Effect of topical application of diclofenac liposomal suspension on experimentally induced subcutaneous inflammation in horses

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Objective—To determine whether 1% diclofenac liposomal suspension (DLS) ointment would be absorbed transdermally and attenuate experimentally induced subcutaneous inflammation in horses.

Animals—7 healthy adult horses

Procedure—Inflammation was produced by injecting 1% sterile carrageenan into subcutaneously implanted tissue cages 8 hours before (time –8) and at the time of application of test ointment. A crossover design was used. Horses received 1 of 2 treatments (topically administered control or DLS ointments) during 48 hours of carrageenan-induced subcutaneous inflammation. A single application of test ointment (7.2 g) was applied over each tissue cage (time 0). Samples of transudate and blood were collected at –8, 0, 6, 12, 18, 24, 30, 36, and 48 hours. Plasma and transudate diclofenac concentrations were determined by use of high-performance liquid chromatography. Transudate concentrations of prostaglandin E_2 (PGE_2) were determined with a competitive enzyme immunoassay.

Results—DLS was absorbed transdermally. The highest concentration (mean ± SEM, 76.2 ± 29 ng/mL) was detectable in tissue-cage fluid within 18 hours after application. Minimal concentrations of diclofenac were detectable in plasma. Application of DLS significantly decreased transudate concentrations of PGE_2 at 6 and 30 hours. Decreases in PGE_2 concentration were observed in the DLS group at all collection times.

Conclusions and Clinical Relevance—A single topical application of DLS resulted in concentrations of diclofenac in transudate within 6 hours and significantly attenuated carrageenan-induced local production of PGE_2. Results of this study suggest that DLS is readily absorbed transdermally and may be efficacious for reducing subcutaneous inflammation in horses. (*Am J Vet Res* 2004;65:271–276)

Musculoskeletal injuries in horses are a common cause of poor performance or loss of athletic function. Depending on the severity, these injuries can result in delayed return to performance or return at a lower plane of performance. More than 50% of horse farms in the United States had 1 or more lame horses during the preceding 12 months.[1] The incidence of breakdown injuries in the racing population that involve soft tissue injuries ranges from 5% to 61% depending on type of horse, age of horse, track surface, and geographic location.[2] Musculoskeletal injuries result in substantial economic losses to the equine industry. The most common soft tissue injuries in equine athletes are tendonitis of the superficial and deep digital flexor tendons and desmitis of the suspensory ligaments.[3]

Management of the inflammation associated with these conditions is a challenge to equine practitioners. **Nonsteroidal anti-inflammatory drugs (NSAIDs)** are traditionally the most commonly used systemically administered chemotherapeutics in horses with musculoskeletal pain, with phenylbutazone being the cornerstone since its introduction in the 1950s.[4-7] Unfortunately, the use of high doses of NSAIDs for a prolonged period, or concurrent use in conditions such as dehydration, can result in adverse effects such as gastrointestinal ulcers and renal papillary damage.[7-9] Therefore, given the narrow safety index for therapeutic use of commonly administered NSAIDs, topical administration of NSAIDs may provide an alternate route of administration for the alleviation of pain and lameness associated with musculoskeletal conditions in horses. Development of an effective and safe NSAID for topical administration may be of tremendous therapeutic and economic benefit in the treatment of performance horses with acute and subacute musculoskeletal diseases, joint synovitis, or osteoarthritis.

Diclofenac sodium is a potent dual **cyclooxygenase (COX)** and lipoxygenase inhibitor that has been extensively studied in humans.[10,11] It is commonly administered orally, but IV, IM, and topical routes of administration have also been evaluated.[11] Diclofenac sodium in various topical formulations has been readily absorbed at therapeutic concentrations during in vitro and in vivo studies[12-14] in humans and in vivo studies[15] in rodents. To the authors' knowledge, transdermal absorption of diclofenac sodium in a liposomal suspension and its efficacy in attenuating a local inflammatory response have not been evaluated in horses.

Subcutaneously implanted tissue cages have been used to investigate mechanisms, time course, and severity of acute nonimmune inflammatory responses in many species.[16-20] In those studies, injection of the tissue cages

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with a mild irritant (ie, carrageenan) generated an acute inflammatory response. Serial measurements of inflammatory mediators, leukocyte influx, and protein concentration of the inflammatory exudate have been used to quantitate the magnitude and duration of the inflammatory response.[16,21] Furthermore, tissue-cage models have been used to determine the ability of various systemically administered therapeutic agents to attenuate the inflammatory response. Specifically, these techniques have been used extensively to investigate the mechanism of action of steroidal agents and NSAIDs.[18,22-27] In pharmacokinetic and efficacy studies[22,24] of various NSAIDs, decreased concentrations of **prostaglandin E_2 (PGE_2)** in tissue-cage exudate have been used as an indicator of inhibition of COX-1 and -2.

The purpose of the study reported here was to evaluate the ability of a topically administered NSAID (ie, 1% **diclofenac sodium liposomal suspension [DLS]** ointment), to minimize experimentally induced subcutaneous inflammation in horses. We hypothesized that topically applied DLS would be absorbed transdermally and would substantially attenuate experimentally induced subcutaneous inflammation in horses.

Materials and Methods

Animals—Seven adult horses (5 mares and 2 geldings, consisting of 5 Quarter Horses, 1 Quarter Horse crossbreed, and 1 Thoroughbred) were used in the study. Mean ± SD age of the horses was 13.1 ± 9.1 years (range, 4 to 26 years), and mean weight was 452 ± 42 kg (range, 386 to 508 kg). A routine physical examination was performed, and all horses were determined to be healthy. Horses were housed separately in stalls during the study, and coastal Bermuda hay and water were provided ad libitum. All procedures were approved by the university animal care and use committee.

Implantation of tissue cages—We used a method for inducing acute inflammation and collecting subcutaneous inflammatory exudate that has been described elsewhere.[17,19,21,28,29] Multiple-perforated tissue cages were fabricated from medical-grade silicone tubing[a] (inner diameter, 9.5 mm). Tubing was cut into pieces (each piece was 4 cm in length), and 2 staggered rows (10 holes/row) were made in each end of the tubing by use of a 3-mm biopsy punch. Ends of the tissue cages were then crimped and sealed with silicone adhesive. Tissue cages were packaged and sterilized by use of gas plasma sterilization.[b]

Four weeks before initiation of the study, each horse was administered a single dose of procaine penicillin G (22,000 U/kg, IM) and phenylbutazone (4.4 mg/kg, IV). Tissue cages were implanted in standing sedated horses by use of detomidine (40 µg/kg, IV) and locally administered anesthetic (2% solution of lidocaine hydrochloride). Eight tissue cages were implanted in a row in the subcutaneous tissues on each side of the neck of each horse (16 cages/horse). Blunt dissection was used to create subcutaneous pouches 5 cm apart on the horizontal axis of the neck. The sterilized tissue cages were inserted, and primary closure of incisions was achieved by use of 2-0 polypropolene.[c] Incision sites were allowed to heal before experimental procedures were performed.

Experimental design—A crossover design was used in which horses received a single application of 1 of 2 treatments (control ointment or 1% DLS[d] ointment) 8 hours after initiation of an experimentally induced acute inflammatory response. Horses were randomly assigned an initial treatment (DLS or control ointment). After a 4-week wash-out period, the other treatment was applied to each horse.

A 5 × 5-cm area over each tissue cage was prepared by clipping the hair and cleansing with 2% chlorhexidine gluconate and isopropyl alcohol. At the initiation of each test period, an acute inflammatory reaction was induced by injection of 0.5 mL of 1% sterile carrageenan[e] solution into each tissue cage of each horse. The inflammatory reaction was maintained by an additional 0.5-mL injection of carrageenan 8 hours after the initial injection. Immediately after the second injection of carrageenan, a 7.6-cm strip (7.2 g) of the appropriate ointment was topically applied to the skin over each tissue cage (time 0). Total dose of diclofenac sodium applied to treatment horses was approximately 1.15 g (7.2 g/tissue cage × 16 tissue cages/horse × 1 g of diclofenac sodium/100 g of ointment). Investigators did not have knowledge of the treatments administered to each horse during each test period.

Transudate was collected from tissue cages by direct-needle aspiration. A 20-gauge, 3.75-cm needle and 3-mL syringe were used for collections. Transudates were collected immediately before the first carrageenan injection (time –8); immediately before the second carrageenan injection and application of ointment (time 0); and 6, 12, 18, 24, 30, 36, and 48 hours after application of the ointment. For the sample obtained at time 0, ointment was applied to the skin overlying the tissue cages, and then the entire site was immediately cleansed and aseptically prepared by use of 2% chlorhexidine gluconate and isopropyl alcohol, effectively removing any ointment before the sample was obtained. This procedure was performed to identify and quantitate possible contamination of the transudate with residual diclofenac on the skin surface.

Aspiration sites for the tissue cages were aseptically prepared by use of 2% chlorhexidine gluconate and isopropyl alcohol before sample collection to prevent bacterial contamination of tissue cages, and minimize transdermal contamination of the sample with residual ointment on the skin surface. Transudates were harvested aseptically in duplicate at each time point (samples obtained from 2 separate cages/time point) by direct-needle aspiration; samples were then immediately placed into tubes containing 12.5 µL of 100mM EDTA and 10mM meclofenamate to inhibit ex vivo production of eicosanoids. Samples were obtained from each tissue cage only once during each treatment period, and a consistent order of sample collection was used throughout the experimental period. All transudate samples were stored at –70°C until diclofenac and PGE_2 concentrations were assayed. A blood sample (10 mL) was obtained from a jugular vein of each horse and placed into lithium heparin tubes at –8, 0, 6, 12, 18, 24, 30, 36, and 48 hours. After centrifugation (2,000 × *g* for 5 minutes at 4°C), plasma was harvested and stored at –70°C until analyzed for drug concentrations.

Diclofenac assay—Concentrations of diclofenac sodium in transudate samples and plasma were measured as described elsewhere.[30-32] Briefly, 50 µL of 2.5 N H_3PO_4 and 20 µL of a stock solution (10 µg/mL) of flurbiprofen were added as an internal standard to 100 µL of transudate or plasma. Methylene chloride (2 mL) was then added, and the mixture was vortexed and centrifuged (2,000 × *g* for 20 minutes at 4°C). The organic layer was transferred to a clean tube and evaporated at 25°C by use of a stream of nitrogen. The residue was reconstituted with 200 µL of mobile phase consisting of a 50:50 (vol:vol) mixture of acetonitrile and 25mM H_3PO_4 (pH, 3.0). Samples (50 µL) were then injected onto a C^{16} column[f] (150 × 4.6 mm; inside diameter, 5 µm) for high-performance liquid chromatography[g] with an acetonitrile-25mM H_3PO_4 (50:50 solution [vol:vol]) mobile phase (pH, 3.0) at a flow rate of 1 mL/min. Wavelength for the detector was 280 nm. Peak concentrations were determined by comparing concentrations in samples with concentrations on a

standard curve created by spiking samples of tissue fluid (ie, transudate) and plasma with known amounts of diclofenac. Sample recovery was corrected by comparison with recovery of the flurbiprofen internal standard.[30-32] Minimum detectable concentrations of diclofenac for the plasma and transudate samples were 1 ng/mL. Intra-assay variation was < 5% for transudate and plasma samples.

PGE_2 assay—Concentrations of PGE_2 in transudates were determined by use of a competitive enzyme immunoassay.[h] Briefly, frozen samples were thawed at 25°C and centrifuged at 3,000 × *g* for 10 minutes. Assay buffer was used to make serial dilutions of samples. Sample aliquots were added to duplicate wells on an assay plate. Assay reagents were added, and plates were incubated for 18 hours at 4°C. Developer reagent was added, and the plates were evaluated spectrophotometrically[i] at 405 nm. Color intensity was inversely proportional to the amount of PGE_2 contained in a sample.

Data analysis—Mean ± SEM was calculated for all data collected. Data were logarithmically transformed before statistical analysis. Comparison of transudate and plasma concentrations of PGE_2 between time points was performed by use of a repeated-measure ANCOVA, with tissue-cage concentrations of PGE_2 at time 0 as the covariate and treatment group as the explanatory variable. Comparisons between treatments were made by use of least-squared means. Tissue-cage concentrations of diclofenac were compared by use of paired *t* tests. Significance for all tests was set at a value of $P < 0.05$.

Results

Tissue cages and sample collection procedures were tolerated well by all horses, and discomfort associated with the implanted tissue cages was not observed. Implantation sites of tissue cages healed without complication in all horses. Throughout the course of the 2 treatment periods, 3 of the 112 (2.7%) tissue cages became infected and were surgically removed. Four to 6 hours after carrageenan injection, a localized, well-circumscribed plaque of edema developed at the site of the tissue cages, but it completely resolved by 48 hours.

We were consistently able to aspirate between 0.5 and 1 mL of clear, straw-colored transudate from the tissue cages at each time point. A few of the samples were slightly contaminated with blood. This was most likely a result of redirecting the needle when attempting to locate the fluid pocket within the center of the tissue cages.

None of the horses had evidence of adverse local or systemic reactions after topical application of the control or DLS ointment. The 1% DLS ointment was absorbed transdermally, and the highest mean ± SEM concentration (76.2 ± 29 ng/mL) was detectable in transudates within 18 hours after topical application (**Fig 1**). Diclofenac was detectable in transudate at time 0 (32.2 ± 6.6 ng/mL), even after immediate cleansing and aseptic preparation. Analysis of these data indicates small amounts of sample contamination from residual DLS on the skin surface. Although concentrations of diclofenac at 6, 12, and 18 hours were not significantly different from concentrations at time 0, they did increase slightly at these time points. Given the transdermal contamination as a result of needle penetration through residual DLS on the skin surface, diclofenac concentrations in transudate at all time points > 18 hours were consistent with the aforementioned contamination, suggesting clearance of diclofenac from the transudate after 18 hours to concentrations below residual contamination. Small concentrations of diclofenac were detectable in plasma samples obtained 6 (2.6 ± 1.2 ng/mL), 12 (2.2 ± 1.7 ng/mL), 18 (3.3 ± 2.2 ng/mL), 24 (3.8 ± 2.4 ng/mL), 30 (2.9 ± 2.6 mg/mL), 36 (2.4 ± 2.2 ng/mL), and 48 (4.3 ± 3.6 ng/mL) hours after topical application.

Injection of carrageenan induced a comparable inflammatory response in all horses, as indicated by similar increases in PGE_2 concentrations within horses and among treatment periods (**Fig 2**). Minimal PGE_2 concentrations (0.9 ± 0.2 ng/mL) were measurable in transudate obtained from tissue cages before inflammation was induced. Prostaglandin E_2 concentrations were not significantly different between control (9.7 ±

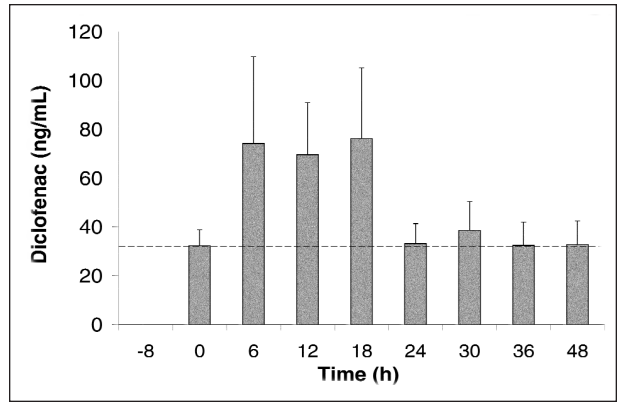

Figure 1—Mean ± SEM diclofenac concentrations at various time points before and after a single application of 1% diclofenac sodium liposomal suspension (DLS) in samples of transudate collected from tissue cages surgically implanted in 7 horses. The DLS was applied topically at time 0. Dashed horizontal line represents diclofenac concentrations that may have been attributable to translocation of residual DLS that remained on the skin.

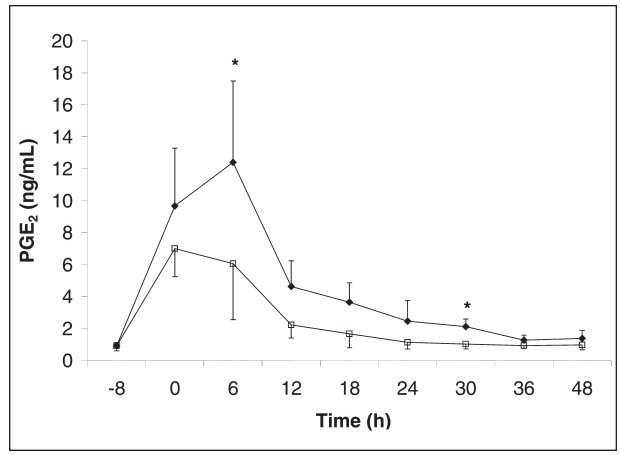

Figure 2—Mean ± SEM prostaglandin E_2 (PGE_2) concentrations at various time points before and after a single topical application of control ointment (solid diamonds) or 1% DLS ointment (open squares) in transudate collected from tissue cages surgically implanted in 7 horses. Tissue cages were injected with 0.5 mL of sterile 1% carrageenan at time –8 and time 0 to induce an acute inflammatory response. The control ointment or 1% DLS ointment was applied topically at time 0. *Within a time point, values differ significantly ($P < 0.05$) between treatment groups.

3.6 ng/mL) and DLS (7.0 ± 1.8 ng/mL) treatments at time 0. Application of DLS significantly decreased carrageenan-induced inflammation at 6 and 30 hours, compared with inflammation for the control ointment.

Discussion

Tissue-chamber methods were adapted by researchers to enable them to investigate the roles of arachidonic acid metabolites in acute inflammation and the mechanisms of action of corticosteroids and NSAIDs in attenuating the inflammatory response.[16,17,19,21,29] Subcutaneous implantation of tissue cages in the neck of horses was tolerated well and resulted in a reliable method that can be used for the evaluation of drug pharmacokinetics. Carrageenan, a seaweed-derived polysaccharide, has been used extensively in a number of species to induce a reproducible, mild, transient inflammatory response that is localized to the site of administration.[16,17,19-21,28,29] Because of the localized, mild nature of the inflammatory response, the tissue-cage method inflicts minimal stress to the animals and is ethically acceptable. Furthermore, sequential sample collection from individual animals and the use of crossover designs minimize interanimal and genetic variation.[19,21,24] This method has been used extensively for determining the efficacy of parenterally administered or topically applied NSAIDs through analysis of transudate samples.

Diclofenac sodium is a phenylacetic acid-class NSAID with potent COX inhibitory activity.[11,33] It also can limit the amount of arachidonic acid entering the COX and lipoxygenase pathways by reducing arachidonic acid release and increasing arachidonic acid uptake.[10,34] Diclofenac is an effective analgesic that is used extensively in the treatment of humans with various arthritic conditions.[11,13,14,33] Unfortunately, it does not spare COX-1, and gastrointestinal complications are the most commonly described adverse effect of the use of diclofenac.[11] An in vitro study[35] of diclofenac in dogs found that it was a nonselective COX inhibitor; however, COX selectivity of diclofenac has not been assessed in horses. The development of an effective topical formulation of diclofenac should be advantageous for the treatment of horses with subcutaneous joint or tendon inflammation while avoiding the adverse effects observed with systemic administration.

A topically applied NSAID must meet various criteria to be clinically efficacious in alleviating subcutaneous inflammation. The compound must be absorbed through the skin and penetrate the deeper tissue layers, and it requires a sufficiently long local residual time to attenuate inflammation between subsequent applications.[36,37] The most superficial layer of skin, the stratum corneum, is the most difficult layer for a topically applied compound to penetrate.[38] Some of the factors that determine effectiveness of an NSAID are the pharmacokinetic and physiochemical properties (eg, drug half-life, molecular mass, ionization, lipophilic-hydrophilic nature, and solubility), formulation of the ointment, site of application, and biological properties of the patient (eg, integrity of the skin and species-specific variations).[36,37]

In the study reported here, a single topical application of 1% DLS ointment resulted in peak transudate accumulations of diclofenac 18 hours after application (Fig 1). Liposomal formulations have been used to target or transport a drug to a specific site of action, localize a drug close to the site of application, or provide a slow-release vehicle for a drug.[37] The active drug is released from the liposomal suspension when the bilayers of the liposome become permeable to the drug or when the bilayer is decomposed. Lipophilic drugs are released when the phospholipid molecules of the outer bilayer are hydrolyzed or oxidized. Hydrophilic drugs are released when the outermost aqueous layer breaks down or the membrane becomes more permeable. Additionally, when a drug is encapsulated in the liposome, it cannot be metabolized; consequently, liposomes may act as a slow-release vehicle.[37] Although the epidermal layer is primarily aqueous, diclofenac sodium is not readily absorbed transdermally.[15,39] Formulating diclofenac sodium in a liposomal suspension substantially increases cutaneous penetration and transdermal absorption of the compound.[40]

Production of transudate inflammatory mediators, leukocyte concentration, protein concentration, and alterations in cutaneous temperature have all been used to quantitate the efficacy of steroidal and NSAIDs in various experimental models.[16,17,24,29] Studies[16,17,20-22] have documented that PGE_2 is released during carrageenan-induced inflammation in ponies. Higgins and Lees[16] documented no apparent relationship between concentrations of PGE_2, thromboxane A_2, and leukocyte numbers by use of a tissue-cage inflammation method in ponies, suggesting other cells or tissues may be involved to a greater extent in production of these mediators. Therefore, in the study reported here, we chose to use local production of PGE_2 as an index of inflammation.

In this study, injection of carrageenan induced comparable inflammatory responses in all horses, as indicated by similar increases in PGE_2 concentrations within horses and among treatment periods. Topical application of DLS significantly attenuated carrageenan-induced local production of PGE_2 at 6 and 30 hours after application. Although significant differences in PGE_2 concentrations were detected only at 6 and 30 hours, PGE_2 concentrations in treated horses were less than those of control horses at all time points after ointment application. It is likely that the significant difference at 30 hours can be attributed to less variation in the samples at this time point. These findings should not be overinterpreted. It is not likely that a single application of DLS remains effective 30 hours after application, and additional studies evaluating multiple applications are necessary to determine the appropriate dosing interval.

Detection of diclofenac sodium within tissue cages at time 0 may be explained by the translocation of residual drug on the skin into samples during aspiration. Ointments were applied to the skin over each tissue cage, including the first cages from which we collected samples (samples obtained at time 0) to account for possible translocation of drug from the skin into samples during sample aspiration. The ointment was applied at time 0, the skin was immediately prepared

for sample collection, and the sample was withdrawn via needle aspiration. It is possible that the diclofenac concentrations measured at time 0 reflect translocation of a small amount of drug within the needle during sample collection and were detected as a result of the sensitivity of the high-performance liquid chromatography. Therefore, the concentrations at 24 through 48 hours may represent residual skin contamination, and the actual concentrations in transudate obtained from the tissue cages are likely to be less than we reported. However, analysis of the data does establish that even after accounting for contamination, DLS was absorbed into the tissue cages between 6 and 18 hours after application at higher concentrations than could be explained by entrance into the tissue cages via the plasma or during sample collection by residual contamination of the skin.

Analysis of results of this study suggests that DLS is readily absorbed transdermally and is effective at reducing subcutaneous inflammation, as indicated by transudate PGE_2 concentrations. On the basis of these findings, DLS may be a useful treatment, alone or in combination, with conventional systemically administered NSAID for the treatment of horses with soft tissue-associated inflammation. Additional studies to evaluate repeated or periarticular application of DLS are needed and may document efficacy for the treatment and management of superficial pain and inflammation associated with musculoskeletal injuries in horses.

[a]Nalgene 50 platinum-cured silicone tubing, Nalge Nunc International, Rochester, NY.
[b]Sterrad, Advanced Sterilization Products, Johnson & Johnson Medical Inc, Irvine, Calif.
[c]Prolene, Ethicon Inc, Somerville, NJ.
[d]Surpass, IDEXX Pharmaceuticals Inc, Greensboro, NC.
[e]Lambda carrageenan, Sigma Chemical Co, St Louis, Mo.
[f]Discovery RP-Amide C16, Supelco, Bellefonte, Pa.
[g]Shimadzu LC-10A HPLC, Shimadzu, Kyoto, Japan.
[h]Prostaglandin E_2 enzyme immunoassay, Caymen Chemical, Ann Arbor, Mich.
[i]Vmax kinetic microplate reader, Molecular Devices, Menlo Park, Calif.

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