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Analysis of the biocompatibility of a biocelulose and a poly L- lactic acid membrane

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The use of selective barriers as resorbable membranes has become a routine clinical procedure for guided bone regeneration. Therefore, the production of membranes with a low inflammatory potential during their resorption process has become the goal of a considerable number of researches. **Aim:** The purpose of the present study was to evaluate the biocompatibility of poly (L- lactic acid) (PLLA) and biocelulose membranes (BC) inserted in the subcutaneous tissue on the dorsum of rats. **Methods:** Fifteen animals underwent surgical procedures for the insertion of 4 types of membranes: COL (Collagen membrane) – Control Group; BC (Biocellulose membrane); BCAg (Biocellulose membrane impregnated with Silver); PLLA (Poly (L-lactic acid) membrane). All membrane types were inserted into each animal. Animals were euthanized after 3, 7, and 15 days of the surgical procedure. Descriptive histological analyses were carried out to investigate host tissue reaction to membrane presence by assessing the anti-inflammatory process composition associated with the membrane resorption and the presence of foreign-body reaction or encapsulation. **Results:** The BC membranes showed a higher degree of inflammation and poor pattern of integration with the surrounding tissues than the PLLA and COL membranes. **Conclusion:** The PLLA and COL membranes present better biocompatibility than the BC membranes.

Keywords: Biocompatible materials. Inflammation. Membranes. Celulose. Polyesters.

Introduction

Guided bone regeneration (GBR) is a technique that consists in applying a mechanical barrier in order to isolate a bone defect from neighboring tissues to favor the formation of bone rather than that of other tissues with faster proliferation. For this purpose, it is necessary to use occlusive, semi-permeable, biocompatible, and preferably, a resorbable membrane in order to maintain the space for the occurrence of bone regeneration[1]. Several materials have already been suggested for use as resorbable membranes, such as collagen[2], polymers[3], hydroxyapatites[4], and latex[5], among others. However, the search for resorbable membranes that present a controlled resorption process, with the least possible inflammatory reaction, good mechanical properties, and that allow the association with osteoinductive or antimicrobial substances is still necessary[1,2].

Collagen membranes are traditionally the most frequently used among the resorbable membranes[2,6], and have presented good clinical outcomes throughout the recent years[7-9]. However, despite the consistent data presented by the collagen membranes, some alternatives for this type of membrane have been proposed in order to provide a material with similar predictability but with an alloplastic origin, as opposed to that of the natural collagen membranes[10].

In this context, this study highlights and investigates the behavior of biocellulose (BC) and poly (L-lactic acid) (PLLA) as mechanical-barrier membranes, as it has previously been proposed[11]. BC membranes are made of a nanomaterial produced by several bacteria in culture, especially *Acetobacter Xylinum* (or *Gluconacetobacter xylinus*), which has been used in the industry due to its ability to produce cellulose in a large scale, in a culture medium containing carbon and nitrogen sources, whether in a stirred or static environment[12]. Recent studies have showed promising potential for the use of BC as a temporary skin substitute biomaterial in the treatment of wounds that are difficult to heal[13]. These researches have also motivated the use of the BC membrane in guided bone regeneration procedures[11,14]. BC alone has no antimicrobial activity to prevent wound infection[15]. In this sense, recently, a great effort has been devoted to the development of membranes with antimicrobial potential, especially BC membranes containing silver particles[12]. The PLLA membranes are polymers synthetized by the combination of citric acid with glycolic copolymers and lactic polymers[16]. The use of the PLLA is widespread in clinical medicine, in processes of bone fixation[17], surgical sutures[18] and in the control of drug release[19]. However, these PLLA and PLGA-based membranes degrade into acid during their resorption process, and an inflammatory reaction can be generated due to the accumulation of this by-product in living tissues[16].

The array of reactions caused by a material when implanted in the body can predict the success or failure of the procedure. For this reason, preclinical studies are necessary to analyze the biocompatibility of resorbable membranes during their degradation process in order to ensure that the material contributes to the formation of bone tissue and/or soft tissue healing without bring-

ing any harmful side-effect to the host. Thus, the aim of this study was to evaluate the biocompatibility of PLLA and BC membranes with and without the addition of silver inserted in subcutaneous tissue on the dorsum of rats by means of a histological description.

Material and Methods

Animals and ethical considerations

In this study, 15 adult male rats (Rattus Norvegicus, variation albinus, Holtzman) approximately 3 months old, with body mass between 250- 300 grams were used. The animals were kept in an environment with humidity, controlled light and temperature cycles. The animals were fed with solid rat chow and had access to water *ad libitum*, before and throughout the experimental period. The study was approved by the ethical committee of our institution (CEUA-15/2015) and was conducted in accordance with the ARRIVE protocol for conducting pre-clinical studies.

Surgical procedure

All rats were anesthetized by a combination of ketamine with xylazine, in the proportion of 0.08 ml/100g of body weight for ketamine (Ketamine Hydrochloride - Francotar - Virbac do Brasil Ind. e Com. Ltda.) and 0.04 ml/100g of body mass for xylazine (Xylazine Hydrochloride - Virbaxyl 2% - Virbac do Brasil Ind. e Com. Ltda.). Subsequently, the animals were submitted to trichotomy in the dorsal region where the implantation of the membranes occurred. The surgical field antisepsis was performed with sterile gauze soaked in povidone-iodine solution.

The animals were placed in the prone position on the operating table. Four linear cutaneous incisions of approximately 2 cm in length were made; then the soft tissues were separated by divulsion with the aid of small blunt-ended scissors and a dissection forceps, until the surgical bed allowed the subcutaneous implantation of the disk-shaped membranes (1 cm in diameter and 1 mm in thickness). One membrane from each group was randomly implanted in the surgical sites: COL (Collagen membrane – Bio Gide®, Geistlich, Wolhusen, Switzerland) – control group; BC (Biocellulose membrane - Procell, São Carlos, Brazil); BCAg (Biocellulose membrane impregnated with Silver - Procell, São Carlos, Brazil); PLLA (Poly (L-lactic acid) membrane, Experimental MW: 128.000 gmol; MN: 70.000 gmol)[20] (Figure 1). The surgical sites were marked with tissue marker to guide the future removal of the samples. The insertion site for each membrane was determined randomly by lottery after the surgical site was made. After implantation of the membranes, the surgical sites were sutured with 4.0 silk thread (Ethicon, Johnson & Johnson, Brazil) and the animals were medicated postoperatively with an intramuscular dose of multibiotic (0.1ml/kg), and an intramuscular dose of Ketoflen (0.3ml/kg).

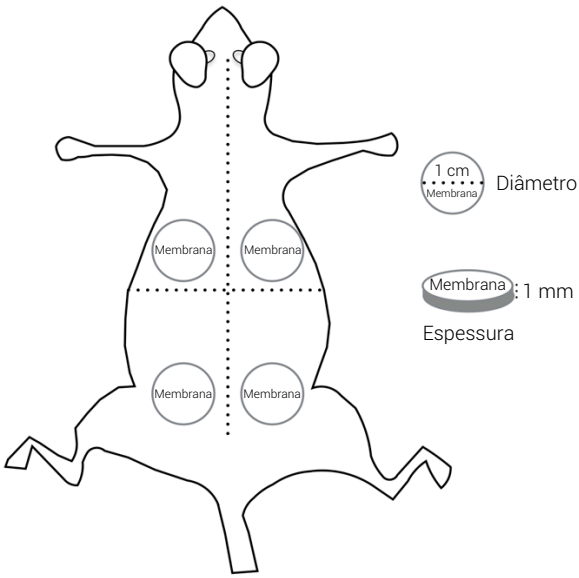

Figure 1. Scheme of the placement of the different membranes subcutaneously in the rat's dorsum.

Retrieval of the biopsies and descriptive histological analysis

The animals were euthanized by anesthetic overdose at the periods of 3, 7 and 15 days after the surgical procedure. The soft tissue samples were removed *en bloc* and fixed in 4% paraformaldehyde for 48 hours. Subsequently, the samples were submitted to laboratory processing for inclusion in paraffin. Then, 5 µm-sections were captured and stained by the hematoxylin /eosin technique.

A total of 3 sections that were 750 µm apart from each other were evaluated. The sections were analyzed by a blinded evaluator at 25X, 100X and 400X magnifications and described according to histopathological findings. After the initial description, the same evaluator performed the review of the slides to confirm the findings described. The histological features evaluated were the presence or absence of inflammatory infiltrate and the severity of the inflammation when present, taking into account the cellular and tissue characteristics surrounding the area of interest. Other pertinent characteristics were described as general characteristics within the analyzed groups.

Results

Collagen Membrane (COL Group)

The COL group presented epithelial discontinuity and necrotic foci in all specimens from the studied periods of 3 days while the subsequent periods presented fibrosis characteristic of the healing process. In general, only residual portions of the membrane were present in a few specimens after 7 days. The chronic inflammatory process varied from slight at the period of 3 days to absent at the periods of 7 and 15 days. After the 7-day period, it was already possible to note remis-

sion of the granulation tissue and the beginning of the tissue reorganization process, showing a vascularized area rich in fibroblasts, compatible with the healing process (Figure 2).

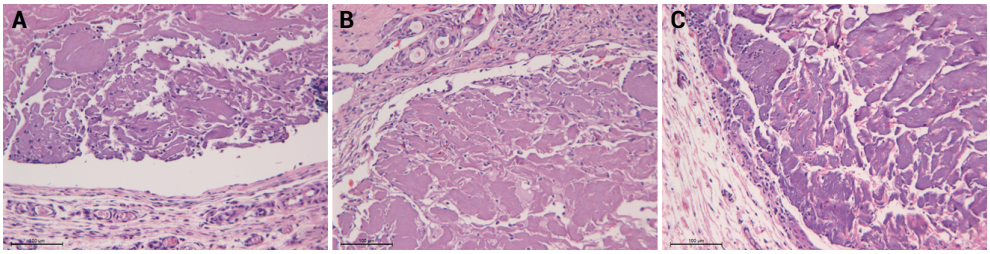

Figure 2. COL group: A)3- day period; B)7-day period; C)15-day period. The COL group presented chronic inflammatory infiltrate varied from slightly at the period of 3 days to the absent at the periods of 7 and 15 days. After the 7-day period, it was noted the beginning of the tissue reorganization process, showing a vascularized area rich in fibroblasts, compatible with the healing process.

Biocellulose membrane (BC Group)

The BC group presented an inflammatory infiltrate with characteristics of chronic inflammation, rich in mononuclear cells and its intensity varied from moderate at the period of 3 days to mild or absent at the subsequent periods. Some specimens of all the periods showed a foreign body-like reaction or a large area rich in granulation tissue around the membrane, with the inflammatory cells arranged in an epithelioid arrangement compatible with the general characteristics of epithelial discontinuity, probably due to suture dehiscence, and exposure of the membrane. At the 15-day period, histological characteristics compatible with the membrane resorption process were observed (Figure 3).

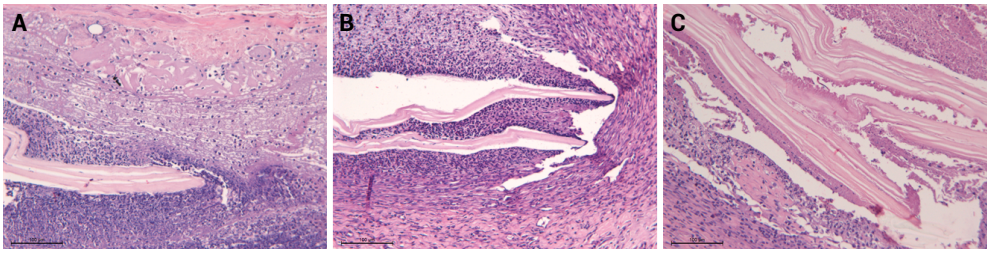

Figure 3. BC group: A)3- day period; B)7-day period; C)15-day period. The BC group presented an inflammatory infiltrate varied from moderate at the period of 3 days to mild or absent at the subsequent periods. Some specimens of all the periods showed a foreign body-like reaction or a large area rich in granulation tissue around the membrane.

Biocellulose impregnated with silver (BCAg Group)

In most specimens in the BCAg group from the three studied periods, the membrane was associated with a moderate chronic inflammatory infiltrate, sometimes with a

foreign body-like reaction characteristic. All specimens at the 15-day study group showed discontinuity of the epithelium, compatible with suture dehiscence and this finding was accompanied by the visualization of fields with necrotic foci associated with the surgical procedure (Figure 4).

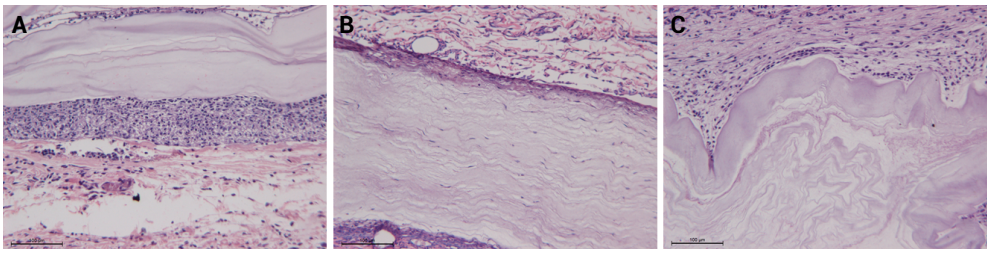

Figure 4. BC/Ag group: A)3- day period; B)7-day period; C)15-day period. The specimens of the BC/AG group was associated with a moderate chronic inflammatory infiltrate, sometimes with a foreign body-like reaction characteristic.

Poly (L-lactic acid) membrane (PLLA)

The PLLA group showed detectable membrane residues in some specimens from all periods. The findings of epithelial discontinuity and small necrotic foci were associated with specimens at the 3-day period. At the 7- and 15-day periods, the histological findings showed a healing variation with the formation of fibrosis or tissue reorganization. No large amount of granulation tissue or foreign body reaction was observed in any of the periods studied. A significant finding was the detection of cell population in regions of the remaining membrane with no significant inflammatory infiltrate (Figure 5).

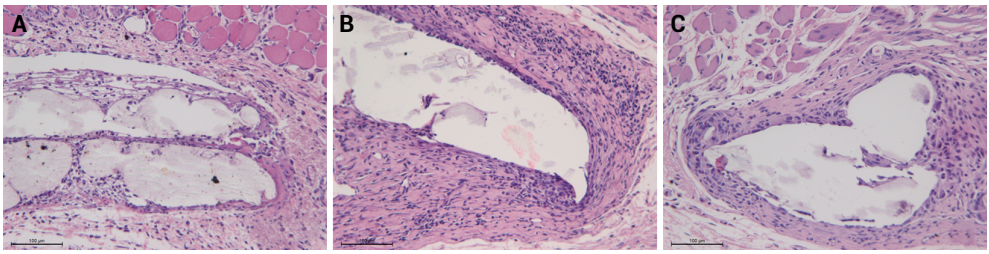

Figure 5. PLLA group: A)3- day period; B)7-day period; C)15-day period. The PLLA group showed detectable membrane residues in some specimens from all periods. No large amount of granulation tissue or foreign body reaction was observed in any of the periods studied. A significant finding was the detection of cell population in regions of the remaining membrane with no significant inflammatory infiltrate.

Discussion

In this study, it was demonstrated that BC-based membranes induced inflammatory reactions in greater severity and longer duration than COL and PLLA membranes. Despite the previous results that showed that this membrane is a good candidate

for GBR material[13,15], these findings were not confirmed in this study. In contrast, PLLA-based polymeric membranes demonstrated a pattern of resorption, tissue integration and induction of inflammatory reactions similar to COL membranes that are traditionally used in the GBR technique.

The use of resorbable membranes has been described as advantageous in relation to the use of non-resorbable membranes since it is not necessary to perform a second surgical procedure for its removal[8,9]. However, some factors such as mechanical strength, handling properties, chemical stability, and the biological effects that occur in the connective tissues during the membrane degradation must be determinate during the assessment of a resorbable membrane[21]. The porcine collagen membranes seem to present the best properties, among the resorbable membranes[8,21], and this justifies its use as a control group in our study. Indeed, this study showed that the porcine collagen membranes presented reduced signs of inflammatory reaction, restricted to the 3-day period, and after this period it showed signs of a progressive process of reorganization on the connective tissue around the membrane, good tissue integration, and adequate wound healing at the 15-day period. These findings corroborate with the results of previous pre-clinical studies[21,22] that demonstrated the good biocompatibility pattern of this type of membrane and this fact may be the reason for the good outcomes of the GBR in clinical studies performed with collagen membranes[7-9].

In an attempt to demonstrate the applicability of BC membranes in GBR, previous studies have obtained promising results in relation to some tested characteristics such as cytotoxicity[15], and in the healing of soft tissues[23]. Another pre-clinical study where the BC was associated with silver as a scaffold presented good outcomes in bone formation[24], as well as in the healing of soft tissues[25]. However, in this study the BC and BC/Ag membranes induced the occurrence of a moderate inflammatory infiltrate at the 3-day period that changed the profile to a chronic inflammatory characteristic at the 15-day period, and maintained a higher level of inflammation than the COL and PLLA groups. In addition, the maintenance of the integrity of the membrane could not be verified in any of the periods, probably due to the lack of mechanical resistance. The analysis of BC/Ag membranes at 15 days also showed necrotic portions of the connective tissue associated with the discontinuity of the epithelium associated with a moderate, but persistent chronic inflammatory infiltrate that is characteristic of a foreign body-like reaction. This fact possibly motivated the complete or partial expulsion of the membranes and justifies its absence in the final evaluation periods. The outcomes in this study could be reflected by the thickness of the membrane since thinner BC membranes (0.10mm vs. 0.15mm) have been reported to promote better outcomes and bone formation[14]. The membranes used in this study were thicker than this previous study in order to improve their handling properties. Furthermore, the coating of the BC membrane with other types of bioactive molecules may improve the biologic properties of this membrane[15].

It was observed in the PLLA group that the presence of remnants of this membrane was associated with the presence of foci of necrosis and epithelial discontinuity restricted to the period of 3 days. However, in the other periods, a progressive deg-

radation of the membranes was observed with the presence of granulation tissue, reorganization of the connective tissue and cell population in the regions where the membrane remained. Therefore, the pattern of biocompatibility observed with this membrane was similar to that founded in the COL group. Although a previous pre-clinical study showed that the polymetric membranes are related to the presence of inflammatory reactions[16], the use of PLLA has been shown to have less intense reactions[3,17]. In addition, this material has proven to be a good vehicle for coating with bioactive substances that can further improve its biocompatibility, degradation and tissue integration properties, which consequently can benefit the formation of bone tissue in GBR techniques[3,4,17].

This study presents some drawbacks that must be considered when interpreting the exposed data. The analysis was limited to the assessment of biocompatibility only, though the effects of these specific membranes on GBR techniques still require further information. Lastly, it is known that biological membranes can be associated with bioactive molecules, such as growth factors or anti-microbial materials. A possible improvement obtained by an eventual anti-microbial coating on the BC and BC/Ag groups should be taken into consideration.

In conclusion, the BC and BC/Ag groups presented reduced biocompatibility since a foreign body-like reaction was perceived, and in some cases these membranes were expelled by the host. The PLLA membrane presented characteristics of biodegradation similar to the COL membranes that were characterized by a mild and reduced inflammatory process and good tissue integration. PLLA seems to be a suitable material to be used in clinical procedures as guided bone regeneration.

Author Contribution

RM – Developed the biocelulose membrane, participate of the conception of the research; AM and ED - Developed the poly L- lactic acid, participate of the conception of the research; JD – Performed the experimental procedure and writing the paper; GJO – Conception of the study, analysis of the histological sections and writing of the paper; CM - Conception of the study, writing of the paper and supervision of the project. All the authors read and approved the final version of this manuscript.

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