Evodiamine Promotes Autophagy and Alleviates Oxidative Stress in Dry Eye Disease Through the p53/mTOR Pathway

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Purpose. This study aims to explore the therapeutic efficacy of evodiamine (EVO) in the treatment of dry eye disease (DED).

Methods. Mouse models of DED was developed using benzalkonium chloride eye drops and subcutaneous atropine injections. Corneal epithelial defects were assessed by fluorescein sodium staining, and tear secretion was measured with the phenol red thread test. For the in vitro model, human corneal epithelial cells were cultured in a sodium chloride–enriched medium. Phenotypic and mechanistic analyses were conducted using real-time quantitative PCR, Western blotting, flow cytometry, and immunofluorescence staining.

Results. The administration of EVO eye drops significantly enhanced tear secretion in mice, ameliorated ocular surface damage, decreased the expression of corneal inflammatory factors, and increased the density of conjunctival goblet cells. Furthermore, EVO reduced oxidative stress by promoting autophagy. Mechanistically, EVO-induced autophagy was mediated via the p53/mammalian target of rapamycin pathway.

Conclusions. These findings suggest that EVO is a potential therapeutic agent for the treatment of DED, with its beneficial effects attributed to the activation of autophagy through the p53/mammalian target of rapamycin pathway.

Keywords: dry eye disease, evodiamine, autophagy, oxidative stress

Dry eye disease (DED) occurs when tear production fails to lubricate the ocular surface adequately, prevent infection, and promote wound healing.[1,2] The 2017 International Symposium on Dry Eye Disease defined DED as "a complex, multifactorial ocular surface disease characterized by a loss of tear film homeostasis, accompanied by ocular symptoms such as dryness, itching, and stinging of the eyes." Key etiological factors in DED include tear film instability, elevated osmolarity of the ocular surface, inflammation, and injury.[3] DED affects approximately 5% to 50% of the global population,[1] with a greater prevalence in women than men and an increased incidence with age.[4] As a major public health issue, DED severely impacts patients' quality of life.[5–7] However, current treatments do not resolve DED completely.

Autophagy, a self-degradation process, is crucial for maintaining cellular and organismal homeostasis. It removes misfolded proteins, damaged organelles, and intracellular pathogens, contributing to cellular repair and survival[8,9] Research has shown that moderate activation of autophagy can alleviate DED symptoms.[10] Enhancing autophagy may, therefore, provide therapeutic benefits in DED management.[11–14]

Oxidative stress, resulting from excessive production and accumulation of reactive oxygen species (ROS), is another key factor in DED pathogenesis. The accumulation of ROS can damage cellular components, leading to inflammation and further contributing to DED progression. The imbalance between ROS and antioxidant enzymes also exacerbates oxidative damage, promoting inflammation and

aggravating DED.[15–17] Therefore, strategies aimed at reducing ROS accumulation and restoring redox balance are important in DED treatment.[14,18,19]

Autophagy and oxidative stress are inter-related. Autophagy plays a protective role by degrading and recycling damaged cellular components through lysosomal activity, thereby reducing oxidative damage and maintaining redox homeostasis.[20] This interplay suggests that autophagy modulation could be a promising approach to mitigating oxidative stress in DED.

Current pharmacological treatments for DED include artificial tears, autologous serum, umbilical cord serum, topical cyclosporine, corticosteroids, mucin secretagogues, and anti-inflammatory agents.[21,22] However, these treatments are unsatisfactory. Artificial tears provide only temporary relief, and corticosteroids can cause significant side effects.[23] Evodiamine (EVO), an active compound from the traditional Chinese medicine *Wu Zhu Yu*, is a quinoline alkaloid with diverse pharmacological properties.[24] EVO exhibits anti-inflammatory, antiproliferative, and antioxidant effects and has been used in the treatment of various diseases.[25] Notably, EVO has been reported to activate autophagy in colitis model,[26] although its effects in DED and its interaction with oxidative stress and autophagy remain unclear.

The tumor suppressor protein p53 is a transcription factor that regulates cellular processes, including metabolism, apoptosis, and the tumor microenvironment.[27] It also plays an important role in autophagy and ferroptosis.[28] The mammalian target of rapamycin (mTOR) is a serine/threonine kinase and a classic upstream regulator of autophagy.[29,30] Modulating the p53/mTOR pathway is essential for autophagy activity. However, it remains unclear whether EVO influences autophagy through the p53/mTOR pathway in the context of DED.

This study investigated the therapeutic effects of EVO on DED and explored its underlying mechanisms. Our findings indicated that EVO reduced corneal damage, enhanced tear secretion, alleviated corneal inflammation, and increased conjunctival goblet cell density in the mouse model of DED. Additionally, EVO was found to reduce cell apoptosis and ROS accumulation, while enhancing antioxidant enzyme expression in human corneal epithelial cells (HCECs). Furthermore, we found that EVO regulated autophagy levels in DED, potentially via the p53/mTOR pathway.

Materials and Methods

Cell Culture and In Vitro Cell Models

The HCECs (ZQ1003, Shanghai Zhong Qiao Xin Zhou Biotechnology) were cultured in DMEM/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). The cells were maintained in a humidified incubator at 37°C with 5% CO_2. To stimulate a hyperosmotic environment akin to DED, sodium chloride was added to the culture medium to achieve a final osmolarity of 500 mOsm/L.

Cytotoxicity Assay and Cell Viability Rescue Experiment

The cytotoxicity of EVO was assessed using a Cell Counting Kit-8 (CCK-8; Vazyme, Nanjing, China). HCECs were seeded into 96-well plates. Cells were then treated with various concentrations of EVO (analytical standard with a purity of > 99%, catalog number: E101966-20 mg, Aladdin Scientific, Shanghai, China), which was initially dissolved in dimethyl sulfoxide and subsequently diluted in complete medium. After 24 hours, 10 µL of CCK-8 reagent was added to the medium and incubated for 1 to 2 hours. Absorbance at 450 nm was measured using a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland).

For the cell viability rescue experiment, HCECs were pretreated with different concentrations of EVO for 24 hours. After this pretreatment, the cells were exposed to hyperosmotic medium. In a parallel setup, HCECs were pretreated with hyperosmotic medium for 24 hours, followed by treatment with different concentrations of EVO. Cell viability was then assessed using the CCK-8 assay.

Measurement of Cell Apoptosis Rate

HCEC were seeded in 6-well plates and treated with EVO or hyperosmotic medium. Apoptosis was measured using the Annexin V-FITC/PI Apoptosis Detection Kit (KGA1109–20, KeyGEN BioTECH, Jiangsu, China). Data were acquired using the Accuri C6 flow cytometer (BD, Franklin Lakes, NJ, USA), and the total number and proportion of late apoptotic cells were analyzed with FlowJo V10 software (FlowJo, Ashland, OR, USA).

RNA Extraction and Real-time Quantitative PCR

Total RNA was extracted from samples using FreeZol reagent (R711, Vazyme). One microgram of total RNA was reverse transcribed into cDNA using the HiScript gDNA Removal RT MasterMix (CW2020, CWBIO, China Medical City, China). Real-time quantitative PCR was performed using the MagicSYBR Mixture (CW3008, CWBIO, China) on the ABI QuantStudio 6 Flex system (Invitrogen, Carlsbad, CA, USA). β-Actin was used as an internal control. The relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.[31] The genes included in the analysis are presented in Supplementary Table S1.

Measurement of ROS

HCECs were treated with EVO and hyperosmotic medium. Subsequently, the cells were stained with the DCFH-DA probe (Beyotime, Shanghai, China) at 37°C for 30 minutes, followed by washing with PBS. Fluorescence intensity was measured at an excitation wavelength of 488 nm using the Leica Thunder system (Leica, Wetzlar, Germany), Accuri C6 flow cytometer (BD), and Tecan SPARK fluorescence microplate reader (Tecan Austria GmbH, Grödig/Salzburg, Austria). Data processing was performed using ImageJ software (NIH) and FlowJo V10 software (FlowJo).

Detection of Oxidative Stress Indicators: Superoxide Dismutase (SOD), Malondialdehyde (MDA), and Catalase (CAT)

Cells samples were lysed using ultrasonication and centrifuged to obtain the supernatant. The contents of SOD, MDA, and CAT were measured according to the instructions provided by the manufacturer (Beyotime). These measurements reflected the oxidative stress levels in the DED model.

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Construction of the DED Mouse Model

Female C57BL/6 mice, aged 6 to 8 weeks, were purchased from the Experimental Animal Center of Yangzhou University and housed in the Animal Experiment Center of Drum Tower Hospital, Affiliated Hospital of Medical School, Nanjing University. The mice were divided randomly into 6 groups, each consisting of 10 mice. One group served as the control group without intervention. The remaining mice received 0.075% benzalkonium chloride eye drops to induce DED. After this period, the mice were assigned to the following groups: DED group, low-dose group (500 µM EVO), high-dose group (1 mM EVO), vehicle group (dimethyl sulfoxide diluted to one-thousandth in PBS), and an artificial tears group. The DED group continued to receive benzalkonium chloride eye drops, and the low-dose EVO, high-dose EVO, vehicle, and artificial tears solution eye drops were administered four times daily, ensuring that at least 1 hour elapsed between modeling and treatment.

For the scopolamine-induced DED model, mice were subjected to a controlled environment chamber maintaining a relative humidity of less than 20%, temperature of 20°C to 22°C, and airflow of 15 L/min, 24 h per day for 14 days. Concurrently, these mice received subcutaneous administrations of 0.5 mg/0.1 mL scopolamine hydrobromide (Aladdin), at a frequency of four times daily. Follow-up treatments were conducted as described elsewhere in this article. Finally, all mice were euthanized, and samples were collected for subsequent experiments.

Immunofluorescence Staining

Cells pretreated with EVO and hyperosmotic medium were first fixed with 4% paraformaldehyde for 30 minutes. After fixation, cells were permeabilized by incubating in 0.5% Triton X-100 solution (Abs9155, Absin, Shanghai, China) for 1 hour. A blocking solution containing 5% bovine serum albumin was applied at room temperature for 30 minutes. After washing with PBS, cells were incubated with p53 monoclonal antibody (T40060F, 1:200, Abmart, Shanghai, China) overnight at 4°C. After primary antibody incubation, cells were washed and then incubated with Alexa Fluor 568 fluorescent secondary antibody (Servicebio, Hubei, China; GB23303) at room temperature for 1 hour. To facilitate nuclear observation, DAPI staining was performed for 5 minutes. Finally, images were captured and analyzed using the Leica Thunder system (Leica).

Western Blot

Protein extraction was conducted using RIPA lysis buffer (Beyotime) on samples. The samples were incubated with the lysis buffer for 10 minutes to facilitate lysis. The lysate was then centrifuged at 13,000 rpm, and the supernatant was collected as the total protein solution. Nuclear and cytoplasmic proteins were further extracted and separated using a nuclear and cytoplasmic protein extraction kit. Protein concentration was determined using a BCA protein assay kit (Vazyme). Proteins were separated by SDS-PAGE gels and subsequently transferred to polyvinylidene fluoride membrane (Vazyme) for Western blotting analysis. The primary antibodies used include: β-actin (81115-1-RR, 1:20,000; Proteintech, Rosemont, IL, USA), LC3A/B (12741S, 1:1,000, CST), p62 (5114S, 1:1,000, CST), p53 (T40060, 1:1,000, Abmart), phosphorylated p53 (p-p53; T40061, 1:1,000, Abmart), mTOR (CY5306, 1:1,000, Abways, Shanghai, China), phosphorylated mTOR (p-mTOR; CY6571, 1:1,000, Abways), TNF-α (HA722022, 1:1000, HUABIO, Woburn, MA, USA), IL-6 (R1414-2, 1:1000, HUABIO), IL-1β (HA601036, 1:1000, HUABIO), and Histone H3 (CY6587, 1:1000, Abways). After incubation with primary antibodies, the membrane was washed with TBST and incubated with horseradish peroxidase–conjugated secondary antibodies (FDM007, 1:10,000; FDR007, 1:10,000, both from FDbio, San Francisco, CA, USA). Bands were exposed using ECL detection reagents (Vazyme). The intensity of each band was quantified using ImageJ software (NIH).

Measurement of LC3 Autophagic Flux

To assess autophagic flux, HCEC were infected with mRFP-GFP-LC3 tandem fluorescent protein lentivirus (GENECHEM, Shanghai, China) for 12 hours (viral titer 1.0×10^8 TU/mL, multiplicity of infection = 100). GFP and mRFP served as markers for tracking LC3 to observe changes in autophagic levels. In the merged images, yellow signals (GFP-RFP) marked autophagosomes, indicating early autophagy. When autophagosomes fuse with lysosomes and forms autolysosomes, the acidic environment quenches GFP fluorescence, resulting in only RFP signals, representative of late autophagy. After infecting for 72 hours, HCEC were treated accordingly. The cells were analyzed using the Leica Thunder imaging system (Leica) to observe GFP-LC3 and mRFP-LC3. The number of yellow and red puncta within the images were quantified.

Conjunctival Goblet Cell Periodic Acid-Schiff Staining

The eyes and eyelids of mice were removed and placed in an eye ball fixation solution (G1109, Servicebio). The eyeballs were then dehydrated and embedded in paraffin, and sections approximately 5 µm thick were prepared and placed on glass slides. Staining was performed using the periodic acid-Schiff staining kit (G1360, Solarbio, Beijing, China) to observe the density of conjunctival goblet cells. The samples were then examined using a Nikon digital optical microscope, and quantification was performed using ImageJ software (NIH).

Corneal Fluorescein Staining and Tear Measurement

On days 0, 7, and 14 of the DED mouse model, corneal staining with fluorescein was performed. A total of 2 µL of 0.25% fluorescein sodium solution (Tianjin Jingming, China) was instilled into the conjunctival sac of each mouse. After 30 seconds, the eyes were rinsed with PBS, and the degree of corneal damage was photographed under cobalt blue light for scoring. The cornea was divided into four quadrants, with each quadrant rated from 0 to 3 points, resulting in a maximum total score of 12. Each quadrant was scored based on the extent of damage, and the overall corneal score was the sum of the scores from the four quadrants.[14]

On days 0, 7, and 14, a segment of phenol red cotton thread was placed in the conjunctival sac of the mice and held in place for 3 minutes. The length of the area wetted by tear fluid was measured. The results of the scopolamine-

induced DED model are presented in Supplementary Figure S1.

Data Analysis

Data were presented as the mean ± SD of three independent experiments. The *t* test was used for comparisons between two groups, while one-way ANOVA was used for comparisons involving more than two groups. A *P* value of less than 0.05 was considered statistically significant. All data analyses were conducted using GraphPad Prism 9.0 software (GraphPad, San Diego, CA, USA).

Results

EVO Alleviated Symptoms in the DED Mouse Model

To assess the efficacy of EVO in alleviating DED symptoms in mice, a benzalkonium chloride–induced DED mouse model was established. The results presented in Figure 1 demonstrate that both EVO and artificial tears treatment significantly mitigated corneal epithelial defects, reducing corneal fluorescein sodium scores and restoring tear secretion in DED mice (Figs. 1A–1C). Furthermore, periodic acid-Schiff staining results revealed a significant increase in the density of conjunctival goblet cells in the EVO treatment groups (Figs. 1D, 1E). The pathogenesis of DED is often associated with elevated levels of inflammatory factors and infiltration of inflammatory cells. Figures 1F to 1I illustrate that EVO effectively decreased the messenger RNA and proteins levels of key inflammatory cytokines, including TNF-α, IL-6, and IL-1β in the corneas. These findings suggest that EVO effectively alleviated symptoms in DED mice, demonstrating its therapeutic potential.

EVO Alleviated HCEC Damage Induced by Hyperosmolarity

In the in vitro model, we treated HCEC with EVO at concentrations of 0.001, 0.010, 0.100, 1.000, and 10.000 µM for 24 hours. Cell viability was assessed using the CCK-8 assay to evaluate potential drug toxicity. The results presented in Figure 2A indicate that none of these concentrations caused significant cytotoxicity. To investigate whether EVO could rescue hyperosmolarity-induced damage in HCECs, we additionally performed hyperosmotic treatment. We found that EVO rescued the decline in cell viability induced by hypertonic stress in a dose-dependent manner (Figs. 2B, 2C), with the optimal concentration being 0.1 µM. Additionally, we observed that EVO reduced the levels of inflammatory cytokines TNF-α, IL-6, and IL-1β after hyperosmolar treatment (Figs. 2D–2G). Furthermore, investigation into the apoptotic response of HCEC was conducted using Annexin-V FITC/PI staining. The results in Figure 2H demonstrate that EVO could reduce the late apoptosis rate in HCEC after hyperosmolar treatment.

EVO Alleviated Hyperosmolarity-Induced Oxidative Imbalance in HCEC

Oxidative stress has been linked closely to DED. Consequently, we explored the effect of EVO on oxidative stress levels in HCECs. Oxidative stress occurs when the balance between ROS and antioxidant enzymes is disrupted. Using DCFH-DA staining, we detected an increase in ROS fluorescence intensity under hyperosmolar conditions. However, pretreatment with EVO and N-acetylcysteine significantly reduced ROS levels (Figs. 3A–3C). SOD and CAT are important antioxidant enzymes that mitigate oxidative stress,[32] and MDA is a terminal product of lipid peroxidation, indicative of oxidative damage and antioxidant capacity.[33] Our results demonstrate that hyperosmolar conditions elevated MDA levels and decreased the expression of SOD and CAT in HCEC. However, EVO significantly ameliorated these effects (Figs. 3D–3F). These findings suggest that EVO could alleviate oxidative stress in HCECs effectively.

EVO Promoted Autophagy in HCEC to Alleviate Oxidative Stress

We further explored the relationship between EVO, autophagy levels, and oxidative stress in HCECs. ROS, generated by oxidative stress, can trigger autophagy, a process that degrades and recycles damaged macromolecules and organelles, thereby reducing oxidative damage.[34] Western blot analysis revealed that, under hyperosmotic conditions, the expression of LC3II increased and p62 decreased in HCECs, indicating a slight elevation in autophagy. EVO treatment significantly enhanced the process (Figs. 4A, 4B). Additionally, we used a fluorescently tagged GFP-RFP-LC3 lentivirus to infect HCEC and observe the changes in autophagic flux. Our results showed that, under hyperosmotic stimulation, HCECs exhibited increased yellow and red dots, indicating more autolysosome formation. After EVO intervention, these dots significantly increased, suggesting that EVO promoted autolysosome formation and enhanced autophagic flux in HCEC (Fig. 4C). Furthermore, the addition of the autophagy inhibitor chloroquine reversed the mitigating effect of EVO on ROS in HCEC (Figs. 4D, 4F). Additional studies showed that chloroquine also reversed the inhibitory effect of EVO on the apoptosis rate (Fig. 4G). These results collectively suggest that EVO reduced oxidative stress in HCEC by promoting autophagy.

EVO Induced Autophagy in HCEC Through the p53/mTOR Pathway

To further elucidate the mechanism underlying EVO-induced autophagy, we performed Western blot analysis. Our findings indicated that hyperosmotic stress led to a decrease in cytoplasmic phosphorylated p53 and an increase in its nuclear expression, while simultaneously inhibiting phosphorylated mTOR activity. EVO treatment significantly enhanced the process (Figs. 5A, 5B).

When we used the p53 inhibitor pifithrin-α (Pα), the effects of EVO were reversed. (Figs. 5C, 5D), suggesting that EVO may enhance the nuclear translocation of p53 in HCEC, thereby regulating mTOR levels and promoting autophagy. Blocking p53 nuclear translocation with Pα results in more p53 remaining in the cytoplasm, thereby inhibiting the autophagy-promoting effect of EVO.

To assess p53 nuclear translocation, we conducted immunofluorescence staining, with red fluorescence indicating p53 and blue fluorescence indicating DAPI. The colocalization of these signals produces a purple signal. As shown in Figure 5E, EVO enhanced the nuclear presence of

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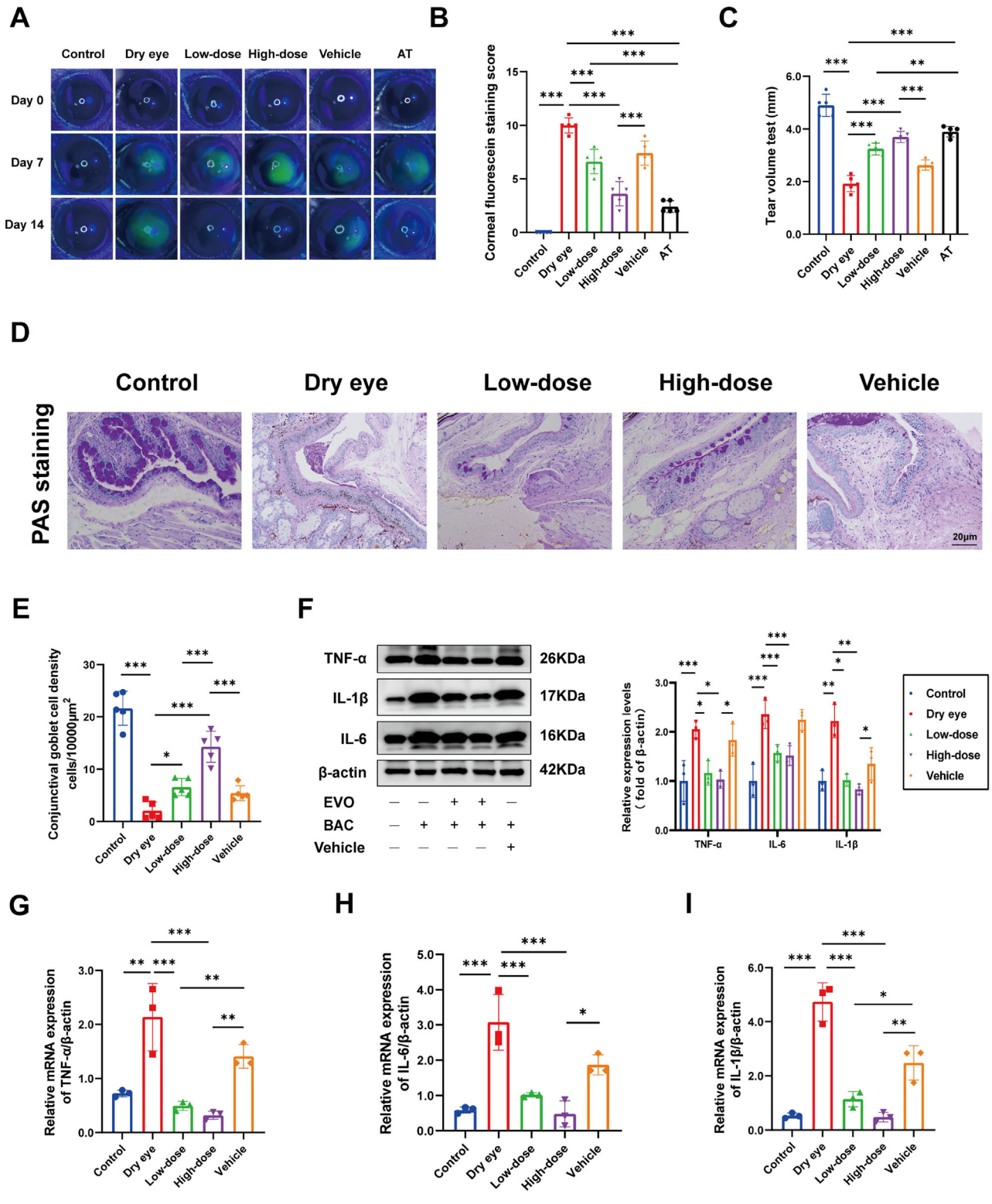

Figure 1. EVO alleviated symptoms in DED mouse model. (**A**) Sodium fluorescence staining of mice from different groups at days 0, 7, and 14, assessing the degree of corneal epithelial damage. (**B**) Quantitative analysis of corneal fluorescein sodium staining ($n = 5$). (**C**) Measurement of tear secretion ($n = 5$). (**D**, **E**) Density of conjunctival goblet cells observed and quantified through periodic acid-Schiff staining in different groups ($n = 5$). (**F**) Western blot analysis of the expression and quantification of TNF-α, IL-6, and IL-1β in corneal tissues from different treatment groups ($n = 3$). (**G–I**) Messenger RNA expression levels of TNF-α, IL-6, and IL-1β ($n = 3$). Data are presented as the mean ± SD from at least three independent experiments Statistical significance is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

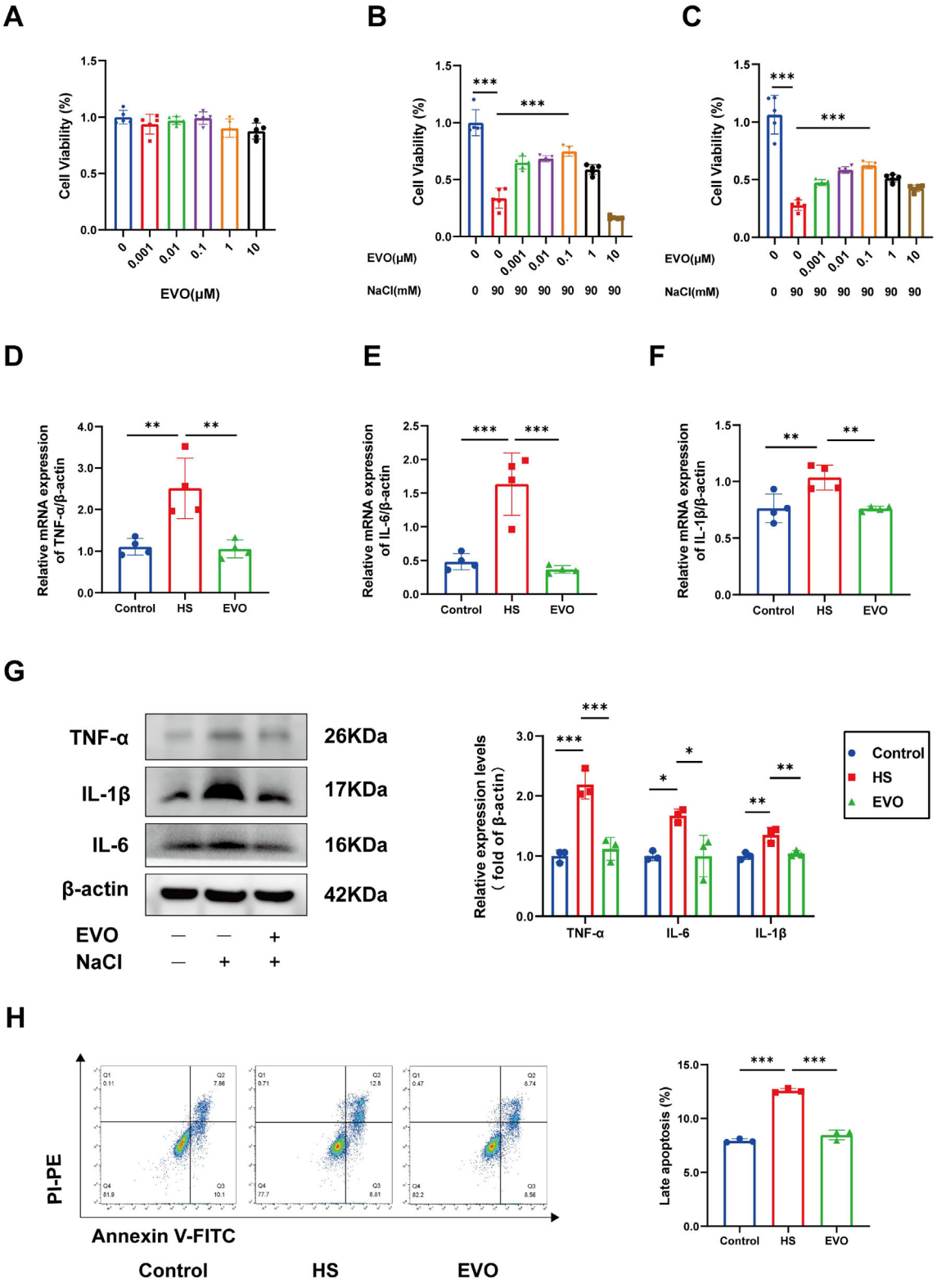

Figure 2. EVO alleviated HCECs damage induced by hyperosmolarity. (**A**) Effect of 24-hour of EVO treatment at various concentrations on HCEC viability, assessed by the CCK-8 assay ($n = 5$). (**B**) Cell viability was measured by pretreatment with EVO followed by hypertonic stimulation ($n = 5$). (**C**) Cell viability was measured by hypertonic stimulation followed treatment with EVO ($n = 5$). (**D–F**) The messenger RNA (mRNA) expression of TNF-α, IL-6, and IL-1β in HCECs from different treatment groups, measured by qRT-PCR ($n = 4$). (**G**) Western blot analysis of the expression and quantification of TNF-α, IL-6, and IL-1β from different treatment groups ($n = 3$). (**H**) Detection and quantification of apoptosis rates in HCEC from different treatment groups using flow cytometry ($n = 3$). HS: hyperosmolarity. Data are expressed as mean ± SD of at least three independent experiments. Statistical significance is indicated as follows: ** $P < 0.01$, *** $P < 0.001$.

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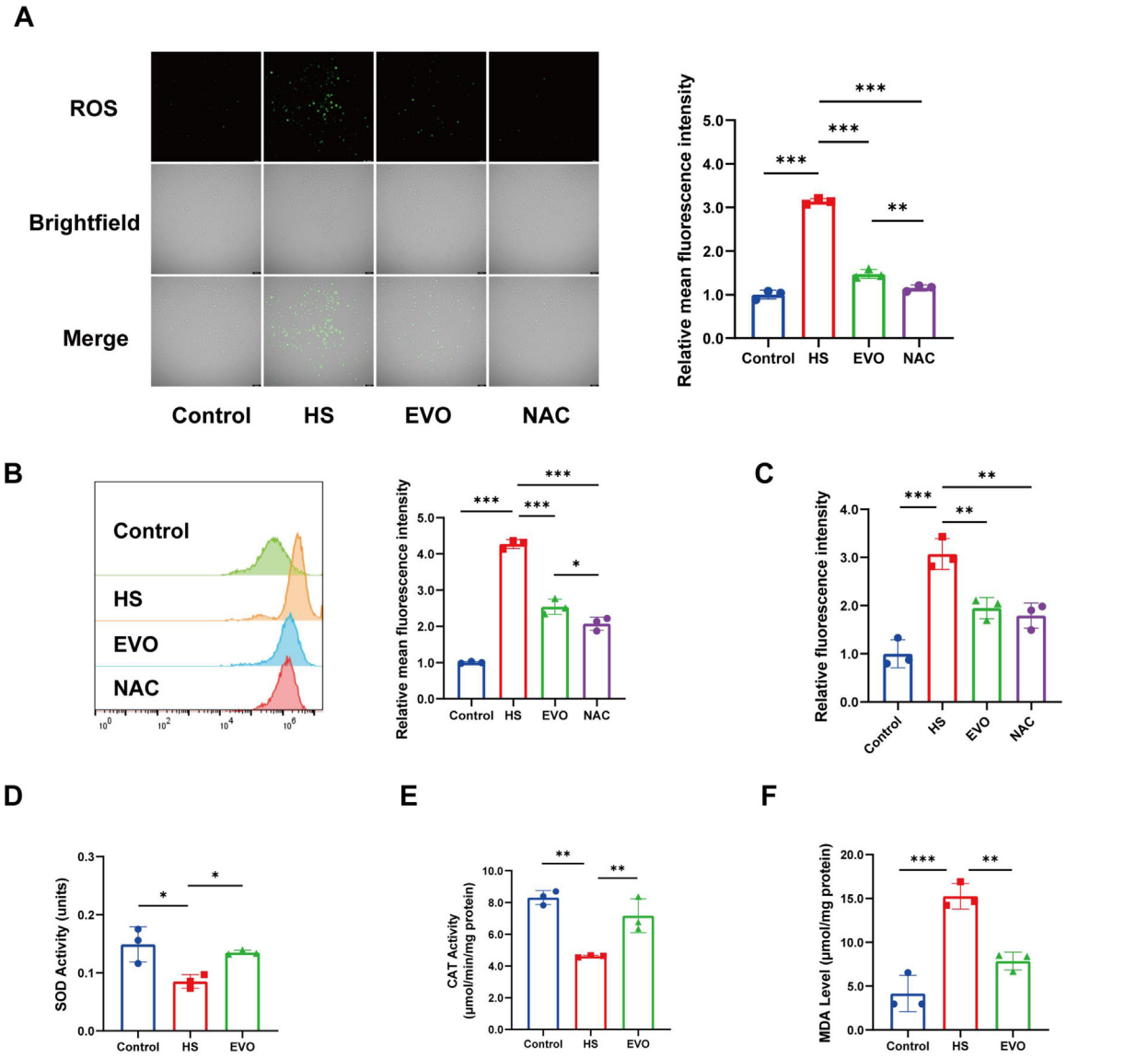

Figure 3. EVO alleviated hyperosmolarity-induced oxidative imbalance in HCEC. (**A**, **B**) ROS fluorescence in HCEC from different treatment groups was measured and quantified using fluorescence microscopy and flow cytometry ($n = 3$). (**C**) ROS fluorescence in HCEC from different treatment groups was measured using a fluorescence microplate reader ($n = 5$), with the ROS detection wavelength set at 488 nm. (**D–F**) Levels of antioxidant enzymes SOD (**D**), CAT (**E**), and lipid oxidizing metabolite MDA (**F**) were examined in different treatment groups of HCEC ($n = 3$). HS, hyperosmolarity; NAC, N-acetylcysteine. Data are expressed as mean ± SD of at least three independent experiments. Statistical significance is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

p53, an effect that was reversed by Pα. Additionally, observation of GFP-RFP-LC3 fluorescence revealed that Pα reduced the intensity of LC3 fluorescence compared with the EVO group (Figs. 5F, 5G). Moreover, Pα reversed the antioxidant effects of EVO (Fig. 5H). These results suggest that EVO induced autophagy in HCEC through the p53/mTOR pathway, contributing to the reduction of oxidative stress.

EVO Eye Drops Activated the p53/mTOR/Autophagy Pathway in the Corneas of DED Mice

To evaluate the effect of EVO on the p53/mTOR/autophagy pathway in vivo, mice were divided into five groups. Western blot analysis revealed that, compared with the DED group, treatment with EVO eye drops significantly increased the expression of nuclear phosphorylated p53 and LC3II. Conversely, the levels of cytoplasmic phosphorylated p53, phosphorylated mTOR, and p62 was decreased (Figs. 6A–F). These results were consistent with our in vitro findings (Fig. 5), indicating that EVO enhanced autophagy in the corneas of DED mice through modulation of the p53/mTOR pathway.

Discussion

DED is a prevalent and debilitating condition in ophthalmology, yet effective treatments remain limited.[35] Current therapeutic options mainly focus on replenishing tears or reducing ocular surface inflammation,[36] often with unsatisfactory results. In this study, we explored the potential therapeutic

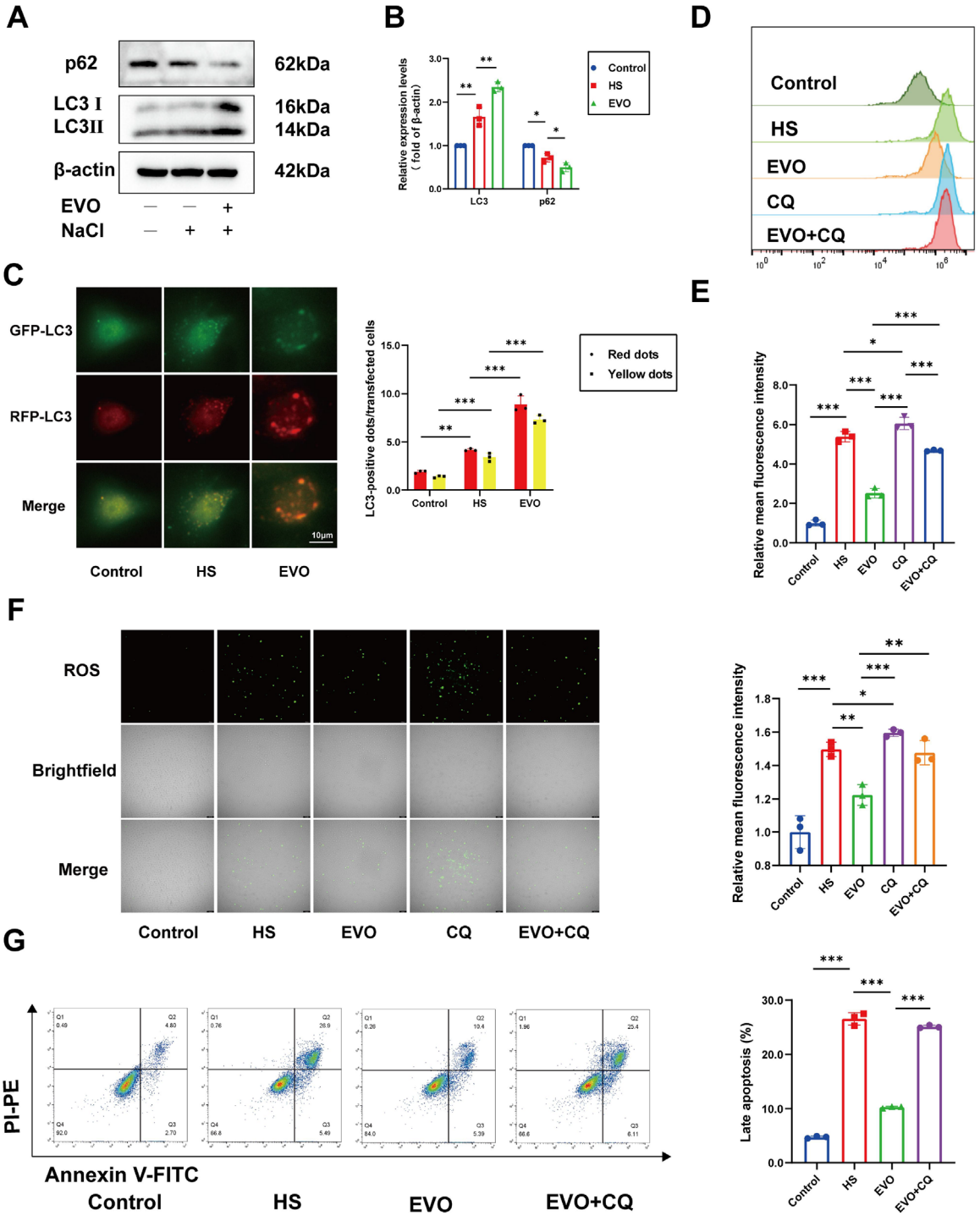

Figure 4. EVO promoted autophagy in HCEC to alleviate oxidative stress. (**A**, **B**) Effect of EVO on the expression and quantification of LC3II and p62 levels in HCEC under hyperosmotic conditions ($n = 3$). (**C**) Expression and quantification of LC3 fluorescence in HCEC after transfection with GFP-RFP lentivirus, observed via fluorescence microscopy ($n = 3$). (**D**, **E**) Expression and quantification of ROS fluorescence in HCEC using flow cytometry ($n = 3$). (**F**) Expression and quantification of ROS in HCEC via fluorescence microscopy ($n = 3$). (**G**) Observation and quantification of apoptosis in HCEC using flow cytometry ($n = 3$). CQ, chloroquine; HS, hyperosmolarity; NAC, N-acetylcysteine. Data are expressed as mean ± SD of at least three independent experiments. Statistical significance is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

effects of EVO in both in vitro and in vivo models of DED. Our findings demonstrated that EVO eye drops not only reduced corneal damage in DED mice, but also restored tear secretion, increased the goblet cell density in the conjunctiva, and decreased the expression of inflammatory factors on the ocular surface (Fig. 1). In vitro, EVO was found to promote autophagy via the p53/mTOR pathway, alleviating hyperosmolarity-induced damage to HCECs, reducing oxidative stress, decreasing the apoptosis rate, and restoring cell viability (Figs. 2–5).

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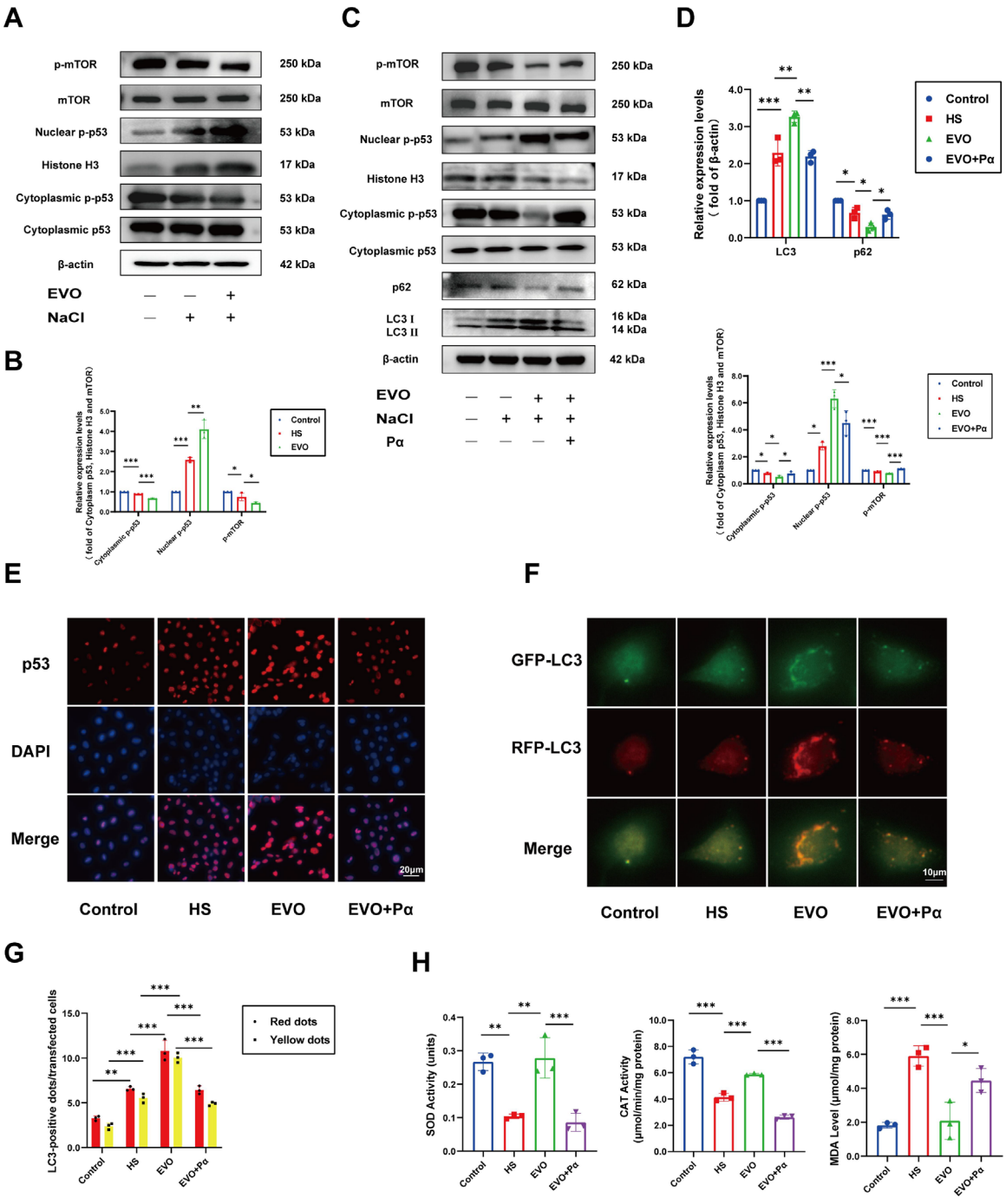

Figure 5. EVO induced autophagy in HCEC through the p53/mTOR pathway. (**A**, **B**) Western blot analysis showing the effect of EVO on the expression and quantification of cytoplasmic and nuclear p53, cytoplasmic and nuclear phosphorylated p53 (p-p53), mTOR, and phosphorylated mTOR (p-mTOR) in different treatment groups ($n = 3$). (**C**, **D**) Western blot analysis of the expression and quantification of cytoplasmic and nuclear p53, p-p53, mTOR, and p-mTOR in HCEC after treatment with the p53 inhibitor Pα ($n = 3$). (**E**) Immunofluorescence staining showing nuclear translocation of p53 in HCEC under different treatments, with a scale bar of 20 μm. (**F**, **G**) Expression and quantification of LC3 fluorescence in HCEC, observed by fluorescence microscopy after lentiviral transfection ($n = 3$). (**H**) Expression levels of the antioxidant enzymes (SOD and CAT) and the lipid oxidation marker MDA in HCEC under different treatments ($n = 3$). HS, hyperosmolarity. Data are expressed as mean ± SD of at least three independent experiments. Statistical significance is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Oxidative stress plays a crucial role in the progression of DED.[37] The onset of DED leads to an increase in ROS, disrupting the balance between oxidative and antioxidative systems, leading to cell apoptosis and ocular surface inflammation. This imbalance exacerbates ROS production and elevates inflammatory factors, creating a vicious cycle that worsens DED symptoms.[38] Previous studies have shown that EVO can reduce toxicity in the peripheral nerves by decreasing ROS and MDA levels.[39] It has also been reported to mitigate oxidative stress, potentially preventing traumatic brain

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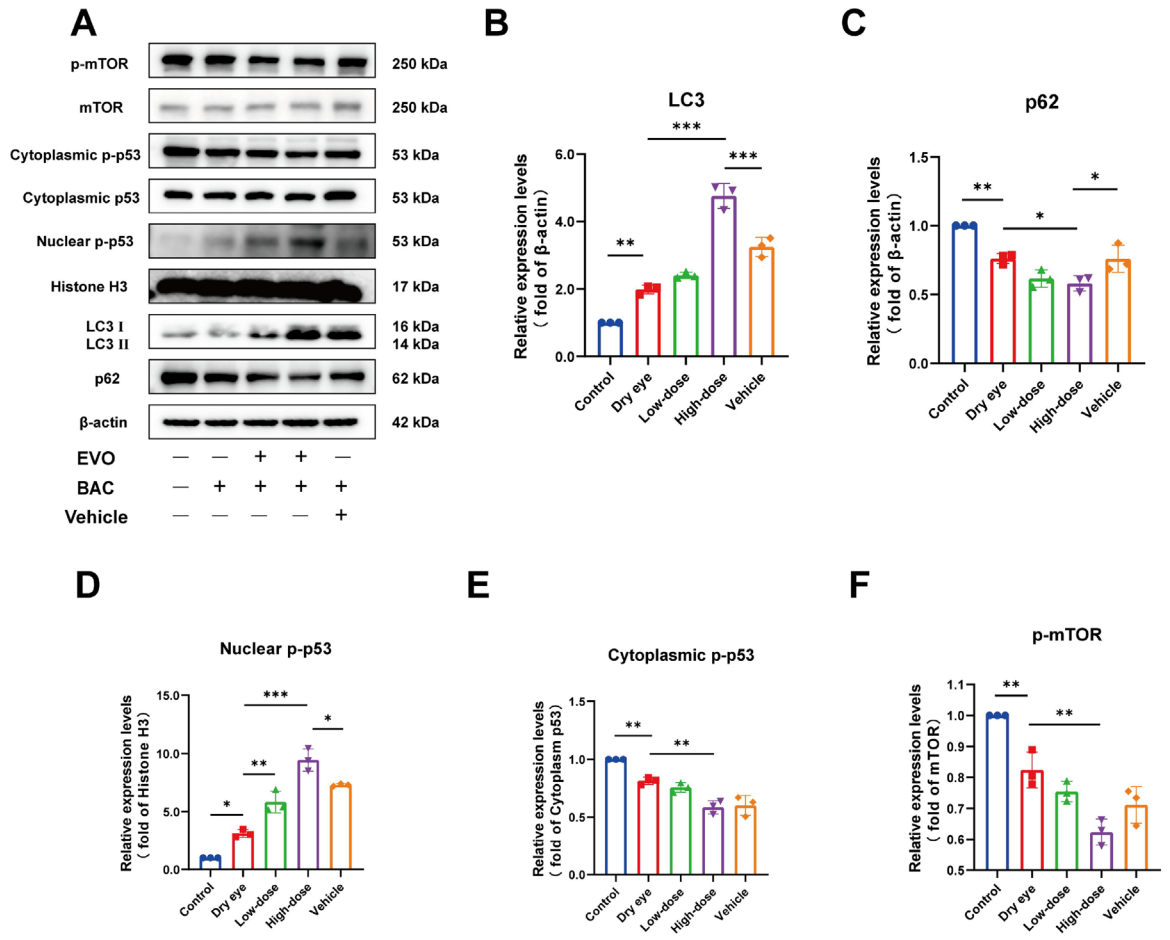

Figure 6. EVO eye drops activated the p53/mTOR/autophagy pathway in corneas of DED mice. (**A**) Western blot analysis showing the expression levels of cytoplasmic p53, cytoplasmic phosphorylated p53 (p-p53), nuclear p53, nuclear phosphorylated p53 (p-p53), mTOR, phosphorylated mTOR (p-mTOR), LC3II, and p62 in different treatment groups. (**B**) Quantification of LC3II expression ($n = 3$). (**C**) Quantification of p62 expression ($n = 3$). (**D**) Quantification of nuclear p-p53 expression ($n = 3$). (**E**) Quantification of cytoplasmic p-p53 expression ($n = 3$). (**F**) Quantification of p-mTOR expression ($n = 3$). Data are expressed as mean ± SD of at least three independent experiments. Statistical significance is indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

injury,[40] reducing ROS accumulation, and enhancing antioxidant activity, such as increasing messenger RNA levels of SOD and HO-1.[41] Our results are consistent with these findings; we observed that EVO alleviated oxidative stress in DED by reducing ROS and MDA levels and simultaneously elevating the activities of SOD and CAT. The relationship between EVO and oxidative stress in ocular research is novel, and we present, for the first time, evidence that EVO can alleviate oxidative stress and protect HCECs from damage in the context of DED. Despite the promising results, the small sample size in both in vitro and in vivo experiments is one of the shortcomings of this study. Additionally, although we verified the effect of EVO on oxidative stress in HCEC, the detailed mechanism underlying its action remains to be fully elucidated. These gaps present valuable directions for future research.

Autophagy, a vital cellular process for maintaining homeostasis, involves the degradation of misfolded or aggregated proteins, damaged organelles, and foreign pathogens.[8] It is considered a protective mechanism against disease, although excessive autophagy can lead to cellular damage and death.[42] In DED, most studies have suggested that autophagy serves as a beneficial mechanism to maintain cellular function.[34] Our previous research has demonstrated that activating autophagy can be beneficial to the treatment of DED.[14] Moreover, autophagy activation has been implicated in regulating potassium ion efflux and alleviate cellular pyroptosis in HCECs.[43] EVO has also been reported to alleviate experimental colitis through the activation of autophagy.[26] Our study similarly demonstrates that EVO activates autophagy in DED, thereby reducing oxidative stress and apoptosis levels. To further validate the role of autophagy in EVO's modulation of oxidative stress, we employed the autophagy inhibitor chloroquine, which reversed the beneficial effects of EVO, confirming that EVO modulates oxidative stress and cellular apoptosis in an autophagy-dependent manner.

The mechanistic pathway through which EVO induces autophagy involves the p53/mTOR signaling pathway. mTOR is a serine/threonine kinase that regulates cellular metabolism and also inhibits autophagy.[44] p53, a dual function protein primarily located in the nucleus and cytoplasm, has been shown to inhibit autophagy when it aggregates in the cytoplasm.[45] Conversely, when p53 translocates to the nucleus, it promotes autophagy.[46] The relationship between EVO and the p53/mTOR pathway in DED has not yet been

reported. Our study, for the first time, demonstrates that EVO promotes the nuclear translocation of p53, leading to a reduction in mTOR activity and enhancing the autophagic process in HCEC. Using the p53 inhibitor Pα reversed these effects, indicating that the p53/mTOR pathway plays a critical role in the regulation of autophagy by EVO to inhibit oxidative stress.

Notably, pyroptosis, a highly inflammatory form of programmed cell death, has been recognized as another key contributor to DED pathogenesis.[47] The hypertonic environment of DED activates NLRP3 inflammasome signaling, subsequently triggering caspase-1 activation, gasdermin D cleavage, and excessive ROS generation, which collectively drive pyroptotic cell death and disease progression. Although our data suggest that EVO demonstrates inhibitory effects on pyroptosis-related pathways, possibly through modulation of ROS-NLRP3 axis as reported in other models,[48] this study primarily focused on investigating EVO's therapeutic mechanisms concerning autophagy regulation and oxidative stress management in DED. The potential interaction between autophagy and pyroptosis in ocular surface disorders remains underexplored, although emerging evidence suggests such crosstalk in other inflammatory conditions.[43] EVO may promote autophagy while simultaneously attenuating pyroptosis. Further investigation into this dual effect, along with a deeper understanding of EVO's impact on various forms of cell death, will be essential for optimizing its therapeutic application in DED treatment. This work remains a critical direction for future research.

Nevertheless, our study had several limitations. First, we concentrated on the p53/mTOR pathway in regulating EVO-induced autophagy and oxidative stress. Although we validated this pathway using Western blot, the small sample size and limited methods necessitate further validation using a broader range of techniques and a larger sample size. Furthermore, numerous interactions between autophagy and oxidative stress have been reported,[49] and we did not explore the full spectrum of molecular interactions involved. Future studies should investigate other pathways that may contribute to EVO's effects in DED. Second, our in vivo validation was focused primarily on the cornea and conjunctiva of DED mice, with limited exploration of other ocular structures such as the lacrimal glands, meibomian glands, lymph nodes, and the trigeminal ganglion. A more comprehensive analysis of EVO's effects across these tissues is a potential research direction. Last, EVO's poor water solubility and low bioavailability in the eye pose challenges for clinical application. Future efforts should focus on optimizing drug delivery methods, such as formulating EVO into liposomes or microspheres, to improve its ocular bioavailability and therapeutic efficacy.

In conclusion, our study indicates that EVO holds promise as a potential therapeutic agent for DED. It alleviates oxidative stress, promotes autophagy, and reduces inflammatory markers, thereby improving ocular surface health. However, further investigation into its molecular mechanisms, clinical applicability, and optimization of drug delivery systems is warranted to fully realize EVO's potential in DED treatment.

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