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Zidovudine protects hyperosmolarity-stressed human corneal epithelial cells via antioxidant pathway

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ABSTRACT

Dry Eye Disease (DED) is a very common disorder that can result in severe disability and vision loss. Although the pathogenesis of DED is not fully understood, hyperosmolarity, inflammation, and tear film instability are recognized as hallmarks of DED. Recently, Nucleoside Reverse Transcriptase Inhibitors (NRTIs), a class of medication used to treat HIV, have been shown to inhibit inflammation in a mouse model of retinal atrophy. In this study, we investigated whether Zidovudine (AZT) can inhibit human corneal epithelial cell (HCEC) inflammatory responses under hyperosmotic conditions. HCECs were cultured in hyperosmotic media containing AZT. Cell viability, cytokine production, and reactive oxygen species (ROS) production were measured. We found that AZT decreased nuclear factor kappa B (NF-κB) and Interleukin-6 (IL-6) levels, increased Superoxide Dismutase 1 (SOD1) production, decreased ROS production, and increased cell viability. These results support the novel use of AZT in the reduction of ocular surface inflammation and the promotion of corneal health in the context of DED.

1. Introduction

Dry Eye Disease is a common, multifactorial inflammatory disorder that affects over 16 million people in the United States and results in irritation, blurred vision, and tear film instability with damage to the cornea and conjunctiva [1,2]. It is estimated that up to 25% of patients seen in ophthalmology clinics report symptoms of DED, making it one of the most common conditions seen by ophthalmologists, and a growing public health concern [3]. Common DED symptoms include but are not limited to: eye irritation, blurred vision, feelings of eye dryness, and overall discomfort. In more severe cases, DED complications may lead to corneal ulcerations, corneal scarring, irreversible vision loss, and blindness [4]. Although symptom relief can be provided through the use of topically applied hypo-osmotic or iso-osmotic tears, these treatments are palliative and often do not prevent disease progression [5]. As evidenced by numerous research studies highlighting the importance of inflammatory mediators in the progression of DED, development of therapeutic strategies that effectively inhibit key inflammatory pathways may provide more effective treatment for patients with DED [6–9].

Most cases of DED are secondary to a wide variety of conditions and disorders that can disturb the intricate homeostatic balance of the ocular surface - resulting in changes in tear film stability and osmolarity [6]. Tear hyperosmolarity has been shown to play a crucial role in the pathogenesis of DED, resulting in tears with an osmolality greater than that of the surrounding epithelial cells. This process leads to reduced epithelial cell volume and increased concentration of intracellular solutes, which in turn results in increased oxidative stress, ROS production, and cellular DNA damage [10,11]. The resulting cascade of inflammatory events leads to the production of several pro-inflammatory cytokines, ultimately

Abbreviations: DED, Dry Eye Disease; NRTI, Nucleoside Reverse Transcriptase Inhibitor; HCECs, Human Corneal Epithelial Cells; AZT, Zidovudine; ROS, Reactive Oxygen Species; SOD1, Superoxide Dismutase 1; NF-kB, Nuclear Factor Kappa B.

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resulting in the death of epithelial surface cells - contributing to the pathogenesis of DED. Although the pathogenesis of DED is not understood in its entirety, inflammation is recognized as a hallmark in the development and amplification of DED.

Previous *in vitro* and *in vivo* studies have demonstrated a strong link between hyperosmolarity and DED [7,8,10–12]. Specifically, past studies have shown that exposure to hyperosmotic stress results in increased expression of several pro-inflammatory cytokines and disrupts the balance of oxygenases and anti-oxidative enzymes, resulting in molecules that stimulate and maintain an inflammatory response leading to the development of DED [13]. Pharmacological regulation and inhibition of these key inflammatory pathways may provide safe and effective treatment of DED.

Recently, multiple papers have highlighted the anti-inflammatory properties of antiviral drugs [14–17]. One class of antiviral drugs, termed Nucleoside Reverse Transcriptase Inhibitors (NRTIs), are a class of drugs which are widely used in combination as the mainstay of treatment for HIV. NRTIs have been shown to inhibit inflammation in mouse models of geographic atrophy and graft-versus-host disease, supporting the idea that NRTIs may have broad therapeutic value in the treatment of other inflammation based diseases [18]. Although previous studies clearly illustrate the anti-inflammatory properties of NRTIs, there are few reports on the protective effects of Zidovudine (AZT), one of the most commonly used NRTIs, in ocular surface diseases—particularly DED. To explore the therapeutic relevance of NRTIs in the treatment of DED, we hypothesize that AZT may be effective as an anti-inflammatory agent. As a widely used, inexpensive, and readily available drug, novel repurposing of AZT for the treatment of DED would provide an alternative therapy for patients who are unable to tolerate the traditional topically applied medications currently used as the primary treatment for DED.

2. Materials & methods

2.1. Cell cultures and treatments

HCECs were used from a previously established SV40-immortalized HCE cell line kindly provided by Deepak Shukla, PhD (University of Illinois, Chicago). The HCECs were cultured in Minimum Essential Media (MEM, Corning) supplemented with 1% Penicillin/Streptomycin (Gibco) and 10% fetal bovine serum (FBS, Sigma). HCECs were maintained at 5% CO_2 and 37 °C. Medium was changed once every three days and the cells were split upon reaching 90% confluence. HCECs grown between passages 2–6 were used to conduct experiments. Cells were pretreated in the presence or absence of Zidovudine (Sigma) for 1 h. HCECs were then exposed to media containing 70 mM NaCl, and cell culture supernatants and lysates were collected after 24 h.

2.2. Enzyme-linked immunosorbent assay

HCECs were plated at a concentration of 25,000 cells/ml in triplicate in a 24-well tissue culture plate and were allowed to adhere overnight. Cells were pretreated with 25 μM or 50 μM AZT for 1 h. HCECs were then exposed to media containing 70 mM NaCl and cell culture supernatants were collected at 24 h. Sandwich ELISA for IL-6 was performed to determine concentration of pro-inflammatory cytokines in media treated with various concentrations of AZT and/or 70 mM NaCl. 96-well plates were coated with 2.5 μg/mL purified IL-6 antibody overnight. Plates were washed three times in PBST (Phosphate buffered Saline and 0.05% Tween-20) and dried after the last wash. Supernatants from HCECs treated in the absence or presence of both AZT and/or 70 mM NaCl were diluted 1:4 in 5% blocking buffer and added to each well. After 1.5 h, the supernatants were washed and 2 μg/mL biotinylated IL-6 was added to each well for another 1.5 h. Wells were washed and HRP Streptavidin was added to each well for 1 h. HRP was washed out and TMB substrate was added to each well – monitoring for color change every 5 min up to 30 min. After sufficient color change, 1M HCl (stop solution) was added to each well. Absorbance was read by a microplate reader via endpoint analysis at 450 nm using an ExL800 (Biotek) plate reader.

2.3. NF-κB assay

Cellular NF-κB activation was measured using an NF-κB p65 Total SimpleStep ELISA Kit (Abcam). HCECs were plated at a concentration of 25,000 cells/ml in triplicate in a 24-well tissue culture plate and were allowed to adhere overnight. Cells were pretreated with 50 μM or 100 μM AZT for 1 h. HCECs were then exposed to media containing NaCl at 70 mM for 24 h 150 μL of Cell Extraction Buffer included in the kit was added to each well to prepare the nuclear lysates. Equal amounts of protein measured by Bradford assay were mixed with 50 uL of Antibody Cocktail included in the kit. The protein/antibody mixture was then sealed and incubated at room temperature for 1 h on a plate shaker set at 400 RPM. Wells were then washed and TMB was added to each well for 15 min in the absence of light. After adding stop solution, color change was measured at 450 nm. Data is reported as percent change in NF-κB expression relative to control.

2.4. Western Blot Analysis

HCECs were plated at a concentration of 25,000 cells/ml in triplicate in a 24-well tissue culture plate and were allowed to adhere overnight. Cells were pretreated with 50 μM and 100 μM AZT for 1 h. HCECs were then exposed to media containing 70 mM NaCl and cell lysates were collected at 24 h using RIPA buffer. Equal amounts of protein measured by Bradford assay were mixed with 6X SDS reducing sample buffer. Samples were then boiled for 10 min and kept on ice for 3 min. The proteins were loaded at 20 μg/lane, separated on an SDS polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% nonfat milk in PBS-T (Phosphate buffered Saline and 0.05% Tween-20) for 1 h. The membranes were incubated with primary antibodies against SOD1 (1:200, Santa Cruz) or β -actin (1:1000, Biolegend) at 4 °C overnight, washed, and then incubated with HRP conjugated goat anti-mouse IgG (1:1000, Biolegend) for 1.5 h. The membrane was then washed and treated with SuperSignal West Pico Chemiluminescent substrate (ThermoFisher) for 3 min devoid of light. Afterwards, band density was measured using a FluorChem E machine (proteinsimple).

2.5. LDH assay

HCECs were plated at a concentration of 7500 cells/well in triplicate in a 96-well tissue culture plate and were allowed to adhere overnight. Media was then changed, and the cells were pretreated with 50 μM or 100 μM of AZT for 1 h and subsequently exposed to media with 70 mM NaCl for 24 h. The LDH reaction was performed using a Pierce LDH Cytotoxicity Assay Kit (Thermo-Scientific) following the manufacturer's instructions. 50 μl of supernatant from each sample were transferred to a clean well in a new plate and 50 μl of reaction mixture was added. Samples were incubated at room temperature for 30 min devoid of light. After the allotted time, 50 μl of stop solution were added to each sample and the plate was read at 490 nm and 680 nm using a Spectramax Plus 384 plate reader (Molecular Devices). Results are shown as percentage cell viability relative to positive and negative controls.

2.6. Measurement of cellular ROS production

Cellular ROS production was measured using DCFDA assay kit (abcam). HCECs were plated at a concentration of 20,000 cells/well in a 96-well black opaque walls tissue culture plate. The cells were allowed to grow overnight to reach 90% confluency. Media was then removed and 100μL/well of 25 μM DCFDA was added and incubated at 37 °C for 45 min. After removing the DCFDA solution, the cells were pretreated with AZT at 50 μM and 100 μM for 1 h. Cells were then exposed to 70 mM NaCl and fluorescence intensity was read every 10 min continuously for 2.5 h using a BioTek SYNERGY reader. Fluorescence data is expressed as fold increase over control cells.

3. Results

3.1. AZT decreases pro-inflammatory cytokine production by HCECs

To determine if AZT can suppress production of IL-6 induced by NaCl treatment, we cultured HCECs in the absence or presence of either NaCl or AZT, or in combination, and measured IL-6 production by ELISA. As seen in Fig. 1, in the presence of salt alone, the HCECs secreted 48.43 ± 1.153 pg/mL of IL-6. When HCECs were pretreated with either 25 μM or 50 μM AZT prior to the addition of 70 mM NaCl, we observed a dose-dependent decrease in IL-6 secretion. IL-6 expression decreased to 12.58 ± 2.878 pg/mL for HCECs pretreated with 25 μM AZT and 6.37 ± 1.547 pg/mL in cells pretreated with 50 μM AZT. The latter condition produced IL-6 secretion levels similar to those of the control − 7.406 ± 1.153 pg/mL. It should be noted that the addition of AZT alone had very little change on control IL-6 secretion levels - 8.441 ± 0.36 pg/mL.

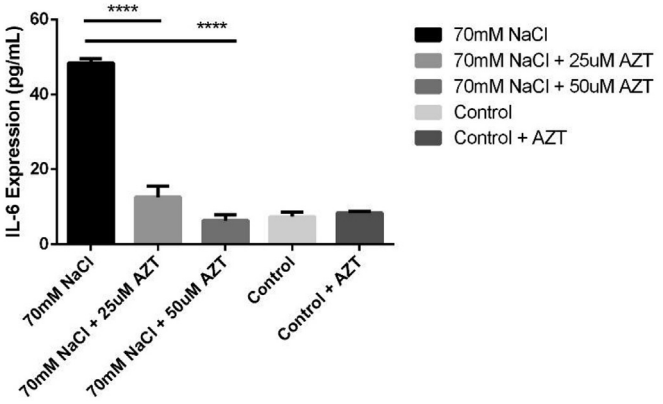

Fig. 1. AZT reduces expression of pro-inflammatory cytokine IL-6 in cells treated with sodium chloride. HCECs were pre-treated with either 25 mM or 50 mM AZT for 1.5 h. AZT was removed and 70 mM sodium chloride was added to 24-well plates overnight. IL-6 Expression was measured via ELISA. Significance was analyzed by Two-way ANOVA (n = 3). **** - P < 0.0001. Data are presented as mean ± SEM.

3.2. AZT decreases activation of NF-κB in HCECs

To determine if NaCl induces IL-6 production via NF-κB activation, we used an NF-κB p65 Total SimpleStep ELISA Kit (abcam) to detect the presence of the p65 subunit of NF-κB in nuclear lysates. As seen in Fig. 2, when treated with 70 mM NaCl, the HCECs exhibited 126.83 ± 3.79% expression of NF-κB relative to control activation levels of 100%. To test if AZT suppresses this activation, we treated cells with NaCl in the absence or presence of AZT. When pre-treated with 50 μM or 100 μM AZT and subsequently exposed to NaCl, we observed 91.46 ± 2.99% and 87.99 ± 1.53% expression of NF-κB in HCECs, respectively.

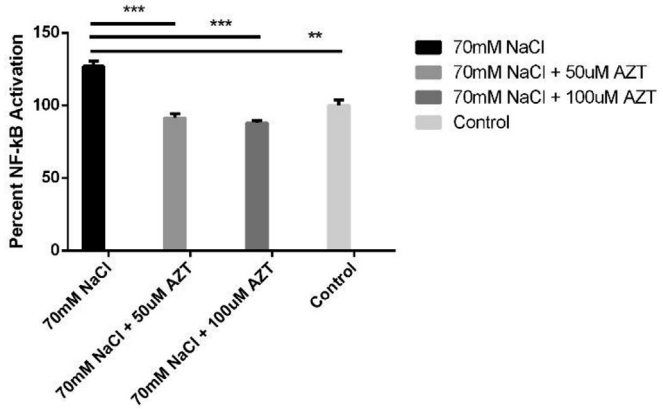

Fig. 2. AZT decreases activation of NF-κB. HCECs were pre-treated with increasing concentration of AZT for 1.5 h and were then treated with NaCl overnight. NF-κB p65 Total SimpleStep ELISA Kit (abcam) was used to detect the presence of the p65 subunit of NF-κB in total nuclear lysates. Significance was analyzed by Two-way ANOVA (n = 3). ** - P < 0.01, *** - P < 0.001. Data are presented as mean ± SEM.

3.3. AZT decreases ROS production and restores Superoxide Dismutase 1 activity in HCECs

As the presence of ROS may contribute to activation of NF-κB and the eventual production of IL-6, we next sought to detect all forms of ROS generated by HCECs treated with NaCl in the absence or presence of AZT. To do so, we used a DCFDA Cellular ROS Detection Assay (abcam). DCFDA fluorescence intensity revealed that treatment with NaCl robustly induced ROS production. As seen in Fig. 3A, treatment with 70 mM NaCl increased ROS production to 6590.33 ± 821.62 relative fluorescence units (RFU) after 150 min of treatment. Control cells receiving no treatment produced 4170.50 ± 129.04 RFU. After we treated the cells with AZT, we observed a dose-dependent decrease in production of ROS. After 2.5 h, HCECs pre-treated with 50 μM or 100 μM AZT and subsequently exposed to 70 mM NaCl produced 5507 ± 433.279 RFU and 5016.67 ± 327.06 RFU, respectively. We further proceeded to analyze the enzymes that regulate levels of ROS in HCECs by measuring the expression level of antioxidant enzyme SOD1 using Western Blot Analysis. As seen in Fig. 3B, in HCECs treated with NaCl, we found that SOD1 was only expressed at 39.10% of the level it is expressed in control cells. When AZT was added, however, we saw an increase in SOD1 expression. Cells treated with 50 μM or 100 μM AZT prior to treatment with NaCl displayed increased SOD1 expression levels at 49.5% and 65.1%, respectively, relative to control SOD1 expression based at 100%.

3.4. AZT increases viability of HCECs

To determine the effects of AZT and NaCl on cellular viability, we used an LDH Cytotoxicity Assay (Roche). As seen in Fig. 4, when treated with 70 mM NaCl for 24 h, 8.47 ± 0.70% of HCECs did not survive. We further tested to see if AZT protected the cells against hyperosmolarity-induced cell death. When HCECs were pre-treated with AZT at either 50 μM or 100 μM and further challenged with 70 mM NaCl, cell death decreased to 4.28 ± 0.8127% and 3.60 ± 0.44%, respectively. Both of these conditions represent percent cell death similar to that of control levels − 4.147 ± 0.8772%. It should be noted again that the addition of AZT to the cells alone had very little change on cell viability − 3.99 ± 0.9327%.

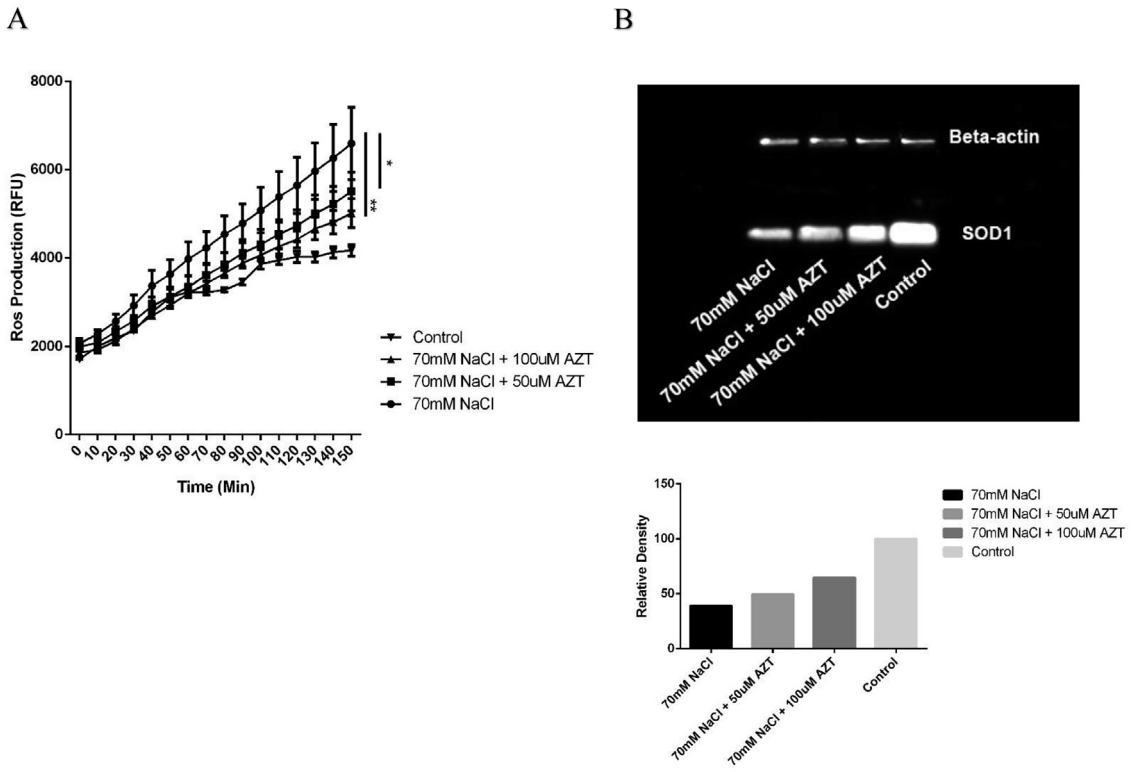

Fig. 3. AZT decreases ROS production in HCECs. HCECs were pre-treated with 50 mM or 100 mM AZT for 1.5 h. AZT was removed and 70 mM Sodium chloride was added to 24-well plates overnight. (A) ROS production was measured every 10 min for 2.5 h via fluorescence spectrophotometer. (B) Cells were lysed and subjected to immunoblot to detect for presence of SOD1. Quantification of band intensity was measured using ImageJ. Significance was analyzed for ROS production over time data by Two-way ANOVA (n = 3). * - P < 0.05, ** - P < 0.01. Data are presented as mean ± SEM.

4. Discussion

In this study, we found that AZT conferred significant anti-inflammatory effects on cultured HCECs which included the following: an increased expression of SOD1, reduced hyperosmolarity-induced ROS production, decreased activation of NF-κB and production of pro-inflammatory cytokine IL-6, and improved cell viability.

NF-κB is a transcription factor that has been shown to act as a key regulator in ocular surface inflammation—activating downstream pro-inflammatory cytokines and modulating intracellular antioxidant protein levels [10,11,19,20]. Culturing of HCECs in hyperosmolar media has been found to activate specific "hyperosmotic sensors" which lead to the stimulation of the NF-κB signaling pathway. Recent studies have found that inhibition of NF-κB activation and translocation to the nucleus can prevent the transcription of downstream inflammatory mediators, and most notably, allows for decreased ocular surface inflammation and reduced DED symptoms [21–23]. In this study, exposure of HCECs to hyperosmotic media indeed stimulated NF-κB activation and increased IL-6 expression. Prophylactic treatment with 50 μM or 100 μM of AZT, however, significantly decreased NF-κB activation and IL-6 production, suggesting reduced expression of inflammatory mediators. Furthermore, we found that pretreatment with AZT significantly increased the viability of HCECs relative to controls exposed to hyperosmolar media. Overall, targeting of NF-κB activation may prove to be a crucial strategy for the suppression of inflammation that contributes to the development of DED as well as for the promotion of ocular health.

In addition to NF-κB activation, oxidative stress has been shown to play key roles in the pathogenesis of DED. Oxidative stress refers

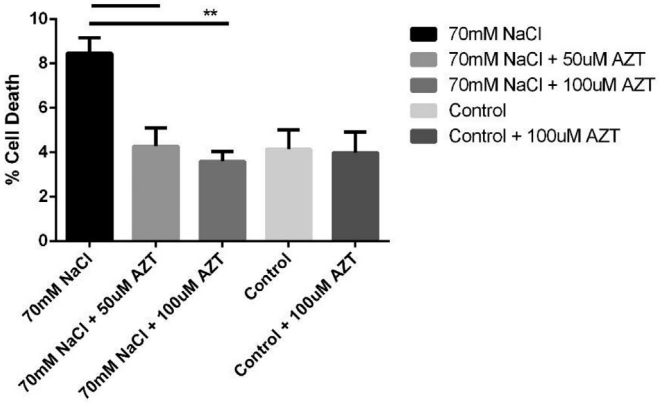

Fig. 4. AZT increases viability of HCECs treated with sodium chloride. HCECs were pre-treated with 50 mM or 100 mM AZT for 1.5 h. AZT was removed and 70 mM sodium chloride was added to 24-well plates overnight. Percent cell death was measured via a Pierce LDH cytotoxicity assay (ThermoFisher). Significance was analyzed by Two-way ANOVA (n = 4). * - P < 0.05, ** - P < 0.01. Data are presented as mean ± SEM.

to the excessive production of ROS relative to the cell's ability to neutralize the intermediate components or repair the resulting damage caused by the elevated ROS levels. Previous studies have shown that exposure of HCECs to hyperosmotic stress increases ROS production and decreases levels of antioxidant enzymes like SOD1, as measured by a DCFDA kit. These results suggest that oxidative damage may be due to an imbalance of oxygenase and antioxidant levels. In this study, we observed a similar response of increased ROS production and decreased SOD1 levels in HCECs

exposed to hyperosmolar media. However, pretreatment of HCECs with 50 μM or 100 μM of AZT showed reduced levels of ROS production and restored levels of antioxidant enzyme SOD1 back to near-control levels. These results suggest that AZT may confer its anti-inflammatory actions by directly inhibiting the production of ROS - leading to decreased antioxidant consumption of SOD1 in HCECs exposed to hyperosmolar media. It is important to note that the use of a DCFDA fluorescence kit in our study measures total oxidative stress rather than the presence of any specific reactive species [24]. Numerous potential molecules may contribute to the oxidative stress seen in DED. In this study, SOD1 levels were measured to shed light on possible identities of upregulated ROS, i.e. superoxide. Further investigation into the upregulation of specific reactive oxygen species in DED would be beneficial.

It is important to note that the blood levels of AZT in patients regularly taking the drug range from 3.46 to 8.18 μM [25]. In our cell culture experiments, we used >10 times this concentration of AZT. Because the cornea has no blood vessels, blood-derived drugs normally reach the cornea via the aqueous humor – an action which normally reduces the effective concentration of drug interacting with corneal surface [26]. Due to this limitation, we plan to use AZT topically. Topical therapy is effective in treating conjunctival, corneal, and iris diseases [27]. Moreover, the use of topical ocular drug emulsions has been shown to allow better interaction of drugs with the corneal surface [28]. Currently, cyclosporine is administered both as an oil in water emulsion and orally. After oral uptake in patients, blood concentrations of the drug range from 500 to 600 ng/mL in whole blood samples [29]. However, when delivered topically, the concentration of cyclosporine remains uniform at 0.5 mg/mL across the cornea [30]. To ensure that the levels of AZT measured *in vitro* be achieved *in vivo* as well, we propose AZT be applied topically as a drug emulsified in oil. Our results have also indicated no change in IL-6 production or cellular viability in cells treated with AZT alone – indicating the safety of using this drug at our working concentrations in the eye.

In summary, this study supports the anti-inflammatory activity of AZT in a hyperosmolar-induced *in vitro* DED model. More importantly, it indicates that AZT activity may confer protective effects by reducing ROS production and modulating NF-κB activation, resulting in decreased production of downstream pro-inflammatory mediators such as IL-6. These results support the novel use of AZT in the reduction of ocular inflammation and the promotion of corneal cell health.

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Conflicts of interest

The authors declare no conflict of interest.

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