Research Article

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Properties of a bovine collagen type I membrane for guided bone regeneration applications

<https://doi.org/10.1515/epoly-2021-0021>

received December 12, 2020; accepted January 22, 2021

Abstract: Dental implant treatment requires an available bone volume in the implantation site to ensure the implant's mechanical stability. When the bone volume is insufficient, one must resort to surgical means such as guided bone regeneration (GBR). In GBR surgery, bone grafts and membranes are used. The objective of this work is to manufacture and characterize the *in vitro* and *in vivo* properties of resorbable collagen type I membranes (Green Membrane®) for GBR. Membrane surface morphology was characterized by SEM and roughness was measured using an interferometric noncontact 3D system. *In vivo* skin sensitization and toxicity tests have been performed on Wistar rats. Bone defects were prepared in 24 adult male rats, filled with biomaterials (Blue Bone® and Bio Oss®) and covered with collagen membranes to maintain the mechanical stability of the site for bone regeneration. The incisions were closed with simple stitches; and 60 days after the surgery, the animals were euthanized. Results showed that the analyzed membrane

was homogeneous, with collagen fiber webs and open pores. It had no sign of cytotoxicity and the cells at the insertion site showed no bone morphological changes. There was no tissue reaction and no statistical difference between Blue Bone® and Bio Oss® groups. The proposed membrane has no cytotoxicity and displays a biocompatibility profile that makes it suitable for GBR.

Keywords: membrane, collagen, bone regeneration, biomaterials

1 Introduction

In dentistry, membranes are used for guided bone regeneration (GBR) and guided tissue regeneration (GTR). The purpose of GBR is to restore large bone defects in orthopedic and maxillofacial surgery. In these surgery procedures, membranes play an important role. The membrane used together with a bone graft provides mechanical stability to a biomaterial inside the defect site. The membrane prevents epithelial and connective cells from invading the site during the postsurgical wound healing phase while allowing periodontal cells to selectively migrate into the defect (1,2).

The success of GBR depends on the mechanisms involved in the proliferation and differentiation of mesenchymal cells (MSCs) in the surgery site. When the interactions of proteins with the biomaterial are rapid and the differentiation of cells into fibroblasts or preodontoblasts occurs in large numbers, cell differentiation occurs in immature odontoblasts and finally in mature odontoblasts in less time (3). The main function of odontoblasts is to produce an extracellular matrix, which at the beginning of the reactions is formed by collagen type I. Collagen type I has the function of improving cell growth and migration (4). The use of collagen type I membranes in GBR helps to create the most surgery favorable conditions so that the cells involved in the process can perform their function without unwanted tissue invagination or the action of an external agent that

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impairs the natural progress of the process (5). The type I collagen used in the membrane is formed by a polysaccharide protein, which contains a small amount of galactose and glucose (4). Collagen is the main component that forms the extracellular matrix and allows for better cell growth and migration during healing.

Several natural and synthetic membranes have been commercialized for biomedical applications (6,7), including polytetrafluoroethylene (PTFE), expanded PTFE, natural collagen, freeze-dried fascia lata, freeze-dried dura mater allografts, polylactic acid, polyglycolic acid, polyorthoester, polyurethane, polyhydroxybutyrate, calcium sulfate, titanium mesh, and titanium foils; but natural type-I collagen is the preferred choice (8,9). The membrane helps to create a space for fibroblasts and osteoblasts to remodel the damaged tissues (7). Collagen membranes are preferred due to biocompatibility, the capability of promoting wound healing, and the fact that a second surgery is not necessary to remove it. Fibroblasts are responsible for the synthesis of collagen fibrils that provide physical support for the cellular matrix (10), while osteoblasts, thanks to the barrier created by the collagen membrane, do not have to compete with connective tissues and improve the deposition of bone matrix (8). Regarding clinical results, the use of resorbable collagen membranes in GBR is comparable with nonresorbable membranes (9).

Chia-Lai et al. (11) studied *in vivo* cellular reactions for several types of collagen membranes. The data showed two kinds of cellular reactions that depend on the physicochemical properties and processing techniques. The membranes that induce a physiological reaction using mononuclear cells undergo an integration process and maintain their structure for 60 days. The reaction of collagen-based materials is dominated by mononuclear cells, which lead to their integration into the host tissue (12).

The advantages of collagen membranes over other natural and synthetic materials are the hemostatic functions that allow early wound stabilization; capable of promoting cell attachment and proliferation; capable of attracting fibroblasts, fibroblasts, and osteoblast, which can attach to its surface; capable of integrating with soft tissues; and permeability that facilitates the diffusion of nutrients (12). Another important advantage is that natural resorbable membranes do not require a second surgery for removal. When the collagen membrane is used, the cell activity starts 3–5 days after the surgery (13,14).

Collagen is of more than 20 different types, and they change their structure and composition based on the location and function. Collagen from types I to IV are

the most common in the human body. Type I collagen consists of 90% total collagen and is found in the main connective tissues such as tendons, ligaments, skin, bone, periodontal connective tissue, and cornea. Type II collagen is mainly found in cartilage and intervertebral discs. Type III collagen is found in the cardiovascular system and granulation tissues, and type IV collagen is mostly found in the basal membrane (15).

In the market, a variety of natural membranes are available, which possess different times of resorption, i.e., from 8 to 38 weeks. Each membrane has advantages and disadvantages (9,16,17). The collagen type I membranes can be derived from different sources such as bovine tendon, porcine pericardium, porcine submucosa, equine tendon, and equine pericardium. In the present work, a new bovine tendon membrane was developed. The advantages of collagen membranes are biocompatibility, lack of rigidity, good malleability during surgery, manipulation, and the capacity to absorb blood clots. The purpose of this work was to develop and characterize the *in vitro* and *in vivo* properties and the potential of resorbable collagen type I membranes for GBR procedures.

2 Materials and methods

In the present work, an experimental bovine collagen membrane was prepared for GBR. This membrane is a natural type I collagen made from a bovine tendon.

The membrane biocompatibility was analyzed *in vitro* and *in vivo* tests. Scanning electron microscopy (SEM) and 3D interferometry microscopy analyzed the surface morphology. The surface roughness parameters and wettability were measured. *In vivo* tests have been performed in rats.

2.1 Membrane preparation

The experimental collagen membranes with a long resorption time (60 days) were prepared in the laboratory of Regener Biomateriais (Curitiba, Brazil) based on highly purified collagen type I fibers derived from bovine Achilles tendons. The collagen underwent purification and processing procedures including the use of sodium hydroxide to inactivate pathogens. The purification and processing procedures followed the Brazilian and international standards for handling and supplying animal tissues.

2.2 Surface morphology, roughness, and wettability

The surface structure morphology was characterized by SEM using a Field Emission Gun FEI QUANTA FEG 250[®] (FEI Corporate, Hillsboro, OR, USA).

The membrane surface roughness was investigated using an interferometry noncontact 3D surface measurement system (New View 7100 Profilometer; Zygo Co., Middlefield, CT, USA). The parameters for numerically characterized roughness were the arithmetic mean of the absolute values of roughness (R_a), the peak-to-valley roughness (R_z), the mean of the third maximum peak-to-valley height (R_{3z}), the root square value of average roughness (R_q), the highest peak to valley (PV), root mean square (R_{ms}), area above (A_a), and area below (A_b).

The surface wettability was determined by measuring the contact angle with a goniometer First Ten Angstroms model FTA-100 (First Ten Angstroms Co., Portsmouth, VA, USA). The contact angles were determined by averaging the values obtained at five different areas on the two sample surfaces using distilled water.

2.3 *In vitro* cytotoxicity testing

NCTC Clone 929 cell lines and mouse connective tissue cells (ATCC CCL 1), at a concentration of 3.0 to 105 cells/mL, were seeded in Petri dishes. Membrane fragments (10 × 10 mm) were incubated for 48 h at 37°C in a humidified incubator with an atmosphere of 5% CO₂ to form a cell monolayer. The liquid culture medium was replaced with a solid covering medium composed of equal parts of concentrate and agar at 1.8% with 0.01% neutral red.

Membrane fragments (10 × 10 mm) were placed on this covering medium before its complete solidification. The plates were stored at 37°C in an atmosphere of 5% CO₂ for 24 h. For the negative control, nontoxic filter paper discs with 5 mm in diameter were used. As a

positive control, Biocure[®] (Pele; Nova Biotecnologia, Co., Brazil) latex fragment with 5 × 5 mm of proven toxic nature was used. The samples were tested in triplicate on separate plates. The plates were analyzed microscopically for cell integrity and macroscopically for the presence of a halo.

The cytotoxicity was measured by the diameter of the light halo, which is classified according to the reactivity scale shown in Table 1.

2.4 Tests for irritation and skin sensitization

Intracutaneous tests on rats were carried out to evaluate the irritation and skin sensitization of the membrane. Two incisions were made in the skin of each animal's calvaria. The skin was raised and sutured. The first incision was used to introduce a sample of the membrane under the skin. The second incision was left as a control. The degree of irritation and skin sensitization are quantified by the index shown in Table 2.

The biocompatibility classification was based on a comparison of the observed erythema, edema, and necrosis effects with those of the control incision. The tissue reaction index on the side with the membrane was subtracted from the reaction index on the control side. Table 2 shows the tissue reaction index classification.

2.5 Tissue reaction *in vivo* testing

The *in vivo* study was carried out at the Instituto de Biologia Roberto Alcântara of Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil. Twelve male adult Wistar rats weighing approximately 350 g were selected. The research was approved by the ethics committee (Process 001/2019). The rats were trichotomized and underwent a triangular incision in the calvaria

Table 1: Cytotoxicity classification

Description of the reactivity zone	Cytotoxicity	Classification
No detectable zones around or under the sample	None	0
Some malformed or degenerate cells under the sample	Light	1
Zone limited to the area under the sample	Mild	2
Zone extends from 0.5 to 1.0 cm beyond the sample	Moderate	3
Zone extends more than 1.0 cm beyond the sample	Strong	4

Source: BS EN ISO 10993-10 – Biological evaluation of medical devices.

Table 2: Tissue reaction index

Reaction	Classification
Formation of erythema and bedsores	
Without erythema	0
Very mild erythema	1
Well-defined erythema	2
Moderate erythema	3
Severe erythema	4
Edema formation	
Without edema	0
Very mild edema	1
Well-defined edema	2
Moderate edema	3
Severe edema	4
Maximum possible irritation score	
Other adverse changes must be recorded and reported	

Source: BS EN ISO 10993-10 – Biological evaluation of medical devices. Tests for irritation and skin sensitization.

region (Figure 1a). Two surgical sites were prepared in each animal using a sterilized punch (cutting edge Ø 3 mm). The bone fragments were carefully removed to avoid damage to the dura mater. In each animal, one surgical site was filled with Bio Oss® (Geistlich, Switzerland) and the other was filled with Blue Bone® (Regener, Brazil). After the biomaterial was inserted in the bone defect (Figure 1b and c), a membrane was placed in the region (Figure 1d) to cover the entire surgical wound. The animal skin was carefully placed and a suture was made with 5.0 silk to close the surgical region (Figure 1e). A control group with 12 animals was subjected to the same procedure but without placing the membrane over the biomaterial.

The rats were placed in separate cages after surgery, and an analgesic was administered (sodium dipyrone, 0.1 mL/100 g orally) for 7 consecutive days. After 60 days, the animals were euthanized according to the animal welfare protocol. The collected fragments were conditioned

in formalin 10% and sent for histological and ultrastructural analyses.

The authors confirm that they have complied with the World Medical Association Declaration of Helsinki regarding the ethical conduct of research involving animals.

After euthanasia, the animal heads were decalcified using 7% EDTA and 0.1 M phosphate buffer (pH 7.4) for approximately 40 days. The samples were washed in distilled water, dehydrated with ethanol (70%, 95%, and 100%), clarified with dimethylbenzene, and embedded in paraffin blocks (Paraplast®) at 65°C.

Ten serial coronal sections of 7 µm thick were made using a microtome (LEICA Co., Ltd., Nussloch, Germany). The sections were mounted on glass slides and subjected to the hematoxylin–eosin (HE) staining technique and Masson’s trichrome staining. The images were obtained with 25× and 40× magnifications using a Carl Zeiss Axiolab optical microscope.

To examine the surface morphology, the membrane samples were cut into 8 × 8 mm pieces, mounted on a support with the aid of adhesive tape, and coated with gold using a sputtering coating machine.

2.6 In vivo intracutaneous biological toxicity/reactivity testing

The intracutaneous biological toxicity/reactivity test aims to determine the biological response of substances inserted through intracutaneous injections. This test was conducted to study the possible harmful effects of the test substance on the calvaria of animals.

Samples of the membrane with a concentration of 0.2 g/mL of polar extraction solution (0.9% saline solution) were used. The samples were placed in sterile borosilicate containers with a capacity of 25 mL and heated in an autoclave at 121°C for 1 h. The same process was

Figure 1: In vivo testing: (a) triangular incision in the calvaria region; (b) bone defect prepared; (c) insertion of biomaterial; (d) collagen membrane in the subcutaneous region; (e) suture with 5.0 silk to close the surgical region.

performed for controls containing only the polar solution. Three female rats were used for each test group.

The animals were kept individually in galvanized steel cages and acclimated to the conditions of the laboratory for at least 5 days before the test. The diet consisted of commercial feed and filtered water, both provided at will.

Subjects and controls were inoculated with 0.2 mL by intracutaneous injection at 5 points on one side of the spine. The animals were kept for 72 h after application and observed for the presence of erythema, edema, and necrosis as well as other changes.

During this period, the environmental conditions were the following: ventilation with 10–15 air changes per hour, temperature between 19°C and 23°C, relative humidity between 30% and 70%, controlled illumination with 12 h of light and 12 h of darkness, the light intensity between 150 and 325 lux, and noise between 50 and 70 dB.

2.7 Number of osteocytes in the regenerated defect

Stereological analysis was used to determine the number of osteocytes. Samples were analyzed using a microscope (Carl Zeiss – JVC TK-1270) with a color video camera and 400× magnification. In this analysis, a stereological grid composed of cycloid segments was placed over the image of the defect after healing. All points that the grid cut the osteocytes were counted. The osteocytes were quantified using Image-Pro Plus for Window, version 7.0.1 (Media Cybernetics).

The number of osteocytes was counted in a semiautomatic way. The researcher decided to define and

identify the number of interceptions of the tissue image with the stereological grid. This procedure was adopted due to the complexity of the image. The double-blind principle was used to circumvent the possible biased assessments. In rehearsals, one person marked the main points in the images and the second person counted. The two people who did the counting did not know the sample groups. In the counts, Buffon's recommendations were observed, a similar procedure has been adopted in previous studies (18–20).

Data on osteocytes in bone defects were analyzed using one-way ANOVA, followed by a Wilcoxon matched-pair test ($p < 0.05$). All analyses were performed by specific software (GraphPad Prism Version 8.0 and BioEstat 5.0).

3 Results

3.1 Membrane surface morphology

Figure 2 shows a scanning electron surface image of the representative membrane structure at increasing magnifications. SEM images showed that the outer membrane surface was homogeneous and not compact (Figure 2), with a tightly woven and fibrous structure.

3.2 Surface morphology and roughness analysis with interferometry

The most characteristic structural morphology of membrane for bone regeneration is the cellular structure.

Figure 2: Samples of the outer surface collagen membrane at different magnifications (from left to right: 500×, 2,500×, and 10,000×). A tightly woven and fibrous morphology is observed. At high magnification, one sees the typical periodic pattern of collagen fibrils.

Typically, collagen membrane morphology characterization is performed by SEM, interferometry, MRI, small-angle XRD, and AFM. In the present work, the membranes were characterized by SEM and interferometry. The membrane characterization using interferometry is not found in the literature. Figure 3 shows a representative membrane surface morphology observed by interferometry. The membrane surface morphology has an irregular shape with valleys and peaks.

Several techniques can be used to measure the material surface roughness, some of which involve contact with the material. In the contact-type instruments, a stylus tip scans the surface of the sample. Direct contact has many disadvantages, among them the fact that the measuring pressure may scratch the surface of a soft sample. Moreover, the stylus cannot measure the roughness properly if the scratch width is smaller than the diameter of the stylus tip. This is the reason a noncontact instrument was used in this work. Another advantage of the laser noncontact equipment is that it provides simultaneous observation of the surface images during a roughness measurement.

Figure 3 shows details of the membrane surface structure; no sample surface damage can be observed due to roughness measurement. Figure 3 shows a surface roughness profile, which is an image like a contact instrument across one direction.

The interferometric analysis showed the 3D structure of the collagen and elastic fibers (Figure 3). It was possible to observe the collagen fibers arranged in stacked layers parallel to the membrane surface. A pattern of curled fibrils with a certain degree of orientation was seen. Some regions displayed a network of long, straight, and uniformly oriented fibrils. Similar morphology was observed by Mostaçõ-Guidolin et al. (21).

The membrane roughness parameters were $R_a = 1.235 \pm 0.125 \mu\text{m}$, $R_z = 2.759 \pm 0.337 \mu\text{m}$, $R_{3z} = 3.065 \pm 0.412 \mu\text{m}$,

Table 3: Cytotoxicity test results

Material	Halo diameter (mm)		
	Sample 1	Sample 2	Sample 3
Membrane	0	0	0
Negative control	0	0	0
Positive control	1.2	1.0	1.1

$PV = 5.246 \pm 0.768 \mu\text{m}$, $R_{ms} = 1.441 \pm 0.114 \mu\text{m}$, area above = $579.49 \pm 68.795 \mu\text{m}^2$, and area below = $121.49 \pm 20.825 \mu\text{m}^2$.

3.3 Surface wettability

Wettability is an important property for resorbable biomaterials. The larger the wettability, the greater are the contact with body fluids and the resorption rate. In the present work, the wettability of the proposed membrane was observed to be very high. A drop of water penetrates quickly into the membrane pores, making it difficult to measure the contact angle. The contact angle of each sample was measured several times and it was found to be smaller than 10° . This result shows that the membrane surface is very hydrophilic.

3.4 In vitro cytotoxicity results

Table 3 shows the results of the cytotoxicity tests. The membrane showed no signs of cytotoxicity. In the tests, no halos of toxicity were observed around or under the samples (Figure 4). The cells showed characteristics without any morphological changes identical to those of the negative control.

Figure 3: Interferometric image of the proposed collagen membrane, showing 3D bundle fibrils and the roughness surface profile.

Figure 4: *In vitro* cytotoxicity testing results. Samples of negative control (filter paper) are marked *. Samples of the membrane are marked +. The tin samples did not show halos of toxicity. The samples from the positive control group (latex) showed halos of toxicity.

In the positive control, toxicity was revealed by the presence of a clear halo with an average diameter of 1.1 mm (Figure 4). This halo is observed when the red dye that was incorporated in the cells is released following cell death.

The sample had a cytotoxic action index of 0, presenting any effect for the cell line NCTC clone 929.

3.5 Tissue reaction *in vivo* testing results

The membrane irritation and skin sensitization classification were given according to the comparison of the observed erythema, edema, and/or necrosis values with those of the control side. Table 4 shows the results of the intracutaneous tests. The tissue reaction index was null. In the present work, the tissue reaction index (0) on the side with the membrane was subtracted from the irritation index (Table 3) on the control side (0). The result was that the tissue reaction had an index equal to 0 and thus was being considered a nonreactive substance when intracutaneously applied in the proportion of 0.2 g/mL of polar extracting solution (0.9% physiological solution) in rats.

Figure 5 shows the micrography from the control group without a membrane and sample from the region of the bone defect filled with Bio Oss[®] and the membrane covering the region. Figure 5a was from the region above the defect. Figure 5c shows a slice of the defect filled with Blue Bone[®] biomaterial and covered with the proposed

membrane. Cellular action can be observed 60 days after the surgery. The histological sections stained for HE was marked with a square showing the area where the membrane acted during tissue regeneration. The microscopic examination showed newly formed bone in the prepared defects with Masson's trichrome staining (Figure 6). The graft particles were surrounded by newly formed bone and improved bone regeneration. Few osteocytes were observed in the marrow bone.

3.6 Osteocyte counting

Figure 7 shows a representative image cut from the Blue Bone[®] group obtained from stereological analysis for counting the number of osteocytes. Ninety-six cycloids were counted in the. Among the cycloids, six intercept some osteocytes.

A statistical difference was observed in the number of osteocytes between the groups covered with the membrane and without a membrane (Table 5). The control group (without membrane) induced a lower number of osteocytes than the group with membranes. Table 5 shows that using Blue Bone[®] biomaterial in the bone defect, it forms a greater number of osteocytes than that in the side with Bio Oss[®].

Table 5 shows the statistical analysis of the number of infiltrated osteocytes in the new bone inside the bone defect observed 60 days after the surgery.

4 Discussion

The purpose of the present work was to develop and investigate the properties of a collagen type I bovine membrane for GBR and guided tissue regeneration (GTG). Collagen is a component of the bone matrix. It is the most abundant protein in the human body and has a significant influence on the adhesion of fibroblasts. The collagen-based membrane has also been reported to influence the cellular functions of fibroblasts, including cell shape, differentiation, and migration, due to the presence of Arginine-glycine-aspartic acid - Arg-Gly-Asp (RGD) and GFOGER (integrin-specific glycine-phenylalanine-hydroxyproline-glycine-glutamate-arginine) sequences (22). Wound healing, which is a complex and multicellular process that aims to restore the structural and functional integrity of bone tissues, follows GBR and GTG procedures. The body can regenerate and repair itself

Table 4: Intracutaneous test results

Side	Animal 1, 2, and 3		
	Erythema	Edema	Bedsore
Membrane	0	0	0
No membrane	0	0	0

Figure 5: Photomicrographs of slides stained with hematoxylin and eosin (HE). (left side picture) Sample cut from the region above the defect without membrane. (middle picture) Samples cut from the defect filled with Bio Oss® biomaterial and covered with an experimental membrane. Sixty days after the surgery. (*) membrane. (right side picture) Samples cut from the defect filled with Blue Bone® biomaterial and covered with an experimental membrane. Sixty days after the surgery. (*) membrane. NB = native bone; Bio = biomaterial; AR = artery. Scale bar = 100 μm, 25× magnification.

Figure 6: Enhanced photomicrograph of slides stained with Masson's trichrome. (left side picture) Removed from the defect filled with Blue Bone® biomaterial and covered with the membrane. Many blood vessels can be observed. (right side picture) Removed from the defect filled with Bio Oss® biomaterial and covered with the membrane. Sixty days after surgery. Blue shows the mineralized new bone. Scale bar = 100 μm, 40× magnification.

Figure 7: Representative images from the stereological analysis. Ninety-six cycloids were counted from the image and only six intercepted some osteocytes (scale bar = 15 μm, 40× magnification). Image cut from the Blue Bone® group.

but only in the case of relatively small defects. Spontaneous bone regeneration involves the migration, proliferation, and differentiation of several kinds of cells, including osteoblasts, fibroblasts, macrophages, and platelets. The process starts with an inflammatory response to the injury and ends with the growth of new bone and

Table 5: Statistical analysis of osteocytes numbers in new bone

	No membrane		With membrane	
	Bio Oss®	Blue Bone®	Bio Oss®	Blue Bone®
Mean	0.94	1.45	2.68	3.99
SD	0.27	0.42	0.77	1.15
P values	P = 0.0001			

SD, standard deviation.

soft tissues. Large bone defects can only be repaired with the help of biomaterials such as scaffolds, graft, and membranes (23,24).

Type I collagen membranes are used in GBR surgeries to guarantee the maintenance of the fully elaborated human intestinal epithelium (25). It is also used in normal corneal endothelial cells producing mainly type I collagen from the basement membrane (26). It helps in the regeneration of the basement membrane of the liver (27), among other indications (28,29). All applications adopt procedures similar to the present study.

Maurer *et al.* characterized four naturally porcine membranes. Their membranes displayed a smooth, compact, and irregularly crumpled surface morphology (30). They observed a finely formed collagen fibrils, loosely arranged, and undulating collagen bundles. The surface showed a single fibril or small bundles interconnecting larger bundles. In some area, a compact collagen arrangement was observed, which was interrupted by clusters of circular discontinuities. Their analyzed membrane surface morphologies were different than the present work showed in Figure 2. The difference is possibly due to the manufacturing membrane processing.

In the present work, the analyzed collagen type I membrane showed a porous and open-cell structure (Figure 2). This surface morphology is important to satisfy critical criteria for membrane, including osteoconductive. Open pores are essential for bone healing and regeneration by allowing adhesion, attachment, migration, a proliferation of protein, and cells. The fibril bundles are interconnected and form larger bundles. Some fibrils showed helical grooves, which are indicative of twisted microfibrils. This mesh is permeable to the macromolecules necessary for providing nutrition for tissue repair of the underlying membrane and is a very retentive surface. Similar morphology was observed by Zenóbio *et al.* (31).

SEM results of the present work (Figure 2) show that the membrane has a three-dimensional structure made of collagen fibrils forming bundles in random orientation. There are no data in the literature about the influence of the surface morphology of a collagen membrane on cell behavior such as differentiation, migration, proliferation, and gene expression. Li *et al.* studied the effects of microgrooved collagen membrane on the behavior of mesenchymal stem cells (32). They observed that microgrooves such as the ones observed in this work (Figures 2 and 3) have a significant effect on the morphology, alignment, and collagen synthesis of the cells.

The membrane roughness ($R_a = 1.235 \mu\text{m}$) and the contact angle (10°) of the membrane explain the good *in vivo* test results. The membrane surface wettability, energy, and roughness are important parameters that influence its performance during bone regeneration. Open porous, rough and chemically activated surfaces provide ideal conditions for direct protein adsorption and facilitate the adsorption of fibronectin and albumin due to modifications in their ionic state. These mechanisms are important for bone regeneration. The basic premise is that the driving force for protein adsorption is the free energy of the membrane, which is higher if the surface is rough (33).

The results of the present work show that the contact angle of this bovine collagen type I membrane is very low and the surface energy is high. This is consistent with the histological results; materials with a high wettability and high surface energy have greater cellular activity (34).

The histological results of the present study corroborate studies in the literature (35). Collagen membranes provide organized cell growth, in addition to presenting the potential for the production of tissue substitutes.

The results of cytotoxicity tests showed that the developed type I collagen membrane is a nontoxic material. This result indicates that this kind of membrane, when used for wound covering, helps tissue regeneration. The membrane can also improve the healing of burns and ulcers (36). In the cytotoxicity tests, no inflammatory reactions were found, showing that the material is nontoxic and does not promote an inflammatory response.

Borges *et al.* carried out a study of the use of collagen membranes with and nonresorbable membranes in the same surgical procedure in the femur of Wistar rats (37). The results showed that the simultaneous use of collagen membranes and nonresorbable membranes in GBR procedures do not improve the bone quality of the rat femur. Tanaskovic *et al.* analyzed the effectiveness of the simultaneous use of collagen membranes and titanium mesh nonresorbable membranes and compared them with the separate use of membranes (38). The results showed that only using the nonresorbable membrane promotes an intense inflammatory reaction in the host tissues, which can cause fibrosis. When the nonresorbable membrane was covered with a collagen membrane, the inflammatory response reduced (39). The best result was obtained only using the collagen membrane.

Toledano *et al.* analyzed the degradation of three types of non-cross-linked resorbable membranes (40). They concluded that some membranes start to degrade in the first 8 h and this early degradation decreases GBR. Fadel *et al.* studied the influence of using collagen membrane in the regeneration of defects in the calvaria of Wistar rats (41). They concluded that defects that were covered with resorbable collagen membranes showed better bone regeneration than defects without a membrane. The present work corroborates the results of the literature (39–42).

Previous work compared the performance of non-cross-linked type I collagen membranes with the nonabsorbable PTFE membrane (43). It has been observed that membranes of non-cross-linked collagens promote a high rate of vascularization 60 days after the surgery. This result was also observed in the in histological analyses of the present study.

The *in vivo* testing shows that the defects that were covered with a membrane had a larger number of blood vessels (Figure 6) and osteocytes (Figures 6 and 7, Table 5) than the defects without a membrane. Similar results were obtained by other researchers who observed that the use of resorbable membrane improves bone repair (44,45).

5 Conclusions

The results of this work showed that:

- (a) The structure of the developed collagen type I membrane exhibited wide cross-linked fibrils of various thicknesses forming collagen bundles.
- (b) The fibrils have various thicknesses arranged in several directions and forming highly interconnected cross-links.
- (c) The proposed membrane does not have cytotoxicity and does not promote inflammatory reactions.
- (d) The use of the developed membrane to keep the graft stable significantly increases the number of osteocytes during bone regeneration.
- (e) The developed membrane morphology is adequate for GBR surgery and has characteristics suitable for use in GBR.

Research funding: The authors thank the Brazilian Agencies CNPq and FAPERJ for the financial support, and Regener Co for using their facilities.

Author contributions: Igor S. Brum was involved in experimental testing and original draft preparation; Carlos N. Elias was in charge of conceptualization, the surface structure morphology analysis, roughness measurements, results analysis, and writing original draft preparation; Jorge J. de Carvalho contributed to the intracutaneous biological toxicity/reactivity test; Jorge L. S. Pires was involved *in vivo* testing; Mario J. S. Pereira performed the *in vivo* study and cytotoxicity testing; and Ronaldo S. de Biasi contributed to revision and concepts.

Conflict of interest: Authors state no conflict of interest and declare any personal circumstances or interests that may be perceived as inappropriately influencing the representation or interpretation of reported research results.

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