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Research paper

Ocular tolerance of preservatives on the murine cornea

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Abstract

We investigated the effects of instilling 13 commonly used preservatives on the murine cornea in vivo. Due to the instillation of preservatives, micro-lesions are formed on the cornea and can be selectively marked by fluorescein. The sum of the resulting fluorescent areas was measured using an episcopic microscope coupled to an image processing system. All the tested preservatives proved to be well-tolerated by the eye at commonly used concentrations. However, in some cases, increased concentrations of preservatives or combinations resulted in significant increase of the amount of corneal damage. With increasing the concentration, corneal lesion increased the most in the case of cetylpyridinium. While a combination of chlorobutanol 0.5% and phenylethylalcohol 0.5% did not result in an increase in corneal damage (when compared to the use of each separately), the associations of thiomersal 0.02% and phenylethylalcohol 0.4% on one hand and of edetate disodium (EDTA) 0.1% and benzalkonium 0.01% on the other, resulted in significant increases in the amount of corneal damage. However, in none of the tested combinations, the increase in the observed damage exceed the limit of ocular intolerance we had defined beforehand: thus, they were all deemed relatively well-tolerated. In the last part of the study, we investigated the effects of combining several preservatives, at usual concentrations, with an anesthetic solution of oxybuprocaine and found no notable increase in ocular damage.

Keywords: Ocular tolerance; Preservatives; Corneal epithelium; Eye drops

1. Introduction

Ophthalmic preservatives are chemical agents which can be added to ophthalmic preparations to prevent their microbiological degradation [1]. Their function is either to destroy microorganisms accidentally introduced in the ophthalmic solution after opening due to daily use (bactericide effect) or at least to prevent their growth (bacteriostatic effect) [2,3]. An ideal preservative should satisfy numerous criteria [4,5]: it should have a broad antimicrobial spectrum combined with a good activity at low concentration; it should be sufficiently soluble in the formulation; it should act rapidly and independently of pH; it should be chemically stable, i.e. resistant to autoclaving; and finally, it should meet the general requirements for excipients, i.e. it should be compatible with the drugs and other vehicles present in the formulation, free of undesirable therapeutic activity, and harmless to tissues. Unfortunately, none of the preservatives fulfills all these requirements. Thus, there is no ideal preservative which can be used universally [6]. For each formulation, the preservative which meets at best the above mentioned criteria must be chosen individually.

Numerous adverse effects of various preservatives on the cornea have been described like corneal erosions, allergic reactions, destabilization of the tear film, corneal deposits or delayed wound healing rate [7]. These corneal adverse effects have been investigated by the variety of techniques

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– in vitro, ex vivo or in vivo – and using different animal models – rabbit or mice. Thus, the comparison between the results is the more difficult since various concentration ranges are tested and since no study investigates all currently used preservatives at the same time.

We have established a quantitative objective method scaled down on mice eyes for the evaluation of ocular surface lesions caused by topical applications of cytotoxic preservatives by measuring the change of the corneal permeability for fluorescein subsequent to the alteration of the tissue integrity [8–12].

The purpose of this paper is to test by a standardized protocol the ocular irritation potential of 13 commonly used preservatives for ophthalmic preparations on the murine cornea. The present study examines the effects on the corneal tolerance of different factors: the nature of the preservative, its concentration, the combination of two preservatives and finally the addition of a preservative to a local anesthetic.

2. Materials and methods

2.1. Chemicals

The quaternary ammoniums, benzalkonium chloride and cetrimide were purchased from Fluka Chemie AG (Buchs, Switzerland); cetylpyridinium chloride was provided by Sigma (St. Louis, MO, USA). The organic mercurial preservatives, thiomersal and phenylmercury nitrate, were obtained from Fluka Chemie AG. The alcoholic compounds, chlorobutanol, phenylethyl alcohol and 2-phenoxyethanol, were supplied by Fluka Chemie AG; benzyl alcohol was purchased from Sigma. We chose sodium bisulfite as antioxidant (Sigma). Other miscellaneous preservatives: methyl 4-hydroxybenzoate, propyl 4-hydroxybenzoate, sorbic acid and sodium edetate were supplied by Fluka Chemie AG. Chlorhexidine digluconate was obtained from Sigma. Sodium fluorescein was purchased from Reactolab (Servion, Switzerland). Oxybuprocaine hydrochloride (Benoxinate) was obtained from Sigma. All other chemicals used for the preparation of the instilled solutions were of analytical grade.

All the solutions were freshly prepared in bidistilled water. All solutions were adjusted to the isocryoscopicity of tears by addition of sodium chloride except in the case of digluconate chlorhexidine and phenylmercury nitrate solutions, which were adjusted with sodium D-digluconate and sodium nitrate, respectively. The cryoscopicity of the solutions measured with a vapor pressure osmometer (Wescor 5500, Baumann-Medical, Switzerland) ranged between 285 and 300 mmol/kg. The solutions were unbuffered, their pH ranges between 4.0 and 7.5 except for the sorbic acid solution (pH = 3.3).

2.2. Animals and treatment

NMRI albino mice weighing 30–40 g (Biological Research BRL, Füllingsdorf, Switzerland) were used for the test. All animals were healthy and free of clinically observable ocular abnormalities. They were allowed free access to food and water. They were treated according the Swiss laws on the use of animals in scientific experimentation. The protocol of the experiment was approved by local ethics committees and has been described in detail previously [11–13]. Briefly, the protocol schedule consists in the instillation of the test solution (1 μl) in the right eye of mice at an interval of 2.5 h, 4 times per day for 3 days and once on the 4th day just before measurement. After the last instillation, the mice were anesthetized by i.p. injection of 0.1 ml pentobarbital 2%. The animal is then installed on a thermostatised stage-plate and 4 μl sodium fluorescein 0.1% are instilled into the eye. Fluorescein was used to reveal selectively the injured areas. The eye is then rinsed for 1 min with a NaCl 0.9% solution at 37°C. Each test was carried out on six mice (three males and three females).

2.3. Optical device and image processing system

The fluorescent image of the mouse cornea was detected by means of an opto-electronic device consisting in a high-sensitivity video camera (C2400; Hamamatsu Photonics, Jocho-cho, Japan) mounted on a dark-field episcopic microscope (Leica AG, Glattbrugg, Switzerland) equipped with a binocular tube (FSA; 0.50; 50%), oculars (Periplan 6.3) and a dark-field objective (Ultropack 6.5). A xenon lamp (XBO 75W/2; Osram AG Munich, Germany) served as a light source. Sets of color filters (BG 12 and K 530, Leica, Glattbrugg, Switzerland) and polarized filters (POL 513711, Leica) are placed on the light path to select out the excitation and emission wavelength and to eliminate reflected parasitic light, respectively. The video signal was digitalized by a Synergy framestore card (Synoptics, Cambridge, UK) plugged into an IBM AT-compatible computer. A high resolution color monitor (Mitsubishi HF-1400, Japan) connected to the card was used to visualize the digital image. Image processing enabled a measurement of the surfaces of fluorescent areas representing the injured corneal zones. The sum of the fluorescent areas was then expressed in percent of the total corneal surface. Image processing has been described in detail elsewhere [12].

2.4. Statistical evaluation

All data were analyzed by Student's *t*-test (unpaired samples). The 0.05 probability level was used for all comparison. Calculations were made with a Microsoft Excel 7.0 program.

3. Results

3.1. Irritation potential of preservatives at therapeutic concentration

In order to make a direct comparison possible, we used our test (see above) to investigate 13 most frequently used preservatives at their therapeutic concentrations, and all under the same conditions. The corneal damage expressed by the relative area of fluorescence following to the instillation of the preservative solutions is shown in Fig. 1. A sodium chloride solution (0.9%) was used as a control. The compounds are grouped according to their chemical classification in four categories: quaternary ammoniums, mercurials, alcohols and miscellaneous [1,14]. It appears that none of the preservatives at the tested concentration produces a fluorescent surface higher than 16% of the total corneal surface. A validation study carried out previously with the same technique showed that test solutions producing damage of up to 25% of corneal surface can be considered well-tolerated [15].

A detailed examination of Fig. 1 reveals that, among the chemical classes, the alcohols are in general the least-tolerated preservatives. Within the chemical classes no significant differences can be observed, except in the group of quaternary ammoniums in which cetrimide 0.01% produced a significantly higher score than its analogues ($n = 6$, $P > 0.05$). Among the miscellaneous additives, the fungistatic agent sorbic acid is the best-tolerated compound despite the pH of its solution (pH = 3.31) ($n = 6$, $P > 0.05$). Five preservatives (cetylpyridinium 0.02%, benzalkonium 0.01%, thiomersal 0.01%, phenylmercury nitrate 0.002% and sorbic acid 0.1%) were not more damaging to the cornea than the physiological saline solution which was used as a control ($n = 6$, $P > 0.05$). In contrast to these solutions, cetrimide 0.01%, parabens 0.1%, sodium bisulfite 0.1% (an antioxidant), chlorhexidine 0.01% and the alcohols were significantly more irritating to the cornea than the NaCl solution. In conclusion, the tested preservatives are safe at therapeutic concentrations, but at higher concentrations, they could be less-tolerated. Therefore, we decided to look into the relationship between the degree of irritation and the drug concentration administered with a view to establish a margin of safety in the use of preservatives.

3.2. Concentration effects

Since no comprehensive study of series of preservatives has been published, we decided to investigate the ocular effects of increasing concentration on the irritation potential

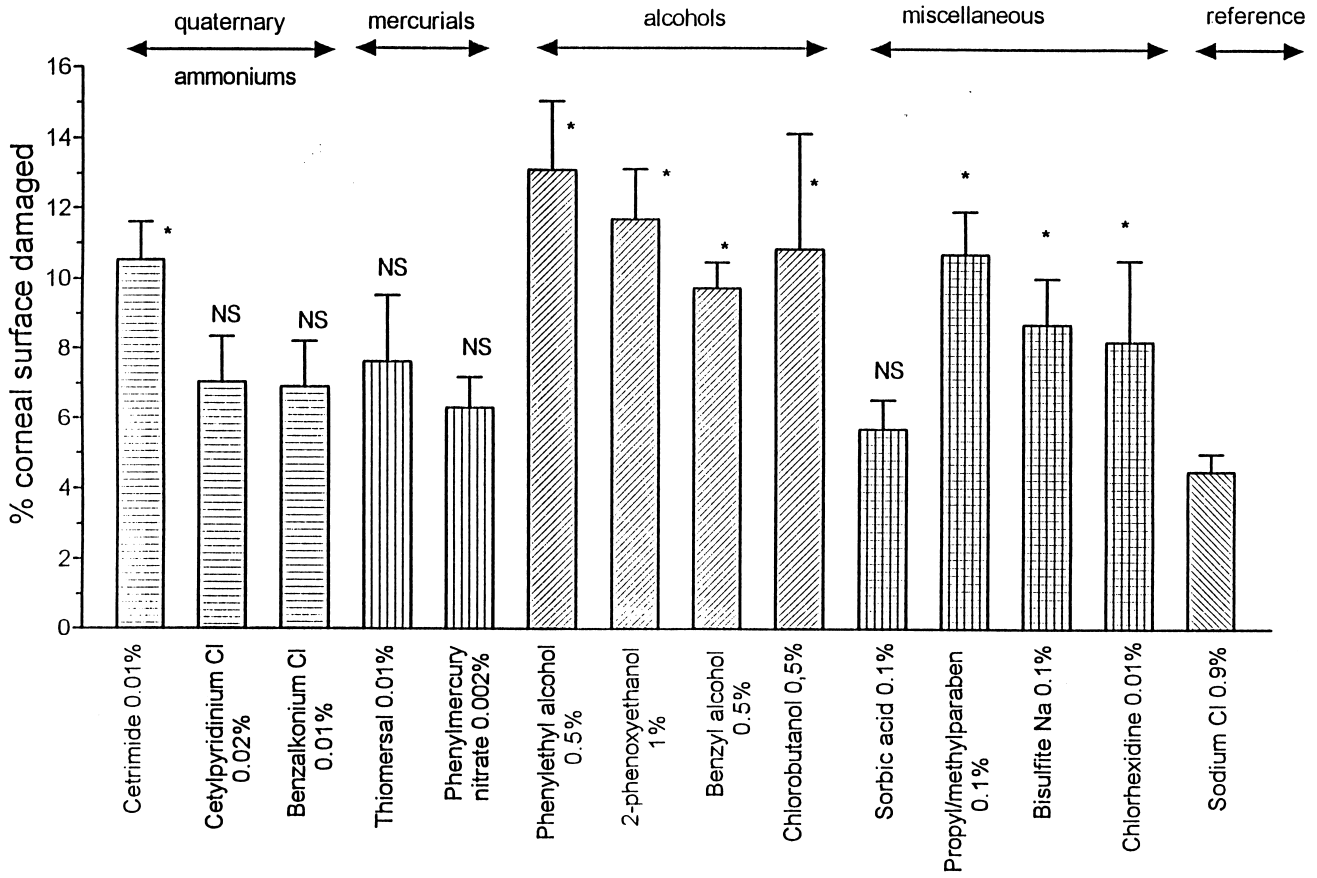

Fig. 1. Extent of corneal surface damage produced by instilling the most common preservatives in ocular solutions at therapeutically used concentration – compared to a sodium chloride solution (0.9%). Mean ± SD ($n = 6$), Student's t-test: NS, not significant; *P>0.05.

of four common preservatives (benzalkonium, thiomersal, cetylpyridinium and chlorhexidine) using our standardized protocol. The tested concentrations range from the generally used concentration up to 10 times that if the aqueous solubility of the preservative is sufficient. Fig. 2 shows that the irritation index of all four compounds increases with the concentration. At concentrations below 1.5 mM all dose–response relationships are similar. In this range, all preservatives appear to be well-tolerated. At higher concentrations, above 1.5 mM, the different preservatives can be separated into three groups. Firstly, for thiomersal, increasing concentrations have little effect on corneal damage. The second group, chlorhexidine and benzalkonium, show a more marked concentration effect. Finally, cetylpyridinium displays the most marked increase in corneal lesion with increasing concentrations. Nevertheless, for all tested preservatives, a 10-fold increase in the generally used concentration does not produce a pronounced irritation. Indeed, in our test, a result with a fluorescent surface of under 40% indicates that the solution is relatively well-tolerated.

3.3. Influence on the irritation potential of preservatives in combination

Two preservatives are often combined to broaden the antimicrobial spectrum. In order to assess whether the combination of two preservatives could affect the irritation potential or not, we tested three common combinations at currently used concentrations. The three different situations are presented in Figs. 3–5 which show the extent of damage to corneal surface produced by instilling two combined preservatives; the effects of the combinations is compared to the effect of saline solution as well as to the effects of each preservative administered separately. In the first case (Fig. 3), the combination of the two preservatives does not add to the level of irritation that would be produced by each preservative separately: chlorobutanol 0.5% associated with phenylethyl alcohol 0.5% does not produce significantly higher corneal lesions than phenylethyl alcohol alone ($n = 6$, $P > 0.05$). Each preservative instilled alone or the combination of both is nonetheless more irritating than the physiological saline solution.

In the second case (Fig. 4), the combination results in an increase of the level of irritation. The association of EDTA 0.1% and benzalkonium 0.01% produces significantly higher scores than each preservative alone, whereas neither the first nor the second compound is more damaging to the cornea than the NaCl solution.

The third case is displayed in Fig. 5. The irritation effect of the preservatives taken in combination is the sum of the irritation effects of each preservative taken alone. The combination of thiomersal 0.02% with phenylethyl alcohol 0.4% causes a significantly greater irritation to the corneal surface than either both preservative taken alone. Nevertheless, it should be noted that the overall effect is still below the threshold of 25% of corneal surface damaged and, therefore, the preservatives can be considered relatively well-tolerated.

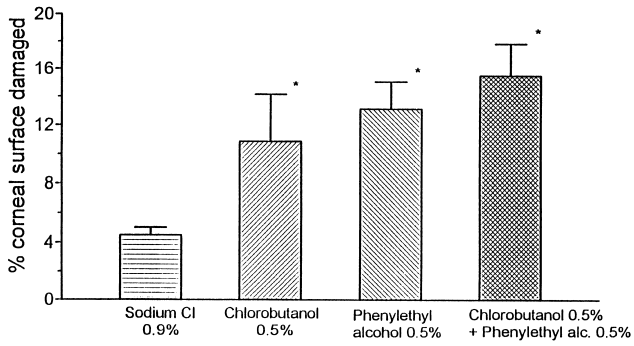

Fig. 3. Extent of damages of corneal surface induced by instilling a combination of chlorobutanol and phenylethyl alcohol compared to saline solution and to the individual preservative. Mean ± SD ($n = 6$), Student's *t*-test: NS, not significant; $*P > 0.05$.

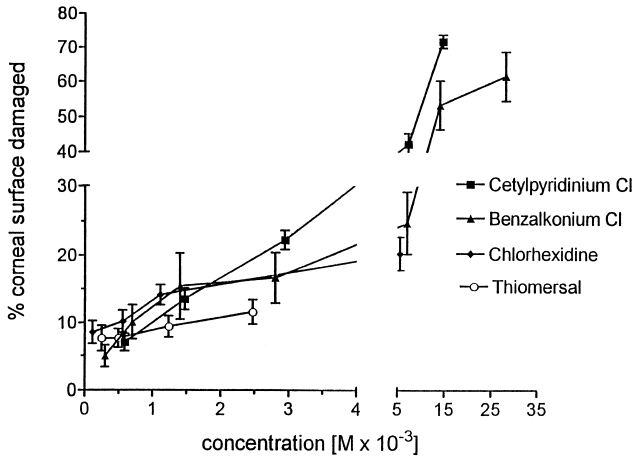

Fig. 2. Extent of corneal surface damage vs. the instilled concentration of four common preservatives (benzalkonium, cetylpyridinium, thiomersal and chlorhexidine). For all compounds, the tested concentration range covers the currently used and at least a 10-fold increased concentration. Mean ± SD ($n = 6$).

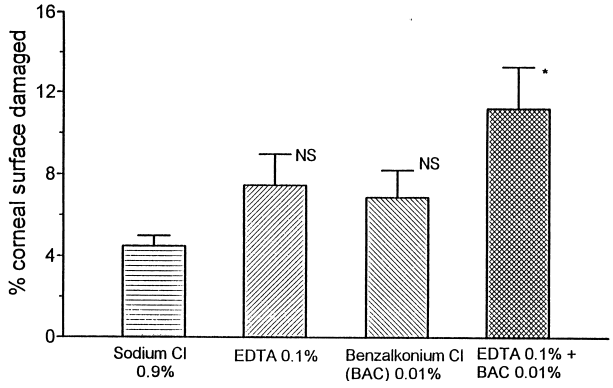

Fig. 4. Extent of damages of corneal surface induced by instilling a combination of EDTA and benzalkonium (BAC) compared to saline solution and to the individual preservative. Mean ± SD ($n = 6$), Student's *t*-test: NS, not significant; $*P>0.05$.

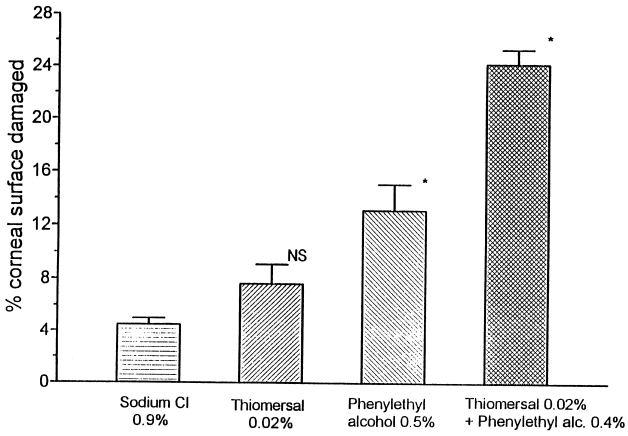

Fig. 5. Extent of damages of corneal surface induced by instilling a combination of thiomersal and phenylethyl alcohol compared to saline solution and to the separated preservative. Mean ± SD (n = 6), Student's t-test: NS, not significant; *P>0.05.

3.4. Effect of the association of preservatives and a local anesthetic on the irritation potential

Most drugs products are associations of excipients and one or more therapeutic agent. Ideally, the excipients should not hamper the pharmacological action nor increase the toxicity of the therapeutic agent. We investigated the ocular tolerance of an association of some commonly used preservatives (benzalkonium, chlorhexidine, thiomersal and chlorobutanol) with a model drug, oxybuprocaine, a local anesthetic known to produce ocular lesions after prolonged use [16,17]. The results are presented in Fig. 6. Except in the case of chlorhexidine, it appears that the addition of a preservative increases significantly (n = 6, $P < 0.05$) the damaged surface of the cornea compared to the damage produced by oxybuprocaine alone. However, none of the associations produced injuries which might be of concern in general therapeutic practice.

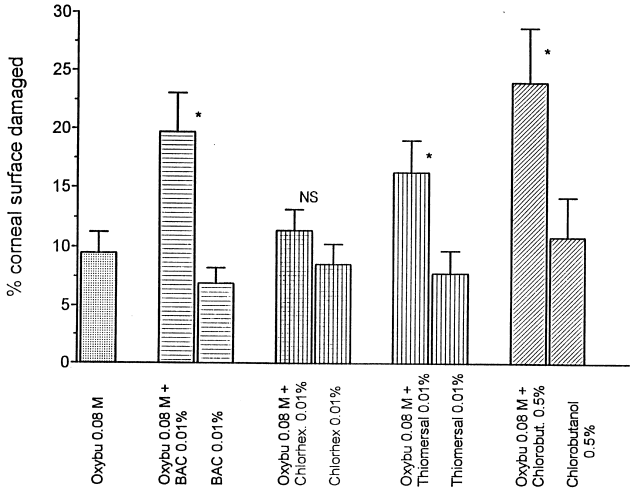

Fig. 6. Extent of damage of corneal surface produced by instilling preservatives added to oxybuprocaine (0.08 M), a common local anesthetic – compared to oxybuprocaine alone. Mean ± SD (n = 6), Student's t-test: NS, not significant; *P>0.05.

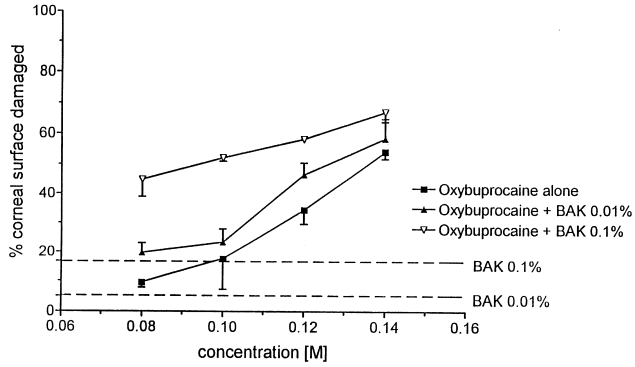

Fig. 7. Effect of increasing concentration of oxybuprocaine alone (closed square) or associated with benzalkonium (BAK) used at the current concentration of 0.01% (closed triangle) or a 10-fold increased concentration (open triangle) on the degree of corneal lesions. Mean ± SD (n = 6).

In addition to the combinations at therapeutic concentrations of oxybuprocaine with various preservatives, we investigated the effect of two concentrations – at therapeutic level and 10 times that concentration – of benzalkonium on the dose–irritation potential relationship of oxybuprocaine. We chose benzalkonium as preservative because it is by far the most commonly used preservative [18]. Fig. 7 shows the effect of changing concentrations on the extent of corneal damage. For oxybuprocaine alone, increasing concentrations of the instilled solution increases the percentage of damage to the corneal surface. For the combination of oxybuprocaine with benzalkonium 0.01% – the normally used concentration of the preservative – we note a slight increase in corneal damage which is not significantly different (n = 6, $P < 0.05$) than the effect of the unpreserved anesthetic solution alone. Hence, the dose–response curves of oxybuprocaine alone and of oxybuprocaine combined with benzalkonium 0.01% are very close together. However, when a benzalkonium solution that is 10 times more concentrated is added to the anesthetic, the resulting irritation is significantly higher than the one caused by unpreserved oxybuprocaine.

4. Discussion

4.1. Irritation potential of preservatives at therapeutic concentration

There have been many attempts to determine the ocular irritation potential of chemicals and especially preservatives [7]. However, it is difficult to draw general conclusions about the oculotoxicity induced by preservatives because the techniques, animal models and methods used are so different from one another. Furthermore, the numerous grading systems used in order to quantify ocular toxicity have often relied on a subjective evaluation [19–23]. Thus, there has been a need to develop an objective test allowing rapid screening under standardized conditions.

The present test permits an objective evaluation of cor-

neal tolerance since the percentage of fluorescent areas representing the lesions is measured without any subjective intervention by the operator. A rapid screening of the corneal irritation potential of ophthalmic products is also possible [24]. By extending the duration of the test (over a period of months instead of weeks), adverse ocular reactions caused by long-term instillation of eye drops can be assessed: chronic toxicity is indeed an important consideration when choosing a preservative. Phenylmercuric salts, for example, seem to be well-tolerated in this 4-day study, but long-term instillation of eye drops containing this preservative can lead to mercurialentis [25,26].

Another eye tolerance test on mice eye has been described by Maurice et al. [27–29]. They assessed acute corneal toxicity by measuring corneal permeability to fluorescent dye (sulforhodamine B). The advantage of using such an animal model is the low cost, the ease to handle and the availability of standardized strain. Furthermore, the little size of the mouse head allows the use of standard microscopes; it's not necessary to modify the stage.

Another animal model, namely, the albino rabbit, is very often used for the evaluation of the potential irritancy of cosmetics, toiletries, household products and chemicals [30]. But the use of living rabbits for the testing of acute eye toxicity has come under considerable attack on the grounds that it is cruel and that the extrapolation of animal data to human is difficult due to anatomical and physiological differences from the human eye (presence of a nictitating membrane, no well-defined Bowman's membrane, thinner cornea, less effective tearing mechanism, slower blinking rate, more alkaline tear pH, slower regeneration capability of the corneal epithelium) [27,31–34]. Thus, there are no perfect animal model in terms of valid predictability to human situation. A comparative study has demonstrated that mice were not worse model than rabbits [15].

In eye toxicology, considerable work has been directed toward developing, for use in ocular safety assessment, in vitro assays which use isolated ocular or non-ocular tissues, cultures of cells, invertebrate organisms, fertilized hen's eggs or physicochemical systems [35–38]. However in vitro tests have some drawbacks. They are unable to reproduce the complete irritation reaction observed in living eye and they can not assess chronic toxic effects on the cornea [39]. In conclusion, none of the current available in vitro have shown to be a valid replacement for in vivo assays. It is then preferable to use an in vivo irritation test on mice cornea rather than a test on fertilized hen's eggs.

4.2. Concentration effects

So far only few studies have attempted to investigate the influence of increasing preservative concentration influences ocular toxicity [7]. The present study (Fig. 2) demonstrates that at the tested concentrations, thiomersal is better-tolerated than three other selected preservatives – benzalkonium, chlorhexidine and cetylpyridinium. This difference in tolerance can be explained by the fact that thiomersal is not ionized and has no surfactant properties. Cationic surfactants like benzalkonium and cetylpyridinium are known to emulsify the lipid layer of the tear and to disrupt the lachrymal film barrier [40–43]. Our finding that cetylpyridinium is the most damaging to the eye is consistent with a previous study by Green et al. [44]. Exposing isolated rabbit corneas to benzalkonium and cetylpyridinium, they found a more pronounced progression in the dose–response curve for cetylpyridinium.

Thus, when developing ocular preparations, it is important to plot dose–response curves of their components to establish a margin of safety in the use of the preparations.

4.3. Influence on the irritation potential of preservatives in combination

The combination of two or more preservatives mostly serves to enlarge the antibacterial spectrum [45]. Indeed, EDTA has been shown to enhance the efficacy of benzalkonium chloride (BAK) against *Pseudomonas aeruginosa* and it can even reverse the resistance of the bacterium to BAK [46]. The mechanism of this antibacterial potentialization can be explained by the fact that both preservatives have different targets: benzalkonium disrupts the bacterial external membrane whereas EDTA disorganizes the cell envelope. By the same mechanism, EDTA, a chelator of calcium which is a cation required for the assembly of the cellular cytoskeleton, can disorganize the cytoskeleton of mammalian cells. It is therefore possible that the instillation of EDTA in the eye may result in a cytotoxic effect [47,48]. Such an effect has in fact been observed in vitro and in vivo: EDTA induces a loss of the cellular membrane in isolated rabbit corneas [49]; the instillation of EDTA 0.1% produced corneal edema in rabbit eye [50]. The same concentration applied to the eyes of human volunteers caused severe stinging [51].

Some studies have investigated the antibacterial spectrum of preservatives, but few have assessed the influence of the combination of preservatives on the irritation potential. In our experiment, the combination of EDTA 0.1% and BAK 0.01% led to a potentialization of ocular lesions. This finding concurs with the results of two previous studies. Indeed, Collin and Caroll [52] demonstrated that a mixture of EDTA 0.1% and BAK 0.01% resulted in markedly greater damage to rabbit corneal cells than BAK alone at 0.02%. Colin [53] found a slower healing rate of the rabbit cornea after keratectomy when BAK (0.01%) and EDTA (0.1%) were given in combination, whereas there was no significant effect on corneal healing after the instillation of each preservative alone. Our results show that the effects of preservatives in combination can vary. In some cases, the combination is no more irritating to the eye than the components taken separately (Fig. 3). In other cases, the resulting overall irritation potential of the combination exceeds individual irritation potentials of each of its components (Figs. 4 and 5). How-

ever, for the tested combinations, the increased levels of irritation still fall within the limits of ocular tolerance.

It is therefore clear from our observations that the benefits of increasing antibacterial spectrum by combining preservatives can be diminished by the increased damage to the cornea caused by the combination.

4.4. Effect of the association of preservatives and a local anesthetic on the irritation potential

The present study shows that oxybuprocaine is a relatively well-tolerated topical anesthetic, but that the addition of preservatives can increase the irritation potential of the preparation. Several authors have investigated the effect produced by the addition of a preservative to a drug. Ramselaar et al. [54] have observed that, while oxybuprocaine instilled without a preservative has no effect on corneal permeability to fluorescein in humans, when combined with benzalkonium 0.01%, it produces a significant increase in corneal permeability. Pfister and Burstein [55] have shown that benzalkonium 0.01% with pilocarpine 2% administered to excised human corneas caused significant injury to the plasma membrane, desquamation of the epithelium and cell death, whereas pilocarpine 2% alone produced only a moderate level of injury to the plasma membrane.

The use of preservatives in topical ophthalmic medications and in contact lens solutions presents an obvious dilemma. On the one hand, preservatives provide a necessary prevention of microbial growth in ophthalmic solutions, but on the other hand they may be toxic to the ocular tissues. Preservative-free solutions can be an alternative, but they have some drawbacks: short shelf life, conservation under refrigeration or necessity of expensive unit dose package [56]. Thus, preservatives remain the easiest way for the conservation of multi-dose eye drops. For selecting a suitable preservative, consideration must be given to its microbiological effectiveness as well to its possible adverse effects on the tissue to which they are applied.

5. Conclusions

No preservative is perfect in terms of maximal antibacterial activity and absence of side effects. Each preservative must be evaluated on a risk to benefit ratio with regard to long-term safety and toxicological consideration [57]. The present work shows that the commonly used preservatives induce little or no ocular damage provided they are applied at their recommended concentrations. Among the tested preservatives the best-tolerated are cetylpyridinium (0.2%), benzalkonium (0.01%), thiomersal (0.01%), phenylmercury nitrate (0.002%), sorbic acid (0.1%), parabens (0.1%), sodium bisulfite (0.1%) and chlorhexidine (0.01%). At higher concentrations, however, the preservatives produce a greater amount of corneal damage. In some cases combining preservatives can influence ocular irritability: the association of thiomersal (0.02%) and phenylethyl alcohol (0.4%) results in significantly more pronounced microlesions than each of the preservatives alone. But all the combinations tested remained within the limits of an acceptable tolerance. The addition of preservatives to an oxybuprocaine solution did not affect the ocular tolerance of the preparation. However, one has to keep in mind that the present test has been carried out in mice and that further experiments lack to investigate the possible extrapolation to the human eye. Currently, we are investigating the irritation potential of preservatives on the rabbit and human eyes using confocal microscopy. This procedure will enable to better understand the validity of animal models for the extrapolation to human situation.

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