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Chapter

In Vitro Cytotoxicity Screening as a Criterion for the Rational Selection of Tear Substitutes

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Abstract

A large number of artificial tears are currently available in the pharmaceutical market. Selecting the right drug for the patient remains a challenge for both the doctor and the patient. Comparing the cytotoxicity of artificial tears is one of the criteria for the rational selection of a drug that promotes maximum clinical efficacy and a higher safety profile. It is known that cells grown in vitro retain many metabolic features of the parent host tissues and at the same time lack tissue and organ interrelations and regulatory effects of the nervous and endocrine systems and have very limited compensatory capabilities. These features of cell cultures provide an opportunity to investigate the interaction of chemical agents directly with the cell itself, to identify changes in cellular and subcellular structures that can be masked in whole-organism settings. This study presents the results of assessing the cytotoxicity of tear substitutes, which demonstrate that these drugs can have a cytostatic effect in vitro and differ in their cytotoxic potential. In recent years, the problem of drug therapy of patients with dry eye syndrome has been attracting increasing attention of ophthalmologists, so screening the cytotoxicity of a wide range of tear substitutes using cell culture-based test systems can promote the rational selection of these drugs.

Keywords: cell cultures, cytotoxicity, artificial tear, preservatives, buffers, cornea, ocular surface epithelium

1. Introduction

Tear substitutes are widely used in ophthalmology today and are the first-line treatment of multifactorial causes that occur in various cases of irritation of the ocular surface, including dry eye syndrome (DES). Artificial tears contain the following substances as active ingredients: sodium hyaluronate, carbomer, hydroxypropyl methylcellulose (HPMC), carmellose sodium, trehalose, as well as a combination of polyvinyl alcohol and povidone and HPMC together with dextran. In addition to the active ingredient, various preservatives are added to maintain the stability of the drops and suppress microorganism growth: benzalkonium chloride (BAC), cetrimide, cetalkonium chloride, Polyquad®, polyhexanide, and also Oxide®, Purite®, and OcuPure®. Substances that increase the viscosity (prolongators) reduce the rate of

removal of the substance from the ocular surface. These include povidone, polyvinyl alcohol, glycerol, propylene glycol, gelatin, methylcellulose, dextran 70, and carboxymethylcellulose. The next component of eye drops is antioxidants; they prevent the decomposition of the active ingredient by atmospheric oxygen. The most commonly used antioxidants are EDTA, bisulfite, thiosulfate, and metabisulfite. Eye drops may contain buffer substances (systems), which allow maintaining the pH of the drug in the range of 6–8. This is necessary to ensure that the pH of the drops is similar to the normal acidity of a human tear (7.14–7.82). With such similarity, the active substances can easily penetrate the cornea into the anterior chamber of the eye, without causing discomfort during instillation. Examples of buffers are citrate, phosphate, borate, and Tris buffers. Another important component of eye drops is osmotic agents: propylene glycol, glycerol, dextrose, and dextran. These substances provide isotonicity of eye drops in relation to the tear film and maintain osmotic pressure at the level of 305 mOsm/L. Isotonic solutions are better absorbed and well tolerated by the patient.

Thus, in addition to the main pharmaceutical ingredient, eye drops contain a number of excipients, some of which can have an adverse effect on the ocular surface, such as preservatives, buffer system components, and antioxidants.

The inclusion of preservatives in the composition of eye drops is necessary to maintain sterility and prevent their bacterial contamination. According to international standards, the addition of preservatives is mandatory in the manufacture of multidose dosage forms for topical use.

The concentration of the latter in the composition of the eye drops is relatively low; however, the cumulative dose over the entire period of use, especially with their frequent and prolonged use, can be quite high. This is especially important to remember in the context of the development of side effects that can be caused by some of the excipients in eye drops, including artificial tears [1, 2]. The preservatives in the composition of eye drops can be divided into three main types: detergents, oxidizers, and ion buffer systems. Detergent-type preservatives have a broad spectrum of antimicrobial action, which makes them quite toxic for corneal and conjunctival cells [3]. Oxidizing preservatives are less toxic than detergents, while they are effective against bacteria even at low concentrations, which minimizes their adverse effects on conjunctival and corneal epithelial cells [4]. The ion buffer preservative with an antibacterial and antifungal effect is similar to oxidizing agents in its mechanism of action [5]. It is less cytotoxic for the cells of the ocular surface than conventional preservatives but is not yet included in the composition of tear substitutes currently [6].

In the United States, many comparative clinical studies have been conducted to assess the efficacy of tear substitutes. In the published report of the Dry Eye Workshop, it was noted that despite the fact that many tear substitutes improved the ocular surface condition, there was no reliable evidence that any of the drugs was superior to another, while the inflammation of the ocular surface could worsen in the presence of preservatives in their composition [7, 8].

Recently, studies of not only developed but already available drugs have been increasingly using in vitro test systems, among which models with monolayer cell cultures are the simplest and most accessible ones [9–11]. Cells grown in vitro retain many metabolic features of the parent host tissues and at the same time lack tissue and organ interrelations and regulatory effects of the nervous and endocrine systems and have very limited compensatory capabilities. These features of cell cultures provide an opportunity to investigate the interaction of chemical agents directly with the cell itself, to identify changes in cellular and subcellular structures that can be masked in whole-organism settings. It is known that cells affected by various biologically active substances can undergo changes in morphology, cell growth rate, time of death, and degree of disintegration; therefore, it is advisable for each dosage form to assess its effect on cell survival [12]. In toxicology studies,

various cell cultures are used. They differ in origin, belonging to a particular tissue type, sensitivity to various xenobiotics, as well as in their ability to proliferate. The selection of a test system in each case depends on the purposes and objectives of the study. To assess the safety of ophthalmic drugs, the most informative are test systems based on human eye tissue cells [13–18].

The purpose of this study was to comparatively analyze the cytotoxic effect of 21 tear substitutes on human corneal epithelial cells in vitro.

2. Materials and methods

2.1 Test drugs

The study object was 11 tear substitutes with various systems of preservatives (**Table 1**) and buffer systems (**Table 2**), Systane® Ultra, Artelac® Balance, Optive®, Cationorm®, Vismed® Light, Blink® contacts, Stillavit®, Ophtolique®, Lacrisifi®, Hypromellose®-P, and Slezin®, and 7 preservative-free tear substitutes, Hylabak®, Thealoz®, Thealoz Duo®, Hylo-Comod®, Hyloparin-Comod®, Hylosar-Comod®, and Hylomax-Comod® (**Table 2**).

2.2 Cell cultures used

Cells of the immortalized human corneal epithelial cell (HCE) line were used as a test system. This test system has a higher sensitivity to the action of tear substitutes, compared with a test system based on the permanent human conjunctival cell line (Chang conjunctiva, clone 1-5c-4) [17].

2.3 Study design and methods

The effect of artificial tear eye drops on the viability of human corneal epithelial cells was studied in vitro during culturing the cells in Keratinocyte-SFM growth medium (Gibco, USA) containing the test drugs at a concentration of 10% of the

Chemical glass of preservative	Name of preservative/antioxidant	Trade name of tear substitute
Detergents	BAC, EDTA	Slezin®
	BAC, EDTA	Hypromellose®-P
	BAC, EDTA	Lacrisifi®
	BAC, EDTA	Ophtolique®
	Polyhexanide, EDTA	Vismed® Light
	Polyquad®	Systane® Ultra
	Cetalkonium chloride	Cationorm®
Oxidants	Stabilized oxychloro complex	
	Purite®	Optive®
	OcuPure®	Blink® contacts
	Stabilized chlorite complex	
	Oxide®	Artelac® Balance
Other	EDTA	Stillavit®

Table 1.
The main groups of preservatives/oxidants in the tear substitutes.

Buffer systems	Tear substitutes
Citrate buffer (C)	Hylo-Comod®, Hyloparin-Comod®, Hylomax-Comod®, Hylosar-Comod®
Phosphate buffer (P)	Lacrisifi®
Borate buffer (B)	Systane® Ultra, Blink® contacts, Slezin®
Tris buffer	Cationorm®, Hylabak®, Thealoz®, Thealoz Duo®
Combination of buffers	Optive® (B + C), Vismed® Light (P + C), Stillavit® (B + P)
No buffer	Artelac® Balance, Ophtolique®

Table 2.
Buffer systems in the study of tear substitutes.

medium volume at 37°C in a CO_2 incubator in an 5% CO_2 atmosphere. Cells cultured under standard conditions without the addition of drugs were used as control cells. The concentration of tear substitutes for the experiment was selected on the data of clinical use of the test drugs and our own cytotoxicity studies of artificial tears on cell cultures [17]. Cell viability was assessed by their morphology and functional activity using phase-contrast microscopy (FCM) methods, MTT test, and xCELLigence system.

The morphology of the cells in the course of their culturing with the test drugs was evaluated using an inverted Nikon Eclipse TS100 microscope equipped with a camera. To evaluate the effect of tear substitutes on the metabolic activity of human corneal epithelial cells by MTT method, the cells were inoculated in 96-well plates in 200 μl of the growth medium containing the test drugs and cultured as usual for 2 days. After the culturing period, MTT test was performed. The absorbance of the solutions was measured using Fluorofot Charity analyzer (Russia) at a wavelength of 570 nm and a reference wavelength of 630 nm. Mathematical processing of the data was performed by variation statistics methods using Microsoft Excel 2007. Differences were considered significant at $p < 0.05$.

To evaluate the effect of tear substitutes on the adhesion and proliferative activity of corneal epithelial cells using xCELLigence system, 1×10^4 HCE cells were inoculated per well of the E-plate in 100 μl of the growth medium containing the test drugs. The plates were placed in the real-time cell analyzer dual purpose (RTCA-DP) (ACEA Biosciences), and adhesion and cell proliferation dynamics was monitored in real time for 24 h. The results were analyzed using RTCA Software 1.2.1 (Roche). The change in impedance at microelectrodes due to cell attachment and spreading was expressed as Cell Index; the value of which is automatically calculated by the program: Cell Index = (RnRb)/t, where Rb is the initial impedance value in the well containing the cell growth medium only (negative control) and Rn is the impedance value at any time t in the well containing the test cells (positive control) in addition to the growth medium. The Cell Index thus reflects changes in the number of cells, the quality of cell attachment, and the morphology of the cells in the well, which may vary over time. The data were presented as the mean value (M) ± standard deviation, the significance of differences was calculated by the Mann-Whitney U-test, and differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1 MTT test

The MTT test, commonly known as a screening method for measuring cell survival and included in most protocols of molecular biology and medicine [19],

revealed differences in the effect of the test tear substitutes on the metabolic activity of the corneal epithelial cells. The results of the MTT test are presented as a histogram, where the viability of cells cultured in growth media with the addition of eye drops is expressed as a percentage relative to the control (**Figure 1**).

The MTT test showed that the tear substitutes containing preservatives from the group of detergents, especially BAC at various concentrations, have the greatest toxicity to corneal epithelial cells: Lacrisifi® (BAC 0.1 mg/mL), Slezin® (BAC 0.075 mg/mL), Ophtolique® (BAC 0.1 mg/mL), Hypromellose®-P (BAC 0.1 mg/mL), and Cationorm® (cetalkonium chloride). Cell viability in the presence of these drugs was close to zero. The exceptions in this group were Vismed® Light (polyhexanide), which did not have a toxic effect at the studied concentration, and Systane® Ultra (Polyquad®), which had a moderate toxic effect. This was probably due to the fact that Vismed® Light contains the preservative polyhexanide, which is rarely included in the composition of tear substitutes. This preservative has a limited antifungal activity and no irritating effect on the human corneal epithelial cells [5]. The eye drops Systane® Ultra contain Polyquad® (polydronium chloride), which is a detergent-type preservative derived from BAC. It is unique in that bacterial cells attract Polyquad®, while corneal epithelial cells tend to repel it. Despite the occurrence of some superficial epithelial lesions, it is better tolerated than other detergent-type preservative agents [20]. Extremely high toxicity to corneal epithelial cells was also shown by the tear substitutes containing oxidants as preservatives: the stabilized oxychloro complex Optive® (Purite®) and the stabilized chlorite complex Artelac® Balance (Oxide®). The least toxic in this group was the Blink® tear substitute with OcuPure® as a preservative. It is thought that oxidative preservatives have a mild cytotoxic effect and are well tolerated and safe [21]; however, it has been found that this group of preservatives can cause superficial punctate keratitis with prolonged use [22]. The group of tear substitutes consisting of only one drug (Stillavit®), which showed moderate toxicity compared to artificial tears with detergent- and oxidative-type preservatives, includes the antioxidant EDTA (sodium edetate). It is a chelating agent, which, while not being a true preservative, can increase the antimicrobial activity of the main disinfectant while reducing its concentration. It chelates the divalent cations of calcium and magnesium, making the microorganisms more vulnerable to the preservative. Since EDTA chelates calcium and magnesium ions, it can also have a slight toxic effect on the corneal

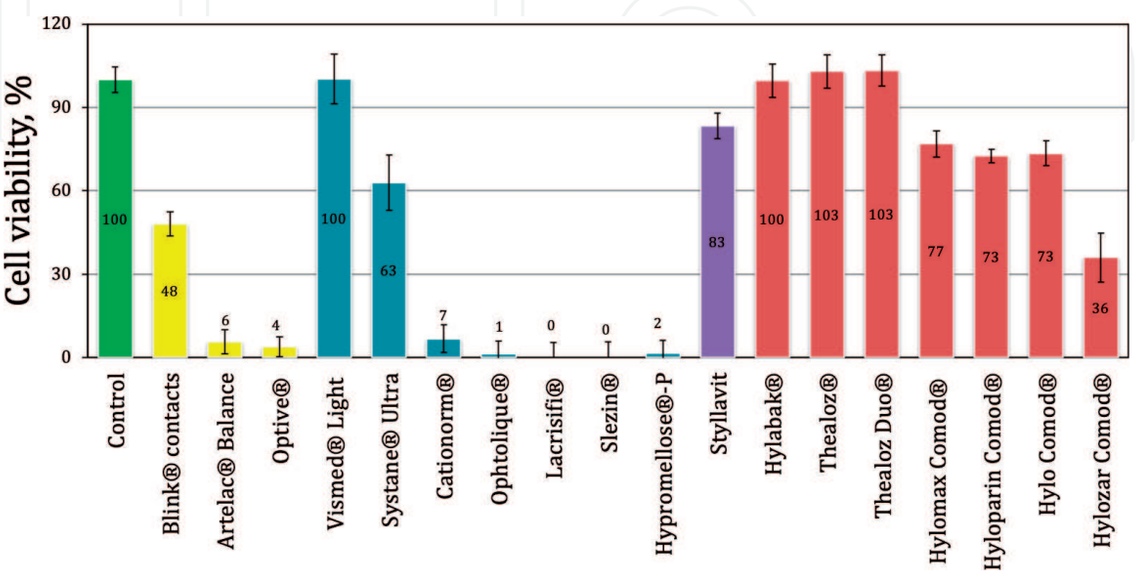


Figure 1.
HCE viability histograms on day 3 of culturing in the medium with a concentration of the test drugs of 10% of the growth medium volume. The drugs were added to the growth medium at the time of inoculating the cells.

cells, which need these ions for metabolism. Although EDTA does not generally have a pronounced toxic effect, there is evidence that patients with severe DES often complain of discomfort after using drugs containing EDTA [23].

The results of the study indicate the cytotoxic effect of tear substitutes with different chemical groups of preservatives on corneal epithelial cells. In scientific literature, this problem is currently covered quite objectively and completely. In addition to the active ingredient, preservatives, and some other excipients, tear substitutes contain various buffer systems (**Table 2**), which can have an adverse effect on corneal and conjunctival epithelial cells. Information on the comparative toxicity of the buffer systems included in the eye drops is almost not available. Nevertheless, separate reports present data on the occurrence of keratopathy and deposition of calcium hydroxyapatite in a transparent layer of the cornea, after using eye drops containing phosphate buffer [24, 25]. Phosphate-containing tear substitutes are widely used in the composition of ophthalmic dosage forms in EU countries, about a third of all buffered drugs contain phosphates as a buffer. The European Committee on Human Medicinal Products (CHMP) gives preference to the use of phosphate-containing drugs, reasoning that the risks do not exceed adverse reactions that occur during their use, since the proportion of complications is less than 1 case per 10,000 sold vials of tear substitutes. Calcification is a multifactorial complication and can occur without the use of phosphate-containing drugs. Preference in selecting phosphate-containing tear substitutes should be given if it is consistent with the low risk of corneal calcification, especially in serious pathology, and on an individual case basis. It is not currently clear what phosphate concentration is critical for the onset of corneal calcification. Quite recently, preference has been given to borate buffers, which possess antimicrobial activity, showed good biocompatibility with the ocular surface both in vivo and in vitro and are considered safer [26, 27]. Tris buffers are also included in some dosage forms and have been found to be effective and low toxic [28].

As our studies have shown, the viability of human corneal epithelial cells depends, among other things, on the composition of the buffer system used in tear substitutes containing no preservatives. The lowest metabolic activity of cells in the study of a line of preservative-free tear substitutes was observed in the presence of Hylosar-Comod® containing citrate buffer together with dexpanthenol. The pronounced cytotoxic effect of Hylosar-Comod® on corneal epithelial cells can be due to the sensitivity of this test system to the combination of the drug ingredients. This question requires further investigation. A higher level of metabolism in cells was detected in the presence of three preservative-free tear substitutes containing citrate buffer (Hylomax-Comod®, Hyloparin-Comod®, and Hylo-Comod®). The average level of cell viability in this case ranged from 73 to 77%. The preservative-free tear substitutes Hylabak®, Thealoz®, and Thealoz Duo® with Tris buffer showed no toxicity to corneal epithelial cells in our studies (**Figure 1**).

3.2 xCELLigence analysis

Based on the data obtained in the MTT test, eight products with various types of preservatives were selected from a wide range of tear substitutes for xCELLigence analysis: Artelac® and Blink® (oxidants), Ophtolique® and Systane® Ultra (detergents), Stillavit® (EDTA), Hylo-Comod®, Thealoz Duo®, and Hylosar-Comod® (preservative free). These drugs within their groups showed varying degrees of toxicity to the metabolic activity of corneal epithelial cells. The xCELLigence real-time cell analyzer (RTCA) technology is based on the use of microelectronic cell sensors integrated into the bottom of the wells of special culture plates (E-Plate). The resistance measured between the electrodes in a separate well depends on the

geometry of the electrode, the concentration of ions in the well, and whether the cells are attached to the electrodes. In the absence of cells, the electrode resistance is mainly determined by the ionic environment both at the electrode/solution interface and in the entire volume. The cells attached to the electrode surfaces act as insulators and thus change the local ionic medium at the electrode/solution interface, which will increase the resistance. Thus, the more cells spread on the electrodes, the higher the resistance of the electrodes. The presence of cells on the electrodes in the wells of the E-Plate affects the local state of the ionic environment, which leads to a change in the resistance on the electrodes. The Cell Index value is an indicator of electrical potential, which reflects the cell status. The Cell Index can be used for real-time monitoring of cell viability: their morphology, degree of adhesion, cell growth (proliferation) dynamics, and other important parameters [12]. Continuous monitoring of the effect of tear substitutes on the HCE cell line in real time using the xCELLigence system revealed that the studied drugs at a concentration of 10% of the growth medium volume manifested varying degrees of toxicity to the cultured cells. In the course of monitoring, Cell Index-cell cultivation time plots were obtained, that made it possible to assess the cell viability by the degree of their spreading and proliferative activity (**Figure 2**).

As can be seen from the plots, of all the drugs tested using the xCELLigence system, the tear substitute Ophtolique® with BAC has the highest toxicity to the corneal epithelial cells (**Figure 2B**). The Cell Index equal to zero throughout the observation period indicates that cell adhesion has never occurred. In the presence of Artelac® Balance (**Figure 2A**) containing the preservative Oxide®, adhesion occurs within 1 h, but after 5 h, the cells begin to detach from the bottom of the wells, and after 10 h, no viable cells were detected. The xCELLigence analysis revealed an adverse effect of preservative-free Hylosar-Comod® containing citrate buffer with dexpanthenol on the cell culture (**Figure 2D**). This drug turned out to be the most toxic drug from the group of preservative-free tear substitutes; its cytotoxic effect was comparable to that of Blink® containing an oxidative-type preservative (**Figure 2A**). In the presence of these drugs, the cells could only adhere and spread. In the presence of Hylo-Comod® (**Figure 2D**) and Systane® Ultra (**Figure 2B**), the corneal epithelial cells adhered and spread, but their proliferation dynamics was low. Cell proliferation in the presence of Stillavit® (**Figure 2C**) was slightly lower than in the control, and the presence of Thealoz Duo® (**Figure 2D**) was comparable to the control, indicating that Stillavit® was moderately toxic and Thealoz Duo® did not exhibit toxicity at the given concentration to corneal epithelial cells.

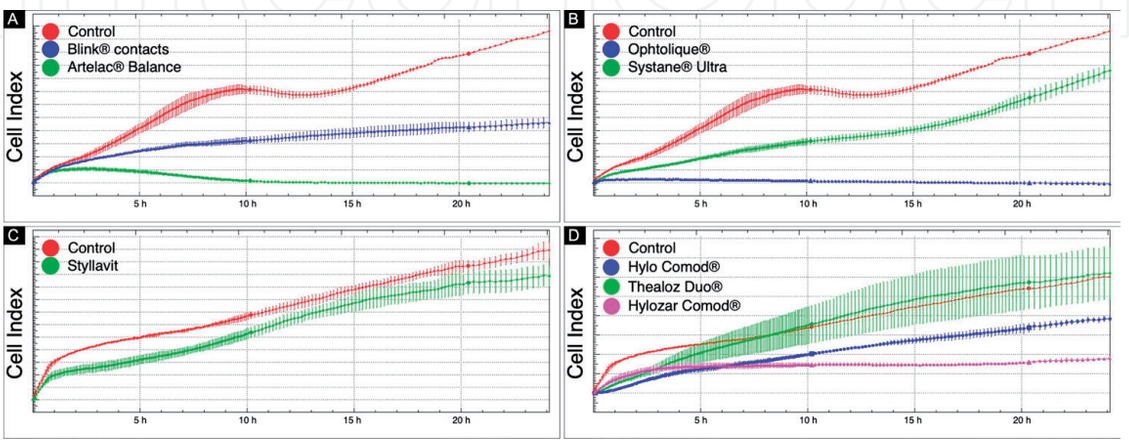


Figure 2.
Monitoring of the effect of tear substitutes with various types of preservatives ((A) oxidants, (B) detergents, (C) EDTA, (D) preservative-free drugs) on the viability of HCE cells in real time (proliferation curves). xCELLigence cell analysis.

3.3 Observation of the cells morphology

The results of observation of the HCE cell morphology during their culturing in media containing 10% PCTs with various preservative systems are shown in **Figure 3**. In the pictures presented, the human corneal cells in the control are well spread, have a typical epithelium-like morphology, and have formed a confluent monolayer on cultivation day 3 (**Figure 3E**).

The morphology of the cells in the presence of the tear substitute Thealoz Duo® is comparable to the control. A slightly less dense monolayer than in the control is formed by the cells in the presence of Stillavit® in the growth medium. With Hylo-Comod®, the monolayer of cells is less dense than in the presence of Thealoz Duo® and Stillavit®. Most of the cells in the presence of the former drug are well spread, but their cytoplasm has a granular structure; a lot of unattached cells are observed. In the presence of the tear substitutes Blink® and Systane® Ultra, the monolayer is formed by about 50%; the cells have a vacuolated granular cytoplasm, which indicates their supressed state. With Hylosar-Comod®, a part of the cells have adhered, and intercellular contacts are found between them; in the presence of Artelac®, only single adherent cells are detected, and in the presence of Ophtolique®, no spread cells are found at all; most of the cells have a rounded shape. The cell structure is granular, with vacuoles; invagination of the cytoplasmic membrane is observed. Many cellular fragments are found in the growth medium. These observations suggest a launch of cell death processes. Thus, the results of the MTT test are consistent with the results of the xCELLigence cell analysis and the cell morphology analysis using phase-contrast microscopy methods.

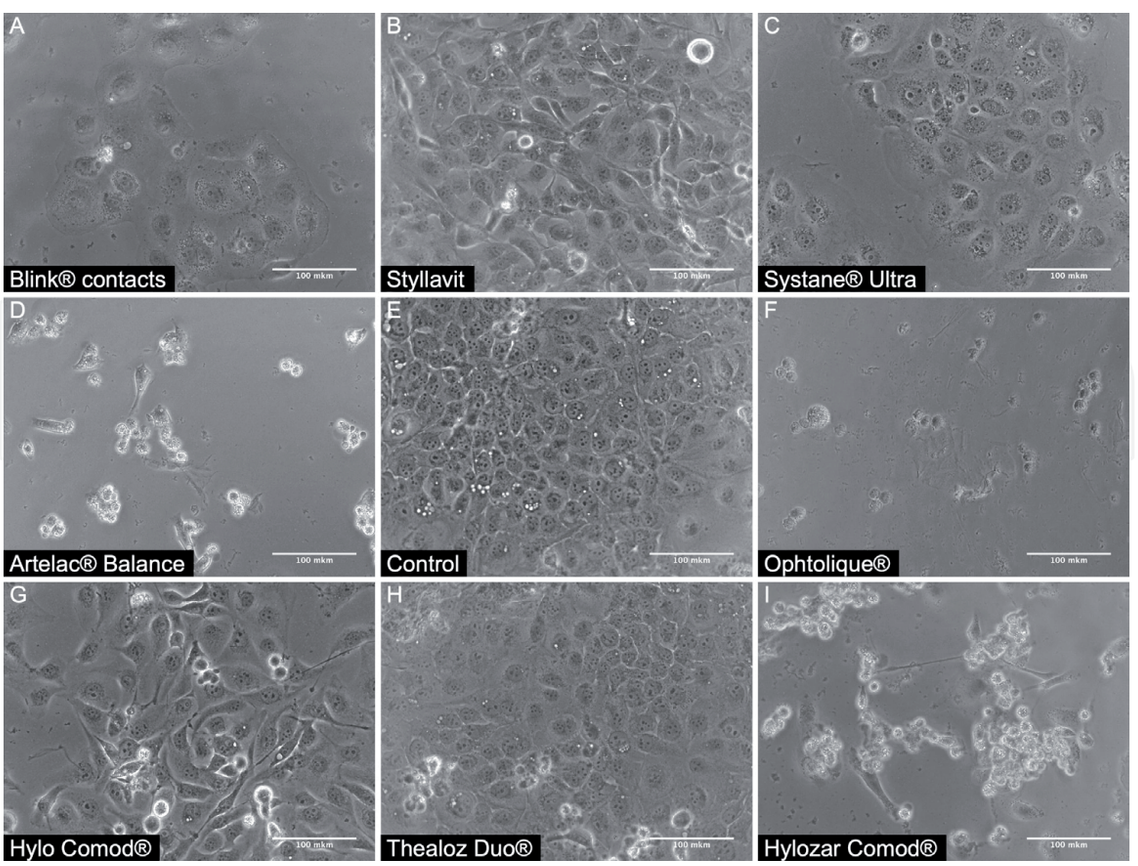


Figure 3.
Morphology of HCE cell line on the third day of culturing in a growth medium containing 10% of the test PCTs with various types of preservatives: oxidants (A, D), detergents (C, F), EDTA (B), and preservative-free drugs (G, H, I). Scale ruler 100 nm (20×). Phase-contrast microscopy.

4. Conclusion

A large number of artificial tears are currently available in the pharmaceutical market. Selecting the right drug for the patient remains a challenge for both the doctor and the patient. This study presents the results of assessing the cytotoxicity of tear substitutes, which demonstrate that these drugs can have a cytostatic effect in vitro and differ in their cytotoxic potential. Comparing the cytotoxicity of artificial tears is necessary for the rational selection of a drug that promotes maximum clinical efficacy and a higher safety profile. The tear substitutes Hylabak®, Thealoz®, and Thealoz Duo® that do not contain preservatives in their composition had not cytotoxic effect on the cells. Vismed® Light containing the preservative polyhexanide was not toxic, either. It was found that the so-called mild preservatives can also have an adverse effect on the ocular surface. Among the artificial tears, the greatest toxic effect on corneal epithelial cells was observed in the tear substitutes Ophtolique®, Lacrisifi®, Hypromellose®, Slezin®, and Cationorm® containing BAC at various concentrations as preservative and in the artificial tears Artelac® Balance (Purite®) and Optive® (Oxide®). The study showed the possibility in principle to use in vitro systems for the comparative assessment of the cytotoxic effect of tear substitutes. It should be noted, however, that the test system under consideration has a number of limitations due to the very nature of the method. In particular, studies on cell cultures cannot take into account such aspects that are important in terms of general toxicology as the route of delivery of a chemical agent into the body, its distribution, elimination, and other toxicodynamics issues. Like with the use of other model test systems, extrapolation of the results obtained to the whole body requires great caution, especially when it comes to quantitative indicators. However, in vitro testing provides information on the potential effects of drugs and their specific effects. Given that the problem of drug therapy of patients with DES has been recently attracting increasing attention of ophthalmologists due to both the increasing prevalence of DES and the increasing range of "artificial tear" drugs, screening the cytotoxicity of a wide range of tear substitutes using test systems based on cell cultures can promote the rational selection of these drugs.

Conflict of interest

The author declares no competing interests.

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References

[1] Baudouin C, Labbe A, Liang H, Pauly A, Brignole-Baudouin F. Preservatives in eye drops: The good, the bad and the ugly. Progress in Retinal and Eye Research. 2010;**9**:312-334. DOI: 10.1016/j.preteyeres.2010.03.001

[2] Whitson J, Petroll W. Corneal epithelial cell viability following exposure to ophthalmic solutions containing preservatives and/or antihypertensive agents. Advances in Therapy. 2012;**29**:874-888. DOI: 10.1007/s12325-012-0057-1

[3] Tu E. Balancing antimicrobial efficacy and toxicity of currently available topical ophthalmic preservatives. Saudi Journal of Ophthalmology. 2014;**28**(3):182-187. DOI: 10.1016/j.sjopt.2014.06.006

[4] Elder D, Crowley P. Antimicrobial preservatives part one: Choosing a preservative system. American Pharmaceutical Review. 2012. Available from: http://www.americanpharmaceuticalreview.com/Featured-Articles/38886-Antimicrobial-Preservatives-Part-One-Choosing-a-Preservative-System/

[5] Freeman P, Kahook M. Preservatives in topical ophthalmic medications: Historical and clinical perspectives. Expert Review of Ophthalmology. 2009;**4**(1):59-64

[6] Ammar D, Noecker R, Kahook M. Effects of benzalkonium chloride-preserved, polyquad-preserved, and sofZia-preserved topical glaucoma medications on human ocular epithelial cells. Advances in Therapy. 2010;**27**(11):837-845. DOI: 10.1007/s12325-010-0070-1

[7] Schiffman R, Christianson M, Jacobsen G, Hirsch J, Reis B. Reliability and validity of the ocular surface disease index. Archives of Ophthalmology. 2000;**118**:615-621

[8] Management and therapy of dry eye disease: Report of the management and therapy Subcommittee of the International Dry Eye WorkShop (2007). The Ocular Surface. 2007;**5**(2):163-178

[9] Danchenko EO. Evaluation of cytotoxicity of pharmaceutical substances using cell cultures. Immunopathology, allergology, infectology=Immunopatologija, Allergologija, Infektologija. 2012;**2**: 22-31. (In Russ.)

[10] Eropkin MJ, Eropkina EM. The Cell Cultures as a Model System Toxicity Studies and Screening of Cytoprotective Drugs. SPb.: Morsar AV; 2003 (in Russ.)

[11] Anikina LV, Puhov SA, Dubrovskaja ES, Afanas'eva SV, Klochkov SG. Comparative determination of cell viability using the MTT and Resazurin. Fundamental Research = Fundamental'nye issledovaniya. 2014;**12**:1423-1427. (In Russ.)

[12] Urcan E, Haertel U, Styllou M, Hickel R, Scherthan H, Reichl F. Real-time xCELLigence impedance analysis of the cytotoxicity of dental composite components on human gingival fibroblasts. Dental Materials. 2010;**26**(1):51-58. DOI: 10.1016/j.dental.2009.08.007

[13] Aleksandrova OI, Okolov IN, Takhtaev YV, Khorolskaya YI, Khintuba TS, Blinova MI. Comparative evaluation of the cytotoxicity of antimicrobial eye drops. Ophthalmological Bulletin. 2015;**8**(1):89-97. (In Russ.)

[14] Aleksandrova OI, Khorolskaya YI, Maychuk DY, Blinova MI. A study of the overall cytotoxicity of aminoglycoside and fluoroquinolone antibiotics on

cell cultures. Ophthalmology Bulletin. 2015;**5**:39-48. (In Russ.)

[15] Aleksandrova OI, Okolov IN, Khorolskaya YI, Blinova MI, Churakov TK. Evaluation of the effect of benzalkonium chloride on the cytotoxicity of Nettacin and Tobrex eye drops in vitro. Modern Technologies in Ophthalmology. 2016;**3**:163-166. (In Russ.)

[16] Aleksandrova OI, Okolov IN, Khorolskaya YI, Panova IE, Blinova MI. Possibilities of cellular technologies for rational pharmacotherapy of ocular pathologies. Modern Technologies in Ophthalmology. 2017;**7**:5-7. (In Russ.)

[17] Aleksandrova OI, Okolov IN, Khorolskaya YI, Panova IE, Blinova MI. Evaluation of cytotoxicity of tear substitutes using an in vitro system. Ophthalmology. 2017;**14**(1):59-64. DOI: 10.18008/1816-5095-2017-1-59-66. (In Russ.)

[18] Aleksandrova OI, Okolov IN, Khorolskaya YI, Panova IE, Blinova MI. The effect of non-steroidal anti-inflammatory eye drops on corneal and conjunctival epithelial cells in vitro. Ophthalmology. 2017;**15**(3):251-259. DOI: 10.18008/1816-5095-2017-3-251-259. (In Russ.)

[19] Langdon SP, editor. Cancer Cell Culture: Methods and Protocols. Ser. Methods in Molecular Medicine. Vol. 88. Totowa, NJ: Humana Press; 2003. p. 165-169

[20] Epstein S, Ahdoot M, Marcus E, Asbell P. Comparative toxicity of preservatives on immortalized corneal and conjunctival epithelial cells. Journal of Ocular Pharmacology and Therapeutics. 2009;**25**(2):113-119

[21] Noecker R. Effects of common ophthalmic preservatives on ocular health. Advances in Therapy. 2001;**18**(5):205-215

[22] Schrage N, Frentz M, Spoeler F. The ex vivo eye irritation test (EVEIT) in evaluation of artificial tears: Purite-preserved versus unpreserved eye drops. Graefe's Archive for Clinical and Experimental Ophthalmology. 2012;**250**(9):1333-1340. DOI: 10.1007/s00417-012-1999-3

[23] Samar K. Basak preservatives and ocular surface diseases. Kerala Journal of Ophthalmology. 2016;**18**(4):311-316

[24] Schrage N, Frentz M, Reim M. Changing the composition of buffered eye-drops prevents undesired side effects. The British Journal of Ophthalmology. 2010;**94**:1519-1522. DOI: 10.1136/bjo.2009.177386

[25] Mueller-Lierheim W. Traenenersatz- und Kontaktlinsenbenetzungsloesungen. In: Köln Biermann, editor. Aktuelle Kontaktologie; 2015. 8-15

[26] Houlsby R, Ghajar M, Chavez G. Antimicrobial activity of borate-buffered solutions. Antimicrobial Agents and Chemotherapy. 1986;**29**:803-806

[27] Lehmann D, Cavet M, Richardson M. Nonclinical safety evaluation of boric acid and a novel borate-buffered contact lens multi-purpose solution, Biotrue™ multi-purpose solution. Contact Lens & Anterior Eye. 2010;**33**(Suppl 1):S24-S32. DOI: 10.1016/j.clae.2010.06.010

[28] Graupner O, Hausmann C. The alternation of the pH in the anterior chamber of the rabbits eye burned with smallest volumes of high concentrated acid and base [in German]. Albrecht von Graefes Archiv für Klinische und Experimentelle Ophthalmologie. 1968;**176**:48-53