Ultrasound in Med. & Biol., Vol. 36, No. 8, pp. 1298–1305, 2010

0301-5629/$ - see front matter

ELSEVIER

doi:10.1016/j.ultrasmedbio.2010.05.005

● *Original Contribution*

THE EFFECT OF VARIABLE WAVEFORM LOW-INTENSITY PULSED ULTRASOUND IN A FOURTH METACARPAL OSTEOTOMY GAP MODEL IN HORSES

S. R. McClure,* K. Miles,* D. VanSickle,† and T. South*

*Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Iowa State University, Ames; and †Department of Basic Medical Sciences, School of Veterinary Medicine, Purdue University, West Lafayette, IN

(*Received* 12 *November* 2009; *revised* 2 *April* 2010; *in final form* 5 *May* 2010)

Abstract—The objective of this study was to evaluate the effects of variable waveform low-intensity ultrasound on the healing of a fracture gap of the fourth metacarpal bone in horses. A randomized, blinded, controlled trial was conducted in eight healthy adult horses. In each horse, a 1-cm osteotomy of the fourth metacarpal bone was created. One randomly selected metacarpal gap was treated daily with a 40-min session of ultrasound and the opposite gap was managed similarly with an inactive transducer. The fourth metacarpal bones were radiographed weekly. Fluorescent markers were administered at 14, 28, 56 and 70 d. At the completion of the study at day 84, the bones were harvested and evaluated with peripheral quantitative computed tomography (pQCT) and histology. There were no significant differences between treated and control bones for any of the radiographic, pQCT or histologic parameters evaluated. These findings suggested that low-intensity ultrasound did not affect bone formation in a fracture gap model in the horse. (E-mail: mcclures@iastate.edu) © **2010 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Horse, Low-intensity ultrasound, Fracture gap healing.**

INTRODUCTION AND LITERATURE

Delayed healing or nonunion of bone after fracture is a clinical problem in multiple species. In horses, delayed fracture healing is associated with high morbidity that may include laminitis, secondary fractures and failure of the contralateral limb (Ducharme and Nixon 1996). In humans, 5–10% of fractures develop delayed or nonunions (Einhorn 1995). Mechanisms used to prevent or treat delayed bone healing are similar between species.

Autogenous bone grafts are used in multiple species to aid fracture healing (Markel 1996; Fossum 2007). Bone grafts enhance bone healing by osteogenic, osteoinductive and osteoconductive capacities and may also provide mechanical support. Donor site morbidity, increased surgery time and availability may limit the use of bone grafts. Allografts decrease the morbidity, but to prevent disease transmission and minimize immune response, they are often devoid of cells (Hofer et al. 2003; Borden 2003). Demineralized bone matrix lacks cells and structural integrity but is a readily available osteoinductive and osteoconductive material (Hofer et al. 2003; Borden 2003).

Growth factors such as bone morphogenic proteins, transforming growth factor–β family and platelet-derived growth factors also contribute to fracture healing. These cytokines show clinical promise for stimulation of fracture repair. Growth factor–based biologic products are an area of much current research (Cheung et al. 2003). However, much more work is needed to identify appropriate levels and combinations necessary to avoid undesirable expression after their use.

Physical methods have been investigated as a relatively noninvasive mechanism to stimulate bone healing. The application of electric current during the early phases of fracture repair have shown a 20–25% increase in bone healing rate. This does not justify routine application, but has been used for delayed and nonunion fractures (Ostrum et al. 1994). The use of direct current, alternating current and pulsed electromagnetic fields have all been approved by the United States Food and Drug Administration for stimulation of fracture repair. More recently, shock wave therapy has shown promise as a physical method of stimulation of bone healing. In a study using a nonunion model in dogs, 5/5 shock wave–treated dogs progressed

Address correspondence to: Dr. S. R. McClure, Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Iowa State University, Ames, IA 50010-1250. E-mail: mcclures@iastate.edu

to union compared with 1/5 untreated control dogs (Johannes et al. 1994). Shock wave treatment for osteonecrosis of the femoral head resulted in a more effective treatment than decompression and bone grafting (Wang et al. 2005). Nonunions are routinely treated with shock wave therapy (Schaden et al. 2001) and recent publications demonstrate the benefit of shock wave application to stress fractures (Taki et al. 2007; Moretti et al. 2009) and alveolar bone regeneration (Sathishkumar et al. 2008).

Ultrasound is another physical modality that has been shown to stimulate the healing of fresh fractures in double-blind, controlled clinical trials (Heckman et al. 1994; Kristiansen et al. 1997). A meta-analysis of low-intensity pulsed ultrasound therapy in humans concluded that it was an effective therapy, with an improvement in healing time of 64 d compared with similar untreated fractures (Busse et al. 2002). Animal studies indicate that low-intensity ultrasound application results in a stronger and stiffer callus formation, with acceleration in endochondral ossification (Millis and Jackson 2003). In a rabbit model using midshaft tibial osteotomies, pulsed ultrasound treatment at an intensity of 30 mW/cm^2 accelerated the recovery of torsional strength and stiffness (Pilla et al. 1990).

Absorption of ultrasound energy can result in tissue heating. Therapeutic ultrasound with energy intensity ranging from 1–3 W/cm^2 can be used to warm tissue and decrease joint stiffness and muscle spasm. Low-intensity ultrasound with energy output in the milliwatt level can cause small increases in temperature, which can stimulate some enzymes such as collagenase or MMP-1, which are sensitive to temperature variations (Welgus et al. 1981). Ultrasound pressure also creates mechanical stresses within bone. This mechanical stress is a much lower magnitude of strain than that induced by loading; however, the loading rate is much higher (Rubin et al. 2001). In normally functioning bone, low-energy, high-frequency stresses create regulatory signals even during fracture healing (Fritton et al. 2000; Huang et al. 1999; Goodship et al. 2009).

Various signal intensities have been used in ultrasound treatment. A typical treatment is a 20-min period of 1 MHz sine waves with a 200-μs pulse width and an average intensity of 30 mW/cm^2 (Claes and Willie 2007). This application has been shown to be effective in research models and clinical trials (Pilla et al. 1990; Busse et al. 2002). An ultrasound treatment that delivered 200 mW/cm^2 accelerated the healing of tibial fractures in rabbits by 18% compared with a nontreated group (Klug et al. 1986). Signal intensities up to 500 mW/cm^2 showed a greater mineral apposition rate (Tsai et al. 1992). It has also been shown that excessively high intensity can be deleterious. A signal intensity of 1.0 W/cm^2 was found to be deleterious to a fracture model in rats (Tsai et al. 1991).

To date, there are no blinded, controlled bone gap model studies in the horse to measure the effect of low-intensity pulsed ultrasound on bone healing in the horse. The objective of this study was to asses the affect of multivariant waveform, low-intensity pulsed ultrasound treatments on bone healing in a fourth metacarpal bone osteotomy gap model in the horse.

MATERIALS AND METHOD

Eight clinically normal female adult horses including four Quarter Horses, three American Paint Horses, and one Arabian were used in this study. The median age was 5 y (range, 2 to 9) and mean weight was 424 kg (range, 383 to 500). Horses were considered healthy based on a normal physical examination and absence of detectable lameness. There were no palpable abnormalities of the metacarpal bones; preoperative radiographs confirmed the absence of abnormalities of the metacarpal bones. Horses were maintained in stalls from one week before the surgery to the completion of the study. The study was approved by the Iowa State University Animal Care and Use Committee.

Gentamicin (6.6 mg/kg) and phenylbutazone (4.4 mg/kg) were administered intravenously before induction of anesthesia. Horses were sedated with xylazine (0.5 mg/kg intravenously) and anesthesia induced with a combination of glyceryl guaiacolate (10% solution) and thiopental (0.4% solution) to effect. Horses were positioned in dorsal recumbency and anesthesia maintained with isoflurane in oxygen *via* endotracheal intubation. Both forelimbs were circumferentially prepared for aseptic surgery from the distal radius to the distal metacarpus. After draping, a 20-gauge needle was used to identify the proximal aspect of the fourth metacarpal bone. A longitudinal 3-cm incision was centered 7 cm distal to the proximal aspect of the fourth metacarpal bone (MC IV) and a 1-cm section of the bone from 7–8 cm from the proximal aspect was marked. The periosteum and ligamentous attachments were cut with a scalpel and the bone transected with an oscillating saw. The segment of bone was removed, resulting in a 1-cm gap in MC IV in both forelimbs created at the same time. The incisions and gap were lavaged with sterile saline and the subcutaneous tissue and skin sutured. A sterile bandage was applied.

The gentamicin and phenylbutazone treatments were repeated daily for two more treatments. The bandages were removed 2 d after surgery. One MC IV gap was randomly assigned to be treated and the contralateral MC IV gap served as the untreated control. Each ultrasound generator had one active port and one inactive port. These were color-coded, and all investigators remained blinded to treatment or control until after the study

was complete. The ultrasound generator was hung on a surcingle and the ultrasound probe was attached by wires to the generator (Statison V, Statison Medical Inc., Arcadia, CA, USA). Each probe was placed in a neoprene leg wrap with Velcro closure that fit snugly on the leg. The 2-cm diameter transducer was coated with contact gel and placed directly over the osteotomy gap (Fig. 1). The treated and control legs were both managed similarly. Treatments were applied daily until the completion of the study. The bandages were replaced after treatment for the first two weeks after surgery, when sutures were removed. Bandaging was then discontinued.

A 40-min treatment cycle with a 44 mW/cm^2 spatial average–temporal average intensity output with a range of 30–80 mW/cm^2 was used. With a 1.5-MHz, transducer, there was a 2-min period of 2-ms pulse at 166.7 Hz (33% duty cycle), a 3-min period of a 200-μs pulse at 1000 Hz (20% duty cycle), 2 min of a 400-μs pulse at 833 Hz (25% duty cycle) and 3 min of a 200-μs pulse at 1000 Hz (20% duty cycle). The cycle was repeated four times for the 40-min treatment period.

Digital dorso, 30° lateral-palmaromedial oblique radiographs of the metacarpal bones were obtained before surgery, 24 h after surgery (week 0) and weekly for the next 12 weeks (EDR-3, Sound-Eklin, Carlsbad, CA, USA). Radiographs were measured for fracture gap (mm), bone callus width (dorsoplantar, mm) and bone callus length (proximal-distal, mm) and scored by a blinded investigator for bone remodeling and bone healing. The score was based on a 0 = no callus formation; 1 = callus at margin only, not in gap, or <25%; 2 = 25–50% gap filled; 3 = 50–75% gap filled with callus; and 4 = 75–100% osteotomy filly with radiographically visible callus.

Fig. 1. The 2-cm diameter transducer (*small white arrows*) was in a neoprene leg wrap that was fastened around the limb with Velcro closures. The wrap is designed with multiple locations for transducer placement (*large white arrows*) so the transducer can be placed in a different location within the bandage to best maintain contact by the transducer, or multiple transducers could be used.

On days 14, 28 and 70, post osteotomy, the horses were given tetracycline (25 mg/kg) and on day 56 they were given calcein (20 mg/kg) to label active osteons. At the completion of the project on day 84, horses were euthanatized with a barbiturate overdose. The fourth metacarpal bones were dissected and radiographed. The bones were fixed to a tongue depressor to prevent distortion and placed in 10% neutral-buffered formalin solution for 48 h. They were then transferred into a 600-mL bottle filled with 70% alcohol for peripheral quantitative computed tomography (pQCT) analysis.

The pQCT scanner (XCT-3000, Stratec Medizintechnik, Pforzheim, Germany) used for analysis was done with the parameters recommended by the software specialists (Bone Diagnositics, Inc., Fort Atkinson, WI, USA). The threshold for trabecular bone density was set at 169 mg/cm^3 and the threshold for cortical bone set at 500 mg/cm^3. Slice thickness was 2.2 mm and voxel size 0.137 mm. A scout view was conducted to locate the center of the defect and the detailed scan 10 cm in length was performed. Fifteen slices, with slice 8 at the center of the defect, were used for analysis.

Data obtained from the analysis were total bone content per 1-mm slice (mg/mm), total bone density mg/cm^3, total bone attenuation (1/cm), cortical and subcortical content per 1-mm slice (mg/mm), cortical and subcortical density (mg/cm^3), cortical and subcortical attenuation (1/cm), trabecular content per 1-mm slice (mg/mm), trabecular density (mg/cm^3), trabecular attenuation (1/cm), cortical content per 1-mm slice mg/mm), cortical density (mg/cm^3) and cortical attenuation (1/cm).

Data were evaluated for five groups of 2.2-mm slices. Slices 7–9 included the center of the defect; slices 6–10 extended to the ends of the ostectomized bone; slices 4–12 included most of the callus formation; and slices 1–15, which included return to normal bone, were evaluated.

The bones, including periosteum, were embedded in polymethylmethacrylate (Polysciences, Warrington, PA, USA), sectioned in a saggital plane and then ground to 75–100 μm (Exakt Technologies, Oklahoma City, OK, USA). Then 5–10 μm of the surface was etched with 1% formic acid to remove the polymethylmethacrylate to allow Wright's-Giemsa/toluidine blue stain penetration for 3–5 μm. One slide was made to determine osteonal activity as evaluated by fluorescent microscopy.

All sections were subjectively evaluated by a blinded investigator. Comments were recorded on the static histology as evaluated on the Wright's Giemsa/toluidine blue–stained slides. Similarly, the fluorescent microscopy was evaluated subjectively and an objective score was given to each bone. Each osteogenic surface (proximal end MC IV, distal end of MC IV, medial periosteal surface, lateral periosteal surface) was given 1 point, unless there was a small amount that was given 0.5. There

was 1 point for a completely full osteotomy gap and 0.5 for a partially-filled gap.

Data analysis

All objective data collected at the termination of the study (pQCT, radiographic measurements) were analyzed with a paired *t*-test. Scored data (radiographic and histologic) were expressed as median and range and analyzed using the Mann-Whitney *U* rank test. Differences were considered significant at $p \leq 0.05$

RESULTS

All eight horses had successful 1-cm segmental ostectomies of the fourth metacarpal bone and completed the 84-day study. Horses were in pain throughout the study period and all incisions healed by primary intention with minimal swelling. There were no clinically apparent complications associated with the treatment. The horses tolerated the daily treatments well.

At the completion of the study, there were no significant differences between treated and control groups for the radiographic score ($p = 0.29$), osteotomy gap ($p = 0.21$), callus width ($p = 0.23$) and callus length ($p = 0.23$) (Figs. 2 and 3). There were no significant differences between treatment and control bones found for any of the parameters evaluated by pQCT (Table 1). Osteopenia of the distal fragments of both treated and control bones was noted as early as two weeks (Figs. 4 and 5).

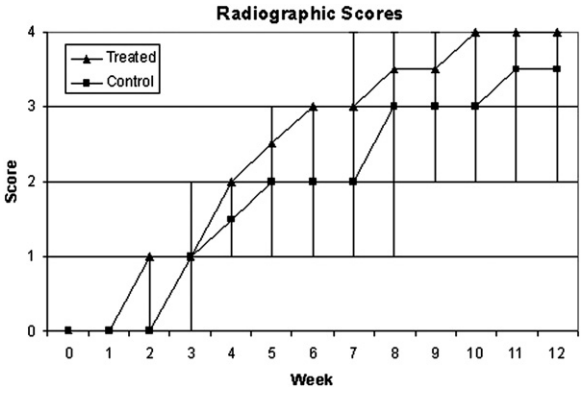

Fig. 2. The median and range for the radiographic scores for the treated (▲) and control (■) groups over the 12-week study period. There were no significant differences at any time point.

The subjective static histologic findings included osteotomy gaps that were filled with primarily dense irregular connective tissue with variable amounts of woven

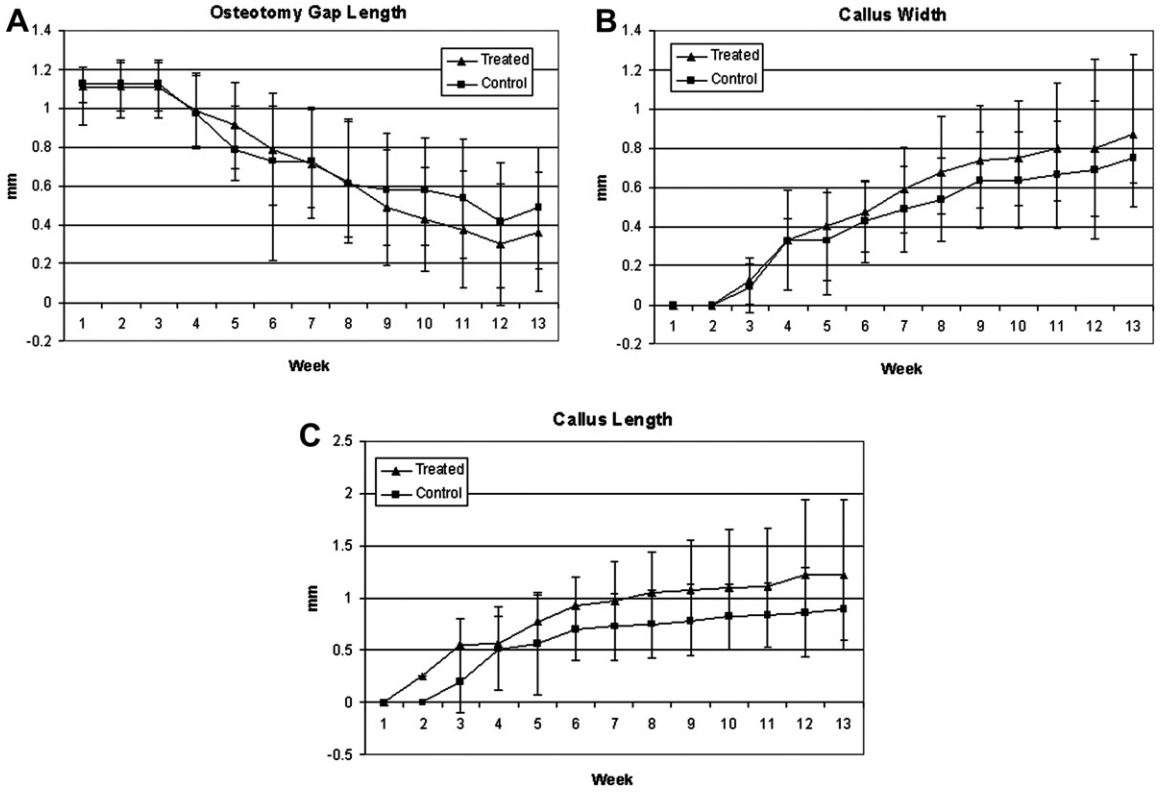

Fig. 3. The mean and variance for the radiographic measurements for the treated (▲) and control (■) groups over the 12-week study period. There were no significant differences for the fracture gap (2A), callus width (2B) or callus length (2C) at any time point.

Table 1. The pQCT data for each of the parameters evaluated

		Slices			
		7–9	6–10	4–12	1–15
Total bone content (mg/mm)	Treated	20.6	25.4	36.3	43.3
	Control	27.3	33.6	44.5	50.4
	p-value	0.71	0.65	0.62	0.61
Total bone density (mg/ccm)	Treated	520.9	56809	688.9	448.8
	Control	463.1	546.3	676.7	771.5
	p-value	0.592563	0.780029	0.826035	0.861027
Total attenuation (1/cm)	Treated	0.54	0.6	0.69	0.75
	Control	0.49	0.58	0.67	0.74
	p-value	0.630033	0.651726	0.703144	0.747239
Cortical and subcortical content (mg/mm)	Treated	16.58	20.6	32.4	40.3
	Control	24.17	30.1	40	47.7
	p-value	0.667543	0.592882	0.638871	0.59044
Cortical and subcortical density (mg/ccm)	Treated	627.1	685.1	784.5	847.5
	Control	559.1	648.6	762.9	843.8
	p-value	0.569966	0.662273	0.689093	0.908959
Cortical and subcortical attenuation (1/cm)	Treated	0.62	0.67	0.75	0.8
	Control	0.55	0.63	0.72	0.78
	p-value	0.511372	0.576675	0.637834	0.6628
Trabecular content (mg/mm)	Treated	4.03	4.73	3.9	2.98
	Control	3.1	3.54	3.27	2.66
	p-value	0.499683	0.314173	0.399634	0.531575
Trabecular density (mg/ccm)	Treated	326.9	344.3	357.6	365.5
	Control	288.1	321.7	344.2	359.3
	p-value	0.470087	0.489034	0.441574	0.544057
Trabecular attenuation (1/cm)	Treated	0.43	0.44	0.46	0.47
	Control	0.37	0.42	0.44	0.46
	p-value	0.43	0.44	0.42	0.47
Cortical content (mg/mm)	Treated	16.95	21.29	33.11	40.9
	Control	24.6	30.48	41.59	48.2
	p-value	0.67	0.06	0.6	0.59
Cortical density (mg/ccm)	Treated	644.9	693.7	787.7	856.7
	Control	597.5	669.8	777.1	851.3
	p-value	0.697431	0.780294	0.851013	0.887164
Cortical attenuation (1/cm)	Treated	0.64	0.68	0.75	0.8
	Control	0.59	0.6	0.74	0.79
	p-value	0.661949	0.335345	0.781162	0.813939
Total bone area (mm^2)	Treated	33.86	39.9	48.1	51.3
	Control	34.1	42.4	53	56.3
	p-value	0.99	0.9	0.77	0.7
Cortical and subcortical area (mm^2)	Treated	22.1	26.3	37	42.8
	Control	26.1	32.6	43.7	48.8
	p-value	0.83	0.73	0.67	0.64
Trabecular area (mm^2)	Treated	11.7	13.5	11.1	8.4
	Control	8.8	9.8	9.3	7.4
	p-value	0.48	0.3	0.42	0.5
Cortical area (mm^2)	Treated	22.8	27.4	38	43.6
	Control	26.7	33.2	44.2	49.5
	p-value	0.830693	0.741974	0.691735	0.646434

Osteotomies were oriented so the center of the osteotomy was at the middle (slice 8) of the region of interest.

and lamellar bone. The distal portions of the MC IV were consistently osteoporotic. The predominance of the osteogenesis was from the proximal end of the osteotomy and the periosteal surfaces. Revascularization of the osteotomy was split between periosteal and the proximal end of MC IV. There were no differences in inflammation noted and no indication of necrosis or tissue injury beyond the osteotomy.

In the dynamic histologic study, all of the fluorescent labels were visible. As noted with the static findings, the predominance of the bone formation was at the proximal end of the osteotomy and the periosteal surfaces. The 14- and 28-d labels were seen in the woven bone mostly adjacent to the proximal osteotomy surface. The labeled woven bone was remodeling into lamellar bone when the 56- and 70-d labels were administered (Fig. 6). The 56- and 70-d labels were consistently seen at the proximal aspect of the filling defect and along the periosteal surfaces proximal and distal to the gap. Subjectively, there were no differences noted between treated and untreated bones. The median (range) osteogenic scores for the treated MC IV were 3.56 (1.5 to 5) and the control MC

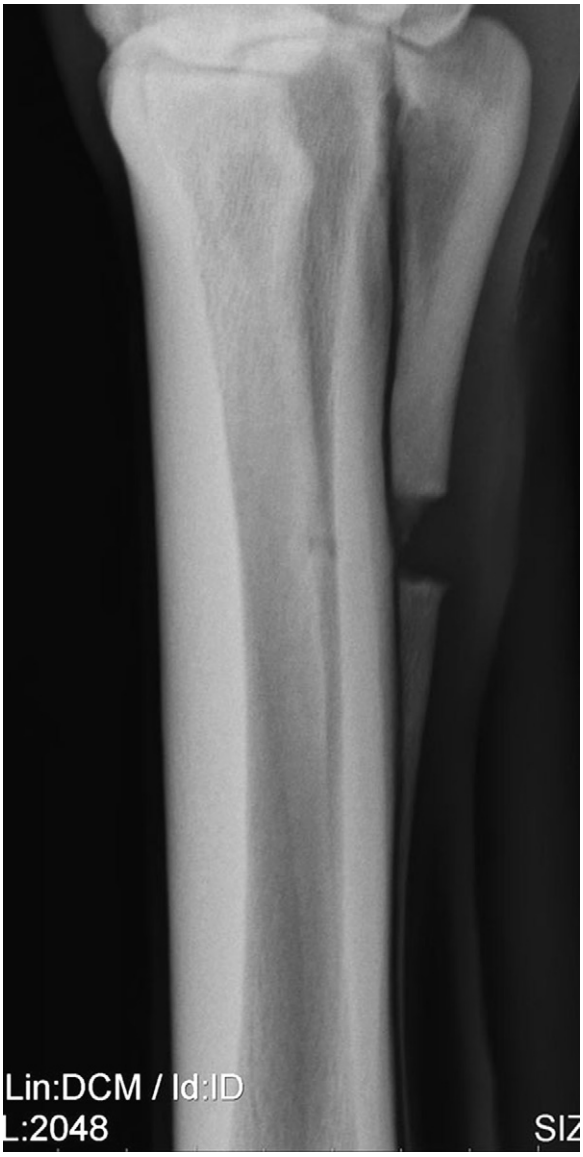

Fig. 4. A DLPMO radiographic view of an untreated control fourth metacarpal bone seven weeks after ostectomy. There is little osseous reaction in the fracture gap. The distal segment of MC IV is osteopenic.

IV 2.5 (0 to 4). These values were not significantly different ($p = 0.21$).

DISCUSSION AND SUMMARY

Management of equine fractures is challenging, and the potential to use a noninvasive mechanism to stimulate fracture healing is appealing to clinicians. The results of this study demonstrate no significant difference in degree of healing of an osteotomy gap model in MC IV of the horse after low-intensity pulsed ultrasound therapy. A model with a 1-cm gap was selected to provide an adequate time frame for healing to occur and any differences created by the ultrasound to become apparent during the study period. There were no significant differences noted at the end of the study period. Increasing the treatment time or applying the treatment multiple times per day are factors that might have affected the study outcome. The treatment period and interval used were those recommended by the manufacturer.

Previous investigations have shown improvement in bone healing with low-intensity pulsed ultrasound. A meta-analysis of human fracture patients identified 158 homogeneous cases treated in a randomized, blinded, controlled fashion. A 64-d decrease in healing time was identified after low-intensity ultrasound treatment (Busse et al. 2002). The spatial average temporal intensity of patients in the meta-analysis was 30 mW/cm^2 compared with 44 mW/cm^2 used in this study, which was recommended by the manufacturer. Tissue heating has been a suggested mechanism of stimulation. However, in a rabbit model, minimal temperature increases were seen at 50 mW/cm^2 for 15 min (Chang et al 2002). It is possible that tissue heating, potentially to detrimental levels, could occur at 44 mW/cm^2 for 40 min, which may affect the outcome in this study. However, in this clinical study, no attempt was made to quantitate temperature increase.

The mechanisms by which ultrasound stimulates bone healing are more likely nonthermal and a number of nonthermal effects have been proposed. A direct effect on cell membrane permeability (Dyson and Brookes 1983) and direct stimulatory effects on cells (Dinno et al. 1989) may be involved. The effects may be mediated by cavitation and subsequent acoustic streaming (Dyson 1982). At this time, a number of potential mechanisms exist; however, the exact mechanism of stimulation remains unknown.

It has been suggested that the low-intensity ultrasound may affect angiogenesis (Rawool et al. 2003). In this model, neovascularization was predominantly from the proximal end of the osteotomy as well as the periosteum from the proximal fragment, with a smaller contribution from the periosteum of MC III. The limited neovascularization from the distal fragment may have delayed the healing response. In a previous study of clinical cases where segmental ostectomies were performed after MC II and MC IV fractures (Jenson et al. 2004), no sequestration of the distal segments was observed. A hypothesis that periosteal and soft-tissue vasculature maintained perfusion of the distal segment was proposed. The proximal fragment and the periosteum contributed the majority of the blood supply to the ostectomy gap in our model.

The outcome may also have been affected by the 1-cm osteotomy gap model selected. This model with some variations has been used previously (Southwood

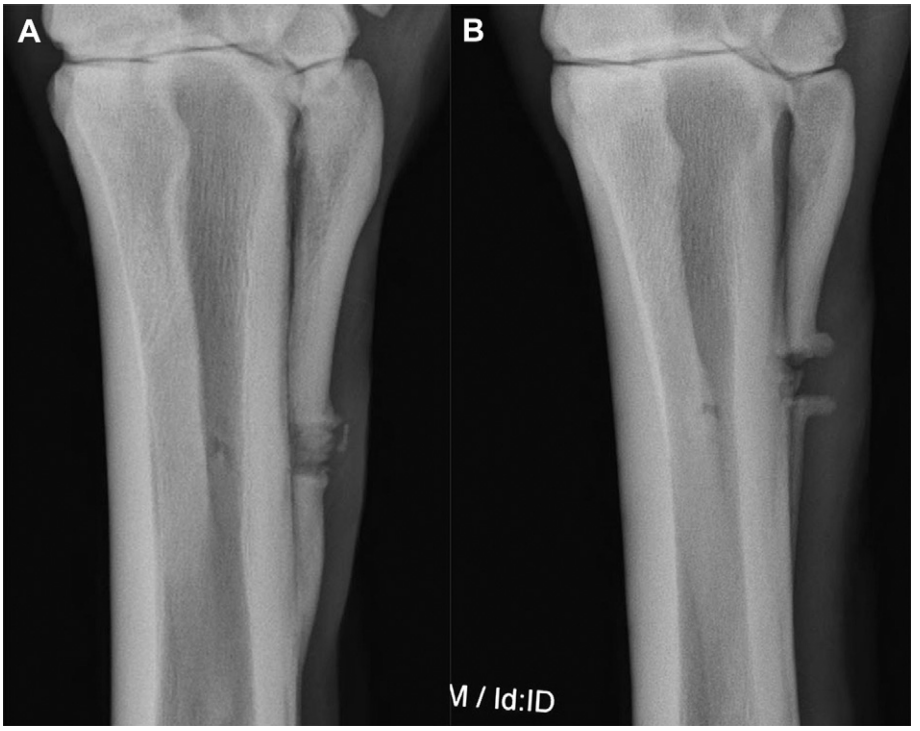

Fig. 5. A DLPMO radiographic view of a treated MC IV at 12 weeks that had a complete bridging callus (A) and a DMPLO view of a different treated leg with a gap remaining (B).

et al. 2006; Waselau et al. 2007; Perrier et al. 2008). In this model, we chose a 1-cm gap to provide an adequate gap to allow for a longer period of evaluation. It is possible that the gap size was beyond what ultrasound stimulation was capable of affecting. However, as seen in Fig. 4, a bridging callus did form in some gaps. We expected the gap to heal by secondary bone healing with callus formation. This was chosen because this is the situation in the horse when a method to stimulate healing, such as ultrasound, would be most valuable. Of note, strain across the fracture gap has a significant effect on healing (Claes et al. 1997) and we made no effort to quantify the strain across the gap in this model.

The radiographically evident osteopenia of the distal segment and the histologic evidence of osteoporosis was consistent in all bones. The unloading of the bone produced by the ostectomy gap, in addition to the stall confinement throughout the study period likely contributed to this finding. In a study of clinical cases of segmental ostectomies for splint bone fractures, the horses had resumed training or normal activity by eight weeks (Jenson et al. 2004). The osteotomy gaps were still present in all horses in our study at eight weeks. Based on the clinical findings, the horses could have had increased exercise over the 12-week study period with the potential for stimulated healing in these cases.

There was no stimulation of healing across a 1-cm osteotomy gap model in the eight horses included in this study after variable waveform low-intensity pulsed ultrasound therapy. Noninvasive mechanisms to stimulate fracture healing are still needed in difficult fracture patients, indicating that further refinement of treatment parameters should be investigated.

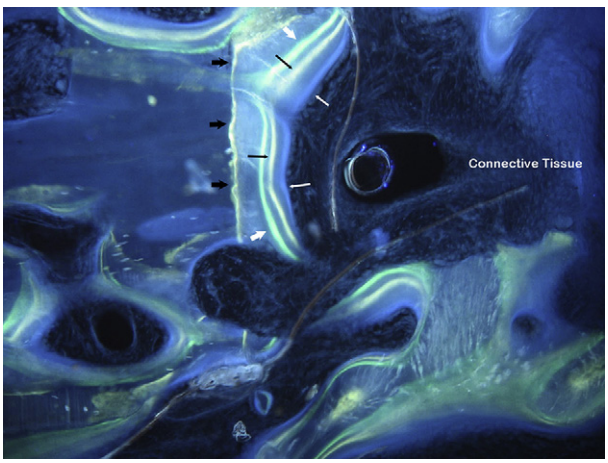

Fig. 6. The proximal aspect of MC IV is on the left and the gap is on the right. The straight line was the location of the proximal cut and it was labeled with second tetracycline administered on day 28 (*thick black arrows*). The day 56 calcein (*thick white arrows*) and the day 60 tetracycline labels (*narrow black arrows*) are seen. The new bone deposited after the day 60 tetracycline can be seen (*narrow white arrows*). The osteotomy gap is to the right and filled with connective tissue and some woven bone in the lower right (125x, unstained).

Acknowledgment—This study was funded by Statison Medical, Inc., Arcadia, California.

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