Evaluation of species differences and the effects of storage duration and temperature on the anticollagenase efficacy of canine, feline, and equine serum on in vitro corneal degradation

Emily D. Conway BVMS, MS

Jean Stiles DVM, MS

Wendy M. Townsend DVM, MS

Hsin-Yi Weng BVM, MPH, PhD

Received November 10, 2014.
Accepted March 6, 2015.

From the Departments of Veterinary Clinical Sciences (Conway, Stiles, Townsend) and Comparative Pathobiology (Weng), College of Veterinary Medicine, Purdue University, West Lafayette, IN 47907.

Address correspondence to Dr. Stiles (stilesj@purdue.edu).

OBJECTIVE
To evaluate species differences and effects of storage duration and temperature on the anticollagenase efficacy of canine, feline, and equine serum on in vitro corneal degradation.

SAMPLES
Corneas and serum from dogs, cats, and horses.

PROCEDURES
Clinically normal corneas from dogs, cats, and horses were harvested within 2 hours after euthanasia. Serum samples from dogs, cats, and horses were collected and pooled by species. Corneal specimens were incubated with collagenase derived from *Clostridium histolyticum*, 5mM calcium chloride in saline (0.9% NaCl) solution, and feline, canine, or equine serum that had been stored for 0, 30, 90, or 180 days at –20° or –80°C. Following incubation, the corneal weight loss percentage and hydroxyproline concentration in the incubation fluid were calculated and compared among experimental combinations.

RESULTS
Feline serum was more effective than canine or equine serum for minimizing corneal weight loss. Incubation with feline or equine, but not canine, serum significantly reduced hydroxyproline production. Serum storage duration did not affect corneal weight loss, but the hydroxyproline concentration was greater for corneal specimens that were incubated with serum that was stored for 90 days, compared with that for corneal specimens incubated with serum that was stored for 0, 30, or 180 days. Serum storage temperature did not affect corneal weight loss or hydroxyproline concentration.

CONCLUSIONS AND CLINICAL RELEVANCE
Results suggested that serum reduced corneal degradation in vitro, and the duration and temperature at which serum was stored did not affect its anticollagenase efficacy. (*Am J Vet Res* 2015;76:989–995)

Corneal ulceration is a common clinical problem in domestic animal species. Corneal ulcers that affect only the epithelium often heal with minimal clinical intervention, whereas ulcers that involve the corneal stroma may require substantially more treatment.[1–3] Complications resulting from destruction of stromal collagen can cause rapid progression of an ulcer to a descemetocele or perforated cornea.[2–5]

In humans,[6,7] rabbits,[2] dogs,[5] and horses,[1,8,9] enzymes capable of protein degradation have been implicated in the progression of corneal ulcers. The nomenclature for such enzymes varies among sources, and the distinctions between names of proteolytic enzymes can be confusing.[10,11] Terms commonly used in the literature include collagenases, MMPs, proteinases, proteases, peptidases, gelatinases, and stromeolysins. Although those terms are frequently used interchangeably, for the purpose of the study reported here, collagenase is used to describe an enzyme capable of corneal collagen degradation. Rabbit corneal collagenase, which is described as a tissue collagenase because it cleaves collagen into 3/4- and 1/4-length fragments, is associated with corneal ulceration in rabbits, and human corneal collagenase degrades collagen in a similar manner.[7] Collagenases are produced by bacteria or leukocytes or from the growth of corneal epithelium and keratocytes.[1–4,7–9,12] Regardless of the inciting cause, progression of ulcers affecting the corneal stroma is mediated by collagenases that break down stromal collagen.[1–4,6–8,13,14] In dogs[5] and horses[9] with unilateral corneal ulceration, MMP concentrations in the affected eye were higher than those in the unaffected eye and decreased significantly as the ulceration healed. Consequently, inhibition of collagenase

ABBREVIATIONS

MMP	Matrix metalloproteinase
NAC	N-acetyl cysteine

may have an important role in halting the progression, minimizing adverse sequelae, and promoting the healing of corneal ulcers.[1–3,6,8,12,13]

Clinically, topical ophthalmic agents such as EDTA, NAC, and tetracycline have been used to inhibit corneal collagenase.[1–4,6,14] In human medicine, serum has been described as an effective inhibitor of corneal collagenase,[6,7,13] and its inhibitory effect is attributed to α_2 macroglobulin, which forms tight complexes with collagenases and inhibits their activity.[6,7,15,16]

In veterinary medicine, topical administration of autogenous or homologous serum is used to treat ulcers affecting the corneal stroma because of its anticollagenase properties.[1–4,8] Despite the widespread topical use of serum as a collagenase inhibitor, to our knowledge, information regarding the anticollagenase efficacy of serum that has been stored > 7 days or between heterologous and homologous serum is lacking.

The purpose of the study reported here was to evaluate species differences and the effects of storage duration and temperature on the anticollagenase efficacy of canine, feline, and equine serum on in vitro corneal degradation. We hypothesized that the anticollagenase efficacy of heterologous serum would not differ from that of homologous serum. We also hypothesized that the anticollagenase efficacy of serum stored for up to 6 months at –20° or –80°C would not differ from that of fresh serum.

Materials and Methods

Corneal specimens

All study procedures were approved by the Purdue University Animal Care and Use Committee. Corneas were obtained from dogs, cats, and horses without evidence of corneal disease as determined by examination with a direct light source that were euthanized for reasons unrelated to the study at the Purdue University Veterinary Teaching Hospital or a local animal shelter. The corneas were harvested and sectioned into 4 (canine and feline) or 6 pieces (equine) of roughly equal size within 2 hours after euthanasia. Each piece was placed in an individual container that was labeled with the species of origin and stored frozen at –80°C until use. Because of the extended duration of the study, corneal specimens were stored for varying lengths of time up to 6 months.

Serum samples

Serum was obtained from healthy dogs (n = 4), cats (6), and horses (4). The dogs and cats were owned by employees of the Purdue University College of Veterinary Medicine, and the owner of each animal provided consent for the use of his or her pet prior to study initiation. The horses were teaching animals owned by Purdue University. Cats were anesthetized with isoflurane gas via box induction for blood collection, whereas dogs and horses were manually restrained. All cats weighed > 5 kg, whereas all dogs weighed > 17 kg. The volume of blood drawn from each animal represented no more than 10% of its circulating blood volume (30 mL from cats, 40 mL from dogs, and 100 mL from horses). Samples were collected by jugular venipuncture and placed into serum separator tubes, allowed to clot for 60 minutes, and then centrifuged at 4,200 × *g* for 5 minutes to obtain serum. The serum was removed from each tube, pooled by species, and then divided into 10-mL aliquots. The aliquots were used fresh (ie, used within 24 hours) or stored for 30, 90, or 180 days at either –20° or –80°C. Aseptic technique was used to collect and process all samples; however, bacteriologic culture was not performed on any of the serum samples.

In vitro corneal degradation

In vitro corneal degradation was incited by the use of clostridial collagenase as described by Haffner et al.[12] Briefly, each corneal specimen was placed in a plastic weigh boat and dried in an oven at 40°C for 3 hours. After the specimen was dried, it was weighed and the result was recorded as the pretreatment corneal weight. Incubation fluid was prepared by the addition of collagenase derived from *Clostridium histolyticum*[a] (800 U/mL) to 5 mL of 5mM calcium chloride in saline (0.9% NaCl) solution. Five milliliters of the resultant solution was added to a 10-mL conical tube, then 500 µL of serum (100 µL/mL) from the appropriate species and storage duration–temperature combination was added to each tube. Negative control samples of incubation fluid consisted of 5 mL of 5mM calcium chloride in saline solution only, whereas positive control samples consisted of 5 mL of 5mM calcium chloride in saline solution and clostridial collagenase (800 U/mL) without any serum. Negative and positive controls were included for each experimental condition. A dried corneal specimen was added to each tube, and the tube was incubated with agitation at 40°C for 4 hours. After incubation, a 1.8-mL aliquot of incubation fluid was obtained from each tube and stored at –80°C until analyzed for determination of hydroxyproline concentration. The corneal specimen within each tube was then retrieved by pouring the remaining incubation solution through filter paper[b] (pore size, 11 µm). Each cornea specimen was again dried in an oven at 40°C for 3 hours, then weighed with the result recorded as the posttreatment corneal weight.

Experimental conditions

Corneal samples from each species were incubated in incubation fluid prepared from homologous serum and 2 heterologous serum samples (eg, corneal specimens obtained from cats were incubated with feline [homologous] serum as well as canine and equine [heterologous] serum) that had been stored for 0 (fresh serum), 30, 90, or 180 days at –20° or –80°C. Thus, there were 63 combinations for species of origin for corneal specimens, species of origin for serum, serum storage duration, and serum storage tempera-

ture. Each combination was considered an experimental condition, and each experimental condition was performed in triplicate.

Determination of hydroxyproline concentration

Hydroxyproline is a product of collagen degradation and determination of its concentration in the fluid in which corneal specimens were incubated was used as a measure of corneal degradation in another similar study.[12] A commercially available spectrophotometric assay[c] was used to determine the hydroxyproline concentration in each 1.8-mL aliquot of incubation fluid that was obtained after 4 hours of incubation with the assigned cornea specimen. The assay was performed in accordance with the manufacturer's specifications except each sample was diluted 1:2 with ultrapure water prior to analysis to ensure that the measured hydroxyproline concentrations would fit on the standard curve and samples were centrifuged at 10,000 × *g* for 10 minutes. A new standard curve was calculated for each plate.

Statistical analysis

For each corneal specimen, the extent of corneal degradation was measured by 2 methods, the percentage of corneal weight loss between pretreatment and posttreatment measurements and the hydroxyproline concentration in the incubation fluid. The distribution of the data for each outcome of interest (corneal weight loss percentage and hydroxyproline concentration) was evaluated for normality by the Shapiro-Wilk statistic. Results indicated that the data for corneal weight loss percentage had a parametric distribution, whereas the data for hydroxyproline concentration had a nonparametric distribution. Therefore, the mean (SD) corneal weight loss percentage and the median (range) hydroxyproline concentration were reported for each experimental condition. A general linear model or a Kruskal-Wallis test with a Bonferroni adjustment was used to compare mean corneal weight loss percentage and median hydroxyproline concentration, respectively, among experimental conditions. All analyses were performed with statistical software,[d] and values of $P < 0.05$ were considered significant.

Results

Percentage corneal weight loss

Corneal specimens weighed substantially less following incubation with clostridial collagenase, which suggested that the collagenase caused collagen degradation within the corneal specimens. The addition of serum to the incubation fluid appeared to inhibit corneal degradation as evidenced by the fact that the percentage of corneal weight loss for specimens incubated with serum in addition to the clostridial collagenase was less than that for positive control specimens that were incubated with the clostridial collagenase without serum. The serum storage duration ($P = 0.074$) and temperature ($P = 0.526$) did not significantly affect the percentage of corneal weight loss. The mean (SD) percentage of weight loss for corneal specimens incubated with serum that was stored for 0 (fresh), 30, 90, and 180 days was 63% (13%), 48% (22%), 58% (19%), and 60% (22%), respectively, whereas that for corneal specimens incubated with serum that was stored at –20° and –80°C was 56% (23%) and 55% (20%), respectively. However, the percentage of corneal weight loss was significantly ($P = 0.028$) associated with the species of origin for both the corneal specimen and serum **(Table 1)**. Feline serum was more effective at inhibiting corneal degradation (ie, the percentage of weight loss was less) than either canine or equine serum, regardless of the species from which the corneal specimen was obtained. Additionally, the percentage of corneal weight loss was significantly ($P = 0.004$) less for equine corneal specimens incubated with equine serum than those incubated with canine serum.

Hydroxyproline concentration

The hydroxyproline concentration was significantly higher in the incubation fluid obtained from corneal specimens incubated with clostridial collagenase only (positive controls) than that from corneal specimens incubated without clostridial collagenase (negative controls). The hydroxyproline concentration for corneal specimens incubated with feline or equine serum was significantly ($P < 0.001$ for both) lower, whereas that for corneal specimens incubated with canine serum did not differ significantly ($P =$

Table 1—Mean (SD) weight loss percentage for feline, canine, and equine corneal specimens that were incubated in feline, canine, or equine serum or negative or positive control incubation fluid for 4 hours at 40°C.

	Species from which corneal specimen was obtained		
Incubation fluid	**Feline**	**Canine**	**Equine**
Feline serum	52 (16)*[a]	55 (17)*[a]	24 (15)*[a]
Canine serum	74 (10)[b]	74 (17)[b]	62 (12)[c]
Equine serum	66 (10)[b]	63 (6)[b]	39 (16)[b]
Negative control	6 (4)	6 (5)	4 (2)
Positive control	77 (10)	93 (5)	72 (12)

Values represent the mean (SD) for 21 replicates for each experimental condition and 12 replicates for positive and negative controls. Corneal specimens obtained from each species were incubated with feline, canine, or equine serum that had been stored for 0 (fresh), 30, 90, or 180 days at –20° or –80°C, and each cornea–serum–storage time–storage temperature combination (along with negative and positive control samples) was replicated in triplicate. The incubation fluid for each combination consisted of 5 mL of 5mM calcium chloride in saline (0.9% NaCl) solution with collagenase (800 U/mL) derived from *Clostridium histolyticum* and 500 µL of serum from the designated species. The negative control incubation fluid consisted of 5 mL of 5mM calcium chloride in saline solution only, and the positive control incubation fluid consisted of 5 mL of 5mM of calcium chloride in saline solution with clostridial collagenase (800 U/mL). *Within a column, value differs significantly ($P < 0.05$) from the corresponding value for the positive control. [a–c] Within a column, values with different superscripts differ significantly ($P < 0.05$).

Table 2—Median (range) hydroxyproline concentration (μg/mL) in the incubation fluid of feline, canine, and equine corneal specimens following incubation with feline, canine, or equine serum or negative or positive control incubation fluid.

	Species from which corneal specimen was obtained		
Incubation fluid	**Feline**	**Canine**	**Equine**
Feline serum	0.040 (0–0.086)*	0.025 (0–0.056)*	0.036 (0–0.138)*
Canine serum	0.032 (0.009–0.112)	0.042 (0.028–0.101)†	0.055 (0.027–0.134)
Equine serum	0.039 (0.016–0.058)*	0.033 (0.008–0.060)*	0.038 (0.005–0.087)*
Negative control	0.0003 (0–0.003)	0 (0–0.009)	0.0014 (0–0.006)
Positive control	0.04 (0.03–0.09)	0.04 (0.02–0.11)	0.08 (0.04–0.149)

Values represent the median (range) for 21 replicates of each experimental condition and 12 replicates for positive and negative controls.

† Within a column, value differs significantly ($P < 0.05$) from the corresponding value for specimens incubated with feline serum.

See Table 1 for remainder of key.

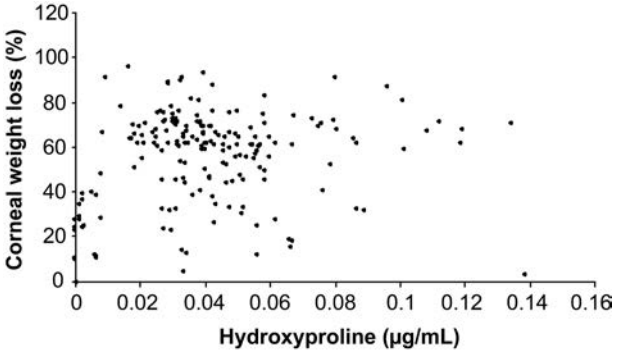

Figure 1—Scatterplot of corneal weight loss percentage versus hydroxyproline concentration in the incubation fluid of feline, canine, and equine corneal specimens following 4 hours of incubation at 40°C with 5 mL of 5mM calcium chloride in saline (0.9% NaCl) solution with collagenase (800 U/mL) derived from *Clostridium histolyticum* and 500 μL of feline, canine, or equine serum that had been stored for 0, 30, 90, or 180 days at –20° or –80°C. Each cornea–serum–storage time–storage temperature combination was replicated in triplicate. There was a weak (Spearman ρ, 0.12) but nonsignificant ($P = 0.096$) positive correlation between hydroxyproline concentration and corneal weight loss percentage.

0.229), compared with the hydroxyproline concentration for positive control specimens **(Table 2)**.

The median (range) hydroxyproline concentration for corneal specimens following incubation with serum that was stored for 90 days (0.055 μg/mL [0.022 to 0.138 μg/mL]) was significantly ($P < 0.001$) higher than that for specimens that were incubated with serum that was stored for 0 (0.034 μg/mL [0.005 to 0.086 μg/mL]), 30 (0.033 μg/mL [0 to 0.079 μg/mL]), and 180 days (0.032 μg/mL [0.016 to 0.086 μg/mL]). The median (range) hydroxyproline concentration did not differ significantly ($P = 0.132$) among corneal specimens incubated with fresh serum (0.034 μg/mL [0.005 to 0.086 μg/mL]) or serum that had been stored at –20°C (0.040 μg/mL [0 to 0.134 μg/mL]) or –80°C (0.039 μg/mL [0 to 0.138 μg/mL]). There was a weak (Spearman ρ, 0.12) but nonsignificant ($P = 0.096$) positive correlation between corneal weight loss percentage and hydroxyproline concentration **(Figure 1)**.

Discussion

Results of the present study indicated that incubation of corneal specimens with feline, canine, or equine serum effectively inhibited corneal weight loss in an in vitro model in which corneal degradation was induced by incubation of corneal specimens with clostridial collagenase. Incubation of corneal specimens with feline or equine, but not canine, serum also significantly reduced hydroxyproline (a product of collagen degradation) production, compared with the hydroxyproline production of specimens incubated with clostridial collagenase alone. Feline serum was more effective than either canine or equine serum for inhibiting corneal weight loss, regardless of the species from which the corneal specimen was obtained. The percentage of corneal weight loss was not significantly affected by serum storage duration or temperature.

Proteinases are enzymes that degrade proteins and are subdivided into cysteine, aspartic, and serine proteinases and metalloproteinases.[10,17] Matrix metalloproteinases are a group of zinc-dependent enzymes (which include collagenases) capable of degrading collagen and other matrix proteins.[1,2,18,19] In the cornea, MMPs are required for general repair and remodeling of the stroma and are counterbalanced by endogenous enzyme inhibitors. Dysregulation in the balance between MMPs and endogenous enzyme inhibitors can result in uncontrolled collagen degradation.[2,19,20] Degradation of corneal collagen, or keratomalacia, is caused by an increase in collagenases from various endogenous (corneal epithelial cells, leukocytes, and tear film) and exogenous (bacterial or fungal) sources.[1–4,7,8,12,20]

Many compounds have been investigated for efficacy as corneal anticollagenolytic agents. Collagenase inhibitors are classified as specific or nonspecific. Specific collagenase inhibitors block only 1 type of proteinase (eg, serine), whereas nonspecific collagenase inhibitors such as α macroglobulins block all 4 types of proteinases.[16,17,21] Effective topical collagenase inhibitors include NAC, EDTA, tetracycline, tetanus antitoxin, and autogenous serum.[1,2,4,8,12] Both NAC and

EDTA chelate zinc or calcium, which are required cofactors for MMPs.[2–4,22] Tetracycline chelates zinc and calcium, inhibits endogenous antitrypsin degradation, and may inhibit leukocyte migration.[2–4,22] Serum contains α_2 macroglobulin, a nonspecific collagenase inhibitor, and α_1 antitrypsin.[1–4,6,7,13,14,17] Although human α_1 antitrypsin does not inhibit human corneal collagenase, it does inhibit rabbit corneal collagenase, and α_2 macroglobulin in both human and rabbit sera inhibits collagenase.[6,7,13] In humans, α_2 macroglobulin is produced in the liver, accounts for 8% to 10% of serum proteins, and inhibits both endogenous and exogenous collagenases by forming tight complexes with collagenase.[13,16,17] Results of multiple kinetic studies[2,13,16,17,21] indicate that α_2 macroglobulin is an important inhibitor of collagenases. In 1 study,[23] > 90% of the anticollagenase activity of serum was attributed to α_2 macroglobulin. The anticollagenase effects of equine serum, tetanus antitoxin, and NAC did not differ significantly in an in vitro corneal degradation model.[12] Doxycycline, EDTA, NAC, ilomastat, and equine serum all inhibit latent and active forms of MMP.[8] Serum has several advantages when compared with other anticollagenases; it is well tolerated when topically administered in eyes and can be readily obtained from healthy donors. Thus, serum is frequently used as an anticollagenase for the treatment of corneal ulcers in both human and veterinary medicine.

Despite the widespread use of serum for the treatment of corneal ulcers, few guidelines exist regarding the storage of serum for topical ophthalmic use, and only a few of those[24–26] that are available contain data to support recommendations regarding serum storage duration or temperature. Investigators of 1 study[26] recommend that serum be stored at 4°C for no more than 16 hours before use or frozen at –20°C for up to 3 months, and serum stored frozen for 6 months should be used or discarded within 1 day after being thawed. Findings of a study[24] conducted to investigate the effects of various preparation methods on the epitheliotrophic factors in human serum suggest that blood samples should be allowed to clot at room temperature (approx 22°C) for 2 hours and then centrifuged at 3,000 × *g* for 15 minutes before serum is harvested for topical ophthalmic administration. The harvested serum should be diluted 1:4 with physiologic saline (0.9% NaCl) solution and stored at –20°C for no longer than 3 months[24]; however, the effects of storage duration and temperature were not investigated in that study. In another study,[25] the concentrations of epithelial growth factor, vitamin A, and transforming growth factor β1 in fresh serum did not differ significantly from those in serum that had been stored at 4°C for 1 month or frozen at –20°C for 1 or 3 months. The effect of storage on serum α_2 macroglobulin concentration was not evaluated in that study.[25]

Published recommendations for the storage of serum for veterinary ophthalmic use vary. Serum can reportedly be stored frozen indefinitely, or it can be stored at room temperature or refrigerated and replaced every 8 days because of microbial contamination.[2,4] Storage of equine serum at –18°, 4°, and 23°C for 7 days did not alter its ability to inactivate MMPs in equine tears over a 7-day period.[e] Results of the present study indicated that storage of serum for ≤ 180 days at –20° or –80°C did not significantly affect the percentage of corneal weight loss in vitro and were consistent with the findings of other studies,[25,e] although the storage duration evaluated in the present study was longer than that of those studies and we only investigated the anticollagenase efficacy of serum. Because we did not evaluate the anticollagenase efficacy of serum stored at room temperature or 4°C, we cannot draw any conclusions regarding those storage conditions. Further studies are necessary to evaluate the maximum duration that serum can be stored at various temperatures without losing its efficacy.

Hydroxyproline is a by-product of collagen degradation, and quantification of its concentration can be useful for determining the extent of collagen degradation.[27] Results of the study[12] by Haffner et al indicate that corneal weight loss and hydroxyproline concentration are highly and positively correlated (r = 0.80). In the present study, however, there was only a weak (Spearman ρ, 0.12) positive correlation between corneal weight loss percentage and hydroxyproline concentration. Results of the present study also indicated that feline and equine, but not canine, serum significantly inhibited hydroxyproline production, compared with that produced by corneal specimens incubated with clostridial collagenases without serum (ie, positive controls), which suggested that canine serum was not as effective at inhibiting collagenase as were feline and equine sera. Storage temperature did not significantly affect the hydroxyproline concentration. The median hydroxyproline concentration for specimens incubated with serum that had been stored for 90 days was significantly higher than that for specimens incubated in fresh serum or serum that had been stored for 30 or 180 days, but we could not determine a reason for this finding. The percentage weight loss for corneal specimens incubated in serum that had been stored for 90 days did not differ significantly from that for corneal specimens incubated in fresh serum or serum stored for 30 and 180 days. Therefore, that finding may be spurious or not clinically relevant.

In the present study, serum inhibited the corneal weight loss percentage in vitro as expected. Interestingly, feline serum appeared to be more protective than was either canine or equine serum, regardless of the species from which the corneal specimen was obtained. Given that α_2 macroglobulin is the serum molecule responsible for its anticollagenase activity, it is tempting to speculate that the concentration of α_2 macroglobulin in feline serum is greater than that in canine or equine serum; however, the serum α_2 macroglobulin concentration was not measured in the present study. Differences among species in regards to the concentration and protein binding capacity of α_2 macroglobulin still need to be elucidated. The pre-

dicted protein sequence for isoforms X1 and X2 of the α_2 macroglobulin of cats, dogs, and horses is available on the National Center for Biotechnology Information website and can be compared to the known sequence for human α_2 macroglobulin.[f] We used a Basic Local Alignment Search Tool[g] to compare isoforms X1 and X2 among species. In dogs, there is 99% domain conservation between isoforms X1 and X2 of the predicted α_2 macroglobulin molecule.[g] When the predicted α2 macroglobulin X1 and X2 isoforms were compared among species, there was 86% domain conservation between dogs and cats, 82% domain conservation between dogs and horses, 81% domain conservation between cats and horses, 81% domain conservation between humans and horses, and 80% domain conservation between humans and both dogs and cats.[g] Those findings suggest that the α_2 macroglobulin molecule should perform similarly among species, although that has yet to be definitively determined. Preferential use of feline serum for the topical treatment of corneal ulcers in other species cannot be recommended without further investigation. Also, because cats can be infected with contagious viruses such as FeLV and FIV, use of serum from cats with an unknown virus status should not be used to treat corneal ulcers in other cats.

It is possible that the variation in the percentage of corneal weight loss among species in the present study was a function of interspecies differences in the corneal specimens rather than in serum efficacy. The type and thickness of corneal collagen vary with age, sex, and species.[28–31] In the present study, we evaluated the percentage of corneal weight loss in response to treatment and did not evaluate absolute corneal weight or composition. Therefore, we cannot conclude that corneal weight loss was not associated with collagen type.

In the present study, the efficacy of serum against in vitro corneal collagen degradation did not vary significantly among serum samples stored at either −20° or −80°C for up to 180 days. Although the anticollagenase activity of feline serum appeared to be greater than that of either canine or equine serum, further research is necessary to elucidate differences in the structure and function of the α_2 macroglobulin molecule among those 3 species.

Acknowledgments

This manuscript represents a portion of a thesis submitted by Dr. Conway to the Purdue University Department of Veterinary Clinical Sciences as partial fulfillment of the requirements for a Master of Science degree.

Supported by a Veterinary Clinical Sciences Graduate Student Competitive Research Grant.

Presented in abstract form at the 45th Annual Meeting of the American College of Veterinary Ophthalmology, Fort Worth, Tex, October 2014.

The authors thank Dr. Lynn Guptill and Anisa Dunham for technical assistance.

Footnotes

a. Collagenase from *Clostridium histolyticum,* type XI, Sigma Aldrich, St Louis, Mo.
b. Whatman filter paper grade 1, VWR International, Radnor, Pa.
c. Hydroxyproline assay kit, Sigma Aldrich, St Louis, Mo.
d. SAS, version 9.2, SAS Institute Inc, Cary, NC.
e. Brooks DE, Ollivier FJ, Schultz FJ, et al. Duration of in vitro activity of equine serum against equine tear film matrix metalloproteinases (abstr). *Invest Ophthalmol Vis Sci* 2003;44: e-abstract 902.
f. Protein database [database online]. Bethesda, Md: National Center for Biotechnology Information, 2015. Available at: www.ncbi.nlm.nih.gov/guide/proteins/. Accessed Jan 15, 2015.
g. BLAST, National Center for Biotechnology Information, National Institutes of Health, Bethesda, Md. Available at: blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed Jan 15, 2015.

References

1. Brooks DE. Inflammatory stromal keratopathies: medical management of stromal keratomalacia, stromal abscesses, eosinophilic keratitis, and band keratopathy in the horse. *Vet Clin North Am Equine Pract* 2004;20:345–360.
2. Brooks DE, Ollivier FJ. Matrix metalloproteinase inhibition in corneal ulceration. *Vet Clin North Am Small Anim Pract* 2004;34:611–622.
3. Ollivier FJ. Medical and surgical management of melting corneal ulcers exhibiting hyperproteinase activity in the horse. *Clin Tech Equine Pract* 2005;4:50–71.
4. Slatter DH, Severin GA. Collagenase inhibitors in veterinary ophthalmology. *Aust Vet Pract* 1975;5:174–176, 179.
5. Wang L, Pan Q, Xue Q, et al. Evaluation of matrix metalloproteinase concentrations in the precorneal tear film from dogs with *Pseudomonas aeruginosa*–associated keratitis. *Am J Vet Res* 2008;69:1341–1345.
6. Berman MB. Collagenase inhibitors: rationale for their use in treating corneal ulceration. *Int Ophthalmol Clin* 1975;15:49–66.
7. Berman MB, Barber JC, Talamo RC, et al. Corneal ulceration and the serum antiproteases I. a1 antitrypsin. *Invest Ophthalmol Vis Sci* 1973;15:759–770.
8. Ollivier FJ, Brooks DE, Kallberg ME, et al. Evaluation of various compounds to inhibit activity of matrix metalloproteinases in the tear film of horses with ulcerative keratitis. *Am J Vet Res* 2003;64:1081–1087.
9. Ollivier FJ, Brooks DE, Van Setten GB, et al. Profiles of matrix metalloproteinase activity in equine tear fluid during corneal healing in 10 horses with ulcerative keratitis. *Vet Ophthalmol* 2004;7:397–405.
10. McDonald JK. An overview of protease specificity and catalytic mechanisms: aspects related to nomenclature and classification. *Histochem J* 1985;17:773–785.
11. Barrett AJ, Rawlings ND, Salvesen G, et al. Introduction. In: Rawlings D, Salvesen G, eds. *Handbook of proteolytic enzymes*. 3rd ed. London: Elsevier, 2013;3–18.
12. Haffner JC, Fecteau KA, Eiler E. Inhibition of collagenase breakdown of equine corneas by tetanus antitoxin, equine serum and acetylcysteine. *Vet Ophthalmol* 2003;6:67–72.
13. Berman M, Gordon J, Garcia LA, et al. Corneal ulceration and the serum antiproteases. II: complexes of corneal collagenases and alpha-macroglobulins. *Exp Eye Res* 1975;20:231–244.
14. Berman MB, Kerza-Kwiatecki AP, Davison PF. Characterization of human corneal collagenase. *Exp Eye Res* 1973;15:367–373.
15. Barrett AJ, Starkey PM. The interaction of alpha 2-macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. *Biochem J* 1973;133:709–724.
16. Borth W. Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics. *FASEB J* 1992;6:3345–3353.
17. Meyer C, Hinrichs W, Hahn U. Human α2 macroglobulin—another variation on the venus flytrap. *Angew Chem Int Ed Engl* 2012;51:5045–5047.
18. Matrisian LM. Matrix metalloproteinases. *Curr Biol* 2000;10:R692.
19. Fridman R. Matrix metalloproteinases. *Biochem Biophys Acta* 2010;1803:1–2.

20. Ledbetter EC, Gilger BC. Diseases and surgery of the canine cornea and sclera. In: Gelatt KN, Gilger BC, Kern TJ, eds. *Veterinary ophthalmology.* 5th ed. Ames, Iowa: Wiley-Blackwell, 2013;976–1050.
21. Nagase H, Itoh Y, Binner S. Interaction of alpha 2–macroglobulin with matrix metalloproteinases and its use for identification of their active forms. *Ann N Y Acad Sci* 1994;732:294–302.
22. Herring IP. Clinical pharmacology and therapeutics, part 3: mydriatics/cycloplegics. Anesthetics, ophthalmic dyes, tear substitutes and stimulators, intraocular irrigating fluids, topical disinfectants, viscoelastics, fibrinolytics and antifibrinolytics, antifibrotic agents, tissue adhesives and anticollagenase agents. In: Gelatt KN, ed. *Veterinary ophthalmology.* 4th ed. Ames, Iowa: Blackwell, 2007;332–354.
23. Wooley DE, Roberts DR, Evanson JM. Small molecular weight beta 1 serum protein which specifically inhibits human collagenases. *Nature* 1976;261:325–327.
24. Liu L, Hartwig D, Harloff S. An optimised protocol for the production of autologous serum eyedrops. *Graefes Arch Clin Exp Ophthalmol* 2005;243:706–714.
25. Tsubota K, Goto E, Fujita H, et al. Treatment of dry eye by autologous serum application in Sjögren's syndrome. *Br J Ophthalmol* 1999;83:390–395.
26. Geerling G, MacLennan S, Hartwig D. Autologous serum eye drops for ocular surface disorders. *Br J Ophthalmol* 2004;88:1467–1474.
27. Kivirikko KI, Laitinen O, Prockop DJ. Modifications of a specific assay for hydroxyproline in urine. *Anal Biochem* 1967;19:249–255.
28. Montiani-Ferreira F, Petersen-Jones S, Cassotis N, et al. Early postnatal development of central corneal thickness in dogs. *Vet Ophthalmol* 2003;6:19–22.
29 Lee RE, Davison PF. Collagen composition and turnover in ocular tissues of the rabbit. *Exp Eye Res* 1981;32:737–745.
30. Gilger BC, Whitley RD, McLaughlin SA, et al. Canine corneal thickness measured by ultrasonic pachymetry. *Am J Vet Res* 1991;52:1570–1572.
31. Moodie KL, Hashizume N, Houston DL, et al. Postnatal development of corneal curvature and thickness in the cat. *Vet Ophthalmol* 2001;4:267–272.