Effect of Topically Applied Azithromycin on Corneal Epithelial and Endothelial Apoptosis in a Rat Model of Corneal Alkali Burn

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**Purpose:** To investigate the antiapoptotic effect of topically administered azithromycin (AZM) on corneal epithelial and endothelial cells in a rat model of corneal alkali burn.

**Methods:** Twenty-four Wistar albino rats were divided into 4 equal groups as pseudovehicle (group 1), control (group 2), alkali burned (group 3), and treatment (group 4) groups. Alkali injury was induced only in the right corneas of rats belonging to groups 3 and 4 using 1N NaOH. The rats in group 3 and the rats in group 4 were respectively treated either with an artificial tear gel or with 1.5% AZM eye drops for 5 days. At the fifth day of the experiment, the apoptosis in the corneal epithelium and endothelium of all rats was assessed using a terminal dUTP nick-end labeling (TUNEL) assay. In addition, tumor necrosis factor-alpha (TNF-α) density in the corneal epithelium was measured in all rats.

**Results:** The mean numbers of TUNEL+ cells in the corneal epithelium and endothelium of rats in group 3 were 117.1 ± 23.8 and 34.6 ± 11.3, respectively, whereas in group 4, they were 75.8 ± 15.7 and 14.7 ± 3.5, respectively. Also the mean TNF-α densities in the corneal epithelium in group 3 and group 4 were 2.65 ± 1.3 and 1.65 ± 1.1, respectively. There was a significant decrease in the mean number of TUNEL+ cells in the corneal epithelium and endothelium and in the mean TNF-α density in the corneal epithelium of rats in group 4, when compared with group 3.

**Conclusions:** Topically applied AZM can decrease TNF-α–induced apoptosis in corneal alkali burn.

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Alkali burn of the ocular surface is one of the most serious injuries of the eye that requires urgent medical intervention to decrease the risk of devastating complications, such as ocular perforation, corneal neovascularization, and ocular scarring.1,2 Because these complications mainly occur as a result of extreme inflammation, corticosteroids are administered as a mainstay therapy to suppress the inflammation in the acute phase of corneal alkali burn. However, because long-term usage of corticosteroids can be related to the formation of cataract and glaucoma, and may remain inadequate for suppressing inflammation, different therapeutic modalities devoid of these harmful effects have been the subject of research in the management of corneal alkali burn. Increased expression of various inflammatory cytokines, including interleukin-1 (IL-1), IL-6, IL-10, and tumor necrosis factor-alpha (TNF-α), have been associated with corneal damage and repair during the early stages in the alkali-burned cornea. Moreover, topical applied infliximab, which is a monoclonal antibody that neutralizes the biological activity of TNF-α, has been found to be safe and effective to reduce corneal perforation and opacity in a mouse model of corneal alkali burn.4

Because of the favorable results obtained by topically used infliximab in the treatment of experimental corneal alkali burn, utilization of drugs that possess anti–TNF-α property seems to be reasonable in the treatment of acute corneal alkali burn. Aside from their antimicrobial effect, macrolide antibiotics have been reported to inhibit TNF-α secretion in several studies.5–8 Among the macrolide antibiotics, only azithromycin (AZM) has an approved topical form, and this eye drop has been shown to be effective in the treatment of bacterial conjunctivitis and blepharitis.9,10 Apart from ameliorating the infectious inflammation of the conjunctiva, topical AZM has been demonstrated to suppress the noninfectious corneal inflammation by decreasing the expression of IL-1β and TNF-α in a mouse model.11 Topical AZM has also been revealed to be related with the improvement of corneal graft survival because of its antiinflammatory aspect in a rat model of corneal transplantation.12 Increased production of TNF-α is not only involved in ocular surface
inflammation but has also been found to cause increased apoptosis in the corneal epithelium and endothelium in a mouse model of corneal transplantation.\textsuperscript{13} On the basis of these data, we believe that topically administered AZM may also be beneficial against increased corneal epithelial and endothelial apoptosis associated with extreme production of TNF-\(\alpha\) such as in a corneal alkali burn.

In this study, we aimed to evaluate the effect of topical AZM on apoptosis in the corneal epithelium and endothelium, along with investigating its effect on TNF-\(\alpha\) density expressed in the corneal epithelium in a rat model of corneal alkali burn.

**MATERIALS AND METHODS**

**Animals and Groups**

A total of 24 right eyes of 24 male Wistar albino rats weighing between 250 and 300 g, obtained from Experimental Research and Administration Center of Canakkale Onsekiz Mart University (COMU), were included in this study. The study protocol was approved by the Committee on the Ethics of Animal Experiments of COMU. The rats were housed in cages with free access to standard food and drinking water and they were maintained under controlled conditions that consisted of a 12-hour light/dark cycle (08:00–20:00 light; 20:00–08:00 dark), temperature of 23–25°C, and humidity in the range of 55%–60% in the COMU Laboratory Animal Research Center. All rats were divided into 4 equal groups. The first group (\(n = 6\)) was used as a pseudovehicle group and only artificial tear gel (Lipotears opht gel; Bausch & Lomb), which contains middle-chained triglycerides, was instilled onto the right corneas, just after inducing the alkali burn of the cornea. Instillation of pseudovehicle was continued 5 times daily for 5 days.

The eyes were collected and fixed with 10% neutral buffered formalin and embedded in paraffin. Corneal sections (5 \(\mu\)m) were prepared and then stained with hematoxylin and eosin for light microscopic observations. The histopathological grading of the severity of corneal alkali injury was made by considering the EsoHisto guidelines that are used for describing the histopathological severity of microscopic esophagitis.\textsuperscript{14} This histopathological grading system was modified for corneal alkali injury by scoring epithelial loss (0 = none, 1 = minimal splitting, 2 = mild erosion, 3 = moderate ulceration, and 4 = strong erosion), corneal hemorrhage (0 = none, 1 = subepithelial, 2 = subepithelial and upper stromal tissue, 3 = subepithelial and partial stromal tissue, and 4 = total stromal tissue), and inflammation (number of polymuclear leukocytes (PNL) in magnified area: 0 = none, 1 = 1 to 5 cells, 2 = 6 to 15 cells, 3 = 16 to 25 cells, and 4 = more than 25 cells). The mean histopathologic score ranging from 1 to 4 was correspondingly related with the severity of corneal alkali injury, such as minimal = 1, mild = 2, moderate = 3, and severe = 4.

**Measurement of TNF-\(\alpha\) Density in the Corneal Epithelium**

The corneal epithelium was treated with specific monoclonal anti–TNF-\(\alpha\) antibody (1:100, ab6671; Abcam) according to the avidin–biotin complex technique described by Kasprzk et al.\textsuperscript{15} Immunocytochemical staining of the corneal epithelium with anti–TNF-\(\alpha\) antibody was evaluated by the semiquantitative technique, relating the score of 0–4 points to the fraction of stained cells (score 0, 0% cells; 1, less than 5% cells; 2, 5%–20% cells; 3, 20%–20% cells; and 4, more than 40% positive cells).\textsuperscript{15} The preparations were examined under a light microscope at \(\times400\) magnification.

**TUNEL Staining**

Apoptotic cells were stained by the TUNEL assay using an apoptosis detection kit (Calbiochem, San Diego, CA), as previously reported.\textsuperscript{16} To determine the numerical distribution of TUNEL+ cells in the corneal samples stained with TUNEL kit, cells were examined under a light microscope (\(\times400\)). In each section, the numbers of positive cells in 10 different areas selected at random were counted in random high-power sections using a light microscope (Olympus BX51; Olympus, Japan) and incorporating a software analysis system (version 2.11.5.1; Argenit Kameram, Istanbul, Turkey). Finally, all...
counts were converted to number of TUNEL+ cells per unit area (in square millimeters).

Statistical Analysis
Data were expressed as mean ± SD. The Bartlett test was used to determine whether the data were heterogeneous or homogeneous. A Bonferroni multiple comparison test was applied to identify differences between mean values. Differences were considered statistically significant at \( P < 0.05 \).

RESULTS

Histopathological Findings
In group 1 and group 2, the corneas of healthy rats had normal appearance in terms of epithelium, stroma, and endothelium. However, there was severe acute corneal inflammation characterized with polymorphonuclear leukocyte infiltration, increased degenerative cells, stromal edema, and stromal hemorrhage in rats with corneal alkali burn belonging to group 3 and group 4. The mean histopathological score in corneal stroma was zero in both group 1 and group 2. However, the mean histopathological score in corneal stroma was 3.7 ± 1.2 in group 3, whereas it was 1.9 ± 0.9 in group 4 (\( P < 0.05 \)) (Fig. 1).

Changes in the Severity of Apoptosis in the Corneal Epithelium and Endothelium of Rats With Alkali Burn After the Use of Topical AZM
The number of TUNEL+ cells detected per square millimeter in all examined corneal epithelia was found to be significantly more in alkali-burned corneas belonging to group 3 and group 4, when compared with healthy ones belonging to group 1 and group 2 (\( P < 0.05 \)). The mean numbers of TUNEL+ cells in the corneal epithelium were 1.4 ± 0.6, 1.7 ± 0.1, 117.1 ± 23.8, and 75.8 ± 15.7 in group 1, group 2, group 3, and group 4, respectively (Fig. 2).

Regarding the mean number of TUNEL+ cells in the corneal epithelium, the statistical comparison between group 3 and group 4 showed a significant decrease in apoptosis in group 4 (117.1 ± 23.8 in group 3 vs. 75.8 ± 15.7 in group 4, \( P < 0.05 \)) (Fig. 3).

The number of TUNEL+ cells detected per 100 cells in all examined corneal endothelium was found to be significantly more in alkali-burned corneas belonging to group 3 and group 4, when compared with the healthy ones belonging to group 1 and group 2 (\( P < 0.05 \)). The mean numbers of TUNEL+ cells in the corneal endothelium were recorded as 3.0 ± 0.2, 3.4 ± 0.8, 34.6 ± 11.3, and 14.7 ± 3.5 in group 1, group 2, group 3, and group 4, respectively.

Regarding the mean number of TUNEL+ cells in the corneal endothelium, the statistical comparison between group 3 and group 4 showed a significant decrease in apoptosis in group 4 (34.6 ± 11.3 in group 3 vs. 14.7 ± 3.5 in group 4, \( P < 0.05 \)) (Fig. 4).

Effect of Topical AZM on TNF-α Density Increased in the Alkali-Burned Corneal Epithelium
In comparison with the density of TNF-α detected in the epithelium of the healthy corneas belonging to group 1 and group 2, significantly increased TNF-α density was detected in the epithelium of alkali-burned corneas belonging

FIGURE 1. A, Group 1 (pseudovehicle) and (B) group 2 (control); normally appearing cornea. C, Group 3 (alkali burned); distinct hemorrhage, inflammation, and edema in corneal stroma. D, Group 4 (treatment); significant decrease in hemorrhage, inflammation, and edema in corneal stroma (hematoxylin and eosin staining, all magnifications: ×400).
The mean TNF-α densities in the corneal epithelium were 0.60 ± 0.2, 0.55 ± 0.1, 2.65 ± 1.3, and 1.65 ± 1.1 in group 1, group 2, group 3, and group 4, respectively (Fig. 5).

There was a statistically significant decrease in terms of TNF-α density measured in the corneal epithelium in group 4, when compared with that in group 3 (2.65 ± 1.3 in group 3 vs. 1.65 ± 1.1 in group 4, \( P < 0.05 \)) (Fig. 6).

DISCUSSION

The mechanism of corneal injury due to alkali burn significantly depends on the expression of inflammatory cytokines and matrix metalloproteinases (MMPs) that are the consequence of recruitment of inflammatory cells into the injured cornea. Among the numerous inflammatory cytokines, an increased production of TNF-α and IL-1 has been suggested to involve in the formation of sight-threatening complications of corneal alkali burn such as corneal neovascularization and ocular surface scarring. Along with TNF-α, increased intracorneal levels of MMP-2 and MMP-9 that are called gelatinase A and B, respectively, and responsible for corneal epithelial and stromal degradation have been reported in the alkali-burned cornea. Although no substantial change has been reported in the expression of MMP-2 in human corneal epithelial cells after being exposed...
to various cytokines, the upregulation of MMP-9 by IL-1 and TNF-α has been demonstrated in human corneal epithelial cells. Moreover, in the same study, it has been found that the abolishment of upregulated MMP-9 could be achieved using IL-1 and TNF-α antagonists. In an experimental research, because of TNF-α induction, increased activity of MMP-2 and MMP-9 has been revealed in the corneal epithelium. Aside from leading to ocular surface inflammation, alkali burn has been implicated in TNF-α–induced corneal and retinal apoptosis, which were shown to be impeded using infliximab. Given these data, treatment options aiming to reduce the increased cytokine and MMP level seem to have significant importance in the management of corneal alkali burn injury.

Macrolide antibiotics have been reported to exert antiinflammatory activity as a result of decreasing cytokine expression. Apart from systemically used macrolide antibiotics, a topical form of AZM, which is a second-generation macrolide antibiotic, has been found to display immunomodulatory effects by causing a decrease in the level of IL-6, nuclear factor-kappa B (NF-κB), and MMP-2 activity in a rat model of conjunctivitis. Moreover, Li et al have demonstrated the suppression of the production of TNF-α, IL-1β, MMP-1, MMP-3, and MMP-9 secreted by human corneal epithelial cells using topical AZM, and this aspect of AZM has been attributed to its inhibitory effect on NF-κB activation. The decrease in the NF-κB activity provided by AZM has also been noted in airways of mice exposed to cigarette smoke. In a corneal alkali burn model in mice, using topical inhibitory agent of NF-κB, Saika et al have reported reduction in the incidence of epithelial defect and ulceration, as well as a decrease in the activity of MMP, basement membrane destruction, and cytokine expression. Therefore, topical AZM may be beneficial for ameliorating corneal inflammation due to corneal alkali burn by inhibiting the NF-κB pathway, which is used by a number of cytokines and growth factors for signaling on ligand binding to cell surface receptors that are upregulated in corneal cells.

In this study, different from its immunomodulatory effect, we investigated whether topically used AZM also possesses antiapoptotic effect on corneal epithelial and endothelial cells, as a decrease in corneal stromal apoptosis with the administration of topical AZM has been previously reported in an experimental model of refractive surgery. In this study, we determined that the number of TUNEL+ cells

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**FIGURE 5.** TNF-α immunohistochemical staining. A, Group 1 (pseudovehicle); (B) group 2 (control); (C) group 3 (alkali burned), and (D) group 4 (treatment). The expression of TNF-α was observed as highest in the corneal epithelium and stroma of group 3. Besides that, TNF-α density was noticed as decreased in the corneal epithelium of group 4 (all magnifications: ×400). Thin and thick black arrows were, respectively, used to indicate the examples of TNF-α–stained cells in corneal epithelium and stroma.

**FIGURE 6.** Comparisons of the mean TNF-α density measured in the corneal epithelium in all groups. *P < 0.05; when compared separately with group 1 and group 2; **P < 0.05; when compared separately with group 1, group 2, and group 3; P > 0.05; when compared group 1 with group 2.
in the corneal epithelium of rats with alkali burn was significantly decreased with the topical application of AZM. Moreover, we found that topical AZM was capable of reducing the TNF-α density increased in the corneal epithelium as a result of alkali burn. According to the previous data, suggesting the anti–TNF-α property of AZM, this result of this study may not be surprising because the susceptibility of corneal epithelial cells to TNF-α–induced apoptosis is known from a previous study. It has been reported that the suppression of corneal epithelial apoptosis can contribute to the acceleration of epithelial wound healing in corneal alkali burn. This relation can be important because it has been hypothesized that corneal stromal ulceration in alkali burn is initiated as a consequence of proteases secreted by the defective corneal epithelium. So that inhibitory effect of topical AZM on corneal epithelial cell apoptosis in alkali burn may promote epithelial healing, which can subsequently lead to a decrease in stromal degradation.

Because of inadequate allocation in our institute, apart from lack of the evaluation of NF-kB pathway in corneal layers, we could not also evaluate the TNF-α density in the corneal endothelium in this study, and we consider these as important shortfalls of this investigation. However, the determination of increased TNF-α density in the endothelium of immune-rejected corneal allografts in a previous study can support the significant role of TNF-α expression in the induction of corneal endothelial cell apoptosis. The exact mechanism by which increased TNF-α expression can lead to corneal endothelial apoptosis remains unknown, but Sagoon et al have suggested that TNF-induced activation of NF-kB can cause corneal endothelial apoptosis by triggering the synthesis of nitric oxide. Therefore, although we do not have an opinion about TNF-α density in the corneal endothelium, we consider that topically applied AZM may have given rise to a decrease in corneal endothelial cell apoptosis by inhibiting the TNF-α–induced activation of NF-kB in our corneal alkali burn model. Topical AZM has been previously demonstrated to be effective in the promotion of corneal graft survival in mice corneas that have undergone experimental corneal transplantation, but its mechanism of action in this relation has not been fully elucidated yet. Nevertheless, according to the data belonging to previous studies, and to this study, it may be reasonable to benefit from the application of topical AZM as an auxiliary medication in the prevention of apoptotic endothelial cell death, in addition to main immunosuppressive regimens administered for corneal transplantation surgery.

Because the ocular surface distribution and pharmacokinetics of topically used AZM were not the objectives of this study, we could not measure the level of AZM in the cornea. However, Akpek et al have reported that a high concentration of AZM could be achieved in the corneas of rabbits within 5 minutes after the instillation and it could persist for up to 6 days. In this study, because we considered that the usually recommended treatment duration for topical AZM, which is twice a day for 3 days, may not be sufficient in the management of corneal alkali burn, we randomly administered topical AZM 5 times daily for 5 days. However, because Sano et al have reported that corneal graft rejection can occur within 2 weeks after being transplanted on neovascularized graft bed, sustaining the application of topical AZM for at least up to 2 weeks may be reasonable, if corneal transplantation is considered for the complication of corneal alkali burn that usually results in severe corneal neovascularization.

In conclusion, the administration of topical AZM, which was shown to reduce both corneal epithelial and endothelial cell apoptosis in the corneal alkali burn model of rats in this study, may provide benefit in the management of acute and subacute complications of corneal alkali burn such as epithelial defect and stromal melting, as well as in the management of further complications of the corneal alkali burn such as poor corneal graft survival. However, further studies involving the assessment of toxicity of topical AZM on ocular tissues are required for the safe and long-term use of this drug for these mentioned purposes.

REFERENCES


