Changing the composition of buffered eye-drops prevents undesired side effects

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ABSTRACT

Purpose The Ex Vivo Eye Irritation Test (EVEIT) is used to analyse the clinical observations of corneal calcification attributed to the presence of phosphate within applied eye-drops used in treating glaucoma, Still—Chauffard syndrome, ocular burns and dry eyes.

Method Live corneas from abattoir rabbit eyes were cultured in order to study epithelial healing following mechanical abrasion of the corneal surface combined with repeated exposure to various eye-drops.

Results Obvious corneal calcification of the wound area along with a complete epithelial healing covering the calcified area was observed following exposure to phosphate hyaluronate eye-drops. Epithelial healing without calcification was achieved using citrate hyaluronate eye-drops.

Conclusion Clinical observations show that topical use of artificial tears containing phosphate on injured eyes may lead to sight-threatening corneal complications. Simulation of such treatment conditions by the EVEIT convincingly demonstrates that changes in the composition of the pharmaceutically used treatments can prevent this undesired side effect. Although considerable healing was achieved during the repeated application of eye-drops, using either a phosphate or citrate buffer, only the drops containing citrate did not develop corneal calcification on the eye. The authors therefore recommend discontinuing the use of phosphate-buffered eye-drops, or other topically applied solutions, to avoid further injury to the patient.

INTRODUCTION

It is well known that the phosphate concentration is very low in the stroma of human corneas.[1] This is also true for the stroma of rabbit corneas,[2] making them an excellent animal model for analysing the effects of ophthalmological treatments on corneas. The development of calcified patches on corneas is usually slow under physiological conditions, sometimes lasting months or years, as been reported by Huige *et al* for phosphate-containing steroids.[3] Nevertheless, Bernauer *et al*[4] described the development of calcification in conjunction with corneal wounds which have been treated with phosphate-buffered artificial tears in conjunction with corneal wounds. The same effects were observed previously[5] during the treatment of corneal burns with phosphate-containing drugs[6] and in an experimental eye burn model on rabbit corneas treated with phosphate buffer rinsing. Recent reports[4 7] from clinical practice concerning calcified human corneas have finally led to considerable changes in the formulation of artificial tears[8] by replacing phosphate with citrate in treatment buffers.

In this study, we aimed to evaluate the presence or absence of corneal calcification after clinical use of such eye-drops by taking advantage of the Ex Vivo Eye Irritation Test (EVEIT) approach. In studying the morphological changes in the cornea during the wound-healing process, determining residual clarity and the appearance of calcification or other types of turbidity is a simple but convincing approach in the evaluation and development of new ophthalmological treatments. By exposing ex vivo cultured corneas to pharmaceutical preparations, we have shown that removing phosphate from these solutions prevents the calcification found with use of phosphate-containing solutions.

MATERIALS AND METHODS

Ex Vivo Eye Irritation Test (EVEIT)

EVEIT is a non-animal-consuming, self-healing system that uses living corneal tissue. This procedure uses corneas taken from animals which are otherwise slaughtered for food preparation. The heads are decapitated, and the eyes are treated with Polyspectran drops (Alcon Pharma, Freiburg, Germany) and sealed with plaster. The heads are immediately transported at 4°C and prepared for experimentation within no more than 8 h.

After reopening the eye lids for preparation, the corneas were excised with a 12 mm trephine, and adhering tissues were gently removed. The corneas were placed in special culture chambers (ACTO, Aachen, Germany). The chambers were gently filled with serum-free minimal essential medium (MEM) (Cat No T031-05, Biochrom AG, Berlin, Germany, containing 200 mg $CaCl_2$ and 140 mg $NaH_2PO_4 \bullet 2H_2O$ in its Earle salt composition), supplemented with Piperacillin (2 mg/ml; Ratiopharm GmbH, Ulm, Germany), Amikacin (0.2 mg/ml; Fresenius Kabi Deutschland GmbH, Bad Homburg vor der Höhe, Germany) and Nystatin (400 U/ml; Valeant Pharmaceuticals Germany GmbH, Eschborn, Germany). The medium was continuously replenished by a micropump with an entrance pH of 7.4±0.2 and a flow rate of 6 µl/min. The culture chambers were held at 32°C in normal air without supplementary CO_2 and 100% relative humidity for 24 h before the explanted corneas were used for further experiments.

Examination techniques

The epithelial surface was microscopically examined daily for the duration of the experiment. Surface staining was performed with fluorescein solution (1.7 mg/ml fluorescein; Sigma-Aldrich, St Louis, Missouri, in 0.9% saline). The corneas were briefly flushed with isotonic Ringer solution (DeltaSelect

GmbH, Pfullingen, Germany) to remove excess fluorescein prior to being photographed. Samples of MEM from the artificial anterior chamber outflow were taken according to our protocol and analysed for glucose, lactate and pH (Bayer Automatic Analyser; Rapid Lab 860; Siemens Diagnostics, Fernwald, Germany) to monitor the corneal metabolism.

Healing measurements and corneal calcification

After 1 day of stabilisation in the EVEIT culturing system and establishment of the epithelial integrity by fluorescein staining of the surface, we applied four small abrasions (3–6 mm^2) with a small dental drill (Arkansasschleifer 638XF, Meisinger, Stuttgart, Germany). All corneas were then cultured and treated with either eye-drops or MEM (serving as control) for the following 3 days, and all defects were monitored daily with fluorescein staining. The green fluorescence (figure 1) indicates the areas of epithelial defects. The areas were photographed and measured by means of Diskus software (Hilgers, Königswinter, Germany). Corneal calcifications were visually defined as distinct white, opaque structures and interpreted as either present or absent. To substantiate the asserted calcification by histological examination, all corneas were snap-frozen at the end of the experimental time (4 days). Cryosections were prepared and stained according to von Kossa to detect calcium deposits within the exposed corneas. Here, a grading scheme to quantify the calcium deposits within the cornea was applied, ranging from 0 (no staining) to 4 (complete staining of epithelium and upper stroma).

Application of test substances

We used an artificial tear replacement solution adjusted to this citrate content of 14.581 mmol/l Ca^{2+} in 0.9% saline solution (Delta Select, Germany). This solution was applied once per hour onto the corneas, providing 11.7 µg of Ca^{2+}.

During the experiment, we compared the old phosphate-containing HYLO-COMOD and HYLO-CARE with HYLO-LASOP, which contained citrate buffer (all Ursapharm Arzneimittel GmbH, Saarbruecken, Germany). At present, all of these commercially available products have been reformulated to contain citrate.

Each of these unpreserved hyaluronate drops was applied, along with the artificial tear replacement by using two separate dropping devices, exactly over the apex of the cornea. Controls were represented by corneas treated with MEM and artificial tear replacement in the same manner.

A 20 µl drop of each solution was applied hourly for 3 days, with a time difference of 30 min between applications of the test substance and the artificial tears. A soft-tipped cannula, applying continuous suction, was placed on the lower side of the cornea in culture to remove excess fluid.

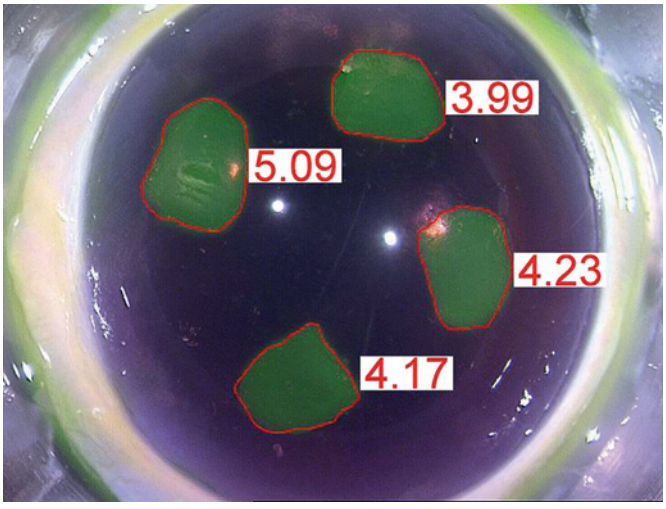

Figure 1 Fluorescein-stained cornea with measurements of injured area (mm^2).

Osmometry

The osmolarities of all eye-drops were measured with the Osmomat 030 (Gonotec, Berlin, Germany). The applied substances were in the range of 260–390 mOsmol/kg (hyaluronate phosphate eye-drops 262±1.9 mOsmol/kg; hyaluronate citrate 272±1.5 mOsmol/kg; MEM 383±3.3 mOsmol/kg; artificial tears containing $CaCl_2$ 317±1.3 mOsmol/kg).

RESULTS

All 10 corneas which were treated with hyaluronate phosphate and artificial tears showed distinct white opaque spots at all 40 abraded areas, suggesting strong corneal calcifications. Coincidently, as shown in figure 2A, the calcified corneas showed significant closure of the epithelial defects after 3 days (as determined by negative fluorescein staining; figure 2A). Histological analysis of these corneas via von Kossa staining confirmed the visual appearances and highlighted the dense calcifications especially at the location of epithelial scrapings (figure 2B).

However, the five corneas treated with hyaluronate citrate and artificial tears showed a completely clear, smooth surface devoid of any visual calcification after 3 days of treatment. A slight positive fluorescein staining of the surface was still detectable at the end of the experiment, which indicates that some epithelial damage remained (figure 2C).

None of the corneas exposed to these drops showed a positive result for the von Kossa stain (figure 2D).

Throughout the 3 days of culture, all six controls (24 drops MEM/day) showed considerable healing down to approximately half of the surface area of the initial abrasions (figure 2E), and no calcification was visually apparent.

Although the MEM contains a small amount of phosphate (0.9 mmol/l), calcification is therefore possible but was not detectable in our experimental conditions. No calcium deposits in the corneal stroma were visually observed, as compared with that seen in the cases of phosphate-buffered eye-drop (50.9 mmol/l) treatment,[4] and only the histological analysis showed trace evidence of calcium deposits or calcium salts (figure 2F).

All treated corneas, as displayed in table 1, showed predominately good healing. Slower healing was detected for MEM compared with advanced healing achieved by using HYLO-LASOP and HYLO-COMOD ($p<0.01$, Fisher test). However, there was no statistical difference ($p>0.05$) between the hyaluronate treatments, buffered with either phosphate or citrate.

The dense, white opacifications, macroscopically apparent at the sites of injury and whose damage is further highlighted by light microscopy, as demonstrated by the von Kossa stains, could only be due to the application of phosphate containing hyaluronate eye-drops.

No opacification caused by stromal calcification was found in any other group. Statistically, the staining of the hyaluronate phosphate treated corneas was shown to be significantly different ($p<0.001$, Dunn multiple comparison test) from the results achieved after treatment with the citrate-buffered hyaluronate eye-drops.

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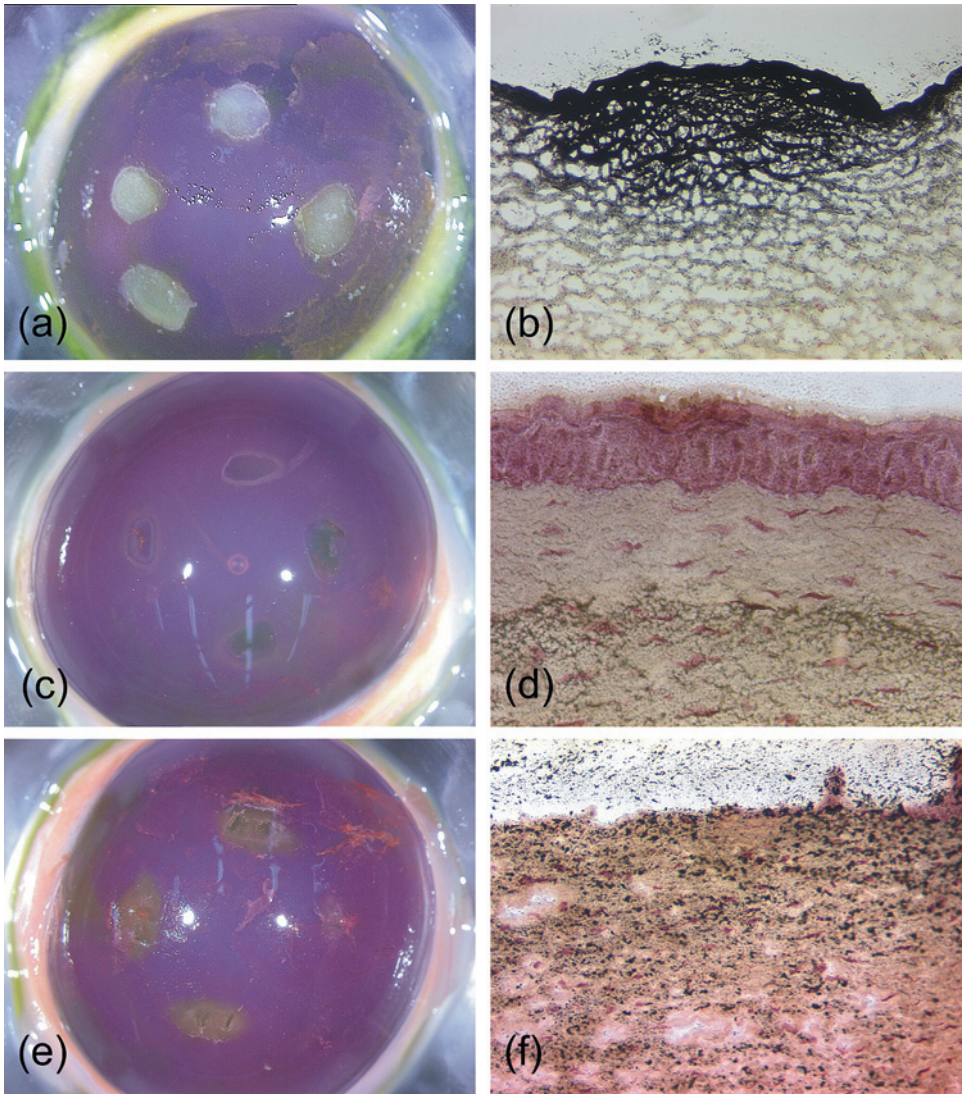

Figure 2 Visual appearances of representative corneas after 3 days of treatment. (A) Healed but calcified spots caused by dropping the corneal surface with phosphate containing eye-drops and artificial tears (1/h). (B) Positive von Kossa stain of one of the calcified areas. (C) Citrate-buffered hyaluronate eye-drops and artificial tears producing a smooth and clear corneal surface. (D) Photomicrograph, showing that no excessive calcium deposits are visible with von Kossa, and showing complete healing of the formerly wounded epithelium. (E) Abraded cornea after treatment with medium and artificial tears. (F) Histological analysis showed trace evidence of calcium deposits or calcium salts.

The Fisher test revealed a highly significant correlation in the achieved corneal calcification to the presence of phosphate in the used eye-drops ($p<0.001$). However, a histological analysis of the MEM treated corneas demonstrated very slight amounts of surplus calcium.

During the experimental period, the measured pH values in all groups always stayed in the defined range of 7.4 ± 0.2. Monitoring the corneal metabolism according to our protocol by measuring the lactate production in the outflow medium of chamber showed that all corneas in each group exceeded the determined lowest value of 1.0 mMol/l.

DISCUSSION

There is no doubt that mixtures of phosphate and calcium ions immediately form calcium phosphate or calcium apatite. Both compounds have a very low water solubility which decreases with increasing pH or temperature. Stanley,[9] Leopold[10] and Caldeira[11] found the calcium content in tears to be in the order of 0.0211—0.041 µg/µl with a basic secretion rate of 120—240 µl/h[12]: this results in the introduction of calcium onto the corneal surface in the range of 2.53—9.84 µg/h for the healthy eye. As the tear secretion rate is upregulated during eye injury as simulated here, values above this range are expected.

A limitation to the EVEIT experimental system is the obvious absence of natural tear production, which provides the corneal surface with calcium. This absence would undermine the natural emergence of possible calcified spots while using phosphate-buffered eye-drops. Additionally, the citrate buffer in the new formulated hyaluronate drops can withdraw calcium ions from the corneal surface, thereby affecting epithelial healing. To

Table 1 Summary of all performed experiments

Sample	No of corneas	Mean healing (percentage of initially damaged area)	Von Kossa (0—4)	Visual calcification
HYLO-COMOD phosphate*	2	94.5 ± 0.70	4/4	+/+
HYLO-CARE phosphate*	8	75.25 ± 4.23	4/4/4/4/4/4/4/4	+/+/+/+/+/+/+/+
HYLO-LASOP citrate	5	84.4 ± 9.81	0/0/0/0/0	–/–/–/–/–
Minimal essential medium	6	57.8 ± 12.73	1/1/1/1/1/1	–/–/–/–/–/–

*Current composition is citrate buffered.

overcome these obstacles, we introduced artificial tears containing a physiological amount of calcium.

The capability of the EVEIT in effectively and reproducibly predicting the potential toxic effects of topical application on the eye has been demonstrated in earlier experiments,[13–15] and here we could exemplify the practicability of the EVEIT in the evaluation of pharmaceutical products. This study revealed that the addition of phosphate in eye-drops is an important factor in inducing calcification of the corneal surface following injury. We were able to support experimentally the clinical findings of Bernauer *et al*,[4 7] who observed this in cases of keratoconus,[16] dry eye[17] and vernal conjunctivitis,[18] and demonstrate that excessive use of phosphate-containing drugs is associated with corneal calcification. These results also confirm that calcified deep stromal tissue is related to early changes in the mineral composition of the cornea. This has similarly been shown for eye burns[2 6] due elevated phosphate concentrations in combination with the normal tear calcium content[10] leading to undesirable side effects. We have demonstrated that using a citrate-based buffer instead of a phosphate-based buffer in eye-drop formulations is a better and safe choice of therapy. Patients who require even extremely high rates of citrate-buffered eye drops, as described by Bernauer *et al*,[7] will not exceed the calcium supply of normal tear fluid.[11 12]

Additionally, we observed distinct stages of corneal healing following the various types of repeated applications. These results led us to the assumption that hyaluronate eye-drops improved epithelial healing after deliberate damages, even in large areas of corneal damage such as that used in these particular experiments.

With these available ophthalmological lubricants, relevant epithelial defects such as those caused by severe dry eyes, mechanical scraping, photorefractive and phototherapeutic excimer ablations, corneal ulcers and eye burns can also be treated successfully without any risk of calcification.

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