

Effect of Cyclosporine on Conjunctival Mucin in a Canine Keratoconjunctivitis Sicca Model

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PURPOSE. To test the hypothesis that beneficial effects of Cyclosporin A (CsA; Sandimmune; Sandoz, Basel, Switzerland) in treating keratoconjunctivitis sicca (KCS) include an effect on the mucin-producing conjunctival goblet cells independent of CsA's effect on lacrimation.

METHODS. Keratoconjunctivitis sicca was induced bilaterally in six dogs after removal of orbital and nictitans lacrimal glands. Two weeks after induction of KCS, either 2% CsA or vehicle was applied twice daily to each surgically altered eye until 6 weeks after KCS induction. Eyes of three control dogs without surgically altered eyes were treated twice daily with vehicle only. Incisional biopsy specimens of ventral fornix conjunctiva were collected before gland removal (baseline) and at 2, 4, and 6 weeks after KCS induction. At each sampling time, eyes were photographed, and color images were subsequently graded for degree of conjunctivitis and characteristics of ocular discharge. Intracellular mucin stores in conjunctival epithelia were estimated using computer-assisted morphometry of biopsy specimen cross sections, and clinical and morphometric findings were correlated.

RESULTS. Lacrimal gland removal resulted in induction of KCS in dogs by 2 weeks, with mean Schirmer tear test (STT) values of 5 mm/min or less occurring in surgically altered eyes compared with STT values of 22.5 mm/min before surgery and 22.9 mm/min in unaltered control eyes at 2 weeks. In surgically altered eyes, STTs remained low during the 6-week study, independent of topical treatment. Intracellular mucin stores were quantified from conjunctival samples collected from each eye at baseline and 2, 4, and 6 weeks. At 4 and 6 weeks (after 2 and 4 weeks of topical treatment), intraepithelial mucin quantities were significantly greater ($P < 0.05$) in CsA-treated KCS eyes (14.4 and 13.1 $\mu\text{m}^2/\mu\text{m}$, respectively) compared with pretreatment KCS (7.4 $\mu\text{m}^2/\mu\text{m}$) eyes and vehicle-treated KCS eyes (7.3 and 8.5 $\mu\text{m}^2/\mu\text{m}$, respectively). KCS eyes treated with CsA had lower conjunctivitis and ocular discharge scores than did vehicle-treated KCS eyes.

CONCLUSIONS. Topical 2% CsA restored in vivo conjunctival mucin stores to control levels over a 4-week period, determined by computer-assisted morphometry of sequential conjunctival biopsy specimens from eyes of dogs with surgically induced KCS. Degree of conjunctivitis and severity of mucus discharge were decreased in KCS eyes treated with CsA. Because lacrimal tissues were removed from animals in this study, conjunctival responses occurred independent of lacrimogenic effect(s). These results indicate that restoration of conjunctival

goblet cell mucin production, i.e., the balance between synthesis and secretion of mucin glycoproteins, may play an important role in the beneficial effect of CsA in treating KCS. (*Invest Ophthalmol Vis Sci.* 2001;42:653-659)

Mucins are a heterogeneous group of O-linked glycoproteins, synthesized and secreted primarily by goblet cells (GCs), which coat and protect mucosal epithelia. Ocular surface mucin, consisting predominantly of conjunctival goblet cell (CGC) secretions, adheres to the glycocalyx of conjunctival and corneal epithelial cells and enhances wettability of the cornea by serving as an interface between the hydrophobic corneal epithelium and the aqueous tear fluid.¹⁻³ CGC mucin provides a transparent and optically smooth covering to the anterior corneal surface, has an affinity for IgA, and serves as a barrier to microbial invasion. It also shields conjunctival and corneal cells from surface debris and noxious substances.

Instability of the tear film with resultant drying and inflammation of the conjunctiva and cornea, ocular pain, corneal ulceration, and corneal opacification characterize mucin-deficient ocular disease.³ A marked reduction in frequency or absence of GCs is a morphologic feature of conjunctival biopsy specimens from human patients affected by ocular surface mucin deficiencies.^{4,5} In disorders of aqueous tear deficiency (dry eye syndromes), aqueous and mucin tear disturbances may occur simultaneously.^{6,7} With dehydration of the ocular surface, qualitative changes have been observed in mucous glycoproteins with a shift from mucin rich in sialic acid to highly sulfated mucin substantiating interaction between these tear components.⁸

Cyclosporine is a naturally occurring fungal metabolite widely used as an immunosuppressant in human organ recipients. Immunosuppressive mechanisms of cyclosporine relate to binding of specific nuclear proteins required for initiation of T-cell activation, thus preventing T-cell production of inflammatory cytokines such as interleukin (IL)-2 and IL-4 and thereby disrupting immune-mediated processes.⁹ More specifically, cyclosporine forms a complex with the cytosolic protein cyclophilin and inhibits the phosphatase activity of calcineurin, which regulates nuclear translocation and subsequent activation of nuclear factor of activated T cells (NFAT) transcription factors.¹⁰ Recent studies indicate that cyclosporine also blocks the activation of the signaling pathways, JNK and p38, which are triggered by antigen recognition.^{11,12} Because of its highly specific inhibition of T-cell activation, cyclosporine has been used to treat autoimmune and immune-mediated disorders, including a number of ocular diseases.¹³

In addition to its widely accepted immunomodulating effects, cyclosporine also has recognized lacrimostimulant and lacrimomimetic properties. Systemic cyclosporine has been shown to increase tear flow in human patients without lacrimal gland disease.¹⁴ In spontaneous canine keratoconjunctivitis sicca (KCS), topical Cyclosporin A (CsA; Sandimmune; Sandoz, Basel, Switzerland) significantly increases tearing, with 75% to 82% of idiopathic cases showing improvement.¹⁵⁻¹⁷ Lacrimomimetic effects of CsA on tear proteins in canine KCS were

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investigated by Fullard et al.¹⁸ who reported a trend toward return to normal tear protein profiles after topical CsA treatment.

Based on clinical observations that spontaneous canine KCS is also characterized by squamous metaplasia of conjunctival epithelium and loss of CGCs, we hypothesize that part of CsA's beneficial effect in treating KCS results from restoration of CGC function independent of lacrimal effects. To test this hypothesis, an experimental canine model of induced KCS was induced and, after manifestations of aqueous tear deficiency, affected eyes were treated topically with CsA or a placebo. Eyes were photographed for comparison of clinical parameters, sequential specimens of ventral fornix conjunctiva were obtained by incisional biopsy, and intracellular conjunctival epithelial mucin stores were estimated using computer-assisted morphometry. After topical treatment, mucin quantities were significantly greater in conjunctiva of CsA-treated KCS eyes compared with vehicle-treated KCS eyes or pretreatment KCS eyes. CsA-treated KCS eyes had less severe clinical changes—that is, changes in conjunctivitis and ocular discharge scores—than did vehicle-treated KCS eyes.

METHODS

KCS Induction

KCS was induced bilaterally in six mongrel dogs by removing the orbital lacrimal glands (OLGs) and the nictitans lacrimal glands (NLGs). Three dogs with unaltered eyes served as controls. Before dogs were admitted to the study, eyes of each prospective canine subject were screened using a hand-held slit lamp biomicroscope (SL-2; Kowa, Tokyo, Japan) and a binocular indirect ophthalmoscope (Keeler, Reading, PA). Schirmer I tear test strips (Alcon Laboratories, Fort Worth, TX) were inserted into the ventral conjunctival fornix, and results were recorded in each eye of each animal. Dogs used in these experiments were handled in adherence to institutional Animal Care and Use Committee guidelines and to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Lacrimal tissues were removed aseptically in six dogs after administration of general anesthesia. Sterile preparation of the eyes and periocular areas and previously described surgical techniques were used.¹⁹ Briefly, for removal of the OLGs, after surgical preparation the skin was incised over the dorsolateral rim of the orbit, and the orbital septum was transected, separating it from the orbital rim and orbital ligament. After exposed soft tissues were depressed, the orbital lacrimal gland was identified and dissected from its supporting tissue and removed. Wound closure was performed by apposing the orbital fascia with 5-0 absorbable sutures and the skin was sutured with 4-0 nonabsorbable sutures. The NLGs were removed by incising the conjunctiva on the posterior third eyelid and dissecting the gland from the associated cartilage and surrounding connective tissues. After removal of the NLGs, the conjunctival wound was closed with 7-0 absorbable sutures using sterile instruments. Systemic analgesia (Torbugesic; American Home Products, Animal Health Division, Fort Dodge, IA) and topical triple antibiotic ophthalmic ointment (Neosporin; Burroughs Wellcome, Research Triangle Park, NC) were administered immediately after surgery.

Topical Treatments

Because effects of gland removal occur by 2 weeks after surgery, topical treatments were initiated at that time and consisted either of 2% CsA in corn oil or corn oil vehicle only, applied topically twice daily to each affected eye for 4 weeks (6 weeks after KCS induction). Unaltered eyes of three control dogs were similarly treated with vehicle. Schirmer tear test (STT) values were recorded twice weekly after surgery until the termination of the study. Animals were euthanized at 6 weeks after surgery by intravenous barbiturate.

Clinical Assessment

All eyes were photographed before any treatments (baseline) and at 2, 4, and 6 weeks after KCS induction and/or topical treatments. Before conjunctival sampling (see Tissue Sampling and Processing section) external ocular photographs were taken of each eye of each experimental subject with a hand-held fundus camera (RC-2; Kowa). Photographs were logged and subsequently projected and evaluated independently by each of two blinded observers and scored for conjunctival inflammation and characteristic of the mucus discharge. Scores for conjunctival inflammation ranged from 0 to 3, with 0 indicating normal conjunctiva; 1, mild hyperemia without chemosis; 2, moderate hyperemia with mild chemosis; and 3, intense hyperemia with moderate to severe chemosis. Scores for mucus discharge also ranged from 0 to 3, with 0 indicating no visible mucus or clear mucus thread; 1, scattered nonadherent mucopurulent strands; 2, moderate adherent mucopurulent strands covering up to 25% of the cornea; and 3, diffuse extensive adherent mucopurulent discharge covering 25% to 50% of the cornea. Numerical scores from the two blinded observers for conjunctivitis and mucus discharge were averaged and then statistically evaluated both within and among groups.

Tissue Sampling and Processing

Specimens were obtained by incisional conjunctival biopsy before gland removal (baseline) and at 2, 4, and 6 weeks after KCS induction. After anesthetic was administered, conjunctiva from the ventral fornix anterior to the third eyelid was gently grasped and tented using 0.3-mm mouse-tooth thumb forceps. This anatomic location has previously been determined to be the most reliable sampling site for quantifying canine CGCs.²⁰ A 3-mm³ specimen was excised with curved conjunctival scissors and fixed for 2 hours at room temperature in 2% freshly depolymerized paraformaldehyde in 70 mM NaCl, 30 mM HEPES, and 2 mM CaCl₂ (pH 7.4). After three rinses in HWB (70 mM NaCl, 30 mM HEPES [pH 7.4]) and dehydration with an ethanol series, biopsy specimens were infiltrated and embedded in methacrylate resin (JB-4; Polysciences, Warrington, PA) in a manner that allowed the epithelial layer to be cross-sectioned.

Morphometric Analysis

Semithin cross-sections (0.9 μ m) were stained with 50 μ g/ml of fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA), a lectin with high affinity for glycoproteins with terminal *N*-acetylglucosamine or sialic acid residues, and 50 μ l/ml propidium iodide (Sigma, St. Louis, MO) in HWB containing 0.1% gelatin (Sigma). FITC-conjugated WGA stained intracellular mucin stores of all CGCs intensely. Propidium iodide, which is commonly used as a DNA or DNA-RNA stain, was intentionally used at a concentration that caused high background staining of the epithelial layer so that this layer could be easily distinguished and measured with the image analysis software. A series of photographs of the entire length of the epithelial surface of all specimens was prepared using a CCD camera (model 200; VideoScope, Washington, DC) and microchannel plate image intensifier (model KS-1381; VideoScope) mounted on a microscope (Diaphot; Nikon, Melville, NY) equipped with a \times 20 planar apochromatic objective (numeric aperture, 0.75).

The propidium iodide images were then opened in image management software (PhotoShop; Adobe, San Jose, CA) and the lasso function used to outline and delete all nonepithelial areas from the image. The resultant mask of nonepithelial tissue was then overlaid on the corresponding FITC-WGA image to delete the same regions from these images. The pair of modified images were then exported to an image analysis system (Image 1; Universal Imaging, West Chester, PA), and the thresholding and area functions were used to quantify total area of intracellular mucin stores in the FITC-WGA image and total epithelial area in the propidium iodide image. The arc function was used to determine the length of the apical and basal margins of the epithelial area. After quantification of mucin area/apical (epithelial surface)

length (Ma/Al) and total epithelial area/apical length (Ea/Al), data (in square micrometers per micrometer) were statistically analyzed.

Statistical Methods

Two-way analysis of variance (ANOVA) for repeated measures of time was performed on STT data and morphometric parameters. Because of concerns about statistical independence, eye data were combined for each dog, and therefore the experimental animal became the unit analyzed. STT data were normally distributed ($P < 0.025$) when a square root transformation was performed. Tukey's multiple comparison test was performed when the ANOVA indicated a significant difference occurring among means. Because multiple comparisons increase type I error, means for the three data sets (STTs and morphometric data) were considered to be statistically significant if $P < 0.017$. Correlations between clinical parameter means (conjunctivitis and mucus scores) and between these mean scores and volumetric data were conducted using Spearman rank correlation coefficients.

RESULTS

KCS Induction

The mean presurgical STT value for nine canine subjects (18 eyes) was 22.5 mm/min, consistent with normal aqueous tear levels in dogs.^{20,21} Before surgery there were no significant differences among groups in STT values ($P > 0.83$). The STT values in intact eyes remained constant over all time periods, with mean values at 2, 4, and 6 weeks of 22.9, 23.8, and 24.8 mm/min, respectively ($P > 0.45$). Removal of OLGs and NLGs induced KCS in surgically altered eyes by 2 weeks, producing characteristic clinical features of conjunctival hyperemia and accumulation of tenacious discharge (Fig. 1). During the 2-week induction period, mean STT values were reduced to 5 mm/min or less in surgically altered eyes and remained significantly lower ($P < 0.01$) at 2, 4, and 6 weeks after surgery compared with baseline values (Fig. 2). At 2, 4, and 6 weeks after surgery, STT values in surgically altered eyes were significantly lower ($P < 0.001$) than in unaltered eyes.

Clinical Parameters

Respective conjunctivitis and mucus discharge scores were combined for right and left eyes of each animal. Mean conjunctivitis and mucus discharge scores for each of the three groups at each sampling time are shown in Figures 3 and 4, respectively. In each KCS group, conjunctival inflammation scores at 2 weeks ranged from 1 to 3, with a mean score of 1.71 ± 0.14 (SEM) for the six dogs with surgically altered eyes. At 6 weeks (after 4 weeks of topical treatment), conjunctival inflammation scores were increased in the vehicle-treated KCS group to a mean of 2.25, whereas conjunctival scores for CsA-treated eyes decreased to a mean value of 1.08 at 6 weeks. Conjunctival inflammation scores of CsA-treated KCS eyes were less than in vehicle-treated KCS eyes at 6 weeks (Fig. 3). Compared with control dogs at 2 weeks (before topical treatments), mucus discharge scores increased in the two KCS groups, with scores ranging from 1 to 3, with a mean score of 1.67 ± 0.20 (SEM). In the vehicle-treated KCS group, mucus discharge increased in severity to a mean score of 2.0 at 4 weeks and 2.167 at 6 weeks. By contrast, mucus scores for CsA-treated KCS eyes decreased in severity to a mean value of 0.92 at 4 weeks (Fig. 1C) and returned to baseline (score of 0) at 6 weeks (Fig. 4).

A significant positive correlation ($\rho = 0.877$) was found between conjunctivitis scores and mucus discharge scores. The morphometric measurement Ma/Al (see following section) was significantly negatively correlated ($\rho = -0.448$) with mucus discharge scores.

Morphometry

Conjunctival epithelium with intracellular mucin stores was stained with FITC-conjugated WGA, allowing identification of epithelial mucin (green) within CGCs (Fig. 5A). Epithelial areas were outlined and processed with image management software (Photoshop; Adobe) to remove subepithelial connective tissue. Image analysis software (Image 1; Universal Imaging) was used to threshold and quantify the remaining WGA-stained mucin areas (Fig. 5B). Computer-assisted morphometric analysis was used to quantify intracellular mucin stores (Ma/Al = square micrometers of intracellular mucin stores per micrometer of apical length) for each treatment group at baseline and 2, 4, and 6 weeks. Among the three groups (two surgical and one nonsurgical) there were no significant differences between Ma/Al values before surgery ($P > 0.26$). At 2 weeks after surgery (before topical treatments), Ma/Al values ($7.4 \mu\text{m}^2/\mu\text{m}$) in surgically altered eyes were significantly less ($P < 0.017$) than at surgery ($13.1 \mu\text{m}^2/\mu\text{m}$). At 4 weeks after surgery (2 weeks after CsA treatment), CsA-treated KCS eyes had significantly ($P < 0.017$) greater Ma/Al values ($14.4 \mu\text{m}^2/\mu\text{m}$) than vehicle-treated KCS eyes ($7.3 \mu\text{m}^2/\mu\text{m}$). In CsA-treated eyes, Ma/Al values also increased at 6 weeks after surgery (i.e., after 4 weeks of topical treatment) to $13.1 \mu\text{m}^2/\mu\text{m}$, which was significantly greater ($P < 0.017$) than the 2-week (before CsA treatment) values ($7.4 \mu\text{m}^2/\mu\text{m}$) but the same as values before surgery ($13.1 \mu\text{m}^2/\mu\text{m}$), indicating a return to normal equilibrium between mucus synthesis and secretion (Fig. 6).

For surgically altered eyes treated with vehicle only, data at 4 and 6 weeks were not significantly different from 2 weeks after surgery (before vehicle treatment; $P > 0.037$), indicating no restoration of the normal balance in mucus production in the vehicle-treated group. In nonsurgical dogs, no significant differences ($P > 0.86$) for Ma/Al values at sampling intervals were present. At 4 and 6 weeks, intraepithelial mucin quantities were significantly greater in CsA-treated KCS eyes than in either pretreatment KCS eyes or vehicle-treated KCS eyes. Morphometry data for mucin area per apical length of epithelium for each group at baseline and each of the three subsequent treatment times are presented in Figure 6.

Total epithelial area per apical length of epithelium (Ea/Al) was also morphometrically determined by analyzing the area between the epithelial basement membrane and the epithelial surface. Mucosal sections were intentionally overstained with propidium iodide (red) to nonspecifically highlight the entire epithelial area (Fig. 5C). Epithelial areas were outlined and processed with image management software (PhotoShop; Adobe) to remove that portion of the image representing the subepithelial connective tissue. Image analysis software (Image 1; Universal Imaging) was used to threshold and quantify the remaining propidium iodide-stained epithelial areas (Fig. 5D) and to measure apical and basal length of the epithelium.

Nonsurgical eyes had Ea/Al values that did not change significantly over the three time periods. After surgery, largest mean Ea/Al values were noted (Fig. 7) in surgically altered eyes treated with CsA, with $72.0 \mu\text{m}^2/\mu\text{m}$ and $63.3 \mu\text{m}^2/\mu\text{m}$ at 2 weeks and 4 weeks after CsA treatment, respectively, compared with $50.9 \mu\text{m}^2/\mu\text{m}$ at baseline. Ea/Al values were significantly greater at 2 weeks and 4 weeks in both CsA-treated and vehicle-treated surgically altered eyes ($P < 0.017$) compared with presurgical values, indicating that surgical induction of KCS resulted in increased total cross-sectional epithelial area. At 6 weeks, Ea/Al of CsA-treated KCS eyes tended to remain elevated ($63.3 \mu\text{m}^2/\mu\text{m}$), whereas vehicle-treated KCS dogs were indistinguishable from nonsurgical vehicle-treated controls at $51.0 \mu\text{m}^2/\mu\text{m}$ and $50.9 \mu\text{m}^2/\mu\text{m}$, respectively, suggesting a possible CsA-induced epitheliotropic effect.

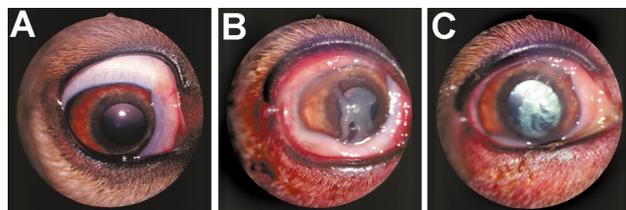


FIGURE 1. Right eye of dog (A) before induction of KCS, (B) 2 weeks after induction of KCS, and (C) 2 weeks after topical treatment with 2% CsA (4 weeks after induction of KCS). Reduction in severity of conjunctival inflammation and changes in the amount and clarity of ocular mucus were observed after topical treatment with CsA (compare C with B).

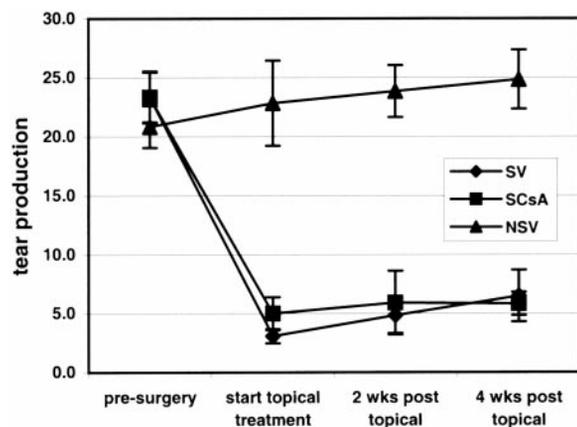


FIGURE 2. STT data in control dogs and two groups of KCS dogs ($n = 3$ per group). Surgically altered eyes of dogs in one KCS group were treated with 2% CsA beginning 2 weeks after induction of KCS. Eyes of dogs in the second KCS group and the non-KCS group received topical placebo (corn oil) treatments for the same time period and at the same frequency (twice daily). STT values were significantly lower ($P < 0.001$) than in nonsurgical (control) eyes. SV, surgery with vehicle; SCsA, surgery with CsA; NSV, no surgery with vehicle.

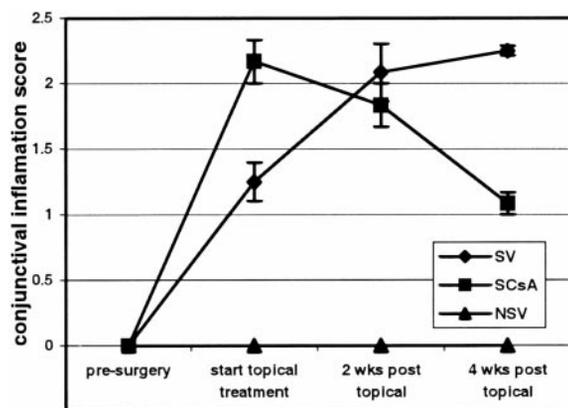


FIGURE 3. Mean conjunctival inflammation scores in eyes of dogs in non-KCS and vehicle, KCS and CsA, and KCS and vehicle groups ($n = 3$ per group). A continuous increase was observed in severity of conjunctival changes (higher mean conjunctivitis scores) in KCS eyes treated with vehicle compared with KCS eyes treated with topical CsA. Non-KCS eyes treated with placebo maintained at baseline (0 score; see Methods section for definition of conjunctivitis scores used in this figure and in Figures 4, 6, and 7). Abbreviations are defined in Figure 2.

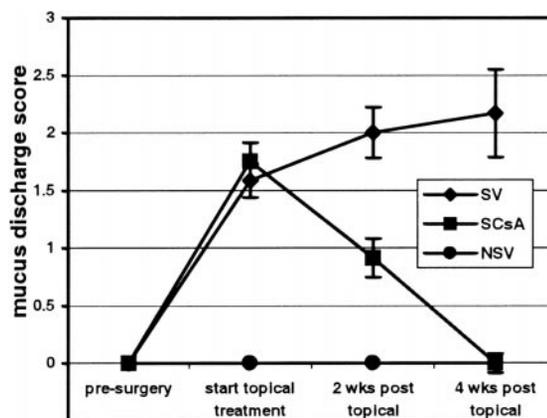


FIGURE 4. Mean mucus discharge scores in eyes of dogs in non-KCS and vehicle, KCS and CsA, and KCS and vehicle groups ($n = 3$ per group). A continuous increase was observed in severity of mucus discharge (high mean mucus discharge scores) in KCS eyes treated with vehicle compared with KCS eyes treated with topical CsA. Mean mucus discharge scores of KCS eyes treated with CsA returned to baseline at the end of the 4-week topical treatment period. Non-KCS eyes treated with placebo maintained baseline scores (0). A significant positive correlation ($\rho = 0.877$) was found between conjunctivitis and mucus discharge scores. Abbreviations are defined in Figure 2.

DISCUSSION

We hypothesized that in KCS, pathologic events effecting the conjunctival mucosa result in a compromise in the quantity of mucosubstance produced by the conjunctival epithelium. We further hypothesized that detrimental effects could be reversed or improved by topical CsA treatment. After experimental induction of KCS in dogs and using morphometric methods to analyze conjunctival biopsy specimens, significant reductions in intraepithelial mucin volumes were determined. In eyes with surgically induced KCS, morphometric assessment of sequential conjunctival samples revealed that CsA restores the

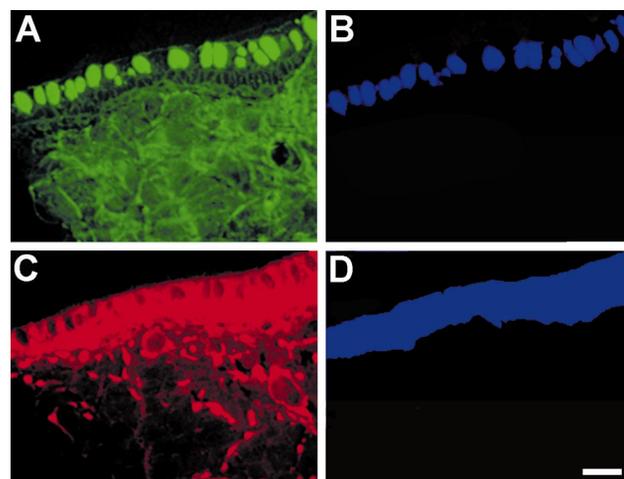


FIGURE 5. CGCs were profiled (green) by staining conjunctival epithelial sections with FITC-conjugated WGA (A). Image analysis software was used to threshold the epithelium-producing blue goblet cell images (B), allowing morphometric determination of mucin area per apical length of epithelium. Morphometric determination of total Ea/Al was determined after mucosal sections were intentionally overstained with propidium iodide (red) to nonspecifically highlight the entire epithelial area (C). Using the software, the entire conjunctival epithelial area for a given section was then highlighted and segregated (blue) allowing morphometric analysis of total epithelial area (D).

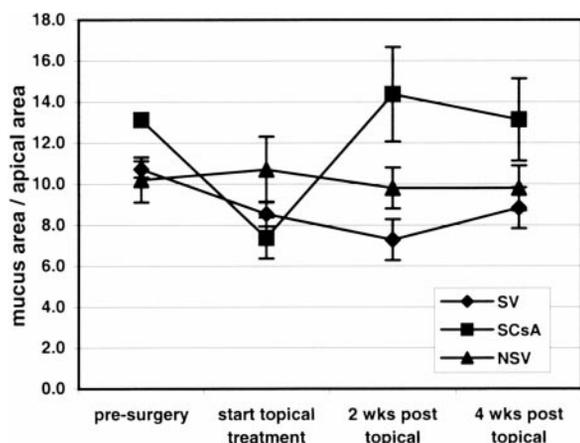


FIGURE 6. Ma/AI, as effected by KCS and topical treatments ($n = 3$ per group). Ma/AI increased in KCS eyes after 2 and 4 weeks of topical CsA treatments ($P < 0.017$). Abbreviations are defined in Figure 2.

balance in CGC mucin synthesis and secretion as intraepithelial mucin quantities returned to pre-KCS levels.

Although the role of CGC mucin in KCS and other human dry eye syndromes has been somewhat controversial, Lemp et al.²² provided evidence that some cases of dry eye were secondary to a mucus deficiency leading to decreased tear breakup time. CGC density was found to decrease by 86% in human KCS and even more in extreme types of dry eye, such as ocular pemphigoid and Stevens-Johnson syndrome.⁶ A recent study using an antibody against a mucinlike glycoprotein present on the surface of stratified ocular epithelia including conjunctiva found an increased staining of goblet cells in patients with dry eye.²³ The increase in the number of positively stained cells was believed to reflect altered mucin glycosylation in a diminished population of CGCs.

This study appears to be the first report to demonstrate quantitatively an in situ reduction of the conjunctival mucin contents in a dry eye state. Removal of lacrimal glands in this study allowed demonstration of the effect of CsA on CGCs independent of a lacrimostimulatory effect. The clinical improvement of KCS eyes in the absence of increased lacrimation suggests that, in this canine model, the CGCs play an important role in the recovery of eyes from KCS. This observation is consistent with an earlier report that the goblet cell density is a relatively sensitive indicator of the severity of ocular surface disease.⁵

Administration of topical CsA to human patients with severe KCS associated with Sjögren's syndrome has resulted in increased tearing and improved clinical findings.²⁴ CsA's effect of stimulating aqueous tear production is widely recognized in the treatment of canine KCS.^{12,13,21} Several mechanisms have been proposed to explain CsA's efficacy in the treatment of KCS. Because lymphocytic infiltration of lacrimal gland acini and ducts is a hallmark of canine KCS, the principal rationale for the use of CsA initially was its well-characterized effect as an immunosuppressant of T-cells.²⁵ The 3 to 56 days required for the onset of the therapeutic benefit of topical CsA in spontaneous canine KCS was consistent with an immunosuppressive mechanism of action.²⁵ It was soon realized, however, that CsA had T-cell-independent effects on lacrimation that may have contributed to its therapeutic effect. CsA's ability to increase lacrimation in normal beagles and in ex vivo glandular preparations suggests a direct effect on lacrimal glands, independent of T-cell effects.²⁵

A possible role of CsA in modulating apoptosis in treatment of canine dry eye was recently investigated by using an apo-

ptosis assay and immunohistochemical analysis for various apoptosis mediators.²⁶ Although normal canine lacrimal gland and conjunctival epithelial cells exhibit limited apoptosis when conventional apoptotic markers are used, in canine KCS these cells undergo increased apoptosis. By contrast, lymphocytes in canine KCS demonstrated decreased apoptotic activity compared with lymphocytes of unaffected dogs. After topical administration of CsA to eyes of KCS-affected dogs, apoptosis was induced in the lymphocytes and suppressed in the acinar and conjunctival epithelial cells. It was concluded that CsA facilitates the re-establishment of normal apoptotic balance as a possible additional therapeutic mechanism.

Results of the present study demonstrate that CsA has a stimulatory effect on mucin production by CGCs in KCS eyes. The mechanism by which CsA increases intracellular stores of mucin in CGCs remains unknown. Clinical evaluations of placebo and CsA-treated KCS eyes in this study revealed greater amounts of visible mucus correlated with decreased amounts of intracellular mucin stores, which could suggest that excessive secretion of mucins in placebo-treated eyes leads to decreased intracellular stores. CsA may act through its classic target the T cell, inhibiting T-cell release of cytokines that act directly or indirectly to increase mucin secretion. For example, CsA reduces secretion of tumor necrosis factor (TNF)- α from T cells.²⁷ TNF- α is known to increase mucin secretion from respiratory epithelial cells²⁸ and, therefore, represents a potential mucin secretagogue for GCs in the inflamed conjunctiva.

Alternatively, CsA's inhibition of T-cell cytokine secretion may alter the presence and activity of diverse inflammatory cells that could potentially release other mediators that drive mucus hypersecretion. Inflammatory mediators that have been shown to increase mucin secretion include IL-1 β and IL-6,²⁹ platelet-activating factor,³⁰ histamine,³⁰ and prostaglandins.³¹ A third possible mechanism may exist by which CsA directly modulates mucin production in CGCs. We have recently found that CsA causes a dose-dependent increase in both the number of GCs and the amount of mucin stored within individual GCs in a human adenocarcinoma cell line grown in vitro.³² This raises the intriguing possibility that CsA may act directly on the conjunctival epithelium to modulate distribution of mucins and/or the relative proportions of distinct mucin protein species or glycoforms being synthesized. Such a hypothesis is consistent with the conclusions of Danjo et al.²³ that alterations in mucin distribu-

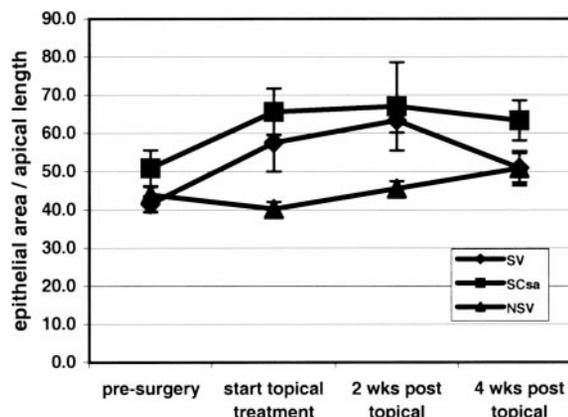


FIGURE 7. Ea/AI, as effected by KCS and topical treatments ($n = 3$ /group). Note effect of KCS in increasing epithelial area. In placebo-treated KCS eyes, epithelial area returned to baseline 4 weeks after treatment, whereas CsA-treated eyes were noted to maintain greater relative areas, suggesting a possible epitheliotropic effect of topical CsA in KCS eyes ($P < 0.017$). Abbreviations are defined in Figure 2.

tion and glycosylation accompany the pathologic signs of dry eye and the analysis by Carrington et al.³³ of secreted ocular mucins in canine KCS, wherein mucus accumulation was paralleled by an increase in sialylation of mucins. The concept that CsA has a direct epitheliotropic effect is also consistent with an earlier observation that CsA's ability to directly modulate proliferation and differentiation of keratinocytes contributes to its therapeutic benefit in the treatment of psoriasis.³⁴

Although the mechanisms explaining apparent differences between the amounts of mucus produced in canine KCS and human KCS are not known, the relatively larger amount of mucus produced in canine KCS may relate, at least in part, to a greater surface area of canine conjunctiva, which results in an increased amount of mucus production in both normal and KCS states. Given the depth of canine conjunctival fornices and the amount of conjunctiva covering the canine nictitating membrane, total conjunctival area in dogs appears to be substantially greater than in humans. Some species-dependent local pathophysiological changes in the normal tear film could also exist that may affect the physiochemical properties of mucin. For example, while noting qualitative changes in ocular mucin in canine KCS (i.e., increased sialylation), Carrington et al.³⁴ suggested that the mucus accumulations may reflect alterations in the viscoelastic properties of the gel layer of the tear film that could then influence the elimination of mucus from the ocular surface. It is also possible that a relative difference in the intensity of the disease process—that is, accelerated inflammatory reaction in this acute canine KCS model versus a slower onset and more slowly progressive disease in humans—is also a factor.

The present study has established that topical CsA, independent of any direct effect it may have on lacrimal secretory cells, exerts an effect on mucin production by CGCs and thereby may contribute to the overall therapeutic effect in its use for the treatment of KCS. Further investigations focusing on possible mechanisms by which CsA upregulates conjunctival mucin production and ways CsA may modify the types of mucin produced in KCS-affected eyes are warranted. By raising questions about the dynamics of conjunctival mucus production (i.e., synthesis, storage, and delivery), results of this study invite specific future investigations to further define the temporal quantitative and qualitative secretory events of the conjunctival mucus system in normal and dry eye states and possible pharmacologic manipulation of those events.

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