Influence of topically applied cold treatment on core temperature and cell viability in equine superficial digital flexor tendons

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Objective—To determine rate and degree of cooling for the superficial digital flexor tendon (SDFT) during a standard cryotherapy application in horses and evaluate in vitro effects of cooling on survival of tendon cells.

Sample Population—6 limbs of 5 adult horses and cultured cells obtained from SDFT of 3 adult horses during necropsy.

Procedure—In vivo data were acquired by use of a thermocouple temperature probe inserted into the SDFT of a forelimb of each standing sedated horse. After baseline temperatures were recorded, a commercial compression splint with circulating coolant was placed on each selected limb, which was then exposed to cold treatment for 60 minutes. Temperatures were recorded at 30-second intervals. Mean minimum core temperature was calculated and used to design a protocol for in vitro cold treatment of cells. Specimens were obtained from the SDFT of horses during necropsy; tendon cells were cultured in suspension and exposed to 1-hour of cold treatment that mimicked the in vivo procedure. Viability of cells after cold treatment was compared with viability of cells maintained at body temperature.

Results—After 1 hour of cold treatment, SDFT core temperature was reduced by a mean of 21.8°C, reaching a mean minimum temperature of 10°C. Viability did not differ significantly between cold-treated and control cells.

Conclusion and Clinical Relevance—Results indicated that topical application of cryotherapy significantly reduced core SDFT temperature in standing sedated horses. Temperatures achieved in vivo during cold treatment were not detrimental to the in vitro viability of tendon cells. (Am J Vet Res 2003; 64:835–844)

Topically applied cold treatment (ie, cryotherapy) is widely used in horses and is commonly recommended by veterinarians for treatment and prevention of various musculoskeletal injuries in horses that range from tendonitis to laminitis. Unfortunately, there is little controlled experimental evidence verifying the beneficial effects of cryotherapy in horses and virtually no agreement on the optimal method of application. Despite this fact, many reports have provided detailed recommendations regarding duration, frequency, or ideal temperature required for cold treatment of specific conditions in horses. Guidelines for cryotherapy of injured tendons and ligaments are often particularly comprehensive, but authors offer conflicting advice. For example, in 1 report, clinicians are cautioned to avoid the use of cold treatments more than 24 to 48 hours after the injury occurred, whereas in another report, clinicians are instructed to treat patients for 30 minutes 3 times/d for at least 4 to 5 days after the injury. Whereas the authors of 1 report suggest that injured tendons be treated with ice for 3 to 4 hours/d for the first 72 hours after injury, an author in another report recommends application of cold for periods of only 20 to 30 minutes. Most of the specific clinical recommendations are extrapolated from human medical protocols, relayed from personal clinical experience, or based on pure conjecture. To further confuse the issue, it has been speculated that cooling of tendons and ligaments may progress faster than that of better vascularized tissues and that particular care must be used to prevent thermal injury to these tissues. However, a specific safe recommendation cannot be made for effective cold treatment of tendons in horses because of the lack of available background information.

Several years ago, it was suggested in an editorial that clinical and basic scientific research was needed to document the effects of cryotherapy in horses. Since that time, a few studies have been conducted to evaluate the effects of cold treatment on skin and tissue temperatures in the limbs of horses. Two reports described the effects of 30 minutes of cold treatment on surface temperature of the distal aspect of the limbs in horses. Those investigators used thermography to document temperature differences between treated and untreated limbs and found that treatment with a nylon-covered gel wrap resulted in a temperature differential of 6°C, the cold effect was sustained for 4 hours, and adjacent surface areas were also cooled. In another study, investigators used indwelling thermistor probes to compare superficial and deep tissue temperatures during 30 minutes of immersion in ice water with temperatures documented during a 30-minute application of a commercial cold pack. That study documented a decrease in deep tissue temperature of 16.3°C for ice-water immersion, and the authors concluded that immersion was significantly more effective for cold treatment of limbs than application of...
the commercial cold pack. In an additional study, investigators evaluated the effects of external application of heat and cold on perfusion of the digits in horses. They reported a mean decrease in laminar tissue temperatures of 11.6°C during a 30-minute period of cold treatment in which a horse's foot was bathed in ice water. Finally, I report described the use of cryother-apy to treat experimentally induced synovitis in horses. Investigators in that study concluded that twice-daily treatment for 2 hours did not effectively control inflammation in horses with lipopolysaccharide-induced synovitis. Despite these reports, more in-depth investigations of the use of cryotherapy in equine athletes are required before specific treatment recommendations can be established.

Although there is little detailed practical information supporting the use of cryotherapy in equine patients, there is a great deal of empirical evidence advocating its beneficial effects in other species, including humans. A number of clinical studies have documented the analgesic and anti-inflammatory effects of topical cold application, particularly for postoperative management of orthopedic injuries. However, despite the clinical evidence of its beneficial effects, there are still many questions about the mechanism of action of cryotherapy for treatment of any mammalian species, and there is little agreement about the length or duration of treatment that should be used or even the best method of application. Experimental evidence obtained from appropriately designed veterinary studies could help to clarify specific guidelines and recommendations regarding duration and frequency of treatment in human patients. In addition to the proposed benefits of topically applied cold treatments, a number of adverse effects have been described. In fact, when physiologic responses to cryotherapy are examined experimentally, many of the reactions that are detected are extremely different from those expected. For example, prolonged application of cold can result in reflex vasodilatation that enhances inflammation and increases edema. Inadvertent thermal injury .10 In addition, many current protocols for cold treatment in horses exceed reported recommendations for humans with regard to duration of exposure and application method. Clearly, there is a need to define effective and safe protocols for cold treatments in equine patients. We hypothesized that use of a commercial cooling device that combined circulation of cold liquids with compression was likely to decrease the temperature of the tendon core to less than 15°C but that the resulting temperatures would be unlikely to affect the viability of resident tendon cells.

The purpose of the study reported here was to measure the core temperature in the superficial digital flexor tendon (SDFT) in the forelimb of horses during 1 hour of topically applied cryotherapy accomplished by use of a clinically accepted combination method of cold treatment and compression. In addition, a second objective was to determine whether temperatures achieved during this hour of cold treatment would adversely affect viability of tendon cells. Overall, the study was designed to quantitate the effects of topical application of cold treatment at the tissue and cellular levels, with the ultimate goal being to improve cryotherapy protocols designed for clinical use in horses.

Materials and Methods

In vivo determination of core temperature of the SDFT during cold treatment—Two sets of experiments were conducted during the study. The first involved determining the effects of cold treatment on core temperature in the SDFT in the forelimbs of horses.

Animals—Five horses (4 geldings and 1 mare) between 7 and 25 years of age (mean, 14.2 years) were included in the study. These horses had no history or clinical evidence of lameness or other musculoskeletal problems. The experimental protocol was approved by the University of California–Davis Animal Care and Use Committee.

Experimental procedure—The right or left forelimb of
each of the 5 horses was randomly selected. Thermocouple temperature-measuring probes were used to measure skin and SDFT core temperature in the midmetacarpal region. In 1 horse, both the right and left forelimbs were tested at 2 separate times; thus, there was a total of 6 limbs tested.

Horses were sedated by IV administration of detomidine (0.01 mg/kg) and butorphanol tartrate (0.01 mg/kg) and restrained in a standing position in stocks. The plantar surface of the metacarpal region was clipped, shaved, and disinfected by use of iodophors and alcohol. A small area of skin overlying the palmar surface of the SDFT at the midpoint between the proximal and distal ends of the third metacarpal bone was infiltrated with 4 mL of a 2% solution of lidocaine hydrochloride, and an 18-gauge, 3.5-in spinal needle was inserted into the core of the SDFT at this location. The thermocouple used to measure tendon temperature was a 635-µm-diameter flexible implantable probe designed for tissue implantation, which was inserted through the spinal needle into the SDFT and manually held in place as the spinal needle was removed from the tendon. The temperature probe was maintained in place for the duration of the experiment by use of a butterfly adhesive of commercial duct tape affixed to the horse. A sterile antimicrobial incision draped was placed around the metacarpus and secured with a preattached adhesive patch. A paper clip was used to secure the remaining length of the probe to the upper part of the forelimb for additional stabilization.

Ultrasoundography was used to confirm appropriate placement of the temperature probe within the core of the SDFT. Ultrasound coupling gel was applied to the region to be evaluated. The SDFT and probe were examined by use of a 7.5-MHz multiple-frequency tendon probe set at 10 MHz; a helmet standoff was used. The tendon and probe were viewed in the transverse and longitudinal planes.

A 1-m-long copper constantan disposable thermocouple was attached to the freshly shaved palmar surface of the metacarpus, secured with a preattached adhesive patch. A sterile antimicrobial incision drape was placed around the metacarpus to protect the temperature probe from contamination during the application of cold treatment. Rectal temperature was recorded by use of a custom-built 3-m-long flexible thermocouple that was 635 µm in diameter. The temperature probe was inserted into the rectum of the horse and secured to the base of the tail with tape.

Thermocouple probes for the rectum, skin, and SDFT were attached to extension cables and plugged into a 16-channel adapter that transmitted multiplex data to a thermometer data acquisition card installed in a personal computer. The data card recorded thermocouple voltages, and associated spreadsheet converted these values to temperature measurements with stability of 0.1°C. A paper clip was used as a metal conductor to record ambient room temperature via the in-line thermometer every 2.5 minutes. Degree of sedation in each horse was monitored during application of cold treatment, and additional sedative was administered as needed.

After 1 hour of cold treatment, the cooling-and-compression splint and all temperature probes were removed, and the distal portion of the forelimb was wrapped in sterile disposable bandage material. These support wraps were left in place for 12 hours after the application of cold treatment, and the horses were monitored for 24 hours after treatment. At 12 and 24 hours after the 60-minute cold treatment, horses were observed while walking and trotting in a straight line to detect visible signs of lameness. In addition, the treated limb was palpated to detect swelling, heat, or sensitivity to digital pressure applied over the previously treated SDFT.

Statistical analysis—Mean, median, and SD values of temperatures recorded at each site were calculated for each of the 6 limbs. Temperature data were fitted to a regression curve by use of polynomial regression analysis, and regression coefficients were determined. This allowed curvilinear relationships to be established among horses, time points, and skin and core tendon temperatures. For all analyses, values of \( P < 0.05 \) were considered significant.

In vitro determination of viability of tendon cells during cold treatment—The second set of experiments was conducted to determine viability of cells obtained from the SDFT and exposed in vitro to cold treatment.

Collection and culture of tendon cells—One forelimb was harvested from each of 3 recently euthanized horses. These horses had no history of musculoskeletal problems or clinically detectable evidence of tendon injury. The selected forelimb of each cadaver was amputated at the distal aspect of the radius. The metacarpus was shaved and aseptically prepared by use of iodophors and alcohol, and the amputated limb was placed in a protected enclosure for tissue culture dissection. Skin overlying the middle portion of the metacarpus was reflected by use of an 1-shaped incision along the palmar surface of the SDFT, and subcutaneous connective tissues were separated from the underlying tendon. A 5-cm section of the SDFT was collected from a point midway between the proximal and distal ends of the third metacarpal bone. The tendon segment was immediately placed into a 50-mL polypropylene centrifuge tube that contained 30 mL of tissue culture maintenance medium consisting of Dulbecco’s modified Eagle medium-Ham’s F-12 nutrient medium (DMEM F-12) supplemented with 5% fetal calf serum, 2.5 µg of amphotericin B/mL, 50 µg of ascorbate/mL, 100 U of penicillin/mL, 100 µg of streptomycin/mL, and 50 µg of gentamicin sulfate/mL.

The tendon segment was transferred to a class-II laminar-flow hood and placed in a 90-mm culture dish that contained fresh tissue culture maintenance medium. A scalpel blade was used to cut the tendon specimen into fragments that consisted of 0.5-cm cubes. Tendon fragments were rinsed with 30 mL of fresh DMEM F-12 and transferred to a 50-mL centrifuge tube. Twenty milliliters of filter-sterilized maintenance medium containing 0.1% (wt/vol) type-XIV bacterial protease was added, and tendon fragments were incubated at 37°C with gentle agitation for 1 hour. Then, the...
solution was replaced with 30 mL of filter-sterilized maintenance medium containing 0.1% (wt/vol) type-II collagenase, and fragments were incubated overnight.

Tendon cells were centrifuged (1,500 X g for 5 minutes), harvested, resuspended in 20 mL of fresh DMEM F-12, and filtered through a sterile 70-µm nylon cell strainer to remove extracellular matrix and debris. Tendon cells were pelleted by use of the same centrifugation protocol, resuspended in maintenance medium, and counted in a Neubauer hemacytometer. Cells were plated on a 90-mm tissue culture plate at a concentration of 1 X 10^4 cells/mL. After incubation for 24 hours, the cells were adherent and had the characteristic appearance of fibroblasts. Once tendon cells reached confluence, they were released by incubation with 0.05% trypsin in sterile PBS solution, pelleted by centrifugation (230 X g for 5 minutes), resuspended in maintenance medium, and transferred in 1-mL aliquots to 1.5-mL (39 X 10-mm) microcentrifuge tubes (final concentration, 2 X 10^6 cells/mL).

**Exposure to cold treatment**—Six microcentrifuge tubes were used for each of the 3 horses. Three control tubes were incubated at 37°C without agitation for 1 hour. The remaining 3 tubes were exposed to cold treatment without agitation for 1 hour in a thermodielectric cold-block incubator set at 10°C. After 1 hour of exposure to the designated temperature, cell suspensions from each of the 6 microcentrifuge tubes were transferred to separate wells in a 24-well tissue culture plate and incubated for an additional 24 hours at 37°C in a humidified atmosphere of 5% CO2 and 95% air. During this period, viable cells formed an adherent monolayer on the plate surface.

Rate of temperature change in the microcentrifuge tubes exposed to cold treatment was determined by placing 3 separate microcentrifuge tubes containing 1 mL of tissue culture medium into the thermodielectric cold-block incubator, setting the unit at 10°C, and measuring the temperature of the medium by use of a 635-µm-diameter flexible thermocouple probe. The medium was exposed for 1 hour with core temperatures recorded at 30-second intervals; medium was then removed from the incubator and warmed at 23°C for an additional 30 minutes. This step was used to ensure that the rate of temperature reduction in the tendon cell cultures resembled that detected in the SDFT during the in vivo phase of the study.

**Assay of cell viability**—After the 24-hour incubation, remaining viable adherent cells were quantified by use of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Supernatant medium was removed from each treatment and standard well and replaced with 0.5 mL of filter-sterilized DMEM F-12 medium containing 0.6 mg of MTT dye/mL. The 24-well plate was wrapped in aluminum foil to exclude ambient light and incubated for 1 hour at 37°C. Following incubation, the dye was aspirated from each well and discarded. Two milliliters of dimethyl sulfoxide and 0.1 mL of Sorensen buffer (consisting of 0.1M glycine and 0.1M sodium chloride [pH, 10.5]) were added sequentially to each well. Uniform mixing of the reagents and cells was ensured by placing the plate on an orbital shaker at 4 X g.

Samples (200 µL) from each well were placed in triplicate into a 96-well microplate, and absorbance was measured at 570 nm on a microplate reader. Absorbance values were converted to relative cell concentrations by use of reference to values for a standard curve. New standard curves for cell concentration were created for each of the 3 horses by plating cells in a range of concentrations between 1.25 X 10^4 and 1.0 X 10^6 cells/mL.

**Statistical analysis**—All statistical analyses were performed by use of standard software. Alterations in cell viability associated with cold treatment were determined by comparing the concentrations of viable adherent cells remaining following incubation at 10°C with concentrations of viable adherent cells remaining following incubation at 37°C. A 2-way, cross-classified ANOVA with interference was performed to evaluate significance, and when a significant difference was detected, post-hoc Student t tests were used to determine differences between groups. For all analyses, values of P < 0.05 were considered significant.

**Results**

**In vivo determination of SDFT core temperature during cold treatment**—None of the horses had signs of discomfort during insertion of the microprobe or the subsequent 60-minute period of cold treatment. However, horses were sedated during the procedures. Ultrasonography confirmed accurate placement of the thermocouple probe within the core of each SDFT at a point midway between the proximal and distal ends of the third metacarpal bone. Each probe remained in place for the duration of the treatment period and was not displaced by movements of the horses in the stocks.

Each of the 6 limbs had a similar pattern for the response to cold treatment (Fig 1). Specifically, core temperature of the SDFT and temperature of the palmar metacarpal skin surface were similar at the outset of treatment, but skin and tendon temperatures decreased significantly within 5 minutes after onset of cold treatment. However, in all cases, core temperature of the SDFT decreased more rapidly and was lower than that of the skin surface within a mean time of 2 minutes. Absolute difference between tendon and skin surface temperatures varied among horses, but tendon temperature was consistently lower than skin temperature during cold treatment.

Data for the 6 limbs were compiled. Mean ± SD core temperature of the SDFT prior to cold treatment was 32.2 ± 1.6°C, and mean temperature within the SDFT core after 60 minutes of cold treatment was 10.4 ± 3.7°C, which represented a mean decrease in temperature of 21.8°C (Fig 2). As expected, there was a sig-

![Figure 1](https://example.com/figure1.png)
significant association between time and temperature, with temperature decreasing in a predictable pattern over time. There also was significant variation among horses, suggesting that each horse is likely to respond differently to identical cold treatment. Based on regression analysis, a regression coefficient was calculated (mean ± SEM, 0.008894 ± 0.00027), and the mean predicted decrease in SDFT temperature for 1 hour of cold treatment with the cooling-and-compression splint was 14.0°C. Temperature of the SDFT decreased significantly for the first 45 minutes of cold treatment, after which time the temperature maintained a plateau.

Mean ± SD temperature of the palmar metacarpal skin surface prior to cold treatment was 31.4 ± 2.0°C, and mean temperature decreased to 11.8 ± 4.5°C after 60 minutes of cold treatment. Similar to the pattern for tendon temperature, there was a significant association between time and temperature, and the temperature decreased significantly for the first 45 minutes of cold treatment before stabilizing; mean ± SEM regression coefficient was –0.77022 ± 0.0169.

Cold treatments and temperature recordings were performed on 6 separate days during the course of a 2-month period. Although all treatments were performed in the same enclosed air-conditioned facility, ambient room temperature did vary (range, 21.7 to 28.5°C; mean ± SD, 24.8 ± 2.5°C). However, room temperature did not change substantially during the course of the 60-minute treatment for each limb, and we did not detect a correlation between room temperature and tendon temperature for these experimental conditions.

Rectal temperature ranged from 36.9 to 37.6°C (mean, 37.3°C) and was not correlated with room temperature. Furthermore, rectal temperature did not decrease significantly during the course of the 60-minute cold treatment applied to the distal portion of the selected limb.

During the 24-hour observation period following the 60-minute cold treatment, none of the horses had adverse effects from insertion of the probes or application of cold treatment. We did not detect evidence of lameness, and none of the horses developed swelling or edema of the selected forelimbs.

Cell viability during in vitro cold treatment—Temperature of cultured tendon cells exposed to cold treatment in a cold-block thermoelcetric incubator was monitored (Fig 3). An MMT assay was used to monitor cell viability (Fig 4). Viability of cultured tendon cells exposed to cold treatment (10°C for 1 hour) was not significantly different from that of cells maintained at 37°C. Specifically, tendon cells from each of the 3 horses yielded a mean ± SD cell concentration following cold treatment of 1.99 ± 0.47 × 10⁴ cells/well, whereas mean cell concentration of tendon cells from aliquots not exposed to cold treatment was 1.88 ± 0.28 × 10⁴ cells/well. These concentrations of viable cells did not differ significantly.

![Figure 2](image1.jpg)
Figure 2—Mean ± SD temperatures recorded for the skin surface of the plantar aspect of the metacarpus and superficial digital flexor tendon (SDFT) during a 60-minute cold treatment in 6 forelimbs of 5 clinically normal horses. Temperatures were measured at 30-second intervals by use of thermocouple probes applied directly to the skin surface or inserted in the tendon core but are reported at 2.5-minute intervals.

![Figure 3](image2.jpg)
Figure 3—Representative results for rate of cooling and warming of suspensions of tendon cells exposed in vitro to cold treatment. One milliliter of tissue culture medium containing 2 × 10⁴ cells/mL was pipetted into a microcentrifuge tube (39 × 10 mm), maintained at 10°C for 60 minutes in a thermoelectric cold-block incubator, and then allowed to warm for 30 minutes at room temperature. Cells were subsequently assayed to determine viability and cell survival.

![Figure 4](image3.jpg)
Figure 4—Standard curve for a dye-based viability assay of tendon cells. First-passage equine tendon cells cultured as monolayers in densities ranging from 1.25 × 10⁴ to 10 × 10⁴ cells/mL were exposed to tetrazolium dye, and formazan production was measured as absorbance at a wavelength of 570 nm. OD = Optical density.
Discussion

Analysis of results of the study reported here suggested that the SDFT can be cooled effectively during a 1-hour period of cold treatment to temperatures below those that reportedly provide analgesia and decrease metabolic enzyme activity. Furthermore, these temperatures were apparently maintained without causing detrimental effects. In addition, analysis of results for the in vitro portion of the study suggested that the minimum temperatures achieved in the tendon core during that 1-hour treatment period would be unlikely to affect viability of tendon cells.

When equine athletes participate in strenuous competitions, such as racing, the SDFT and other supporting soft-tissue structures in the metacarpal region are prone to serious injury. These injuries typically consist of lesions within the tendon core that initiate an inflammatory response characterized by an increase in blood flow, edema, and subsequent pain. Topically applied cryotherapy is frequently prescribed to minimize the potentially adverse and painful effects of the associated inflammatory response. However, there is surprisingly little evidence of the efficacy of cold treatment in equine patient, and we are unaware of studies documenting optimal frequency or duration of treatment. Nevertheless, many veterinary textbooks provide detailed recommendations for topical application of ice without an explanation as to how those protocols were established. In 1 text, it even states that anecdotal evidence suggests percutaneous cryotherapy may enhance soft-tissue healing in horses. In contrast, the potential misuse of cold treatment as an analgesic has also created concern in equine athletes.

There is little published guidance in human medical literature regarding an optimal method or duration of cold treatment application. The apparent controversy and confusion in clinical practice surrounding the therapeutic benefits and application protocols of cold modalities strongly suggest that more information is needed to help equine clinicians improve clinical recommendations and enhance the benefits obtained from use of cryotherapy protocols in equine athletes.

The study described in this report provides specific temperature data for 1 cryotherapy technique applied to the limb of horses. This study also provides a simple, minimally invasive method of temperature measurement that could be readily adapted for more extensive comparisons of various cold treatment techniques. Core temperature of the SDFT in the midmetacarpal region was selected for this study because of the prevalence of serious injuries in this location. We were interested in documenting tissue temperatures in an area of the limb that would be treated clinically. In addition, we believed that surface skin temperatures alone did not accurately reflect temperatures in deeper tissues. Thus, skin surface temperature was simultaneously recorded to allow subsequent comparisons. Analysis of the data supports the fact that surface skin temperature during cold treatment parallels that documented in deeper tendon tissues in the distal portion of the limb of horses, but it also indicates that measuring skin temperature alone is likely to underestimate the temperature decline of underlying tendons.

As a result, thermography may not be an accurate measure of the efficacy of cold treatment for affected limbs of horses, and owners should be cautioned that skin temperature may not accurately reflect the temperatures of deeper tissues.

The striking decrease in temperature of the tendon core documented in this study can be explained by the lack of subcutaneous fat or other insulating tissues overlying the flexor tendons in this part of the equine limb and by the relatively poor vascularity of this tissue. Similar experiments to measure muscle temperatures in the more proximal portions of the forelimb would be likely to produce extremely different results because of the delayed cooling and protective vasodilatory effects anticipated in that region. In a study in humans, skin surface temperature was found to be a weak predictor of intramuscular temperature during cryotherapy. An alternative explanation for the lower temperatures recorded in the tendon core of horses in our study would be that the thermocouple probes themselves were acting as a cold sink and conducting cold directly to the tendon core. However, the thermocouples used in this study were specifically selected on the basis of their design for negligible thermal conduction, which minimized this source of error. The thermocouples were encased in a fluoride coating, which has a thermal resistive value of 190 that allows it to serve as a thermal insulator.

Cryotherapy is believed to reduce pain, swelling, and inflammation by several mechanisms including vasoconstriction, reduction in cellular metabolic demand, reduction of inflammatory mediators, reduction of nerve conduction velocity, and enhancement of collagen stiffness. Evaluation of the clinical implications of the results of the study reported here requires reference to established critical amounts of tissue cooling required for effective treatment. For instance, 1 of the major purported benefits of cold treatment is the associated reduction in tissue metabolism, cell activity, and enzyme reactions. Metabolic enzymatic activity decreases by half for every 10°C decrease in tissue temperature, and temperatures between 10 and 11°C have been suggested for effective inhibition of metabolism and minimization of secondary tissue damage. In addition, local analgesic benefits are believed to result from the apparent cooling of deeper tendons. Extrapolation of these critical temperatures to horses implies that the temperatures reached in the SDFT by use of cryotherapy in accordance with this experimental protocol would be expected to have clinical benefit for inflamed tissues.
Unfortunately, in contrast to the beneficial effects of cryotherapy, there appears to be a crucial temperature below which additional tissue cooling causes adverse effects including local tissue damage, frostbite, and nerve palsy. Determining a safe temperature for clinical use has proven to be controversial: the temperature at which deleterious effects may be observed ranges from 25 to 10°C. Motor performance is impeded at temperatures of ≤18°C, whereas inflammation and edema reportedly increase, with an accompanying increase in lymphatic permeability, at temperatures <15°C. In addition, cold treatment can influence tissue deformity by increasing collagen stiffness, resulting in a loss of dexterity and increased susceptibility to injury; however, to our knowledge, a critical temperature has not been established for that complication. Based on those reports from the human literature, the temperatures recorded from the SDFT of horses in the study reported here would be expected to result in some tissue damage. Although we did not identify any post-treatment complications in this study, the limbs were wrapped for 12 hours following cold treatment, and these wraps may have prevented obvious swelling and edema. In addition, motor performance was not evaluated, because the horses were sedated during the course of the experiment. Furthermore, although the in vitro portion of the study did not document cell death in equine chondrocytes exposed to cold treatment at 10°C for 1 hour, additional studies would be required to ensure that adverse effects of this cold treatment were not obscured by the study design.

Equipment that was selected to provide cold treatment for this study incorporated temperature-regulated continuous flow of coolant and compression techniques in 1 commercially available unit. Continuous-flow cold treatment was selected, because it is superior to the use of crushed ice for control of postoperative pain in human patients. Also, it provided us with the ability to monitor and record the temperature at the point of delivery. Many of the reusable gel-filled cold packs currently being marketed have noticeable temperature fluctuations as the product begins to warm immediately after contact with the skin, and the heat abstraction properties of gels are less effective than for ice. Similarly, ice applied directly to the body can melt and warm at the skin surface, whereas continuous-flow treatment ensures delivery of a consistent temperature.

Another reason a compression splint was selected for this study is that studies in humans indicate that the use of compression in conjunction with cold leads to greater reductions in tissue temperature, presumably by inhibiting the influx of rewarmed blood. In fact, the analgesic properties of cold applied in combination with compression yielded the result that compression was more critical than cold treatment for control of pain and edema. One report alludes to the importance of compression in physical therapy in horses. The role that compression played in the decrease in tendon temperature was not addressed in this study design but would certainly warrant further investigation.

A 1-hour cryotherapy protocol was selected in this study to investigate the frequently stated risks of prolonged cold treatment. It has been suggested that horses should receive multiple short-term treatments for acute soft-tissue injuries and avoid treatment sessions of more than 20 to 30 minutes, whereas other authors have recommended several hours of continuous cold treatment. Human physical therapists have also failed to reach a consensus regarding optimal duration of treatment, providing guidelines that vary from 20 to 45 minutes to as long as 48 hours; however, it is also cautioned that superficial tissue damage and nerve palsy can result from ice application exceeding 20 minutes. Analysis of data from the study reported here indicates that cold treatment accomplished by circulation of coolant can be comfortably applied for periods of at least 1 hour without producing obvious complications; however, a temperature plateau was reached after 45 minutes of this type of cryotherapy.

Thermocouples and thermistors are the most common methods for monitoring temperatures in deep tissues in cryotherapy experiments, and both are considered reliable methods. In this study, SDFT temperature was measured by use of thermocouples that consisted of pairs of dissimilar electrical conductors that generated a net thermoelectric voltage between the open ends in accordance with the size of the temperature difference along the length of the conductors. We elected to use thermocouples in this study because of their small size, which allowed for relativelyatraumatic implantation. In theory, this small size should also have prevented erroneous measurement calculations that have been observed as a result of a deformed temperature field that can be caused by tissue puncture from larger thermistors.

Temperature responses documented in this study were more dramatic than those documented in another report in which investigators compared the effects of ice-water immersion and commercial cold packs on tissue temperatures in the metacarpal region of 1 horse. In that report, thermistor probes were used to measure temperatures in subcutaneous and intertendinous tissues and at the skin surface. After 30 minutes, the minimum temperature difference detected between the SDFT and deep digital flexor tendon during ice-water immersion was 20°C. Several differences between that study and the current investigation may have accounted for the differences in response to cold treatment. Techniques for cold treatment differed, duration of treatment was substantially shorter in the other report, and location of the temperature sensors differed (in the other report, it was located between tendons rather than within the tendon core). In another cold-treatment study in horses, investigators recorded results similar to those reported by Kaneps et al. That study was designed to evaluate the effects of heat and cold on equine laminar temperature and perfusion. During cold treatment, the hooves of 6 horses were immersed in a bath of ice water, and within 30 minutes, temperature in the laminar tissues of the dorsal hoof wall had decreased by a mean of only 11.6°C. Unfortunately, differences in treatment protocols make it difficult for us to directly compare data between those reports and the study reported here. Nevertheless, the striking difference among results of all these studies emphasizes the need for additional research to compare popular techniques for cold treatment.
One factor that may confound accurate interpretation of results of this investigation is the unknown influence of systemically administered sedatives on limb circulation and recorded cooling rates. In this study, horses were sedated by IV administration of a combination of detomidine and butorphanol, and the skin overlying the insertion site for the thermocouple probe was infiltrated with lidocaine hydrochloride. This protocol for sedation and analgesia was required during implant placement to meet university guidelines on animal care and use. In addition, a preliminary study performed to develop these techniques suggested that it was not feasible to implant the thermocouple probe in the SDFT core and subsequently allow the horse to move around freely (it typically resulted in dislodging of the probe). As a result, we elected to keep the horses lightly sedated and restrained in stocks during the course of the study to prevent movement or dislodging of the thermocouple probe. Subsequent clinical experience with the same cooling-and-compression splint for treatment of the metacarpal and metatarsal regions of hospitalized equine patients has indicated that the application of cold treatment for up to 1 hour in accordance with the protocol used in this study is tolerated well in unsedated equine patients.

The impact of systemically administered sedatives on limb perfusion and the results reported here is unknown. However, hemodynamic effects of detomidine and butorphanol have been studied. Detomidine can cause a decrease in heart rate and cardiac index as well as increases and decreases in arterial blood pressure in horses. In another study, arterial hypotension was not observed in horses given a dose of detomidine comparable to that administered to the horses of our study. Direct effects of this α2-agonist on digital vessels have also been studied. Equine digital arteries exposed in vitro to detomidine had a contractile response of variable intensity. In addition, IV administration of xylazine, another α2-agonist, induces a transient decrease in digital arterial blood flow in the digits of horses. However, the influence of detomidine on digital blood flow has not been reported. Similarly, the effect of butorphanol on digital blood flow in horses has not been examined directly. Extrapolation from studies on the cardiopulmonary effects of butorphanol and its effects on intestinal blood flow indicates that the doses administered in our study would be unlikely to have substantial impact on peripheral hemodynamic variables. It is clearly possible that the sedation techniques used in the study reported here influenced digital blood flow, and variations in digital blood flow would be expected to affect the reported results. In contrast, other studies on the effects of cold treatment in horses were able to avoid the use of systemically administered sedatives during temperature-recording sessions. However, considering data in the literature regarding the cardiopulmonary effects of the doses of these drugs, it is likely that the hemodynamic effects of direct cold treatment of limbs were substantially more profound than those related to sedation alone.

As designed, the study reported here did not address the issue of warming of the tissues after cold treatment. In a preliminary study used to establish techniques for the experiments reported here, we did monitor limb temperature for 2 hours after discontinuing the 60-minute period of cold treatment. Despite gradual warming, temperature of the tendon core did not return to baseline values within 2 hours. In another study, investigators recorded a rapid return of surface, subcutaneous, and deep tissue temperatures to baseline values following a 30-minute period of immersion in ice water. In contrast, other investigators found that tissue temperatures did not return to pretreatment values within 30 minutes after a 30-minute cold treatment or within 2 hours after a 60-minute cold treatment. Again, it is difficult to accurately compare data among these studies. However, evidence of prolonged cooling of the distal portion of the limbs in horses after cold treatment reinforces the need for studies to investigate possible detrimental effects of commonly used modes of physical therapy, including cryotherapy. In addition to determining warming and cooling rates of limbs after thermal manipulation, such studies should address concerns about possible perfusion or reperfusion injury.

A temperature of 10°C was chosen for the in vitro viability assay on the basis of data generated in vivo documenting that this was the lowest temperature recorded during the 1-hour cold treatment. Although it is certainly possible that lower temperatures may be achieved with other treatment modalities, other studies in horses do not suggest that temperatures <10°C are routinely achieved during therapeutic cold treatment. Exposure of cells to cold treatment in an in vitro system as performed in this study is not completely analogous to the situation in vivo, because the cells are not surrounded by matrix or exposed to circulating inflammatory mediators, and cell survival in these experiments cannot directly predict in vivo results. However, the rate of cooling in vivo was similar to that observed in vitro, and the lack of detrimental effects of cryotherapy on posttreatment cell viability was not unexpected considering the resiliency of other equine musculoskeletal cell types that were exposed to temperatures much lower than those investigated here. Nevertheless, resistance of equine tendon fibroblasts to hypothermic damage is biologically important and, to our knowledge, has not been documented before. Similar in vitro experiments have also found that equine tendon cells are highly resistant to hyperthermia, suggesting that there may be selection pressure for cells capable of withstanding wide temperature fluctuations in these poorly vascularized and anatomically exposed structures.

The MTT assay used in the experiments reported here yields a relatively rapid and accurate assessment of cell viability. Following cold treatment and prior to determination of cell viability, we cultured control and treated cells on plastic tissue culture plates for 24 hours to ensure that only cells capable of adhering and surviving a minimum of 24 hours after cold exposure would be considered viable. Other methods of viability testing, such as the trypan blue dye exclusion test, are not as reliable, because cells may appear viable immediately following exposure but then may subsequently die. It is possible that the reproductive capability of the cells considered viable in our study was damaged, and
this type of long-term damage would only be detected in long-term studies. However, it is generally accepted that measuring the cell fraction able to reestablish growth under short-term culture conditions can be used to accurately assess lethal cell damage following exposure to temperature extremes.\textsuperscript{23}

Data from the experiments reported here should provide a useful standard for comparing use of various cryotherapy protocols in horses. One inherent limitation of this study was that the temperature responses were conducted in normal limbs in horses at rest. Different absolute temperature values would be expected in limbs after exercise or after injury. However, similar patterns of response probably would be expected in traumatized tissues.\textsuperscript{24} In fact, it would be reasonable to expect greater absolute changes in temperature as a result of an increased temperature gradient between the tendon and cryotherapy unit.\textsuperscript{25} Therefore, although direct application of the results from this in vivo study to a clinical population may be limited, the most effective but safe method of topically applied cold treatment may ultimately be determined by use of these methods. Ultimately, experiments involving the use of horses with traumatized tissue or pathologic conditions would permit accurate extrapolation of results to clinical situations. Additional in vitro studies should also be performed in parallel to in vivo studies to establish better guidelines regarding the critical temperatures that tissues should not exceed during treatment.

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