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Human Amniotic Membrane Extracts have Anti-Inflammatory Effects on Damaged Human Corneal Epithelial Cells *In Vitro*

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Abstract: The human amniotic membrane has therapeutic potential for several diseases such as cardiac ischemia, liver fibrosis, and ocular surface disorders. In the treatment of ocular surface disorders, human amniotic membrane transplantation promotes epithelial wound healing and suppresses inflammation. The objective of this study was to determine whether human amniotic membrane extracts (HAE) help damaged corneal epithelial cells recover from an inflammatory response. Human corneal epithelial cells (hCEC) which were induced inflammation were treated with human amniotic membrane extracts, and then the levels of inflammatory cytokines were measured. Human amniotic membrane extracts had an anti-inflammatory effect on damaged human corneal epithelial cells. More importantly, homogenized human amniotic membrane extracts of less than 3 kDa had a greater capacity for reducing inflammation and secretion of interleukin-6 (IL-6) and interleukin-8 (IL-8). Thus, these results indicate that the use of human amniotic membrane extracts is a promising treatment for ocular surface disorders accompanied by inflammation.

Key words: Human amniotic membrane extract (HAE), Human corneal epithelial cell (hCEC), Anti-inflammatory effect, Interleukin-6 (IL-6), Interleukin-8 (IL-8)

Introduction

Dry eye syndrome (DES), also known as keratoconjunctivitis sicca (KCS), is a common disorder associated with increased osmolality of the tear film. DES can potentially damage to the ocular surface and results in various symptoms like ocular irritation, mucoid discharge, and excessive tearing with multifactor causes. DES also leads to an increase the risk of secondary infections¹⁾. Research has demonstrated that inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor- α (TNF- α) are highly expressed in tears of patients with multi-factorial DES²⁻⁷⁾. Moreover, current research suggests that neurogenic inflammation induced by neuropeptides may be closely associated with both the initial immune response and chronic inflammation in the disease⁸⁾. Thus, suppression of ocular inflammation and enhancement of tear film stability may be a therapeutic option in the treatment of DES.

The amnion is a membrane that protects the embryo during development. The human amniotic membrane has long been used

clinically as biological dressings to heal skin wounds⁹⁾. Since the 1940s, human amniotic membranes have been applied as grafts or as patches for the management of ocular surface disorders¹⁰⁾. Human amniotic membrane extracts (HAEs) consist of a variety of molecules such as sugars, lipids, proteins, and peptides¹¹⁾. Many studies have reported that the function of the amniotic membrane is known to exert anti-inflammation and anti-scarring effects and promote epithelial wound healing¹¹⁻¹³⁾. Also, amniotic epithelial cells are reported to secrete immunosuppressive factors in previous studies. These studies showed that ocular surface inflammation was inhibited by the amniotic membrane¹⁴⁻¹⁸⁾. Despite the beneficial effects of amniotic membranes, their current clinical application requires transplantation, which is an invasive procedure.

In this study, we evaluated whether a suspension of amniotic membranes could have therapeutic effects. Human amniotic membranes were extracted with a homogenizer. We then tested whether the extract had an anti-inflammatory effect on damaged human corneal epithelial cells (hCECs). Finally, we investigated the anti-inflammatory effect of small molecule components of HAEs, which were isolated using a 3 kDa cut-off centrifugal filter.

Materials and Methods

Cell culture

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The human corneal epithelial cell (hCEC) line, CRL-11135 (ATCC, VA, USA), was cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Invitrogen, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin at 37°C in an atmosphere containing 5% CO₂. The cell culture plates were precoated with a mixture of 0.01 mg/mL fibronectin (Sigma, Louis, MO, USA), 0.03 mg/mL bovine collagen type I (Sigma), and 0.01 mg/mL bovine serum albumin (BSA) (Sigma). The medium was changed every 3 days.

Ethics

This study was approved by the Ethics Committee of Chung-Ang University Hospital. All procedures described here comply with Ethics Committee guidelines. The placenta with fetal membrane was obtained from Unimed Pharm. Inc. (Seoul, Korea). Informed consent was obtained from the mother, and the use of human placenta was approved by the clinics.

Homogenization of human amniotic membranes

The placenta was first cleaned with sterile phosphate-buffered saline (PBS) to remove blood clots and other contaminants. The separated amniotic membrane was thoroughly washed repeatedly with PBS supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). The amniotic membrane was stored in chilled PBS containing a protease inhibitor cocktail (Sigma). The tissue was kept moist with chilled PBS throughout the entire procedure. The excised tissues were homogenized by homogenizer (Rose Scientific, Alberta, Canada) in chilled PBS containing a protease inhibitor cocktail. Homogenates were centrifuged at 3,000 rpm for 10 min and then at 12,000 rpm for 20 min to obtain a clear supernatant. Extracts were immediately filtered through a 0.2 µm filter (Sartorius-Stedim, Göttingen, Germany) to remove any microorganisms. Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). The extract was then stored in aliquots at -70°C until use.

Separation of human amniotic membrane extracts (HAEs)

HAEs were separated by size by using a Microcon centrifugal filter device (Millipore, Billerica, MA, USA), according to the manufacturer's protocol. Filtered HAEs were separated into 2 groups: those larger than 3 kDa and those smaller than 3 kDa.

Cell proliferation assay

The cell proliferation assay was carried out using the Cell Counting Kit 8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). Briefly, cells were cultured in 96 well culture plates. Cells were incubated with the homogenized HAEs for 72 hr at 37°C. After treatment, 10 µl of the CCK-8 solution was added to each well, and the 96 well plates were incubated at 37°C for 2 hr. Optical

densities (OD) of all wells were examined at a wavelength of 450 nm to determine cell viability.

Preparation of chemically damaged hCECs and treatment with HAEs

To generate chemically damaged hCECs, cells (1×10^4 cells/cm²) were incubated with 200 µl of 15% ethanol (EtOH) for 30 sec. The wells were washed, and the cells were cultured for 24 hr. The cells were then cultured for 24 hr in conditioned media containing different concentrations of HAEs. After the treatment, the cells were washed with PBS and cultured for 24 hr.

Caspase assay

To measure caspase 3/7 activity, hCECs were seeded in triplicate in a 96 well plate and incubated with 15% EtOH for 30 sec. After 72hr, caspase 3/7 activity was assayed with a Caspase-Glo assay kit (Promega, Fitchburg, WI, USA), which includes a luminogenic caspase 3/7 substrate, according to the manufacturer's protocol.

Cytokine quantification

Media from cultured hCECs were harvested, and IL-6 and IL-8 levels were measured using a commercially available ELISA kits (Koma Biotech, Seoul, Korea), according to the manufacturer's instructions.

Statistical analysis

Data were presented as the mean $\pm$ SD. Comparisons of continuous variables between more than two groups were performed by one way ANOVA, and t-tests were used to specify differences between groups. $P < 0.05$ was considered statistically significant. The SPSS software package was used for statistical tests (12.0.1).

Results

Induction of inflammation in human corneal epithelial cells by using EtOH

To generate the chemically-damaged hCECs, cells were incubated with 15% EtOH for 30 sec¹⁹⁾. To confirm the cell viability and inflammation effect of hCECs by 15% EtOH, viable cells were quantified by a CCK-8 assay and the levels of IL-6 and IL-8 were measured by ELISA. Morphology of the damaged cells was not changed (Fig. 1). However, as shown in Fig. 2a, cell viability decreased from 100% to 73% after exposure to EtOH. Chemical injury significantly increased IL-6 and IL-8 levels in the hCECs growth media (Fig. 2c, d) compared with the control group. In the cells treated with EtOH, IL-6 and IL-8 secretion increased from 100% to 121% and from 100% to 144%, respectively.

When normal cells face environmental stress such as chemical

Figure 1. Cell morphology of hCECs (a) and chemically-damaged hCECs (b). (Scale bar = 100 μ m).

Figure 2. Inflammation is induced in the chemically injured human corneal epithelial cells (hCECs) by using 15% EtOH. (a) hCEC viability was measured by a cell proliferation assay. (b) Apoptosis in chemically damaged hCECs was determined by a caspase 3/7 assay. For the purpose of comparison, cell viability, levels of IL-6 and IL-8, and caspase 3/7 activity expressed by the control were considered to be 100%. (c and d) The levels of IL-6 and IL-8 in hCECs culture media were detected by ELISA. Control group: non-treated hCECs, 15% EtOH group: hCECs treated with 15% EtOH for 30 sec. Each bar represents the mean $\pm$ SD of 3 independent experiments. Significant differences were determined using Student *t*-test (* $P < 0.05$).

exposure, high or low temperatures, and pH change, they undergo apoptosis. To determine whether our model of chemical injury had induced apoptosis, we measured the expression levels of caspase 3/7, a reliable indicator of cellular apoptosis. As shown

in Fig. 2b, caspase 3/7 levels did not change significantly (from 100% to 98%) in hCECs treated with EtOH, indicating that this chemical injury did not influence caspase 3/7 related apoptosis. Taken together, our data demonstrate that EtOH treatment induced

Figure 3. Human amniotic membrane extracts (HAEs) have an anti-inflammatory effect on hCECs. (a) hCECs were cultured in conditioned media containing 0.02, 0.04, 0.06, or 0.08 µg/ml homogenized HAE. Epithelial growth factor was used as a positive control. For comparison, the cell viability expressed by the control (non-treated hCECs) was considered to be 100%. (b) The EtOH-treated group was cultured in media for 24 hr after chemical injury. The homogenized HAE-treated group was cultured in conditioned media containing homogenized HAE (0.05 µg/ml) for 24 hr after chemical injury. The levels of IL-6 secretion were measured using ELISA. For the purpose of comparison, the amount expressed by the control (15% EtOH-treated group) was considered to be 100%. Each bar represents the mean $\pm$ SD of 3 independent experiments. Significant differences were determined using Student *t*-test (* $P < 0.05$).

Figure 4. HAEs smaller than 3 kDa have a greater anti-inflammatory effect than HAEs larger than 3 kDa. Chemically damaged hCECs were cultured in conditioned media containing homogenized HAEs (0.05 µg/ml), homogenized HAEs larger than 3 kDa, and homogenized HAEs smaller than 3 kDa for 24 hr after chemical injury. The levels of IL-6 (a) and IL-8 (b) secretion were measured using ELISA. For the purpose of comparison, the amount expressed by each of the control (15% EtOH-treated group) was considered to be 100%. Each bar represents the mean $\pm$ SD of 3 independent experiments. Significant differences were determined using one-way ANOVA (* $P < 0.05$).

inflammation without caspase-dependent apoptosis in hCECs.

Human amniotic membrane extracts (HAEs) have an anti-inflammatory effect on hCECs

We investigated whether HAEs have anti-inflammatory effects on chemically damaged hCECs. First, we examined whether HAEs are toxic to hCECs. hCECs were cultured with homogenized HAEs at 4 different concentrations (0.02, 0.04, 0.06 and 0.08 µg/µl) for 72 hr. In this experiment, epithelial growth factor (EGF) was used as a positive control, and a solution containing no extracts was used as a negative control. As shown in Fig. 3a, HAEs have no significant effect on the proliferation rate of hCECs, and there is no variation in this proliferation rate according to HAE concentration. Thus, HAEs at 0.05 µg/µl were used in the next experiment.

Next, we directly examined the anti-inflammatory effects of homogenized HAEs. We treated hCECs, which had been previously treated with 15% EtOH to induce inflammation, with

homogenized HAEs (0.05 µg/µl) for 24 hr. As shown in Fig. 3b, homogenized HAEs significantly decreased IL-6 secretion from 100% to 71% in chemically injured hCECs. Therefore, we concluded that HAEs reduce inflammation in damaged hCECs.

HAEs smaller than 3 kDa have a greater anti-inflammatory effect than HAEs larger than 3 kDa

To determine which fraction of homogenized HAEs contains anti-inflammatory factors, we used a Microconcentrifugal filter to separate the homogenized HAEs into 2 fractions: those larger than 3 kDa and those smaller than 3 kDa. Chemically damaged hCECs were cultured with conditioned media containing homogenized HAEs, HAEs larger than 3 kDa, or HAEs smaller than 3 kDa. The IL-6 secretion from each group was then measured. As shown in Fig. 4a, IL-6 levels in HAE-treated groups were significantly decreased compared to those in the control (15% EtOH treated group). In addition, we measured IL-8 secretion levels in the control (15% EtOH treated group) and HAE-treated

groups. IL-8 secretion was slightly reduced in hCECs treated with whole HAEs and HAEs over 3 kDa. Interestingly, hCECs treated with HAEs smaller than 3 kDa showed a significant decrease in IL-8 secretion, from 100% to 83% (Fig. 4b). These data indicate that HAEs smaller than 3 kDa had a greater anti-inflammatory effect than whole HAEs or HAEs greater than 3 kDa. Therefore, we concluded that small molecules present in HAEs are important factors in reducing the inflammatory reaction of hCECs.

Discussion

The mechanisms of DES are related to tear hyperosmolality and tear film instability. Tear hyperosmolality, which arises from excessive water evaporation at the exposed ocular surface, leads to ocular surface inflammation with a cascade of inflammatory events. This result in tear film instability is considered as one of the symptoms in DES because it causes alterations of the tear film²⁰. Previous reports showed that tear concentrations of inflammatory cytokines IL-6 and IL-8 were significantly higher in dysfunctional tear syndrome. Detection of elevated IL-6 concentrations suggests an existence of inflammation induced disease because significant correlation was observed between IL-6 and irritation symptoms²¹⁻²². Therefore, treatments reducing inflammation of damaged corneal epithelia may be helpful in alleviating the symptoms of DES.

In the present study, we induced inflammation in hCECs *in vitro* with EtOH. This chemical injury reduced cell viability greatly induces inflammation via significantly increasing IL-6 and IL-8 levels. Next, we investigated whether HAEs have an anti-inflammatory effect on damaged hCECs and we discovered that the anti-inflammatory effect of HAEs (0.05 µg/µl) was superior to that of the negative control. Hence, this indicates that HAEs can alleviate inflammatory reactions. Finally, we attempted to identify which fraction of HAEs acts as an anti-inflammation mediator. HAEs smaller than 3 kDa have an anti-inflammatory effect by predominantly suppressing IL-6 and IL-8. We concluded that HAEs smaller than 3 kDa are the most beneficial.

Recently, the use of amniotic membranes as a graft for ocular surface reconstruction has increased in popularity¹². Many studies have revealed amniotic membrane transplantation as a temporary or permanent graft that induces epithelial wound healing. It also possesses anti-inflammatory and anti-scarring effects on the ocular surface^{11,13}. Because of these beneficial effects, amniotic membranes are clinically used as a graft or a patch in severe case of ocular surface disease. Furthermore, current research suggests that amniotic membrane suspensions aid corneal epithelial wound healing by promoting cell migration and proliferation²³. Thus, we hypothesized that amniotic membranes in a suspension form would also reduce the inflammatory reaction. As described in the Results section, HAEs exerted an anti-inflammatory effect on corneal epithelia without inducing apoptosis. This suggests that

HAEs may be a therapeutic option for epithelial defects accompanied by inflammation.

In this study, we demonstrated that HAEs smaller than 3 kDa, generated using a 3 kDa molecular weight cut-off membrane, have greater anti-inflammatory activity than HAEs larger than 3 kDa. The HAE filtrate consists of diverse molecules, such as peptides, amino acids, and nucleotides. Previous studies have reported that peptides smaller than 3 kDa in size possess antioxidant activities²⁴⁻²⁶.

The suspension form of amniotic membranes can have therapeutic effects on corneal epithelia, suppressing the inflammatory response. Specifically, HAEs smaller than 3 kDa in size significantly contribute to its anti-inflammatory effect. Although further investigation is needed, these findings may provide a therapeutic approach for mild ocular surface disorders accompanied by inflammation. In particular, the use of small molecules may ultimately provide a new, targeted approach for the therapeutic treatment of mild ocular surface disorders accompanied by inflammation.

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Conflict of Interest

The authors have no COI existed.

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