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Application of in vivo confocal microscopy to the objective evaluation of ocular irritation induced by surfactants

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Abstract

An ocular irritation test using confocal laser scanning ophthalmoscopy has been developed in which corneal lesions subsequent to instillation of surfactants are specifically marked by fluorescein and assessed by digital image processing. The sum of the observed fluorescent corneal areas is taken into account as an endpoint of ocular irritation. Eight currently used nonionic, cationic and anionic surfactants were applied onto the cornea of rabbits and mice, four times per day during 3 days at various concentrations. Benzalkonium chloride, a cationic surfactant, at a concentration range of 0.01–0.5%, was tested in the same manner. The cornea was evaluated in vivo for ocular tolerance by confocal microscopy. In both rabbits and mice, the test revealed following irritation rankings: cationic > anionic > nonionic surfactants. Furthermore, in both animal models, the ocular damage increased with the concentration of benzalkonium. The test was sensitive enough to detect ocular microlesions at concentrations of surfactants as low as 0.01% for benzalkonium. These findings demonstrate the usefulness of confocal microscopy for the non-invasive, in situ evaluation of ocular tolerance. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Confocal microscopy; Cornea; Draize eye test; Surfactants; Preservatives; Image analysis

1. Introduction

The tests currently used to evaluate the safety of xenobiotics in human eyes with vertebrate animals have been heavily criticized because of ethical and scientific considerations. Indeed, the Draize rabbit eye test (Draize et al., 1944), a test adopted worldwide by regulatory bodies for the assessment of ocular irritancy, is based mainly on scoring of observed macroscopic changes in the rabbit cornea, conjunctiva and iris after exposure to a test compound. These observations have been criticized because of their subjectivity (Buehler, 1974; Heywood and James, 1978), the high dose of test material used (Griffith et al., 1980; Walker, 1985; Williams, 1985; Freeberg et al., 1986;

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Chambers et al., 1993; Lambert et al., 1993), their intra- and interlaboratory variability (Russel and Hoch, 1962; Rieger and Battista, 1964; Weil and Scala, 1971; Burton, 1972; Marzulli and Ruggles, 1973; McDonald and Shaddhuck, 1977), their over-predictiveness of human responses (Marzulli and Simon, 1971; Buehler, 1974), and because they harm animals (Rowan, 1984; Zbinden, 1985).

Considerable work has been devoted to developing alternatives for ocular safety testing. The principal alternative to animal testing is in vitro testing. The numerous in vitro assays developed generally use cell, tissue or organ cultures and measure a wide range of end-points such as cell proliferation, cytotoxicity, membrane permeability or metabolism (Bruner, 1992; Chu and Toft, 1993). Their advantages over live animal testing include cost, effectiveness, simplicity, reproducibility, and reduced suffering in animals (Rohde, 1997). However, although there are numerous screening tests available, some of which have been well-validated (Balls et al., 1995), none has yet gained full acceptance (Jester et al., 1996; Schneider et al., 1997). The reason for this lack of replacement is in part due to the difficulty in correlating in vitro data with subjective in vivo test results (Jester et al., 1996). Thus, there is a need to develop refined in vivo tests that provide better risk assessment information with the aid of objective and reliable data. Some in vivo techniques have been proposed in order to increase the sensitivity of detection of injury by the use of non-invasive objective techniques (Daston and Freeberg, 1991; Ballantyne, 1995). These techniques include pachymetry, i.e. the measurement of the corneal thickness after instillation of chemicals (Burton, 1972; Conquet et al., 1977; Morgan et al., 1987; Jacobs and Martens, 1989), tonometry, i.e. the measurement of the intraocular pressure which can vary under the influence of drugs (Ballantyne et al., 1972; Walton and Heywood, 1978), and corneal permeability studies, i.e. the recording of the modification of the corneal permeability to fluorescein or sulforhodamine B (Etter and Wildhaber, 1985; Magada et al., 1993; Maurice and Brooks, 1995; Kälin et al., 1996). These in vivo methods have the advantage of monitoring a functional physiological system with quantifiable evaluations.

Recent progress in optical physics has enabled the development of confocal microscopes, technical aids which have proved very useful for the non-invasive clinical evaluation of the eye. Confocal microscopy permits optical sectioning through a living intact tissue with resolution and contrast superior to conventional optical microscopy and without the artifacts induced by the preparation of the specimen in electron microscopy (Wilson, 1986; Wright et al., 1993). It has found numerous ophthalmic applications including the assessment of ocular toxicology (Furrer et al., 1997; Jester et al., 1998).

The aim of the present work was to develop a method for evaluating possible corneal effects following repeated application of new ophthalmic preparations. A new in vivo method for assessing ocular irritation potential in mice and rabbit eyes using confocal laser scanning microscopy and corneal fluorescein staining was described. The ability of this test to quantify objectively surfactant-induced eye injury and the influence of the type of surfactants and their concentration on the ocular irritation potential were evaluated. Surfactants or quaternary ammonium preservatives with surfactant properties were chosen as test materials because they are widely used in industry and at home for a variety of purposes (as preservatives, detergents, absorption enhancers, or emulsifying agents) and because they are known to cause varying ocular irritation (Olson et al., 1962; Maurer et al., 1997).

2. Material and methods

2.1. Materials and animals testing

Eight surfactants were selected, representing different chemical classes (anionic, cationic and nonionic surfactants) and different levels of ocular irritation. Surfactants obtained from Fluka Chemie (Buchs, Switzerland) included benzalkonium chloride and cetrimide, two cationic quaternary ammoniums, and Tween® 20, a nonionic surfactant. Sodium lauryl sulfate (an anionic surfactant also called sodium dodecyl sulfate) was supplied by Merck (Darmstadt, Germany).

Pluronic F127 (Poloxamer 407, also called Lutrol F127), a nonionic surfactant, was generously donated by BASF (Ludwigshafen, Germany). Sodium cholate, an anionic bile salt, was purchased from Sigma (St Louis, MO). Two nonionic surfactants: Brij 35P (CTFA-Name: Laureth-23) and Mirj 51 were kindly provided by ICI (Essen, Germany). Sodium fluorescein was obtained from Reactolab (Servion, Switzerland). All other chemicals used for the preparation of the instilled solutions were of pharmaceutical grade and used as received, without further purification.

All solutions were freshly prepared with bidistilled water. The solutions were adjusted to the isocryoscopicity of tears by addition of sodium chloride. The cryoscopicity of the prepared solutions measured with a vapor pressure osmometer (Wescor 5500, Baumann-Medical, Switzerland) ranged between 285 and 300 mmol/kg. The solutions were not buffered; their unmodified pH ranged between 5.1 and 6.3.

Male New Zealand white rabbits weighing approximately 4–5 kg (University Medical Center, Geneva, Switzerland) and NMRI albino mice weighing 30–40 g (Biological Research BRL, Füllingsdorf, Switzerland) were used in this study. Animals were individually housed in stainless steel cages and maintained in a 12-h light, 12-h dark cycle at 19 ± 1°C. They were allowed water and food ad libitum. All animals were healthy and free of clinically observable ocular abnormalities. All experiments were performed in accordance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research (ARVO, 1994), and were approved by the local veterinary authority for animal experimentation.

2.2. Treatment

The protocol schedule consisted of instillation of the test solution (10 μl for rabbit, 1 μl for mice) directly applied onto the right cornea of the animal at an interval of 2.5 h, four times per day for 3 days and once on the 4th day just before the examination. The instilled amount for the rabbit was chosen according to the low-volume test (Lambert et al., 1993), and for the mice, the smallest volume that could be instilled accurately was chosen. The dosing schedule was the one used in previous work (Kälin et al., 1996) and corresponded to the usual frequency of instillation of eye drops such as those used in antiglaucomateous preparations. After the last instillation, the animals were either sedated or anesthetized. Rabbits were sedated with an intramuscular injection of ketamine HCl (15 mg/kg body wt.) and xylazine (3 mg/kg) and placed on a adjustable-height trolley in front of the camera head. Mice were anesthetized by intrapeeritoneal injection of 0.1 ml pentobarbital 2% and installed in a stand in front of the camera. Before the microscopic observation, a rapid macroscopic examination of the animal eye was made in order to detect any clinical evidence of irritation (corneal opacity, conjunctival redness or chemosis, discharge). A sodium fluorescein solution 0.5% (10 μl for rabbit, 4 μl for mice) was instilled in the eye to be tested. Fluorescein allowed the injured areas to be selectively marked. The eye was then rinsed during 1 min with a NaCl 0.9% solution at 37°C. After the experiment, the rabbit was replaced to rest in the cage. The rabbits were reused after 4 weeks when ocular examination by confocal microscopy showed complete healing. The mice were sacrificed by an overdose of pentobarbital. Each test was carried out on six mice or three rabbits. The number of animals corresponded to the recommendation in the literature (see Section 4).

2.3. Optical device and image processing system

A confocal laser scanning ophthalmoscope (CLSO® Zeiss, Oberkochen, Germany) was modified by addition of a set of lenses in order to allow the examination of the cornea (Massig et al., 1994; Furrer, 1999). The light source was an argon ion laser (wavelength: 488 nm). The microscope objective was an Epiplan-Neofluar $5 \times /0.15$ (Zeiss, Germany). The images displayed on a monitor were continuously recorded on a Super VHS videotape. The ophthalmoscope focusing was controlled via a computer during the image acquisition. After the three-dimensional reconstruction of the digitized images with an image-processing system (Semper6, Synoptics, UK), the

fluorescent zones, representing the injured corneal areas, were determined. For both animal models, a 4.43 mm^2 area was observed; for the mice, the area represents the whole cornea, whereas for the rabbit, central regions of the cornea was examined. Preliminary studies have shown that the measured zone is representative for the whole corneal surface.

2.4. Statistical evaluation

Comparison were made with Student's t-test (unpaired samples). The statistical significance level was fixed at $P < 0.05$. Calculations were made with Microsoft® Excel 7.0 program.

3. Results

3.1. Corneal damage in mice and rabbits

Table 1 shows the percentage of corneal surface damaged after instillation into the mouse and rabbit eyes of surfactants according to the three chemical classes of surfactants: nonionic, anionic and cationic. A sodium chloride solution (0.9%) served as a control. All the tested surfactants were used at 0.5% concentration, except for Pluronic F127 which was assessed at 20%, a concentration at which it forms a thermoreversible gel (Schmolka, 1972). The nonionic surfactants, Tween® 20, Pluronic F127, Mirj 51 and Brij 35, were not more damaging to the mouse or rabbit cornea than the physiological saline solution. The two tested anionic surfactants behaved differently. On one hand, sodium lauryl sulfate (0.5%) had an unacceptable tolerance (about 36% of the corneal surface is damaged in the mouse and 48% in the rabbit). On the other hand, sodium cholate (0.5%) was well tolerated and did not cause higher lesions than nonionic surfactants or the saline control solution in both species. Concerning the oculotoxicity of cationic surfactants, benzalkonium chloride (0.5%), a cationic quaternary ammonium, caused the most marked lesions in both mice or rabbits.

In comparing the irritation potential of surfactants in mice and rabbit eye, it should be noted that despite the anatomic and physiological differences between the two animal species, the results were comparable. In both models, the cationic surfactants were the least tolerated and the ocular irritation induced by benzalkonium increased with concentration.

3.2. Influence of the concentration on the irritation in mice and rabbits

Fig. 1 displays the relative area of fluorescence on mouse and rabbit cornea after instillation of

Table 1
Extent of corneal surface damage in mice and rabbits produced by instilling seven common surfactants in aqueous solutions, compared to a sodium chloride solution (0.9%)[a]

Surfactant products		% of corneal surface damaged			
		Mouse		Rabbit	
Reference	NaCl (0.9%)	7.28 ± 0.24		1.15 ± 0.23	
Nonionic	Tween 20 (0.5%)	7.78 ± 0.53	NS	3.56 ± 0.22	NS
Nonionic	Pluronic F 127 (20%)	8.67 ± 0.60	NS	3.57 ± 0.13	NS
Nonionic	Mirj 51 (0.5%)	9.25 ± 0.92	NS	5.24 ± 0.99	NS
Nonionic	Brij 35 (0.5%)	9.37 ± 0.76	NS	2.52 ± 0.51	NS
Anionic	Cholate Na (0.5%)	10.12 ± 2.05	NS	3.67 ± 0.58	NS
Anionic	Laurylsulfate Na (0.5%)	36.12 ± 1.39	*	47.78 ± 1.31	*
Cationic	Benzalkonium Cl (0.5%)	50.55 ± 1.66	*	57.00 ± 2.00	*

[a] All solutions were not buffered and isoosmotic to tear fluid. Mean ± SD ($n = 6$ mice; $n = 3$ rabbits), Student's t-test: NS, not significant.

* Student's t-test: $P < 0.05$ compared to NaCl 0.9% solution.

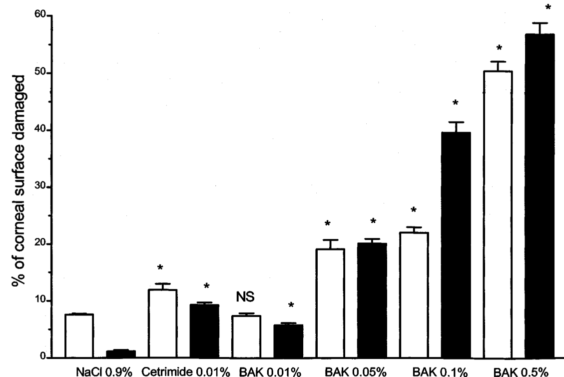

Fig. 1. Extent of corneal surface damage in mice (open columns) or rabbits (closed columns) versus the instilled concentration of benzalkonium chloride (BAK), a quaternary ammonium salt, compared to a sodium chloride solution (0.9%) and a cetrimide solution (0.01%), another cationic surfactant. The tested concentration range of BAK covers the currently used concentration in eye drops (0.01%) and a 50-fold increased concentration (0.5%). Mean $\pm$ S.D. ($n = 6$ mice; $n = 3$ rabbits), Student's t-test: NS, not significant; $*P < 0.05$ versus NaCl 0.9%.

increasing doses of benzalkonium (BAK), a cationic surfactant. A sodium chloride solution (0.9%) served as control; a cetrimide solution (0.01%), another cationic surfactant, was added for comparison. Fig. 1 shows clearly that the irritation index of BAK increased with the concentration of the surfactant. At the lowest concentration (0.01%), the concentration at which the compound is commonly used as preservative for aqueous eye drops, BAK was not more irritating to the mice eye than the saline solution. In the case of rabbit, BAK 0.01% was only slightly more irritating than the saline reference (5.78% of corneal surface fluorescent vs. 1.15%). For cetrimide (0.01%), the resulting irritation in both species was slightly higher than for benzalkonium 0.01%.

4. Discussion

4.1. Corneal damage in mice and rabbits

The test developed has permitted the ranking of the irritation potency of some surfactants according to their ionic nature. The findings that cationic surfactants are the most irritating compounds and nonionic are the least irritating are consistent with other studies. Etter and Wildhaber (1984) showed that it is possible to classify surfactants according to their increasing irritancy potential in mice as follows: nonionic Tween® 80 (1%) < anionic sodium lauryl sulfate (1%) < cationic benzalkonium (1%). Maurice and Brooks (1995), using an acute ocular toxicity test on mouse cornea stained with sulforhodamine B, also noticed the innocuity of Tween® (score similar to NaCl) and the relative toxicity of sodium lauryl sulfate (score twice as high as NaCl). As in the case of mice eyes, the ranking order in rabbit eyes is nonionic surfactants < anionic surfactants < cationic surfactants. This result is consistent with data from previous rabbit studies. Draize and Kelley (1952) reported that the maximal tolerated concentrations were 0.5% for benzalkonium chloride, 20% for sodium lauryl sulfate and 100% for Tween® 20. Kennah et al. (1989) reported Draize scores of 56 — out of a maximum score of 110 — for benzalkonium chloride (1%), 16 for sodium lauryl sulfate (3%) and 0 for Tween® 20 (1%). Finally, Grant and Acosta (1996) using a low-volume modification of the Draize rabbit test (instillation of 0.01 ml) obtained almost the same score with benzalkonium (1.9%) and sodium lauryl sulfate (19%), showing that a 10-fold diluted benzalkonium solution is as irritating as sodium lauryl sulfate. The confocal microscopy test provides the same ranking as the Draize test, but is more sensitive and less painful to animals as the concentrations tested are lower. Surfactants have been used in the test as standards, but the sensitivity of the test allows an assessment of active constituents of medicines.

The toxic action of the surfactants on the cell components depends on their chemical class. The cationic surfactants precipitate cellular proteins, the anionic surfactants dissolve the cell membranes whereas the nonionic surfactants have neither of these effects (Grant, 1986). Benzalkonium, a cationic surfactant, has been shown to induce morphological ocular adverse effects in the form of a corneal epithelial desquamation and hence increase the corneal permeation of drugs (Green, 1992). Sodium lauryl sulfate, an anionic surfac-

tant, induced a corneal epithelial erosion as demonstrated by confocal investigations on rabbit eyes (Maurer et al., 1997).

In comparing the irritation potential observed in mouse and rabbit eye, a similar conclusion has been drawn about the irritation ranking of surfactants according to their chemical classes. However, despite a similar tendency, a difference is clearly observed with the saline reference solution (NaCl 0.9%). Indeed the control solution causes about 7% of the corneal surface to be fluorescent in the mouse, whereas only about 1% appears fluorescent in the rabbit. These damaged areas are due to a physiological desquamation process; they are observed even in healthy eye and described as micropunctate fluorescein staining (Norn, 1970; Josephson and Caffery, 1988). The natural exfoliation has been reported to be less than 1% in the rabbit (Doughty, 1992), but no comparison has been made with mice. The greater sensitivity of the mouse eye compared to the rabbit may be explained by the thinner epithelium (20–30 μm vs. 30–40 μm in rabbit) and the lower number of cell layers (3–5 vs. 5–7 in rabbit) or this difference of vulnerability may reflect difference in the turnover rate of epithelial cells in rabbits and mice (Ehlers, 1970; Tonjum, 1975). The respective advantages and drawbacks of these two animal models in eye irritancy testing have been discussed previously (Furrer et al., 1999). Similarly, Jester et al. (1996) and Maurer and Parker (1996) suggested that rodents such as rats could be used as a surrogate for the rabbit in studies of surfactant-induced eye irritation.

4.2. Influence of the concentration on the irritation in mice and rabbits

In both species, the irritation index of benzalkonium chloride increases with the concentration. A dose–response relationship in the form of sigmoid curves has already been described for surfactants instilled in mice eyes (Furrer et al., 1999).

The test described here indisputably presents many advantages: it is relevant, objective and sensitive.

4.2.1. Relevancy

Firstly, the most obvious advantage lies in the relevance of the test which is carried out on the live eye. Indeed many in vitro procedures on perfused eye or cultured cells do not reproduce the complexity of the physiological mechanisms involved in the response of ocular tissues to an irritation (lacrimation, blinking, chemosis, inflammation, vascularization, chemical composition of tears). Furthermore, some alternative systems even use nonocular tissue like rabbit ileum or chorioallantoic membrane of chick embryo, with the explanation that the cell-toxicity is a non-specific mechanism. But it is clearly preferable to rely on tests carried out on cornea, the relevant target tissue, having all protecting functioning systems. The proposed test provides a direct measurement of the corneal toxicity. The cornea, in general, and the corneal epithelium in particular, serve as a functional barrier to toxicant entry into the eye (Kruszewski et al., 1997). Thus, disruption of the integrity of this tissue is a relevant endpoint for a toxicological evaluation. There are three types of methods used to evaluate this functional integrity of the cornea: firstly, measurement of the transepithelial electrical resistance, secondly, measurement of radiolabeled ion movements and finally, permeability to fluorochromes (generally fluorescein) (Carter et al., 1973; Green, 1976; Tchao, 1988). Transepithelial permeability to fluorescein is appropriate to reveal epithelial defects since fluorescein has been shown to be a nontoxic indicator of a loss of permeability barrier (Maurice, 1967; Maurice and Singh, 1986). The use of fluorescein permeability gives direct information on the state of the corneal integrity. Etter and coworkers (Etter and Wildhaber, 1985; Magada et al., 1993) have investigated chemically-induced lesions on mouse eye by means of an opto-electronic system using fluorescein for labeling the corneal wounds.

4.2.2. Objectivity and reproducibility

The test gives objective numerical responses. There is no subjective element in the result of the assay. Because of this objectivity of the evaluation, the test is more reproducible than the Draize test. Moreover, this non-invasive test enables a

follow-up of the ocular reactions after stopping the instillation of the chemical under study. Thus it is possible to evaluate not only the degree of eye irritation, but also the reversibility of the injury and consequently the healing of the damaged areas, an important endpoint for eye tolerance which most in vitro techniques are not suitable to establish (Chan and Hayes, 1989).

4.2.3. Sensitivity

The method is sufficiently sensitive to assess either feebly irritating substances or irritating compounds at low concentration, two situations where the Draize test failed to be discriminating. Furthermore, in these conditions, the experimentation has proved to be painless for animals. Indeed, it has been shown that the instillation of low-volumes, 10 μl instead of 100 μl in the Draize test, is less stressful to rabbits, permits the discrimination between similar materials and becomes more predictive of human ocular irritancy than the standard Draize method (Griffith et al., 1980; Walker, 1985; Freeberg et al., 1986; Lambert et al., 1993; Gettings et al., 1996). The low-volume test also allows reduction of the number of animals used, since three animals provide results similar to those obtained from six animals (DeSousa et al., 1984; Talsma et al., 1988; Bruner et al., 1992; Springer et al., 1993).

Unlike the official Draize test which evaluates the ocular effects of chemicals on the cornea, iris, nictitating membrane and conjunctiva, i.e. all observable external parts of the eye, the present ocular irritation test focuses on corneal damage. Indeed the majority of the irritants act directly on the outer ocular surface and in most cases trauma to the corneal epithelium is the most immediate and significant effect (Maurice and Brooks, 1995). Additionally, changes in the cornea have always been of more concern in assessing potential ocular irritation as evidenced by the fact that the standard Draize system applies to the cornea a maximum score of 80 out of the overall maximum irritancy score of 110 (Maurer et al., 1997). There is a merit to providing such gravity for corneal involvement, since injury to the cornea may directly lead to impairment or total loss of vision (Daston and Freeberg, 1991).

It should be emphasized that the ophthalmoscope used in this test is easily available but requires a special modification. This modification can be quickly dismantled to revert to the original instrument. Therefore if used efficiently, the ophthalmoscope turns out to be versatile: in its original design, a large number of tests can be carried out like fluorescein angiography and fundus topography; in the modified version, the observation and the evaluation of the cornea are possible.

In conclusion, these results demonstrate the ability of confocal microscopy to generate non-invasive optical sections of ocular structures at the cellular level. The chief advantage of this powerful new technology is that it provides images of the live eye with excellent contrast and resolution. This study also indicates that the in vivo monitoring of fluorescein corneal permeability by confocal microscopy is a useful method for assessing ocular irritation potential of surfactants in the rabbit or the mouse, since it can give us a better understanding of the factors affecting eye irritancy and wound healing. This method provides a means of objective quantification of ocular damage after exposure to drugs or pharmaceutical excipients. As a consequence, the test described here is a promising contribution to the formulation of ophthalmic preparations that are better tolerated by patients.

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