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Influence of topical NPH-insulin use on wound healing and oxidative stress in alkali corneal burns: an experimental study in a rabbit model

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Abstract

Background: In acute alkali corneal burns, the routine treatment protocol includes suppressing inflammation using corticosteroids. In recent years, there has been a growing interest in the role of insulin in wound healing research due to its remarkable positive effects. **Aims:** The present study aimed to compare the healing effects of dexamethasone -the conventional approach- and NPH-insulin on alkali corneal burns. **Methods:** An alkali corneal burn was created using $Ca (OH)_2$ in all subjects. One group (n=6) was treated with an ophthalmic ointment containing dexamethasone (group I), and another group (n=6) was treated with an insulin ointment (group II). The control group (n=6) received no treatment (group III). Clinical changes in the corneal burn areas were monitored on days 3, 7, 14, and 21. The animals were euthanized on day 21. The excised corneas were examined for histopathological, immunohistochemical, and biochemical changes. **Results:** A more successful clinical recovery graph was drawn for group II. Particularly, a significant (P<0.05) improvement was detected in group II on day 21. The highest positivity in MMP-9 corneal staining was found in group I. Group II had a significant (P<0.05) increase in total antioxidant capacity (TAC) values. The treatment groups showed a significant (P<0.05) decrease in total oxidant content (TOC) values and a significant (P<0.05) increase in reduced glutathione (GSH) levels as compared with the control group. **Conclusion:** Topical NPH-insulin provided rapid and uncomplicated clinical recovery of alkali corneal burns. Insulin and dexamethasone showed similar effects like increased antioxidant molecules and decreased oxidant substances which indicated that insulin may prevent free radical formation in the cornea.

Key words: Corneal alkali burn, Insulin, Oxidative stress, Rabbit, Wound healing

Introduction

Chemical burns comprise a large proportion of corneal injuries in human and veterinary medicine. Alkali and acidic substances can cause burns (Gkountelia, 2021; Lorenzana-Blanco *et al*., 2023). Alkali substances penetrate faster than acidic ones and cause more severe burns. Tissues can be damaged in different degrees according to the effect time of chemical substances, affecting agent, and degree of penetration. Corneal epithelium, basement membrane, stroma, nerve endings, endothelium, and surrounding tissues such as conjunctiva, iris, and lens can be affected. Damaged tissues secrete proteolytic enzymes due to the inflammatory response, which increases the damage. As a result of these rapidly occurring events, even irreversible intraocular damage may occur in a short time (Saroglu and Arikan, 2002; Fish and Davidson, 2010).

The routine treatment protocol involves rapidly removing the chemical agent, suppressing inflammation, preventing infection, and enabling rapid re-epithelization in acute alkali corneal burns (Fish and Davidson, 2010; Gkountelia, 2021). In the acute phase, corticosteroids are used to control inflammation (Pfister and Pfister, 2005; Lorenzana-Blanco *et al*., 2023). Reactive oxygen species are formed during the formation of ocular chemical burns, and the balance between inflammatory and anti-inflammatory responses is impaired. Therefore, treatment agents with antioxidant properties are useful (Moghadam *et al*., 2020). In the chronic phase, the aim is to manage complications and provide a healthy ocular surface. In cases requiring surgical intervention, anti-glaucoma treatment, tissue adhesives, keratoplasty, and limbal stem cell transplantation are used to support recovery in corneal burns (Fish and Davidson, 2010; Choe *et al*., 2019; Kesper *et al*., 2023). As is well known, alkaline burns, which create a sterile wound, carry the risk of secondary infection due to the open corneal epithelium

and the stromal surface that begins to melt. The fact that serious injuries require long-term steroid therapy also increases the risk of infection (Fish and Davidson, 2010).

Several studies have reported the use of insulin in wound healing successfully (Chen *et al*., 2012; Hrynyk and Neufeld, 2014; Ozaydin *et al*., 2018). Some reports were encountered on using insulin for also corneal wound healing. However, the available literature does not include detailed information about the effects of insulin on corneal burns (Wang and Xu, 2020). Insulin contributes to healing by acting at the receptor level. Insulin plays an important role in tissue regeneration and increasing bio-macromolecules. It is associated with novel vessel formation in severe burn injuries (Hrynyk and Neufeld, 2014). Therefore, the present study aimed to reveal the differences between a conventional corticosteroid treatment and a topical NPH insulin treatment in healing experimental corneal alkali burns in rabbits.

Materials and Methods

Ethical approval

The study was approved by the Local Ethics Committee for Animal Experiments of Kafkas University (approval No.: KAU-HADYEK/2018-048).

Experimental animal and study design

A total of 18 female New Zealand white rabbits weighing 2.5-3.0 kg were used in the study. The rabbits were fed standard food and water *ad libitum* for one week for adaptation.

Experimental procedures were performed under dissociative anesthesia with a combination of intramuscular xylazine HCl (5 mg/kg) (Rompun®, Bayer, Germany) and ketamine HCl (35 mg/kg) (Ketasol® 10%, Richter Pharma AG, Austria). Only the right eye of each animal was used for the experiment. A blepharostat was used to open the eyelid after shaving, asepsis, and antisepsis. To limit the injury area, an absorbent filter paper disc with a diameter of 6 mm, soaked in 1 mol/L $Ca(OH)_2$ (Merck, Germany), was placed on the corneal surface, and a moderate alkali burn was created by waiting 2 min. Then, all chemical remnants were removed by rinsing the cornea with sterile NaCl 0.9% for 2 min (Saroglu and Arikan, 2002; Bizrah *et al*., 2019). Following the alkali burn, the animals were randomly sorted into three groups of six each: groups I-III. A dexamethasone-containing ophthalmic ointment was applied (Maxidex®, Alcon Pharmaceuticals, Alcon-Couvreur, Puurs, Belgium) to the animals in group I. For group II, a prepared insulin ointment was applied. Groups I and II were the treatment groups. The insulin ointment was prepared by homogenously mixing 5 ml of NPH insulin (Humulin® N NPH, Lilly France SAS, Fegersheim, France) and 95 g lanoline. Group III was the control group and received no treatment. For pain control, 0.5 mg/kg IM Meloxicam, Melox®, NOBEL was administered to all subjects for 5 days in the postoperative period.

Treatment protocol and clinical evaluation

The day of creating the experimental corneal alkali burns was considered day 0 of the study. The treatment protocols were performed only in groups I and II. While a dexamethasone-containing ophthalmic ointment was applied in group I, insulin ointment was applied in group II. Applications were topically made in the same way for both groups. To apply eye ointments, the animals' head was held stable, and gently pulled down the lower eyelid to make a pouch. A half-inch strip of ointment was placed into the pouch. The eye was gently closed and waited to spread the medication. Treatment commenced on day 1 and continued for 15 days with one application every day. Dexamethasone dosage was slowly reduced from day 10 to 15 to avoid potential melting corneal ulcers. The dose was reduced by decreasing the amount of ointment used. Every 48 h, it was reduced by half the amount previously used. And the treatment was terminated. To observe clinical outcomes, corneal burns were photographed with a direct light source and a slit-lamp biomicroscope (BQ 900 LED Slit Lamp, Haag-Streit, USA) after fluorescein staining (Fluorescite® 10%, Alcon Pharmaceuticals, Alcon-Couvreur, Puurs, Belgium). They were evaluated for the condition of the epithelium, limbal changes, and the grade of corneal burns on days 3, 7, 14, and 21. The Roper-Hall classification system was used to assess the severity of corneal burns. In this system, based on the amount of corneal haze and the rate of ischemic perilimbal conjunctiva, burn grading was done as follows: Corneal epithelial damage and no limbal ischemia, grade I; Corneal haze with visible iris details and <1/3 limbal ischemia, grade II; Total epithelial loss, stromal haze, with obscured iris details and 1/3 to ½ limbal ischemia, grade III; Opaque cornea, with obscured iris and pupil and >1/2 limbal ischemia, grade IV (Soleimani and Naderan, 2020). The assessment of the grade of burns between three groups was performed using the averages of each group.

On day 21, all animals were euthanized with intravenous pentobarbital sodium (125 mg/kg). The ocular bulb enucleated and then corneas were excised for histopathological and immunohistochemical examinations.

Histopathological evaluation

Corneas were fixed in a 10% formalin solution for 72 h and then washed under running tap water. To dehydrate the corneas, they were kept in alcohol solutions of increasing concentration, from 50% to 70%, 80% to 96%, and 99% for 1 h in each batch. After dehydration, the tissues were kept in xylene for 5-10 min to achieve transparency. The corneas were placed in molten liquid paraffin to be filtered and then embedded in paraffin blocks. Serial sections of 5 µm thickness were taken from each tissue block with a microtome (Leica RM2125RTS). Hematoxylin-eosin (H&E) and immuno-histochemical staining were performed on the sections.

Immunohistochemical evaluation

Immunohistochemical staining was performed according to the manual protocol. Sections were kept at 65°C for 20 min for deparaffinization. After the sections were washed with phosphate buffer solution (PBS), they were kept in a citrate buffer solution for 60 min at 95°C for antigen retrieval. After washing with PBS, the tissues were treated with 3% H_2O_2 to inhibit endogenous peroxidase activity, washed with PBS, incubated with a blocking solution, and washed again with PBS. Next, the primary antibody matrix metalloproteinase-9 (MMP-9) (Abcam ab74277, 1:250 dilution) was applied for 60 min. Then, a secondary antibody (TL-060-HD, UltraVision LP Detection System HRP/DAB Plus Chromogen) with HRP solution was applied for 10 min. The sections were washed with PBS, DAB chromogen was applied for 2 min, the reaction was stopped by adding dH_2O, and then counterstained with hematoxylin. The sections were dehydrated with the alcohol series and sealed with Entellan (Bayram *et al.*, 2022). Cell scoring was performed in five different fields on each slide. Each field was scored for staining intensity as follows:
Negative=0
Low positive=1
Positive=2
High positive=3

Pixel ratios were determined for staining intensities between 0-100% (Bacus *et al.*, 1988). Photographs were taken with an Olympus bx43 microscope and an Olympus dp21 camera.

Biochemical evaluation

Changes in oxidative stress parameters were evaluated by measuring reduced glutathione (GSH), total oxidant content (TOC), total antioxidant capacity (TAC), tumor necrosis factor (TNF-α), and total protein (TP) in all groups.

Tissue homogenization

After washing the corneal tissues in phosphate buffer, they were frozen in liquid nitrogen as quickly as possible to prevent protein degradation by proteases. The frozen tissues were placed in Eppendorf tubes, and 250 µL of lysis buffer (150 mM sodium chloride, 1.0% NP-40; Triton X-100 can be substituted for NP-40) and 50 mM Tris (pH 8.0) were added to each 5 mg tissue sample, and the tissues were homogenized on ice with a mechanical homogenizer. The samples were centrifuged at 12,000 rpm at 4°C for 30 min, and the supernatant was stored at -20°C for later use. Following the completion of protein analysis on the supernatant, it was stored for further analysis.

Protein determination with the bradford method

The total protein level in the cornea was determined according to the Bradford (1976) method using bovine serum albumin (BSA) as a standard at 595 nm.

Total oxidant/antioxidant capacity analysis

The total corneal oxidant/antioxidant capacity was measured with a spectrophotometer using a commercial kit (Rell Assay, Gaziantep, Turkey).

Reduced glutathione analysis

Glutathione analysis was performed according to Beutler *et al.* (1963) method. All proteins not containing sulfhydryl (-SH) were precipitated in the liver, kidney, and colon tissues and were homogenized in a lysis buffer. In the obtained clear liquid, the absorbance of the yellow complex formed by -SH groups with 5.5´-(2-dithiobis nitrobenzoic acid; DTNB) was read at 412 nm.

TNF-α Analysis

TNF-α levels were determined in homogenized corneal tissue samples using commercial enzyme-linked immunosorbent assay kits (ELISA; Sunred Biological Technology Co. Ltd., Shanghai, China).

Statistical analysis

Statistical analysis was performed using SPSS 20.0 for Windows (SPSS Inc.). The data were compared with the Kolmogorov-Smirnov normality test. Mean values between the groups were determined using a one-way analysis of variance (ANOVA). The significance level was set at $P<0.05$. The results are expressed as mean±standard deviation (x±SD).

Results

Clinical results

The daily variation in corneal burn degrees is presented as between-group statistics (Fig. 1). On day 7, lesions of the treatment groups (I and II) improved more than the control group (III). On days 14 and 21, group II (Insulin) showed a statistically significant improvement compared with both groups I (Dexamethasone) and III (untreated). Figure 2 shows changes in corneal burn lesions observed under cobalt blue, following fluorescein staining. Opacification occurred in the corneas of all subjects following contact with alkali substances. However, this opaque field did not hinder the observation of details in the iris. Partial limbal ischemia developed in all cases.

The ophthalmologic examination revealed that photophobia and blepharospasm continued until day 5 and disappeared after that in all groups. However, in group II (Insulin), the severity of symptoms decreased more rapidly. No tear discharge was observed during the follow-up. Corneal perforation was not detected in any of the subjects. No allergic reactions or infections were observed.

Histopathological and immunohistochemical findings

The greatest corneal epithelium thickening was observed in group II (Insulin) and the lowest in group III (untreated). Stromal defects were the most common in group III and the least common in group I (Dexamethasone) (Fig. 3). Immunohistochemistry MMP-9 corneal staining was the most positive in group I, less

in group II, and the least in group III. A statistically significant difference was detected between the groups (P<0.05). The stroma was the thinnest in group III, which was a statistically significant difference (P<0.05). However, there was no significant difference between groups I and II (P>0.05).

Biochemical findings

A statistically significant difference was detected for total antioxidant capacity (P<0.05; Table 1). It was much higher in group II (Insulin) than groups I
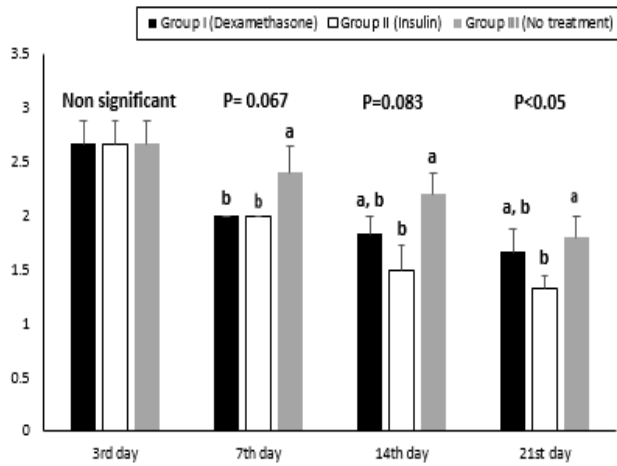

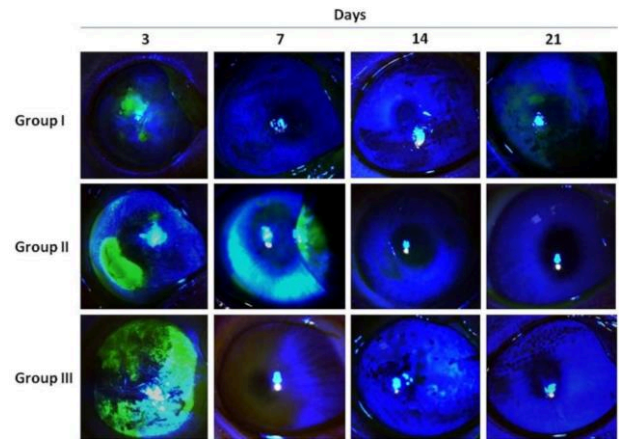**Fig. 1:** Inter-group comparison of the changes in the grade of corneal alkali burns in time

Fig. 2: Slit lamp photographs of the experimental corneal alkali burn of rabbit eyes in the treated and control groups with cobalt blue light, stained by fluorescein (group I: Dexamethasone, group II: Insulin, group III: Untreated); On day 3, epithelial defects are seen as fluorescein staining areas in all groups. The area in group I is minimum and in group III is maximum. On day 7, conjunctivalisation is seen in group I. There is a local staining at the edge of third eyelid in group II, and limbus can visible. Group III has still fluorescein staining covering the cornea and limbus. On day 14, conjunctivalisation is seen in group I, however, group III has a fibrovascular pannus appearance. In group II, limbus is clearly seen, but there is a superficial punctate on the cornea. On day 21, local staining can be seen still with conjunctivalisation and punctate in group I. Group III has superficial punctate, too. At the same day a transparent cornea is seen in group II
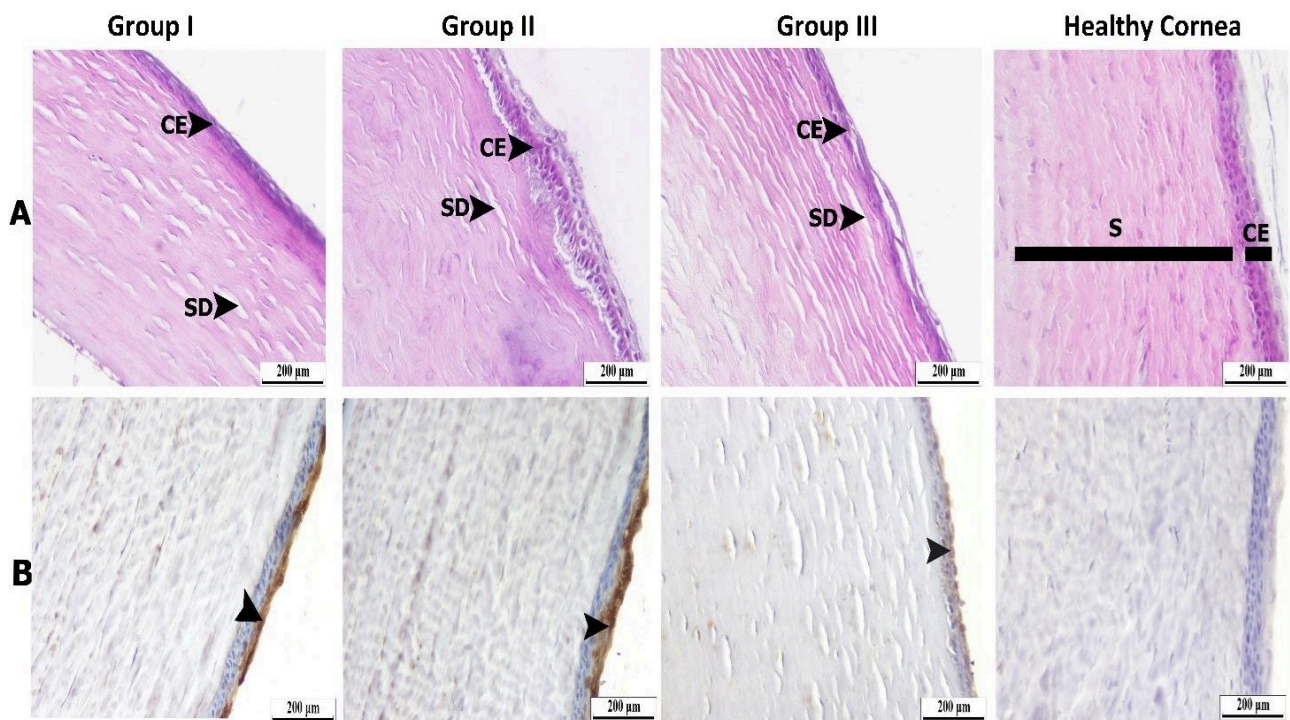

Fig. 3: (**A**) Cornea (hematoxylin-eosin, scale bar, 200 µm). CE: Corneal epithelium (arrow), S: Stroma, and SD: Stromal defects. Corneal epithelial thickening is most common in group II (P<0.05). The stroma is thinner in group III (P<0.05), and (**B**) Cornea (immunohistochemistry MMP-9 staining corneal epithelium (arrow), scale bar, 200 µm). It is seen as the most positively active in group I (P<0.05). The healthy stroma and epithelium of the cornea is seen in the last column (group I: Dexamethasone, group II: Insulin, and group III: Untreated)

Table 1: Corneal tissue TAC, TOC, GSH, TNF-α, and TP values in all groups (mean±SD)

Parameters	Group I (Dexamethasone)	Group II (Insulin)	Group III (Untreated)	ANOVA P-values
Total antioxidant capacity (mmol Trolox Eqv./mg protein)	0.072 ± 0.002^b	0.099 ± 0.004^a	0.074 ± 0.001^b	P=0.01
Total oxidant capacity (μmol H_2O_2 Eqv./mg protein)	1.969 ± 0.167^b	1.994 ± 0.094^b	2.413 ± 0.052^a	P=0.049
GSH (nmol/mg protein)	1.810 ± 0.026^a	1.711 ± 0.077^{ab}	1.540 ± 0.035^b	P=0.027
TNF-α (pg/mg protein)	41.010 ± 0.208^b	43.778 ± 0.475^a	43.751 ± 0.399^a	P=0.030
Total protein (mg/ml)	12.55 ± 0.44	12.36 ± 0.17	12.60 ± 0.14	NS P=0.757

[a, b] The difference between the groups with different letters on the same line is significant (P<0.05), and NS: there is no statistical difference between the groups (NS: Non-significant)

(Dexamethasone) and III (untreated) (P<0.05). Total oxidant capacity levels were higher in group III than groups I and II (P<0.05; Table 1). GSH levels were the highest in group I and the lowest in group III (P<0.05; Table 1). TNF-α levels were high in groups II and III and the lowest in group I (P<0.05; Table 1). There were no statistically significant differences in total protein levels (Table 1).

Discussion

The integrity and vitality of the cornea are preserved by various cells. Cell imbalances may lead to severe eye problems (Bruze *et al*., 2000). Chemical burns, which comprise 12% of all ocular traumas, may lead to severe and irreversible anterior chamber complications through rapid, progressive, and destructive corneal damage (Gkountelia, 2021). The present study was compared the effectiveness of dexamethasone, a routinely used agent for treating corneal burns, and insulin ointment -our own formulation- against the control group.

The severity of corneal injury-related complications is related to the concentration of the exposed agent, duration of exposure, pH of the agent, and amount of agent that penetrates the ocular surface and tissues (Fish and Davidson, 2010; Gkountelia, 2021). In our study, to create an experimental alkali burn, the chemical substance was kept on the cornea for 2 min. Hughes (1946) reported that 30 s of contact between the chemical substance and the cornea was sufficient to create a moderate alkali burn; however, the burns were not at sufficient depth, so 2 min of waiting period was preferred according to Saroglu and Arikan (2002).

Chemical burns are the most common cause of limbal stem cell insufficiency and are characterized by non-healed epithelial defects, stromal inflammation, neovascularization, corneal opacification, and conjunctivalization, and may result in blindness (Choe *et al*., 2019). In the treatment of chemical burns, removing the agent from the ocular surface and suppressing inflammation are essential in the acute phase. In the chronic period, the aim is to eliminate complications and restore the health of the ocular surface. Complications can be prevented with primary precautions, including proper eye protection and first aid (Fish and Davidson, 2010; Gkountelia, 2021). In our study, we assessed treatment outcomes of corticosteroid application, a conventional option. Corticosteroids may help prevent

collagenase accumulation in ocular alkali burn (Kim *et al*., 2021). However, over 10 days of use may cause corneoscleral weakness, leading to perforation (Fung *et al*., 2020). Our study shows that this treatment (group I/Dexamethasone) is superior to untreated group (group III), which is consistent with the literature (Kim *et al*., 2021). Although the corticosteroid dose was reduced beginning on day 10, group I did not show clinical progress when compared with group II (Insulin) on days 14 and 21.

Studies have shown that using topical insulin in acute and chronic wounds is beneficial for re-epithelialization and stimulation of angiogenesis (Chen *et al*., 2009; Wang and Xu, 2020). Insulin is used in modern dressing methods and especially for burn wounds. It has been reported that it improves cellular signaling and eliminates metabolic problems (Chen *et al*., 2012; Hrynyk and Neufeld, 2014). This potential of insulin motivated us to investigate the effects of insulin as a treatment option for alkali corneal burns. Topical insulin application consists of subcutaneous injection or insulin spray and cream or dressing methods. It seemed that controlled and long-term bioactive insulin could not be delivered to tissues using these methods (Hrynyk and Neufeld, 2014; Wang and Xu, 2020). However, in our study, we used insulin in the form of NPH, and in a vehicle substance of high consistency by a direct application on the lesion. This method allowed insulin to influence that area for a longer time. While clinical findings showed similarities in terms of blepharospasm and photophobia in all groups, the insulin group (group II) exhibited a faster recovery graph. This result indicated that the regional effect of insulin was sufficient. Comparing the burn degrees on the same days, group II (Insulin) showed a better recovery than group I (Dexamethasone).

In corneal burns, healing of the epithelium may be delayed due to damage to epithelial stem cells (Gkountelia, 2021). In the histopathological examinations performed in our study, the best corneal epithelial integrity was in group II (Insulin). Group III (untreated) had more defects and less integrity than other groups. Consistent with the literature (Gkountelia, 2021), this shows that both treatment groups (I and II) fared better than the control group. It also reveals the positive effect of insulin on epithelial integrity. The burns created in our study were considered second-degree burns due to the presence of epithelial damage alone. Third- and

fourth-degree burns are characterized by the development of thrombosis in the vessels (Lorenzana-Blanco *et al*., 2023).

MMP-9, a member of the matrix metalloproteinase family, effectively degrades collagen. Constant high levels of MMP enzymes contribute to the chronicity of the wound (Lobmann *et al*., 2002). Therefore, suppressing MMP enzymes is important for treating chemical burns. Medical agents such as tetracyclines, synthetic peptides, acetylcysteine, and Na-EDTA can suppress the action of MMPs (Saroglu and Arikan, 2002; Kato *et al*., 2006). In light of this, the fact that MMP-9 was lower in group II (Insulin) than that in group I (Dexamethasone) suggests that insulin may be better than dexamethasone in suppressing inflammation in alkali corneal burns, at least in this study. However, it is noteworthy that MMP-9 expression was minimal in group III as compared with the treatment groups. Studies argue that there is only a thin line between physiological and pathological conditions governed by different mechanisms that directly or indirectly affect MMP expression (Singh *et al*., 2012). Mohan *et al*. (1998) has shown that MMP-9 expression promotes the migration of basal epithelial cells and is associated with remodeling of the subepithelial basement membrane region. Therefore, given the clinical results of our study, it seems that the low level of MMP-9 encountered in the untreated group (group III) is not due to being suppressed by medical agents, but rather because of the insufficiency in expression. In order to explain this contradiction, in addition to experimental studies, there is a need for clinical studies to evaluate a larger number of cases and examine both the presence and activity of MMP.

Alkali agents reportedly mediate the formation of oxidant substances in the cornea due to their toxic nature and damage living cells (Yuan *et al*., 1994; Hakami *et al*., 2020). Consistent with previous studies, our study showed a higher TOS level in group III (untreated), where alkali burns were created, and no treatment was performed. Similar to Paschalis *et al*. (2017) findings, both treatment groups (I and II) had lower TOS levels than the control group (III). Furthermore, Ruban *et al*. (2019) determined that the level of malondialdehyde (MDA), an important oxidative stress marker, was high in the group where only topical saline was administered. These data suggest that the eye, which has an active metabolic structure, causes the destruction of redox potential as a result of alkali damage, triggering an increase in TOS by forming oxidative stress. In our study, the lower TOS level may be explained by the anti-inflammatory effect of dexamethasone administration in group I and insulin improving the ocular surface due to rapidly regulating blood glucose levels in group II.

It has been reported that oxidative stress increases and antioxidant levels decrease due to factors harming the cornea, such as chemicals, high temperatures, and ultraviolet light (Kolesnikov *et al*., 2020). Treating corneal burns is difficult, and antioxidant substances provide effective treatment by triggering antioxidant enzyme activity in the cornea (Chen *et al*., 2009;

Kolesnikov *et al*., 2020). Some researchers have proposed that antioxidant medications or endogenous antioxidant substances are effective in eliminating and/or correcting oxidant-substance-induced damage (Gakhramanov, 2005; Makarov *et al*., 2005; Williams, 2008; Günay *et al*., 2020). In our study, the TAC level was higher in group II (Insulin) than in group III (untreated). Likewise, TAC levels were higher in group II than in group I (Dexamethasone). Similar to our results, Bashkaran *et al*. (2011) found that TAC levels were lower, and the MDA level and lipid peroxidation parameter were higher.

Our study showed lower GSH levels in group III (untreated) than in group II (Insulin), which agrees with Salman *et al*.'s (2011) results. They also found that GPx levels were significantly lower than controls in alkali corneal burns. Similar to our study, other studies have reported high oxidant parameters and low antioxidant parameters in corneas with alkali burns (Ozturk *et al*., 2000; Schramm *et al*., 2003; Mohamed and Ali, 2018). Additionally, we found that GSH levels were higher in group I (Dexamethasone) than in groups II (Insulin) and III (untreated). This suggests that insulin has a comparable effect to dexamethasone in alkali corneal burns due to increased antioxidant levels in the group receiving insulin therapy. The low value of lipid peroxidation products in group II (Insulin) clearly demonstrates the protective role of insulin as an antioxidant against oxidative stress caused by alkali chemical damage. Antioxidants are resistant to free radicals and maintain cell integrity. GSH, an intracellular antioxidant, may have had a regulatory effect, especially in the first stages of wound healing, due to its sensitivity to tissue damage. Moreover, increased GSH levels suggest that the burn may have provided healing by supporting the regeneration of epithelial cells in the cornea.

It has been reported that various cytokines are induced at the site of inflammation caused by corneal damage by alkali burns. Although alkali burns cause a strong inflammatory reaction involving the invasion of various inflammatory cells, the exact mechanisms of their repair have not definitively been explained (Poon *et al*., 2020). Cade *et al*. (2014) propose that TNF-α plays a critical role and TNF-α inhibition may provide important protection for both the cornea and posterior segments. Another study noted that the TNF-α mRNA transcript levels were significantly higher in the keratitis group than in controls (Ruban *et al*., 2019). Our results are consistent with these results, as well as those in the study by Yao *et al*. (2012). In our study, high TNF-α levels in the non-treated control group may be due to burns inducing cytokine release. Another remarkable point of our study is that while TNF-α release was reduced by dexamethasone administration, insulin administration did not change TNF-α release. This suggests that insulin is not very active in TNF-α inhibition.

In conclusion, this study indicates that applying topical NPH insulin in ointment form in alkali corneal burns may be a new treatment option to accelerate

healing with minimal sequelae. The study results show that the formation of free radicals in the cornea can be prevented by increasing the level of antioxidant molecules and decreasing the level of oxidant substances. Although our study has only been conducted on a certain species, it may pioneer future studies on the subject. In this way, promising improvements can be made in alkali corneal burns, which are common in farm animals exposed to barn pesticides or small animals exposed to chemicals used in household cleaning, or in chemical laboratory and factory workers, agricultural and construction workers.

Acknowledgement

Conflict of interest

The authors have no conflicts of interest to declare.

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