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## Comments

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# Effect of High Glucose on Ocular Surface Epithelial Cell Barrier and Tight Junction Proteins

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**PURPOSE.** Patients with diabetes mellitus are reported to have ocular surface defects, impaired ocular surface barrier function, and a higher incidence of corneal and conjunctival infections. Tight junctions are critical for ocular surface barrier function. The present study was designed to investigate the effect of high glucose exposure on human corneal and conjunctival epithelial cell barrier function and tight junction proteins.

**METHODS.** Human corneal and conjunctival epithelial cells were exposed to 15 mM and 30 mM glucose for 24 and 72 hours. The barrier function was measured using transepithelial electrical resistance (TEER). The cell migration was quantified using scratch assay. The cells were harvested for protein extraction and mRNA isolation. Gene and protein expression of claudins, zonula occludens (ZOs), and occludin was quantified using real-time PCR and Western blot.

**RESULTS.** Glucose caused a significant decrease in TEER after 72 hours of exposure in both corneal and conjunctival epithelial cells. Glucose did not cause any notable change in migration of either corneal or conjunctival epithelial cells. Glucose exposure did not cause any notable change in protein expression of claudin-1, ZO-1, ZO-2, ZO-3, or occludin. On the other hand, 15 mM glucose caused an increase in gene expression of claudin-1, claudin-3, ZO-2, ZO-3, and occludin, a likely response to osmotic stress since 15 mM mannitol also caused consistently similar increase in gene expression of these proteins.

**CONCLUSIONS.** High glucose exposure causes impairment of corneal and conjunctival epithelial cell barrier function, but this detrimental effect is not caused by a decrease in expression of tight junction proteins: claudin-1, ZO-1, ZO-2, ZO-3, and occludin.

**Keywords:** corneal epithelium, conjunctival epithelium, tight junction, high glucose, diabetes mellitus

The ocular surface is perpetually exposed to the external environment, which makes it vulnerable to microbes, allergens, and chemicals. Corneal and conjunctival epithelial cells form the outermost layer of the ocular surface and provide a strong barrier against these environmental insults. These cells have been shown to express components of tight junction complexes.<sup>1,2</sup> One of the major functions attributed to the tight junctions is to restrict the paracellular movement of large molecules and pathogens, thus significantly contributing to the barrier function of epithelial cells.<sup>3-5</sup> Tight junctions are localized on the apical-lateral surface of epithelial cells and contain an assembly of integral transmembrane proteins that are further connected to the peripheral membrane proteins present in the cytosol. Four transmembrane proteins present in the tight junctions include claudins, occludin, junctional adhesion molecule, and tricellulin. The peripheral proteins are zonula occludens (ZO)-1, ZO-2, and ZO-3, and they serve to connect the transmem-

brane proteins to the cytoskeletal proteins.<sup>3-5</sup> The claudins protein family consists of 27 members that share a common feature of four transmembrane spanning regions forming two extracellular loops and cytosolic C and N terminals. Claudins demonstrate a tissue-specific distribution with various combinations of the 27 subtypes being expressed in the epithelial cells of various organs.<sup>6,7</sup> Claudin-1, -2, -4, -7, -9, and -14 expression has been detected in both human corneal and conjunctival epithelial cells while claudin-3 and claudin-10 were detected only in corneal epithelial cells and conjunctival epithelial cells, respectively.<sup>1</sup> Expression of other components of tight junctions, including occludin and ZOs, has also been detected in corneal and conjunctival epithelial cells.<sup>1,2</sup>

Recent evidence suggests that diabetes mellitus has many detrimental impacts on the anterior segment of the eye, including tear film dysfunction, dry eye, corneal keratopathy, and corneal epithelial defects.<sup>7-10</sup> Abnormal-

ities in epithelial cell density and maturation have also been detected in the cornea of patients with diabetes mellitus.<sup>11–13</sup> Clinical studies using fluorometry-assisted tracking of fluorescein penetration have consistently demonstrated an impairment of corneal epithelial barrier function in diabetic patients showing a correlation between the barrier impairment and HbA1c levels.<sup>14,15</sup> In addition to these corneal abnormalities, patients with diabetes mellitus have been reported to have conjunctival defects as well. Abnormalities in conjunctival capillaries, including dilation, increased tortuosity, and leakage, have been reported in patients with diabetes mellitus.<sup>16,17</sup> The incidence of infections in the conjunctiva and cornea of diabetic patients is also higher compared with nondiabetics, suggesting a compromise in the barrier function of the ocular surface epithelium.<sup>18,19</sup> Although many of these clinical studies have consistently demonstrated diabetes-associated impairment in the corneal and conjunctival barrier function, there are no published data on how diabetes mellitus affects corneal and conjunctival epithelial cell tight junction proteins. The present study was designed to investigate the effect of high glucose exposure on human corneal and conjunctival epithelial cell barrier function and tight junction proteins: claudin-1, ZO-1, ZO-2, ZO-3, and occludin.

## MATERIALS AND METHODS

### Human Corneal Epithelial and Conjunctival Epithelial Cell Culture

Telomerase-transformed human corneal (provided by Dr. James V. Jester, School of Medicine, University of California, Irvine) and human conjunctival epithelial cells (provided by Dr. Ilene Gipson, Harvard Medical School, Boston, MA) were used for the experiments. The identity of the cell lines was further confirmed by short tandem repeats (STR) profiling from ATCC (Manassas, VA). The human corneal epithelial cells were cultured in keratinocyte growth medium supplemented with 4  $\mu$ L/mL bovine pituitary extract, 0.125 ng/mL human epidermal growth factor, 5  $\mu$ g/mL insulin, 0.33  $\mu$ g/mL hydrocortisone, 0.39  $\mu$ g/mL epinephrine, 10  $\mu$ g/mL transferrin, and 0.15 mM calcium chloride (PromoCell GmbH, Heidelberg, Germany).<sup>20,21</sup>

Human conjunctival epithelial cells were cultured in keratinocyte serum-free medium supplemented with 25  $\mu$ L/mL bovine pituitary extract, 0.2 ng/mL human epidermal growth factor, and 0.4 mM calcium chloride (Gibco, Thermo Scientific, Rockford, IL).<sup>21,22</sup>

For high glucose treatment, the cells were exposed to their respective growth media supplemented with 15 mM and 30 mM glucose for 24 hours and 72 hours. Mannitol treatment was used to determine whether the observed changes were due to the metabolic effects of glucose or purely due to osmotic stress. For mannitol treatment, cells were exposed to respective media supplemented with 15 mM and 30 mM mannitol for 24 hours and 72 hours. Each experiment was conducted in triplicate.

### Transepithelial Electrical Resistance

For measurement of transepithelial electrical resistance (TEER), the cells were seeded onto collagen-coated 3.0- $\mu$ m polytetrafluoroethylene Costar transwell membrane inserts (Corning, New York, NY) placed onto 12-well plates. The cells were grown to produce confluent monolayer. The TEER

was measured using the chopstick electrode method by placing the electrodes on the top and bottom side of transwells (Molecular Devices, San Jose, CA).

### Scratch Assay

The cells were plated in 12-well plates. The scratch was performed on the cells in two perpendicular straight lines using a 1000- $\mu$ L pipette tip. The dislodged cells were immediately removed using media change. The cells were imaged at the same position just above where the two perpendicular scratch lines intersected at time points immediately after scratch (0) and then at 24, 48, and 72 hours using a phase contrast lens on a bright-field microscope (Keyence BZ-X 710, Itasca, IL). The reepithelization of the scratch width was quantified by measuring the distance between the two edges of the scratch lines using ImageJ software (National Institutes of Health, Bethesda, MD).

### Isolation of mRNA and Preparation of cDNA

The total RNA from corneal and conjunctival epithelial cells was isolated using a commercially available kit (RNeasy Mini Kit; QIAGEN, Valencia, CA) as per manufacturer's instructions. The RNA was immediately reverse transcribed to cDNA using a commercially available kit (SuperScript III First-Strand; Invitrogen, Carlsbad, CA).

### Gene Expression Quantification

The gene expression of the tight junction proteins claudin-1, -2, -3; ZO-1, -2, -3; and occludin was quantified using real-time PCR. The sequence of forward and reverse primers for each of these target genes is listed in the Table. A 20- $\mu$ L reaction mixture containing 2  $\mu$ L cDNA and 18  $\mu$ L SYBR Green Master Mix was run at a universal cycle (95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 55°C for 60 seconds) using a QuantStudio 3 Real-Time thermocycler (Applied Biosystems, Thermo Fisher Scientific, Singapore).  $\beta$ -Actin was used as the housekeeping gene. The relative change in gene expression was calculated using the  $\Delta\Delta$ Ct method.

### Immunoblotting

The protein extracts were prepared by adding radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor (Thermo Fisher Scientific, Rockford, IL) to the cells. The cells were incubated in RIPA buffer on a shaker followed by the removal of any adherent cells using a cell scraper. The supernatant was collected as total cell lysate, and protein concentration was determined by the BCA method using a commercially available kit (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific). The protein extracts were suspended in loading buffer containing  $\beta$ -mercaptoethanol (Bio-Rad Laboratories, Carlsbad, CA) and heated at 95°C for 5 minutes. Protein samples were resolved by loading on 10% SDS-PAGE gels (Bio-Rad Laboratories) and run at 100 V for about 85 minutes using 1 $\times$  TRIS-glycine-SDS buffer. The resolved proteins were transferred from gel onto the polyvinylidene difluoride membranes using wet transfer at 100 V for 60 minutes. The membranes were blocked in 5% blocking buffer and then incubated with primary antibodies. The primary antibodies for human claudin-1, -2, -3; ZO-1, ZO-2, ZO-3; and occludin were

TABLE. Sequence of Forward and Reverse Primers Used for Real-Time PCR Quantification of Gene Expression

Gene	Forward Primer	Reverse Primer
Claudin-1	5'-CCA GTT AGA AGT GGT AGT GTG AAT-3'	5'-CAG CCA GCT GAG CAA ATA AAG-3'
Claudin-2	5'-CCT CCA TCC CAC TCT TGT TAT G-3'	5'-CAT CCT GCA TCC TGC TTT CT-3'
Claudin-3	5'-CCA AGG CCA AGA TCA CCA T-3'	5'-GGT TGT AGA AGT CCC GGA TAA TG-3'
ZO-1	5'-GCA GCC ACA ACC AAT TCA TAG-3'	5'-GAA AGG TAA GGG ACT GGA GAT G-3'
ZO-2	5'-AGG ATG CCG TTC TCT ACC T-3'	5'-CAC AAG CCA GGA TGT CTC TAT AC-3'
ZO-3	5'-GGC GGG AAA GTT CAG TAG ATT-3'	5'-GGC ACT CTG TAG ATG TCA TAG C-3'
Occludin	5'-GGA AGG TTC TGG TGT GAA CTA A-3'	5'-CTG AAA GGT GGT TGA GAG GAT TA-3'
$\beta$ -actin	5'-GGA CCT GAC TGA CTA CCT CAT-3'	5'-CGT AGC ACA GCT TCT CCT TAA T-3'

purchased from Thermo Fisher Scientific. The membranes were washed three times with 0.1% TBST (Tween 20 in Tris-buffered saline), followed by incubation with donkey anti-rabbit, horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, MA).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene (Thermo Fisher Scientific). The blot was developed using enhanced chemiluminescence reagents (Lumigen, Inc., Southfield, MI) and imaged using a chemiluminescence gel imager (Bio-Rad Laboratories). The digital quantification of Western blots was performed by densitometric analysis using ImageJ software. For each sample, the pixel density of the target protein band was normalized by dividing it by the pixel density of the corresponding GAPDH bands. The fold change was calculated by dividing the normalized target protein/GAPDH ratio of test samples by the normalized target protein/GAPDH band ratio of control samples. The respective control and high glucose exposed samples for the corneal and conjunctival epithelial cell extracts from the same gel were used for comparison but have been rearranged in the figures to represent the data in a logical fashion. The Western blots were run in triplicate. In separate pilot experiments, the validity of GAPDH as a housekeeping gene was confirmed by comparing fold change in GAPDH compared with  $\beta$ -tubulin. We did not detect any change in GAPDH protein expression in cell extracts exposed to 15 mM or 30 mM high glucose for 24 hours and 72 hours as compared with the control samples cells exposed to media containing regular 5 mM glucose. Three Western blots each for corneal and conjunctival epithelial cells were performed for this validation (data not shown).

### Statistical Analysis

The data are presented as mean  $\pm$  SEM. Statistical analysis was performed using GraphPad Prism software (version 8; GraphPad Software, San Diego, CA). The data were analyzed using two-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. A *P* value of <0.05 was considered statistically significant.

## RESULTS

### Effect of High Glucose on TEER

To test whether high glucose causes impairment of corneal and conjunctival epithelial cell barrier functions, we cultured a monolayer of the cells on a transwell membrane and measured the TEER using the chopstick method. In human corneal epithelial cells, 15 mM glucose caused 35%  $\pm$  4%, and 30 mM glucose caused 15%  $\pm$  5% decrease in the TEER

after 72 hours of exposure, but no significant change in TEER was observed after 24 hours (Fig. 1A). In conjunctival epithelial cells also, 15 mM glucose caused 15%  $\pm$  1%, and 30 mM glucose caused 8%  $\pm$  1% decrease after a 72-hour exposure but caused no notable effect after 24 hours (Fig. 1C). The decrease in TEER in both the cell lines with the two tested concentrations noted only at 72 hours suggests that a persistent exposure to high glucose may be required to cause impairment of barrier functions. The more pronounced decrease of TEER noted in the corneal epithelial cells suggests their likely higher susceptibility to glucose-mediated detrimental effect compared with the conjunctival epithelial cells.

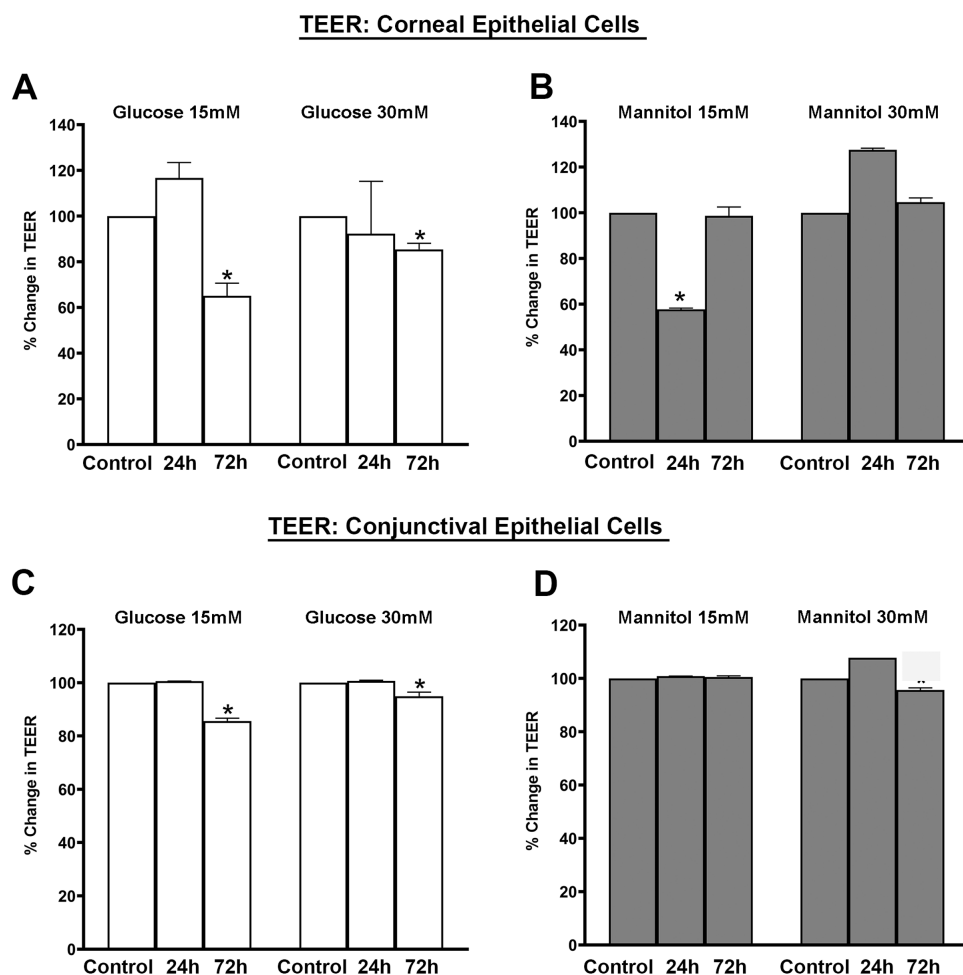
In contrast to glucose, mannitol did not cause any decrease in TEER in corneal epithelial cells at 72 hours, but a significant decrease was noted after a 24-hour exposure with the 15-mM dose only in corneal epithelial cells, suggesting that these cells may be more sensitive to the moderate acute osmotic stress caused by mannitol (Fig. 1B). Further, mannitol exposure did not cause any significant decrease in TEER in the cultured conjunctival epithelial cells (Fig. 1D).

### Effect of High Glucose on Cell Migration

Corneal and conjunctival epithelial cells migrate from limbus to sclera and cornea. An impairment of epithelial cellular migration can significantly compromise the barrier functions. Therefore, we tested the effect of high glucose exposure on the migration of corneal and conjunctival epithelial cells. High glucose exposure did not affect the migration of corneal epithelial cells (Fig. 2A) but caused a notable albeit statistically insignificant increase in the migration of conjunctival epithelial cells at the 30-mM concentration (Fig. 2C). On the other hand, mannitol at the 30-mM concentration caused a statistically significant increase in the migration of both corneal and conjunctival epithelial cells (Figs. 2B, 2D).

### Effect of High Glucose on Claudin Family Gene and Protein Expression

Tight junctions play an important role in maintaining the cellular barrier functions.<sup>3-5</sup> The claudin family of proteins is an integral part of tight junctions. Claudin-1 has high expression in the corneal and conjunctival epithelial cells.<sup>1</sup> Therefore, we next tested the effect of high glucose exposure on the protein and gene expression of claudin-1. However, Western blot data show that high glucose exposure for 24 or 72 hours did not cause any notable change in claudin-1 protein expression in corneal epithelial cells (Fig. 3C) or conjunctival epithelial cells (Fig. 3F). On the other hand, real-



**FIGURE 1.** Effect of glucose (15 mM; 30 mM) and mannitol (15 mM; 30 mM) on barrier function of corneal (A, B) and conjunctival (C, D) epithelial cells quantified using TEER. \* $P < 0.05$  compared to control (cells exposed to media containing no mannitol and regular 5 mM glucose).

time PCR quantification of gene expression demonstrated that glucose exposure caused a  $2.33 \pm 0.4$ -fold increase at 15 mM and a  $1.88 \pm 0.3$ -fold increase at 30 mM in the gene expression of claudin-1 in corneal epithelial cells after 72 hours (Fig. 3A). Glucose exposure at 15mM also caused a  $4.8 \pm 0.3$ -fold increase in claudin-1 gene expression in conjunctival epithelial cells (Fig. 3D).

Mannitol also did not cause any notable change in claudin-1 protein expression, and as was noted with glucose, mannitol caused a significant increase in claudin-1 gene expression in both cell lines after 72 hours (Figs. 3B, 3E).

Previous studies have reported the detection of claudin-2 and claudin-3 transcripts in human corneal and conjunctival epithelial cells.<sup>1</sup> Our data confirm these previous findings<sup>1</sup> and demonstrate the presence of claudin-2 and claudin-3 transcripts in human corneal and conjunctival epithelial cells. Additionally, our data show that high glucose exposure did not cause any notable change in the gene expression of claudin-2 in human corneal and conjunctival epithelial cells (Fig. 4, left panel). On the other hand, 15-mM glucose exposure for 72 hours caused a  $2.1 \pm 0.66$ -fold and  $1.17 \pm 0.2$ -fold increase in the gene expression of claudin-3 in corneal and conjunctival epithelial cells, respectively (Fig. 4, right panel). High mannitol exposure caused a significant increase in the gene expression of both claudin-2 and claudin-3 in both the

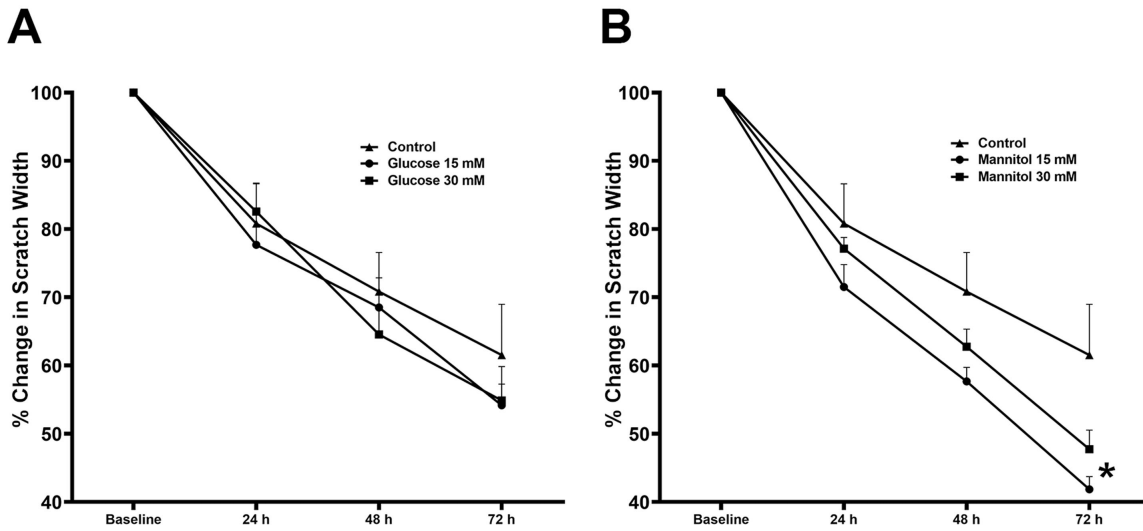
corneal and conjunctival epithelial cells (Fig. 4). However, Western blot quantification did not detect protein expression of claudin-2 and claudin-3 in human corneal or conjunctival epithelial cells, and our data are supported by a previous report that did not detect claudin-2 and claudin-3 in human corneal and conjunctival epithelial cells using immunostaining.<sup>1</sup>

### Effect of High Glucose on ZO Family Gene and Protein Expression

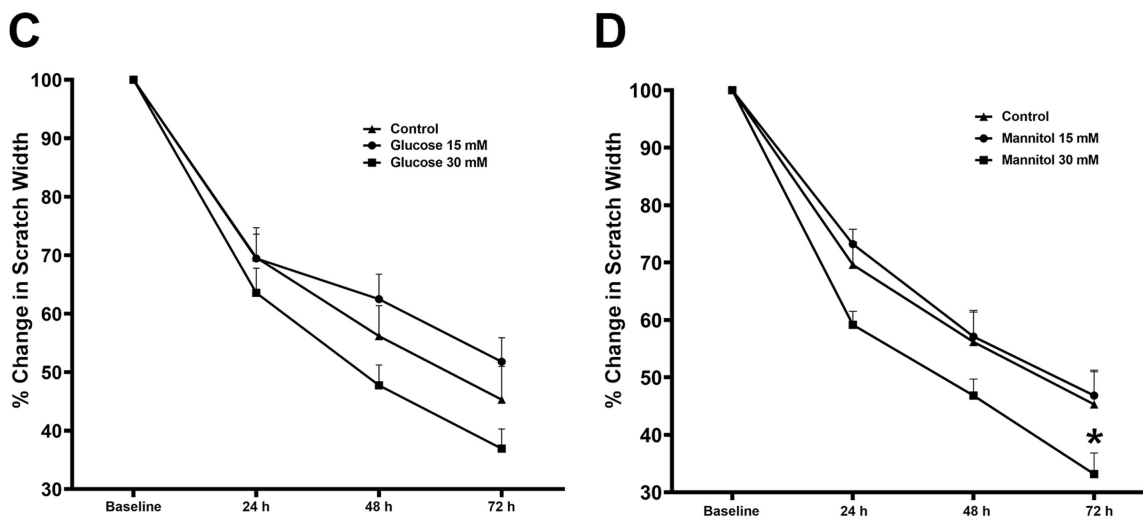
The ZO family of proteins tethers the cytoplasmic chain of claudin proteins to the cytoskeletal actin filaments.<sup>3-5</sup> Therefore, we next tested the effect of high glucose exposure on the ZO family of proteins. Glucose exposure at either of the tested concentrations did not modulate the ZO-1 gene or protein expression in corneal (Figs. 5A, 5C) real time PCR and conjunctival epithelial cells (Figs. 5D, 5F) as quantified by Western blotting, respectively. Just like glucose, mannitol also did not have any notable effect on the protein or gene expression of ZO-1 in corneal (Figs. 5B, 5C) or conjunctival epithelial cells (Figs. 5E, 5F).

Next, high glucose exposure did not modulate the protein expression of ZO-2 also in either corneal (Fig. 6C) or

**Scratch Assay: Corneal Epithelial Cells**



**Scratch Assay: Conjunctival Epithelial Cells**



**FIGURE 2.** Effect of glucose (15 mM; 30 mM) and mannitol (15 mM; 30 mM) on cellular migration of corneal (A, B) and conjunctival (C, D) epithelial cells quantified using scratch assay. \**P* < 0.05 compared to control (cells exposed to media containing no mannitol and regular 5 mM glucose).

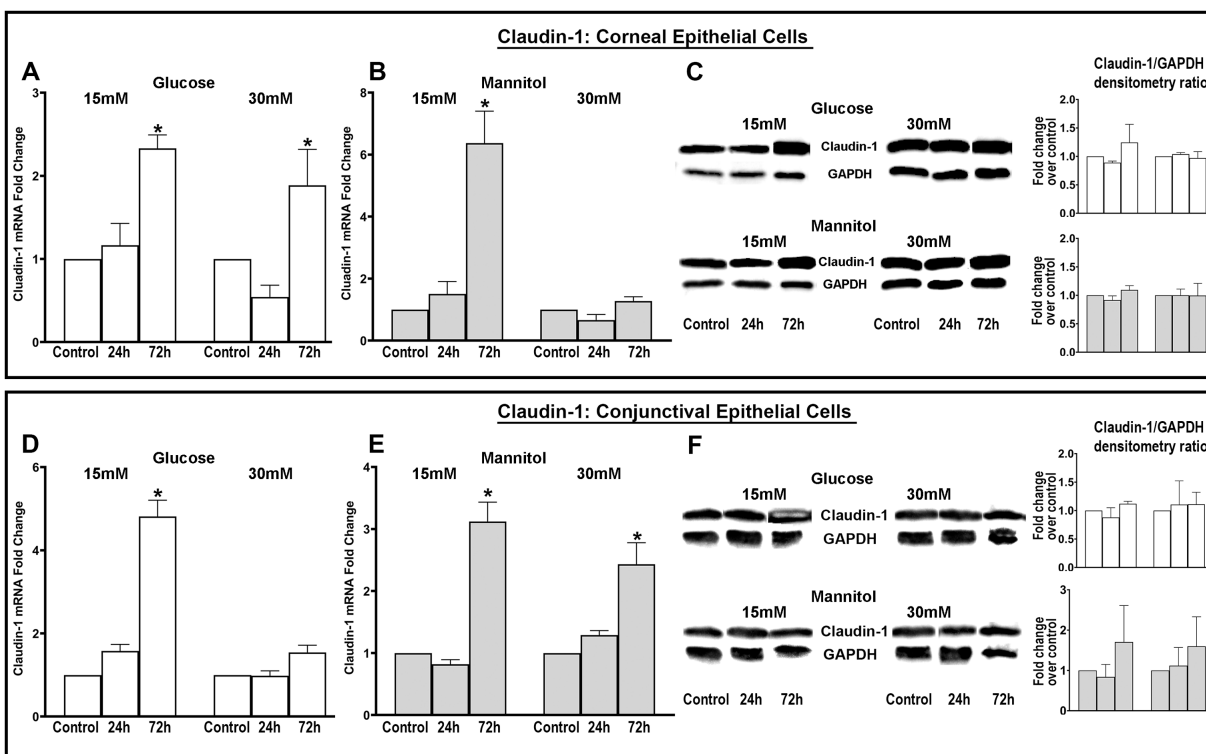
conjunctival epithelial cells (Fig. 6F). However, 15 mM glucose caused a  $2.3 \pm 1.3$ -fold and  $3.7 \pm 0.3$ -fold increase in the gene expression of ZO-2 in corneal (Fig. 6A) and conjunctival epithelial cells (Fig. 6D), respectively, after a 72-hour exposure. In congruence with the effects noted with glucose, mannitol also did not cause any change in the protein expression of ZO-2 but caused a notable increase in the gene expression of ZO-2 in conjunctival epithelial cells (Fig. 6E).

Lastly, high glucose did not cause any notable change in the protein expression of ZO-3 as well in either corneal (Fig. 7C) or conjunctival epithelial cells (Fig. 7F) but at a 15-mM concentration did cause a  $3.7 \pm 0.4$ -fold and  $4.7 \pm 0.15$ -fold increase in ZO-3 gene expression in corneal (Fig. 7A) and conjunctival epithelial cells (Fig. 7D), respectively, after a 72-hour exposure. As noted with glucose, mannitol

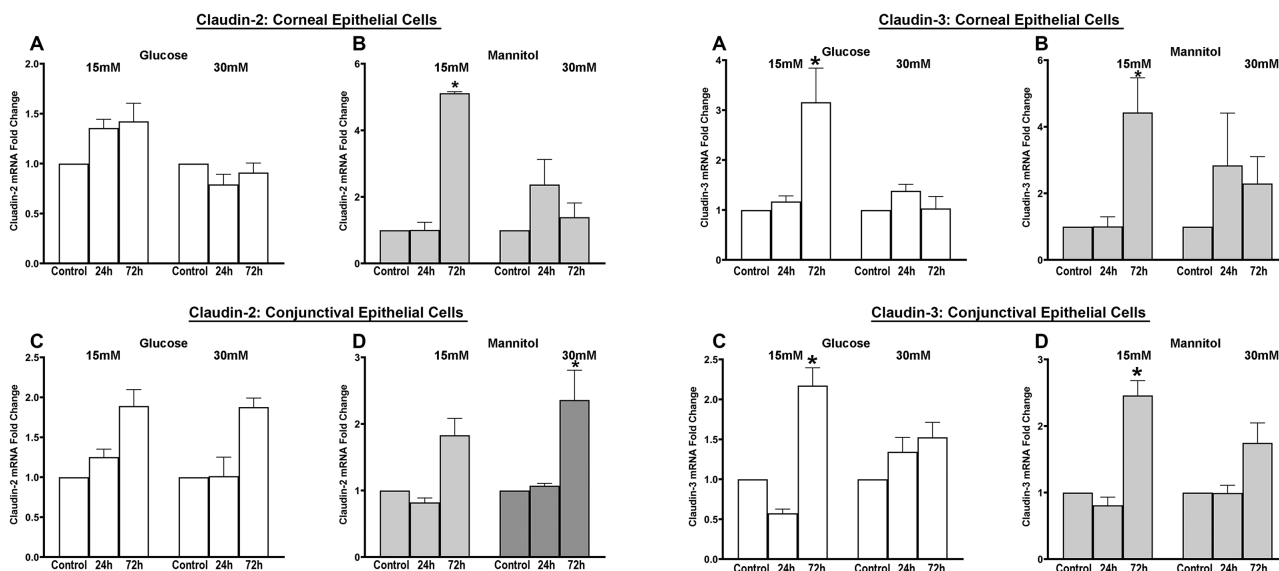
also did not cause any change in the protein levels of ZO-3 but caused an increase in the gene expression of ZO-3 in the corneal (Fig. 7B) and conjunctival epithelial cells (Fig. 7E).

**Effect of High Glucose on Occludin Gene and Protein Expression**

Just like claudin-1 and ZOs, high glucose did not cause any significant change in occludin protein expression either in corneal (Fig. 8C) or conjunctival epithelial cells (Fig. 8F), but 15 mM glucose caused a  $3.2 \pm 0.2$ -fold and  $2.67 \pm 0.17$ -fold increase in the gene expression of occludin in corneal (Fig. 8A) and conjunctival epithelial cells (Fig. 8D), respectively, after a 72-hour exposure.



**FIGURE 3.** Effect of glucose (15 mM; 30 mM) and mannitol (15 mM; 30 mM) on claudin-1 gene and protein expression in corneal (A–C) and conjunctival (D–F) epithelial cells quantified using real-time PCR and Western blot. \**P* < 0.05 compared to control (cells exposed to media containing no mannitol and regular 5 mM glucose).



**FIGURE 4.** Effect of glucose (15 mM; 30 mM) and mannitol (15 mM; 30 mM) on claudin-2 (left panel) and claudin-3 (right panel) gene expression in corneal and conjunctival epithelial cells quantified using real-time PCR. \**P* < 0.05 compared to control (cells exposed to media containing no mannitol and regular 5 mM glucose).

Mannitol also did not affect the protein expression of occludin in either cell line but caused a significant increase in the gene expression of occludin in conjunctival epithelial cells at a 15-mM concentration after 72 hours of exposure (Fig. 8E).

**DISCUSSION**

Experimental diabetes mellitus and high glucose exposure have been shown to cause an impairment of barrier function.<sup>23–29</sup> A recent study reported a decrease in TEER in human corneal epithelial cells exposed to 30 mM glucose.<sup>30</sup>



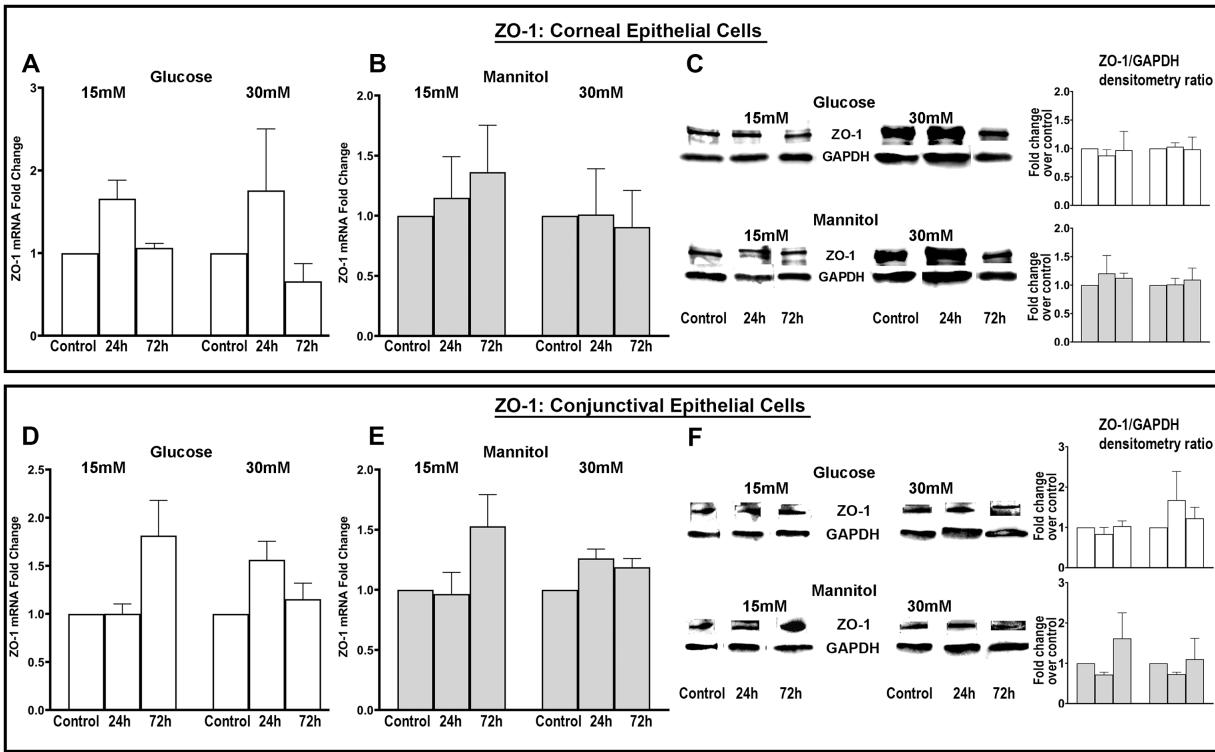


FIGURE 5. Effect of glucose (15 mM; 30 mM) and mannitol (15 mM; 30 mM) on ZO-1 gene and protein expression in corneal (A–C) and conjunctival (D–F) epithelial cells quantified using real-time PCR and Western blot.

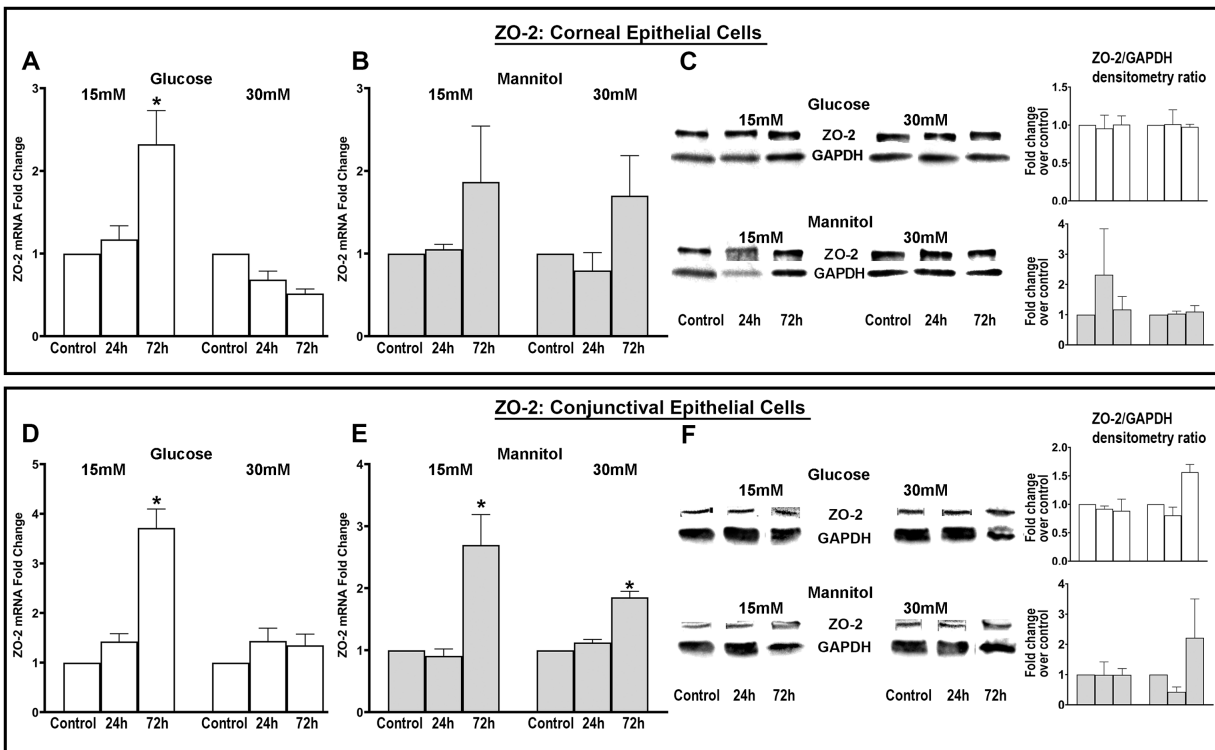


FIGURE 6. Effect of glucose (15 mM; 30 mM) and mannitol (15 mM; 30 mM) on ZO-2 gene and protein expression in corneal (A–C) and conjunctival (D–F) epithelial cells quantified using real-time PCR and Western blot. \* $P < 0.05$  compared to control (cells exposed to media containing no mannitol and regular 5 mM glucose).

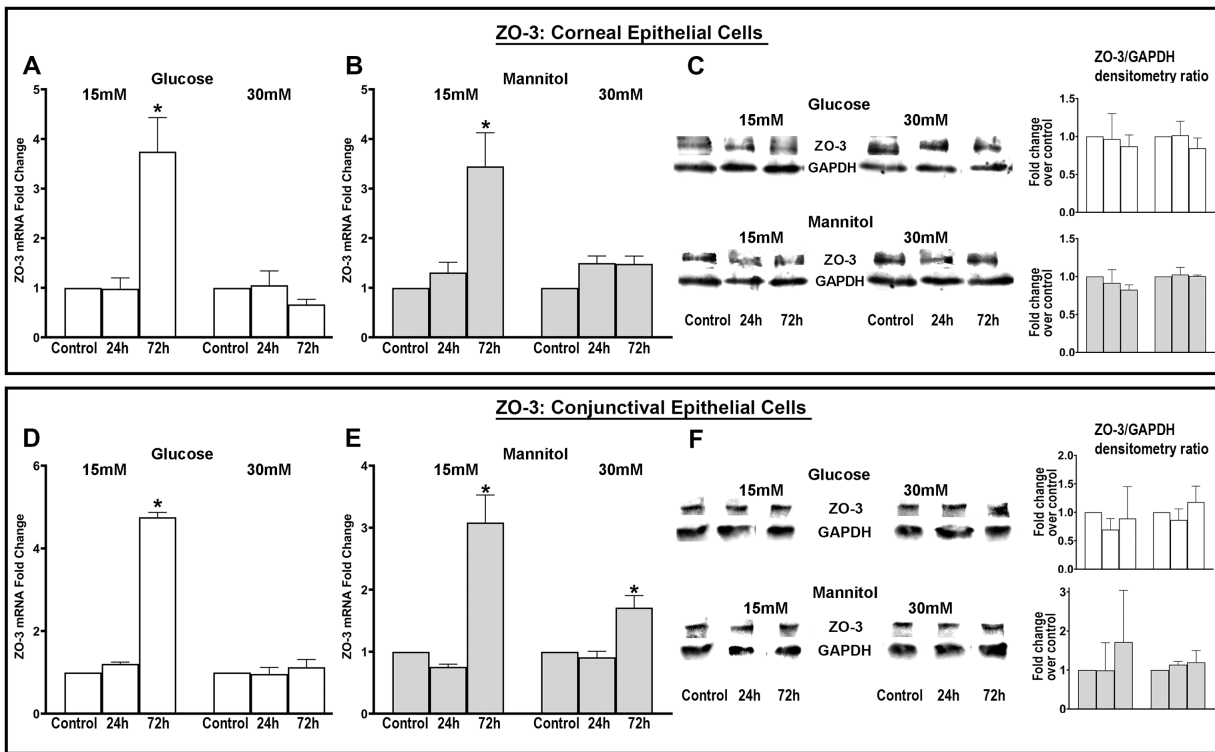


FIGURE 7. Effect of glucose (15 mM; 30 mM) and mannitol (15 mM; 30 mM) on ZO-3 gene and protein expression in corneal (A–C) and conjunctival (D–F) epithelial cells quantified using real-time PCR and Western blot. \* $P < 0.05$  compared to control (cells exposed to media containing no mannitol and regular 5 mM glucose).

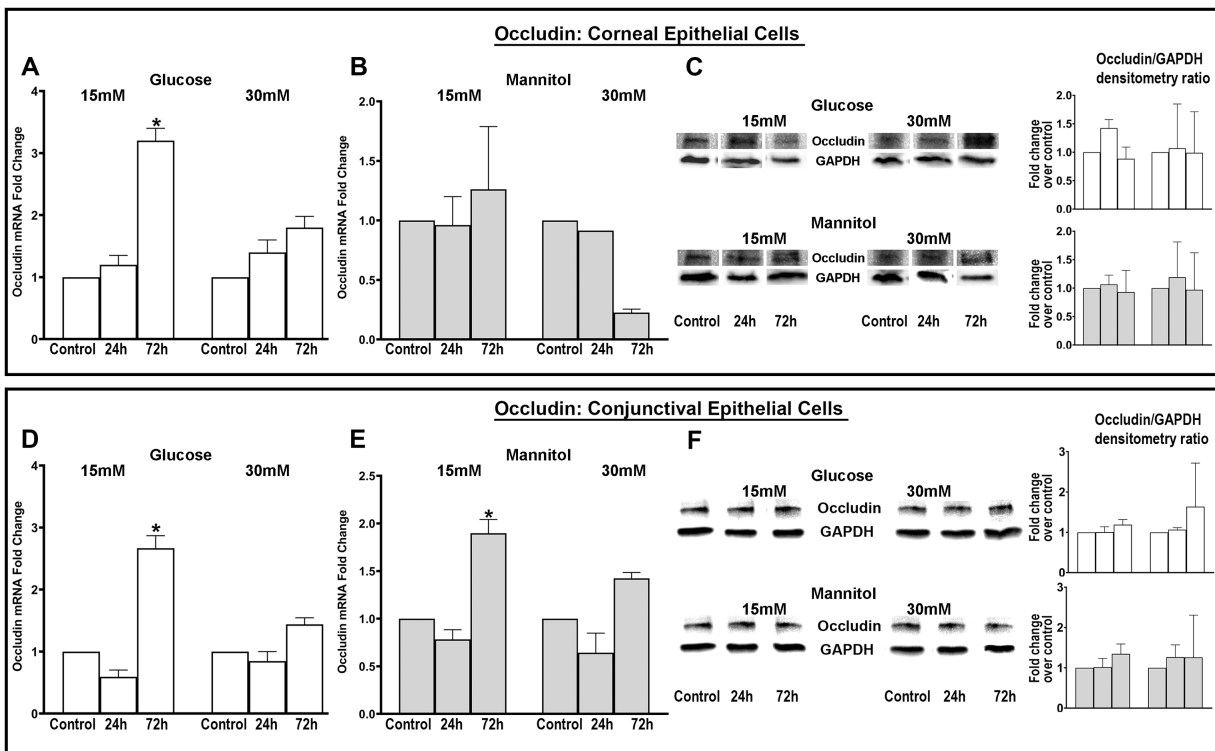


FIGURE 8. Effect of glucose (15 mM; 30 mM) and mannitol (15 mM; 30 mM) on occludin gene and protein expression in corneal (A–C) and conjunctival (D–F) epithelial cells quantified using real-time PCR and Western blot. \* $P < 0.05$  compared to control (cells exposed to media containing no mannitol and regular 5 mM glucose).

Our data are in agreement with this report<sup>30</sup> and additionally demonstrate that corneal epithelial barrier function can also be compromised by moderately high glucose (15 mM). Furthermore, our results also show that high glucose compromises human conjunctival epithelial cell barrier function too. It is worth noting that both 15 mM and 30 mM glucose caused an almost equivalent decrease in TEER in both corneal and conjunctival epithelial cells but only after 72 hours of exposure. These observations tentatively suggest that the detrimental effects of high glucose exposure are likely dependent on the duration of exposure rather than the concentration. Corneal and conjunctival epithelial cells are continually replenished by cells migrating from limbus. A literature survey reveals that the effect of high glucose exposure on corneal epithelial cell migration is equivocal with studies reporting a delay, an augmentation, or no change.<sup>30–32</sup> Variables such as the use of SV40, telomerase-immortalized, or primary cell types; inclusion/exclusion of serum; and variable epidermal growth factor (EGF) concentrations used in these studies likely contribute to the reported equivocal effect of high glucose exposure on corneal epithelial cell migration. However, our data demonstrate that high glucose exposure does not significantly affect the migration of corneal or conjunctival epithelial cells.

Tight junctions play a crucial role in maintaining cellular barrier function and are composed of claudins, occludin, junctional adhesion molecule, and tricellulin.<sup>3–5</sup> Corneal and conjunctival epithelial cells have been shown to express claudin-1, but protein expression of claudin-2 and claudin-3 could not be detected in human corneal and conjunctival epithelial cells.<sup>1</sup> In accordance with these observations, our results also show the presence of claudin-1 in corneal and conjunctival epithelial cells but did not detect claudin-2 and claudin-3 protein expression. The PDZ domains in claudins interact with the intracellular ZO molecules, which are further connected to cytoskeletal proteins for anchoring function.<sup>6,7</sup> Occludin in the tight junctions acts as a zipper to hold the adjacent cells.<sup>33</sup> Therefore, we tested whether alterations in tight junction proteins claudin-1, ZOs, and occludin may be responsible for high glucose-mediated impairment of ocular surface epithelial cell barrier function. Qualitative immunostaining data in a previous study show that high glucose decreases ZO-1 protein levels in human corneal epithelial cells. In contrast to this study, quantitative Western blot analysis used in the present study demonstrated that high glucose exposure does not cause any notable decrease in ZO-1 in corneal or conjunctival epithelial cells. Besides ZO-1, the present study is the first one to demonstrate that high glucose exposure does not affect the protein levels of claudin-1, ZO-2, ZO-3, and occludin in human corneal and conjunctival epithelial cells. Therefore, a decrease in these tested tight junction proteins does not seem the likely underlying cause for high glucose-mediated compromise of ocular surface epithelial cell barrier function. Our data are further supported by similar results noted in retinal pigmented epithelial cells where high glucose caused a decrease in the barrier function but without causing any change in claudin or occludin proteins.<sup>29</sup> Besides claudin-1, human corneal and conjunctival epithelial cells have also been reported to express other claudins such as claudin-4 and claudin-10.<sup>1</sup> It may be possible that a high glucose-mediated decrease in claudin-4 or claudin-10 may contribute to the noted decrease in barrier function and may be addressed by future studies. The role of junctional adhesion molecule and tricellulin in

barrier functions of tight junctions is not completely understood yet, it is possible that high glucose may impact these proteins. Lastly, our study did not test the impact of high glucose on the chemical or conformational changes of tight junction proteins. Such alteration can also contribute to high glucose-mediated compromise of barrier function without causing any decrease in the protein levels.

In the current study, we also observed a highly consistent increase in the gene expression of claudin-1, ZO-2, ZO-3, and occludin in both corneal and conjunctival epithelial cells when exposed to 15 mM glucose for 72 hours. This observed increase in the gene expression of the tight junction proteins may be a compensatory cellular response to mild or moderate osmotic stress since 15 mM mannitol also caused consistently similar changes as observed with 15 mM glucose. It is hard to explain why 30 mM glucose or mannitol did not cause any increase in gene expression, but it may be possible that high osmotic stress may initiate a diverse set of compensatory responses.

In summary, the results of the present study demonstrate that high glucose exposure causes impairment of corneal and conjunctival epithelial cell barrier function, but this detrimental effect is not caused by a decrease in the expression of tight junction proteins, including claudin-1, ZO-1, ZO-2, ZO-3, and occludin.

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