Olopatadine enhances recovery of alkali-induced corneal injury in rats

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ABSTRACT

Aims: The alkali-induced corneal injury is an ocular emergency that required an immediate and effective management to preserve the normal corneal functions and transparency. Olopatadine is a fast, topically-effective anti-allergic drug, which exhibited potent anti-inflammatory and anti-angiogenic abilities in different allergic animals’ models. Therefore, this study aimed to evaluate the therapeutic effect of olopatadine on alkali-induced corneal injury in rats.

Materials and methods: Corneal alkali injury (CI) induced in the right eyes of an eight-week-old male Wister rats, by application of 3 mm diameter filter-papers, soaked for 10 s in 1 N-NaOH, to the right eyes’ corneal centers for 30 s, afterward, the filter paper removed, and the rat right eye rinsed with 20 ml normal saline. For treatment of CI, either 0.2% or 0.77% olopatadine applied topically daily for 14 days, starting immediately after the induction of CI.

Key findings: Olopatadine, in the present work, effectively and dose-dependently enhanced the corneal healing after alkali application, with significant reduction of the corneal opacity and neovascularization scores, besides, it suppressed the augmented corneal IL-1β, VEGF, caspase-3 levels, and nuclear NF-κB expression, meanwhile it abrogated the corneal histopathological changes, induced by alkali application.

Significance: Olopatadine appears to be a potential treatment option for alkali-induced corneal injury.

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1. Introduction

Chemical injuries of the eye account for 11.5–22.1% of all the ocular traumas, where two-thirds of which affecting young men. Most of these injuries occur as a result of industrial accidents in agriculture, laboratories, fabric mills and automotive repair facilities, while the minority occurs at home, and as a secondary assault. The alkali-induced ocular injury is account nearly for 60% of all chemical ocular injury, and considered as an ocular emergency, where immediate and effective management is needed to preserve the normal corneal functions and transparency, otherwise, severe corneal injury, with slow re-epithelialization, ulceration, neovascularization, and opacification will be settled, with subsequent corneal and anterior chamber destruction that ends ultimately into loss of vision [1–4]. The common alkali that causes corneal injury are lye, ammonia, potassium and magnesium hydroxide, and lime, which present in many cleaning agents, drain cleaners, building material, staining dyes, fertilizer, laboratory reagents, and firework sparklers, where, lime is considered the most common agent, and ammonia and lye, which contains NaOH, are the most dangerous ones [3, 4].

Furthermore, alkali agents are lipophilic in nature, thus can penetrate tissues very rapidly, with consequent saponification of the cell membranes’ fatty acids [5]. Moreover, the hydroxyl group present in most alkalis penetrates the corneal stroma, destroying its proteoglycan ground substance, as well as the collagen bundles, with subsequent production of proteolytic enzymes from the damaged tissue, as a consequence of the inflammatory response, leading to further chemical penetration [2, 6–8]. Additionally, the exposure of the eye to alkali affect the ocular surface epithelium, in addition to its adnexa, with special effect on the cornea leading to its apoptosis, inflammation, perforation, neovascularization, and opacification, with recruitment of inflammatory cells, mesenchymal cells, activated kerocytes and macrophages that orchestrating the processes of corneal injury, repair, and wound healing, through the release of various cytokine, angiogenic and anti-angiogenic factors [7, 9]. Where, the imbalance between the angiogenic and anti-angiogenic factors, with predominance of angiogenic factors such as vascular endothelial growth factor (VEGF), and the increased release of the pro-inflammatory cytokines, such as interleukin (IL)-1β, with activation of caspase and nuclear factor-kappa B (NF-κB) cascades are thought to be the main pathogenesis of corneal opacity and neovascularization, following the alkali injury [9–11].

Accordingly, by a proper understanding of the alkali corneal injury's...
epithelial transplantation, or corneal transplantation complicated with epithelium is associated with the release of proteolytic enzymes, with intervention of the corneal injury by debridement of corneal necrotic pruritus, and erythema exudative multiform [13, 15]. It is a potent dual acting mast cell stabilizer, and selective histamine-1 receptor antagonist, so, it inhibits type I immediate hypersensitivity reaction, and re-acting mast cell stabilizer, and selective histamine-1 receptor antagonist, so, it inhibits type I immediate hypersensitivity reaction, and re-acting mast cell stabilizer, and selective histamine-1 receptor antagonist, so, it inhibits type I immediate hypersensitivity reaction, and re-acting mast cell stabilizer, and selective histamine-1 receptor antagonist, so, it inhibits type I immediate hypersensitivity reaction, and re-acting mast cell stabilizer, and selective histamine-1 receptor antagonist, so, it inhibits type I immediate hypersensitivity reaction, and re-acting mast cell stabilizer, and selective histamine-1 receptor antagonist, so, it inhibits type I immediate hypersensitivity reaction, and re-acting mast cell stabilizer, and selective histamine-1 receptor antagonist, so, it inhibits type I immediate hypersensitivity reaction. Consequently, new approaches are needed to improve the management of corneal alkali injury as regards for the inflammatory, apoptotic and angiogenesis-related factors.

Olopatadine is a fast topically effective anti-allergic drug, used in the treatment of allergic conjunctivitis, rhinitis, urticaria, dermatitis, pruritus, and erythema exudative multiform [13–15]. It is a potent dual acting mast cell stabilizer, and selective histamine-1 receptor antagonist, so, it inhibits type I immediate hypersensitivity reaction, and releases the strain of mast cell inflammatory mediators like histamine, tryptase and prostaglandin D2, with subsequent suppression of inflammatory and pro-inflammatory cytokine release, and inflammatory cells and conjunctival epithelium recruitment [13, 16–18]. Furthermore, it exhibited potent anti-inflammatory, anti-edematous and anti-angiogenic abilities in a mice and rat models of allergic conjunctivitis and rhinitis respectively, through the suppression of the pro-inflammatory and inflammatory cytokine, such as IL-1β, and the angiogenic factors, such as VEGF, production [14, 19]. Additionally, topical ocular application of olopatadine as a single daily dose documented clinically to be effective and safe for long-term use in the treatment of allergic conjunctivitis [13, 18, 20, 21]. Therefore, this study aimed to evaluate the therapeutic effect of olopatadine on the corneal opacity and neovascularization, through the assessment of its anti-inflammatory, anti-angiogenic and anti-apoptotic activities, as well as its abrogative potentiality on the corneal histopathological changes and immunohistochemical NF-κB expression, in a rat model of alkali-induced corneal injury.

2. Materials and methods

2.1. Drugs and chemicals

Olopatadine hydrochloride and diaminobenzidine purchased from Sigma, St. Louis, MO, USA, ketamine from Sigma, Nasr City, Cairo, Egypt, xylazine from Advia, Obour City, Cairo, Egypt, hematoxylin stain from Oxford, India, eosin stain from Jahangir, India, and sodium pentobarbionate from Abbott Lab., Chicago, IL, USA. Moreover, phosphate-buffered saline (PBS), Tris-HCl buffer, formalin-buffered saline, Canada balsam, periodic acid, Schiff's reagent, hydrogen peroxide, methanol and sodium hydroxide (NaOH) obtained from El Gomhuria Co., Tanta, El-Gharbeya, Egypt. However, serum-free protein blocking solution (Block Ace) bought from DS Pharma Biomedical Co., Ltd., Osaka, Japan and normal saline from Otsuka Egypt Co., Nasr City, Cairo, Egypt.

2.2. Animals

Eight-week-old male Wister rats, weighing 150–200 g, obtained from Tanta Faculty of Medicine animal house, Egypt, used in the present study. Rats housed in meshed plastic cages under standard laboratory conditions, with free access to standard laboratory food and water Ad libitum. Animals permitted to adapt for one week before experimentation start. All animals’ management followed the Association for Research in Vision and Ophthalmology (ARVO) animals’ guidelines, which were in accordance with Tanta Faculty of Medicine guidelines for care and use of Animals in research, with the approval of Tanta Faculty of Medicine’s Animal Experiment Ethics Committee, Egypt.

2.3. Induction of alkali corneal injury

Alkali corneal injury induced in the right eyes of rats only, with sparing of the left one to ensure the animals welfare. It induced according to Arikan et al. [22], briefly, 3 mm diameter filter paper soaked in 1 N NaOH for 10 s (sec), then the excess NaOH removed, and applied to the corneal center of the rat right eye for 30 s, after the anesthesia of rat with intraperitoneal injection of 75 mg/kg ketamine and 5 mg/kg xylazine. Later, the filter paper removed, and the rat right eye rinsed with 20 ml normal saline. Whereas, normal saline instead of NaOH applied to the rats’ right eyes of the control group [22].

2.4. Experimental design

Sixty Wister rats randomly distributed into 5 groups of 12 rats each. Group I (CON), was normal rats. Group II (CI), was NaOH-induced corneal injury rats. Group III (VEH), was corneal injury induced rats, treated topically with 10 μl/eye/day PBS. Group IV (OL), was corneal injury induced rats, treated topically with 10 μl/eye/day olopatadine 0.2% (dissolved in PBS) [23]. Group V (OH) was corneal injury induced rats, treated topically with 10 μl/eye/day olopatadine 0.77% (dissolved in PBS) [23]. All treatments were freshly prepared daily and applied once daily for 14 days between 10:00 A.M. and 12 P.M., starting immediately after the induction of corneal injury.

2.5. Corneal opacity and neovascularization scores

The corneal opacity and neovascularization scores assessed at 3rd, 7th, 10th and 14th day of treatment, after the application of NaOH, by an ophthalmoscope (HS-OP10, Belson, China). The corneal opacity score determined with the method described by Ke et al. Where, grade 0, normal transparent cornea, grade 1, minimal corneal opacity, grade 2, mild corneal opacity with pupil margin and iris vessels were visible, grade 3, mild corneal opacity with only pupil margin was visible, grade 4, moderate corneal opacity with only pupil still visible, grade 5, complete corneal opacity [24]. However, the corneal neovascularization score evaluated according to the method reported by Dana and Streilein. Shortly, the cornea divided into 4 quadrants, then neovascularization of each quadrant graded between 0 and 3, according to the neovascular branch centripetal extension from the corneoscleral junction. Where, grade 0, no neovascularization, grade 1, the neovascularization < 1/3 of corneal radius, grade 2, neovascularization between 1/3 and 2/3 of corneal radius, grade 3 the neovascularization > 2/3 of the corneal radius. Afterward, the grade of all quadrants summed to get 0 to 12 corneal neovascularization score for each eye [25]. Both corneal opacity and neovascularization scores expressed as arbitrary units.

2.6. Samples collection

Twenty-four hours after the last treatment, rats sacrificed by cervical dislocation. Then the rats’ right eyes dissected and their cornea harvested. The corneas of six rats per each group immediately
processed for histopathological and immunohistochemical evaluation. However, each cornea of the other six, instantly homogenized in 1 ml of 0.1 mmol Tris·HCl buffer (pH = 7), and centrifuged at 5000 rpm for 10 min, afterward, the supernatant collected and stored at −80 °C for further assessment.

2.7. Assessment of corneal IL-1β level

The corneal IL-1β level estimated according to the manufacturer's instruction of an enzyme-linked immunosorbent assay (ELISA) kit purchased from Bio Vendor Laboratorní medicína A.S., Karasek, Czech Republic, with a minimum detection limit of 4 pg/ml. The absorbance of the samples determined using an automated ELISA plate reader (Stat Fax 2100, Fisher Bioblock Scientific, BP., Illkirch Cedex, France), and the corneal IL-1β level expressed as pg/ml.

2.8. Assessment of corneal VEGF level

The corneal VEGF level determined with an ELISA kit obtained from CUSABIO, Donghu, Hi-Tech, Wuhan, Hubei Province, China, with a minimum detection limit of 0.97 pg/ml, as described by its producer. The samples' optical densities analyzed with an automated ELISA plate reader (Stat Fax 2100, Fisher Bioblock Scientific, BP., Illkirch Cedex, France), and the corneal VEGF level expressed as pg/ml.

2.9. Assessment of corneal caspase-3 level

An ELISA kit acquired from Sunred Bio. Tec., Shangai, China, with minimum detection limit 0.045 ng/ml used to estimate the corneal caspase-3 level, according to the manufacturer's protocol. The samples' absorbance assessed by an automated ELISA plate reader (Stat Fax 2100, Fisher Bioblock Scientific, BP., Illkirch Cedex, France), and the corneal caspase-3 level expressed as ng/ml.

2.10. Assessment of corneal histopathological changes

Corneal samples prepared according to [26]. Concisely, specimens took form 6 rats per each group and directly fixed in 10% formalin-buffered saline. Then, dehydrated, cleared and impregnated into paraffin. Finally, a rotary microtome (Olympus, Tokyo, Japan) used to obtain sections of 5 μm, used for the histological and immunohistochemical study [26].

2.10.1. Hematoxylin and eosin (H & E) staining

Corneal specimens' sections deparaffinized, hydrated and stained with hematoxylin for 30 min (min), then with 1% eosin for 10 min. Finally, sections dehydrated, cleared, mounted in Canada balsam and examined with a light microscope (Olympus, Tokyo, Japan).

2.10.2. Periodic acid Schiff's (PAS) staining

Specimen's deparaffinized and hydrated, then, immersed in 1% periodic acid followed by Schiff's reagent for 15 min, after that dehydrated, cleared and mounted in Canada balsam. Lastly, magenta color staining of basement membranes saw using a light microscope (Olympus, Tokyo, Japan).

2.10.3. Immunohistochemical localization of the NF-κB-p65

In short, sections deparaffinized, rehydrated, then, the endogenous hydrogen-peroxidase activity blocked by placing sections in 0.3% hydrogen peroxide/methanol for 20 min, then, washed with PBS. Afterward, a serum-free protein blocking solution (Block Ace) added to the sections and incubated for 20 min, later, the sections incubated with the rabbit anti-NF-κB-p65 antibody (1:200, Santa Cruz Biotechnology, California, U.S.A.) overnight at 4 °C. Next, sections washed with PBS and incubated with the secondary antibody (Dako North America, Inc., CA, USA) for 10 min. Finally, 1-2drops of diaminobenzidine added to each slide for 5–10 min, then, counterstained with hematoxylin, dehydrated, cleared and examined by a light microscope (Olympus, Tokyo, Japan). Nuclear and cytoplasmic brown reaction got. The negative control specimens obtained through the addition of PBS rather than the primary antibody [27]. Furthermore, the nuclear and cytoplasmic NF-κB-p65 expression each alone arbitrary scored according to Guo et al. [28]. In brief, the percentage of cells that expressed a positive reaction in either cytoplasm or nucleus each alone determined per 200 cells in ×400 magnified images per each rat, and scored arbitrary, where, 0, 0–9%, 1, 10–25%, 2, 26–50% and 3, > 50% of cells expressed a positive reaction. Moreover, the positive reaction intensity, in the same cells, scored arbitrarily as follow, 0, no reaction, 1, weak reaction, 2, moderate reaction and 3, strong reaction. Then, the total immunohistochemical score was estimated by the following formula “percentage of positive reaction cells score X positive reaction intensity score”, where the score ranged from 0 to 9 arbitrary units, for the nuclear and cytoplasmic NF-κB-p65 expression each alone [28].

2.11. Statistical analysis

The data of the present study expressed as mean ± SD. The statistical difference between the different groups evaluated by one-way ANOVA (followed by Tukey’s test as a post hoc test) or Kruskal-Wallis’s test (followed by Mann-Whitney’s U test as a post hoc test), after analysis of variances with Bartlett’s test. However, the corneal opacity and neovascularization scores’ data analyzed by two-way ANOVA, followed by Tukey’s test as a post hoc test. The significance of data considered when the P value < 0.05.

3. Results

3.1. Effect of olopatadine on the corneal opacity and neovascularization scores

Alkali application significantly induced a severe corneal injury, which could be revealed by a coarse corneal opacity that deepened with time. Furthermore, the healing process, after NaOH application, associated with a progressive growth of neovascular branches from the corneoscleral junction toward the corneal center. In contrary, the daily topical treatment with olopatadine effectively and dose-dependently enhanced the corneal healing after alkali application, with a significant reduction of the corneal opacity and neovascularization scores, when compared with CI group. Additionally, the reduction of the corneal opacity by olopatadine showed no significant difference throughout the time course of its assessment, in contrary to the neovascularization scores where there is a significant increase of it by the 14th day of the experiment, when compared with the 3rd day (Fig. 1).

3.2. Effect of olopatadine on the corneal IL-1β level

In parallel with the severe corneal opacity and neovascularization that induced by NaOH application, the corneal IL-1β level significantly augmented after alkali application, as compared to the control group. The daily treatment with topical olopatadine efficiently suppressed the amplified corneal IL-1β level, induced by alkali application, in a dose-dependent manner (Fig. 2).

3.3. Effect of olopatadine on corneal VEGF level

In correspondent to the neovascularization score results, the corneal VEGF level significantly enhanced, in the corneal injury induced by alkali application, when compared to the control group. However, the daily treatment with topical olopatadine application, after alkali-induced corneal injury, powerfully suppressed the enhanced corneal VEGF level dose-dependently (Fig. 2).
3.4. Effect of olopatadine on the corneal caspase-3 level

The corneal apoptosis significantly increased by NaOH topical application, as indicated by significant elevation of the corneal caspase-3 level, when compared with the caspase-3 level of the control group. The daily topical application of olopatadine, for treatment of the corneal alkali injury, effectively and dose-dependently reduced the elevated corneal caspase-3 level induced by alkali injury. Furthermore, the OH group showed a significant elevation of the caspase-3 level, when compared with the control group (Fig. 3).

3.5. Effect of Olopatadine on corneal histopathological changes

3.5.1. H & E stained sections

Corneal H & E stained sections of the control group showed five distinct layers arranged from outside inward as follow, the 1st layer was the corneal epithelium that was non-keratinized stratified squamous epithelium resting on a basement membrane, and consisting of basal columnar cells with oval basal nuclei, however, the 2nd layer consisted of two to three layers of intermediate polygonal cells, with rounded nuclei, and one or two layers of superficial squamous cells, with flattened nuclei, moreover, the 3rd layer was a cellular homogenous Bowman's membrane, in addition to the 4th layer, which was stroma or...
substantia propria that appeared as avascular parallel lamellae of dense collagenous fibers, with flattened fibroblasts (keratocytes) scattered in-between them, the last layer was flattened endothelial cells that lined the inner surface of the cornea, and supported by Descemet's membrane that appeared as a thick homogenous basement membrane. The examination of CI & VEH groups' sections revealed a severe corneal injury that evidenced by the presence of deeply stained epithelial cells' nuclei that surrounded by vacuolated cytoplasm, with irregularities of Bowman's membrane. Moreover, the stroma exhibited areas of widely separated collagen lamellae, as well as mononuclear cellular infiltration, and stromal vascularization. Furthermore, some of the endothelial cells appeared swollen with some focal parts stratified, with focal separation from the Descemet's membrane. Conversely, OL & OH groups revealed nearly restoration of the normal corneal histological structure dose-dependently (Fig. 4).

3.5.2. PAS-stained sections

The PAS-stained sections of the control group showed a PAS-positive magenta colored epithelial basement membrane and Descemet's membrane. Whereas the CI & VEH groups revealed focal interruption of the epithelial basement membrane, that proved by the interruption or even absence of the PAS-positive reaction in some areas, while, the Descemet's membrane displayed a normal PAS positive reaction. On the other hand, the OL & OH groups demonstrated a dose-dependent enhancement of PAS-positive reaction of the epithelial basement membrane to be apparently like the control group in the high-dose treated olopatadine group (Fig. 5).
nuclear because of increased NF-κB p65 cytoplasmic in the control group, while, in CI & VEH groups, it became dependent elevation of cytoplasmic and reduction of nuclear NF-κB p65. Moreover, in olopatadine treated groups, there is a significant elevation of cytoplasmic and significant elevation of nuclear NF-κB p65 immunohistochemical expression, when compared with the control group.

3.6. Effect of olopatadine on the corneal epithelial NF-κB-p65 immunohistochemical expression

Corneal epithelial NF-κB-p65 immunohistochemical expression showed different localizations in the experimental groups, by which it is cytoplasmic in the control group, while, in CI & VEH groups, it became nuclear because of increased NF-κB-p65 nuclear translocation. Moreover, in olopatadine treated groups, there is a significant and dose-dependent elevation of cytoplasmic and reduction of nuclear NF-κB-p65 immunohistochemical expression, when compared to CI group. Nevertheless, the OH group showed a significant reduction of cytoplasmic and significant elevation of nuclear NF-κB-p65 immunohistochemical expression, when compared with the control group (Figs. 3 & 6).

4. Discussion

The cornea is an avascular transparent part of the eye that performs an important role in the maintenance of clear eye vision and is considered as a notable immune site in the body. Moreover, the corneal epithelium considered as the first defensive line of the eye against pathogens-associated molecular pattern recognition that initiates strong innate immune responses. The exposure of the eye to the alkali, induce a chronic immune-mediated inflammation, with subsequent disruptions of the ocular surface structure and function [29, 30].

Our model in the present study is a complete corneal alkali-burn, being used for assessment of the new medication effectiveness [22], as the currently used therapeutics for corneal injuries are disappointing, insufficient, and not so effective in restoration of normal corneal functions and transparency. Moreover, most of the medications used have many adverse effects, and its clinical outcomes are not satisfactory. Furthermore, therapies that rapidly and effectively suppress inflammation and angiogenesis are critical for the treatment of corneal chemical burns [1, 9, 12, 31].

The present work showed that alkali application was significantly induced a coarse corneal opacity that deepened with time and associated with a progressive evolution of neovascular branches from the corneoscleral junction toward the corneal center. In addition, it significantly elevated corneal IL-1β, VEGF and caspase-3 levels as compared to the control group. Furthermore, it altered the histological structure of the cornea, with widely separated collagen lamellae, stromal mononuclear cellular infiltrations, and neovascularization in H&E stained sections, and focal interruption of corneal epithelial basement membrane in PAS-stained section. In parallel with these findings, the corneal cytoplasmic and nuclear NF-κB immunohistochemical expression significantly reduced and elevated by NaOH application to the cornea, respectively. In contrary, olopatadine in the present study dose-dependently abrogated these changes.

These previous findings can be explained by the fact that the application of alkali to the cornea induces corneal catarization, with protein denaturation, and dysregulation of cellular fluid homeostasis. Additionally, it promotes the local corneal inflammation, with requirement of inflammatory cells, mesenchymal cells, activated keratocytes and macrophages, which secrete local chemokines, pro-inflammatory cytokine, such as IL-1β, and angiogenic factors, such as VEGF, also, it induced corneal cell apoptosis, with subsequent cells death, corneal opacification and neovascularization [5, 11, 32]. Moreover, the induced saponification creates a softened or liquid environment that disrupts the cell membrane, and consequently, enables the alkali to spread over and over with impairment of the adjacent and inner tissues [3, 29].

Additionally, as a result of corneal epithelial insult, the damaged epithelium and its interrupted basement membrane, secrete pro-inflammatory cytokines, mainly IL-1β, which stimulate corneal stromal keratocyte to release Fas ligand that induces apoptosis of the nearby keratocytes, thus augment IL-1β secretion, with subsequent stromal requirement of macrophages, T-lymphocytes and neutrophils, which phagocytosed the damage cell, and myofibroblast activity that enhance VEGF, also, it induced corneal IL-1β secretion, with subsequent cells death, corneal opacification and neovascularization [5, 11, 32]. Moreover, the induced saponification creates a softened or liquid environment that disrupts the cell membrane, and consequently, enables the alkali to spread over and over with impairment of the adjacent and inner tissues [3, 29].

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into corneal damage [34–37]. Furthermore, IL-1β enhances expression and activation of NF-κB-p65, with further amplification of inflammatory cascades, hence, corneal damage, opacification, and neovascularization [11, 38, 39]. Also, IL-1β reported triggering corneal neovascularization directly, independent of angiogenic factors, such as VEGF, via enhancement of vascular endothelial cells migration and capillary tubes development [40]. Additionally, treating of corneal alkali injury with IL-1 receptor antagonist suppresses corneal inflammation, with the promotion of corneal healing, re-epithelialization, thus, restoration of its transparency [41].

Moreover, corneal cells alkali insult induces excessive expression and secretion of VEGF, promoted by IL-1β, which in turn stimulates VEGF receptor that mediates activation of phosphatidylinositol-3-kinase/protein kinase b, extracellular signal-regulated kinase/mitogen-activated protein kinase and protein kinase A/endothelial nitric oxide synthase activation, with subsequent promotion of growth, survival, differentiation, proliferation and migration of the endothelial cells, as well as, capillary tube formation, thus considered as the major modulator of corneal wound healing and neovascularization [42, 43]. Also, the exposure of the eye to alkali cause corneal hypoxia, with subsequent poor nutrition, as well as poor regeneration of the corneal epithelium results in corneal opacity, besides, it enhances the production of hypoxia-inducible factor 1-alpha, which accumulates in the nucleus, with consequent promotion of VEGF transcription and translation, thus, enhances corneal angiogenesis, that plays a fundamental role in the late corneal healing stage, however the tremendous angiogenesis induced by inflammatory reaction in alkali application results into corneal scarring, with subsequent corneal opacification and neovascularization [5, 19, 31, 44]. Furthermore, VEGF plays a pivotal role in the development of corneal inflammation following alkali injury, through the promotion of macrophages corneal recruitment, thus initiates and/or augments signals essential for corneal pathological neovascularization [40, 45]. Additionally, Choi et al. [6] added that neovascularization in turn contributes to further opacification, through disruption of the regular collagen fibers, and edema because of vascular extravasation [6].

However, the present work revealed that olopatadine is effectively and dose-dependently enhanced the corneal healing after alkali application, with a significant reduction of the corneal opacity and neovascularization scores, as well as suppression of the augmented corneal IL-1β and VEGF levels, besides, restoration of the normal corneal histological structure when compared with CI group. These findings are parallel with earlier studies, which proved that the treatment with olopatadine reduces the inflammatory reactions, as well as the IL-1β and VEGF levels in antigen-induced allergic conjunctivitis and rhinitis in mice and rats [14, 19, 46]. In addition, olopatadine has a suppressor effect on several inflammatory cells as it affects human eosinophils and neutrophils, with inhibition of ionophore A23187-induced release of peptide-leukotrienes, suppression of thromboxane B2, and leukotriene B4 release from human eosinophils and neutrophils [47]. Olopatadine, also, is a potent mast cell stabilizer, as well as anti-histaminic drug, as it inhibits the release of histamine and prostaglandins from mast cells through the inhibition of mast cell Ca²⁺ influx, which is important for mast cell lysosomal exocytosis, consequently, inhibits the induction of histamine, with subsequent inhibition of recruitment and activation of T lymphocytes, hence suppresses the secretion of the pro-inflammatory mediators, such as IL-1β, and the angiogenic factors, such as VEGF [17, 48]. Moreover, the pro-inflammatory cytokine, IL-1β, increase VEGF expression, then, VEGF induces a number of pro-inflammatory genes that in turn promotes transendothelial migration of neutrophils, therefore, the inhibition of their secretion by olopatadine, attenuated the neutrophil-mediated inflammatory responses, with subsequent improving the corneal structure and function [49]. Nevertheless, the significant increase of the neovascularization score by the end of the 14th day, as compared to the 3rd-day score, could be clarified as that the summit of the expressed molecules in response to alkali injury is the 14th day after the injury, then its levels fad later on [37, 50, 51].

Furthermore, caspases are a family of important signaling molecules, when activated, multiple cellular damages settled. Caspase-3 is an executioner pro-enzyme, activated by caspase-8 and caspase-9, when activated, it initiates the “death cascade”, and so, it is considered as an important marker of the apoptotic signaling pathway that leads to morphological cell changes ending with apoptosis [52, 53]. In alkali-induced corneal injury, caspases play pleiotropic role in induction of corneal cells apoptosis, as well as, in induction of inflammatory cascades, through upregulation and activation of NF-κB that in turn promotes IL-1β excessive secretion [11, 29, 54–56]. Additionally, IL-1β
corneal wound healing, corneal opacity in expression and activation of NF-κB by that these parameters measured at its expression peak-time, where avascular transparency. However, the significant increase in corneal NF-κB expression, hence, promotes corneal wound healing, with the restoration of normal corneal epithelium [38].

NF-κB-p65 expression, in the present study, exposed nuclear rather than cytoplasmic immunohistochemical reaction, in CI group, when compared to the control group. These findings are consistent with the other findings of our present research, which proved that prolonged and sustained NF-κB-p65 upregulation is associated with corneal angiogenesis, as well as, ocular inflammation and opacification. This could be explained by the fact that NF-κB is a heterodimer complex consisting of two subunits, p50 and p65, that exist in the cytoplasm in an inactive form associated with an inhibitory subunit IκBα. Stimulation of NF-κB by the inflammatory cytokines induces dissociation of NF-κB from its IκBα inhibitory subunit [59]. So, allows NF-κB to migrate from the cytoplasm to the nucleus, where it binds to the promoters of NF-κB-regulated genes, initiating gene transcription, with augmentation of IL-1β expression [55]. Other studies, demonstrated that IL-1β promotes expression and activation of NF-κB-p65, thus entering into vicious circle of inflammatory reactions amplification, with consequent delaying of corneal wound healing, corneal opacification, neovascularization that end eventually into loss of vision [38, 39, 43]. Additionally, the inhibition of inflammatory cascades mediated by NF-κB endorses corneal alkali wound healing, through suppression of the augmented IL-1β expression [60]. Also, the increased NF-κB-p65 levels proved to be associated with allergic eye diseases such as allergic keratoconjunctivitis which is triggered by degranulation of mast cells [38].

Data of the present work showed that olopatadine, in a dose-dependent manner endorsed the suppression of the expression, activation, and translocation of nuclear NF-κB from the cytoplasm to the nucleus, thus, repressed IL-1β expression, hence, promoted corneal alkali injury healing, with the restoration of corneal transparency, and hindering corneal neovascularization. The downregulation of NF-κB by olopatadine can be explained by the activation of IκBα inhibitory subunit which has an intrinsic nuclear localization signal, that enters to the nucleus, and then displaces NF-κB from its nuclear DNA binding sites, so transports NF-κB back to the cytoplasm [59]. By suppression of the NF-κB activation, the corneal epithelial proliferation encouraged causing corneal wound healing, with the restoration of normal corneal avascular transparency. However, the significant elevation of caspase-3 level and nuclear translocation of NF-κB-p65 by the end of the 14th day in the OH group, as compared to the control group, could be elucidated by that these parameters measured at its expression peak-time, where the healing process is at its summit, and before its repression [11, 22, 37, 50, 51, 56].

In conclusion, the daily topical olopatadine therapy effectively and dose-dependently attenuated alkali-induced corneal injury through its anti-inflammatory, anti-angiogenic, and anti-apoptotic activities, as well as its suppressive ability on NF-κB-p65 expression and activation, which subsequently suppressed the corneal inflammation, opacity, and neovascularization, with abrogation of the histopathological changes induced by alkali injury. Moreover, further investigations are in progress to evaluate the corneal histopathological changes, the progression of wound healing and rats’ visual function throughout the course of olopatadine therapy. So, olopatadine could be a potential medication to treat the alkali-induced corneal injury.

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Conflict of interest
The authors declare that they have no conflict of interest.

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