Caspase 3/GSDME-Mediated Corneal Epithelial Pyroptosis Promotes Dry Eye Disease

Ling Wang,[1,2] Ye Tian,[2] Hui Zhang,[1,2] Yongxiao Dong,[3] Xia Hua,[4,5] and Xiaoyong Yuan[1,2]

[1]Clinical College of Ophthalmology, Tianjin Medical University, Tianjin, China
[2]Tianjin Key Laboratory of Ophthalmology and Visual Science, Tianjin Eye Institute, Tianjin Eye Hospital, Tianjin, China
[3]Department of Ophthalmology, The First People's Hospital of Xianyang City, Xianyang, China
[4]Aier Eye Institute, Changsha, China
[5]Tianjin Aier Eye Hospital, Tianjin, China

Correspondence: Xiaoyong Yuan, Clinical College of Ophthalmology, Tianjin Medical University, No. 4 Gansu Road, Heping District, Tianjin 300020, China; yuanxy_cn@hotmail.com.
Xia Hua, Tianjin Aier Eye Hospital, 102 Fukang Road, Nankai District, Tianjin 300191, China; cathayhuaxia@163.com.

LW and YT contributed equally to this study and are to be considered co-first authors.

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Purpose. Dry eye disease (DED) is a common ocular surface inflammatory disease with a complex pathogenesis. Herein, the role and effect of gasdermin E (GSDME) in DED pathogenesis were explored.

Methods. In vitro, flow cytometry, Cell Counting Kit-8 (CCK-8) and lactate dehydrogenase (LDH) release assays were used to determine the effects of hyperosmotic stress on pyroptosis, apoptosis, and cell viability in human corneal epithelial cells (HCECs). Quantitative PCR (qPCR) and Western blot assays were used to detect GSDME expression in HCECs and in those transfected with si-GSDMD. In vivo, GSDMD-knockout (KO) mice were used to study the role of GSDME in DED pathogenesis. The qPCR, Western blotting, and immunofluorescence were used to explore the effects of GSDME on HCEC apoptosis, pyroptosis, and the expression of related genes and proteins in GSDMD-KO mice with scopolamine-induced dry eye.

Results. Pyroptosis and cell membrane rupture occurred, and caspase-3 and GSDME protein expression increased after HCECs were treated with 312 to 500 mOsm sodium chloride. GSDME gene and protein expression levels were increased in HCECs from both si-GSDMD- and GSDMD-KO mice. Although caspase-3 expression was increased in the dry eye group of GSDMD-KO mice, HCEC apoptosis and the apoptosis-related factors PARP were not detected. The gene and protein expression levels of the pyroptosis-related factors ASC and IL-1β were greater than those in GSDMD-KO mice without dry eye.

Conclusions. GSDME is involved in DED pathogenesis by mediating inflammation via the pyroptosis pathway, GSDME inhibition may be a therapeutic target for DED.

Keywords: dry eye disease (DED), gasdermin E (GSDME), pyroptosis, caspase-3, human corneal epithelial cells, GSDMD-KO mice

Dry eye disease (DED) is a common ocular disease affecting tens of millions of people. The incidence and prevalence of DED are greater in Asia than in Europe and North America, with a prevalence of 5% to 50%.[1–3] The clinical symptoms are discomfort, including dryness, burning, foreign body sensation, heavy eyelids, pain, increased sensitivity to light, itching, and eye strain, or visual disturbance.[4,5] DED is a multifactorial ocular disease characterized by the loss of tear film homeostasis, in which tear film instability, hyperosmotic pressure, ocular surface inflammation and injury, and nerve pressure play etiological roles,[6] which may be related to human corneal epithelial cell (HCEC) pyroptosis.[4] Pyroptosis, an important process in the pathogenesis of DED, is a type of cytolytic programmed necrosis characterized by cell swelling and cell membrane bullae that is involved in inflammatory diseases caused by a variety of pathogen infections and injuries.[7,8]

Studies have shown that treating HCECs with sodium chloride to construct a dry eye model can activate Toll-like receptor 4 (TLR4) receptors on the cell membrane and activate caspase-8 via signal transduction, which then enables Nod-like receptor family CARD domain containing protein 4 (NLRC4) and NLRP12 assembly with ASC and pro-caspase-1, forming inflammasomes.[9] These proteins activate inflammatory caspases and further cleave GSDMD and promote the maturation of interleukin-1β (IL-1β) and IL-18.[10] After GSDMD cleavage, the exposed gasdermin-N domain oligomerizes to form membrane pores, which decrease cell osmotic pressure and lead to cell swelling and eventual dissolution.[7] IL-1β and IL-33 exit the cell via pyropyrotic pores, and IL-33 further activates caspase-8 and the NLRC4 inflammasome to form a complex.[9] The inhibition of GSDMD expression may be beneficial for alleviating DED. Zhang et al. reported that calcitriol can reduce hyperosmolar-induced HCEC damage by inhibiting the NLRP3-ASC-Caspase-1-GSDMD pyroptotic pathway.[11]

GSDME was first described in 2017,[12] and it was later confirmed that caspase-3-mediated cleavage of GSDME leads to cell death and cell membrane rupture.[13] The prominent role of pyroptosis caused by caspase-3-mediated cleavage of GSDME has gradually become recognized in various tumor diseases[14] and immune diseases, such as dermatomyosi-

tis, Crohn's disease, acute liver failure, and drug-induced myocardial injury,[15–18] as well as in eye disease. Cai et al. reported that the inhibition of the GSDME-mediated photoreceptor cell pyrosis and apoptosis can provide therapeutic targets for inhibiting dry age-related macular degeneration and Stargardt's disease,[19] but whether GSDME is involved in the pathogenesis of dry eye through the mediation of HCEC pyroptosis has not been reported. Interestingly, the baseline expression of GSDMD is generally greater than that of GSDME. Xu et al. noted that GSDME-mediated pyroptosis plays a much greater role in cholestatic hepatitis than does GSDMD.[20] To explore the function of GSDME more accurately, we studied the gene and protein expression of GSDME and its associated inflammatory factors by knocking down GSDMD in cellular and animal models. The mechanism of its involvement in the pathogenesis of DED was also examined.

Materials and Methods

DED Mouse Model

In this study, female 6 to 8-week-old C57BL/6 mice and 6 to 8-week-old GSDMD-knockout (GSDMD-KO) mice (C57BL/6 background) were purchased from Weitonglihua (Beijing, China) and GemPharmatech LLC (Jiangsu, China), respectively. The experiment started after 7 days of adaptive feeding in the animal room of Nankai Hospital. The dry eye model was induced by subcutaneously injecting desiccation stress (DS)-treated mice with scopolamine hydrobromide (0.6 mg/250 µL; Sigma Aldrich, Allentown, PA, USA) 4 times a day for 7 days.[9,21] The signs of successful modeling included obvious defects in the corneal central epithelium and a significant reduction in tear secretion. The eyeballs were removed after the first injection on day 8 for the corresponding experiments.

This experimental protocol was approved by the Experimental Animal Ethics Committee of Nankai Hospital (NKYY-DWLL-2021-059), in line with ARVO's Statement on the Use of Animals in Ophthalmology and Vision Research.

Cell Culture and siRNA Experiments

HCECs were purchased from Cellcook Biological Technology (Guangzhou, China). The cells were incubated in a cell incubator at 37°C with 5% carbon dioxide. DMEM/F12 (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) was used as the culture medium. HCECs were treated with 312 to 500 mOsm sodium chloride to construct dry eye models. For GSDMD gene knockdown in HCECs, the HCECs were transfected with siGSDMD or siNC and RNAiMAX transfection reagent (Thermo Fisher Scientific), according to the manufacturer's instructions.

CCK-8 Cell Activity Experiment

A CCK-8 assay was used to measure the activity of HCECs treated with different concentrations of sodium chloride. The cells were inoculated in 96-well plates for 24 hours and treated with different concentrations of sodium chloride, and 100 µL of serum-free medium (SFM) and 10 µL of CCK-8 solution were added to each well (GLPbio, USA). After incubation at 37°C for 2.5 hours, the absorbance of each well was detected and compared at a wavelength of 450 nm using a microplate reader (BioTek, Burlington, VT, USA). Each experiment was independently repeated five times.

Flow Cytometry Assay

A flow cytometry assay was performed to detect pyroptosis and apoptosis in HCECs inoculated in 6-well plates for 24 hours, which were then treated with 312 mOsm or 450 mOsm sodium chloride for another period of 24 hours. An Annexin V Apoptosis Test Kit (88-8005-74; Thermo Fisher Scientific, Waltham, MA, USA) was used for the assay. After digestion with trypsin without EDTA,[22] according to the manufacturer's instructions, the samples were collected, centrifuged, and washed twice with PBS. Then, 1 × binding buffer was added for cell counting in accordance with the instructions for the Annexin V Apoptosis Test Kit (88-8005-74; Thermo Fisher Scientific, Waltham, MA, USA). The samples were subsequently incubated with Annexin V-FITC and propidium iodide for 20 minutes in the dark. Afterward, the cells were added to the flow tube and analyzed with a flow cytometer (BD LSR Fortessa; BD Biosciences, Franklin Lakes, NJ, USA), and the experimental results were acquired with FlowJo_V10. According to the previous description, pyroptosis is related to positive PI (Q1 + Q2),[20] apoptosis is related to positive Annexin (Q3), and the normal group is Q4. Each experiment was repeated three times independently.

LDH Release Assay

An LDH release assay was performed to detect cell membrane rupture. After HCECs were inoculated in a 96-well plate for 24 hours, the cells were treated with 312, 400, 450, or 500 mOsm sodium chloride. The LDH release reagent was added to the positive control group after 23 hours, and the cells were incubated in the cell incubator for 1 hour. After incubation, the well plate was centrifuged at 400 × g for 5 minutes. Next, 120 µL of the resulting cell culture supernatant was added to a new well plate, and 60 µL of LDH working solution was added. The mixture was incubated in a horizontal shaker at room temperature in the dark for 30 minutes. The absorbance of each sample at 490 nm was detected with a microplate reader.

Western Blot Analysis

Proteins from HCECs treated with different concentrations of sodium chloride for 24 hours or from wild-type or GSDMD-KO mice treated with scopolamine for 7 days were extracted with RIPA lysis buffer containing protease inhibitors, and a BCA assay was performed to measure the protein concentration. Proteins were added to precast PAGE gels (GenScript, Jiangsu, China). After electrolysis and electrical transfer, the PVDF membranes (Miltenyi Corp., Billerica, MA, USA) containing the transferred proteins were incubated with the following primary antibodies: GSDME, procaspase-3, cleaved caspase-3, PARP, IL-1β, GAPDH, TUBULIN, and β-Actin at 4°C overnight. After washing three times with TBST, the PVDF membrane was incubated with goat anti-rabbit or goat anti-mouse secondary antibodies at room temperature for 1 hour. After washing with TBST, the proteins on the PVDF membrane were detected by chemiluminescence using enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA). The primary and secondary antibodies used for Western blotting are listed in Supplementary Table S1. Each experiment was repeated three times independently.

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Quantitative PCR Assay

After the HCECs were treated with sodium chloride or the wild-type or GSDMD-KO mice were treated with scopolamine for 7 days, an EZ-press RNA Purification Kit PLUS (EZBioscience, Roseville, MN, USA) was used to extract the RNA from the samples. TransScript II All-in-One First-Strand cDNA Synthesis SuperMix (TransGen Biotech) was used for reverse transcription (RT) for quantitative PCR (qPCR). PerfectStart Green qPCR SuperMix (TransGen Biotech) and corresponding primers were used for RT-qPCR. Each experiment was independently repeated five times. The sequences of the primers used were as follows: GAPDH, 5′-GAGTCAACGGATTTGGTCGT-3′ and 5′-AATGAAGGGGTCATTGATGG-3′; GSDMD, 5′-GAGTGTGGCCTAGAGCTGG-3′ and 5′-GGCTCAGTCCTGATAGCAGTG-3′; GSDME, 5′-ACATGCAGGTCGAGGAGAAGT-3′ and 5′-TCAATGACACCGTAGGCAATG-3′, CASP3, 5′-CATGGAAGCGAATCAATGGACT-3′ and 5′-CTGTACCAGACCGAGATGTCA-3′; PARP, 5′-TGGAAAAGTCCCACACTGGTA-3′ and 5′-AAGCTCAGAGAACCCATCCAC-3′, and IL1B, 5′-ATCAGCACCTCTCAAGCAG-3′ and 5′-AGTCCACATTCAGCACAGG-3′.

Corneal Fluorescein Staining Assay

Seven days after the hypodermic injection of scopolamine, each eye was stained with fluorescein to observe whether the dry eye model was successfully established. One microliter of 0.1% fluorescein solution was dropped into the conjunctival sac of each mouse. After 90 seconds, corneal epithelial injury was observed under a slit lamp using a cobalt blue filter.

Mouse Tear Secretion Assay

After C57BL/6 or C57BL/6, the GSDMD-KO mice were injected subcutaneously with scopolamine for 1.5 hours. A phenol red-impregnated cotton thread was placed on the side of the conjunctival sac of each mouse for 1 minutes, and the wet length of the thread was then measured. If the wet length of the thread was less than 2 mm, the scopolamine dosage was considered effective at reducing tear secretion and could be used in subsequent experiments.

Mouse Corneal Epithelial Cell Apoptosis Detection via TUNEL

Eyeballs were fixed with FAS eyeball fixative solution (G1109; Servicebio, Wuhan, China), dehydrated, paraffin embedded, sliced, dewaxed, and then incubated in DNA-free protease K at room temperature for 20 minutes. After washing with PBS, the sections were incubated with 3% hydrogen peroxide solution at room temperature for 20 minutes. The sections were then washed with PBS, TUNEL working solution (Beyotime, Shanghai, China) was added, and the samples were incubated at 37°C for 1 hour in the dark. After washing with PBS, mounting medium was added, and the sections were imaged. Each experiment was independently repeated five times.

Immunofluorescence

The corneal paraffin sections from the different groups were washed with PBS after antigen repair and blocked with 10% goat serum for 1 hour. After washing with PBS 3 times for 5 minutes each, the sections were incubated with primary antibodies against ASC, IL-1β, and K12 overnight at 4°C. The samples were washed with PBS 3 times and then incubated with goat anti-rabbit and goat anti-mouse secondary antibodies at room temperature in the dark for 1 hour. Finally, after incubation with DAPI (Solarbio, Beijing, China) at room temperature for 15 minutes, the images were taken with a laser confocal microscope (Leica, Germany). The primary and secondary antibodies used for immunofluorescence are listed in Supplementary Table S2.

Statistical Analysis

All the data were independent samples and are expressed as the mean ± standard deviation. A 95% bilateral confidence interval was used, and a P value less than 0.05 was considered to indicate statistical significance. The data were analyzed with GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). The Shapiro–Wilk and Kolmogorov–Smirnov methods were used to test normality. A homogeneity test of variance was subsequently performed. The statistical methods used were as follows: two independent samples were tested for normal distribution with homogeneous variances according to the unpaired t-test. Two independent samples were also tested for normal distribution with uneven variance according to Welch's t-test. Independent samples were also tested for conformation to the normal distribution according to the Mann–Whitney U test. Multiple independent samples were also examined for conformations to a normal distribution according to 1-way ANOVA.

Results

Cell Pyroptosis, Decreased Cell Activity, and Cell Membrane Rupture Were Observed in HCECs in the Dry Eye Model

Flow cytometry was used to observe pyroptosis and apoptosis in HCECs, and the results revealed that the number of pyroptotic cells was significantly greater in the 450 mOsm sodium chloride treatment group than in the healthy group (312 mOsm; Figs. 1A, 1B). The results of the CCK-8 assay revealed that the viability of HCECs gradually decreased with increasing sodium chloride concentration (Fig. 1C). In addition to cell apoptosis, cell membrane rupture is an important characteristic of pyroptosis.[23] An LDH release assay was performed to examine the effect of hypertonic treatment on the rupture of the HCEC membranes. A gradual, concentration-dependent increase in LDH release was observed after sodium chloride treatment (Fig. 1D).

Caspase-3 and GSDME Gene and Protein Expression Levels Increased in the HCEC DED Model

To determine whether caspase-3 and GSDME are involved in the pathogenesis of dry eye, the gene and protein expression levels of caspase-3 and GSDME were detected in the HCEC DED model. The qPCR results revealed that, compared with those in the healthy group, the expression levels of the caspase-3 and GSDME genes increased after HCECs were

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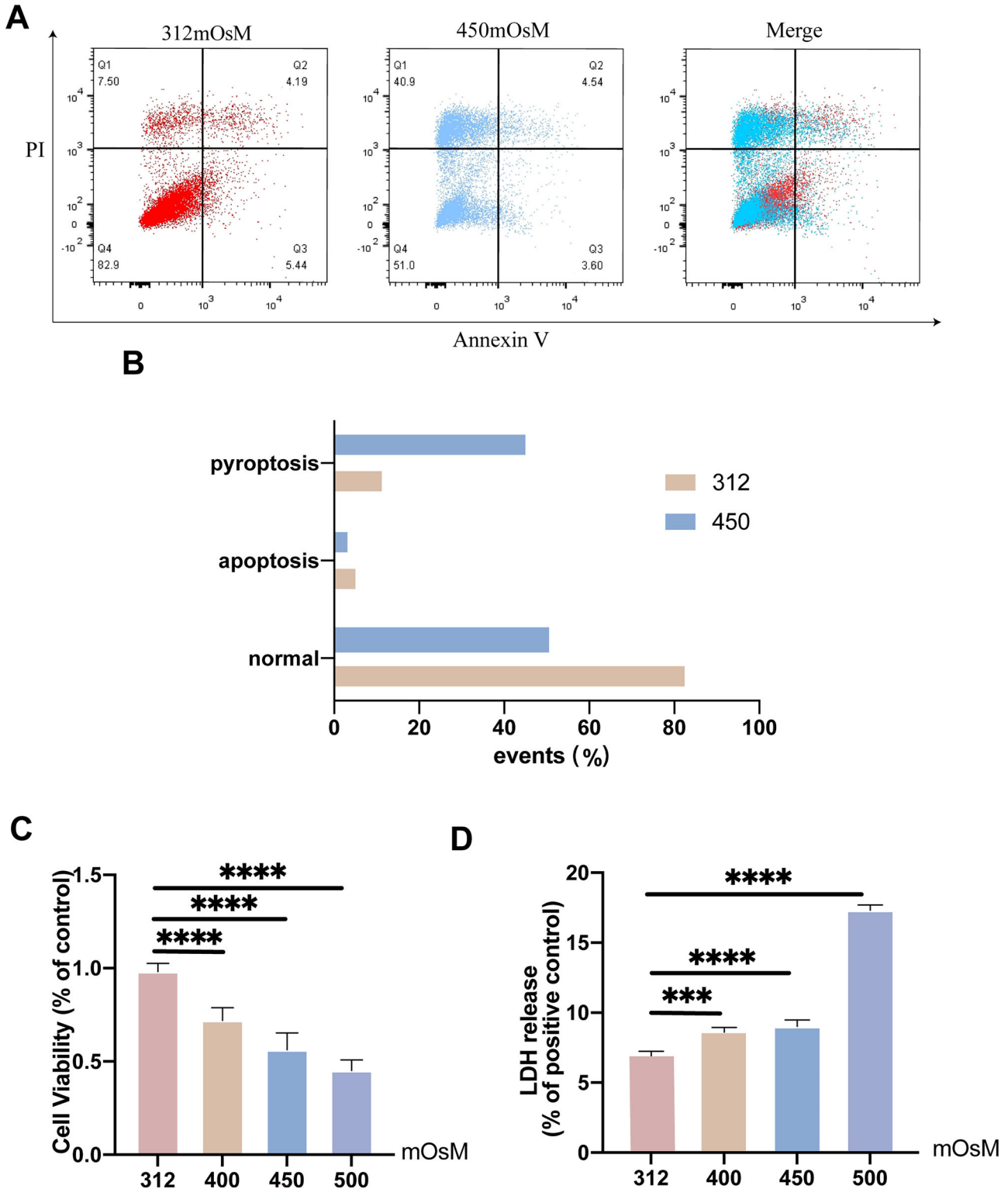

Figure 1. Pyroptosis, a decrease in cell viability and cell membrane rupture occurred in human corneal epithelial cells after hypertonic treatment. (**A**, **B**) A flow cytometry assay was used to detect corneal epithelial cell pyroptosis after HCECs were treated with 312 and 450 mOsm sodium chloride. (**C**) A CCK-8 assay was used to detect the effects of different concentrations of sodium chloride on the viability of corneal epithelial cells. (**D**) An LDH release assay was used to detect corneal epithelial cell membrane rupture after treatment with different concentrations of sodium chloride. ***$P < 0.001$.

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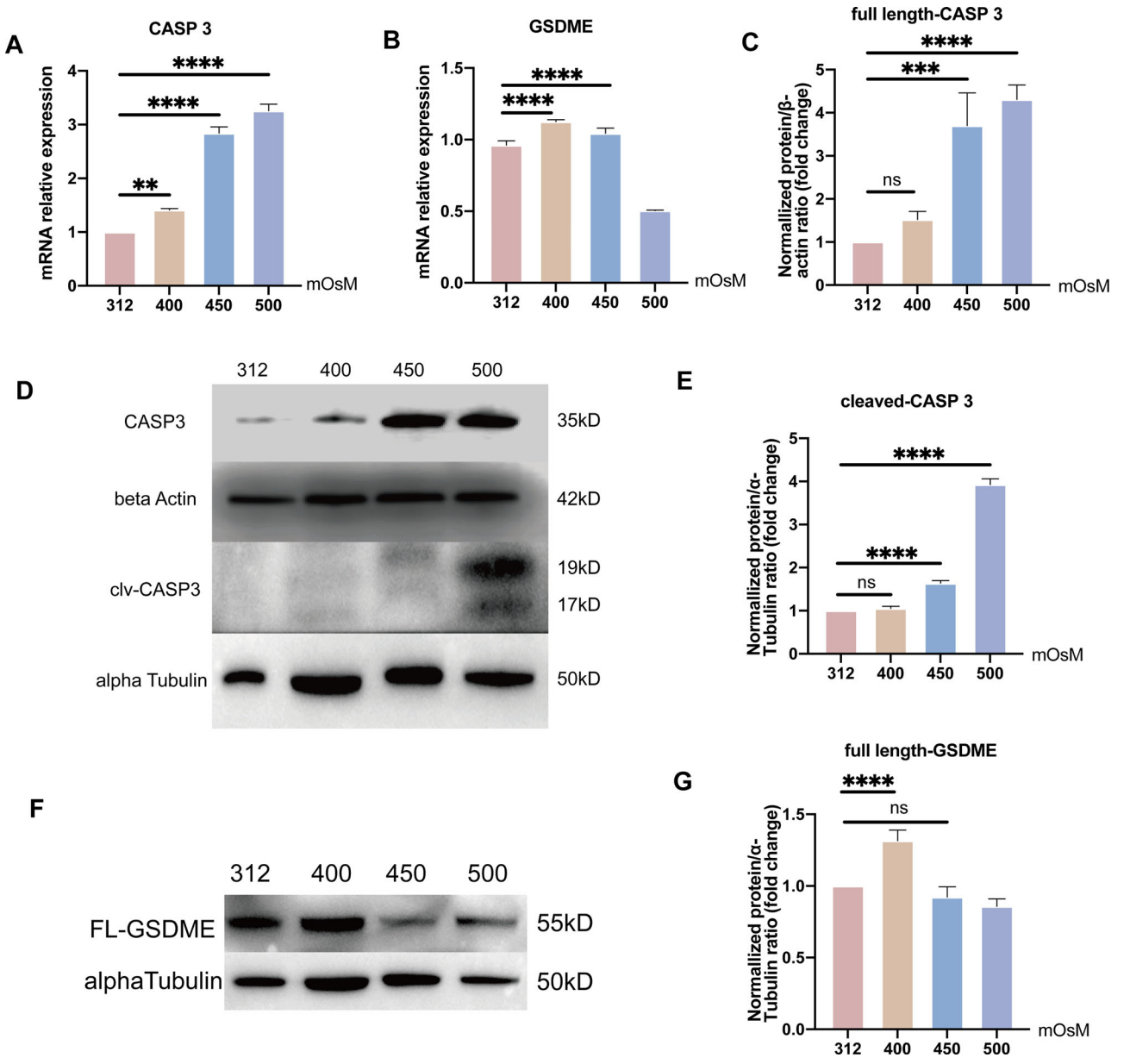

Figure 2. Increased expression levels of the caspase-3 and GSDME genes and proteins in human corneal epithelial cells treated with different concentrations of sodium chloride. (**A**, **B**) A qPCR assay was used to detect the expression levels of the caspase-3 and GSDME genes in human corneal epithelial cells treated with different concentrations of sodium chloride. (**C–G**) Western blotting was used to detect the protein expression of caspase-3, cleaved caspase-3, and GSDME in HCECs treated with different concentrations of sodium chloride. NS, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

treated with sodium chloride (Figs. 2A, 2B). Additionally, the Western blot results revealed that the expression levels of the caspase-3, cleaved caspase-3, and GSDME proteins increased after hypertonic treatment. Caspase-3 expression was dose dependent, and the gene and protein expression of GSDME began to decrease after peaking.

GSDME Expression Increased After GSDMD Knockdown in HCECs and in a Mouse Model

The basal expression of GSDMD is generally higher than that of GSDME (Fig. 3A), which means that GSDMD is likely to mask the effects of GSDME. Therefore, GSDMD was knocked down to determine the function of GSDME. To this end, HCECs were transfected with siGSDMD, and the effects on GSDME expression were observed. The qPCR revealed that the expression of GSDME increased after GSDMD was knocked down in HCECs (Figs. 3B, 3C). In C57BL/6 mice, tear secretion decreased and corneal fluorescein sodium staining was positive after the mice were injected subcutaneously with scopolamine (Figs. 3D, 3E), which confirmed that the DED mouse model was successfully established. The qPCR revealed that the gene expression levels of GSDME in the DS-treated and GSDMD-KO mice were greater than those in the wild-type mice (Fig. 3F). Western blot analysis revealed that the protein expression levels of full-length GSDME and cleaved GSDME increased in DS-treated mice

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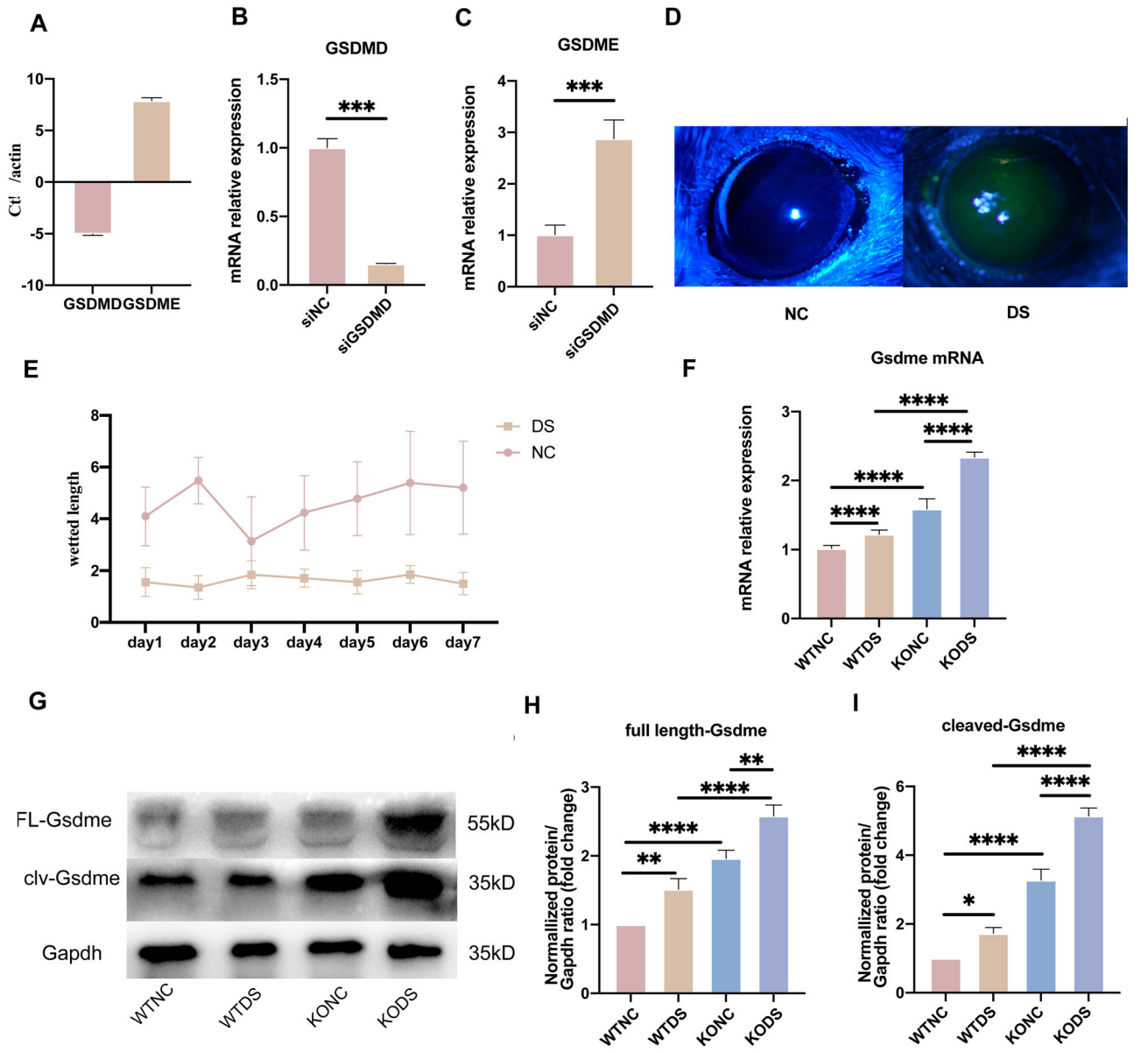

Figure 3. Increased expression of the GSDME gene and protein in corneal epithelial cells and mice after GSDMD knockdown. (**A**) Ct values of actin homogenized by GSDMD and GSDME in the normal control group (312 mOsm). (**B**, **C**) A qPCR assay was used to detect the gene expression levels of GSDMD and GSDME knocked down by GSDMD in human CECs. (**D**) Corneal fluorescein staining was used to analyze the integrity of the corneal epithelium. E: A mouse tear secretion assay was used to detect tear secretion after dry eye modeling in C57BL/6 mice. (**F**) A qPCR assay was used to detect GSDME gene expression after GSDMD-KO mice were treated with DS. (**G–I**) Western blotting was used to detect the protein expression levels of FL-GSDME and cle-GSDME in DS-induced wild-type mice and GSDMD-KO mice with dry eye. WTNC, wild-type mouse; WTDS, DS-treated wild-type mouse; KONC, GSDMD-KO mouse; KODS, DS-treated GSDMD-KO mouse. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

and GSDMD-KO mice (Figs. 3G-I) compared with those in wild-type mice, indicating that the expression of GSDME, which may be the main pyroptosis-related protein, increased after GSDMD knockout.

GSDMD Depletion Increased Caspase-3 Expression But Did Not Affect Apoptosis

Western blot analysis revealed that, compared with that in wild-type mice, the protein expression of caspase-3 in GSDMD-KO mice increased after treatment with DS (Fig. 4A). To explore whether this effect was related to the apoptosis of corneal epithelial cells, Western blot analysis was performed to detect the expression of PARP. There was no significant difference in the expression of full-length Parp in the GSDMD-KO mice treated with DS. The expression levels of the cleaved PARP protein were also low and not significant (see Figs. 4A, 4B), and the results of the TUNEL apoptosis detection assay revealed that the four groups did not exhibit strong fluorescence (Fig. 4C), which indicated that although the expression of caspase-3 increased in the GSDMD-knockdown group treated with DS, apoptosis was not affected.

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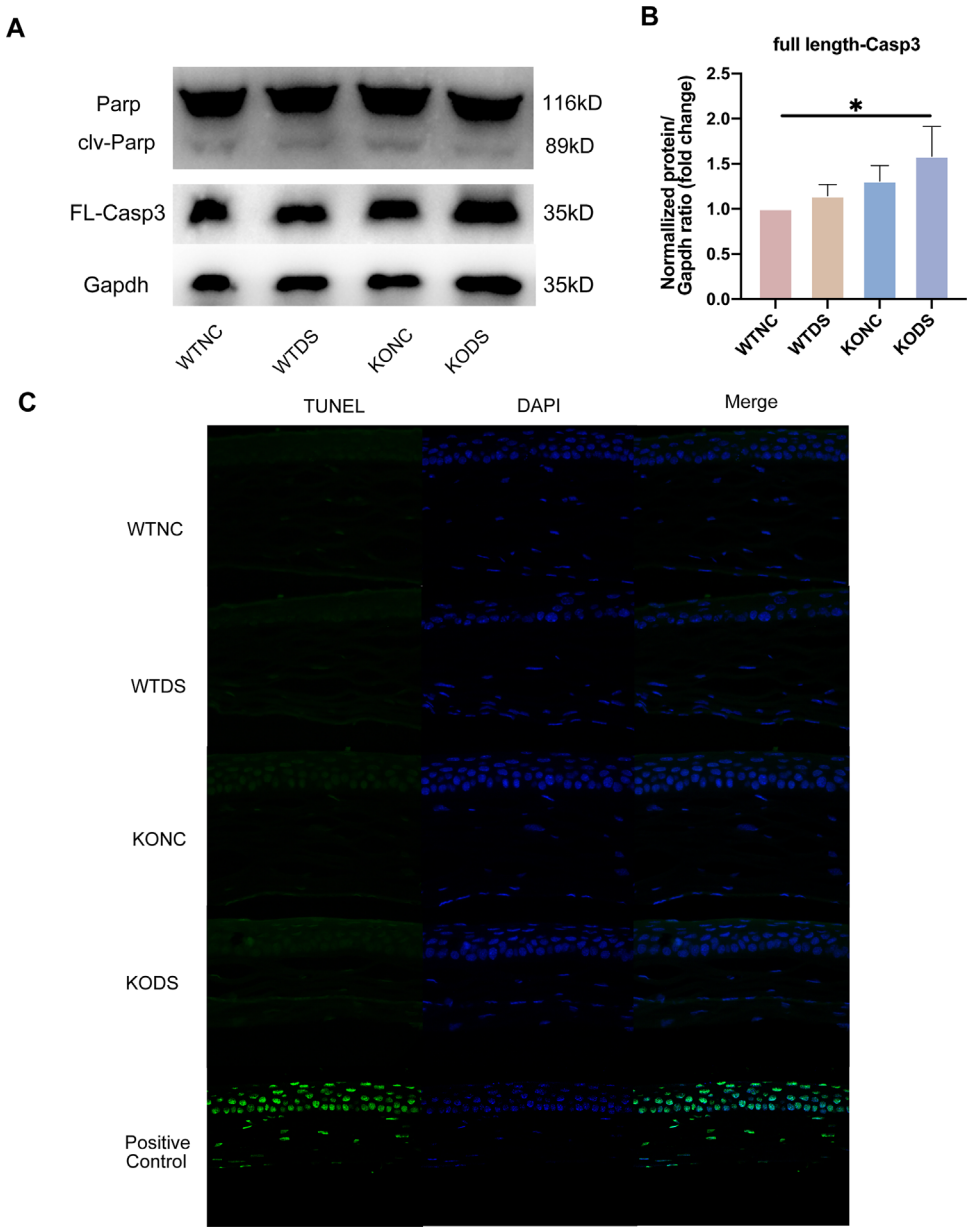

Figure 4. GSDMD depletion increased caspase-3 expression but did not affect apoptosis. (**A, B**) Western blot analysis was used to detect the expression levels of the FL-caspase-3, Parp, and cle-Parp proteins in DS-induced wild-type and GSDMD-KO dry eye model mice. (**C**) A TUNEL assay was used to detect corneal epithelial cell apoptosis in DS-induced wild-type and GSDMD-KO dry eye model mice. WTNC, wild-type mouse; WTDS, DS-treated wild-type mouse; KONC, GSDMD-KO mouse; KODS, DS-treated GSDMD-KO mouse. *$P < 0.05$.

ASC and IL-1β Gene and Protein Expression Levels in GSDMD-KO Mice Following Subcutaneous Injection With Scopolamine Were Still Greater Than Those in the Normal Group

Immunofluorescence experiments targeting ASC, IL-1β, and K12 (epithelial markers) revealed that the expression levels of ASC and IL-1β in DS-treated wild-type mice or DS-treated GSDMD-KO mice were significantly greater than that in wild-type or GSDMD-KO mice (Figs. 5A, 5B). The results of qPCR and Western blotting revealed that the expression level of IL-1β in DS-treated GSDMD-KO mice was greater than that in untreated GSDMD-KO mice, although the increase was not as high as that in wild-type mice (Figs. 5C–E). In conclusion, a significant inflammatory response still occurs through the GSDME-mediated pyroptosis pathway after GSDMD knockout in a DS-induced dry eye model in mice.

Discussion

DED has a high incidence worldwide and is the main reason why ophthalmic patients seek treatment.[24] In addition to dryness, foreign body sensation, and visual fatigue,[4] patients with severe disease may suffer from visual impairment.[25] Moreover, long-term eye discomfort may also affect the patients' physical and mental health,[26,27] which has attracted

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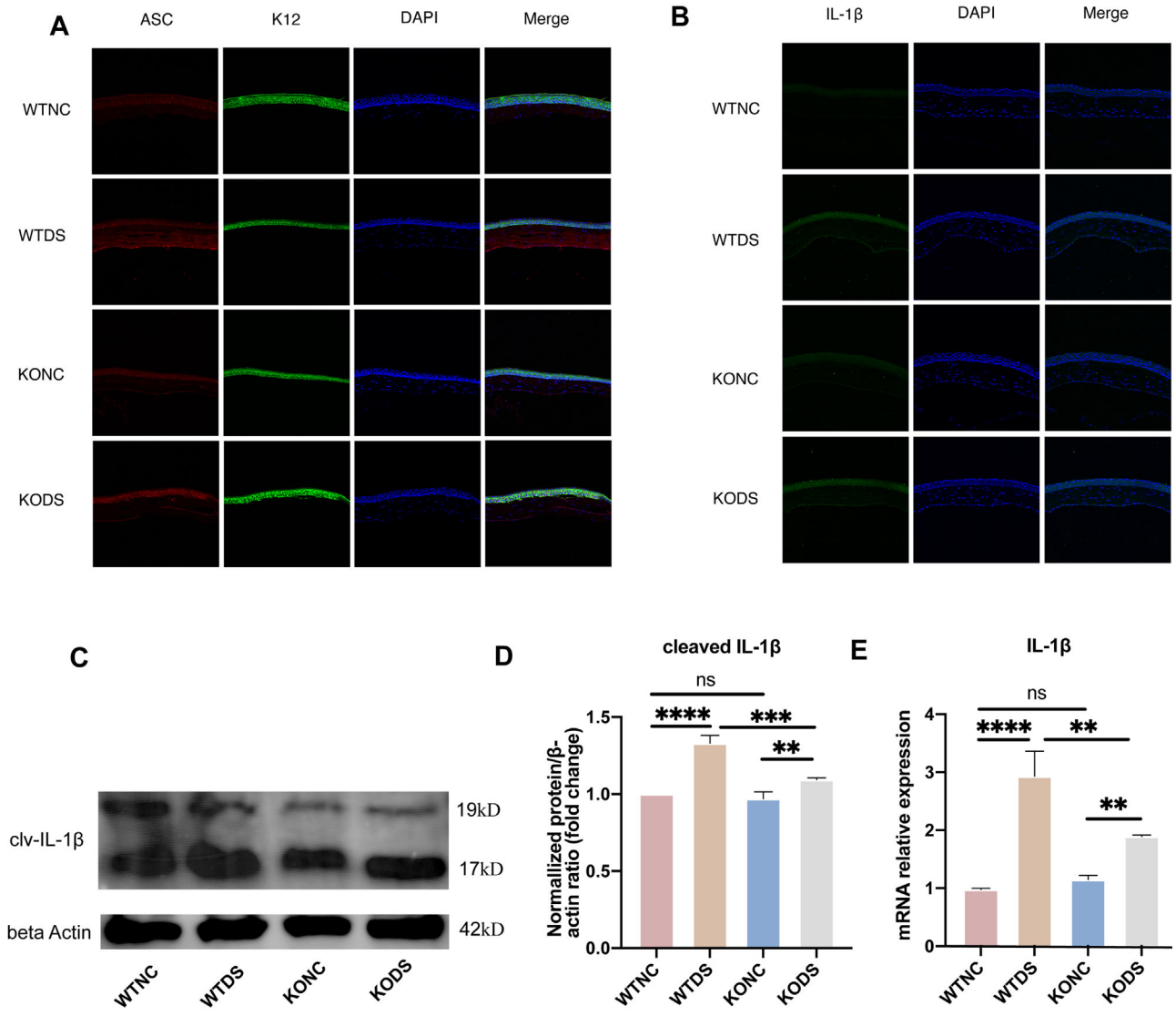

Figure 5. The expression levels of ASC and IL-1β in the DS-induced DED GSDMD-KO mice were greater than those in the wild-type mice. (**A, B**) Immunofluorescence was used to detect the protein expression levels of ASC, IL-1β, and K12 (markers of the epithelium) in DS-induced wild-type and GSDMD-KO dry eye model mice. (**C, D**) Western blot analysis was used to detect the protein expression of levels IL-1β in DS-treated wild-type and GSDMD-KO dry eye model mice. (**E**) The qPCR was used to detect the gene expression of IL-1β in DS-induced wild-type mice and GSDMD-KO mice with dry eye. WTNC, wild-type mouse; WTDS, DS-treated wild-type mouse; KONC, GSDMD-KO mouse; KODS, DS-treated GSDMD-KO mouse; ns, not significant, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

considerable attention from the academic community. The onset of DED may involve desiccation and osmotic stress caused by one or more factors.[28] Inflammation caused by ocular surface injury and hyperosmolarity affects corneal epithelial cells and immune cells.[29] Disruption of inflammatory factor secretion further leads to reduced mucus secretion and unstable tear film, aggravating the occurrence of dry eye.[30] Pyroptosis, a type of programmed cell death that can affect the inflammatory cycle, is considered a key process induced by GSDMD. This process is regulated by caspase-1 and plays an important role in the pathogenesis of dry eye.[31] A variety of inflammation- and pyroptosis-related factors, including ASC, procaspase-1, IL-1β, and IL-18,[32] regulate the downstream immune response,[33] activate caspase-1 to lyse GSDMD and ultimately lead to pyroptosis.[34,35] Chen et al. reported that NLRP4 and NLRP12 cooperate synergistically to promote pyroptosis and aggravate DED in a GSDMD-dependent manner.[9] GSDME was originally identified in 2017.[12] Later, Rogers et al. confirmed that the cleavage of GSDME by caspase-3 leads to cell death and cell membrane rupture.[13] Researchers have subsequently focused on the role of pyroptosis caused by the cleavage of GSDME by caspase-3 in treating tumors and immune diseases.[15,16,36] Notably, the extent of GSDME-mediated pyroptosis is much greater than that of GSDMD in cholestatic hepatitis.[20] Therefore, the role of GSDME-mediated pyroptosis in DED is worthy of further exploration.

In this study, positive PI was related to pyroptosis,[20] whereas apoptosis is more likely to be associated with positive Annexin and is distinguished from cell rupture. Recently, some researchers have referred to GSDME-mediated necrosis as secondary necrosis.[37] Pyroptosis is defined as a type of

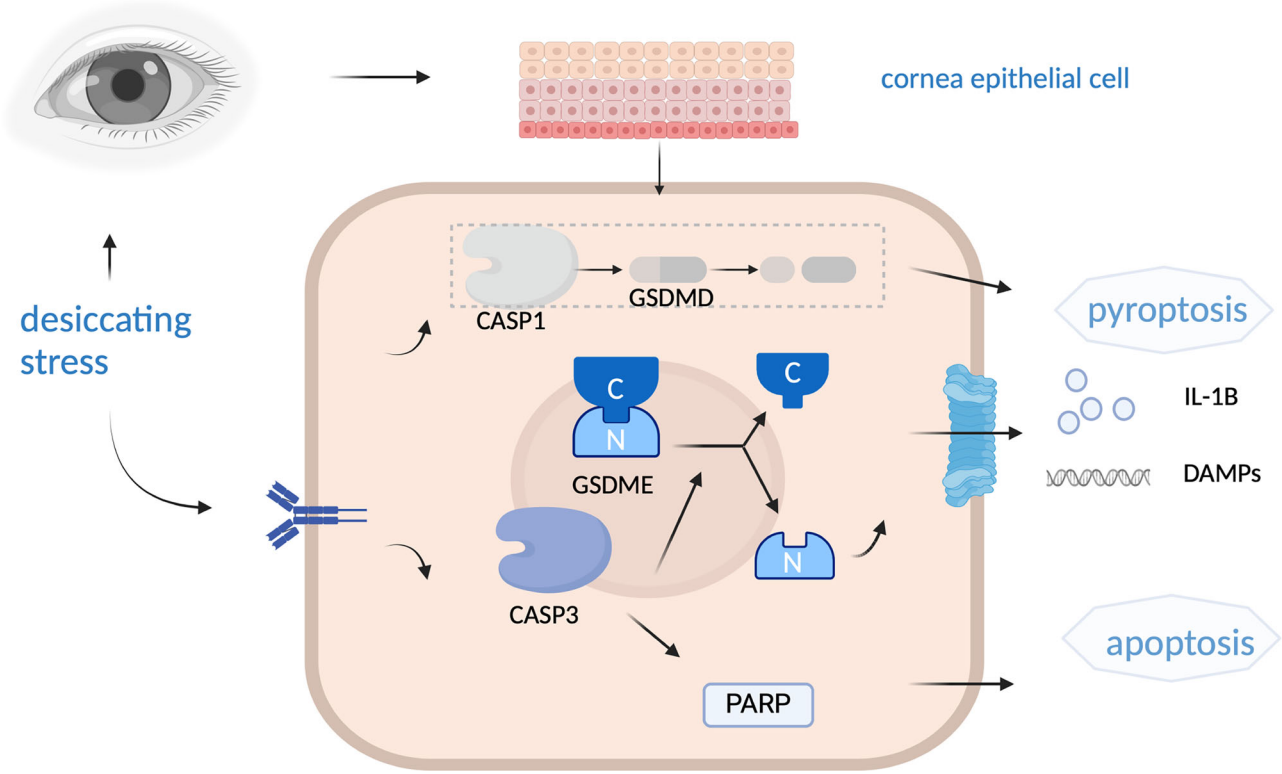

Figure 6. Diagram illustrating that DS induces caspase-3/GSDME axis-mediated HCEC pyroptosis rather than apoptosis, which exacerbates DED development after GSDMD being knocked down. This figure is created by Biorender.

cytolytic programmed necrosis characterized by cell swelling and cell membrane bullae. Further confirmation of pyroptosis by transmission electron microscopy in the future will complement this view, whereas other scholars have also reported that the changes were significantly positive for Annexin and PI through chemotherapy-induced cell pyroptosis,[12,18] which may be related to the strong modeling conditions, and our previous study aimed to simulate the death of aseptic inflammatory disease caused by chronic injury-related DAMPs. In DED, the corneal epithelium undergoes obvious pyroptosis. Our results revealed that the gene and protein expression levels of caspase-3 and GSDME were elevated in both in vitro and in vivo DED models. We also observed that GSDME expression was not dependent on the dosage of sodium chloride in HCECs and that the increase in GSDME expression was not as strong as that in caspase-3 expression. This may be related to the mutation of GSDME, and the mutation site is near the site cut by caspase-3 so that GSDME cannot be cut.[38] However, studies have shown that GSDME epigenetic inhibition is more common than the GSDME mutation.[39,40] Compared with that in healthy tissues, GSDME expression levels in gastric cancer, colon cancer, breast cancer, and other tumor cells are decreased,[41] which may be due to methylation of the GSDME promoter, resulting in the normal transcription of GSDME.[42,43] Thus, it is difficult to activate pyroptosis in most tumor cells,[44] suggesting that GSDME methylation is a tumor marker.[45,46]

We found that the baseline expression of GSDMD was much greater than that of GSDME. In the presence of GSDMD, pyroptosis caused by GSDME may be masked. Therefore, analysis of pyroptosis caused by GSDME in the presence of GSDMD requires more complex techniques. However, knockout of GSDMD makes the study much more intuitive. Therefore, GSDMD-KO mice and HCECs transfected with si-GSDMD were used in the present study. The results revealed that the GSDME gene and protein expression increased after GSDMD knockout, which suggests that GSDME may be able to replace GSDMD as the main protein involved in pyroptosis (Fig. 6).

The gene and protein expression of caspase-3 still increased in the GSDMD-KO mice after they were injected with scopolamine, and the apoptosis rates of the corneal epithelial cells and the levels of apoptosis-related factor PARP did not change significantly. Cell destruction during apoptosis is mediated mainly by inflammatory caspases.[47] A series of different stimuli lead to the continuous activation of caspases, leading to different cell destruction processes,[48] including DNA cleavage by DNase, chromatin coagulation, and the production of apoptotic bodies.[49] Full-length PARP can repair damaged DNA, deactivation of PARP after cleavage leads to apoptosis, and overactivation of PARP can also lead to cell death.[50] However, in our study, there was no significant difference in full-length PARP levels among the groups, and no positive results were found in the TUNEL assay, indicating that the level of apoptosis induced by caspase-3 after GSDMD knockout was extremely weak. The modeling conditions did not cause significant apoptosis but also indicated that the activation of caspase-3 was closely tied to the GSDME-mediated pyroptosis pathway. However, DED models of different severities are needed to verify these conclusions further.

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When different pathogenic factors affect HCECs, after a series of signal transduction pathways, ASC and procaspase-1 are recruited and assemble with NLRP3, NLRP6, NLRC4, and NLRP12, and other NOD-like receptor family proteins to form inflammasomes,[9,51] which provide response sites for subsequent reactions. The expression levels of ASC and IL-1β can directly predict and reflect the current level of inflammation in cells, respectively. The results revealed that the expression levels of the ASC and IL-1β genes and proteins increased after dry eye modeling in the GSDMD-KO mice, indicating that obvious inflammation was still generated through the GSDME pyroptosis pathway after GSDMD knockout. However, the present study could not directly prove the existence of cleaved GSDME oligomerization from pyropores. Notably, IL-1β and other proteins are known to exit pyroptosis pores and cause cascade inflammation, which is an important feature of pyroptosis.[34,52] Finally, the presence of IL-1β can be used to infer the presence of pyropores. In summary, this study revealed that GSDMD knockdown cannot completely interrupt the positive feedback loop of pyroptosis-induced inflammation and that GSDME-mediated pyroptosis is also an important step involved in pyroptosis pathogenesis.

In this study, we observed increased GSDME expression in HCECs and GSDMD-KO model mice with dry eye, and caspase-3/GSDME played an important role in promoting dry eye through mediating pyroptosis. We proposed and observed an important role of GSDME in the pathogenesis of dry eye. However, in the future, GSDME should be further knocked down to observe its ability to relieve dry eye and its feedback effect on other inflammatory molecules, such as IL-1β. Furthermore, we hope that knocking down GSDMD and GSDME at the same time to identify key proteins and regulate GSDMD- and GSDME-mediated pyroptosis to inhibit dry eye syndrome will be an important direction of future research.

Dry eye is classified as aqueous-deficient dry eye, evaporative dry eye, or mixed dry eye.[53] In this study, we revealed the effect of GSDME on HCEC pyroptosis in scopolamine-induced DED models. This is currently the most widely studied model of dry eye disease,[9,21] whereas the role of GSDME in other types of DED models also needs to be further explored. NLRP3 inflammatory body activation has been reported to be associated with environmentally induced dry eye,[54] and Lou et al. reported that suppression of GSDMD-mediated corneal epithelium pyroptosis is helpful for inhibiting benzalkonium chloride (BAC)-induced DED. It is highly important to further explore the role of GSDME and whether reducing GSDME can inhibit BAC-induced dry eye.[55]

This study has several limitations, such as not being able to further confirm whether GSDME is the final link of pyroptosis in the pathogenesis of dry eye. Additionally, the relationships among other promoters, such as IL-1β and GSDME, have not been fully elucidated.

Conclusions

In this study, we found that caspase-3/GSDME mediates pyroptosis rather than the apoptosis pathway in the occurrence and development of dry eye by promoting inflammation. The inhibition of GSDME may be a potential therapeutic target for DED.

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Author Contributions: Ling Wang conceived and designed the project, methodology, investigation, and writing - original draft preparation. **Ye Tian** conceived and designed the project, data curation, and visualization. **Hui Zhang** was responsible for the resources and supervision. **Yongxiao Dong** was responsible for the formal analysis, software, and investigation. **Xia Hua** and **Xiaoyong Yuan** were responsible for the funding acquisition, resources, supervision, and writing - review and editing. All the data were generated in-house, and no paper mill was used. All the authors agree to be accountable for all aspects of the work, ensuring its integrity and accuracy.

Availability of Data and Material: The data that support the findings of this study are available upon reasonable request from the corresponding author.

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