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Evaluation of the Contraction of Cutaneous Wounds in *Wistar* Rats Treated with Brazilian Green Propolis Gel

Telmo. J. MEZADRI [1], Mateus F. LEITE [2], Marcos C. STAACK JÚNIOR [1], Paulo H. COLCHON [1], Alexandre B. BALAN [1], Matheus L. ZANATTA [1], Imyra C. COSTA [1], Caroline PERIN [1], David R. TAMES [1], João P.B.SOUSA [2], Jairo K. BASTOS [2] & Sergio F. ANDRADE [3]*

[1] *Centro de Ciências da Saúde, Universidade do Vale do Itajaí, Itajaí, SC, Brazil.*

[2] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.*

[3] *Programa de Mestrado em Ciências Farmacêuticas, Núcleo de Investigações Químico-Farmacêuticas (NIQFAR), Universidade do Vale do Itajaí – UNIVALI, Rua Uruguai, 458, Bloco 27, Itajaí, Santa Catarina, Brazil.*

SUMMARY. In this work the effects of topic use of Brazilian green propolis gel in the contraction of wounds performed in rat's skin was evaluated. In ten female *Wistar* rats were done four wounds in dorsal region. Two wounds in each rat were treated daily with Brazilian green propolis gel during 10 days. The wounds were photographed daily, and the images were analyzed with ImageJ software, in order to measure wound areas and evaluate wound contraction. In the graphic analysis, treated group and control group had similar behavior, and both evolved for complete closure in 10th experimentation day. The wound clots fell down before in treated wounds. Green propolis in the gel formulation at 5% in topical use had not effect in the process of wound contraction. The macroscopic visualization of the contraction of wounds, by itself, does not seem to be a good indicator of the process of tissue repair.

INTRODUCTION

Cutaneous damages can expose organism to risk factors, like pathogen microorganisms, and cause a homeostatic imbalance. The nature of lesions, patient's physiologic state, wound degree of infection and contamination and other disease processes can interfere in the cutaneous wound healing [1].

The wound repair is a dynamic process that involves physiologic and biochemical phenomenon, comprehending different processes like inflammation, cell proliferation and extracellular matrix synthesis. Thus, it consists in a perfect and coordinated cascade of cellular and molecular events that interacts to perform the reconstitution of the tissue [2].

The use of medicines and plants in various diseases, even to favor the wound healing, is an ancient practice. Thus, it's employed the use of several propolis extracts, including Brazilian green propolis. Brazilian green propolis is a resinous substance, collected by *Apis mellifera* bees from the plant *Baccharis dracunculifolia,* native from Brazil. In the presence of this plant, bees produce preferentially the Brazilian green propolis, independent on season or plant growth period [3].

Extracts of this kind of propolis generally contain 30% wax, 10% aromatic and essential oils, 5% pollen, 5% other substances and about 50% *B. dracunculifolia* resins [4]. Analysis on its chemical composition have already identified at least 300 compounds that may be organized in main groups like: aliphatic acids and esters, aromatic acids and esters, sugars, alcohols, aldehydes, fatty acids, aminoacids, steroids, ketones, chalcones and dihidrochalcones, flavonoids (flavones, flavonols e flavonones), terpenoids, proteins, vitamins B1, B2, B6, C, E, and also diverse minerals [5,6]. Regarding all these groups of compounds, the one which has more called the attention of researchers is the group of flavonoids [5].

Five main flavonoids (drupanin, artepelin C,

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* Author to whom correspondence should be addressed. *E-mail:* faloni@univali.br

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baccharin, caffeic acid and coumaric acid) isolated from propolis samples showed antioxidant activity [3].

Recent studies have shown the efficacy of Brazilian green propolis in different activities, as for example, antioxidant [7], fungicide [8], immunomodulatory [9], antiulcer [10,11] and also in the treatment of bacterial infections [12,13] in prevention of retina damages [14], against *Leishmania* species [15] and as neuroprotective substance in ischemic damages [16].

Although a lot of benefits from propolis have been described, further investigation are necessary before the commercialization of this substance as a medicine itself or combined with other drugs [9], even as its topic use in cutaneous wounds. Therefore, the aim of this study is to evaluate the action of topic use of Brazilian green propolis in the contraction of wounds performed in rat's skin, with macroscopic observation e measure of the wound areas by digital images.

MATERIAL AND METHODS

Preparation of Brazilian green propolis gel

Propolis samples were collected in Franca, São Paulo state, Brazil, in December 2005 from *Apis mellifera* hives. Samples were air-dried at 40 °C for 48 h. The dried propolis was kept in a freezer overnight, powdered (100 g) and submitted to exhaustive extraction with ethanol: water (7:3) (v/v) at room temperature by maceration. After filtration, the extract was concentrated under reduced pressure, yielding 5.2 g of dried crude green propolis extract.

The gel used in the experimentation was obtained by incorporation of Brazilian green propolis crude extract (5%) in carboximetilcellulose gel. This concentration was selected on the basis of preliminary studies carried out by our group and according to bibliographic information that consideres this concentration as not toxic for topic administration.

Animals

Ten *Wistar* rats (Rattus norvegicus albinus), females, aged between 3 and 4 months, weighing 200-250 g, were provided by Central Animal House of the Universidade do Vale do Itajaí (UNIVALI), Itajaí, Santa Catarina state, Brazil. The animals were housed in individual cages covered with a net, during the ten days of experimentation at ambient temperature, in 12-h dark/12-h light cycles, with both food and water *ad libitum*. Animals used in the present study were housed and cared in accordance with the Federal Government legislation on animal care, after authorization by the Research Ethics Committee of UNIVALI, with the protocol number 319/08.

Cutaneous wounds

Animals were anesthetized with intramuscular injection of Ketamine Chlorhydrate 100 mg (0,1 ml/kg). In anesthetic period, it was realized manual depilation of the cervical-dorsal region, and was performed 4 wounds, distant 2,5 cm from each other (Fig. 1), with a biopsy punch of 5 mm of diameter. The wounds were done avoiding the muscle plan.

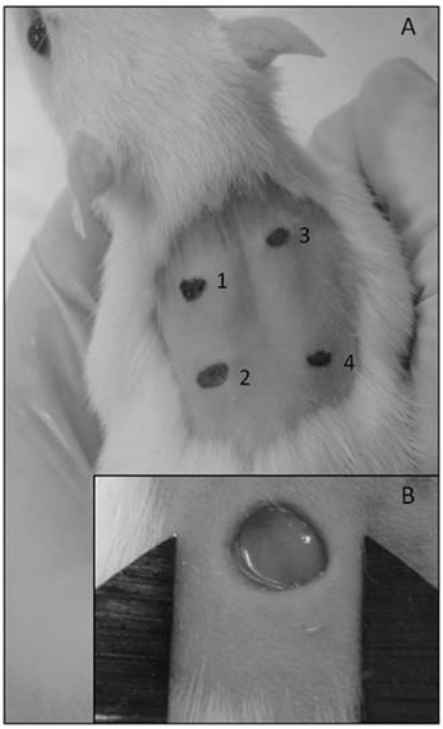

Figure 1. **A**: injuries (1, 2, 3 and 4), in cervical-dorsal region of the animal, 2.5 cm distance between them, after 2 days of treatment. It is present more developed clot in the wounds 1 and 4 (treated) and granulation tissue in wounds 2 and 3 (control); **B**: pachymeter used as scale (10 mm) in the same plan of the object's image after making the injury.

Wounds treatment

The four wounds were identified as superior-left (1), superior-right (3), inferior-left (2) and inferior-right (4) (Fig. 1). The wounds number 1 and 4 received topic treatment with Brazilian green propolis gel, while the wounds 2 and 3 (control) did not receive any substance. The topic treatment begun immediately after the

punch incisions and was hold during 10 days, in 24 h intervals. It was applied 34 mg by day with a syringe.

Wound photography

For photography, the animal was immobilized by Camila's Method [17]. A digital pachymeter open in the measure of 10 mm was positioned near to the wound (Fig. 1), as it served as scale and reference to the photo parallelism. The image was obtained with the camera Sony CyberShot 7,2 MP at maximum resolution, positioned about 15 cm over the wound, taking care for that the image axis made a right angle with the wound and pachymeter plan. Each wound was photographed daily for 10 days, before the topic treatment. It totals 400 images that were used in the evaluation of wound contraction.

Image analysis

For image analysis, it was used the graphic analysis software ImageJ, which is an open-source software for digital image analysis, based in Java and free distributed by National Institutes of Health (NIH) in the site *http://rsbweb.nih.gov/ij/index.html*. It's an alternative for the paid softwares, and it's already much utilized in biologic research [18]. ImageJ has more than 5000 plugins that make it able to a wide use, like in the measuring of human liver volume by computerized tomography image analysis [19], measuring of neuronal dimensions by the analysis of fluorescent image of neuron culture [20], in quantitative detection of Nuclear Factor κB (NF-κB), by microscopic immunofluorescence digitalized images [21], and others methods.

The software was fed with the scale of 10 mm of the pachymeter in the image, before the area measurement of each wound. The tool Polygon Selections was used to delimit the wound, with the marking of several points in the wound's margin. The software automatically links the points, forming an irregular polygon, which has its area measured.

Each one of the 400 images was measured by 6 different observers, giving a total of 2400 measurements. So, each wound in the final results is represented by the arithmetic mean of the 6 measures, what diminish statistics variation.

For statistic and graphic analysis, the software Microsoft Excel 2007® was used. The areas measurements was turn into percentages as it follows: the initial wound was considered 100%, the subsequent days values represented the percent fraction of the initial wound, as the entire closure was represented by 0%. The percentages of contraction were put in a dispersion graphic, and the software gave us a tendency line based on a 1st degree equation ($y = bx + a$). The tendency line permitted the evaluation of the wound contraction behavior.

RESULTS AND DISCUSSION

The experimentation consisted in the evaluation of Brazilian green propolis macroscopic activity in the wound contraction process, during 10 days. The means of the daily measurements of treated and control wounds, as well as the contraction percentage, are shown in Table 1 and the statistic analysis, by the regression model, in Figure 2.

In Table 1, it is verified that in the first 24 hs the wound contraction has its bigger amount of contraction in both groups equally (34,9% in treated group and 36,9% in control group). It

Day	Treated		Control	
	Area (mm²)	% of initial wound	Area (mm²)	% of initial wound
1	17,773	100,000	19,519	100,000
2	11,570	65,097	12,317	63,101
3	10,166	57,196	11,374	58,273
4	8,491	47,774	10,019	51,329
5	7,482	42,099	8,956	45,883
6	5,278	29,695	7,068	36,211
7	4,072	22,911	4,519	23,149
8	2,550	14,348	2,576	13,196
9	1,596	8,981	1,902	9,745
10	0	0	0	0

Table 1. Comparison of wound contraction in treated and control wounds during the 10 days of experimentation. Results obtained from the mean of 120 measurements (20 wounds photographed per day and measured by 6 observers, in each group).

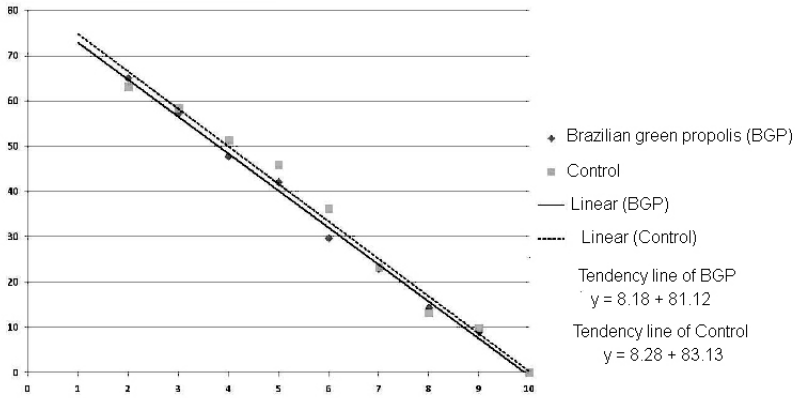

Figure 2. Percentage of wound contraction in treated and control groups during 10 days. Data obtained from table 1, showed in points, with tendency line traced by a first degree equation given above. The comparison shows the similar behavior of the tendency lines.

may be explained by the natural retraction of the wound that occurs due to elastic forces and skin tension, and that does not undergo treatment influence. This initial contraction is influenced by the underlying contractile connective tissue, which shrinks in size to bring the wound margins toward one another [22].

In Figure 2 the initial wound areas (100%) were unconsidered because it's a theoretical parameter to compare the contraction percentage in subsequent days. Also, the treatment doesn't influence in the first day of contraction. Using the percentage values of areas after the first 24 h, until the 10th day we could create an equation rule for the contraction. This rule is expressed by a linear equation [**1**].

$$y = bx + a \qquad [1]$$

where *"b"* represents the *mathematical* value of daily contraction, *"x"* is the number of days, and *"a"* is the theoretical value to correct the equation to real results *("y")*. This equation gives us a linear tendency line, as shown in the Figure 2, that allow us to evaluate the behavior of contraction.

The treated group tendency line is expressed by the equation $y = -8.18x + 81,12$; it means that the wound contraction occurs in reason of 8,18% a day. The control group tendency line is expressed by the equation $y = -8.28x + 83.13$; in this case the reason of contraction is 8.28%. The difference of 0.1% between groups is disposable. Corroborating with this, the tendency lines also shows a similar behavior in both groups. This is in agreement with other authors who did similar experiment [23].

At the final 10th day, the wound complete closure happened at the same time in both treated and control wounds. In the wound site, inflammatory cells are recruited, and neutrophils and monocytes are attracted to the lesion by a huge variety of chemotactic signals. These cells are the first on the scene of the injury. Both neutrophils and monocytes are recruited from the circulating blood in response to molecular changes in the surface of endothelial cells lining capillaries at the wound site [22].

An experiment demonstrated that a neutrophil depletion influenced an accelerated reepithelization in rats, but it had not effect in tension strength or in collagen amount. It could mean that, in a non infected wound, neutrophils delay the wound closure by inhibiting its reepithelization, but not altering overall quality of the dermal healing process [24]. The presence of neutrophils, that secrete proteases and reactive oxygen species causing tissue damage, has demonstrated negative effect on wound repair [25].

It is presumed that Brazilian green propolis, with its anti-inflammatory, antimicrobial and immunomodulatory properties, sustains the wound healing in an organized manner, with no lack or excess of cytokines, inflammatory mediators and cells [26,27]. Although it is described, we didn't observed difference between groups in macroscopic study. However, a positive finding in macroscopic visualization was the fall of the wound clot.

The bigger difference among the wound areas means occurred in the 6th experimental day, when achieved 1,79 mm² (6.51%). This situation is attributed to the fall of the wound clot, what happened faster in the treated wounds.

The wound clot is formed by the bleeding originated with the original lesion and it occurs by platelet aggregation and arrangement of a mesh of crosslinked fibrin fibers. This fibrin clot serves as a temporary shield to the damaged tissues and provides a provisory matrix over and through which cells can migrate during the repair process. The clot still serves as a reservoir

of cytokine and growth factors that are released as activated platelets degranulate. This concentration of these substances acts as a trigger for the wound closure process: chemotatic signals attract inflammatory cells, angiogenesis is stimulated and is initiated the tissue movements of reepithelialization and connective tissue contraction [22]. Moreover, the clot has another importance to the wound contraction: as it dehydrates, it shrinks and makes that the epithelial tissue to it adhered be centripetally moved, approximating wounds margins [28].

The anticipated fall of the clots of the treated wounds may suggest that cellular and chemical processes that experience influence of the clot presence were facilitated by Brazilian green propolis.

Studies which analyses the wound healing process utilize, basically, three general parameters, which is wound histological analysis, including cell and extracellular matrix observation; quantification of reepithelized area; and macroscopic wound qualification, observing the presence of clot, secretions, hyperemia and hair [29-32]. The present study aimed the quantification of reepithelized area and also the macroscopic observation of the clots fall.

There are some methods described in literature to quantify the reepithelization process in wound healing. These methods are since rudimentary techniques, until digital processes, easier and with greater precision. A method uses a digital pachymeter with a magnifying glass to measure the wound diameter. Other uses decal in a paper to compare wounds areas. More recent studies employ digital photography and softwares for wounds area quantification [29-32].

Our study directed to the quantification of the reepithelized area and macroscopic observation of the fall of the crust, through the analysis of digital images. It bounced a some concerns about the methodology, which can provide more reliable data, minimizing false-positive results: 1) it is important to keep the parallelism among camera lens and the wound plan; 2) scale must be kept in the same plan of wound; 3) the distance between camera and object must follow a pattern; 4) the object must be centralized in image, also be photographed with better focus as possible. In addition, the statistic variance can be reduced if used more evaluators, because the method is observer-dependent. These concerns are important for that distortion, at times inherent to the method, become null or at least diminished.

CONCLUSION

The results of this study indicate that the Brazilian green propolis in the gel formulation at 5 % in topical use had not effect in the process of wound contraction, by macroscopic visualization. It had a similar behavior between treated and control groups, and both healed completely on the tenth trial day. The macroscopic visualization of the contraction of wounds, by itself, does not seem to be a good indicator of the process of tissue repair.

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