

Diquafosol ophthalmic solution enhances mucin expression via ERK activation in human conjunctival epithelial cells with hyperosmotic stress

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Purpose: To evaluate the effect of diquafosol tetrasodium on the expression of secretory and membrane-associated mucins in multi-layered cultures of primary human conjunctival epithelial cells (HCEC) using intracellular extracellular signal regulated kinase (ERK) signaling.

Methods: HCECs were treated with hyperosmotic stress (400 mOsm/l) for 24 h after air-liquid interface cell culture followed by treatment with diquafosol. HCECs were stimulated for 1 h with or without PD98059, an ERK inhibitor, then treated with diquafosol for 6 h and 24 h. Mucin 1 (MUC1), mucin 16 (MUC16), and MUC5AC mRNA and protein expression levels were analyzed, and cell viability was detected using an MTT assay. Western blot analysis was used to examine p44/42 MAPK (Erk1/2) and phosphorylated p44/42 MAPK (Erk1/2) expression.

Results: Hyperosmotic stressed HCECs demonstrated increased MUC5AC secretion and gene expression when treated with diquafosol. MUC1 mRNA levels increased significantly at 24 h ($p < 0.01$), and expression of MUC16 mRNA levels increased at 6 h and were maintained until 24 h ($p < 0.05$). There was no significant difference in cell viability compared to the control group. Immunostaining results for MUC1, MUC16, and MUC5AC in diquafosol tetrasodium-treated HCECs at 24 h showed more positive cells than in the control group. Phosphorylation of p44/42 MAPK (Erk1/2) signaling molecules significantly increased from 5 min to 60 min ($p < 0.05$). The effects of diquafosol on mucin expressions in hyperosmotic stressed HCECs were significantly inhibited by PD98059, an ERK inhibitor, at 6 h and 24 h.

Conclusions: ERK signaling may regulate the expression levels of MUC1, MUC16, and MUC5AC induced by diquafosol in hyperosmotic stressed HCECs.

The conjunctival mucous epithelium, a stratified squamous non-keratinizing epithelium with 5 to 10 cell layers, is critical in protecting the eye from external stimuli and maintaining a healthy ocular surface [1]. Along with mucin 5AC (MUC5AC) secreted by conjunctival goblet cells, membrane-associated mucin 1 (MUC1), mucin 4 (MUC4), and mucin 16 (MUC16) of apical corneal and conjunctival epithelium can protect and hydrate the ocular surface [2-4]. Both secreted and membrane-associated mucins play important roles in the care of the ocular surface, such as by supplying lubrication, providing a barrier, forming a smooth spherical surface for good vision, and removing pathogens and debris [5].

Diquafosol tetrasodium (Diquas® ophthalmic solution 3%; Santen Pharmaceutical Co., Ltd., Osaka, Japan), a stabilized derivative of a UTP dimer (P1,P4-bis[50-uridy] tetraphosphate), shows agonist activity against the P2Y2 receptor (P2Y2R) [6]. In the conjunctival epithelium, diquafosol can facilitate the secretion of soluble MUC5AC from goblet cells and accelerate fluid efflux via chloride channel activation after increasing $[Ca^{2+}]$ [7]. Many previous reports have demonstrated that the expression of secreted MUC5AC increases after diquafosol administration [8-11]. Shigeyasu et al. found a significant increase in sialic acid, a mucin-like substance, in the tears of healthy human subjects after application of diquafosol [11]. Choi et al. and Hori et al. observed an increased concentration of tear MUC5AC and a decreased percentage of periodic acid-Schiff (PAS)-positive goblet cells in rat tear fluid and rabbit tear fluid, respectively [8,9]. Recently, Moon et al. reported that diquafosol can upregulate both secreted and membrane-associated mucins and contribute to conjunctival goblet cell recovery in mice [10].

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A previous study evaluated the effects of diquafosol on corneal epithelial wound healing in vivo and on P2Y2R-related downstream signaling pathways in vitro [12]. The study showed that topical diquafosol, a P2Y2R agonist, promotes corneal epithelial wound healing in vivo and induces cell proliferation and migration through intracellular calcium ($[Ca^{2+}]_i$)-dependent extracellular signal regulated kinase (ERK) activation in vitro [12].

Tear hyperosmolarity is a major mechanism of dry eye pathogenesis, which is caused by tear deficiency or excessive tear evaporation [13]. Hyperosmotic stress induced by an increase in extracellular osmolarity occurs during a normal cellular response in human conjunctival and corneal epithelial cells [14,15]. Tear hyperosmolarity is considered a causative factor in ocular surface inflammation, cell damage, and irritation symptoms in patients with dry eye [16]. Hyperosmotic stress has been used in previous experimental models to investigate the relationship between dry eye disease and hyperosmolarity. Therefore, hyperosmotic stressed human conjunctival epithelial cells (HCECs) are regarded as ex vivo dry eye models [14,17,18].

The objective of this study was to determine whether diquafosol could increase membrane and goblet cell mucins via intracellular ERK signaling in hyperosmotic stressed human conjunctival epithelial cells.

METHODS

Human conjunctival epithelial cell culture: Six 3×2 mm conjunctival specimens were obtained from post-keratoplasty discards of human corneal-limbal tissues from unidentifiable cadavers at Seoul St. Mary's Hospital Eye Bank (Seoul, Korea). The Institutional Review Board has determined that the use of these tissues does not constitute research on human subjects. As shown in Figure 1, conjunctival specimens were incubated for 16–20 h at 4 °C with 0.1% protease (Sigma-Aldrich, St Louis, MO) at a 1:1 mixture of a Dulbecco's modified Eagle's and Ham's nutrient mixture F12 (DMEM/F12, Gibco, NY) supplemented with 1% penicillin-streptomycin (Gibco). Loosened cells were scraped with a pipette, washed three times with DMEM/F12 supplemented with 1% penicillin-streptomycin (Gibco) and 10% fetal bovine serum (FBS; Gibco), and suspended in the same medium. Suspended epithelial cells were seeded at a cell density of 3×10⁴ cells per dish in 60 mm plastic culture dishes. The culture medium used was bronchial epithelial growth medium (BEGM; Lonza, Walkersville, MD) supplemented with 10 ng/ml of epidermal growth factor (EGF) and 1.5% bovine serum albumin (BSA). When these cells reached 60%–70% confluence, they were harvested and seeded onto Costar®

Transwell-clear 3450 culture inserts (Corning, NY) using 10⁵ cells per insert in culture media. At confluence (5–7 days of culture), the medium was removed to establish an air-liquid interface (ALI) culture to promote differentiation and stratification [19–23]. The medium used for the ALI culture was a 1:1 (v/v) mixture of BEGM and DMEM with EGF concentration reduced to 0.5 ng/ml and BSA. After airlift culturing for two weeks to induce growth of multi-layered human conjunctival epithelial cells (HCECs), hyperosmotic stress (400 mOsm/l) was applied by adding an appropriate volume of 5 M NaCl to ALI media with diquafosol tetrasodium (100 μM) for 24 h [18]. ALI media not under hyperosmotic stress were used for controls. The formation of multi-layered HCEC was confirmed by immunofluorescence staining with CK19 [19–23].

RNA isolation and quantitative real-time PCR: The expression levels of membrane-associated mucin genes (MUC1 and MUC16) and secreted mucin gene (MUC5AC) were analyzed using reverse transcription-polymerase chain reaction (RT-PCR). For analysis of mucin gene expression, HCECs were treated with hyperosmotic stress (400 mOsm/l) for 24 h followed by treatment with diquafosol tetrasodium (100 μM) at different time periods (6, 12, 24 h). TRIzol reagent (Gibco-Invitrogen, Grand Island, NY, USA) was used to isolate total RNAs from HCECs. Synthesis of cDNA strands (cDNAs) was achieved using SuperScript III reverse transcriptase (Invitrogen) and random hexamers. Real-time PCR was performed using SYBR Green I. GAPDH was used for internal calibration of average threshold cycle (CT) values. For relative quantification, the 2^{-ΔΔCt} method [19] was used. The primers used in this study were as follows: for MUC1, Forward: 5'-AGA CGT CAG CGT GAG TGA TG-3', Reverse: 5'-CAG CTG CCC GTA GTT CTT TC-3'; for MUC16, Forward: 5'-AGC ATC CTG GAC GTA ACC AC-3', Reverse: 5'-CAG GTG GAA GGG TGT TCT GT-3'; for MUC5AC, Forward: 5'-ACT GGG GAG AAA TAT CGC ATG-3', Reverse: 5'-CTG ATC AAA GGG CCT GAT AGC-3'; for GAPDH, Forward: 5'-ATT GCC CTC AAC GAC CAC T-3', Reverse: 5'-ATG AGG TCC ACC ACC CTG T-3'.

Cell proliferation/cytotoxicity assay: To determine the cell proliferation/cytotoxicity of diquafosol to be used in the experiments, cell viability was detected using a MTT cell proliferation kit (Roche, Penzberg, Germany) in which the MTT is converted into formazan granules in the presence of molecular oxygen. The intracellular formazan can be solubilized and quantified by spectrophotometric means. Triplicate samples in a 96-well plate were incubated in a medium containing 5 mg/ml MTT for 4 h at 37 °C in a humidified 5% CO₂ atmosphere. Solubilized formazans with DMSO were put

in a 96-well plate and optical densities were measured using an ELISA plate reader (SPECTRA max; Molecular Devices, Sunnyvale, CA) at a wavelength of 550 nm. Each experiment included a blank control (culture medium). All experiments were performed three times in triplicate.

Immunohistochemistry and confocal microscopy: When the HCEC cells reached confluence on the transwell insert, the medium was removed to establish an ALI culture for promoting differentiation and stratification. After airlift culturing for two weeks to induce multi-layers of HCECs with or without hyperosmotic stress (400 mOsm/l) and diquafosol tetrasodium (100 μ M) for 24 h, HCECs on the insert membrane were immunostained. Cells were fixed with cold methanol, permeabilized with 0.1% Triton X-100, and incubated with 10% goat serum for 1 h to block nonspecific reactions. Then, cells were incubated with MUC1 (Abcam), MUC5AC (Abcam), MUC16 (Abcam), and P2Y2R (Santa-cruz) antibodies and incubated with Alexa Fluor 488–conjugated anti-rabbit IgG antibody. The immunostains were captured using confocal microscopy (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany).

Western blot analysis: To investigate the effect of diquafosol tetrasodium on intracellular signaling molecule ERK, HCECs were treated with hyperosmotic stress (400 mOsm/l) for 24 h followed by treatment with diquafosol tetrasodium (100 μ M) for the indicated time period (0–60 min) after ALI culturing for two weeks. To further investigate the significance of the regulation of ERK signaling pathways in the expression of mucins in hyperosmotic stress (400 mOsm/l)-stimulated HCECs for 24 h, cells were stimulated with or without PD98059, an ERK inhibitor, for 1 h. Cells were then treated with diquafosol tetrasodium (100 μ M) for 6 h and 24 h. Total protein concentrations were determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific) following the manufacturer's protocol. Samples at equal amount of proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). PBS containing 5% nonfat milk and 0.1% Tween-20 (PBST) was used to block the membrane for 1 h. The membrane was then incubated with primary rabbit polyclonal antibodies targeting p44/42 MAPK (Erk1/2; 1375F5; 1:1000; #4695S, Cell Signaling Technology,

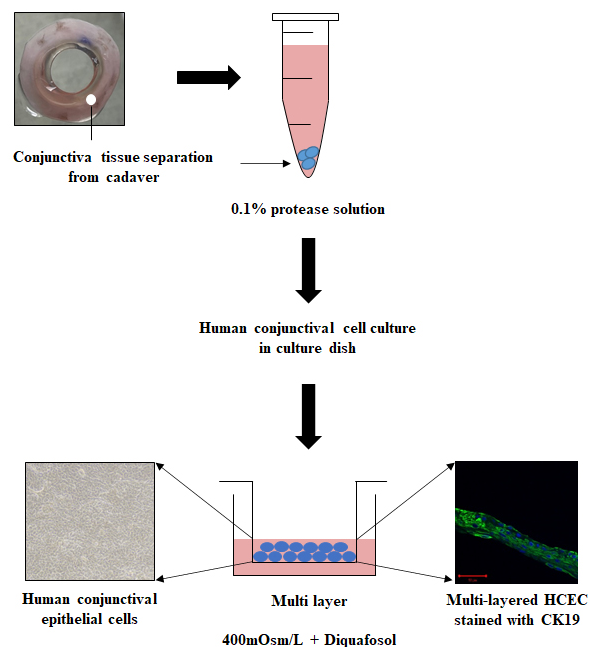


Figure 1. Flow diagram for the production of human conjunctival epithelial cells from cadavers. Conjunctival specimens from cadavers were incubated at 4 °C with 0.1% protease. Suspended epithelial cells were seeded in culture dishes. When these cells reached 60%–70% confluence, they were harvested and seeded onto transwell inserts using 10^5 cells per insert in culture media. At confluence, the medium was removed to establish an air-liquid interface (ALI) culture to promote differentiation and stratification. After airlift culturing for two weeks to induce multi-layer human conjunctival epithelial cells (HCECs), hyperosmotic stress (400 mOsm/l) was applied by adding an appropriate volume of 5 M NaCl to ALI media with diquafosol tetrasodium. Immunofluorescence microscopy shows multi-layered HCECs stained with CK19 shown in green. Bar = 50 μ m.

Danvers, MA), phospho-p44/42 MAPK (Erk1/2; Thr202; 1:2000; #4370S, Cell Signaling Technology), MUC1 (1:1000; ab-45167, abcam, UK), or MUC16 (1:10,000; ab-134093, abcam) at 4 °C for 18 h. After three washes with PBST, membranes were incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:10,000, Thermo Fisher Scientific) at room temperature (RT) for 1 h. After washing three times with PBST, enhanced chemiluminescence reagent (ECL; Amersham Biosciences, Sweden) was added to the membranes to detect protein bands. All membranes were then subjected to stripping and reprobing processes with mouse monoclonal anti- β -actin antibody (Santa Cruz Biotechnology, Dallas, TX) to obtain data for normalization. Each experiment was normalized against β -actin to obtain relative expression levels by image analysis. Three independent experiments were performed.

ELISA for human MUC5AC: The concentrations of MUC5AC in the HCECs were quantitatively analyzed using ELISA (MyBioSource, San Diego, CA) according to the manufacturer's instructions. After airlift culturing HCECs for two weeks with ALI media, hyperosmotic stress (400 mOsm/l) was applied for 24 h. Then 100 μ M of diquafosol tetrasodium (Diquafosol®, Santen Pharmaceutical Co., Ltd., Osaka, Japan) was applied for different time periods (1, 3, 6, 12, and 24 h). The supernatant of the cells was collected after centrifuging at 1,000 \times g for 15 min at 4 °C and then stored at -70 °C until analysis. The supernatant was treated with α 2-3,6,8,9 neuraminidase A (New England BioLabs Japan Inc., Tokyo, Japan) for 1 h at 37 °C to enhance MUC5AC mucin recognition in ELISA for human MUC5AC [24]. For human MUC5AC detection, samples were analyzed in triplicate.

Statistical analysis: The data are presented as average \pm standard deviation. The statistical significance between groups was examined with one way ANOVA (ANOVA) test using SPSS version 17.0 (SPSS Inc., Chicago, IL). We regarded $p < 0.05$ as significant and $p < 0.01$ as highly significant.

RESULTS

Diquafosol tetrasodium induces expression of mucins in hyperosmotic stressed HCECs: To assess the effect of diquafosol tetrasodium on HCECs, concentrations of secreted MUC5AC were examined using ELISA. As shown in Figure 2A, treatment with diquafosol tetrasodium increased the secretion of MUC5AC in HCECs. The maximal secretion of MUC5AC was noted at 6 h (320 \pm 26 ng/ml). After hyperosmotic stress (400 mOsm/l) for 24 h, MUC5AC mRNA level was suppressed in Figure 2B. However, treatment with diquafosol tetrasodium significantly increased the expression

of MUC5AC gene in HCECs from 6 h to 24 h (increase of 2.63 \pm 0.29 folds at 24 h, $p < 0.01$; Figure 2B). Interestingly, the mRNA levels of the membrane-associated mucin MUC1 were also significantly increased at 24 h (2.40 \pm 0.64 folds at 24 h, $p < 0.01$). MUC16 mRNA levels were increased at 6 h. The increase was maintained until 24 h (1.79 \pm 0.41 folds at 24 h, $p < 0.05$, Figure 2C). We performed cell proliferation/cytotoxicity assays in HCEC cells with various concentrations of diquafosol to determine the range of effective or toxic concentrations in vitro. The cell viability through absorbance values of wells treated with 1, 5, 10, 50, 100, and 200 μ M diquafosol (84.35 \pm 5.29, 82.19 \pm 4.92, 82.71 \pm 4.12, 87.135 \pm 4.81, 88.38 \pm 3.82, and 87.4 \pm 11.65 at 48 h, respectively) showed no significant difference compared to those of the vehicle after hyperosmotic stress (400 mOsm/l, 84.18 \pm 5.29, Figure 2D). Immunostaining results demonstrated fewer positive cells for MUC1, MUC16, MUC5AC, and P2Y2R in hyperosmotic stress-stimulated HCECs. Interestingly, mucin positive cells were significantly increased in diquafosol tetrasodium-treated HCECs at 24 h. P2Y2R was weakly stained in the control, whereas it was strongly stained in the vehicle after hyperosmotic stress and the addition of diquafosol-treated cells (Figure 2E).

Diquafosol tetrasodium induces p-ERK expression in hyperosmotic stressed HCECs: To determine the signaling pathways involved in the modulation of mucin expression in diquafosol tetrasodium-treated HCECs, the phosphorylation of p44/42 MAPK (Erk1/2) expression was analyzed under hyperosmotic stress conditions. As shown in Figure 3, the phosphorylation of p44/42 MAPK (Erk1/2) signaling molecules was increased by treatment with diquafosol tetrasodium in a time-dependent manner under hyperosmotic stress. The ratio of phospho-p42/total p42 and phospho-p44/total p44 expression also significantly increased from 5 min to 60 min, respectively.

ERK signaling regulates mucin expression enhanced by diquafosol tetrasodium in HCECs: To investigate the effect of ERK signaling regulation on mucin gene expression, hyperosmotic stress (400 mOsm/l)-stimulated HCECs were treated with or without PD98059, an ERK inhibitor, followed by stimulation with diquafosol tetrasodium (100 μ M) at different time points (6 h and 24 h). As shown in Figure 4A, diquafosol tetrasodium-treated HCECs showed increased mRNA expression of MUC1, MUC16, and MUC5AC. This effect was blocked by PD98059, an ERK inhibitor. The protein expression patterns of mucins were also regulated by the ERK signaling pathway. Protein expression levels of MUC1 and MUC16 were significantly inhibited by PD98059 at 6 h and 24 h in HCECs (Figure 4B). Additionally, concentrations of

MUC5AC in HCECs were quantitatively analyzed by ELISA. Significant inhibition of MUC5AC secretion was observed in HCECs treated with PD98059 compared to those that were not (Figure 4C).

DISCUSSION

The current study demonstrated for the first time that diquafosol tetrasodium can increase the expression of membrane-associated mucin and goblet cell-secreted mucin MUC5AC in HCECs via the intracellular ERK signaling pathway. Human epithelial mucins are classified into two families: secreted

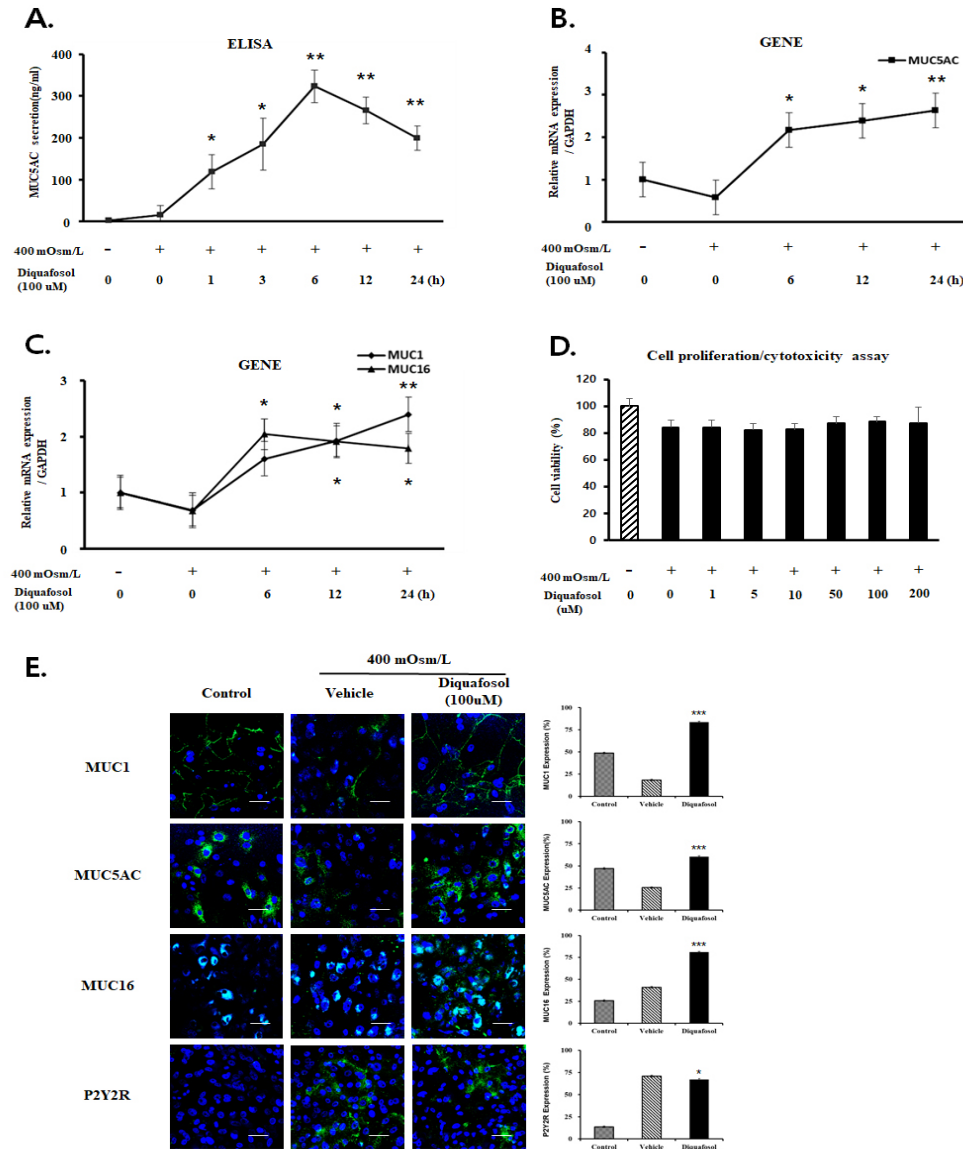


Figure 2. Diquafosol tetrasodium-induced expression of mucins in hyperosmotic stressed HCECs. **A:** Concentrations of secreted MUC5AC by ELISA before and after hyperosmotic stress (400 mOsm/l) with diquafosol tetrasodium. **B:** Gene expression of MUC5AC before and after hyperosmotic stress with diquafosol tetrasodium. **C:** Transcription of membrane-associated mucin MUC1 and MUC16 genes in HCECs induced by diquafosol tetrasodium. MUC1 and MUC16 mRNA levels are shown. **D:** MTT cell proliferation/cytotoxicity assay of HCECs with various concentrations of diquafosol tetrasodium. **E:** Representative confocal images of HCECs immunostained for MUC1, MUC5AC, and MUC 16. Bar = 20 μ m. The values in these graphs represent the mean of three experiments; the error bars represent the standard deviation. * $p < 0.05$, ** $p < 0.01$.

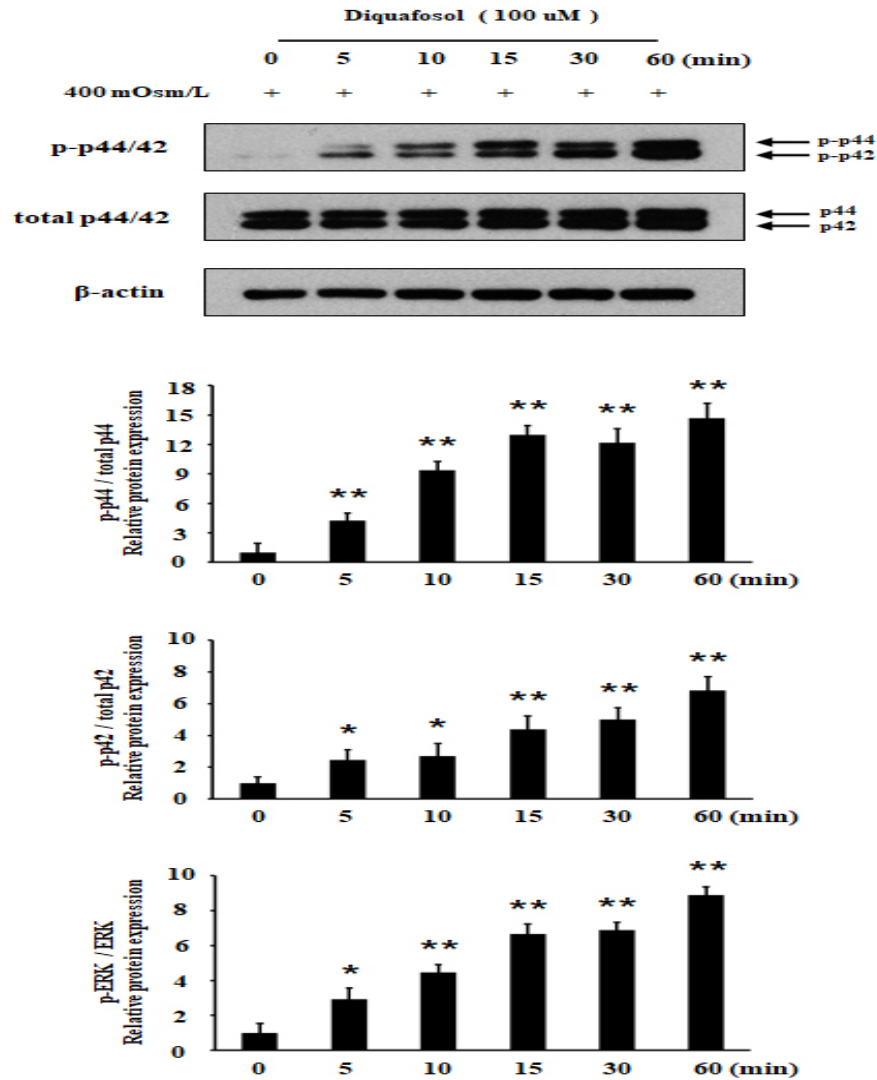


Figure 3. Diquafosol tetrasodium induces p-ERK in hyperosmotic stressed HCECs. Representative photograph of p-ERK and ERK protein levels and relative expression results for both markers. All relative signal intensities were normalized against β -actin. Expression levels of p-ERK and ERK were obtained from the same gel in western blot. * $p < 0.05$, ** $p < 0.01$ versus control.

mucins and membrane-associated mucins. At least four subfamilies of secreted mucins (MUC5AC, MUC7, MUC2, and MUC19) [4, 25–27] and four subtypes of membrane-associated mucins (MUC1, MUC4, MUC16, and MUC20) [28–31] are expressed on the ocular surface. Ocular secreted mucin MUC5AC is produced by goblet cells. It has no transmembrane-spanning domains [25]. It is believed to be expressed most dominantly on the ocular surface and secreted into the tear film [32]. Among membrane-associated mucins, MUC1, MUC4, and MUC16 are produced in both corneal and conjunctival epithelia [30,33].

Diquafosol tetrasodium (Diquas® ophthalmic solution 3%; Santen Pharmaceutical Co., Ltd., Osaka, Japan), a

uridine 5'-triphosphate (UTP) derivative, is a potent P2Y2 purinergic receptor agonist that can stimulate tear fluid and mucin secretion from conjunctival epithelial cells and goblet cells with high clinical efficacy and has a good safety profile [8,9,11,12,34-38]. Many previous studies have demonstrated that the expression of secreted mucin MUC5AC is increased after diquafosol administration [8,9]. Hori et al. reported that the MUC5AC level in rabbit tears increases 15 min after instillation of 3% diquafosol eyedrop [9]. Choi et al. reported that the administration of diquafosol is effective in stimulating mucin secretion in both normal and keratoconjunctivitis sicca rat models [8]. The peak time for tear MUC5AC concentration was found to be 15 min after instillation [8]. Our study showed that the peak time of MUC5AC

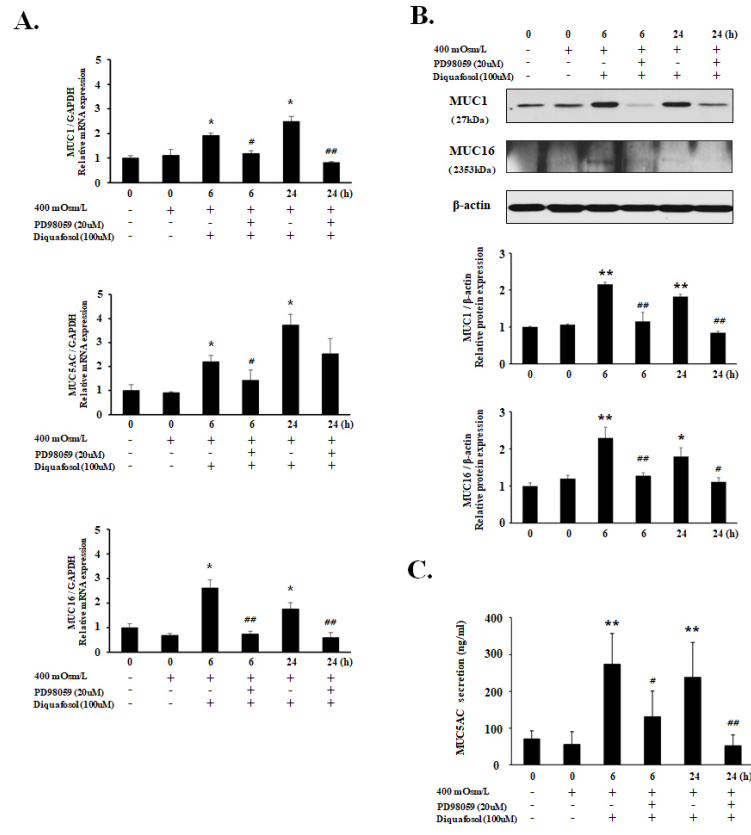


Figure 4. ERK signaling regulates mucin expression enhanced by diquafosol tetrasodium in HCECs. **A:** Inhibition of MUC1, MUC16, and MUC5AC mRNA levels by ERK inhibitor. **B:** Representative photograph of MUC1 and MUC16 protein levels and relative expression results for both markers with ERK inhibitor. All relative signal intensities were normalized against β-actin. **C:** Inhibition of MUC5AC secretion by ERK inhibitor in HCECs. Concentrations of MUC5AC in HCECs were quantitatively analyzed by ELISA. * p<0.05, ** p<0.01 versus control; # p<0.05, ## p<0.01 versus without ERK inhibitor.

concentration in culture supernatant of HCECs was at 6 h after treatment with diquafosol. Also, gene expression levels increased from 6 h until 24 h under hyperosmotic stress to mimic dry eye conditions.

In regards to membrane-associated mucins, a previous study reported that diquafosol can increase the mRNA expression levels of MUC1, MUC4, and MUC16 in human corneal epithelial cells 3 h after treatment [39]. Recently, Moon et al. showed that topical diquafosol treatment can upregulate MUC1, MUC4, and MUC16 at both the mRNA and protein levels in a dry eye-induced mouse model and that such upregulation contributes to conjunctival goblet cell recovery [10]. Our study also showed that the gene expression levels of MUC1 and MUC16 increased in conjunctival epithelial cells after treatment with diquafosol, consistent with the results of previous studies. The gene expression of

MUC1 was increased from 6 to 24 h. Interestingly, MUC16 gene expression levels reached peak time at 6 h and was maintained until 24 h. Protein levels and immunostaining of positive cells for MUC1 and MUC16 were also increased in the HCECs hyperosmotic stressed by diquafosol treatment.

Our findings demonstrated that hyperosmotic stress (400 mOsm/l) on HCECs leads to the inhibition of membrane-associated mucin gene (MUC1 and 16) expression. Although hyperosmotic stress (400 mOsm/l) did not induce reactive oxygen species (ROS) generation significantly (Appendix 1), the inhibition of mucin gene expression was confirmed before treatment with diquafosol tetrasodium (100 μM). The expression levels of the mucin genes in HCEC with hyperosmotic stress induced by diquafosol tetrasodium increased after 6 h. This finding suggests that hyperosmotic stress on HCECs is closely involved in the expression of membrane-associated

mucins. A previous study by Kim et al. showed that diquafosol inhibits nuclear factor-kappa B signaling and inflammatory mediators induced by hyperosmotic stress in human corneal epithelial cells, thus conferring protective properties on hyperosmotic stress-induced inflammation [40]. These results indicate that diquafosol tetrasodium may have protective effects on dry eye patients with a history of hyperosmotic stress conditions by inducing mucin genes expression.

It is generally believed that dry eye is a disease of decreased mucin secretions. Previous studies reported reduced expression of membrane mucins in dry eye [41,42]. From this point of view, the use of diquafosol for dry eye patients can promote the increase of mucin, so it has potential as a promising treatment option. However, there have been reports that membrane mucins such as MUC1 and MUC16 are somewhat increased in patients with Sjogren's syndrome (SS) dry eyes compared to patients with non-SS syndrome dry eyes and controls. Caffery et al. demonstrated an excess of both MUC1 and MUC16 in the tear film and increased mRNA levels in conjunctival epithelial cells in SS dry eyes, but not in non-SS dry eyes and the control group [43,44]. In addition, ocular cicatricial pemphigoid (OCP) patients showed increased mucin production [45]. It seems that a cautious approach is required in the use of diquafosol in diseases accompanied by ocular surface inflammation, such as SS or OCP [43-45].

The mechanism for promoting mucin expression through diquafosol, a uridine 5'-triphosphate (UTP) derivative, has yet to be elucidated, although many previous reports have identified the mechanism of action of diquafosol involved in corneal epithelial healing. Nucleotides such as adenosine 5'-triphosphate (ATP) and UTP can elicit strong calcium responses and the phosphorylation of EGFR and ERK via P2YR-induced downstream signaling that can stimulate epithelial cell migration and proliferation [12,46,47]. Byun et al. reported that diquafosol can stimulate corneal epithelial cell proliferation and migration through the $[Ca^{2+}]$ -induced EGFR-ERK signaling pathway in the same way as extracellular nucleotides [12]. Our study confirmed that the phosphorylation of ERK expressions in conjunctival epithelial cells with hyperosmotic stress that are known to mimic dry eye conditions was increased by diquafosol treatment in a time-dependent manner. This result indicates that diquafosol can activate $[Ca^{2+}]$ -induced EGFR/ERK signaling pathways in conjunctival epithelial cells with hyperosmotic stress.

To investigate the effect of ERK signaling regulation on mucin expression induced by diquafosol, mucin expression was evaluated after treatment with PD98059, an ERK inhibitor. Our results revealed that the expression levels of

MUC1 and MUC16 were markedly enhanced by diquafosol at different time points (6 h and 24 h). However, their expression levels were effectively attenuated by PD98059, an ERK inhibitor. Goblet cell mucin MUC5AC gene expression and secretion were also significantly enhanced by diquafosol at different time points (6 h and 24 h). These levels were notably attenuated by PD98059, an ERK inhibitor. These results indicate that ERK signaling is crucial in regulating mucin expression levels enhanced by diquafosol treatment. However, our study showed that mucin expression levels in PD98059 treated-HCECs were slightly increased after diquafosol treatment at some time points. This suggests that ERK-independent signaling pathways in mucin secretion might be stimulated by diquafosol. Lippestad et al. studied cultured rat conjunctival goblet cells and reported that proresolution mediator resolvin D1 can stimulate mucin secretion from goblet cells by increasing intracellular Ca^{2+} via multiple signaling pathways, including phospholipases (C, D, and A2) and their signaling components ERK 1/2 and Ca^{2+} /CamK [48]. Further studies are needed to clarify the multiple signaling pathways involved in mucin regulation.

The current study had some limitations. First, we could not evaluate the expression of MUC4, one of the major ocular membrane-associated mucins [30,33], because the MUC4 protein was not detected by the anti-MUC4 monoclonal antibody in our cultured primary conjunctival epithelial cells. Further studies on MUC4 are necessary to clarify this finding. In addition, studies on the long-term application of diquafosol are needed to observe changes in mucin expression under hyperosmotic conditions. In conclusion, this study demonstrated that ERK signaling might be important in regulating the expression of MUC1, MUC16, and MUC5AC induced by diquafosol tetrasodium in hyperosmotic-stressed HCECs known to mimic dry eye conditions.

APPENDIX 1. SUPPLEMENTARY FIGURE 1.

To access the data, click or select the words “[Appendix 1.](#)” Cellular reactive oxygen species production of human conjunctival epithelial cells under hyperosmolar conditions (400 mOsm/l) and effects of diquafosol tetrasodium (100 μ M).

ACKNOWLEDGMENTS

This research was supported by the grant (2020R1A2B5B01002407) of the Basic Science Research Program through the National Research Foundation (NRF) funded by the Ministry of Education, Republic of Korea and by the Research Grant of Seoil University in 2022.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 30 June 2022. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.