MAST CELL POPULATION ON ORAL ULCERS OF RATS TREATED BY BRAZILIAN PROPOLIS

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Summary

Propolis has been used as a pharmacological agent due to therapeutic properties. The aim of this study was to analyze numeric changes of mast cell population during wound healing of oral ulcer of rats treated by propolis. An oral ulcer was chemically induced on the tongue and treated topically with 30% propylene glycol solution of propolis (experimental group) and saline solution (control group). A tissue fragment of the treated area was processed in laboratory and mast cells were counted in epithelial/connective tissue and submucosa areas using ImagePro analysis program 4.0.1. Mast cell numbers for epithelial/connective tissue region in experimental group were $86 \pm 38,8$ cells/mm^2, $44,8 \pm 15,7$ cells/mm^2, $48 \pm 9,9$ cells/mm^2, and $52,4 \pm 35,6$ cells/mm^2 at 2, 7, 14, and 21 post-treatment days, respectively. The numbers of mast cell in submucosa region were, respectively, $70,8 \pm 39$ cells/mm^2, $62,6 \pm 21$ cells/mm^2, $76,4 \pm 21,5$ cells/mm^2, and 79 ± 12 cells/mm^2 at 2, 7, 14, and 21 post-treatment days. There was a significant increase in mast cell numbers at second day of post-treatment (Tukey test, *P<0.05*). This study revealed that 30% propylene glycol solution of propolis was able to increase mast cell population during wound healing of oral ulcer of rats. According to these results, propolis is able to accelerate the wound healing.

Keywords: Propolis; mast cell; inflammation; wound healing; ulcer.

Introduction

Most cases of oral ulcers presenting in general practice are due to recurrent aphthous ulceration, infection or trauma. However, many of the reports in the literature have not been validated in controlled clinical trials. Ulcers related to trauma usually resolve in about a week after removal of the cause and use of widely pharmacological agents (28).

Propolis is a natural product collected by bees from plant sources that is used to seal holes and repair many structures in the hive (11). The biological activities of propolis include antibacterial, antifungal, antiprotozoan, antitumoural, immunomodulation, anti-inflammatory, and others therapeutic properties. Propolis has been used for ulcers treatment by folk medicine for a thousand years. Although a number of reports dealing with this point have been published, very few have focused the effect of propolis on mast cells population in ulcers.

In these circumstances, the ulcer is repaired through growth and re-development of tissue remodeling, growth of new blood vessels (angiogenesis), and re-innervation of the mucosa by the extrinsic and intrinsic nerves. Enhanced numbers of inflammatory cells, mainly mast cells, are well known to occur during angiogenesis and tissue remodeling (2). The mast cell is a tissue-based inflammatory cell of bone marrow origin that responds to danger signals of innate and acquired immunity with immediate and delayed release of inflammatory mediators (31). Direct examination of the spatial distribution of mast cells in normal oral mucosa revealed that mast cells are distributed preferentially about the superficial lamina propria, in close proximity to the abluminal surface of vascular endothelium and to nerves (32). Human mast cells are capable of synthesizing and secreting an array of cytokines. The cytokines appear to vary on conditions of culture, type of disease, and the degree and type of stimulus. Others cytokines reported to be produced by human mast cells include IL-4, associated with T_H2 cell differentiation and IgE synthesis; IL-3, GM-CSF, and IL-5, critical for eosinophil development and survival; and IL-6, IL-8 and IL-16 (22).

The aim of this study was to investigate the effect of propolis in mast cell population during the wound healing of oral ulcers chemically induced.

Materials and methods

The experimental protocol of the present study was approved by Ethics Committee on Animal Experimentation at Universidade Tuiutí do Paraná – Curitiba/PR, Brazil.

Animals and drugs
Seventy 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), an ulcerated lesion was chemically induced on the tongue using 40% sodium hydroxide. The animals were maintained in individual cages and received standard solid food and water *ad libitum.*

Two experimental groups were prepared:

i)   Control – thirty-five animals received daily topic application of saline solution for seven days.
ii) Experimental - thirty-five animals received daily topic application of a 30% propylene glycol solution of propolis (3 ml of pure extract of propolis dissolved in 7 ml of propylene glycol) for seven days.

The animals were killed under general anesthesia induced with thiopental sodium® (Cristália, Brazil, 20mg/Kg) in groups of seven after post-treatment periods of 2, 7, 14, and 21 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 0,2% toluidine blue.

Mast cell counting
Mast cells were counted separately 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 analysis program 4.0.1 (Media Cibernetics, Atlanta, GA, USA). Mast cells were counted in 4 counting fields per section at 100× magnification. In order to assess distribution, the counting fields were distributed in two areas located at the epithelial/connective tissue and submucosa (25 adjacent counting fields per area) by one calibrated observer blinded to the objectives of this study (25). Results were expressed as cells/mm^2 (mean ± SEM).

Statistical analysis

All data were tabulated and statistical tests were performed with SPSS for Windows 13.0 (SPSS Inc., Chicago, Illinois, USA). Significant statistical differences between groups were examined using Tukey test. Differences were considered statistically significant when $P < 0.05$.

Results

Abundant mast cells were found immediately under basement membrane in all sections studied (mostly in the lamina propria, within inflammatory infiltrates, perivascularly and in the deep connective tissue). However, intraepithelial mast cells could not be detected. The average mast cell number in the epithelial/connective tissue region was greater in experimental group compared to control sections at 2, 7, and 21 post-treatment days, as shown in Table 1. This increase in mast cell count was significant only at 2 post-treatment days ($P<0.05$).

The numbers of mast cells were abundantly increased for both groups in the submucosa area when compared to epithelial/connective tissue region except in the animals treated by propolis for 2 days. The average mast cell number in the submucosa region was greater in experimental group compared to control sections at 2, and 21 post-treatment days, as shown in Table 2.

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 $P<0.05$.

TABLE 1 – Number of mast cells at the epithelial/connective tissue.

PERIOD	EXPERIMENTAL GROUP (mean ± sd)	CONTROL GROUP (mean ± sd)	*P value*
2 days	86.0±38.8	36.8±10.4	0.018900*
7 days	44.8±15.7	41.8±14	0.9999981
14 days	48.0±9.9	51.4±7.9	0.2432682
21 days	52.4±35.6	50.8±12.5	1

*Tukey test: P<0.05

TABLE 2 – Number of mast cells at the submucosa.

PERIOD	EXPERIMENTAL GROUP (mean ± sd)	CONTROL GROUP (mean ± sd)	*P value*
2 days	70.8±39	50.2±18.9	0.808764
7 days	62.6±21	64.2±9.4	1
14 days	76.4±21.5	93.6±21.3	0.913081
21 days	79.0±12	63.4±18.8	0.946181

*Tukey test: P<0.05

Discussion

Evaluation of the biological activities of compounds in propolis and elucidation of the mechanisms of their functions provide substantial clues for the development of new drug candidates. In previous studies was demonstrated that propolis accelerates the wound healing in dental pulp, skin and oral mucosa (3,5,19,20).

This study analyzed the effect of a Brazilian propolis on the mast cell population during the wound healing of oral ulcer in rats. In Brazilian propolis other classes of bioactive components, instead of flavonoids, have been described, such as prenylated phenolic acids and specific terpenoids (7). Chemical studies of propolis have showed the presence of flavonoids and related compounds such as phenolic acids, which may be responsible for its antibacterial, antifungal, antiviral and anti-inflammatory activities (11,16,21,26).

The propolis sample used was obtained in the state of Paraná (South of Brazil). This kind of propolis is rich in 2,2-Dimethyl-8-prenyl-2H-1benzopyran-6-propenoic acid, 3,5-Diprenyl-4-hydroxycinnamic acid, 3-Prenyl-4-hydroxycinnamic acid, and Cinnamic acid derivative (7). This sample of propolis was dissolved in propylene glycol in order to minimize the effects of an undesirable alcoholic vehicle on the wound healing.

The inflammatory process involves production and/or release of mediators from neurons, cells, and damaged tissues, which are responsible for different responses aiming the healing. Mast cells play an important role in this process. Mast cells possess high-affinity receptors for IgE (FcεRI) in their plasma membranes. Cross-linking of these receptors is essential to trigger the secretion of histamine and other preformed granule-associated mediators and to initiate the generation of newly formed phospholipids-derived mediators (9). Various flavonoids have been shown in several systems to inhibit this secretory process (23). Quercetin, kaempferol, and myricetin were found to inhibit the release of rat mast cell histamine (24). Several others studies have also described inhibition of histamine release from mast cells by certain flavonoids (1,6,8,12,15).

Although interesting, the finding of increased mast cell number induced by 30% propylene glycol solution of propolis raised several obvious questions. Firstly, which is the probable mechanism that mediates such increasing? It seems surprising that there is a significant increase in mast cell numbers epithelial/connective tissue region within 2 days. For this reason, it is difficult to ascribe these changes to mast cell differentiation

from bone marrow mast cell precursors. It is believed that some propolis compound could induce mast cell migration from other anatomic sites. Secondly, why does mast cell number increase? Mast cell clearly also play a role in many pathologic events associated with hypersensitivity (4). However, mast cells also play a protective role in mucosal defense; thus, they have both positive and negative effects but, at present, the mechanisms that control the balance of these various effects are poorly known (30). The increased number of mast cell population observed in this study serves to enhance the production of cytoprotective mediators, such as nitric oxide or TNF-α (tumour necrosis factor-α).

Human mast cells are capable of synthesizing and secreting an array of cytokines. The cytokines appear to vary on conditions of culture, type of disease, and degree and type of stimulus. Through diverse effects on many different cell types, these products can directly or indirectly result in both acute changes and chronic effects. Studies in mast cell-deficient mice or others lines of evidence indicate that when conditions suitable for mast cell activation persist over long periods of time, mast cells can contribute to such tissue changes as enhanced proliferation of epithelial cells, increased production and deposition of collagen and others interstitial proteins, angiogenesis, enlargement of smooth muscle cells and remodeling of bone and joints (10).

It has long been speculated that pro-inflammatory cytokines play an important role in wound repair. However, little is known about the temporal and spatial expression pattern of these cytokines during normal and impaired wound healing. Hubner et al. have showed a strong and early induction of interleukins 1 alpha and beta (IL-α and β) and of tumour necrosis factor alpha (TNF-α) expression after cutaneous injury. Highest levels of these cytokines were seen as early as 12-24 h after wounding. After completion of the proliferative phase of wound healing, mRNA levels of these cytokines returned to the basal level. During the early phase of wound repair, proinflammatory cytokines were predominantly expressed in polymorphonuclear leukocytes, suggesting a novel function of these cells in the initiation of wound healing. At later stages of the repair process,

expression of IL-1 α, IL-1 β and TNF-α was also seen in macrophages. Furthermore, TNF-α was detected in the hyperproliferative epithelium at the wound edge and IL-1 α was found in keratinocytes. Induction of these cytokines after injury was significantly reduced during wound repair in healing-impaired corticosteroids-treated mice (13). This finding demonstrates that wound healing defects are associated with impaired cytokine expression and suggests that the early induction of these genes is important for normal repair.

TNF-α, according to all studies, appears to be a major cytokine produced by human mast cells. It appears to be both stored and synthesized after mast cell activation. TNF-α upregulates endothelial and epithelial adhesion molecules. Other cytokines reported to be produced by human mast cells include IL-4, associated with T_H2 cell differentiation and IgE synthesis; IL-3, GM-CSF, and IL-5, critical for eosinophil development and survival; and IL-6, IL-8 and IL- 16 (22). Human mast cells are also documented to produce chemokines, such as macrophage inflammatory protein-α. Chemokines are a family consisting of at least ten distinct novel 8-10 kD cytokines. The cysteine-cysteine (C-C) chemokines are chemoattractant and activators for monocytes, T cells and mast cells. RANTES is the prototype of the C-C chemokine subfamily, purified from different sources with chemoattractant and activator properties. Kempuraj et al. believe that presence of mast cells for the production of RANTES in the inflammatory process and contribute to an understanding of the mechanism by which RANTES profoundly affects inflammatory responses in vivo (14).

The gamisopoonghwanghyul-tang (GSPHHT) has been used as a traditional Korean medicine for the treatment of inflammatory diseases; this herbal has a terpenoids, which acts on production of inflammatory cytokines by activated human mast cell. Because the mast cell contains potent mediators, including multifunctional cytokines, its potential contributions to the processes of inflammation and matrix degradation (29). Our results suggest that the propolis regulates production of inflammatory cytokines from activated mast cells, because it has high levels of terpenoids.

A series of phenolic acid esters from herbals have been synthesized. The effects of phenolic acid derivatives on antiinflammatory activity induced production of superoxide anion, an inflammatory mediator produced by neutrophils (17). These results reforce propolis effects (rich in phenolics) in mast cells expression. Concluiding phenolic acid derivatives may exert their antiinflammatory action through inhibiting superoxide generation.

The effects of herbal flavonoids have been studied on LPS-induced (lipopolysaccharide) delay in spontaneous apoptosis and adhesion molecules. As one of the pro-inflammatory factors, LPS aggravates inflammation through priming neutrophils to synthesize/release cytotoxic contents and prolonging functional lifespan of neutrophils by delaying the spontaneous apoptosis. The flavonoids decreased the susceptibility of neutrophils to pro-inflammatory factors (e. g. LPS) (18). Our results showed that propolis increased mast cell population suggesting that propolis pro-inflammatory factors could have inhibitory effects.

The present research represents the first study suggesting that a standardized propolis extract could increase the mast cell population on chemically induced ulcers during the wound healing. This fact reinforces the use of propolis in therapy with oral ulcerated lesions.

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