QUANTITATIVE ANALYSIS OF MATURE AND IMMATURE COLLAGENS DURING ORAL WOUND HEALING IN RATS TREATED BY BRAZILIAN PROPOLIS

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Abstract

Immature and mature collagens presents in oral lesions of rat were evaluated. Oral ulcer was chemically produced on the tongue of 56 male rats and, then, treated by topic application of solution of propolis (experimental group) and saline solution (control group). The animals were treated for seven days and sacrificed at 2nd, 7th, 14th, and 21th days. Samples of the treated area were processed in laboratory and stained by picrosirius. Mean areas of immature collagen in the experimental group at 2nd, 7th, 14th, and 21th days were, respectively, 60.41 µm ± 18.3, 67.2 µm ± 16.2, 90.77 µm ± 6.64 and 56.48 µm ± 7.56. Mean areas of mature collagen evaluated for this same time were: 39.58 µm ± 18.31, 32.8 µm ± 16.2, 9.23 µm ± 6.64 and 43.53 µm ± 7.56.

There was a significant difference between the mean areas of mature and immature collagens between groups at 2nd and 14th days (Tukey's test, p≤0.05). Propolis used in this study had contributed positively to the wound healing of oral ulcers of rats, probably, due to concentration and alcoholic vehicle.

Experimental article (J Int Dent Med Res 2011; 4: (3), pp. 106-110)

Keywords: Propolis, Collagen, Inflammation, Wound healing, Oral ulcer.

Received date: 13 August 2011 Accept date: 05 Nowember 2011

Introduction

The ability of the body to replace injured or dead cells and to repair tissues after an injury is critical to survival[1]. The entire wound healing process involves a complex series of events that begins at the moment of injury and can continue for months to years. Wound healing in skin or oral mucosa proceeds by various stages that can be defined as a primary hemostatic event followed by an inflammatory response, a proliferative phase in which new and extracellular matrix components are produced, and finally a remodeling phase involving reorganization of the matrix to give functional tissue[2].

Oral ulcerations are common complaints of patients attending out-patient clinics. These lesions which affect oral mucosa are classified as acute and chronic[3]. Most acute oral ulcers heal spontaneously without specific therapy being necessary, but an understanding of the cause of the ulcer is reassuring to the patient and guides the clinician in management to prevent recurrent episodes of oral ulceration, or chronicity of ulcers[4].

Meanwhile, the use of natural medicines to heal these lesions faster has been the goal of several researches. Some studies have examined the wound healing in several tissues treated by propolis[5-7]. Propolis is a resin-like compound extracted from plants by bees. The chemical composition of propolis is complex and several compounds already were identified and represent alcohols, aldehydes, aliphatics acids and esters, amino acids, aromatic acids and esters, flavonoids, ketones and sugars[8].

A study realized in animals has demonstrated that caffeic acid phenethyl ester (CAPE) of propolis is able to increased the submucosal collagen content during wound healing after esophageal caustic injuries[9].

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Meanwhile, there is no evidence if propolis is able to modify collagen production during wound healing of oral mucosa.

The present study was undertaken to examine the mature and immature collagen production which accompany the healing of chemically-induced wounds in oral mucosa of rats treated by Brazilian propolis.

Materials and methods

The experimental protocol of the present study was approved by Ethics Committee on Human and Animal Research of the Pontifícia Universidade Católica do Paraná - PUCPR.

Animals and drugs

Fifty-six male albino rats (Rattus norvegicus albinus, Rodentia, Mammalia) weighing 200-250 g were used in this study. After general anesthesia induced with thiopental sodium® (Cristália, Brazil, 20mg/Kg, i.p.), an ulcerated lesion was topically induced on the tongue using 40% sodium hydroxide solution. The animals were maintained in individual cages and received standard solid food and water *ad libitum*.

Two experimental groups were prepared:

i) Control – twenty-eight animals were treated daily by topic application of saline solution for seven days.

ii) Experimental – twenty-eight animals received daily topic application of a solution alcoholic of propolis (3 mL of pure extract of propolis dissolved in 7 mL of ethylic alcohol) for seven days.

The animals were killed under general anesthesia induced with thiopental sodium® (Cristália, Brazil, 20mg/Kg; i.p.) in groups of seven after post-treatment periods of 2nd, 7th, 14th, and 21th days. The tongues were removed and fixed in 10% formalin solution and subjected to routine laboratory studies after sectioning at a thickness of 6 μm and staining with hematoxilin and eosin and picrosirius.

Collagen analysis

Collagen was analyzed by Picrosirius (Sirus Red) histochemical method under light and polarized microscopy. Collagen analysis was done in sections of ulcerated area using a microscope of binocular light OLYMPUS BX50 equipped with an objective PLAN 10X/0,25 (Olympus, Japan), ocular WH10X-H/22 (Olympus, Japan) and connected to video camera (Color video camera CCD-IRIS of SONY) and ImagePro Plus analysis program 4.0.1 (Media Cibernetics, Atlanta, GA, USA). Results were expressed as μm^2 (mean ± SD).

Statistical analysis

All data were tabulated and statistical tests were performed with SPSS for Windows 13.0 (SPSS Inc., Chicago, Illinois, USA). For each group, mean ± SEM was calculated and the data was analyzed by Tukey's test. Differences were considered statistically significant when $p < 0.05$.

Results

Tests of normality (Kolgomorov-Smirnoff test) and homogeneity of variances (Levene's Test) in function of all the variables were used. All the tests had accused normality of the data and homogeneity of variance between the treatments to a level of probability of $p<0.05$.

The quantity of collagens present in the cicatricial tissue of the oral ulcers treated by propolis has showed a significant variance according to the time. The mean areas of immature and mature collagen are showed in the tables 1 and 2, respectively.

	2 days Mean ± SD	7 days Mean ± SD	14 days Mean ± SD	21 days Mean ± SD
E	60,4 μm² ±18,3*	67,2 μm² ±16,2	90,77 μm² ±6,6*	56,5 μm² ±7,5
C	84 μm² ±5,0*	69,94 μm² ±12,9	66,64 μm² ±7,6*	64,7 μm² ±11,2

Table 1. Mean values of immature collagen area in cicatricial tissue of oral ulcers treated by propolis (E) or saline solution (C) in different time intervals. F= 11.87. p<0.01 *Tukey test:(p=0.05)

	2 days Mean ± SD	7 days Mean ± SD	14 days Mean ± SD	21 days Mean ± SD
E	39,6 μm² ±18,3*	32,8 μm² ±16,2	9,23 μm² ±6,6*	43,5 μm² ±7,5
C	16 μm² ±5,0*	30,06 μm² ±12,9	33,36 μm² ±7,6*	35,3 μm² ±11,2

Table 2. Mean values of mature collagen area in cicatricial tissue of oral ulcers treated by propolis (E) or saline solution (C) in different time intervals. F= 11.87. p<0.01 *Tukey test:(p=0.05)

The mean area of immature collagen was significantly higher in control group during the

second day post-treatment. Meanwhile, the experimental group has showed the highest mean area of immature collagen at the 14th day of post-treatment.

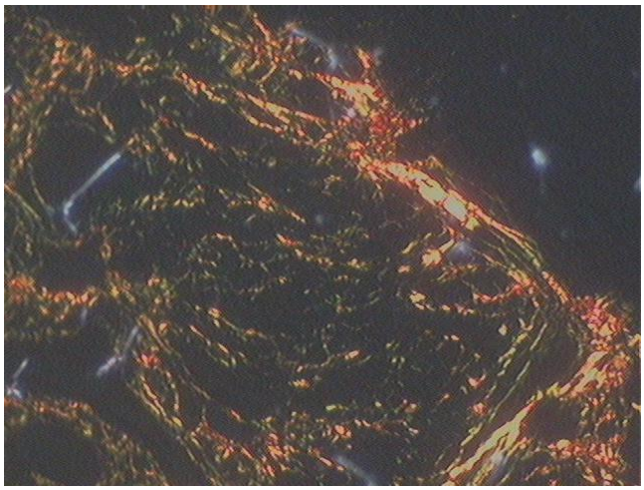

Figure 1. Aspect of the cicatricial tissue treated by propolis during 2 days showing the beginning of the immature collagen (Picrosirius, 20x).

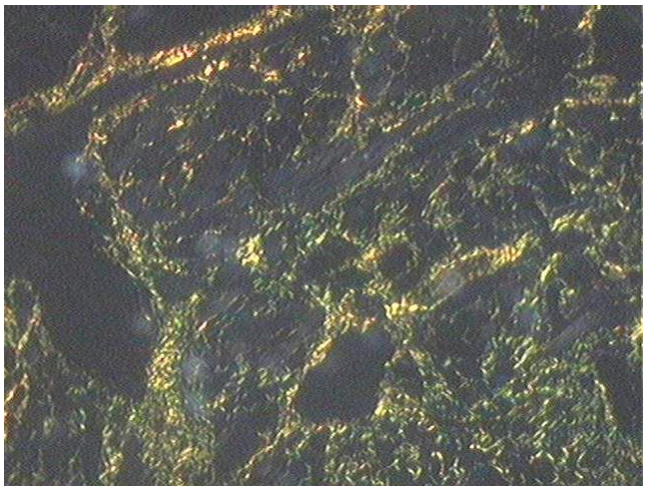

Figure 2. Aspect of the cicatricial tissue treated by propolis during 7 days showing the highest quantity of immature collagen (Picrosirius 20x).

The quantity of mature collagen was greater in experimental group compared to control sections at 2nd, 7th, and 21th post-treatment days, as shown in Table 2. This increase in mature collagen was significant only at 2nd post-treatment days ($p<0.05$). Only at the 14th day of post-treatment, the mature collagen was higher in the control group.

Histologically, the slices of the experimental group showed the beginning of the immature collagen production. This type of collagen was characterized by delicate fibrils (figure 1). The production of immature collagen was accentuated at 7th day post-treatment with propolis and the bundles of fibers were denser (figure 2). At the 14th day post-treatment, the collagen persists denser and stained in red colour. This fact indicates its maturation. The mature collagen predominates at 21th day of post-treatment. It was characterized by denser bundles of red fibers (figure 3).

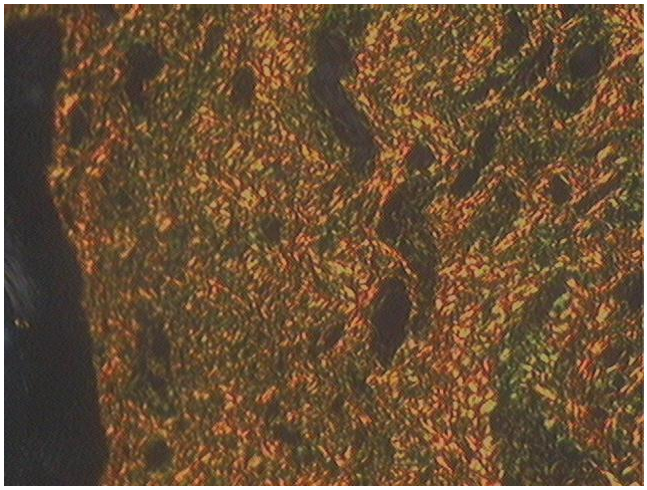

Figure 3. Aspect of the cicatricial tissue treated by propolis during 21 days showing the highest quantity of mature collagen (Picrosirius 20x).

Discussion

Dermal disruption in the cutaneous or mucosal wound triggers a cascade of inflammatory events involving cellular mediators, cytokines, and growth factors. Inevitably, the end result is dense collagen deposition and scar formation[10].

The entire wound healing process is a complex series of events that begins at the moment of injury and can continue for months to years. The final part of the proliferative phase is granulation tissue formation. In this phase, fibroblasts differentiate and produce ground substance and then collagen. The ground substance is deposited into the wound bed; collagen is then deposited as the wound undergoes the final phase of repair[1].

Propolis, a naturopathic substance derived from bees wax extract, has recently been praised for its antimicrobial, anti-inflammatory, and cicatrization-enhancing properties[12]. This study tested a bee-hive product propolis as a drug to treat oral ulcers of rats and analyzed its

effects on the mature and immature collagen production which accompany the healing of chemically-induced wounds in oral mucosa of rats.

The distribution of immature collagen between groups has showed an increase in the mean areas of the 2nd until 14th day post-treatment. This fact can be associated to the collagen maturation. Differently, there was a decreasing of immature collagen in control group. The quantity of immature collagen was more expressive in experimental group during the 14th day post-treatment. This result can be reflex of a pharmacologic event called of taquifilaxy which cumulative doses would add to a therapeutic potency on propolis solution.

The action of different alcoholic propolis solution was evaluated on cultures of fibroblast. The results showed that lower concentration of propolis (1%, 2% or 4%) were more efficient and no toxic to the fibroblasts[13]. These concentrations are considerably lower than one used in this study. Probably, the 30% propolis solution has induced fibroblast death and this reflected in a slower wound healing.

The development of a scar involves in the influx of inflammatory cells into the wound, and the production of growth factors for fibroblast, such as transforming growth fibroblast ß [14]. In response to these, fibroblasts migrate into the wound and synthesize collagen. Collagen is the most common protein in the animal world, providing the extracellular framework for all multicellular organisms. The collagens are synthesized by fibroblasts and are composed of a triple helix of three polypeptide α chains, having gly-x-y repeating sequence. Initially, type III collagen is the principal collagen synthesized, but this is gradually replaced by type I collagen[1]. Whereas type I collagen is found in all dermal layers, the main part of type III collagen can be found within the adventitial dermis[11].

Many other different cytokines are involved in the proliferative phase of wound repair[1]. The steps and the exact mechanism of control have not been elucidated. Transforming growth factor- ß stimulates fibroblast chemotaxis and the production of collagen by cells, while inhibiting collagen degradation by decreasing proteases and increasing protease in inhibitors[1]. Propolis and its constituents (flavonoids hesperidin, quercetin and caffeic acid phenethyl ester) are able to increase the production of TGF-ß by T lymphocytes[15].

Polarization microscopy and sirius red staining was used in this study. It is considered an ideal method in analyzing the distribution and arrangement of the collagen in scars[16]. Visualization by crossed polaroid filters allowed estimation of collagen type I, seen as thick yellow, orange or red coloured fibers; collagen type III fibers are thinner and stained in pale green shades.

Conclusions

Our preliminary results showed that more mature collagen is deposited when oral wounds are treated with propolis solution. In general, wounds treated with propolis products consistently showed less inflammation and more rapid cicatrization. Propolis appears to have a beneficial effect on the healing of partial thickness burn wounds[17]. The results of this study provide several insights into the healing which occurs following chemically-induced trauma of oral mucosa treated by propolis. Most of these wounds are ulcers relatively superficial, and while the epithelium was destroyed, there was little involvement beyond the lamina propria and tongue musculature.

Acknowledgements

The authors would like to profusely thank UNIMEL for propolis samples.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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