Corneal Sensitivity Following Lacrimal Gland Excision in the Rat

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PurPOSE. Dry eye disease (DED) produces ocular pain and irritation, yet a detailed characterization of ocular sensitivity in a preclinical model of DED is lacking. The aim of the present study was to assess nociceptive behaviors in an aqueous tear deficiency model of DED in the rat.

Methods. Spontaneous blinking, corneal mechanical thresholds, and eye wipe behaviors elicited by hypertonic saline (5.0 M) were examined over a period of 8 weeks following the unilateral excision of either the exorbital lacrimal gland or of the exorbital and infraorbital lacrimal glands, and in sham surgery controls. The effect of topical proparacaine on spontaneous blinking and of systemic morphine (0.5–3.0 mg/kg, subcutaneous [SC]) on spontaneous blinking and eye wipe responses were also examined.

Results. Lacrimal gland excision resulted in mechanical hypersensitivity and an increase in spontaneous blinking in the ipsilateral eye over an 8-week period that was more pronounced after infra- and exorbital gland excision. The time spent eye wiping was also enhanced in response to hypertonic saline (5.0 M) at both 1- and 8-week time-points, but only in infra- and exorbital gland excised animals. Morphine attenuated spontaneous blinking, and the response to hypertonic saline in dry eye animals and topical proparacaine application reduced spontaneous blinking down to control levels.

Conclusions. These results indicate that aqueous tear deficiency produces hypersensitivity in the rat cornea. In addition, the increase in spontaneous blinks and their reduction by morphine and topical anesthesia indicate the presence of persistent irritation elicited by the activation of corneal nociceptors.

Keywords: corneal sensitivity, dry eyes, lacrimal gland excision
Corneal Sensitivity Post–Lacrimal Gland Excision

RESULTS

Tear Levels

Tear measurements were conducted weekly after sham surgery, single and double LGE. While sham surgery had no effect on tear levels ($P > 0.05$), a significant reduction was found after both single and double LGE (Fig. 1). After single LGE, a two-way ANOVA with repeated measures indicated significant effects of both time after surgery ($F [7.84] = 8.06, P < 0.001$) and side of gland excision ($F [1.84] = 25.64, P < 0.001$) but no interaction between these two factors ($F [7.84] = 2.06, P = 0.06$). Post hoc analysis revealed lower tear levels on the side of the gland excision during 4 of the 8 weeks (Fig. 1B). Single LGE produced a 42% to 64% reduction in tear levels over the first 2 weeks after surgery; however, this reduction was not consistently maintained in subsequent weeks. The removal of both the infra- and exorbital lacrimal glands also affected tears levels. A two-way

Methods

Animals

A total of 150 male Sprague-Dawley rats (200–250 g) were obtained from Charles River Laboratories (Cambridge, MA, USA) and housed in an environment with free access to food and water and a controlled 12-hour light/dark cycle. Animals were treated according to the policies and recommendations of the National Institutes of Health guidelines for the handling and use of laboratory animals and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were approved by the committee on animal research at the University of New England.

Surgery

Unilateral lacrimal gland excision was performed under 2% to 3% isoflurane anesthesia. The left exorbital lacrimal gland was removed in animals receiving single LGE surgery, whereas double LGE included the removal of both the left exorbital and infraorbital lacrimal glands.27 For sham surgeries, incisions were made and glands were partially exposed on the left side. Lidocaine (lidocaine HCl 1% and epinephrine 1:100,000) was injected subcutaneously into the cheek prior to gland excision to attenuate bleeding. Carprofen was administered subcutaneously (5 mg/kg) once per day for 3 days to provide postsurgical analgesia. Morphine (gift from National Institute on Drug Abuse) was administered subcutaneously 30 minutes prior to eye wipe measurements. Proparacaine hydrochloride ophthalmic solution (0.5%, 10 μL, Alcon, Fort Worth, TX, USA) was topically applied to the eye.

Statistical Analysis

Multiple group means of parametric data sets were compared using either a one- or two-way ANOVA after it was determined that data conformed to a normal distribution with equal variances. A Tukey post hoc test was performed if an overall significance was found. The Kruskal-Wallis one-way ANOVA on ranks test with post hoc comparisons (Dunn’s method) was applied to nonparametric data sets (fluorescein scores and mechanical thresholds) and in parametric data when tests for normality or equal variance failed. Analyses were performed using commercial software (Sigma Stat version 3.5; Systat Software, Chicago, IL, USA). All results are expressed as mean ± SEM. Values of $P < 0.05$ were considered to be statistically significant.

Results

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ANOVA with repeated measures indicated a significant effect of side of the gland excision ($F_{[1.98]} = 31.65, P < 0.001$), but no effect of time after surgery ($F_{[7.98]} = 0.67, P = 0.70$) and no interaction between these factors ($F_{[7.98]} = 0.780, P = 0.59$). Post hoc analysis revealed significantly lower tear levels in the ipsilateral eye over each of the 8 weeks examined (Fig. 1C). Compared with the contralateral side, tear levels were reduced by 42% to 86%, with the greatest reduction observed during the first 2 weeks after surgery.

**Fluorescein Staining**

The ability of lacrimal gland excision to affect the corneal epithelium was examined using a slit lamp ophthalmoscope after the application of fluorescein (Fig. 2). Following double LGE, cornea fluorescein scores escalated throughout the duration of the study, from an initial score of 1.0 ± 0.2 recorded 1 week after surgery to 2.8 ± 0.2 observed at week 8. The increase in fluorescein scores between 1 and 8 weeks after double LGE was statistically significant (Mann-Whitney sum rank test, $P < 0.001$). While not as severe, single LGE also produced significant fluorescein staining, which increased from 0.14 ± 0.1 one week after surgery and peaked at 1.14 ± 0.18 after 8 weeks (Mann-Whitney sum rank test, $P < 0.05$). Sham surgery had no effect on cornea fluorescein scores ($P = 0.93$). At the 1-week time point, a one-way ANOVA on ranks test revealed a significant difference between treatment groups ($P < 0.01$) with post hoc analysis indicating lower fluorescein scores after single LGE when compared with double LGE (Fig. 2). In addition, at the 8-week time point, a one-way ANOVA on ranks indicated a significant difference between treatment groups ($P < 0.001$) with post hoc analysis showing lower fluorescein scores after single LGE when comparing with double LGE (Fig. 2).

**Eye Blinking**

Spontaneous eye blinking was quantified over the 8-week observation period in animals undergoing single LGE, double LGE, and sham surgery. In the case of sham-treated animals, a two-way ANOVA revealed no significant effect of surgery side ($P = 0.18$), no effect of time after surgery ($P = 0.50$), and no interaction ($P = 0.60$; Fig. 3A). In contrast, after single LGE, a two-way ANOVA indicated a significant effect of surgery side ($F_{[1.91]} = 9.88, P < 0.01$) and interaction ($F_{[7.91]} = 2.60, P < 0.05$), but no effect of time after surgery ($F_{[7.91]} = 1.36, P = 0.23$). Post hoc analysis indicated significantly higher eye blinks ipsilateral to the side of gland excision at 1, 3, and 5 weeks post surgery when compared with the contralateral side (Fig. 3A). While single LGE produced a greater than 2-fold increase in ipsilateral eye blinks over the first few weeks, the effect of double LGE on the number of eye blinks was even more dramatic (Fig. 3C). A two-way ANOVA indicated a significant effect of surgery side ($F_{[1.98]} = 53.76, P < 0.001$), but no effect of time after surgery ($F_{[7.98]} = 2.05, P > 0.05$) and no
interaction (F[7.98] = 1.77, P = 0.102). Post hoc analysis revealed a significant elevation in ipsilateral eye blinks across the entire 8-week period after double LGE when compared with the contralateral side (P < 0.001).

In order to determine the contribution of primary afferent neuronal activity in driving the increase in blinking, the topical anesthetic proparacaine (10 μL) was applied to the eye 8 weeks after animals had undergone double LGE. A 2-way ANOVA indicated a significant effect of time after application of drugs (F[2.12] = 1.77, P < 0.001) and a significant interaction (F[2.12] = 6.77, P < 0.05) but no effect of drug (F[1.12] = 0.62, P < 0.46) (Fig. 4). Post hoc analysis demonstrated that proparacaine produced a significant reduction in the number of eye blinks when compared to both baseline and the animals treated with vehicle control (P < 0.05). The effect was short lasting, with blinking coming back up to control levels by 25–30 minutes after drug application.

**Corneal Sensitivity**

Mechanical sensitivity of the corneal surface was examined using a Cochet-Bonnet esthesiometer. Baseline thresholds were obtained prior to surgery, with thresholds for all treatment groups averaging between 4.3 and 5.5 g/mm² (Fig. 5A). In animals receiving both single and double LGE, mechanical thresholds were reduced beginning the second week after surgery (P < 0.01, Kruskal-Wallis one-way ANOVA; Fig. 5A). By week 6, these differences were no longer significant, although still numerically lower than baseline. In sham-treated animals, a decrease in corneal thresholds was observed 1 week after surgery; however, this decrease did not reach significance, and values increased toward baseline values in subsequent weeks.

Sensitivity of the cornea to hypertonic saline was assessed by quantifying wipe behaviors in response to the application of 5 M saline to the eye at 1 and 8 weeks post surgery in separate groups of animals. At the 1-week time point, a one-way ANOVA did not indicate a significant difference between treatment groups (P = 0.34, Kruskal-Wallis one-way ANOVA; Fig. 5A). However, at the 8-week time point, double LGE produced a significant increase in eye wipe behaviors when compared with sham and single LGE (P < 0.05, Kruskal-Wallis one-way ANOVA; Fig. 5B).

**Effect of Morphine on Blinking and Eye Wipe Behavior**

Spontaneous blinking and 5 M saline-evoked eye wipe behaviors were quantified after the systemic administration of morphine in sham and double LGE animals (Fig. 6). Morphine produced a dose-dependent decrease in spontaneous blinking in double LGE animals, reaching significance after 2.0 mg/kg (P < 0.05, Kruskal-Wallis one-way ANOVA; Fig. 6A). In sham-treated animals, morphine did not produce a significant difference in the number of blinks (P = 0.36). In these same animals, morphine suppressed eye wipe behavior.
DISCUSSION

While LGE has been used to produce aqueous tear deficiency in rodents, this is the first study to directly compare the effects of single and double LGE on tear levels and corneal pathology associated with DED. Tear measurements decreased and corneal fluorescein scores increased in a graded fashion, with greater signs of epithelial cell damage associated with double LGE. The excision of both the infra- and exorbital gland also produced a greater increase in spontaneous blinking when compared with single LGE and sham treatment throughout the 8-week observation period. This increase in blinking was reduced by the application of the topical anesthetic proparacaine and systemic morphine. Furthermore, LGE increased sensitivity to mechanical stimuli and hyperosmotic saline applied to the cornea, indicating the sensitization of nociceptive neurons in this model of DED.

The composition of tears includes water, electrolytes, mucins, and other glycoproteins and proteins. The lacrimal gland is the major supplier of the aqueous components, including water, electrolytes, and proteins, whereas conjunctival goblet cells are the primary source of mucins. Dry eye disease can result from multiple factors, including reduced or altered composition of tears secreted from glandular tissues. The reduction in tears produced by lacrimal gland excision likely results in aqueous tear deficiency, a specific subclass of DED marked by inadequate tear volume. Evaporative DED, in contrast, may result from decreased lipid content.

Previous studies have examined ocular conditions following excision of the exorbital lacrimal gland in rats, using this model of aqueous tear deficiency to test the effectiveness of various potential therapies for DED. Fluorescein staining was commonly used as the primary endpoint to determine the overall condition of the ocular surface. To this end, compounds that have targeted oxidative stress and purinergic receptors, and the nonsteroidal anti-inflammatory drug diclofenac were all demonstrated to reduce ocular surface damage produced by LGE. The severity of DED symptoms after excision of the exorbital gland—as measured by corneal fluorescein staining—in these studies appears to be greater than that found in the present study. The reasons for these differences are unclear, but may be related to rat age or relative humidity levels in the vivarium. In the present study, the severity of dry eye was much greater after double LGE. Furthermore, the effects of double LGE were also more...
sustained throughout the 8-week observation period, perhaps indicating that the infraorbital gland may have the ability to compensate for the loss of the exorbital gland over time. If this is the case, then excision of the exorbital lacrimal gland may be an ideal model for studying plasticity in the infraorbital lacrimal gland.

The reduction in blinking following the application of a topical anesthetic in double LGE animals indicates that although these blinks are often referred to as “spontaneous,” they are driven by the activation of corneal afferents.24 The class of afferent neurons responsible for eliciting eye blinks under this condition remains unknown. At least three types of corneal afferents have been characterized according to their receptive field properties.38–48 Mechanoreceptive afferents respond exclusively to mechanical stimulation, polymodal nociceptive afferents are activated by mechanical, noxious thermal, and chemical (e.g., low pH, capsaicin) stimulation, and cold receptive afferents are sensitive to innocuous cooling. All three categories of corneal afferents project to the brainstem region in the trigeminal nucleus that regulates tearing and blinking through projections to preganglionic parasympathetic neurons.5,40–54

Cold receptors are responsible for the regulation of secretions and possibly blinking that occurs in response to the constant evaporative cooling on the ocular surface.43,46 Mechanoreceptors and polymodal nociceptors are more likely involved in noxious stimulation evoked reflexive tearing and blinking as well as the perception of irritation and pain.48,55,56 Lacrimal gland excision has been shown to sensitize corneal cold receptors to cooling and menthol stimulation, suggesting that these neurons may be involved in the increased blinking observed in dry eye animals.25 The suppression by morphine, however, may implicate the activation of nociceptors in blinking following LGE. Morphine has been demonstrated to increase the activity cold cells recorded in the trigeminal nucleus region that regulates tearing and blinking as well as in second-order neurons located in the spinal dorsal horn.57,58

Corneal cold receptors are sensitized following LGE, yet the properties of corneal mechanoreceptors and polymodal nociceptors have not been examined under similar conditions. The lower mechanical thresholds and increased responses to hypertonic saline are signs that corneal nociceptors may also be sensitized in this model of DED. This result is in contrast to reports on corneal sensitivity in human subjects with DED, in which decreased sensitivity to mechanical, thermal, and acidic conditions has been reported.59–61 In contrast to these findings, other studies have observed lower mechanical thresholds in subjects with DED.62–66 Possible reasons for the discrepancy in these results include potential differences in the severity or etiology of the disease among the study participants, as well as differences in the methods used for assessing corneal sensitivity.

Comparing the results from human studies with the present findings in rats is complicated by several factors, including the cause and duration of the dry eye and the absence of any treatment provided to the animals. One possibility is that corneal hypersensitivity occurs during the initial phase of DED, with nerve degeneration and hypoesthesia taking place during later stages of the disease. In order to explore this possibility, changes in corneal sensitivity would need to be carried out over a longer period of time and correlated with morphological alterations in corneal nerves following LGE. Alterations in corneal nerve morphology, including reduced nerve fiber density in the subbasal nerve plexus, have previously been reported in individuals with DED and may be correlated with the occurrence of corneal hypoesthesia.60,64,67–70

Lacrimal gland excision and other preclinical models of DED have been utilized in evaluating the efficacy of novel therapies.16,18,23 These studies typically examine tear levels, tear break-up time, and the overall condition of the corneal epithelium. Signs of ocular discomfort and pain are not generally quantified, despite the importance of treating these symptoms in patients with DED and their common use as endpoints in clinical studies.71–73 The assessment of corneal sensitivity following LGE now provides an opportunity to examine preclinically the effectiveness of potential therapies in treating ocular pain and irritation. Importantly, these treatments should be tested under conditions in which corneal nociceptors have become sensitized, providing an advantage over previous studies that have examined acute nociceptive responses under healthy ocular conditions.74–76

In summary, unilateral excision of the exorbital lacrimal gland or removing both the infraorbital and exorbital lacrimal glands produced graded symptoms of DED in the rat. These symptoms included increased corneal fluorescein staining and spontaneous eye blinks on the side ipsilateral to gland excision. In addition, signs of persistent sensitization developed, including a decrease in corneal mechanical thresholds and increased eye wipe behaviors in response to hypertonic saline over the course of 8 weeks. These features indicate that LGE may provide a useful model for preclinical testing of potential treatments for DED and lead to a more mechanistic approach in the development of novel therapeutics for ocular pain.

**Acknowledgments**

Supported by the National Eye Institute Grant R01EY021230 and the National Institute of General Medicine Grant P20GM103643 (IDM). The authors declare no competing financial interests.

Disclosure: I.D. Meng, None; S.T. Barton, None; N.E. Mccum, None; M. Kurose, None

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