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Evaluation of the subconjunctival injection of Hesperidin with or without olive oil on the healing of alkali burn corneal ulcer in rabbits

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ABSTRACT

Corneal ulcers represent an anxious problem in animals and humans. The alkali burn corneal ulcer is severe and may be associated with damage to most of the corneal structure. The healing of the corneal ulcer is mainly complicated by the impairment of vision. The striving to find a new therapy that promotes the healing of corneal injuries with the maintenance of the power of vision is the main aim of most studies. The current study was conducted to evaluate the effect of hesperidin with or without olive oil after its deposition under the bulbar conjunctiva on the healing of induced alkali burn corneal ulcers. For carrying out the study, 18 New Zealand albino rabbits were included. They were divided into three equal groups. Group I (control) received 0.5 ml of normal saline 0.9% under the bulbar conjunctiva 5 times at one-week intervals. Group II (H) received 0.5 ml of hesperidin nanovesicles subconjunctivally 5 times at one-week intervals. Group III (HO) received 0.5 ml of nanovesicles of hesperidin with olive oil under the bulbar conjunctiva 5 times one week apart. The right eye of animals was subjected to induction of corneal ulcer using 1% NaOH before the commencement of treatment. The left eye was used as a negative control one. The animals were examined clinically (lacrimation, neovascularization, pus formation, corneal perforation, measurement of corneal ulcer), and with fluorescein test staining every week just before each treatment. The animals were examined on days 1, 8, 15, 22, 29, 36 post corneal ulcer induction. At the end of the experiment, the treated and nontreated eye samples were collected for histopathological and electron microscopy examination. The results showed an improvement in the total clinical score in groups H and HO especially in the fifth week, while the control group displayed increasing in the inflammatory process of the injured eye throughout the time of experiment. There was a significant difference between both of H and HO groups and the control group in the third, fourth, and fifth weeks. The results of the histopathological and electron microscopy revealed the superiority of hesperidin with olive oil over hesperidin alone in promoting the healing of corneal ulcers (p= 0.045). The current study concluded that the subconjunctival injection of hesperidin with or without olive oil has a beneficial and promoting effect in the healing and regeneration of alkali burn corneal ulcers in rabbits. Moreover, subconjunctival injections can ensure long-term drug maintenance compared to topical methods, which in turn saves time and effort.

Introduction

It is common to experience chemical burns of the eye when an acidic or alkaline corrosive material attaches to the ocular surface. Medical attention is urgently needed for this type of injury (Ramponi, 2017). It is not uncommon for alkaline burns to occur in ocular tissues due to the widespread use of alkaline substances in home cleaning solutions and industrial applications (Pargament *et al*., 2015). In alkali injuries of the cornea, treatment aims to improve epithelial integrity and stromal stability, reduce inflammation, and prevent or manage complications as soon as possible. Traditional treatment plans have included a variety of biological medications and surgical procedures. New therapies for treating ocular ulcers include autologous and umbilical cord serum preparations, platelet-rich plasma, amniotic membrane transplantation, limbal stem-cell transplantation, and anti-angiogenic agents (Garg *et al*., 2001). There is a newly published study that used Hesperidin as a sole agent or combined with olive oil for topical management of the alkali burn corneal ulcers in rabbits (Ali *et al*., 2024). Hesperidin is a flavonoid that has anti-bacterial, anti-inflammatory, anti-apoptotic, and antioxidant properties. It is extracted from the citrus fruits. Olive oil is a natural source of polyphenolic compounds that have anti-inflammatory, angiogenetic, antioxidant, and antibacterial activities. As a result of the conventional ocular delivery systems which result in low bioavailability and therapeutic effectiveness due to rapid drug elimination through the nasolacrimal duct and high lacrimal fluid turnover, the frequent topical application of the drugs is required, which is associated with patient discomfort (Ahsan and Rao, 2017). It is essential to find a technique that maintains the drug's effect for a long time in the ocular system. Therefore, the current study investigated the effect of subconjunctival injections of nanovesicles of Hesperidin alone or Hesperidin-olive oil combination on healing alkali burn corneal ulcers in rabbits.

Materials and methods

This study included 18 New Zealand albino rabbits that were in good health. Among them, 12 were males, and six were females who were neither pregnant nor nursing. The rabbits' body weight and age varied between 2000-2500g and 6-8 months old. The standards of the ethical committee for the care and use of laboratory animals were taken into consideration. The National Ethical Committee of the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, approved the study, with the ethical approval number being aun/vet/1/0001.

The rabbits underwent eye examination thoroughly to detect any lesions or abnormalities. The assessment included shape, movement, position, secretions, and the menace response. As well as, the outer ocular structures such as eyelids, cornea, conjunctiva, iris, and pubil were examined (Stoppini and Gilger, 2016). The animals received 200 µg/kg of ivermectin (Arab veterinary industrial co. (AVICO); Naour - Alquds St. – Jordan) and were vaccinated against rabbit hemorrhagic viral disease (Inactivated vaccine against rabbit hemorrhagic disease (LAPIMUN GEM, BioTestLab; Ukraine; 10 doses bottle) with 1m/animal. Both were administered through the subcutaneous injections. For anesthesia, a mixture of

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xylazine HCl (5 mg/kg; xyla-ject 2%, Adwia, Egypt) and ketamine HCl (25 mg/kg; ketamine 10% alfasan, Elyoser Egypt) was injected intramuscularly. The preparation of nanovesicles Hesperidin and Hesperidin olive oil combination was carried out according to Ali *et al.* (2024).

An alkali burn-induced ulcer was created in the right eye of all rabbits using a sterile cotton swab of 1% NaOH on the central cornea for 60 seconds. The surface of the eye was then washed with 60 ml of physiological saline for 2 minutes, and the induced lesion was ascertained using the fluorescein dye test (Hosny *et al*., 2022).

Animals were divided into three different groups, each consisting of six rabbits. Group I (+ve control group): received 0.5ml of standard saline solution (0.9%) subconjunctivally after induction of corneal ulcer in the right eye. Group II (H group): received 0.5 ml of sterilized Hesperidin nanovesicle in the subconjunctival space. Group III (HO group): received 0.5 ml of Hesperidin-olive oil combination in the nanovesicle preparation into the subconjunctival space. Five injections were carried out in each group with one week apart. The animals were slaughtered at the 6th-week post induction of corneal ulcer. Eyes specimens were sent for histopathological and electron microscopical examinations. The left (intact) eye was used as a negative control sample.

Morphological evaluation

The eye examination was carried out weekly just before each treatment. Naked eye external and detailed ophthalmic investigations of each eye were carried out just after the completion of 1, 2, 3, 4 and 5 weeks after the corneal ulcer induction. Eyes were examined for the presence of perforation, vascularization, lacrimation, pus, corneal opacity and size of corneal ulcer.

The total clinical score includes the evaluation of the following: The degree of corneal neovascularization was marked from 0 to 3 (Basu *et al*., 2018), where 0= no neovascularization, 1= confined to the limbus of the cornea, 2= extending up to the margin of the pupil, and 3= extending beyond the margin of the pupil into the central cornea. Corneal opacity was scored as grade 0= clear or a trace haze; grade 1= mild opacity; grade 2= moderately dense opacity partially obscuring the details of the iris; and grade 3= severely dense opacity obscuring the details of the intraocular structure (Fantes *et al*., 1990). Lacrimation was graded from 0 to 3; where 0 refers to the absence of lacrimation, and 1, 2, and 3 refers to mild, moderate, and severe lacrimation respectively. Pus formation is either absent (0) or present (mild (1), moderate (2), and severe (3)). The perforation of cornea (nonperforated (0); or perforated (1)). Therefore, the total clinical score for each animal ranged from 0 to 13. Where, 0 refers to normal and healthy, and 13 indicates highly damaged tissue.

The test of Fluorescein staining was performed following an ophthalmic inspection before the treatments as the morphological investigations just after the completion of 1, 2, 3, 4 and 5 weeks. 2% fluorescein solution (One drop) was installed into the eye and left for 1 min. Sterile normal saline was used for rinsing to get rid of the extra stain (Petroutsos and Pouliquen, 1984). The eye was photographed just after the completion of 1, 2, 3, 4 and 5 weeks after corneal ulcer initiation by a digitalized camera after rinsing with normal saline. Two photographs were obtained, once after completing an external ocular examination and once after applying fluorescein staining. The ImageJ 4.48v software, developed by the National Institutes of Health in the USA, was used to measure the surface area of the corneal ulcer.

Histopathological examination

Samples of corneal tissue were fixed in 10 % neutral buffered formalin, then dehydrated by ascending grades of alcohol, cleared by xylene, and incorporated in paraffin. Tissue slicing with 4-5 µm thick and stained with hematoxylin and eosin stains (H&E) (Banchroft *et al*., 1996).

Scanning electron microscopy (SEM) representative corneal samples were promptly rinsed with normal saline. After that, set in a mix of 5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M sodium phosphate buffer with a pH of 7.3 at 4°C for 24 h. Subsequently, it was washed with 0.1 M sodium phosphate buffer with a pH of 7.3, then dried utilizing an ascending series of ethanol 30, 50, 70, and 90 for two hours, 100% for two days, and then to amyl acetate for two days. Critical point drying was applied to the samples by using liquid carbon dioxide. Each sample was stuck on metallic blocks using silver paint. Employing gold sputter coating apparatus, samples were evenly coated with gold at a thickness of 15nm. Specimens were investigated through the use of a JEOL (JSM 5400 LV) scanning electron microscope 15- 25 kV and photographed within an electron microscope unit at Assiut University, Egypt (Bozzola and Russell, 1999) Digital coloring of scanning electron microscopic images. The scanning electron microscopic images were colorized digitally using the Photo Filter 6.3.2 program to recognize different types of cells and structures.

The healing of the ulcerated cornea was evaluated using the histopathological examination, and electron microscopy through the summation of the following items: epithelium (healthy (0), or necrosed (1); non-desquamated (0), or desquamated (1), nonulcerated (0), small ulcer (1), or large ulcer (2), no colonies of bacteria (0), presence of colonies of bacteria (1)), Stroma (normal (0), or swollen (1), no inflammatory cells (0), presence of inflammatory cells (1)), and Decemet's membrane (normal (0), ruptured (1)). The total score of histopathology and electron microscopy examination ranged from 0 to 8. Zero refers to the normal corneal tissue, while 8 refers to the highly damaged and deteriorated tissue.

Statistical Analysis

The statistical analysis was performed using IBM SPSS Statistics version 22. The data are expressed as mean± SE. One-way ANOVA is used to analyze the variance of means of the total clinical score of animals of different groups in each week. One-way ANOVA was used to compare the different clinical signs in different groups along 5 weeks, As well as Two-way ANOVA was used to determine the effect of both treatment and time on the different clinical signs. P value is considered significant when it was lower than 0.05.

Results

The results of the clinical parameters were summarized in Tables 1 and 2. The opacity was obvious in the control group from the 3rd to 5th weeks. On the other hand it markedly decreased at the 3rd, 4th, and 5th weeks in the hesperidin group. It was noticed improved in the hesperidin-olive oil group from the 4th week till the end of experiment. The new-vascularization was clearly developped and detected in the control group from the 3rd week till the end. There was a fluctuation of the displaying of new-vascularization in the hesperidin group. It was not detected in the hesperidin- olive oil group. The lacrimation was gradually increasing in the control group to the end of experiment, However it decreased markedly in the hesperidin group. It was not detected in the third group except in one animal, which did not show lacrimation in the 5th week. The appearance of pus increased gradually in the control group but not significantly. In the hesperidin group, the pus was developped in the 1st week, but it markdely decreased afterthat till the end of the experiment. There was only one animal which showed pus at its eye in the 1st week in the hesperidin-olive oil group. Later on the pus was subsided in that animal, and it was not develooped in others in the same group till the end of study. Finally, the perforation of the cornea was detected in two animals in the control group from the 3rd week and persisted to the end of the study. The corneal perforation was developped in one animal in the hesperidin group from the beginning to end of the study. It was not noticed in the hesperidin-olive oil group.

In the control group, there were severe and significant detrimental changes in the total clinical score from the third week to the fifth week.

However, in the Hesperidin group and the Hesperidin olive oil group, there was an improvement in the total clinical score as the treatment progressed, with the most significant seen in the fifth week. According to the statistical analysis, there was no substantial difference in the total clinical score between the different groups in the first two weeks. However, after the third week of treatment, both the Hesperidin and Hesperidin-olive oil groups showed an improvement in the total clinical score compared to the control group (P< 0.05). Figure 1 shows the different clinical signs a long 5 weeks in different group. The difference in the total clinical score in different groups in 5 weeks of treatment was displayed in Figure 2.

Results of fluorescein staining of the corneal tissue

The animals in the control group retained the fluorescein dye throughout the five weeks of treatment. The dye was clear and diffused within the corneal tissue in this group. The animals in the Hesperidin group and the Hesperidin olive oil group retained the fluorescein dye in the first two weeks of treatment. However, the preservation of the fluorescein dye decreased in both the third and fourth weeks of treatment. By the fifth week of treatment, there was no presence of the dye within the corneal tissue. Figure 3, displays the effect of fluorescein stain on the corneal tissue in different groups throughout 5 weeks of treatmnet. The measured corneal ulcer in different groups after fluorescein staining at the end of experiment was recorded in Table 3. There were no significant changes between the different groups along five weeks in the measured corneal ulcer. Hesperidin-olive oil significantly improved corneal ulcer healing, especially in the third, fourth, and fifth weeks. On the other hand, the size of the ulcer increased in the control group towards the end of the experiment. According to the results of the experiment, the corneal ulcer size did not significantly change in the hesperidin group during the 5 weeks

The findings of the histopathological investigations

The statistical analysis of the total histopathological score in different groups revealed a significant difference between the hesperidin group

Table 1. The results of statistical analysis of the different clinical signs of animals of different groups over five weeks.

Clinical sign	Treatment	Week1	Week 2	Week 3	Week 4	Week 5
Opacity	Control	0.83 ± 0.31^{aA}	1.67 ± 0.42	2.17 ± 0.42^{B}	2.17 ± 0.42^{aB}	2.17 ± 0.42^{aB}
	Hesperidin	2.17 ± 0.48^{bA}	2.00 ± 0.52^{C}	0.83 ± 0.31^{BD}	0.67 ± 0.21^{bBD}	0.33 ± 0.21^{bBD}
	Hesperidin- olive oil	2.60 ± 0.20^{bA}	$1.67\pm0.42^{*C}$	1.67 ± 0.42^{E}	$0.67\pm0.33^{bB*D}$	$0.33\pm0.21^{bB*D}$
New vascularization	Control	0.00 ± 0.00^{A}	0.33 ± 0.21^{C}	1.83 ± 0.30^{aBDE}	2.7 ± 0.33^{aBDF}	2.67 ± 0.33^{aBDF}
	Hesperidin	0.33 ± 0.21^{A}	1.00 ± 0.37	$1.67\pm0.56^{*B}$	0.83 ± 0.48^{b}	0.83 ± 0.34^{b}
	Hesperidin- olive oil	0.00 ± 0.00	0.17 ± 0.17	$0.00\pm0.00^{b*}$	0.67 ± 0.49^{b}	0.0 ± 0.00^{b}
Lacrimation	Control	1.00 ± 0.52^{A}	1.67 ± 0.42^{a}	2.00 ± 0.37^{a}	2.17 ± 0.4^{a}	2.33 ± 0.33^{aB}
	Hesperidin	1.83 ± 0.48^{A}	1.17 ± 0.48^{C}	0.17 ± 0.17^{bBD}	0.20 ± 0.16^{bBD}	0.00 ± 0.00^{bBD}
	Hesperidin- olive oil	0.33 ± 0.21	0.17 ± 0.17^{b}	0.17 ± 0.17^{b}	0.17 ± 0.17^{b}	0.00 ± 0.00^{b}
Pus formation	Control	0.67 ± 0.33	0.83 ± 0.48	1.67 ± 0.42^{a}	1.67 ± 0.49^{a}	1.33 ± 0.56^{a}
	Hesperidin	0.83 ± 0.48^{A0}	0.00 ± 0.0^{B}	0.17 ± 0.17^{bB}	0.0 ± 0.00^{bB}	0.0 ± 0.00^{bB}
	Hesperidin- olive oil	0.17 ± 0.17	0.00 ± 0.00	0.333 ± 0.21^{b}	0.0 ± 0.00^{b}	0.0 ± 0.00^{b}
Perforation	Control	0.00 ± 0.00	0.00 ± 0.00	0.33 ± 0.21	0.33 ± 0.21	0.33 ± 0.21
	Hesperidin	0.167 ± 0.17	0.17 ± 0.17	0.17 ± 0.17	0.17 ± 0.17	0.17 ± 0.17
	Hesperidin-olive oil	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.0 ± 0.00	0.0 ± 0.00

Data are presented as Mean±SE.
The significant difference (P< 0.05) in the same treatment allover 5 weeks was represented with superscripted different consecutive capital letter (the same row), while the significant difference between different treatment and control (the same column) at certain time was represented by superscripted different consecutive small letter or asterisk.
Note: There was significant difference between the values that were superscripted with different letters in the alphabet arrangement. Fore example; there was significant difference between A and B, C and D.

Table 2. Total clinical score in different groups a long five weeks of treatment.

Group	1st week	2nd week	3rd week	4th week	5th week
Control	2.4 ± 1.29^{a}	4.4 ± 1.568^{a}	8.0 ± 1.50^{bA}	9.0 ± 1.79^{bA}	9.0 ± 1.92^{bA}
Hesperidin	5.4 ± 1.77^{a}	4.4 ± 1.50^{a}	3.0 ± 1.26^{aB}	1.8 ± 0.97^{abA}	1.4 ± 0.68^{bB}
Hesperidin olive oil	3.2 ± 0.58^{a}	2.0 ± 0.70^{a}	2.2 ± 0.86^{aB}	1.4 ± 0.87^{aA}	0.4 ± 0.25^{bB}

Data are presented as mean±SE.
Values followed with different superscript letters (lower case) in the same row indicate significant difference at P<0.05. Values followed with different superscript letters (upper case) in the same column indicate significant difference at P<0.05.

Table 3. Measured corneal ulcer diameter (centimeter) in different groups a long 5 weeks.

Time	Control (cm)	Hesperidin (cm)	Hesperidin-olive oil (cm)
1st week	0.47 ± 0.12	0.29 ± 0.104	0.84 ± 0.24^{ABCD}
2nd week	0.19 ± 0.07^{ab}	0.26 ± 0.073	0.46 ± 0.06^{A}
3rd week	0.48 ± 0.051	0.15 ± 0.03	0.13 ± 0.07^{B}
4th week	0.63 ± 0.18^{a}	0.17 ± 0.03	0.14 ± 0.03^{C}
5th week	0.70 ± 0.09^{b}	0.17 ± 0.08	0.27 ± 0.08^{D}

Data are expressed as Mean± Std. Error. Values were superscripted with the same letter either the lower and upper case in the same column indicate significant difference at P<0.05

and the hesperidin olive oil group compared to the control group (P= 0.045 and 0.00 respectively). There were significant changes between the hesperidin group and the hesperidin olive oil group (P= 0.045).

Histopathological examination of the corneal stained by H&E in the control positive group revealed necrosed epithelium, desquamation, and multiple ulcerated areas covered with colonies of bacteria. The stroma widened due to edema and infiltrated with inflammatory cells (neutrophils), and the Descemet layer was damaged (Figure 4 A, B, C).

The rabbits' cornea that were treated with hesperidin showed necrosed epithelium and ulceration. The stroma revealed proliferated keratocytes and inflammatory cell infiltration (lymphocytes) (Figure 4 A, B).

The hesperidin olive oil-treated group revealed necrosed epithelium with a small area of ulcer, but with normal stroma (Figure 4 ep, st).

Scanning electron microscopy

Scanning electron microscopical examination of the cornea in the control positive group revealed multiple areas of ulceration and denuded epithelium, colonies of bacteria in the ulcerated area and stroma revealed widening with leucocytic cell infiltration (Figure 5A-D). However, in the Hesperidin-treated group showed small ulcers and normal stroma (Figure 5D, E). Hesperidin olive oil-treated group revealed normal surface epithelium of the cornea with no ulceration and normal stroma (Figure 5 F, G).

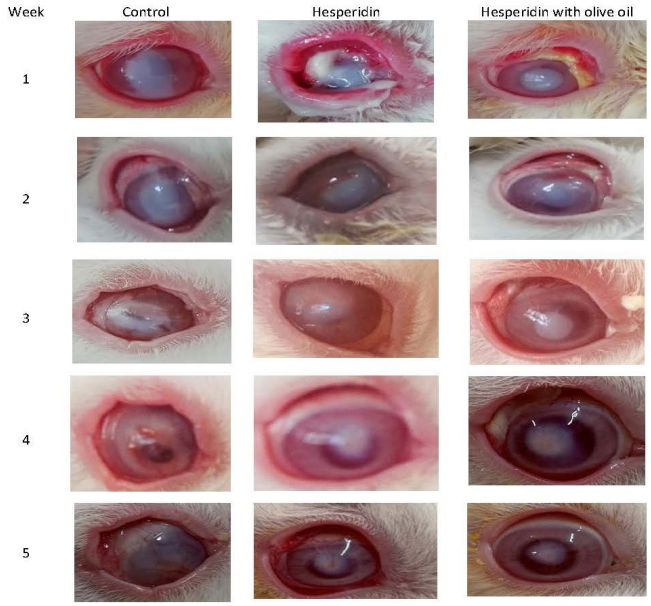

Fig. 1. Clinical pictures for the of the three different groups a long 5 weeks (without fluorescein).

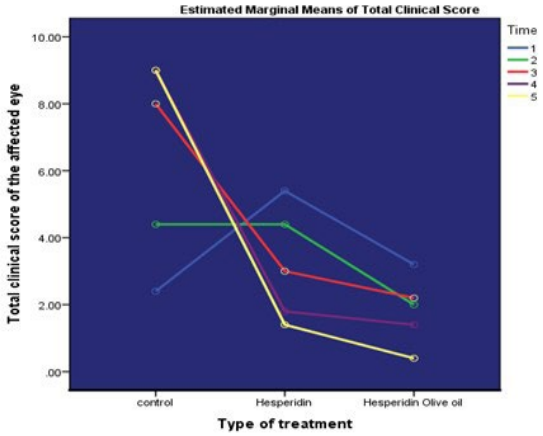

Fig. 2. Chart depicts the interaction between number of treatment (1st - 5th week) and type of the treatment on the total clinical score in animals. Notice at the 3rd, 4th, and 5th treatment (weeks) the total clinical score of the eye with alkali burn ulcer was so high (8-10) in the control group compared to the hesperidin and hesperidin olive oil groups (very low).

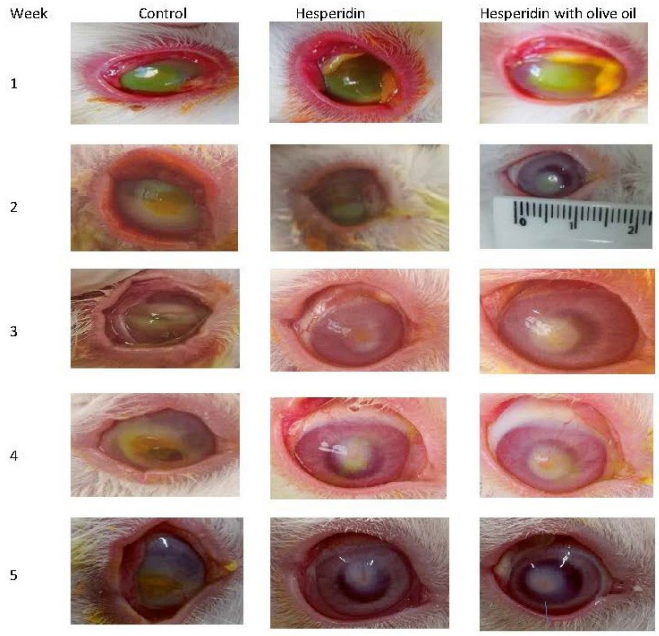

Fig. 3. Clinical pictures and ulcer staining in different groups throughout the 5 weeks after using of fluorescein stain.

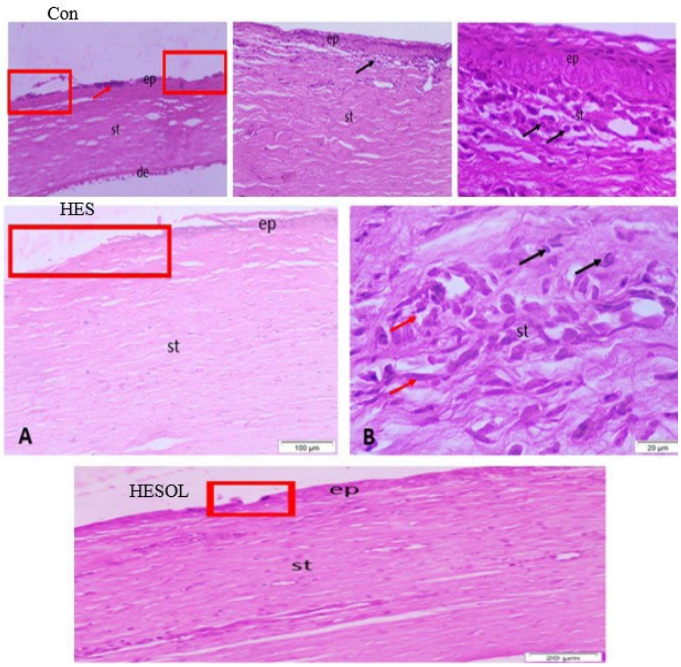

Fig. 4. Histopathological examination using H&E stain of the cornea in control group (Con, upper figures) showed necrosed epithelium, desquamation and ulceration (Red rectangle), cellular infiltration in the stroma (arrows), and white spaces refers to stroma edema. Histopathological examination using H&E stain of the cornea in hesperidin group (HES) A and B showed necrosed epithelium and ulceration. The stroma revealed inflammatory cell infiltration (black arrows) and edema of the stroma (red arrows). Histopathological examination using H&E stain of the cornea in hesperidin olive oil group (HESOL) showed necrosed epithelium (ep) with a small area of ulcer (red rectangle) with normal stroma (st).

Discussion

The present study aimed to evaluate the effect of hesperidin and hesperidin olive oil on the healing of an experimentally induced corneal ulcer in rabbits. This study is the first of its kind in rabbits to investigate the healing process of the corneal ulcer after subconjunctival injection of one of the above subjects in different periods.

The study found that all the rabbits developed signs of eye inflammation after being given corneal ulcers and throughout the experiment. These signs were caused by the irritating effect of Sodium hydroxide, which was used to induce ulcers in the rabbits. The effect of sodium hydroxide on the cornea in animals was reported in previous studies (Hosny

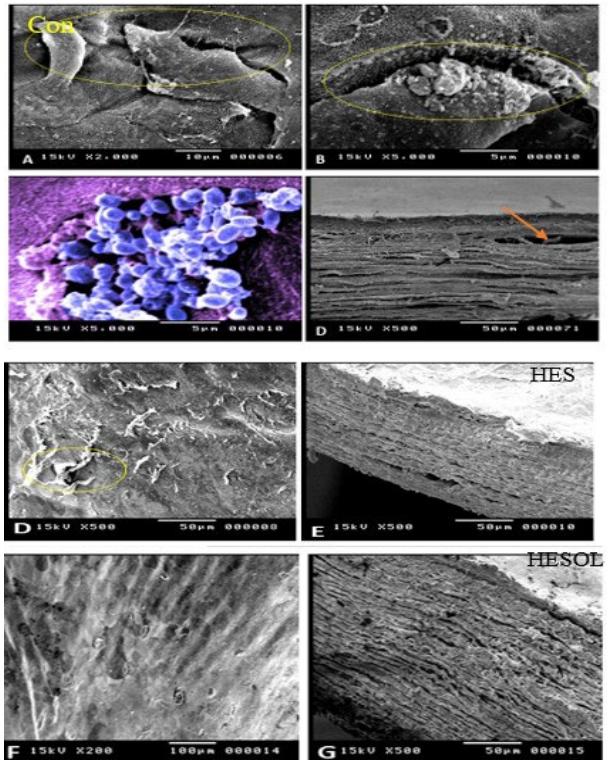

Fig. 5. Scanning electron microscopical examination of the cornea in the control group (Con) from A to D showed ulceration (yellow circles), colonies of bacteria (light blue spheres) and widening in stroma (orange arrow). Scanning electron microscopical examination of the cornea in the Hesperidin-treated group (HES) D and E showed small ulcers (yellow circle) and normal stroma. Scanning electron microscopical examination of the cornea in the hesperidin olive oil-treated group (HESOL) F and G displayed normal epithelium of the cornea without ulceration and the stroma is normal.

et al., 2022; Sobhy, 2016). Corneal alkali burns can cause a strong inflammatory reaction, leading to cell infiltration and the production of proteolytic enzymes and cytokines (Christmas, 1991; Pfister *et al*., 1971; Sotozono *et al*., 1999).

Ophthalmic disorders such as corneal opacity, excessive tearing, pus presence, and corneal neovascularization were observed in the control group, and these symptoms became more pronounced from the third week until the end of the experiment. This refers to the highly irritating effect of sodium hydroxide, which causes severe destruction of the corneal tissue (Hosny *et al*., 2022; Sobhy, 2016). These harmful changes could not be repaired by the animal itself.

On the other side, the rabbits in the Hesperidin group and the Hesperidin olive oil group, showed an improvement in the total clinical score as the treatment progressed, with the most significant improvement seen in the fifth week compared to the signs of the first week. Also, there was no substantial difference in the total clinical score between the different groups in the first two weeks. However, after the third week of treatment, both the Hesperidin and Hesperidin-olive oil groups showed an improvement in the total clinical score compared to the control group ($P< 0.05$). The findings demonstrate the effectiveness of hesperidin, either alone or combined with olive oil, in treating alkali-burn corneal ulcers. However, it's important to note the severe damage caused by sodium hydroxide to the cornea. A previous study found that subconjunctival injection of oxytetracycline for the treatment of alkali burn corneal ulcers did not have a positive effect, except for preventing bacterial colonization on the surface of the ulcer (Abdelhakiem *et al*., 2023). A newly published study pointed out the efficacy of hesperidin with/out olive oil after its topical instillation for the treatment of alkali burn corneal ulcers (Ali *et al*., 2024). The authors of that study displayed the advantages of hesperidin and olive oil in the process of healing. A previous study that was conducted by da Silva *et al*. (2019) revealed the beneficial effect of hesperidin in the healing of ulcerated gastric mucosa in rats. This means that the hesperidin could be effective in the acidic media. As well as, Hesperidin had a crucial role in the healing of diabetic foot wounds in rats as reported by Wang *et al*. (2018). The authors of the present study attributed the good and positive results of hesperidin in the healing of different tissues in different conditions (acidic media, diabetes, and alkali burn ulcers) to its antibacterial and anti-inflammatory properties as was reported by Köksal Karayıldırım (2017); Martin *et al*. (1953) and Salgado and Green (1956).

According to the results of the present study, the addition of olive oil to hesperidin had a powerful and synergistic effect in the healing of corneal ulcers in rabbits. This is clear through the follow-up of the clinical findings and preservation of the fluorescein dye. The results of the present study did not show a significant difference between the hesperidin and hesperidin-olive oil clinically. However, the significant difference was clear through the histopathological and electron microscopy examination. The addition of olive oil to hesperidin had a beneficial effect on the regeneration of the corneal epithelium as was suggested by Ali *et al*. (2024). The regenerating effect of olive oil might be attributed to the bio-stimulation of fibroblasts, enhancing their proliferative capacity and its migration, upper-regulation of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and transforming growth factor beta 1 (TGFβ1) (Melguizo-Rodríguez *et al*., 2021).

In addition to the antibacterial and anti-inflammatory effect of hesperidin (Köksal Karayıldırım, 2017; Martin *et al*., 1953; Salgado and Green, 1956), Olive oil also has the same properties (Medina *et al*., 2007; Rosillo *et al*., 2014; Wongwarawipat *et al*., 2018). The antibacterial effect of hesperidin with/ out olive oil is more obvious in this study clinically through the decrease of pus formation gradually from the beginning to complete disappearance at the end of the experiment. Furthermore, no bacterial colonies were found on the corneal surface as compared to the control group on both histopathological and electron microscope examinations. These results verified the outcomes of previous studies (Ali *et al*., 2024; Köksal Karayıldırım, 2017; Melguizo-Rodríguez *et al*., 2021).

Comparing the total clinical score of the hesperidin and hesperidin olive oil groups to the control group, it was noted a decrease in the total clinical score mainly due to the decrease in inflammation. The results indicated the potent anti-inflammatory effect of both hesperidin and olive oil. These results were consistent with the findings of previous studies (Ali *et al*., 2024; Köksal Karayıldırım, 2017; Medina *et al*., 2007; Rosillo *et al*., 2014; Wongwarawipat *et al*., 2018).

Comparing the results of the present study with those of a previous one (Ali *et al*., 2024), it was found that topical instillation twice daily for five weeks resulted in more favorable healing. Although five injections were administered, the traumatic effect of needle insertion could have adverse effects. The authors believe that subconjunctival injection may have both advantages and disadvantages. The advantages include saving time by using only five injections at a one-week interval and the maintenance of the injected drug for a relatively long time. However, the disadvantages include the traumatic injection of the drugs that may adversely affect the healing, the necessity for complete anesthesia for injection, the formation of blebs after the injection that may last for a long time, which may prevent the palpebral fissure from closing completely, and the loss of transparency of the bulbar conjunctiva at the injection site. There were some limitations to the present study, including: 1. The short duration of the experiment (6 weeks). According to the authors, a long duration may result in more satisfactory results. 2. Although the induction of the corneal ulcer in all animals was carried out by the same person, there were some changes in the size and depth of the induced ulcer. The authors recommended that further studies on the induced corneal ulcer should be conducted on deeply anesthetized animals, and a ring should be applied on the surface of the cornea to prevent the diffusion of the 1% NaOH outside the borders of that ring.

Based on the outcomes of this study, it can be concluded that the subconjunctival injection of hesperidin with or without olive oil promotes the healing of alkali burn corneal ulcers in rabbits. The anti-inflammatory and anti-bacterial effects of hesperidin with or without olive oil may be

the main factors behind their satisfactory effects and results. Additionally, hesperidin with olive oil is superior to hesperidin alone in controlling and managing alkali burn corneal ulcers.

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Conflict of interest

The authors have no conflict of interest to declare.

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