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Research article

Optimization and validation of an existing, surgical and robust dry eye rat model for the evaluation of therapeutic compounds

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ABSTRACT

The aim of this research was to optimize and validate an animal model for dry eye, adopting clinically relevant evaluation parameters.

Dry eye was induced in female Wistar rats by surgical removal of the exorbital lacrimal gland. The clinical manifestations of dry eye were evaluated by tear volume measurements, corneal fluorescein staining, cytokine measurements in tear fluid, MMP-9 mRNA expression and $CD3^+$ cell infiltration in the conjunctiva. The animal model was validated by treatment with Restasis® (4 weeks) and commercial dexamethasone eye drops (2 weeks). Removal of the exorbital lacrimal gland resulted in 50% decrease in tear volume and a gradual increase in corneal fluorescein staining. Elevated levels of TNF-α and IL-1α have been registered in tear fluid together with an increase in $CD3^+$ cells in the palpebral conjunctiva when compared to control animals. Additionally, an increase in MMP-9 mRNA expression was recorded in conjunctival tissue. Reference treatment with Restasis® and dexamethasone eye drops had a positive effect on all evaluation parameters, except on tear volume.

This rat dry eye model was validated extensively and judged appropriate for the evaluation of novel compounds and therapeutic preparations for dry eye disease.

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1. Introduction

Dry eye disease (DED) or keratoconjunctivitis sicca (KCS) can be broadly defined as a group of disorders that affect various components of the lacrimal functional unit, resulting in the dysfunction of the ocular tear film and/or the integrity of the ocular surface (Dieckow, 2011). This multifactorial disease exhibits symptoms of discomfort, visual disturbance and tear film instability with potential damage to the ocular surface, as defined by The International Dry Eye Workshop. Although DED is strongly related with age, the prevalence in the younger population is rising due to increased contact lens wear, low quality air and low humidity and spending more time using computers and smartphones (Stern et al., 2013). Treatment options are very limited and often unsatisfactory.

To study the complex pathology and the multifactorial origin of DED, several animal models for different forms of dry eye have been developed (Schrader et al., 2008; Barabino and Dana, 2004). The characteristics and practicability of dry eye models differ considerably and strongly depend on the animal species and induction method (Schrader et al., 2008; Barabino and Dana, 2004). Animal

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models have indeed contributed significantly to our current understanding of DED pathogenesis and are necessary to further unravel DED pathophysiology and to evaluate novel therapies. However, a lot of these models are time consuming in maintaining dry eye, are reversible or fail to simulate the underlying mechanisms of dry eye (Suwan-Apichon et al., 2006). In addition, several models have not been thoroughly validated or have limited evaluation parameters, making them in our opinion less suitable for drug discovery and evaluation.

The DED induction method employed in the present study was the surgical removal of the exorbital lacrimal gland to obtain a tear-deficient dry eye model as already described in several animals (Sanson and Barnett, 1985), including the rat (Katagiri et al., 2015; Fujihara et al., 2001). However, it was necessary to further optimize and validate this model for optimal use in drug discovery. Apart from standard tear volume measurements, fluorescein staining and histological analysis, several clinically relevant evaluation parameters were included. For example, T-cell infiltration in the conjunctiva has been proven to be one of the driving forces in the self-perpetuating inflammatory cycle of dry eye (Stern et al., 2013, 2002, 2005). Hence, immunohistochemical analysis of $CD3^+$ cells in palpebral conjunctiva was assessed. Furthermore, key inflammatory cytokines in tear fluid (IL-1α and TNF-α) and MMP-9 expression in the cornea, all or which play a key role in dry eye pathogenesis, were evaluated (Stern et al., 2013; Massingale et al., 2009; Stevenson et al., 2012; Pflugfelder et al., 2013; Lan et al., 2012).

The specific aim of this study was the development and validation of a non-pharmaceutical, robust animal model with fast rate of induction and a permanent state of dry eye. Known ocular anti-inflammatory drugs Restasis® and dexamethasone were used as reference treatments.

2. Methods

2.1. Animals

Female Wistar rats (200–300 g, Janvier, Roubaix, France) were kept under standard pathogen-free conditions. Husbandry conditions: room temperature 20–25 °C, humidity 50–60% and a day–night cycle of 12 h light/12 h dark. Food and water were available ad libitum. All *in vivo* manipulations were approved by the Animal Ethical Committee of the University of Antwerp (2013-67) and are in accordance to the ARRIVE guidelines for the use of animals in ophthalmic and vision research.

2.2. Anesthesia

To remove the exorbital lacrimal gland, animals were anesthetized with an intraperitoneal injection of 25 mg/kg ketamine (Anesketin®, Eurovet, Bladel, Netherlands) and 2.5 mg/kg xylazine (Rompun®, Bayer, Leverkusen, Germany). Routine manipulations (tear collection, fluorescein staining) were performed after induction with 5% isoflurane (Halocarbon®, New Jersey, USA), followed by a maintenance dosage of 1.5%.

2.3. Induction of dry eye

DED was induced by removal of the exorbital lacrimal gland, located subcutaneously, on top of the masseter muscle and inferior to the nervus ophthalmicus. To remove the gland, a small incision was made in the skin 10 mm in front of and inferior to the tragus below the right ear. The exorbital lacrimal gland is located easily due to its relatively large size and location just under the skin. For a skilled technician, the whole procedure doesn't take more than 10–15 min without the need for a surgical microscope. The lacrimal glands of the left eye were kept intact, leaving each animal with its own individual control and eliminating the need for extra control groups. Progression of dry eye was monitored for 28 days, starting 2 days after surgery and tear sampling, tear volume measurements and fluorescein staining were performed once a week.

2.4. Anti-inflammatory drugs

Restasis® (2.5, µl 2x/day during 28 days, 0.05% cyclosporine A, Allergan, Irvine, USA), the only FDA-approved anti-inflammatory treatment for DED and Monofree Dexamethasone (2.5 µl, 4x/day for 14 days, 1 mg/ml, Théa, Wetteren, Belgium), a potent anti-inflammatory corticosteroid, were utilized to treat DED derived ocular inflammation. Restasis® or Monofree Dexamethasone was administered directly onto the ocular surface using a pipette. Use of dexamethasone eye drops was restricted to 14 days to avoid side effects.

2.5. Measurement of aqueous tear production

A phenol red thread (Zone Quick®, Menicon Co. Ltd, Nagoya, Japan) was placed in the lateral cantus of the conjunctival fornix for 15 s. Absorption of tear fluid resulted in a color shift from yellow to red and tear distance was measured in millimeters. Tear fluid volumes were measured once a week.

2.6. Evaluation of ocular surface damage by fluorescein staining

Sodium–fluorescein (1%, Sigma–Aldrich, Seelze, Germany) in phosphate buffered saline (PBS, Gibco® by LifeTechnologies Europe, Gent, Belgium) was administered topically to the surface of the eye. To avoid false positives, eyes were rinsed after 1 min with PBS and excess fluorescein was removed by placing filter paper in the lateral cantus of the eye. The eye was photographed with a microscopic lens (Photo adapter 1.0 MC 80 DX – Axiovert 25 CA, Carl Zeiss AB, Göttingen, Germany) in a darkened room under cobalt blue light. Using the Oxford fluorescein grading scale, scores from 0 to 5 were given to each eye, depending on ocular staining intensity. Semi-quantitative scoring was done in a blind manner by three independent observers. Evaluation of ocular surface damage was performed every week.

2.7. Tear collection

Tear fluid was collected once a week with 10 µl capillaries (Blaubrand® Intramark, Wertheim, Germany) connected with a flexible tube to a syringe. When creating a weak vacuum, more tear fluid could be collected in comparison to conventional collection methods. Immediately after collection, capillaries and tear fluid were stored at −80 °C, for immunological analysis.

2.8. Tear fluid analysis

TNF-α and IL-1α concentrations in tear fluid were measured flow cytometrically (FACS calibur, BD Biosciences), using Cytometric Bead Array (CBA) according to the manufacturers protocol (Rat IL-1α and TNF-α CBA flex sets, BD Biosciences, Erembodegem Belgium. ± 1 µl of undiluted tear fluid is extracted per (untreated) rat with DED. Tear fluid of all animals (N = 5) of the same treatment group was pooled per time point prior to flow cytometric analysis. 3 µl per treatment group per time point was then diluted to 15 µl with assay diluent. Control tear fluid (every left eye) was pooled in the same manner per treatment group and mean values were calculated.

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