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

Hui Liu [a, b, 1], Frank Gambino Jr. [c, 1], Crystal Algenio [b], Charles Bouchard [b], Liang Qiao [c, d, e, 2], Ping Bu [b, e, 2], Shaozhen Zhao [a, *, 2]

[a] Tianjin Medical University Eye Hospital, Tianjin Medical University Eye Institute, College of Optometry and Ophthalmology, Tianjin Medical University, Tianjin 300384, China
[b] Department of Ophthalmology, Stritch School of Medicine, Health Sciences Division, Loyola University Chicago, Maywood, IL 60153, USA
[c] Department of Microbiology and Immunology, Stritch School of Medicine, Health Sciences Division, Loyola University Chicago, Maywood, IL 60153, USA
[d] Biotherapy Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China
[e] Institute of Precision Medicine, Jining Medical University, Jining, Shandong 272067, China

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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.

* Corresponding author.
E-mail address: Zhaosz1997@sina.com (S. Zhao).

[1] Denotes Equal Contribution (Co-First authors).
[2] Denotes Equal Contribution.

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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.