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The incorporation of Brazilian propolis into collagen-based dressing films improves dermal burn healing

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ABSTRACT

Ethnopharmacological relevance: Hydroalcoholic solutions of propolis, a resinous product produced by bees, have been currently employed in improving the cicatricial repair. Biological activity of propolis might be related to its antimicrobial, anti-inflammatory and immunomudalatory properties.
Aim of this study: Investigate the suitability of the collagen-based films containing hydroalcoholic extracts of two different varieties of Brazilian propolis (green and red ones) on the dermal burn healing in rodent model.
Materials and methods: The hydroalcoholic extracts of red propolis (RP) or Green propolis (GP) were incorporated into collagen-based dressing films (COL). Burn wounds were performed in the dorsum of *Wistar* rats and dressing with COL, COL+GPa (0.5%), COL+GPb (1,0%) or COL+RP (0.5%). A control group (CTR) was performed keeping the wound undressed. The histological analyses were carried out after 3, 7, 14, 21 and 30 days for histological assessment of the inflammatory response, epithelization rates (ER), myofibroblastic count (MC) and collagenization pattern.
Results: GPa, GPb and RP provided significant decrease of the inflammatory severity, improved the ER in GPa in 7 ($p=0.000$), 14 ($p=0.000$), 21 ($p=0.005$) and 30 days ($p=0.015$), and induced earlier replacement of type-III for type-I collagen ($p<0.05$) than COL and CTR. In all the groups, the MC increased progressively from 3 to 14 days, and then started to decrease slowly until 21 days. Although no significant difference was observed among the groups in 3, 7 and 30 days, the MC was significantly increased in RP in 14 ($p=0.0001$) and 21 days ($p=0.04$), as well as grosser interlacement of the collagen bundles compared with the other groups.
Conclusion: The incorporation of hydroalcoholic extracts of Brazilian propolis improved the biological events associated to burn healing without toxic effects, but the red variety provided the best results. Therefore, these collagen-based containing natural apicultural products films may be considered a promising new dressing for wound occlusion and tissue repairing.

1. Introduction

The skin works as a barrier to the environment, which is responsible for protecting the organism from water loss and penetration of harmful substances. If this barrier is disturbed by mechanical or thermal action, the skin starts a complex repair mechanism known as wound healing (Fu et al., 2007). The analysis of the kinetic of this biological process in response to different forms of dermal substitution is important for the development of efficient therapeutic products capable of stimulating the wound healing (Alborova et al., 2007).

Despite the fact that there have been many recent advances in dermal substitution and wound healing research areas of medicine, neither the commercially available products nor the materials currently described in experimental studies are able to fully substitute for natural living skin (Shakespeare, 2001). However, healing of dermal wounds with macromolecular agents such as natural polymers is preferred to skin substitutes owing to many advantages such as biocompatibility, nonirritant and nontoxic properties, and ease and safety of the application on dermis (Sezer et al., 2007).

Collagen films have been employed to improve the cicatricial repair of mechanical and chemical damages (Gopinath et al., 2005). Furthermore, in addition to the excellent biocompatibility properties presented by these films, it has been demonstrated that collagen matrices stimulate biological phenomena involved in the success of wound healing, such as myofibroblastic differentiation and fibroblastic proliferation (Helary et al., 2006).

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Some studies have been carried out in order to incorporate bioactive compounds (synthetic or natural) within implantable materials, such as type-I collagen films (Gopinath et al., 2005). These modified films seem to provide controlled liberation of the incorporated product directly within the damaged tissue, and promote acceleration of the granulation tissue development and epithelization process (Helary et al., 2006).

Hydroalcoholic solutions of propolis, a resinous product produced by bees, have been currently employed in improving the cicatricial repair (Ramos and Miranda, 2007). Biological activity of propolis might be related to its antimicrobial, anti-inflammatory and immunomudalatory properties (Moura et al., 2011; Orsatti et al., 2010; Sforcin, 2007; Silva et al., 2012). Research relating propolis and wound healing has been carried out by using different vehicles, such as sponges (Moura et al., 2011), ointments (Sehn et al., 2009) and collagen-based dressing films (Albuquerque-Júnior et al., 2009), but the majority of the papers are focused in the Brazilian green propolis, so that only some few studies pointing at the biological effects of the Brazilian red variety have been reported (Albuquerque-Júnior et al., 2009; Marquele et al., 2005).

Therefore, the aims of this study were to investigate the suitability of the collagen-based films containing hydroalcoholic extracts of two different varieties of Brazilian propolis (green and red ones) on the dermal burn healing in rodent model.

2. Material and methods

2.1. Assessment of the flavonoids content

Flavonoids in propolis hydroalcoholic extracts were expressed as quercetine equivalent. Quercetine (Sigma, Germany) was used to make the calibration curve (standard solutions of 6.25, 12.5, 25.0, 50.0, 80.0 and 100.0 g mL^{-1} in 80% ethanol (V/V)). 0.5 mL of a product (ethanolic solutions of propolis) was mixed with 1.5 mL 95% ethanol (V/V), 0.1 mL 10% aluminum chloride (m/V), 0.1 mL of 1 mol L^{-1} potassium acetate and 2.8 mL water. A volume of 10% (m/V) aluminum chloride was substituted by the same volume of distilled water in blank. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm.

2.2. Preparation of the films containing propolis

Collagen was obtained from bovine tendon through treatment with NaCl, acetic acid and pepsin (Cardoso, 2005). Hydroalcoholic extracts of red propolis was solubilized in polyethylene glycol 400, which was employed as cosolvent and plasticizer. This solution was mixed to 1% collagen dispersion in acetic acid (0.5 mol/L) and the films were obtained by casting process. The final propolis concentration in the film was 0.5 and 1.0%. After solvent evaporation, the films were cut off in square shape (2×2 cm), and sterilized using UV rays (20 min).

2.3. Burning procedures and groups formation

A hundred and twenty-five male Wistar rats (250 ± 50 g), supplied with food and water ad libitum in a temperature and humidity-controlled environment, were anesthetized with intraperitoneal ketamine-xylazine (100 mg/kg–5 mg/kg). Second-degree burn wounds were performed in the back of the animals by the contact of a heated 1 cm^2 standard-sized square-shaped copper plate with the skin for 10 s. 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 ($n=25$): according to the dressing used: CTR (burn wounds), COL (collagen-based dressing), GPa and GPb (collagen-based films containing 0.5 and 1.0% of green propolis, respectively) and RP (collagen-based films containing 0.5% of green propolis). After 3, 7, 14, 21 and 30 days five animals of each group were euthanized in CO_2 chamber and the burned areas were removed, formalin-fixed and paraffin-embedded according to routine laboratorial techniques. Serial 5 μm thick histological sections were obtained and stained by histochemical and immunohistochemical techniques.

2.4. Assessment of the inflammatory profile (IP) and epithelization rates (ER)

The IP was classified as acute (predominance of polymorphonuclear cells) and chronic (predominance of mononuclear cells), and graded as mild/absent (1), moderate (2) or severe (3). The ER was evaluated by measuring the epidermal migration from the normal wound margin to the point where the migrating epithelium stopped processing by using a morphometry software (ImageTool®). ER (%) was determined by the relation between the new epithelium present in the burn wound surface and total area of the burn wound surface.

2.5. Determination of the mean of myofibroblasts count (MC)

Myofibroblasts were detected by using a monoclonal antibody against the α-smooth muscle actin antigen (clone 1A4; 1:200, 12 h, Dako, Glostrup, Denmark). After washing in PBS, slides were incubated with biotin-labeled antimouse secondary antibodies (Vector Laboratories Inc., Burlingame, CA), then washed in PBS, and incubated with peroxidase-labeled streptavidin (DAKO). The reaction products were visualized by immersing the slides in freshly prepared diaminobenzidine (Dojindo, Kumamoto, Japan). Ten histological sections ($\times$ 40, 10 ocular, 0.739 mm^2 per field) were randomly selected and the mean of immunostained cells was assessed.

2.6. Assessement of the collagen deposition

Histological sections stained in picrosirius and analyzed under polarized light were used to the descriptive analysis of the collagen deposition. 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 disposition (parallel-arranged or interlaced).

2.7. Statistical analysis

Statistical significance of the quantitative measurements was assessed by analysis of variance (one-way ANOVA) and Tukey test. Each time point was analyzed separately, and two-tailed α-levels of $P < 0.05$ were regarded as significant.

2.8. Ethics aspects

In accordance to the institution's guidelines outlined in “Guide for the Care and Use of Laboratory Animals”, it is hereby assured that all animals received humane care during all the steps of the experimentation. Furthermore, the study protocols were approved by our National Research Council prior to the beginning of the experiments.

3. Results

3.1. Propolis extract yield

The yield of the hydroalcoholic extraction process of the green propolis was 41.43%, whereas the red propolis was 48.8%. On the