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Lacrimal osmolarity and ocular surface in experimental model of dry eye caused by toxicity

Osmolaridade lacrimal e superfície ocular em modelo de olho seco por toxicidade

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ABSTRACT

Purpose: Describe an animal model of dry induced by topical instillation of BAK and evaluate ocular surface biomarkers and histological findings. **Methods:** Male Wistar rats were used. Topical instillation of 0.2% BAK eyedrops twice a day during 7 days, in the right eye of each animal, while the other eye was taken as control. After 7 days treatment, we performed evaluation of tear film osmolarity, the red phenol thread and ocular surface staining with fluorescein and lissamine green. Afterwards, the animals were sacrificed for tissue extraction and histological evaluation under optical microscopy and H&E staining. **Results:** Compared with untreated controls, the BAK-group presented tear secretion significantly decreased, increased ocular surface staining by fluorescein and lissamine green and tear film hyperosmolarity (p <0,05). Histological evaluation revealed epithelial thinning and estromal oedema. **Conclusion:** A toxicity animal model of dry eye induced by topical instillation of benzalkonium chloride, which presents corneal and ocular surface alterations, decreased tear film volume and tear hyperosmolarity as seen in dry eye condition.

Keywords: Dry eye, syndromes/chemically induced; Osmolar concentration; Tears/metabolism; Bezalkonium compunds/toxicity; Models animal; Rabbits

RESUMO

Objetivo: *Descrever um modelo animal de olho seco induzido pela aplicação tópica de cloreto de benzalcônio (BAC) e avaliar marcadores de integridade da superfície ocular e os achados histológicos.* ***Métodos:*** *Foram utilizados ratos wistar machos adultos. Foi realizada a administração tópica de colírio de BAC 0,2% no olho direito de cada animal duas vezes por dia, durante 7 dias, sendo o olho contralateral tido como controle. Após o tratamento foi realizada a avaliação da osmolaridade do filme lacrimal, o teste de fenol vermelho e a coloração com fluoresceína e lisamina verde. Os animais foram sacrificados e os tecidos extraídos para o estudo histológico da córnea, por microscopia óptica, corada com hematoxilina eosina (H&E).* ***Resultados:*** *Comparados com os controles não tratados o grupo BAC apresentou diminuição significativa na secreção lacrimal, defeitos na integridade epitelial da superfície ocular marcada por corantes vitais, fluoresceína e lisamina verde além do aumento da osmolaridade do filme lacrimal (p < 0,05). À avaliação histológica observou-se diminuição da espessura do epitélio e edema estromal induzidos pela aplicação de BAC.* ***Conclusão:*** *O modelo animal de olho seco por toxicidade induzido pela aplicação tópica de cloreto de benzalcônio apresentou alterações estruturais da córnea e da superfície ocular, diminuição do volume lacrimal e hiperosmolaridade da lágrima características dessa condição.*

Descritores: *Síndromes do olho seco/induzido quimicamente; Concentração osmolar; Lágrimas/metabolismo; Compostos de benzalcônio/toxicidade; Modelos animais; Coelhos*

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Introduction

Dry eye is a multifactor high-prevalence ocular disease involving changes in tears and inthe ocular surface, and results in symptoms of visual discomfort and disorder and instability of the lacrimal film with potential risk to the integrity of the ocular surface[1-3].

The potential damage to the ocular surface caused by changes in the quantity and/or quality of the lacrimal film associated with dry eye has been studied exhaustively[4-7]. These changes can be justified by the accumulation of inflammatory mediators, hormonal changes, and instillation of preservatives or drugs with toxic or allergenic potential[8-10].

The lacrimal film is fundamental to optical quality, comfort and maintenance of the ocular surface, and can be altered by autoimmune diseases, scarring and degenerative processes, besides comorbidities such as diabetes, ocular allergy, menopause and senility processes, use of medication of either topic or systemic administration[11-16]. It is essential to have a harmonious integration among the lacrimal glands, the ocular surface (cornea, conjunctiva and meibomian glands), the eyelids and the sensory and motor nerves. Damage or disease of any of these components can destabilize the lacrimal film and evolve to the dry eye condition[17].

There is great variability in symptoms and severity of damage to the ocular surface in dry eye cases[17, 18]. Physiopathology of dry eye is based on hyperosmolarity and inflammation of the ocular surface[19, 20]. Hyperosmolarity is a change in tear composition, due to increased solute concentration and it occurs in the dry eye, through volume reduction of the aqueous fraction, and has been considered a possible biomarker for dry eye diagnostic. Increased tear osmolarity leads to abnormal differentiation and accelerated loss of epithelial cells and induction of inflammatory biomarkers, film instability, cellular oedema and change in the antimicrobial barrier[21, 22].

Inflammation of the ocular surface can be involved both in the induction and in the maintenance of dry eye: lacrimal gland dysfunction changes tear composition, leading to a state of hyperosmolarity and stimulating the production of inflammatory mediators which in turn lead to dysfunction of the secretory glands, lysis of the epithelial basal membrane and of intercellular junctions, and cell exfoliation process with consequent epithelial erosion[10].

Preservatives are important components of ophthalmological preparations, promoting antimicrobial activity and preventing decomposition of the active drug. However, significant cytotoxic effects on the ocular surface have been associated with the chronic use of these substances[23]. The most common preservative in commercial ophthalmological preparations for glaucoma and ocular surface diseases (irritations, allergies and infections) is benzalkonium chloride (BZK),usedin concentrationsvarying from 0.004% to 0.02% in multidose topical solutions[24]. BZK is a quaternary ammonium compound with detergent, antibacterial and antifungal action as well as the ability to break the epithelial barrier of the ocular surface at the intercellular junctions, which would theoretically increase penetration and action of the active principle. Various studies have demonstrated that BZK acts on the ocular surface as cytotoxic, pro-apoptoticand pro-inflammatory, reducing cellular viability and stimulating the expression of inflammatory cytokines[25]. Exposition to BZK can play an important role in precipitation and acceleration of the dry eye syndrome installation process and in worsening of preexisting dry eye, affecting the cornea as well as the conjunctiva. The physiopathological mechanism responsible for these deleterious effects caused by BZK are related to lacrimal film unbalance, ocular surface inflammation and epithelial damage[23].

Regardless of the initial stimulus, there is an inflammatory vicious circle on the ocular surface, leading to gradual dysfunction of secretion or tear retention. What is not clear is whether, with so many different mechanisms and even without inflammation in the initial stages, there would be a common diagnostic examination with a similar cutoff value in different disease groups, and what would be the correlation of this parameter with others used in clinical practice and its limitations[26].Furthermore, we do not today have curing measures to reverse dry eye, only chronic and palliative treatments[27].Faced by these doubts, it becomes necessary to improve animal models for the study of the disease and to improve knowledge of the disease mechanisms and definitive therapeutic strategies.

The objective of this study was to describe an animal model for dry eye caused by toxicity using BZK and to evaluate the clinical parameters used in the dry eye diagnostic and the histological changes induced by toxicity to BZK.

Methods

This study was approved by the Committee for Ethics in Animal Experimentation, Faculty of Medicine of RibeirãoPreto, USP (CETEA-FMRP), and follows international guidelines for studies with animals.

Dry eye was induced by topical instillation of benzalkonium chloride, as described previously[25].We used 8-week male Wistar rats obtained from the Central Animal Laboratory of the Faculty of Medicine of RibeirãoPreto (RibeirãoPreto, São Paulo; Brazil). The animals had free access to food and water. The animals (n=5) received topic instillation in the right eye of 5 µl benzalkonium chloride at 0.2% without additives (OphthalmosLtda, São Paulo, Brazil), twice daily, (at 7amand7 pm) for 7 days. The contralateral eye was preserved for use as control.

The animals were subjected to propedeutic evaluation of the ocular surface, in an environment with controlled humidity and temperature, and by the same examiners, without using anaesthetic eyedrops, after seven days of topic treatment with 0.2% BZK eyedrops. Evaluations with vital stains were photographed and subsequently quantified by two examiners in double blind. The techniques used in this study to evaluate the ocular surface and the lacrimal film are described below and were carried out in this sequence[28].

Evaluation of ocular surface

1. Tear osmolarity

Tear samples were obtained from the animals through collection without stimulation or need for eyedrops, through approximation of a delicate collector for a volume of around 50-200 nanoliters of tears by capillarity, and the measurements were carried out using a Tearlab Osmolarity System® osmometer[29].

2. Staining with fluorescein for quantification of areas with irregularities and disepithelization of the corneoconjunctival surface. After instilling one drop of fluorescein at 2% (OphthalmosLtda, São Paulo, Brazil) an evaluation was performed, using a slit lamp with blue filter. Impregnation of the dye was quantified in central, superior, inferior, nasal and tem-

poral regions (0-3) according to intensity, and could therefore vary from 0 to 15

3. Staining with lissamine green for quantification of squamous metaplasia of the optical surface. After instilling one drop of lissamine green at 1% at the bottom of the conjunctival sac (OphthalmosLtda, São Paulo, Brazil), the conjunctiva and the cornea were examined using a lamp with white light. Impregnation of the dye was quantified in central, superior, inferior, nasal and temporal regions (0-3) according to intensity, and could therefore vary from 0 to 15

4. Phenol red test to quantify lacrimal volume: the thread soaked in phenol red (Zone Quick Phenol Diagnostic Thread, MeniconLtda) was placed at the edge of the lower eyelid for 15 seconds, followed by the measurement in millimeters of the extent of tear absorption and the consequent color change of the thread.

5. Optical microscopy: after the ocular surface evaluation, the animals were sacrificed using an excessive dose of the combination of anesthetics ketamine and xylazine applied intraperitoneally, and the eyeballs were removed and fixed for histology. Histological sections were made and stained with hematoxylin and eosin (H&E) and were subjected to evaluation of corneal epithelial thickness after the images were scanned.

Statistical analysis

Statistical analysis to compare the values obtained in the propedeutic and histological examinations of the group treated with BZK and of the control group was done by means of the Mann-Whitney U test with Prism 5.0® (GraphPad, California) application, and the significance value adopted was $p<0.05$.

Results

Osmolarity of lacrimal film: we found a statistically significant difference in lacrimal film osmolarity of the studied groups ($p<0.05$), demonstrating tear hyperosmolarity in the group treated with BZK (306.4±9.1 mOsmol/L), compared with the control group (284.8±7.3 mOsmol/L). (Figure 1)

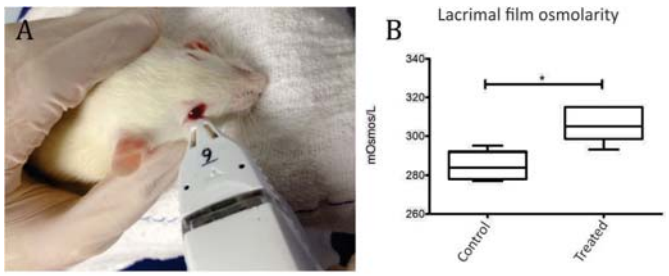

Figure 1: Demonstrative image of lacrimal osmolarity measurement usingTearlab Osmolarity System® osmometer (A) and chart showing increased osmolarity in the group treated with BZK (B) (*$p<0.05$).

Lacrimal volume: using the phenol red test we evaluated the production of aqueous fraction of the lacrimal film. Here we observed reduced ($p<0.05$) lacrimal volume in eyes subjected to BZK instillation (3.4±1.5 mm) as opposed to in untreated eyes (7.6±4.5 mm) (Figure 2).

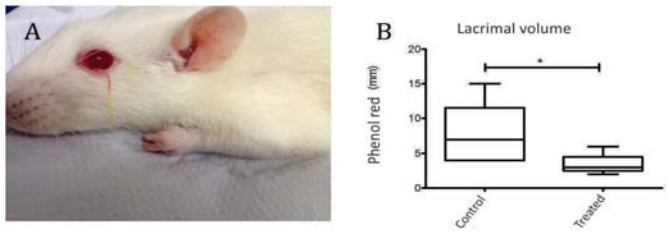

Figure 2: Demonstration of phenol red test (A) and chart showing reduced lacrimal secretion in the group treated with BKZ (B) (*$p<0.05$).

Vital stains: Eyes treated with BZK showed higher impregnation both by fluorescein and by lissamine green as observed in Figure 3 ($p<0.05$). In the control group we observed staining at 1.0±1.7 e 2.2±1.6 for fluorescein and lissamine, respectively. Whereas in eyes treated with BZK the stain impregnation was 8.4±3.0 para fluorescein and 9.8±3.3 for lissamine. (Figura 3)

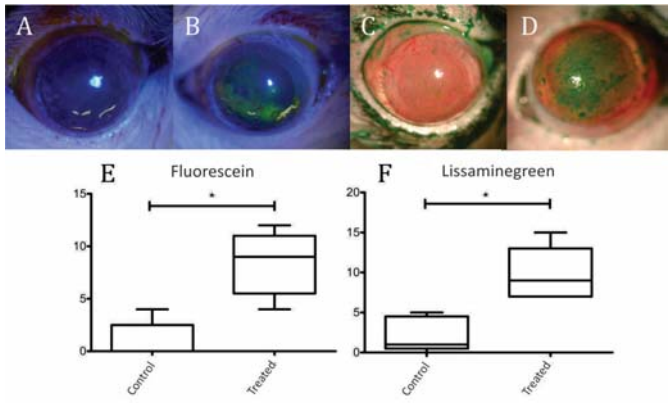

Figure 3: Representative photographs of untreated eyes and eyes treated with BZK, stained with fluorescein (A and B, respectively) and stained with lissamine green (C and D, respectively) and charts quantifying the greater impregnation by the dye in the group of animals treated with BZK (E and F) (*$p<0.05$)

Optical microscopy: In the evaluation of the corneal epithelial thickness, we noted a significant reduction of this measurement in treated animals compared to the values observed in the untreated group ($p<0.05$) (Figura 4).

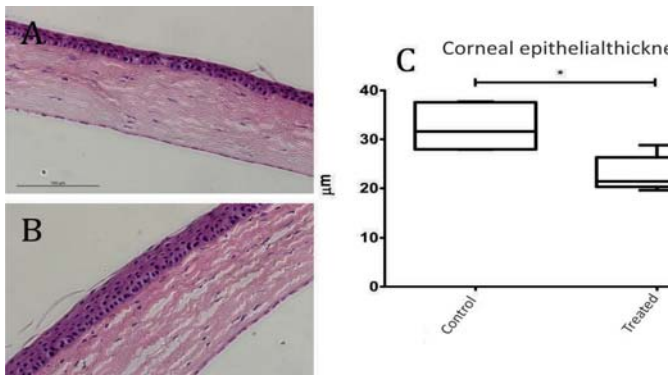

Figure 4. Representative images of histological sections of corneas of treated (A) and untreated (B) animals, and evaluation of corneal epithelial thickness of both groups (C) (C*$p<0.05$).

Discussion

The present study describes an animal model of dry eye induced by topical application of benzalkonium chloride in rats. Toxic action on the ocular surface caused by this compound was evaluated according to several parameters used in clinical practice, such as the vital dyes fluorescein and lissamine green, lacrimal volume and tear osmolarity. Furthermore, we described histological changes in the cornea of treated animals as compared to controls.

Dry eye is a multifactor, complex condition, and our findings can help to elucidate the physiopathology of the dry eye syndrome, and serve as a model for pre-clinical therapeutic tests.

Hyperosmolarity is considered an important factor in the vicious circle governing the dry eye physiopathology, both as an initial sign and as a disease perpetuator, since it is a catalyst for inflammation and it reduces subsequent lacrimal secretion[22, 30]. In this way, an animal disease model which reproduces the pattern of osmolarity increase becomes valuable for the study of dry eye mechanisms and treatment.

The measurement of lacrimal film osmolarity has been carried out in humans with the Tearlab Osmolarity System® osmometer, with great ease of operation, since tear collection in the lower lacrimal meniscus is done by capillarity in a quick and minimally invasive manner, the reading being taken immediately[31]. In the animal model described, we were able to obtain these propedeutically valuable data with good reproducibility and homogeneous distribution in the groups, using the same equipment and techniquestandardized for use in humans; this resource will allow prospective studies to follow up disease evolution and treatment results, with an animal model of simple reproducibility and rapid induction.

Corroborating the above, the data obtained in this study demonstrate the viability of evaluation of lacrimal film osmolarity in the experimental field with no major difficulties in its application in animal models, requiring minimal animal restraint and no sedation, thus extrapolating to the animal model characteristics already observed in clinical practice.

We believe that the dry eye induction mechanism reproduced in this experiment is probably due to partial denervation of the cornea, inflammation and reduced secretion production. Nevertheless, future trials are necessary for better understanding of the ways related to the mechanisms in the model in question Such events probably occur in chronic users of eyedrops containing preservatives based on this quaternary ammonium compound, since similar findings are described, for example, in patients afflicted by glaucoma undergoing prolonged topical therapy with multiple doses[32].

In the propedeutics available today, there is fluorescein staining as a detector of irregularities and of disepithelization of the corneoconjunctival surface which appears in several pathologies that harm the ocular surface, and in the dry eye due to inflammation and toxicity imposed on the corneoconjunctival epithelium, demonstrated in this study by greater impregnation of the eyedrops in ocular surfaces of eyes treated with BZK. Similarly, we proceeded with lissamine green staining and, once again as expected, in the dry eye animal model this impregnation was more intense than in the eyes of the control group. Beside these tools, reduced lacrimal volume, already well consolidated as a sign of dry eye syndrome, was also observed in this dry eye animal model. Furthermore, we carried out an analysis of histological parameters of the corneal sections of the animals treated and not treated with BZK, quantified in this study by measurement of the corneal epithelium, which, not surprisingly, proved to be thinner in the eyes of the animals belonging to the treated group, which derives, among other factors, from the direct lesion to the cellular layers of this tissue and from the reduced density of populations (apoptosis stimulus) of cells with germinative potential which generate epithelial cells, due to inflammation present in the disease. In this way, the findings observed in the animal model described in this study reflect the clinical parameters used in the propedeutics of lacrimal dysfunction and of the ocular surface described in dry eye.

BZK, as already mentioned, is an important preservative frequently used in ophthalmic formulae. The role of preservatives is of extreme relevance mainly in medications of prolonged use and of multidose presentation, such as hypotensive medication. The toxic potential for the ocular surface comes from its detergent action on the lacrimal film, besides the pro-inflammatory and pro-apoptotic action, and has been acknowledged in several studies[23].

Conclusion

In conclusion, the present study made headway in the demonstration of a dry eye syndrome model induced by toxicity of topical application of BZK on rats and on the novel use in animals of tear osmolarity measurement through a method already incorporated in clinical practice in humans, as well as surface dyes and measurement of lacrimal volume and tear osmolarity. In the future, it is hoped that these findings are used for studies of physiopathological mechanisms and proposed definitive and efficient treatment of the disease.

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Errata

In the scientific article "Lacrimal osmolarity and ocular surface in experimental model of dry eye caused by toxicity", the authors: David Lazarini Marques, Monica Alves, Carolina Maria Module , Lilian Eslaine Costa Mendes da Silva, Peter Reinach and Eduardo Melani Rocha, published in journal of Ophthalmology in the March - April 2015 (Rev Bras Oftalmol. 2015; 74 (1): 24-9) on page 68, the English title has an error which reads: osmolaritu , read: osmolarity .