Thin membrane sections were analyzed using a JEOL JEM-1011 transmission electron microscope (JEOL, Ltd., Akishima, Tokyo, Japan), operating at 60 kV. Digital micrographs were

captured using an ORIUS CCD digital camera (Gatan, Inc., Pleasanton, CA, USA) at 8000×, 10,000× and 25,000× magnification.

The preparation of the samples for TEM analysis was as follows:

- (a) fixation in 2.5 wt % glutaraldehyde diluted in 0.1 M cacodylate buffer solution (overnight);
- (b) washing in 3 baths in cacodylate buffer solution (0.1 M), for 15 min each bath;
- (c) dehydration in 30 vol% acetone bath (15 min), 50 vol% acetone, 70 vol% acetone (15 min), 90 vol% acetone (15 min), 100 vol% acetone (15 min), and 100 vol % acetone (15 min);
- (d) infiltration in acetone + epon mixture (2:1) for 2 h; acetone + epon (1:1) for 2 h; acetone + epon mixture (1:2) for 2 h, infiltration in pure Epon (overnight);
- (e) inclusion in Epon and polymerization between 48 and 72 h at 60 °C;
- (f) plate cuts with a thickness of 1 micrometer and staining with toluidine blue;
- (g) cutting with ultramicrotome to obtain 70 nm slides, which were collected on 300 mesh copper grids;
- (h) contrasting of the slides with uranyl acetate (for 20–30 min); and
- (i) TEM observation.

Immunohistochemical Analysis (In Vitro)

For the preparation and analysis of the samples, the sequence of steps was as follows:

- (a) deparaffinized the sections in 3 xylol baths (5 min for each bath);
- (b) hydrate the sections (100%, 90%, 70% ethanol, distilled water - 5 min each bath);
- (c) incubate the sections in 3% hydrogen peroxide diluted in distilled water for 15 min, protected from light to inhibit endogenous peroxidase;
- (d) wash in 3 baths of TBS (Tris Buffered Saline) pH 7.4 buffer (5 min each bath);
- (e) perform antigen retrieval in Tris/EDTA (ethylenediaminetetraacetic acid) buffer pH 9.0 at 95 °C for 20 min;
- (f) cool and wash in 3 baths of TBS buffer pH 7.4 (5 min each bath); and
- (g) block non-specific sites with 3% TBS/BSA for 20 min.

The final steps were: incubate with the primary anti-Collagen I antibody (Santa Cruz), diluted in TBS/BSA at 1% (1:200), overnight, in a refrigerator (4 °C), in a humid chamber; wash in 3 baths of TBS buffer pH 7.4 (5 min each bath); incubate with biotinylated secondary antibody (VECTASTAIN® Universal Quick HRP Kit) for 30 min at room temperature; wash in 3 baths of TBS buffer pH 7.4 (5 min each bath); incubate with streptavidin VECTASTAIN® Universal Quick HRP Kit) for 15 min at room temperature; wash in PBS buffer pH 7.2; develop with DAB (diaminobenzidine); stain with hematoxylin; dehydrate (distilled water, 70%, 90% and 100% ethanol—5 min each bath); and clarify in 3 xylol baths (5 min each bath) and assemble with Entellan.

Results

Figure 6 shows the histomorphometric analysis results using Goldner trichrome staining. Blue-Bone® + Membrane Group (Perio) had a higher content of collagen type 1.

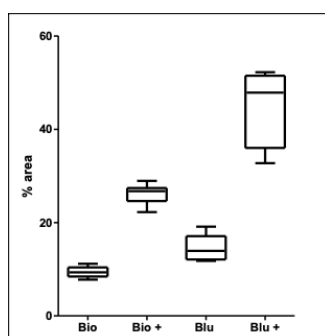


Figure 6. Histomorphometric analysis of Goldner trichrome staining showed higher collagen type 1 content in Group Perio (Blue-Bone® + membrane). A statistical difference was also found between Group Mucrograft (BioOss® + membrane) ($p = 0.0313$).

As results we can observe that the Blue-Bone® + Green Membrane Group (Perio) obtained a significant difference when compared to the other groups, including compared to the Bio-Oss® + Membrane group (Mucograft®).

Table 1 shows the statistical analysis differences among the groups. All groups were statistically different, including those where membranes were used to improve type 1 collagen formation.

Table 1. Statistical analysis results.

Mann Whitney test		
P value	0,0022	P < 0.01
P value summary	**	
Medians significant different (P < 0.05)	Yes	
Column C	Blue	
vs	vs	
Column D	Blue-Bone® + membrane	
Mann Whitney test		
P value	0,0022	P < 0.01
Exact or approximate P value	Exact	
Column B	BioOss® + membrane	
vs	vs	
Column D	Blue-Bone® + membrane	
Mann Whitney test		
P value	0,0022	P < 0.01
Exact or approximate P value	Exact	
Column A	BioOss®	
vs	vs	
Column C	Blue-Bone®	
Mann Whitney test		
P value	0,0022	P < 0.01
Exact or approximate P value	Exact	

The statistical analysis showed differences among the analyzed groups. All groups were significantly relevant, including the groups in which membranes were used to improve the type 1 collagen range.

Transmission Electron Microscopy (TEM)

Transmission electron microscopy reveals the bundles of thin, well-defined collagen fibers characteristic of type 1 collagen.

Figure 7 shows a type 1 collagen membrane. Collagen fiber bundles (arrow) characteristic of type 1 collagen (B) were observed.

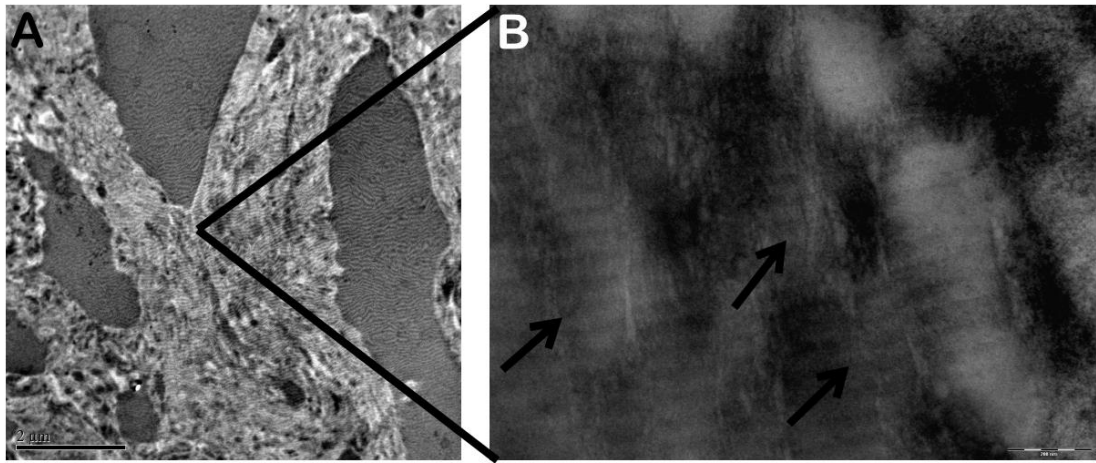


Figure 7. TEM and SEM pictures. Type 1 collagen membrane (A); delimit the bundles of collagen fibers (arrow) characteristic of type 1 collagen (B).

Immunohistochemistry

Figure 8 shows the morphology of type 1 collagen immunohistochemistry analysis. The negative control test showed no stains, certifying the efficiency of the manufacturing process.

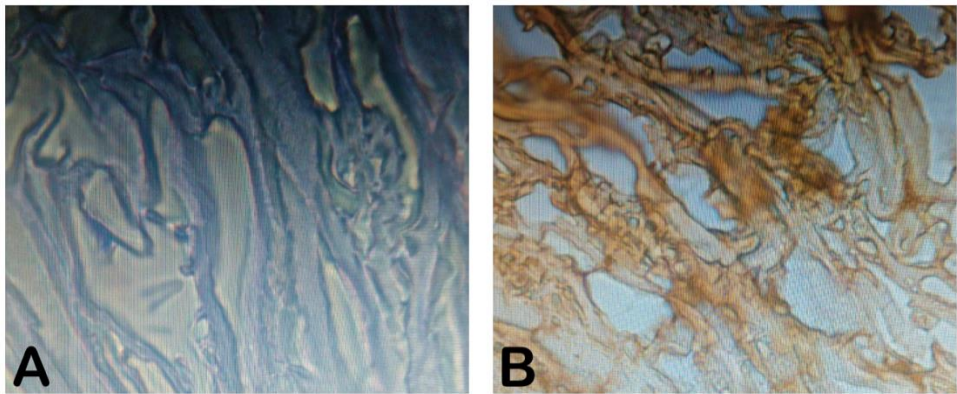


Figure 8. Morphology of type 1 collagen immunohistochemistry (A). The immunostained in shades of brown, proving no other substances were present (B). Scale bar of 100 μm, magnification of 400×.

Scanning Electron Microscopy

Figure 8 shows the surface morphology of the membranes.

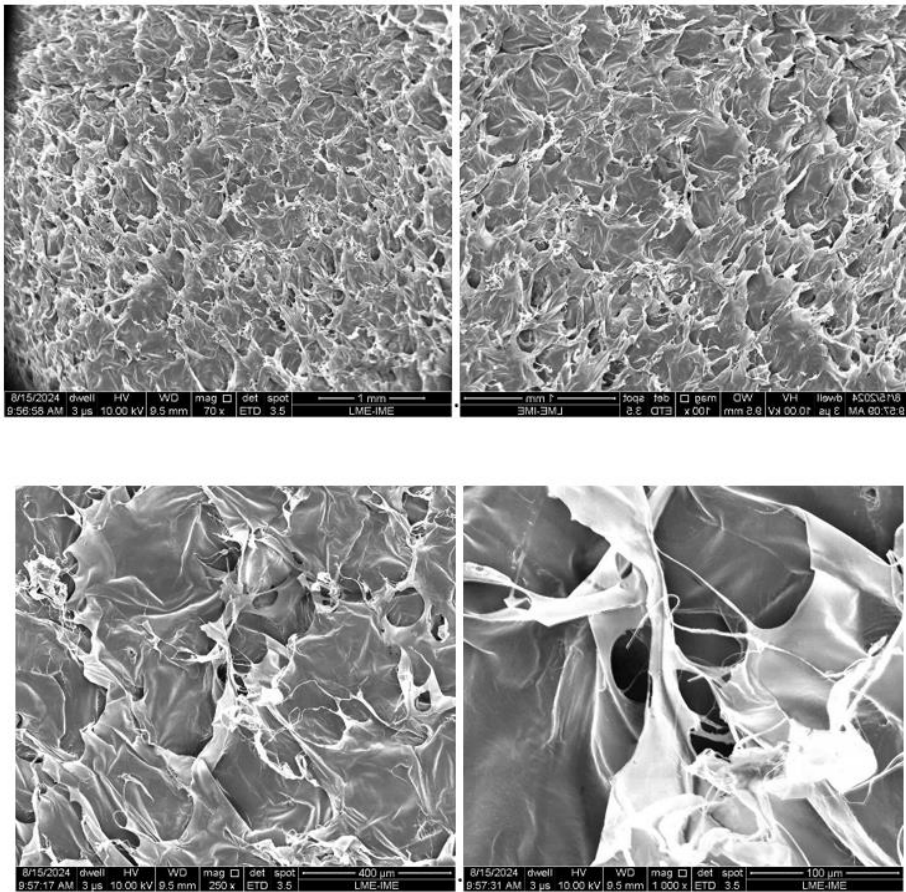


Figure 9. Type 1 collagen Green Membrane Perio®.

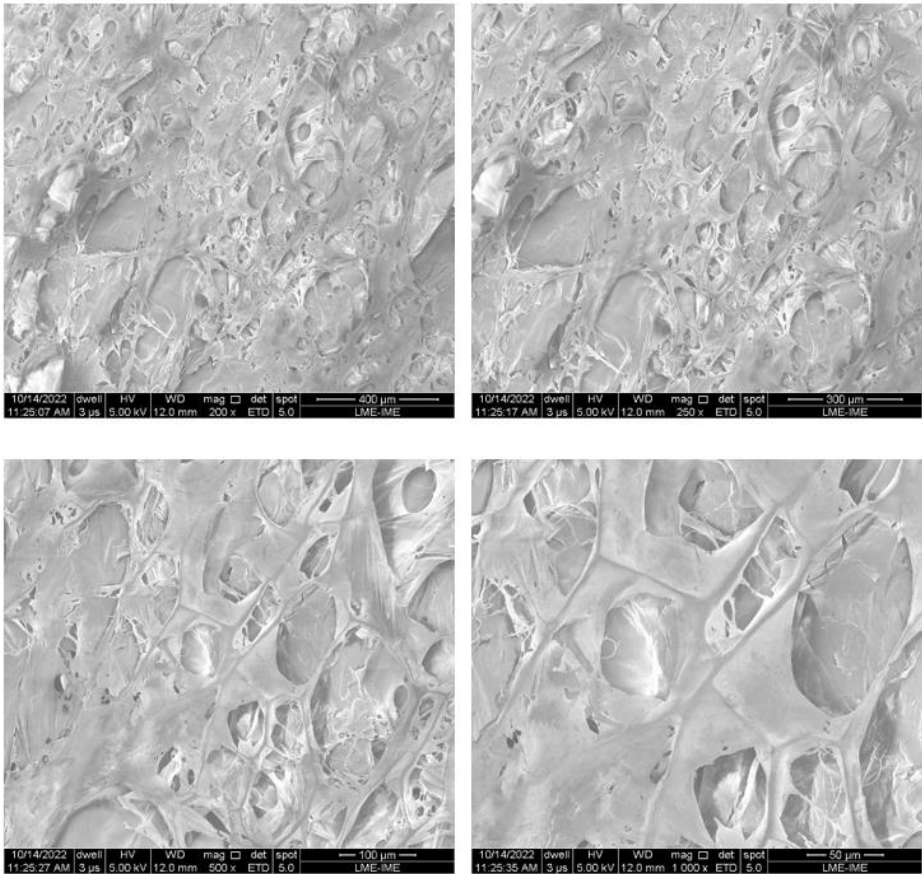


Figure 10. Surface morphology of Mucrograft® - Geistlich collagen membrane.

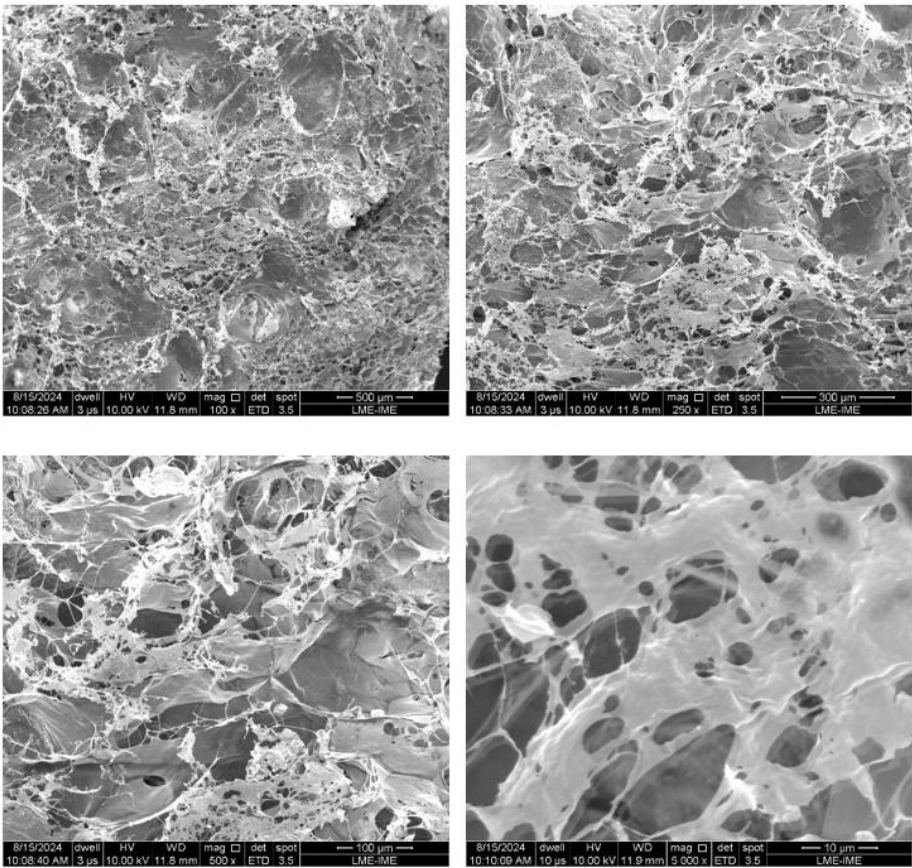


Figure 11. Surface morphology of Green Membrane Perio®.

Roughness

Table 2 shows the measured membrane surface roughness parameters. Figure 12 shows the surface morphologies of tested membranes.

Table 2. Measured membrane surface roughness parameters.

Membrane		Ra (μm)	Rsk (μm)	RMS (μm)	Rku (μm)	PV (μm)	Rpk (μm)	R3z (μm)
Mucograft	Mean	8.273	-1.115	10.72	4.282	97.109	5.53	7.23
	StdDev	1.428	0.191	1.689	0.469	17.824	1.95	14.44
Green	Mean	6.55	-0.237	8.244	3.342	76.746	7.61	63.98
	StdDev	0.939	0.117	1.096	0.472	11.56	1.05	12.94

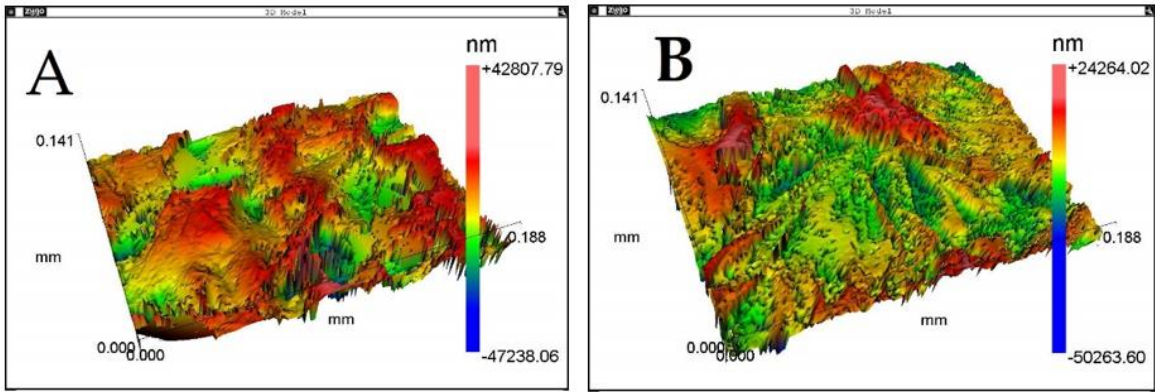


Figure 12. Surface morphologies of tested membranes. (A) Mucograft Geistlick® membrane. (B) Gree membrane®. Interferometry images.

Discussion

Collagen membranes are widely used to improve tissue healing, and one study examined the efficacy of type 1 collagen for the healing of prematurely ruptured fetal membranes. Although injection of PBS into the ruptured fetal membranes resulted in 40% closure, application of type 1 collagen improved closure rates to 90% within 72 h, [12]. These data are interesting because the groups using collagen membranes had a more efficient healing process than those without membranes.

The extracellular matrix has numerous components. Therefore, some tests are essential to characterize these elements. A study used SEM and TEM to determine the pattern of collagen fibers and thus determine how fibroblasts behave to their degradation or deposition. The present work showed that the more similar the pattern of collagen fibers is concerning the extracellular matrix, the greater the stability of the collagen fiber, [13]. Corroborating the results presented, the characteristic fibrillar pattern of collagen was found.

Membrane roughness is crucial for conducting tissue regeneration, mainly because it prevents the rapid growth of soft tissues in bone defects. A study that evaluated the activity of macrophages on the surface of different collagen membranes determined that different topographies did not induce changes in macrophage morphology or release of pro and anti-inflammatory cytokines. This result showed that the effect of surface roughness on macrophage behavior could depend on other factors, [14]. These findings show that the different roughness between the Bio-Oss® + Membrane group (Mucograft®) and Blue-Bone® + Membrane Group (Perio) did not prevent both from producing collagen satisfactorily and that another characteristic is probably crucial for one to stimulate more collagen than the other, as also described by other authors, [15,16].

Histology is one of the main alternatives to determine the percentage of collagen in a sample. Several studies have shown that among the stains procedure, the Goldner's trichrome and Masson's trichrome are the two prominent stains to determine the perception of collagen [17–19]. In a study that analyzed the subcutaneous implantation of a collagen membrane in 25 rats at different times, it was observed through Masson's - Goldner 's staining of the interaction, incorporation, and degradation of the material until the final stage of regeneration, [20]. And we can consider the evaluation in a single stage of time, as a limitation of this study, however the Goldner's trichrome proved to be very efficient for statistical verification between the groups analyzed.

Conclusion

Based on results of the present work it is concluded that:

- a) the membrane type 1 collagen has roughness similar to the extracellular matrix.
- b) the ultrastructural characteristics were consistent with the collagen pattern, certified by immunohistochemistry that in the sample proved to have only the presence of collagen
- c) the histology showed that the Blue-Bone® + Membrane Group (Perio) promoted a higher percentage of collagen than the Bio-Oss® + Membrane group (Mucograft).

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