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An In Vitro Evaluation of the Effect and Protection of Artificial Tear Formulations on Human Corneal Epithelial Cells in Normal and Dry Eye Disease States

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Abstract

Background: Dry eye disease (DED) is characterized by tear film instability and a hyperosmolar ocular surface, which significantly impacts ocular health. Artificial tear solutions (ATSs) have been effective frontline treatments for DED, yet current commercially available products often provide only temporary relief, necessitating frequent daily administration. Significant efforts have been made to develop next-generation ATSs that can provide prolonged protective effects for DED. High-molecular-weight sodium hyaluronate (HA) is more commonly used in multi-dose preservative ATSs due to its longer chain lengths and rheological properties that can provide an enhanced retention time and clinical comfort and effects. The current methods to evaluate ATSs have largely focused on human biocompatibility and rheological testing and often overlook the dynamic nature of cellular phenotypes or the protective mechanisms at a cellular level. Therefore, this study developed novel in vitro mammalian cell assays involving human corneal epithelial cells (HCECs) to comprehensively assess ATSs with HA for biocompatibility and efficacy. **Methods:** We evaluated cellular viability across varying severities in two distinct DED models: desiccation and hyperosmotic stress. Simultaneously, time-lapse imaging coupled with computational image analyses quantified subtle, yet significant, cellular morphological changes under these stress condition. **Results:** Our assays revealed that ATSs provide significant, yet varying, protection against mild, medium, and harsh desiccation stress, as well as hyperosmotic conditions. This study also made a key insight that was the observation that DED conditions induce drastic HCEC morphological changes, including significant cellular monolayer breakage, which were effectively mitigated by the ATS products used in this work. **Conclusions:** The assays presented here provide a robust standard for ATS testing, ultimately guiding the selection of more effective next-generation therapies and aiding in a greater understanding of DED pathogenesis.

Keywords: dry eye disease; artificial tear solutions; corneal epithelium; desiccation; hyperosmotic dry eye; ocular drug discovery

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1. Introduction

Dry eye disease (DED) is a multifactorial ocular surface disorder that is characterized by an unstable or insufficient tear film. Globally, it is estimated that 5% to 50% of individuals over the age of 40 are affected [1]. DED is a self-perpetuating cycle of tear film instability, hyperosmolarity, and inflammation, all of which further damage the ocular surface [2–4]. This can lead to patient discomfort and visual impairment, and, in severe cases, corneal ulcerations or even blindness can occur [5–7]. Clearly, DED significantly impairs patient quality of life. Artificial tear solutions (ATSs) remain the cornerstone and primary first-line treatment for managing mild-to-moderate DED [8–10]. While there is a wide array of ATSs available for DED patients, their fundamental function is to supplement the compromised ocular tear film, thereby providing moisture, improving ocular surface lubrication, and offering relief from discomfort and pain, albeit temporary [8].

The complexity of DED extends beyond a mere lack of moisture on the ocular surface. This is highlighted by the diverse range of ATS products available on the market, including aqueous solutions, gels, and emulsions [5,8,11,12]. Each of these products are specially tailored to target distinct aspects of tear film instability. Indeed, current products have a wide array of ingredients incorporated, which include not only viscosity enhancers for prolonged retention but also electrolytes to maintain osmotic balance, lubricant agents to reduce evaporation, osmoprotectants to protect cells from hyperosmolar stress, antioxidants, and wound-healing agents [8–10]. Despite their widespread use and varying compositions, current ATS efficacy is limited [13,14]. Many formulations are rapidly cleared from the ocular surface due to tear film turnover and blinking, necessitating the frequent administration of ATSs to maintain patient relief [15,16]. This can lead to patient inconvenience and poor patient compliance. Although ATSs have been critical to the management of DED signs and symptoms, there is an urgent need to develop next-generation formulations that provide more efficacious and longer-lasting ocular protection.

Hyaluronic acid (HA) is a naturally occurring glycosaminoglycan throughout the body and is a key ingredient in many ATS products due to its strong hydrophilic properties [17,18]. It serves critical roles in maintaining hydration in several tissues including the skin and eyes, it provides structural support in the extracellular matrix, and it lubricates bone joints. These attractive properties of HA resulted in its use in ATS where it enhances tear viscosity and improves hydration and lubrication of the ocular surface [8,17,19,20]. Furthermore, its shear-thinning behavior mimics natural tears, reducing friction and blurring. Interestingly, HA has been demonstrated to promote corneal healing by binding the CD44 receptor on epithelial cells and may possess anti-inflammatory and antioxidant functions [21]. It should be noted that HA can have different biophysical and biological properties depending on its molecular weight [22]. High-molecular-weight HA is currently more commonly used in multi-dose preservative ATSs due to its longer chain lengths and rheological properties that can provide an enhanced retention time and clinical comfort and effects [23]. These types of parameters must be carefully considered when designing an HA ATS for clinical efficacy in patients. Given that the composition of the ATS is crucial for clinical performance, there remains a need for novel cellular assays to properly evaluate the protective effects of ATS products and aid in delineating the benefits between closely related compounds, such as HA.

Despite the clinical importance of ATSs, especially those containing HA, current in vitro models often lack the physiological relevance and high-throughput capabilities for comprehensive evaluations of their cytoprotective effects [24,25]. Furthermore, few studies evaluate the mechanism by which an ATS provides protection at the cellular level. Although previous assays using a desiccation model for DED have been instrumental in guiding measurements of the extent of ATS protection [26], their use has been limited as

they address a single feature of DED, dehydration. This study aims to address this gap by developing novel cellular assays that accurately simulate the dynamic ocular surface.

This study's objective is to identify and characterize the protective effects of artificial tear solutions, particularly formulations containing HA, against desiccation- and hyperosmolarity-induced cellular damage. It should be noted that the formulations studied in this work are complex mixtures, and the observed protective effects may be due to the interaction of all components. Nevertheless, these advanced assays will set a standard for in vitro DED models and will provide deeper insights into how HA-containing artificial tears protect human corneal epithelial cells, moving beyond symptomatic relief.

2. Materials and Methods

2.1. Materials and ATS Product Acquisition

All ATS products/eye drop formulations purchased and used in this study are commercially available and preservative-free (Table 1).

Table 1. List of ATS products used in this study.

ATS Name	Composition	Manufacturer
OPTASE Intense	Active Ingredients: Glycerin 0.2% Inactive Ingredients: Hydrochloric acid, sodium hyaluronate, sorbitol, trometamol, water	Scope Ophthalmics (West Sussex, UK)
Systane HYDRATION	Active Ingredients: Polyethylene Glycol 400 0.4%, Propylene Glycol 0.3% Inactive Ingredients: Aminomethylpropanol, boric acid, hydroxypropyl guar, potassium chloride, purified water, sodium borate, sodium chloride, sodium hyaluronate, sorbitol	Alcon (Geneva, Switzerland)
iVIZIA	Active Ingredients: Povidone 0.5% Inactive Ingredients: Hydrochloric acid, purified water, sodium chloride, sodium hyaluronate, trehalose, tromethamine	Théa Pharma, Inc. (Waltham, MA, USA)

2.2. Human Corneal Epithelial Cell (HCEC) Culture

HPV-immortalized HCECs were gifted from Dr. Maud Gorbet at the University of Waterloo. HCECs were maintained in DMEM/F12 medium (Thermo Fisher Scientific, A4192001, Waltham, MA, USA) supplemented with 1% fetal bovine serums (FBS, Thermo Fisher Scientific, A5256701, Waltham, MA, USA) and 1% penicillin/streptomycin (P/S, Thermo Fisher Scientific, 15140122, Waltham, MA, USA) at 37 °C, 95% humidity, and 5% CO_2. HCECs were passaged when they reached ~90% confluency. HCECs were seeded into 96-well or 6-well polystyrene plates and maintained for 16–24 h at a density of 15,000 cells or 250,000 cells per well, respectively, prior to DED treatment conditions.

2.3. Inducing DED Conditions Using Desiccation

Upon reaching ~90% confluency, HCECs were washed briefly with 1× PBS (Thermo Fisher Scientific 10010023, Waltham, MA, USA) and exposed to desiccating conditions as previously described [26]. For ATS treatments, the cell culture medium was removed, and HCECs were rinsed once with 1× PBS. ATS products of interest were added to HCECs such that a thin layer of the eye product covered the cells. For example, cells cultured in a 96-well plate were treated with only ~40–50 µL of ATS. HCECs were then incubated with ATS for 15 min at 37 °C, 5% CO_2, and 95% humidity. For assays involving PBS washing

to simulate blinking, HCECs were rinsed with 1× PBS, with gentle aspiration. After the last PBS wash, HCECs were immediately placed in a desiccation chamber (37 °C, 5% CO_2, and 45% humidity) for the indicated times specified in each experiment. After exposure to desiccating conditions, HCECs were immediately incubated with 1× alamarBlue™ reagent (Thermo Fisher Scientific, DAL1025, Waltham, MA, USA).

2.4. Inducing DED Conditions Using Hyperosmolar Stress

Once HCECs reached ~90% confluency, cells were rinsed with 1× PBS and cells were subsequently treated with ATS products or a PBS control for 15 min. Next, ATS or PBS was removed, and HCECs were rinsed with 1× PBS once, requiring slow and gentle aspiration of the PBS from of the culture plate. HCECs were subsequently treated with hyperosmotic medium for 16–24 h at 37 °C, 5% CO_2, and 95% humidity. The hyperosmotic medium was prepared using serum-free DMEM/F12 with 1% P/S, with the osmolarity adjusted to ~500 mOsM using sodium chloride (NaCl, Sigma-Aldrich, S9888, St. Louis, MO, USA). To prepare hyperosmotic DMEM/F12 medium, 1 mL of 5 M NaCl was added to 49 mL of DMEM/F12. HCECs treated with serum-free isosmotic medium was used as the control. After cells were treated with isosmotic or hyperosmotic conditions, HCECs were imaged under brightfield settings using the Cytation 5 plate reader (Agilent BioTek Instruments, Winooski, VT, USA).

2.5. Live-Cell Brightfield Microscopic Imaging

To perform live-cell imaging, HCECs at ~70–90% confluency were treated with conditions of interest in a 96-well plate. The 96-well plate was placed in an Imaging Multimode Plate Reader (Cytation 5), set to image each well in the 96-well plate for 15–30 min, with 1 min time intervals. The Cytation 5 plate reader was set for 37 °C throughout the imaging protocol.

2.6. Computational Quantification of Live-Cell Microscopy

Time-lapse images were acquired and exported as TIF images from the multimode plate reader and analyzed using the CellProfiler software (https://cellprofiler.org/, version 4.2.8). An image-analysis pipeline was generated that identified cell objects based on brightfield microscopy and based on the pixel intensity. For each single-cell object identified, the cell length along the major axis of the identified object was quantified. This measurement was used as a proxy for cellular health and morphology throughout the analyses in this study. Elongated cellular shapes and morphology are associated with HCECs growing in normal conditions. This study associated more rounded cellular shapes with stress conditions, which could be captured by measuring the change in cell lengths. All images were quantified using the exact same pipeline, and the resulting data was analyzed and graphed using R (https://cran.rstudio.com/, version 4.5.1).

2.7. Cellular Viability

HCECs were seeded in 96-well plates at a density of 15,000 cells per well and incubated for 16–24 h. After assay treatments with ATS products and desiccation or hyperosmotic stress conditions, 1× alamarBlue™ reagent was added to HCECs, and cells were incubated at 37 °C with 5% CO_2 and 95% humidity between 2 and 4 h. Next, the fluorescence was measured using the Imaging Multimode Plate Reader (Cytation 5) with excitation and emission settings of 540 nm and 590 nm, respectively.

2.8. Reverse Transcription–Quantitative Polymerase Chain Reaction (RT-qPCR)

HCECs were seeded in 6-well polystyrene plates at a density of 250,000 cells per well and incubated for 24 h in cell culture medium. HCECs were exposed to the DED conditions

described above. After treatment, HCECs were washed twice with warm 1× PBS, and RNA was extracted using TRIzol™ Reagent (Thermo Fisher Scientific, 15596026, Waltham, MA, USA). Reverse transcription was performed using iScript™ cDNA Synthesis Kit (BioRad, 1708890, Hercules, CA, USA) according to the manufacturer's instructions. The expression level of inflammatory genes of interest was measured using Sso-Fast EvaGreen (BioRad, 1725201, Hercules, CA, USA). The gene expression of interleukin-1β (IL-1β), interleukin-8 (IL-8), interleukin-6 (IL-6), and matrix metallopeptidase 9 (MMP9) was performed and normalized to GAPDH using the specific primers described in Table 2, purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA).

Table 2. Primers used in this study for RT-qPCR to determine gene expression changes.

Primer Name	Primer Sequence (5′–3′)
IL-1b-Forward	CCTGTCCTGCGTGTTGAAAGA
IL-1b-Reverse	GGGAACTGGGCAGACTCAAA
IL-8-Forward	TTTCAGAGACAGCAGAGCACACAA
IL-8-Reverse	CACACAGAGCTGCAGAAATCAGG
IL-6-Forward	CACAGACAGCCACTCACCTC
IL-6-Reverse	TTTTCTGCCAGTGCCTCTTT
GAPDH-Forward	GAAGGTGAAGGTCGGAGTC
GAPDH-Reverse	GAAGATGGTGATGGGATTTC
MMP9-Forward	GCCACTACTGTGCCTTTGAGTC
MMP9-Reverse	CCCTCAGAGAATCGCCAGTACT

2.9. Quantification and Statistical Analyses

Statistical analyses were performed using GraphPad Prism version 9.4.0 (GraphPad, La Jolla, CA, USA), R (https://cran.rstudio.com/, version 4.5.1), and/or Python (https://www.python.org/, v3.11.3). Significance tests for multiple comparisons of groups were performed using a one-way analysis of variance (ANOVA), as described in the figure legends. Results with $p < 0.05$ were considered statistically significant: * $p < 0.05$, ** $p < 0.005$.

3. Results

3.1. Biocompatible Commercial Eyedrop Formulations Differentially Affect HCEC Morphology

A critical requirement for all ATS formulations intended for human use is to pass cytotoxicity testing. In this study, the selected ATS products (OPTASE® Intense, Systane® HYDRATION, and iVIZIA®) demonstrated high biocompatibility with HCECs in a desiccation model (Figure 1). Across all tested conditions, the ATS products did not reduce metabolic activity, a proxy for cell viability, indicating their non-toxic nature. Interestingly, some ATS products consistently increased cellular viability compared to the phosphate-buffered saline (PBS) control at varying levels (Figure 1A). This suggests a potential beneficial effect beyond simple cytocompatibility.

To further understand the dynamic cellular responses to continuous ATS treatment, HCECs were observed via brightfield microscopy after 30 min of ATS exposure (Figure 1B). A computational image-analysis pipeline was employed to quantitatively measure the cellular shape and morphology, calculating a cell morphology index value for each cell to facilitate comparisons between treatment groups (Figure 1C). As anticipated, HCECs maintained in regular DMEM/F12 cell culture medium exhibited the characteristic flat and elongated shapes of healthy epithelial cells. In contrast, HCECs treated with PBS, OPTASE Intense, and Systane HYDRATION displayed less elongated, more uniformly rounded cellular shapes, resulting in lower cell lengths (Figure 1C–E). These changes were also observed in the PBS control condition, suggesting that HCECs removed from their normal culturing conditions can induce cellular morphology changes. Treatment with both

OPTASE Intense and Systane HYDRATION further accelerated these cellular morphology changes (Figure 1D,E), which was more pronounced with Systane HYDRATION-treated cells. These observed morphological changes, even in the absence of reduced viability, highlight that ATS products can induce cellular responses beyond simple cytotoxicity. It is important to note that the nature and biological relevance for these unexpected morphological changes are unknown, for example if they are adaptive cellular responses and thus warrant future work to understand if they are adaptive cellular responses. Nevertheless, these morphological effects, in addition to viability measurements, may be important for maximizing the biocompatibility of eye products and ensuring the optimal maintenance of ocular surface homeostasis.

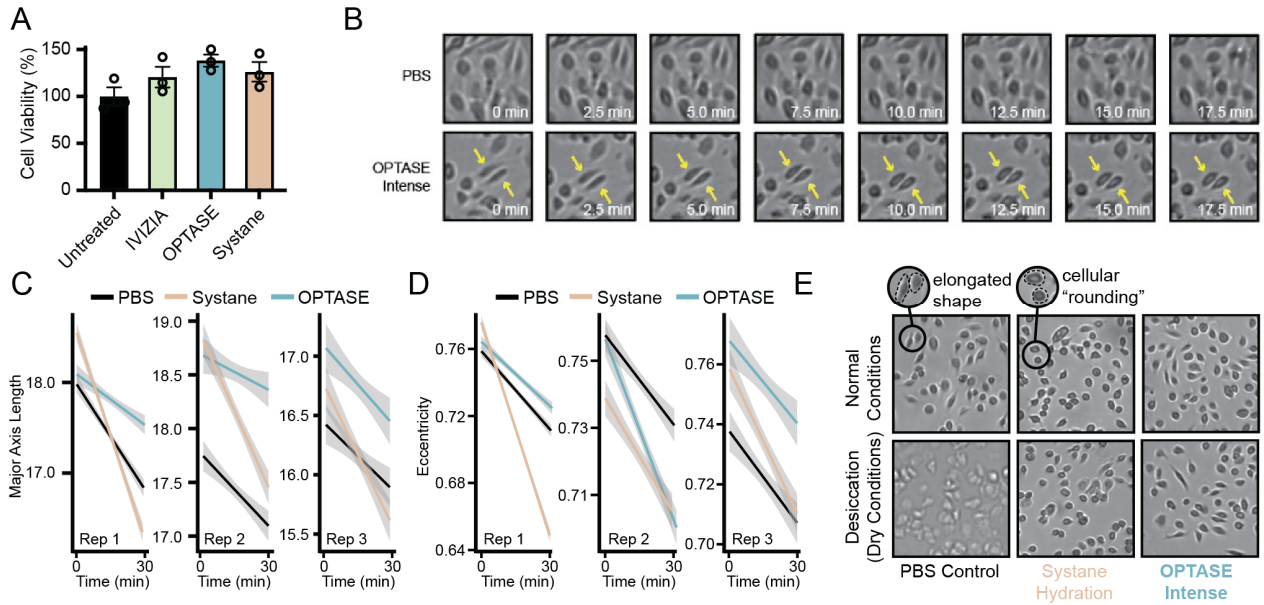

Figure 1. Biocompatible ATS products can affect HCEC cell morphology. (**A**) Cell viability of HCECs following 15 min treatment with PBS control and indicated ATS products. (**B**) Representative bright-field time-lapse micrographs of HCECs during treatment with PBS control or OPTASE Intense. Yellow arrows indicate cells that are undergoing significant cellular morphology changes during exposure to the ATS product. Representative images acquired at 10× magnification. (**C**) Computational quantification of cellular length along the major axis length and how it changes during incubation with ATS products of interest (n = 3 biological replicates). (**D**) Computational quantification of cellular circularity (eccentricity) and its changes during incubation with ATS products of interest (n = 3 biological replicates). Values closer to 0 indicate a more circular morphology. (**E**) Representative images of HCECs and their cellular morphology before and after desiccation conditions (low humidity, 45%) with and without pre-treatment with the indicated ATS products at 10× magnification. (n = 2 biological replicates).

3.2. ATSs Protect Against Mild, Medium, and Harsh Desiccation Stress

DED is characterized by an inadequate tear film and a dry ocular surface. This condition can be effectively modeled in vitro by desiccating HCECs (Figure 2A). To establish a robust desiccation model for DED, HCECs were incubated at 37 °C in low humidity conditions (45%) for durations ranging from 0 to 10 min (Figure 2B). As expected, cellular viability decreased proportionally with an increasing exposure time to low humidity. A significant reduction in HCEC viability was observed after just 4 min of desiccation stress ($p < 0.05$, one-way ANOVA). These findings guided the selection of desiccation exposure times for the remainder of this study, defining mild (2 min), medium (5 min), and severe (10 min) DED conditions.

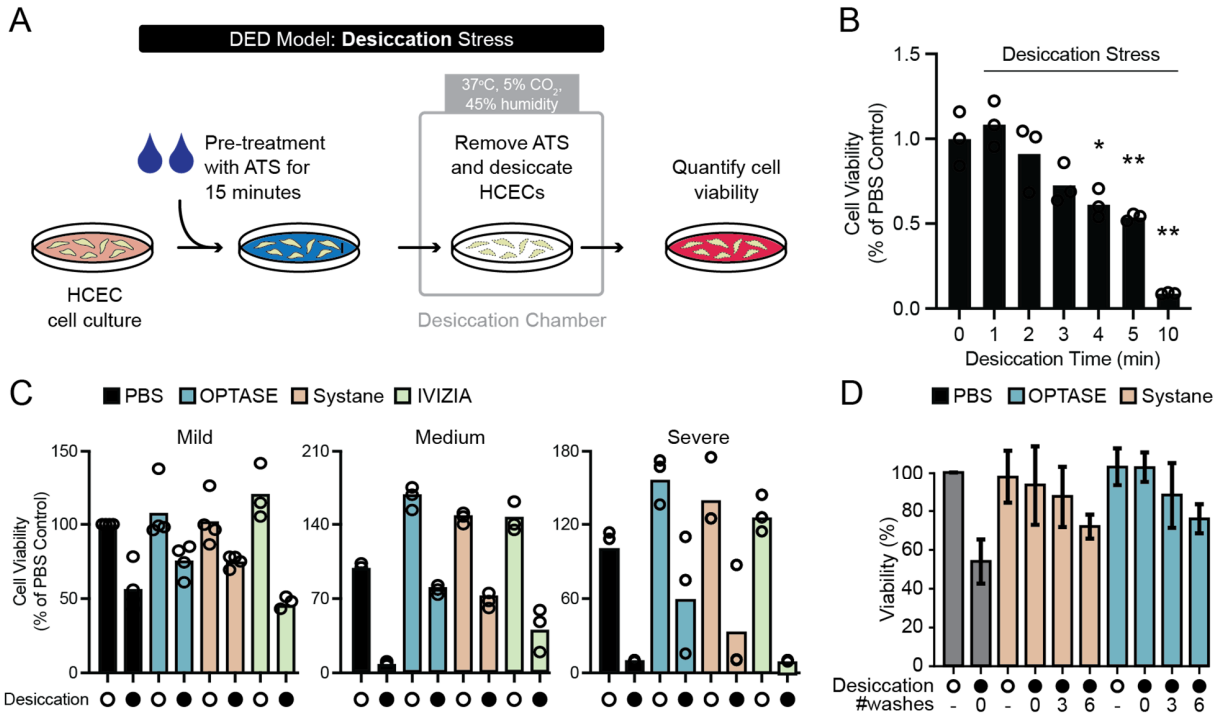

Figure 2. ATSs protect HCECs from desiccation stress. (**A**) Experimental workflow of desiccation stress as an in vitro model for DED. Briefly, cells are pre-treated with the ATS prior to exposure in low-humidity conditions. (**B**) HCEC viability for cells without pre-treatment with ATS and incubated in low-humidity (45% humidity) conditions for varying times, between 1 to 10 min. Open circles represent biological replicate data points (n = 3). (**C**) Quantification of cell viability before and after exposure to desiccation stress with different severities and with and without pre-treatment with the indicated ATS products. Open circles below the plot indicate no desiccation stress (cells remained hydrated), and closed circles indicate 5 min desiccation stress at 37 °C with 5% CO_2 (n = 3 biological replicates). (**D**) Cell viability of HCECs before and after 5 min desiccation stress, with and without pre-treatment with the indicated ATS products and with the indicated number of PBS wash steps following ATS pre-treatment and before exposure to low-humidity conditions (n = 3 biological replicates). Statistical significance determined via one-way ANOVA with Tukey multiple comparisons test * $p < 0.05$, ** $p < 0.005$.

Next, the protective efficacy of the ATS products under varying DED severities was assessed by measuring HCEC viability after exposure to different desiccation times (Figure 2C). Under mild desiccation stress, cellular viability was reduced to approximately 56% in the untreated control. Notably, only OPTASE Intense and Systane HYDRATION provided protection, resulting in HCEC viabilities of 61% and 69%, respectively. Similar trends were observed under medium desiccation stress; untreated HCECs displayed only 44% viability, while HCECs treated with OPTASE Intense and Systane HYDRATION demonstrated statistically significantly higher viabilities (67% and 59%, respectively). iVIZIA-treated HCECs showed minimal protection, with viability at 49%. Interestingly, the relative protective efficacy between OPTASE Intense and Systane HYDRATION differed between mild and medium desiccation conditions, with OPTASE Intense showing less protection under mild DED conditions, but greater protection when exposed to medium DED conditions, compared to Systane HYDRATION. Strikingly, both OPTASE Intense and Systane HYDRATION provided substantial protection even in severe desiccation conditions, where PBS-treated HCECs exhibited a mere ~9% cellular viability.

To further simulate the impact of the dynamic environment of the ocular surface on the retention of protective effects, where tear fluid and blinks constantly wash away eye products, we incorporated PBS washes following ATS treatment into the in vitro

desiccation model for DED. HCEC viability was measured only for OPTASE Intense and Systane HYDRATION under medium DED conditions, as iVIZIA previously showed limited protection at this severity (Figure 2D). As predicted, the highest HCEC viability in desiccation conditions was observed when HCECs were not washed with PBS after ATS treatment. This protective effect decreased as the number of washes increased for both OPTASE Intense and Systane HYDRATION. An intriguing observation was that even after extensive washing (more than six washes), HCEC viability did not return to the same low level as with HCECs without any ATS treatment (PBS control). One possible explanation is that protective components of the ATS products may be retained on the surface of ocular cells even after excessive washing. However, this requires further investigation to conclusively determine if there are protective agents retained.

3.3. ATSs Protect Against Hyperosmotic Conditions

DED is also characterized by tear film hyperosmolarity, a key pathophysiological feature [8]. To determine if ATSs provide protective benefits beyond simple lubrication and hydration, specifically against high-salt conditions on the ocular surface, HCECs were exposed to hyperosmolar conditions with differing salt concentrations, and their viability was assessed in the presence and absence of commercially available ATS (Figure 3A). As expected, HCECs exposed to medium hyperosmotic stress conditions (100 mM NaCl) displayed a significant reduction in viability (Figure 3B, approximately 38% reduction compared to isosmotic conditions). Notably, HCECs pre-treated with either Systane HYDRATION or OPTASE Intense provided substantial protection against 100 mM hyperosmotic stress, reducing HCEC viability by only 24% and 19%, respectively, compared to the untreated hyperosmotic group. This demonstrates their capacity to mitigate osmotic stress-induced cellular damage.

3.4. Hyperosmolar Treatment Causes Drastic Changes to HCEC Cellular Morphology and Monolayer Integrity

Consistent with observations made earlier in this study, HCECs exhibited drastic changes in their cellular morphology in response to different treatments and stresses. Therefore, the cellular morphology of HCECs under hyperosmotic stress was meticulously imaged (Figure 3C,D). Strikingly, during persistent exposure to hyperosmotic stress, the HCEC morphology changed significantly, and the characteristic cellular monolayer formed under isosmotic conditions was severely disrupted, with readily observable gaps forming between cells. This study was interested in measuring these changes in the "monolayer integrity", which is defined in this work as a confluent, uniform sheet of adherent cells on the surface. To measure this, the surface area occupied by HCECs and the total surface area of the gaps formed in hyperosmotic stress conditions were quantified using image analysis. Interestingly, pre-treatment with both OPTASE Intense significantly reduced the formation of these gaps compared to HCECs without ATS pre-treatment, which is shown by the greater surface area occupied by HCECs with the OPTASE Intense treatment compared to the DED condition alone, as well as the reduced surface area in the gap-formation analysis (Figure 3D,E). The degree of protection against cellular monolayer breakage varied between the products, with HCECs pre-treated with OPTASE Intense exhibiting fewer gaps in their monolayer, suggesting a potentially superior ability to maintain monolayer integrity under hyperosmotic challenge.

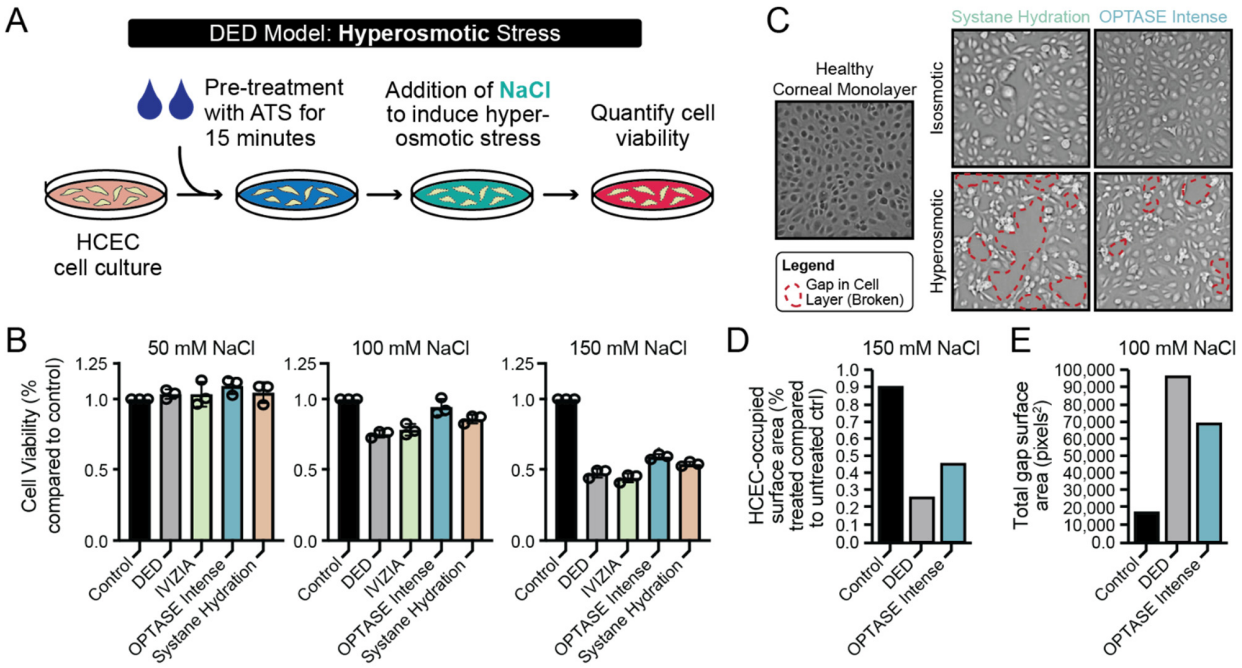

Figure 3. ATSs protect HCECs from hyperosmotic stress. (**A**) Experimental workflow of desiccation stress as an in vitro model for DED. Briefly, cells are pre-treated with the ATS prior to treatment with cell culture medium supplemented with NaCl to create hyperosmotic conditions. (**B**) HCEC viability for cells without pre-treatment with indicated ATS products and exposed to low (50 mM NaCl; ~400 mOsM)-, medium (100 mM NaCl; ~500 mOsM)-, and high (150 mM NaCl; ~600 mOsM)-salt conditions for 16–24 h at 37 °C with 5% CO_2 ($n = 3$ biological replicates). (**C**) Representative brightfield micrographs of HCECs grown in a monolayer in standard conditions (left) and in hyperosmolar conditions, with and without pre-treatment with indicated ATS products (right). The dotted red line indicates regions in the monolayer where there are observable gaps due to breakage of the cellular monolayer at 10× magnification. (**D**) Quantification of the surface area occupied by HCECs in the field of view of the microscope represented as a percentage of HCECs treated with the indicated condition compared the non-treated control. (**E**) Quantification of the surface area (in pixels squared) of gaps and HCEC monolayer breakage in hyperosmolar conditions in the indicated conditions.

3.5. Desiccation and Hyperosmotic Stress Conditions Recapitulate Dry Eye Disease States

To validate the physiological relevance of the described in vitro desiccation and hyperosmotic DED models, the expression of the pro-inflammatory markers IL-1β, IL-6, IL-8, and MMP-9, which are commonly upregulated in DED patients, was measured via RT-qPCR. Notably, the levels of all tested cytokines increased when HCECs were exposed to desiccation and hyperosmotic stress (Figure 4), confirming that our in vitro models successfully induce an inflammatory response characteristic of DED [27]. More importantly, the pre-treatment of cells with OPTASE Intense reduced the expression of several inflammatory markers in HCECs exposed in DED conditions to differing degrees. Most notably, for HCECs exposed to desiccating conditions, pre-treatment with OPTASE Intense prevented significant increases in pro-inflammatory gene expression for all biomarkers tested. In contrast, these observed anti-inflammatory effects were less pronounced in HCECs experiencing hyperosmotic stress. Interestingly, pre-treatment only provided a significant anti-inflammatory effect with IL-6. However, a reduced, but not significant, reduction in IL-1β and MMP9 was observed (Figure 4). Overall, these finding suggest that certain ATS products may not only provide physical protection but also possess DED-dependent anti-inflammatory properties, offering a more comprehensive clinical benefit for DED patients.

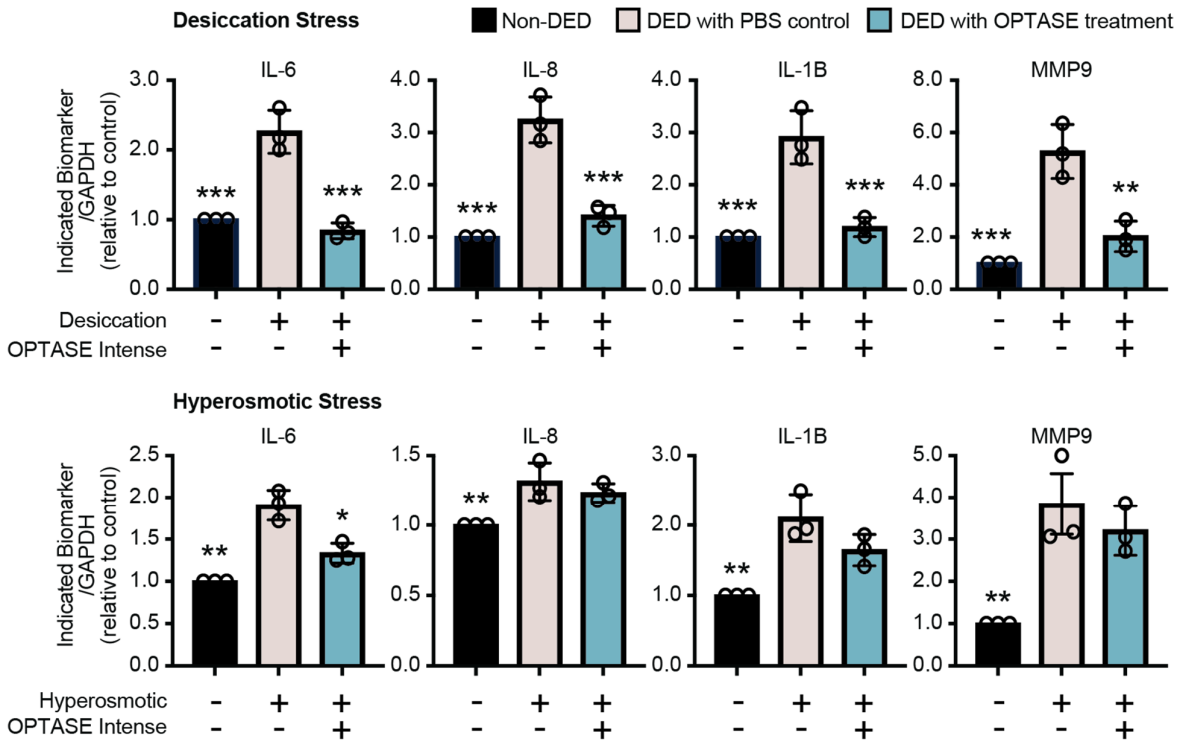

Figure 4. HCECs in DED models show increase in inflammatory gene expression. Inflammatory biomarkers were measured via RT-qPCR in HCECs before and after the indicated DED conditions, either treatment with desiccation stress or hyperosmolar stress. Significance tests are comparisons to the DED treatment. Statistical significance determined via one-way ANOVA with Tukey multiple comparisons test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ ($n = 3$ biological replicates).

4. Discussion

4.1. Improving the Methods to Evaluate ATS Toxicity and Efficacy

The novel in vitro assays described in this study significantly improve the methods to evaluate ATS toxicity effects on cell viability and efficacy for treating DED phenotypes, adding to the current repertoire of standardized testing protocols and advancing our understanding of DED pathology. Current industry standards often rely on simple cytotoxicity tests with single end time points and often overlook the dynamic responses exhibited by human cells to ATS formulations [24,25]. The assays presented here go beyond simple viability measurements by quantitatively assessing cellular morphology and monolayer integrity under various stress conditions. Specifically, this study developed a computational image-analysis methodology to quantify changes in HCEC morphology, which provided insight into the cellular responses to ATS treatment and DED exposure. Notably, this imaging method provides a novel metric for cellular health beyond traditional endpoint measurements. Importantly, this approach is a substantial improvement over more qualitative assessments, offering quantifiable measurements that will be essential for comparative studies between different ATS products. Furthermore, the DED models presented here included wash steps to more accurately mimic the dynamic ocular surface, where tear film turnover and blinking constantly remove the ATS, ultimately affecting the retention and efficacy of eye drops. The inclusion of this washing regimen was to purposely create more physiologically relevant models that can help guide future work in uncovering novel protective molecules and formulations that can enhance the retention and protective effects to enhance patient long-term comfort and reduce dosing needs. One limitation is that this study utilized PBS to simulate natural tears. However, the composition of natural tears contains salts, peptides, and lipids. Future work can integrate synthetically formulated tear

solutions that more accurately reflect the ocular surface environment. Finally, the models used in this study recapitulate the pro-inflammatory cellular response commonly observed in DED patients, providing further support that the assays established here are powerful tools to evaluate ATS toxicity and efficacy.

4.2. Cell Morphology and Monolayer Breakage as Underappreciated Cellular Responses to DED

Extending beyond simple product evaluation, the assays developed here have uncovered a previously underappreciated cellular response to DED stresses, particularly the breakage of the cellular monolayer following hyperosmotic stress. Although ocular surface hyperosmolarity is a known hallmark of DED [2,28], its direct and rapid impact on the human corneal epithelial monolayer in vitro has not been well characterized. This study's findings demonstrate that hyperosmotic stress not only reduces cell viability but can also cause striking disruptions to the HCEC morphology and the monolayer that they create, leading to the formation of visually observable gaps. Importantly, this provides insight into the pathogenesis of DED, suggesting that hyperosmolarity may directly impair the corneal barrier in a patient. The disruption in the cell monolayers may also suggest an uneven, non-smooth surface, which could be linked to faster tear breakup times, as is often observed clinically in dry eye patients [4,29]; however, substantial further work is needed to validate these correlations. The ATS products tested here (OPTASE Intense and Systane HYDRATION) were able to mitigate this monolayer breakage, highlighting another protective mechanism of these formulations that once again go beyond simple hydration. This includes osmoprotection, barrier stabilization, and/or anti-inflammatory effects. Altogether, this study presents new avenues for investigating the precise molecular mechanisms involved in DED related to hyperosmolarity and how ATS intervention can counter this damage.

4.3. Different ATSs Have Different Performance

Despite being marketed for similar purposes, this study demonstrated that commercially available ATS products can exhibit differences in their toxicity and protective efficacy. By comparing the cellular effects of OPTASE Intense, Systane HYDRATION, and iVIZIA under desiccation and hyperosmotic conditions, this study illustrates that not all products perform equally, and each may have specific use cases. For example, OPTASE Intense and Systane HYDRATION consistently provided significant protection in desiccation and hyperosmotic stress, whereas iVIZIA offered limited protection. More interestingly, subtle differences between the performance of OPTASE Intense and Systane HYDRATION was observed. OPTASE intense exhibited greater protection under mild desiccation and also against hyperosmotic tress, as evidenced by both cell viability measurements and cell monolayer breakage, demonstrating that differences in the ATS composition can lead to different clinical outcomes. These findings challenge the perception that ATS products are interchangeable.

4.4. Guiding the Development of Future ATSs

The insights gained from these assays lay the groundwork for future research and development that will be critical in guiding the next-generation ATS development. First, investigation into specific ATS ingredients responsible for their protective effects, specifically which aspect of DED they target, is warranted, for example, testing the ability of specific compounds to maintain cell monolayer integrity. Second, the work presented here could be expanded to other cell types that are also impaired in DED, including conjunctival and goblet cells. Furthermore, the incorporation of immune cells would be important since the immune response is a large contributor to DED symptoms and would provide a more holistic representation of the ocular surface environment [3,30]. Lastly, exploring the

long-term effects of ATS on cellular morphology and the gene expression of biomarkers of interest (e.g., inflammatory markers) would provide valuable insight into chronic DED management. For example, visualizing specific protein markers to assess changes in the cytoskeleton and key junctional proteins could provide greater mechanistic insight into the biological relevance of these cellular morphological changes. All these potential future directions will require a robust assay platform that can differentiate between different ATSs and accelerate research and development and market entry. This study presents advancements in the ways an ATS is tested for toxicity and efficacy that can be used to develop specific protection profiles of existing and future products that will help inform on evidence-based ATS recommendations for patients, providing more personalized care. Going even further, these assays can be used on the individual components in the ATS, aiding in understanding the contribution of components to DED protection. This will ultimately contribute to improving DED patient outcomes and quality of life.

5. Conclusions

The study presented here successfully developed and implemented novel in vitro assays that model dry eye disease (DED). These assays provide a more comprehensive evaluation for artificial tear solutions (ATSs) and have demonstrated that biocompatible eyedrop formulations can exert varying degrees of protection against desiccation and hyperosmotic stress. The in vitro models for DED established here can effectively distinguish between the clinical efficacies of current and future ATS products. They can offer invaluable insight into understanding the mechanism of protection, identifying superior compounds and formulations, and aid in the development of more targeted and effective therapies for the challenges of DED.

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