

*Full Length Research Paper*

# Effect of the maltodextrin-induced chemical reticulation on the physical properties and healing potential of collagen-based membranes containing Brazilian red propolis extract

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The study evaluated the physical properties and healing potential of chemically modified collagen-based membranes containing hydroalcoholic extract of Brazilian red propolis (HERP) in rodents. The collagen chemical modification was carried out through Maillard's reaction using maltodextrin as reticulant agent, and HERP was incorporated at 0.5%. The HERP-incorporated collagen-based membranes were evaluated regarding the mechanical properties, water vapor permeability and swelling rates. For the wound healing assay, surgical wounds were performed on the back of 100 rats, assigned into five groups (n = 20), whose wounds were dressed as follows: (C) – original membranes; CM – modified membranes; CP – HERP-incorporated original membranes; CMP – HERP-incorporated modified membranes; CTR – undressed wounds (control). Five animals of each group were euthanized at 3, 7, 14 and 21 days and the wounded areas were macroscopic and microscopically analyzed. The CMP-membranes showed lower thickness ( $p < 0.05$ ), permeability ( $p < 0.001$ ) and swelling ( $p < 0.05$ ) than the C-membranes. The CMP-membranes promoted increased wound contraction rates at 3 ( $p < 0.01$ ), 14 ( $p < 0.001$ ) and 21 days ( $p < 0.01$ ) compared to CTR, and provided earlier granulation tissue and cutaneous appendages formation, as well as better organization of the collagen deposition. In conclusion, the CMP-membranes presented advantageous properties to be used as wound dressing and improved wound healing in rodent model.

**Key words:** Collagen, chemical modification, propolis, wound healing.

## INTRODUCTION

Wound healing is a pathophysiological event that occurs in three overlapping biological phases described by inflammatory, proliferative and remodeling phases

(Broughton et al., 2006). The inflammatory phase is characterized by vascular events, which culminates with the influx of leukocytes into the wound site, in order to

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promote microbial and necrotic tissue elimination (Li et al., 2007). The proliferative phase refers to the proliferation of fibroblasts and endothelial cells to form the granulation tissue, and further primary collagen scar, as well as the migration of keratinocytes from the wound edges to restore the epithelial lining of the wound surface (Diegelmann and Evans, 2004). The remodeling phase is characterized by increased collagen turnover and wound contraction in an attempt to restore skin morphological and functional integrity (Rangaraj et al., 2011).

Many studies have been performed in an attempt to find new biomaterials able to accelerate the biological events involved in wound healing, with no deleterious effects on the organism (Vinhas et al., 2007; Nunes et al., 2011; Sargeant et al., 2012; Seth et al., 2012). In this regard, the application of bioactive membranes produced from biocompatible, biodegradable and nontoxic materials have been successfully used as wound dressings in order to improve the healing process of a wide range of dermal wounds (Boateng et al., 2008). The type I collagen extracted from bovine tendon has been applied in the production of such membranes, not only due to its satisfactory biological properties, such as biocompatibility and atoxicity (Neel et al., 2012), but also because collagen-based membranes can act as a matrix for cell proliferation and as a suitable delivery system for drugs, isolated molecules or natural products with biological properties into the wounded area (Albuquerque-Júnior et al., 2009; Nunes et al., 2011; Riella et al., 2012).

It has been reported that the red propolis produced in the Brazilian northeastern presents distinct biological properties and chemical composition from those samples collected from other regions of the country (Nunes et al., 2009). Studies have demonstrated that these samples of Brazilian red propolis display antimicrobial, anti-oxidant (Alencar et al., 2007) and antitumor (Frezza et al., 2013). In addition, we have previously demonstrated that the incorporation of hydroalcoholic extracts of Brazilian northeastern red propolis into collagen-based dressing membranes improves wound healing in rodent model, probably by modulating the dynamics of the inflammatory response and collagenization process (Albuquerque-Júnior et al., 2009).

In the last decade, studies have proposed the chemical reticulation of the collagen chains in attempt to improve some physical properties of collagen-based membranes, such as the low mechanical strength, resulting from the extraction process, and rapid degradation by endogenous enzymes (Sionkowska et al., 2006; Becker et al., 2009).

In this sense, the Maillard reaction, a chemical incorporation process of carbohydrate molecules to residues of hydroxylysine, has been successfully employed to reticulate protein polymers (Cardoso et al., 2011). Therefore, the goal of this study was to assess the effects of the Maillard reaction-induced chemical modification of the mechanical and healing properties of collagen-based membranes containing hydroalcoholic extract of Brazilian

northeastern red propolis in murine model.

## MATERIALS AND METHODS

### Extraction of collagen

The collagen was extracted according to the method described by Albuquerque-Júnior et al. (2009), from bovine tendon which was chopped into small pieces and treated with acetone to remove fatty material. Subsequently, 5 g of tendon were washed with distilled water and placed in 10% NaCl solution (w/v) for 24 h at 4°C. After this period, the tendon was again washed with distilled water and placed in a citrate buffer solution of 0.02 mol/L pH 4.3 for 48 h at room temperature, for swelling of the tissue. The swollen tissue was homogenized in 500 ml of an acetic acid solution 0.5 mol L<sup>-1</sup> in the presence of pepsin at a ratio of 1:50 (w:w) in relation to the initial mass of the material. The gel formed was maintained for 24 h at 4°C. After this period, the protein was precipitated by salting out the gel by adding 5% NaCl (w/v). The collagen precipitate was dialyzed for 72 h against distilled water.

### Modification of polymer using maltodextrin (CM)

The modification of the polymer was carried out by the reaction of collagen with a polysaccharide (maltodextrin (Nidex)). Collagen has been placed to react with 250 ml of 3% maltodextrin, at room temperature for 30 days (Cardoso, 2005), 0.2% methylparaben was added to the middle in order to conserve the system. After this period, the material was dialyzed.

### Hydroalcoholic extract of red propolis (HERP)

The sample of propolis gathered from the region of the Brejo Grande/SE/Brazil (10° 25' 28" S 36° 27' 44" O) was previously crushed and homogenized. The extraction was carried out using the method of maceration. The maceration was performed using 10 g of powdered material in 1000 ml of 70% ethanol during 24 h at room temperature, with constant stirring. After the extraction period, the alcohol was evaporated and a dry extract was obtained.

### Bioactive membranes preparation

To obtain bioactive membranes, collagen (C) and modified (CM) were dispersed in acetic acid 0.5 mol L<sup>-1</sup>. The final polymer concentration was 1%. The extract of propolis has been previously solubilized in propylene glycol (plasticiser) and added to the dispersion of collagen. The concentration of plasticizer and HERP were 20 and 0.5%, respectively on the mass of the polymer. The membranes were produced by casting process, the dispersion being poured into a polypropylene plate to allow solvent evaporation in a stove at 40°C. Membranes were produced with and without HERP.

### Mechanical characterization

#### Tensile testing

The mechanical analysis was carried out in a tensile strength apparatus (TA-TX2, Stable Micro Systems, England). Samples of films were cut into strips of 25 mm × 10 mm. Each strip was measured at three points with a digital micrometer (Micrometer external digital Pantec, accuracy ± 0.001 mm) to record the

**Table 1.** Description of the experimental groups according to the wound dressing applied in the animals.

Group Nomination (n=20)	Dressing Type Applied
CTR	Undressed
C	Original collagen
CM	Modified (reticulated) collagen
CP	HERP-incorporated original collagen
CMP	HERP-incorporated modified (reticulated) collagen

thickness. Each experiment was carried out at room temperature, and 10 replicates were performed. The measure speed used was  $1.0 \text{ mm s}^{-1}$  and the initial gauge length was 10 mm. The calculations were made by:

$$\sigma = F/A$$

Where,  $\sigma$  is tensile strain in Newton  $\text{mm}^{-2}$ ,  $F$  is force in Newton, and  $A$  is cross-section area in  $\text{mm}^2$ .

#### Water vapor permeability rates

The permeability of membranes was determined by water vapor loss in a gravimetric cup film sealed method under known RH given by saturated solutions in contact with non-dissolved salt (KBr 84% RH) placed in a desiccator containing silica in a dehumidified room. Each experiment was performed with five replicates during 48 h. The permeability was calculated by:

$$WVP = (w \times e) / (t \times A \times PV)$$

Where  $WVP$  is the water vapor transmission,  $w$  is lost mass in mg and  $e$  film's thickness,  $t$  is time,  $A$  is the area of the film and  $PV$  is the difference between the vapor pressure of water inside and outside the container.

#### Swelling test

The film swelling studies were conducted using a neutral environment (phosphate buffered saline (PBS) pH 7.2) and an acid medium (PBS pH 1.2). Each film sample (surface area  $2 \times 3 \text{ cm}^2$ ) was pre-weighed and submerged into 30 ml medium in a plastic container. The weight of the film was determined at predetermined intervals after removal of excess surface water with a paper. The test was done in triplicate. The degree of swelling was calculated by:

$$I\% = (Wt \times 100) / W_o$$

Where  $Wt$  is the weight of film at time  $t$ , and  $W_o$  is the weight of dry film.

#### Animals and surgical procedures and groups

The animals used in this study were adult male *Rattus norvegicus albinus*, Wistar lineage, weighing 300 to 350 g. The rats were housed in clear plastic cages with solid floors and loose hardwood chip bedding, and supplied with food and water *ad libitum* in a temperature and humidity-controlled environment. Experimental protocols and procedures were approved by the University

Tiradentes Animal Care and Use Committee (CEUA n° 030308). One hundred rats were anesthetized with intraperitoneal ketamine-xylazine (100 to 5 mg/kg), surgical wounds were then performed with standardized dimensions of  $1 \text{ cm}^2$ . Animals were handled in accordance with the principles of aseptic chain in order to avoid bacterial contamination. Subsequently, rats were randomly assigned into five groups of 20 animals each as described in Table 1. After three, seven, 14 and 21 days, five animals of each group were euthanized in  $\text{CO}_2$  chamber, and the healing/scar area was surgically removed, formalin-fixed and paraffin-embedded for further histological examinations.

#### Assessment of the wound contraction rates (WCR)

For the macroscopic analysis and assessment of the wound contraction rates, after three, seven, 14, and 21 days, the craniocaudal and latero-lateral measures of each wound were assessed by a digital caliper (precision 0.01 mm), prior to the excision of the wounds, and the final wound areas were obtained through the equation:

$$WCR = (A_1 - A_2) / A_1 \times 100$$

Where  $A_1$  is the initial wounded area and  $A_2$  is the final wounded area.

#### Histological procedures and morphological analysis

Serial  $5 \mu\text{m}$  thick sections were obtained from the paraffin-embedded samples and stained in hematoxylin-eosin. The intensity of the inflammatory response was assessed as described in Table 2. For the assessment of the collagen deposition, histological sections stained in Sirius red and analyzed under polarized light were used for the descriptive analysis. Collagen fibers were analyzed according to their birefringence pattern (greenish/yellow-greenish or orange, orange-reddish), morphological appearance (wavy or stretched, thin or thick, short or long), and architectural arrangement (reticular, parallel or interlaced) (Rich and Whitaker, 2005). All readings were performed by investigators blinded to treatments (six histological sections/animal).

#### Statistical analysis

The average values obtained from analysis to characterize the bioactive membrane, as well as the quantitative analysis of the diameter of wounds were compared between experimental groups prefixing the level of significance with 95% ( $p < 0.05$ ). Test was applied by analysis of variance (ANOVA) followed by Tukey's test to verify existence of statistically significant differences between groups, whereas the analysis of the inflammatory infiltrate was carried out by Kruskal-Wallis test.

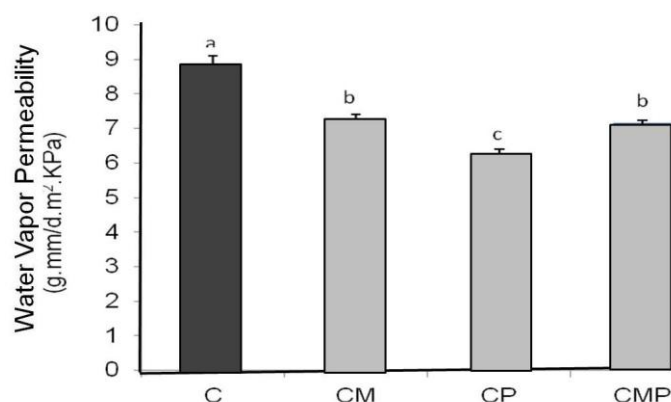
**Table 2.** Description of the parameters for histological assessment of the intensity of the inflammatory response in the wounded areas.

Score of the inflammatory response	Semiquantification of the inflammatory response	Classification of the inflammatory response
0	Absent	Absent
1	Mild	Chronic (predominance of lymphocytes and histiocytes)
2	Moderate	
3	Intense	
4	Intense	Subacute (balance of neutrophils, lymphocytes and histiocytes)
5	Intense	Acute (predominance of neutrophils)

**Table 3.** Assessment of the mechanical properties of C (original collagen), CM (modified-reticulated-collagen), CP (HERP incorporated original collagen) and CMP(HERP incorporated modified-reticulated- collagen) membranes.

Membranes	Thickness ( $\mu\text{m}$ )	Deformation (%)	Rupture tension (MPa)	Young module (MPa)
C	38.9 $\pm$ 5.9 <sup>a</sup>	3.7 $\pm$ 1.5 <sup>a,c</sup>	47.3 $\pm$ 16.6 <sup>a,b</sup>	1308.8 $\pm$ 232.0 <sup>a</sup>
CM	22.2 $\pm$ 1.0 <sup>b</sup>	2.1 $\pm$ 1.5 <sup>b</sup>	36.7 $\pm$ 16.9 <sup>b</sup>	1972.7 $\pm$ 664.8 <sup>b</sup>
CP	28.1 $\pm$ 1.2 <sup>c</sup>	6.4 $\pm$ 2.8 <sup>a</sup>	75.6 $\pm$ 25.5 <sup>a</sup>	1232.2 $\pm$ 263.7 <sup>a</sup>
CMP	19.3 $\pm$ 2.0 <sup>a</sup>	2.0 $\pm$ 1.0 <sup>b,c</sup>	17.9 $\pm$ 5.9 <sup>c</sup>	1010.6 $\pm$ 368.4 <sup>a</sup>

Different letters in the same column express significant different values ( $p < 0.05$ ).

**Figure 1.** Assessment of the water vapor permeability (WvP) of the C (original collagen), CM (modified-reticulated-collagen), CP (HERP incorporated original collagen) and CMP (HERP incorporated modified-reticulated- collagen) membranes. Different letters represent significantly different WvP values ( $p < 0.05$ ).

## RESULTS

### Assessment of the mechanical properties

According to Table 3, both the chemical modification and propolis incorporation procedures reduced the membranes thickness in comparison to C group ( $p < 0.05$ ),

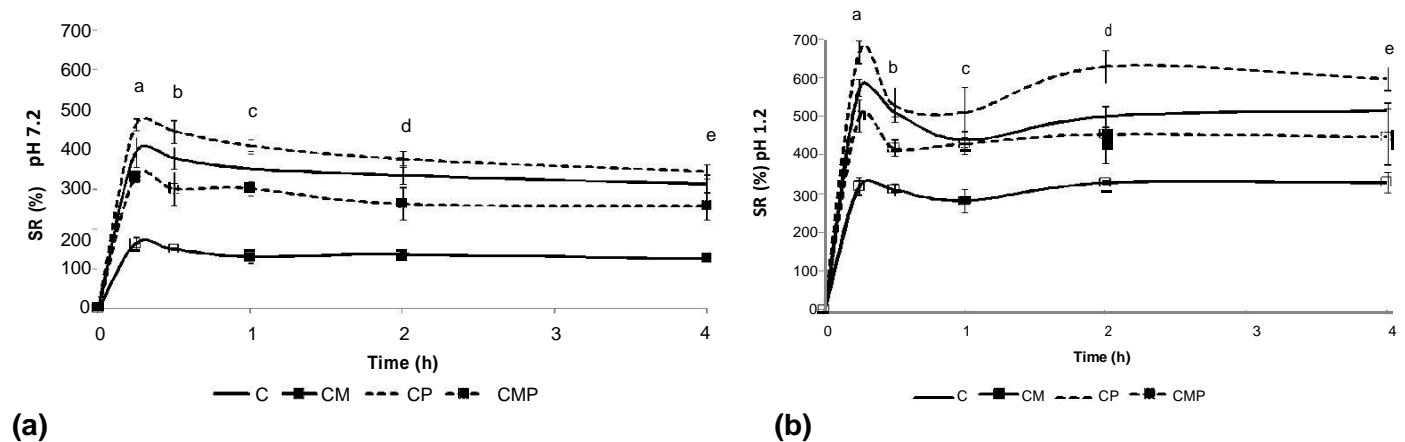
and the CMP were shown to be the thinner samples. In addition, although the tension values of CMP were significantly lower than C, deformation and Young module presented no significant difference between these groups.

### Assessment of the water vapor permeability

As shown in Figure 1, the chemical modification of the collagen and the incorporation of the HERP reduced significantly the WvP compared to the original collagen-based membranes (C) ( $p < 0.001$ ). However, no significant difference was observed in the WvP of CMP and CM ( $p > 0.05$ ).

### Assessment of the swelling rates

Figure 2 presents the swelling rates (SR) of the membranes at neutral (Figure 2a) and acid (Figure 2b) pH. In both environments, the chemical modification of the collagen reduced significantly the SR of the membranes (CM) all over the time course of the experiment. The incorporation of HERP increased the SR of the collagen-based membranes at the initial experimental times, but from 1 h, CP and CMP presented SR similar to those observed in C (original collagen-based membrane) in both pH.



**Figure 2.** (a) Assessment of the Swelling Rates (SR) of the membranes over the time. Where C (original collagen), CM (modified-reticulated-collagen), CP (HERP incorporated original collagen) and CMP (HERP incorporated modified-reticulated-collagen). (a) C is significantly different from CM ( $p < 0.001$ ), CP ( $p < 0.05$ ) and CMP ( $p < 0.001$ ). (b) C is significantly different from CM ( $p < 0.001$ ) and CMP ( $p < 0.05$ ). (c) C is significantly different from CM ( $p < 0.001$ ). (d) C is significantly different from CM ( $p < 0.001$ ). (e) C is significantly different from CM ( $p < 0.001$ ). (b) Assessment of the Swelling Rates (SR) of the membranes over the time. Where C (original collagen), CM (modified-reticulated-collagen), CP (HERP incorporated original collagen) and CMP (HERP incorporated modified-reticulated-collagen). (a) C is significantly different from CM ( $p < 0.001$ ) and CP ( $p < 0.05$ ). (b) C is significantly different from CM ( $p < 0.001$ ) and CMP ( $p < 0.05$ ). (c) C is significantly different from CM ( $p < 0.01$ ). (d) C is significantly different from CM ( $p < 0.01$ ). (e) C is significantly different from CM ( $p < 0.01$ ).

### Macroscopic analysis and assessment of the wound contraction rates (WcR)

Figure 3 presents the macroscopic aspects of the wounds over the time course of the experiment, showing no sign of abscess formation either in the early phases (3 and 7 days) or hypertrophic scars in the final ones (14 and 21 days). The WcR of all the groups increased progressively over the time course of the experiment (Figure 4). CMP presented increased WcR compared to C at three ( $p < 0.01$ ) and 14 days ( $p < 0.001$ ), whereas at 21 days, all of the groups (CM, CP and CMP) showed higher WcR in relation to C ( $p < 0.01$ ). No difference between the groups was seen at seven days ( $p > 0.05$ ).

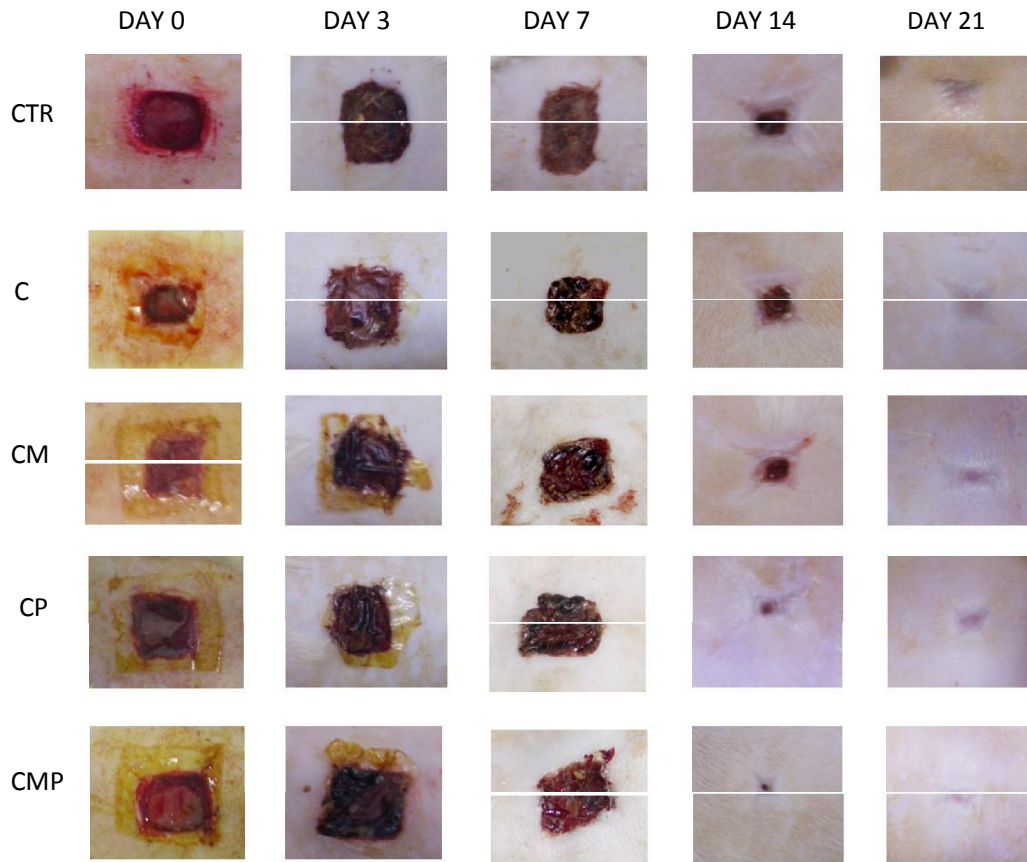
### Analysis of the inflammatory response

As demonstrated in Table 4, at three days the content of neutrophils infiltrate was significantly lower in CP than in CTR ( $p < 0.01$ ), C ( $p < 0.01$ ) and CM ( $p < 0.01$ ), but there was no difference in relation to CMP ( $p > 0.05$ ). No difference between the groups was observed in seven days ( $p > 0.05$ ). At 14 days, the inflammatory score means observed in CTR were significantly higher than those seen in CM ( $p < 0.05$ ) and CMP ( $p < 0.001$ ), but no difference was evidenced between the different wound-dressed groups, irrespective to the membrane applied ( $p > 0.05$ ). At 21 days, although all the groups exhibited only scarce residual inflammatory infiltrate, the mean scores of the wound-dressed groups (C, CM, CP and CMP)

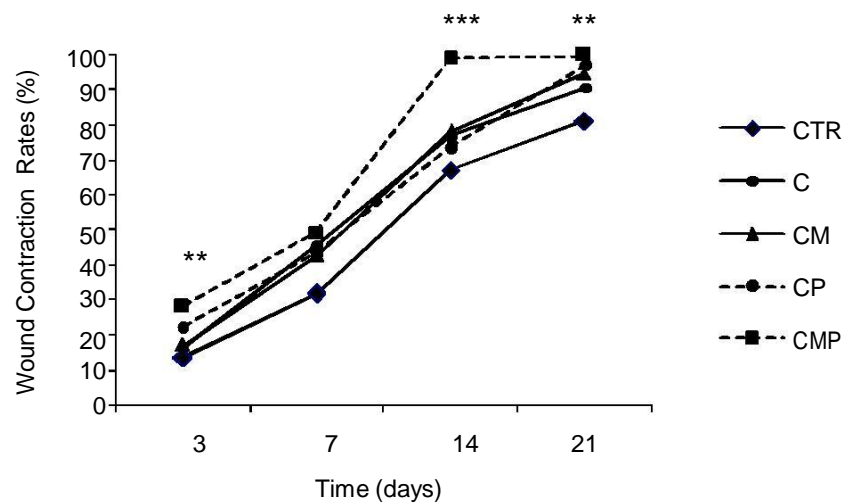
were significantly lower than CTR ( $p < 0.05$ ).

### Morphological analysis of the time course healing process

At three days, the inflammatory response was composed of intense neutrophils infiltrate, particularly in the center of the wounded areas, regardless the group (Figure 5a to e). At seven days, the wounds were filled with an exuberant granulation tissue, with remarkable spindle-shaped cells proliferation (fibroblasts and angioblasts) and formation of irregular and marrowed capillary vessels (Figure 5f to j). Clusters of amorphous eosinophilic material consistent with hyalinized collagen, interpreted as remnants of the chemically modified collagen-based membranes, were observed only in CM and CMP (Figure 5h to j). The epithelization was still incipient in all the groups, corresponding to less than 30% of the wound surface. At 14 days, there was a marked reduction of the vascular-endothelial content, and formation of a primary fibrous scar (Figure 5k to o). The epithelization was advanced and corresponded to more than 80% of the wound surface in all the groups. In CP and CMP, the epidermis was thicker and expressively orthokeratinized, but only in CMP the formation of cutaneous appendages rudiments, such as hairy follicles, was observed in the scar margins (Figure 5o). At 21 days, there was quite a fibrous scar and a mature and intensely orthokeratinized epidermal tissue in all the groups. Cutaneous appendages, however, were seen in the marginal areas of CP,



**Figure 3.** Macroscopic features of the wounds of the different groups over the time-course of the experiment, showing no abscess or hypertrophic/atrophic scar formation. Where CTR (undressed wounds), C (original collagen), CM (modified-reticulated-collagen), CP (HERP incorporated original collagen) and CMP (HERP incorporated modified-reticulated-collagen).



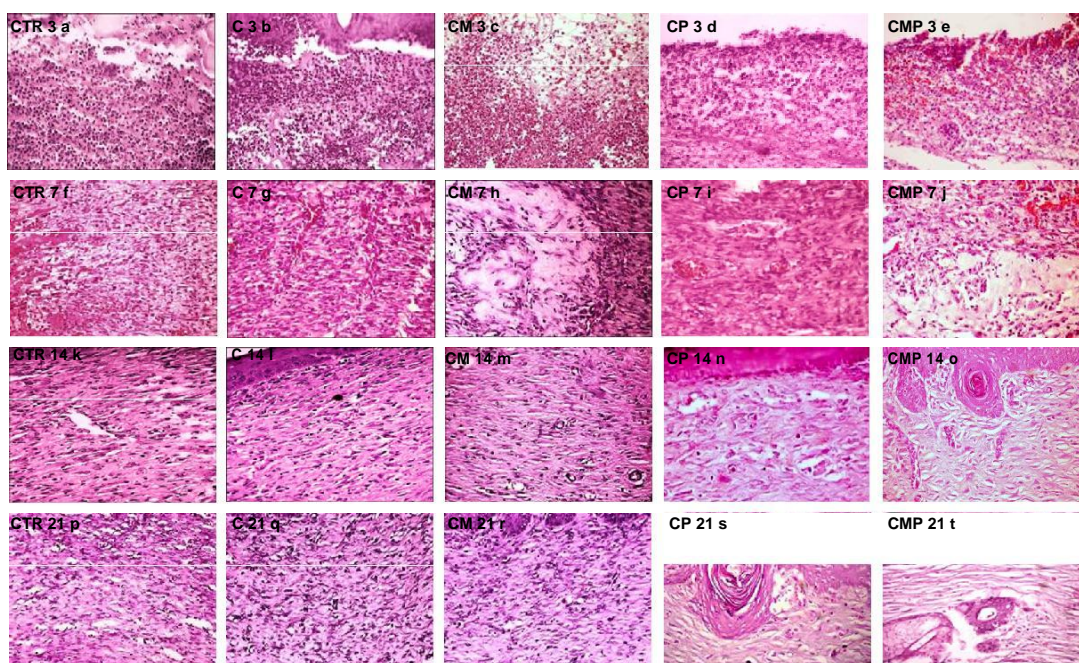
**Figure 4.** Assessment of the Wound Contraction rates (WcR) in the experimental groups over the time course of the wound healing biological assay. \*\*CMP is significantly different from CTR ( $p < 0.01$ ). \*\*\*CMP is significantly different from CTR ( $p < 0.001$ ). CTR (undressed wounds), C (original collagen), CM (modified-reticulated-collagen), CP (HERP incorporated original collagen) and CMP (HERP incorporated modified-reticulated-collagen).



**Table 4.** Histological assessment of the scores (mean± standard deviation) of the inflammatory response intensity in the experimental groups over the time course of the wound healing biological assay.

Time (days)	Groups				
	CTR	C	CM	CP	CMP
3	5.0±0.0 <sup>a</sup>	5.0±0.0 <sup>a</sup>	5.0±0.0 <sup>a</sup>	4.4±0.54 <sup>b,c</sup>	4.6±0.54 <sup>a,c</sup>
7	3.0±0.0 <sup>a</sup>	3.0±0.0 <sup>a</sup>	3.0±0.0 <sup>a</sup>	2.8±0.44 <sup>a</sup>	3.0±0.70 <sup>a</sup>
14	2.2±0.44 <sup>a</sup>	1.4±0.54 <sup>a,d</sup>	1.2±0.44 <sup>d</sup>	1.4±0.54 <sup>a,d</sup>	0.8±0.83 <sup>d</sup>
21	1.4±0.54 <sup>a</sup>	0.4±0.54 <sup>d</sup>	0.4±0.54 <sup>d</sup>	0.4±0.54 <sup>d</sup>	0.4±0.54 <sup>d</sup>

Different letters in the same line represent significantly different values ( $p < 0.05$ ). Where CTR (undressed wounds), C (original collagen), CM (modified-reticulated-collagen), CP (HERP incorporated original collagen) and CMP (HERP incorporated modified-reticulated-collagen).



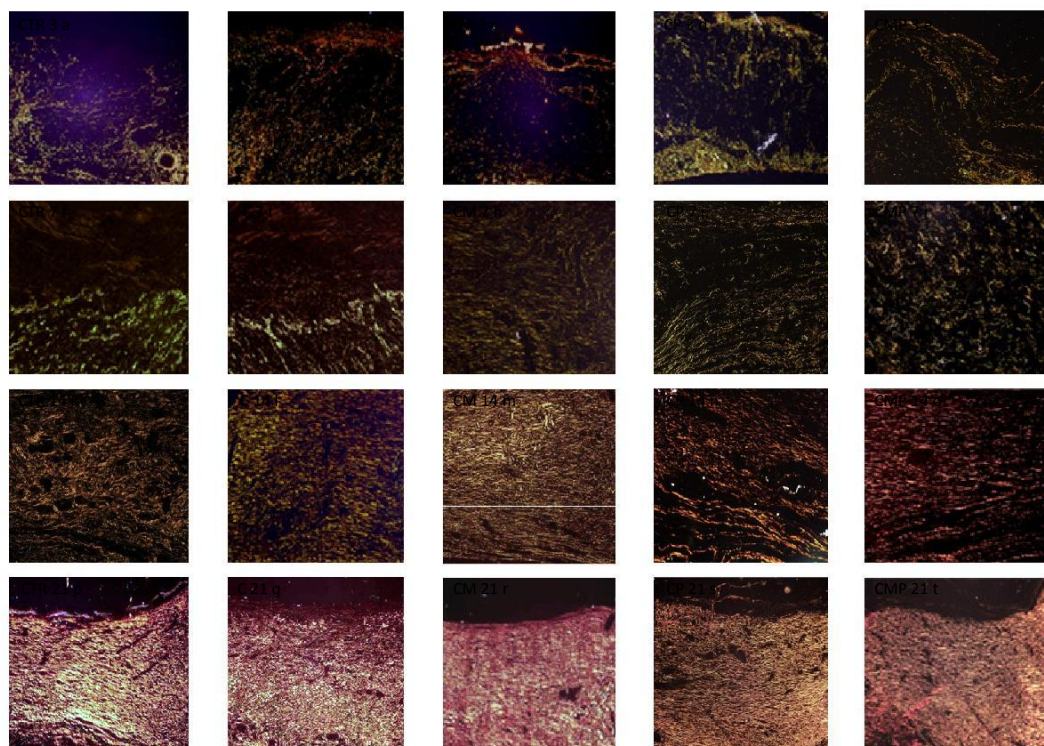
**Figure 5.** Histological sections of the groups (wounded areas) over the time course of the wound healing biological assay. Intense neutrophils infiltrate is seen at three days, and exuberant granulation tissue at seven days (note the presence of amorphous hyalinized material consistent with remnants of collagen-based membranes in CP and CMP (\*). At 14 days, replacement of the granulation tissue by a primary fibrous scar is seen in all the groups, but in CMP is observed the development of a rudimentary hairy follicle. At 21 days, the fibrous scar tissue is denser in all the groups, whereas hairy follicles are evidenced in CP, and sebaceous glands are seen in CMP (HE, 400x magnification). Where CTR (undressed wounds), C (original collagen), CM (modified-reticulated-collagen), CP (HERP incorporated original collagen) and CMP (HERP incorporated modified-reticulated-collagen).

whereas in CMP these structures were found throughout all the extent of the healed areas (Figure 5s to t).

### Morphological analysis of the collagenization

At three days, extremely delicate greenish or yellow-

greenish fibrillar structures (type-III collagen) with varied dimensions and reticular dispositions were observed in all the groups. In seven days, type-III collagen fibers thicker and denser, concentrated in bottom of the wounds in CTR, C and CM, but more homogeneously disposed in CP and CMP. At 14 days, there was replacement of type III for type I collagen fibers (yellow, golden and red



**Figure 6.** Collagen deposition in the experimental groups during the time-course of the study. There is a predominance of thin delicate type III collagen fibrils (greenish birefringence) at three and seven days, whereas thicker and longer type I collagen fibers are seen at 14 days. Ggrossly interlaced type I collagen fibers are observed in all the groups at 21 days (Sirius Red/Polarized Light, 400× magnification). Where CTR (undressed wounds), C (original collagen), CM (modified-reticulated-collagen), CP (HERP incorporated original collagen) and CMP (HERP incorporated modified-reticulated- collagen).

birefringence), which were short, thin, delicate and irregularly disposed in CTR but longer and arranged in a parallel disposition in C, but presented a clear parallel arrangement in the other groups. Moreover, the fibers were longer and thicker in CP and CMP. At 21 days, all the groups presented a dense collagen-rich fibrous scar, with gross parallel arranged and interlaced fibers.

## DISCUSSION

The reduction in the thickness of the HERP-incorporated reticulated collagen-based membranes is likely a result of interactions between chemical groups present in both the reticulant agent (maltodextrin) and the HERP. In this study, the HERP-incorporated reticulated collagen-based membranes were thinner, but did not change the elasticity in relation to the original membrane (COL). It has been previously reported that the reticulation process enhances the interaction forces between the polypeptidic chains, reducing the thickness (Henrique et al., 2008) and elasticity of the membranes. As the incorporation of HERP produced even thinner membranes, but with elasticity modules similar to COL, we hypothesize that

interactions between alkaloid groups present in the HERP and carboxylate and amine groups of the polymer, likely through either hydrogen bond or eletrostactical interactions, which might have approached the polymer, reducing the membrane thickness, while also working as a plasticizer. However, further investigations using nuclear magnetic resonance methods are necessary to clarify the precise intercatenary chemical interactions profiles underlying the mechanical effects on the mechanical properties of the HERP-incorporated collagen-based membranes.

Lower permeability rates are advantageous feature of dressing membranes, as long as prevent wound dehydration (Franco and Gonçalves, 2008). A decrease in the permeability of the CMP membranes was observed in this study. Other investigations have reported the reduction of the water vapor permeability of different polymeric films resulting from the incorporation of propolis extract (Pastor et al., 2010; Bodini, 2011). It has been proposed that the incorporation of HERP increased the concentration of composites into the membranes, increasing the sinuosity of the path taken by the water vapor to cross the membrane (Sinha and Okamoto, 2003).



Low swelling rates are desirable properties in wound dressings, as providing longer-term humidity to the wounds and minimizing the risk of dressing membrane rupture (Assis and Albertini, 2002). We also observed that the swelling of the CMP membranes was reduced at the first hour of the experiment. It has been proposed that narrower spaces between the macromolecular chains, as occurring in the CMP membranes, hamper the interaction of polar molecules (such as water vapor) with the polymeric matrices (Boanini et al., 2010), which would determine lower swelling rates.

In wound healing assays, the wound contraction rates represent valuable parameters to evaluate the dynamics of the wound repair. Such biological event is closely associated to the differentiation fibroblasts into myofibroblasts, a phenotypic change mediated by transforming growth factor beta (TGF- $\beta$ ), a cytokine widely released by mononuclear inflammatory cells within the wound (Thannickal et al., 2003). In this study, the wound contraction rates of CMP were significantly increased in CMP at three, 14 and 21 days.

It has been reported that the peak of myofibroblastic differentiation occurs around the seventh day of wound repair, while the number of such cells tended to decrease dramatically from the 14th day (Ribeiro et al., 2009). However, at the third day, the healing process was still in the acute inflammatory phase, when myofibroblastic differentiation was not supposed to occur yet. On the other hand, previous studies have demonstrated that Brazilian red propolis is rich in flavonoids with anti-inflammatory activity, such as quercetin, formononetin and daidzein (Joshi et al., 2012; Lai et al., 2012; Choi et al., 2012). Therefore, it is possible to suggest that the higher wound contraction rates observed at three days is likely a result of reduced edema instead of myofibroblast-induced wound contraction. Notwithstanding, the increased wound contraction rates observed in CMP at 14 days suggest a possible stimulatory role played by HERP on the myofibroblast differentiation. Supporting this theory, it has been demonstrated that formononetin, a isoflavonoid found in Brazilian samples of red propolis, enhances the local release of TGF- $\beta$ , resulting in increased myofibroblast differentiation (Huh et al., 2011).

The fact that similar findings were not observed in CP groups is suggestive that the chemical reticulation of the collagen might have promoted a prolonged release of the propolis-derived chemical compounds into the wound. In fact, only in CMP clusters of amorphous eosinophilic material consistent with hyalinized collagen, interpreted as remnants of the modified collagen-based membranes, were observed at seven days, suggesting a longer-term persistence of the wound dressing. Supporting our findings, the proteolysis of collagen molecules has been demonstrated to be slower after chemical reticulation compared to wild polypeptides (Yamauchi et al., 2001).

Earlier development of cutaneous appendages was observed in CP and, particularly, in CMP. These findings are suggestive that the chemical compounds play an important role in the proliferation of keratinocytes, as well as in the differentiation of such epidermal cells into cutaneous appendages. Supporting our findings, it has been recently demonstrated that other natural products rich in isoflavonoids, such as formononetin and daidzein, were able to stimulate the differentiation of cutaneous appendages in dermal lesions (Lipovac et al., 2011). Therefore, it is possible to suggest that the CMP membranes improved keratinocytes maturation and differentiation, accelerating the recovery of the epidermal tissue.

Despite the best results observed in CMP group, the fact that all the other groups presented better histological features than CTR is strongly suggestive that the application of collagen-based dressing membranes, irrespective of the presence of HERP or the chemical modification of the collagen is able to improve wound healing. These results are likely associated to the biological properties of the collagen-based wound dressing membranes, such as working as organic matrices for cell attachment and proliferation (Gopinath et al., 2004; Sionkowska et al., 2006). Moreover, wound dressings also provide a mechanical barrier to prevent bacterial contamination and sustain the humidity of the wound environment (Boanini et al., 2010).

The pattern of collagenization was quite similar in the groups over the time course of the experiment, with greenish fibers consistent with type III collagen predominance in three and seven days, and golden type I collagen at 14 and 21 days. Such collagenization pattern is likely related to the fact that type III collagen is formed in the initial phases of wound healing in order to provide a fibrillar network which orientates endothelial growth and blood vessels formation for the granulation tissue development. These delicate fibrils are further replaced by gross type I collagen fibers to provide tensile force and mechanical stability for the connective tissue of the fibrous scar (Albuquerque-Júnior et al., 2009; Nunes et al., 2011; Riella et al., 2012). Nevertheless, the application of the HERP-incorporated collagen-based membranes apparently induced a more regular and homogeneous collagen arrangement at seven and 14 days, but did not promote excessive collagen formation in the final stages of the healing process, as observed in the macroscopic analysis of the wounds. Similar findings were previously reported in previous investigations using non-reticulated collagen-based membranes containing HERP (Albuquerque Júnior et al., 2009), and suggests that this natural product might favour fibroblast proliferation and collagen deposition, and influence the pattern of collagenization, without causing excessive collagenization and hypertrophic scar formation.

## Conclusion

The membranes formed with HERP and reticulated collagen presented suitable thickness, elasticity, permeability and swelling features. In addition, the application of these new membranes was successful in improving wound healing by favoring the wound contraction and reducing the magnitude of the inflammatory response. Therefore, these membranes may be considered a promising new dressing for wound occlusion and tissue repairing.

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