J Stomatol Oral Maxillofac Surg 124 (2023) 101607

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Original Article

Surface topography of resorbable porcine collagen membranes, and their effect on early osteogenesis: An *in vitro* study

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ARTICLE INFO

Article History:
Received 20 July 2023
Accepted 12 August 2023
Available online 13 August 2023

Keywords:
Bone regeneration
Guided tissue regeneration
Collagen membranes

ABSTRACT

Objective: Guided tissue regeneration (GTR) is based on the use of different membranes that function as sealants and barriers in specific clinical situations. Among the several tissue production methods and origins, resorbable porcine-derived membranes are the most commonly used. Because these membranes are so diverse, and have several different clinical applications, doubts linger as to their effect in stimulating osteogenesis. The objective of this study was to make an in vitro evaluation of the viability and differentiation of osteoblastic cells cultured on the surface of the following collagen membranes: Jason® (Botiss Biomaterials), Collprotect® (Botiss Biomaterials), and Bio-Gide® (Geistlich).
Material and methods: Fragments of the 3 resorbable collagen membranes (5 × 5 mm) were used, and pre-osteoblastic SAOS-2 cells (ATCC, USA) were plated on their porous surfaces. Evaluation of the membranes was performed at 3, 5 and 7 days, considering the following parameters: (1) topographic analysis of the different surfaces by scanning electron microscope; (2) cellular viability by MTT, (3) quantification of type I collagen and osteopontin by Elisa. The quantitative analyses were carried out using a significance level of 5%.
Results: Collprotect® and Jason® membranes presented a rough surface with an irregular aspect on both sides, while double-layered Bio-Gide® had one layer with a smooth surface and the other with a rough surface along each respective length. The viability assays revealed that the cells cultured the cells grown on Collprotect® showed higher viability than those grown in Bio-Gide® or Jason®, especially after 5 and 7 days. After 3 and 5 days, evaluation of type I collagen showed that the cells plated on the Jason® and Collprotect® surfaces had greater collagen secretion than those plated on BioGide®. After 7 days, an increase in osteopontin levels was observed when the cells were plated on all the experimental membranes, compared with the control group.
Conclusion: All the tested membranes were suitable for use in GTR clinical procedures. Their indication in specific regenerative cases depends on the mechanical and biological properties of their originating tissues, thus enabling better results and assertive choices by dental professionals.

1. Introduction

Guided tissue regeneration (GTR) treats intraosseous periodontal defects with a combination of membranes and biological barriers to produce clinically significant effects on the clinical attachment level and probing depth, hence improving periodontal treatment prognosis and effectiveness [1]. Membranes help stabilize blood clots, activate primary intention healing, isolate the defect in relation to gingival soft tissues, and preserve space [2], thereby preventing epithelial and connective tissues from invading and growing into bone defects [3]. Membranes with many layers of nanofibers have recently been developed to encourage bioactivity and cellular, tissue, and vascular stimulation, and also support collagen matrices that can be used as soft tissue graft alternatives for periodontal plastic surgery [4].

Collagen has several significant properties, including biocompatibility and biodegradability [5]. Another is hemostasis, whereby tissue healing is promoted by induced chemotaxis of cells such as fibroblasts [6]. Human and porcine collagen matrices have been developed as successful alternatives for standard conjunctival grafting [4]. The porous structure of nanofibrous membrane plays a significant role in the osteoconductive properties of the material, enabling

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https://doi.org/10.1016/j.jormas.2023.101607
2468-7855/

osteoblast adhesion, proliferation and differentiation, and tissue neoformation [7].

The collagen membranes used in dental procedures are obtained primarily from tendon, dermis, skin, or pericardium, and are mostly derived from bovine or porcine sources [8]. Structural variances among the membranes have been identified, given the numerous sites of possible porcine tissue extraction from the animal. The Jason® membrane is derived from the pericardium, whereas Bio-Gide® is derived from the peritoneum, and Collprotect®, from the periderm.

The Jason® membrane is composed of porcine-derived type I and III collagen, and is produced in a multistage standard cleaning process that removes non-collagenous cells and components, while retaining the original and porous nature of the tridimensional collagen structure. Tolerance studies and clinical trials have demonstrated its biocompatibility [9]. Bio-Gide® is a double-layered membrane derived from pig peritoneum. It has received the most widespread attention, and is regarded as the gold standard in comparison with other materials. It exhibits delayed resorption and aids in periodontal regeneration [10]. Lastly, Collprotect® is a native collagen membrane that is derived from pig dermis, and that retains the inherent hemostatic effect of collagen, which promotes early wound stability and natural healing. It adapts well to the surface, and has good tissue integration, thus functioning well in most situations requiring intermediate stability and ease of handling [11].

Given the multiple sources and clinical applications of collagen membranes, the purpose of this study was to make an in vitro evaluation of the surface topography of different porcine collagen membranes, and assess the effect of these membranes on human osteoblastic cell culture, taking into account cell viability, and secretion of type I collagen and osteopontin, which are important proteins in the early stages of bone neoformation.

2. Material and methods

2.1. Sample groups and cell culture

The study comprised the following three porcine-derived membranes, measuring 30×40 mm each: Bio-Gide® (Geistlich Biomateriais), Collprotect® (Botiss Biomateriais), and Jason® (Botiss Biomateriais). Human osteoblastic cells (Saos-2, HTB-85TM) obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were plated on the porous surface of each membrane after approval by the Ethics Committee of the São Leopoldo Mandic Research Institute, Campinas, Brazil (#2019/0271). The cells were cultured in McCoy's 5A medium (Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (Cultilab®, Campinas SP, Brazil) and 1% antibiotic-antimycotic solution (penicillin-streptomycin) (Sigma, St. Louis, MO, USA). The culture medium was replaced every two days, and culture progression was monitored with an inverted microscope (Nikon Eclipse TS100, Tokyo, Japan). The cells were kept at 37 °C throughout the culture period, in a humidified environment containing 5% CO_2 and 95% humidity.

2.2. Topographic analysis

The ultrastructural morphology of the samples was evaluated using a high-resolution field emission scanning electron microscope (FE-SEM) (Zeiss FEG Auriga and FEI SEM Magellan 400 L), with secondary electrons accelerated to 5 kV in high vacuum. The samples were metallized with a 20 nm gold conductive film and mounted on stubs [12]. Three specimens of each surface were photographed at magnifications ranging from 500 X to 1000 X.

2.3. Cell viability assay

After 24, 48 and 72 h of cell plating, 10 μL of MTT solution (5 mg/mL, Sigma, USA) diluted in McCoy's 5A serum-free medium was added to the cultures, which were then incubated for 3 h at 37 °C. Following incubation, 100 μL of 10% DMSO solution (Dimethylsulfoxide, LGC, São Paulo, SP, Brazil) was added [13]. After the crystals were solubilized, an ELX800 microplate reader (BioTek Epoch Instruments) was used for quantification at 590 nm, and the optical density (OD) measurements were obtained. Cells plated on polystyrene surfaces were used as the control. All the experiments were performed in blind fashion, in biological quadriplicate.

2.4. Enzyme-linked immunosorbent assay (ELISA)

After 3, 5 and 7 days, the type I collagen and osteopontin secreted by the cells plated on the different surfaces were quantified by the enzyme-linked immunosorbent assay (ELISA). The supernatant was aspirated and centrifuged at 5000 g for 15 min at 4 °C, and aliquots of each sample were assayed by means of ELISA to determine the type I collagen and osteopontin levels, according to the manufacturer's recommendations (R&D Systems, Minnesota, MN, USA). The results were calculated using the standard curves created in each assay and measured in a spectrophotometer (BioTek Epoch, Winooski, VT, USA) at a wavelength of 450 nm. All the experiments were performed in blind fashion, in biological triplicate.

2.5. Statistical analysis

Descriptive and exploratory analyses of all the data were performed. Since the data did not meet the assumptions of analysis of variance (ANOVA), generalized linear models were applied considering the main effects and the interaction among them. The analyses were conducted using the R program, with a significance level of 5%.

3. Results

3.1. Surface topography

The ultrastrucutral morphology of the surfaces is shown in Fig. 1. All the surfaces exhibited porous and fibrillar characteristics, which allowed internal cell proliferation upon grafting. Collprotect® (A, B) displayed collagen fibers organized on the surface in a loose, crosslinked mesh configuration. On the other hand, Jason® (C, D) demonstrated a homogeneous crosslinked surface with honeycomb features. Lastly, the topography of Bio-Gide® (E, F) revealed two distinct sides, one with smooth, homogeneous, and non-crosslinked collagen fibers, and the other with crosslinked collagen fibers and homogeneous characteristics.

3.2. Cell viability

Cell viability results for all the groups are shown in Table 1. The cells grown in polystyrene (control group) presented more viability than the other cell groups at all the time periods ($p < 0.05$). As for the membranes evaluated, the cells grown in Collprotect® showed higher viability than those grown in Bio-Gide® or Jason®, especially after 5 and 7 days ($p < 0.05$).

3.3. Quantification of type I collagen and osteopontin

The quantification of the proteins secreted by the osteoblasts plated on the membranes is depicted in Table 2. At 3 days, the cells plated on Jason® showed greater secretion of type I collagen than those plated on the other membranes ($p < 0.05$). At 5 days, the cells plated on Collprotect® and Jason® surfaces showed greater secretion

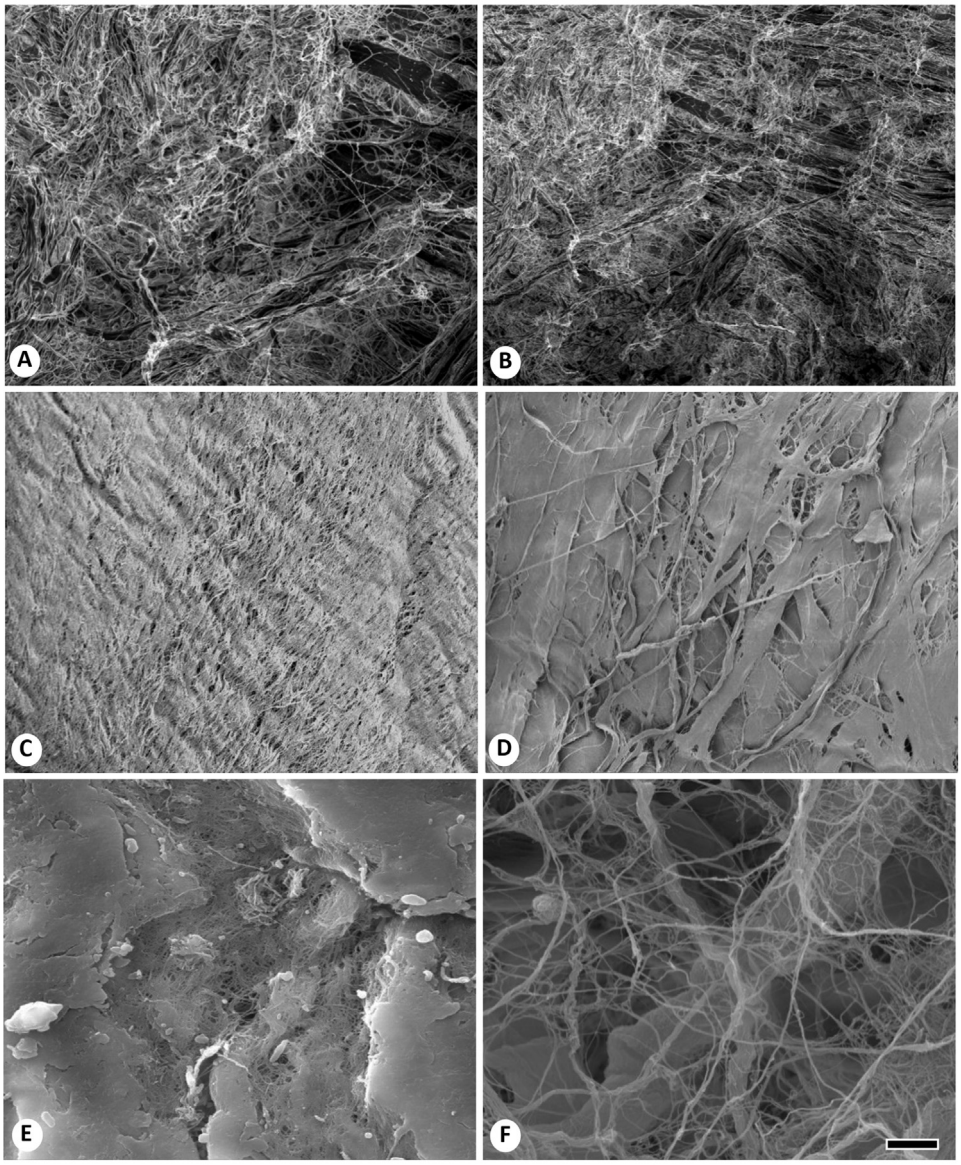

Fig. 1. Representative SEM image of Collprotect® (A, B), Jason® (C, D) and Bio-Gide® (E, F) membranes. Bars: A, C = 10 μm; B, D and *E* = 5 μm; *F* = 1 μm.

of type I collagen than those plated on Bio-Gide® ($p < 0.05$). At 7 days, the collagen levels were lower for cells plated on all the experimental membranes than on control group ($p < 0.05$).

The results for osteopontin quantification are shown in Table 3. The lowest levels for osteopontin were observed for the cells plated on polystyrene (control) at all the experimental time points evaluated ($p < 0.05$). At 3 days, the cells plated on Collprotect® exhibited higher osteopontin concentration than the other cell groups ($p < 0.05$). At 5 days, the highest osteopontin concentration was observed when the cells were plated on Bio-Gide® ($p < 0.05$). After 7 days, an increase in osteopontin levels was observed when the cells were plated on all the experimental membranes, compared with the control group ($p < 0.05$).

Table 1
Quantification of cell viability in osteoblastic cells plated on the collagen membranes at 3, 5 and 7 days. Data is presented as a mean (standard deviation) expressed in Arbitrary Units (AU).

Group	Time (days)		
	3	5	7
Control	0.293 (0.053) Ca	0.374 (0.010) Ba	0.653 (0.093) Aa
Bio-Gide®	0.145 (0.007) Ac	0.139 (0.006) Ac	0.148 (0.027) Ac
Jason®	0.174 (0.016) Ab	0.144 (0.007) Bc	0.145 (0.024) Bc
Collprotect®	0.189 (0.008) Ab	0.184 (0.015) Ab	0.181 (0.018) Ab

p(group)<0.0001; p(time)<0.0001; p(interaction)<0.0001. Different letters (uppercase horizontally and lower case vertically indicate statistically significant differences (p<0.05).

4. Discussion

Resorbable membranes were developed with the aim of avoiding a possible second surgery, thereby reducing morbidity and improving tissue recovery. However, these membranes have certain drawbacks, such as the significant variance in the acceptable lifetime of the barrier function due to degradation [14]. This is an important consideration, given that the membranes must be kept in place for at least 4 to 6 weeks to accomplish effective tissue regeneration [15].

The mechanical and biological properties of the membranes should offer a favorable environment for bone neoformation, both in terms of barrier occlusiveness and receptor site protection, particularly as regards admitting nutrition into the regeneration area. Accordingly, this study investigated the characteristics of the

Table 2
Quantification of type I collagen secreted by osteoblastic cells plated on the collagen membranes at 3, 5 and 7 days. Data is presented as a mean (standard deviation) expressed in pg/mL.

Group	Time (days)		
	3	5	7
Control	927.129 (42.105) Ba	1057.761 (67.489) ABa	1224.051 (63.944) Aa
Bio-Gide®	54.841 (44.829) Ac	52.856 (4.145) Ab	6.505 (2.033) Ab
Jason®	966.759 (171.692) Aa	1121.955 (263.811) Aa	5.632 (2.590) Bb
Collprotect®	401.926 (74.316) Bb	1078.698 (383.995) Aa	5.530 (2.382) Cb

p(group)<0.0001; p(time)<0.0001; p(interaction)<0.0001. Distinct letters (upper case horizontally and lower case vertically indicate statistically significant differences (p<0.05).

topography, as well as the viability and osteogenic differentiation of cells cultured on different porcine membranes. The results found significant structural variations between the membranes. Collprotect® and Jason® both had rough surfaces with an irregular aspect on both sides. On the other hand, Bio-Gide® is double-layered, with one smooth side and another rough side, along each respective length. These findings highlight the variation in porcine tissues found in the membranes, inasmuch as Bio-Gide® is derived from the peritoneum, Collprotect®, from the periderm, and Jason®, from the pericardium.

Collagen membranes have structural variances that depend on their source, and that are indicated for distinct clinical situations. Jason®, for example, has slow enzymatic degradation that provides an extended time barrier, thus making it suitable for treating larger defects, such as extended ridge augmentations and maxillary sinus elevation with additional lateral augmentation [9,16,17]. On the other hand, although Bio-Gide® has a second layer, it has a faster resorption period, averaging 2 to 3 months when a single layer is employed [10]. Lastly, Collprotect® has a collagen structure with a naturally rough surface that promotes cell adhesion and migration, as well as angiogenesis [18].

The three membranes used in this investigation are all natural, with no artificial cross-linking, which could result in diminished tissue integration and vascularization, as well as increased risk of foreign body reactions [19]. Additionally, when compared with the synthetic ones, collagen membranes possess low stiffness and rapid enzymatic degradation in vivo [20]. In order to overcome some clinical shortcomings such as the mechanical and biodegradable stability, biological cross-linking methods have been introduced to cross-link collagen [21,22]. However, artificial fiber cross-linking reduces biocompatibility, tissue integration, revascularization, and degradation, and causes more postoperative complications than natural membranes, including suture dehiscence and oral exposure [23,24,25]. In comparison, the membranes evaluated showed biocompatibility, excellent clinical management, and satisfactory results in terms of support and a physiological barrier function providing protection to the tissue regeneration area. This makes the membranes suitable for use in current surgeries [26,27].

Table 3
Quantification of OPN secreted by osteoblastic cells cultured on the collagen membranes at 3, 5 and 7 days. Data is presented as a mean (standard deviation) expressed in pg/mL.

Group	Time (days)		
	3	5	7
Control	46.380 (14.088)Cc	72.823 (2.071)Bc	112.199 (27.904)Ab
Bio-Gide®	76.439 (8.287)Cb	176.688 (12.178)Ba	214.657 (31.034)ABa
Jason®	72.773 (16.351)Cb	119.783 (4.233)Bb	233.743 (7.574)Aa
Collprotect®	103.058 (20.232)Ba	117.523 (19.454)Bb	197.581 (40.136)Aa

p (group) <0.0001; p(time)<0.0001; p(interaction)<0.0001. Different letters (uppercase horizontally and lower case vertically indicate statistically significant differences (p<0.05).

Cells plated on Collprotect® and Jason® had higher viability than those plated on Bio-Gide®, notably after 3 and 5 days. The surface of both membranes was irregular and porous, characteristics that contribute to cell adhesion and spread. These findings highlight the importance of topographical aspects in cell proliferation and viability parameters. In fact, surface roughness may promote maximum cell-substrate interaction through the formation of focal adhesion sites, which are required for sustained adherence, cell growth, and proliferation [28,29]. Furthermore, collagen scaffolds mimic extracellular matrix and favor the adhesion to the substrate of various cell types, and stimulates cell proliferation and differentiation [30].

In terms of type I collagen synthesis, the cells plated on Collprotect® and Jason® secreted more collagen than those plated on BioGide®. Type I collagen is a critical protein in the development of mineralized matrix and hydroxyapatite crystal nucleation. The synthesis of type I collagen is an early event in the development of osteoblasts. After cells secrete collagen, alkaline-phosphatase, osteocalcin and bone sialoprotein are all expressed sequentially [31].

Osteopontin is a non-collagenous protein found on the surface of the bone matrix, and plays a role in osteoblast and osteoclast mineralization and adhesion. It is also found in various tissues. In addition to promoting osteoblast adhesion, it aids in managing hydroxyapatite crystal formation [32]. In general, osteopontin is described as a multifunctional protein that engages in physiologic processes such as tissue healing, bone formation and remodeling, and that appears in the early and late stages of mineralization [33].

The results of the study revealed that osteopontin secretion was higher on the cells plated on the surface of Jason® and Collprotect® than on the control group surface. Furthermore, Collprotect® stimulated higher osteopontin secretion than that of the control group after 3 days. The cells plated on the Bio-Gide® surface showed higher levels of osteopontin than those plated on the other groups, at all the experimental time points, thus showing that its characteristics have the chemical potential of participating effectively in bone formation, which is an inherent property of collagen membranes [34].

This in vitro study indicated that membranes of different porcine origin, such as Jason® and Collprotect®, presented satisfactory results, and can thus be considered for clinical procedures that require membranes to improve tissue regeneration. In vivo experimental models should be used in future investigations to determine which membrane is optimal for tissue healing, as well as the therapeutic implications. Furthermore, it is important to evaluate the impact of these collagen scaffolds on diverse osteoblastic-like cell lineages in order to validate the current study's findings.

Declaration of Competing Interest

The authors state no conflicts of interest.

CRediT authorship contribution statement

Dalton Marques: Conceptualization, Methodology, Formal analysis, Investigation, Supervision, Visualization, Writing – original draft,

Writing – review & editing. **Lucas Novaes Teixeira:** Supervision. **Carlos Nelson Elias:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Alexandre Barboza Lemos:** Methodology, Formal analysis, Writing – review & editing. **Elizabeth Ferreira Martinez:** Conceptualization, Methodology, Formal analysis, Investigation, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Acknowledgments

The authors would like to thank Dr. Rafael Bovi Ambrosano for helping with the statistical analysis of the data, and also acknowledge NCristina Martorana for promptly volunteering to review this manuscript for its English language content.

Funding

This research did not receive any specific grant from any funding agencies in the public, commercial, or not-for-profit sectors.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jormas.2023.101607.

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