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Experimental eye research

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.

Keywords: Dry eye disease, KCS, DED, animal model, rat

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[1]. 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[2]. 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[3-4]. The characteristics and practicability of dry eye models differ considerably and strongly depend on the animal species and induction method[3-4]. Animal 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[5].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[6], including the rat[7-8]. 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[2,9,10]. 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[2, 11-14].

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 (200-300g, 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 12h light/12h 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 minutes 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, 1mg/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 seconds. 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 one minute 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. 3µl per treatment group per time point was then diluted to 15µl with assay diluent. Control tear fluid (every left eye) was pooled in the same manner per treatment group and mean values were calculated.

2.9 Immunohistochemistry

T-cell infiltration in the palpebral conjunctiva was analyzed immunohistochemically, using primary antibodies against CD3 (Abcam, Cambridge, United Kingdom). Eyelids were excised, and frozen in optimal cutting temperature (OCT, TissueTek®, Sakura® Finetek Inc., Torrance, USA). Histological sections (12µm) were transferred to poly-L-lysine coated slides and incubated with 0.05% thimerosal, 0.01% NaN_3, 0.1% bovine serum albumin, 1% triton-X100 and 10% normal horse Serum in PBS. The samples were incubated with anti-CD3 primary antibody (Abcam, Cambridge, UK) followed by goat anti-rabbit CyTM3 (Jackson ImmunoResearch Europe Ltd, Suffolk, UK). A nuclear counterstaining was applied using 4′,6-diamidino-2-phenylindole (DAPI, LifeTechnologies Europe, Gent, Belgium). All slides were analyzed using a Zeiss observer Z1 AX10 fluorescence microscope (Carl Zeiss AB, Göttingen, Germany).

2.10 RNA preparation and qRT-PCR

Total RNA from cornea and palpebral conjunctiva was isolated using the RNeasy® Plus Mini Kit (Qiagen, Venlo, The Netherlands). cDNA was obtained by the AccuScript Hi-Fi Reverse Transcriptase kit (Agilent Technologies Europe, Boeblingen, Germany) following the manufacturer's instructions. Expression of MMP-9 relative to the endogenous reference gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was determined using TaqMan® gene expression assays (Applied Biosystems™, California, USA). RT-PCR reactions were set up in triplicate and were performed on a StepOnePlus™ Real-Time PCR system (Applied Biosystems, California, USA). Amplification conditions consisted of 10 minutes at 95°C, 40 cycles of 30 seconds at 95°C and 60 seconds at 60°C.

2.11 Statistical evaluation

Statistical analysis was performed with GraphPad using non-parametric tests. Different experimental groups were compared by a Kruskall-Wallis test. Only when p-values ≤ 0.05 were obtained, post-hoc pairwise comparisons by means of Mann Whitney U multiple comparison test was performed. *p<0.05, **p<0.01, ***p<0.001.

2.12 Experimental design

Tear fluid and corneal tissue damage were evaluated once a week. Animals were treated with Restasis® for 4 consecutive weeks (2x/day) or dexamethasone for 2 weeks (4x/day). Mean values of 5 dry eye experiments (with 5 animals per group for each experiment) and 2

dexamethasone and Restasis® treatment experiments (with 5 animals per group for each experiment) ± SEM are displayed in the results section below.

3 Results

3.10 Tear volume

Removal of the exorbital lacrimal gland resulted in a significant tear volume reduction of about 50% compared to control eyes. Treatment with Restasis® or dexamethasone displayed no significant effect on tear secretion compared to untreated dry eyes (Figure 1).

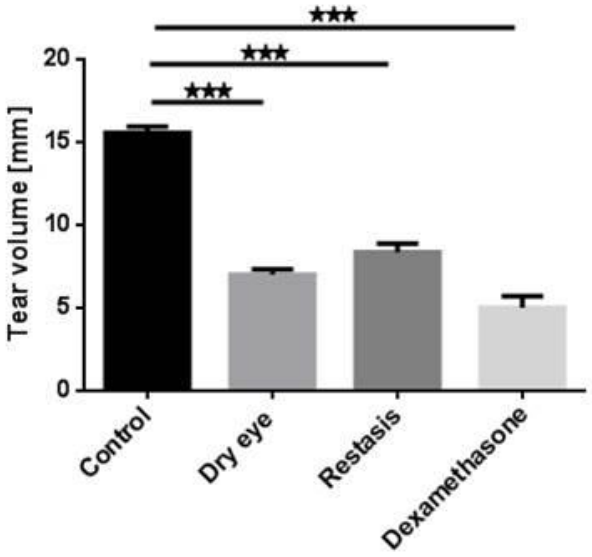

Figure 1: Tear volume of control eyes, untreated dry eyes and Restasis® (4 weeks) and dexamethasone (2 weeks) treated eyes. Data represent mean values ± SEM. *p<0.05, **p<0.01, ***p<0.001. 5 repeats dry eye experiments, 2 repeats Restasis® (4 weeks) and dexamethasone (2 weeks); (N=5)

3.11 Evaluation of ocular surface damage

To evaluate the effect of exorbital lacrimal gland removal on corneal surface damage, control eyes and dry eyes were stained with Na-fluorescein once a week. (Figure 2 and 3). Animals treated with Restasis® or dexamethasone showed a decrease in ocular surface damage compared to untreated dry eyes, however this was only significant for Restasis® (Figure 3).

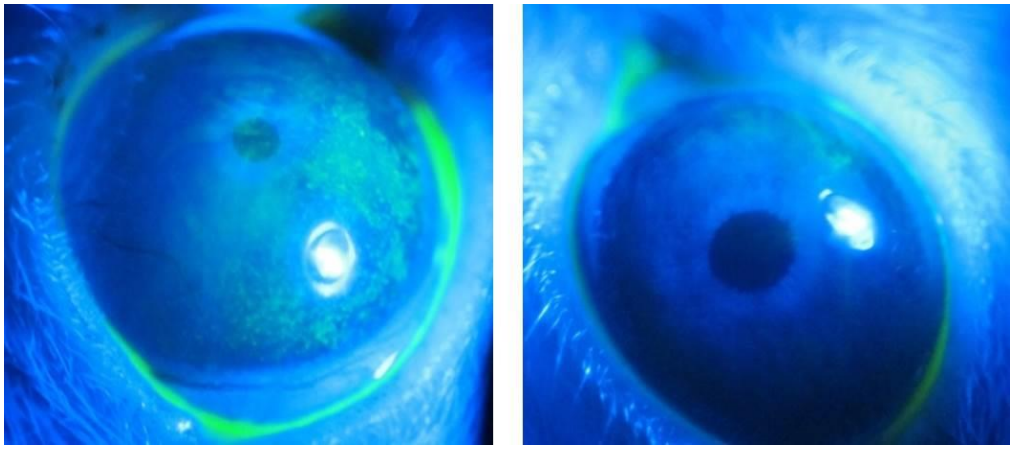

Figure 2: Fluorescein staining of the ocular surface.

Left: Untreated dry eye, 21 days post induction. Right: Control eye, 21 days post induction

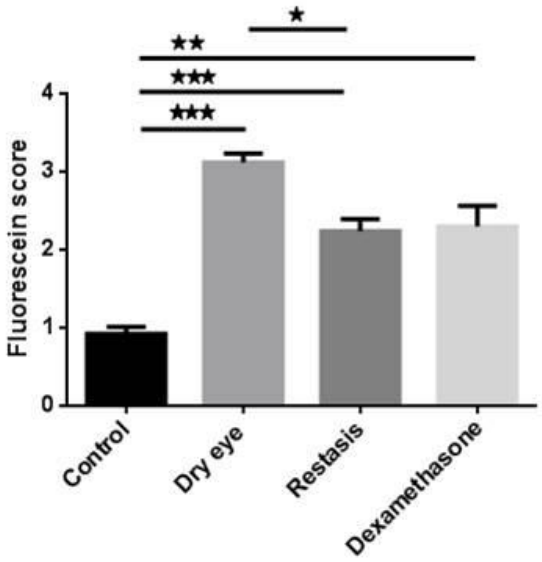

Figure 3: Fluorescein staining of control eyes, untreated dry eyes and Restasis (4 weeks) and Dexamethasone (2 weeks) treated eyes. Data represent mean values ± SEM. *p<0.05, **p<0.01, ***p<0.001. 5 repeats dry eye experiments, 2 repeats Restasis® (4 weeks) and dexamethasone (2 weeks); (N=5)

3.12 Elevated levels of pro-inflammatory cytokines

To demonstrate the inflammatory nature of the dry eye model, pro-inflammatory cytokines were measured in tear fluid of control and dry eye induced animals. Both TNF-α and IL-1α concentrations were significantly elevated in untreated dry eyes compared to control eyes over the course of the experiment (Figure 4).

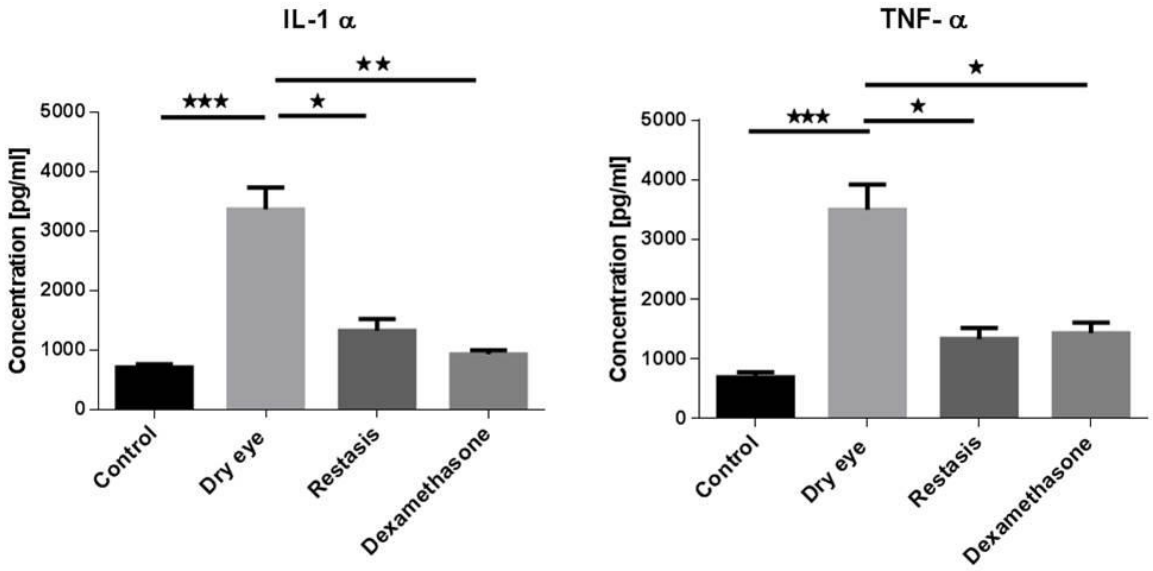

Figure 4: IL-1α and TNF-α concentrations [pg/ml] of control eyes, untreated dry eyes and Restasis (4 weeks) and Dexamethasone (2 weeks) treated eyes. Data represent mean values ± SEM. *p<0.05, **p<0.01, ***p<0.001. 5 repeats dry eye experiments, 2 repeats Restasis® (4 weeks) and dexamethasone (2 weeks); (N=5)

Elevated levels of both cytokines were observed from week one onwards (Figure 5). Treatment with Restasis® and dexamethasone reduced TNF-α and IL-1α levels significantly (Figure 4 - 5).

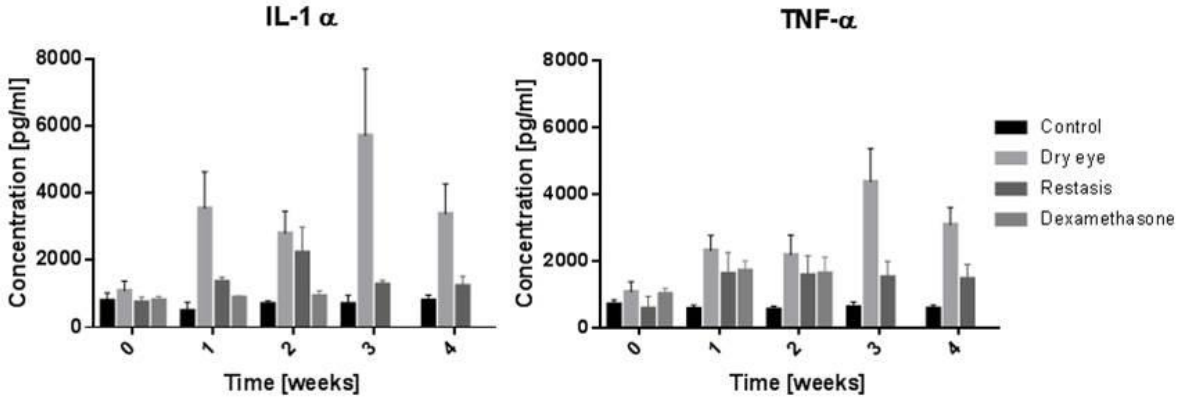

Figure 5: IL-1α and TNF-α concentrations [pg/ml] of control eyes, untreated dry eyes and Restasis (4 weeks) and Dexamethasone (2 weeks) treated eyes in relation to time. Data represent mean values ± SEM. 5 repeats dry eye experiments, 2 repeats Restasis® (4 weeks) and dexamethasone (2 weeks); (N=5)

3.13 CD3+ cell infiltration in palpebral conjunctiva

Untreated dry eyes exhibited dense areas of $CD3^+$ cell populations (81 $CD3^+$ cells/500 cells counted) in palpebral conjunctival tissue compared to control, Restasis® and dexamethasone treated eyes (<8 $CD3^+$ cells/500 cells counted - Figure 6 A, B, C, D).

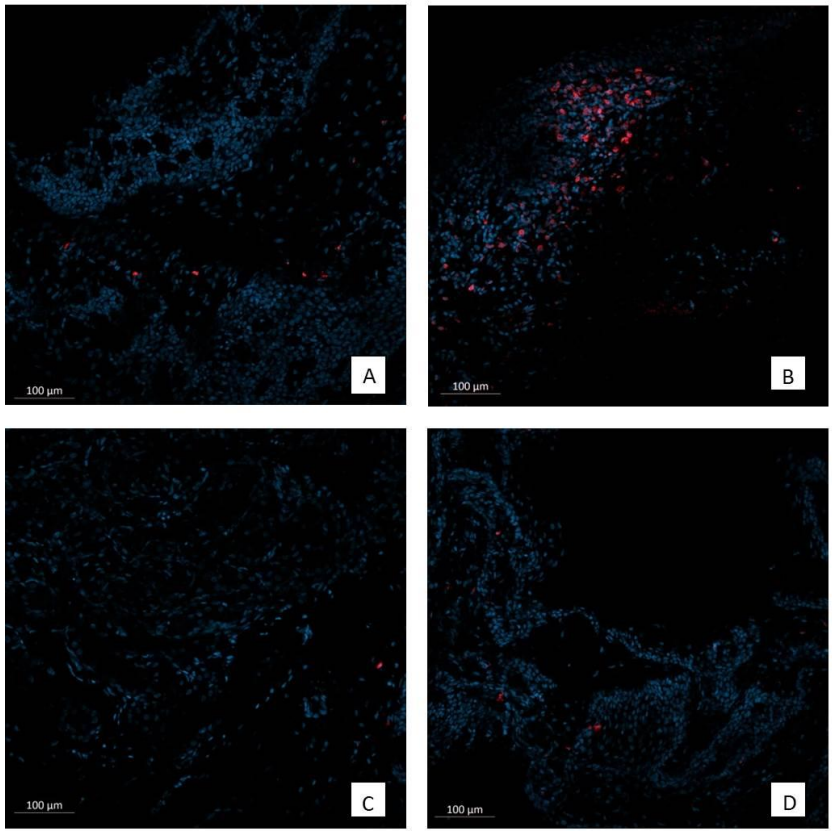

Figure 6: $CD3^+$ cell infiltration (red) and DAPI stained nuclei (blue) in palpebral conjunctiva. A: Untreated control; B: Untreated dry eye; C: Restasis® D: dexamethasone Magnification x200.

3.14 MMP-9 expression in the cornea

Compared to control eyes, a 6-fold increase in MMP-9 expression (mRNA), a prominent biomarker for dry eye, was observed in corneas from untreated dry eyes. Treatment with Restasis® and dexamethasone effectively reduced MMP-9 expression (Figure 7).

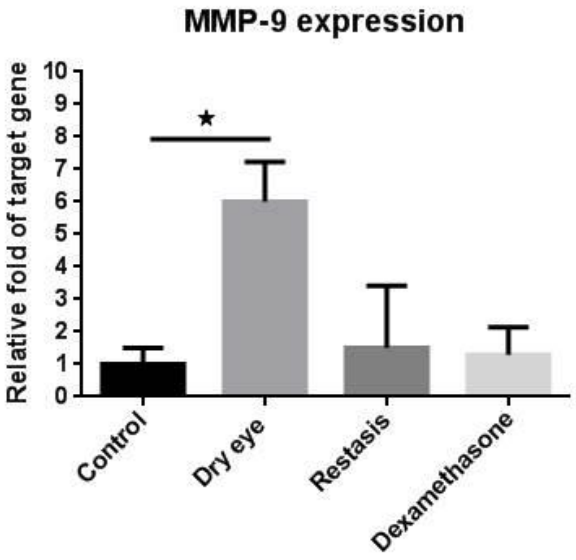

Figure 7: 6 fold increase of MMP-9 mRNA in cornea's from dry eyes, compared to Restasis® and dexamethasone treated animals and control. Data represents mean values ±SEM; N=10.

4 Discussion

To elucidate the pathogenesis of dry eye and to evaluate novel potential treatments for DED, standardized, validated, robust and user friendly animal models for dry eye disease are a major added value in this type of research.

Multiple methods for tear collection have been described[15,16]. Because rodents yield low tear sample volumes, eye fluid washings are generally taken instead of undiluted tear fluid. Although tear collection using eye washings is easier and yields higher volumes, this method complicates the determination of exact protein concentrations. Furthermore, small volumes are highly susceptible to evaporation, even for very limited periods of time. For these reasons, capillaries are preferred to the use of absorbent material. Their use in combination with a very weak negative pressure results in the collection of satisfactory volumes of undiluted tear fluid. Multiplex assays such as CBA, require just a fraction of the volume for the detection of multiple analytes in comparison to conventional techniques (e.g. ELISA). Due to volume restrictions, duplicates are not possible for rats with DED with only 5 animals per group. Increasing the amount of animals per group makes it possible to include duplicates. In this study, control tear fluid has very low inter and intra assay variability (<10 %). High variation (20-40%) in interleukin levels is registered in DED-induced animals. Fluctuations in interleukin levels are well known and the influence of reflex tearing during tear collection cannot be underestimated. These limitations are also observed in human patient studies[17]. A very clear difference (at least 300%) between control tear fluid and tear fluid from (untreated)

DED-induced rats is always observed however, which facilitates the evaluation of potential treatments.

Apart from different animal species, many different strategies exist for the induction of dry eye. Topical administration of benzalkonium chloride (BAC) or other chemicals have a toxic effect on the ocular surface[18]. However, Chen et al demonstrated that BAC can also impair the whole cornea without occurrence of dry eye. Furthermore, the direct toxic effect on the whole cornea should be taken into account if BAC is used to establish an animal dry eye model[18]. In our opinion ocular inflammation and tissue damage should develop as a result of tear film deficiencies or reduced tear secretion, rather than exposure to irritating chemicals. Topical applications of DED-inducing agents may also interfere with topical treatments, especially as BAC is administered multiple times a day[18-19] More clinically relevant options for the induction of dry eye include anticholinergic treatments such as scopolamine or trans-conjunctival botulinum toxin (BTX) B injections. Single BTX-B injections provide a state of dry eye for at least four weeks[20]. This induction method is less suitable for rats because of a mutation in synaptobrevin, reducing the anticholinergic effect of BTX[21]. Scopolamine injections are another option, but a systemic effect and intensive follow-up remains an issue as injections are needed three times a day[22,23]. Several knock-out models are also available, each with different specific causes of dry eye. These models are an added value to demonstrate the mechanistic involvement of certain genes and their related expression products in the dry eye pathogenesis. They are, however, less suitable for routine evaluation of novel compounds, due to their high cost and limited availability and because they usually fail to represent the DED pathology as a whole[5].

The most commonly used animals to mimic a variety of different pathophysiological mechanisms for the induction of dry eye include: mice, rabbits and dogs. Due to the large ocular surface (accessibility) and longer lifespan, rabbits and dogs are most suitable for evaluation of signs of dry eye. On the other hand, using rodents, has several advantages, such as easier handling, reduced costs, fewer prerequisites for housing and maintenance and better availability of reagents. The downsides are low sample volumes and small ocular dimensions to study clinical dry eye signs.

Surgical Induction of DED in this study is fast, permanent and no follow up is needed. Removal of the extraorbital lacrimal gland results in a significant reduction in tear secretion, resulting in an increase in ocular surface damage and an elevation of inflammatory markers in

tear fluid, suggesting reduced tear volume is sufficient to excite ongoing activity of moisture-sensitive corneal afferent fibers that increase during ocular surface dryness[24]. Removal of the exorbital lacrimal gland may interfere with the study of potential tear secretion stimulating agents. The intra orbital and accessory lacrimal glands may be sufficient to register an increase in tear secretion. This, however has to be investigated further. We are aware that this dry eye model mimics tear deficient dry eye and are currently investigating a similar model for evaporative dry eye.

In this study , mRNA analysis revealed a 6-fold increase in MMP-9 in corneas from dry eye groups in comparison to control eyes. Furthermore, an increase in $CD3^+$ cell infiltration in the palpebral conjunctiva was observed in untreated dry eye. Human clinical studies confirm conjunctival $CD3^+$ cell infiltration and elevated IL-1α and TNF-α levels in tear fluid[10-12]. MMP-9 is also a major human biomarker for DED and plays a pivotal role in dry eye pathogenesis[14,25,26]. These findings confirm the clinical relevance of this animal model.

This surgical method of DED induction has been immunologically evaluated in mice[27], but to improve clinical monitoring and to obtain larger sample volumes, female Wistar rats were selected as animals of choice[28]. For model validation, the animals were treated with two established anti-inflammatory agents, e.g. cyclosporine A (Restasis®) and dexamethasone. Restasis® and dexamethasone eye drops displayed anti-inflammatory properties and significantly reduced IL-1α and TNF-α concentrations in tear fluid. Moreover, both treatments reduced MMP-9 expression in the cornea. Fluorescein staining, for the detection of ocular surface damage, was significantly diminished. Few to no $CD3^+$ cell infiltration was observed in the two treatment groups. Depending on the area of research, additional targets, detectable with qRT-PCR, immunohistochemistry and tear fluid analysis, could be easily implemented.

5 Conclusion

This animal model strives to be as robust and low-maintenance as possible. Immunologically relevant evaluation parameters were included and the model was validated with the two most commonly known anti-inflammatory treatments for dry eye. Due to the fast induction and onset of ocular damage and inflammation, this model can be used for the quick evaluation of potential therapeutic compounds or preparations for DED and other related ocular inflammatory disorders.

6 Acknowledgements

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