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Research Article

Chitosan and Xanthan Membrane in Periosteal Injury in the Calvary of Rats

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Abstract

Introduction: The periosteum is a complex structure composed of an external fibrous layer that confers structural integrity and an internal layer, which has osteogenic and repair potential. When an extensive injury occurs, which does not allow spontaneous repair of the periosteum, there is a difficulty in repairing the wound. With this, there is a search for substitute biomaterials, with reparative capacity. Research with bioresorbable polymers, of natural origin such as chitosan, alginate, gelatin, cellulose and their derivatives, combined or used individually, has been applied in tissue engineering in the development of membranes or dermal dressings.

Objective: To evaluate the membrane composed of Chitosan and Xanthan in a periosteal skin lesion in the calvaria of rats.

Method: 24 male Wistar rats, weighing an average of 360g and aged 10 weeks, were used. All rats were subjected to excision of 10 mm^2 of skin and periosteum from the underlying calvaria and the covering of the lesion or not. They were distributed in 2 groups: Control group (CG), without chitosan/xanthan membrane, n = 12 and Experimental group (EG), with chitosan/xanthan membrane, n = 12. The rats were euthanized at 2, 4 and 6 weeks after surgery, and specimens of the calvaria were obtained, where macroscopic analysis of the open area was performed. In the macroscopic analysis, using an ImageJ software, the mean values of the areas of the periosteal lesion in the unhealed calvaries were obtained. For the analysis of intra-group variables between 2, 4 and 6 weeks, the Kruskal-Wallis test was used and for inter-group analysis at 2, 4 and 6 weeks, the Wilcoxon test was used. Values of $p < 0.05$.

Results: In the macroscopic analysis, the amount of raw area, in the 2^{nd} week, the values expressed for the CG and EG were 93.7% and 81.3%, respectively. In the 4^{th} and 6^{th} weeks, the bloody area values of the EG were 52.3% and 11.3% respectively, and in the CG they were 78.8% and 69.9%.

Conclusion: The chitosan/xanthan membrane, in a periosteal skin lesion in the calvaria of rats, accelerated the healing process, being favorable when compared with the control group in the interval of 4 and 6 weeks.

Keywords: Chitosan; Xanthan Membrane; Periosteal Injury; Calvary of Rats

Introduction

The regenerative capacity of the periosteum is known, and this tissue has an important role in bone and tissue growth and repair, and has an important impact, when any aggression to the periosteum occurs, requiring a repair [1]. The greater and better adaptive and reactive capacity of the periosteum promotes local phenomena, and is dependent on a more severe aggression against the periosteum or mild and long-lasting aggression, producing media-

tors, which at higher levels, induce resorptive phenomena and the maintenance of its structural and functional integrity, preserving its cells and the circulatory structure of the periosteum to the bone, which are fundamental to produce new bony and tissue layers, on the surface of the cortices [2].

Surgical procedures in the oral cavity occasionally result in bone exposure, and this is true in cases that involve partial resection of soft tissues due to trauma, surgery, oral cancers or precancerous lesions [3]. When removal of the periosteum is necessary, exposure of the bone surface can be problematic, leading to acute inflammatory processes that are difficult to repair [3,4]. Adequate coverage of the exposed periosteum or bone surface is often necessary to prevent complications [4,5].

The periosteum is important in the complex process of bone healing and tissue repair. Within 24 to 48 hours after a periosteal lesion, an acute inflammatory reaction can be observed [6]. Subsequently, the periosteal cells begin to proliferate and a thickening of the periosteum is observed after these 48 hours. This process is defined as periosteal activation [7]. The cells of the bone marrow or endosseous remain in the medullary cavity and do not migrate to form bone consolidation [7]. In fact, the activation of progenitor cells derived from periosteum can induce chondrogenesis and osteogenesis, accompanied by induction of angiogenesis, which may eventually lead to vascularization and remodeling of bone grafts, with which there will be tissue repair [8,9].

One of the first steps, through tissue bioengineering, has been the selection of different types of polymeric materials of natural or synthetic origin, which have been used, as an example, in the production of dermal dressings [10,11]. Some intrinsic properties of the biomaterial, such as biocompatibility, biodegradability, mechanical resistance, pore size and shape, have been reported as relevant, as well as allowing cell adhesion, growth, migration and cell differentiation [9,12]. The use of bioresorbable polymers of natural origin such as chitosan, alginate, gelatin, cellulose and their derivatives [3,12,13], prepared separately or in association, have been studied and applied in tissue engineering, as well as the use of xanthan, which represent an alternative for the production of these frameworks [14-16].

The complexation of xanthan with chitosan, through interactions between the amino groups of chitosan and xanthan carboxyl, makes it possible to obtain membranes that have high absorption of aqueous solutions and with proven stability in biological fluids [17,18]. The results showed that the membranes are not mutagenic and present a favorable architecture for cell adhesion, maintenance and proliferation. They also showed a significant acceleration of the healing of the dermo-epidermal wound, leading to the conclusion that this membrane-cell association can be considered viable in tissue engineering [12,19].

The use of a membrane composed of chitosan and xanthan as a substitute biomaterial for periosteal tissue has not yet been described in the literature.

Objective of the Study

Thus, the objective of this study is to evaluate the use of a chitosan and xanthan membrane in terms of periosteal skin lesions in the calvary of rats at intervals of 2, 4 and 6 weeks. The tested null hypothesis is that the membrane does not present a difference when compared to surgeries without the use of the membrane.

Materials and Methods

In vivo study

Twenty-four male Wistar rats, 10 weeks old, weighing an average of 360 g (320 to 380g), from the Center for the Development of Experimental Models (CEDEME) Unifesp, were used. The animals were divided into two groups: I Control Group (CG) - animals with periosteal lesions that remained without association of the chitosan/xanthan membrane. These were distributed according to the evaluation time: (CG2) - analysis in the 2nd week n = 4; (CG4) - analysis at the 4th week n = 4 and (CG6) - analysis at the 6th week (n = 4).

Experimental group (EG) - animals had periosteal excision at calvaria, associated with chitosan/xanthan membrane coverage, which were distributed according to the time of analysis: (EG2) - analysis at the 2nd week n = 4; (EG4) - analysis at the 4th week n = 4 and (EG6) - analysis at the 6th week (n = 4). The animals went through a period of one week to adapt to environmental conditions, and were housed in individual cages, standard CEDEME/Unifesp and kept in an environment controlled by temperature and humidity (23º + 1º C and 60 + 10%). During the experimental period the animals received water and feed *ad libitum*.

Materials

To obtain the membrane, Chitosan reagents of medium molecular weight (190 to 310 kDa) with an 82% deacetylation degree,

Xanthomonas campestre Xanthan gum, 100% acetic acid (glacial) were used. All the water used was obtained using Millipore's Direct-Q®3 system.

Membrane preparation

The Chitosan-Xanthan solution (Chi/Xan) was prepared based on the procedures described by Bueno E Moraes [13], Veiga E Moraes [10] and Bellini E Moraes [12] where 500 ml of 1% chitosan solution in acetic acid 2% were poured into 500 ml of 1% aqueous xanthan solution, using a rate of 10 ml/min with the aid of a peristaltic pump, maintaining a rotation of 1.400 rpm on a mechanical stirrer at 1.400 rpm for 50 minutes. After the solutions were mixed, the agitator speed was increased to 1.600 rpm for another 10 minutes. 80 ml of the chi/xan solution were added to the polystyrene plates and taken to an oven at 37ºC for drying for 48 hours. After this period, the membranes were washed with 500 ml of ultrapure water for 30 minutes. This washing procedure was repeated 3 times. The membranes were again taken to the oven at 37ºC for another 24 hours for final drying.

Operative technique

The animals were anesthetized with an anesthetic combination of 1 ml/kg, in the proportions of 80% Ketamine Hydrochloride and 20% Xylazine Hydrochloride. Xylazine Hydrochloride was used in the proportion of 13 mg/kg and the Ketamine Hydrochloride in the proportion of 33 mg/kg, intramuscularly. Anesthesia was performed using a 5 ml syringe and a 30 mm x 7 mm hypodermic needle. The rats had the parietal region trichotomized by an electric shearing machine with a 0 size cut. Subsequently, the trichotomy was performed on the animals' calvaria. After trichotomy and asepsis, the craniometric points were demarcated, which delimited and standardized the lesion area by 10 mm^2. All animals, after being anesthetized, were placed ventrally on cork boards and received a metallic fixation on the front and rear legs for total immobilization. The head was also immobilized, so that the surgeries could take place in a stable manner. The area to be operated on the calvaria was isolated by a disposable fenestrated surgical field. After placing the disposable fenestrated field, the following points were marked: A (midpoint of the base of the right ear); A1 (midpoint of the base of the left ear); B (medial corner of the right eye); B1 (medial corner of the left eye) and the following lines: Line A-A1; B-B1. After marking these lines, the following points were marked: C (midpoint of line A-A1); D (midpoint of line B-B1). After marking these points, the C-D sagittal line was marked. Afterwards, points C1 and C2 were marked respectively 5 mm away from point C to the right and left, in the same way, points D1 and D2 were marked respectively 5 mm away from point D to the right and left. Afterwards, the lines C1-D1 and C2-D2 were marked. From point C1 and D1, a point 10 mm was measured and the corresponding line was made. In this way, the design of an area of 10 mm^2 was obtained (Figure 1).

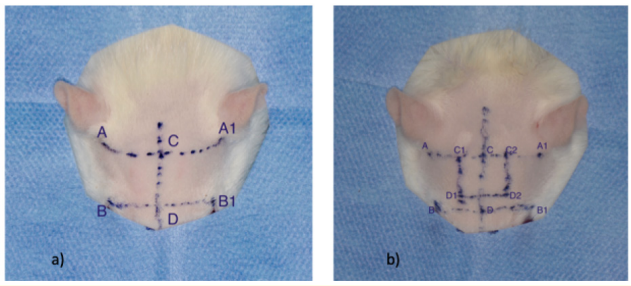

Figure 1: a) Determination of the craniometric points, based on the anatomical references for the excision of the 10 mm^2 periosteal lesion b) Determination of the central points, to delimit the final lesion.

After determining the design of the lesion to be created of 10 mm^2 based on anatomical references, and before the cutaneous and periosteum excision, subperiosteal anesthesia was administered with Lidocaine Hydrochloride with 2% epinephrine 1: 100.000 for bleeding control (Figure 2).

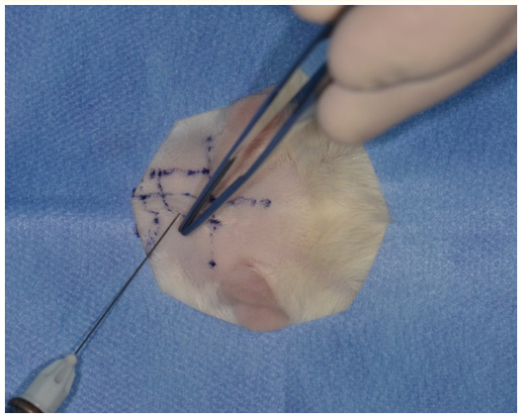

Figure 2: Local anesthesia with lidocaine hydrochloride 1: 100.000 - 2% epinephrine.

Using a scalpel no. 3 and blades 15c, 14 cm Iris scissors, and the aid of a 14 cm Dietrich forceps, cutaneous and periosteum excision was promoted (Figure 3 and 4).

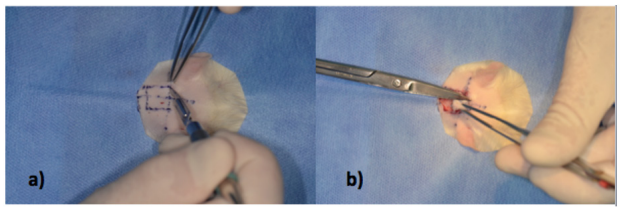

Figure 3: a) Incision of the flap marked using a handle and scalpel blade size 15c. b) Cutaneous excision with the aid of scissors.

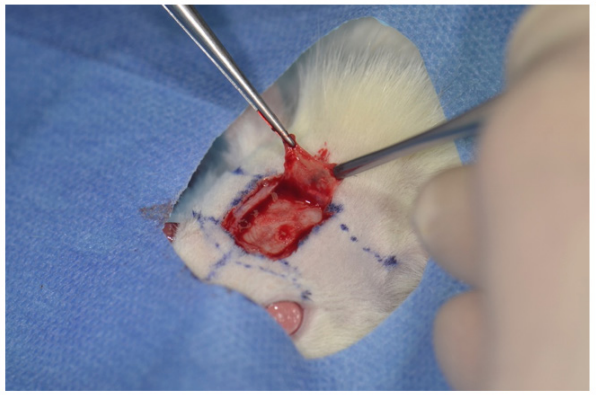

Figure 4: Removal of the periosteum after cutaneous excision, in the size of the lesion in the demarcated area.

In the animals of the CG control group, the raw areas were kept without cover. In the animals of the experimental group EG, the raw areas were covered with chitosan/xanthan (chi/xan) membranes sutured to the periosteum of the lesion margins. Chi/xan membranes, 10 mm^2 in size, were obtained using scissors and sterile metal ruler. The sutures of the membranes near the periosteum of the lesion edges were performed with 4 symmetrical stitches with 6-0 Nylon Ethicon thread (Figure 5).

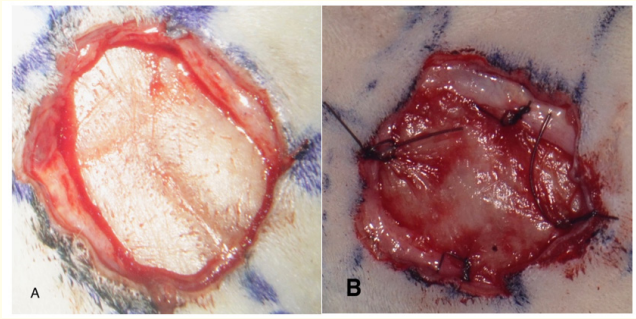

Figure 5: A) CG - open area, without a chitosan/xanthan membrane; B) EG - Bloody area with a chitosan/xanthan membrane, sutured next to the periosteum of the lesion margins.

During the first three postoperative days, the animals received an intramuscular route, a daily dose of 1 mg/kg of anti-inflammatory, Diclofenac sodium 75 mg and 24000 IU/kg of benzyl benzylpenicillin, procaine benzylpenicillin and associated potassium benzylpenicillin.

Macroscopic analysis

Painlessly induced death of the animals of all groups was carried out by administering, by intraperitoneal route, overdose of Ketamine 300 mg/kg + Xylazine 30 mg/kg. All animals, after death was confirmed, were placed ventrally on cork boards and received a metallic fixation on the front, rear and head paws for the photographs. The time intervals for specimen analyzes were determined at 2, 4 and 6 weeks, for both groups, CG and EG. The analysis of the wound closure of both groups was performed by a blind examiner and the specimens were chosen randomly.

The animals of all groups were photographed with the use of a Canon Rebel T5i camera and a Canon Macro model EF 100 mm f/2.8L lens with focus aperture and exposure of 1/125 and ASA 100, with the aid of a support tripod at a focal length of 30 cm measured from the lens to the area of the rat's parietal region.

Measurements of the means and the standard deviation of the values of the areas of the periosteal lesion in the calvaries were performed, after the periods of 2, 4 and 6 weeks, obtained by pho-

tographs. The analyzed areas were defined according to the closure of the lesion, always trying to delimit the margins of the repaired lesion at the respective times of 2, 4 and 6 weeks. The ImageJ software was used and presented in mm^2 (Figure 6).

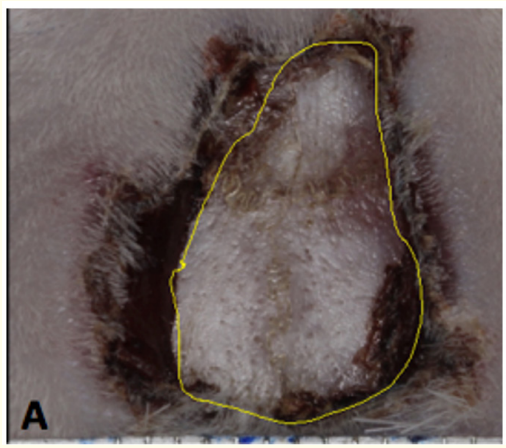

Figure 6: Demonstration of the delimitation of the raw area analyzed using the ImageJ software, to obtain the means and standard deviation of the values of the non-healed tissue areas.

Statistical analysis

For the analysis of intra-group variables between 2, 4 and 6 weeks, the Kruskal-Wallis test was used and for inter-group analysis at 2, 4 and 6 weeks, the Wilcoxon test was used. Values of $p < 0.05$ were considered significant.

Results

Macroscopic analysis

Chitosan/xanthan membranes: It was observed in the macroscopic analysis that the mixture of chitosan and xanthan produced a translucent, homogeneous, flexible, transparent and wavy surface membrane (Figure 7).

Quantitative analysis of the remaining raw area

The measurements of the areas of the raw, unhealed areas are summarized in table 1. The data were expressed as mean ± standard deviation (SD).

Discussion

In this study, a membrane produced by combining chitosan and xanthan in a 1: 1 mass ratio was used. The preparation of the membranes was carried out according to the established by Rodrigues., *et al*. [15] for chitosan/alginate membranes, Veiga and Moraes [10] in the production of chitosan/carrageenan, chitosan/pectin and chitosan/xanthan membranes, and by Bellini., *et al* [12]. In the works mentioned above, the porous lamellar membranes of Chi and Xan were tested and applied as dermal dressings and support for tissue engineering. The results showed that membranes prepared with the same mass proportions of Chi and Xan, without the presence of surfactants, have low cytotoxicity *in vitro*, have tensile

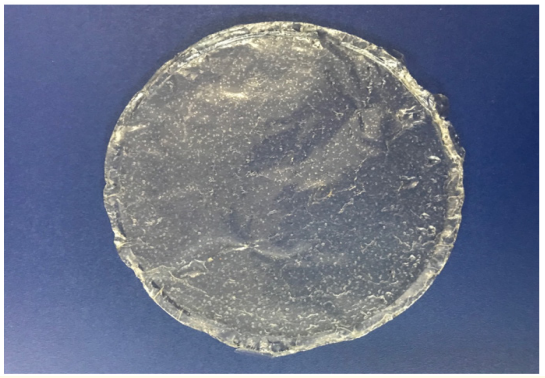

Figure 7: Final aspect of the produced chitosan/xanthan membrane, showing translucency.

	Time			P Value
Groups	**2 weeks**	**4 weeks**	**6 weeks**	
GC	93,7 ± 12,5	78,8 ± 12,7	69,9 ± 11,7	0,03*
GE	81,3 ± 25,7	52,3 ± 16,7	11,3 ± 3,7	0,03*
P Value	0,4	0,04*	0,02*	

Table 1: Means and standard deviation of the values of raw, unhealed areas, obtained by photographs and analyzed by the ImageJ software, with the values expressed in relative values.

CG: Control Group (without chi/xan coverage) n = 12; EG: Experimental Group (with chi/xan coverage) n = 12 Intra-group analysis between 2, 4 and 6 weeks - Kruskal-Wallis test Inter-group analysis between 2, 4 and 6 weeks - Wilcoxon test.

Data are shown as mean ± standard deviation (mm^2). Values of $p < 0.05$ were considered significant and were marked with *.

strength, compatible with human skin, a high capacity to absorb large amounts of fluids physiological and adequate stability in its presence.

Bellini., *et al*. [19] using membranes with the combination of Chi/Xan in the proportion of 1.2/0.8 obtained membranes with other characteristics, denominating them as porous membranes since the addition of surfactants Tween® 80 or Pluronic® was used F68 [12,13,16]. One factor reported by these authors was that the porous membranes would be thicker and could increase the difficulty of cell adhesion. In the present study, membranes called "dense" were used, that is, without the use of surfactants, due to the fact that surfactants can leave cytotoxic residues and decrease retention, which was shown in the results found in the work of Bellini., *et al*. [19] with the use of the Pluronic68® surfactant.

In the present study, a murine "*in vivo*" experimental model was used, which allowed a quantitative analysis of periosteal regeneration. This model involved the total excision of skin and periosteum of the calvaria of Wistar rats in a measure of 10 mm^2, considered here of critical size. "Critically-sized lesions" have been an experimental approach to *in vivo* evaluation of tissue reconstructions and can be defined as "a defect that does not regenerate without intervention" [20] or a defect that will not heal spontaneously during the life of the animal or the experiment [3,21,22].

In this study, the analysis of the raw area, it was found that there was no significant difference in the healed area, between the control and experimental groups in the two-week analysis period. There was a significant difference between the other control groups CG4 and CG6 and experimental groups EG4 and EG6, that is, in the analysis periods of four weeks after surgery and six weeks after surgery, with the area of non-scar tissue being greater in the control groups in relation to experimental. When we compare the absolute values of the control group in two, four and six weeks with the values found in the study by Koshinuma., *et al*. [3] it can be seen that in the present study the area of non-scar tissue was close to that found by these authors, showing that there is a beneficial action next to the tissue to be healed and showing that the membrane interacts with the adjacent tissue, improving the repair of the defect.

The results of Koshinuma., *et al*. [3] in mice, demonstrated that membranes of collagen origin are reabsorbed in four weeks, while those originating from PGA (polyglycolic acid) are reabsorbed by hydrolysis and metabolic activity in approximately 15 weeks. They also reported that PGA membranes that were not reabsorbed, could inhibit the formation of periosteum and the proliferation of osteoblasts and thus, delay bone remodeling and, finally, wound healing [23]. In the present study, comparing with what was observed by Koshinuma., *et al*. [3] the chi/xan membranes, which remained on the rats' calvaria, throughout the experiment, allowed the lesion to close, showing that in the observational analysis, this membrane shows itself as an ally in the repair of the critical wound. Based on the time analyzed, future studies, with the possibility of analyzing it in longer times, may show greater closure and repair of critical periosteal defects.

The production of membranes with different proportions between the chitosan and xanthan polymers can help in the search for dressings for tissue and periosteal defects, since in the macro analysis of wound closure, it proved to be efficient, directly helping in the repair of critical defects. As future perspectives for new studies as well, promote analysis of cellular signaling pathways and inflammation and tissue regeneration, as well as prolong the analysis time. With more information in hand and the science of limitation in animal model studies, try to take these future studies to the applicability of this membrane in humans [24-29].

Conclusion

The chitosan/xanthan membrane, in a periosteal skin lesion in the calvary of rats, showed an acceleration of the healing process of wounds of critical size.

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Volume 5 Issue 6 June 2021