Original Article

Role of hyaluronate containing artificial tears in mitigating markers of dry eye disease using *in vitro* models

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Purpose: Ocular surface discomfort and dry eye disease (DED) are the most common conditions addressed by ophthalmologists worldwide. Artificial tear substitutes are used as the first line of treatment management for DED patients. The present study was performed to understand the role of artificial tear formulation namely Soha Liquigel (0.18% sodium hyaluronate with trehalose) and Soha (0.1% sodium hyaluronate) for the treatment of DED *in vitro*. Human corneal epithelial (HCE) cells were used in adapted cell culture conditions which induce relevant cellular and molecular modifications thus mimicking the DED. **Methods:** Artificial tears containing either sodium hyaluronate (SH) (Soha 0.1%, Sun Pharma) or a combination of SH with trehalose (Soha Liquigel 0.18%, Sun Pharma) were compared with respective controls to analyze the effect on desiccation-induced stress or oxidative stress or hyperosmolarity induced stress on HCE cells. Cellular viability was evaluated using the trypan blue assay, while epithelial morphology was observed under light microscopy. Real-time polymerase chain reaction (RT-PCR) was utilized to analyze the transcriptional profile of a specific set of gene signatures, namely S100A7, FOS, SOD-2, COX2, TonEBP, IL6, MCP1, and IL10. **Results:** The response of HCE cells to desiccation stress (24 hr) was observed through alterations in their cellular morphology, which were subsequently restored by applying Soha Liquigel. Oxidative stress was induced using 100 nM of H_2O_2 on HCE cells (short- 24 h and long-term 5 days) and measured using increased expression of S100A7, an oxidative stress-responsive gene. Oxidative-stressed HCE cells after treatment with Soha Liquigel showed reduced pro-oxidant gene and COX2 expression and elevated anti-oxidant genes, FOS, and SOD levels. HCE cells were subjected to +100mOsmol and +200mOsmol NaCl-containing media, inducing hyperosmolar stress that imitates the symptoms of DED. Further, these hyperosmolar stressed cells were treated with Soha Liquigel and Soha eye drops for 24 h and 5 days. Both eye drops rescued the cell morphology under hyperosmolar conditions in both short- and long-term treatments. Increased TonEBP levels confirm the osmotic stress in HCE cells. Reduction in IL6, MCP1, TonEBP, and elevated expression of IL10 in hyperosmotic stressed HCE cells treated with either of the artificial tears indicates their osmo-protection properties. **Conclusion:** By using desiccation, oxidative, and hyperosmolar stress simulated in HCE cells in culture, we observed that SH-containing artificial tears provided bio-protection, osmo-protection, and anti-oxidant benefits that were further strengthened with SH and trehalose combination.

Key words: Anti-oxidant, artificial tears, bio-protection, dry eye, inflammation, ocular surface, Osmo-protection, Soha Liquigel

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Dry eye disease (DED) and ocular surface discomfort are mainly caused by loss of homeostasis of the tear film.[1,2] Although being a multi-factorial disease, the key drivers include oxidative stress, hyperosmolarity, inflammation, and lack of aqueous phase of the tears or ocular dryness.[3] These conditions result in visual disturbance and fatigue and affect the quality of life for patients across all age groups and thus pose a significant economic burden on the society.[4] Various treatment modalities are available for DED patients, and the use of topical artificial tear substitutes is usually the first option for restoring the natural tear film.[5] Studies indicate a notable rise in the utilization of artificial tear substitutes over the last 20 years for managing DED.[5,6]

Artificial tears are made up of elements that increase viscosity, extend retention time, and enhance lubrication of the ocular surface.[6] Understanding their biological roles based on their composition is crucial in selecting an appropriate tear substitute for a specific type of DED. Corneal epithelium

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forms the outermost ocular surface layer directly involved in discomfort and pain leading to DED.[7] Trehalose, a naturally occurring disaccharide, has garnered attention for its numerous therapeutic benefits, particularly in managing DED and supporting corneal epithelial cell health.[8-10] Trehalose effectively scavenges reactive oxygen species (ROS) and protects the corneal epithelial cells from oxidative stress, a key contributor to DED and cellular damage.[11] In comparison with animal studies and cell culture models, *in vitro* cells provide a more manageable human-based model that closely mimics human epithelial physiology.[12] In addition, *in vitro* cells have higher permeability than *in vivo* tissue, making it more advantageous for creating sensitive experimental models in understanding the early impacts of sub-toxic doses.[13] We hypothesize that this new formulation of SH-artificial tears, with and without trehalose as a modulator, interacts with and differentially influences the corneal epithelium (ocular surface) to counteract the pathology of DED, thereby exhibiting enhanced osmo-protection, anti-oxidant, and agents that support bio-protection. Although each compound present in these artificial tears has been extensively studied individually regarding DED, there is a scarcity of studies investigating their comparative effects in model systems. Henceforth, we established the culture conditions to induce relevant morphological, cellular, and molecular changes associated with dry eye symptoms, including desiccation stress, oxidative stress, and hyperosmolarity stress-associated response in HCE cells. Therefore, the current study's aim was to examine the potential modulatory effect of trehalose and sodium hyaluronate (SH) in combination against SH alone on experimentally induced DED-related molecular conditions using human corneal epithelium (HCE) cells. Consequently, our study will offer a better understanding of the treatment options for DED by highlighting their relative differences in function on relevant molecular pathways.

Methods

Human corneal epithelial (HCE) cells culture

HCE cells were cultured on Primaria™ treated culture dishes (Becton Dickinson, Franklin Lakes, NJ) in DMEM/F-12 media supplemented with 10% (FBS) fetal bovine serum and 1% antimycotic antibiotic solution and maintained at 37°C in 5% CO_2. Cells below passage 30 were used for all experiments.

Artificial tears: Two formulations of SH eye drops (Sun Pharma Pvt Ltd) were used in the present study, namely;

a) Soha Liquigel (0.18%) composition has SH Ph. Eur. (1.8 mg), stabilized oxychloro complex (0.1 mg as preservative) with trehalose, carbomer, and tonicity adjusting agents as excipients per mL of the solution.
b) Soha (0.1%) eye drop has SH (0.1% w/v), stabilized oxychloro complex (0.01% w/v as preservative) with glycerine IP, boric Acid IP, calcium chloride IP, erythritol USP-NF, levocarnitine IP, potassium chloride IP, borax BP, and sodium citrate IP.

Cell morphology, viability and treatments

The morphology of HCECs was observed using a bright field microscope (EVOS FL cell imaging system; Thermo Fisher Scientific, Waltham, MA) under various stress conditions. Cell viability was calculated using the trypan blue staining (0.4%, Gibco) for a clear identification of the most bio-available and non-toxic dose of the test products, respectively.

For the calculation of cell viability, the following formula: 100*(live cells)/(dead cells + live cells) was used for each condition.

For determining the non-toxic dose of the test products, namely Soha Liquigel and Soha on HCE cells, cell viability and cell morphology assessments were performed. Based on these, we established 0.002% per mL of Soha Liquigel (0.18%) and Soha (0.1%) dosage as the non-toxic dose for all the further experiments [Supplementary Figs. 1 and 2].

In vitro desiccation stress model and Soha Liquigel treatment: HCE cells were cultured for 2–3 days to reach 95% confluence. The media was completely aspirated from HCE cells and air dried for 10 min at room temperature (25°C) and humidity of 40%, as reported earlier.[14] Further, the growth media was replenished, and desiccated HCE cells were treated with 0.002% of Soha Liquigel for 24 h.

In addition, a pre-treatment of 0.002% Soha Liquigel for 3 h was performed. After completion of 3 h, the pre-treated HCE cells underwent desiccation stress for 10 min at room temperature (25°C) and humidity of 40%. After 24 h, an assessment of the HCE cells from both conditions was performed for the cell morphological changes and viability using light microscopy and trypan blue staining, respectively.

Oxidative stress *in vitro* model and Soha Liquigel treatment: An increasing concentration of hydrogen peroxide (H_2O_2) ranging from 1 nM till 100 nM in DMEM/F-12 media was carried out (data not shown).[15] Oxidative stress media was prepared by adding 100 nM of H_2O_2 in DMEM/F-12 media and added to HCE cells every 24 h. HCE cells were cultured for 3 days to reach 90% confluence for each experiment. Cells were treated with 0.002% of Soha Liquigel under oxidative stress stimulus for 24 h (short-term treatment). For the long-term treatment, 0.002% of Soha Liquigel was added to the HCE cells for 5 days.

Hyperosmolar stress induction and SH-containing artificial tears treatment: HCE cells are cultured for 3 days to 85–90% confluence, and pre-treated with the 0.002% Soha Liquigel or with Soha eye drops for 3 h before NaCl is added (+100mOsmol and + 200mOsmol NaCl to obtain hyperosmotic culture media, as shown earlier[16]) for 24 h (short-term treatment). For the long-term treatment, 0.002% of Soha Liquigel eye drop or Soha eye drops and NaCl were added (as explained above, to obtain hyperosmotic culture media), for an exposure of 15 min duration, at the same time every 24 h for 5 days to the HCE cells (starting cell confluence will be 30–40% in this experiment). Further, this hyperosmotic media was replaced with the normal (growth) media (i.e., after 15 min of exposure to hyperosmotic stress) every day for 5 days of the experiment.

Isolation of RNA, synthesis of complementary DNA, and analysis of mRNA expression

Cells were harvested after each timepoint, and total RNA was extracted by using TRIZOL reagent-based protocol (Invitrogen, Carlsbad, CA). After measuring RNA yield, 1 µg of the total RNA was converted to cDNA (Bio-Rad, Philadelphia, PA). CFX connect real-time PCR (Bio-Rad) was used to assess mRNA expressions of TonEBP (marker for hyperosmolarity),

interleukins IL6, IL10, MCP1 (inflammatory markers), pro-oxidant gene, cyclooxygenase-2 (COX2), oxidative stress response gene S100 calcium-binding protein A7 (S100A7), and anti-oxidant genes like FOS proto-oncogene and superoxide dismutase (SOD2).

Statistical analysis

All the statistical analysis and the graphs were plotted using GraphPad Prism 9.0. Kruskal-Walli's test was performed to obtain the statistical significance among the conditions.

Results

Morphological and cytotoxic effects of artificial tears in human corneal epithelial cells

Soha Liquigel (0.18%) was administered on HCE cells after dilution at five different concentrations ranging from 0.002% to 0.01% per mL of media. HCE cells are cuboidal shaped and retained their morphology at 0.002% and 0.004% Soha Liquigel per mL of media till 24 h. A higher dosage (0.006%, 0.008%, and 0.01% per mL) has resulted in loss of cuboidal shape and cell death within 24 h. A gradual reduction in cell viability was observed between various dosages of Soha Liquigel on HCE cells for 24 h [Supplementary Fig. 1]. Similarly, Soha (0.1%) eye drops were administered on HCE cells after dilution at five different concentrations ranging from 0.002% to 0.01% per mL of media. Lower dosage (0.002% and 0.004%) of Soha showed no morphological changes in HCE cells till 24 h. Increasing dosage of Soha eye drops exhibited loss of cuboidal shape and cell death as early as 6 h. Cell viability assay using trypan blue staining shows a gradual reduction in the percentage of live (viable) cells at 24 h upon various dosages of Soha on HCE cells [Supplementary Fig. 2].

Percentage viability and morphological analysis of HCE cells treated with these various concentrations of Soha Liquigel and Soha eye drop showed 0.002% per mL as the non-toxic bioactive dosage respectively for further experiments.

Bio-protection aspects of Soha Liquigel

HCE cells were desiccated for 10 min and post-termination of desiccation, 0.002% Soha Liquigel was administered to the desiccated HCE cells. HCE cells with cubodial morphology serves as untreated control for the experiment [Fig. 1a]. Upon desiccation, HCE cells lose its original morphology and become more spindled shaped, and shrinkage was observed with 70% cell viability showing induction of desiccation stress model [Fig. 1b]. Administration of 0.002% Soha Liquigel on these desiccated HCE cells was able to regain their cuboidal morphology within 1 h and 78% cells were viable till 24 h [Fig. 1c]. The pre-treatment of 0.002% Soha Liquigel was performed on HCE cells followed by desiccation for 10 min. Interestingly, no morphological changes were observed in these HCE cells within 1 h [Fig. 1d]. After the termination of incubation for 24 h, 82% of cells were viable, indicating the bio-protective aspects of Soha Liquigel [Fig. 1e].

Anti-oxidant properties of Soha Liquigel

Oxidative stress was achieved by using hydrogen peroxide (H_2O_2) dissolved in culture media. We have analyzed the gene expression for short-term (24 h) and long-term (5 days) oxidative-stressed HCE cells treated with 0.002% of Soha Liquigel. Oxidative stress response gene and S100A7 expression increased in both short- and long-term oxidative-stressed cells compared with the untreated control, indicating the oxidative stress model was achieved in HCE cells [Figs. 2a and e]. Reduction in S100A7 expression was observed for cells treated with 0.002% Soha Liquigel at basal ($P = 0.008$) and short-term oxidative-stressed cells compared with only oxidative-stressed HCE cells [Fig. 2a]. We found a significant 6-fold ($P = 0.04$) increase in the expression of S100A7 in cells under oxidative stress (long-term) compared with untreated control HCE cells. S100A7 was reduced when treated with 0.002% Soha Liquigel at basal ($P = 0.006$) and oxidative-stressed cells compared with cells under oxidative stress [Fig. 2e].

Anti-oxidant gene FOS expression was significantly increased by 5-folds in short-term oxidative stress cells treated with 0.002% Soha Liquigel compared with basal condition ($P = 0.002$) and to untreated and only oxidative-stressed cells [Fig. 2b]. A significant 5.6-fold increased expression of FOS was observed in long-term oxidative-stressed cells treated with 0.002% Soha Liquigel compared with basal condition ($P = 0.04$) and untreated control ($P = 0.04$) [Fig. 2f]. Superoxide dismutase, SOD2, an anti-oxidant gene, also exhibited a similar trend. A significant 4-fold increased expression of SOD2 in short-term oxidative-stressed cells treated with 0.002% Soha Liquigel was observed when compared with basal cells ($P = 0.006$) and with untreated control (by 5 folds, $P = 0.04$), respectively [Fig. 2c]. SOD2 showed enhanced expression in oxidatively stressed cells treated with 0.002% Soha Liquigel for 5 days compared with cells at basal, untreated, and only stressed cells [Fig. 2g]. An increase in pro-oxidant gene COX2 expression in cells under short-term (6.8 folds, $P = 0.03$) and 3.8 folds in long-term oxidative stress was observed compared with untreated control cells [Fig. 2d and h]. Upon treatment with 0.002% Soha Liquigel, a significant reduction in COX2 expression ($P = 0.0008$) was observed in basal cells compared with H_2O_2 stressed cells [Fig. 2d]. While administration of 0.002% Soha Liquigel reduced COX2 expression at basal and oxidative-stressed cells ($P = 0.002$) compared with only oxidative-stressed cells [Fig. 2h]. The data strongly indicates the anti-oxidant properties of Soha Liquigel as a reduction in pro-oxidative and oxidative response genes along with elevated anti-oxidant genes were observed in HCE cells for both short- and long-term treatments.

Osmo-protection and SH-containing artificial tears

Hyperosmolarity stress was induced using 50 or 100 mM of NaCl to obtain culture media with increased osmolarity of +100mOsmol, or +200mOsmol for short-term (24 h) and long-term (5 days) treatment followed by rescue using a bioactive non-toxic dosage of SH-containing artificial tears in HCE cells. Microphotograph show the cell morphology at 1 h, 12 h, and 24 h at all the conditions for short-term treatment. HCE cells under +200mOsmol condition exhibited stressed morphology with long elongated cells throughout the experimental time frame, indicative of stress condition. While these cells were pre-treated with 0.002% of Soha Liquigel showed minimum changes in the morphology when encountered with +200mOsmol condition. This suggests the osmo-protection nature of Soha Liquigel [Fig. 3a]. These experimental cells were further used for isolation of RNA, and gene expression analysis was performed. We observed a significant 6-fold increase in TonEBP expression ($P = 0.02$) of cells under +200mOsmol condition compared with the untreated HCE cells (basal condition, 24 h). Upon treatment with 0.002% Soha Liquigel, HCE cells under basal condition significantly reduced TonEBP expression ($P = 0.0001$)

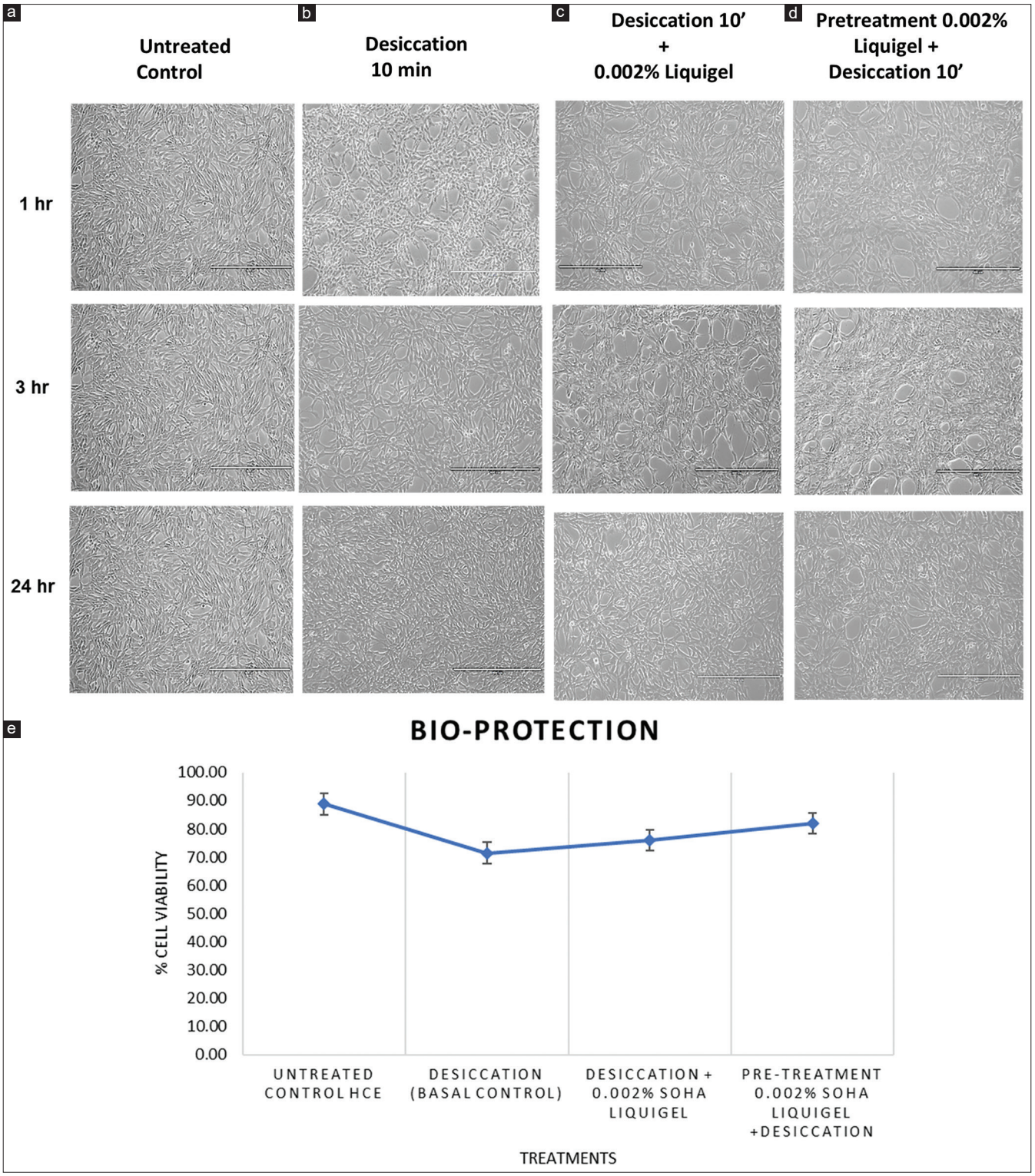

Figure 1: Microphotographs of (a) HCE cells (b) under desiccation stress (c) treated with 0.002% of Soha Liquigel post 10 min of desiccation stress and (d) pre-treatment of 0.002% of Soha Liquigel for 3 h followed by desiccation stress at 1 h, 3 h, and 24 h. Magnification 10x in EVOS microscope. One representative photograph from three independent experiments was shown. (e) Cell viability using trypan blue assay of HCE under desiccation stress and treated with 0.002% per mL of Soha Liquigel for 24 h. Graph represents the data from three independent experiments

compared with cells under +200mOsmol condition. Interestingly, 0.002% Soha Liquigel was able to significantly reduce the TonEBP expression of cells at +100mOsmol ($P = 0.001$) compared with + 200mOsmol without drops and +200mOsmol ($P = 0.009$) compared with the basal condition [Fig. 3b]. Interleukin, IL6 expression increased linearly based on the osmolarity media in HCE cells compared with untreated control. 0.002% Soha Liquigel treated +100mOsmol media cells showed significant reduction

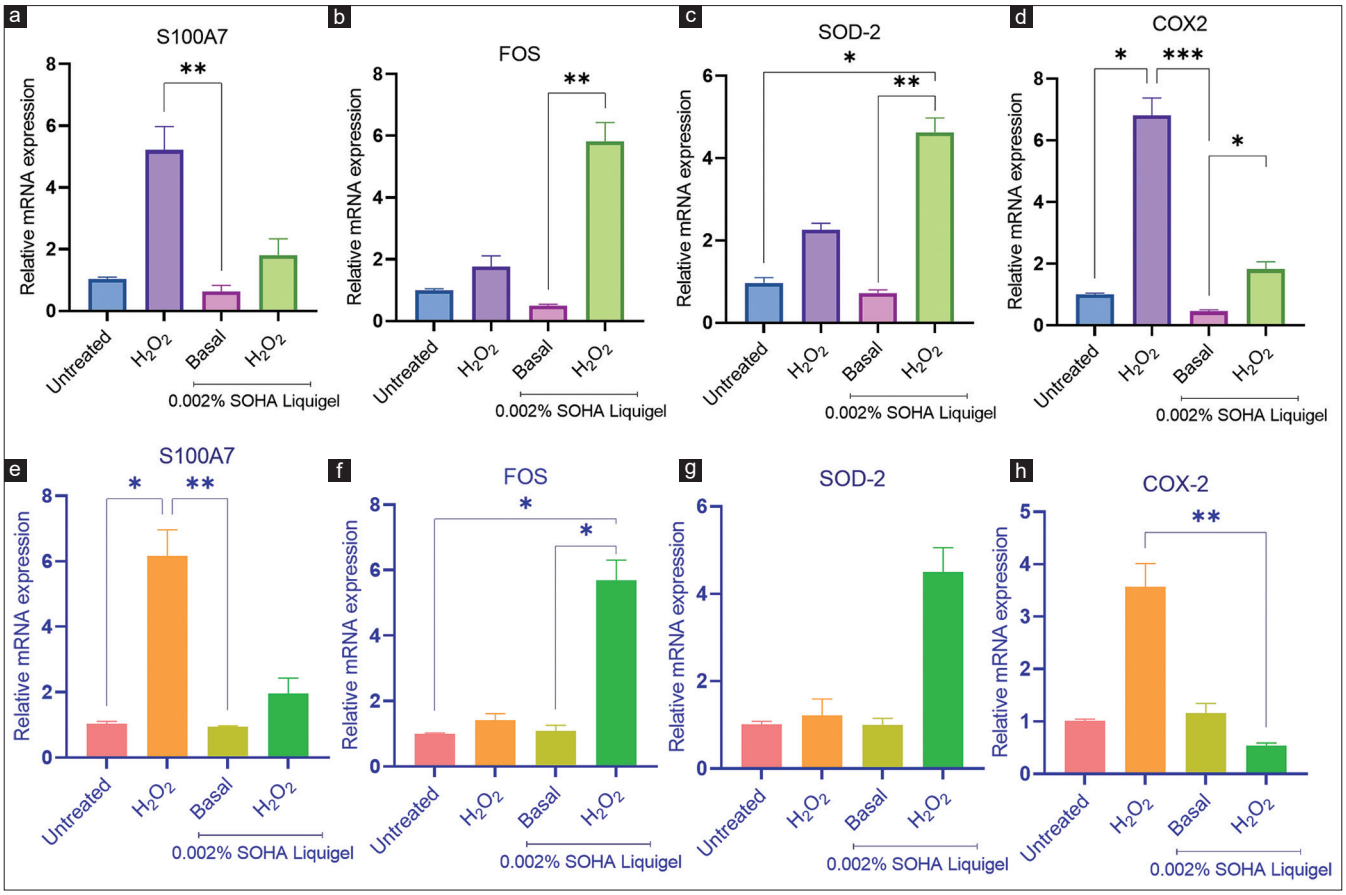

Figure 2: Relative gene expression of S100A7, FOS, SOD-2, and COX2 from HCE cells for (a-d) short-term oxidative stress treatment conditions and (e-h) for long-term/5 days oxidative stress treatment conditions. ***P* value > 0.005; Kruskal-Walli's test was performed to obtain the statistical significance among the conditions. Each graph is a cumulative representation of *n* = 3 repeats for each experiment

compared to only hyperosmolar stressed cells (+100mOsmol, $P = 0.003$ and +200mOsmol, $P = 0.006$). Similarly, +200mOsmol media stressed cells treated with 0.002% Soha Liquigel showed reduced expression of IL6 compared to +100mOsmol, $P = 0.002$; and +200mOsmol, $P = 0.005$ stressed HCE cells [Fig. 3c]. MCP1 expression was reduced when hyperosmolar stressed cells were treated with 0.002% Soha Liquigel. Cells under +200mOsmol treated with 0.002% Soha Liquigel showed a significant ($P = 0.008$) decrease in MCP1 expression when compared to its counter condition of +200mOsmol without any drops [Fig. 3d]. Anti-inflammatory, IL10 gene expression showed an increasing trend upon 0.002% Soha Liquigel administration on hyperosmolar stressed cells. Upon comparison with +100mOsmol cells, significantly increased expression of IL10 was observed for 0.002% Soha Liquigel treated +100mOsmol cells ($P = 0.002$) and 0.002% Soha Liquigel treated +200mOsmol cells ($P = 0.001$). IL10 expression in +200mOsmol cells treated with 0.002% Soha Liquigel increased significantly ($P = 0.04$) compared with +200mOsmol cells [Fig. 3e].

HCE cells were grown for 5 days, and morphological changes were observed for each day under various conditions. On day 1, due to lesser cell numbers the morphology of HCE cells, is more elongated and sparsely seen. On day 2, 0.002% Soha Liquigel treated HCE cells showed no morphological changes in hyperosmolar and basal conditions. On day 3, HCE cells attained their cuboidal morphology, and we observed more elongated, stressed, round cells in +200mOsmol condition, and lesser stressed in +100mOsmol condition. 0.002% Soha Liquigel treated cells in the presence and absence of hyperosmolar condition showed no significant changes in the cellular morphology. On day 4, HCE cells attained approximately 70–75% confluence and 0.002% Soha Liquigel treated cells were able to retain the epithelial morphology. On day 5, HCE cells achieved full confluency, and treatment with 0.002% Soha Liquigel was able to rescue the cellular morphology in the hyperosmolar stressed cells [Fig. 4a]. Molecular expression was found to be dysregulated in long-term treatment to a greater extent. Significant reduction in TonEBP (+100mOsmol $P = 0.01$; +200mOsmol $P = 0.001$) expression of 0.002% Soha Liquigel treated cells under long-term of hyperosmolarity condition compared with hyperosmolarity stressed cells. A similar decrease in expression signature was observed for IL6 expression with significance ($P < 0.01$), and MCP1 ($P < 0.0001$) and IL10 expression increased significantly ($P = 0.01$) in treated 0.002% Soha Liquigel cells compared without treatment under hyperosmolar condition [Fig. 4b-e]. Collectively, administration of 0.002% Soha Liquigel on hyperosmolarity (short- or long-term) induced HCE cells showed a reduction in inflammation and osmolarity markers, indicating the osmo-protection properties without any significant changes in cellular morphology.

Similarly, we created the hyperosmolar condition using two dosages of NaCl in culture media and observed elongated HCE

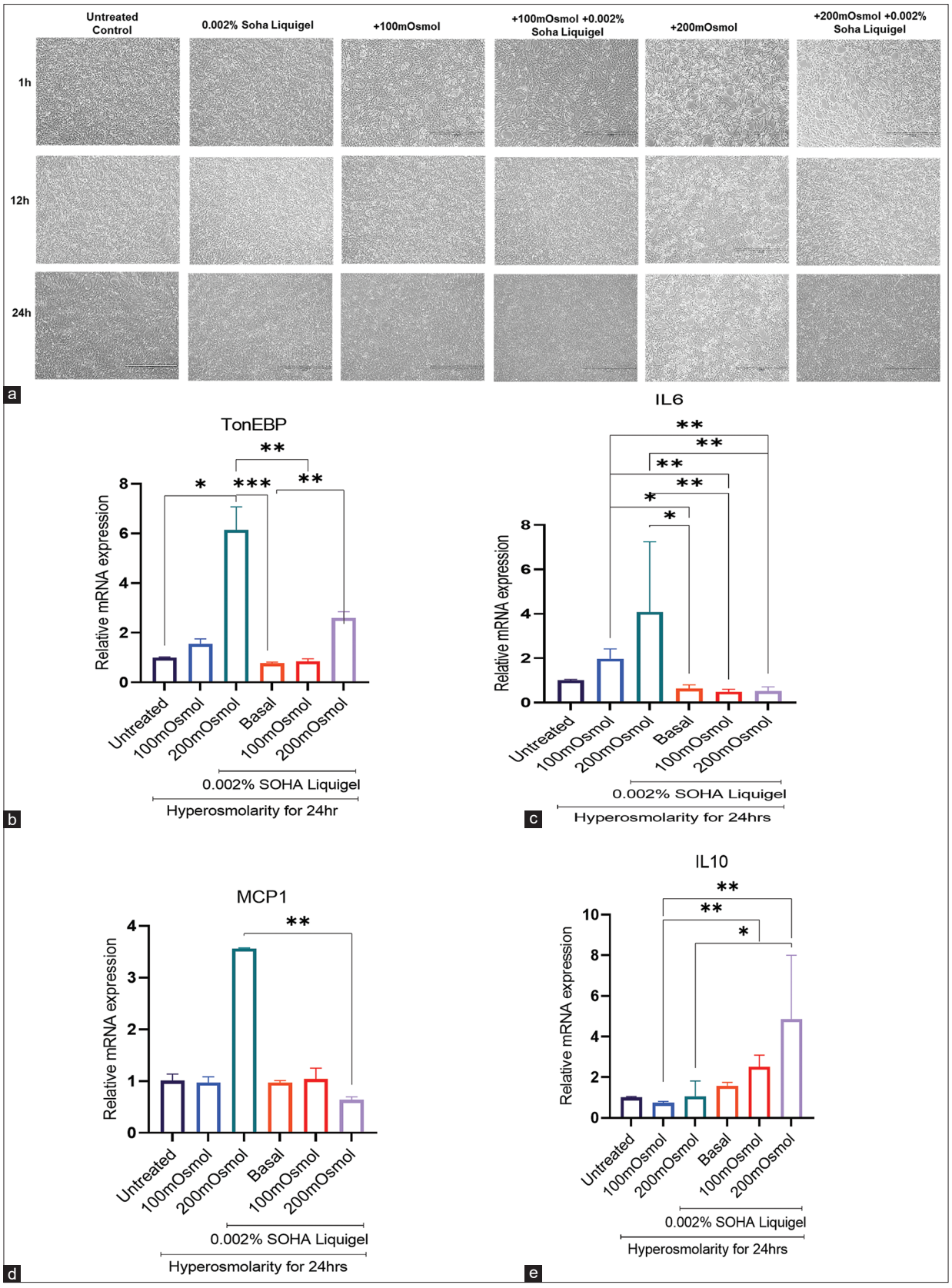

Figure 3: Panel (a) microphotographs of HCE cells untreated control, treated with 0.002% Soha Liquigel eye drops, treated with +100mOsmol, pre-treatment of 0.002% of Soha Liquigel 3 h followed by +100mOsmol stress, treated with +200mOsmol and pre-treatment of 0.002% of Soha Liquigel 3 h followed by +200mOsmol stress at 1 h, 12 h, and 24 h. Magnification 10x in EVOS microscope. One representative photograph from three independent experiments was shown. Relative gene expression of (b) TonEBP, (c) IL6, (d) MCP1, and (e) IL10 from HCE cells for short-term hyperosmolarity treatment conditions. $^{**}P$ value < 0.005; Kruskal-Walli's test was performed to obtain the statistical significance among the conditions. Each graph is a cumulative representation of $n = 3$ repeats for each experiment

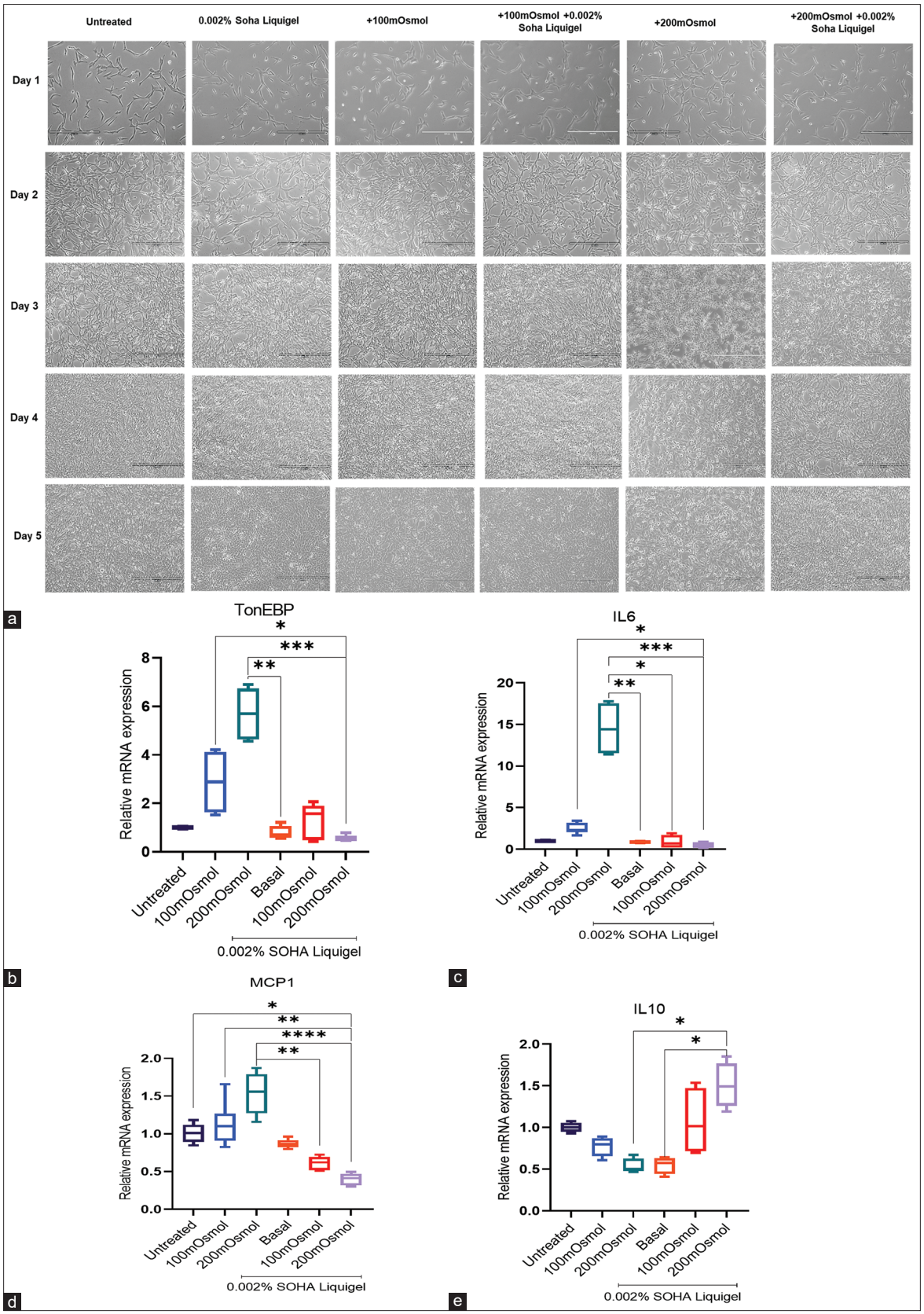

Figure 4: Long-term treatment panel (a) microphotographs of HCE cells untreated control, treated with 0.002% Soha Liquigel eye drops, treated with +100mOsmol, 0.002% of Soha Liquigel +100mOsmol stress, treated with +200mOsmol and 0.002% of Soha Liquigel +200mOsmol stress at day 1, day 2, day 3, day 4, and day 5. Magnification 10x in EVOS microscope. One representative photograph from three independent experiments was shown. Relative gene expression of (b) TonEBP, (c) IL6, (d) MCP1, and (e) IL10 from HCE cells for long-term/5 days hyperosmolarity treatment conditions. **P* value = 0.05, ***P* value = 0.005; ****P* value = 0.001, and *****P* value < 0.001; Kruskal-Walli's test was performed to obtain the statistical significance among the conditions. Each graph is a cumulative representation of *n* = 3 repeats for each experiment

cells showing a response to the stress and noted the rescue upon a non-toxic bioactive dose of 0.002% of Soha eye drops. 0.002% Soha eye drop was able to rescue the cells morphology under hyperosmolar conditions in both short- and long-term treatments [Supplementary Figs. 3a and 4a]. A significant reduction in expression of osmotic stress marker, namely TonEBP (P = 0.008, by 20 folds) and inflammatory marker, IL6 (by 28 folds, P = 0.007) was observed when +100mosmol stressed cells were treated with 0.002% Soha eye drop for short-term (24 h) treatment compared with hyperosmolar stressed cells without drug administration. MCP1 expression was reduced significantly by 37 folds (P > 0.005) in cells treated with 0.002% Soha eye drop compared with a hyperosmolar group [Supplementary Fig. 3b-d]. An increase in the expression of anti-inflammatory marker and IL10 (by 10 folds, P > 0.005) was observed in hyperosmolar stress cells treated with short-term 0.002% Soha eye drop compared to only hyperosmolar stressed cells [Supplementary Fig. 3e]. A significant increase of 5.4 folds TonEBP (P = 0.04) expression in cells under long-term hyperosmolar treatment for 5 days compared with the experimental control was observed, indicative of hyperosmolar stress model. A significant reduction was observed in cells treated with 0.002% Soha eye drop (by 6.8 folds, P = 0.006, long-term) and hyperosmolar stress cells + treated with 0.002% Soha eye drop (by 7.5 folds, P = 0.0004) compared to only hyperosmolar stressed cells [Supplementary Fig. 4b]. A significant reduction of IL6 (P = 0.008, P = 0.0007) expression in hyperosmolar cells treated with 0.002% Soha eye drops was observed when compared with only hyperosmolar stressed cells without any drug treatment. Long-term hyperosmolar stress followed by 0.002% Soha eye drop showed a significant increase in the expression of the anti-inflammatory gene IL10 (P < 0.001) compared with hyperosmolar stressed cells without any drug treatment, indicating that Soha eye drop anti-inflammatory properties have enabled osmo-protection over a period of 5 days as well [Supplementary Fig. 4c-e].

Further, a significant reduction in TonEBP, IL-6, and MCP-1, and a significant increase in IL-10 expression was observed in hyperosmolar cells treated with 0.002% Soha eye drops indicating its anti-inflammatory and osmo-protection properties.

Discussion

Artificial tears are employed in the management of DED globally.[17] In the present study, we have explored the bio-protection, anti-oxidant, and osmo-protection properties of SH-containing eye drop formulation in HCE cells. We established a desiccation stress model and observed more stressed HCE cells with spindle shape morphology compared to the cells without stress conditions. Pre- and post-treatment with 0.002% Soha Liquigel on these desiccated HCE cells regained their cellular cuboidal morphology, indicating bio-protection of corneal epithelial cells. Panigrahi *et al*.[14] have explored the protective role of trehalose in desiccated HCE cells *in vitro*. By regulating key signaling pathways, trehalose prevents apoptosis and promotes cell survival. Its ability to induce autophagy further supports cellular repair and resilience during stress.[18,19] Oxidative stress is one of the major factors in DED etiology.[20] Administration of drugs with anti-oxidant properties aids in managing the disease. We have evaluated the anti-oxidant property of Soha Liquigel on oxidative stress-triggered HCE cells. Upon treatment with 0.002% Soha Liquigel, a significant reduction in expression of S100A7 and COX2, while an increase in FOS and SOD-2 indicates the ability of the drug to maintain the balance between pro-oxidant and anti-oxidant mediators in HCE cells. S100A7 (Psoriasin) expression is reported in corneal and limbal epithelial cells and has a role in ocular surface inflammatory disease.[21,22] Various studies have reported higher expression of S100A7 in viral infections,[23] delayed wound healing,[24] and ROS induction.[25] COX2 elevated levels were observed in the corneas, conjunctiva, lacrimal, and meibomian glands of dry eye-induced mice model.[26] Kessal *et al*.[27] has shown FOS expression in the conjunctiva of DED subjects as one of the transcription factors. Administration of anti-oxidant enzymes such as Bacillus-derived SOD was reported as a complimentary treatment for DED in a murine model.[28]

Increased tear film osmolarity and reduced aqueous tear flow form a vicious cycle in DED pathology.[29] The application of osmo-protectants on corneal cells will aid protection in DED conditions.[30] Trehalose serves as an osmoprotectant, by maintaining cellular homeostasis under hyperosmolar conditions.[31] Our data shows that the hyperosmotic stressed corneal epithelial cells morphology was rescued upon treatment with Soha and Soha Liquigel within 24 h till 5 days *in vitro*. We have found a significant increase in the expression of TonEBP, an osmo-responsive factor,[32] which remarkably showed reduced expression upon Soha and Soha Liquigel administration. Higher expression of TonEBP indicates that epithelial cells are exposed to increased osmolarity and act as a marker of hyperosmolarity.[33] The association of inflammation with DED is well known, and the cellular and molecular genes such as IL6, MCP1, and IL10 are the known markers,[34,35] explored in the present study. Additionally, trehalose attenuates the inflammation by lowering the cytokine levels and mitigates the discomfort associated with dry eye.[36] A substantial reduction in inflammatory genes (IL6, MCP1) levels and an increased anti-inflammatory gene, IL10 were observed in hyperosmotic stressed epithelial cells treated with Soha and Soha Liquigel. Collectively, the data suggests that the ability of both artificial tears, Soha, and Soha Liquigel has the ability of osmo-protection on corneal epithelial cells. A detailed signaling mechanism underlying these targeted markers was not evaluated under the scope of the present study and poses as one of the limitations. In the future, a cross-talk study between inflammatory, oxidative stress, and hyperosmolarity will help uncover the functional aspects of SH-based artificial tears with and without trehalose.

Conclusion

Our findings suggest that Soha Liquigel eye drops with trehalose were able to mitigate desiccation stress along with oxidative stress factors in HCE cells. Both artificial tears, namely Soha Liquigel and Soha eye drops, protect HCE cells from hyperosmotic stress-associated response genes, thereby preventing cellular death and maintaining the ocular surface homeostasis.

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Conflicts of interest: There are no conflicts of interest.

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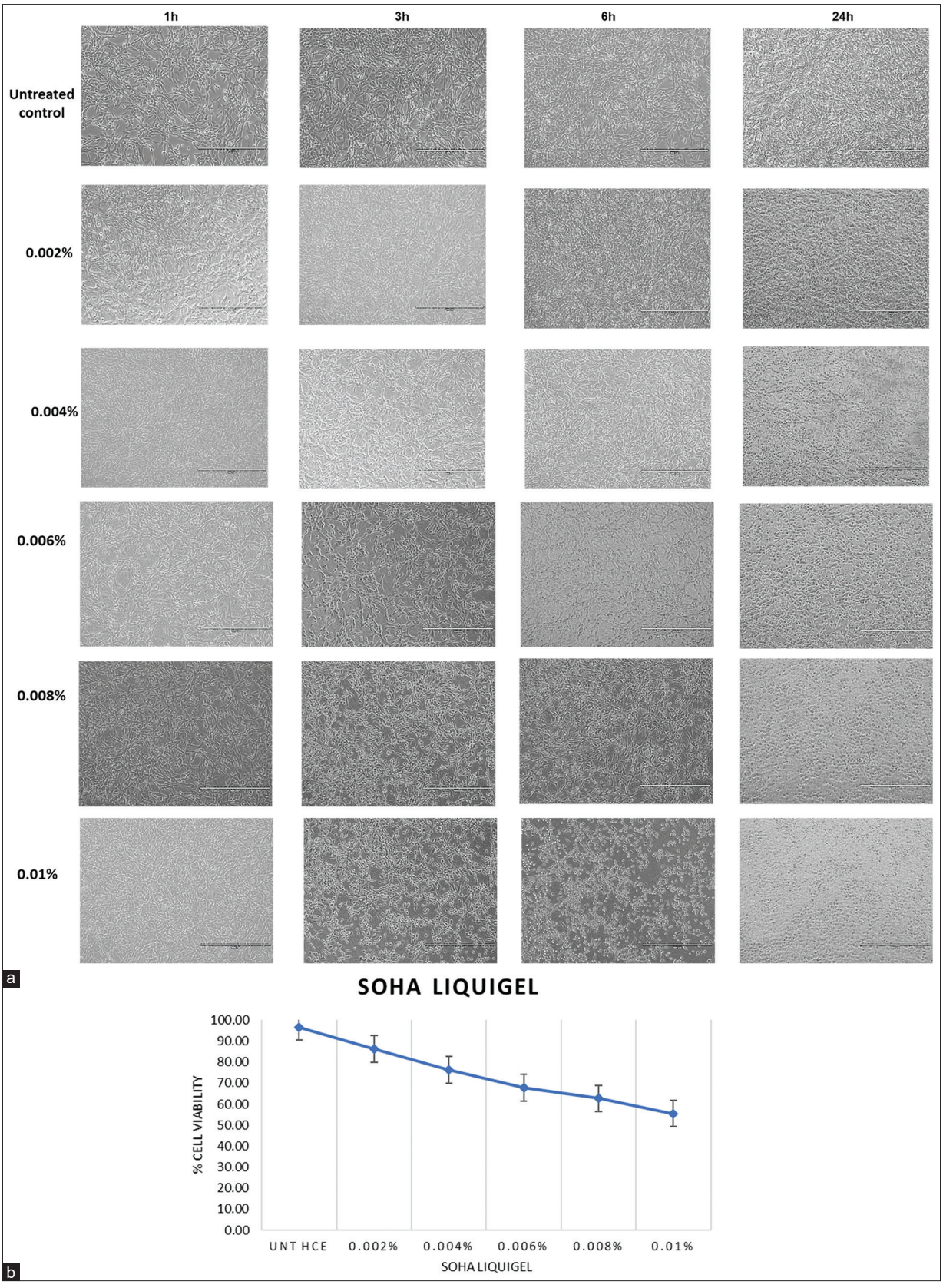
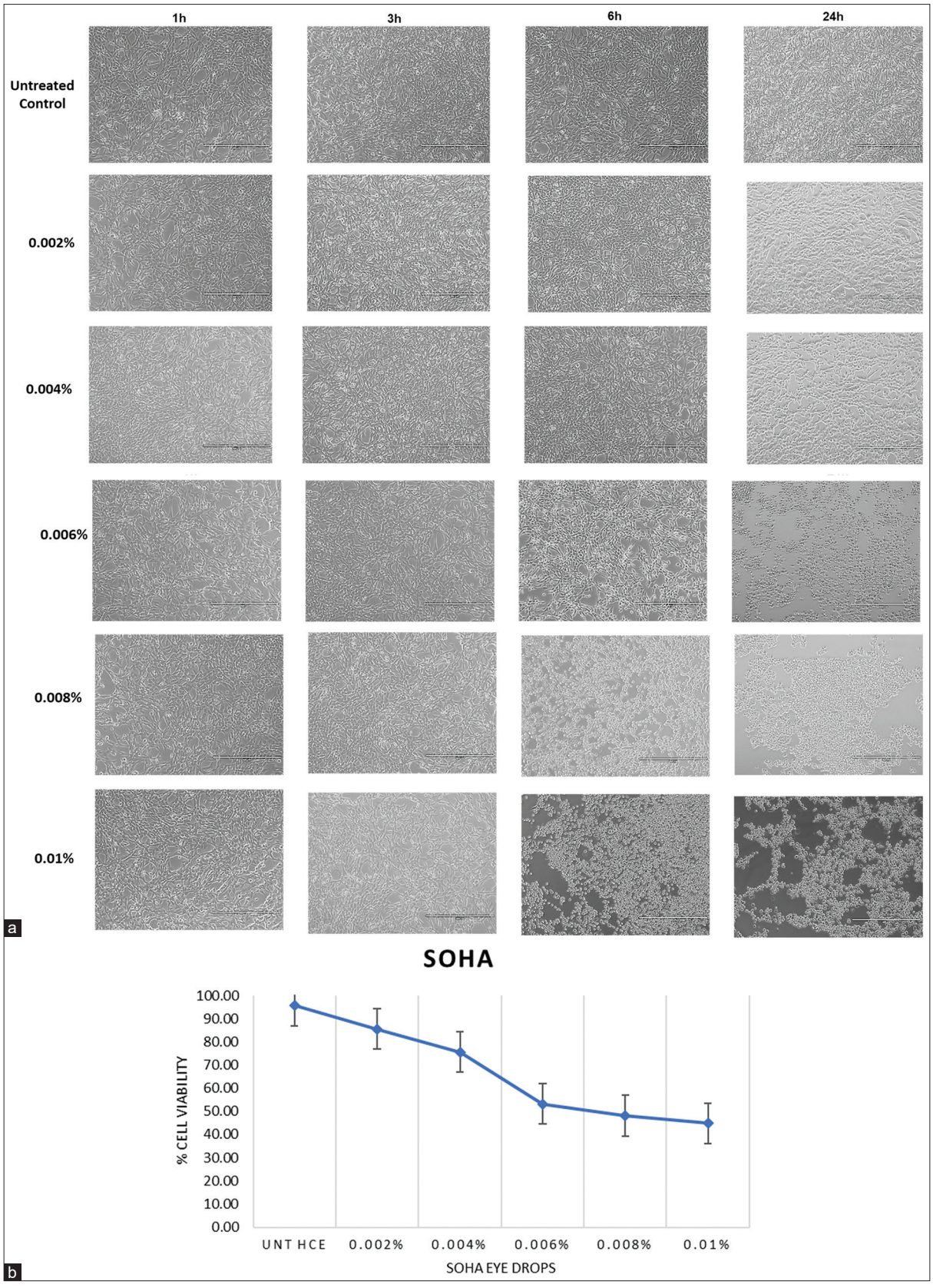
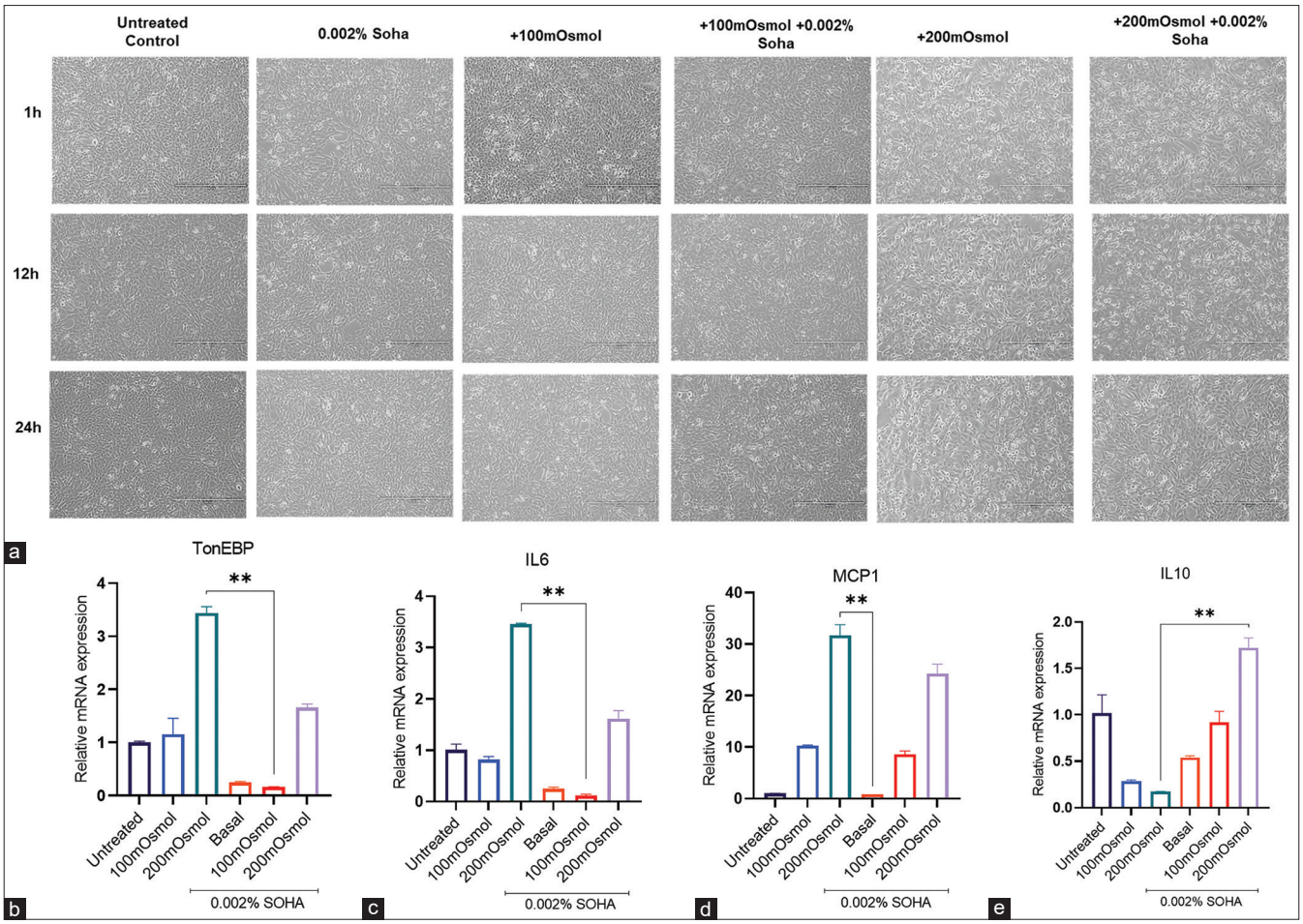


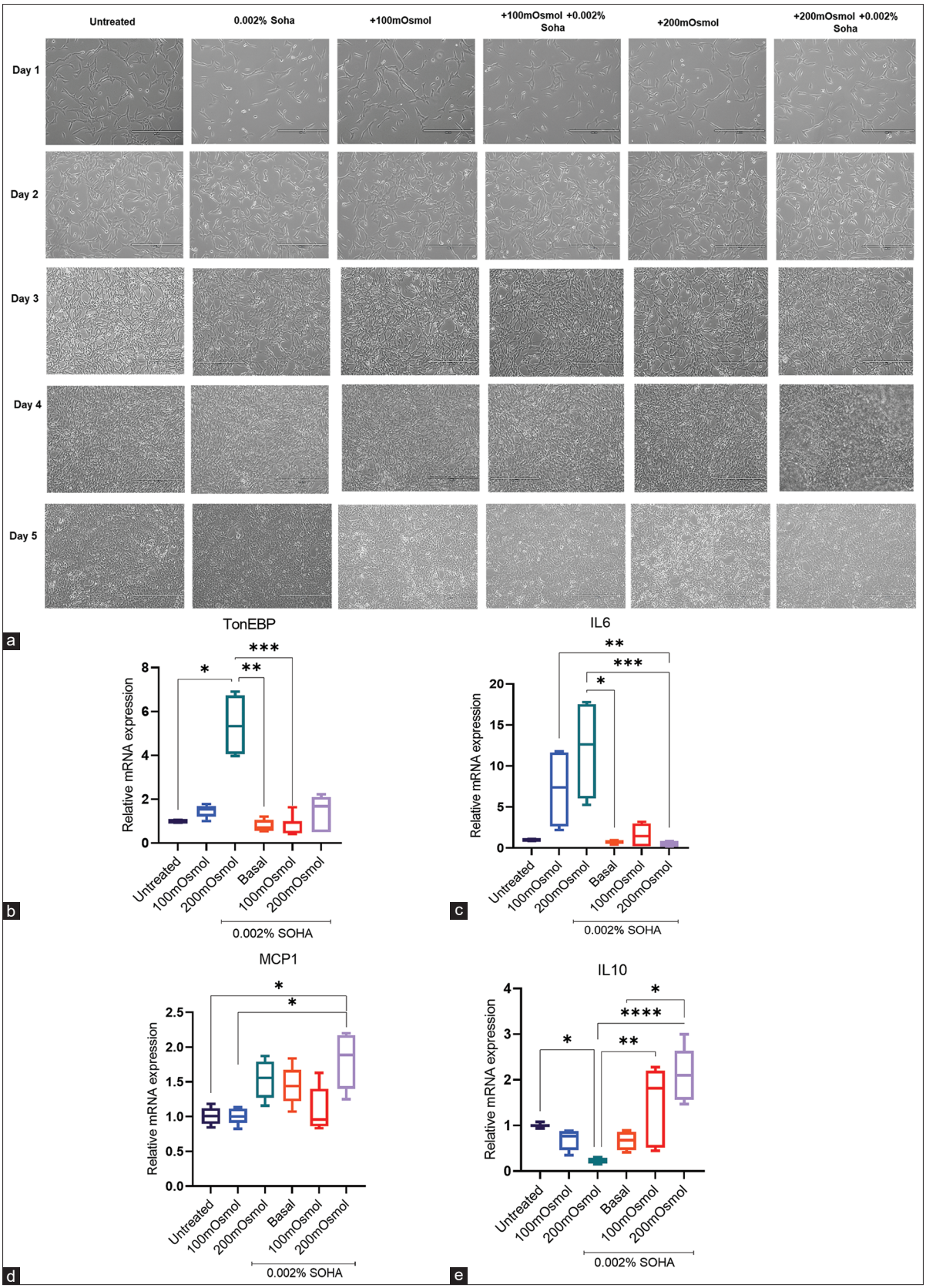
Supplementary Figure 1: (a) Microphotographs of HCE cells treated with Soha Liquigel at various dosages (0.002%, 0.004%, 0.006%, 0.008%, and 0.01%) at 1 h, 3 h, 6 h, and 24 h. Magnification 10x in EVOS microscope. One representative photograph from three independent experiments was shown. (b) Cell viability using trypan blue assay of HCE treated with various dosages of Soha Liquigel eye drops for 24h



Supplementary Figure 2: (a) Microphotographs of HCE cells treated with Soha at various dosages (0.002%, 0.004%, 0.006%, 0.008%, 0.01%) at 1h, 3h, 6h, and 24h. Magnification 10x in EVOS microscope. One representative photograph from three independent experiments was shown. (b) Cell viability using trypan blue assay of HCE treated with various dosages of Soha eye drops for 24h



Supplementary Figure 3: (a) Microphotographs of HCE cells untreated control, treated with 0.002% Soha eye drops, treated with +100mOsmol, pre-treatment of 0.002% of Soha 3h followed by +100mOsmol stress, treated with +200mOsmol and pre-treatment of 0.002% of Soha 3h followed by + 200mOsmol stress at 1 h, 12 h, and 24h. Magnification 10x in EVOS microscope. One representative photograph from three independent experiments was shown. Relative gene expression of (b) TonEBP, (c) IL6, (d) MCP1, and (e) IL10 from HCE cells for short-term hyperosmolarity treatment conditions. $^{**}P$ value < 0.005; Kruskal-Walli's test was performed to obtain the statistical significance among the conditions. Each graph is a cumulative representation of $n = 3$ repeats for each experiment



Supplementary Figure 4: (a) Microphotographs of HCE cells untreated control, treated with 0.002% Soha eye drops, treated with +100mOsmol, 0.002% of Soha +100mOsmol stress, treated with +200mOsmol and 0.002% of Soha +200mOsmol stress at day 1, day 2, day 3, day 4, and day 5. Magnification 10x in EVOS microscope. One representative photograph from three independent experiments was shown. Relative gene expression of (b) TonEBP, (c) IL6, (d) MCP1, and (e) IL10 from HCE cells for long-term/5 days hyperosmolarity treatment conditions. **P* value = 0.05, ***P* value = 0.005; ****P* value = 0.001, and *****P* value < 0.001; Kruskal-Walli's test was performed to obtain the statistical significance among the conditions. Each graph is a cumulative representation of *n* = 3 repeats for each experiment