Immunohistochemical evaluation of substance P and calcitonin gene-related peptide in skin and periosteum after extracorporeal shock wave therapy and radial pressure wave therapy in sheep

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Objective—To evaluate the effect of focused extracorporeal shock wave therapy (ESWT) and radial pressure wave therapy (RPWT) on immunohistochemical staining for substance P and calcitonin gene-related peptide (CGRP) in the skin and periosteum of sheep.

Animals—36 sheep.

Procedures—All 4 limbs of 36 sheep were treated with ESWT, RPWT, or a sham treatment. For 14 days after treatment, at least 2 sheep were euthanized daily and tissue was harvested for histologic evaluation of nerves via staining for substance P and CGRP in the skin and periosteum.

Results—No effects of ESWT or RPWT were observed on the number of nerves with stain uptake for substance P or CGRP in the skin or periosteum.

Conclusions and Clinical Relevance—Substance P- and CGRP-containing nerve fibers are not disrupted by ESWT or RPWT. Further studies are needed to identify the mechanism of analgesia observed in association with these treatment modalities. (Am J Vet Res 2007;68:323–328)

Abbreviations
ESWT Extracorporeal shock wave therapy
RPWT Radial pressure wave therapy
CGRP Calcitonin gene-related peptide
sP Substance P

Extracorporeal shock wave therapy involves the focused application of shock waves. The high-energy shock waves are focused on a point distal to the applicator.1 Radial pressure wave therapy involves deposition of lower-energy pressure waves on the skin surface at the applicator with dissipation of the waves beyond the end of the applicator.1 Recognition of the potential benefits of ESWT and RPWT for musculoskeletal applications has lead to the investigation of the usefulness of these modalities for treatment of numerous diseases.2-7

In multiple studies8-10 conducted to evaluate the effect of ESWT and RPWT, analgesia has been identified in association with the treatments. Mechanisms by which these treatments promote analgesia have not been definitively determined. Among the proposed mechanisms are an endogenous central pain-control mechanism acting by the gate-control theory via opioid (eg, β-endorphins, enkephalins, and dynorphin)11 or nonopioid systems,11,12 shock wave–induced cell damage of peripheral nociceptors preventing generation of membrane potentials,13 shock wave–induced damage to nerves,9,10 and depletion of peripheral neurotransmitters.14

Data have been published concerning the direct effects of ESWT and RPWT on nerves. In 1 study10

in horses, a decrease in sensory nerve conduction velocities in palmar digital nerves after application of RPWT was reported. Morphologic damage to peripheral nerves included disruption and separation within the myelin sheath of medium-to-large myelinated axons. However, in other studies9,15 involving electrical stimulation in horses, no analgesia was found after ESWT or RPWT on nerves. Repetitive generation of action potentials created during application of ESWT in isolated frog sciatic nerves has been reported,16 a result of interaction between cavitation bubbles and the nerves.

The endogenous opioid systems (eg, β-endorphins, enkephalins, and dynorphins) in spinal cords of rats evaluated after ESWT application to the right hind foot were found to be unaffected by the treatment.12 There were no differences between treated or sham-treated groups in dynorphin or enkephalin immunoreactivity in the left or right side of the lumbar portion of the spinal cord after single or repetitive ESWT application. Similarly, another investigation11 in rats revealed no change in c-Fos expression after ESWT application to the hind foot. In the dorsal horn of the spinal cord, c-Fos is an inducible transcription factor and is used as a marker for neuronal activity. Results of these studies indicate that ESWT is unlikely to induce analgesia by the action of peripheral nerves on the CNS.
Substance P and CGRP are released from activated nociceptive terminals and cause neurogenic inflammation while transmitting information to the spinal cord. When ESWT was applied to a footpad on the left hind foot of rats, a decrease in CGRP immunoreactivity in ipsilateral dorsal root ganglion neurons was detected. The CGRP was only detected in small- and medium-sized neurons. Because a behavioral examination of pain was not performed in that study, the relevance of those findings is unclear. Another study in rats was performed to investigate the influence of low-energy ESWT on expression of sP and CGRP in the lumbar segment of the spinal cord after ESWT was applied to rat feet. No differences in expression of those 2 neuropeptides in the C-fiber terminals of the dorsal horn were seen between the treatment and sham-treatment groups after 1 treatment, repeated treatments, or treatments that consisted of different energy flux densities. Investigators in that study concluded that ESWT has no influence on sP and CGRP release in the spinal cord.

Substance P is a polypeptide of the tachykinin family and consists of 11 amino acids. Substance P is released at central and peripheral terminals of sensory nociceptive neurons after stimulation. Calcitonin gene-related peptide is a 37-amino acid neuropeptide. In peripheral nerves, CGRP is expressed in finely myelinated sensory A-δ fibers and unmyelinated C fibers. Calcitonin gene-related peptide and sP are found in 50% of dorsal root ganglion neurons and are expressed in 35% of primary sensory nerve fibers. Substance P and CGRP immunoreactive nerve fibers have been observed in periosteum, ligament, muscle, connective tissue, bone, and bone marrow. Substance P and CGRP are involved in nociception in both the peripheral and central nervous systems. The sP and CGRP immunoreactive nerve fibers in periosteum are thought to be directly involved with pain mediation, whereas thicker nerve fibers are thought to be involved in proprioceptive impulse conduction. Most sP immunoreactive nerve fibers in the periosteum contain CGRP, and most CGRP immunoreactive nerve fibers contain sP. However, larger sensory nerves have more CGRP immunoreactivity. The 2 neurotransmitters may have overlapping but different roles in neurotransmission.

Some studies have investigated a potential link between neurotransmitters and posttreatment analgesia after ESWT. After ESWT application to a rabbit femur, sP release from the periosteum was increased 6 and 24 hours after the treatment, compared with control femurs, and sP concentrations were decreased 6 weeks after ESWT. In that study, the increase in sP occurred when signs of pain were typically detected following ESWT administered for tendon disease, and the decrease in sP occurred when signs of pain relief were typically seen.

Analgesia associated with depletion of neurotransmitters at the treatment site in rat feet has been reported. The epidermis of healthy rats is heavily innervated by nerve fibers immunoreactive for protein gene product and CGRP. In this study, there was nearly complete degeneration of epidermal nerve fibers in the shock area.
wave–treated skin, as indicated by loss of immunoreactivity for CGRP and protein gene product. The epidermis was reinnervated 2 weeks after treatment. These findings indicated that relief of pain after shock wave application to the skin results from rapid degeneration of intracutaneous nerve fibers. In another study, concentrations of sP and CGRP in sheep skin and perios- teum after ESWT and RPWT varied. The skin and periosteum were emulsified to determine neurotransmitter concentrations, and it is possible that the variability could have been a result of the tissue sample containing a larger-diameter sP- or CGRP-containing nerve fiber. Histologic evaluation of immunostained samples may provide a more accurate assessment of sP- and CGRP-containing nerve fibers after ESWT and RPWT.

Determination of the mechanism of analgesia will yield the information necessary to continue investigation of the potential mechanisms of healing after ESWT or RPWT. The objective of the study reported here was to evaluate the effect of ESWT and RPWT on sP and CGRP concentrations in perios- teum and skin in sheep. We hypothesized that ESWT and RPWT would decrease immunohistochemical stain uptake for sP and CGRP.

Materials and Methods

Animals—Thirty-six white-faced sheep approximately 6 months of age and ranging in weight from 22 to 44 kg (mean, 31 kg) were used. The sheep's limbs were the units of randomization: each sheep had 1 control limb; 1 limb treated with ESWT; 1 limb treated with RPWT; and 1 limb treated with ESWT, RPWT, or the control treatment (ie, no treatment). Sheep were sedated by administration of xylazine (0.1 mg/kg, IV) to facilitate ESWT or RPWT treatment. Wool was clipped and shaved from a circular treatment area 3 cm in diameter on the middorsal portion of the third metacarpal and third metatarsal bones. Treatments were 1,000 pulses at 0.15 mJ/mm² for ESWT, 1,000 pulses at 0.16 mJ/mm² for RPWT, or no treatment (control). Two sheep were euthanized daily for 14 days except on days 2, 4, and 6, when 4 sheep were euthanized. The skin from the 3-cm treatment areas was removed en bloc, and 6-mm punch biopsy specimens were immediately placed into optimal cutting temperature compound and stored at –70°C. Samples of periosteum from the metacarpal and metatarsal regions of the limbs were obtained by dissecting away the connective tissue with a scalpel blade and cutting 5-mm squares, then elevating the periosteum off the surface of the bone. Periosteum samples were immediately placed in optimal cutting temperature compound and stored at –70°C. The study protocol was approved by the university animal care and use committee.

Paraffin embedding—Samples were thawed at ambient temperature in neutral-buffered 10% formalin for 24 hours while being stirred on an agitator. Tissue samples were placed in alcohol for dehydration and paraffin embedding that was completed by an automated system. After embedding, samples were sectioned to 5-µm thickness for immunohistochemical staining.

Avidin-biotin complex immunohistochemical staining for sP and CGRP—Immunohistochemical staining was performed manually. Tris-buffer saline (NaCl) solution (pH, 7.6) was used as the rinse solution unless otherwise indicated. After heating for 30 minutes at 60°C, slides underwent rehydration, first in propylene-based glycol ether for 13 minutes, then in 100%, 95%, 80%, and 70% ethanol and distilled water for 10 seconds each. Endogenous peroxidase activity in tissues was quenched by application of 3% H₂O₂ for 10 minutes. Sections were rinsed with de-
ionized water, and slides were assembled into coverplates and rinsed. Tissue sections on the slides were incubated with rabbit anti-sP or rabbit anti-CGRP antibody. Trials on tissue known to contain sP- and CGRP-containing nerve fibers (from sheep ileum) had optimum stain uptake at a primary antibody dilution of 1:20,000 when incubated at 4°C for 96 hours for the rabbit anti-sP primary antibody, whereas optimum staining with the rabbit anti-CGRP primary antibody was at a 1:20,000 dilution when incubated at 4°C for 48 hours. Primary antibodies were diluted in antibody diluent. Normal rabbit serum at the dilution, time, and temperature described previously served as a negative control sample. After incubation, slides were rinsed and biotinylated anti-rabbit mouse IgG secondary antibodies were applied and allowed to incubate for 10 minutes. Slides were rinsed, and an immunoperoxidase system applied to tissue sections for 10 minutes. Slides were rinsed, and sites of immunostaining were developed by use of a red peroxidase substrate applied to the sections for 5 minutes and washed with deionized water. Slides were counterstained with hematoxylin for 1 minute, washed thoroughly with deionized water, dehydrated through graded concentrations of ethyl alcohol, and cleared. Finally, the sections were covered by glass coverslips and air-dried.

**Evaluation of nerve fiber staining**—Evaluators of the slides (JMA and MJY) were blinded as to the treatment or time of the sample until after evaluation was completed. Three layers of skin were evaluated separately. The layers were the superficial portion of the dermis, from the epidermis to the base of the hair follicles; the deep portion of the dermis, from the base of the hair follicles to the subcutis; and the subcutis, at the level of transition from dermal collagen to subcutaneous adipose tissue. The large vessels at the dermal-subcutis junction were included in the subcutis category. The periosiium was evaluated as a single layer of tissue.

The widest part of the biopsy specimens was used for evaluation. This allowed the sP- and CGRP-staining nerve fibers to be counted in each layer of the skin across the center of the 6-mm biopsy sample at 400× magnification and totaled. Similarly, in periosiium, the number of sP- and CGRP-staining nerve fibers was counted across the 5-mm center of the biopsy sample at 400× magnification.

**Statistical analysis**—Analysis of the number of neuropeptide-staining fibers was evaluated as a study design with 2 factors, treatment (ESWT, RPWT, or control treatment) and time (days 0 to 14). Analyses of variance consisted of a significance test for the main effects of treatment and time and the group-by-time interaction. If the group-by-time interaction was not significant, only the main effects were reported. For analysis, each treatment (ESWT or RPWT) was compared with an untreated control by a mixed-effects model with sheep as the random effect for each of the 3 locations in the skin, the sum of the 3 sites in the skin, and the periosiium. Graphs of the data were plotted and analyzed. For all analyses, values of P < 0.05 were considered significant.

**Results**

**sP in skin**—No treatment effects were found for superficial dermis (P = 0.789), deep dermis (P = 0.739), subcutaneous layer (P = 0.606), or the sum of the 3 sites (P = 0.655; Figure 1). Number of days after treatment did not affect the number of sP-staining fibers in the deep portion of the dermis (P = 0.576), the subcutaneous layer (P = 0.225), or the sum of the 3 layers (P = 0.128). Number of days after treatment did have a significant (P = 0.028) effect on the superficial portion of the dermis (Figure 2), although the correlation was poor (R² = 0.05) because of substantial within-group variation in the data.

**CGRP in skin**—No treatment effect was found for the superficial portion of the dermis (P = 0.988), deep portion of the dermis (P = 0.267), subcutaneous layer (P = 0.864), or sum of the 3 sites (P = 0.776; Figures 1 and 3). Number of days after treatment did not effect the number of CGRP-staining fibers in the superficial portion of the dermis (P = 0.327), deep portion of the dermis (P = 0.077), subcutaneous layer (P = 0.77), or sum of the 3 sites (P = 0.149).
sP in periosteum—No effect of number of days after treatment was found in the periosteum ($P = 0.718$) or for number of sP-staining fibers in the periosteum ($P = 0.322$; Figures 4 and 5).

CGRP in periosteum—No treatment effect was found in the periosteum ($P = 0.757$; Figure 3). Number of days after treatment did not affect the number of CGRP-staining fibers in the periosteum ($P = 0.904$).

Discussion

Results did not support the hypothesis that the number of nerve fibers with immunoreactivity for sP or CGRP in skin or periosteum would be significantly altered after ESWT or RPWT. These findings were similar to findings in a previous study\(^9\) in sheep in which concentrations of sP and CGRP were measured in tissue after ESWT and RPWT. The sensitive method of counting sP and CGRP immunoreactive nerve fibers used in the present study together with results of that earlier study\(^9\) suggest that depletion of these fibers following ESWT or RPWT is not a mechanism for analgesia in sheep.

Results of the present study differ from those in a previous study\(^9,15\) in which the numbers of nerve fibers in rat feet with stain uptake for CGRP after ESWT were measured. Treated rat feet had a significantly lower number of CGRP-staining fibers until day 7 after treatment. Interestingly, in that study,\(^14\) the decrease in CGRP staining was not associated with analgesia as measured by thermal stimulation. The reason for the variation between studies is unclear, but substantial anatomic differences in thickness and innervation of the rat feet and the skin and periosteum of sheep may play an important role. Rats in the earlier study were treated with 1,000 pulses at 0.08 mJ/mm\(^2\). The same number of pulses delivered at a higher energy level was used in the present study and should have induced a similar, if not greater, response. Pulses in the present study were distributed over an area 3 cm in diameter, which is substantially larger than a rat foot.

The periosteum was included in analyses in the present study because it contains the innervation and serves the sensory function for the underlying bone.\(^20,26\) For a musculoskeletal abnormality involving the bone, the periosteum would be affected in any local analgesic treatment effect. Substance P- and CGRP-containing fibers were detected in the periosteum in control and treated samples. The finding of high numbers of sP-containing nerve fibers in the periosteum and joint capsule has been associated with lameness in horses with osteoarthritis,\(^23\) in which associated inflammation resulted in increased synthesis of sP. Therefore, it is possible that depletion of sP is associated with analgesia after ESWT or RPWT. Although sheep were used as a surrogate for horses in the present study, it is expected that sheep and horses would respond similarly to treatment.

Local cutaneous analgesia after ESWT has been reported in multiple studies.\(^8,9,23\) The local analgesia induced and absence of changes in local CGRP and sP concentrations indicate that the likely mechanism is related to other neurotransmitters or to nociceptors. Damage to local nociceptors from ESWT or RPWT and prevention of development of membrane potential are possible mechanisms that warrant further investigation. The treatments could also inhibit pain impulses by affecting other neurotransmitters or via local free radical production.

The data are conflicting regarding differences between ESWT and RPWT in presence and duration of analgesia. Application of ESWT has resulted in analgesia for 3 days following treatment when evaluated by electrical stimulation.\(^8\) In another study,\(^22\) 9 horses with osteoarthritis or navicular syndrome had 2 days of analgesia after treatment when evaluated with force plate analysis. Similarly, in a horse with desmitis of the proximal portion of the suspensory ligament, analgesia of 3 days’ duration was repeatedly induced.\(^8\) However, in another study\(^26\) evaluatng analgesia associated with RPWT in horses with navicular syndrome, no analgesia was found.

The direct effects of ESWT and RPWT on nerve fibers appear to be different. Sheep that underwent ESWT in the present study had no detectable histologic changes in nerves. However, in another study,\(^9\) axonal swelling and increased numbers of inflammatory cells were detected after RPWT. After RPWT of the palmar nerve in horses in a different study,\(^10\) sensory nerve conduction velocity was lower 3 and 7 days after treatment and disruption of the myelin sheath persisted for as long as 35 days. Despite these conflicting findings in nerves, 2 in vivo studies\(^9,15\) in horses failed to confirm induction of cutaneous analgesia in tissues adjacent to the coronary band after treatment of the palmar nerve at the level of the proximal sesamoid bones with either ESWT or RPWT.\(^8,15\) It is possible that ESWT and RPWT induce analgesia via different mechanisms.

The immunohistochemical staining techniques used in the present study were similar to those used in previous studies.\(^14,17,18\) Uptake of immunostain for sP and CRGP in small nerve fibers validates the method. Because each sheep had at least 1 limb that served as an untreated control, the number of control specimens was adequate to verify that the staining techniques were consistent throughout the study.

The mechanism of analgesia induced by ESWT and RPWT remains unknown at present. Our results

Figure 5—Photomicrograph of a section of sheep periosteum treated with RPWT and stained immunohistochemically. Notice the 3 nerve fibers with stain uptake for sP (arrows).
indicate that SP- and CGRP-containing nerve fibers are not disrupted by these treatment modalities, but further investigation is needed to identify the mechanism of analgesia.

References