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Investigation of the healing effectiveness of pine resin in experimentally induced corneal wound in rats

Osman Bulut [1] Tolga Meriç Dümbek [1] Ali Sorucu [2] Zehra Avci Küpeli [3]

[1] *Department of Surgery, Faculty of Milas Veterinary Medicine, Muğla Sıtkı Koçman University, Muğla, Türkiye*
[2] *Department of Pharmacology, Faculty of Milas Veterinary Medicine, Muğla Sıtkı Koçman University, Muğla, Türkiye*
[3] *Department of Pathology, Faculty of Veterinary Medicine, Bursa Uludağ University, Bursa, Türkiye*

Correspondence: *Osman Bulut (obulut@mu.edu.tr)*

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ABSTRACT

Objective: Pine resin is a product obtained from plants belonging to the Pinaceae family and traditionally used in the treatment of wounds. The aim of this study is to determine the effectiveness of pine resin in corneal wounds.

Material-Methods: In this study, three groups of 7 male Wistar Albino rats (n=7), each 2 months old, were established. To create the corneal wound model, the rats were anesthetized and the borders of the wound to be created on the corneal surface were determined using a 3 mm punch biopsy, then the first two layers of the cornea were removed with a corneal knife. Then, the first group was considered as the control group and no treatment was performed. The second group was determined as the pine resin group and applied once a day. The third group was considered as the drug group and was administered once a day. Fluorescein staining was performed every day for three days and the results were recorded.

Results: Pine resin group showed the fastest recovery. On the third day, the rats were euthanized, and their eyes were enucleated. The collected eyes were sent for histopathologic examination and stained with hematoxylin-eosin. The lesions in the examined specimens were evaluated under microscope for hyperemia, vascularization, cellular infiltration and corneal edema.

Conclusion: As a result of the study, ulceration was observed in the pine resin group. The study concluded that pine resin reduces clinical symptoms and promotes healing in corneal wounds.

Keywords: *Fluorescein, Natural, Rosin, Terpenoids, Ulceration*

INTRODUCTION

The corneal epithelium defends the eye from pathogen entry and is crucial to maintaining corneal clarity. As a result, cornea is extremely susceptible to the blinding effects of serious illness or trauma (Fernandes-Cunha et al., 2019). External traumas to the cornea frequently result in abrasions, lacerations, and corneal ulcers, which impair vision. Pathogens such as viruses, bacteria, and fungi can cause corneal ulceration and infection, which can result in corneal blindness (Demir et al., 2022). Reduced vision is frequently caused by corneal ulceration. Due to the severity of corneal lesions and the ways in which they are repaired during various morbid processes, prompt implementation of efficient therapeutic measures is required (Alfaris et al., 2009).

The squamous epithelium protects the cornea from environmental trauma while preserving optical clarity. The cornea typically re-epithelializes right away after damage to reduce the chance of infection, opacification or perforation. Delay in re-epithelialization, however, may result in a slow healing of the injured corneal epithelium and ultimately the loss of corneal barrier function in conditions like dry eye syndrome, diabetic keratopathy, and chemical injury. If the injury is not treated properly, it invades the cornea, eventually leading to corneal melting, corneal perforation, and blindness. Healing a corneal wound is a difficult process that requires three ongoing procedures. These are complete re-modelling of the adhesive structure, active migration of superficial cells to cover the wound surface, and cell proliferation, differentiation, and stratification. The first step in the sequential procedures that quickly heal the wounds is corneal epithelial migration. Therefore, it is crucial to create a therapeutic plan to encourage epithelial migration and thus corneal re-epithelialization (Kibar Kurt and Belge, 2021; Hu et al., 2023).

Resins are liquids produced by trees to protect themselves against external factors, to heal wounds on their trunks or to protect newly developing shoots from the effects of rain and sun rays (Dell and McComb, 1979; Alihosseini, 2016). This liquid usually contains substances with important physiological properties such as phenolic compounds, alkaloids, terpenes, saponins, glycosides as secondary metabolites (Alihosseini, 2016). Pine resin is a natural resin secreted by members of the Pinaceae family (Güzel, 2019). It is secreted especially from the injured parts of the trunks of pine trees or during the maturation of the cones and provides protection and healing of the tree. The resin, which is initially secreted as liquid, solidifies white and yellow upon contact with air and dries on the surface (Rodrigues-Corrêa et al., 2012). The chemical composition of pine resin includes many secondary metabolites, mainly terpenoids (Park et al., 2017). Most of these are volatile monoterpenes and sesquipenes (Rodrigues-Corrêa et al., 2012; Güzel, 2019). Many traditional medicine systems use pine resin as medicine in various forms to treat wounds (Khmelnitskii et al., 2002; Shah, 2011; Rodrigues-Corrêa et al., 2012; Park et al., 2017). Turpentine obtained from pine resin is widely used in medicine (Shah, 2011; Sharma et al., 2018). Tar, which is obtained by burning various pine trees and contains a large amount of terpenes, is used locally to treat various wounds on animals (Barnes and Greive, 2017). Apart from wounds, it is used to protect the hooves of animals (Barnes and Greive, 2017; Sinmez et al., 2018). Especially terpenoids and other secondary metabolites in pine resin accelerate wound healing by various mechanisms (Pérez-Recalde et al., 2018; Romo-Rico et al., 2022; Venkata et al., 2022).

Pine resin, also referred to as rosin, is the natural resin secreted by Pinaceae family members including Pinus longifolia Roxb., Pinus sylvestris L., and Pinus palustris Mill. It is also known as Colophony, Pine Resin, and Resina Pini. Many conventional medical systems use pine resin as a drug to treat wounds. Pine resin is used in Unani medicine to treat suppurated wounds because it decreases exudation and boosts local perfusion (Park et al., 2017). Abietanes and pimaranes are the two main types of diterpenoids found in fresh resin. Pimaranes are more stable than abietanes, which are more likely to isomerize and yield abietic acid (Beltran Sanchidrian, 2016).

Studies on the effectiveness of pine resin on corneal wounds could not be found in the literature. Therefore, the aim of this study is to investigate the effectiveness of pine resin on experimentally induced corneal wounds in rats.

MATERIALS and METHODS

The preparation of pine resin

Pine resins to be used in the study were collected from red pine (Pinus brutia Ten.) forests in Muğla region. The collected resin was dried in the shade, then hardened in the freezer and finally homogenized by pulverizing with a grain grinder. The powder resin was weighed 100 g and mixed with 500 ml of ultrapure water. It was shaken for 5 min twice a day for 10 days using a vortex mixer, then filtered with Whatman No. 1 filter paper, transferred to falcon tubes and stored at +4°C until the experimental phase was performed. The pH of pine resin is between 2-7. Since the pH of the eye is 7.4, the pH of the extraction was adjusted to 7.4 with $NaHCO_3$ before the application (Angin and Ertas, 2021).

Animals

Twenty-one male Wistar albino rats (230-250 g/60 day old) were used in study. The rats were fed with ad libitum water and food under a 12/12-hour light/dark environment at a temperature of 22±2°C during the study. Rats were obtained from Muğla Sıtkı Koçman University, Experimental Animals Application and Research Center, Muğla, Turkey. The study was conducted with the approval of the Muğla Sıtkı Kocman University Animal Experiments Local Ethics Committee under permit number 24.08.23/20-23

Experimental procedure

Rats were acclimatized for two weeks prior to the study. After 0. hours in study, all rats were anesthetized by administering 10mg/kg Xlazine hydrochloride (Rompun, Bayer, 23.32 mg/mL, Barmen, Germany) followed by 70 mg/kg Ketamin hydrochloride (Ketalar, Parke-Davis, 50 mg/mL, Brooklyn, New York, ABD) injection intramuscularly. The right eyes of the anaesthetized rats were prepared sterilely for the operation. The borders of the wound to be created with a 3 mm punch biopsy were then determined and the first two layers of the cornea were removed with a corneal knife (Zagon et al., 2000; Nagai et al., 2009). After 21 male Wistar albino rats were divided into three groups randomly. Groups: Control (no medication or treatment was applied to the rats in this group from the time of corneal wound formation), Pine resin (Pine resin was applied one a day with one drop to the rats in this group until the day of sacrifice at the cornea), Drug (The antibiotic drug - Exocin, Allegan, 3mg/mL, Ireland) was applied one times in a day with one drop to the rats in this group until the day of sacrifice at the cornea). Following the operation, the corneal wound site was measured in terms of length and within mm until the day of sacrifice and documented by taking photographs. For this purpose, before the measurements each rat was applied fluorescein test. The corneal wound surface areas were calculated by transferring the photographs to the "Image j" program in the computer environment. Total wound healing was found by looking at the difference between days in terms of wound areas.

Histopathologic analyses

After the formation of the corneal wound, rats were euthanized by cervical dislocation under general anesthesia on third day. The corneal tissue sample was taken from each euthanized rat with enoculation bulbi procedures. The cornea samples taken were fixed in a 10% formaldehyde solution for histopathological examination. After fixation, the tissues were subjected to a tissue processing procedure consisting of alcohol and xylene series, and then embedded in paraffin blocks. 4µm thick sections transferred from the paraffin blocks to slides were stained with hematoxylin-eosin, and then microscopic examination was performed on these sections.

Statistical analysis

All the data was presented as mean ± standard error of mean. Statistical Package for Social Sciences (SPSS) version 22.0 for Windows (SPSS Inc., Chicago, IL) was used for analysis. For corneal wound sizes used to compare data across groups using one-way Analysis of Variance (ANOVA) and post hoc Tukey honestly significant difference tests were conducted. Kruskal-Walli's test was used for histo-pathological examination. Paired comparisons of variables were performed using Mann-Whitney U. P-values below 0.05 were considered as statistically significant results.

RESULTS

Corneal wound sizes

When daily fluorescein staining images were examined macroscopically, a gradual decrease was observed in all groups. The corneas of the rats in the pine resin group showed complete disappearance of dye uptake at the end of the 48th hour (Figure 1).

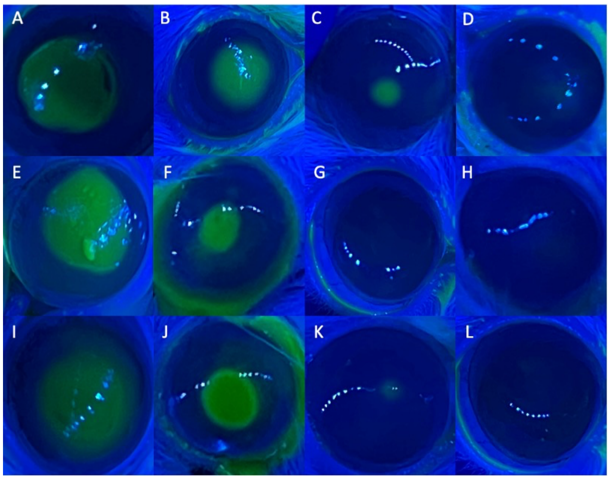

Figure 1. (A) 0.hours in control group (B) 24.hours in control group (C) 48.hours in control group, (D) 72.hours in control groups, (E) 0.hours in pine resin groups, (F) 24.hours in pine resin groups, (G) 48.hours in pine resin groups, (H) 72.hours in pine resin groups, (I) 0.hours in drug groups, (J) 24.horus in drug groups, (K) 48.hours in drug groups, (L) 72.hours in drug groups.

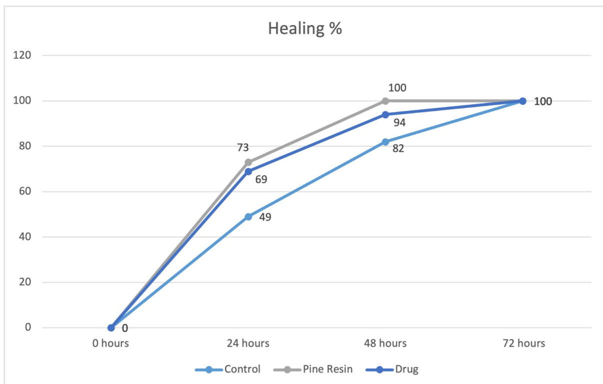

Figure 2. Daily healing percentages of corneal wounds.

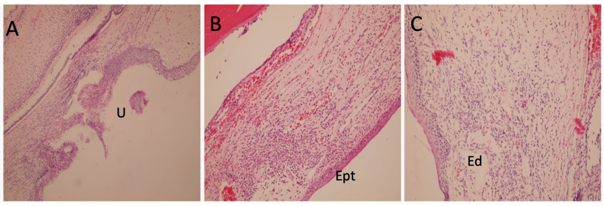

Figure 3. Histopathological images. A. Showed ulceration area in control group. U: Ulceration. B. Showed remodeling area in pine resin group. Ept: Epithelium Layer. C. Showed edema at stroma in drug group. Ed: edema.

The fluorescein staining measurements were subjected to an ANOVA analysis test. According to the analysis, at the 24th hour, the pine resin and drug groups showed significantly faster improvement compared to the control group. At the 48th hour, there is a significant difference among the three groups, with the pine resin group showing the fastest improvement, while the control group exhibited the slowest improvement ($p<0.05$). At 72 hours, all three groups have fully improvement (Table 1).

Table 1: Results of fluorescein staining 0, 24, 48, and 72 hours after surgery of the wound. The values are given in mm^2 (square millimeters).

Time	Control	Pine Resin	Drug	P value
0. hours	9.956±0.227	9.772±0.170	10.009±0.225	0.706
24. hours	5.034±0.197^a	2.569±0.245^b	3.038±0.135^b	<0.001
48. hours	1.759±0.273^a	0.000±0.000^c	0.522±0.137^b	<0.001
72. hours	0.000±0.000	0.000±0.000	0.000±0.000	-

a-c: Different letters in the same line are statistically significant ($p<0.001$).

Table 2: Histopathological examination results (0: none, 1: mild, 2: moderate, 3: severe)

Findings	Control	Pine Resin	Drug	p value
Corneal Edema	2.125 ±0.295^a	0.875±0.295^b	1.750±0.250^a	0.029
Bleeding	1.750±0.250	1.375±0.182	1.750±0.313	0.375
Vascularization	1.875±0.295	1.625±0.263	1.500±0.267	0.728
Cell Infiltration	2.250±0.163	2.000±0.267	1.750±0.163	0.238
Ulceration	0.625±0.182^a	0.000±0.000^b	0.500±0.188^a	0.028

The mean extent of healing in the groups was calculated as percentages. The control group showed an average of 49% healing at 24. hour, 82% at 48 hour and 100% at 72 hours. Pine resin group showed 73% healing at 24. hour and 100% healing at 48 hours. The drug group showed 69% improvement at 24. hour, 94% at 48 hour and 100% at 72 hour (Figure 2). The amounts of corneal wound healing (%) were calculated according to this equation (Nagai et al., 2010);

Corneal wound healing (%) = (wound area0h – wound area24-36-72h) / wound area0h x 100

Histopathological examination

The sections stained with hematoxylin-eosin were taken for microscopic examination. In the histopathological examination, the groups were evaluated semi quantitatively for the presence of bleeding, vascularization, cellular infiltration, corneal edema, and ulcer formation (0: none, 1: mild, 2: moderate, 3: severe).

When histopathological images were examined, microscopic ulceration areas were observed in the control group within the epithelial layer. In the pine resin group, it was noted that the epithelial layer exhibited a smoother healing compared to other groups, and cell infiltration was observed in the subepithelial layer, while microscopic bleeding foci were visible near the endothelial layer. In the drug group, edematous areas were observed in regions close to the epithelium, and bleeding foci were observed in subendothelial layer (Figure 3).

Histopathologically, all animals were examined for corneal edema, bleeding, vascularization, cell infiltration and ulceration. The tests revealed that the pine resin group exhibited significantly better healing in terms of corneal edema and ulceration compared to the control and drug groups. However, there was no difference between the drug group and the control group in terms of corneal oedema and ulceration. No significant differences were detected among the groups in terms of bleeding, vascularization and cell infiltration (Table 2).

DISCUSSION

Due to its structure, the cornea is extremely important for the continuity of vision. Therefore, corneal wounds should be treated quickly and normal corneal structure should be restored (Martin et al., 2013; Chandler et al., 2019). Physical injuries of the cornea occur in animals due to factors such as foreign bodies, tear problems, loss of eyelid function, anomalies, infection and trauma (Demir et al., 2022). Various methods such as scraping the layers of the cornea (physically) or exposing the cornea to chemical agents can be applied to experimentally create corneal wounds (Ho et al., 2013; Fernandes-Cunha et al., 2019; Kibar Kurt and Belge, 2021). Physically induced corneal injuries in rats have been used in studies for the development of corneal healing drugs (Zagon, 2007; Nagai et al., 2010). Therefore, we physically induced corneal injury to examine the efficacy of pine resin extract on corneal injury.

Resins have important biological activities such as antibacterial, antifungal, antiviral, antiviral and antiparasitic thanks to the substances in their content (Alkan et al., 2016). One of the effective examples of this is propolis, which bees obtain by collecting plant resins (Salatino et al., 2011). Pine resin is a natural resin found in injured areas on the trunks of pine trees (Rodrigues-Corrêa et al., 2012; Güzel, 2019). Traditional medicine systems have used pine resins to treat wounds (Khmelnitskii et al., 2002; Shah, 2011; Rodrigues-Corrêa et al., 2012; Park et al., 2017). Pine resins, which are frequently used in traditional medicine, are now included in ointments used for medicinal purposes. These ointments are seen as a promising treatment for burns, wounds (stage 1 of the wound process), purulent diseases of the skin and subcutaneous tissue (Simbirtsev et al., 2002e). The terpenoids and other secondary metabolites it contains have been reported to accelerate wound healing by various mechanisms (Pérez-Recalde et al., 2018; Venkata et al., 2022).

Studies on the direct wound healing effect of pine resin are quite limited wounds (Khmelnitskii et al., 2002; Shah, 2011; Rodrigues-Corrêa et al., 2012; Park et al., 2017). In addition, there are no studies in the literature on the effectiveness of pine resin on corneal wounds. However, honey, which has been used together with resins for wound healing since ancient times, and propolis, which is also obtained from resins, have been reported to accelerate healing on corneal wounds (Martin et al., 2013; Park et al., 2017; Abd Rashid et al., 2022; Bulut et al., 2023). Based on these results, we also examined the healing accelerating activity of pine resin on corneal wounds.

Alcohol-based substances have been used in previous studies to purify pine resin (Boudjelal et al., 2022) and it is known that good efficiency in the extraction of terpenoids in it is obtained with solvents such as alcohol and benzene (Angin and Ertas, 2021). However, it is not appropriate to use alcohol and benzene in eye application (McDonald et al., 1970) , so we preferred to use pure water for the extraction of pine resin in our study. The normal pH value of pine resin is between 2-7. For this reason, after extraction with pure water, the pH value was adjusted to around 7.4 with NaHCO3, which is suitable for use in the eye. 7.4, which is suitable for use in the eye (Peyman et al., 2007).

Simbirtsev et al. (2002a; 2002b; 2002c; 2002d; 2002f) carried out various studies on pine resin and ointments containing pine resin. As a result of these studies, pine resin increased macrophage, neutrophil and leukocyte activity (Simbirtsev et al., 2002f), showed bacteriocidal activity (Simbirtsev et al., 2002d), inhibited humoral response (Simbirtsev et al., 2002a), suppressed free radicals (Simbirtsev et al., 2002b), showed immunomodulatory activity (Simbirtsev et al., 2002c). Rozbahani et al. (2019) examined the effectiveness of pine resin with their study on the skin and stated that pine resin showed a similar healing rate to the positive control group. However, in our study on corneal wounds, the use of pine resin accelerated healing compared to antibiotic use. Previous studies have indicated that the excipients in antibiotic drops used in the treatment of corneal wounds may affect corneal healing (Lin and Boehnke, 2000). The difference between the two studies is thought to be due to this reason. Pine resin has shown these effects in studies on skin. However, the effects of pine resin on the cornea are not fully known.

This study is pioneering as it is the first study to investigate the efficacy of pine resin extract on physically induced corneal wounds. Therefore, it was not compared with a previous study using pine resin. However, it has been reported in various studies that propolis species accelerate healing in corneal injuries compared to propolis obtained from resins (Ozturk et al., 1999; Martin et al., 2013; Abd Rashid et al., 2022).

Martin et al. (2013) used propolis in corneal wounds caused by alkaline substances in their study and revealed that corneal wounds were completely healed macroscopically at the end of the 120th hour. In our study, corneal wounds in animals treated with pine resin were completely healed macroscopically at the end of the 48th hour. It has been reported in previous studies that physically induced corneal wounds heal faster than corneal wounds induced by alkaline substances (Ho et al, 2013). The difference in healing time between the two studies is thought to be due to the difference in methods of corneal wound creation. In both studies, the fastest healing occurred within the first 24 hours.

Öztürk et al. (1999) compared propolis and dexamethasone on the corneas of rabbits and reported that they showed similar effects in terms of anti-inflammatory activity. Abd Rashid et al. (2022) reported that propolis obtained from honey and resins, which have been frequently used with resins since ancient times, accelerated healing in corneal wounds. The results of our study are compatible with these studies and pine resin, which is also used in propolis production, accelerated healing in physically induced corneal injuries.

When compared histopathologically with Propolis studies, Martin et al. (2013) reported that the use of Propolis at 24 and 48 hours reduced cell infiltration in corneal wounds. Bulut et al. (2023), in their study examining the effects of propolis and nanopropolis on corneal wounds, reported that propolis did not reveal a significant difference in terms of corneal edema, bleeding, vascularization, cell infiltration and ulceration compared to the control group. In our study, pine resin decreased corneal oedema and ulceration, but did not cause a significant change in cell infiltration.

CONCLUSION

In conclusion, in the study, pine resin accelerated the healing of experimentally induced corneal wounds in rats. Histopathologically, corneal oedema and ulceration were reduced. However, there is a need for further studies on the efficacy of pine resin by increasing the amount, frequency and duration of application.

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