Topical Green Propolis Improves Corneal Wound Healing and Inflammation in Rats following Alkaline Burns

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Running head: Effect of green propolis in corneal inflammation and regeneration.

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ABSTRACT

Background: The objective of this study was to investigate the effects of green propolis, a widely used folk medicine, in corneal wound healing and inflammation.

Methods: Epithelial defects of 1mm in diameter were made in the right corneas of Wistar male adult rats by cauterization with silver nitrate sticks. Subsequently, they were divided in two groups (n=40 rats/group): group green propolis (GP), which received topical application of eye drops containing microemulsion of 1% green propolis, and group vehicle (VH), which received eye drops without propolis. The area of injury was photographed and measured at t=0 (wound induction), and after 12, 24, 48 and 120 hours of treatment. The inflammatory response was compared histologically by leukocytes counts. The rate of regeneration between the groups was compared by expression of Ki-67 in basal epithelial cells of the cornea by immunohistochemistry. Comparisons were made using the Kruskal-Wallis and the Mann-Whitney U test.

Results: In the GP group significantly smaller wounded areas were found at 12, 24 and 48 hours, and the corneal total neutrophil infiltration was lower at 24 and 48 hours ($P<0.05$). Concomitantly, Ki-67 was highly expressed in the GP group at 12 and 24 hours ($P<0.05$).

Conclusions: Topically applied green propolis accelerated wound healing and reduced inflammatory response after silver nitrate-induced alkali burns in rats.

Key words: Green propolis, alkali burn, cornea regeneration, corneal inflammation, Ki-67.
Background

Corneal epithelium plays important roles in the maintenance of corneal function and integrity. Corneal epithelial defects resulting from several clinical conditions (e.g. chemical burns) may cause corneal opacity, neovascularization, infection, and visual loss [1]. Although patients with these complications are frequently seen in eye clinics and the molecular mechanisms to revert them are known from translational studies, there are no commercially available products to effectively treat delayed corneal re-epithelization [1,2].

Herbal therapy constitutes the largest proportion of the anecdotal complementary and alternative medicines used in the United States of America, accounting for more than 15 million consumers [3]. Taking the spreading of their consumption into account, non-industry sponsored researchs are warranted to clarify the safety and efficacy of the herbal medications.

Propolis (honeybee glue), a resinous product consisting of sap, bark and bee excreta, accumulates in beehives. It is currently used as a dietary supplement for the treatment of various diseases [4-7]. Indeed, it has been shown to have a wide range of biological activities, principally attributable to the presence of flavonoids and caffeic acid phenethyl ester [4]. Hence, the putative therapeutic properties of propolis could be related to its antibacterial [5,6], anti-inflammatory [7] and antioxidant activities [8,9].

Propolis has a variety of botanical sources, and its chemical composition can also be variable. *Baccharis dracunculifolia* DC (Asteraceae), a native plant from Brazil, is the most important botanical source of Brazilian propolis, known as green propolis because of its color [10,11]. Brazilian propolis is used extensively in foods and beverages with the aim of maintaining or improving human health [12,13].
However, considering the lack of commercially available products to promote corneal wound healing, the anti-inflammatory and healing properties of propolis, as well as the fact that ocular therapeutical use of green propolis has never been investigated before, and there is no information about the frequency of its use with this purpose in the community, the aim of the present work was to evaluate its effect on cornea inflammation and wound healing after alkaline injury.
Methods

Animals and procedures

All experimental procedures were approved by the Ethics and Animal Experiments Committee at the University of Ribeirão Preto, São Paulo University and animals were treated in accordance with guidelines provided in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Wistar male rats 250–300 g (n=80), sourced from the Central Bioterium of the University of São Paulo (Ribeirão Preto Campus), were anaesthetized with halothane (4%, in air), and had the centers of their right corneas cauterized with a silver nitrate applicator stick (75% silver nitrate, 25% potassium nitrate; Graham-Field Inc, Hauppauge, NY). The applicator was held in contact with the cornea for 2 s, producing a discrete grayish-white lesion of 1 mm in diameter. The cauterized eye was then rinsed several times with room temperature saline. Rats recovered fully from anesthesia within a few minutes, and exhibited no outward signs of distress.

After wounding, animals from a subset of each group (n=40 rats/group) were examined with a slit lamp. A drop of fluorescein was used to stain the damaged area and to measure the lesions. The rats were held in the focus position by a fixation device commonly used for stereotaxic surgical procedures, attached to a plastic base. Photographs were taken at five time points: immediately after lesion-induction (time 0), and 12, 24, 48 and 120 hours post-injury. Animals were labeled on the inferior eyelid, with a paper designating their group, number and time after lesion. This paper also contained a 1mm ruler for posterior measurement of wounded areas and conversion from pixels to millimeters.
In the slit lamp, cobalt blue light and a zoom of 16 times were used (Carl Zeiss - Ltda, Germany). Photographs were taken by a digital camera (DSC-W5, Sony Ltda, Japan) connected to the slit lamp by an optical system (D.F. Vasconcelos, Brazil). Images were downloaded to a desktop computer and corneal wound areas were measured, using an image software (Image J - 1,33u, NIH, USA).

**Green propolis preparation and use**

Green propolis (GP) used in this study is produced by *Scaptotrigona sp.* and was obtained from the Northeast region of Brazil (Barra do Corda, MA, Brazil).

Dried GP was diluted in a solution of polyethylene-6-caprylate/caprate, polyglyceryl-6-dioleate, glycerides caprylate/caprate (10.0%) (Mackaderm MicroExpress - McIntyre), benzalkonium chloride (0.01%) and distilled deionized water produced by a MilliQ system (MilliQ ultrapure water purification system, Millipore, USA). A 1% microemulsion of GP was then prepared.

Subsequently, GP and vehicle (VH) (to work as placebo in the control group) were filtered separately using a filtering membrane of 0.22µm (Milipore, MA, USA) and stored in eyedropper polyethylene bottles. Eye drops manipulations were made in sterile conditions, using a laminar flux camera irradiated with ultraviolet light (MiniFlow 1 – Marconi, São Paulo, Brazil). All material was autoclaved before use at 121°C, for 15 minutes.

Both, the GP and VH groups received eye drops immediately after corneal cauterization, at a frequency of four times a day until the rats were sacrificed after 12, 24, 48 and 120 hours (n=10/subgroup per time point).

**Tissue processing and data analysis**

At each time point, animals were anesthetized and corneas were examined with the slit lamp. After being photographed, still under influence of anesthesia, the
rats were sacrificed in a carbon dioxide chamber. Corneas were harvested, fixed with formalin, and blocked with paraffin. Histological cuts of 5µm were performed, transferred to slides, and stained with Hematoxylin and Eosin (HE).

Slides were analyzed with an optic microscope and at 400x magnification (Olympus BX40 light microscope, Olympus Corporation, Tokyo, Japan) and pictures were taken with a digital camera (Olympus Q-color 5). Three fields were used for cell counting at 400X magnification, with posterior calculation of the number of cells per mm². The center of the lesion was determined and counting of leukocytes was performed in this area. Right and left adjacent fields were excluded and the next peripheral fields were also analyzed, both in the right and in the left borders of the lesion (Figure 1). The procedure was performed for two slides from the center of paraffin block from each cornea harvested (per time point).

The analysis of leukocytes migration was performed by calculating two parameters. The first was the ratio between the number of leukocytes in the center of the lesion, counted in the central histological field, and the total number of leukocytes, counted in the three fields examined. This ratio was called "center/total". The second parameter was the ratio between neutrophil counts in the center and in the borders of injured tissue and was called "center/border" ratio. The number of leukocytes in the center and border fields was counted for the four time points (i.e., 12, 24, 48 and 120 h, n=10/group per time point).

For quantification of mitosis as a measure of regeneration, quantitative immunohistochemical analysis of Ki-67 protein expression was performed. In brief, paraffin was removed from the slides throughout re-hydration, endogenous peroxidase was blocked with PBS, and nonspecific antibody binding was blocked with normal horse serum. IgG anti rat Ki-67 was used as primary antibody, diluted
1:200 (Novocastra, Newcastle Upon Tyne, UK) followed by incubation with a secondary antibody (Vectastain Elite ABC kit Universal, Vector, CA, USA), immunoperoxidase with streptavidin/biotin, and DAB (NovoLink, Novocastra, Newcastle Upon Tyne, UK) and counter-staining with HE. Afterwards, slides were mounted and, using the same optical microscope mentioned above, the number of epithelial cells with brown stained nuclei in the corneal basal layer was determined in five serial sections of samples from each animal. The intensity of staining presented slight variation among the different histological sections and for counting analysis; only the positively stained cells in the first row of all cornea extension were considered.

**Statistical analysis**

Data are expressed as means ± SEM. Comparisons were made using the Kruskal-Wallis test for continuous data comparing several time points, and the Mann-Whitney U test for continuous data comparing GP and VH parameters at each time point (Graphpad 5.0 software, Prism, San Diego, CA). The level of significance used was $P<0.05$. 
**Results**

**Wounded area**

Digital analysis of the wounded area stained with fluorescein was used to calculate the size of the injured areas. There was a significant statistical difference between the wound sizes in groups GP and VH at 12, 24 and 48 hours post-injury ($P<0.01$). No differences were found at time 0, confirming that a similar lesion was made in both groups. At 120 hours after injury all animals presented complete re-epithelization (Table 1).

**Leukocytes migration**

Histology revealed the presence of inflammatory cells in almost all corneal layers and in all the extensions of corneal stroma. We additionally found an area of central necrosis in corneal epithelia and anterior stroma, associated with silver impregnation, in both groups (Figure 1).

No statistically significant difference was found in the number of leukocytes present in corneas between groups GP and VH at 12 hours after injury. At 24 and 48 hours, a significant lower number of leukocytes was found in the group GP ($P<0.01$). After 120 hours of lesion, leukocytes count was lower in GP, but not statistically different between groups (Table 2).

As after injury, leukocytes migrate from the limbus to the center of the cornea, both center/total and center/borders ratios were used to compare migration of leukocytes involved in the inflammatory response in both GP and VH groups. Although faster migration was observed in the GP group between 24-48 hours by both counting methods, there was no statistical significance, neither in the slope of the
curve nor in the comparison of each time point between both groups (Figure 2A and 2B).

**Immunohistochemistry (Ki-67)**

Immunohistochemical assays showed Ki-67 staining of epithelial cells primarily in the basal layers of the epithelia in both GP and VH groups (Figure 3A and 3B). A significant higher number of Ki-67 positive cells was found in group GP at both 12 and 24 hours (P<0.0001 and P<0.001, respectively). However, this difference was not maintained as no difference was found after 48 hours of injury (table 3). At 120 hours after injury, no significant detection of stained cells was observed, due to the complete re-epithelization.

**Discussion**

Corneal surface injuries are among the most frequent traumas of the eye [14]. The prompt recovery of such injuries is critical to the maintenance of corneal transparency. Although previous publications [15,16] addressed therapeutic options, no commercial eye drops have been approved for this specific purpose, nor has GP been clinically tested to the best of our knowledge.

Aqueous extracts obtained from GP have antioxidative properties and inhibitory actions on certain enzymes, and are greater than those obtained with ethanolic extracts. These extracts are rich in terpenoids and derivatives of coumaric, caffeoylquinic and cinnamic acids, and it is probable that these components have synergistic effects on healing [15-17].

It is known that red propolis causes damage to the corneal epithelial cells of rats when at concentrations of 7.81 mg/mL [18]. Thus, although there are no specific
studies on corneal toxicity of GP, the concentration of 1% (0.01 mg / mL) is far below than the one used with red propolis and as indicated here, safe to corneal epithelium.

The mechanisms by which GP healing effects have been focus of previous works [4-9]. In the eye, GP reduces both neuronal death and apoptosis of retinal ganglion cells [17,19]. Furthermore, it is associated with the reduction of the amount of free radicals, generating protection against oxidative stress induced by lipid peroxidation [19].

Mechanical desepithelizations usually regenerate faster than those caused by alkali burn [20,21]. Such difference may be explained by peculiarities of chemical burns, which involve different types of mediators [22-24], inflammatory responses greater than those observed in mechanically injured corneas, and oxidative damage, causing degradation of the stromal matrix [20,25]. These pathological findings were mostly considered in the choice of the experimental model used in the present work.

In the present work, it was observed that complete re-epithelization occurred between 48 and 120 hours. Although the exact mean re-epithelization time was not determined, it seemed closer to 48 than to 120 hours, once the VH and GP group had 81% and 92% of the wound areas healed, at 48 hours, respectively. These findings are consistent with the literature findings cited above.

The replacement of epithelial cells occurs by mitotic cell divisions that occur in the basal epithelial layer and in the limbus, where stem cells begin proliferating after detection of a corneal lesion [26,27]. In this study only mitotic events in cells in the basal layer of the corneal epithelia was measured. There, Ki-67 staining indicated an intense cell proliferation in the group GP, especially at 12 hours after injury, suggesting that wounds are more rapidly healed in the presence of GP.
The indirect measurements of inflammation by counting the total number of leukocytes in the injured region showed an increase of this cell population at 12 hours, in both groups. As the number of leukocytes in the cornea of rats is small under normal conditions [28,29], one can conclude that a significant neutrophil migration occurred in the first 12 hours after injury and that GP drops did not prevent or inhibit this migration, since there was no statistically significant difference between GP and VH. However, since at 24 hours the counts in both groups increased by more than 100% and in the VH group these counts reached values 50% higher when compared to the GP group, suggests that GP has significant anti-inflammatory effects in this model. Moreover, this difference persists even after 48 hours. At 120 hours, there is a reduction of approximately 80% of leukocytes comparing to 48 hours and no statistical differences are found between GP and VH groups.

The time-course of neutrophil infiltration after corneal injury was consistent with most findings in the literature, in which the peak of neutrophil count was observed between 18 and 24 hours [30-32]. The use of GP did not inhibited or changed the time-course or migration pattern, as observed by the lack of statistical differences when center/total and center/borders ratios were compared between the GP and control group.

In summary, the present work suggests that an anti-inflammatory action of propolis preserves the migration pattern of leukocytes involved in the inflammatory process, but reduces its intensity while simultaneously accelerating epithelial healing. Although other substances have shown similar results in pre-clinical studies and randomized clinical trials are challenging due to conditions related to cornea abrasion or delayed re-epithelization, it will be necessary to go further, in order to improve therapeutical options [31,33-35]. The clinical usefulness of a treatment with topical
GP in chemical burns, and potentially in other aseptic keratitis, is based in the cicatrizing and anti-inflammatory effects. Combining these actions, GP has the pro-healing advantage over the current anti-inflammatory medications (e.g. corticosteroids) available for the treatment of eye conditions.
Conclusions

Topically applied GP accelerated wound healing and reduced inflammatory response after silver nitrate-induced alkali burns in rats. The clinical usefulness of a treatment with topical GP in ocular surface diseases associated with corneal epithelial defects should be explored further as an alternative for the prevention of these potentially blindness conditions.
Competing interests

Financial support: São Paulo Research Foundation - FAPESP.

Financial interest statement: The authors have no financial competing interests in this study.
Authors’ contributions

LM carried out the experiment, participated in the statistical analysis and drafted the manuscript. JP: participated in the design of the study, as well as the statistical analysis and drafted the manuscript. ER participated in the design of the study and revision of the manuscript. SG carried out the histology and immunohistochemistry analysis. All authors read and approved the final version of the manuscript.
Authors’ information

SG has been studying, in contribution with other authors, the effects of green propolis in different tissues. As an associate professor and a former chief of the Department of Pathology of the University of São Paulo, he carried out the histology and immunohistochemistry analysis of multiple studies, which correlated green propolis with inflammation and wound healing in several tissues, including skin and bones.

JP and ER are associate professors of ophthalmology in the Department of Ophthalmology, Otorhinolaringology, Head and Neck Surgery of the University of São Paulo, and they both have been working with experimental corneal models, related to ocular surface disease in dry eye syndrome, and other ocular inflammation conditions. JP was a former chief of the Division of Ophthalmology of this Department, and ER is the current chief of the same Division.

LM is a young ophthalmologist who had been working with SG since medical school, before ophthalmology residency. After finishing residency of ophthalmology and a one-year fellowship in cornea, he was invited by those professors to develop the present study, as part of his PhD.
Acknowledgements

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References


34. Lin M, Carlson E, Diaconu E, Pearlman E: CXCL1/KC and CXCL5/LIX are selectively produced by corneal fibroblasts and mediate neutrophil infiltration to the corneal stroma in LPS keratitis. *J Leukoc Biol* 2007, **81**:786-792.

Figure Legends

**Figure 1.** Photomicrograph of a cornea with 12 hours of injury in an animal of green propolis (GP) group, showing a brownish central lesion with loss of epithelium and superficial stroma. The circles represent, schematically, the histological fields. Of the five fields represented, the count was made in the central and the peripheral areas, represented in dark blue. The intermediate fields, represented in light blue, were discarded (Hematoxylin & Eosin; original magnification: 100X).

**Figure 2.** Leukocytes counting ratio in histological slides of cornea tissue from groups VH [vehicle (black circle)] and Green Propolis [GP (black square)], displayed as mean±SD. In the left side (A), the ratio was obtained counting the number of leukocytes in the center area and in the peripheral area, adding both and dividing the number obtained in the center by the sum. In the right side (B), the ratio was obtained counting the number of leukocytes in the center area and in the peripheral area, and dividing the number obtained in the center by the number obtained in the periphery (n=10/group per time point).

**Figure 3.** Immunohistochemical Ki-67 analysis of cornea tissue cauterized with Silver Nitrate, and treated with VH (vehicle - A) or GP (green propolis - B) eye drops. Photographs were taken at time-point 12 hours with 400X (original magnification).
Table 1. Area of corneal epithelial defect at various time points after injury on groups GP (green propolis) and VH (vehicle) (mean ± SEM).

<table>
<thead>
<tr>
<th>TIME (h)</th>
<th>VH (mm²)</th>
<th>GP (mm²)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0389 ± 0.0017</td>
<td>0.0341 ± 0.0014</td>
<td>0.1978</td>
</tr>
<tr>
<td>12</td>
<td>0.0291 ± 0.0012</td>
<td>0.0160 ± 0.0030</td>
<td>0.0032</td>
</tr>
<tr>
<td>24</td>
<td>0.0163 ± 0.0021</td>
<td>0.0076 ± 0.0022</td>
<td>0.0007</td>
</tr>
<tr>
<td>48</td>
<td>0.0075 ± 0.0009</td>
<td>0.0031 ± 0.0007</td>
<td>0.0019</td>
</tr>
<tr>
<td>120</td>
<td>0.00</td>
<td>0.00</td>
<td>N/A</td>
</tr>
</tbody>
</table>

NA: Not applicable.
Table 2. Neutrophil count in corneas at various time points after injury in groups GP (green propolis) and VH (vehicle) (mean ± SEM).

<table>
<thead>
<tr>
<th>TIME (h)</th>
<th>VH (cells/field)</th>
<th>GP (cells/field)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>150.4 ± 9.0</td>
<td>150.3 ± 10.8</td>
<td>1.000</td>
</tr>
<tr>
<td>24</td>
<td>448.6 ± 21.7</td>
<td>354.7 ± 9.7</td>
<td>0.0006</td>
</tr>
<tr>
<td>48</td>
<td>408.0 ± 16.5</td>
<td>326.7 ± 13.3</td>
<td>0.0014</td>
</tr>
<tr>
<td>120</td>
<td>82.2 ± 20.0</td>
<td>56.9 ± 10.0</td>
<td>0.3752</td>
</tr>
</tbody>
</table>

NA: Not applicable.
**Table 3.** Positive Ki-67 stained cells in the corneal basal layer in both groups (VH – vehicle and GP – green propolis) at the different time point studied (mean±SEM).

<table>
<thead>
<tr>
<th>TIME (h)</th>
<th>VH (No. of cells)</th>
<th>GP (No. of cells)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>46.7 ± 0.02</td>
<td>63.7 ± 0.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>24</td>
<td>64.2 ± 0.02</td>
<td>74.15 ± 0.0195</td>
<td>0.0067</td>
</tr>
<tr>
<td>48</td>
<td>61.7 ± 0.0130</td>
<td>58.05 ± 0.0209</td>
<td>0.1412</td>
</tr>
<tr>
<td>120</td>
<td>0.0</td>
<td>0.0</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: Not applicable.
Figure 2

(A) Leukocyte Center/Total Ratio vs. Time (h)
- VH
- GP

(B) Leukocyte Center/Border Ratio vs. Time (h)
- VH
- GP