• Basic Research •

Hyperosmolarity disrupts tight junction *via* TNF-α/MMP pathway in primary human corneal epithelial cells

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Abstract

● **AIM:** To investigate the mechanism of the tight junction (TJ) disruption and the association between tumor necrosis factor (TNF)-α and matrix metalloproteinase (MMPs) under hyperosmotic condition in primary human corneal epithelial cells (HCECs).

● **METHODS:** The cultured HCECs were exposed to media which adding sodium chloride (NaCl) for hyperosmolar stress or adding rh-TNF-α (10 ng/mL). NF-κB inhibitor (5 μmol/L) or GM-6001 (potent and broad spectrum MMP inhibitor, 20 μmol/L) was added 1h before that treatment. The integrity of TJ proteins was determined by immunofluorescent (IF) staining. The mRNA levels of TNF-α and MMPs were evaluated by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and the protein expression by enzyme-linked immunosorbent assay (ELISA).

● **RESULTS:** TJ proteins ZO-1 and Occludin were disrupted in primary HCECs exposed to hyperosmotic medium. The mRNA expression and protein production of TNF-α increased significantly in hyperosmotic media at 500 mOsM. TNF-α mediated the expression and production of MMP-1, MMP-13, MMP-9, and MMP-3 stimulated by hyperosmotic stress. The production of MMPs in hyperosmolar media were increased through the increase of TNF-α. GM-6001 prevent the destruction of ZO-1 and Occludin in hyperosmolar stress and rh-TNF-α treated medium. TNF-α induced activation of MMPs was involved in the TJ disruption by hyperosmolarity.

● **CONCLUSION:** TJ proteins ZO-1 and Occludin are disrupted by hyperosmolar stress and TNF-α, but protected by MMP inhibitor (GM-6001). It suggests that TNF-α/MMP pathway mediates the TJ disruption in primary HCECs exposed to hyperosmotic stress.

● **KEYWORDS:** dry eye; hyperosmolarity; cornea epithelium; tight junction
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INTRODUCTION

Dry eye disease is a developing health problem resulting in serious impacts on general state of health, functional vision, economy and life quality[1-3]. The primary pathogenesis of dry eye disease contains, tear film flimsiness, causing and went with by tear hyperosmolarity, driving to epithelial inflammation and damage[1,4-8]. Recent evidences have documented that the levels of pro-inflammatory cytokines and chemokines increase in the tear fluid of dry eye[7,9-11]. In dry eye conditions tear clearance decreases, which can also cause increased concentrations of proinflammatory cytokines; whereas before these studies, dry eye disease was thought to be caused simply by insufficient tear production. Nowadays, dry eye disease is also known as an inflammatory disorder.

Tear film disorders are followed by matrix metalloproteinase (MMP) intervened corneal disease[12]. Collagenases (MMP-1, MMP-13), gelatinases (MMP-9), and stromelysins (MMP-3) have been recommended to play a critical part within the disruption of corneal epithelium under hyperosmolar model[13-14].

In ocular surface, the corneal epithelium forms a barrier to noxious stimuli and prevents foreign material within the outside environment[15]. Four kinds of junctions have been identified to form this barrier, including tight junction (TJ), desmosomes, adherens junctions, and gap junctions[16]. The foremost layer is the TJ between adjoining cells. The TJ proteins ZO-1 and Occludin form the very first barrier for the eyeball. Recent studies have found that MMP-9 is able to disrupt one of the proteins, Occludin[14]. TNF-α has been found to induce the destruction of ZO-1 from the neighboring human corneal epithelial cells (HCECs)[17]. Disruption of these TJ proteins leads to numerous pathological situations, resulting in infection, or melting of the cornea[18].

But the physiological mechanism of the disruption of the TJ proteins, as well as the association between tumor necrosis factor (TNF)-α and MMPs under hyperosmotic dry eye condition, remains unclear.

HCECs are the most broadly used models for studies of dry eye disease[19]. Hyperosmolar stress is widely applied to these primary cells to mimic dry eye hyperosmotic conditions[7,11,20-22]. The latest studies showed that dry eye disease models were treated with hyperosmotic saline solution in 500 mOsM/L both *in vitro* and *in vivo*[11]. We hypothesized that the TJ may be disrupted in hyperosmolarity dry eye condition by inflammation, and we used nuclear factor-κB (NF-κB) inhibitor and MMP inhibitor to identify the roles of TNF-α and MMPs in this process.

MATERIALS AND METHODS

HCECs Culture Fresh corneoscleral tissues of human (<72h after death), donors aged 18-70 years old, after the central cornea had been used for keratoplasty. Primary HCECs (Figure 1A) were cultured on 8-chamber slides for immunofluorescence (IF) staining and in 12-well plates for RNA extraction or enzyme-linked immunosorbent assay (ELISA) for cytokine proteins. Primary corneal epithelial cells migrate from limbus. We used the explants from corneal limbus in supplemented hormonal epidermal medium (SHEM) with 5% fetal bovine serum (FBS) according to the protocol of previous publications[23-25].

Treatment Primary HCECs cultured for 14-19d were exchanged with serum-free medium for 24h, then adding 44, 69 or 94 mmol/L sodium chloride (NaCl) for hyperosmolar stress group (400, 450 and 500 mOsM) or adding rh-TNF-α (10 ng/mL) as TNF-α group. NF-κB inhibitor (5 μmol/L) was added 1h before that treatment (500 mOsM or TNF-α group) to antagonize the effect of TNF-α. MMP inhibitor GM-6001 (20 μmol/L) was also added 1h before that treatment (500 mOsM or TNF-α group) to antagonize the effect of MMP.

HCECs treated for 4h were used for mRNA expression. HCECs treated for 24h were used for IF staining or ELISA.

Quantitative Reverse Transcription Polymerase Chain Reaction After treatments, total RNA was extracted using extraction kit (RNeasy Micro RNA kit) with protocol. RNA concentrations were measured with NanoDrop® ND-2000 Spectrophotometer. Reverse transcription was performed by RT using Ready-To-Go You-Prime First-Strand Beads. (25℃ for 10min and 37℃ for 60min). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed essentially as described in detail previously[26]. In short, 50℃ for 2min and 95℃ for 10min, 40 cycles of 95℃ for 15s and 60℃ for 1min. TaqMan gene expression assays of primer were: GAPDH (Hs99999905_m1), MMP-1 (Hs00899658_m1), MMP-13 (Hs00942589_m1), MMP-9 (Hs00234579_m1), MMP-3 (Hs00233962_m1), TNF-α (Hs00174128_m1).

Enzyme-Linked Immunosorbent Assay The cell culture supernatants were collected after different treatments[27]. ELISA for TNF-α and MMPs (BioLegend, San Diego, CA, USA) were performed by manufacturers' protocols and previously reported methods[24]. Absorbance was studied at 450 nm and 570 nm using Infinite M200 microplate reader (Tecan US, Inc., Morrisville, NC, USA).

Immunofluorescence Staining The cultured HCECs on 8-chamber slides were exchanged with FBS-free SHEM and were treated with 90 mmol/L of NaCl to make hyperosmolar media (500 mOsM) for 24h, and the untreated media served as control group (312 mOsM). MMP inhibitor GM-6001 (20 μmol/L) was added 1h before. Cells on slides were fixed in cold acetone (for TJ proteins ZO-1, Occludin staining), then blocked with 20% goat serum (Abcam, Cambridge, MA, USA). Slides were then incubated at 4℃ overnight with rabbit anti-mouse ZO-1 and Occludin antibodies (Invitrogen, dilution 1:200), then incubated with secondary antibodies and DAPI for one hour.

Statistical Analyses Data analysis was done using GraphPad Prism 8.0.2 (California, USA). Student's *t*-test was used to infer statistical significance between two groups. $P<0.05$ were considered to be statistically significant.

RESULTS

ZO-1 and Occludin in HCECs Exposed to Hyperosmotic Medium To evaluate the subcellular distribution of TJ proteins in dry eye, hyperosmotic media at 500 mOsM was used in HCECs. IF staining was done to evaluate the integrity of major TJ proteins, ZO-1 and Occludin. As shown in Figure 1B, the immunoreactivities staining of these two proteins showed a contiguous network morphology in the untreated (UT) group (SHEM with iso-osmolarity 312 mOsM).

However, these two junction proteins were significantly disrupted when exposed to hyperosmotic media at 500 mOsM after 24h, appearing their inadequate connections and irregular staining of the damaged network between cells.

TNF-α Expression in HCECs Exposed to Hyperosmotic Medium As assessed by RT-qPCR (Figure 2A), TNF-α mRNA level was osmolarity-dependently upregulated to 4.11±1.67 ($P<0.05$), 11.98±4.19 ($P<0.05$) and 17.17±5.12 ($P<0.01$) fold, respectively, when HCECs were exposed to different hyperosmolar media (400, 450 or 500 mOsM) compared with UT group. To quantify the protein levels of TNF-α in HCECs, ELISA was used. TNF-α levels in the supernatants increased significantly from 28.80±10.84 pg/mL in UT group to 189.49±27.12 pg/mL ($P<0.0001$) in hyperosmolar stress group (500 mOsM; Figure 2B).

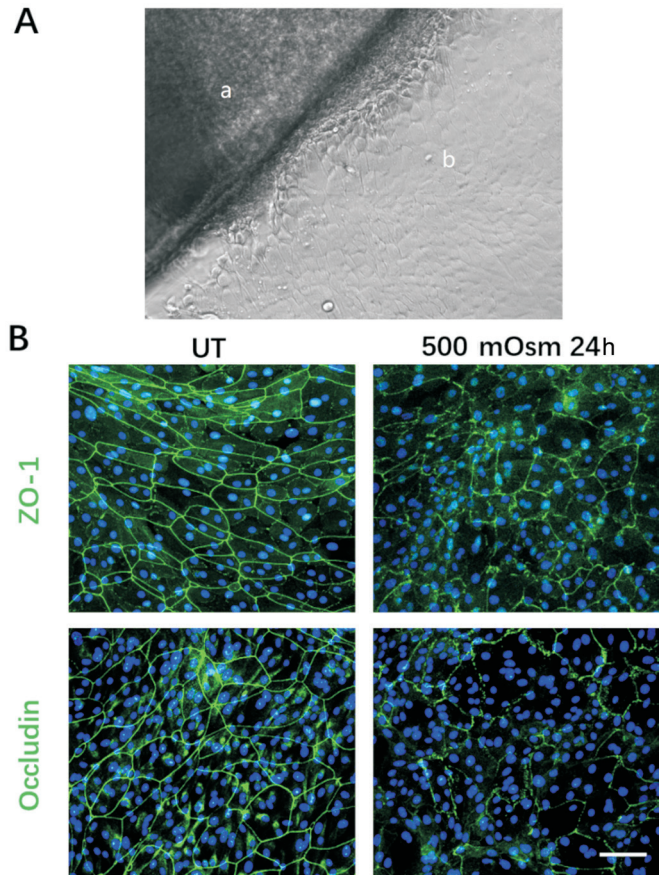

Figure 1 The changes of TJ proteins (ZO-1 and Occludin) in HCECs exposed to hyperosmotic medium (500 mOsm) A: Fresh corneoscleral tissue of human (a), cultured primary HCECs (b). B: ZO-1, and Occludin showed contiguously network morphology in UT group (SHEM with iso-osmolarity 312 mOsM). These two junction proteins were significantly disrupted when exposed to hyperosmotic media at 500 mOsM after 24h appearing their inadequate connections and the irregular staining of damaged network between cells (bar=100 μmol/L). TJ: Tight junction; HCECs: Human corneal epithelial cells; UT: Untreated.

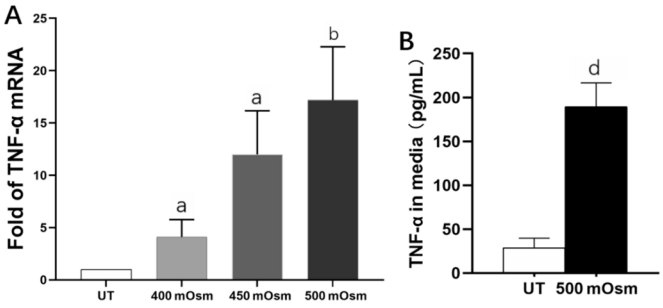

Figure 2 TNF-α increased in HCECs by hyperosmotic medium A: The mRNA expression of TNF-α increased by hyperosmolarity with osmolarity-dependent manner (400, 450, and 500 mOsM); B: The protein level of TNF-α increased significantly in hyperosmotic media at 500 mOsM. [a]*P*<0.05, [b]*P*<0.01, [d]*P*<0.0001. TNF-α: Tumor necrosis factor; HCECs: Human corneal epithelial cells; UT: Untreated.

Collagenases, Gelatinase, and Stromelysin Expression in HCECs Stimulated by Hyperosmotic Stress HCECs were treated with either hyperosmolar media (500 mOsM) or rh-TNF-α (10 ng/mL) for 4h or 24h with or without NF-κB inhibitor (5 μmol/L) added 1h before.

The mRNA level of MMP-1 increased to 4.80±0.47 and 3.96±0.65 fold, respectively, when exposed to hyperosmolar stress (500 mOsM) and rh-TNF-α (*P*<0.0001, *P*<0.001). MMP-1 level decreased, with addition of NF-κB inhibitor, to 2.05±0.23 fold (*P*<0.001, 500 mOsM group) and 1.75±0.44 fold (*P*<0.001, TNF-α group). The protein level of MMP-1 in HCECs increased from 21.39±5.63 to 73.55±10.46 and 64.03±12.39 ng/mL (*P*<0.005, *P*<0.01) when exposed to hyperosmolar stress (500 mOsM) and rh-TNF-α, but decreased to 26.79±6.93 (*P*<0.005, 500 mOsM group) and 31.51±7.81 ng/mL (*P*<0.001, TNF-α group) with addition of NF-κB inhibitor (Figure 3A). The mRNA level of MMP-13 increased to 8.05±0.81 and 6.49±0.58 fold, when exposed to hyperosmolar stress (500 mOsM) and rh-TNF-α, (both *P*<0.0001), but decreased to 2.62±0.45 fold (*P*<0.001, 500 mOsM group) and 2.79±0.40 fold (*P*<0.0001, TNF-α group) with addition of NF-κB inhibitor. The MMP-13 protein level increased from 159.82±25.00 pg/mL to 966.53±101.79 and 806.19±143.41 pg/mL (*P*<0.001, *P*<0.005) when exposed to hyperosmolar stress (500 mOsM) and rh-TNF-α, but decreased to 255.18±57.87 pg/mL (*P*<0.001, 500 mOsM group) and 367.49±74.03 pg/mL (*P*<0.005, TNF-α group) with addition of NF-κB inhibitor (Figure 3B).

The mRNA level of MMP-9 increased to 4.68±0.71 and 3.85±0.74 fold, when exposed to hyperosmolar stress (500 mOsM) and rh-TNF-α (*P*<0.001, *P*<0.005), but decreased to 2.08±0.36 fold (*P*<0.001, 500 mOsM group) and 2.40±0.44 fold (*P*<0.005, TNF-α group) with addition of NF-κB inhibitor. The MMP-9 protein level increased from 7.43±1.55 pg/mL to 24.86±2.82 and 25.15±3.55 pg/mL (*P*<0.001, *P*<0.001) when exposed to hyperosmolar stress (500 mOsM) and rh-TNF-α, but decreased to 9.80±2.10 pg/mL (*P*<0.0001, 500mOsM group) and 8.95±2.33 pg/mL (*P*<0.001, TNF-α group) with addition of NF-κB inhibitor (Figure 3C).

The mRNA level of MMP-3 increased to 4.90±0.60 and 4.06±0.42 fold, when exposed to hyperosmolar stress (500 mOsM) and rh-TNF-α (*P*<0.001, *P*<0.0001), but decreased to 2.25±0.60 fold (*P*<0.0001, 500 mOsM group) and 2.00±0.64 fold (*P*<0.0001, TNF-α group) with addition of NF-κB inhibitor. The MMP-3 protein level increased from 19.54±5.32 pg/mL to 87.61±15.20 and 73.83±12.03 pg/mL (*P*<0.001, *P*<0.001) when exposed to hyperosmolar stress (500 mOsM) and rh-TNF-α, but decreased to 39.47±7.35 pg/mL (*P*<0.01, 500 mOsM group) and 33.16±7.51 pg/mL (*P*<0.01, TNF-α group) with addition of NF-κB inhibitor (Figure 3D).

NF-κB is a key mediator of TNF-α actions. The effect of TNF-α was antagonized by NF-κB inhibitor (NF-κB-I). The

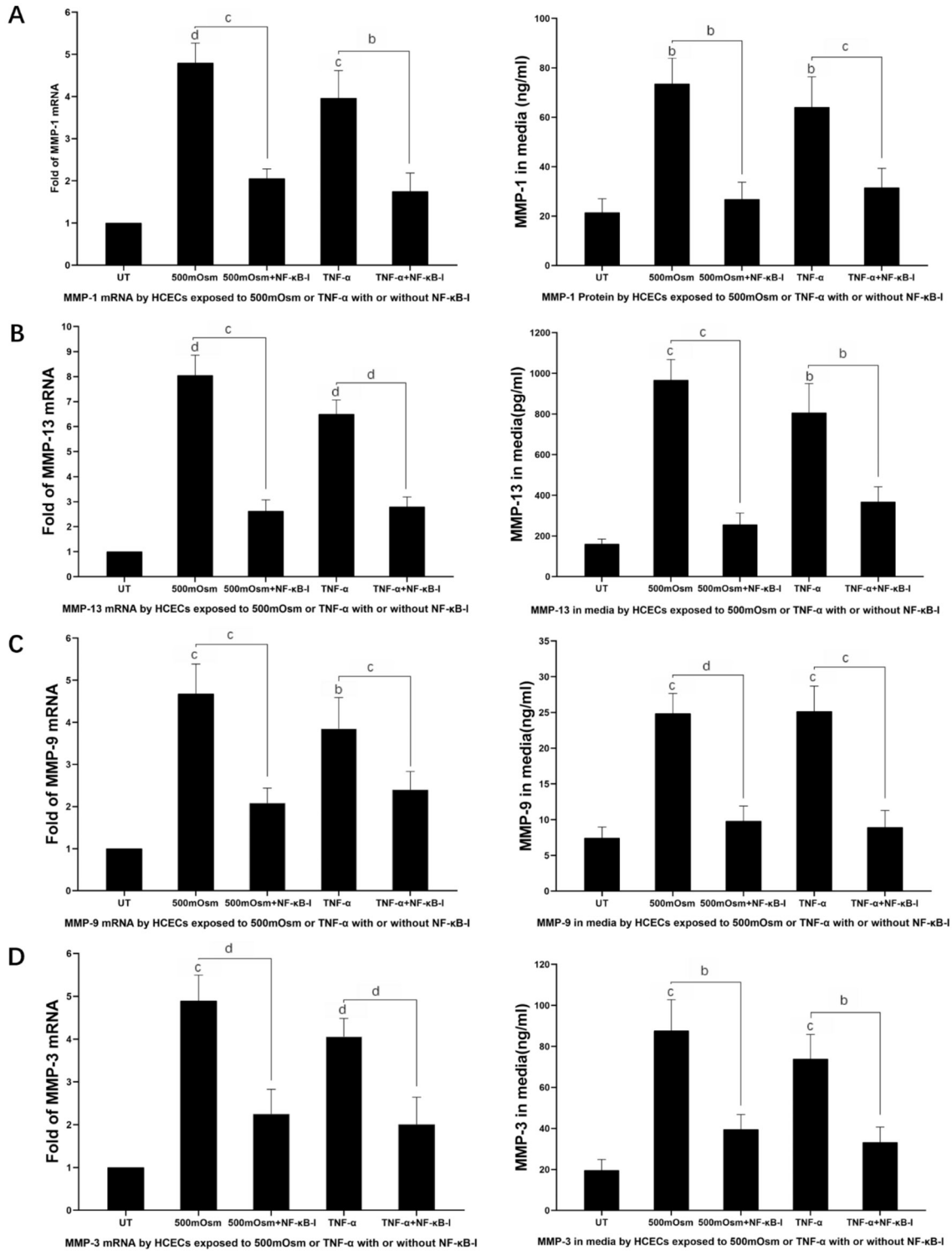

Figure 3 The productions of MMP-1 (A), MMP-13 (B), MMP-9 (C) and MMP-3 (D) increased at both mRNA level and protein expression in hyperosmolar stress When in rh-TNF-α treated medium, MMPs also increased. These stimulated MMPs were suppressed by NF-κB inhibitor (NF-κB-I). NF-κB inhibitor was added 1h before. $^{a}P<0.05$; $^{b}P<0.01$, $^{c}P<0.001$, $^{d}P<0.0001$, compared with hyperosmolar stress (500 mOsM) or rh-TNF-α group. MMP: Matrix metalloproteinase; UT: Untreated.

levels of MMPs increased significantly when HCECs were exposed to hyperosmolar stress. The expression levels of these MMPs also increased in HCECs treated with rh-TNF-α. The stimulated MMPs by hyperosmolarity and TNF-α were largely suppressed by NF-κB inhibitor.

From these results, we infer that TNF-α mediated the mRNA and protein level of MMPs stimulated by hyperosmotic stress. That hyperosmolarity stimulates the level of MMPs through the increased expression of TNF-α.

Activation of MMPs by TNF-α Involved in Tight Junction Disruption GM-6001 (potent and broad spectrum MMP inhibitor) protected cells from destruction of ZO-1 and

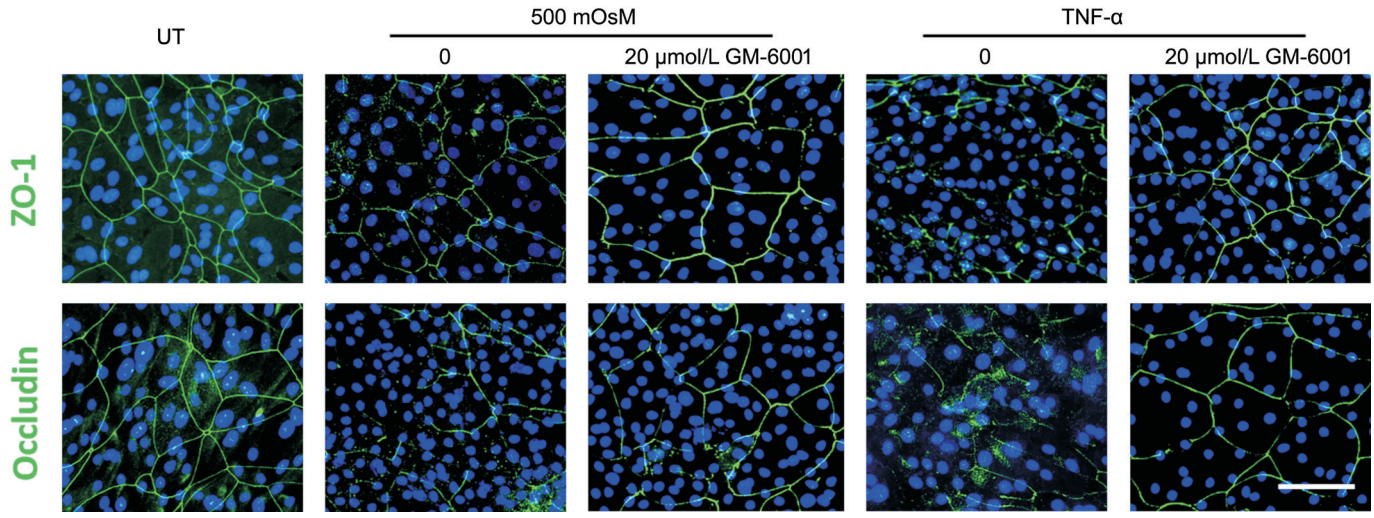

Figure 4 The structure of ZO-1 and Occludin proteins was disrupted when exposed to 500 mOsM or rh-TNF-α When GM-6001 (potent and broad spectrum MMP inhibitor) pre-added in 500 mOsM medium or rh-TNF-α treated medium, the disruption of ZO-1 and Occludin was prevented (bar=100 μmol/L). MMP: Matrix metalloproteinase; TNF-α: Tumor necrosis factor-α; UT: Untreated.

Occludin by hyperosmolar stress and rh-TNF-α.

When exposed to 500 mOsM or rh-TNF-α, the structure of ZO-1 and Occludin proteins was disrupted. However, 20 μmol/L of GM-6001 pre-added in 500 mOsM medium 1h before, restored most of those disruption. Interestingly, this phenomenon also happened in rh-TNF-α treated medium. When 20 μmol/L GM-6001 pretreated, the disruption of ZO-1 and Occludin was prevented in rh-TNF-α treated HCECs (Figure 4).

Taken together, these results suggest that hyperosmolarity disrupts the TJ proteins ZO-1 and Occludin *via* TNF-α/MMP pathway in HCECs.

DISCUSSION

The corneal epithelial barrier is formed by four kinds of junctions. Among them, the TJ is the major intercellular intersection structure, with ZO-1, Occludin, and others as essential component proteins. Studies showed that the TJ play an imperative role in protecting the ocular surface against adverse external conditions[17,28-30]. Another important role for TJ was the control of cell proliferation and gene expression[31].

We found that these important TJ proteins were significantly disrupted, and the net-structure stain of TJ proteins was destroyed when exposed to hyperosmotic media after 24h. However, the physiological mechanism of the disruption of the TJ proteins in hyperosmolarity condition is still elusive.

In dry eye conditions proinflammatory cytokines, such as TNF-α increased[9-10]. MMPs were proposed to play the important role in the disruption of corneal epithelium under hyperosmolar stress[13-14]. But the relationship between TNF-α and MMPs in the process is not clear.

We observed that TNF-α and MMPs both increased by hyperosmolar stress at mRNA and protein level. Interestingly, the stimulatory effect of MMPs was repressed by NF-κB inhibitor under hyperosmolar stress. NF-κB is a key mediator of TNF-α actions[32-33]. TNF-α activates the NF-κB signaling pathway in HCECs[17]. TNF-α production and activation of the NF-κB/IκBα pathway were measured to evaluate inflammatory effects *in vitro* dry eye disease model[34].

To confirm that TNF-α can cause the increase of MMPs, we added rh-TNF-α in medium and found that in rh-TNF-α treated HCECs, MMPs also increased. These stimulated MMPs were suppressed by NF-κB inhibitor. These findings reveal that the hyperosmolar stress stimulated production of MMPs was through increasing TNF-α expression.

Then we tried to determine if the disruption of TJ is in connection with this TNF-α/MMP pathway in HCECs under hyperosmolar stress. When exposed to 500 mOsM or rh-TNF-α treated medium, the integrity structure of ZO-1 and Occludin was disrupted. While, 20 μmol/L GM-6001 (MMP inhibitor) pre-added in 500 mOsM medium one hour before, prevented most of this disruption.

Interestingly, this phenomenon also occurred in rh-TNF-α treated medium. When 20 μmol/L GM-6001 pre-added in rh-TNF-α treated medium, the disruption of ZO-1 and Occludin was reduced, suggesting TJ disruption in hyperosmolar stress and TNF-α was protected by MMP inhibitor GM-6001. Taken together, hypertonicity-induced TNF-α upregulation leads to disruption of TJ proteins through MMP activation.

In conclusion, our findings demonstrate that hyperosmolarity disrupts the TJ proteins ZO-1 and Occludin *via* TNF-α/MMP pathway in HCECs, further suggesting that drugs targeting MMPs upregulation may be a protective option to the ocular surface in dry eye disease. Although, HCECs are the most broadly used models *in vitro* for studies of dry eye disease[19]. Some data from animal models or human patients would be

significant. However, animal experiments are proceeding very slowly at present, due to the pandemic of COVID-19. These findings would further validated using an animal model in our future work.

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