10 years of preservative-free eyedrops

Volume 2
Experimental evidence

Prof. Christophe Baudouin, Quinze-Vingt National Hospital Center for Ophthalmology, Paris, France

volume 2

In 2004, we asked Professor Christophe Baudouin, who is an authority on the ocular surface, to review the international literature and provide a synthesis of the experimental, clinical and epidemiological studies of the effects of preservatives.

After publishing a **first volume about the theoretical aspects**, which you have told me, has either strengthened your grasp of the importance of using preservative-free eye drops in your day-to-day practice or made you aware of it for the first time, I am pleased to be able to present **this second volume concerning the experimental evidence**.

A forthcoming third volume will discuss the clinical evidence, thus completing this work on "10 years of the preservative-free revolution" that we have tried to make as clear and as exhaustive as possible.

I hope you enjoy reading it,

Henri Chibret

Already published:

Vol. 1 – Grounds for concern

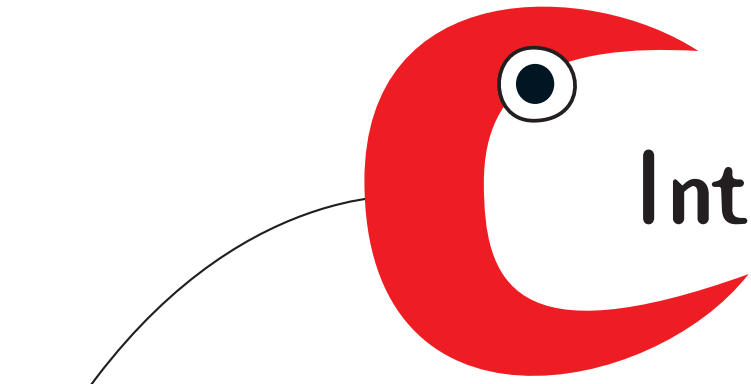
Forthcoming:

Vol. 3 – Clinical evidence

10 years of the preservative-free revolution

Vol. 2 – Experimental evidence

Prof. Christophe Baudouin, CHNO des XV-XX, Paris

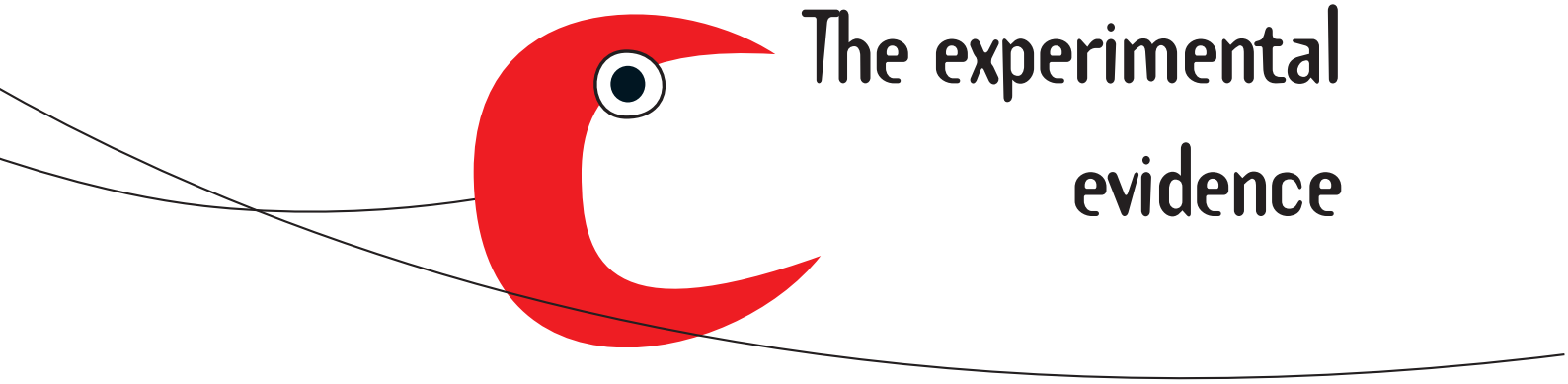


Introduction

In order to avoid introducing contaminants and allowing the growth of microorganisms, nearly all the eye drops on the market contain preservatives, which are bactericidal, bacteriostatic and/or antifungal substances compatible with the other constituents of the eye drops. The anti-infectious properties of preservatives are based on a non-specific biological activity that results in membrane solubilization, an increase in ionic permeability and/or the inhibition of cell metabolism. The use of preservatives in eye drops is not therefore risk-free, notably with regard to the corneo-conjunctival surface.

Although the eye drops on the market have all been subjected to preliminary preclinical and clinical tests to demonstrate their low ocular toxicity, it is not rare for patients to complain of stinging or burning sensations or of discomfort, irritations or dry eye. More rarely, conjunctivitis or corneal damage can occur, particularly during chronic treatment or the repeated instillation of several different eye drops.

The preclinical studies carried out *in vitro* demonstrate that preservatives can be extremely cytotoxic, even at low concentrations. Animal studies have made it possible to evaluate the superficial or deep morphological changes that may often not be clinically detectable, and to determine the respective contributions of the preservative and of the active substance to these reactions.



The experimental evidence

1. Nature and properties of the various preservatives: the different chemical classes

The preservatives found in ophthalmic preparations differ from each other with regard to their physico-chemical properties, their compatibility with the other constituents of the eye drops, their spectrum of activity, their bacteriostatic or preferably bactericidal potential, their virulence against pathogenic species, their ocular toxicity and their allergenic potential.

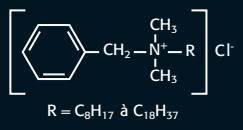
1.1- Quaternary ammoniums

The most commonly used quaternary ammonium is benzalkonium chloride.

Ophthalmic preparations may also contain cetrimonium bromide (cetrimide), benzododecinium bromide, cetylpyridinium chloride, and polyquaternium (polyquad). They are bipolar compounds, which are highly hydrosoluble, and which have surfactant properties. They act mainly via their detergent activities, which vary in strength and which dissolve the bacterial walls and membranes, and destroy the semi-permeable cytoplasmic layer. Their bactericidal activity is rapid and is greatest at 37°C in an alkaline medium. Benzalkonium chloride is usually used at concentrations of between 0.004% and 0.02%. The spectrum of activity is mainly focused on Gram + bacteria (Staphylococcus) even at very low concentrations [9].

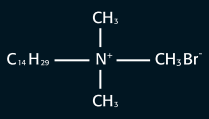
Its activity versus Gram - bacteria (*Pseudomonas aeruginosa*) is increased when it is combined with EDTA 0.1% [53]. The quarternary ammoniums are also excellent fungicides, and are particularly active against *Candida albicans* [32] and *Aspergillus fumigatus* [34]. Finally, the quaternary ammoniums are potent spermicides [62], and as well as being used in ophthalmic preparations, they are used in a wide range of commonly-used products (soaps, cosmetics, cleaning products, disinfectants…).

Quaternary Ammoniums

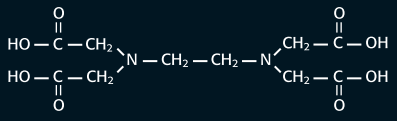


R = C_8H_{17} à $C_{18}H_{37}$

Benzalkonium chloride

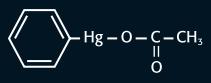


Cetrimide

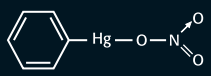


Ethylenediaminetetracetic acid (EDTA)

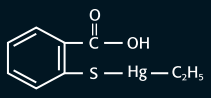
Mercury derivatives



Phenylmercuric acetate



Phenylmercuric nitrate

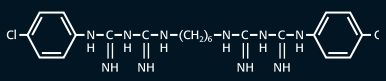


Thiomersal

1.2-Organo-mercurial derivatives

These consist of phenylmercuric compounds (acetate, borate or nitrate), mercurobutol and sodium mercurothiolate (thiomersal or thimerosal). They act as a result of the sulfur-removing properties of the mercuric ion. They act by combining with the sulfhydril groups of proteins to precipitate bacterial proteins by forming proteinates of mercury. Their spectrum of activity includes the pathogens that use an enzyme containing the sulfhydril group in the course of their metabolism, in particular Gram + bacteria and non-sporulating organisms. Thimerosal is the only one currently in use. The usual concentrations range from 0.001% to 0.004%. It is particularly active in slightly acid media.

Amidines

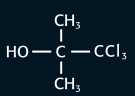


Chlorehexidine

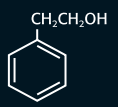
1.3- Amidines

The main compound used is chlorhexidine, a cationic agent that belongs to the family of the bis-diguanides. It is used in digluconate form, which is soluble in water and is active in neutral or slightly alkaline media (pH 8). It acts by destroying the semi-permeable layer of the cytoplasmic membrane and produces its antimicrobial activity mainly against cocci and Gram + bacteria, and some Gram - bacteria. The most resistant bacteria are Serratia, Proteus and Pseudomonas. This compound also has fungistatic activity. It has little activity against *Mycobacterium tuberculosis*, and is neither sporicidal nor virucidal.

Alcohols



Chlorobutanol



Phenylbutanol

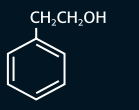
1.4- Alcohols

The alcohols most commonly used are chlorobutanol and phenylethanol. Chlorobutanol acts by increasing lipid solubility, and its antimicrobial activity is based on its ability to cross the bacterial lipid layer. At the usual concentrations (0.2 to 0.5%), it has bacteriostatic and antifungal activity. It is active against both Gram + and Gram – organisms (*Pseudomonas aeruginosa*) and *Candida albicans*.
Phenylethanol has little activity of its own, but does exhibit synergistic activity when combined with other preservatives (chlorobutanol, benzalkonium chloride, chlorhexidine).

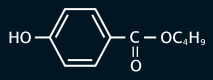
1.5- Parabens

These are the esters of parahydroxybenzoic acid. It is possible to distinguish between the ethyl, methyl, butyl and propyl esters. Their activity targets molds and fungi rather than bacteria (principally Gram + bacteria). They are active at concentrations close to the limits of their solubility. They are more active in acid medium.

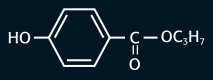
Parabens



Methylparaben



Butylparaben



Propylparaben

1.6- The oxychlorinated complexes

Oxidative preservatives, such as the stabilized oxychlorinated complexes (Purite®), have come into use more recently. These are small molecules that easily penetrate within the membranes, and they disrupt cell function by modifying the lipids, proteins or DNA. Stabilized oxychlorinated complexes consist mainly of chlorite (NaClO2), a small proportion of chlorate and traces of chlorine. Chlorite acts by producing a high degree of oxidation of glutathione, thus reducing the cell's defenses against oxidative stress. It is therefore particularly effective against species that contain low levels of glutathione, such as *Staphylococcus aureus*. In contrast, it is less effective against *Pseudomonas aeruginosa, Candida albicans* and *Alternaria alternata* [32].

Points to remember:

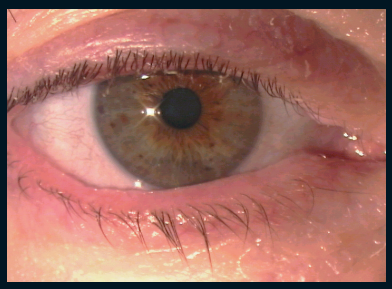
Several different classes of preservatives are available.

They have differing bactericidal potentials.

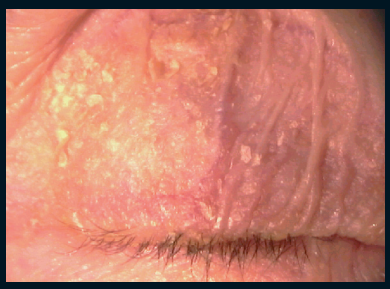
Most of them have a non-specific detergent effect and as a result can also act against and damage eukaryote cells.

2. Preservatives: toxicity or allergy?

The ocular reactions induced by eye drops, such as irritation, corneal damage, conjunctival redness and inflammation, and lacrymation tend to lead to the suspicion of an allergic reaction. The possibility that a toxic mechanism may have been induced by preservatives is less often envisaged. In fact, toxic reactions are far more common, and account for most of the adverse clinical effects.



Mild allergic blepharitis



Chronic eczema

2.1- Allergic reactions

The repeated and prolonged application of preservatives can induce sensitization, and the onset of allergic reactions.

Sensitization towards preservatives is tending to increase, because they are found not only in ophthalmic preparations, but also in all sorts of commonly-used products (soaps, cosmetics, disinfectants…) [17].

Mercurial products are strongly allergenic (13 to 37% of skin tests positive in the various studies) [5, 41, 58]. The salts of benzalkonium are classified as being moderately allergenic (4 to11% in the various data sets) [5, 27]. Sensitization towards other preservatives (chlorhexidine, chlorobutanol) is more unusual.

From a clinical standpoint, allergy towards a preservative usually takes the form of a conjunctivitis-type condition: this may consist of simple hyperemia of the conjunctiva or of papillary conjunctivitis, with or without eczema of the eyelids. The reactions observed often consist of contact allergies. These are delayed, type-IV hypersensitivity reactions that can be detected by skin tests [61, 65].

These contact allergic reactions have been reproduced experimentally in animals [1, 21]. Indeed it is possible to sensitize guinea pigs to benzalkonium chloride [21]. In rabbits that had been sensitized towards thiomersal, Baines *et al.* [1] induced a giganto-papillary conjunctival reaction by applying contact lenses containing traces of thiomersal.

The inflammatory reaction is characterized by the infiltration of polymorphonuclear and mononuclear cells into the corneo-conjunctival tissues. Depending on the severity of the reaction, the following may be observed: hyperemia and edema of the conjunctiva, production of significant quantities of mucus, edema and neovascularization of the cornea, inflammation of the iris and infiltration of the anterior chamber.

Points to remember:

The induction by preservatives of ocular reactions of an allergic nature is becoming increasingly common.

These are usually contact allergies corresponding to delayed, type-IV hypersensitivity phenomena.

Allergy towards preservatives usually takes the form of a conjunctivitis-like condition: this may consist of simple hyperemia of the conjunctiva, or of papillary conjunctivitis with or without eyelids eczema.

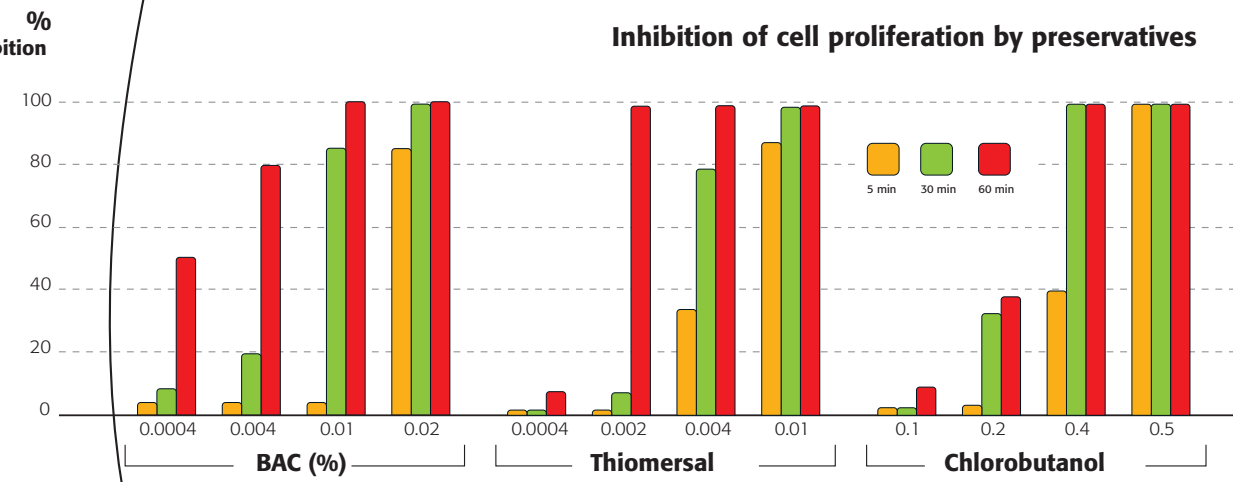
2.2- Toxicity of preservatives

Preservatives are potentially toxic towards all the structures of the eye, both those on the surface (conjunctiva, cornea) and the internal structures (trabeculum, lens, retina).

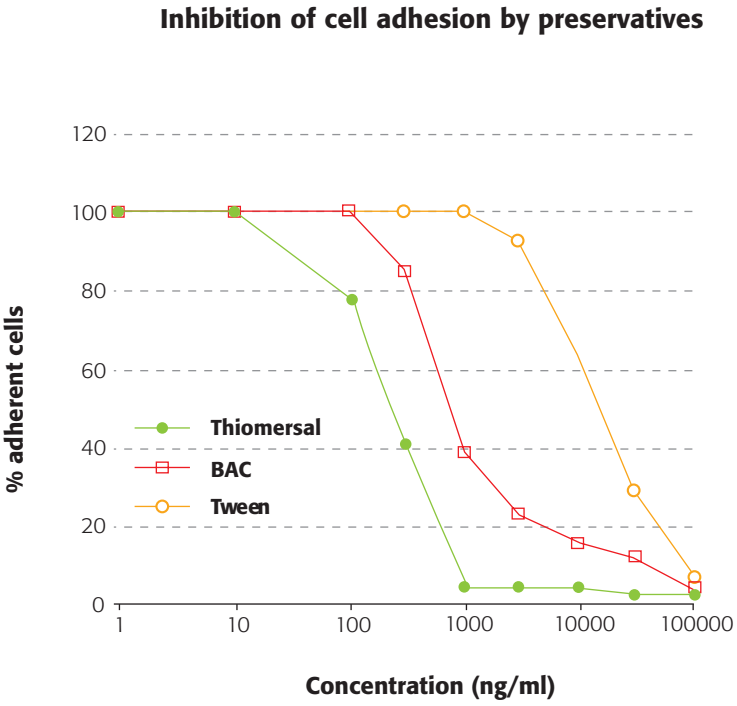
In vitro toxicity

In vitro, the cytotoxicity of preservatives has been demonstrated using various types of cell cultures: epithelial cells of the cornea or of the conjunctiva, keratocytes [36, 50. 56], endothelial cells of the cornea [56], fibroblasts from Tenon's capsule [63], trabecular cells [26, 56] and the epithelial cells of the lens [22].
The *in-vitro* cytotoxicity of preservatives particularly affects cell viability (impaired integrity of the plasma membrane or of the mitochondrial energy metabolism) [10. 14, 50. 54], proliferation [31, 38, 43] or cell adhesion [63]. These cytotoxic effects increase with the concentration of the preservative, and the duration of exposure (Figure 1). They occur at concentrations lower than those contained in commercial preparations [36, 55, 57]. At high concentrations, preservatives produce cytotoxic effects within minutes of being applied [10. 12, 31]. Some of the cellular modifications are irreversible, and eliminating the preservative may not always be enough to enable the cells to recover [12, 57, 60].

Figure 1



Inhibition of thymidine incorporation by the epithelial cells of the rabbit cornea in primary culture exposed for 5, 30 or 60 minutes to various concentrations of benzalkonium chloride (BAC), thiomersal or chlorobutanol. After Imperia *et al.* [31]



Human sarcoma cells were cultured in the presence or absence of thiomersal or of benzalkonium chloride (BAC) at various concentrations for 2 hours. These two preservatives were compared to Tween 80, a detergent. The figures are expressed as a percentage of the control cultures without preservative. Mean of three independent experiences.

After Salonen *et al.* [55]

Figure 2

The quaternary ammoniums are the most toxic preservatives. They demonstrate similar cytotoxicity *in vitro*, producing rapid impairment of membrane integrity (15 minutes) at concentrations of 0.005 and 0.01%. [13]. In the rabbit, the concentration of benzalkonium choride producing 50% cell death (LD50) after exposure of primary cultures for one hour is estimated to be 0.0003% for the epithelial cells of the cornea and 0.001% for the keratocytes [36]. Inhibition of cell adhesion can be observed at concentrations 40 to 200 times lower than the concentrations of the proprietary products on the market. In comparison, thiomersal can be active at concentrations 30 times lower (Figure 2).

Using videomicrography, Tripathi *et al*. have shown that a single dose of benzalkonium chloride (0.01%), of thiomersal (0.001%) or of chlorobutanol (0.5%) produces immediate inhibition of the cytokinesis and mitotic activity of corneal epithelial cells in primary culture [59, 60]. Cell degeneration is observed after 2 hours of exposure to benzalkonium chloride, and after 9 hours of exposure to chlorobutanol or thiomersal.

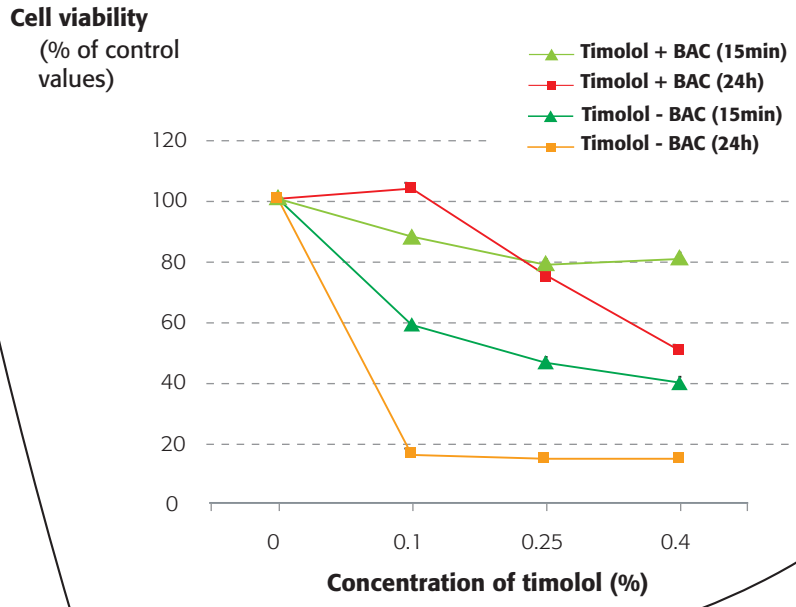
In-vitro studies suggest that much of the toxicity of the ophthalmic preparations available is due to preservatives.

De Saint Jean *et al*. [11] have shown that the viability of cultured conjunctival cells was much more severely affected by preparations of timolol (0.1 to 0.25%) containing benzalkonium chloride (0.01%) than by preservative-free preparations of timolol (Figure 3). Hamard *et al*. [25] have shown that the cytotoxicity of preparations of betaxolol on human trabecular cells was markedly potentiated by the presence of benzalkonium chloride. Williams *et al*. [63] have reported similar findings concerning the proliferation of fibroblasts in Tenon's capsule in primary culture in the presence of timolol containing benzalkonium chloride.

The experimental conditions used *in vitro* can sometimes seem to be more exaggerated and severe than the clinical reality. Given that the

Figure 3

Effect of timolol in the presence or absence of benzalkonium chloride 0.01% on the viability of cultured conjunctival cells



Human conjunctival cells were incubated for 15 minutes with various concentrations of timolol in the presence or absence of benzalkonium chloride (BAC) 0.01%. The cellular viability was measured by staining with neutral red, a marker of membrane integrity, just after the incubation (15 min.) or after culturing for 24 hours. The reduction in cell viability produced by preserved timolol is statistically significant ($p<0.01$) compared to non-preserved timolol. After Saint Jean *et al*. [11].

concentration of eye drops is immediately diluted after instillation into the eye by the basal volume of the tears, the concentration really in contact with the corneo-conjunctival cells differs from that applied. The effects produced in vitro by low concentrations of preservatives over very short periods of time (within minutes or even seconds after applying the preservative) are therefore probably closer to the clinical reality. Thus, Takahashi *et al*. [57] demonstrated that the exposure time to benzalkonium chloride resulting in 50% cell damage was 30 seconds at a concentration of 0.01%, which is similar to the estimated contact time with the human eye after instilling one drop of ophthalmic solution [49]. At lower concentrations, the contact time producing 50% damage is greater (90 seconds and 190 seconds for the concentrations of 0.005% and 0.0025% respectively). Exposures lasting for several minutes or several hours would be closer to the long-term use of eye drops in certain clinical situations, such as glaucoma or dry-eye syndromes.

Points to remember:

***In vitro,* preservatives are cytotoxic towards conjunctival and corneal cells at concentrations considerably lower than those used in commercial eye drops.**

The toxic effect is dose dependent and increases with the duration of exposure.

Morphological changes

The morphological changes produced by preservatives or preserved eyedrops are widely documented by *ex-vivo* observations using electronic scanning microscopy [16, 42, 51] or *in-vivo* observations using confocal microscopy [29].

The changes in the corneal epithelium have been demonstrated using extreme experimental conditions, such as prolonged exposure lasting several hours or using preservative preparations containing high concentrations. This has made it possible to demonstrate a series of events typical of the cytotoxicity of preservatives on the corneal epithelium:
1) loss of the microvillosities on the surface of the epithelial cells,
2) loss of contact with the adjacent cells,
3) marginalization of the cells and cell death characterized by puckering of the plasma membrane,
4) desquamation of the surface layers exposing the cells in the other layers of the cornea [16, 51].

Dormans *et al*. [16] have reported that the first effects of instilling one drop of benzalkonium chloride 0.01% appeared within less than 10 minutes and depended on the concentration. The first thing seen was the swelling of the epithelial cells and the loss of the microvillosities. After exposure for 30 minutes, the cornea is covered by swollen cells, and the first two layers of the epithelium are severely affected. There is a complete loss of the microvillosities, degenerative changes in the membrane, cell death and desquamation of the first two surface layers after exposure for 3 hours. These effects peak after 2-3 hours. Optical microscopy reveals a progressive reduction in the number of layers of epithelial cells after 30 minutes (5-7 layers), 2 hours (5-6 layers) and 8 hours (4-5 layers).

Benzalkonium chloride produces the most severe effects amongst the preservatives studied, due to its greater penetration capacity.
However, changes in the microvillosities and in the epithelial intracellular junctions has been described in the presence of thiomersal (0.004% and 0.0025%) in the rabbit after prolonged experimental exposure (30 to 60 minutes) [42]. Under similar conditions, chlorobutanol (0.4%) may produce desquamation of the epithelial cells in the rabbit [42].

These results have been corroborated by *in-vivo* observations in the rabbit using confocal microscopy revealing slight swelling of the epithelial cells as soon as benzalkonium chloride 0.005% is instilled, and then desquamation of the surface layer after one hour of observation. The desquamation is greater the higher the concentration of the benzalkonium chloride. However, the underlying keratocytes appear to be intact, as do the endothelial cells and the basal membrane [29].

Points to remember:

In animals, prolonged application of preservatives to the ocular surface induces:

1) a loss of the microvillosities of the epithelial cells

2) a loss of contact with the adjacent cells

3) marginalization and cell death

4) desquamation of the superficial epithelial layers. The higher the concentration of the preservative, the greater the desquamation.

Mechanisms responsible for the toxicity of preservatives

To a large extent, the physical and chemical properties of preservatives account for their toxicity. At high concentrations, they can cause cell lysis by dissolving the membranes by means of detergent effects (cell necrosis). At lower concentrations, they can prevent the intercellular interactions essential for cell survival. By being intercalated into the cell membranes, some preservatives (benzalkonium chloride in particular) can induce secondary degeneration as a result of a biological cascade leading to apoptosis. Indirectly, the cell damage, the denaturing of proteins and the metabolic changes can trigger and maintain an immuno-inflammatory reaction with the risk of the development of scarring.

— Detergent effect

Most preservatives (quaternary ammoniums, chlorhexidine, alcohols, parabens) are detergents. The quaternary ammoniums have the greatest cytotoxicity. They have a positively-charged hydrophilic head, and an uncharged hydrophobic part, which allows them to be anchored within the membranes. Ionic interactions severely disrupt the lipid bilayer of the plasma membranes. In this way, the detergent preservatives can create openings allowing aqueous or ionic substances to penetrate into the intracellular or intercellular spaces [47]. These effects are sufficient to produce severe damage to the epithelial cells and to produce excessive penetration of fluid into the stroma, its hydration and the development of corneal edema [47].

The severe ocular reactions that can be induced by the quaternary ammoniums are illustrated by the work of Jester *et al*. [33] using confocal microscopy. In the rabbit and rat, the effect of one drop of a high

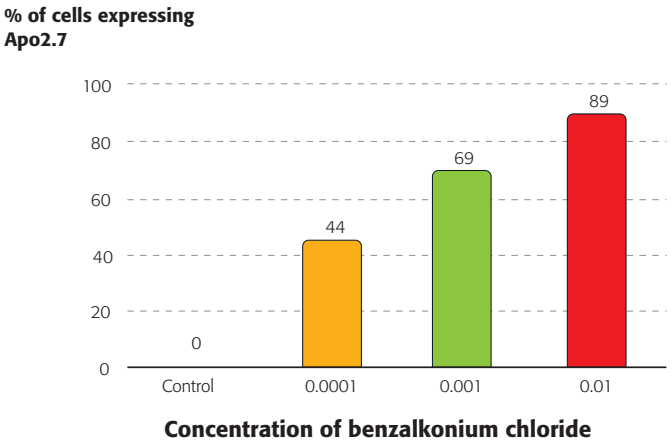
concentration of a cationic surfactant (50% cetyltrimethylammonium chloride) on the corneal epithelium produces severe irritation (lacrymation, hyperemia, photophobia and edema) combined with a reduction in the thickness of the epithelial layer of the cornea, thickening of the cornea, lysis of the keratocytes, and damage of the corneal endothelium. The mechanisms of cellular regeneration that are induced can be observed after three days: the presence of keratocytes, disappearance of superficial features, the presence of an exsudate of polymorphonuclear cells in the endothelium. On Day 35, calciform cells could be observed on the surface of the epithelium, indicating abnormal conjunctivalization of the cornea. Neovascularization and fibrosis (the presence of a retrocorneal fibrous membrane) could be seen.

— Necrosis/apoptosis

Two possible mechanisms for cell death have been suggested, depending on the concentration of the preservative. **At high concentrations of quaternary ammoniums** (0.01% and 0.05%), the loss of cell viability and the cell death observed in cultures of human conjunctival epithelial cells involve changes typical of **cell necrosis** [15]: the cells are lysed and membrane debris are visible in the cultures, the cells are very small and irregular in volume, the pattern of DNA migration on agar gel is characteristic.

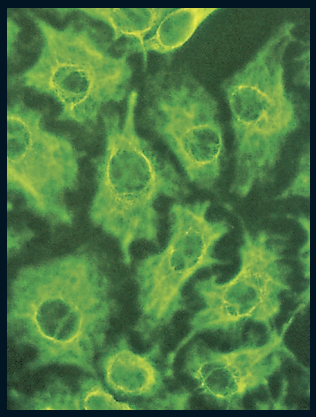
Figure 4

Apoptosis triggered by benzalkonium chloride



Human corneal epithelial cells were cultured with or without (control) various concentrations of benzalkonium chloride for 10 minutes. 24 hours later the apoptosis marker Apo2.7 was measured by immunofluorescence.

After De Saint-Jean *et al.* [10]



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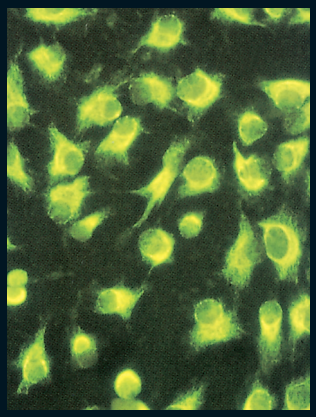

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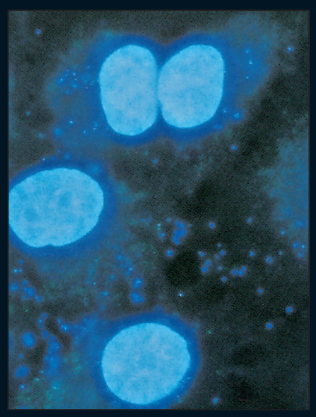

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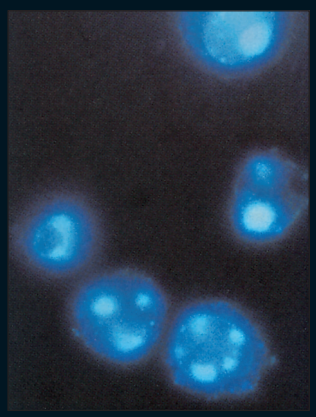


D

Human conjunctival cells in culture

***A** : Cytoskeleton of corneal cells*

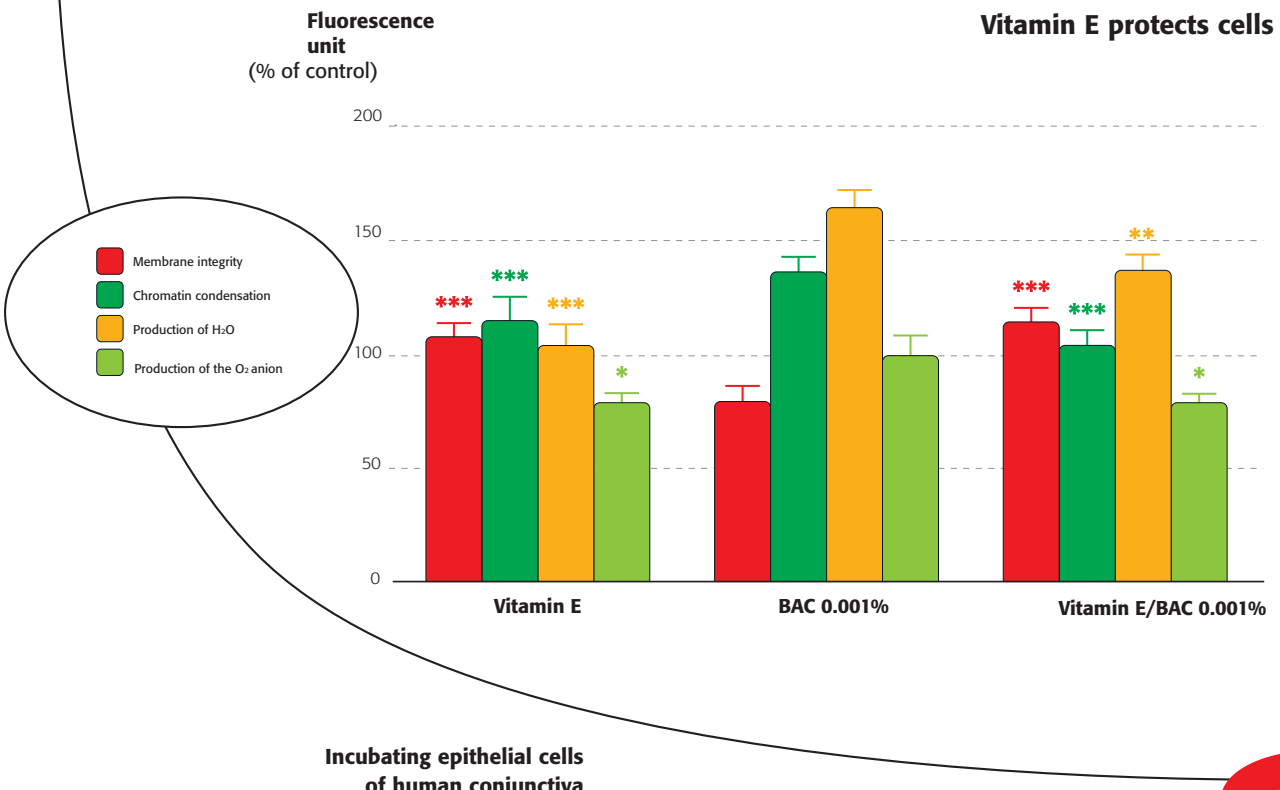
***B** : Cytoskeleton of the cells 24 hours after exposure to a 0.01% solution of benzalkonium chloride for 10 minutes*

***C** : Nucleus (DAPI staining) of the control cells*

***D** : Condensation and fragmentation of chromatin, 24 hours after exposure to a 0.01% solution of benzalkonium chloride for 10 minutes*

At low concentrations, the quaternary ammoniums stop cell growth and trigger a process of programmed cell death. Cell death occurs after some delay, with the morphological and metabolic changes characteristic of apoptosis (cell retraction, chromatin condensation, DNA fragmentation and the expression of apoptosis markers (Figure 4)) [15].

Other metabolic changes can be linked to apoptosis. Thus, in primary cultures of epithelial cells of rabbit cornea, Grant *et al*. [20] report finding a 70% reduction in the intracellular calcium, and a significant rise in the intracellular pH combined with a reduction in cell viability after exposing the cells to low concentrations of benzalkonium chloride (0.0001%).



Incubating epithelial cells of human conjunctiva for 1 hour with vitamin E protects the cells against the effects of benzalkonium chloride (BAC), and against the production of reactive species of oxygen (hydrogen peroxide and the superoxide anion), and preserves membrane integrity and chromatin condensation. * $p < 0.005$ ** $p < 0.001$ and *** $p < 0.0001$ versus BAC 0.001%.

After Debbash *et al.*[15]

Figure 5

Oxidative Stress

The superoxide anion O2.- has a cytotoxic effect on cultured cells: it can break down polysaccharides and DNA, alter the structure of the membranes by lipid peroxidation, impair vascular permeability, and potentiate infammatory reactions. Recently, Debbash *et al.* [15] have shown that **eyedrops containing quaternary ammoniums (0.01%) generate significantly more superoxide anions than preservative-free eyedrops.** The generation of the superoxide anion was correlated with the loss of membrane integrity and apoptosis of the cells in the presence of benzalkonium chloride. Pre-incubation with an antioxidant (vitamin E) protects the cells in culture, and significantly reduces the membrane damage and apoptosis induced by benzalkonium chloride 0.001% (Figure 5).

— Inflammation

In the rat, Baudouin *et al*. [2] demonstrated that inflammatory cells infiltrate the conjunctiva and trabeculum following instillations of timolol containing a preservative for one month. This infiltration was not observed with preservative-free timolol, which strongly suggests that the preservative plays a role in the onset of the inflammatory reaction. It is highly probable that the application of preservatives to the ocular surface could denature cellular proteins and stimulate immunocompetent cells. There are Langerhans cells in both the conjunctival epithelium and the corneo-conjunctival chorion. They are in the front line when ophthalmic products are applied. After being activated, they could migrate from the sub-epithelial spaces to perpetuate an immuno-inflammatory reaction and the onset of sub-conjunctival fibrosis.

Points to remember:

Cytotoxicity can involve several different mechanisms:

1) **lysis of the membranes and denaturing of the cell proteins by a detergent effect**
2) **cell death as a result of apoptosis**
3) **induction of oxidative stress**
4) **activation and perpetuation of an immunoinflammatory process and sub-conjunctival fibrosis.**

Pharmacokinetic parameters

The penetration, metabolism and elimination of benzalkonium chloride instilled in the rabbit as a single dose or repeated doses have been investigated by Champeau *et al.* [6] and Green *et al.* [24]. These studies carried out in the rabbit reveal that there is a considerable accumulation of benzalkonium in the corneo-conjunctival epithelium and in the stroma (Figure 6). Benzalkonium is also detected in the deepest structures: the lens, iris, vitreous, choroid and retina. It is broken down slowly and has a long half life (estimated to be 20 hours in the epithelium, 11 hours in the conjunctiva and 8 hours in the stroma following a single instillation) [6].

The conjunctival and corneal epithelium acts as a reservoir: it is very rapidly saturated and can then gradually release the preservative and redistribute it to the tear film or other ocular tissues.

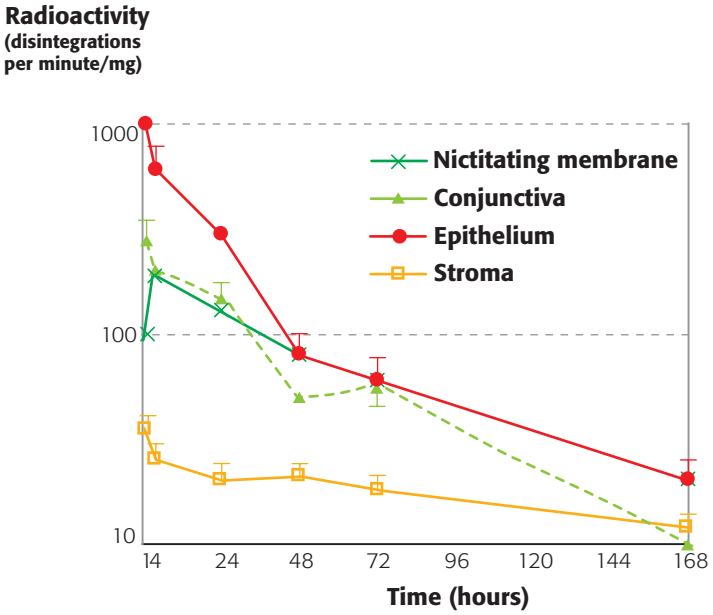
Benzalkonium chloride exhibits extremely high affinity towards the cornea and the conjunctiva, which is considerably greater than that of the anionic detergents. This genuine "appetite" of the ocular tissues towards benzalkonium chloride could partly explain why benzalkonium chloride induces more severe corneal lesions than the anionic detergents [24].

Points to remember:

Preservatives can accumulate to a marked extent at the ocular surface.

They are metabolized slowly and can act as reservoirs feeding into the internal tissues of the eye.

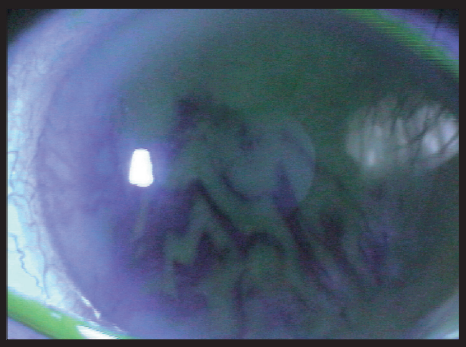
Incorporation and elimination of benzalkonium chloride



A drop of carbon^{14} –labelled benzalkonium chloride was instilled into the rabbit eye. Benzalkonium chloride is rapidly incorporated by the superficial ocular tissues . Elimination is slow. Mean ± standard deviation of 8 to 10 experiments. After Champeau *et al.* [6]

Figure 6

3. Cytotoxicity in the superficial ocular tissues



Destabilisation of the tear film

3.1- Changes in the tear film

The tear film is a nutrient layer that protects the epithelium by acting as a lubricant. As a result of their organoleptic properties, detergent preservatives are able to dissolve the lipid layer of the tear film.

This rapidly breaks up the film, facilitating the evaporation of water and thus leading to ocular dryness. The instillation of three drops of benzalkonium chloride, even at a very low concentration (0.0001%), reduces the BUT (the break-up time of the tear film) by more than 50% [64]. Benzalkonium chloride is more aggressive than chlorhexidine, chlorobutanol or thiomersal. At concentrations of over 0.005%, its surface tension is lower than that of the tear film [40], which prevents the lipid secretions from the Meibomius glands from spreading over the surface aqueous phase of the tear film [35]. A significantly greater reduction in the break-up time of the tear film was demonstrated by Pisella et al. [52] in albino rabbits treated for 60 days by preserved beta-blocker compared to that in rabbits treated with a preservative-free beta-blocker.

Points to remember:

Due to their detergent properties, preservatives are able to destabilize the tear film by dissolving the lipid layer.

3.2- Conjunctival cytotoxicity

Instilling preservatives can have several closely-linked consequences on the conjunctiva: cytotoxicity, activation of an infraclinical immuno-allergic reaction, onset of sub-conjunctival fibrosis, which can lead to gradual conjunctival healing [4]. The impact on the lachrymal apparatus (loss of mucus cells, dissolution of the lipid component of the tear film, dry eye) can be serious and result in dry eyes or compromise the success of filtering surgery in glaucoma patients.

Loss of mucus cells

A reduction in the density of the mucus cells has been observed following the instillation of eyedrops containing a preservative both in human subjects [66] and in animals [3]. The first consequence of this cell loss is a change in the composition and quality of the tear film.

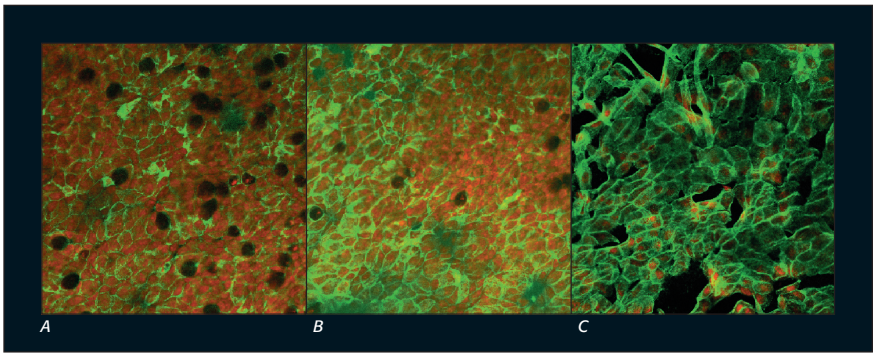
Conjunctival imprints using confocal microscopy

The mucus cells correspond to the dark patches

A - Untreated patient:
Numerous mucus cells

B - Prolonged single-drug therapy:
Fewer mucus cells

C - Multidrug therapy:
metaplasia and the disappearance of the mucus cells

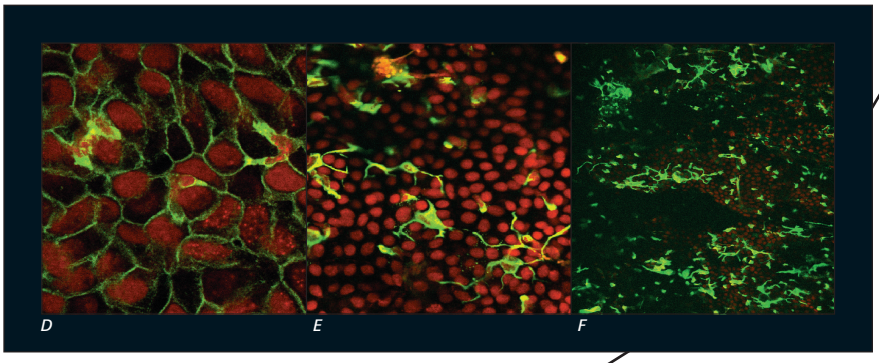


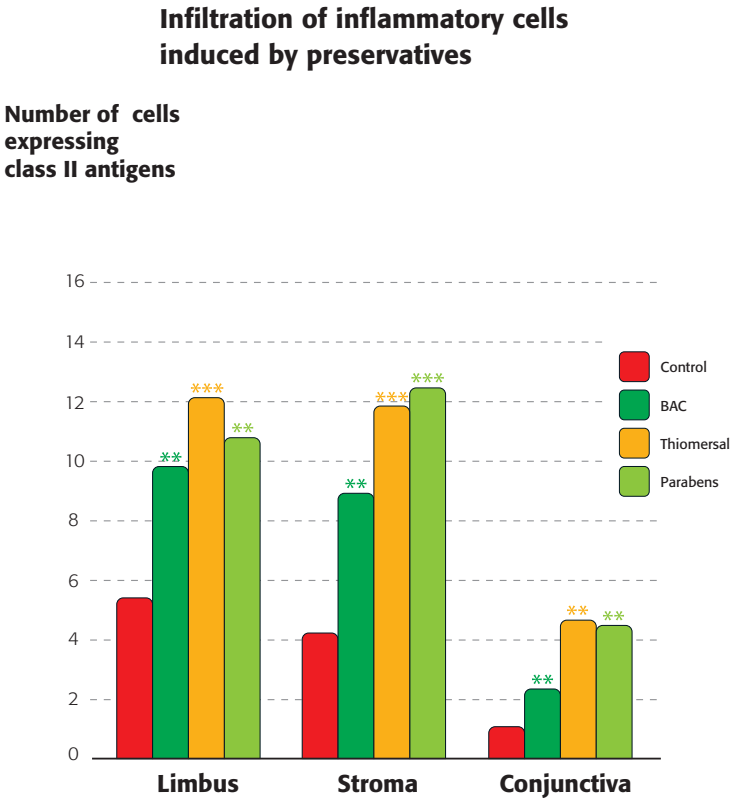
The cells expressing the markers of inflammation are stained green

D - Untreated patient:
paucity of immune cells

E - Prolonged single–drug therapy:
moderate inflammatory infiltrate

F - Multidrug therapy:
very numerous immune cells





Several preservatives (benzalkonium 0.01% [BAC], thiomersal 0.004%, paraben 0.05%) were instilled in the rat (3 drops per day for 30 days). After euthanasia, the corneas and temporal and nasal bulbar conjunctivae were isolated and thin sections were prepared for immunohistochemical staining. The data correspond to the mean numbers of cells/0.1 mm² obtained from 5 rats (10 eyes). * p< 0.05, ** p< 0.01 and *** p < 0.001 versus controls.

After Becquet *et al.* [3]

Figure 7

Inflammation and sub-conjunctival fibrosis

Becquet *et al*. [3] demonstrated that in rats treated for one month with various preservative solutions (in particular: bezalkonium chloride 0.01%, methyl parahydroxybenzoate 0.05% and thiomersal 0.004%), there was an infiltration of immunocompetent cells into the limbus and bulbar conjunctiva (Figure 7). These cells expressed class II and CD11b membrane HLA antigens (leukocyte integrin) in particular. This reaction was also associated with severe damage of the ocular surface: loss of goblet cells, keratinization, and an increase in the superficial epithelial layers.

Similar results have been reported by Baudouin *et al*. [2] in rats treated by timolol 0.5% containing benzalkonium chloride (0.01%). In comparison, preservative-free timolol did not induce any significant histopathological differences versus control animals, which confirms that

most of the toxicity of commercial preparations is due to the preservative.
Similarly Noecker *et al*. [48] have demonstrated the infiltration of lymphocytes into the various conjunctival layers (epithelium, surface and deep stroma) in rabbits treated for 30 days with various different antiglaucoma preparations containing benzalkonium chloride.

Various animal studies have demonstrated that an infiltration of fibroblasts and the onset of chronic fibrosis is induced by preservatives. Mietz *et al*. [45] have demonstrated that the instillation of metipranolol 0.3% preserved in benzalkonium chloride (one drop b.i.d. for 6 months), produced deterioration of the composition of the extracellular matrix and the organization of the conjunctival stroma, combined with an increase in the number of activated subepithelial fibroblasts, in the deposits of collagen and the thickening of the basal membrane of the endothelium. Similar results have been observed with pilocarpine 2% preserved in cetrimonium chloride 0.004% [44]. These changes appear to be permanent and irreversible, and could be partly attributable to preservatives [44].

These studies corroborate the initial findings published by Young *et al*. [67] demonstrating proliferation of the conjunctival fibroblasts increased after filtering surgery in rabbits previously treated by eyedrops containing preservatives (timolol 0.5%, pilocarpine 4%, or artificial tears).

Points to remember:

Preservatives can induce an immuno-inflammatory reaction with sub-conjunctival fibrosis combined with severe damage to the ocular surface: loss of mucus cells, keratinization, and an increase in the superficial epithelial layers.

3.3- Corneal cytotoxicity

The instillation of eyedrops containing preservatives can induce morphologic modifications of the cornea such as a loss of microvillosities or the rupture of intercellular junctions increasing the permeability and thus the penetration of ionic solutions, lipophilic substances and microorganisms. The consequences for an unhealthy eye can be serious: thickening of the cornea, corneal edema, damage of the endothelium, opacity of the cornea.

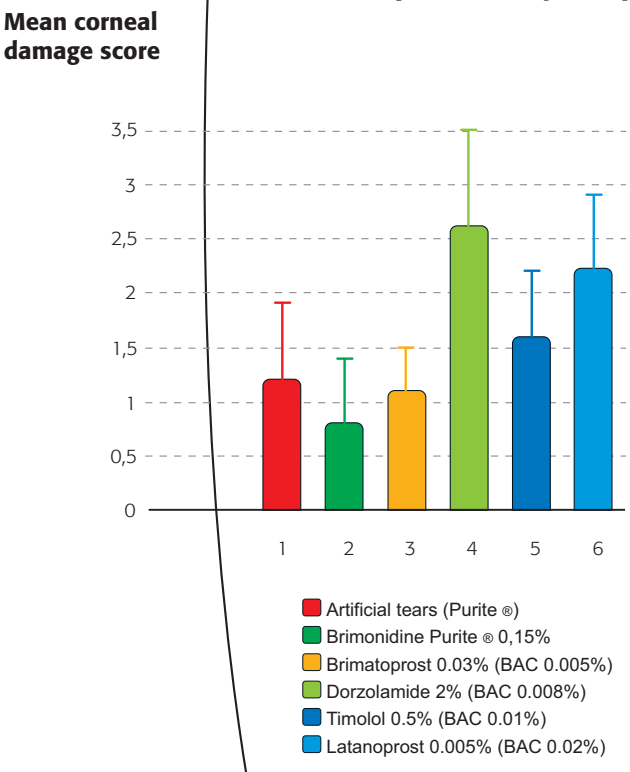
Corneal distress

The experiments carried out by Furrer *et al*. have demonstrated that instilling preservatives (quaternary ammoniums, mercury derivatives, alcohols, chlorhexidine, parabens) in mice produced microlesions that were detected using fluorescein staining [18]. In the rabbit, the application of a beta-blocker preserved in benzalkonium chloride 0.01% or benzododecinium bromide 0.012% produced microlesions that could cover nearly 15% of the surface of the cornea after 28 days of treatment (one or two drops per day). This effect has been attributed to the preservative, since preservative-free beta-blockers do not produce any specific toxicity [19]. Imayasu *et al*. [30] have demonstrated that in the rabbit repeated instillation (2 drops at 5-minute intervals for one hour) of benzalkonium chloride (0.005 to 0.02%) or of chlorhexidine digluconate (0.01 to 0.03%) produces a dramatic release of lactate dehydrogenase and albumin into the tears. This release, a sign of corneal distress, was correlated to lesions on the ocular surface that were observed using a slit lamp.

Using scanning electron microscopy, Noecker *et al*. [48] have recently demonstrated that applying various eyedrops (one or two drops per day for 30 days) preserved in benzalkonium chloride (0.005% to 0.02%) or in a stabilized oxychlorinate complex (Purite®) could result in a variable degree of loss of the microvillosities, with puckering of the plasma membranes (a sign of cell necrosis) and partial erosion of the cells in the first epithelial layer (Figure 8).

Figure 8

Morphological changes of the corneal surface induced by instilling various preserved eye drops in the rabbit



The data are the mean ± standard deviation of 5 observations (5 eyes).
BAC: benzalkonium chloride.

After Noecker *et al*. [48]

Score 0: No corneal change

Score 1: Slight, diffuse or peripheral loss of microvillosities; puckering of the plasma membranes < 10% of the cells, cell erosion > 2% of the cells; increase in epithelial perforations, increased number of dark cells.

Score 2: Moderate and diffuse loss of microvillosities, puckering of the plasma membranes > 10% and < 50% of the cells, cell erosion > 2% and < 25% of the cells ; loss of the hexagonal shape (smoothing).

Score 3: puckering of the plasma membranes > 50% of the cells ; diffuse cell erosion > 25% of the cells; retraction of the limits of the cell membranes.

Score 4: Loss of the superficial cell layer; second layer of cells left intact.

Score 5: Erosion of the second layer of cells.

Rupture of the epithelial barrier

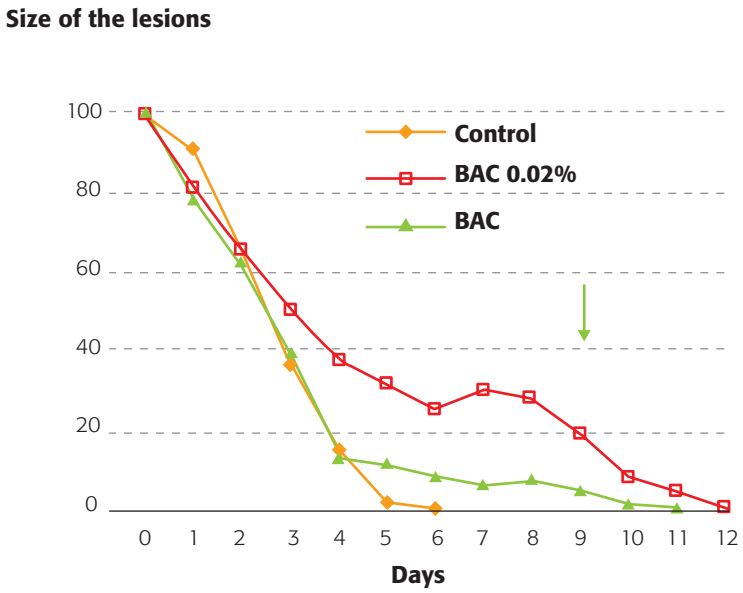
The erosion of the epithelial layer can result in disruption of the epithelial barrier and exposure of the deepest corneal cell layers.

In the rabbit, prolonged contact (1.5 to 3 hours) between the cornea and artificial tears containing benzalkonium chloride (0.01%), increases the uptake of carboxyfluorescein (which is more hydrophilic than fluorescein and so exhibits limited penetration into the pericellular spaces) by a factor of 10 to 100 [39]. Artificial tears containing thiomersal 0.004% and polyquad 0.001% also increase this uptake, but to a more moderate extent (up to fourfold).

Using ruthenium red (a stain that is bound to the anionic groups of the mucopolysaccharides on the surface of the lateral membranes of the epithelial cells), Lopez Bernal *et al*. [39] were able to demonstrate evidence of the complete destruction of the epithelial barrier with the loss of the most superficial layers of cells. In rabbits' eyes, contact with benzalkonium chloride 0.01% produced accumulation of ruthenium red in the intercellular spaces of all the epithelial layers, indicating the deep penetration of the benzalkonium chloride. The cells had lost their normal morphological appearance, numerous vacuoles could be seen within the cells. In contrast, with the other preservatives investigated (thiomersal 0.004%, polyquad 0.001%) the stain remained localized in the surface layers. The degree of penetration of the ruthenium after exposure to benzalkonium chloride indicates that the damaged corneas could be susceptible to invasion by pathogens.

Figure 9

Effect of benzalkonium chloride on corneal healing



Rabbits were subjected to central keractectomy, and were then given drops of benzalkonium chloride (BAC) four times a day until they had recovered. The size of the lesions was measured on the photographs after fluorescein staining. Each point corresponds to the mean of 6 experiences. The arrow indicates the end of the instillation of BAC 0.02% . After Collin and Grabsch [8].

Corneal repair – re-epithelialization

Repeated applications of benzalkonium chloride to the cornea can lead to the loss of cells from layers down as far as the least differentiated layers of the epithelium and to retarding or even inhibiting cell regeneration and the repair of the epithelial barrier.

In an experimental model of lesions, created *in vitro* on a monolayer of epithelial cells of the dog cornea in primary culture, the epithelial cells around the edges of the lesion display characteristic pseudopods extending towards the lesions [28]. It is possible to see a progressive reduction in the area of the lesion in cultures not exposed to benzalkonium chloride 0.0025% or to timerosal 0.025%. In the cultures containing benzalkonium chloride, the cells do not develop pseudopods and migration is inhibited [28].

The process of re-epithelialization is facilitated by the fact that the cells hook onto the extracellular matrix. Salonen *et al*. [55] have shown that benzalkonium chloride and thimerosal at concentrations 40 to 200 times lower than the concentrations used in commercial preparations could inhibit the adherence of the cells to a layer of fibronectin, and thus compromise the corneal repair process.

The experiments conducted in rabbits after keratectomy, demonstrated delayed healing when the eyes had been exposed to benzalkonium chloride 0.01% in the presence of EDTA 0.1% [8] (Figure 9). At a higher concentration (0.02%), the improvement in the size of the lesions was still only partial by day 6. Complete healing requires the discontinuation of the instillations of benzalkonium chloride 0.02%.

These findings suggest that treating corneal ulcers with substances containing a preservative could tend to reduce the re-epithelialization.

Points to remember:

Preservatives can induce morphological changes in the corneal epithelium with the appearance of microlesions and desquamation of the surface layers, or even the rupture of the epithelial barrier. In a damaged cornea, they slow the recovery and healing process.

4. Cytotoxicity in the deep ocular tissues

In some situations, in particular when the corneo-conjunctival surface is severely affected, the penetration of the eyedrops, and therefore of the preservative may be increased, and the deep tissues of the eye may be affected.

4.1- Trabeculum

Clinical experience has led to the suspicion that preservatives may play a role in the failure of trabeculectomies in patients receiving long-term antiglaucoma treatment with preserved eyedrops [37]. The trabecular cells seem to be very sensitive to preservatives. *In vitro*, benzalkonium chloride inhibits the growth of human trabecular cells after exposure for seven days to particularly low concentrations (10^{-7} to 10^{-5}%) [56].

The studies performed by Hamard *et al*. [25, 26] have shown that benzalkonium chloride could induce apoptosis in trabecular cells after brief exposure (15 minutes) to a low concentration (0.0001%). This effect is specific to benzalkonium chloride, since apoptosis was not triggered in cultures of cells exposed to preservative-free eyedrops.

The toxic effects of benzalkonium chloride on the cells of the trabeculum could partially account for the trabecular changes in glaucoma patients who have been receiving treatment with eyedrops containing benzalkonium chloride for several years.

Points to remember:

The cytotoxicity of preservatives may lead to trabecular changes in glaucoma patients receiving long-term treatment with preserved eye drops.

Stimulation by benzalkonium chloride of the secretion of proinflammatory mediators in cultures of lens cells

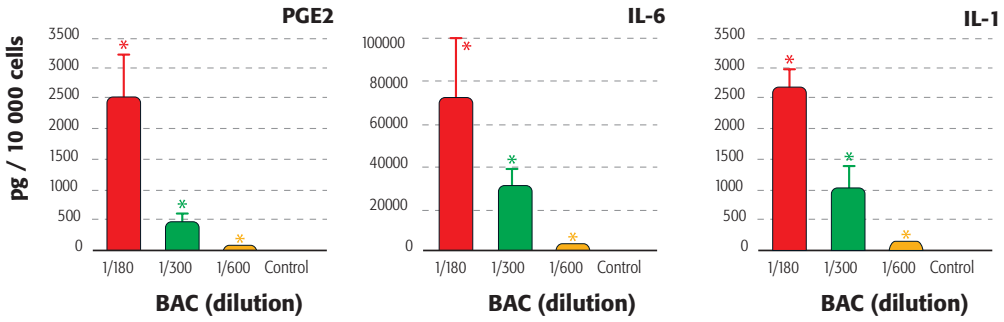


Figure 10

The cells were incubated for 7 days with various concentrations of benzalkonium chloride 0.02% or without the benzalkonium chloride (control). PGE2, IL-1 and IL-6 were assayed in the supernatant of the culture by immunofluorescence. Mean ± standard deviation of 6 independent experiments. * $p < 0.05$ versus control

After Goto *et al.* [22]

4.2- Lens

Patients receiving long-term antiglaucoma treatment, tend to develop macular cystoid edema more easily after a cataract surgery [46]. This effect is observed with various types of eyedrops (epinephrine, dipivefrin, timolol and latanoprost) containing a preservative. The causes of this induction have not been clearly identified. A possible link with inflammatory reactions has recently been suggested. The mechanism that is probably involved includes the release of pro-inflammatory mediators (prostaglandins, cytokines) during surgery. Thus, Goto *et al.* [22] have shown that benzalkonium chloride has the greatest dose-dependent toxic effect against human lens cells in culture by strongly inducing the expression of soluble chemical mediators (PGE2, IL-1±, and IL-6) (Figure 10).

Points to remember:

The inflammatory reaction induced by preservatives could account for the development of cystoid macular edema after a cataract surgery in patients treated long-term with preserved eye drops.

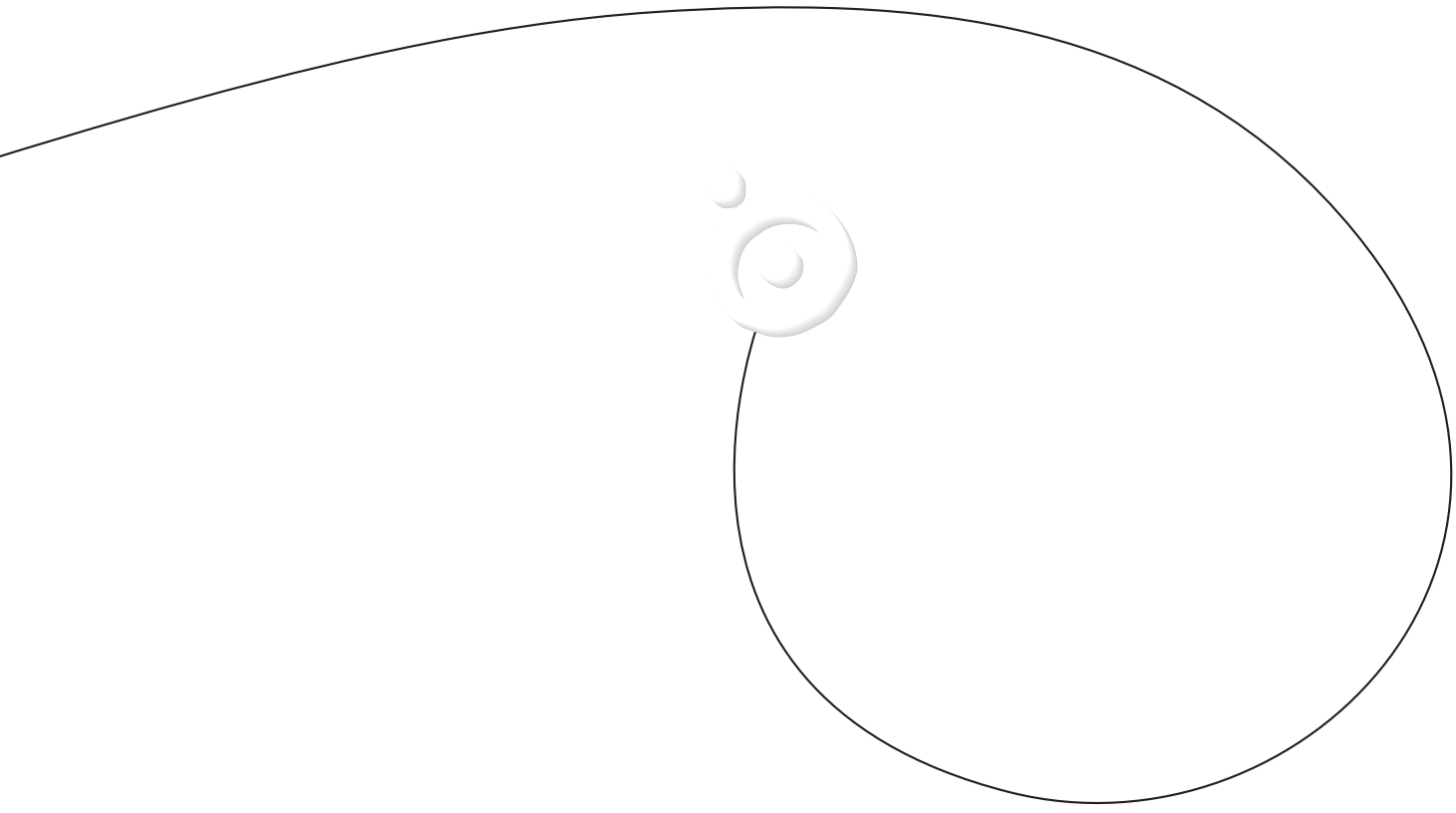
4.3- Retina

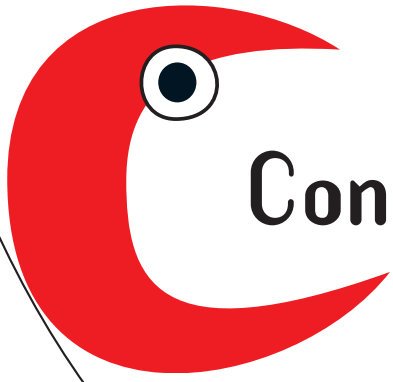
In pigmented rabbits, the sub-conjunctival injection (200 µl per day for 2 weeks) of eyedrops (timolol 0.5% or befunolol 1%) containing benzalkonium chloride produces retinal lesions that can be detected on the electroretinogram as a 50% reduction in the a and b waves after exposure for one week [7]. This is followed by detachment of the retina, a loss of visual acuity and the atrophy of the pigmented epithelium of the retina and the choroid. More particularly, the disappearance of the granules of melanin from the pigmented epithelial cells was observed, together with the disappearance of the internal and external segments of photoreceptor cells.

These effects seem to be specific to the preservative, since eyedrops containing preservative-free timolol or befunolol displayed only non-significant effects.

Points to remember:

Exposure of the retina to preservatives could produce severe retinal lesions





Conclusion

In addition to the evidence suggesting the toxicity of preservatives that we discussed in the first volume, experimental *in-vitro* and *in-vivo* studies have demonstrated that in situations involving prolonged exposure to the preservative, even if at low concentrations, there is a risk of histological, inflammatory and toxic changes at the surface of the eye, in particular if it is unhealthy, and therefore vulnerable.

These studies also make it possible to distinguish between the contributions of the preservative and the active substance in the eye drops in some of the toxic effects observed in Man.

These findings obtained using cell cultures or animals need to be interpreted with caution, since they are only experimental models, but they are being confirmed by increasingly numerous clinical studies. The most recent (Manni *et al*. [68]), published in the American Journal of Ophthalmology in January 2005 has just added another piece to the puzzle.

All the clinical data available on this subject will be discussed in the forthcoming third volume.

However, we can already state that all this data confirms that it is advisable to favour preservative-free forms whenever these are available on the market.

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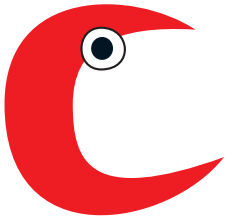

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