Biocompatibility of propolis in subcutaneous tissue of rats: a possible biomaterial for cavity cleansing

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ABSTRACT
OBJECTIVE: The aim of this study was to histopathologically analyze, in subcutaneous connective tissue in rats, a propolis solution for cavity cleansing and its toxicity through hemolytic and Artemia franciscana tests.

METHODS: Fifteen male rats were selected and randomly distributed in three experimental periods (07, 30 and 45 days), in which each animal received the four treatment groups in rounds: Group I – Propolis I; Group II – Propolis II; Group III – Calcium Hydroxide Water and Group IV – 2% Chlorexidine; the sides of the tube were the control group. The data were analyzed using descriptive statistics. The results showed, in terms of biocompatibility, that all materials presented a significant reduction of the inflammatory infiltrate and an increase of the thickness in the collagen fibers. It may be suggested, in decreasing order of biocompatibility, the use of following materials: calcium hydroxide-water, 2% chlorexidine, propolis I and propolis II.

RESULTS: In the cytotoxicity test using A. franciscana, the propolis extract showed high toxicity when tested at concentrations and in the hemolytic activity test the propolis I extract showed greater activity than propolis II.

CONCLUSION: The present study suggests the use of propolis as a cavity cleansing solution for shallow and medium cavities similar to 2% chlorexidine.

Key words: Materials testing; Propolis; Dental cavity lining; Detergents

RESUMO
OBJETIVO: O objetivo deste estudo foi analisar histopatologicamente, em tecido conjuntivo subcutâneo de ratos, uma solução de própolis para a limpeza de cavidades e sua toxicidade através dos testes de hemólise e Artemia franciscana.

METODOLOGIA: Foram utilizados 15 ratos machos, selecionados e distribuídos aleatoriamente em três grupos (n=5) em períodos experimentais (7, 30 e 45 dias), em que cada animal recebeu os quatro grupos de tratamento em forma de rodízio: Grupo I – Própolis I; Grupo II – Própolis II; Grupo III – Água de Hidróxido de Cálculo e Grupo IV – Clorexidina a 2%; as laterais do tubo foram o grupo controle. Os dados foram analisados pela estatística descritiva.

RESULTADOS: Todos os materiais apresentaram uma redução significativa do infiltrado inflamatório e aumento da espessura das fibras colágenas. No teste de citotoxicidade de Artemia franciscana, o extrato de própolis apresentou alta toxicidade e no teste de atividade hemolítica o extrato de Própolis I mostrou-se mais ativo que o da Própolis II.

CONCLUSÃO: O presente estudo mostrou a biocompatibilidade da própolis, sugerindo seu uso como solução de limpeza em cavidade cavidades rasas e médias semelhante à clorexidina a 2%.
INTRODUCTION

The general principles of cavity preparation were altered because of the bond restorative materials to tooth structure. Carious dentin must be removed until a leathery consistency layer of dentin is reached promoting a successful and long-term survival of the restoration [1]. Whenever there is an abrasion or cut in the dental structure, a surface called smear-layer is formed, comprised of organic and inorganic materials, blood, saliva, oils from rotatory instruments, microorganisms and their products [2].

The cavity cleansing aims to effectively remove most of the residues that can harm adaptation, marginal sealing and at the same time reduce the amount of microorganisms and their products [3].

Propolis is a complex mix of resinous, gummy and balmy substances gathered by Apis mellifera bees from sprouts, flowers and plant exudates, in which the bees add salivary secretions, wax and pollen to generate the final product [4]. It has a bactericide, antimicrobial [5], antioxidant [6], anti-fungal [7], healing, anti-inflammatory [8], anti-cancerous, anti-HIV [9] and anti-curious biological function [10].

The propolis components vary according to the collection location and the vegetal species used by the bees in its production process. Chemically, propolis presents 160 components. Among the identified compounds, we can note flavonoids (flavones, flavolones, flavonones), chalcones, benzoic acid and derivates, benzealdehydes, alcohols, acetones, fenolics, heteroaromatics, cinnamic alcohol and derivates, diterpen and triterpen acids, minerals and other elements [6].

In order to enable the clinical use of this new biomaterial, extensive research is needed, including evaluating the biological compatibility of the product, to prove its safety and to enable its use in dentistry. The toxicity of a dental material can be evaluated by in vitro tests in animals and humans. When a new product is developed, it is necessary to perform laboratorial tests with it, allowing safe clinical applications, and providing subsides for its use by health professionals and clinics proving its non-toxicity to tissue [11,12].

The purposes of this study were to analyze the biocompatibility of a propolis solution cavity cleansing in rat subcutaneous connective tissue and the toxicity through hemolytic and Artemia franciscana tests.

METHODS

The propolis (Apis mellifera) samples were collected in two apiaries in the State of Amazonas, Brazil, in four different beehives using the scraping method. The samples were obtained with 8% ethanolic extract following guidelines directions of the Brazilian Pharmacopeia for an appropriate cavity cleansing solution [13].

This research was approved by the Animal Research Ethics Committee of the Federal University of Amazon (55/2011). Fifteen male young adults Rattus norvegicus rats, Rodentia mammalian, Wistar lineage were used, weighing on average 180 to 220 grams [14].

For the surgical procedure, the animals were anesthetized with ketamine hydrochloride 10%, 0.15 mL/100mg (Ketamine®, Syntec, São Paulo, Brazil), and muscle relaxant (Rompum®, Xylazine hydrochloride 2%, 0.01 mL/100mg – Bayer from Brazil S/A, São Paulo, Brazil), followed by anti-sepsis with iodated alcohol and trichotomy of the dorsal area. At the mean line, two incisions were made, one pelvic and the other scapular, of approximately 1 centimeter in length, followed by divulsion.

Polyethylene tubes were implanted on rounds in each side of the animal and kept parallel to the incision, and each animal (N=15) received the four experimental groups. The test groups were denominated Group I: propolis I (PI); Group II: propolis II (PII); Group III: Calcium Hydroxide Water (CHW); Group IV: 2% Chlorhexidine® (CH) (FGM, Joinville, Santa Catarina, Brazil). The test specimens were retained in situ for experimental periods of 7, 30 and 45 days (n=5).

To begin the study, the animals were observed until full recovery. Later, they were put in cages, taken to a mouse facility and fed a balanced diet and water ad libitum. After the experimental periods, the rats were once again anesthetized and, after locating the tubes, the tissue fragments that contained them were removed with a wide safety margin, and then immersed in 10% buffered neutral formalin and sent for histopathologic processing with 6 µm-wide cuts and then dyed with Hematoxylin and Eosin [14]. The animals were anesthetized and sacrificed.

Histopathologic analysis was performed with a microscope and the following cellular elements were considered for evaluation of the analysis: presence of inflammatory cells, neutrophils, eosinophils, lymphocytes, macrophages and giant cells, deposits of collagen fibers and abscess formation. The cellular events were classified according to scores (Table 1).

Table 1. Scores according to the intensity of the inflammatory process

<table>
<thead>
<tr>
<th>Score</th>
<th>Intensity of the inflammatory process</th>
<th>Description</th>
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<tbody>
<tr>
<td>1</td>
<td>Absent</td>
<td>no inflammatory cells</td>
</tr>
<tr>
<td>2</td>
<td>Mild</td>
<td>presence of inflammatory cells very sparsely or in small groups</td>
</tr>
<tr>
<td>3</td>
<td>Moderate</td>
<td>presence of inflammatory cells in groups, but not dominating the microscopic field</td>
</tr>
<tr>
<td>4</td>
<td>Intense</td>
<td>presence of inflammatory cells dominating the microscopic field and near the tested material</td>
</tr>
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The fibrous formation was classified according to established scores [15] (Table 2).

The abscess, characterized by the presence of degenerated neutrophils (piocites) in an area of the microscopy field, was also classified according to scores [15] (Table 3).
A study of the toxicity of the extracts was performed using *Artemia franciscana* (*A. franciscana*) as a model. The *A. franciscana* cysts were cultured in a Petri dish (90 mm×15 mm) containing a 3.5% saline solution, for 48 hours at room temperature under continuous luminosity. After 48 hours, the eggs hatched and the larvae were ready for testing. After the hatching period, 1.800 mL of the 3.5% saline solution were added to a microplate (4×6), and in those wells were inserted 10 *A. franciscana* nauplii, and then 20 mL of the 8% propolis ethanolic extract, incubated for 24 hours at room temperature in the dark. This procedure was performed three times. The negative control followed the same procedure, but using 20 mL of dimethylsulfoxide without adding the extract [16]. The mortality rate was determined in \( \% \text{ mortality} = \left( \frac{\text{number of dead individuals}}{\text{total number of individuals}} \right) \times 100 \), and the degree of toxicity was classified according to the mortality observed: 0-9% = non-toxic (NT); 10-49% = slightly toxic (ST); 50-89% = toxic (T); 90-100% = highly toxic (HT) [17].

For the hemolytic test, venous blood was collected first in EDTA and then it was processed at 2500 rpm for 10 minutes. The supernatant was ignored and the infranatant was collected. A total of 1 mL of the infranatant (red blood cells) in 99 mL of a phosphate buffer with pH 7 forming the red blood cell solution. The experimental groups were: Group I – P I; Group II – P II; Group III – Triton X-100 Solution (control) and Group IV – 80% Ethanol (white). The tubes were made homogeneous and incubated at 37°C for 5 minutes. After this period, they were processed at 3000 rpm for 5 minutes. The readings were performed in the microplate reader at 540 nm. The hemolysis of the sample was calculated by the formula:

\[
\% \text{ hemolysis} = 100 - \frac{\text{Absorbance test}}{\text{Absorbance control} \times 100} \times 100
\]

The data were tabulated and analyzed through descriptive statistics.

### RESULTS

The results of the biocompatibility in rat subcutaneous connective tissue were analyzed comparatively evaluating the experimental groups according to the periods of time.

In the 7-day period, the predominance of mild inflammatory infiltrate was observed in groups I (Figure 1) and III, and moderate infiltrate in groups II (Figure 2) and IV, and in group III (control), 100% of the samples presented mild inflammatory reaction. Regarding the fibrous formation, Groups I, II and III presented fine collagen fibers and in Group IV, there was a complete absence of collagen fibers at 80%. Abscess appeared in only 20% of Group II.

![Figure 1. Group I (Propolis I) at 07 day – Mild inflammatory infiltrate (MI) and fine collagen fibers (FCF) 240x.](image1)

![Figure 2. Group II (Propolis II) at 07 day – Moderate inflammatory infiltrate (MdI) and fine collagen fibers (FCF) 240x.](image2)
Analyzing the 30 and 45 day periods, it was observed that in 30 days, the absence of inflammatory infiltrate occurred for groups I and III, mild inflammatory infiltrate in Group II and the predominance of mild inflammatory infiltrate in Group IV. In 45 days, the predominance of absence of inflammatory infiltrate was observed in Groups I (Figure 3) and III (Figure 4), and predominance of mild inflammatory infiltrate in Group II (Figure 5), and in Group IV (Figure 6), 100% of samples presented mild inflammatory reaction.

Overall, the inflammatory reaction regressed over the experimental periods and the collagen fibers grew thicker, with the Group III (control) presenting a more favorable inflammatory response followed by Groups IV, I and II. The CHW presented fine collagen fibers in all experimental periods.
Comparing histopathologic analysis (inflammatory response, collagen fibers and abscess), the use of following materials can be suggested in decreasing order of biocompatibility: Group III – CHW, Group IV – CH, Group I – PI and Group II – PII.

The result of the toxicity test confirmed that *A. franciscana* was sensitive to 100% of the tested extracts, not only in relationship to PI, but also PII. It was observed that the tested extracts, based on the *A. franciscana* mortality rate, were classified in HT (100%), showing its biologic property in the tested concentration.

The result of the hemolytic activity showed that in Group I (PI) there was no hemolysis in the extract concentrations starting from 0.01%, and in Group II (PII) there was no hemolysis in the concentrations starting from 0.05%.

**DISCUSSION**

When a new material is introduced into the market its properties should be investigated. From a biological standpoint, their biocompatibility must be evaluated, because the eventual toxic components present may cause tissue irritation, degeneration or necrosis of the tissues adjacent to the materials [13].

The research was a study that involved patent application delaying its disclosure, so it was used Arthemia test, which is considered a preliminary study of low cost and easy handling on bioassay of extracts with strong biological activity [19], since the accomplishment of the lethality test allows the evaluation of the toxicity involving only one parameter: life or death [20].

This assay is simple, fast, practical and does not require aseptic technique and allows a large number of samples to be processed properly and these bioassays are useful for evaluating the exposure of a wide variety of extracts [21].

Silva et al. [22] affirmed that subsequently, another cytotoxicity test, such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, should be performed, since it is highly sensitive and has been indicated as a high-throughput screening assay.

The results comparing the inflammatory response of the tested materials on Day 7 show a more intense inflammatory infiltrate was found close to the tube opening in all groups and absence of collagen fibers. This may be due to responses to initial irritation in short periods when in touch with the tested material, or due to the surgical procedure [23]. Different results were observed by Nelson Filho et al. [24], who reported that calcium hydroxide induces less inflammatory infiltrate in the initial hours, progressing to a moderate degree after longer periods, and inducing subsequent tissue repair.

Garcia et al. [25] assessed the biocompatibility of two endodontic pastes based on calcium hydroxide and propolis, with two vehicles—non-fractionated Copaiba-oilresin (A) and volatile fraction of Copaiba-oilresin (B), in the connective tissue of rats, using the same methodology of this study. Tissue reaction ranged from slight (7/21 days) to no inflammation (42 days) for the control group, concluded that both pastes presented satisfactory tissue reaction in the connective tissue of rats, which is in accordance to the results found in this study, since the inflammatory reaction regressed over the experimental periods.

Analyzing the compatibility of dental adhesives All-bond 2® and Scotchbond MP®, Costa et al. [26] observed that in the final periods, the histopathologic events regressed, showing a repARATION process with intense presence of fibroblasts and collagen fibers, similar to this study.

Studies on biocompatibility of propolis are rare in the literature, and funding is lacking for comparing the results of this research. However, Geraldini et al. [27] suggested that because of propolis’ antibacterial activity in the dentinal cavity, where there is a strict relationship between the dentin and the pulp, it can result in a favorable pulpal response, partially disagreeing with the histological findings of this study, where it was noticed that the proposed solution is similar to CH, being indicated for shallow and medium cavities at 8% concentration.

About this, Bandeira et al. [3] evaluated the morphology of the dentin surface cut and treated with copaiba oil emulsions (CO) and suspension of ethanol extract of propolis (EP) through scanning electron microscopy (SEM), and the results suggest that copaiba oil emulsions (CO) and suspension of ethanol extract of propolis (EP) have feasibility to be used as bioactive dental cleaning agents.

New studies suggest the need for reducing the solution’s concentration, aiming the analysis in deep cavities and in cavities with pulpal exposure in vivo.

**CONCLUSION**

The outcomes of the present study showed that in the biocompatibility test Propolis I and II were irritating to the rats subcutaneous connective tissue, enabling their application in shallow and medium cavities, similarly to 2% chlorhexidine and in the cytotoxicity test using *A. franciscana* the propolis extract presented high toxicity in the tested concentration and in the hemolytic activity test the Propolis I extract showed more activity than Propolis II. The inflammatory response of the calcium hydroxide solution reinforced its recommended use for cleaning deep cavities and cavities with pulpal exposure, being less toxic to tissues. Further research is necessary to determine the clinical behavior of propolis as a cavity cleaning in dentistry therapy.

**ACKNOWLEDGMENTS**

This research was funded by the Brazilian National Council for Scientific and Technological Development (CNPq) grant nº 575752/2008-4 and registered at the National Institute of Industrial Property.

**REFERENCES**


