Abstract. The aim of the current study was to investigate whether olopatadine and naphazoline hydrochloride reduce allergic conjunctivitis in mice through alterations in inflammation, NGF and VEGF. An allergic conjunctivitis mouse model was established using histamine or an antigen (ovalbumin), following which mice were treated with 1% olopatadine solution and/or 0.2 mg/ml of naphazoline hydrochloride. Histamine or antigen-induced conjunctival vascular hyperpermeability was examined and the levels of inflammatory factors, cytokines, IgE, GMCSF and NGF were analyzed using ELISA in antigen-induced conjunctival hyperpermeability mice. In addition, VEGF protein expression was measured using western blotting in antigen-induced mice. The results indicated that olopatadine and naphazoline hydrochloride significantly suppressed conjunctival dye leakage in mice with histamine or antigen-induced conjunctival hyperpermeability. In addition, treatment with olopatadine and naphazoline hydrochloride was able to reduce the levels of inflammatory factors (TNF-α, IL-1β and IL-6), cytokines (IFN-γ and IL-4), IgE, GMCSF, and NGF in antigen-induced conjunctival vascular hyperpermeability mice. The protein expression levels of VEGF in antigen-induced conjunctival vascular hyperpermeability mice were reduced following treatment with olopatadine and naphazoline hydrochloride. These results suggest that treatment with olopatadine and naphazoline hydrochloride reduces conjunctivitis in mice via effects on inflammation, NGF and VEGF.

Introduction

Allergic conjunctivitis is a common ocular allergic disease, with a high incidence of ~20% of the total population in China (1). It predominantly occurs as a result of type I and IV hypersensitivity, of which the main symptoms include ocular itching, frequently with conjunctival hyperemia and edema (2). Allergic conjunctivitis is divided into the following clinical subtypes: i) Seasonal allergic conjunctivitis; ii) perennial allergic conjunctivitis; iii) vernal keratoconjunctivitis; iv) atopic keratoconjunctivitis; and v) giant papillary conjunctivitis (3). Diagnosis and classification of allergic conjunctivitis is predominantly based on clinical features, laboratory or pathological tests (4).

The early reactions in the pathogenesis of allergic conjunctivitis are mediated by mast cells and T cells. Following contact with the allergen, the antigen is combined with specific immunoglobulin E (IgE), resulting in mast cell degranulation and the release of inflammatory mediators (5). Mast cell degranulation activates endothelial cells, promoting the expression of chemokines and adhesion molecules (6). This attracts inflammatory cells to the conjunctival membrane, and activates conjunctival fibroblasts and epithelial cells to participate in the generation of conjunctivitis, with this process occurring within a few seconds following contact with the antigen, and the effects lasting from tens of minutes to several hours (7). In addition, interleukins (ILs) are released by fibroblasts, and mast cells are activated and release secondary messengers, promoting the allergic reaction to enter the late phase (8). The released cytokines, including IL-4, IL-5, IL-6, IL-8, IL-13, tumor necrosis factor-α (TNF-α) and vascular cell adhesion molecule-1, act on the conjunctiva and recruit inflammatory cells, including eosinophils, basophils, neutrophils and helper T lymphocytes, producing the second peak of immune inflammatory reaction (9).

Naphazoline hydrochloride is an adrenergic drug, stimulating adrenergic α-receptors resulting in vasoconstriction (10). Clinically, it is predominantly used for allergic and inflammatory nasal congestion, acute and chronic rhinitis and eye congestion. Additionally, it is also used for bacterial and allergic conjunctivitis and reduces blepharospasm (11). Olopatadine is a drug with dual effects, as a selective...
antagonist of histamine 1 receptors and a stabilizer of mast cells, and works faster than non-steroidal anti-inflammatory agents and mast cell stabilizers (12). However, the effects and mechanisms of olopatadine and naphazoline hydrochloride on allergic conjunctivitis remain to be fully elucidated. The current study hypothesized that olopatadine and naphazoline hydrochloride are able to reduce allergic conjunctivitis in mice, with the mechanism involved associated with effects on inflammation, nerve growth factor (NGF) and vascular endothelial growth factor (VEGF).

Materials and methods

Animals. A total of 40 female wild-type BALB/c mice (4-5 weeks; 18 g ± 2 g) were housed in the facilities of the Health Sciences Center of The people's Hospital of Guangxi (Guangxi, China) and maintained following the Use of Animals in Research and the internal animal use guidelines (13). All mice were maintained at 23±2°C and 55% humidity with a 12/12 h light/dark cycle, and received sterilized food and water ad libitum. The present study was approved by the Ethics Committee of Guangxi People's Hospital.

Study groups. The mice were divided into five groups: i) Control group (Con; n=8), mice received physiological saline [0.1 ml/100 g, intraperitoneal injection (i.p.)]; ii) allergic conjunctivitis model group (AC; n=8), mice were induced by histamine (30 µl, 0.1 mg/ml; Sigma-Aldrich) or ovalbumin (OVA; 30 µl); iii) olopatadine (Sigma-Aldrich, St. Louis, MO, USA) group (OLO; n=8), allergic conjunctivitis mice received 0.1% olopatadine solutions (10 µl per eye) (14); iv) naphazoline hydrochloride (Sigma-Aldrich) group (NH; n=8), allergic conjunctivitis mice received 0.2 mg/ml naphazoline hydrochloride (10 µl per eye) (15); v) olopatadine and naphazoline hydrochloride group (OLO + NH; n=8), allergic conjunctivitis mice received 1% olopatadine solutions and 0.2 mg/ml of naphazoline hydrochloride (10 µl per eye) (11).

Histamine-induced conjunctival vascular hyperpermeability in mice. The mice were narcotized with pentobarbital (50 mg/kg, i.p.), then injected with 30 µl of histamine (0.1 mg/ml) in the upper subconjunctiva following the intravenous injection of 1.5% w/v Evans blue solution. At 30 min following treatment, the mice were sacrificed by decollation following anesthesia with pentobarbital sodium (50 mg/kg; Sigma-Aldrich) and the treated eye was immediately removed. The tissue sample was extracted using formamide at 45°C for 5 min and the absorbance was determined using a spectrophotometer (3550; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 625 nm. Values were analyzed as per weight of each eye.

Antigen-induced conjunctival vascular hyperpermeability in passively sensitized mice. The mice were narcotized with pentobarbital (50 mg/kg, i.p.), then injected with 30 µl anti-OVA antisera (cat. no. C6534; rabbit anti-chicken; Sigma-Aldrich) in the upper subconjunctiva. Subsequently, 48 h later the animals were subjected to a challenge by an intravenous injection of OVA (2 mg/ml) with 1.5% w/v Evans blue solution. At 30 min following treatment, the mice were sacrificed and the treated eye was immediately removed.

Normal saline (10-20 µl) was applied to the tissue sample for 30 min and the absorbance was determined using a spectrophotometer (3550; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 625 nm. Values were analyzed as per weight of each organization.

Measurement of inflammation. The peripheral blood was collected from the tail vein and centrifuged at 12,000 x g for 10 min at 4°C and the supernatants were collected. TNF-α, IL-1β and IL-6 levels were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Lianshuo Biological Technology Co., Ltd., Shanghai, China).

Measurement of cytokine levels. The peripheral blood was collected from the tail vein and centrifuged at 12,000 x g for 10 min at 4°C and the supernatants were collected. Interferon (IFN)-γ and IL-4 levels were measured using commercially available ELISA kits according to the manufacturer's instructions (Lianshuo Biological Technology Co., Ltd.).

Measurement of IgE levels. The peripheral blood was collected from the tail vein and centrifuged at 12,000 x g for 10 min at 4°C and the supernatants were collected. The IgE levels were measured using a commercially available ELISA kit according to the manufacturer's instructions (Lianshuo Biological Technology Co., Ltd.).

Measurement of granulocyte-macrophage colony-stimulating factor (GMCSF). Conjunctivae were removed at room temperature. The removed tissue was immediately homogenized in phosphate-buffered saline (pH 7.4) containing a protease inhibitor (Shanghai Sangon Biological Engineering Technology, Shanghai, China). The samples were centrifuged at 12,000 x g for 10 min at 4°C and the supernatants were collected. The GMCSF level was measured using a commercially available ELISA kit according to the manufacturer's instructions (Lianshuo Biological Technology Co., Ltd.).

Measurement of NGF level. Conjunctivae were removed at room temperature. The removed tissue was immediately homogenized in phosphate buffered saline (pH 7.4) containing a protease inhibitor. The samples were centrifuged at 12,000 x g for 10 min at 4°C and the supernatants were collected. The NGF level was measured using a commercially available ELISA kit according to the manufacturer's instructions (Lianshuo Biological Technology Co., Ltd.).

Western blot analysis. Conjunctivae were removed at room temperature and immediately homogenized in phosphate buffered saline (pH 7.4) containing a protease inhibitor. The samples were centrifuged at 12,000 x g for 10 min at 4°C and the supernatants were collected. The protein content of the samples were centrifuged at 12,000 x g for 10 min at 4°C and the supernatants were collected. The samples were centrifuged at 12,000 x g for 10 min at 4°C and the supernatants were collected. The NGF level was measured using a commercially available ELISA kit according to the manufacturer's instructions (Lianshuo Biological Technology Co., Ltd.).

Western blot analysis. Conjunctivae were removed at room temperature and immediately homogenized in phosphate buffered saline (pH 7.4) containing a protease inhibitor. The samples were centrifuged at 12,000 x g for 10 min at 4°C and the supernatants were collected. The protein content of the samples was quantified using a bicinchoninic acid assay (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Equal amounts of proteins (50 µg) were separated using 12% sodium dodecyl sulfate polyacrylamide gel (Sangon Biotech Co., Ltd., Shanghai, China) electrophoresis and transferred to a nitrocellulose membrane (EMD Millipore, Billerica, MA, USA). The membrane was incubated with antibodies against goat
polyclonal anti-VEGF (1:1,000; cat. no. sc-1876; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and goat polyclonal β-actin (1:500; cat. no. sc-1616; Santa Cruz Biotechnology, Inc.) overnight at 4˚C with agitation. The proteins were detected using horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:5,000; cat. no. sc-2793; Santa Cruz Biotechnology, Inc.) at room temperature and visualized with an enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ, USA).

**Statistical analysis.** Values are presented as the mean ± standard error. SPSS software, version 17 (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis. Statistical analysis was performed using a one way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Olopatadine and naphazoline hydrochloride reduce histamine-induced conjunctival vascular hyperpermeability in mice.** To investigate the effects of olopatadine and naphazoline hydrochloride on histamine-induced conjunctival vascular hyperpermeability, mice were induced with histamine. The amount of conjunctival dye leakage following the injection of histamine was increased. Treatment with olopatadine or naphazoline hydrochloride reduced the levels of conjunctival dye leakage compared with the AC group (Fig. 1). Combined treatment with olopatadine and naphazoline hydrochloride further reduced conjunctival dye leakage in histamine-induced mice, compared with the olopatadine alone group (Fig. 1).

**Olopatadine and naphazoline hydrochloride reduce antigen-induced conjunctival vascular hyperpermeability in mice.** To investigate whether treatment with olopatadine and naphazoline hydrochloride reduces antigen-induced conjunctival vascular hyperpermeability, mice were induced using OVA. The amount of conjunctival dye leaked following the injection of the OVA antigen was significantly increased. Treatment with olopatadine or naphazoline hydrochloride reduced the level of conjunctival dye leakage compared with the AC group (Fig. 2). Combined treatment with olopatadine and naphazoline hydrochloride further reduced conjunctival dye leakage in antigen-induced mice, compared with the olopatadine alone group (Fig. 2).

**Olopatadine and naphazoline hydrochloride reduce inflammation in mice with antigen-induced conjunctival vascular hyperpermeability.** The effect of olopatadine and naphazoline hydrochloride on inflammatory factors was investigated in mice with antigen-induced conjunctival vascular hyperpermeability. Following OVA antigen induction, the levels of TNF-α, IL-1β and IL-6 were observed to increase. Treatment with olopatadine or naphazoline hydrochloride reduced the levels of the inflammatory factors compared with the AC group (Fig. 3). Combined treatment with olopatadine and naphazoline hydrochloride further reduced the levels of the inflammatory factors in antigen-induced mice, compared with the olopatadine alone group (Fig. 3).

**Olopatadine and naphazoline hydrochloride reduce cytokine levels in mice with antigen-induced conjunctival vascular hyperpermeability.**
hyperpermeability. To further explore the effects of olopatadine and naphazoline hydrochloride, the cytokine levels were measured in mice with antigen-induced conjunctival vascular hyperpermeability. The IFN-γ and IL-4 levels were increased in antigen-induced mice. Treatment with olopatadine or naphazoline hydrochloride resulted in a reduction in the cytokine levels, however this was not a statistically significant difference compared with the AC group (Fig. 4). Combined treatment with olopatadine and naphazoline hydrochloride significantly reduced the levels of IFN-γ and IL-4 in antigen-induced mice, compared with the olopatadine alone group (Fig. 4).

Olopatadine and naphazoline hydrochloride reduce the levels of IgE in mice with antigen-induced conjunctival vascular hyperpermeability. Fig. 5 indicates that the levels of IgE were increased in antigen-induced mice. Treatment with olopatadine or naphazoline hydrochloride reduced the levels of IgE compared with the AC group (Fig. 5). Combined treatment with olopatadine and naphazoline hydrochloride further reduced the levels of IgE level in antigen-induced mice, compared with the olopatadine alone group (Fig. 5).

Olopatadine and naphazoline hydrochloride reduce the levels of GMCSF level in mice with antigen-induced conjunctival vascular hyperpermeability. Fig. 6 indicates that the levels of GMCSF were increased in antigen-induced mice. Treatment with olopatadine or naphazoline hydrochloride reduced the levels of GMCSF compared with the AC group (Fig. 6). Combined treatment with olopatadine and naphazoline hydrochloride further reduced the levels of GMCSF in antigen-induced mice, compared with the olopatadine alone group (Fig. 6).

Olopatadine and naphazoline hydrochloride reduce the levels of NGF in mice with antigen-induced conjunctival vascular hyperpermeability. Fig. 7 indicates that the levels of NGF were increased in antigen-induced mice. Treatment with olopatadine or naphazoline hydrochloride reduced the levels of NGF compared with the AC group (Fig. 7). Combined treatment with olopatadine and naphazoline hydrochloride further reduced the levels of NGF in antigen-induced mice, compared with the olopatadine alone group (Fig. 7).

Olopatadine and naphazoline hydrochloride reduce the expression levels of VEGF in mice with antigen-induced conjunctival vascular hyperpermeability. To investigate the effects of olopatadine and naphazoline hydrochloride upon VEGF protein expression in antigen-induced mice, the VEGF expression levels were measured using western blot analysis.
Fig. 8 indicates that antigen induction increased the expression levels of VEGF. Treatment with olopatadine or naphazoline hydrochloride suppressed the elevation of VEGF protein expression compared with the AC group (Fig. 8). Combined treatment with olopatadine and naphazoline hydrochloride further reduced the VEGF expression levels in antigen-induced mice, compared with the olopatadine alone group (Fig. 8).

Discussion

Conjunctivitis is a common ocular surface disease with various etiologies, which present with similar symptoms including eye irritation, eye watering, increased secretions and congestion (16). The symptoms of conjunctivitis have a rapid onset and may result in a degree of damage to the ocular tissue, and in severe cases may result in blindness, and additional adverse consequences (17). Severe bacterial keratitis is a sight-threatening disease, which may result in various degrees of vision loss, with lesions in the central corneal area potentially resulting in reductions in vision, and in severe cases, corneal ulcers and corneal perforation, which may eventually require corneal transplant (18). The present study demonstrated that olopatadine and naphazoline hydrochloride significantly reduced the level of conjunctival dye leakage in mice with histamine or antigen-induced conjunctival vascular hyperpermeability.

Conjunctival infection may result in systemic inflammatory responses, and induce increases in the levels of the inflammatory cytokines, IL-1β, IL-6 and TNF-α (19). Previous studies have indicated that IL-1β is an important inflammatory cytokine, contributing to inflammatory injuries and the occurrence of autoimmune diseases, and participating in an autocrine loop composed of inflammatory mediators including IL-6, TNF-α and neutrophils (20-22). The cytokines IL-6 and TNF-α exhibit important biological activities and are generated by a variety of immune cells in the body, such as monocyte-macrophage cells (23). In the present study, TNF-α, IL-1β and IL-6 levels were reduced following treatment with olopatadine and naphazoline hydrochloride in mice with antigen-induced conjunctival vascular hyperpermeability. Murota et al (24) demonstrated that olopatadine reduced the elevated levels of inflammatory markers in NC/Nga mice (24). Tamura et al (25) showed that olopatadine significantly reduced IL-1β and IL-6 levels induced by the repeated topical application of oxazolone in rats. However, in the present study the effect of naphazoline hydrochloride on inflammatory factors was unremarkable. The effect of olopatadine solutions and naphazoline hydrochloride on inflammatory factors may be predominantly concerned with olopatadine.

Previous studies have demonstrated that cluster of differentiation (CD)4+ T cells may be further divided into Th1-type and Th2-type cells, according to the surface identity and function of the T lymphocyte cell subsets (26-28). Under normal conditions, the Th1/Th2 system of the body maintains a state of equilibrium; Th1 cell subsets secrete IL-2, IFN-γ and TNF-α to activate macrophages, with these cells involved in mediating delayed hypersensitivity responses, which may inhibit the function of Th2 cells in stimulating B lymphocytes for IgE synthesis (29). Th2 cells generate IL-4, 5, 6, 13, 10 and GMCSF, which induces the differentiation and recruitment of eosinophils, inducing the transformation of B cell immunoglobulin subtypes and promoting IgE synthesis and mediating humoral immune responses (30). The present study demonstrated that the combined treatment with olopatadine and naphazoline hydrochloride reduced cytokine levels (IFN-γ and IL-4) in mice with antigen-induced conjunctival vascular hyperpermeability. Tamura et al (25) previously demonstrated that olopatadine is able to ameliorate IFN-γ and IL-4 levels in a rat model of experimental cutaneous inflammation (31).

Allergic conjunctivitis is a type-1 hypersensitivity, mediated by IgE, and eosinophil cationic protein is the biomarker of eosinophil activity and is able to damage mucosa epithelium (32). In addition, eosinophilic basic protein may result in severe allergic damage (31). A previous study observed that the levels of total IgE and specific IgE are raised in the plasma and tears of patients with allergic conjunctivitis. In addition, B lymphocytes that express CD23, CD21 and CD40 in conjunctival lymphoid follicles were activated, suggesting that this type of B lymphocyte may be the precursor cells for IgE synthesis, and the conjunctiva may serve a catalytic role in IgE synthesis (33). In the present study, it was observed that the IgE levels in the olopatadine and naphazoline hydrochloride treated group was lower compared with the olopatadine or naphazoline hydrochloride alone groups. Cook et al (34) reported that olopatadine inhibits the levels of IgE in human conjunctival mast cells.

GMCSF is able to stimulate the proliferation and differentiation of early pluripotent hematopoietic stem cells and
granulocyte-monocyte progenitor cells, and enhance the collaboration capabilities of mature neutrophils, eosinophils and monocyte-macrophages (35,36). Additionally, GMCSF stimulates the growth of erythroid and granulocyte-macrophage progenitor cells in addition to proliferative hematopoietic progenitor cells, which prolong the survival time of macrophages and enhance the anti-tumor capabilities. Furthermore GMCSF is able to stimulate endothelial cell growth and prevent the apoptosis of various cells (37). GMCSF is an important indicator of inflammatory stress within the body (38). In the present study, the GMCSF levels in the olopatadine and naphazoline hydrochloride alone groups. Tamura et al (39) indicated that olopatadine hydrochloride reduced the inflammatory rebound phenomenon via the suppression of IL-1β, IL-4, IL-18 and GMCSF levels in mice with chronic contact hypersensitivity.

Under normal physiological conditions, NGF is a neurotrophic factor (40). The elevated levels of NGF in inflammatory reactions have been indicated to result in the aggravation of pain (41). Previous studies have indicated that proinflammatory cytokines (IL-1β, TNF-α) are closely associated with NGF, and promote an increase in the levels of NGF (42-44). In addition, NGF may act on proinflammatory cytokines in turn (45). It has been demonstrated that NGF is able to inhibit inflammation in inflammatory bowel disease, with increased levels of NGF serving a protective role in the inflammatory reaction, and suggesting that NGF may in turn act on proinflammatory cytokines, to inhibit the excessive expression of proinflammatory cytokines in a potential bio-feedback regulation mechanism (45-47). In the current study, the NGF levels were reduced by the treatment with olopatadine and naphazoline hydrochloride in mice with antigen-induced conjunctival vascular hyperpermeability. Tamura et al (48) demonstrated that repeated pretreatment with olopatadine inhibited NGF and VEGF production in rats (48). VEGF is a growth factor with heparin-binding activity, which was purified from the substrate of follicular stellate cells of bovine pituitary by Ferrara in 1989 for the first time (49,50). VEGF exerts a strong mitogenic effect on endothelial cells and is regarded as a vascular endothelial cell-specific mitogen, inducing the proliferation of vascular endothelial cells and promoting angiogenesis (51). The biological activity of VEGF depends on the expression levels, the distribution of the target cell receptors and its integration with the receptors (52). Previous studies have demonstrated that hypoxia results in the high expression of VEGF, and that the alteration in retinal microvascular permeability in diabetic retinopathy is associated with VEGF (52,53). In addition, VEGF and the corresponding receptor are integrated, resulting in retinal endothelial cell proliferation, migration and contributing to the formation of new blood vessels which destroy the blood-retina shielding protection in retinal endothelial cells (54). The current study observed that olopatadine and naphazoline hydrochloride were able to reduce the expression levels of VEGF in mice with antigen-induced conjunctival vascular hyperpermeability. Tamura et al (55) suggested that the anti-allergic activity of olopatadine reduced VEGF levels in sensitized rats.

In conclusion, the current study demonstrated that treatment with olopatadine and naphazoline hydrochloride reduces histamine or antigen-induced conjunctival vascular hyperpermeability in mice. In addition, treatment with olopatadine and naphazoline hydrochloride reduces inflammatory reactions and the levels of IL-1β, IL-6, IFN-γ and IL-4. Furthermore, treatment with olopatadine and naphazoline hydrochloride reduces the levels of IgE, GMCSF, NGF and VEGF in antigen-induced conjunctival vascular hyperpermeability mice. These results suggest that olopatadine and naphazoline hydrochloride are potential treatments for non-bacterial conjunctivitis.

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