

## Original Article

# High osmotic pressure increases reactive oxygen species generation in rabbit corneal epithelial cells by endoplasmic reticulum

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Received October 28, 2015; Accepted December 4, 2015; Epub February 15, 2016; Published February 29, 2016

**Abstract:** Tear high osmotic pressure (HOP) has been recognized as the core mechanism underlying ocular surface inflammation, injury and symptoms and is closely associated with many ocular surface diseases, especially dry eye. The endoplasmic reticulum (ER) is a multi-functional organelle responsible for protein synthesis, folding and transport, biological synthesis of lipids, vesicle transport and intracellular calcium storage. Accumulation of unfolded proteins and imbalance of calcium ion in the ER would induce ER stress and protective unfolded protein response (UPR). Many studies have demonstrated that ER stress can induce cell apoptosis. However, the association between tear HOP and ER stress has not been studied systematically. In the present study, rabbit corneal epithelial cells were treated with HOP and results showed that the production of reactive oxygen species increased markedly, which further activated the ER signaling pathway and ultimately induced cell apoptosis. These findings shed new lights on the pathogenesis and clinical treatment of dry eye and other ocular surface diseases.

**Keywords:** High osmotic pressure, endoplasmic reticulum, endoplasmic reticulum stress, reactive oxygen species, apoptosis

## Introduction

Human tear film stability is essential for maintaining the normal structure and function of the ocular surface and plays an important role in maintaining a healthy visual function. Tear high osmotic pressure (HOP) is the core mechanism causing ocular surface injury. Measurement of tear osmotic pressure has become the gold standard for the diagnosis of dry eye [1]. Our previous study showed that HOP could increase the production of intracellular reactive oxygen species (ROS) in rabbit corneal epithelial cells (RCECs), which in turn regulated CD95/CD95L by activating the JNK signaling pathway and inducing cell apoptosis [2].

The endoplasmic reticulum (ER) is an intracellular organelle responsible for the protein folding and assembly. Various physiopathologic factors may induce the accumulation of unfolded proteins within ER, causing ER stress. To maintain homeostasis of protein synthesis within ER, eukaryotic cells undergo an unfolded

protein response (UPR) to protect ER [3]. When factors causing cell injury are not removed or further increase, and UPR is unable to maintain the balance of proteins and calcium within ER, the apoptosis pathway is activated, inducing cell apoptosis [4]. There have been studies reporting that reactive oxygen species (ROS) generation under various conditions is an event causing ER stress [5, 6]. Seo et al. [7] used transmission electron microscopy (TEM) to investigate the lacrimal acinar cells in a dry eye mouse model and found severe cystic expansion of ER in these mice as compared to normal mice. Western blot assay showed that the expressions of CHOP, eIF2 $\alpha$  and p-PERK were up-regulated significantly in the lacrimal acinar cells of these mice, indicating that there are UPR and ER stress in dry eye.

Our previous study showed that ROS generation increased in RCECs following HOP treatment. This study was to further explore the regulatory effect of ROS in the presence of HOP on the ER stress and evaluate the association between ER stress and cell apoptosis.

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## Materials and methods

### *Ethics statement*

The whole protocol was approved by the Animal Care Committees in School of Medicine of Tongji University. All the animal procedures were conducted in accordance with the Association of Research for Vision and Ophthalmology statement for the use of Animals in Ophthalmic and Vision Research. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize the suffering of animals.

### *Cell culture*

RCECs were cultured as described previously [2]. The epithelial cells of 1<sup>st</sup> or 2<sup>nd</sup> generation were used in the following experiments. Cells of 80% confluence were washed thrice with PBS and then maintained in a serum-free keratinocyte-SFM medium containing 100 U/ml penicillin and 100 mg/ml streptomycin overnight. All experiments were performed at least three times in triplicate.

### *Transfection*

RCECs were seeded into a 6-well plate at a density of  $4 \times 10^5$  cells/well and transfected with siRNA in the presence of Lipofectamine<sup>TM</sup> 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). When cells confluence reached 70%, the medium was removed and cells were washed with PBS twice, followed by addition of 1.5 ml of Opti-MEM (Invitrogen) to each well; 5  $\mu$ l of Lipofectamine<sup>TM</sup> 2000 was added to 250  $\mu$ l of Opti-MEM, and tranquillized at room temperature for 5 min; after extracting 250  $\mu$ l of Opti-MEM, siRNA at a final concentration of 200 nM was added, tranquillized at room temperature for 5 min, mixed with the above reagent, and tranquillized for 20 min; cells were washed, and then 500  $\mu$ l of transfection fluid was added. After 6-h transfection, basic culture medium was added, followed by incubation at 37°C for 24 h. Following incubation in serum-free medium for 24 h, cells were used in subsequent experiments.

### *Detection of ROS*

Intracellular ROS were detected in RCECs under HOP as described previously by using

the DCFH-DA fluorescence probe (Invitrogen, Carlsbad, CA) [2].

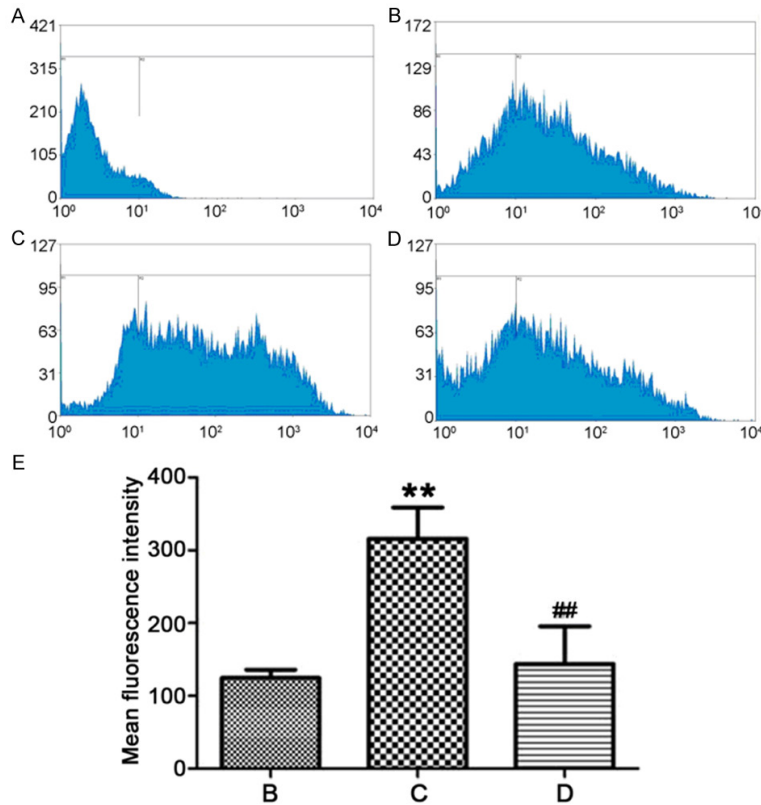
### *Determination of ROS location in ER*

Cells were seeded at  $2.5 \times 10^4$  cells/ml and maintained in serum-free medium for 24 h. Cells were divided into different groups: normal osmotic pressure (312 mOsM) group (NOP group), HOP (500 mOsM) group (90mM sodium chloride [NaCl] was used) (HOP group) and HOP+NAC group (N-Acetyl-L-cysteine [NAC; Sigma-Aldrich, St.Louis, MO, 10 mM] was added 40 min before the addition of NaCl). After 24-h treatment, the medium was removed and cells were washed with PBS thrice, fixed in 10% paraform (pH 7.4) for 20 min, and mixed with an appropriate amount of anti-quenching agent (Sigma-Aldrich). The ER Tracker<sup>TM</sup> RED probe (Millipore) was pre-heated at 37°C. Then, 2  $\mu$ mol/L ROS probe DCFH-DA (Invitrogen) and 500 nM ER Tracker<sup>TM</sup> RED probe were added to each well, followed by incubation at 37°C for 30 min in dark. After removing the medium, cells were washed with PBS thrice and observed under a confocal microscope (Zeiss 510, Göttingen, Germany). The intracellular ROS probe DCFH-DA exhibits green fluorescence and the ER Tracker<sup>TM</sup> RED probe exhibits red fluorescence.

### *Detection of cell proliferation*

RCECs were seeded into a 96-well plate (4000 cells/well). The experiment was conducted in two parts: 1) time intervention: HOP (500 mOsM) treatment for 12-72 h; and 2) medical intervention in five groups: NOP (312 mOsM) group, HOP (500 mOsM) group, HOP+10 mM NAC group, HOP+10 nM GSK2606414 (PERK inhibitor; Millipore) group, HOP+control siRNA group and HOP+CHOP siRNA (Sigma-Aldrich) group. Treatment was conducted for 24 h. At the end of treatment, the medium was removed and cells were rinsed with PBS thrice, and cells were maintained in serum-free Keratinocyte-SFM medium containing 100 U/ml penicillin and 100 mg/ml streptomycin overnight. Then, 10  $\mu$ l of MTT working solution was added, followed by incubation at 37°C for 4 h. After removing the medium, 150  $\mu$ l of DMSO (Sigma) was added to each well. After a constant shaking for 10 min at 37°C, the absorbance was detected at 490 nm. Experiment was conducted in duplicate for each group.

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**Figure 1.** ROS generation detected by flow cytometry. Only a negative peak was detected in the blank control group (A). The fluorescence intensity in HOP group (C) was significantly stronger than that in NOP group (B). The fluorescence intensity in HOP with NAC group (D) was weaker than that in HOP group. Statistical analysis (E) showed that the fluorescence intensity of ROS in 90 mM NaCl HOP group (C) was increased significantly as compared with that in NOP group (B) ( $P < 0.01$ ), and the fluorescence intensity of ROS in HOP with NAC group (D) was decreased significantly as compared with that in HOP group ( $P < 0.01$ ).

### Detection of cell apoptosis

Cells were divided into five groups: NOP (312 mOsm) group; HOP (500 mOsm) group, HOP+10 mM NAC group, HOP+10 nM GSK2606414 group, HOP+control siRNA group and HOP+CHOP siRNA group. Cell apoptosis was detected with the annexin V-FITC/propidium iodide (PI) apoptosis kit (Invitrogen, USA) according to the manufacturer's instructions. Cells were incubated for 24 h, washed with PBS thrice after removing the medium, and maintained in serum-free Keratinocyte-SFM medium containing 100 U/ml penicillin and 100 mg/ml streptomycin overnight. After incubation with 0.05% pancreatin at 37°C for 5 min, serum-containing medium was added to terminate the digestion. The cell suspension was transferred into a flow test tube, centrifuged at 800 rpm for 3 min, and re-suspended with the binding buffer after

removing the supernatant. Cells were gently mixed with 5  $\mu$ l of Annexin V, followed by incubation for 15 min in dark. After addition of 5  $\mu$ l of PI, cells were incubated at room temperature for 5 min in dark. Apoptotic cells were detected by flow cytometer (BD FACSCanto™II) within an hour.

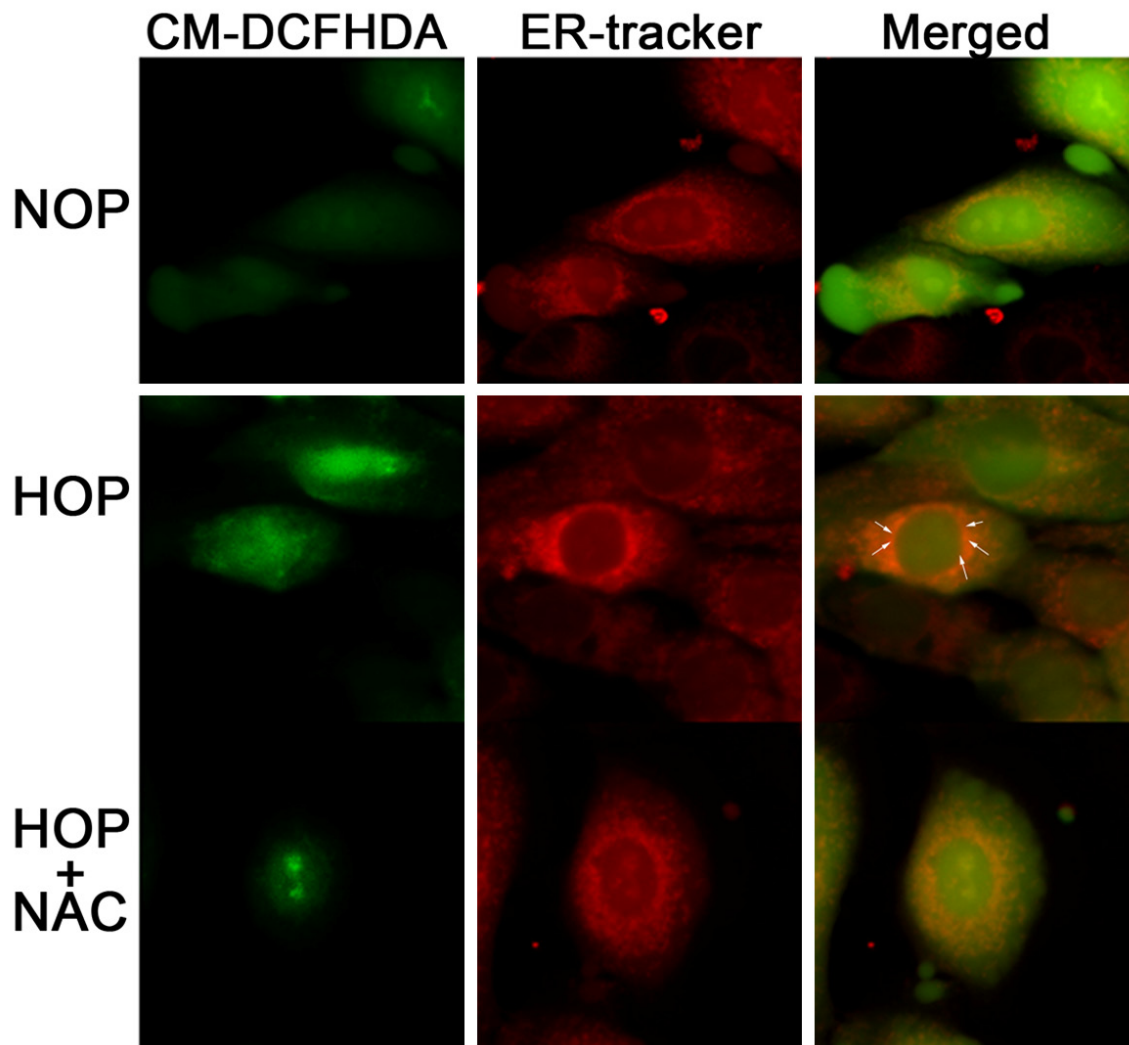
### Western blot assay

Proteins were harvested by centrifugation using the total protein extraction method, washed with PBS thrice, added with protein cleavage fluid, lysed on ice for 30 min, and centrifuged at 4°C at 14000 rpm for 10 min. After retaining the supernatant, the protein concentration was detected using the BCA method (Thermo, USA). Then, 30  $\mu$ g of total protein was mixed thoroughly with 5 $\times$  SDS loading buffer, and heated at 100°C for degeneration. After separation by 6% or 10% SDS polyacrylamide gel, proteins were transferred onto an NC membrane, blocked in 5% non-fat milk for 1 h, and incubated with primary antibody (1:500-1000) overnight.

Primary antibodies against GRP78 (Abcam, UK), CHOP, PERK, p-PERK, Akt, Actin (Cell Signaling Technology, Beverly, MA) and p-Akt (Millipore, USA) were used in this study. Membranes were rinsed in TBST for 10 min thrice, incubated with horseradish peroxidase conjugated secondary antibody against rabbit IgG (Dako, Glostrup, Denmark) (1:1000) for 2 h. After rinsing in TBST for 10 min (4 times), the membrane was exposed by SuperSignal West Pico Chemiluminent Substrates (PIERCE), and analyzed for the optical density (OD) using the Gel-Pro Analyzer. Actin served as an internal control.

### Statistical analysis

All the data are expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was per-



**Figure 2.** ROS location in the ER. Green fluorescence indicates ROS, red fluorescence indicates ER and yellow fluorescence indicates ROS in ER (merging of green and red fluorescence). There was more yellow fluorescence in HOP group as compared to NOP group, while less yellow fluorescence was found in HOP+NAC group as compared with HOP group

formed with the SPSS version 17.0. ANOVA was employed for the comparisons among groups and Pearson correlation analysis was used to evaluate the correlation between parameters. A value of  $P < 0.05$  was considered statistically significant.

## Results

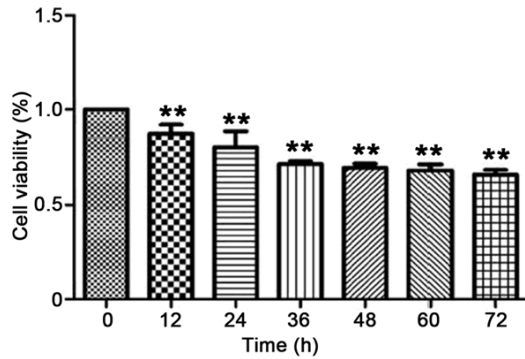
### ROS generation

Cell fluorescence was detected by flow cytometry. In blank control group, only a negative peak was detected under the FITC fluorescence condition (**Figure 1A**), while positive peaks

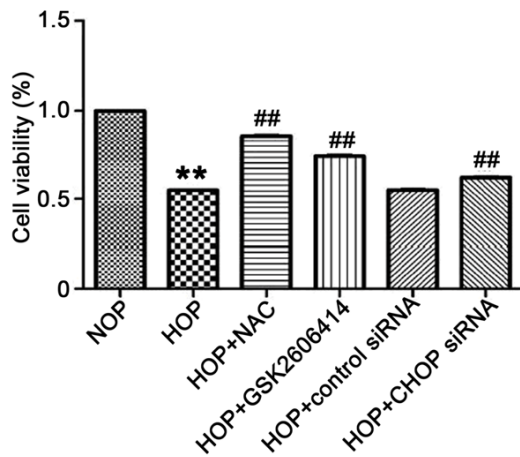
were detected in remaining three groups without negative peaks. The fluorescence intensity in HOP group was significantly stronger than in NOP group. As shown in **Figure 1**, the positive peaks right-deviated in HOP group (**Figure 1B, 1C**). The fluorescence intensity in HOP+NAC group was weaker than in HOP group, and the positive peak left-deviated (**Figure 1D**). The fluorescence intensity of ROS in HOP group increased significantly as compared to NOP group ( $P < 0.01$ ), and the fluorescence intensity of ROS in HOP+NAC group decreased significantly as compared to HOP group ( $P < 0.01$ ) (**Figure 1E**).



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**Figure 3.** Proliferation of RCECs in the presence of HOP. HOP induced growth inhibition of RCECs in a time-dependent manner. \*\* $P < 0.01$  versus 0 h.



**Figure 4.** Viability of RCECs in the presence of HOP. Cell viability after different treatments was detected by MTT assay. RCECs were treated with NOP (normal osmotic pressure), HOP (high osmotic pressure, 90 mM NaCl), HOP+NAC (10 mM), HOP+GSK2606414 (10 nM), HOP+control siRNA (non-targeting siRNA), or HOP+CHOP siRNA for 24 h. Cell viability of HOP group significantly decreased when compared with NOP group, but cell viability after HOP combined with NAC, GSK2606414 or CHOP siRNA significantly increased. \*\* $P < 0.01$  versus NOP group. ## $P < 0.01$  versus HOP group.

### ROS location in ER

Green fluorescence indicates ROS generation, red fluorescence indicates ER, and yellow fluorescence indicates ROS in ER (merging of green fluorescence and red fluorescence). There was more yellow fluorescence in HOP group as compared to NOP group, while less yellow fluorescence was observed in HOP+NAC group as compared to HOP group (**Figure 2**).

### Cell proliferation assay

Results showed that cell viability reduced significantly in a time-dependent manner after 12-h HOP intervention ( $P < 0.01$ ) (**Figure 3**). Cell viability in HOP group and control siRNA group decreased significantly as compared to NOP group ( $P < 0.01$ ), while cell viability increased significantly after intervention with NAC, PERK inhibitor GSK2606414 or CHOP siRNA, as compared to HOP group ( $P < 0.01$ ) (**Figure 4**).

### Cell apoptosis

Compared with NOP group, the percentage of early apoptotic cells in HOP group and control siRNA group increased significantly ( $P < 0.01$ ). In addition, the percentage of early apoptotic cells in NAC group, GSK2606414 group and CHOP siRNA group decreased significantly as compared to HOP group ( $P < 0.01$ ). There was no significant difference in the percentage of early apoptotic cells between control siRNA group and HOP group ( $P > 0.05$ ) (**Figure 5**).

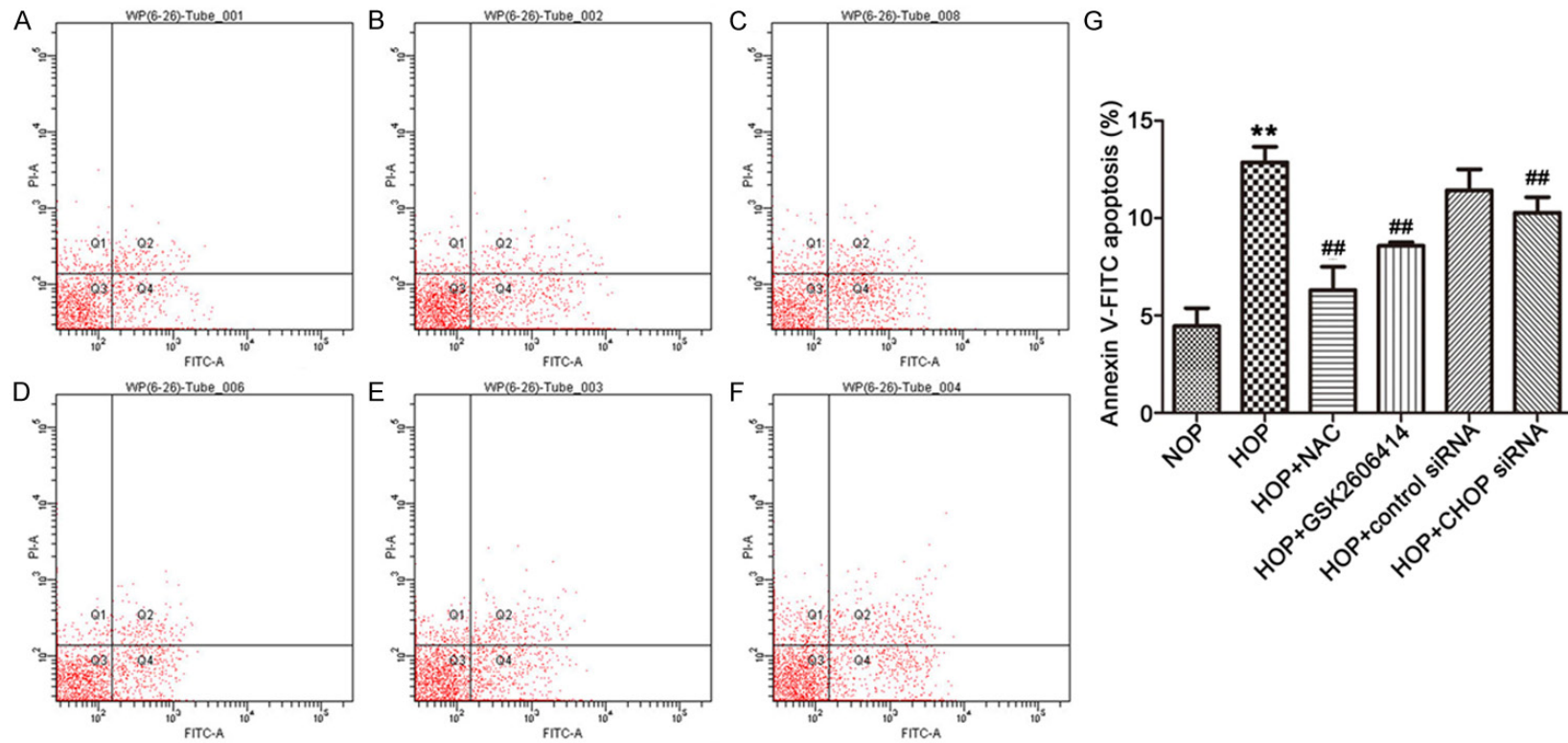
### Protein detection by western blot assay

When compared with NOP group, the expressions of p-PERK, GRP78 and CHOP in RCECs were up-regulated, but p-Akt expression was down-regulated in HOP group. When compared with HOP group, the expressions of p-PERK, GRP78 and CHOP were down-regulated, but p-Akt expression was up-regulated in HOP+NAC group (**Figure 6**), the expressions of p-PERK and CHOP were down-regulated and p-Akt expression was up-regulated in HOP+GSK2606414 group (**Figure 7**), while CHOP expression was down-regulated and p-Akt expression up-regulated in HOP+CHOP siRNA group (**Figure 8**).

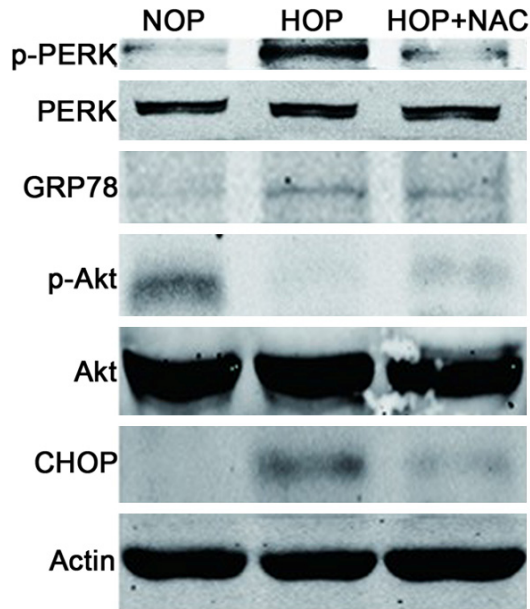
### Discussion

HOP is the key factor in the pathogenesis of ocular surface diseases, and therefore HOP injury to the ocular surface epithelium should not be ignored. Tear HOP is a key factor contributing to the pathogenesis of dry eye [8]. Low secretion and over-evaporation of ocular surface tear are the main causes of ocular surface HOP, which further results in the apoptosis of goblet cells and decreases the secretion of mucus, thus altering the stability of lacrimal film. This instability aggregates ocular surface

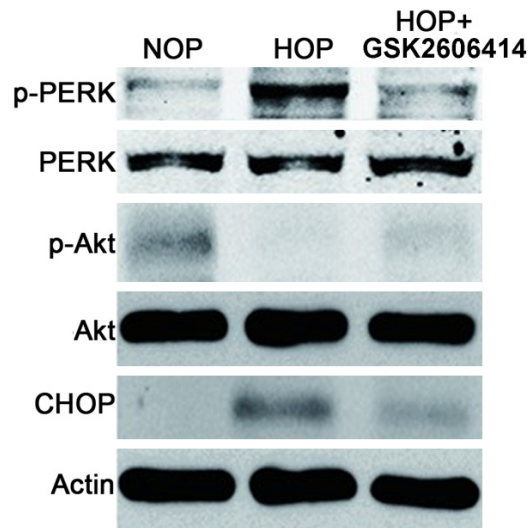
## High osmotic pressure increases reactive oxygen species generation



**Figure 5.** Detection of apoptotic cells with Annexin V assay. A: Normal osmotic pressure group; B: 90 mM NaCl HOP group; C: 90 mM NaCl HOP added with NAC group; D: 90 mM NaCl HOP added with GSK2606414 group; E: 90 mM NaCl HOP added with Control siRNA group; F: 90 mM NaCl HOP added with CHOP siRNA group. The percentage of apoptotic cells in HOP group was significantly higher than in NOP group, but it in HOP+NAC, GSK2606414 or CHOP siRNA group significantly decreased. G: Statistical analysis of result. (mean $\pm$ SD, n = 6). \*\*P<0.01 versus NOP, ##P<0.01 versus HOP.

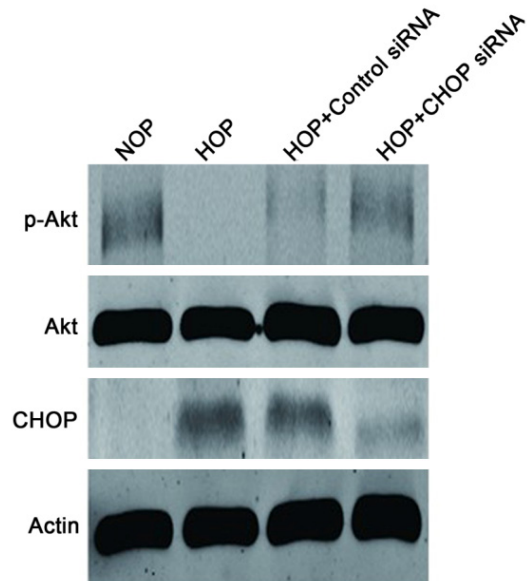


**Figure 6.** HOP induces the expressions of ER stress-related molecules in RCECs. The expressions of p-PERK, PERK, GRP78, p-Akt, Akt, CHOP and beta-actin were detected by Western blot assay.



**Figure 7.** HOP induces the expressions of ER stress-related molecules in RCECs. The expressions of p-PERK, PERK, p-Akt, Akt, CHOP and beta-actin were detected by Western blot assay.

HOP, forming a vicious cycle [9-11]. HOP is a potential pro-inflammatory stressor. Some investigators [12-15] have revealed that when the tear osmotic pressure reaches 440 or 450 mOsm, the corneal epithelial cell-related signaling pathway may be activated, causing cell



**Figure 8.** HOP induces the expressions of ER stress-related molecules in RCECs. The expressions of p-Akt, Akt, CHOP, and beta-actin were detected by Western blot assay.

injury, which further impairs the corneal epithelial barrier function. Our previous study [2] showed that HOP-induced ROS generation in RCECs served as an upstream activator of the Jun N-terminal kinase (JNK) signaling pathway. Activation of JNK signaling pathway may further activate the pro-inflammatory NF- $\kappa$ B and then induce the expressions of inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ , thus resulting in the inflammatory response of the corneal epithelial cells. At the same time, HOP-induced ROS generation may trigger cell apoptosis by regulating CD95/CD95L system via the JNK signaling pathway.

ER is an important eukaryotic organelle. There are various factors that may cause the accumulation of mal-folded and unfolded proteins in the ER and the imbalance of calcium ion, may further trigger ER stress [16, 17]. Unfolded proteins exert their effects through three signaling pathways: activating transcription factor6 (ATF6), type-1 ER transmembrane protein kinase (IRE-1) and PERK. They are in an inactive state when they bind to ER molecular chaperone GRP78. Under stress conditions, GRP78 is separated from the ER cavity, triggering the self-phosphorylation and dimerization of above three receptors, as well as UPR. If these factors that can cause cell injury are not relieved, UPR

is unable to maintain the calcium and protein balance in the ER, thus leading to the activation of apoptosis pathway and subsequent cell apoptosis, mainly via activating PERK to induce the expression of eukaryotic translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), which further enhances the expression of pro-apoptotic transcription factor CHOP, resulting in cell apoptosis [18].

ER Tracker Red™, a fluorescence-labeled glibenclamide mainly binding to ER sulphonylureas receptors (SUR), can be used for the specific fluorescence staining of ER in living cells. A variety of studies about the ROS sources [19] mainly focus on individual organelles (such as mitochondria) by confocal microscopy to make local analysis on ROS generation when mitochondria are under a redox condition. In the present study, a DCFH-DA probe was used to determine the ROS content in RCECs quantitatively, and at the same time, ER Tracker Red™ probe was employed to determine ROS in the ER. Zhang et al. [20] used both DCFH-DA and highly selective ER Tracker Red™ probes to study the coronary artery muscle cells and found that the content of intracellular ROS increased under stress and the generation of ROS in the ER increased markedly in a time-dependent manner. In the present study, results also showed that ROS content in RCECs of HOP group increased significantly as compared to NOP group, and NAC reduced the ROS production in the ER under HOP.

Analysis of cell viability showed that the RCECs viability decreased gradually over time in the presence of HOP. In addition, ROS inhibitor NAC and PERK inhibitor GSK2606414 improved cell viability in HOP group significantly.

The early apoptosis rate increased markedly under HOP, and pretreatment with NAC and PERK inhibitor GSK2606414 decreased the early apoptosis rate in HOP group. Thus, tear HOP plays a key role in the pathogenesis of ocular surface disease and its injury to the ocular surface epithelium should not be ignored.

Studies [21] have demonstrated that oxidative stress is an event before UPR. Lu et al. [22] found that inorganic arsenic could increase the expression of ROS in neurons, which further induced ER stress, causing cell apoptosis. Tagawa et al. [23] used cigarettes to increase the ROS production in airway epithelial cells

and found that antioxidants could effectively reduce the expression of proteins related to ROS-induced ER stress. Yang et al. [24] used sodium fluoride to treat testicular trophoblast cells and found that the intracellular ROS increased, the expressions of GRP78, PERK and CHOP were up-regulated, and NAC was effective to relieve the ER stress and decrease cell apoptosis. In our study, results indicated that the expressions of GRP78, p-PERK and CHOP in HOP group increased significantly as compared to NOP group. As an molecular chaperone in the ER, GRP78 is in an inactive state in normal conditions; when stress occurs, GRP78 is separated from the ER cavity, which symbolizes the occurrence of ER stress [25, 26]. As a specific transcription factor of ER stress, CHOP has been used as a marker of ER stress. The CHOP expression is very low under normal conditions; when ER stress occurs, its expression increases markedly [27, 28]. Our study showed the expressions of GRP78, p-PERK and CHOP decreased significantly in HOP group after pretreatment with NAC, indicating that NAC was able to inhibit ROS generation by attenuating ER stress, which further confirms that ROS generation is an event before ER stress. In addition, pretreatment with PERK inhibitor GSK2606414 decreased the expressions of p-PERK and CHOP in HOP group, which also confirms that PERK is an upstream molecule of CHOP, and the PERK activation may activate the expression of downstream CHOP. Studies [29-31] have revealed that cigarettes can increase the ROS production and induce ER stress in airway epithelial cells and retinal pigment epithelial cells, which further up-regulates the CHOP expression, ultimately causing cell apoptosis. Yang et al. [24] found that silencing of CHOP gene effectively relieved sodium fluoride-induced cell apoptosis as shown by flow cytometry.

Finally, the CHOP expression was silenced using CHOP siRNA and results showed the CHOP expression decreased significantly in HOP group, accompanied by the enhanced viability of RCECs and the decreased early cell apoptosis rate.

The Akt signaling pathway not only plays a key role in regulating cell metabolism, proliferation, survival and many other biological processes, but protects cells against ER stress [32, 33].



There is evidence [34] showing that ER stress may rapidly decrease the Akt phosphorylation, in which the PERK-CHOP-Akt pathway plays an important role. Our study showed the p-Akt expression in HOP group was significantly lower than in NOP group, and pretreatment with NAC or GSK2606414 increased the p-Akt expression. In addition, the p-Akt expression in HOP+CHOP siRNA group was significantly higher than in HOP group. These findings suggest that CHOP plays an adverse role in the Akt activation during the HOP-induced apoptosis of RCECs.

## Conclusions

In summary, our findings demonstrate that HOP-induced ROS generation in RCECs triggers cell apoptosis by activating ER stress pathway. Studies [34, 35] have shown that protective agents against osmotic pressure can effectively attenuate the inflammatory response of human corneal epithelial cells. Our findings inspire us to further determine whether antioxidants can be used as new protective agents against osmotic pressure to prevent inflammation and apoptosis of corneal epithelial cells in the presence of HOP so as to provide a novel strategy for the clinical treatment of HOP-induced ocular surface diseases.

## Acknowledgements

This work was supported by the National Natural Science Foundation, China (81300771)

## Disclosure of conflict of interest

None.

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