Effects of propolis-containing nanofibers on corneal wound in rats

Research Article

Osman Bulut[1a]
Ali Sorucu[2b]
Tolga Meriç Dümbek[1c]
Zehra Avcı[3d]

[1] Muğla Sıtkı Koçman University, Faculty of Milas Veterinary Medicine, Surgery Department, Muğla, Türkiye

[2] Muğla Sıtkı Koçman University, Faculty of Milas Veterinary Medicine, Pharmacology Department, Muğla, Türkiye

[3] Bursa Uludağ University, Faculty of Veterinary Medicine, Pathology Department, Bursa, Türkiye

ORCİD-

[a]0000-0003-2773-8243

[b]0000-0002-0496-9498

[c]0000-0003-1734-3491

[d]0000-0003-1853-4679

Correspondence

Osman BULUT

obulut@mu.edu.tr

ABSTRACT

The cornea is the outermost layer of the eye and is constantly exposed to trauma due to its anatomical location. Propolis is a substance produced by honeybees by mixing the extracts they collect from plants with their secretions. Studies have shown that propolis contains essential biological active substances for the life of organisms, which enhance epithelialization and have strong analgesic, anti-inflammatory, immunomodulatory, antioxidant, antitumor, antibacterial, antifungal, and antiviral effects. In our study, we created a corneal wound with a diameter of 3 mm using a corneal blade. In the experimentally induced corneal wound, no treated the first group, the second group was treated with nanofibers containing propolis produced by the electrospinning method, and the third group treated water-based topical propolis application. Topical propolis was applied once a day for 3 days, while nanofibers containing propolis were applied once following wound formation. Fluorescein staining was performed on the rats eyes every day throughout the study, and photographs were taken to measure the wound sizes. On the third day, the rats were euthanized under general anesthesia, and histopathological examination was performed on their corneas. In terms of bleeding, no significant difference was observed between the propolis and control groups, while a lower level of bleeding was detected in the nanopropolis group. Propolis and nanofibers containing propolis groups showed a significantly positive effect on wound healing compared to the control group.

Keywords: Bee product, experimental, electrospinning, nanopropolis

INTRODUCTION

The cornea is the outermost layer of the eye and is constantly exposed to external factors due to its anatomical location (Ljubimov and Saghizadeh, 2015). Under normal conditions, the cornea is important for maintaining transparency and visual acuity (Khosravimelal et al., 2021). The cornea epithelium, Bowman's layer, corneal stroma, Descemet membrane, and corneal endothelium constitute the five layers of the cornea, with the corneal epithelium being the outermost layer (Nagai et al., 2018). Many corneal injuries involve damage to the epithelial layer (Wilson, 2020).

Normal corneal keratocytes are stable and assist in maintaining corneal transparency (Yi and Zou, 2019). In response to corneal injuries, an healing process occurs, involving the migration of surrounding cells to fill the defect, cell proliferation, cell differentiation, and remodeling. Failure to timely and properly close the defect can lead to corneal ulceration, perforation, or opacification (Wilson, 2020; Nagai et al., 2018).

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Increasing topical lubricants, reducing evaporative tear loss, applying topical antibiotics, shielding the corneal surface with a bandage contact lens, and surgery are all part of the standard treatment for complicated corneal wounds. These measures, however, are frequently ineffective even when taken together (Chandler et al., 2019).

Propolis is a product created by bees through the mixing of plant materials they collect with their own enzymes in order to defend their hives. The word "propolis" is derived from the Greek words "pro" (before) and "polis" (city), referring to its association with hive protection (Forma ve Brys, 2021; Przybyłek and Karpiński, 2019). Bees use propolis for various reasons such as filling gaps in the hive walls, reducing the entrance during cold days, mummifying foreign intruders, and disinfecting the hive (Przybyłek and Karpiński, 2019).

Bees collect resins from buds, exudates, and other parts of plants, mix them with their salivary enzymes and beeswax, and create propolis (Almuhayavi, 2020; Forma ve Brys, 2021; Przybyłek and Karpiński, 2019). The composition of propolis varies depending on the vegetation, season, and climatic zone of the region it is sourced from (Marquez et al., 2023; Przybyłek and Karpiński, 2019). Despite variations in composition, propolis possesses antibacterial, antiviral, antioxidant, anti-inflammatory, immunomodulatory, and antitumor properties (Hossain et al, 2022). Due to these effects, propolis has been used in cases of gastrointestinal disorders, wound healing, dermatitis, corneal wounds, and conjunctivitis (Mele, 2023).

Electrospinning is an electrohydrodynamic process used for the production of polymeric fibers with diameters in the range of a few microns. The properties of polymeric fibers produced by electrospinning can be tailored to various applications, including tissue regeneration and repair in the medical wound (Mele, 2023).

In recent years, various agents have been tried for corneal ulcers, and propolis is one of them. The concept of enhancing the adhesive properties of nanofibers on surfaces through chemical coatings or surface modifications is widely acknowledged. It has been documented in the literature that natural components such as propolis can be combined with nanofibers to create a biologically compatible material. However, nanopropolis obtained through the electrospinning method has never been tested before in corneal wounds. The aim of this study is to investigate the effectiveness of nanopropolis obtained by the electrospinning method in experimentally induced corneal wounds.

MATERIALS AND METHODS

Animals

A total of 24 female Wistar albino rats, with 8 rats in each group, were used in the study. The rats were obtained from the Bursa Uludağ University Experimental Animals Application and Research Center. Throughout the study, the animals were maintained on a 12-hour light-dark cycle and provided with *ad libitum* food and water.

The prepation propolis

The propolis used in the study was collected from an apiary located in the Muğla region through a propolis trap. The collected propolis had a reddish color. All the propolis was first frozen at -20°C and then ground into a powder using a grinder (Lavion grain mill) to achieve a homogeneous consistency. Ultra-pure water was used for the extraction of propolis. 400 g of propolis were mixed with 1 liter of ultra-pure water and left to stand in an orbital shaker for 10 days. During the time it spent in the orbital shaker, it was immersed twice a day in an ultrasonic bath for 30 min each time. The obtained mixture was filtered through Whatman No.1 filter paper to obtain the extract. The extract was sent to the Department of Textile Engineering at Bursa Uludağ University for obtaining the extract tissue.

Electrospinning methods and nanopropolis

In this study, polyethylene oxide (PEO) (Mw: 900 kDa, Sigma Aldrich, USA) was used as the polymer, and propolis was used as the active substance to be added to the polymer solution. No pre-treatment was applied to the materials used in the study. Firstly, a polymer solution was prepared with a weight ratio of 3% PEO in the polymer solution. For this purpose, the PEO polymer was mixed with ethanol and water (1:1) using a magnetic stirrer until completely dissolved. Then, a propolis: ethanol solution was added to the prepared solution, with the propolis ratio in the total solution being 1% of the weight of the PEO solution. The final solution was mixed on a magnetic stirrer for approximately 24 hours to obtain a homogeneous solution.

An electrospinning system from Inovenso was used for the electrospinning process. During electrospinning, the prepared PEO/propolis solution was fed to the system at a rate of 1.5 mL/hour. A voltage of 17 kV was applied to the polymer solution. The distance between the spinneret and the collector was maintained at 15 cm. PEO/propolis nanofibers were collected on a rotating cylinder at a speed of 300 rpm.

Corneal wound model and measurements

The animals were anesthetized with intramuscular injections of 10 mg/kg Xylazine hydrochloride (Rompun®, Bayer, Germany) followed by 70 mg/kg Ketamin hydrochloride (Ketalar®, Parke-Davis, USA). A 3 mm punch biopsy was used to determine the boundaries of the wound to be created under anesthesia. After determining the boundaries, the first two layers of the cornea were removed using a corneal knife. Following the surgery, fluorescein staining was performed daily, and photographs were taken for measurements. The obtained photographs were evaluated using the "ImageJ" (Wayne Rasband National Institutes of Health, USA) program in digital format.

Experimental design

The animals were divided into three groups, with 8 animals in each group. The applications in the groups were performed as follows:

Control Group: No application was performed following wound formation.

Nanopropolis Group: Nanopropolis was applied as a single application following wound formation.

Water-based propolis Group: Water-based propolis was applied one drop (50 µL) once a day for three days following wound formation.

Histopathologic analyses

Three days after creating the corneal wound, euthanasia was performed on the rats under anesthesia by cervical dislocation. Subsequently, enucleation surgery was carried out to collect eye samples. The collected samples were fixed in a 10% formaldehyde solution for histopathological examination. After fixation, the samples were dehydrated through alcohol and xylene series and then transferred to paraffin blocks. The obtained samples were stained with hematoxylin-eosin and examined under a microscope at magnifications of 4x, 20x, and 40x. Based on the examinations, the findings were evaluated as follows: 0; absent, 1; mild, 2; moderate, and 3; severe.

Statistical analysis

All the data was presented as mean ± standard error of mean (SEM). Statistical Package for Social Sciences (SPSS) version 22.0 for Windows (SPSS Inc., Chicago, IL) was used to compare data across groups using one-way Analysis of Variance (ANOVA) and post hoc Tukey honestly significant difference (HSD) tests were conducted. The histopathology results were evaluated using the non-parametric Kruskal-Wallis test. Pairwise comparisons for non-normally distributed variables were performed using the Mann-Whitney U test and evaluated with Bonferroni correction. P values equal or less than 0.05 were considered statistically significant.

RESULTS

Corneal wound sizes

The eye samples, which were stained daily with fluorescein and photographed, were transferred to digital format. Subsequently, using the ImageJ (Wayne Rasband National Institutes of Health, USA) program, the boundaries of the area with dye retention were determined, and the area calculations (mm^2) were performed. The results are shown in Table 1.

Table 1. Fluorescein staining results 0, 24, 48, and 72 hours after wound formation

Time	Control	Nanopropolis	Propolis	P value
0.hours	10.054±0.242	9.827±0.126	9.962±0.203	0.716
24.hours	5.154±0.213[a]	1.074±0.196[c]	2.403±0.24[b]	<0.001
48.hours	1.653±0.201[a]	0±0[b]	0±0[b]	<0.001
72.hours	0±0	0±0	0±0	-

[a-c]: Different letters in the same line are statistically significant (P<0.001). The values are given in mm^2(square millimeters)

When the average healing areas at 24, 48, and 72 hours were calculated among the groups, in the control group, at the end of 24 hours, 49% of the total wound area had healed, 82% had healed at 48 hours, and 100% had healed at 72 hours. In the nano-propolis group, 89% of the total wound area had healed at 24 hours, and 100% had healed at 48 hours. In the propolis group, 75% of the total wound area had healed at 24 hours, and 100% had healed at 48 hours (Figure 1).

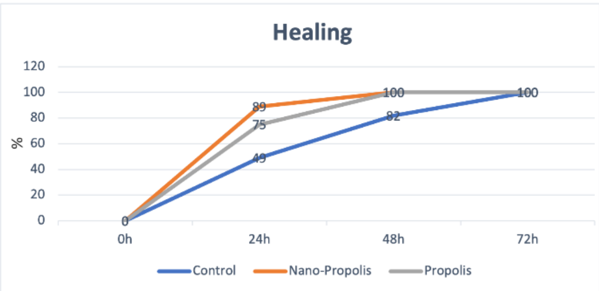

Figure 1. Percentages of corneal improvement at 0, 24, 48, and 72 hours.

The obtained values were subjected to an ANOVA test, and the statistical analysis revealed that there was no significant difference among the groups at 0 hours (P>0.05). At 24 hours, a significant difference was observed among the three groups, with the nano-propolis group showing the best healing (P<0.001). At 48 hours, there was no significant difference between the nano-propolis and propolis groups, but a significant difference was observed between the control group and the study groups (P<0.001). Since all corneas had healed by 72 hours, no statistical analysis could be performed (Table 1). After creating corneal wounds, the healing process was monitored for 72 hours and recorded in a digital format. When fluorescein staining was applied to the recorded photographs, the wounded areas were visually observed (Figure 2).

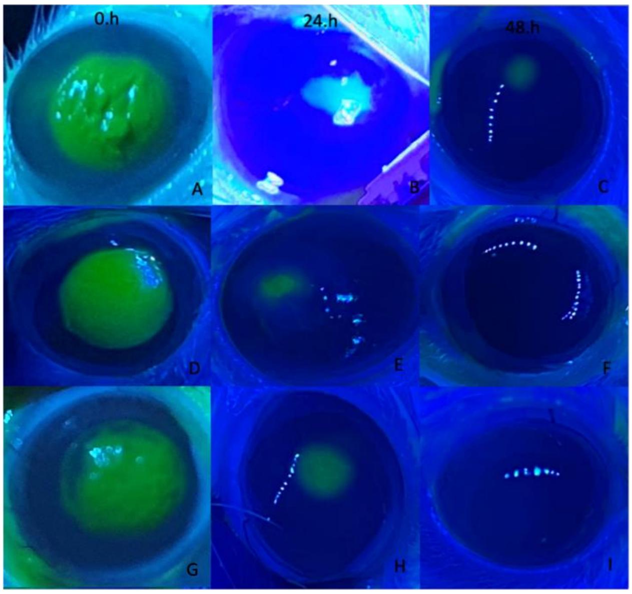

Figure 2. The digital images of the wounded areas. A: 0-hour control group. B: 24-hour control group. C: 48-hour control group. D: 0-hour nano-propolis group. E: 24-hour nano-propolis group. F: 48-hour nano-propolis group. G: 0-hour propolis group. H: 24-hour propolis group. I: 48-hour propolis group.

Histopathologic results

The findings were determined to be concentrated in the regions close to the epithelial layer. Figure 3 (A) illustrates the area of ulceration in the epithelial layer. Figure 3 (B) demonstrates cell infiltration and corneal edema in the region near the epithelial layer.

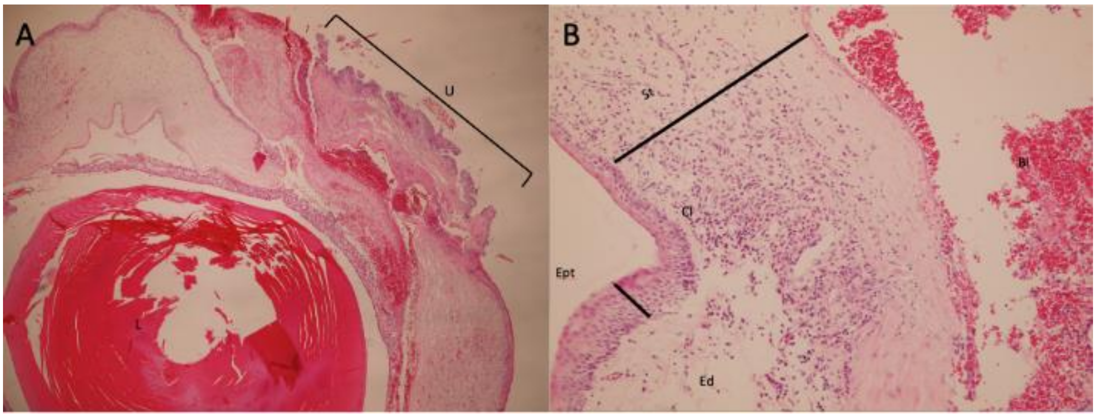

Figure 3. Histopathological images. A: Observation of ulcerated area in the control group under 4x magnification. (L: Lens, U: Ulceration). B: Figure of the propolis group under 20x magnification. (St: Stroma, Bl: Bleeding, Ed: Edema, Ept: Epithelium, Cl: Cell infiltration).

Moderate corneal edema was observed in all groups, but there was no histopathologically significant difference between the groups ($P>0.05$). The groups were evaluated for bleeding, and varying degrees of findings ranging from moderate to severe were observed in the control and propolis group, while the nano-propolis group showed a significantly lower occurrence ($P<0.05$). The groups were assessed for vascularization and cell infiltration, and findings ranging from mild to moderate were observed in all groups, with no significant difference between the groups ($P>0.05$). Furthermore, the groups were evaluated for ulceration, and mild ulceration was observed in all groups, with no significant difference between the groups ($P>0.05$). The results are shown in Table 2.

Table 2. Histopathological examination results. The findings were evaluated as follows: 0; absent, 1; mild, 2; moderate, and 3; severe.

Findings	Control	Nanopropolis	Propolis	P value
Corneal Edema	2.000±0.327	1.750±0,313	1.500±0,327	0.598
Bleeding	2.125±0.295^{b}	1.125±0.295^{a}	2.375±0.263^{b}	0.024
Vascularization	2.000±0.267	1.750±0.250	1.875±0.350	0.777
Cell infiltration	2.125±0.226	1.625±0.323	1.875±0.295	0.512
Ulceration	0.625±0.182	0.250±0.163	0.500±0.188	0.324

$^{a-b}$: Different letters in the same line are statistically significant ($P<0.05$).

DISCUSSION

The transparent cornea serves as the eye's frontal layer, refracting incoming light for vision and shielding the underlying ocular tissues from harm. It is crucial to have a well-coordinated corneal wound healing response after injury to avoid loss of transparency and subsequently vision as well as to keep the barrier of protection in place (Reins et al., 2016). The complex process of healing a corneal wound includes the migration and division of epithelial cells, the death of stromal keratocytes, the regeneration of nerve tissue, and a localized inflammatory response that results in the infiltration of immune cells and the extravasation of platelets from limbal vessels (Nagai et al., 2018). In this study, we investigate the healing effect of nanopropolis on corneal wounds in rat.

Propolis is a well-known resinous material collected by bees from bud and exudates of the plants, mixed with bee enzymes, pollen and wax (Zullkiflee et al., 2022). Propolis can be used in many medical fields. Propolis has been extensively used by several civilizations to treat colds, wounds and ulcers because of its antiseptic and local anaesthetic properties (Sforcin, 2016). Propolis contains a wide variety of phenolic compounds, typically phenolic acids and flavonoids with biological effects. Several pharmacological effects have been attributed to propolis, especially ethanolic propolis extracts, such as antibacterial, antioxidant, antiviral, fungicidal, anti-inflammatory, anticarcinogenic, antiapoptotic, immunomodulatory and gastric protective (antiulcer) effects (Oruç et al., 2017). In veterinary medicine, propolis is reportedly active against fungal otitis and dermatomycosis in dogs (Cruz Sánchez et al., 2014), and may also use for treatment of bovine dermatophytosis (Çam et al., 2009). Propolis extract has been shown to have anti-inflammatory effects in the treatment of canine anal sacculitis (Durgun and Durmuş, 2004). Propolis appears to have a positive impact on the healing of wounds (Yang et al., 2022). More frequent dressing changes would have improved the antimicrobial and wound healing effects. Similar to these findings, the current study's findings demonstrated that propolis in the healing of burn wounds due to its antioxidant, anti-inflammatory, and antimicrobial properties. The wound healing effect of propolis is attributed to its contained aminoacids, phenolic acids, phenolic acids esters, flavanoids, cinnamic acid, terpens and caffeic acid (Kasote et al., 2022). According to our results propolis and nanoprolis showed an improvement corneal wound, for the first 2 days after the injury. However, since the composition of propolis was not examined in this study, it is unclear which specific propolis characteristic led to this improvement.

Re-epithelialization of the rat cornea takes roughly 72 hours after debridement of a 3 mm diameter circular area, leading to complete covering of the injured area with basal epithelial cells (Nagai et al., 2018). Before proliferating to help with re-stratification, epithelial cells must first migrate to cover the wound during re-epithelialization. Cell division is inhibited in the wound area right away to facilitate effective migration (Terai et al., 2011). When the average healing areas over the course of 24, 48, and 72 hours were calculated for each group, the control group had a wound that was 49% healed after 24 hours, 82% healed after 48 hours, and 100% healed after 72 hours. At 24 hours, 89% of the total wound area in the nanopropolis group had healed, and at 48 hours, 100% had. In the propolis group, 100% of the wound area had healed after 48 hours, and 75% of the overall wound area had.

In a study conducted, topically applied propolis has been reported to reduce cell infiltration at 24 and 48 hours (Martin et al., 2013). In our study, at the end of 72 hours, there was no significant difference among the groups regarding cell infiltration. Based on the results of our study, we believe that longer term studies in terms of vascularization should be conducted. Currently, there is no existing study on the use of nanopropolis in the treatment of corneal wounds; therefore, histopathological findings take precedence.

The use of nanofiber structures in wound healing has gained popularity in recent times. The aim is to accelerate healing by incorporating known substances that promote healing into the production of nanofiber structures (Cavalu et al., 2019). Morais and colleagues (2022) reported in their study that collagen nanofibers obtained through the electrospinning method accelerated wound healing. Shi and colleagues (2023) also conducted a study where they produced nanofibers using hydrogel not propolis through the electrospinning method and used them in corneal wounds (Morais et al., 2022; Shi et al., 2023). In their study, they found that edema and inflammation in the corneal stroma significantly decreased. They discovered that nanofibers with hydrogel components accelerated corneal healing in acute alkali wounds. It is speculated that the use of nanofiber structures in corneal wounds will increase in the current era. One advantage of using these nanofibers is that it eliminates the difficulty of continuous application and provides successful results with a single application.

Nanopropolis has emerged as an expanding field with a wide range of applications and effectiveness, similar to propolis. Nanopropolis has become a subject of study in the field of antibacterial, antifungal, and antitumoral properties (Tatli Seven et al., 2018).

CONCLUSION

In our study, nanopropolis prepared using the Electrospinning method was tested for corneal wounds. As a result of our study, it has been concluded that the application of nanopropolis in superficial corneal wounds as a single administration would be beneficial. However, further research is needed in this field. Especially for faster and uncomplicated wound healing, advanced studies are needed to use nanofibers containing propolis in different bases, and to compare these bases, with the aim of particularly investigating their efficacy.

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Conflict of interest: The authors declare no conflict of interest.

Ethical statement: The study was conducted with the approval of the Bursa Uludağ University Animal Experiments Local Ethics Committee under permit number: 2019-06/03.

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