Published in final edited form as:

Invest Ophthalmol Vis Sci. 2007 October ; 48(10): 4468–4475. doi:10.1167/iov.06-1486.

Evaluation of Novel Dry Eye Model: Preganglionic Parasympathetic Denervation in Rabbit

Hiroshi Toshida¹, Doan H. Nguyen², Roger W. Beuerman³, and Akira Murakami¹

¹Juntendo University School of Medicine, Tokyo, Japan

²Louisiana State University (LSU) Eye Center, Lions Eye Research Laboratories, Laboratory for the Molecular Biology of the Ocular Surface, LSU Health Sciences Center, New Orleans, Louisiana

³Singapore Eye Research Institute, Singapore

Abstract

Purpose—To evaluate ocular surface status after interruption of preganglionic, parasympathetic neural control after surgical removal of the greater superficial petrosal nerve (GSPN).

Methods—New Zealand White rabbits underwent unilateral section and removal of a 5-mm portion of the GSPN by a route through the inner ear; no ocular or orbital tissue was involved. Before and 7 days after surgery, all animals underwent preliminary examination, including fluorescein staining, rose bengal instillation, blink rate, tear breakup time (BUT), tear flow, and impression cytology. Total tarsorrhaphy was carried out in four additional rabbits, and another four animals underwent unilateral sham procedures. The GSPN, pterygopalatine ganglion, lacrimal gland, and conjunctiva were evaluated by light and transmission electron microscopy (TEM).

Results—GSPN sectioning resulted in significant changes of the ocular surface after 7 days: intense rose bengal staining of the conjunctiva, fluorescein staining of the cornea, increased blink rate ($P < 0.05$), decreased BUT ($P < 0.005$), decreased tear flow by 26% ($P < 0.005$), and decreased goblet cell density ($P < 0.01$). TEM revealed massive accumulation of secretory granules in lacrimal acinar cells. The changes were also seen after tarsorrhaphy. Neither the contralateral control nor the sham eyes were affected.

Conclusions—The effects of GSPN nerve section led to the rapid onset of a dry eye condition in the rabbits that continued for at least 1 week. The authors suggest that continuous neural drive of the pterygopalatine ganglion is necessary to maintain adequate tear flow and mucin secretion. It is likely the trigeminal system is the afferent origin of this continuous neural tone.

Dry eye, or keratoconjunctivitis sicca (KCS), is an ocular surface disease caused by abnormalities in the quantity or quality and distribution of tear fluid.¹ The diagnosis of dry eye includes a number of functional tests that evaluate the adequacy of tear flow and the status of the ocular surface. These results include vital staining with fluorescein and rose bengal, tear flow with the Schirmer tear test, and tear breakup time (BUT).¹ Vital staining tests are the most commonly used method to screen for dry eye.² The disease is manifested after or with their dysfunction of acinar cells or nerves, trauma, aging, or sex hormones.

Studies have shown that the main lacrimal gland (LG), which is under parasympathetic control, contributes largely to the aqueous component of the tear film.³⁻⁵ Conjunctival goblet cells, which contribute to the mucous layer of the tear film, are also under parasympathetic control.⁶ Parasympathetic innervation of the LG and conjunctival goblet cells is mediated through the pterygopalatine ganglion (PPG). The parasympathetic pathway originates from the superior salivatory nucleus and passes through the geniculate ganglion without synapsing, and the fibers emerge as the greater superficial petrosal nerve (GSPN), which then joins the deep petrosal nerve to form the vidian nerve.⁷⁻¹⁰ The vidian nerve passes through the pterygoid canal and terminates on the cell bodies of the PPG. The output of these ganglion cells, the postganglionic axons, provide the parasympathetic secretory drive to the LG and conjunctiva, including the goblet cells.¹¹⁻¹⁴

Several studies have reported the loss of parasympathetic function, resulting in reduced tear flow in humans.¹⁵⁻¹⁸ Other reports have examined parasympathetic control of lacrimation in experimental animals.¹⁹⁻²¹ Ruskell¹⁹ reports changes in lacrimation and histologic changes in the main LG after parasympathetic denervation in monkeys. Notably, the effect on tear flow was observed to be more variable, probably because of the incompleteness of the surgical procedures. Toda et al.²⁰ reported that stimulation of tear flow is abolished after postsynaptic denervation of the parasympathetic nerve. However, it was unclear to what extent the parasympathetic system contributes to the observed changes because the sectioned lacrimal nerve also contains sympathetic and sensory nerve fibers. Additionally, Butler et al.²¹ report the effect of structural disruption of the PPG on neurotransmitter levels in ocular and orbital tissues, but none of these studies provides a comprehensive evaluation of denervation on the ocular surface and LG.

Recently, neurturin-deficient mice, characterized by decreased parasympathetic innervation of lacrimal and salivary glands, were evaluated.^{22,23} These mice exhibit many clinical symptoms of dry eye, including decreased tear flow and mucin production. However, these mice also had a defect in the sensory nervous system; thus, the specific effect of parasympathetic influence could not be clearly discerned.

In the present study, we sought to determine whether the loss of preganglionic parasympathetic control adversely influences the ocular surface. We focused on the ocular findings caused by acute section of the primary efferent outflow, the GSPN, without injuring or involving the eye, orbital tissues, or facial nerve. Tear flow, goblet cell density, and LG morphology were significantly affected after sectioning of the GSPN, the input to the PPG. Furthermore, the ocular surface findings were not changed or diminished by total tarsorrhaphy. This procedure may be useful in discerning the specific effect of primary efferent neural outflow and in establishing a severe and long-lasting dry eye animal model.

Materials and Methods

Twenty-five male adult New Zealand White rabbits (2.0 -3.5 kg) were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animals underwent screening eye examination with a slit lamp and fluorescein and rose bengal instillation. Animals were randomly assigned to one of three groups. Animals in group 1 ($n = 14$) underwent unilateral section of the GSPN, animals in group 2 ($n = 7$) underwent unilateral section of the GSPN and total tarsorrhaphy of the experimental eye, and animals in group 3 ($n = 4$) underwent a sham procedure that was identical with that of group 1 in all respects except that the GSPN was not sectioned. Animals were evaluated after surgery on days 1, 3, 5, and 7.

Denervation Surgery

Surgical procedures were carried out under deep general anesthesia with intramuscular injections of 100 mg/kg ketamine (Schering-Plough Animal Health, Union, NJ) and 10 mg/kg xylazine (Ben Venue Laboratories, Bedford, OH). Animals were placed in the prone position, and the skin on the temporal side of the head was shaved and wiped with 70% ethanol. The approach through the internal auditory meatus to the GSPN was carried out under a surgical microscope adapted for the anatomy of the rabbit.

A vertical 3-cm incision was made in the skin, close to the external auditory meatus. The facial nerve was followed after removal of the temporal bone with rongeurs, and the facial canal was opened in the middle ear. When the dissection reached the outside of the internal auditory meatus, the bifurcation of the GSPN and the facial nerve was found as previously reported.^{8, 14} The GSPN, which runs in a groove on the front of the petrous temporal bone,²⁴ was sectioned, and a 3-mm piece was removed without cutting or injuring the facial nerve. The sham procedure was carried out in an identical fashion except that the GSPN was identified but not sectioned.

The surgical area was flushed with sterile normal saline, and the field was gently dried with cotton wicks. The muscle was closed with 4-0 chromic gut and the skin with 6-0 silk suture. Each animal was given 4% gentamicin (Elkins-Sinn, Inc., Cherry Hill, NJ) 0.2 mL intramuscularly for 3 days after surgery. Acute denervation and the sham procedure were carried out unilaterally. In the closed-eye group, total tarsorrhaphy was produced using three to four sutures. The surgical procedure included temporary suturing of the upper and lower eyelids to reduce symptoms from excessive conjunctival and corneal surface exposure. The day after surgery, animals were examined to ensure that there were no postsurgical complications. All procedures to evaluate the ocular surface were carried out before surgery and on specific days after surgery.

Evaluation of the Ocular Surface

Seven denervated, seven tarsorrhaphy, and four sham rabbits were used in this study. One week after surgery, slit lamp examinations were performed. Blink rate was determined before instillation of disclosing agents. Each animal was positioned in a special rabbit bag so that both eyes could be observed. Blinks for a 3-minute period were counted for each eye of each animal except tarsorrhaphy rabbits. Noninvasive tear BUT was also measured. Each rabbit was placed behind an instrument for the measurement of tear production (Tearscope; Keeler, Lübeck, Germany) and was made to blink three times before the lids were held open. BUT was then measured in seconds for each eye (Tearscope; Keeler).²⁵

After fluorescein instillation, eyes were evaluated by slit lamp. Rose bengal solution (1%; Clinic Pharmacy and Sick Room Supplies, Inc., New Orleans, LA) was then applied to the inferior cul-de-sac. After blinking, the degree of staining was graded in the medial and lateral bulbar conjunctivae and cornea on a scale of 0 to 3 points by slit lamp biomicroscopy.^{26,27} We used a 0 to 3 scoring system for rose bengal and fluorescein for the medial and lateral bulbar conjunctiva and the cornea. Thus, the maximum score was 9, a combined score from all three areas. Scores for each area of the ocular surface were also noted.

In denervated and sham-operated rabbits, Schirmer tear tests without topical anesthesia were performed to evaluate lacrimation, including reflex tear flow, before surgery and on postoperative days 1, 3, 5, and 7 using Schirmer tear test strips (Alcon Laboratories, Fort Worth, TX). In rabbits with a tarsorrhaphy, Schirmer tear tests were performed before surgery and on postoperative day 7 after suture removal. The strip was placed between the lower lid and the globe for 5 minutes, and the length of wetting was measured with a ruler with 0.5-mm precision.

Four denervated rabbits were anesthetized with a mixture of ketamine and xylazine, described to measure the tear flow from the main LG, which opened into the conjunctival sac. The excretory duct of the LG was cannulated in situ according to techniques previously applied for the collection of LG fluid.^{28,29} Fluid was collected in calibrated 5- μ L glass capillaries from the conjunctival opening of the lacrimal duct. The time to collect each microliter of fluid was counted and averaged, and flow rate per second was calculated.

Conjunctival goblet cell density, evaluated by impression cytology, was carried out on each eye of each rabbit before and 1 week after surgery. Impression cytology was performed on the lower tarsal and upper bulbar conjunctivae in control and experimental eyes of all animals using a nitrocellulose membrane (BioBlot-NC, Cambridge, MA).^{27,30} The specimens were stained with periodic acid-Schiff (PAS) reagent to reveal goblet cells. After computer capture of the light microscopic images, the number of PAS-positive spots was counted by NIH Image software (Freeware; Scion Corp., Frederick, MD). Three areas were randomly selected in each specimen, and the number of goblet cells was counted and averaged. Data per square millimeter was calculated as in a previous report.³¹

Microscopy

Eight denervated rabbits and four tarsorrhaphy rabbits were used for microscopy. The structures of the GSPN and the PPG were examined in the normal rabbit and in the following section of the GSPN. After initial anesthesia, rabbits were killed with overdoses of sodium pentobarbital (Fort Dodge Laboratories, Fort Dodge, IA) on postoperative days 4 and 7. Five millimeters of GSPN, distal to the site of section, was removed for analysis 4 days after section in four denervated rabbits. Seven days after GSPN sectioning, the PPG was removed from its position on the inferior-medial aspect of the maxillary nerve, located deep within the orbit for analysis.^{7,9} LG and lower tarsal conjunctiva were also removed 7 days after surgery from the other four denervated rabbits and four tarsorrhaphy rabbits.

Tissues were immediately immersed in an aldehyde fixative (2% glutaraldehyde, 1.0% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for light microscopy.²⁸ One micron-thick plastic sections of all tissues were prepared and stained with toluidine blue and basic fuchsin for light microscopy. Conjunctival tissues were also stained by PAS for observation of goblet cells.

Images for light microscopy were captured on a research microscope (Eclipse (E600; Nikon, Tokyo, Japan) equipped with a 3-CCD camera (DXC-970 MD; Sony, Tokyo, Japan) controlled by image analysis software (MetaView; Universal Imaging, West Chester, PA) and installed on a personal computer. Color images were converted to gray scale, annotated (PhotoShop version 5; Adobe, Cupertino, CA), and printed on a color printer (8650 PS; Eastman Kodak, Rochester, NY). Thin sections for electron microscopy were contrasted and observed with a transmission electron microscope (EM 10/CA; Zeiss, Oberkochen, Germany), as previously described.³²

Statistical Analysis

Statistical analysis (Student's *t*-test or Mann-Whitney *U* test) was performed (Statistica software; Statsoft, Tulsa, OK) to evaluate the data from experimental, control, and sham eyes, making the appropriate corrections for the correlated sampler.

Results

Evaluation of the Ocular Surface

After surgery, no changes were found in eye position or eye movement. Effects of GSPN sectioning on the ocular surface were apparent after 1 day. Clinical evaluation revealed bulbar conjunctival injection. Corneas appeared dull and without luster because of superficial punctate keratitis in all experimental eyes on the first postoperative day, and this persisted through the termination of the experiment at 7 days (data not shown). These findings were similar to those for dry eyes.

Fluorescein Staining

One week after sectioning of the GSPN, slit lamp examination after fluorescein instillation showed fluorescein staining on the ocular surface of all experimental eyes but not of contralateral control eyes (Figs. 1A, 1B). Fluorescein staining of the ocular surface was also evident in animals that underwent GSPN sectioning and tarsorrhaphy (Fig. 1C). Control eyes for all the experimental (denervation and tarsorrhaphy) animals were without any fluorescein staining. The mean fluorescein score for a cornea after GSPN sectioning compared with the contralateral control eyes was 1.86 ± 0.40 ($P < 0.005$; Fig 1D). Mean fluorescein scores for the medial and lateral bulbar conjunctiva for the denervated eye were 0.43 ± 0.20 and 0.14 ± 0.14 , respectively, which were not significantly different from the scores of the control side. However, the total score of all areas showed a significant difference between the control eye and the denervated eye (2.43 ± 0.43 ; $P < 0.005$). In tarsorrhaphy rabbits, the mean fluorescein score of the cornea was 2.43 ± 0.20 , the mean fluorescein score of the medial bulbar conjunctiva was 0.85 ± 0.34 ($P < 0.05$), and the mean fluorescein score of the lateral bulbar conjunctiva was 0.00 ± 0.00 ($P < 0.005$), all of which are similar to those of the rabbits after parasympathectomy. Total score of all areas showed a significant difference between contralateral control eyes and experimental eyes (3.29 ± 0.42 ; $P < 0.005$). Sham-operated and control eyes were without fluorescein staining.

Rose Bengal Staining

Rose bengal staining revealed differences in the degree of staining in all areas of the ocular surface of control and denervated eyes (Fig. 2). After section of the GSPN, all seven eyes revealed rose bengal staining, and the mean score for the medial conjunctiva was 1.71 ± 0.18 ($P < 0.005$), the mean score for the lateral conjunctiva was 0.86 ± 0.26 ($P < 0.05$), and the mean score for the corneas of denervated eyes was 2.57 ± 0.20 ($P < 0.005$). The total score of the denervated eyes was 5.14 ± 0.51 , which was significantly greater than control (0.14 ± 0.14 ; $P < 0.005$). In tarsorrhaphy animals, the mean rose bengal scores for cornea, medial bulbar, and lateral conjunctivae and the total score of all areas in the eye with tarsorrhaphies were 1.86 ± 0.46 ($P < 0.005$), 0.29 ± 0.18 , 2.43 ± 0.20 ($P < 0.005$), and 4.57 ± 0.57 ($P < 0.005$), respectively. The rose bengal scores were similar to those of rabbits with GSPN sectioning, except for the lateral conjunctiva. As shown in Figure 2D, six of the seven control eyes were graded 0 (no staining) in all areas, and one was graded 1+ for the cornea. The mean score for control corneas was 0.14 ± 0.14 . As with fluorescein results, rose bengal staining was not observed in the sham-operated controls or the contralateral control eyes.

Tear Flow

Sectioning of the GSPN had a dramatic effect on tear flow (Fig. 3A). In fact, it was found that within 24 hours of the procedure, tear flow as measured by the Schirmer tear test declined by approximately 60%. Tear flow continued to decline, albeit more slowly, over the 7-day observation period. There were significant differences in Schirmer values between control and denervated eyes at all observed times ($P < 0.005$). However, there were no differences between

control eyes and the eyes of rabbits undergoing the sham procedure. On day 7, tear flow in eyes undergoing GSPN section was only 26% of the value of contralateral control eyes or approximately 30% of the presurgical levels (Fig. 3A). The results suggested that the neurons of the PPG had little ability to continue to activate LG acinar cells without continued preganglionic input. One week after surgery, decreased tear flow (5.88 ± 0.81 mm) was also found in eyes with tarsorrhaphies compared with contralateral control eyes (18.5 ± 0.35 mm; $P < 0.005$). Sham-operated animals showed no changes in tear flow (Fig. 3B). Corneal sensitivity was tested bilaterally in all animals and was found to be unaffected by the surgical procedure. Additionally, the presence of the blink reflex was noted in all the animals.

Flow rate of the main LG fluid from the cannulated conjunctival duct of the GSPN-section eye (5.02 ± 1.96 nL/s) was significantly decreased compared with contralateral control eyes (51.51 ± 4.77 nL/s). The reduced tear flow rate from the main LG fluid was also evident compared with the tear flow rate before denervation surgery in ipsilateral and contralateral eyes (51.43 ± 1.56 nL/s, 55.94 ± 6.54 nL/s), respectively ($P < 0.005$).

Breakup Time

Tear BUT in the denervated eyes on postoperative day 7 (8.53 ± 3.09 seconds) was significantly shorter than in contralateral control eyes (50.00 ± 5.52 seconds; $P < 0.005$). Similarly, BUT in eyes with tarsorrhaphies on postoperative day 7 (14.20 ± 3.89 seconds) was significantly shorter than in contralateral control eyes (61.90 ± 1.39 seconds; $P < 0.005$). There were no significant differences in the eyes of sham-operated animals (data not shown).

Blink Rate

The blink rate in the denervated rabbit eye (9.25 ± 2.10 blinks/3 min) was significantly higher than in control eyes (1.00 ± 0.41 blinks/3 min; $P < 0.05$). These measurements were made on postoperative day 7. There were no significant differences in eyes of sham-operated animals (data not shown).

Goblet Cell Density

The density of mucin-containing goblet cells in bulbar and tarsal conjunctivae was lower in denervated eyes than in contralateral control eyes 1 week after surgery ($P < 0.005$; Fig. 4). Moreover, the loss of goblet cells in bulbar conjunctivae appeared greater than in tarsal conjunctivae. After combined GSPN sectioning and tarsorrhaphy, the density of mucin-containing goblet cells in bulbar and tarsal conjunctivae was also significantly decreased compared with contralateral control eyes ($P < 0.005$). In sham-operated eyes, no significant changes occurred in conjunctival goblet cell density.

Histologic Evaluation

GSPN and PPG—Light microscopic studies of the normal structure of the GSPN were conducted using 3- to 5-mm segments of the nerve that were removed at the time of surgery. The nerve that sits in the bony groove had a thick connective tissue sheath separating it from the bone. Myelinated axons of variable size were found in abundance, along with many unmyelinated axons (Fig. 5A). Four days after parasympathetic denervation, examination of the remaining GSPN 3 mm from the nerve section showed that few axonal-like structures remained (Fig. 5B). After GSPN sectioning, the PPG revealed that the neurons were still abundant but that there was a loss of the myelinated axons adjacent to them (Figs. 5C, 5D). These findings were observed in every sample taken from denervated animals.

Lacrimal Gland—Seven days after GSPN section, the LG appeared smaller, and wet weight (in grams) of the whole LG was significantly decreased (0.45 ± 0.02 g) compared with the

contralateral side (0.51 ± 0.02 ; $P < 0.05$; $n = 8$). The decreased wet weight in the denervated LG suggested possible loss of acini content, possibly from dysfunction and atrophy.

The structure of the contralateral control LG appeared normal, with a heterogeneous mixture of secretory granules in the cytoplasm of acinar epithelial cells (Fig. 6A). In the denervated LG, the acinar epithelial cells contained many more secretory granules, and the nuclei were displaced closer toward the basal membrane of the acinar cells (Fig. 6B).

TEM of the control LG showed normal appearance of the membrane-limited electron-dense secretory granules (Fig. 6C). Conversely, the numerous secretory granules that were observed by light microscopy in the denervated LG were not membrane limited and appeared incomplete and irregular. A few non-electron-dense secretory granules were also observed (Fig. 6D).

Conjunctiva—Impression cytology of the conjunctiva revealed different patterns of goblet cell density and distribution. In the control eye, goblet cells were found frequently in the conjunctiva of the globe and the tarsal conjunctiva. In contrast, goblet cell density was reduced in the denervated eyes (Figs. 7A, 7B). Moreover, goblet cells in the denervated eye often showed a slender stalk-like appearance, in contrast to those from the control eye, which typically had a rounded appearance.

Discussion

In this study, we demonstrated by functional and structural evaluation of the ocular surface that sectioning of the GSPN, the preganglionic parasympathetic input to the PPG, resulted in a rapidly developing clinical description of dry eye. The specific effect of denervation was also confirmed by total tarsorrhaphy, which was performed to prevent dryness of the ocular surface but did not reduce or prevent these ocular surface changes. On the contrary, staining in the lateral conjunctiva with the use of rose bengal was greater when both tarsorrhaphy and GSPN sectioning were performed than when GSPN sectioning alone was performed. One of the reasons we speculate is that hypoxia caused by tarsorrhaphy may disturb homeostasis and delay wound healing. Furthermore, staining for the same area was usually greater with rose bengal than with fluorescein. Based on these results, we hypothesize that disruption of parasympathetic innervation decreases goblet cell density and prevents the secretion of mucin by goblet cells in the presence or absence of dryness of the eyes.

The postganglionic parasympathetic fibers remained intact and were not subject to interference during the course of the procedure. These results also suggest that the regulation of tear flow and goblet cell activity were under constant neural control because there was little reserve and few alternative means, such as a paracrine mechanism or a general systemic activation, of activation of these important physiological responses to replace the loss of the parasympathetic drive.

We did not begin evaluation studies of the ocular surface until we were able to routinely obtain satisfactory nerve degeneration, as confirmed by light and electron microscopy. Tear volume in the denervated eye, as measured by Schirmer tear test, declined to approximately 24% of intact control eyes at 1 week, and tear flow measured at the main LG from the conjunctival duct also declined to approximately 10% of intact control eyes at 1 week. The first postoperative day revealed a dramatic decline in tear flow, an increase in superficial punctate keratitis, and rose bengal staining of the conjunctiva. Clinical symptoms associated with dry eye in humans, such as fluorescein staining, rose bengal staining, shortened BUT, and increased blink rate, continued at postoperative day 7 in denervated eyes of all operated animals. Acute, severe changes of the ocular surface were unexpected. These changes may be a direct consequence of complete removal of the parasympathetic input to the LG, as previously suggested.³³⁻³⁹

Furthermore, these ocular surface abnormalities were not diminished or eliminated by total tarsorrhaphy. Previous studies had not used this control to validate their results. In fact, previous studies had not used a battery of clinical tests to substantiate a dry eye-like state and, therefore, did not comment extensively on this aspect.

Light microscopy and TEM showed an accumulation of secretory granules in the denervated LG, as had been reported for sensory denervation.⁴⁰ However, the study did not detail changes to the structure of secretory granules. The lack of a complete membrane around secretory granules might have resulted from alterations in their formation at the *trans*-Golgi network. This change might also have been a consequence of alterations to vesicular trafficking from the loss of parasympathetic input to secretion, and the coalescing (and fusion) of secretory vesicles might have led to an increase of vacuole formation.

PAS staining of impression cytology membranes revealed a decrease in goblet cell density in the denervated eye. This change remained even after tarsorrhaphy. Goblet cell activity might have decreased in the denervated eye. This decrease in goblet cell activity was an important aspect of the ocular surface effects and suggested that parasympathetic nerves may be necessary for the discharge of the mucin contents to the ocular surface, as previously suggested by Dartt et al.⁴¹⁻⁴³ As such, the usual standard clinical approach to assess this parameter typically does not show a significant decrease in goblet cell density. Gilbard et al.^{34,44} suggest in their dry eye model that goblet cell loss is associated with decreased tear secretion. However, weeks would be required in this model to detect goblet cell loss. Therefore, we suggest that parasympathetic innervation is necessary to maintain acinar cell and goblet cell function.

We found an increased blink rate in experimental eyes after sectioning of the GSPN. In normal rabbit, the blink rate is generally reported as three times per hour.^{45,46} In our model, the blink rate of the experimental eye was much higher—9.25 blinks every 3 minutes. This result supports the clinical finding that patients with dry eye have a blink rate more rapid than normal.⁴⁷ The increased blink rate, as observed in our rabbit model, arises from an unpleasant sensation caused by the dryness of the ocular surface that triggers increased corneal nerve activity, as documented in dry eye patients. However, these sensations arise through minute breaks in the epithelial barrier function because the corneal surface is not intact, as revealed by fluorescein staining.

Another possibility is that patients with dry eye may have declining nerve function.⁴⁸ The origin of neural activation for the parasympathetic activation of the LG and goblet cells is suggested to be the constant low-level neural outflow of the corneal free-nerve endings.^{3,4,13} However, many aspects of neural function decline with age, and the neural activity necessary to maintain activity in the LG or goblet cells may be lost. Sensitivity of the corneal nerve ending decreases significantly with age, as does the function of the parasympathetic cholinergic system.^{3,13,49-51} In addition, in patients with decreased autonomic nerve function (Lambert-Eaton syndrome), a loss of parasympathetic innervation to the LG is associated with decreased reflex tearing.⁵²

Our data suggest that a steady flow of secreted tears, goblet cell function, and mucin availability depend on an intact nerve supply and a continually functioning parasympathetic neural outflow. This corroborates recent findings.^{3,4} Nevertheless, other aspects of the neural control of tears, such as that provided by the accessory lacrimal glands, are unclear, though reports of associated nerves are available.⁵³ The ocular surface effects seen in our study were more severe than the effects of the total removal of the main LG in monkeys.^{36,54} For example, little rose bengal staining occurred in the conjunctivae of monkeys; thus, goblet cell function did not seem to be compromised.

In summary, our model, involving sectioning of the GSPN leads to the development of an animal model that exhibits most of the clinically measured symptoms of human dry eye disease. This model, which is focused on ocular surface effects and can survive after surgery, may be useful for the development of new drugs for the treatment of dry eye.

Acknowledgments

Supported in part by a Grant-Aid for Young Scientists (B) 16791070 (HT) from the Ministry of Education, Science, and Culture of Japan, United States Public Health Service Grants R01EY12416 (RWB) and P30EY002377 (LSU Eye Center core grant) from the National Eye Institute, and a Challenge Grant from Research to Prevent Blindness, Inc., New York (LSU Eye Center). This publication was made possible by National Institutes of Health Grant P20 RR16456 from the INBRE Program of the National Center for Research Resources. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of National Institutes of Health.

References

1. Nelson JD. Diagnosis and treatment of the dry eye: a clinical perspective. *Adv Exp Med Biol* 2002;506 (pt B):1067–1078. [PubMed: 12614033]
2. Toda I, Tsubota K. Practical double vital staining for ocular surface evaluation. *Cornea* 1993;12(4):366–367. [PubMed: 7687944]
3. Beuerman, RW.; Mircheff, A.; Pflugfelder, SC.; Stern, ME. The lacrimal functional unit. In: Pflugfelder, SC.; Beuerman, RW.; Stern, ME., editors. *Dry Eye and the Ocular Surface Disorders*. Marcel Dekker; New York: 2004. p. 11–39.
4. Stern ME, Gao J, Siemasko KF, Beuerman RW, Pflugfelder SC. The role of the lacrimal functional unit in the pathophysiology of dry eye. *Exp Eye Res* 2004;78(3):409–416. [PubMed: 15106920]
5. Dartt DA. Regulation of tear secretion. *Adv Exp Med Biol* 1994;350:1–9. [PubMed: 8030459]
6. Dartt DA. Regulation of mucin and fluid secretion by conjunctival epithelial cells. *Prog Retin Eye Res* 2002;21(6):555–576. [PubMed: 12433377]
7. Duke-Elder, SS.; Wybar, KC. The peripheral parasympathetic system. In: Duke-Elder, SS., editor. *System of Ophthalmology: The Anatomy of the Visual System*. Henry Kimpton; London: 1961. p. 857–869.
8. Ruskell GL. The orbital branches of the pterygopalatine ganglion and their relationship with internal carotid nerve branches in primates. *J Anat* 1970;106(pt 2):323–339. [PubMed: 4986076]
9. van der Werf F, Baljet B, Prins M, Otto JA. Innervation of the lacrimal gland in the cynomolgous monkey: a retrograde tracing study. *J Anat* 1996;188(pt 3):591–601. [PubMed: 8763476]
10. Toth IE, Boldogkoi Z, Medveczky I, Palkovits M. Lacrimal preganglionic neurons form a subdivision of the superior salivatory nucleus of rat: transneuronal labelling by pseudorabies virus. *J Auton Nerv Syst* 1999;77(1):45–54. [PubMed: 10494749]
11. ten Tusscher MP, Klooster J, Baljet B, van der Werf F, Vrensen GF. Pre- and post-ganglionic nerve fibres of the pterygopalatine ganglion and their allocation to the eyeball of rats. *Brain Res* 1990;517 (12):315–323. [PubMed: 1695865]
12. Goadsby PJ. Sphenopalatine ganglion stimulation increases regional cerebral blood flow independent of glucose utilization in the cat. *Brain Res* 1990;506(1):145–148. [PubMed: 2302552]
13. Yasui T, Karita K, Izumi H, Tamai M. Correlation between vasodilatation and secretion in the lacrimal gland elicited by stimulation of the cornea and facial nerve root of the cat. *Invest Ophthalmol Vis Sci* 1997;38(12):2476–2482. [PubMed: 9375565]
14. Hoshino T, Yoshizaki K, Koyano H, Ishikawa K, Terada N, Konno A. An electrophysiological study of pterygopalatine ganglion neurons in the rabbit. *Acta Otolaryngol Suppl* 1998;537:20–26. [PubMed: 9870644]
15. Ruskin SL. Control of tearing by blocking the nasal ganglion. *Arch Ophthalmol* 1930;4:208–211.
16. Rowbotham GF. Observations on the effects of trigeminal denervation. *Brain* 1939;62:364–380.
17. de Haas EBH. Lacrimal gland response to parasympathomimetics after parasympathetic denervation. *Arch Ophthalmol* 1960;64:64–73.

18. Whitwell J. Denervation of the lacrimal gland. *Br J Ophthalmol* 1958;42(9):518–525. [PubMed: 13572766]
19. Ruskell GL. Changes in nerve terminals and acini of the lacrimal gland and changes in secretion induced by autonomic denervation. *Z Zellforsch Mikrosk Anat* 1969;94(2):261–281. [PubMed: 4984648]
20. Toda N, Ayajiki K, Tanaka T, Okamura T. Preganglionic and post-ganglionic neurons responsible for cerebral vasodilation mediated by nitric oxide in anesthetized dogs. *J Cereb Blood Flow Metab* 2000;20(4):700–708. [PubMed: 10779014]
21. Butler JM, Ruskell GL, Cole DF, et al. Effects of VIIth (facial) nerve degeneration on vasoactive intestinal polypeptide and substance P levels in ocular and orbital tissues of the rabbit. *Exp Eye Res* 1984;39(4):523–532. [PubMed: 6209157]
22. Rossi J, Luukko K, Poteryaev D, et al. Retarded growth and deficits in the enteric and parasympathetic nervous system in mice lacking GFR alpha2, a functional neurturin receptor. *Neuron* 1999;22(2):243–252. [PubMed: 10069331]
23. Song XJ, Li DQ, Farley W, et al. Neurturin-deficient mice develop dry eye and keratoconjunctivitis sicca. *Invest Ophthalmol Vis Sci* 2003;44(10):4223–4229. [PubMed: 14507865]
24. Linberg, JV. Surgical anatomy of the lacrimal system. In: Weinstein, GW., editor. *Contemporary Issues in Ophthalmology: Lacrimal Surgery*. Churchill Livingstone; New York: 1988. p. 1-188.
25. Craig JP, Blades K, Patel S. Tear lipid layer structure and stability following expression of the meibomian glands. *Ophthalmic Physiol Opt* 1995;15(6):569–574. [PubMed: 8594528]
26. van Bijsterveld OP. Diagnostic tests in the sicca syndrome. *Arch Ophthalmol* 1969;82(1):10–14. [PubMed: 4183019]
27. Toshida H, Nakata K, Hamano T, Nakamura M, Nguyen D, Beuerman RW. Effect of gefarnate on the ocular surface in squirrel monkeys. *Cornea* 2002;21(3):292–299. [PubMed: 11917179]
28. Goldstein AM, De Palau A, Botelho SY. Inhibition and facilitation of pilocarpine-induced lacrimal flow by norepinephrine. *Invest Ophthalmol* 1967;6(5):498–511. [PubMed: 6061673]
29. Thorig L, van Agtmaal EJ, Glasius E, Tan KL, van Haeringen NJ. Comparison of tears and lacrimal gland fluid in the rabbit and guinea pig. *Curr Eye Res* 1985;4(8):913–920. [PubMed: 3862512]
30. Thatcher RW, Darougar S, Jones BR. Conjunctival impression cytology. *Arch Ophthalmol* 1977;95(4):678–681. [PubMed: 192184]
31. Egbert PR, Lauber S, Maurice DM. A simple conjunctival biopsy. *Am J Ophthalmol* 1977;84(6):798–801. [PubMed: 596392]
32. Beuerman RW, Pedroza L. Ultrastructure of the human cornea. *Microsc Res Tech* 1996;33(4):320–335. [PubMed: 8652889]
33. Kaswan RL, Martin CL, Chapman WL Jr. Keratoconjunctivitis sicca: histopathologic study of nictitating membrane and lacrimal glands from 28 dogs. *Am J Vet Res* 1984;45(1):112–118. [PubMed: 6703444]
34. Gilbard JP, Rossi SR, Gray KL. A new rabbit model for keratoconjunctivitis sicca. *Invest Ophthalmol Vis Sci* 1987;28(2):225–228. [PubMed: 8591900]
35. Jabs DA, Prendergast RA. Murine models of Sjogren's syndrome. *Adv Exp Med Biol* 1994;350:623–630. [PubMed: 8030545]
36. Maitchouk DY, Beuerman RW, Varnell RJ, Pedroza-Schmidt L. Effects of lacrimal gland removal on squirrel monkey cornea. *Adv Exp Med Biol* 1998;438:619–624. [PubMed: 9634945]
37. Buralassi S, Panichi L, Chetoni P, Saettone MF, Boldrini E. Development of a simple dry eye model in the albino rabbit and evaluation of some tear substitutes. *Ophthalmic Res* 1999;31(3):229–235. [PubMed: 10224507]
38. Fujihara T, Nagano T, Nakamura M, Shirasawa E. Establishment of a rabbit short-term dry eye model. *J Ocul Pharmacol Ther* 1995;11(4):503–508. [PubMed: 8574813]
39. Ruskell GL. The distribution of autonomic post-ganglionic nerve fibres to the lacrimal gland in monkeys. *J Anat* 1971;109(pt 2):229–242. [PubMed: 4997622]
40. Meneray MA, Bennett DJ, Nguyen DH, Beuerman RW. Effect of sensory denervation on the structure and physiologic responsiveness of rabbit lacrimal gland. *Cornea* 1998;17(1):99–107. [PubMed: 9436887]

41. Dartt DA, McCarthy DM, Mercer HJ, Kessler TL, Chung EH, Zieske JD. Localization of nerves adjacent to goblet cells in rat conjunctiva. *Curr Eye Res* 1995;14(11):993–1000. [PubMed: 8585938]
42. Kessler TL, Dartt DA. Neural stimulation of conjunctival goblet cell mucous secretion in rats. *Adv Exp Med Biol* 1994;350:393–398. [PubMed: 7913285]
43. Rios JD, Zoukhri D, Rawe IM, Hodges RR, Zieske JD, Dartt DA. Immunolocalization of muscarinic and VIP receptor subtypes and their role in stimulating goblet cell secretion. *Invest Ophthalmol Vis Sci* 1999;40(6):1102–1111. [PubMed: 10235543]
44. Gilbard JP, Rossi SR, Gray KL, Hanninen LA. Natural history of disease in a rabbit model for keratoconjunctivitis sicca. *Acta Ophthalmol Suppl* 1989;192:95–101. [PubMed: 2554662]
45. Beuerman RW, Schimmelpfennig B. Sensory denervation of the rabbit cornea affects epithelial properties. *Exp Neurol* 1980;69(1):196–201. [PubMed: 7389846]
46. Maurice D. The effect of the low blink rate in rabbits on topical drug penetration. *J Ocul Pharmacol Ther* 1995;11(3):297–304. [PubMed: 8590262]
47. Nakamori K, Odawara M, Nakajima T, Mizutani T, Tsubota K. Blinking is controlled primarily by ocular surface conditions. *Am J Ophthalmol* 1997;124(1):24–30. [PubMed: 9222228]
48. Baum J. Clinical manifestations of dry eye states. *Trans Ophthalmol Soc UK* 1985;104(pt 4):415–423. [PubMed: 3898477]
49. Beuerman, RW.; Thompson, HW. Evaluation of corneal sensitivity. In: Monterc, BR., editor. *Noninvasive Diagnostic Techniques in Ophthalmology*. Springer-Verlag; New York: 1990. p. 211–222.
50. Gill TM, Gallagher M. Evaluation of muscarinic M2 receptor sites in basal forebrain and brainstem cholinergic systems of behaviorally characterized young and aged Long-Evans rats. *Neurobiol Aging* 1998;19(3):217–225. [PubMed: 9661996]
51. Williams RM, Singh J, Sharkey KA. Innervation and mast cells of the rat exorbital lacrimal gland: the effects of age. *J Auton Nerv Syst* 1994;47(12):95–108. [PubMed: 8188988]
52. Clark CV, Newsom-Davis J, Sanders MD. Ocular autonomic nerve function in Lambert-Eaton myasthenic syndrome. *Eye* 1990;4(pt 3):473–481. [PubMed: 2209912]
53. Seifert P, Spitznas M, Koch F, Cusumano A. The architecture of human accessory lacrimal glands. *Ger J Ophthalmol* 1993;2(6):444–454. [PubMed: 8312832]
54. Maitchouk DY, Beuerman RW, Ohta T, Stern M, Varnell RJ. Tear production after unilateral removal of the main lacrimal gland in squirrel monkeys. *Arch Ophthalmol* 2000;118(2):246–252. [PubMed: 10676790]

FIGURE 1.

(A-D) Slit lamp photographs of fluorescein staining in contralateral control eye (A), experimental eye 7 days after acute section of the greater superficial petrosal nerve (B), and sectioning of the GSPN through tarsorrhaphy (C). Although the control eye shows no staining, experimental and tarsorrhaphy eyes reveal staining of the medial and lateral conjunctiva and the cornea. Histogram of fluorescein score for the experimental animals and sham animals ($n = 4$). Bulbar conjunctival regions, medial (■) and lateral (▨), and the cornea (□) are scored separately. A total score (▩) is also presented. Scores for the sham eyes and the contralateral control eyes are not visible because they fall along the baseline. Mean $\pm$ SEM is shown in experimental animals ($n = 7$) and in sham animals ($n = 4$); * $P < 0.05$ and *** $P < 0.005$, respectively.

FIGURE 2.

(A-D) Rose bengal evaluation of the ocular surface for the control side (A), experimental side (B), and with tarsorrhaphy (C) 7 days after GSPN sectioning. The contralateral control eye always revealed little or no rose bengal staining. The experimental side revealed intense rose bengal staining. Rose bengal staining was still evident in the experimental side even after tarsorrhaphy. Histogram of rose bengal score of medial (■) and lateral (▨) conjunctivae regions and of cornea (□) 7 days after GSPN sectioning and after the sham procedure. Total staining score (■) is also presented. Denervated eyes, with or without tarsorrhaphy, showed significantly greater staining than the control or sham-denervated eyes. Mean $\pm$ SEM are shown in experimental animals ($n = 7$) and in sham animals ($n = 4$); $*P < 0.05$ and $***P < 0.005$, respectively.

FIGURE 3.

(**A, B**) Tear flow analysis using the Schirmer tear test for the control and experimental eyes before surgery, at 1, 3, 5, and 7 days after surgery (**A**), and after sham denervation (**B**). Tear flow was significantly reduced at all time points, beginning 1 day after surgery on the experimental side (□) compared with the contralateral control eyes (■). Tear flow represents mean Schirmer tear value at each time point. In the sham-operated animals, tear flow was not different from that of the control eyes at all time points ($n = 4$). Mean $\pm$ SEM are shown in experimental animals ($n = 7$) and in sham animals ($n = 4$); *** $P < 0.005$.

FIGURE 4.

(**A, B**) Histogram of goblet cell density in the bulbar (**A**) and the margin of the tarsal conjunctivae (**B**), before (Pre) and 7 days after (Post) surgery. Goblet cell density declined at both sites, as determined by impression cytology. (■) Contralateral control eyes. (□) Experimental eyes (E). Mean $\pm$ SEM are shown in experimental animals ($n = 7$) and in sham animals ($n = 4$); *** $P < 0.005$.

FIGURE 5.

(A-D) Light micrographs depicting the structure of the GSPN and PPG before (A, C) and after (B, D) acute severing of the GSPN. The GSPN reveals numerous myelinated axons (*asterisks*) and unmyelinated axons (A). Four days after the nerve was severed (B), only traces of the myelinated axons (*asterisks*) remained. Sections were taken 3 mm distal from the site where the suture was tied. In normal PPG, many myelinated (*asterisks*) and unmyelinated axons are evident (C). The typical ganglionic structure of the neurons with prominent nucleoli was easily seen. Seven days (D) after sectioning of the GSPN, the number of myelinated axons in the PPG was reduced. Scale bars, 50 μ m.

FIGURE 6.

(**A-D**) Light and transmission electron micrographs of the acinar cell structure of the main LG in the contralateral control side (**A, C**) and the GSPN severed side (**B, D**). The experimental side experienced a massive accumulation of secretory granules, in contrast to the control side. The nuclei of the acinar cells were displaced more closely toward the basal membrane in the experimental side. At the ultrastructural level, secretory granules were electron dense, the membrane surface appeared rough and irregular, had an undefined membrane, and appeared coalesced. Scale bars, 50 μm (**A, B**) and 0.1 μm (**C, D**).

FIGURE 7.

(**A, B**) Light microscopic images showing the structure of the lower tarsal conjunctiva in the contralateral side (**A**) and 7 days after GSPN (**B**). The number of goblet cells decreased after GSPN section, and the surface of the conjunctivae appeared flat and lacked surface epithelial folding. Scale bars, 50 μ m.