Effect of onion extract on corneal haze suppression after air assisted lamellar keratectomy

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ABSTRACT. This study evaluated the effect of onion extract on corneal haze suppression after applying the air assisted lamellar keratectomy.

The air assisted lamellar keratectomy was performed on 24 canine eyes. They were treated with an artificial tear (group C), prednisolone acetate (group P), onion extract (group O) and TGF-β1 (group T) three times per day from 7 to 28 days after the surgery. Corneal haze occurred on the all eyes and was observed beginning 7 days after the surgery. The haze was significantly decreased in groups P and O from day 14 compared with the group C using the clinical (group P; \( P=0.021 \), group O; \( P=0.057 \)) and objective evaluation method (group P; \( P=0.021 \), group O; \( P=0.039 \)). In contrast, it was significantly increased in group T from day 14 compared with group C based on the clinical (\( P=0.002 \)) and objective evaluation method (\( P<0.001 \)). Subsequently, these eyes were enucleated after euthanasia, and immunohistochemistry with α-SMA antibodies was done. The total green intensity for α-SMA was significantly more expressed in group T and significantly less expressed in groups P and O than in group C. Onion extract could have potential as a therapeutic in preventing corneal haze development by suppressing the differentiation of fibroblasts into myofibroblasts.

KEY WORDS: α-SMA, canine eye, corneal haze, onion extract, TGF-β1

Many corneal diseases are associated with the development of opacities in the stroma. Corneal haze presents as a superficial opacification of the anterior corneal stroma leading to a transient decrease in corneal transparency after lamellar keratectomy for dermoid, corneal inclusion cyst and corneal tumor, corneocutaneous transposition or autologous lamellar keratoplasty for deep corneal ulcer in veterinary ophthalmology. Also, it is one of the most important complications of photorefractive keratectomy (PRK), and its incidence and intensity increase in eyes treated for higher degrees of refractive error in human medicine [12]. In most transparency disorders, corneal haze may be induced by a combination of two or more predominant factors like corneal edema, scarring, accumulated macromolecules and reflective keratocytes [23]. Moreover, the formation of corneal haze involves the apoptosis of keratocytes and the proliferation and transformation of fibroblasts into myofibroblasts [30]. Therefore, one of the most crucial aspects of corneal healing from refractive surgery is the minimization of corneal haze.

The efficacy of mitomycin C (MMC) in reducing the incidence of corneal haze has led to its widespread use in most refractive surgery practices [29]. However, multiple complications, such as limbal/scleral necrosis, abnormal wound healing and loss of keratocytes, are reported with the topical use of MMC [25]. These results encourage the development of newer pharmacologic agents that can effectively inhibit the formation of corneal haze without causing serious side effects. Recent research on trichostatin A (TSA), a histone deacetylase inhibitor, reported that it inhibits TGF-β1-induced accumulation of the extracellular matrix and myofibroblast formation in vitro and markedly decreases haze in vivo [28]. However, there are no commercially available products for clinical use.

Allium cepa (onion) and onion extract have been reported to be effective in cardiovascular disease, because of their hypolipidemic, anti-hypertensive, anti-diabetic and antithrombotic effects, and to possess many other biological activities including antimicrobial, antioxidant, anticarcinogenic and immunomodulatory activities [6]. Especially, flavonoids in onion extract have been used to reduce hypertrophic scar formation [13]. Myofibroblasts are an important cell in connective tissue remodeling that differentiates during wound healing and fibrosis development in the pathogenesis of such diseases as hypertrophic scars, liver or pulmonary fibrosis [8], and corneal haze formation [20]. The myofibroblasts could represent an important target for corneal haze treatment like in the treatment for hypertrophic scar formation. Thus, onion extract could be useful as a therapeutic in preventing the development of corneal haze by suppressing the...
differentiation of fibroblasts into myofibroblasts.

Air assisted lamellar keratectomy is one of the experimental models for the development of corneal haze [18]. In this method, the wound size and depth were standardized by modification of the bubble technique for corneal transplantation. Also, it could induce more corneal haze than the conventional superficial keratectomy.

The aim of this study was to evaluate the efficacy of onion extract ointment in corneal haze development after applying to the haze model with the air assisted lamellar keratectomy for canine eyes. In addition, the effect of onion extract ointment in the down-regulation of myofibroblast expression was examined with immunohistochemistry using the a-SMA antibody.

MATERIALS AND METHODS

Corneal fibroblast culture and cell viability test for onion extract: Corneal fibroblasts were cultured from porcine eyes, which were obtained from a local slaughterhouse, for the cell viability test of the onion extract. The corneal buttons were cut into four small pieces and incubated overnight in a humidified CO₂ incubator at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) (11995-065, Gibco™) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 15630-080, Gibco™) and 1.25 mg/ml collagenase type 1 (17100-017, Gibco™). The digested tissues were mixed with media by pipetting and filtered a 100 µm cell strainer (08-771-19, Falcon™, Franklin Lakes, NJ, U.S.A.). Then, they were centrifuged at 800 g for 5 min and resuspended in 2 ml of DMEM containing 20 mM HEPES, 50 µg/ml gentamicin (15750-078, Gibco™), 1.25 µg/ml amphotericin B (A20678, Gibco™) and 10% fetal bovine serum (10437-028, Gibco™). The kerocyte-containing cell suspension was then seeded on 6-well plastic dishes and incubated in a humidified CO₂ incubator at 37°C. Eighty percent confluent cultures of cornea fibroblasts (passages 1–3) were used for experiments.

Trypan blue dye exclusion test was used to evaluate cell viability of corneal fibroblasts following onion extract treatment. Onion extract (W281719, Sigma-Aldrich, St. Louis, MO, U.S.A.) was treated to the each well at 0, 0.01, 0.1, 1, 10, 50 and 100 µM concentrations diluted with DMSO (AMR-0231-1; Amresco, Solon, OH, U.S.A.) for 24 hr. Then, they were resuspended using 0.05% trypan/0.53 mM EDTA (25300-054, Gibco™), and trypan blue solutions (0.4% wt/vol, 15250-061, Gibco™) were mixed with the resuspension cells. The suspensions were loaded into a hemocytometer and scored with a light microscope. Cells that stained blue were scored as nonviable.

A process of manufacture for the onion extract ointment: A 1% onion extract ointment was made with 10 g white petrolatum (white petrolatum 1 g/g, Sungkwang Pharm., Cheonan, Korea) and 0.1 ml onion extract (W281719, Sigma-Aldrich) mixture in a water bath. The concentration of onion extract ointment depended on the in vitro viability test. Before use in a main study, the onion extract ointment was applied BID for 2 weeks at the normal cornea of six healthy beagle dogs to test the abnormal allergic reactions (blepharospasm, conjunctival hyperemia, corneal epithelial disorders and other ocular abnormalities) by ophthalmic examinations every the other days.

Animals: Twenty-four eyes from 12 healthy beagles were used in this study. Before the experiment, all dogs underwent an ophthalmic examination including slit-lamp biomicroscopy (SL-D7, Topcon, Tokyo, Japan), indirect ophthalmoscopy (Vantage plus, Keeler, Windsor, U.K.), rebound tonometry (Tonovet, Tiolat, Helsinki, Finland), Schirmer’s tear test (Schirmer tear test, Intervet, Summit, NJ, U.S.A.) and fluorescein staining (Fluorescein paper, Haag Streit AG, Koeniz, Switzerland). Dogs with ocular or systemic diseases were excluded. The animal use and experimental protocols were approved by the Institutional Animal Care and Use committee (SNU-121123-10; Seoul National University, Korea). All dogs were divided into 4 groups consisting of 6 eyes in each group: control (Group C; n=6), Prednisolone acetate treatment (Group P; n=6), Onion extract treatment (Group O; n=6) and TGF-β1 treatment (Group T; n=6).

Corneal Haze Generation by the air assisted lamellar keratectomy: Air assisted lamellar keratectomy was performed following a method reported previously [18]. General anesthesia was accomplished by intravenous injection of tiletamine and zolazepam (Zoletil, Virbac, Carros, France; 2.5 mg/kg) for induction and maintained with isoflurane (Ifran, Hana Pharm, Seoul, Korea; MAC 0.5–1.5%). Atracurium (ATRA, Hana Pharm; 0.01 mg/kg, IV) was administrated for central positioning of the cornea during the surgery. The air assisted lamellar keratectomy was performed for all eyes. Briefly, the center of the cornea was trephined 375 µm using an 8 mm diameter trephine (Barron radial vacuum trephine, Katena) and blunt-tipped corneal scissors (Fig. 1D). Blanching was observed (Fig. 1C). The fuzzy region of the cornea stroma at the base of the trephination groove. The tip was introduced parallel to the corneal surface into wards approximately 60°, while the bevel faces up (Fig. 1B). The surgical field was kept dry after the trephination to minimize stromal edema. Four ml of air were injected at the base of the trephination gutter into the corneal stroma using a 30-gauge needle attached to a syringe. The needle was bent 5 mm from its tip so that the terminal segment angled upwards approximately 60°, while the bevel faces up (Fig. 1B). The tip was introduced parallel to the corneal surface into the central stroma at the base of the trephination groove. The plunger of the air-filled syringe was pressed until intrastromal blanching was observed (Fig. 1C). The fuzzy region of the white opaque cornea was removed using a corneal dissector and blunt-tipped corneal scissors (Fig. 1D).

After surgery, one drop of atropine (1%, Isopo Atropine, Alcon, Antwerp, Belgium) was applied for 3 days for the purpose of analgesic effect. Levofloxacin (0.5%, Cravit, Santen, Osaka, Japan) eye drops were administered three times daily until 7 days after the surgery. After the seventh day, artificial tear eye drops (0.1% sodium hyaluronate; Lacure, Samil Pharm., Seoul, Korea) for group C, prednisolone acetate 1% (Pred-Forte, Allergan, Irvine, CA, U.S.A.)
Corneal Haze Grading: The level of haze in the cornea was evaluated by slit lamp biomicroscopy (SL-D7) at 7, 14, 21 and 28 days after the surgery using two kinds of methods: the previously reported clinical grading system [10] and a quantitative haze grading until 21 days after surgery (day 14; \( P = 0.032 \), day 21; \( P = 0.041 \) and day 28; \( P = 0.210 \)) compared with day 7 (Fig. 3). 

Quantification of \( \alpha\)-SMA-positive Cells: The \( \alpha\)-SMA positive cells in six randomly selected, non-overlapping, full-thickness central corneal columns, extending from the anterior stromal surface to the posterior stromal surface were counted following a method previously reported [21]. The diameter of each column was a 400× microscope field. The images were evaluated using digital image analysis.

RESULTS

Cell viability was over 95% at the concentration of 0, 0.01, 0.1, 1 and 10 \( \mu l/ml \) onion extract in vitro evaluation (data not shown). The 1% onion extract ointment showed no adverse effects and allergic reaction, like blepharospasm, conjunctival hyperemia, corneal epithelial disorders and other ocular abnormalities, when applied every 12 hr to the normal beagle cornea for 2 weeks.

Corneal haze developed after the air assisted lamellar keratectomy depending on each treatment group (Fig. 2). Control corneas treated with the artificial tear eye drops (group C) had significant developed corneal haze in clinical grading until 21 days after surgery (day 14; \( P = 0.032 \), day 21; \( P = 0.041 \) and day 28; \( P = 0.210 \)) compared with day 7 (Fig. 3).
Topical application of 1% prednisolone acetate (group P) and 1% onion extract ointment (group O) caused a statistically significant decrease in corneal haze in group P (day 14; \( P = 0.021 \), day 21; \( P = 0.012 \) and day 28; \( P = 0.001 \)) and in group O (day 14; \( P = 0.037 \), day 21; \( P = 0.008 \) and day 28; \( P = 0.003 \)) compared with the same groups for haze grading at day 7. Additionally, corneal haze was significantly increased in the TGF-β1 treated group (group T) (day 14; \( P = 0.002 \), day 21; \( P < 0.001 \) and day 28; \( P < 0.001 \)) compared with group C. Furthermore, the corneal haze was significantly increased in group T at days 14 (\( P < 0.001 \)), 21 (\( P < 0.001 \)) and 28 (\( P = 0.003 \)) compared with group C.

The results of immunohistochemical staining for α-SMA are shown in Fig. 5. In group C, the corneas exhibited high numbers of α-SMA-positive myofibroblast cells, mostly in the anterior stroma below the epithelium. Topical application of prednisolone acetate (group P) and onion extract (group O) significantly reduced the numbers of α-SMA-positive cells in the stroma. In contrast, TGF-β1 application (group T) significantly increased the numbers of α-SMA-positive cells compared with that in group C.

The total green intensity of the entire stroma was significantly enhanced in group T (\( P < 0.001 \)) compared with that in group C (Fig. 6). The total green intensity in groups P (\( P < 0.001 \)) and O (\( P < 0.001 \)) was significantly lower than that in group C.
DISCUSSION

Formation of corneal haze involves the apoptosis of keratocytes [30] and transdifferentiation of keratocytes into myofibroblasts in response to endogenous epithelial derived cytokines [16]. TGF-β1 directly activates keratocytes and leads to the formation of myofibroblasts as well as the subsequent remodeling of extracellular matrix [26]. Myofibroblasts scatter more light than that of undifferentiated fibroblasts or keratocytes, not only from their nuclei, but also from their cell bodies and dendritic processes [23]. In addition, this population of cells participates in extracellular matrix remodeling, resulting in a denser and more disorganized extracellular matrix [15]. Intracellular microfilament fibers, such as F-actin and α-smooth muscle actin (SMA), were expressed much higher in myofibroblasts than in keratocytes. These cellular components were enabled myofibroblasts to
contract and close wounds, but also rendered the cornea less translucent [19]. Collectively, these changes lead to a loss of corneal transparency.

For a clear cornea, mitomycin C (MMC) is widely used intraoperatively by clinicians to prevent PRK-induced corneal haze, although there are several complications reported with its topical use [3]. There are no effective medicines to control corneal haze, except for MMC treatments. Because the application of steroid eye drop occasionally results in rapid corneal stromal melting, use of these drugs for achieving better corneal transparency is restricted. Thus, we have shown the effects of onion extract ointment in corneal haze prevention and suppression of myofibroblasts from stromal ablation using the air assisted lamellar keratectomy for the development of new treatment and prevention strategies. Corneal fibroblasts were viable in the 10 µg/ml concentration of the onion extract. There were no adverse effects or allergic reactions for the 1% onion extract ointments. Therefore, we used this concentration of onion extract ointments in this study to evaluate efficacy of onion extract. According to our results, corneal haze grading and expression of α-SMA-positive cells were significantly decreased in the onion extract treated group compared with the control group. Because α-SMA is a specific marker for myofibroblasts, these results suggest that onion extract ointment has suppressive effects on corneal haze.

Treatment with prednisolone acetate showed significant suppression of corneal haze compared with the artificial tear treatment in this paper. Postoperative use of topical corticosteroids has been controversial after PRK. Topically applied steroids, acting as an anti-inflammatory agent, have effectively suppressed corneal haze formation after excimer laser keratectomy in experimental studies [17, 24]. But, this reduction in haze appears to be due in part to a delay in the wound-healing response [24]. Also, glucocorticoids increase the lytic action of corneal collagenase, suggesting that this effect might be responsible for the corneal deconstruction in clinical conditions [2]. Accordingly, the onion extract ointment could be used more safely than steroid eye drops, which would be induced corneal melting.

Onion (Allium cepae) extract contains a great amount of antioxidant phytochemicals, sulfur and other numerous phenolic compounds [1]. These compounds have been reported to be effective in cardiovascular diseases because of their hypolipidemic, anti-hypertensive, anti-diabetic and anti-thrombotic effects, and to possess many other biological activities including antimicrobial, antioxidant, anticarcinogenic, antimutagenic, antiasthmatic, immunomodulatory and probiotic activities [6]. Especially, onion extract was shown to have fibroblast-inhibiting properties, to reduce proliferative activity, and to produce substances in the extracellular matrix [13]. Recently, commercial products composed of onion extract have been used to reduce scar formation on the skin [13]. According to the results of this paper, onion extract suppressed the differentiation of myofibroblasts, and as a result, corneal haze developed significantly less than that of the control. Onion extract would be a good therapeutic candidate as a new medicine for corneal haze suppression. Mechanical removal of the corneal epithelium and PRK up-regulate TGF-β1 [11]. TGF-β1, a potent profibrotic cytokine, is a key regulator for the differentiation of myofibroblasts during corneal wound healing. It directly activates keratocytes and leads to the formation of myofibroblasts as well as the subsequent reformation of the subepithelial stroma [11]. Consequently, these mechanisms could promote the clinical expression of corneal haze after corneal surgery. In our results, corneal haze was significantly increased by treatments of additional TGF-β1 compared with the control. Furthermore, one experiment showed the prevention of PRK-induced haze through the use of anti-TGF-β1 antibodies [22]. Thus, the suppression of TGF-β1 expression is critical in the prevention of corneal haze.

Fibroblasts differentiate into myofibroblasts through a Smad 2/3 signaling pathway and enhance NADPH oxidases (Nox) 4-derived reactive oxygen species (ROS) signaling cascades [7]. Depletion of Nox4, an essential mediator of Smad2/3 transcription factor activation in response to TGF-β1, down-regulates α-SMA mRNA, and overexpression of Nox4 induces α-SMA expression [5]. The precise mechanisms of onion extract have not yet been completely elucidated. The corneal haze grade was significantly lower in the onion extract treated group than in the control group, and the expression of α-SMA was also down-regulated by the onion extract treatments shown in the results of this study. Among the many flavonoid compounds, quercetin is a major component of onion extract [27], and it has been shown to have powerful antioxidative activity with metal ion binding properties and radical scavenging abilities [9]. In addition, quercetin has a scavenging effect on superoxide anions and hydroxyl radicals, and it prevents lipid peroxidation by blocking the action of xanthine oxidase and chelating iron [14]. These effects could have important roles in the suppressive effect of onion extract against corneal haze formation. These results suggest that onion extract could block TGF-β1 signaling cascades by scavenging ROS to reduce α-SMA expression and subsequently corneal haze development. Further experiments are needed to understand the exact mechanisms of onion extract.

The limitation of this study is that the evaluation time was short and there was not enough to prove exact mechanism of onion extract ointment against corneal haze formation. Therefore, more studies will be needed to understand the mechanisms.

In summary, onion extract ointment could be useful as the therapeutic in the suppression of corneal haze development after applying the air assisted lamellar keratectomy through the down-regulation of fibroblast transdifferentiation into myofibroblasts. This effect could be from the scavenging ability of the onion extract.

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