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Implications for Ophthalmic Formulations: Ocular Buffers Show Varied Cytotoxic Impact on Human Corneal–Limbal and Human Conjunctival Epithelial Cells

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Purpose: To investigate toxicity associated with buffers commonly used in topical ocular drug formulations using a human corneal–limbal epithelial (HCLE) and a human conjunctival epithelial (HCjE) cell model.

Methods: HCLE and HCjE cells were incubated for 10, 30, or 60 minutes with 4 different buffers based on borate, citrate, phosphate, and Tris-HCl at 10, 50, and 100 mM concentrations. To detect possible delayed effects on cell viability, after 60 minutes of buffer incubation, cells were further incubated for 24 hours with a cell medium. Cell viability was determined using a colorimetric XTT–based assay. The morphology of cells was also investigated.

Results: HCjE cells showed more sensitivity to buffer incubation than HCLE cells. The 100 mM phosphate buffer displayed significant delayed effects on cell viability of HCLE 16.8 ± 4.8% and HCjE 39.2 ± 6.1% cells after 60 minutes of exposure ($P < 0.05$). HCjE cell viability was reduced after 60 minutes incubations with 50 and 100 mM citrate buffer to 42.8 ± 6.5% and 39.3 ± 7.9%, respectively, and even lower percentages at the delayed time point (both $P < 0.05$). HCLE cell morphology was distinctly altered by 100 mM phosphate and Tris buffers after 30 minutes, whereas HCjE cells already showed marked changes after 10 minutes of exposure to 100 mM citrate and phosphate buffers.

Conclusions: We observed a time-dependent decrease of viability in both HCLE and HCjE cells exposed to higher buffer concentrations. Therefore, we propose further in vivo studies to translate these finding to humans to discern the real effects of the buffer concentration in eye drops on the ocular surface.

Key Words: ocular epithelium, topical drug delivery, biocompatibility, eye drops

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Ophthalmic drugs are frequently administered topically, facing special challenges at the ocular surface concerning tolerability, drug permeability, and safety. Eye drop formulations often contain buffering agents. The buffer system of eye drops is often a compromise between the necessities of chemical stabilization to achieve acceptable shelf life and the physiological requirements of the product.[1] Ideally, a formulated buffer system should mimic the natural system of the tear film. Tear fluid consists of 3 main elements: a bicarbonate buffer system, various proteins with dissociable groups, and phosphate compounds. The buffering capacity highly varies between individuals, yet the average physiological pH of lacrimal fluid is 7.4,[2] ranging from 6.5 to 7.6.[3,4]

The most common buffer systems used in ophthalmic formulations are citrate, phosphate, Tris-HCl (Tris), and borate buffer (Table 1). No complications after the application of citrate, borate, and Tris buffering systems in humans are reported in the literature. However, there have been selected case studies in which calcific band keratopathy, a calcific degeneration of the superficial cornea characterized by calcium hydroxyapatite deposition, has appeared after instilling phosphate buffer containing ophthalmic medicinal products.[5,6] Especially in eye drops applied after corneal injuries, the buffer composition has been shown to influence the healing process and the development of corneal calcification.[5] As phosphate naturally occurs in the eye, it has been the buffer of choice for a long time.[6] More recently, borate buffers were introduced as ocular buffers because of their antimicrobial activity and were deemed better suited than phosphate buffers for ophthalmic products.[7] Tris buffers were originally tested for experimental ocular acid burns in rabbits

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TABLE 1. Buffer Systems in Use for Ophthalmic Formulations

Buffer System	Components	pH Range	Concentration Range Used in Ocular Products
Citrate buffer	Citric acid ($C_6H_8O_7$)	3.0–6.2	*
	Sodium citrate ($C_6H_5Na_3O_7$)		
Phosphate buffer	Monosodium phosphate (H_2NaO_4P)	5.8–8.0	0.1–160 mM[26]
	Dibasic sodium phosphate (HNa_2O_4P)		
Tris-HCl buffer	Tris (hydroxymethyl) aminomethane ($C_4H_{11}NO_3$)	7.2–9.0	*
	Hydrochloric acid (HCl)		
Borate buffer	Boric acid (BH_3O_3)	7.6–9.1	*
	Sodium hexaborate		

*Concentrations not available.

and were found to be effective at treating ocular acid burns when the time of exposure to acid was short.[8]

Although literature on the toxicity of preservatives as components in eye drops is available,[9] there are no recent studies dealing with the comparative toxicity of buffer systems on a cellular level. Suitable in vitro systems for the evaluation of novel compounds are a critical step in the process of safety screenings for ocular formulations. We decided to use a corneal and conjunctival epithelial model because the integrity of the ocular epithelium is crucial for visual function and one of the first points of contact for eye drops.[10] In addition, these specific cell lines were chosen because they have been thoroughly characterized[11] and have been used in previous studies to determine cytotoxic effects.[12,13] The purpose of this study was to investigate toxicity of conventional buffer systems used in ophthalmic medicinal products and devices using both human corneal–limbal epithelial (HCLE) and human conjunctival epithelial (HCjE) cell line models.

MATERIALS AND METHODS

Cell Culture

Immortalized HCLE and HCjE cells were kindly provided by Ilene Gipson (Schepens Eye Research Institute, Harvard Medical School, Boston, MA) and their characterization has been reported.[11] Both cell lines were maintained in monolayers in a serum-free keratinocyte growth medium (Life Technologies, Paisley, United Kingdom) at 37°C, 5% CO_2, and 95% humidity. The medium was changed every second day, and the cells were passaged at 70% confluence. Cells were harvested by trypsinization (0.05% trypsin/0.02% EDTA in PBS; PAA Laboratories GmbH, Pasching, Austria) and seeded while within passage 2 to 6.

Preparation of Buffer Solutions

The 4 different buffers were prepared at concentrations of 10, 50, and 100 mM, all with a pH of 7.4 ± 0.1. The osmolarity of the 4 buffer solutions was set to 301 ± 18 mOsm/kg using addition of NaCl, and osmolarity was measured using the Osmomat 030-D (Gonotec GmbH, Germany), according to the manufacturer's instructions.

Cytotoxicity Assay

Cell viability was measured to assess the in vitro toxicity of 3 different concentrations of borate, citrate, Tris, and phosphate buffers through mitochondrial lactate dehydrogenase production. Cells were seeded at 1×10^4 cells/well in 96-well plates and incubated at 37°C, 5% CO_2 for 24 hours for HCLE cells and 48 hours for HCjE cells. After aspiration of the cell culture medium, 100 μL of the respective buffer at a concentration of 10, 50, and 100 mM, respectively, were applied to each well and incubated at 37°C, 5% CO_2 for 10, 30, or 60 minutes. Subsequently, the test buffer was aspirated and replaced with a cell culture medium. Control wells were exposed only to the cell culture medium without growth factors for the same time, as experimental wells were exposed to buffers. For immediate readout, 50 μL of XTT/PMS (N-methyl dibenzopyrazine methyl sulfate) solution (AppliChem GmbH, Germany) was added and incubated for 3 hours under standard conditions. During this period, metabolically active cells reduce the XTT tetrazolium salt to formazan.[14] Then, 100 μL of the cell culture medium was transferred from each well into a new plate, and absorption was measured at 450 nm (reference wave length 630 nm) with a photometer (Tecan GENios). To detect possible delayed cell death, cells that had been cultured for 24 hours after incubation using buffers for 60 minutes were analyzed as well.

Calculations Performed

$$\%\ \text{Viability} = \frac{\text{Abs\_sample} - \text{Abs\_CellMedium}}{\text{Abs\_control} - \text{Abs\_CellMedium}} \times 100$$

Abs_control: absorbance of corneal or conjunctival cells exposed only to the medium without growth factors.

Microscopy

Cells were seeded at 1×10^5 cells/well into 4 chamber slides and incubated for 24 hours in the case of HCLE cells and for 48 hours for HCjE cells at 37°C/5% CO_2 to reach the required degree of confluency. Cells were then incubated with the various buffer solutions at a concentration of 10 or

100 mM for 10 or 30 minutes at standard conditions. The cells were fixed with 4% PFA, stained with hematoxylin and eosin (Sigma-Aldrich, St Louis, MO), mounted, and then examined by microscopy (Zeiss Axiovert 200 Cell Observer; Carl Zeiss GmbH, Vienna, Austria).

Statistics

All experiments were performed 3 times, and a type I 2-way analysis of variance with the Tukey multiple comparisons test was used to compare cell viability data considering the 2 factors buffer type and the time of exposure as well as the 2 factors concentration and time of exposure (GraphPad Prism 6).

RESULTS

Viability of HCLE Cells Is Not Significantly Affected by the Choice of Buffer in Low Concentrations and Short Incubation

All buffers were well tolerated at concentrations of 10 and 50 mM at 10 minutes with average percentage viability between 88.5% and 100%. Incubation times over 30 minutes showed a significant decrease in cell viability at all concentrations. Generally, the longer the HCLE cells were exposed to the various buffer solutions, the significantly lower the viability of the cells (Figs. 1A, 1B, 1C; $P < 0.01$).

Apart from a decreased viability of 16.8 ± 4.8% at 24 hours in HCLE cells exposed to 100 mM phosphate buffer for 60 minutes, other buffers showed no continued effects on late stage cell viability at 24 hours (Fig. 1A; $P < 0.05$).

Statistical analysis between concentrations of the different buffers showed significant changes in the phosphate, citrate, and Tris buffer concentrations of $P < 0.05$ (see SDC 1, Supplemental Digital Content 1, http://links.lww.com/ICO/A516).

HCjE Cell Viability Is Significantly Affected by Citrate Buffer at High Concentrations and Longer Incubation Times

Lower viability of HCjE cells was observed with all 4 buffers at a high buffer concentration; in addition, the HCjE cells showed lower viability than HCLE cells after 10 minutes of incubation with all 4 buffers with average viability levels of 58.3% to 79.2%. Citrate buffer was observed as the most cytotoxic buffer to this cell line with 100 mM citrate buffer showing a significant reduction in the percentage of HCjE cell viability after 30 and 60 minutes to average percentages of 60.0 ± 7.1% and 39.3 ± 7.9%, respectively, compared with borate and Tris buffers at the same time points (Fig. 2A; all $P < 0.05$). Furthermore, at the highest concentration, a significantly higher viability was observed after 30 minutes of incubation with Tris buffer compared with phosphate buffer ($P < 0.05$). HCjE cells at 24 hours that had been exposed to 100 mM citrate or phosphate buffer for 60 minutes also showed high levels of cytotoxicity of

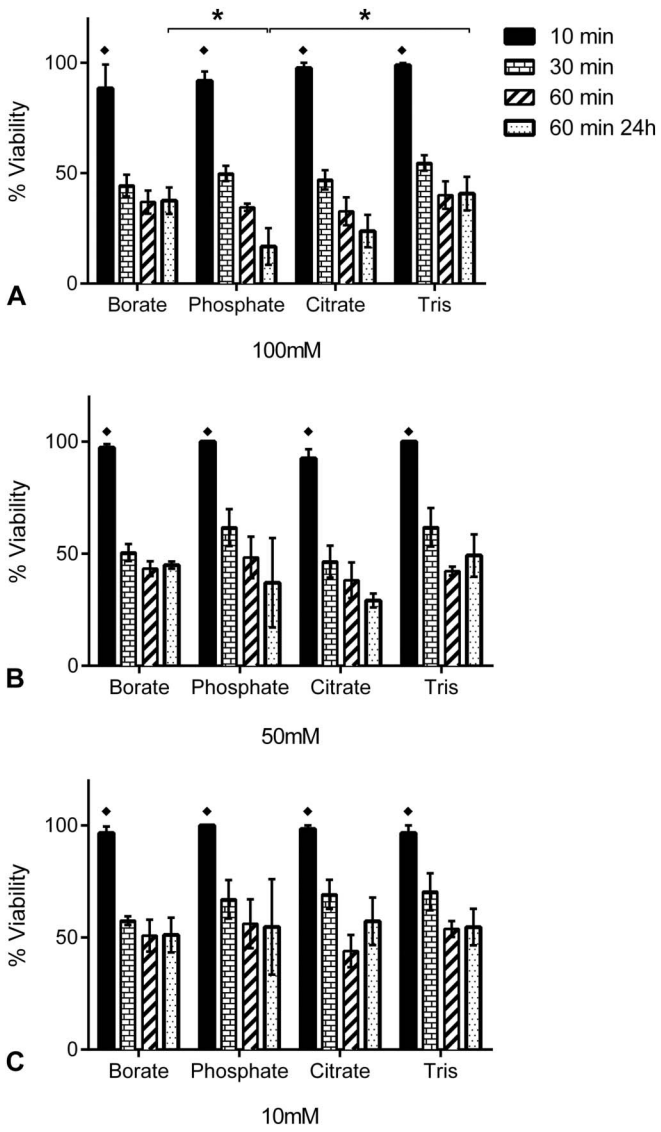

FIGURE 1. Viability of HCLE cells incubated with distinct buffer solutions. HCLE cells were incubated with borate, phosphate, citrate, or Tris buffers at the indicated concentrations for 10, 30, 60 minutes, and 60 minutes followed by a recovery period of 24 hours. Cell viability (XTT assay) was evaluated in comparison with cells incubated in the serum-free keratinocyte cell culture medium, lacking growth factors for the same time. (A) 100 mM; (B) 50 mM; (C) 10 mM. At 10 minutes, all buffers were statistically different from all other time points (♦ $P < 0.01$). Statistical significance of determined differences was tested by a 2-way analysis of variance with the Tukey multiple comparisons test (n = 3; *$P < 0.05$).

30.4 ± 1.1% or 39.2 ± 6.1% compared with the Tris buffer levels of 84.5 ± 12.1% (both $P < 0.05$).

After 60 minutes of incubation and 24 hours after treatment with 50 mM citrate buffer, viability of HCjE cells was 42.6 ± 6.5%, significantly lower than the borate and Tris buffers (Fig. 2B; all $P < 0.05$). The citrate buffer at 24 hours after the 60-minute exposure also showed greater cytotoxicity than at 10 minutes ($P < 0.05$).

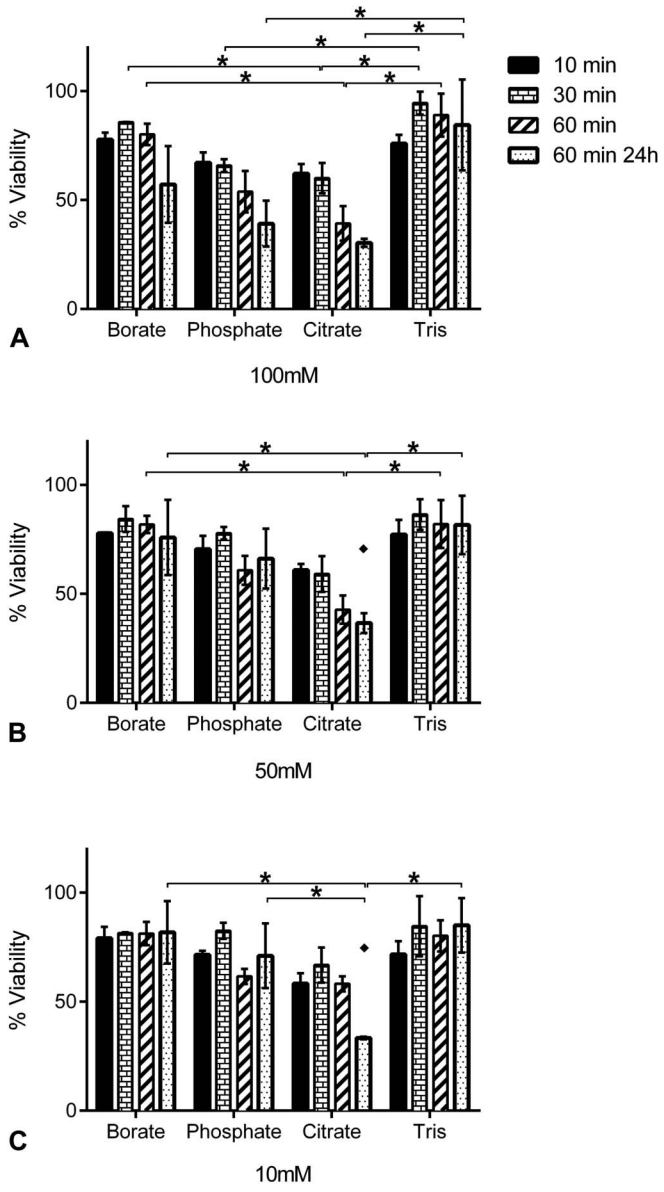

FIGURE 2. Viability of HCjE cells incubated with distinct buffer solutions. HCjE cells were incubated with borate, phosphate, citrate, or Tris buffers at the indicated concentrations for 10, 30, 60 minutes, and 60 minutes followed by a recovery period of 24 hours. Cell viability (XTT assay) was evaluated in comparison with cells incubated in the serum-free keratinocyte cell culture medium, lacking growth factors for the same time. (A) 100 mM; (B) 50 mM; a statistical difference was seen at the 24-hour time point compared with 10 minutes (♦ $P < 0.05$), (C) 10 mM; the 24-hour time point was statistically different from 30 minutes (♦ $P < 0.05$). Statistical significance of determined differences was tested by a 2-way analysis of variance with the Tukey multiple comparisons test (n = 3; *$P < 0.05$).

For the 10 mM buffer concentration, differences were observed between the 24-hour time points after 60 minutes of treatment with citrate buffer compared with all other buffers ($P < 0.05$), showing that already at this low concentration, citrate buffer can produce a cytotoxic effect. In addition, the 24-hour time point of 10 mM citrate buffer showed significantly lower viability of 33.3 ± 0.4% than at the 30-minute time point at which viability was 66.8 ± 8.0% (Fig. 2C, $P < 0.05$).

Statistical analysis of the different buffer concentrations showed significant changes in the borate, phosphate, and citrate buffer concentrations of $P < 0.05$ (see SDC 1, Supplemental Digital Content 1, http://links.lww.com/ICO/A516).

HCLE and HCjE Cell Morphology Is Altered at Higher Buffer Concentrations and Longer Incubation Times

When compared with control cells, all buffers tested showed mild changes in HCLE and HCjE cell morphology at the lowest concentration and time points, with rounding of cells and changes in cell-to-cell contact indicated by prominent cell processes and clearing areas around individual cells (Figs. 3, 4). These changes were exacerbated with longer exposure to the higher concentration buffers.

The higher concentration buffers showed marked differences in cytopathic effects with dark granular bodies and blebbing of epithelial processes. The 30-minute incubation with 100 mM borate buffer showed some condensation of nuclei, whereas the cytoplasm is even with some clearing or thinning between the edges of the cells (Figs. 3A, 4A). The 100 mM citrate buffer at 30 minutes in HCLE cells showed that visible cell-to-cell contact was maintained and showed mild vacuolation of the cytoplasm (Fig. 3B). However, in the HCjE cells, this buffer concentration already showed loss of cytoplasm after 10 minutes, and this was intensified after 30 minutes with very marked pyknosis (Fig. 4B). Both cell lines treated with 100 mM phosphate buffer showed some condensation of nuclei and loss of cytoplasm after 10 minutes, whereas HCjE cells also displayed dark basophilic blebbing at the margins of the cytoplasm. Marked changes were seen after 30 minutes of exposure to phosphate buffer in almost half of the HCLE cells (Fig. 3C) and in all the HCjE cells (Fig. 4C). These changes included pyknotic nuclei, loss of cell-to-cell contact, reduction in cytoplasm, and shrinkage. Incubation with 100 mM Tris buffer showed some clearing between cell margins and condensation of nuclei after 10 minutes, whereas after 30 minutes, moderate changes with some pyknotic nuclei and many prominent dark basophilic bodies in the cytoplasm and in cell processes were observed (Fig. 3D). Interestingly, HCjE cells showed minimal changes in response to the Tris buffer with only mild blebbing after 30 minutes at the 100 mM concentration (Fig. 4D).

DISCUSSION

Topically administered ocular formulations have specific requirements for their buffering system. Buffers are needed to stabilize the pH at a level at which drugs are soluble, active, and tolerable. Because buffer capacity is regulated by its concentration, some formulations use higher dosages to enhance the drug's performance.

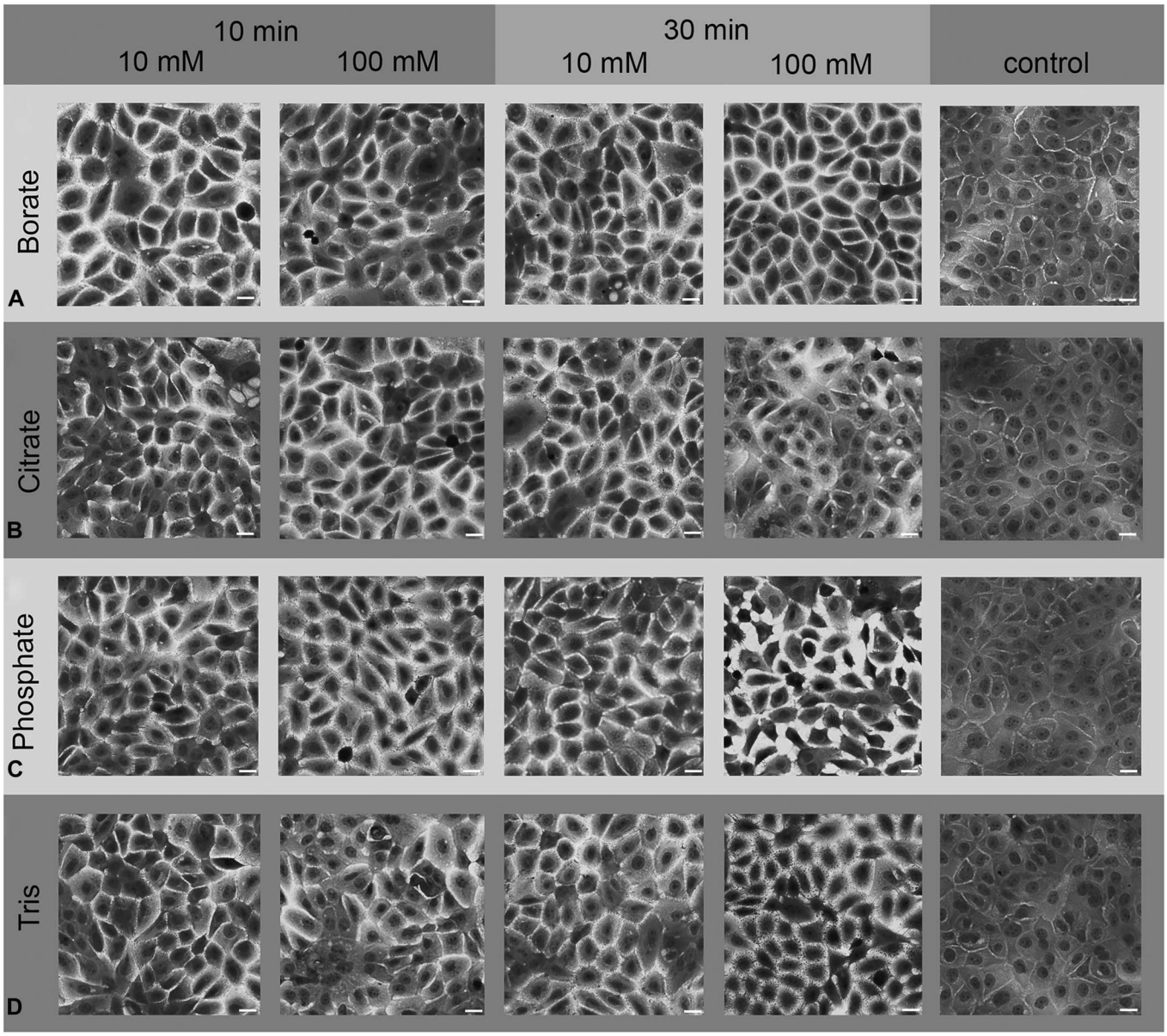

FIGURE 3. Morphological evaluation of HCLE cells treated with ocular buffers. HCLE cells were incubated in 10 mM or 100 mM borate (A), citrate (B), phosphate (C), or Tris (D) buffers for 10 or 30 minutes; the serum-free cell culture medium was used as a control. After staining with hematoxylin and eosin, images were taken with a Zeiss AxioObserver. Original magnification ×20.

In this study, we investigated the toxicity of conventional buffer systems used in ophthalmic medicinal products and devices using 2 ocular cell line models. The 2 cell lines reacted differently to incubations with buffers. In lower concentrations, all tested buffers showed significant loss in HCLE cell viability after 30 minutes of incubation compared with 10 minutes, which is to be expected as the cells were not being kept in the ideal culture conditions needed for survival. Interestingly, this phenomenon was not visible in HCjE cells, in which the 10-minute time point already showed a reduction in viability. These differences could be explained by the different functions these cells fulfill in vivo, in which the avascular corneal epithelium has mostly a barrier function, whereas the conjunctival epithelium acts as a barrier and crucial mediator of the immune response triggered by inflammation.[15] Therefore, we assume that conjunctival cells are quicker to respond to stimuli and are more sensitive than corneal epithelial cells.

No differences were observed in both cell lines between 10 mM buffers for up to 60 minutes of incubation, which suggests that the reduced ability of the cells to survive is due to an absence of culture medium rather than a specific buffer formulation. However, the highest concentration of phosphate buffer resulted in significantly lower HCLE and HCjE cells long-term viability and affected their morphology after 30 minutes of incubation.

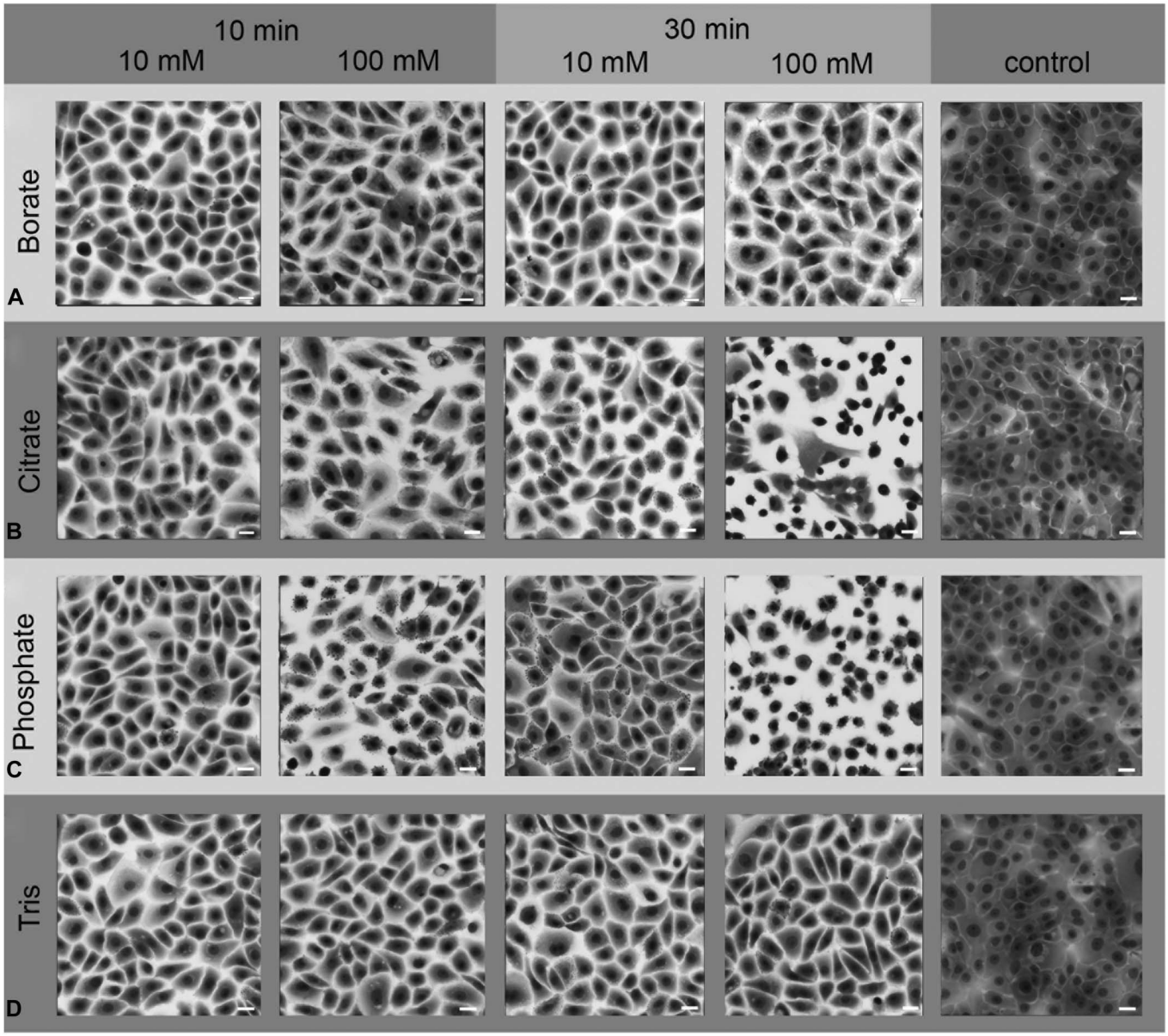

FIGURE 4. Morphological evaluation of HCjE cells treated with ocular buffers. HCjE cells were incubated in 10 or 100 mM borate (A), citrate (B), phosphate (C), or Tris (D) buffers for 10 or 30 minutes; the serum-free cell culture medium was used as a control. After staining with hematoxylin and eosin, images were taken with a Zeiss AxioObserver. Original magnification ×20.

The concentration of phosphate buffer has been investigated in ophthalmic products and in animal models. In a published study on rabbits, adverse effects of higher concentrations of phosphate buffers was reported, in which highly concentrated phosphate-buffered solution permanently altered physiological pH, even after discontinuation.[16] Although some studies showed partly severe sequelae, an evaluation by the European Medicines Agency showed hardly any risk to patients without preexisting corneal defects.[17] In Germany alone, 37% of ocular medicinal products contain phosphate buffers, which are most commonly used in antiglaucoma medications and prostaglandin-containing formulations.[18] The concentration of phosphate in tested artificial tears ranged from <0.1 mM to 68.8 mM. Forty-four percent of the samples revealed phosphate concentrations above the physiological level (1.45 mM), and in 5% of the products, there were phosphate concentrations higher than 50 mM.[19] Within the antiglaucoma medications analyzed, 47% of the formulations showed phosphate concentrations higher than the physiological level, and 19% had concentrations above 100 mM. The concentrations of the tested devices ranged from <0.1 to 160 mM.[20] Unfortunately, most patient information leaflets do not report the concentration of the buffers used.

Our investigation also revealed cytotoxic effects of citrate buffer on HCjE cells; this was observed both in the XTT assay

and morphologically and was shown to be enhanced by the time of exposure and higher concentration. It might be that the mechanism behind the cytotoxicity seen in citrate and phosphate buffers, reported to be implicated in the development of calcific band keratopathy,[6,7] is due to the ocular surface having calcium-dependent channels that maintain the osmotic balance.[21–23] Both citrate and phosphate buffers are not recommended for systems that are highly calcium-dependent, as citric acid and its salts act as calcium chelators, whereas phosphates react with calcium thereby producing insoluble calcium phosphate that precipitates out of the system.

Earlier reports suggested that borate-buffered contact lens multipurpose solutions showed increased cytotoxicity compared with phosphate-buffered multipurpose solution with mere cells[24] or with contact lenses on cells.[25] However, more recent investigations are in agreement with our findings that borate buffer is not toxic to HCLE and HCjE cells. Good biocompatibility was observed both in vitro with a 1% borate buffer (approximately 162 mM) for up to 1 hour,[26] as well as in a rabbit model.[27]

Although we found no information in the literature on Tris buffers for ocular administration, our results indicate that this buffer is the least toxic to HCjE cells from the 4 buffers tested in this study and shows similar effects as borate buffer on HCLE cells.

In summary, both phosphate and citrate buffers revealed significant cytotoxic effects at high concentrations and longer incubation times in ocular epithelial monolayers. Transferring the results of this study to a clinical setting requires thorough in vivo preclinical data, as topical application involves factors such as blinking and tear production that need to be considered. Thus, to translate these finding to humans, further experiments with stratified cells in vitro and preclinical in vivo studies in animals are planned to discern the actual effects of the buffer concentration in eye drops on the ocular surface. This study highlights the relevance of maintaining ocular homeostasis to ensure ocular surface health.

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