Effect of hyperbaric oxygen treatment on incorporation of an autogenous cancellous bone graft in a nonunion diaphyseal ulnar defect in cats

Sharon C. Kerwin, DVM, MS; Daniel D. Lewis, DVM; A. Derrell Elkins, DVM, MS; Julian L. Oliver, DVM, PhD; Giselle Hosgood, BVSc, MS; Robert D. Pechman, Jr, DVM; Sharon L. Dial, DVM, PhD; George M. Strain, PhD

Objective—To determine whether hyperbaric oxygen treatment (HBOT) would affect incorporation of an autogenous cancellous bone graft in diaphyseal ulnar defects in cats.

Animals—12 mature cats.

Procedure—Bilateral nonunion diaphyseal ulnar defects were created in each cat. An autogenous cancellous bone graft was implanted in 1 ulnar defect in each cat, with the contralateral ulnar defect serving as a nongrafted specimen. Six cats were treated by use of hyperbaric oxygen at 2 atmospheres absolute for 90 minutes once daily for 14 days, and 6 cats were not treated (control group). Bone labeling was performed, using fluorochrome markers. Cats were euthanized 5 weeks after implanting, and barium sulfate was infused to evaluate vascularization of grafts. Ulnas were evaluated by use of radiography, microangiography, histologic examination, and histomorphometric examination.

Results—Radiographic scores did not differ between treatment groups. Microangiographic appearance of grafted defects was similar between groups, with all having adequate vascularization. Differences were not observed between treated and nontreated groups in the overall histologic appearance of decalcified samples of tissue in grafted defects. Mean distance between fluorescent labels was significantly greater in cats given HBOT than in nontreated cats. Median percentage of bone formation in grafted defects was significantly greater in cats given HBOT.

Conclusions—Hyperbaric oxygen treatment increased the distance between fluorescent labels and percentage of bone formation when incorporating autogenous cancellous bone grafts in induced nonunion diaphyseal ulnar defects in cats, but HBOT did not affect revascularization, radiographic appearance, or qualitative histologic appearance of the grafts. (*Am J Vet Res* 2000;61:691–698)

Autogenous cancellous bone grafts are commonly used in animals to stimulate bony union after arthrodeses and in animals with multifragmented, delayed, or nonunion fractures.[1-9] Cancellous bone grafts possess 3 properties that augment osseous union: some donor cells survive transplantation and produce bone directly (osteogenesis), the graft acts as a scaffold for ingrowth of new bone from the recipient (osteoconduction), and the graft releases factors that cause transformation of undifferentiated recipient cells into bone-forming cells (osteoinduction).[10] The sequence of graft incorporation is influenced by vascularity of the recipient bed, stability of the repair, and whether there is infection.[2,11] Unfortunately, despite appropriate conventional surgical and medical treatment, including use of autogenous cancellous bone grafts, not all fractures and arthrodeses achieve union. Additional therapeutic modalities that enhance osseous union would be of benefit.[9]

Hyperbaric oxygen treatment (HBOT) consists of providing an environment of 100% oxygen in a chamber in which the pressure is maintained at > 1 **atmosphere absolute (ATA**; greater than pressure at sea level).[12,13] Therapeutic mechanisms and benefits of HBOT have been described[14,15] and include hyperoxygenation of hypoxic tissue,[13] direct stimulation of fibroblasts that causes an increase in collagen synthesis,[16] and enhancement of tissue revascularization.[17,18]

The effect of HBOT on incorporation of autogenous cancellous bone grafts has not been investigated. However, HBOT is considered to be an adjunct to the established treatment modalities of surgical debridement and systemically administered antibiotics in humans with chronic refractory osteomyelitis.[19-21] Hyperbaric oxygen treatment purportedly stimulates osteoclast function, fibroblast proliferation and collagen production, and macrophage production of angiogenesis factor by providing a steep gradient from hyperoxia to hypoxia.[22] A beneficial effect of HBOT on fracture healing in

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From the Departments of Veterinary Clinical Science (Kerwin, Lewis, Elkins, Hosgood, Pechman), Veterinary Pathology (Oliver, Dial), and Veterinary Physiology, Pharmacology and Toxicology (Strain), School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803. Dr. Lewis' present address is the Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL 32610-0126. Dr. Elkins' present address is Indiana Veterinary Surgical Referral Service, 61 E 96th St, Indianapolis, IN 46240-1006. Dr. Dial's present address is Animal Diagnostic Lab Inc, 175 E Fort Lowell, Tucson, AZ 85705.

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laboratory animals (rats, rabbits) has been reported.[23-26] In addition, investigators in another study[27] reported a beneficial effect of HBOT on incorporation of autogenous free corticocancellous grafts in rabbits.

Nonunion fracture models have been developed for the ulna of dogs and tibia of cats.[28-30] On the basis of the positive effects ascribed to HBOT for fracture healing and in the treatment of osteomyelitis, we hypothesized that HBOT would accelerate the incorporation of autogenous cancellous bone grafts in cats, using a nonunion model. The objectives of the study reported here were to duplicate a nonunion diaphyseal ulnar defect in cats that has been documented in dogs, determine whether HBOT would induce healing of a nongrafted experimentally induced nonunion diaphyseal ulnar defect, and determine whether use of HBOT in accordance with a specific protocol would accelerate incorporation of autogenous cancellous bone grafts in an experimentally induced nonunion diaphyseal ulnar defect in cats.

Materials and Methods

Animals—Twelve mature conditioned cats were randomly assigned to 2 groups: 6 cats received HBOT (treated group), and 6 cats did not (nontreated [control] group). Each cat was considered clinically normal on the basis of results of complete physical and orthopedic examinations as well as measurement of PCV, plasma total protein concentration, and concentration of BUN. The protocol used in this experiment was approved by the Louisiana State University Laboratory Animal Care and Use Committee. All cats were housed and cared for in an accredited facility (American Association for the Accreditation of Laboratory Animal Care) that conforms to guidelines established by the National Institutes of Health and the USDA.

Creation of nonunion defect—The procedure for creating a nonunion diaphyseal ulnar defect in the cats was adapted from a procedure described for dogs.[30] On day 1, food was withheld from each cat for 12 hours. Cats were given preanesthetic medication (ketamine hydrochloride; 8 mg/kg of body weight, IM). Anesthesia was induced by IV administration of a combination of diazepam (2 mg/kg) and ketamine (4 mg/kg), followed by mask administration of halothane. Anesthesia was maintained with halothane and oxygen administered via an endotracheal tube. Cats were given lactated Ringer's solution (10 ml/kg/h, IV) throughout the surgical procedure.

The forelimbs of each cat were shaved and prepared for surgery. A caudal surgical approach was used to expose each ulna.[31] Before surgery, the length of each ulna was determined from measurements obtained from radiographs. During surgery, a sterile ruler was used to identify the point at which the proximal and middle third of each ulna met. A full-thickness 1-cm segment of each ulna, including the periosteum, was removed from this point, using a hand-held oscillating saw.[a] A sterile polytetrafluoroethylene spacer,[b] 1 cm in length and 0.75 cm in diameter with a previously drilled central hole, was inserted into each defect. An intramedullary pin (1.57 or 1.93 mm in diameter, dependent on size of the cat and surgeon preference) was placed to retain the spacer and maintain alignment of the ulna. Muscle fascia and subcutaneous tissues were closed, using 3-0 polydioxanone in a simple continuous pattern, and the skin was closed, using 3-0 nylon in a Ford interlocking pattern. Butorphanol tartrate was administered (0.2 mg/kg, IM) immediately after surgery as an analgesic. Placement of spacers was evaluated on lateral radiographs of the antebrachium in each cat (**Fig 1**).

Cats were examined twice daily throughout the remainder of the study. Wounds were inspected for evidence of swelling, discharge, or dehiscence. Rectal temperature of each cat was recorded daily.

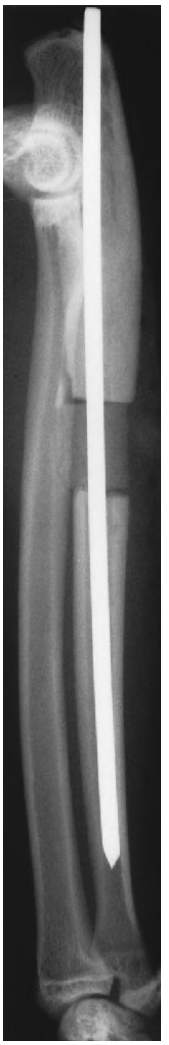

Figure 1—Lateral radiographic view of the forelimb of a cat after the initial surgery. Notice the position of the spacer and intramedullary pin.

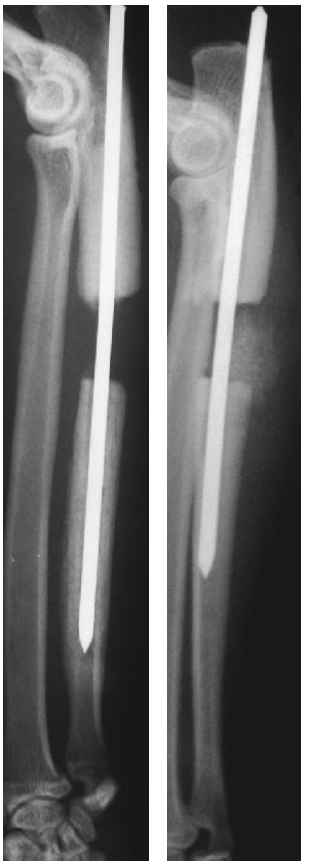

Figure 2—Lateral radiographic views of the forelimb of a cat after removal of the spacer, revealing the open (nongrafted) nonunion (left) and evidence of a cancellous bone graft in the nonunion of the contralateral ulnar diaphysis (right).

Grafting procedure—On day 20, lateral radiographs of the antebrachium of each forelimb were obtained. On day 21, cats were prepared for surgery and anesthetized as described. Cefazolin (22 mg/kg, IV) was administered immediately after induction. Both forelimbs were clipped and aseptically prepared, including the proximal portion of each humerus. The ulnas were exposed by use of the caudal surgical approach described, and intramedullary pins were removed, which allowed removal of polytetrafluoroethylene spacers. New intramedullary pins of equal or greater diameter were placed after removal of the spacers. When the ulna initially had been stabilized with a 1.57-mm-diameter intramedullary pin, a 1.93-mm-diameter intramedullary pin was placed. When a 1.93-mm diameter intramedullary pin had been used initially, a pin of identical diameter was placed. Margins of the ulnar ostectomy were not curetted. An aliquot (0.5 ml) of cancellous bone was harvested from the proximal portion of each humerus.[8] The volume of graft was quantitated by use of a modified 3-ml syringe.

Assignment of the limb in which the graft was placed was determined by use of a complete-block randomized design. An autogenous cancellous bone graft was packed around the intramedullary pin in the defect in the assigned ulna. The defect in the contralateral ulna did not receive a

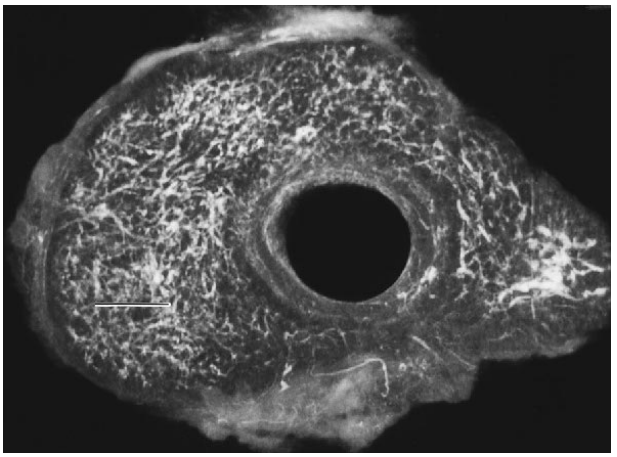

Figure 3—Microangiograph of a section of tissue obtained from the ulna of a nontreated cat 5 weeks after grafting. Notice the adequately vascularized cancellous bone. The section was obtained from the area adjacent to the distal graft-host interface. Not stained. Bar = 150 µm.

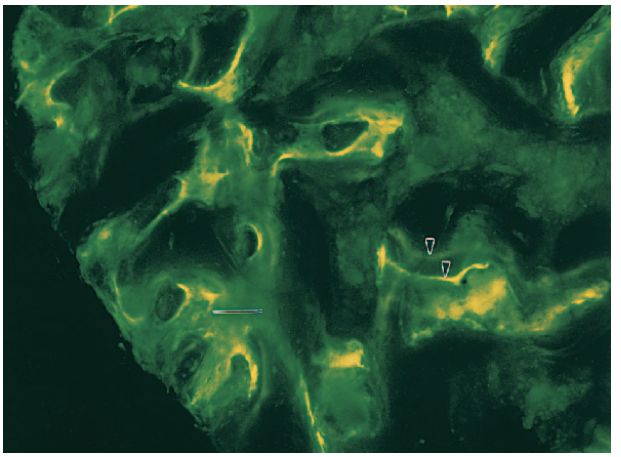

Figure 4—Photomicrograph (obtained by use of a fluorescent microscope) of a section of ulna obtained from a cat after administration of calcein green on day 35 and oxytetracycline on day 42. Two fluorochrome labels, evident as bright parallel bands (arrows), indicate uptake by mineralizing osteoid. Not stained. Bar = 20 µm.

graft. Wounds were closed as described. Butorphanol was administered after surgery as an analgesic, as described. Lateral radiographs of the antebrachium of each forelimb were obtained immediately after surgery (**Fig 2**). Cats were allowed to fully bear weight immediately after the surgeries on days 1 and 21.

Hyperbaric oxygen treatment—The HBOT were performed in an animal hyperbaric chamber (1 m^3).[c] Three cats, each in a separate cage, were treated concurrently. Treated cats were exposed to 90 minutes of 100% oxygen at 2 ATA (202.6 Pa) daily beginning on day 22 (the day after the second surgery) and continuing until day 36 (duration, 14 days). A continuous flow of oxygen (10 L/min) was maintained throughout the procedure. Sedation was not required. Nontreated cats were placed in the chamber daily for 90 minutes and exposed to room air at atmospheric pressure on days 22 to 36.

Administration of fluorochrome label—Oxytetracycline[d] (30 mg/kg, IV) and calcein green[e] (30 mg/kg, IV) were given to all cats to label newly mineralized bone. Oxytetracycline was diluted to a half-strength concentration (50 mg/ml) with sterile water and injected slowly during a 2- to 3-minute period via the cephalic vein on days 28 and 42. Calcein green was prepared as a 1% solution, using sterile buffered deionized water, and diluted to a 0.066% solution in saline (0.9% NaCl) solution; it was injected slowly during a 20-minute period via the cephalic vein on day 35.[32-34]

Radiography—Lateral radiographs of the antebrachium of each forelimb were obtained on days 0 and 20, as described. On day 56 (35 days after grafting), lateral radiographs of the antebrachium of each forelimb were made to evaluate incorporation of the graft and healing of the nongrafted defect. These radiographs were evaluated, using a scoring system reported elsewhere.[35] Briefly, a score of 7 points was possible, using a combination of scores for 3 categories (appearance of graft: 0 = resorbed, 1 = mostly resorbed, 2 = largely intact, 3 = reorganizing; quality of proximal union: 0 = nonunion, 1 = possible union, 2 = radiographic union; quality of distal union: 0 = nonunion, 1 = possible union, 2 = radiographic union). Scoring was performed by a board-certified veterinary radiologist (RDP) who was unaware of the treatment group to which each cat had been assigned.

Vascular infusion—On day 56, cats were sedated with ketamine (8 mg/kg, IM), and a catheter was placed in a peripheral vein. Heparin (300 U/kg, IV) was administered and allowed to disperse in the circulation for 5 minutes prior to induction of anesthesia. Anesthesia was induced, using sodium thiamylal (10 mg/kg, IV), and median sternotomy was used to enter the thoracic cavity. The aortic root was isolated, using a circumferential double loop of 2-0 silk, and cats were euthanatized with an overdose of thiamylal. The aorta was cannulated, using the end of an intravenous infusion set, and radiographic contrast material and formalin were infused, as described elsewhere.[36] The cranial vena cava was transected to allow exsanguination and monitoring of perfusion of the angiography solution. Vasculature was infused via the aorta with a 30% barium sulfate suspension[f] in saline solution at 120 to 140 mm Hg pressure until the venous effluent was pink as a result of the high concentration of barium sulfate. Footpads of the forelimbs were incised, and detection of pink effusion confirmed that the limbs were adequately perfused with the suspension. A second infusion of a solution of 30% barium sulfate diluted with 10% formalin[g] was performed in the same manner. After infusions were completed, forelimbs were removed from each cadaver. Muscles were removed from the ulna on each limb; care was taken to avoid disturbing the fracture callus. Ulnas were labeled for identification and placed in neutral-buffered formalin for 14 days. After removal from formalin, ulnas were deep-frozen (–80 C) to preserve fluorescent bone labels.

Sample preparation for microangiography and histologic examination—The healed callus of those ulnas that had achieved bony union, regardless of whether they had received a cancellous bone graft, was sectioned perpendicular to the long axis of the bone. Sections were cut, using a low-speed diamond-blade saw,[h] and sections were numbered in sequential order from distal to proximal. Alternating thick (approx 1 mm) and thin (approx 200 µm) sections were cut. Thick slices were slowly decalcified, using citrate-buffered formic acid.[37] Microangiographs were made of all decalcified sections, as described elsewhere (**Fig 3**).[36] Thin, undecalcified sections were stored in a solution of 80% ethyl alcohol:20% neutral-buffered formalin for evaluation of fluorescent labels.

Decalcified sections (those originally used for the microangiographs) from each processed ulna were embedded in paraffin, sectioned at a thickness of 6 µm, and stained, using H&E or picrosirius red to enable correlated descriptive

histologic evaluation.[38] Specimens were evaluated qualitatively, with particular attention to appearance and activity of osteoblasts, osteoclasts, evidence of endochondral ossification, and evidence of inflammation or infection. A board-certified veterinary pathologist (JLO) performed the evaluations and was unaware of the treatment group to which each cat had been assigned.

Histomorphometric analysis—Undecalcified sections were manually ground, using 600-grit sandpaper, to a thickness of approximately 70 to 100 µm under running water[39] and mounted on glass slides by use of buffered-glycerol mounting medium.[40] A coverslip was applied, and slides were examined, using a fluorescent microscope[i] linked to a computerized image-enhancement and morphometrics program.[j] The optical-electronic system was calibrated directly by use of a stage micrometer.[k] Distance between adjacent fluorescent bone labels was measured at 20× magnification. Two hundred measurements were made per ulna.[33,41,42] We did not attempt to correct for obliqueness of the sections.[34,42] All measurements were made by the same investigator (SCK), who was unaware of the treatment group to which each cat had been assigned.

The percentage of bone in each section was calculated by examining the same slides used to measure distance between fluorescent labels. Only limbs in which a graft was placed were examined. Histomorphometric analysis was conducted by use of a bone morphometry program.[l] This was accomplished by initially calculating the total area of tissue in the measured section, which was determined on the basis of the measured peripheral circumference of the section. The space occupied by the pin was subtracted from total area to provide actual tissue area. Gaps in the section created during processing or incomplete filling of the nonunion also were subtracted from the total area. Finally, the area of bone in the section was outlined, and the percentage of bone relative to the total area of tissue in the section was calculated ([bone area/total area] – [pin area + gaps]). The remainder of the tissue was cartilage or fibrous tissue. Median percentage of bone was calculated for each group, and comparisons were made.

Statistical analysis—All statistical analyses were performed by use of nonparametric methods. For histomorphometric data, comparisons between groups were made by use of the Wilcoxon-Mann-Whitney test for independent samples. Comparisons within groups were made by use of the Wilcoxon signed-rank test for dependent samples. For radiographic data, frequency of scores was compared between treatments (HBOT vs no HBOT) for grafted and nongrafted limbs, using the Fisher exact test against a 2-sided hypothesis. Frequency of scores was compared within treatment group (between grafted and nongrafted limbs), using the Mantel-Haenszel comparison of repeated categoric data to account for paired data; a 2-sided hypothesis was tested. Mode (most frequent) score was used as a summary measurement for all scores. Significance for all tests was defined at $P \leq 0.05$. Statistical software[m] was used for all analyses.

Results

Clinical evaluation—All cats were ambulatory and able to bear weight on the day after each of the surgeries. None of the cats developed clinical signs consistent with infection (swelling, heat, discharge) at the surgical sites throughout the study. One cat broke both 1.57-mm intramedullary pins several days after the first surgery, and a second cat bent both 1.57-mm intramedullary pins several days after the first surgery. These incidents were accompanied by sudden onset of lameness in the affected limbs. These cats were anesthetized, and surgery was performed as before, except the 1.57-mm intramedullary pins were replaced with 1.93-mm intramedullary pins. Problems were not encountered in either cat after that additional surgery. Examination of lateral radiographs of the forelimbs obtained on day 20 revealed mild caudoproximal displacement (1 to 3 mm) of the proximal ulnar segment of the nonunion in 5 of the other cats in which 1.57-mm intramedullary pins were used. However, these cats used the limbs well, and surgical intervention was not required. After this experience early in the study, 1.93-mm intramedullary pins were used subsequently in all cats.

Adverse effects were not observed in association with HBOT. Three cats vomited immediately after injection of oxytetracycline, and 1 other cat vomited during infusion of the calcein green. Cats had an orange hue, particularly noticeable in the sclera, mucous membranes, and nonpigmented skin, for about 2 hours after infusion of the calcein green. Other adverse effects were not detected, and all cats maintained an excellent appetite throughout the study.

Radiographic evaluation—All pins were within the ulnar medullary canal after all surgeries. All spacers were correctly placed and completely filled each diaphyseal gap. Grafted and open defects were easily distinguishable after the second surgery. Total radiographic scores were not significantly different between treatment groups for grafted ($P = 1.000$) or nongrafted ($P = 0.455$) limbs. Radiographic scores differed significantly within treatment groups when comparing between grafted and nongrafted defects (treated group, $P = 0.014$; nontreated group, $P = 0.002$, respectively). There was 1 cat in the treated group in which the bone graft was almost completely resorbed. That cat received a radiographic score of 1 for the grafted defect and 0 for the nongrafted defect. In the nontreated group, the nongrafted nonunion was almost completely healed in 1 cat; that cat received a radiographic score of 6 for the grafted defect and 5 for the nongrafted defect. The mode of the radiographic scores was 3 for grafted defects in the treated group, 0 for nongrafted defects in the treated group, 2 for grafted defects in the nontreated group, and 0 for nongrafted defects in the nontreated group. Radiographic scores did not differ significantly between treatment groups for comparison of grafted ($P = 0.27$) and nongrafted ($P = 0.9$) defects.

Microangiography—We did not detect a qualitative difference in the appearance of the microangiographs between treatment groups. Defects that did not have sufficient bony callus to be sectioned (11/12 nongrafted defects and 1/12 grafted defects) were not examined. All sections were vascularized throughout the entire cross section of the graft. In a large proportion of the samples in both groups, there was a relatively avascular area adjacent to the pin track.

Histologic examination—Sections of callus from 5 ulnas of the treated group and 7 ulnas of the nontreated group were evaluated. Nonunions that did not have grossly visible reformed bone had a thin (1 to 3 mm)

layer of fibrous tissue surrounding the intramedullary pin and were not examined. Appreciable differences in the global histologic appearance of the decalcified samples between ulnas from cats in treated and nontreated groups were not apparent. All samples had an active osteoblast population and deposition of new bone. Osteoclastic activity was evident, but nominal, in most sections. Bone marrow elements were beginning to form in many sections, and a few sections had a large amount of cartilage undergoing endochondral ossification. A small capsule of fibrous tissue surrounded most of the bones. Additionally, there was usually a small amount of fibrous tissue surrounding the original pin track. Inflammatory change was not observed in any section, nor was there evidence of graft rejection or active bone resorption.

Histomorphometric analysis—The calcein green administered on day 35 and oxytetracycline administered on day 42 were incorporated into bone as fluorescent labels (**Fig 4**). Distance (mean ± SD) between fluorescent labels in treated cats was 35.0 ± 10.80 µm, which was significantly ($P < 0.001$) greater than the distance between fluorescent labels in nontreated cats (29.5 ± 9.17 µm).

Median percentage of bone was compared between treatment groups for the grafted defects. Median percentage of bone was 58.23% for the treated group and 47.06% for the nontreated group. A score of zero was recorded for 1 cat in the treated group that had completely resorbed the graft. Median value for percentage of bone did not differ significantly ($P = 0.07$) between treatment groups when data from all cats were included; however, when the data for the cat that completely resorbed the graft in the treated group was excluded as an outlier, median percentage of bone for the remaining cats in the treatment group was significantly ($P = 0.01$) higher than values for the nontreated group.

Discussion

The nonunion model used in the study reported here was developed on the basis of Key's hypothesis, which states that a segmental long-bone defect 1.5 times the diaphyseal diameter exceeds the regenerative capacity of bone and results in nonunion; this has been documented to be true for a tibial nonunion model in cats.[28,29,43] We were able to duplicate a convenient and reliable diaphyseal ulnar nonunion model in cats similar to that described in dogs.[30] We chose to adapt the bilateral ulnar nonunion model for dogs to cats in this study, because the surgical procedure is less complex than the tibial model, and it carries less morbidity (animals can bear weight immediately after a bilateral procedure, because the radius remains intact).[30,35,44] The spacer used in this study and a study in dogs[30] is a modification of the original ulnar diaphyseal defect model that is commonly used.[45,46] The spacer aids in development of the nonunion by physically blocking ingrowth of capillaries and soft tissues, and it may provide a more uniform diaphyseal defect into which cancellous bone can be placed. However, it may not be necessary for the success of the nonunion model used in this study.

Caudoproximal displacement of the proximal ulnar segment and breakage of intramedullary pins was observed in some cats. We stabilized ulnar ostectomies with 1.57-mm intramedullary pins on the basis of results reported in dogs,[47] but we found the stability afforded by these small-diameter pins to be inadequate. Reasons for this could include the increase in length of the ostectomy, compared with that created in dogs for correction of humeroulnar subluxation, or greater forces placed on the pins by the activities of the cats. It has been suggested that the ulna is relatively larger in cats than dogs and, thus, bears proportionately more weight.[48] Subluxation was not a problem when a 1.93-mm intramedullary pin was used to stabilize the ulnar ostectomy.

It is possible that the problems encountered with the pin breakage in this model may have affected our results, particularly with such a limited number of animals. Alternatively, the proximal ulnar segment could have been transfixed to the proximal portion of the radius by use of a cortical bone screw.[49] Finally, the nonunion could have been created more distally on the ulna, thus negating the pull of the triceps muscle; however, it was believed that the distal aspect of the diaphysis was too small to provide adequate tissue for processing. Further work evaluating and validating this nonunion model, with the above modifications, is indicated.

Union of the nongrafted defect was detected in 1 cat in the nontreated group. Union of nongrafted nonunion defects was not reported in dogs in some studies[29,30] nor in cats with a nonunion of the tibia[29]; however, Heiple et al[5] reported 1 out of 8 unions in the nongrafted limb by use of this model. Inadequate excision of the periosteum at the time of ulnar ostectomy may have been a factor resulting in union of the nongrafted defect, or it may have simply been the result of variation among cats.[50] A union failed to develop in 1 grafted defect in a cat in the treated group, with complete resorption of the graft by 5 weeks. A union also failed to develop in the nongrafted defect in that same cat. It has been reported[5] that even a fresh autogenous cancellous graft placed into a bone defect will not routinely result in successful union, and fibrous tissue and a resorptive response predominate in a small minority of animals.

The use of HBOT at 2 ATA for 90 minutes once daily appears to be safe in mature healthy cats. Adverse effects of HBOT may include neurologic abnormalities (most noticeably seizures) and barotrauma resulting in signs of pain and dysfunction from air trapped in the middle ear, sinuses, teeth, or lungs.[12] We did not observe any adverse effects in the cats treated in this study.

Radiographic evaluation was useful in this study for assessing placement and stability of implants and observing the fate of autogenous cancellous bone grafts. As expected, radiographic scores within groups differed significantly when comparing grafted defects to nongrafted defects. On the basis of these findings, we state that HBOT did not induce union of the nongrafted nonunion defects.

Examination of microangiographs of the grafts did not reveal differences between treatment groups. Autogenous cancellous bone grafts revascularize quickly,[30] and the grafts in both groups were adequate-

ly vascularized. Microangiography is a subjective evaluation of bone vascularization, difficult to quantify, and probably most helpful when there are fairly dramatic differences between treated and control groups. It functions primarily as another method of tissue description and is representative of the vascular pattern at the time of infusion.[36] It may prove more useful in future studies involving the use of allogenous cancellous grafts in which vascularization is not nearly as rapid.[30] A more quantitative technique, such as regional blood flow measurement by use of radioactive microspheres, may have documented a difference between groups.[51]

Fluorochrome markers were administered to allow quantitation of dynamic variables of bone formation.[33] Oxytetracycline and calcein green form complexes with calcium ions and, therefore, are incorporated into newly mineralizing osteoid as it is laid down by active osteoblasts.[32] Fluorochrome labels are incorporated into actively mineralizing bone and will not be incorporated when they are given before osteoid is being mineralized. Two labels were detected when we examined the undecalcified sections with a fluorescent microscope. Because we administered our first dose of oxytetracycline 7 days after the second (grafting) surgery, it is likely that the label was not incorporated. Osteoid generally is not being mineralized in appreciable amounts in cancellous bone autografts until at least 10 to 14 days after grafting.[5]

Distance between fluorescent labels was greater in the grafted defects in cats given HBOT. This reflects a greater amount of osteoid production in the autogenous cancellous bone grafts in the treated group between days 35 and 42 and indicates accelerated graft incorporation during this time. We did not attempt to calculate mineral apposition rate, because we did not measure osteoid volume, percentage of fractional-labeled osteoid, or circumference of fluorescent labels. Those variables are much more difficult to measure in cancellous bone, compared to cortical bone with discrete Haversian systems. Cancellous bone grafts are incorporated by new bone being laid down on necrotic trabeculae.[2,4] The increased distance between fluorescent labels observed in the treated cats could be a function of increased activity of osteoblasts, decreased activity of osteoclasts, or a combination of both.

Osteoblasts are responsible for producing osteoid. Energy required for calcification of osteoid is produced by aerobic conditions; however, calcium release from osteoblast mitochondria requires anaerobic conditions.[52] This implies that aerobic and anaerobic conditions are necessary for bone formation. A study[53] of spontaneously hypertensive rats treated with hyperbaric oxygen (1 hour; 2.8 ATA; 5 d/wk for 30 treatments) revealed a positive effect on osteogenesis, compared with nontreated control rats. Investigators in that study postulated that the production of energy for calcification is enhanced during exposure to HBOT, but the interval of hypoxia (time elapsed between treatments) is essential to allow critical anaerobic bone-forming activities to take place.

Previous in vivo studies[23-25] in which investigators examined the response of bone healing in rats and rabbits subjected to HBOT documented an increase in all variables studied (breaking strength of femur, calcium content of bone, bone ingrowth into a titanium chamber, healing of standardized drill hole) at lower doses of HBOT (range, 2 hours at 2.8 ATA, q 24 h for 14 days to 1 hour at 3 ATA, q 12 h for 30 days). The HBOT protocol chosen for our study was developed on the basis of results obtained by Barth et al.[26] Standardized drill holes in femurs of rats healed more rapidly when exposed to a relatively low dose of hyperbaric oxygen (2 ATA, q 24 h for 90 minutes, 5 d/wk for 20 treatments), compared with healing in nontreated control rats. The positive effects of HBOT on bone healing appear to be dose-dependent. In that same study,[26] it was documented that rats exposed to a higher dose of hyperbaric oxygen (2 ATA for 90 minutes, q 12 h, 5 d/wk for 20 treatments) had pronounced osteoclastic activity and delayed healing of the drill holes, compared with values for daily-treatment and control groups. This is similar to findings reported by Wray and Rogers,[54] who used higher doses of hyperbaric oxygen (5 hours at 2 ATA, q 24 h for 14 days), which resulted in a decrease in breaking strength of bones in a femoral fracture model in rats.

We suggest that an increase in the oxygen supply to the surviving osteoblasts in the graft in the treated group caused an increase in collagen production, which contributed to the overall increased distance between fluorescent labels on the basis that a portion of osteoid is composed of collagen.[55] Additionally, it is possible that there was an increased survival of transplanted osteoblasts in the treated group as a result of higher availability of oxygen.

Little osteoclastic activity was observed in the grafts of treated or nontreated cats in the study reported here. Most activity was overwhelmingly osteoblastic, associated with pronounced production of new bone. This is in contrast with reports about hyperbaric pressures and increased partial pressure of oxygen. It has been reported[56] that prolonged localized pressure (15 mm Hg or 0.020 atmosphere applied to the right bulla for 7 days) alone (in room air) can cause increased systemic osteoclast activity in gerbils. In another study,[57] it was reported that oxygen-derived free radicals stimulate the formation and activation of osteoclasts. Osteoclasts are derived from macrophages, which perform many of their functions via formation of superoxide radicals[57,58] and can have an increase in activity when exposed to hyperbaric oxygen.[59]

Oxygen-derived free radicals are products of normal cellular oxidation-reduction processes. Reactive oxygen species are generated when cancellous bone grafts are procured.[60,61] Under conditions of hyperoxia, oxygen-derived free-radical production increases profoundly and is believed to contribute to the development of oxygen toxicosis under prolonged hyperbaric oxygenation conditions.[62] Stimulation of osteoclast development and activity by oxygen-derived free radicals, plus the increase in pressure involved in HBOT, could explain the massive osteoclastic activity observed at high oxygen concentrations reported elsewhere.[63] It remains unexplained why osteoclast activity

was not prominent in our study or those of others[23-25] and why it appears to be dose-dependent.[26]

Differences could not be detected between treatment groups in the global histologic appearance of the decalcified sections of bony callus. As mentioned, there was active osteoblastic activity and deposition of new bone. Evidence of inflammation, graft rejection, or active bone resorption was not detected. When bone was evaluated more quantitatively, there was a trend, although not significant, toward an increased median value of percentage of bone in all sections of each grafted defect in the treated group, compared with the same value for the grafted defect in the control group. This difference became significant when data for the cat that completely resorbed the graft in the treated group was excluded.

Further investigation will be required to obtain detailed histomorphometric data on the effect of HBOT on the incorporation of cancellous bone grafts, including autogenous and allogenous grafts. Investigation at the cellular level is necessary to determine the exact effects of hyperbaric oxygen on the function of osteoblasts, whether transplanted or in situ, as well as its effects on other cells involved in the production and removal of bone.

[a]Sagittal saw with battery, Dyonics, Andauer, Mass.
[b]Teflon fluorocarbon policeman, Fisher Scientific Co, Pittsburgh, Pa.
[c]United States Air Force Type 2 animal hyperbaric chamber.
[d]Oxyvet 100, Pfizer Inc, Brooklyn, NY.
[e]Calcein, Sigma Chemical Co, St Louis, Mo.
[f]Novopaque, Picker International Inc, Highland Heights, Ohio.
[g]Micropaque, Barkley Medical Products, Anaheim, Calif.
[h]Isomet, Beuhler Ltd, Lake Bluff, Ill.
[i]Axioplan, Zeiss, Oberkochen, Germany.
[j]Image1, Universal Imaging Corp, Westchester, Pa.
[k]Stage micrometer, Leitz, Wetzlar, Germany.
[l]Bioquant, R&M Biometric Inc, Nashville, Tenn.
[m]SAS, version 6.12, SAS Institute Inc, Cary, NC.

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