Anti-inflammatory effects of topical supernatant from human amniotic membrane cell culture on canine deep corneal ulcer after human amniotic membrane transplantation

Tasavarin Wichayacoop,* Pasakorn Briksawan,† Pranee Tuntivanich† and Sirintorn Yibchok-anun*
*Department of Pharmacology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand; †Department of Surgery, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

Address communications to:
S. Yibchok-anun
Tel.: +66 22189726
Fax: +66 22553910
e-mail: sirintorn.y@chula.ac.th

Abstract
The objective of this study was to examine the effect of topically applied human amniotic epithelial cell (HAEC) culture supernatant on corneal inflammatory reaction in dogs. Twenty-five dogs were randomly assigned into five groups. The control group consisted of five dogs with normal cornea. Inductions of corneal ulcers were performed using 0.45 cm trephine and human amniotic membrane was transplanted in 20 dogs. These 20 dogs were assigned into four treatment groups: topical antibiotic, topical corticosteroid, topical mock media and topical culture supernatant from HAEC, respectively. Administrations of the testing agents started at 24 h (h) after transplantation four times daily for nine consecutive days. Tears were collected before an operation 24 h after transplantation, but before application of the testing agents on consecutive odd days following transplantation. The concentrations of interleukin-1β (IL-1β) and nitric oxide (NO) in tear fluid were measured using canine IL-1β ELISA kit and Griess assay, respectively. Our analysis indicates that elevations of IL-1β and NO concentrations are associated with inflammatory conditions in the eyes. Corticosteroid, a reference anti-inflammatory drug, and the culture supernatant from HAEC significantly decreased IL-1β and NO concentrations. In addition, the clinical signs such as conjunctivitis and neovascularization were decreased in both topical corticosteroid and supernatant from HAEC treated groups. Mock and antibiotic solutions failed to decrease NO and IL-1β concentrations. In conclusion, topical application of the culture supernatant from HAEC alleviated inflammation in induced-corneal ulcer of dogs, possibly via inhibition of IL-1β and NO production.

Key Words: corneal ulcer, dog, human amniotic epithelial cell (HAEC), inflammation, interleukin-1β (IL-1β), nitric oxide (NO)

INTRODUCTION
Corneal ulcers occur with a variety of causes including injury, bacterial, fungal, and viral infections, diseases of the eye and eyelid, and a number of other conditions that cause the cornea to ulcerate. Deep corneal ulcers often require both medical and surgical treatment to prevent possible blindness. Methods of surgical treatment for canine corneal ulcers include temporary tarsorrhaphy, third eyelid flap, conjunctival pedical graft, tissue adhesive, corneal-scleral graft and transplantation of natural or synthetic material. Human amniotic membrane has long been used as a surgical material for transplantation in human ophthalmic surgery.1 It is very effective in promoting healing of corneal ulcers.2 Human amniotic membrane transplantation has been performed in treatment of canine corneal ulcers and excellent clinical results were also obtained similar to humans.3 Topical anti-inflammatory agents are also very important for managing postoperative corneal transplantation to suppress inflammation and to prevent scar and granulation tissue formation.4

Topical corticosteroid solution is widely used for postoperative anti-inflammation.5 It is a powerful tool in preventing scarring, maintaining transparency, and treating the immune-mediated inflammation of some forms of keratitis, uveitis, conjunctivitis, scleritis/epikeratitis6 and corneal transplants.7 However, there exists evidence to suggest corticosteroid may increase the risk of infection and worsen stromal melting.8 Recently, culture supernatant from human amniotic epithelial
The human amniotic membrane transplantation, topical antibiotic and topical corneal ulceration and treated with human amniotic membrane transplantation, topical antibiotic and topical corticosteroid. Therefore, the objective of this study was to determine the anti-inflammatory effects of the culture supernatant from HAEC in canine corneal ulcers compared to prednisolone, a corticosteroid anti-inflammatory agent that was used as a reference drug. The changes in nitric oxide (NO) and interleukin-1β (IL-1β) concentrations in tear fluid as well as clinical ophthalmic signs were used as the indicators for this study.

**MATERIALS AND METHODS**

**Animal model**

Twenty-five dogs of various breeds were maintained in animal facility rooms at the Faculty of Veterinary Science, Chulalongkorn University. The protocol was approved from the Ethics Committee of Veterinary Science, Chulalongkorn University. All animals were given physical and ophthalmologic examinations before the study. They were fed twice a day and given water *ad libitum*.

**Human amniotic membrane**

Human amniotic membranes were obtained from the Red Cross Eye Center in Bangkok, Thailand.

**Group classification**

Twenty-five dogs were divided into five groups with each group consisting of five dogs. The pre-study examinations included direct and indirect ophthalmoscopy, Schirmer tear test (STT), pupillary light response (PLR), blink reflex, menace reflex, and dazzle reflex. All dogs were found to be normal.

**Group 1** (Normal control group): Dogs with normal cornea.

**Group 2** (Antibiotic [ABO] alone group): Dogs were induced to have deep corneal ulceration and treated with human amniotic membrane transplantation and topical ABO.

**Group 3** (Corticosteroid group): Dogs were induced to have deep corneal ulceration and treated with human amniotic membrane transplantation, topical antibiotic and topical corticosteroid.

**Group 4** (Mock group): Dogs were induced to have deep corneal ulceration and treated with human amniotic membrane transplantation, topical antibiotic and topical mock media solution containing Dulbecco’s modified Eagle’s medium (DMEM) and 0.5% fetal calf serum (FCS).

**Group 5** (HAEC group): Dogs were induced to have deep corneal ulceration and treated with human amniotic membrane transplantation, topical antibiotic and topical supernatant of HAEC.

**Preparation of topical supernatant of HAECs**

Human amniotic membranes were obtained from normal cesarean sections with approval of the Red Cross Eye Center in Bangkok, Thailand. All placentas were seronegative for human immunodeficiency, hepatitis B and C viruses and syphilis. Sterile human amniotic membrane was transferred to fresh tissue culture dishes and shredded with scissors and incubated in a 0.5% trypsin solution. Cells were collected by gentle centrifugation and resuspended in DMEM. The supernatant was collected 48 h after incubation, filtered through a 0.22 μm polyvinylidene fluoride (PVDF) membrane and stored at 4°C for about 1 week before use.

**Induction of corneal ulceration and transplantation of human amniotic membrane**

Surgery was performed under general anesthesia, premedicated with acepromazine (IM) 0.02 mg/kg and morphine (IM) 0.5 mg/kg, induction with propofol (IV) 3 mg/kg, and maintenance with isoflurane. First, induction of deep corneal ulceration was performed by removing the corneal epithelium and superficial stroma with a surgical blade. Corneal incision completely surrounded the lesion by using the corneal trephine and diamond knife. After the incision was made, the edge of the tissue to be removed was grasped by forceps and corneal dissector. Then, human amniotic membrane was transplanted on the ulcer with epithelial side up and secured with 10–0 nylon suture eight stitches (Inlay technique) at 24 h after induction. Finally, temporary tarsorrhaphy (TT) was performed to protect the surgical site. The TT was applied in a horizontal mattress suture pattern one stitch on an area of lateral canthus for 4 days.

**Application of drugs and test agents**

The administrations with the agents including ABO, corticosteroid, HAEC and mock media solutions started at 24 h after human amniotic membrane transplantation. The ABO alone group received only topical antibiotic solution (tobramycin 0.3%; Tobrex®) four times daily. The corticosteroid, mock and HAEC groups received topical antibiotic and corticosteroid (prednisolone acetate; Predforte®), mock media or HAEC solutions four times daily, respectively.

**Tear fluid collection**

Tear fluid samples were collected from an inferior punctum before and 24 h after the operation, and then every other day using an insulin syringe 50 IU (0.5 CC) connected to a polyester tube. The total volume of collected tear fluid was about 100 μL each time.

**Cytokine IL-1 assays**

The concentration of IL-1β in tear samples was measured using enzyme-linked immunosorbent assay (ELISA) (Rapidbio, Transhold, China), according to the manufacturer’s protocol. Briefly, the samples (50 μL) and distilled water (50 μL) were added in anti-dog IL-1β biotin-coated well plates and incubated at 37°C for 60 min; plates were washed five times...
with washing buffer. Then 100 μL of the horseradish peroxidase (HRP) was added to each well and incubated for 30 min at room temperature and plates were washed five times with washing buffer. TMB (3,3′,5,5′ tetramethylbenzidine) substrate solution (100 μL) was added and incubated for 15 min at room temperature, then 100 μL of stop solution was added and incubated for another 30 min at room temperature. The IL-1β concentration was determined spectrophotometrically at an absorbance of 450 nm and interpolated with a standard curve.

Nitric oxide assays
The concentration of nitric oxide in tear samples was assayed by measuring the accumulation of stable degradation products, nitrate and nitrite. In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite. The metallic cadmium (Cd) was used to convert nitrate to nitrite then, total nitrite concentration was measured using Griess reagents. Briefly, the samples were diluted 20 times with distilled water, mixed with 30% ZnSO₄ and incubated at room temperature for 15 min. The mixtures were centrifuged at 1509 × g for 5 min and the resulting 200 μL supernatant was transferred to a clean microcentrifuge tube and incubated with Cd for 24 h. After incubation, 100 μL of sample, 50 μL of sulfanilamide (p-aminobenzenesulfonamide) and 50 μL of N-(1-Naphthyl) ethylenediamine dihydrochloride were added on well plates. Nitrite concentration was determined spectrophotometrically at an absorbance of 540 nm and interpolated with a standard curve.

Clinical ophthalmic evaluations
Examinations consisting of direct and indirect ophthalmoscopy; STT, PLR, blink reflex, menace reflex, dazzle reflex were performed.

Additionally, fluorescein staining of healing ulcers and development of corneal neovascularization were assessed. The degree of corneal neovascularization was scored based on the maximum reach of the invasion of corneal neovascularization: grade 0, no neovascularization; grade 1, maximum reach less than one-third the distance between the limbus and corneal center; grade 2, maximum reach between one-and two-thirds the distance between the limbus and corneal center; grade 3, maximum reach more than two-thirds the distance between the limbus and corneal center; grade 4, maximum invasion reaching the corneal center.¹¹ Conjunctivitis grading scales was followed by Cornea and Contact Lens Research Unit (CCLRU) grading scales, 0–4 unit scale (0 no conjunctivitis, 1 very slight, 2 slight, 3 moderate, 4 severe).¹²

Statistical analysis
All data were expressed as mean ± standard error of mean (SEM). The data were statistically analyzed for significance using one-way analysis of variance (ANOVA) and Tukey tests. Values of *P* < 0.05 were considered to be statistically significant.

RESULTS
In this study, we measured concentrations of proinflammatory cytokines, IL-1β and NO in the tear fluid of dogs with normal corneas (*n* = 5) and induced-corneal ulcers (*n* = 20) at 24 h after human amniotic membrane transplantation (Day 0). The concentrations of IL-1β and NO in tear fluid of the induced-corneal ulcer dogs were significantly increased compared with those of the normal corneal dogs (*P* < 0.05). The average concentrations of IL-1β and NO in tear fluid of normal corneal dogs were 37.29 ± 1.01 mm and 1.49 ± 0.26 μm, respectively; those of induced-corneal ulcer dogs were 62.80 ± 0.39 mm and 6.60 ± 0.05 μm, respectively (Figs. 1 and 2).

*Figure 1.* The IL-1β concentrations in tear fluid of dogs with normal cornea and induced-corneal ulcer. The samples were collected at 24 h after the induction of corneal ulcer (before transplantation of human amniotic membrane). The data are shown as mean ± SE. *Significant at *P* < 0.05, compared to normal dogs.

*Figure 2.* The nitric oxide concentrations in tear fluid of dogs with normal cornea and induced-corneal ulcer. The samples were collected at 24 h after the induction of corneal ulcer (before transplantation of human amniotic membrane). The data are shown as mean ± SE. *Significant at *P* < 0.05, compared to normal dogs.
Figure 3 shows the concentrations of IL-1β in the tear fluid of induced-corneal ulcer dogs before and after administration of testing agents for nine consecutive days. In all experimental groups, the IL-1β concentrations increased significantly after induction of corneal ulcers (day 1) compared with those in day 0 of the same group. In the ABO alone group, the IL-1β concentrations were gradually increased from day 1 to day 5 and then slightly, but not significantly decreased in days 7 and 9 compared to those in day 1. In the corticosteroid group, after received topical corticosteroid four times daily, the IL-1β concentrations were gradually decreased from day 3 to day 9 and a statistical significant difference was found in day 9 (P < 0.05) compared to those in day 1. In the mock media group, the IL-1β concentrations were gradually increased from day 1 to day 9. Similar to the corticosteroid group, the IL-1β concentrations were gradually decreased every day after received topical HAEC solution four times a day, but the statistical difference was observed only in day 9, compared to that in day 1. When comparing the IL-1β concentrations each day between corticosteroid, mock media and HAEC groups, respectively, with ABO alone groups, the IL-1β concentrations in both corticosteroid and HAEC groups were significantly lower than those in ABO alone group at day 7 and day 9 (P < 0.05). However, in mock media group, the concentrations of IL-1β each day were higher than those in ABO alone group. In addition, the statistical difference was not found between corticosteroid and HAEC groups.

Figure 4 shows the NO concentrations in the tear fluid of induced ulcer dogs before and after an administration of testing agents for nine consecutive days. In all experimental groups, the concentrations of NO increased significantly after induction of corneal ulcer (day 1) compared to those on day 0 of the same group. In ABO alone group, the NO concentrations were gradually increased from day 1 to day 7 and slightly decreased on day 9. In corticosteroid group, the NO concentrations were gradually decreased from day 1 to day 9 after receiving topical corticosteroid four times daily. However, the statistical difference (P < 0.05) compared to day 1 was observed only on day 9. In the mock media group, the NO concentrations were increased after induction of corneal ulcers and remained high throughout the period of experiment (from day 1 to day 9). In the HAEC group, after treatment with topical HAEC solution four times daily, the NO concentrations on day 3 and day 5 were slightly higher than those on day 1; however, at day 7 and day 9, they were slightly low, but not significant compared to those on day 1. When comparing the NO concentrations collected from the ABO alone-treated group with the other testing agent-treating groups, the NO concentrations in both corticosteroid and HAEC groups were significantly lower than those in ABO alone group at day 7 and day 9. However, in the mock media group, although the concentrations of NO each day were lower than those in the ABO alone group, no statistical difference was observed. In addition, a statistical difference was not found among steroid, mock media, and HAEC groups.

Examinations consisting of direct and indirect ophthalmoscopy, STT, PLR, blink reflex, menace reflex, dazzle reflex were normal in every group. In clinical evaluations of corneal ulcers after human amniotic membrane transplantation, the ulcers were completely re-epithelialized within four days in every group. Conjunctival hyperemia was present in all groups within 24 h after operation and gradually improved in 48 h. Purulent ocular discharge was most intense at 24 h after operation in all groups. After treated with each solution

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four times daily, the purulent ocular discharges in the glucocorticoid, HAEC and ABO alone groups were subjectively improved in 48 h. Corneal neovascularization was assessed by slit-lamp biomicroscope. The corneal neovascularization appeared on day 7 in all groups, except corticosteroid group (Fig. 5). In mock (Fig. 6) and ABO alone (Fig. 7) groups, the neovascularization appeared progressively until the end of our investigation. However, the neovascularization gradually improved and disappeared within 14 days in the HAEC group (Fig. 8). The number of vessels in the HAEC group was lower than in the ABO alone and mock groups. The degree of corneal neovascularization was subjectively less in the corticosteroid group than in the HAEC group. The corneal scar formation in ABO alone, mock and HAEC groups was intense on day 7; however, it worsened in the ABO alone and mock groups on day 10. In addition, the scar formation remained throughout the investigation period in these two groups. In the HAEC group, the scar did not appear progressively as in the ABO alone and mock groups. However, the degree of scar formation in the HAEC group was not much improved compared with the corticosteroid group.

Table 1 shows the relationship between concentrations of nitric oxide, IL-1β and clinical parameters on day 9. In the corticosteroid treated group, we found that both NO and IL-1β concentrations decreased significantly compared to those on day 1. By confirming with fluorescein staining, the ulcers were completely re-epithelialized. The signs of inflammation, such as neovascularization and conjunctivitis were not observed; however, scar formation was slightly present (+1). In the HAEC group, NO and IL-1β concentrations were also significantly decreased compared to those on day 1. The ulcers were completely re-epithelialized and conjunctivitis...
was not observed; however, both neovascularization and scar formation still appeared in a mild degree (+1). In mock solution treated groups, NO and IL-1β concentrations were increased compared to those on day 1 and remained in high levels until the end of our investigation. Since the ulcers were transplanted with human amniotic membrane, they were also completely re-epithelialized within 4 days; however, the neovascularization and scar formation in the corneas of these two groups still present in moderate (+2) and severe (+3) degree, respectively.

**DISCUSSION**

Corneal ulceration is a common painful and potentially vision-threatening condition regularly found in dogs. The treatment usually requires medical or surgical treatment or both, depending on the severity of the corneal ulcer, its duration and the suspected underlying cause. Generally, treatment is focused on treating or preventing infection, controlling pain and inflammation, and minimizing scar formation. For superficial ulcers, the treatment usually begins with appropriate topical antibiotic ointment or drops to prevent infection and atropine solution to reduce painful spasm. Deeper ulcers must be treated aggressively to minimize complications and surgery may be needed to place a protective graft over the ulcer. Several surgical techniques are available, such as third eyelid flap, conjunctival graft, tectonic corneal graft, corneo-conjunctival transposition, autogenous lamellar corneal graft and amniotic membrane transplantation.

In our present study, after the induction of corneal ulceration, IL-1β and NO concentrations in tear fluid were much higher comparing to those in normal corneal dogs as well as those during the day before the operation (day 0), indicating that inflammation occurred during surgical process. Inflammation is the body’s response to cellular injury. The chemical mediators that tend to direct the inflammatory response include vasoactive amines (histamine, serotonin), arachidonic acids (prostaglandins, leukotrienes) and cytokines (tumor necrosis factor and IL-1). Our results were similar to the previous reports in which the production of both IL-1 isoforms, IL-1α and IL-1β, are increased in the tear fluid after ocular surface inflammation, and NO is expressed during the corneal inflammation. IL-1 is a potent proinflammatory cytokine that is synthesized by mononuclear cells. It plays an important role in initiating and maintaining inflammation. The main sources of NO in ocular surface tissue are corneal epithelium, fibroblast, endothelium and inflammatory cells. NO is an unstable free radical that is generated by conversion of L-arginine to citrulline by nitric oxide synthase (NOS), which is expressed in various ocular components, including the cornea. An increase in NO concentration is associated with inflammatory conditions within the eyes in which inflammatory cells infiltrate the anterior chamber to produce NO in the local environment. NO plays an important role in damaging the endothelium in inflammation, and also in other physiological functions such as maintaining ocular surface corneal hydration.

Both corticosteroid and culture supernatant from HAEC gradually decreased IL-1β concentrations from day 1 to day 9 and statistical differences in comparison to day 1 were found in day 9. Although the concentrations of IL-1β in tear fluid obtained from the corticosteroid treated group were lower than those obtained from the HAEC group, no statistical difference was observed. In addition, the concentrations of IL-1β after being treated with corticosteroid and culture supernatant from HAEC were lower than those treated with ABO and mock medium solutions. Both corticosteroid and culture supernatant from HAEC also suppressed NO production in tear fluid. The concentrations of NO after being treated with corticosteroid and culture supernatant from HAEC were significantly lower than those treated with ABO alone group.

Corticosteroid is a well-known anti-inflammatory drug that prevents the formation of both PGs and leukotrienes by inhibiting phospholipase A₂ activity, resulting in reduction of arachidonic acid release. It also inhibits the expression of IL-1 and decreases leukotrienes that act as chemoattractant molecules for inflammatory cells such as monocytes and macrophages. IL-1β is produced primarily by monocytes and macrophages, and Sennlaub et al. have reported that neutrophil or inflammatory monocytes are the major source of NO in the corneal inflammation. HAEC has been reported to exert diverse anti-inflammatory effects by inhibiting IL-1 expression, trapping mononuclear and polymorphonuclear inflammatory cells infiltrating the ocular surface of human eyes, reducing keratocyte apoptosis in rabbit corneas, suppressing alloreactive T cells in vitro and expressing mRNA and protein of anti-inflammatory factors, such as IL-1 receptor antagonist and IL-10. Therefore, in similar fashion to corticosteroid, the culture supernatant from HAEC exerted

<table>
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<th>Group/parameter</th>
<th>NO</th>
<th>IL-1β</th>
<th>Fluorescein staining</th>
<th>Neovascularization</th>
<th>Conjunctivitis</th>
<th>Scar/remnant</th>
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<td>Negative</td>
<td>2</td>
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*Significant at \( P < 0.05 \), compared to day 1 of the same group.

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an anti-inflammatory action by inhibiting the inflammatory monocytes and macrophages to produce IL-β and NO. In addition, the culture supernatant from HAEC had a tendency to decrease IL-1β concentration better than NO concentration. It is possible that the culture supernatant from HAEC inhibited the production of IL-1β and NO from the inflammatory monocytes and macrophages. However, NO is also produced from other sources that are not involved in inflammatory processes such as neuronal and vascular endothelial cells. Therefore, the culture supernatant from HAEC worked better in decreasing concentration of IL-1β than NO. Further research should confirm the molecular mechanisms underlying the anti-inflammatory action of culture supernatant from HAEC in dogs. Topical mock solutions failed to decrease either IL-1β or NO concentrations. These results confirmed that the mock solution, which was DMEM plus 5% FCS (v/v), alone did not exert an anti-inflammatory effect. The concentrations of IL-1β in tear fluid obtained from the dogs treated with mock solution were much higher than those from other groups. It is possible that the FCS, a growth factor, in the mock solution were much higher than those from other groups. It is possible that the FCS, a growth factor, in the mock solution may play a role in stimulating synthesis of IL-1β from monocytes and macrophages. The clinical ophthalmic evaluations the authors investigated found that the induced-ulcers in corneas of all dogs were entirely re-epithelialized within 4 days after transplantation of human amniotic membrane. The human amniotic membrane promotes epithelial healing, reduces inflammation and decreases severity of vascularization. The epidermal growth factor present in human amniotic membranes contributes a significant effect to epithelial regrowth. Neovascularization is representative of an inflammation in the cornea. It frequently leads to vision loss due to scarring, persistent inflammation and keratopathy. In this study, the corneal neovascularization and corneal scar formation were noticed in all groups, but the degrees of severity were different. The neovascularization and scar formation in mock and ABO alone groups appeared progressively until the end of our investigation; however, in HAEC and corticosteroid groups, they gradually decreased and disappeared within 14 days. The number of vessels was also lower in the HAEC group than those in the ABO alone and mock groups. Corticosteroid inhibits vascular and cellular characteristics of inflammation by decreasing vasodilation and reducing capillary permeability. It also suppresses the later stages of inflammation by inhibiting formation of fibroblasts and their collagen-forming activity. These results are in agreement with Kobayashi et al in which HAEC exerts anti-angiogenic effects that reduce neovascularization by suppression of bFGF-induced angiogenesis. Moreover, HAEC has expressed four tissue inhibitors of metalloproteases 1, 2, 3 and 4 as well as endothelial cell growth inhibitor endostatin, which contribute to the anti-neovascularization effects. The anti-scarring mechanism of HAEC has been reported as preventing fibroblast activation into myofibroblasts by reduced expression of α-smooth muscle actin, fibronectin-EDA and intergrin α5. Although the results from our experiment revealed that the HAEC culture supernatant exerted anti-inflammatory effects, additional experiments should further investigate whether these effects will be removed after it is immunologically neutralized.

The results in Table 1 show that the clinical parameters, including neovascularization, conjunctivitis and scar formation were in agreement with the concentrations of NO and IL-1β, in which the lower the concentrations of NO and IL-1β, the better the clinical parameters. However, the culture supernatant from HAEC was less effective than corticosteroid in suppressing corneal inflammatory reactions. Since the subjects in this study were not genetically identical, further experiments performed in genetically controlled dogs, such as beagles, need to confirm the anti-inflammatory activity of the culture supernatant from HAEC.

Taken together, we concluded that, similar to corticosteroid, topical application of culture supernatant from HAEC suppressed inflammatory reactions in induced-corneal ulcer dogs via decreased production of proinflammatory cytokine, IL-1β and nitric oxide.

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