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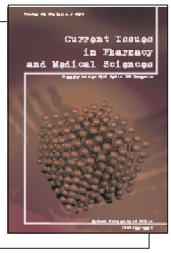

Ultrastructural and morphohistochemical study of the influence of benzalkonium chloride on human corneal limbal epithelial cells in vitro

PAWEŁ BIELIŃSKI[1*], EWA RAKOWSKA[1], ANNA WYSZYŃSKA[1], MONIKA JASIELSKA[1], JERZY A. WALOCHA[2], JERZY MACKIEWICZ[1]

[1] Department of Surgery of the Retina and Vitreous, Medical University of Lublin, Lublin, Poland
[2] Department of Anatomy, Collegium Medicum, Jagiellonian University of Cracow, Cracow, Poland

ABSTRACT

Within the corneal limbal epithelium there exist limbal stem cells (LSC) which under suitable conditions can regenerate their population or differentiate into corneal epithelial cells. However, upon limbal damage, the cells differentiate irreversibly and do not self-renew. One of the causes of the damage of progenitor cells and their niche is a long-term use of eye drops containing preservatives. While the side effects are related to the antimicrobial activity of such eye drops, damage to cellular and cytoplasmic membranes as well as enzymatic reactions can concurrently cause disorders of normal ocular surface tissue. The aim of this study was to evaluate the toxic effects of the preservative used in eye drops – benzalkonium chloride (BAK) – on human corneal limbal epithelial cells in vitro, and to define the mechanisms of acute limbal cell damage caused by the action of BAK. Ten corneoscleras rims, which were not qualified for transplantation by the Eye Tissue Bank, were obtained from 5 deceased donors aged 39 to 43 years. The tissue fragments (explants) containing corneal limbal epithelial cells were immediately after the explantation subjected to the action of the experimental substance being benzalkonium chloride (BAK) in concentrations of 0.005% and 0.01%. The qualitative analysis of microscopic images of the corneal limbus specimens was performed on tissue sections stained with hematoxylin and eosin using the immunohistochemical method for vimentin and with the use of a transmission electron microscope. The structure of the area of corneal limbus, as well as the morphological characteristics and the ultrastructure of the very limbal cells were evaluated with careful attention to the basal epithelial cells of the limbus. The BAK-treated groups of explants in sections stained by H & E featured characteristics of severe structural disorders of the corneal limbus area. Depletion of the epithelial cells was visible and involved both superficial and deep layers. Immunohistochemical staining for vimentin did not show the expression of this protein. This might have been connected with the damage to the cytoskeleton of limbal epithelial cells and large depletion of cells reaching down to the basement membrane. The images obtained with electron microscopy demonstrate serious defects of cell ultrastructure and, indirectly, abnormal cellular metabolism, including water and electrolyte balance and energy metabolism.This experiment confirmed the significant adverse effect of benzalkonium chloride on the limbal epithelial cells and the possibility of their damage.

Keywords: limbal stem cell, preservative, benzalkonium chloride, limbal insufficiency

INTRODUCTION

The cornea is considered one of the most specialized areas of the human body. It consists of five layers: epithelium, Bowman's layer, stroma (*substantia propria*), Descemet's membrane and endothelium.

The corneal epithelium is subject to constant renewal and regeneration. The cells of the superficial layers of the corneal epithelium undergo physiological exfoliation while winking and as a result of minor injuries. In order to retain the structure of the ocular surface they must be replaced by new cells. Proliferation is restricted to the deep layer of cells adjacent to the *membrana propria* of the epithelium located in corneal limbus [5]. Only the cells in contact with the basement membrane have the ability of mitotic activity, whereas their movement to the superficial layers of the cornea devoids them of such ability. Maturing epithelial cells are characterized by both vertical and horizontal movement. The proliferation of the basal epithelial cells of limbus, as well as the movement of the cells in the horizontal and vertical plane, constitute

Corresponding author
* Department of Surgery of the Retina and Vitreous,
Medical University of Lublin, 1 Chmielna Str., 20-079 Lublin, Poland
e-mail: p.a.bielinski@gmail.com

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the dynamic model responsible for maintaining the balance of corneal epithelial tissue [15].

The most active area of the limbus responsible for the regeneration of the epithelium is the transition zone from conjunctival stratified squamous epithelium to corneal stratified squamous epithelium along with the deeper located foldings of the basement membrane, the so-called palisades. They consist of the basal corneal limbal cells, among which we can distinguish limbal stem cells (LSC) and transient amplifying cells (TAC) [18].

In 1971 Evansen and Davenger were the first to present the concept of limbal location of the epithelial stem cells [7]. They claimed that particular areas of the limbus involving papillary structures of the limbal cells' basement membrane contain a specific cell type responsible for the renewal of the corneal epithelium (LSC) and form the so-called palisades of Vogt (Figure 1). Since then, many publications have upheld the concept of limbal location of stem cells [18, 19].

In 1983 Schoefield put forward the hypothesis of the limbal stem cell niche. According to this hypothesis, an appropriate regulation of LSC (survival, rate of proliferation and inhibition of differentiation) is ensured by numerous endogenous and exogenous factors [17]. Microenviromental interactions, such as cell-intercellular substance and intercellular interactions are of significant importance. LSC niche stroma, consisting of *inter alia* stromal (*substantia propria)* cells and numerous blood vessels, also affects normal stem cell renewal by releasing blood-derived cytokines, growth factors and survival factors. Indirectly, an additional effect may also be produced by other components of the microenvironment such as fibroblasts and corneal epithelium, conjunctival fibroblasts and epithelium, as well as vessel endothelium and muscle cells [17, 19].

BENZALKONIUM CHLORIDE

Preservatives, which are widely used in ophthalmic drops, artificial tears or contact lens solutions (e.g. benzalkonium chloride, cetrimide, thimerosal, chlorobutanol, chlorhexidine, EDTA) are used to avert the development of pathogenic microorganisms in these preparations, and thus prevent the infections of the eyeball surface [11].

Benzalkonium chloride (benzyl-dimethyl-tridecyl-azanium chloride) belongs to quaternary ammonium group of optimal activity in the range of pH 4–10. Biological activity of benzalkonium chloride is based on interactions with proteins, fats and guanidine triphosphate binding proteins (G proteins) in cell membranes [11].

In ophthalmic solutions benzalkonium chloride is usually in the concentration of 0.005-0.02%. It has anti-yeast, anti-fungal, anti-Gram-negative and anti-Gram-positive activity [11]. BAK has also a number of side effects, which may initially manifest themselves as intolerance to a specific drug in the mechanism of toxic or immunological reaction [1].

OBJECTIVES

The objective of this study was to assess the toxicity of benzalkonium chloride – preservative widely used in eye solutions – to human corneal limbal epithelial cells during one-hour exposure in vitro, and to determine the mechanisms of the acute limbal cell damage caused by the action of BAK.

MATERIALS

Ten corneoscleral rims from the Eye Tissue Bank at the I Department of Ophtalmology, Medical University of Lublin, not qualified for transplantation (mechanical defects of the transparent cornea) were obtained from 5 deceased donors aged from 39 to 43 (median age – 40.8). Having been removed, the rims were stored in 20ml of Eusol C at 4°C.The time elapsing from the explantation of the rims to treating the explants with the tested substance amounted to 36 hours for all corneas.

Tissue fragments of 2mmx3mm in size (so-called explants) containing corneal limbal epithelium were collected from the areas of corneal limbus of the rims. The explants were subjected to the action of a preservative – benzalkonium chloride (BAK) – commonly used in ophthalmic drops, in the concentrations of 0.005% and 0.01%.

Benzalkonium chloride was dissolved in 0.9% NaCl solution to a reach the concentration of 0.005% and 0.01% (CMC = 0.02%). The solutions of the above mentioned substances were prepared directly before use and used in the volume of 5 ml during the exposure. Control groups were subjected to the action of the same volume of 0.9% NaCl solution.

Immediately after being removed from the corneoscleral rims, the sections were treated for 1 hour with 0.9% NaCl solution (group IA), 0.005% and 0.01% BAK (group IB and IC respectively).

The qualitative analysis of microscopic images of the corneal limbus specimens was performed on the tissue sections stained with hematoxylin and eosin, using immunohistochemical method using Vimentin KIT (Primary Antibody: Mouse Monoclonal Anti-vimentin in buffered saline; Biotinylated Secondary Antibody: Goat Anti-mouse Immunoglobulin in buffered saline; AEC Chromogen: 3-Amino-9-Ethylcarbazole in N,N-Dimethylformamide) in and with the use of a transmission electron microscope. The analysis included 90 sections. The results of immunohistochemical and histomorphological tests were evaluated using light microscopy. Photographic documentation was performed using Carl Zeiss Jenamed light microscope equipped with a digital camera. Ultrathin sections were evaluated and photographic documentation

was prepared using transmission electron microscope Tesla BS-500 in the Electron Microscopy Laboratory of the Department of Histology and Embryology, Medical University of Lublin.

The structure of the area of the corneal limbus and morphological characteristics of limbal epithelial cells were analyzed paying particular attention to the basement membrane cells.

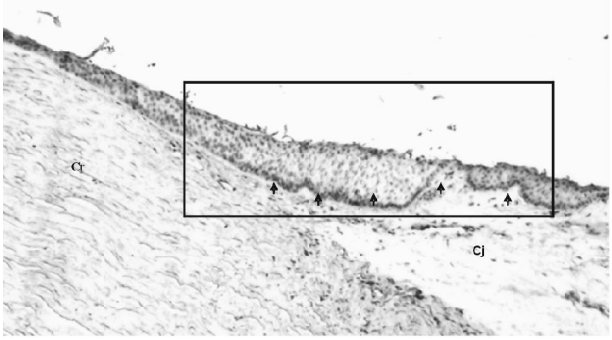

Fig. 1. Cross section through the area of the corneal limbus. Cr-cornea, Cj-conjunctiva; the frame shows the epithelium of the corneal limbus and the palisades of Vogt (←). Light microscope; H&E staining; 80x magnification.

RESULTS

Microscopic images of the sections from groups IA, IB and IC stained with H&E: in all groups of explants limbal epithelium showed characteristics of nonkeratinized stratified squamous epithelium. In samples from Group IA, undamaged structure of the limbal tissue with numerous papillary structures characteristic of the corneal limbal area was observed. The superficial layers showed flat-shaped epithelial cells with light cytoplasm with flattened deep purple nuclei. Depletion of a few cells of the superficial layer was visible. Groups IB and IC were characterized by a significant damage to the structure of the corneal limbal area. Depletion of the epithelial cells involving both the superficial and deep layers was observed. In sections from Group IA, cells located in deeper layers were of a cylindrical shape. Basal cell layer was characterized by high density of cells of different sizes, with vertically-placed nuclei stained darker than in the superficial layers. In Group IC and in some places in Group IB the damage also involved the basal epithelial cells of the limbus (Fig. 2).

In all experimental groups *substantia propria* was characterized by a slight relaxation of the fibers.

The immunohistochemical staining of the explants locally stained light red the cytoplasm of basal corneal limbal epithelial cells but only in Group IA. This pigment detects vimentin – one of the components of the cell cytoskeleton. This also confirmed the existence in this area of cells which might represent the limbal stem cells [13]

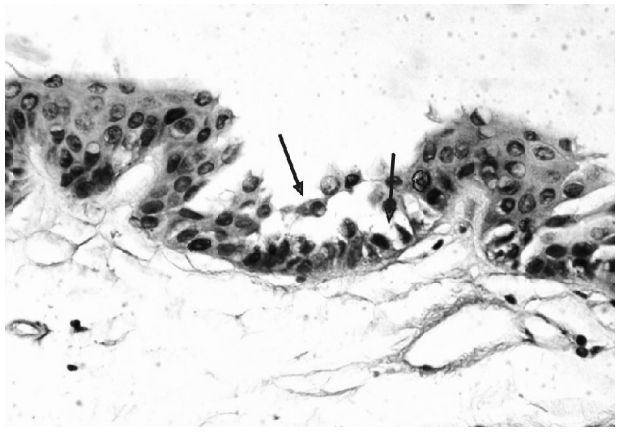

Fig. 2. Cross section through the area of the corneal limbus in Group IC and in some places in Group IB. The damage also involved the basal epithelial cells of the limbus (red arrows). Light microscope; H&E staining; 640x magnification.

(Figure 3). Immunohistochemical staining of the sections from the experimental groups IB, IC showed no expression of this protein. The above might have been associated with damage to the cellular structures of the corneal limbal epithelium and profound depletion of cells reaching down to the basement membrane of limbal epithelium.

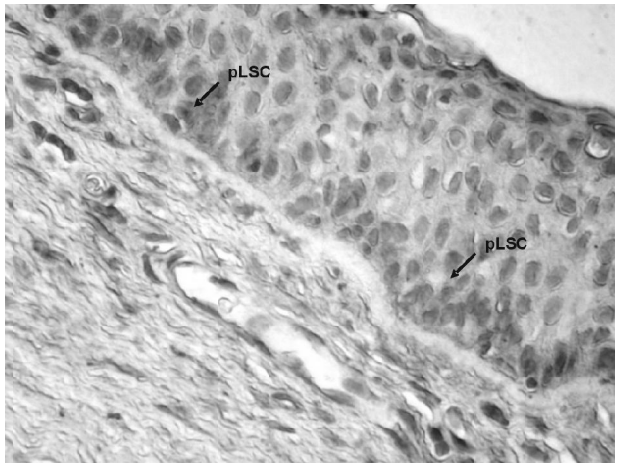

Fig. 3. The immunohistochemical staining (Vimentin KIT) of the explants locally stained light red the cytoplasm of basal corneal limbal epithelial cells but only in Group IA. This pigment detects vimentin – one of the components of the cell cytoskeleton. This also confirmed the existence in this area of cells which might represent the limbal stem cells (pLSC). Light microscope; vimentin staining; 640x magnification.

Ultra-structural studies of sections from groups IA, IB and IC showed a variety of images. In Group IA both cylindrical and circular epithelial cells of the basal layer were observed, as well as closely adjacent cells of the superficial layers (numerous desmosomes and hemi-desmosomes visible). In Group IB cell adhesion was decreased at the level of the cells of superficial and supra basal layers (intercellular space widening) which resulted in visible loss of these cells in the abovementioned layers, whereas profound disturbance of the adhesion in Group IC resulted

in tissue disorganization with visible separation of several cells from the basal layer (Figure 4). Moreover, the images of cells from Group IA were characterized by a clearly visible nucleus and cellular organelles of normal ultrastructure. In groups IB and IC the images of the cells revealed ultra-structural changes including cell membrane damage (Figure 5)., abnormal cytoplasm density, a significant widening of endoplasmic reticulum tubules (Figure 6) and mitochondrial swelling. Many cells from Group IC had also nuclear envelope damaged with prominent chromatin peripheral clumping (Figure 6). Images of cells of Group IB, particularly Group IC, indicate a severe disorder of cellular ultrastructure and, indirectly, cellular metabolic disorders concerning the water and electrolyte balance and energy metabolism.

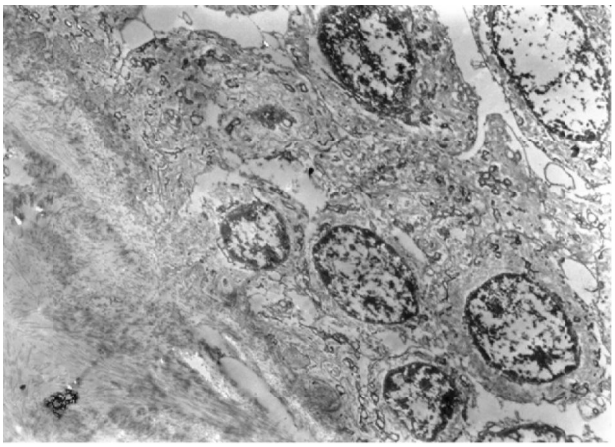

Fig. 4. Ultrastructural studies of sections. Group IC disturbance of the adhesion resulted in tissue disorganization with visible separation of several cells from the basal layer. Transmission electron microscope, 3000x magnification.

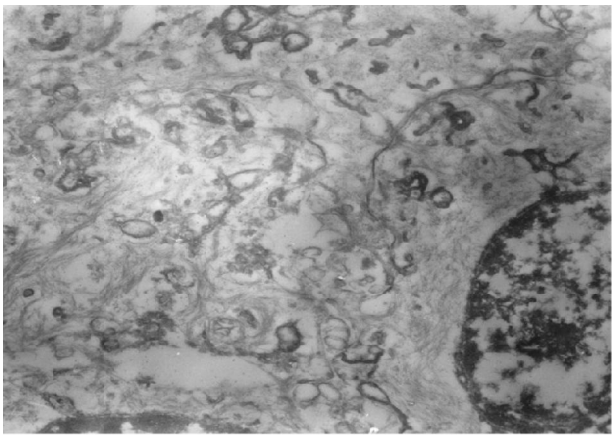

Fig. 5. Group IC – many cells had cell membrane damage with prominent chromatin peripheral clumping. Transmission electron microscope, 18000x magnification.

All these characteristics of the cellular ultrastructure may evidence necrosis caused by the toxic effects of benzalkonium chloride, this being the most likely mechanism of corneal limbal epithelial cells death.

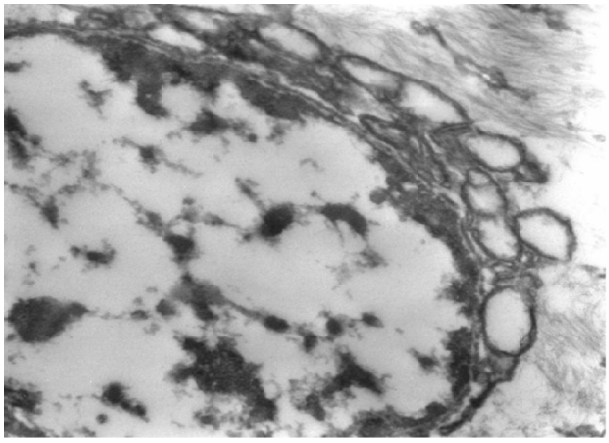

Fig. 6. Group IC – significant widening of endoplasmic reticulum tubules and prominent chromatin peripheral clumping. Transmission electron microscope, 24000x magnification.

DISCUSSION

Preservatives contained in topical ophthalmic drops produce numerous side effects which are associated with their activity against pathogenic microorganisms. In the mechanism of damage to cell membrane (alcohol compounds – chlorobutanol), cytoplasmic membrane (benzalkonium chloride) and enzymatic reactions (chlorhexidine, thimerosal, sorbic acid) they destroy the cells of microorganisms but on the other hand, they may simultaneously cause serious disorders involving normal ocular surface tissue or even the lens [1].

The clinical picture after applying the eye drops containing preservatives, as presented in a number of scientific reports, includes toxic and allergic eye tissue reactions [1].

Toxic reactions include among others effects on the conjunctiva, tear film, cornea, lens, and physiological bacterial flora [1, 2]. In normal conditions conjunctiva plays a crucial role in the secretion of tears and performs a protective function. Scarring (pseudopemphigoid), keratinization, deposits accumulation, dyschromia, hyperemia or edema [2] may be observed ensuing from the use of preservatives. Research has shown that strong antimicrobial activity of preservatives may also significantly affect the natural flora of the ocular surface, thus enabling the growth of pathological microorganisms [2].

Preservatives also influence tear secretion and tear film stability. Benzalkonium chloride damages the goblet cells of the conjunctiva and causes disorders of the lipid layer of the tear film.

Human cornea is particularly susceptible to preservatives. The strongest toxic effect is exerted on the epithelium with the resulting loss of microvilli of the superficial cells, cell membrane damage and abnormal intercellular connections [4]. The excessive shedding of the corneal epithelium secondary to the abovementioned changes can

be observed. We have observed similar changes in our studies as well. The weakening of the connections between cells also results in increased penetration of chemical substances contained in ophthalmic drops (endothelial damage) and increased risk of microorganism penetration. In experimental studies concerning BAK-containing ophthalmic drugs used on animals and humans recurrent ocular irritation, corneal erosion, and prolonged healing time were noticed. According to the authors, the probable cause might have been the toxic damage to the corneal limbal cells [14, 16]. It was also proven that substances such as benzalkonium chloride, thimerosal and chlorhexidine had a toxic effect on the corneal endothelium which led to corneal decompensation as well as its swelling [16]. Studies conducted by Abrams as early as in 1963 demonstrated the connection between thimerosal and the accumulation of deposits of mercury compounds within the eye lens.

Allergic reactions occurring during the use of agents containing preservatives are much rarer than toxic reactions. According to the authors, they represent 3–10% of all ocular adverse reactions to ophthalmic drugs. They also include allergic reactions caused by contact allergy to preservatives (thimerosal, chlorhexidine, EDTA, BAK, sorbic acid). The signs of allergic reactions include hyperemia and edema of the conjunctiva, follicular conjunctivitis, inflammation of the eyelids, periocular skin inflammation. These reactions may particularly afflict people who wear soft contact lenses, especially as pertains to hydroxyethylmethacrylate lenses [1].

Our study revealed significant damage to the tissues in the region of corneal limbus under the influence of BAK within 1 hour. It was also observed that there were prominent differences between the corneal limbal epithelial tissue damage and the concentrations of 0.005% (IB) and 0.01% (IC) BAK. As a result of the exposure to BAK, the tissues in the region of corneal limbus showed profound abnormalities in both light microscope (H&E staining) and in transmission electron microscope. In the sections prepared from explants treated with 0.005% BAK, damage to the structure of the corneal limbal epithelium with depletion of epithelial cells of both superficial and supra basal layers was observed. The concentration of 0.01% BAK caused more severe corneal limbal epithelial cells desquamation along with basal cells loss. Changes in the ultra-structural image of cells of the limbal explants may account for the mechanism of abnormalities in the structure of the corneal area. The loss of microvilli, hemidesmosomes and desmosomes, as well as features of serious cell damage, such as cytoplasm damage, the extension of endoplasmic reticulum tubules (indicating severe fluid and electrolyte disturbances), enlarged mitochondria and the lack of mitochondrial cristae (the result of cell energy processes reduction), fragmentation of the cell membrane and damage to the cell nucleus all support cell necrosis as the mechanism of cell death and explain the severe defects of the layers of corneal limbal epithelium.

Structural changes of the corneal limbal area observed in the abovementioned studies correspond with the images obtained in experiments performed in other research centers.

Sueng-Heon Cha et al. [18] conducted a study in which they cultivated corneal limbal epithelial cells of the rabbit labeled with radioactive ^{51}Cr. They studied the functional disorders, morphological changes and cell death following the use of BAK in different concentrations and with different times of exposure. BAK concentration equaled 0.001%, 0.005%, 0.01%, 0.05% and 0.1% in 5, 10, 30 and 60 minutes. The observed functional disorders of epithelium included substantial weakening of intercellular adhesion and abnormal cell adhesion to the basement membrane.

Cell death rate in the culture was calculated on the basis of ^{51}Cr activity in the cell lysate. Morphological changes were assessed in a transmission electron microscope.

Similarly, Seung-Heon et al. [18] observed changes in the ultrastructure of epithelial cells in the concentration of 0.001% BAK and exposure time of 30 minutes. The changes included wrinkling and lifting of the edges of several cells, loss of microvilli, rupture of the cytoplasmic membrane and features of the cytoplasm damage. In the concentration of 0.1% BAK and exposure time of only 5 minutes the changes included the extension of the cisternae of rough endoplasmic reticulum, swollen mitochondria, loss of microvilli and a significant number of cell membrane tears. Upon the analysis of the results, a significant correlation was found between the concentration of BAK and the time of exposure, and the function disorder, damage and death rate of cultured rabbit corneal epithelial cells. Cell death rate significantly increased in all time intervals (5–60 min) and in concentrations of 0.05% BAK and higher, while in the concentration of 0.005% it had a high level (approx. 35%) after 30 min of incubation. After 60 min of incubation, the cell death rate in concentrations of 0.005%–0.05% showed similar values (approx. 65%–71%). A significant increase in adhesion dysfunction (20%) was stated in the concentration of 0.005% BAK and 30 min of incubation, and 10% in the concentration of 0.01% and the exposure time of 5 minutes.

Deutschle et al. [9] demonstrated the genotoxicity of BAK in the concentrations of 0.005% –0.02% in cell cultures of human bronchial epithelial cells. Genotoxicity is described as the ability of biological, chemical or physical factors to inflict DNA injuries, causing, for example, fragmentation or instability of deoxyribonucleic acid chain. In natural conditions, such DNA injuries are eliminated by cell repair mechanisms. When the repair mechanisms are

overstrained, the risk of mutation increases. Such mutagenesis may result in a malignant transformation of a cell. Deutschle et al. [9] also demonstrated the cytotoxicity of BAK to bronchial epithelial cells. The percentage of viable cells reached 6% after incubating with BAK in the concentration of 0.005% and 0% in the concentration of 0.01% BAK after 2-hour exposure.

In recent years, much research on the toxicity of benzalkonium chloride was carried out on cultured conjunctival cells, which constitute a very good experimental model.

De Saint Jean et al. [8] studied the effect of various concentrations of BAK (0.1–0.0001%) after 10 minutes of exposure time on death rate in cultured conjunctival epithelial cells. Similar to our work, ultra-structural studies at concentrations of 0.05–0.1% revealed characteristics of cell damage indicating progressive cell necrosis (swelling of the cytoplasm and mitochondria, endoplasmic reticulum tubules widening and features of the cell nucleus damage and cell membrane tears). However, the concentrations of 0.0001–0.01% BAK resulted in cytoplasm and nucleus shrinking and strong chromatin condensation, which indicate apoptosis as a mechanism of cell death.

Furthermore, Dutot et al. [10] observed that the increased concentrations of BAK – 0.0025% to 0.001% – and the incubation time of 15 minutes result in the prevalence of necrolysis over apoptosis in cultured epithelial cells of the cornea and conjunctiva.

Buron et al. [3] made an attempt to explain the mechanism of conjunctival epithelial cell death after 24h exposure of the culture to 0.0004% BAK. The results of their experiment not only explain the type of cell death, but also clearly present its mechanism.

The development of diagnostic methods has enabled precise assessment of the damage to the cornea and conjunctival epithelium in vivo brought about by preservatives contained in ophthalmic medications.

On the basis of the above results it may be concluded that the death rate of corneal epithelial cells, including cells of limbal epithelium, depends both on the concentration of BAK and the exposure time. It means that the use of eye drops containing preservatives in a high enough concentration can cause acute toxic injury to the epithelial cells of the ocular surface (usually in the mechanism of necrosis). Moreover, prolonged use of topical drops containing even a small amount of the BAK preservative also causes damage to the surface of the eye through the activation of apoptosis. The unfavorable combination of the extended duration of the toxic substance use and its high concentration can cause swelling and excessive desquamation of epithelial cells of the cornea and limbus, conjunctival epithelium damage, whereas in TEM we can see ultra-structural damage, which can eventually lead to cell death through necrosis and/or apoptosis. As for the corneal limbus, the activity of benzalkonium chloride causes damage to the stem cells (LSC) or their niche, thus leading to what has been termed limbal failure, which is one of the most severe diseases of the ocular surface potentially resulting in the loss of vision. According to the authors [11, 12, 18] and as the conclusions of our studies demonstrate, prolonged use of medications with preservatives (including benzalkonium chloride) may be one of the reasons for this pathology.

In clinical practice, increasingly often we encounter iatrogenic ocular surface damage [1]. Hence, it is vital to prevent it by minimizing the exposure of the eye to the toxic substances, mainly preservatives.

Nowadays there are several alternative methods to preserve solutions for topical use from infectious agents. They include better tolerated and less toxic preservatives of a new generation as well as special systems of eye drops' storage. The new preservatives include *inter alia* quaternary ammonium derivatives such as Polyquad and Dymed. A relatively new preservative is sodium perborate $NaBO_3$ whose activity is based on the formation of hydrogen peroxide with antibacterial properties [6]. As an alternative to BAK authors suggest sorbic acid whose various derivatives are now added as food preservatives [20].

Another option is the use of products not containing preservatives in disposable packaging. The United Kingdom was the first country to introduce in 1965 such small containers [20]. The volume of the solution ranges from 0.1 ml to 1 ml, which makes a therapeutic agent fit for use within no longer than one day. However, negative consequences ensue from the use of such single doses. These include higher price (5-10 times more expensive packaging) [6], the possibility of a patient deliberately keeping an already opened container over its due date, unequal droplet sizes and technical difficulties in unassisted opening of the plastic container. Therefore, some researchers suggest drugs such as antibiotics or alkaloids, which in themselves have antibacterial activity, be put into containers with a seven-day expiration period [20]. The only requirement is to keep such container in the refrigerator. The necessity of dispensing cold drops onto the surface of the eye as well as the incomplete antimicrobial activity at 4–8°C act to the disadvantage of such an alternative. The latest solution and the most promising one at this stage is packaging containing special filters preventing the solution from being contaminated [6]. It is not as expensive as disposable packaging for single doses and can be used with the majority of medicinal. Such systems include among others the ABAK system.

The use of preservatives in ophthalmic medications undoubtedly extends the expiration date of such products and inhibits the growth of pathological microorganisms.

However, due to a relatively high toxicity of preservatives, they are becoming a potential threat to the proper functioning of a healthy eye tissue. As it is reflected by global trends, pharmacology seeks ways to reduce or completely eliminate such a threat. We can only hope that the confirmation of the toxic effects of preservatives on the structures of the ocular surface will contribute to a better understanding of drug action and will allow for the new methods of preserving sterile ophthalmic preparations.

The ocular surface is an extremely complex structure, constantly exposed to detrimental external factors such as mechanical injuries or chemical substances. Based on this study and the research conducted by other authors, it seems that the use of medicinal preparations containing preservatives may prove to be very dangerous to the anterior segment of an eye. Corneal limbus, due to its delicate and complex structure remains a very fragile tissue when exposed to the toxic effect of preservatives while also being difficult to reconstruct. The attempts to replace the products containing preservatives have not been fully satisfying. What is more, treatment of limbal insufficiency such as allo-and autografts as well as the use of tissue transplants cultivated in vitro have yet a very long way to go in order to achieve high rates of treatment success and gain popularity. Hopefully this work will highlight the crucial role of preservatives in afflicting toxic damage to the ocular surface and, in particular, to such an important structure as the corneal limbus.

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