• Basic Research •

High glucose causes apoptosis of rabbit corneal epithelial cells involving activation of PERK-eIF2α-CHOP-caspase-12 signaling pathway

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Abstract

• AIM: To investigate the effect of high concentration of glucose (HCG) on double stranded RNA-activated protein kinase-like ER kinase (PERK)-eukaryotic initiation factor-2α (eIF2α)-transcription factor C/EBP homologous protein (CHOP)-cysteine aspartate specific proteinase (caspase-12) signaling pathway activation and apoptosis in rabbit corneal epithelial cells (RCECs).

• METHODS: RCECs were treated by different concentrations of glucose for 0-48h. The expressions of PERK, p-PERK, eIF2α, p-eIF2α, 78 kDa glucose-regulated protein 78 (GRP78), CHOP, B-cell lymphoma 2 (Bcl-2), B-cell lymphoma-2-associated X protein (Bax) and caspase-12 were determined by Western blot. Apoptosis was detected by TUNEL assay. Meanwhile, the function of PERK-eIF2α-CHOP-caspase-12 signaling pathway activation in high glucose-induced apoptosis was evaluated using PERK inhibitor, GSK2606414.

• RESULTS: HCG significantly promoted the expression of p-PERK, p-eIF2α, GRP78, CHOP, Bax and cleaved caspase-12 in RCECs (P<0.05), while remarkably decreased the expression of Bcl-2 and caspase-12 (P<0.05), and the alterations caused by glucose were in concentration- and time-dependent manners. Meanwhile, PERK and eIF2α expressions were not affected in all groups (P>0.05). TUNEL assay showed that the apoptosis rate of RCECs in the HCG group increased significantly in contrast with that in the normal concentration of glucose or osmotic pressure control group (P<0.05), and the apoptosis rate increased with the increase of glucose concentration within limits (P<0.05). GSK2606414 down-regulated the expression of p-PERK and p-eIF2α in the HCG group (P<0.05), while still did not affect the expression of PERK and eIF2α among groups (P>0.05). Correspondingly, GSK2606414 also significantly reduced the apoptosis rate induced by high glucose (P<0.05).

• CONCLUSION: HCG activates PERK-eIF2α-CHOP-caspase-12 signaling pathway and promotes apoptosis of RCECs.

• KEYWORDS: high glucose; rabbit corneal epithelial cells; PERK-eIF2α-CHOP-caspase-12 pathway; apoptosis

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INTRODUCTION

Dry eye is an irreversible and chronic progressive ocular surface disease characterized by tear film instability and ocular surface damage, accompanying by visual dysfunction and ocular surface discomfort, which seriously affect life quality of the patients[1]. Tear glucose in diabetic patients was relatively higher (about 5-fold than normal). Moreover, tear glucose was positively correlated with blood glucose in diabetic patients[2]. Till now, it is yet to cure this disease completely, especially diabetes-related dry eye. Therefore, finding the molecular mechanism between diabetes and dry eye is the key to prevent and treat diabetes-related dry eye.

Hyperglycemia impacts healthy condition of tear film. Once the stability of its inner environment cannot be compensated, dry eye might occur and develop. Diabetes-related dry eye includes decreased tear secretion, change of tear composition, decreased corneal sensitivity, and prevented corneal epithelial

regeneration[3]. At the same time, tear osmotic pressure is promoted due to the high concentration of glucose (HCG), eliciting ocular surface damage[4]. The incidence of diabetes-related dry eye accounts for about 52.8%-70% at present and high glucose condition was thought to be primary reason for dry eye[5].

Histochemical analysis revealed that goblet cell loss and corneal epithelial cell injury in patients with dry eyes correlate with apoptosis[6], while endoplasmic reticulum stress (ER stress) is an important pathway closely related to the functional failure and apoptosis complicated by various diseases, including diabetes[7]. The accumulation of glycotoxic metabolites in the tears of diabetic patients may lead to emergence of a series of damage factors, such as oxygen free radicals and calcium ion overloading, which may trigger severe ER stress and even final apoptosis of the ocular cells[8]. The conditions within the endoplasmic reticulum are monitored by the unfolded protein response (UPR) signaling pathway[9], and activation of the UPR restores protein folding homeostasis by reducing protein translation, increasing endoplasmic reticulum chaperone expression, and degrading misfolded proteins. However, prolonged ER stress fails to repair protein homeostasis and activates apoptotic signaling pathway once UPR does not sustain the balance of protein production and calcium in the endoplasmic reticulum[10].

Double stranded RNA-activated protein kinase-like ER kinase (PERK)-eukaryotic initiation factor-2α (eIF2α)-transcription factor C/EBP homologous protein (CHOP)-cysteine aspartate specific proteinase (caspase-12) signaling pathway is the key branch of ER stress to activate downstream signal transduction of apoptosis. In this study, we used an *in vitro* model to explore the activation of PERK-eIF2α-CHOP-caspase-12 signaling pathway in high glucose condition and investigate its role in the apoptosis of rabbit corneal epithelial cells (RCECs).

MATERIALS AND METHODS

Ethical Approval The experimental protocol was approved by the Ethics Committee of the Yangpu Hospital, Tongji University Medical School. All animal procedures and experiments were approved by the Animal Care and Use Committee in Tongji University. All animals were cared according to the Association for Research in Vision and Ophthalmology Statement for using animals in ophthalmic and vision research. Surgeries were performed under anesthesia by sodium pentobarbital, and all efforts were made to minimize animal suffering.

Cell Culture Totally forty New Zealand rabbits (weight: 2.25±0.25 kg) were purchased from Central Lab, Tongji University Laboratory Animal Center (Shanghai, China). Corneas were collected to prepare RCECs as previously described[11]. The primary RCECs were then identified according to the experimental protocol described previously[12]. The first generation of cells was used for the subsequent experiments. At 80% of confluency, RCECs were cultured in serum-free keratinocyte medium (ScienCell Research Laboratories, San Diego, CA, USA) supplemented with 100 U/mL penicillin (Gibco, Grand Island, NY, USA) and 100 mg/mL streptomycin (Gibco, Grand Island, NY, USA) at 37°C in a 5% CO_2 humidified atmosphere overnight.

Table 1 Cell groups and treatment

Groups	Treatments
BC	—
NCG	5.5 mmol/L glucose
OPC	27.5 mmol/L D-mannitol+5.5 mmol/L glucose
HCG	33 mmol/L glucose
Tm	100 nmol/L Tm

BC: Blank control; NCG: Normal concentration of glucose; OPC: Osmotic pressure control; HCG: High concentration of glucose; Tm: Tunicamycin.

Groups and Treatments RCECs were seeded at 4×10^5/well into a 6-well plate and maintained in medium as described previously for different treatments[11]. The cells were treated with different concentrations of glucose for different periods (0-48h). The osmotic pressure was equalized using different concentrations of D-mannitol. The function of PERK-eIF2α-CHOP-caspase-12 signaling pathway activation in high glucose-induced apoptosis was evaluated using PERK inhibitor, GSK2606414 (Millipore Corporation, Billerica, MA, USA). Tunicamycin (Tm; Abcam, Cambridge, MA, USA) was used as the positive agent to induce ER stress. Therefore, the experiments were divided into blank control (BC), normal concentration of glucose (NCG, 5.5 mmol/L glucose), osmotic pressure control (OPC, 27.5 mmol/L D-mannitol+5.5 mmol/L glucose), HCG (33 mmol/L glucose) and Tm (100 nmol/L) groups (Table 1). Additionally, in each group, a subgroup with GSK2606414 (10 nmol/L) was set. To observe the concentration-dependent effect of glucose, the experiment was also divided into 0, 5, 15, 25, 35 and 45 mmol/L glucose. To observe the time-dependent effect of glucose, the cells were also treated by 33 mmol/L glucose for 0, 12, 24, 36 and 48h respectively.

Western Blotting Cell lysates of each well were collected directly using 120 μL 1×sodium dodecyl sulphate (SDS) loading buffer (Proteintech, Rosemont, IL, USA) and then sonicated. After heating at 100°C about 10min for denaturing, the samples were stored at -20°C. Each sample was loaded onto 10% to 12% SDS-polyacrylamide gel electrophoresis (PAGE), and electrophoresis was performed for 1.5h at 100 V. Total proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA) and then blocked in 5% nonfat milk or 5% bovine serum albumin

(Beyotime, Shanghai, China) for 1h at room temperature. The membrane was then incubated with primary antibodies diluted in blocking buffer with 0.01% Tween-20 overnight in 4°C. Primary antibodies against glucose-regulated protein 78 (GRP78; 1:2000), eIF2α (1:2000) and B-cell lymphoma-2-associated X protein (Bax; 1:1000) were purchased from ENZO Life Sciences (USA). Primary antibodies against PERK (1:2000), p-PERK (1:500), CHOP (1:1000), p-eIF2a (1:1000) and caspase-12 (1:1000) were purchased from CST (Cell Signaling Technology, Danvers, MA, USA). B-cell lymphoma 2 (Bcl-2; 1:1000) antibody was purchased from Proteintech. β-actin antibody (1:2000) was purchased from Beyotime. After washing three times with Tris buffered saline tween (TBST) for 10min each, the membrane was incubated with goat anti-mouse and goat anti-rabbit horseradish peroxidase conjugated secondary antibodies (Beyotime) in a 1:4000 dilution for 1h at room temperature. The membrane was rinsed three times with TBST for 10min each again, and then detected by enhanced chemiluminescent (ECL) method (Millipore Corporation, Billerica, MA, USA). The fluorescent signals were captured on X-ray films in a dark room. The density was analyzed by densitometry with the Image-J program.

TUNEL Assay RCECs were cultured on sterile coverslips placed in 6-well plates. After 24h treatment, the cell slides were washed with PBS once and fixed with 4% paraformaldehyde (PFA) for 30min. And then, the cell slides were incubated with 0.3% Triton X-100 for 30min at room temperature after washing 3 times. Apoptosis was detected using the TUNEL kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instruction. The cell nucleus were staining with 4',6-diamidino-2-phenylindole (DAPI) at room temperature for 10min in the dark followed by washing 3 times. Finally, the cell slides were sealing and observed. Imaging was obtained using a laser scanning confocal fluorescence microscope (Carl Zeiss AG, Cologne, Germany).

Statistical Analysis All data were presented as means± standard deviation (SD). Statistical and image analysis was performed with GraphPad Prism version 7.00 (GraphPad Software; San Diego, CA, USA) and Image-J program. One-way analysis of variance (ANOVA) with multiple comparisons was used to detect differences between groups. For all tests, a difference was considered to be significant at $P<0.05$.

RESULTS

High Glucose Activated PERK-eIF2α-CHOP-caspase-12 Signaling Pathway RCECs were treated with different concentration of glucose and expression of PERK-eIF2α-CHOP-caspase-12 signaling pathway-related protein was measured by Western blot. As shown in Figure 1, HCG group remarkably increased the expression of p-PERK and p-eIF2α compared with NCG group and OPC group ($P<0.05$), whereas the increase was partially blocked by PERK inhibitor, GSK2606414 ($P<0.05$). As the positive control, Tm also increased the expression levels of p-PERK and p-eIF2α ($P<0.05$), which were also inhibited by GSK2606414 ($P<0.05$). In addition, the expressions of p-PERK and p-eIF2α increased significantly in the OPC group than those in the NCG group ($P<0.05$), while their expression decreased significantly in the OPC+GSK group ($P<0.05$). The PERK and eIF2α expression were not altered in different groups and by GSK2606414 ($P>0.05$).

Osmotic pressure was then set equally using D-mannitol to assess the concentration-dependent effect of glucose on the expression of PERK-eIF2α-CHOP-caspase-12 signaling pathway-related protein in RCECs. As shown in Figure 2, the protein level of p-PERK and p-eIF2α increased firstly then decreased as the concentration of glucose increased ($P<0.05$). In this condition, expression of GRP78, CHOP, Bax, cleaved-caspase-12 kept increasing ($P<0.05$), while expression of Bcl-2 and caspase-12 decreased with the increase of glucose concentrations ($P<0.05$).

We then investigated the time-dependent effect of glucose on the expression of PERK-eIF2α-CHOP-caspase-12 signaling pathway-related protein in RCECs. As shown in Figure 3, 33 mmol/L glucose elevated the protein level of p-PERK and p-eIF2α with time from 0 to 48h ($P<0.05$). In this condition, expression of GRP78, CHOP, Bax, cleaved-caspase-12 kept increasing ($P<0.05$), while expression of Bcl-2 and caspase-12 decreased with the prolonged treatment time ($P<0.05$). These results revealed that the expression of PERK-eIF2α-CHOP-caspase-12 pathway-related molecules in RCECs was significantly promoted or inhibited in a dose- and time-dependent manner.

High Glucose Elicited Apoptosis Through Activating PERK-eIF2α-CHOP-caspase-12 Signaling Pathway The ratio of TUNEL-positive RCECs was both very low in the BC and NCG group basically (Figure 4). Compared with OPC group, the apoptotic rate in HCG group increased remarkably ($P<0.05$). However, GSK2606414 reduced high glucose-induced apoptosis ($P<0.05$). RCECs in OPC and Tm group have the same change after adding GSK2606414 ($P<0.05$). The percentage of TUNEL-positive RCECs in the OPC group increased significantly than that in the NCG group ($P<0.05$). No significant difference in the percentage of apoptotic RCECs were observed among the BC, NCG, BC+GSK and NCG+GSK group ($P>0.05$).

The percentage of TUNEL-positive RCECs increased as concentration of glucose increased when the osmotic pressure was set equally in all group ($P<0.05$; Figure 5). These data furtherly revealed that HCG elicited apoptosis through activating PERK-eIF2α-CHOP-caspase-12 signaling pathway.

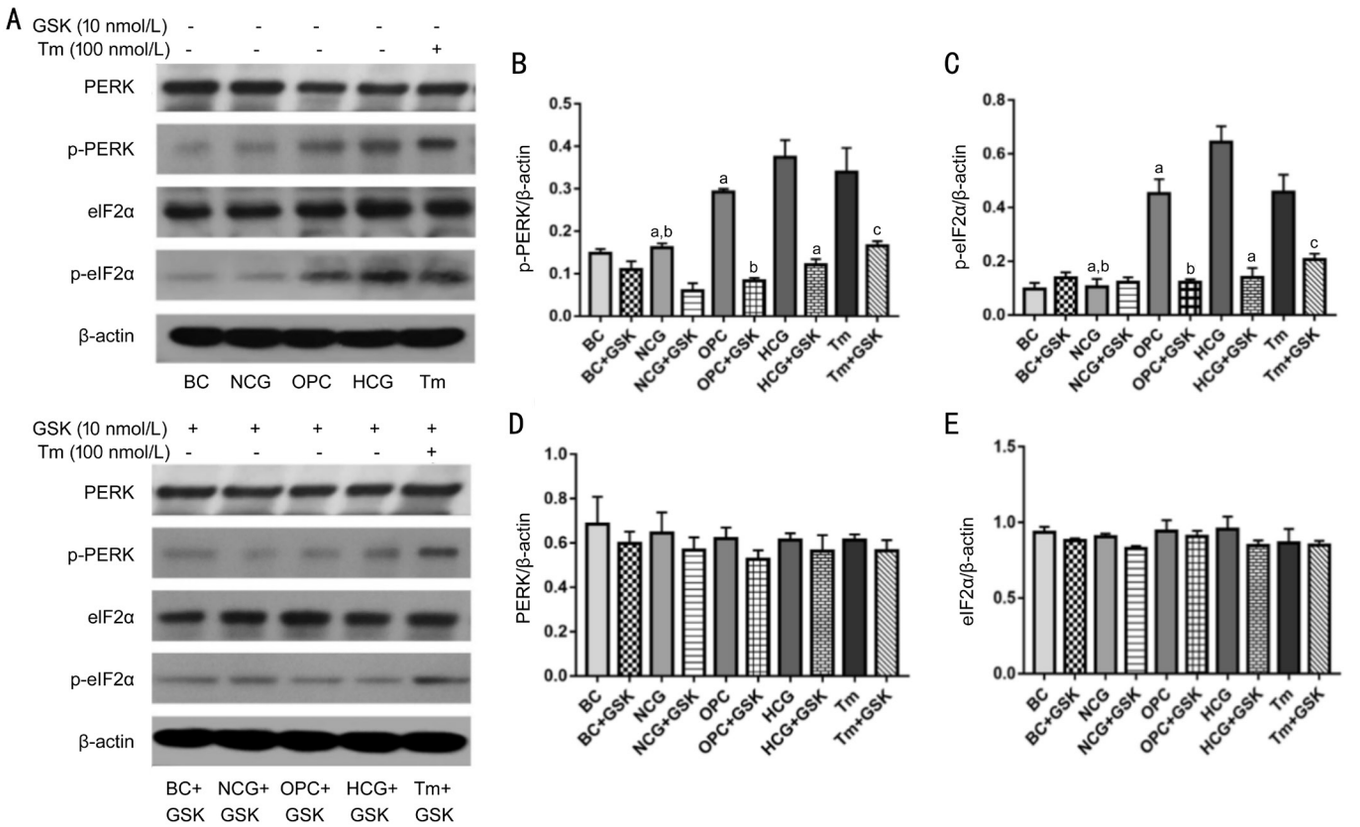

Figure 1 High glucose activated PERK-eIF2α-CHOP-caspase-12 signaling pathway A: Blots of different proteins; B: Quantification of p-PERK; C: Quantification of p-eIF2α; D: Quantification of PERK; E: Quantification of eIF2α. Compared with NCG group and OPC group, the expression level of p-PERK and p-eIF2α in corneal epithelial cells of HCG group was significantly up-regulated, which was reduced by application of GSK2606414 (^{a}P<0.05 *vs* HCG); compared with NCG group, the expression of p-PERK and p-eIF2α in OPC group was up-regulated, and GSK2606414 treatment down-regulated the expression of p-PERK and p-eIF2α (^{b}P<0.05 *vs* OPC); GSK2606414 treatment also reduced the expression level of p-PERK and p-eIF2α in Tm condition (^{c}P<0.05 *vs* Tm); while PERK and eIF2α expressions have no statistically significant difference among groups (P>0.05). Statistical analysis of Western blot was represented as mean±SD. Tm: Tunicamycin.

DISCUSSION

Severe dry eye is featured by delayed epithelial regeneration, persistent corneal epithelial defects, or other complications after ocular surgery[13-14]. Dry eye in diabetic patients is probably more related to diabetic neuropathy[2]. In this study, we systemically assessed the potential effects of high glucose and hyperosmosis on PERK-eIF2α-CHOP-caspase-12 signaling pathway and ER stress-dependent apoptosis in RCECs. Our data revealed that high glucose more than the inner hyperosmosis activated PERK-eIF2α-CHOP-caspase-12 signaling pathway to elicit ER stress-dependent apoptosis of RCECs.

In this study, RCECs were treated with different concentrations of glucose to verify that high-glucose with the impact of inner osmotic pressure excluded can activate PERK-eIF2α-CHOP-caspase-12 pathway but not normal glucose, which induce apoptosis of RCECs. When ER suffers from stress, dissociation of the GRP78 triggers dimerization, oligomerization, autophosphorylation and activation of PERK[15]. PERK phosphorylates the alpha subunit of eIF2α, leading to inhibition of protein synthesis[16-17]. Our previous study demonstrated that p-PERK in high glucose-treated RCECs is significantly higher than those in the normal or the osmotic pressure control group. In the present study, we furtherly demonstrated that the expressions of p-PERK and p-eIF2α increased in high glucose-treated RCECs, which were reduced by PERK inhibitor, GSK2606414. Previous studies on neurons have shown that the activation of PERK-eIF2α pathway can be blocked by GSK2606414, which result in neurological rehabilitation of model animals with neurodegenerative diseases[16-17]. The increase of glucose concentration promoted the expression of p-PERK and p-eIF2α firstly but then turned down those protein expressions. In combination with the time-dependent effect of glucose, these data revealed that the PERK-eIF2α-CHOP-caspase-12 pathway was activated in high glucose-treated RCECs and influenced by the concentration and action time of glucose.

CHOP is the downstream of p-PERK and considered as a marker of cell death[18]. In normal conditions, the expression of CHOP is quite low while the expression of CHOP would be remarkably increased under the ER stress[19]. CHOP interferes the formation of protein disulfide bonds by inducing disorder

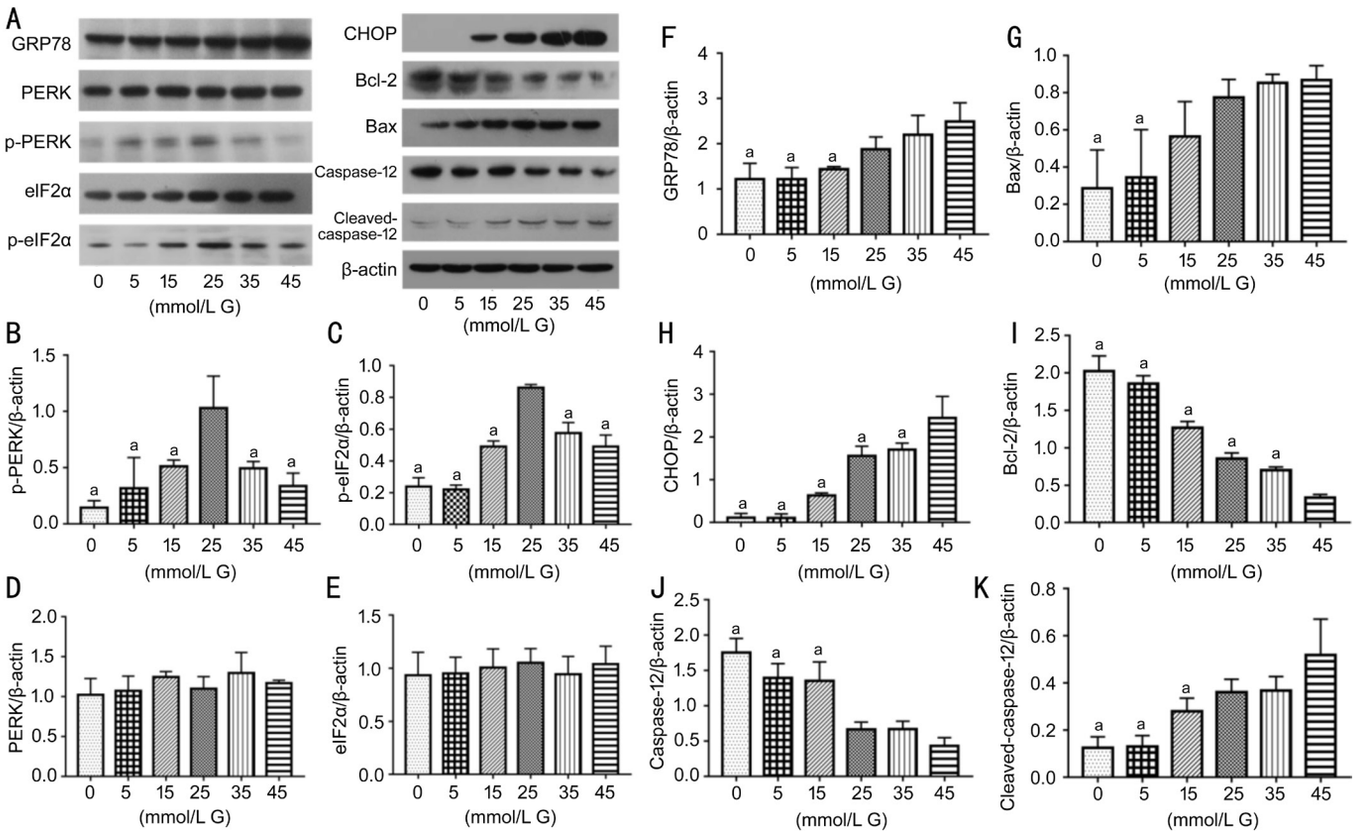

Figure 2 The effects of different concentrations of high glucose on PERK-eIF2α-CHOP-caspase-12 signaling pathway and apoptosis-related proteins A: Blots of different proteins; B: Quantification of p-PERK; C: Quantification of p-eIF2α; D: Quantification of PERK; E: Quantification of eIF2α; F: Quantification of GRP78; G: Quantification of Bax; H: Quantification of CHOP; I: Quantification of Bcl-2; J: Quantification of caspase-12; K: Quantification of cleaved-caspase-12. As glucose concentration increase but not osmotic pressure, the expression of p-PERK and p-eIF2α increased firstly but then decreased, and their expressions peaked when RCECs treated with 25 mmol/L glucose for 24h (^aP<0.05 *vs* 25 mmol/L G). PERK and eIF2α expressions have no statistically significant difference among groups (P>0.05). While the expression of GRP78, CHOP, Bax and cleaved-caspase-12 increased after glucose treatment in a concentration-dependent manner (^aP<0.05 *vs* 45 mmol/L G); Bcl-2 and caspase-12 expressions declined, their changes were correlated negatively with the concentrations of glucose (^aP<0.05 *vs* 45 mmol/L G). Date were represented as mean±SD. G: Glucose.

of the expression of endoplasmic reticulum oxidoreductases 1α (ERO1)[20], which reduce protein folding and lead to the accumulation of unfolded proteins. Meanwhile, CHOP consumes glutathione and promotes the production of reactive oxygen species furtherly[21]. Peroxide status of ER will affect the ion channel function on the membrane. Therefore, calcium inside the ER is released into cytoplasm, which will break the balance of inner environment and activate the ER -associated calcium ATPase, protease and nuclease[22]. The permeability of mitochondrial membrane increases, and its internal components such as cytochrome C, apoptotic protease activating factor 1 (APAF-1) and apoptosis inducing factor (AIF) were released into the cytoplasm, triggering caspase-dependent or independent apoptotic pathways[23]. Procaspase-12 is specifically generated and activated in ER. Calcium homeostasis is broken and intracellular calcium drained away, then cytoplasmic calcium-activated protease calpain cleave procaspase-12 to activate caspase-12 during ER stress. Cleaved-caspase-12 is transported from ER to cytoplasm in order to activate caspase-9 that then lyses caspase-3. Caspase-3 cleaves polyribose polymerase (PMuP) and multiple intracellular substrates, and the fragmentation and inactivation of DNA eventually result in programmed cell death[24-26]. Caspase-12 is therefore considered as the marker molecule of ER dependent apoptosis. Therefore, the apoptosis of high glucose-treated RCECs might result from increasing expression of CHOP, Bax and cleaved-caspase-12 and decreasing expression of Bcl-2 in the cornea. Our present study revealed that the expression of CHOP, Bax and cleaved-caspase-12 increased as concentration of glucose increased or over time, while the expression of Bcl-2 and caspase-12 were in the opposite trend. The results reveal that the expression levels of these signal molecules involved in apoptosis were altered in high glucose-treated RCECs and show time- and dose-dependent manner. Our previous study also demonstrated that the expression of CHOP in high glucose-treated RCECs are significantly higher than those in the normal or the osmotic pressure control group[11].

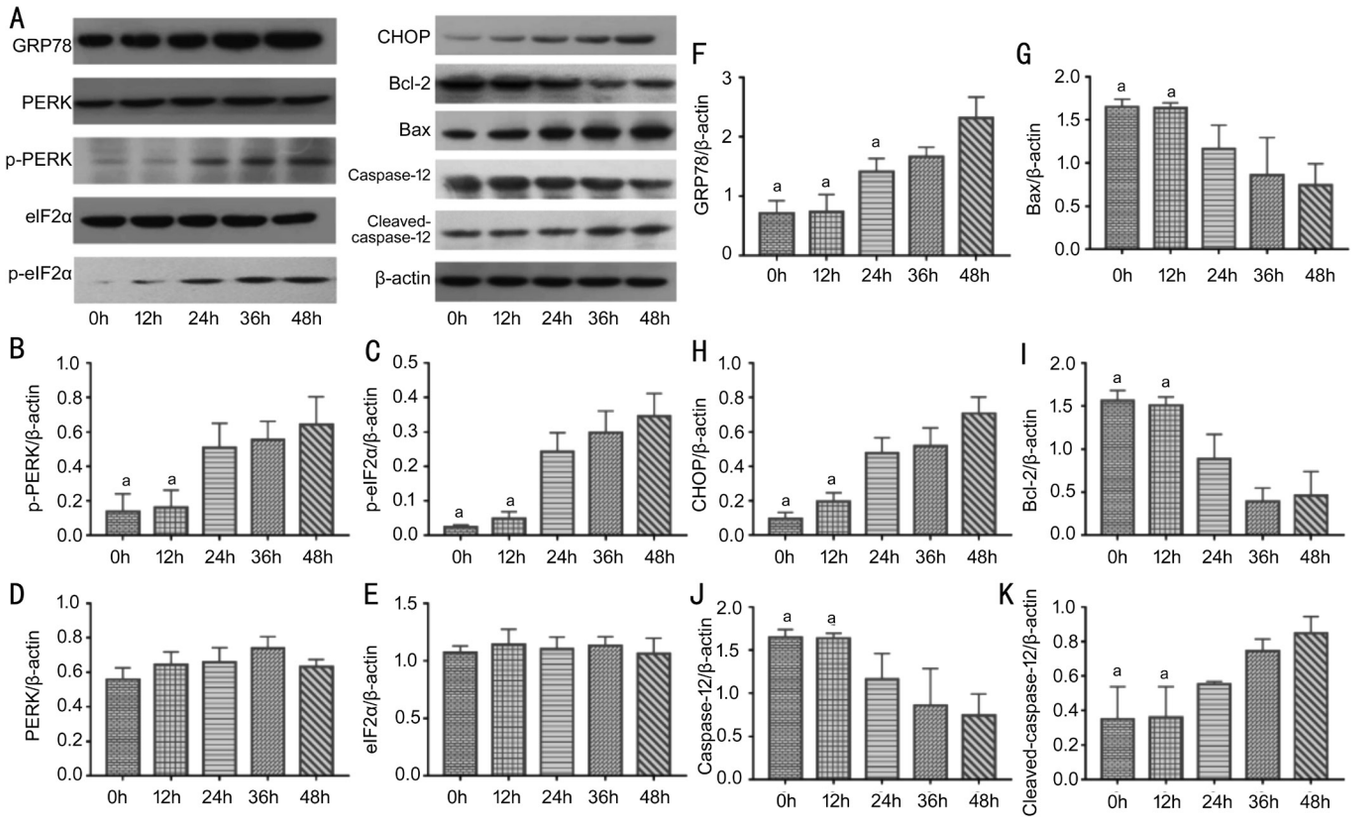

Figure 3 The effects of high glucose on PERK-eIF2α-CHOP-caspase-12 signaling pathway and apoptosis-related proteins after treatment for different periods A: Representative blots of different proteins; B: The expression of p-PERK; C: The expression of p-eIF2α; D: The expression of PERK; E: The expression of eIF2α; F: The expression of GRP78; G: The expression of Bax; H: The expression of CHOP; I: The expression of Bcl-2; J: The expression of caspase-12; K: The expression of cleaved-caspase-12. The expression of p-PERK, p-eIF2α, GRP78, CHOP, Bax and cleaved-caspase-12 increased with time and there was a positive correlation between these molecular expression and time ($^aP<0.05$ *vs* 48h); while Bcl-2 and caspase-12 expression declined, which have a negative correlation with time ($^aP<0.05$ *vs* 48h); PERK and eIF2α expressions have still no statistically significant difference among groups ($P>0.05$). The data were represented as mean±SD.

We found that the longer time RCECs treated with high glucose or the higher the glucose concentration, the more activation of apoptotic signaling pathway. p-PERK and p-eIF2α increased at low concentration of glucose and began to decrease when it reached the peak. The tendency was a bit different from others. Since PERK-eIF2α-CHOP-caspase-12 pathway of ER stress is the upstream signal of cell apoptosis, and it plays a key role in regulating the life and death of cells under stress, it might be that the undue consumption of p-PERK and p-eIF2α in RCECs is not enough to resist the excessive stress of high glucose, so that the expression of apoptotic signaling protein is relatively more active, and triggers apoptotic signal transduction finally.

Our previous study also found that the apoptosis rate in early stage in the HCG group was higher than that in the normal group assayed by flow cytometry[11]. This present study revealed that the apoptotic ratio in high glucose was remarkably higher than that in the normal or osmotic pressure group by TUNEL analysis, and it was significantly reduced by GSK2606414. The results further proved that high glucose condition could facilitate apoptosis of RCECs by activating PERK-eIF2α-CHOP-caspase-12 pathway in profile. We also found that the percentage of apoptotic RCECs increased as concentration of glucose increased in dose-dependent manner. It corresponded to the results of Western blot analysis. Apoptosis was divided into early apoptosis and late apoptosis. The negative charge of phosphatidylserine in early apoptotic cells transfers from the inside to the outside of the cell membrane, and then the surface of cell membrane has changed. Once apoptotic signal transduction is initiated, it will cause multiple morphological changes of cells by inducing genomic DNA fragments, which triggers late apoptosis[27-28]. TUNEL assay detect DNA fragment directly, it was a method to detect late stage of apoptosis. In our present study, there was a significant difference among groups by TUNEL assay, but the apoptosis rate was low in all groups. It was speculated that the HCG is prone to induce early apoptosis of RCECs, which was consistent with the results of the previous study[11].

In summary, PERK-eIF2α-CHOP-caspase-12 pathways were activated in high glucose-induced RCECs and mainly involved in the early stages of cell apoptotic. This might explain why the incidence of dry eye is high in diabetic and dry eye symptoms. The injury severity of RCECs is related to the concentration

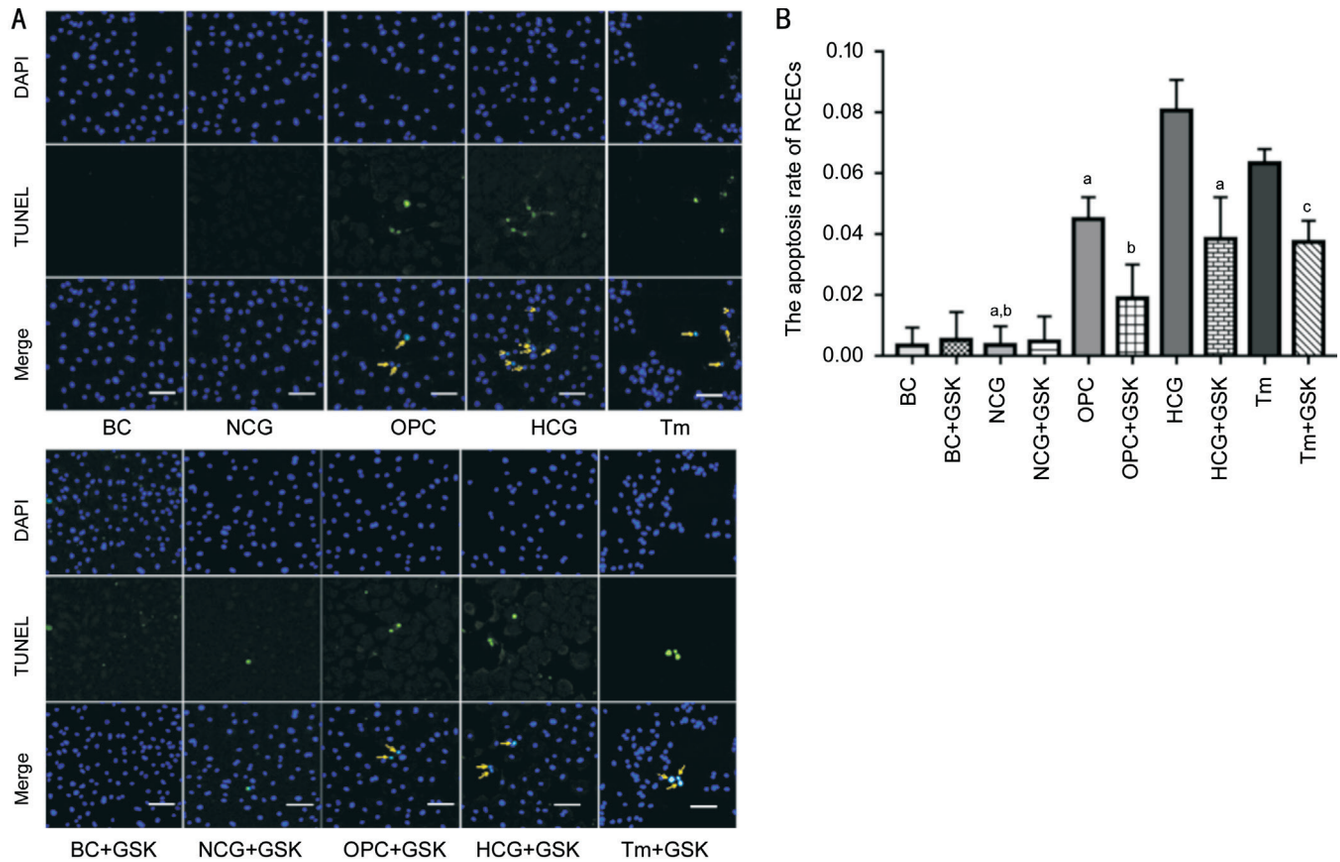

Figure 4 The apoptosis rate of RCECs was evaluated by TUNEL assay A: Representative images of TUNEL staining. The magnification of images were 200×. B: The percentage of apoptotic RCECs in the HCG group was significantly higher compared to those in the NCG group and the OPC group, but it significantly decreased in the HCG+GSK group (^aP<0.05 *vs* HCG). The percentage of TUNEL-positive RCECs in the OPC group was higher compared with NCG and OPC+GSK groups (^bP<0.05 *vs* OPC). The percentage of apoptotic RCECs in the Tm group was significantly higher than those in the Tm+GSK group (^cP<0.05 *vs* Tm). Data were represented as mean±SD. Tm: Tunicamycin.

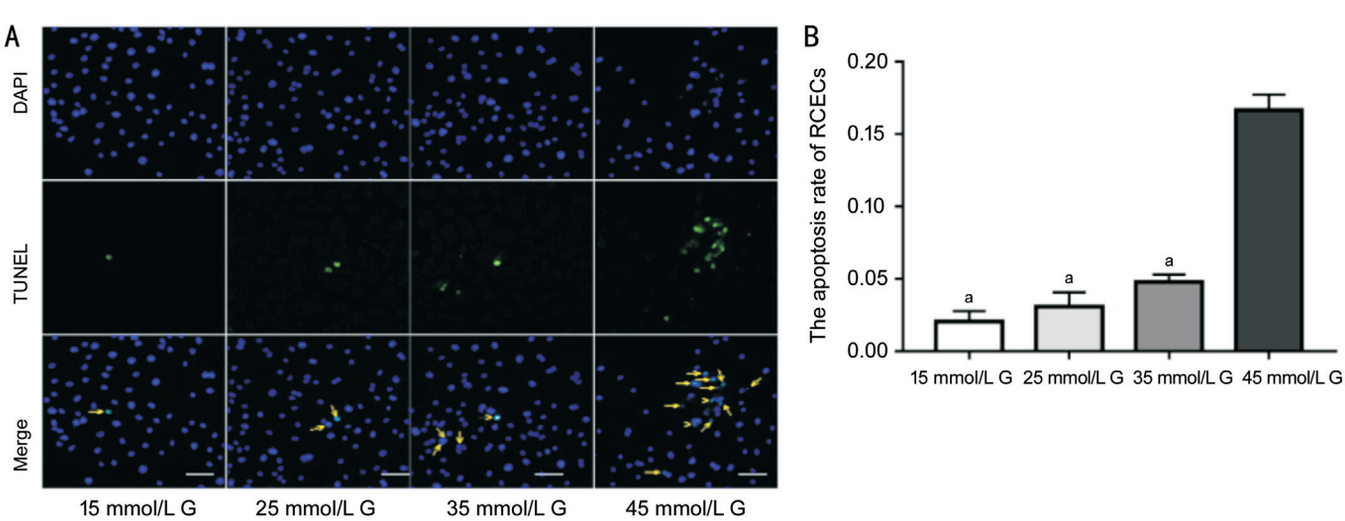

Figure 5 The apoptosis rate of RCECs after treatment with different concentrations of glucose A: Representative images of TUNEL staining. The magnification of images were 200×. B: With the increase of glucose concentration, the percentage of apoptotic RCECs increased (^aP>0.05 *vs* 45 mmol/L G). The data were expressed as mean±SD. G: Glucose.

or action time of glucose. This work will help guide the early prevention and treatment of diabetes-related dry eye.

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