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Combined hyperosmolarity and inflammatory conditions in stressed human corneal epithelial cells and macrophages to evaluate osmoprotective agents as potential DED treatments

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ABSTRACT

Purpose: To develop an easy-to-perform combined model in human corneal epithelial cells (HCECs) and Balb/c mice macrophages J774.A1 (MP) for preliminary screening of potential ophthalmic therapeutic substances.
Methods: HCECs were exposed to different osmolarities (350–500 mOsm/L) and MTT assay was employed for cell survival and flow cytometry to assess apoptosis-necrosis and relative cell size (RCS) distribution. Effectiveness of Betaine, L-Carnitine, Taurine at different concentrations (ranging from 20 mM to 200 mM) was studied. Also, mucoadhesive polymers such as Hyaluronic acid (HA) and Hydroxypropylmethylcellulose (HPMC) (0.4 and 0.8%) were evaluated. Cells were pre-incubated with the compounds (8h) and then exposed to hyperosmotic stress (470 mOsm/L) for 16h. Moreover, anti-inflammatory activity was performed in LPS-stimulated MP.
Results: Exposure to hyperosmotic solutions between 450 and 500 mOsm/L promoted the highest cell death after 16h exposures ($p < 0.0001$) with a drop in viability to 34.96% ± 11.77 for 470 mOsm/L. Pre-incubation with Betaine at 150 mM and 200 mM provided the highest cell survival against hyperosmolarity (66.01% ± 3.65 and 65.90% ± 0.78 respectively) while HA 0.4% was the most effective polymer in preventing cell death (42.2% ± 3.60). Flow cytometry showed that Betaine and Taurine at concentrations between 150-200 mM and 20–80 mM respectively presented the highest anti-apoptotic activity. Also, HA and HPMC polymers reduced apoptotic-induced cell death. All osmoprotectants modified RCS, and polymers increased their value over 100%. L-Carnitine 50 mM, Taurine 40 mM and HA 0.4% presented the highest TNF-α inhibition activity (60%) albeit all of them showed anti-inflammatory inhibition percentages higher than 20%
Conclusions: HCECs hyperosmolar model combined with inflammatory conditions in macrophages allows the screening of osmoprotectants by simulating chronic hyperosmolarity (16h) and inflammation (24h).

1. Introduction

Dry Eye Disease (DED) is a multifactorial pathology affecting the ocular surface and tears that in all cases leads to tear film instability (Aggarwal and Galor, 2018). TFOS DEWS Epidemiology report analyzed series of large-scale international epidemiological studies and concluded that the prevalence of DED was from 5 to 30% in individuals that were over their 50s. In addition, it has been established that DED is boosted with age and females are more affected (Stapleton et al., 2017). Severe clinical signs such as tear film instability and hyperosmolar conditions are given among others, constituting a potential damage to the ocular surface. In fact, one of the most important and well-established causes of

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DED relapses in tear evaporation, that subsequently leads to a raise in osmolarity of the tear film (Baudouin et al., 2013) (Messmer, 2015). Sjögren syndrome (SS), Sjögrens' syndrome-associated keratoconjunctivitis sicca (SS-KCS) or LASIK-induced neurotrophic epitheliopathy (LNE) constitute also some examples of diseases linked to hyperosmolar stress (Pflugfelder et al., 2018). Hyperosmolarity of the surface epithelial cells, results in an imbalance of water and electrolytes between intracellular and extracellular compartments reducing cell volume. A dramatic shrinkage of cells may lead to cell survival alteration, cell membrane and cytoskeletal integrity changes as well as denaturation of cytosolic proteins (Khandekar et al., 2013)' (Jeng, 2013).

An increase in hyperosmolarity of tears can lead to ocular surface impairment by producing a wide amount of proinflammatory markers such as tumor necrosis factor and some matrix metalloproteinases such as MMP-9 or MMP-7 (Stevenson et al., 2012). This environment also produces a decrease in conjunctival globet cells. As the osmolarity of the ocular surface increases the inflammation events persist developing in a vicious cycle (Blalock et al., 2008).

An increase in osmolarity (450, 500 and 550 mOsm/L) of tears has been associated to a subacute inflammation of the ocular surface by triggering some essential cytokines such as tumor necrosis factor alpha (TNF-α) (Luo et al., 2007). TNF-α has been widely studied as the classical proinflammatory factor produced by some important signaling pathways such as MAPK cascades in response to hyperosmolar stress (Luo et al., 2004).

According to TFOS DEWS management and therapy report, tear insufficiency, lid abnormalities and inflammation are some key treatments for DED management (Jones et al., 2017). Treatment of tear insufficiency and supplementation aims to replace tears by artificial tear substitutes, tear production stimulation and tear conservation approaches (Albietz et al., 2002). Hydroxypropylmethylcellulose (HPMC) and Hyaluronic acid (HA) are well known examples of viscosity-enhancing agents able to increase tear film density, thickness, and preserve the surface from damage against desiccation stress, alleviating symptoms (Jones et al., 2017)' (Andrés-Guerrero et al., 2011). For decades, osmoprotection has been demonstrated to play a decisive role in protecting the ocular surface against damage produced as consequence of tear evaporation (Hazarbassanov et al., 2018). According to this, some osmoprotective substances have been studied in the search of useful therapies to manage DED (Corrales et al., 2008; Hua et al., 2015a). Osmoprotectants are osmotically active biocompatible compounds able to modify cellular water uptake. They are usually small, neutral and hydrophilic substances that do not interfere with cellular function (Mateo Orobia et al., 2018). Each osmoprotectant works in a different way with a wide variety of kinetics and cell internalization (Kempf and Bremer, 1998). Cell uptake of osmoprotective substances can be given when a hypertonic environment occurs or if there is a potential cell damage caused by hyperosmotic stress (Sharma et al., 2014). Besides some of them can be internalized in the cells via specific mediated transporters even before hyperosmotic environment (Slama et al., 2015). The amount of substance and period of time that cells can retain them play a critical and important role in their effectiveness (Baudouin et al., 2013). Some clear examples are Betaine (tri methylglycine) and L-Carnitine. They have been well studied for their role as cell membrane stabilizers. Both agents act by avoiding protein misfolding and conformational changes (Corrales et al., 2008). In the ocular surface, BGT-1 (betaine-GABA transporter) is expressed in human corneal epithelial cells and allows betaine to be internalized and accumulated in order to protect cells against osmotic stress (Garrett et al., 2013; Lehre et al., 2011). Moreover, Betaine has been studied to stimulate the intake of osmoprotectants and inhibit p38 pathway resulting in apoptosis inhibition (Garrett et al., 2013). L-Carnitine has also been hypothesized to be crucial during oxidative process by blocking reactive oxygen species (ROS) production in the ocular surface (Hua et al., 2015a) and inhibiting some inflammation pathways such as TRPV1 (transient receptor potential cation channel) (Hua et al., 2015b)' (Hua et al., 2015a). Although it is still unknown how some osmoprotectants inhibit TRPV1 pathway, the efficacy of this protective agents in avoiding its activation has been established (Khajavia et al., 2014). Besides, L-Carnitine has been thought to get into cells through some non-specific transporters, regulating apoptosis, inflammation and osmolytes inside corneal and conjunctival cells (Khandekar et al., 2013) (Fig. 1). In addition, taurine has been studied as an antioxidant and osmoprotective substance capable of preventing damage in the ocular surface, entering cells through different surface taurine transporters (Bucolo et al., 2017; Shioda et al., 2002). Taurine has been hypothesized to upregulate the storage of osmolytes inside the cell therefore regulating volume loss and cell survival (Schaffer et al., 2000). Among all this substances,

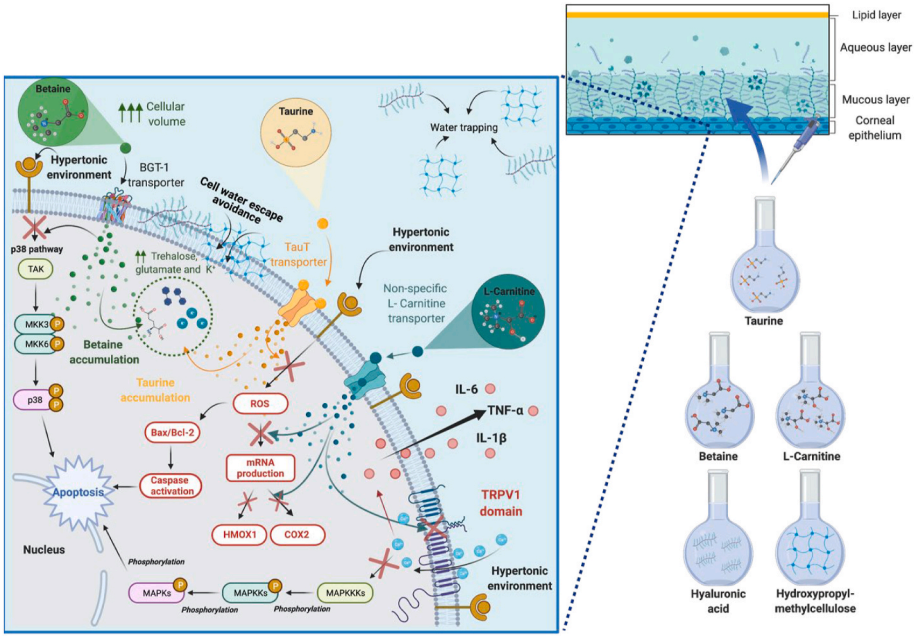

Fig. 1. Proposed mechanism of action of the different potential osmoprotective substances studied according to the bibliography (Corrales et al., 2008; Garrett et al., 2013; Hua et al, 2015a, 2015b, 2015a; Lehre et al., 2011).

L-carnitine is available in the market and HPMC and HA are well known in different commercial formulations but at low concentrations (Jones et al., 2017; Monaco et al., 2011).

Until now, few studies have evaluated osmoprotection in the ocular surface focusing only on specific markers or pathways that play critical roles in DED (Bucolo et al., 2017; Khandekar et al., 2013; Mateo Orobia et al., 2018). The present work aims to recreate some of the DED chronic hyperosmolar conditions in a simple and reproducible *in-vitro* cellular model that allows the study of cell survival and apoptosis. As inflammation is also present in DED, macrophages were used to evaluate the potential anti-inflammatory activity of the osmoprotectants and polymers. The present works aims to develop a useful tool set to screen osmoprotective substances that can be potentially used in DED treatment and also as ocular surface protectants in chronic treatments such as the case of hypotensive topical formulations in glaucoma. To this, an immortalized human corneal epithelial cell line (hTERT-HCECs) was used to carry out osmoprotection studies. hTERT-HCECs expresses typical epithelial cell markers, such as ZO1 (zonula occludens) and KRT3 (keratin 3), present in healthy corneal tissues. HTERT-HCECs was developed without tumorigenic transformation (Kasetti et al., 2016). Apoptosis was also determined by flow cytometry. For the anti-inflammatory studies, Balb/c mice macrophages J774.A1 were stimulated to express TNF-α (De Stefano et al., 2010). We demonstrated that the combination of these cellular models entails a robust mechanism to identify and screen potential therapeutic substances or formulations for ocular surface diseases that undergo with hyperosmolar stress and inflammation.

2. Material and methods

2.1. Reagents

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Trypan-Blue, Dulbecco's phosphate-buffered saline (DPBS), Dimethyl sulfoxide (DMSO), sodium chloride (NaCl) solution 5M, Betaine ≥98%, Taurine ≥98% were purchased from Sigma Aldrich (Madrid, Spain). L-Carnitine was provided by Fagron Ibérica SAU (Barcelona, Spain). Trypsin-EDTA 0.05%, defined trypsin inhibitor, DRAQ5 (647⁄681) and YO-PRO™-1 Iodide (491/509) were supplied by Life Technologies (Madrid, Spain). 7-AAD Viability Staining Solution and cell staining buffer from Bio Legend were obtained from Palex Medical (Madrid, Spain). Hydroxypropyl methylcellulose (HPMC) was supplied by Dismadel, S.L (Madrid, Spain) and MMW (400.000–800.000 Da) hyaluronic acid (HA) was provided by Abarán Materias Primas (Madrid, Spain).

2.2. Cell cultures

Immortalized Human corneal cells (hTERT-HCECs; Evercyte GmbH, Vienna, Austria) were cultured at 37 °C under 5% CO_2 in a humid saturated atmosphere. The cells were seeded in EpiLife® media (Life Technologies, Madrid, Spain) supplemented with EDGS® 1X (Life Technologies, Madrid, Spain) and penicillin-streptomycin 1% (Sigma-Aldrich, Madrid, Spain). The media was changed every 48 h and cells were subcultured at 80–85% confluence, by rinsing with DPBS (Dulbecco's phosphate-buffered saline, Sigma-Aldrich, Madrid, Spain) and detaching with trypsin-EDTA 0.05% (Sigma-Aldrich, Madrid, Spain). Cell passages for *in vitro* studies were in the range of 5–10 (7.5–15 population doubling level).

Murine macrophages J774A.1-TIB67™ (American Type Culture Collection, Manassas, Virginia, USA) were cultured at 37 °C under 5% CO2 as mentioned above. The cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium) (Sigma-Aldrich, Madrid, Spain) supplemented with penicillin-streptomycin 1% (Sigma-Aldrich, Madrid, Spain) and FBS 10% (Fetal Bovine Serum) (Cultek, Madrid, Spain). The media was changed every 48 h and cells were subcultured at 70% confluence. Passages between 4 and 7 (6–10.5 population doubling level) were used for the anti-inflammation studies.

2.3. Preparation of osmoprotectants and polymers

Aqueous isotonic solutions of the osmoprotective substances Betaine, L-Carnitine and Taurine at different concentrations were prepared: Betaine (50 mM, 100 mM, 150 mM and 200 mM); L-Carnitine (50 mM, 100 mM and 150 mM) and Taurine (20 mM, 40 mM and 80 mM). Isotonicity of solutions was adjusted with NaCl. HA and HPMC at two different concentrations (0.4 and 0.8%) were dissolved in NaCl 0.9%. Final sterilization was performed by filtration using 0.22 μm filters.

2.4. In vitro toxicity assessment

The cytotoxicity of osmoprotectant substances and polymers at the selected concentrations was evaluated via MTT assay described previously (Vicario-de-la-Torre et al., 2018). To this, HTERT-HCECs were cultured in 96 well plates (20000 cells/well) and incubated overnight. Following incubation, the cells were exposed to the afore-mentioned substances (Betaine, L-Carnitine and Taurine, HA and HPMC) for 8 h. After that, the supernatant was removed. Then, cells were exposed to an MTT (0.33 mg/mL) mixture in cell culture media and incubated for 4 h. Afterwards, the supernatants were discarded and 100 μL of DMSO were added to each. Finally, the plates were shaken gently for 5 min well for the complete solubilization of formazan crystals in the dark and immediately measured at 550 nm in the spectrophotometer. Benzalkonium chloride at 0.005% was employed as positive control for cell toxicity, since is commonly used as a preservative in artificial tears causing cell dead.

2.5. Hyperosmolar stress simulation in human corneal cells

2.5.1. Cell viability determination under hyperosmolarity

Sodium chloride was employed to reproduce a hyperosmolar environment similar to the one found in DED. Firstly, HCECs cells were seeded in 96 well plates (20000 cells/well) and incubated overnight. Then, the supernatants were removed, and the wells were filled with cell culture media (100 μL) and NaCl 0.9% (300 mOsm/L) (100 μL) for 8 h. After that, the supernatants were discarded and hyperosmolar environment conditions were stablished in each well by using NaCl (350, 400, 450, 460, 470, 480, 490 and 500 mOsm/L). This step was carried out to study the response of HCECs under different hyperosmolar stress to select the most appropriate one. Each hyperosmolar solution was made by mixing NaCl and cell culture media (1:1) (eq. (1)). Finally, the supernatants were removed, and cell viability was measured by MTT as previously described in section 2.4.

The following equation was employed to prepare the different hyperosmolar solutions

$$Stock\ Hyperosmolarity\ (mOsm/L) = \frac{DO\ (mOsm/L)x\ WV1\ (\mu L)\ -\ OM\ (300\ mOsm/L)x\ WV2\ (\mu L)}{HV\ (\mu L)} \quad (1)$$

Stock hyperosmolarity (expressed in mOsm/L) refers to the osmolality concentration needed to be added to the well in order to obtain the final desired osmolality. The desired osmolality of the mixture (DO) is expressed in mOsm/L, WV1 is the total volume of DO in the well (200 μL), OM states for the osmolality of the media (300 mOsm/L), WV2 is the volume of OM added to the well (100 μL) and HV expresses the volume of the stock hyperosmolar solution needed (100 μL). Once the model was optimized, *in vitro* testing of the substances and polymers was evaluated. The hypertonic solution 470 mOsm/L was selected as it provides the optimal cell survival to evaluate osmoprotective protection. Cells were incubated with betaine (50 mM, 100 mM, 150 mM and 200 mM), L-carnitine (50 mM, 100 mM and 150 mM), taurine (20 mM, 40 mM and 80 mM), HA (0.4% and 0.8%) and HPMC (0.4% and 0.8%) for 8 h. After that, all the supernatants containing the substances or polymers were discarded, and hyperosmotic stress conditions (470 mOsm/L) were created in all sample wells according to eq. (1), including the positive control. Then, the cells were incubated for 16 h. Finally, cell viability was determined by the MTT method, as described in section 2.4. Each experiment was made by triplicate (n = 3; 7 wells per compound replicated in 3 separate plates).

2.5.2. Apoptosis and necrosis by flow cytometry

Different dye-mediated parameters such as necrosis and apoptosis were evaluated in hTERT-HCECs in response to different hyperosmolar concentrations (350, 400, 450, 460, 470, 480, 490 and 500 mOsm/L, as described in section 1.5.1). Briefly, 1×10^6 cells/well were seeded in petri dishes and incubated for 24 h until 80% of confluence was reached. Then, the supernatants were removed and 5 mL of cell culture media and NaCl 0.9% mixture was added as described in 2.5.1 section. Cells were incubated for 8 h. After that, the supernatants were discarded, and different hyperosmolar environments were simulated as previously described for 16 h in order to optimize the model. After the incubation time, all the supernatants were withdrawn, and each dish was rinsed with DPBS twice. HTERT-HCECs were detached after 3 min treatment with trypsin-EDTA 0.05%. Cells were centrifuged at 850×*g* for 10 min and re-suspended in 600 μL of cell staining buffer. Finally, 5 μL of 7-AAD viability staining solution and 0.5 μL of YO-PRO™-1 Iodide were added to study the apoptosis-necrosis mechanisms according to the manufacturer instructions. The cell suspension was incubated for 20 min in the dark and taken to the flow cytometer FC 500 (2-laser, 5-color analysis) with FC 500 CXP software (Beckman Coulter, Madrid, Spain) for data acquisition. The different instruments were calibrated weekly by the flow cytometry core personnel. Before use, the system and fluidics were allowed to warm up and stabilize for at least 20 min. All samples were measured at a medium flow rate of <10000 events/s during a time frame of 300s. Briefly, 7-AAD and YO-PRO™-1 Iodide signals were evaluated using the blue/red laser at 535/617 excitation/emission and the blue/green laser at 491/509 excitation/emission respectively.

As cited previously, 470 mOsm/L was selected to perform viability, apoptosis and necrosis studies. Polymers and therapeutic substances at different concentrations (5 mL final volume) were incubated for 8 h as shown previously (section 2.5.1). Then, all the supernatants were discarded. After that, hyperosmolar environment (470 mOsm/L) was achieved in all sample dishes. Samples were prepared as afore mentioned and taken to the flow cytometry core facility. Cells exposed to 470 mOsm/L without previous treatment were established as the positive control. Each experiment was made by triplicate (n = 3).

For apoptosis detection under the fluorescence microscope, cells were seeded in 24 well plates following the same procedure with hypertonic stress when 80% of confluence was reached as previously shown. Cells were stained with YO-PRO1 for 10 min under the dark and examined under the fluorescence microscope with the blue laser (535/617).

2.5.3. Cell size analysis in response to osmotic stress

Cell size of hTERT-HCECs after 16 h exposure to the different hyperosmolarity environments (350, 400, 450, 460, 470, 480, 490 and 500 mOsm/L) were analyzed. A comparison was performed between each concentration and the basal values under isotonic conditions (300 mOsm/L). All sample tubes were analyzed as previously measured (section 2.5.2) at a medium flow rate of <10000 events/s during a time frame of 300s. After selecting the optimal hyperosmolar condition for the model (470 mOsm/L) the fluctuation of cell size was analyzed.

Cell size regulation of the different osmoprotective substances (osmoprotectants and polymers) was tested by their pre-incubation with the cells (8h) prior to the addition of hypertonic solution. Then, the experiments were carried out exactly as described above in section 2.5.2.

2.6. Determination of TNF-α in an LPS-induced inflammation model

A macrophage J774A.1-TIB67™ cell line was used as inflammation model. Briefly, 20000 cells were seeded in 24 well plates and incubated overnight at 37 °C 5% CO_2. After that, all the supernatants were discarded and LPS (12.5 ng/mL) was added in each well. Afterwards polymers or osmoprotective compounds were also included. Negative and positive controls were made by adding isotonic NaCl 0.9% or LPS (12.5 ng/mL) in cell media. After incubation at 37 °C 5% CO_2 (24 h), all the supernatants were collected, centrifuged at 850×*g* 10 min and analyzed. TNF-α was determined through the Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Life Technologies, Madrid, Spain). The plates were measured at 450 nm and 570 nm. Optical densities at 450 nm were subtracted to 570 nm to eliminate background signal according to the manufacturer's instructions. Since TNF-α levels (pg/mL) could vary between assays in samples and controls, the results were normalized in terms of inhibitory effect (described in eq. (2)).

$$Inhibition\ (\%) = \frac{Sample\ TNF-alpha - Negative\ control\ TNF-alpha}{Positive\ control\ TNF-alpha\ (LPS\ 12,5\ ng/mL) - Negative\ control\ TNF-alpha} \quad (2)$$

As above-described (eq. (2)), sample TNF-α levels (pg/mL) were subtracted to those of the negative control (basal levels) since basal levels do not represent an inflammation response. Furthermore, the normalized sample TNF-α values were divided by the normalized positive control TNF-α levels (calculated the same as normalized sample TNF-α levels) and expressed in TNF- α inhibition percentage.

2.7. Statistical analyses

Each experiment was carried out by triplicate (n = 3) and the data are shown as the mean ± SD. Ordinary one-way ANOVA combined with Dunnett's multiple comparisons test was used to determine if specific groups achieved some levels of significance (*; $p \leq 0.05$, **; $p \leq 0.01$, ***; $p \leq 0.001$ or ****; $p \leq 0.0001$) by using GraphPad software Inc. Prism Version 9, US. Beckman Coulter Kaluza Analysis Software, US, was employed to visualize and analyze flow cytometry data.

3. Results

3.1. In vitro toxicity assessment

HTERT-HCECs were exposed for 8 h with solutions of Betaine (50 mM, 100 mM, 150 mM and 200 mM), L-Carnitine (50 mM, 100 mM and 150 mM) and Taurine (20 mM, 40 mM and 80 mM) as potential osmoprotective substances and mucoadhesive polymers such as HA and HPMC at concentrations of 0.4 and 0.8% (Fig. 2).

According to previous studies 80% of cell survival was selected as the minimum tolerance for ocular formulations (Ayaki et al., 2010)' (Gómez-Ballesteros et al., 2019). All Betaine and L-Carnitine solutions presented viability values higher than 89.4% and 93.36% respectively. Taurine resulted in viability values close to 80% with excellent tolerance at 20 mM. HA and HPMC viability values were higher than 80% with the highest ones observed for concentrations of 0.4% in both polymers (94.46 ± 4.90% and 95.31 ± 10.73% for HA and HPMC respectively). Regarding multiple comparison among betaine concentrations, no statistically significant differences ($p > 0.05$) were observed. However, L-Carnitine 100 mM resulted significantly higher in comparison with 150 mM ($p = 0.0022$). Taurine demonstrated high statistically significant results when compared 20 mM against 40 mM and 80 mM ($p < 0.0001$). Regarding polymers, HA 0.4% showed significantly higher viability values in comparison with HA 0.8% ($p = 0.0002$). HA 0.4% also showed significant differences when compared to HPMC 0.8% ($p = 0.0342$). Besides, HA 0.8% was highly significant in comparison with HPMC 0.4% ($p < 0.0001$). Finally, HPMC 0.4% showed significant results when compared to HPMC 0.8% ($p = 0.0108$).

3.2. Hyperosmolar stress simulation in human corneal cells

3.2.1. Cell viability by MTT

Viability of cells exposed under different hyperosmolar NaCl concentrations is shown in Fig. 3. HTERT-HCECs cells were treated with different hyperosmolar concentrations of NaCl for 16 h to simulate chronic hypertonic stress present in DED. All groups were compared with the negative control (isotonic conditions). Viability values showed a slight decrease in cell viability every 50 mOsm/L addition without significant results ($p = 0.9932$ for 350 mOsm/L and $p = 0.5020$ for 400 mOsm/L) compared with isotonic conditions (300 mOsm/L). Cell survival did not significantly changed until 450 mOsm/L (66.66% ± 13.54), which was found to be the threshold concentration at which cell survival linearly decreases with increments of 10 mOsm/L in osmolarity up to 500 mOsm/L (12.77% ± 5.57). All values between 450 and 500 mOsm/L were significantly lower than 300 mOsm/L baseline ($p < 0.0001$).

Cells exposed to 470 mOsm/L exhibited a mean viability of 34.96% ± 11.77 and was chosen as the most appropriate control concentration to screen osmoprotection. This hyperosmolar concentration showed a significant decrease in cell viability and avoided extreme values that could lead to confusion when assessing protection by substances with osmoprotective properties.

Fig. 4 shows the osmoprotection observed for Betaine, L-Carnitine, Taurine, HA and HPMC. Betaine significantly increased viability at all the studied concentrations ($p = 0.0003$ for 50 mM and $p < 0.0001$ for 100, 150 and 200 mM) in comparison with the positive control (470 mOsm/L). Betaine concentrations of 150 mM and 200 mM displayed

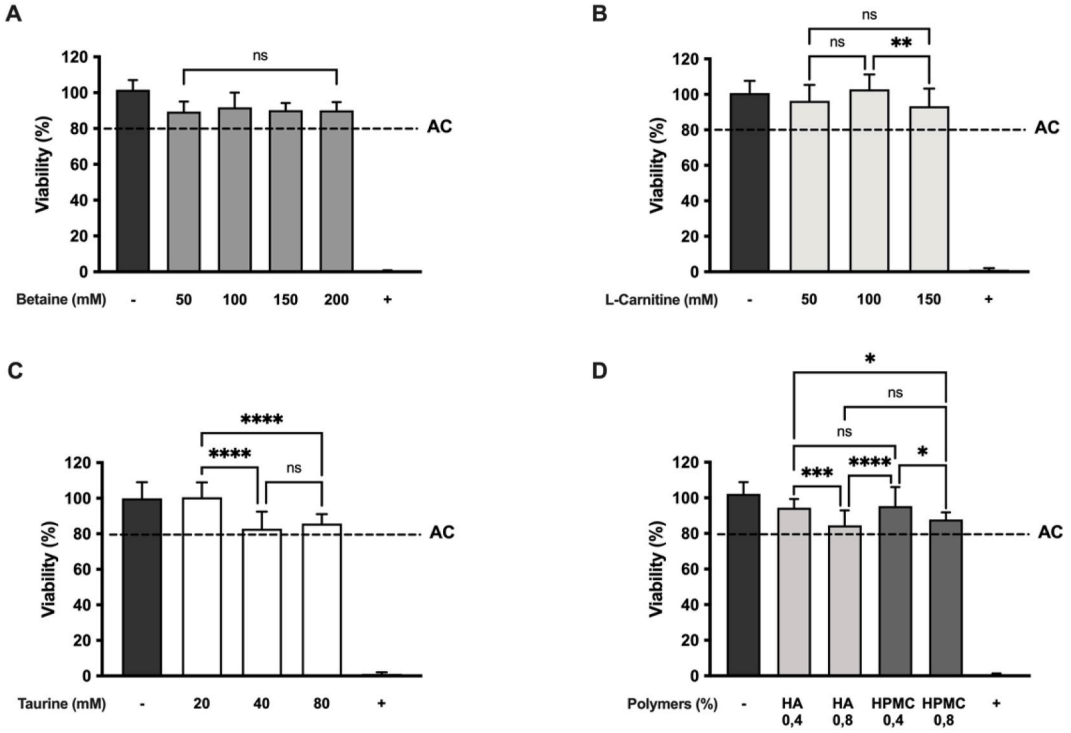

Fig. 2. *In vitro* toxicity evaluation at 8 h of the substances and polymers are illustrated. The negative control (−) represent untreated cells while the positive one (+) are BAK 0.005% treated cells as above mentioned. Broken line AC represents the acceptance criteria for ocular *in vitro* cell viability.

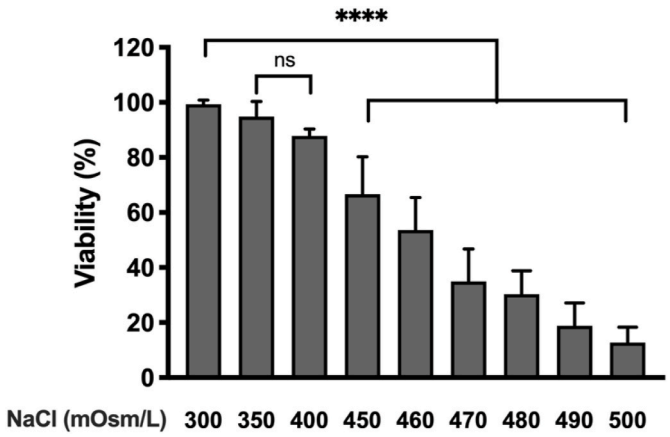

Fig. 3. Decrease of hTERT-HCECs cell survival (%) in different hyperosmolar conditions. 450 mOsm/L shows a threshold concentration where osmolar sensitivity of the cells increase. Hyperosmolar concentrations from 450 to 500 mOsm/L showed statistically significant drop in viability (%) compared to 300 mOsm/L (****; $p < 0.0001$).

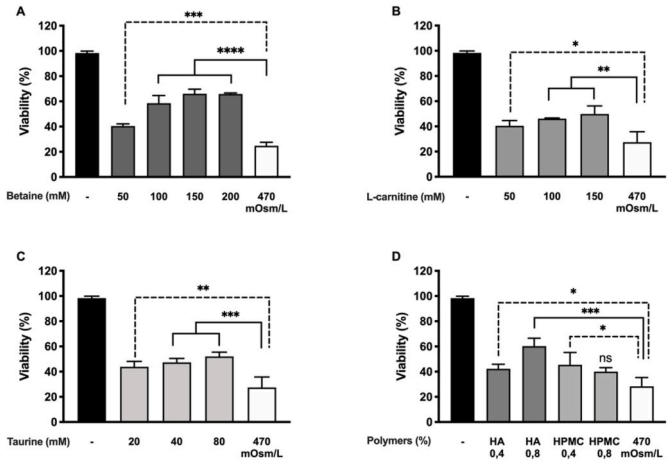

Fig. 4. Osmoprotection of Betaine (A), L-Carnitine (B), Taurine (C) and polymers (HA and HPMC) (D) in response to 470 mOsm/L in hTERT-HCECs cells.

similar percentages (66.01% ± 3.65 and 65.90% ± 0.78 respectively) of viability. L-carnitine, at all concentrations was able to increase cell viability with concentrations of 100 mM and 150 mM exhibiting higher cell viability values (46.2% ± 0.41 and 49.90% ± 6.37, respectively) with p values of 0.0038 and 0.0010, respectively. Cell viability values with taurine exposure resulted similar, albeit the same pattern as previously reported with L-carnitine was shown and high concentrations (40 mM and 80 mM) demonstrated greater statistically significant values (***; $p = 0.0005$ for 40 and $p = 0.0002$ for 80 mM) than the lowest concentration (20 mM) (*; $p = 0.0039$). The conditions provided by HPMC 0.8% were not able to provide an improvement in comparison with the positive control ($p = 0.1070$) while the lowest concentrations (0.4%) of HA and HPMC showed an increase in cell viability (42.2% ± 3.60 and 45.50% ± 9.71 respectively) (*; $p = 0.0498$ for HA 0.4% and $p = 0.0149$ for HPMC 0.4%). HA 0.8% solution increase cell viability values to 60.24% ± 6.29 (***; $p = 0.001$).

3.2.2. *Apoptosis and necrosis by flow cytometry*

Flow cytometry analysis was employed to determine different cell death mechanisms (early, late apoptosis and necrosis) involved in the cellular response under prolonged hyperosmotic stress as well as evaluating the protective properties of the different osmoprotective active substances and polymers. Briefly, side scatter (SSC) was plotted against forward scatter (FSC) to gate cells in order to identify the population of single cells (singlets). Moreover, SSC was plotted against YO-PRO1 to identify the total apoptotic population and 7AAD was represented against YO-PRO1 to identify the different stages involved in cell death (viable cells, early apoptotic, late apoptosis and necrotic).

Firstly, cell recovery and total apoptosis was assessed after 16 h exposure to different hyperosmolar concentrations using the isotonic (NaCl 0.9%) solution as control (100%) (Fig. 5). Regarding cell recovery, 350 mOsm/L was the only concentration considered as non-significant in comparison with isotonic media ($p = 0.1038$). From 400 mOsm/L to 500 mOsm/L a significant decrease was observed with every 10 mOsm/L increase in osmolarity ($p < 0.0001$). With regards to apoptosis, some levels of significance were shown at 460 mOsm/L ($p = 0.0122$) and high levels of significance were exhibited from 470 to 500 mOsm/L ($p < 0.0001$).

Briefly, representative flow plots of cells exposed to 300 mOsm/L or 470 mOsm/L is illustrated in Fig. 6 (A and B respectively). Total apoptosis (YO-PRO1/SSC) for the negative control (A) was 1.41% ± 0.52 and 17.99% ± 5.27 for the selected hypertonic concentration (B).

As shown in Fig. 7, cells exposed to 470 mOsm/L for 16 h after pre-incubation with different concentrations of Betaine (8h) showed a significant drop in apoptosis when compared to pre-incubation with the control solution (NaCl 0.9%). Moreover, betaine appeared to be concentration-dependent with its lowest apoptosis value at 200 mM with a dramatic decrease in early (2.82% ± 1.02) and late apoptosis (1.33% ± 0.03).

In the case of cells preincubated with taurine, early apoptosis was between 2 and 4%, close to the negative control or basal values (Supplementary Fig. 1). Taurine showed the ability to revert the entrance of cells into early stages of apoptosis when compared to the positive control (470 mOsm/L). Taurine at 40 and 80 mM exhibited similar values to those present at basal conditions (300 mOsm/L).

L-Carnitine at all concentrations (50,100,150 mM) decreased apoptosis, but L-Carnitine at 150 mM showed the highest response by

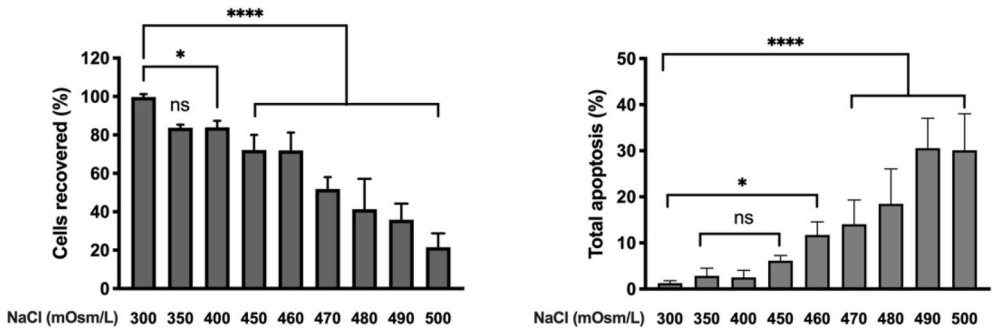

Fig. 5. Total hTERT-HCECs cells recovered following exposure to different hypertonicities (left) and percentage of total apoptosis, measured by flow cytometry analysis.

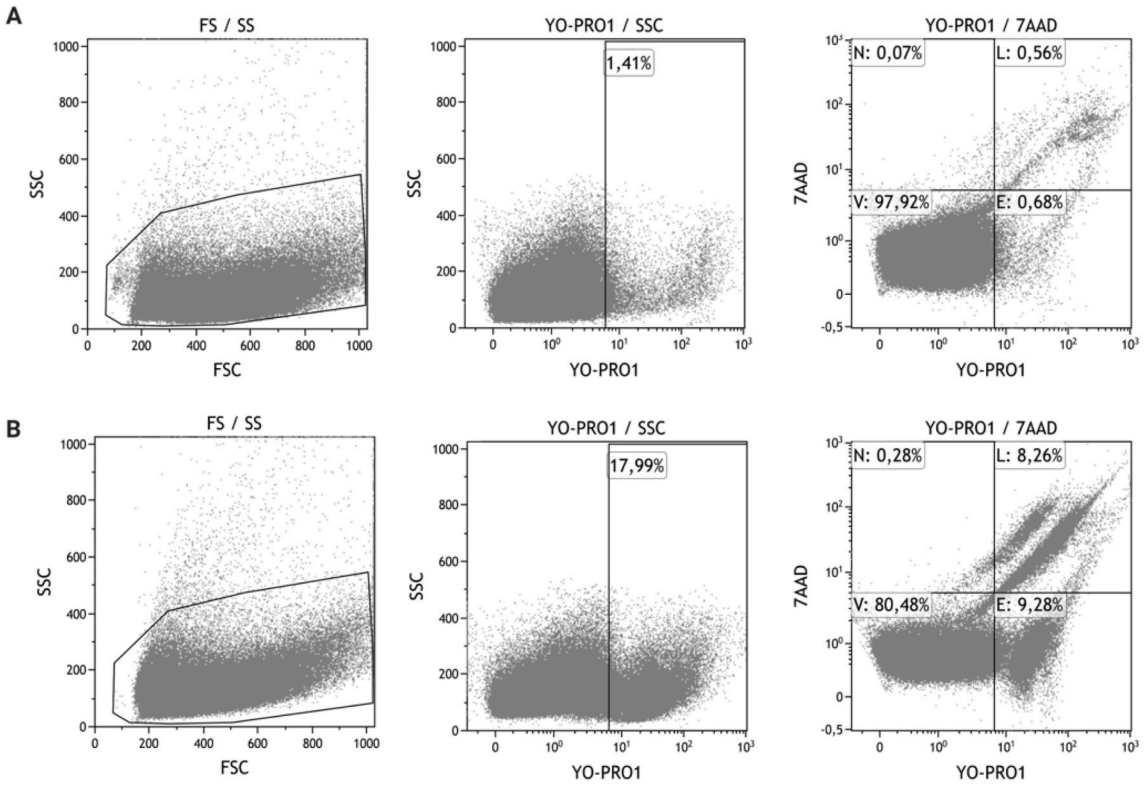

Fig. 6. Representative examples of viable fraction of cells, early apoptosis, late apoptosis and necrosis of cells exposed to 300 mOsm/L (A) and cells exposed to hyperosmolar 470 mOsm/L (B). V: viable; E: early apoptosis; L: late apoptosis and N: necrosis. YO-PRO1/SSC states for the total apoptosis values.

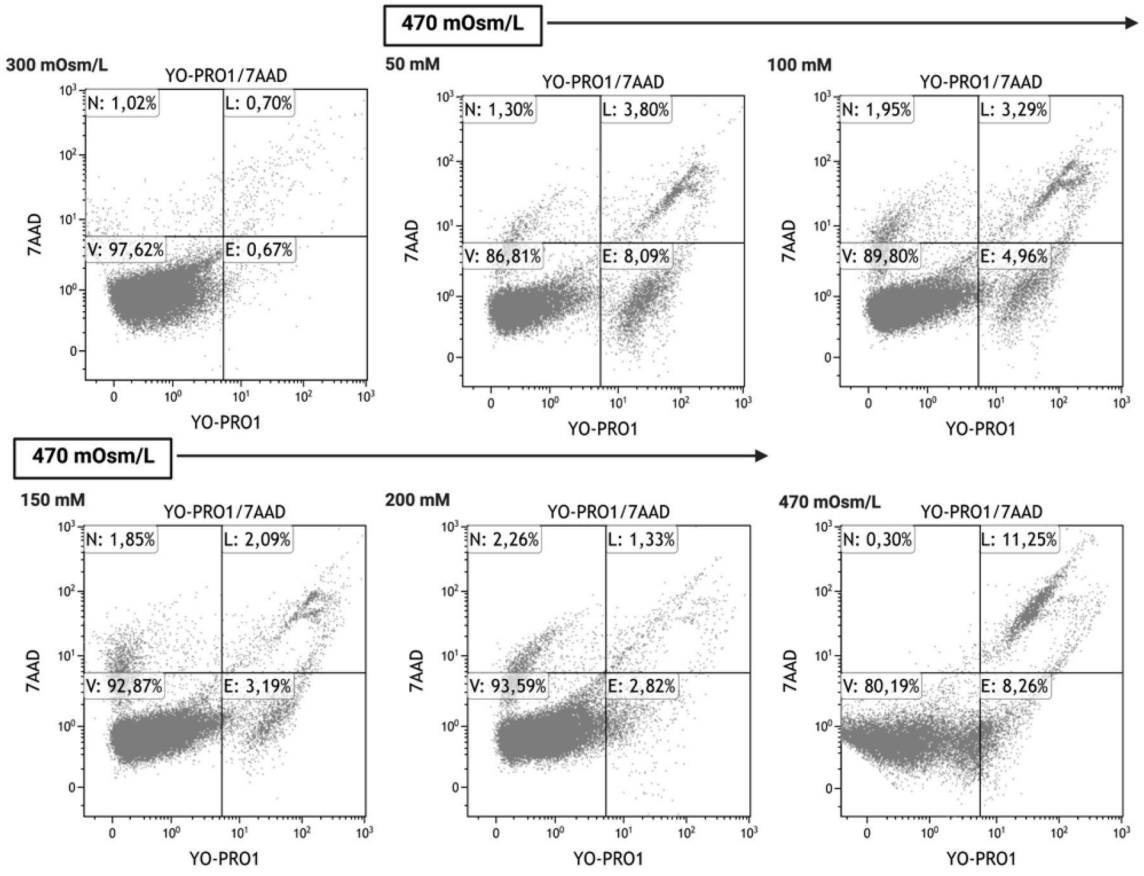

Fig. 7. Cell survival of negative control (300 mOsm/L), untreated cells under osmotic stress (470 mOsm/L) and pre-treated cells with different concentrations of betaine (50–200 mM) later exposed to osmotic stress. V: viable; E: early apoptosis; L: late apoptosis and N: necrosis.

preventing cells from early apoptosis in comparison with the positive control (470 mOsm/L) by more than 4-fold. Besides, at 50 mM and 100 mM early apoptosis was reduced almost 2-fold in comparison with the positive control (Supplementary Fig. 2).

Hyaluronic acid (HA) and hydroxypropyl methylcellulose (HPMC) prevented the cells from entering apoptosis, but HA showed higher ability than HPMC to protect against late apoptosis stages. Cells pre-incubated with HA exhibited early apoptosis values between 2.5 and 3% while those exposed to HPMC showed values ranging from 3.6 to 4.4%. Regarding late apoptosis events, HA showed expressions between 1.5 and 3.3% of apoptosis while HPMC showed values between 4 and 5%. In contrast to other substances, both polymers at different concentrations decrease the ability of 470 mOsm/L to induce late apoptosis stages (Supplementary Fig. 3).

Furthermore, total apoptosis of cells pre-incubated with the osmoprotective substances and polymers was assessed after hypertonic stress exposure by flow cytometry (Fig. 8). Betaine showed a significant decrease in apoptosis ($p < 0.0001$) at all concentrations (50, 100, 150 and 200 mM). Furthermore, betaine exhibited a concentration dependent activity with its maximum at 200 mM. Betaine 50 mM and 100 mM demonstrated similar anti-apoptotic activity (9.47% ± 3.53 and 8.67% ± 1.35). Betaine at 150 mM appeared to be a threshold concentration where its anti-apoptotic activity is increased (4.29% ± 1.34). Finally, Betaine at 200 mM showed the highest anti-apoptotic activity although similar to 150 M (3.31% ± 1.05).

All concentrations of L-Carnitine were also very significant in comparison with the positive control (470 mOsm/L) ($p < 0.0001$). Each one, showed similar protective activity (11.88% ± 0.17 for 50 mM, 11.07% ± 0.35 for 100 mM and 9.98% ± 0.26 for 150 mM). Furthermore, taurine demonstrated higher activity at all concentrations ($p < 0.0001$) although very similar between them. Taurine at 20 and 40 mM were almost identical (4.96% ± 0.43 and 4.35% ± 0.54 respectively). Taurine at 80 mM exhibited higher anti-apoptotic activity (3.65% ± 0.58) than 20 and 40 mM. Finally, both polymers HA and HPMC at 0.4% and 0.8% for each one exhibited similar activity regarding apoptosis inhibition ($p < 0.0001$). On the one hand, HA at 0.4% diminished apoptosis up to 9.78% ± 0.86 and HA 0.8%–8.64% ± 0.92 in comparison with the positive control (24.50% ± 6.26). On the other hand, cells pre-incubated with HPMC 0.4% and 0.8% exhibited similar apoptosis values (10.05% ± 5.05 and 8.72% ± 3.22 respectively).

Visualization of apoptosis under different conditions as previously mentioned (hyperosmotic stress and controls) was performed under the fluorescence microscopy (Fig. 9) confirming the antiapoptotic effect observed by flow cytometry for of different osmoprotective substances and polymers.

3.2.3. *Analysis of cell size in response to different hyperosmolarity*

Taking into consideration that the forward scatter (FSC) is comparable to the size and volume of cells, FSC was evaluated to determine whether osmolarities modified cell volume in response to the studied osmolarities previously mentioned. Cell count was plotted against FSC using the values of cells exposed to 300 mOsm/L as control of a healthy population. The average FSC signal at each concentration was determined and the relative cell size (RCS) in percentage (Fig. 10) was calculated in comparison with the control population (300 mOsm/L). Cell sizes at 350 mOsm/L were practically identical to isotonic conditions (100.4% ± 2.51) with no significant differences compared to isotonic conditions ($p = 0.999$). Moreover, cells exposed at 400 and 450 mOsm/L presented a slightly increase in size (108.5% ± 2.38 and 108.6% ± 2.25 respectively) compared to isotonic control showing significant differences ($p = 0.0092$ and $p = 0.0088$ respectively). Moreover, 460 mOsm/L started to decrease the RCS in a significant manner ($p = 0.0143$). Conversely, a dramatic decrease in cell sizes were observed from 470 mOsm/L to 500 mOsm/L as expected from previous results ($p < 0.0001$). RCS at selected hyperosmolar concentration for the model (470 mOsm/L) was 82.15% ± 2.93. The changes in flow cytometry histograms of RCS in response to different hyperosmolar concentrations can be seen in Supplementary Fig. 4.

Furthermore, cells pre-incubated with Betaine at all concentrations (50, 100, 150 and 200 mM) showed a highly significant normalization ($p < 0.0001$) in cell sizes in comparison with the positive control (470 mOsm/L) presenting similar values (ranging from 99.41% to 97.08%)

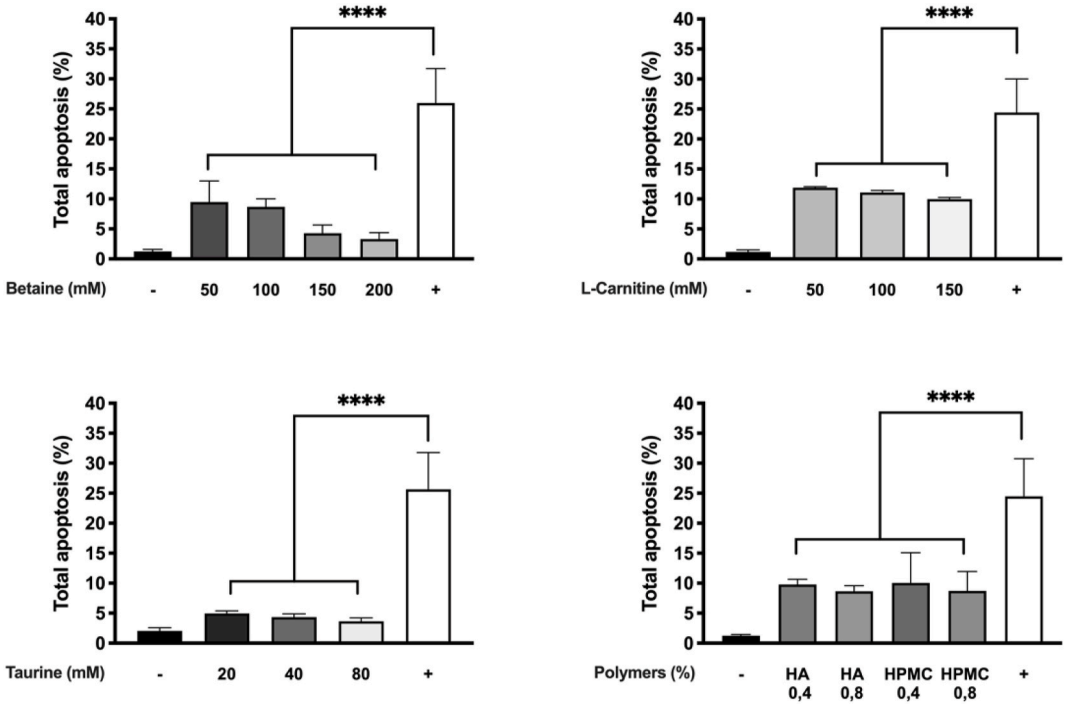

Fig. 8. Total apoptosis of cells pre incubated with the different substances and polymers studied (Betaine, L-Carnitine, taurine, HA and HPMC) at different concentrations in comparison with the positive control (+; 470 mOsm/L). Negative control (−) shows cells pre-incubated with NaCl 0,9% (isotonic).

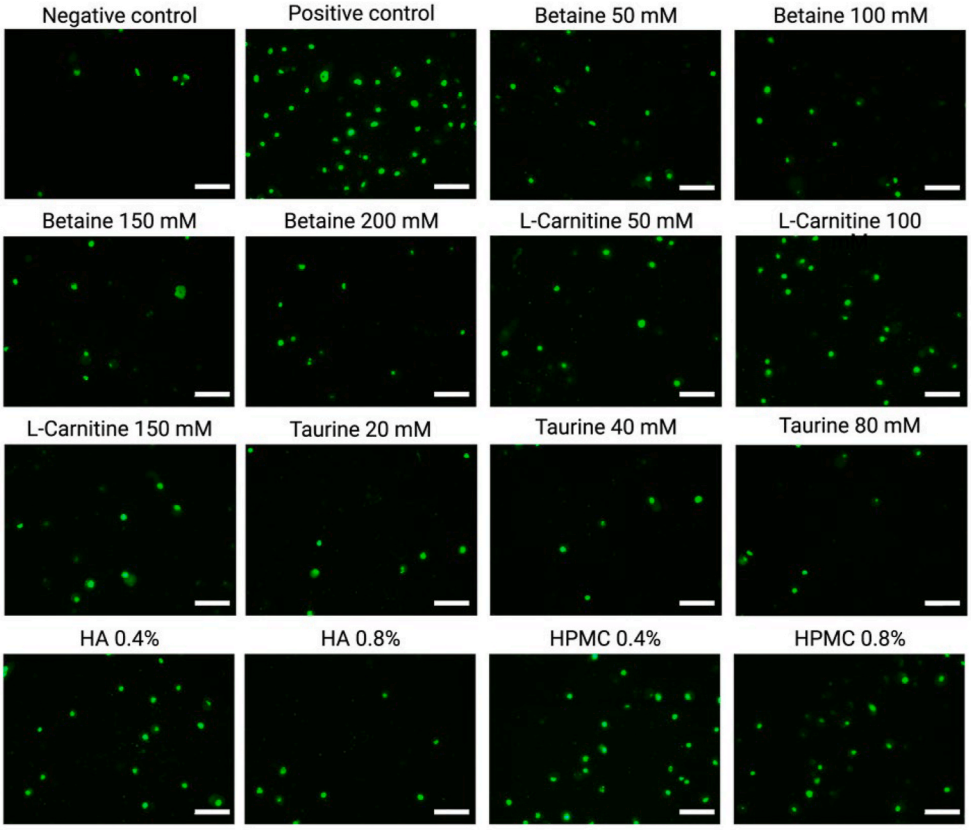

Fig. 9. Apoptosis images of different cell populations pre incubated with osmoprotectants or polymers (Betaine, L-Carnitine, taurine, HA and HPMC), negative control (300 mOsm/L) and positive control (470 mOsm/L). Cells stained with YO-PRO1 and visualized under the fluorescence microscope (scale bar of 50 μm at 20x amplification).

(histogram can be seen in Supplementary Fig. 5). However, those exposed to L-carnitine demonstrated significant changes in cell sizes at 50 mM ($p < 0.0001$) and 100 mM ($p = 0.0005$) (Supplementary Fig. 6). On the contrary, cell sizes from those exposed to L-carnitine concentration of 150 mM showed no significant changes in comparison with the positive control ($p = 0.8640$). Besides, Taurine exhibited a concentration dependent capacity to counteract cell shrinkage with 40 mM and 80 mM being the most effective concentrations ($p < 0.0001$) by increasing cell volume (106.2% ± 3.12 and 106.6% ± 1.34 respectively) (Supplementary Fig. 7). Taurine at 20 mM increased RCS up to 90.52% ± 1.66 being statistically significant in contrast to untreated cells under hyperosmotic stress ($p = 0.0453$). Finally, among all polymers hyaluronic acid at 0.4% increased significantly ($p = 0.0007$) the cell volume

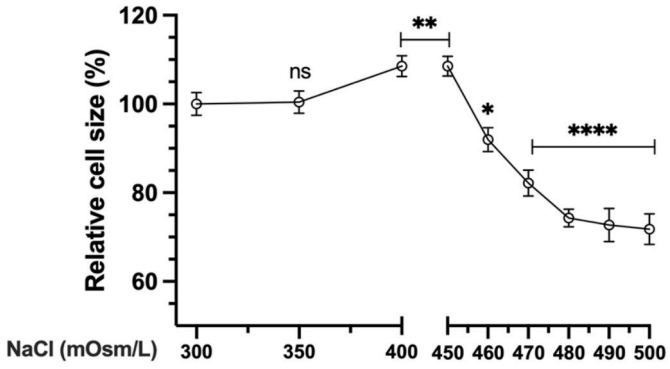

Fig. 10. Representation of normalized FSC (%) in comparison with unstressed cell population (300 mOsm/L) showing the progression of cell size modifications under different hyperosmolar concentrations.

(101% ± 3.97). The highest increase was observed for HA 0.8% and both concentrations of HPMC ($p < 0.0001$). HA 0.8% counteracted the shrinking action by increasing considerably cell volume (113.8% ± 4.11). HPMC increased the RCS up to 106.7% ± 3.84 whereas HPMC 0.8% achieved 110.2% ± 4.73 (Supplementary Fig. 8). All the changes in cell sizes can be seen in Fig. 11.

3.3. Determination of inflammatory markers

Macrophages were incubated with the potential osmoprotective substances or polymers were all exposed to 12.5 ng/mL of LPS for 24 h to mimic inflammatory conditions. The ability of the substances and polymers to reduce the production of TNF-α was expressed as the inhibitory effect in percentage (%) of LPS-induced inflammation (shown in Fig. 12).

Betaine produced a significant decrease in TNF-α at the different concentrations studied (50 mM, 100 mM and 150 mM). All of them had similar inhibitory activities (25.59% ± 1.82, 21.29% ± 2.86 and 25.81% ± 2.63 respectively), showing no statistical difference between them ($p = 0.2611$ for 50 mM vs 100 mM, $p = 0.9994$ for 50 vs 150 mM and $p = 0.2287$ for 100 vs 150 mM). Moreover, 200 mM constituted the highest inhibitory concentration (39.15% ± 2.10) (***; $p = 0.0008$, $p = 0.0003$, $p = 0009$ for 50 vs 200 mM, 100 vs 200 mM and 150 vs 200 mM respectively). Regarding L-carnitine, it also presented a high anti-inflammatory capacity. Furthermore, the lower the concentration showed greater anti-inflammatory activity, with 50 mM (56.39% ± 6.12) being the most effective one, followed by 100 mM (46.69% ± 9.58) and 150 mM (32.37% ± 3.83) respectively. L-Carnitine at 150 mM demonstrated significance when compared with 50 mM ($p = 0.0300$) but no difference was observed when compared with 100 mM ($p = 0.1375$). Taurine showed great anti-inflammatory values at all

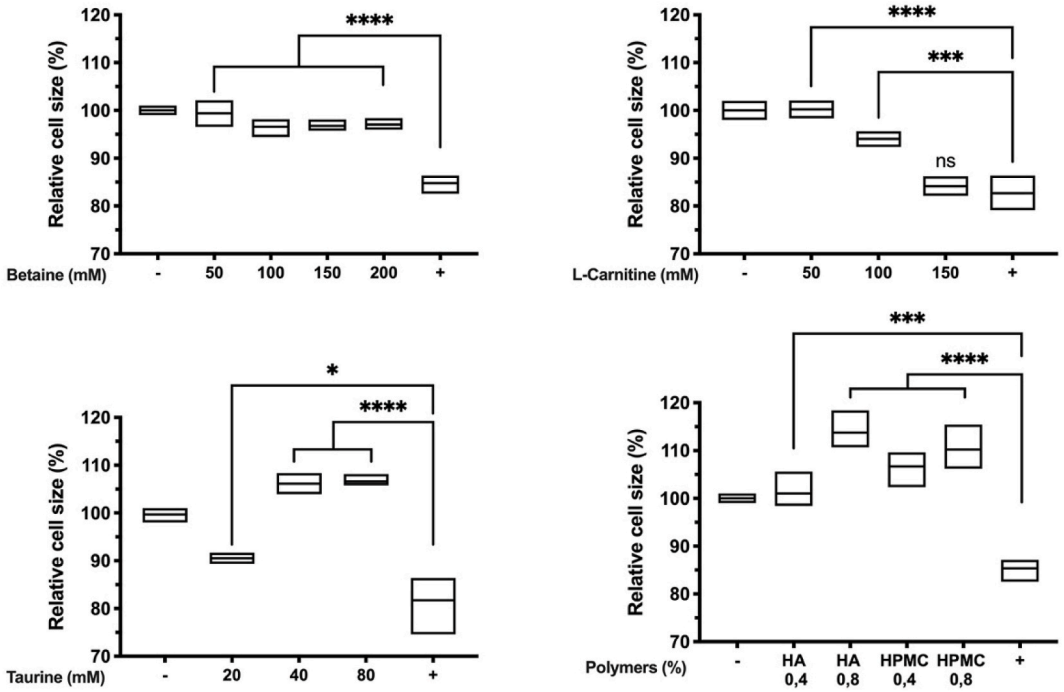

Fig. 11. Activity of different osmoprotective substances and polymers on preventing cell size modification under hyperosmolar concentration (470 mOsm/L).

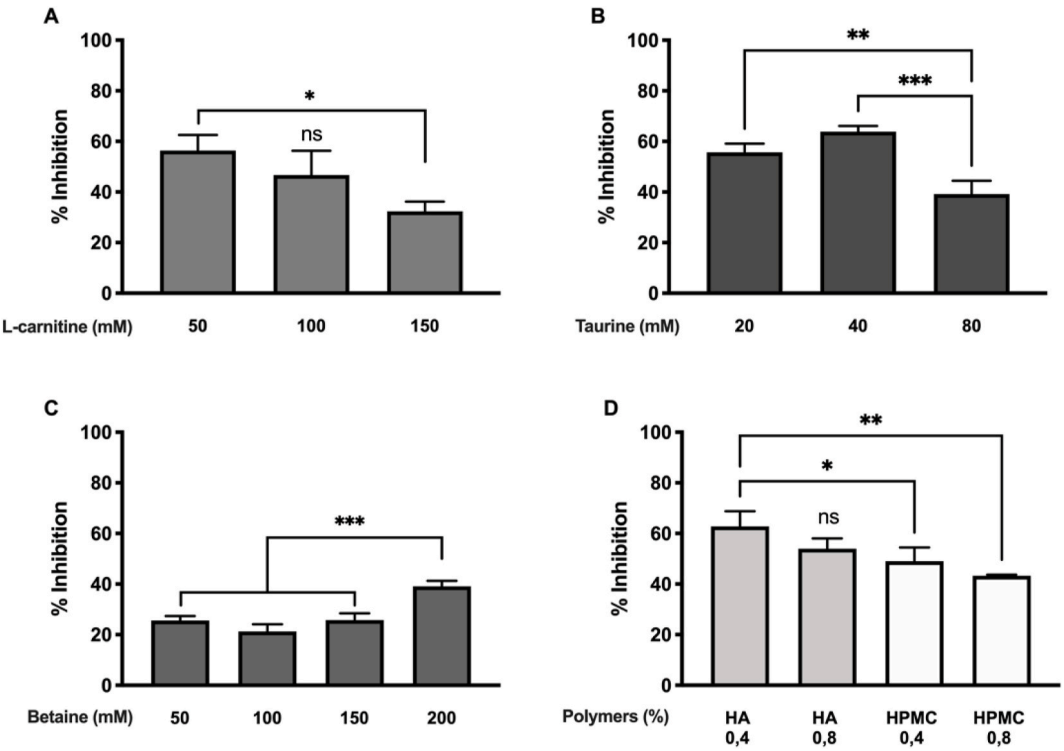

Fig. 12. Inhibition of TNF-α production of L-Carnitine (A), Taurine (B), Betaine (C) and HA and HPMC polymers (D) in response to LPS 12,5 ng/mL in macrophage J774A.1-TIB67™ cell line.

concentrations. Nevertheless, both 20 mM and 40 mM had the highest inhibitory values (55.71% ± 3.43 and 63.85% ± 2.26 respectively) presenting statistically significance in comparison with 80 mM ($p = 0.0046$ for 20 mM and $p = 0.0006$ for 40 mM) in comparison with the highest concentration (80 mM). Conversely, taurine at 20 and 40 mM were similar and presented no differences between them in terms of significance ($p = 0.0907$).

Significant inhibition values were obtained with regards to the above-mentioned polymers. Although polymers at all concentrations exhibited values of inhibition superior to 40%, HA 0.4% had the highest one (62.81% ± 5.96) with high statistically significance in comparison with both concentrations of HPMC $p = 0.0155$ for HA 0.4% vs HPMC 0.4% and $p = 0.0026$ for HA 0.4% vs HPMC 0.8%.

4. Discussion

Hyperosmolarity is one of the most important key elements in DED, responsible for triggering an inflammation cascade, ROS production and apoptosis of corneal and conjunctival cells (Wang et al., 2019). According to some authors, increased osmolarity leads cells to volume modification and shrinking (Garrett et al., 2013). Besides, an increase in solute concentrations cause dysfunction in DNA repair, DNA rupture and consequently cell apoptosis (Baudouin et al., 2013). These highlight the importance of searching for fast and effective screening methods to find and develop potential therapies. With regards to DED screening models and techniques, an ex vivo irritation test to test efficacy of DED therapies has been developed (Spöler et al., 2010). However, the main problem associated to these methods are their costs, due to equipment such as OCT (optical coherence tomography) or a device to maintain the globes with the appropriate conditions of moist, heat and tools to induce lacrimation with the main purpose of mimicking a flow of dry air to resemble DED conditions. Besides, the requirement of animal corneas may hamper the process of rapidly screening potentially useful substances. Another interesting strategy is the development of 3D co-cultures in air-lift conditions mimicking the ocular surface, avoiding the use of animals and increasing reproducibility. *Lu et al*. developed a complex 3D *in vitro* model based on the use of lacrimal spheroids in combination with conjunctival epithelial cells as a way to reproduce the DED pathophysiology associated to inflammatory events (Lu et al., 2017). Besides, *Barabino et* al. developed an air-lift culture with human corneal epithelial cells as a strategy to study the restoration capacity of a new modulator in the ocular surface under changes produced by a mixed sorbitol and desiccation induced hyperosmolarity model (Barabino et al., 2017). In another study, *Puleo* and collaborators developed a corneal micro-culture using collagen-based gelling materials through microfluidic techniques (Puleo et al., 2009). They claimed to have developed an extracellular matrix (ECM) -like tissue for growing corneal cells and study ocular surface processes. Despite the few complex and groundbreaking models that have been studied (Barabino et al., 2020), there is still a need to quickly and easily study the basic features that allow researchers to rapidly screen specific osmoprotective systems or substance so afterwards more complex models that gather specific information can be applied.

According to the results shown in the present study, we have developed a successful combination of cell models to evaluate osmoprotective agents based on tolerability screening, cell survival under osmotic stress by MTT, study of cell death mechanisms through flow cytometry analysis under chronic hypertonic stress (16 h) in human corneal cells as well as anti-inflammatory efficacy (24 h) in macrophages. The use of both cellular models allows to test and screen the potential anti-inflammatory and osmoprotective activity of different substances and provides useful combined evaluated information to check for effective therapeutic approaches. Combination of cell survival by tetrazolium salt reduction and flow cytometry analysis (YO-PRO1/7AAD) provides an overview of cell survival and investigation of apoptosis and necrosis protective mechanisms of the tested compounds. Furthermore, cell volume analysis affords extra information about hypertonic stress counteraction mechanisms. Hypertonic environment causes damaged cells to blow up when detached for flow cytometry analysis, thus combining these data provide us with more comprehensive evaluation (Criollo et al., 2007). Finally, adding TNF-α inhibition in macrophages gives additional value to survival studies and complete the study with more information about each substance.

These combined tools could provide an extremely useful strategy as a first "*in vitro*" step in ocular surface pathologies that progress with hyperosmolarity and trigger a series of inflammatory and cell death events. Among all the parameters that proves useful to check for a system suitability for topical ophthalmic administration are cell tolerability of individual substances, an in-vitro assay that reproduce some environmental parameters of the disease which allows to rapidly check for cell death and protection, an easy but more specific detailed view of cell death insights that provide information about protective mechanisms, morphology and finally determination of anti-inflammatory activity of testing compounds. We selected some compounds that are currently under study by their proposed activity as ocular surface cell protection in the case of Betaine, L-Carnitine and taurine.

Betaine, a known osmolyte by its capacity to protect plant cells against osmotic and temperature stress, has been previously studied for its ability to inhibit the production of interleukins as well as down-regulating the mRNA production of certain chemokines (Hua et al., 2015b). In the present work betaine was well tolerated in corneal epithelial cells after 8h exposure and was also able to decrease the levels of TNF-α in the macrophage inflammation assay at all the concentrations studied (50, 100, 150 and 200 mM). Betaine at 200 mM was demonstrated to have the highest anti-inflammatory efficacy (close to 40%) compared to lower concentrations. Previous studies have shown that betaine was able to protect human epithelial corneal cells from cell death and regulate their volume under hyperosmotic stress (Garrett et al., 2013). Accordingly, the first hyperosmolar model developed in this work, shows that preincubation of corneal cells with betaine is able to provide protection and increase cell survival. Particularly, betaine exhibited a high osmoprotective efficacy in a concentration dependent manner up to 150 mM. From 150 mM to 200 mM, seemed to have similar protective values (around 60%). When apoptosis was assessed in the flow cytometry studies, total apoptosis reduction was more prominent at 150 and 200 mM, but highly significant from the lowest concentration (50 mM). An important finding was that betaine at 200 mM was the most effective in reducing early apoptosis and normalizing late apoptosis at almost basal levels. With regards to cell size, betaine regulated cell size close to normal levels, although 50 mM apparently was closer to the basal levels. Moreover, the rest of the concentrations were also able to revert cell size close to 100% (97–99%).

L-Carnitine has been described by *Hua* et al. in co-culture with a hyperosmolar concentration (NaCl 450 mOsm/L) for its ability to decrease the production of specific oxidative markers such as 8-hydroxy-2' -deoxyguanosine (8-OHdG), 4-Hydroxy-2-nonenal (HNE) or Aconitase-2 protein (Hua et al., 2015a). These authors also demonstrated that L-carnitine was able to reduce the production of specific mRNA for pro-inflammatory mediators such as IL-6, IL-1β and TNF-α respectively. Our results for L-Carnitine are in agreement within the range of well tolerated concentrations (50, 100 and 150 mM). L-Carnitine was able to considerably reduce TNF-α production, particularly at 50 mM. We hypothesize that this could be explained due to transporter saturation or chronic toxicity at long periods of exposure. Besides, L-Carnitine has been also described to regulate cell shrinking produced by hypertonic stress (Khajavia et al., 2014). In this study, L-Carnitine was able to protect cells from death in a concentration dependent manner in the MTT based assay exhibiting its high efficacy at 150 mM. However, apoptosis was mostly decreased at the lowest concentration but very similar in all the three concentrations. Conversely, albeit it was able to regulate cell size at 50 and 100 mM, no differences in cell sizes were observed at 150 mM in comparison with the positive control. These results suggest that one of the main mechanisms of action of L-Carnitine is through inhibition of inflammatory processes and regulation of intracellular pathways involved in apoptosis.

Regarding taurine, it is widely known to be present inside cells in a natural way and has been hypothesized to play an important role in the transport of ions through membrane channel and transporters (Schaffer et al., 2000). Bucolo et al. showed the ability of taurine to decrease ROS production under H_2O_2 stress in corneal epithelial cells, decreasing the level of specific metalloproteinases (MMP-9) as well as normalizing the tear breakup time (TBUT) in a DED rabbit model induced with atropine 1% (Bucolo et al., 2017). We demonstrate that the concentrations tested in this work (20, 40 and 80 mM) when incubated with cells prior to hypertonic exposure are able to prevent cell death. In the cytometry studies we also observed that it is a powerful inhibitor of apoptosis,

particularly at early apoptosis stages. The anti-inflammatory properties increased at 20 and 40 mM in a similar pattern that with L-carnitine but presenting higher activity. Cell sizes, conversely, to betaine and L-carnitine appear to be upregulated when exposed to 40 and 80 mM. We propose that taurine, apart from inhibiting cell death mechanisms through apoptotic pathways is also preventing cell death by increasing cell volume. Apparently, as previously depicted in the illustration (Fig. 1), taurine would penetrate through specific TauT transporter triggering specific pathways.

Moreover, we also studied the features in the model of some polymers that already available in marketed tears such as HPMC or HA. It is also worth mentioning that HPMC concentrations ranging from 0.2% to 0.8% have been employed as mucoadhesive polymers for novel topical antiglaucomatous formulations (Esteban-Pérez et al., 2020) and as lubricants by improving DED symptoms (Jones et al., 2017). Although both concentrations (0.4% and 0.8%) resulted in high viability values, HPMC 0.4% demonstrated to have some protecting effects under hypertonic stress. Although HPMC 0.8% demonstrated higher significance in reducing inflammation, we could argue that HPMC 0.4% would be more suitable than HPMC 0.8% as the cellulose derivative at high concentrations exhibits high viscosities, thus hampering cells survival and nutrients intake without improving osmolar conditions. In fact, HPMC 0.4% seems to have sufficient viscosity to retain enough water to avoid part of the harmful effects caused by hyperosmotic environment.

We have demonstrated that preincubation with HA protected cells by increasing cell survival under osmotic stress. HA also showed an important ability to decrease apoptosis and also increased cell volume, particularly at 0.8%. Besides, we prove that HA at 0.4% and 0.8% is not only well tolerated but also possesses anti-inflammatory properties at both concentrations. HA has been also described as a mucoadhesive polymer showing high viscosity depending on the concentration. These fact makes HA to adhere to the cell surface and prolong its protective efficacy. The use of HA in dry eye treatment has been widely described. *Orobia* et al. exposed the ability of high molecular weight HA to enhance wound healing (Jones et al., 2017), epithelial cell migration, as well as hydrating properties (Mateo Orobia et al., 2018). Also, in a study of patients with glaucoma treated with beta blockers and having tear film instability, sodium hyaluronate was used to increase tear film stability and improve patient discomfort (Monaco et al., 2011).

These findings support the use of both HPMC and HA as co-adjuvant polymers in different therapies to avoid cell surface damage. HPMC acted by upregulating cell volume much more than HA, however HA was able to revert cell death mechanisms in a more effective manner.

Furthermore, this is one of the first studies that show the ability of these specific substances to prevent cells from hyperosmotic damage before being subjected to hypertonic stress. One of the key factors in the management of certain diseases, particularly in DED is the ability of certain substances and therapies to prevent inflammatory processes and cell death mechanisms in response to cell stresses. Many of the present studies, focus on isolated parameters and evaluate the protective capacity of certain substances while osmotic stress administered. However, we demonstrate that exposure to these substances in early stages are able to protect cells from osmotic damage without the need of the substances to be present in the media. According to some authors and as stated in Fig. 1, we hypothesize that the compounds studied in this article excepting polymers, are able to accumulate inside cells and trigger specific inner mechanisms that provide protection against osmotic stress overtime even when the substance is no longer present in the media. According to the results obtained in the present work, the use of osmoprotective active substances and bioadhesive polymers as the ones evaluated in the present study emerges as a useful strategy to prevent DED or treat ocular surface pathologies that develop with hyperosmolarity, ocular inflammation and oxidative stress.

5. Conclusions

Hyperosmolarity can trigger a cascade of inflammatory events as well as cell death mechanisms leading to patient discomfort and ocular surface damage, causing in some circumstances visual impairment. In the present study, we developed a hyperosmolar model based on different techniques that allows to identify well tolerated osmoprotective substances easily and quickly. Besides, we also demonstrated the ability of the osmoprotective substances Betaine, L-Carnitine, Taurine, and the polymers HA and HPMC to protect corneal epithelial cells from cell death under chronic hyperosmolarity or inflammatory stress (after LPS induced conditions in macrophages) but also to prevent them from these events when the substances have already disappeared from the environment. The use of this substances alone or in combination could help in the development of new potential therapies for the treatment of ocular surface pathologies. The present tools developed in this study might also serve as a starting point to continue refining and studying different osmoprotective and anti-inflammatory characteristics of substances and promising formulations. Finally, this could entail a new approach to rapidly screen potential osmoprotective activity before more complex *in vitro* or *in vivo* studies are designed.

Declaration of competing interest

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2021.108723.

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