Development and evaluation of a novel topically applied sildenafil citrate hydrogel and its influence on wound healing in dogs

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OBJECTIVE
To develop a topical sildenafil hydrogel and evaluate its effect on wound healing in dogs.

ANIMALS
6 purpose-bred, sexually intact, adult Beagles.

PROCEDURES
Hydrogels containing sildenafil citrate, N-methyl-2-pyrrolidone, propylene glycol, and poloxamer 407 were developed. Four excision wounds were created along the dorsum of the dogs. Each wound was treated for 21 days with a nonadherent bandage (C) or with a hydrogel containing 0% (G), 5% (5S), or 10% (10S) sildenafil. Daily bandage changes with wound imaging were performed. Biopsy specimens were collected 5 times.

RESULTS
Hydrogels were homogenous at room temperature and released > 90% of the sildenafil within 8 hours in vitro. Time to first granulation tissue was significantly shorter for the sildenafil groups (mean ± SD, 2.8 ± 0.8 days [5S and 10S]), compared with the control groups (5.2 ± 0.4 days [C] and 6.3 ± 1.4 days [G]). The G wounds had a 10% to 14% lower contraction rate, compared with the C, 5S, and 10S wounds. 5S wounds had a total wound area 0.7 ± 0.3 cm² larger than 10S wounds. No significant differences were present when C wounds were compared with 5S and 10S wounds for total wound area, contraction, or epithelialization. Histologic acute inflammatory scores were higher for 5S and 10S wounds in the early and late stages of wound healing, with higher reparative scores on day 7. Neovascularization was higher for 10S wounds on day 7 and 14.

CLINICAL RELEVANCE
The topical sildenafil hydrogel promoted early granulation tissue, which may be beneficial for secondary wound closure in clinical settings.

Traumatic wounds occur commonly in veterinary medicine and can be costly to manage. Having readily available and cost-effective topical treatments proven to benefit wound healing could improve outcomes for wound cases. One product that has been recently evaluated for its wound-healing properties is sildenafil. Sildenafil is a phosphodiesterase-5 inhibitor that enhances tissue blood flow through vascular smooth muscle relaxation. Sildenafil also stimulates the release of nitric oxide, which has been shown to have beneficial wound-healing properties, including potentiating the clotting process, scavenging oxidative stress components, promoting angiogenesis and endothelial cell proliferation, and providing antimicrobial activity. The most common current use of sildenafil in veterinary medicine is for treatment of pulmonary hypertension; adverse effects are uncommon at therapeutic dosages.

The use of sildenafil to aid in wound healing has been explored via several applications. Oral sildenafil has been shown to improve wound healing in rats, skin flap survival in rats and pigs, colonic anastomosis healing in rats, and healing of open surgical wounds in dogs. A small number of cases have shown oral sildenafil to be beneficial in the treatment of chronic or nonhealing ulcers in people. Intraperitoneal injections of sildenafil and sildenafil in combination with fibrin glue have also been reported to...
have beneficial effects on wound healing in rats that have undergone colonic anastomoses or creation of skin flaps, respectively. However, in contrast, daily subdermal administration of sildenafil was noted to decrease viability of skin flaps in rats.

In recent years, topical sildenafil has been investigated for use in wound treatment in various species. Topical sildenafil dressings have been shown to be safe, nontoxic, and nonirritating when applied to rats, rabbits, and mice. Dose-dependent benefits of topical sildenafil formulations have been noted in excisional wounds and skin flaps in rats. One study on caudal skin flaps in rats compared topical and oral sildenafil administration and found topical application to be more effective. Topical sildenafil has also been studied in people and has been shown to provide improved healing of pressure sores.

To the authors’ knowledge, there are limited controlled studies on topical sildenafil administration in rats and humans and no studies in dogs. The objective of this study was to evaluate the effect of a novel topical sildenafil citrate hydrogel on wound healing in dogs. We hypothesized that topical sildenafil hydrogel would accelerate wound healing, compared with the hydrogel alone or a nonadherent bandage. We also hypothesized that systemic concentrations of sildenafil would either not be detectable or would be detectable at extremely low (ie, nontherapeutic) concentrations in the plasma after topical application of sildenafil hydrogel.

Materials and Methods

Preparation of topical sildenafil citrate hydrogel

Solubility studies were performed to select potential dermatologic solvents (Supplementary Appendix S1). Given their high sildenafil solubility, N-methyl-2-pyrrolidone (Pharmasolve) and propylene glycol (Super Refined propylene glycol) were selected for incorporation in poloxamer 407 NF 20% (Fisher Scientific). The viscosity of the hydrogel formulations was tested with a rheometer (MCR 302; Anton Paar) with a sandblasted parallel-plate (PP25/S) geometry of 25-mm diameter and a gap of 1 mm. A texture analyzer (TA HD plus; Stable Micro Systems Ltd) was used to determine the mechanical properties of the hydrogels. Mechanical properties such as firmness or hardness (maximum compressive force), cohesiveness (area of the graph > 0), adhesiveness (area of graph < 0), and springiness (minimum retracting force) were determined with a force-time plot.

Determination of drug content uniformity

A high-performance liquid chromatography (HPLC) system (Alliance e2695 separation module; Waters Corp) equipped with a photo diode array UV detector (2998 PDA detector; Waters Corp) was used to assess drug content uniformity of the hydrogels. The chromatographic separation was performed on a reverse-phase 150 X 4.6-mm column with 5-μm particles (Kinetex EVO C18 column; Phenomenex). The mobile phase consisted of 0.2 M ammonium acetate (pH, 7.0) and acetonitrile (60:40 vol/vol). The absorbance wavelength of sildenafil was set at 240 nm, and the run time was 10 minutes.

In vitro release by dialysis bag method

Dialysis membrane tubing (15 X 2.5 cm) was soaked for 1 hour in the receptor phase prior to the experiment. A 0.5-g sample of each hydrogel formulation was loaded into the tubing with a long needle, the gel was evenly spread to 3 cm of the membrane, and the tubing end was clipped. The surface area of the membrane immersed in the receptor phase was 7.5 cm². The receptor compartment was filled with 250 mL of normal saline solution. The flasks were agitated at 150 rpm and maintained at 37°C in a rotary incubator shaker. Receptor samples (5 mL) were collected at 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hours and analyzed by means of HPLC.

Dogs

The study protocol was approved by the University of Georgia Institutional Animal Care and Use Committee. Six purpose-bred, 1-year-old, sexually intact, male Beagles weighing between 10 and 14 kg were used. Dogs were housed individually and provided food and water twice daily. The dogs were allowed to acclimate for 1 week prior to the study start. A physical examination, complete blood count (CBC), and serum chemistry panel were performed on each dog prior to the study start. After completion of the study, dogs were transferred to another research protocol.

Excision wounds

On day 0, dogs were placed under general anesthesia. The anesthetic protocol included maropitant (1 mg/kg, IV), acepromazine (0.03 to 0.05 mg/kg, IV), and hydromorphone (0.05 to 0.2 mg/kg, IV) for premedication, propofol (2 to 4 mg/kg, IV, to effect) for induction, and a propofol constant rate infusion
for maintenance. Dogs were positioned in sternal recumbency. The dorsum was shaved and aseptically prepared for surgery. A template was then used to create 4 full-thickness, 3 X 3-cm excision wounds 5 cm apart along the dorsum of each dog (Figure 1). Each wound was treated with a nonadherent bandage (Nonadherent pad; McKesson) that did not contain any hydrogel (C) or with the hydrogel containing 0% (G), 5% (5S), or 10% (10S) sildenafil. Treatment site locations were randomized for each dog, and individuals performing wound assessments were blinded to which hydrogel was used on each wound. All wounds were covered with a nonadherent bandage followed by an adhesive bandage (Hypafix adhesive dressing; Patterson Veterinary), cotton-blend cast padding (Cast padding; McKesson), and an elastic bandage (Cohesive bandage; McKesson). All dogs wore an Elizabethan collar to protect the bandages. Transdermal fentanyl patches (2 to 4 μg/kg/h) were placed at the time of surgery. Dogs were evaluated for pain with the Colorado State University canine acute pain scoring system every 4 to 6 hours for the first 24 hours after surgery. Hydromorphone (0.05 mg/kg, IM) was given every 4 to 6 hours if the pain score was > 1.

Figure 1—Photographs of the dorsum of a dog showing the use of a sterile template to create 4 full-thickness 3 X 3-cm excision wounds (A) and showing the wounds immediately after creation (B), after application of 1 mL of a sildenafil hydrogel (C), and after application of nonadherent and adherent bandages prior to placement of a soft, padded bandage (D).

Data collection

Daily bandage changes were performed for 21 days to allow for wound care, subjective wound assessment, image acquisition, and treatment application. Bandage changes were performed without sedation. Aseptic technique was used during bandage changes, and wounds were gently cleaned with sterile saline (0.9% NaCl) solution. At each bandage change, wounds were subjectively characterized by 2 of the authors (SMS and MLW) on the basis of a previously described grading scheme that included evaluations of wound fluid quantity, color, and character as well as periwound tissue status, hydration, and the presence of granulation tissue. Images of each wound were obtained at each bandage change with a ruler held next to the wound for standardized image processing. One milliliter of hydrogel was applied daily to wounds assigned a hydrogel treatment until the wound area had decreased by 50%, after which time the amount of hydrogel was reduced to 0.5 mL.

Daily wound images were masked, batched, and randomized by a single investigator (MLW), and measurements were acquired for total wound area, open wound area, and area of epithelialization with open-access image-processing software.
layer was transferred to another clean glass tube and evaporated at 60°C. The dry residue was reconstructed with 1 mL of normal saline solution and vortexed for 1 minute. A 20-µL aliquot of reconstituted sample was then analyzed by means of HPLC.

The bioanalytical method was validated for linearity, precision, recovery (extraction efficiency), and limit of quantitation. Linearity was assessed by analyzing 3 sets of samples with concentrations ranging from 2 to 100 µg/mL. The acceptance criterion for each concentration was ≤ 15% deviation from the nominal value. The calibration curve was considered acceptable with a correlation coefficient (R²) ≥ 0.99. Precision was determined by analyzing 6 sets of samples with a concentration of 20 µg/mL. The acceptance criterion for precision was < 15%. The extraction efficiency or recovery of sildenafil was evaluated by comparing the mean peak areas of 6 samples before and after the extraction process. Recovery percentage was calculated by obtaining the ratio of mean peak areas after and before extraction; the acceptance criterion was ≥ 80%.

**Statistical analysis**

In vitro differences between hydrogel formulations, including viscosity, texture, and drug release, were analyzed by means of one-way ANOVA with standard software (Prism 9 software; GraphPad), with a value of P < .05 considered significant. All remaining analyses were performed with separate software (SAS, version 9.4; SAS Institute). Linear mixed models were used to analyze total wound area, epithelialization percentage, and contraction percentage. Friedman test was used to compare days to first granulation between treatments, as well as hemorrhage and neutrophil infiltration scores at day 21 (owing to convergence issues with generalized linear mixed models). Wilcoxon signed rank tests were performed for paired comparisons, with the linear step-up false discovery method used to account for multiple comparisons. Generalized linear mixed models were used to analyze histopathology scores, except for those evaluated with the Friedman test.

**Results**

**Characteristics of sildenafil hydrogels**

Grossly, the hydrogels were homogenous at room temperature. The pH of the hydrogels was 5.29, 4.25, and 3.98, respectively, for the formulations containing 0% (G hydrogel), 5% (5S hydrogel), and 10% (10S hydrogel) sildenafil. Mean ± SD viscosity at a constant shear rate was 214.63 ± 7.61 Pa·s (G hydrogel), 166.82 ± 0.63 Pa·s (5S hydrogel), and 173.43 ± 7.07 Pa·s (10S hydrogel). The viscosity profile of all 3 hydrogel formulations decreased with increasing shear rate, thus exhibiting shear-thinning behavior. Viscosity of the SS and 10S hydrogels was significantly higher than that of the 5S hydrogel.

**Bioanalytical method for analysis of plasma sildenafil concentration**

Plasma samples for determination of sildenafil concentrations were collected on days 0, 7, 14, and 21; samples were frozen at −20°C until batch analysis. A modified procedure reported by Quintero et al. was followed for quantifying sildenafil concentration in the plasma samples. Frozen plasma samples were thawed at ambient temperature and centrifuged at 3,000 X g for 10 minutes at 4°C. An aliquot of plasma (460 µL) was placed in a glass tube with 200 µL of 0.1 M Na2CO3 and 5 mL of diethyl ether:dichloromethane (60:40). The mixture was vortexed for 5 minutes and then centrifuged at 3,500 X g for 15 minutes at 4°C. The tubes were frozen at −20°C for 20 minutes, and the organic layer was transferred to another clean glass tube and evaporated at 60°C. The dry residue was reconstituted with 1 mL of normal saline solution and vortexed for 1 minute. A 20-µL aliquot of reconstituted sample was then analyzed by means of HPLC.
(P < .01) less than that of the G hydrogel. The firmness, cohesiveness, adhesiveness, and springiness did not differ significantly between the G and 10S hydrogels. Mean ± SD percentage drug contents of the 5S and 10S hydrogels were 100.05 ± 3.77% and 99.36 ± 3.88%, respectively, suggesting uniform drug distribution within ±4% of the coefficient of variation. In vitro drug release analysis of the 5S and 10S hydrogels confirmed that > 90% of the sildenafil is released within 8 hours (Figure 2), suggesting that

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*Figure 3*—Photographs of the wound appearance for a representative dog following creation of 4 full-thickness 3 X 3-cm excision wounds (day 0) and daily treatment of the wounds with a nonadherent bandage (group C) or with hydrogels containing 0% (group G), 5% (group 5S), and 10% (group 10S) sildenafil.
the entire amount of drug would be available at the wound application sites.

Dogs
Physical examination findings and results of CBC and serum chemistry panels were unremarkable for all dogs. All hydrogels were consistent at room temperature and easy to apply. All dogs tolerated bandage changes and hydrogel application without sedation.

Subjective wound evaluation
Mean ± SD time to first appearance of granulation tissue was significantly (P = .039) shorter for the sildenafil treatment groups (2.8 ± 0.8 days [5S, 10S]), compared with the control groups (5.2 ± 0.4 days [C] and 6.3 ± 1.4 days [G]). No significant differences were present between treatment groups in regard to wound fluid quantity, color, or character. No significant differences were present for wound hydration or periwound status.

A white residue within the wound bed that remained after gentle flushing with sterile saline solution was noted with the 5S and 10S wounds (Figure 3). The residue appeared nonirritating and decreased over time.

Wound planimetry
Overall, G wounds had a 10% to 14% lower contraction rate (Figure 4) and significantly larger wound area (0.8 to 1.5 cm²; P = .000 to .006), compared with the other wound groups. The 5S wounds had a mean ± SD total wound area 0.7 ± 0.3 cm² larger than the 10S wounds (P = .040). Percent epithelialization was significantly higher for C wounds, compared with G wounds, on most days following day 15 (P = .023 to .046). No significant differences in wound area, percent contraction, or percent epithelialization were present between the C wounds and either the 5S or 10S wounds.

Histopathologic findings
HAISs were significantly different on days 3 and 21. The HAIS score for C (2.92 ± 0.92) was significantly lower than 5S (5.67 ± 1.2; P = .000) on day 3. On day 21, both C (1.00 ± 0.55) and G (1.00 ± 0.55) had significantly lower HAIS scores than 5S (3.67 ± 1.60) and 10S (4.00 ± 1.10) (P = .001 to .002). C had significantly less edema (0.83 ± 0.75) compared to G (1.67 ± 0.26), 5S (2.17 ± 0.52), and 10S (1.83 ± 0.41) on day 3 (P = .000 to .011). A significantly higher hemorrhage score was noted for 5S (2.17 ± 0.41) compared to C (1.08 ± 0.20), G (1.42 ± 0.38), and 10S (1.33 ± 0.75) on day 3 (P = .001 to .028). A significantly higher hemorrhage score was noted for 10S (1.83 ± 0.51) compared to C (0.83 ± 0.68), G (1.17 ± 0.41), and 5S (1.33 ± 0.75) on day 14 (P = .011 to .049). Neovascularization was greater for 10S compared to G on day 7 (1.5 ± 0.45 vs 1.25 ± 0.76; P = .047) and greater than C and 5S on day 14 (1.83 ± 0.26 vs 1.16 ± 0.26 and 1.16 ± 0.26; P = .022). 5S and 10S (1.08 ± 0.66 and 1.17 ± 0.26) had significantly greater neutrophilic inflammation compared to C and G (0 ± 0; P = .002) on day 21.

HRS scores differed significantly among groups on days 3 and 7. Mean ± SD HRSs for C wounds (1.5 ± 1.34) were significantly (P = .030) lower than those for G wounds on day 3 (3 ± 1.70). On day 7, mean HRSs were also significantly lower for C wounds (4.92 ± 1.07) than scores for 5S (5.42 ± 0.74; P = .040) and 10S (5.42 ± 1.16; P = .020). No significant differences among groups were seen for collagen density or fibroblast proliferation at any time. Differences in necrosis were not analyzed given the rare occurrence of necrosis in the biopsy samples.

Plasma sildenafil concentration
Plasma sildenafil concentrations were 0 for all dogs on days 0 and 7. On day 14, 1 dog had a plasma sildenafil concentration of 151.93 ng/mL, and all other dogs had concentrations of 0.
Previously, studies\textsuperscript{25,26} have shown pH plays an important role in wound healing, with an acidic pH supporting formation of granulation tissue, modulation of proteolytic activity, infection control, oxygen release, and angiogenesis. Topical acidic solutions such as 1% to 5% acetic acid and 2% to 3% citric acid have been shown effective against \textit{Pseudomonas aeruginosa} as well as \textit{Staphylococcus aureus}, \textit{Escherichia coli}, \textit{Klebsiella} spp, \textit{Proteus} spp, \textit{Citrobacter} spp, \textit{Staphylococcus epidermidis}, streptococci, and enterococci.\textsuperscript{25,28} Topical application of citric acid has also been noted to promote granulation tissue formation and wound contraction in people.\textsuperscript{27} Additionally, honey, with a pH of 3.2 to 4.5, has been shown to be effective in wound healing, which is in part due to its acidic nature.\textsuperscript{28} Information on the pH of previously reported topical sildenafil products is limited. One previous formulation of sildenafil hydrogel applied topically in rats reported a neutral pH.\textsuperscript{19} The pH of the hydrogels in the present study were 5.29, 4.25, 3.98 for the G, 5S, and 10S formulations, respectively. Manipulation of the pH may provide additional benefits to wound healing and should be considered in future studies.

The base hydrogel formulation used in the present study included poloxamer 407 hydrogel along with propylene glycol and N-methyl-2-pyrrolidone as solvents. Each ingredient was selected on the basis of in vitro analyses in an effort to create a homogeneous product that was conducive to topical use. Poloxamer 407 hydrogel is a commonly used commercial product for wound studies, and multiple hydrogel formulations using poloxamer 407 hydrogel have shown benefits in wound healing in rats.\textsuperscript{29–32} One study\textsuperscript{33} noted that a nitric oxide donor-containing poloxamer 407 hydrogel was particularly beneficial in the inflammatory and proliferative phases of wound healing in rats. Poloxamer 407 hydrogel has been studied as a carrier for buccal and oral administration of medications in dogs.\textsuperscript{34–36} However, we did not identify any studies that evaluated poloxamer 407 formulations in wound healing in dogs.

Propylene glycol is a commonly used solvent in drug formulations. A 25% propylene glycol hydrogel was noted to have no effect in wound healing in horses.\textsuperscript{37} An in vitro study\textsuperscript{38} analyzing an antifungal hydrogel formulation on rat skin noted improved transdermal drug permeation with the addition of propylene glycol and N-methyl-2-pyrrolidone. To the authors’ knowledge, there are no studies evaluating the use of topical propylene glycol or N-methyl-2-pyrrolidone for treatment of wounds in dogs. Additional studies are needed to evaluate whether one of these ingredients causes a delay in wound healing in dogs and whether a reformulation would improve the product described in the present study.

Sildenafil was detected in the plasma of 1 dog on day 14 and 4 dogs on day 21 in the present study. A previous study\textsuperscript{39} evaluating plasma concentrations in dogs that received 1 mg of sildenafil/kg by mouth once reported a maximum plasma sildenafil concentration of 117 ng/mL. Another study\textsuperscript{40} found a maximum plasma sildenafil concentration of 124.9 ng/mL.

Discussion

The topical sildenafil hydrogel formulated in this study was consistent at room temperature, had adequate spreadability for wound application, and was nonirritating in dogs. Topical sildenafil promoted early granulation tissue formation, which is consistent with the increase in granulation tissue reported for topical sildenafil in rats and for oral sildenafil in dogs.\textsuperscript{12,19,20} More organized granulation tissue was also noted with topical sildenafil in rats.\textsuperscript{22} Granulation tissue provides protection of the wound bed and is particularly beneficial for secondary wound closure, for which a granulation bed is desired prior to closure. Further study is needed to determine whether secondary wound closure and subsequent healing is improved with use of this hydrogel formulation.

The base hydrogel formulation (G) used in the present study delayed wound closure, but rate of contraction was improved in a dose-dependent manner with the addition of sildenafil in the 5S and 10S formulations. Studies\textsuperscript{14,19,20} in other species have shown a dose-dependent effect of topical sildenafil in wound healing. The G formulation also had a lower rate of epithelialization. Rate of epithelialization was improved with the 5S and 10S formulations, but differences between the G, 5S, and 10S formulations were not statistically significant.

HAISs, which are indicative of acute inflammation, were lower for C wounds, compared with scores for 5S wounds, on day 3, which coincided with the significantly less edema seen histologically on day 3, compared with the appearance of the G, 5S, and 10S wounds. This may represent a greater inflammatory reaction to the hydrogel in the early stages of wound healing. HAISs were significantly lower for G and C wounds, compared with both 5S and 10S wounds, on day 21, which may represent an inflammatory reaction to sildenafil in the later stages of wound healing. HRSS, which are indicative of reparative features, were lower for C wounds than for G wounds on day 3 and lower than scores for 5S and 10S wounds on day 7, supporting reparative effects of the sildenafil-containing hydrogel. The timing of treatment is likely an important factor when using a topical sildenafil hydrogel for wound healing. Higher HAISs and HRSS for 5S and 10S wounds in the early stages of healing may have been a sign of immunomodulatory effects of the topical product in the wound bed. These findings, in conjunction with promotion of granulation tissue, suggest that the product may be most beneficial during the inflammatory phase of wound healing, which is typically the first 3 to 5 days. The delayed effects on wound contraction and epithelialization may indicate that this product is not ideal for the later phases of wound healing, although its effects may be improved with a different base formulation. Additional research is needed to determine the ideal timing and frequency of application.

21, 4 dogs had a plasma sildenafil concentration > 0 (28.57, 69.10. 185.36, and 198.13 ng/mL).
in Beagles given 20 mg by mouth once and maximum concentrations of 366.5 to 831.0 ng/mL when Beagles were given 60 mg (variable-release tablets) by mouth once. Body weights of the dogs in those studies were not provided, but Beagles typically weigh 10 to 15 kg, as was the case in the present study, which would mean that these doses were approximately 1 to 2 and 4 to 6 mg/kg, respectively. The typical dosage of sildenafil used to treat pulmonary hypertension is 1 to 3 mg/kg, PO, every 8 to 12 hours. In the present study, dogs received 5 to 15 mg of sildenafil/kg topically every day (i.e., 5 to 7.5 mg/kg for 0.5 mL of the 5S and 10S formulations and 10 to 15 mg/kg for 1 mL of the 5S and 10S administration). Daily administration of the sildenafil hydrogel led to systemic concentrations of sildenafil potentially within a therapeutic range for pulmonary hypertension for 1 dog at day 14 and 2 dogs at day 21. Despite this, no clinical signs or adverse effects were noted in any dog in this study. All plasma concentrations of sildenafil were within safe ranges.

Limitations of the present study include the small sample size, the use of surgically created wounds, and the limitations in masking associated with a change in the hydrogel color with the addition of sildenafil. Additionally, the use of small biopsy samples taken toward the periphery of each wound may have limited our histopathologic findings.

This study found that topical application of a sildenafil hydrogel promoted early granulation tissue formation in skin wounds of Beagles. The base hydrogel formulation (without sildenafil) delayed wound contraction and epithelization, which sildenafil overcame in a dose-dependent manner. Daily topical administration of sildenafil may lead to therapeutic plasma sildenafil concentrations in some dogs. Further studies are needed to determine the most effective sildenafil hydrogel formulation and the ideal time for application.

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References

Supplementary Material

Supplementary materials are posted online at the journal website: avmajournals.avma.org