Quantifying Massive Allograft Healing of the Canine Femur In Vivo and Ex Vivo

A Pilot Study

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Published online: 24 April 2012
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Abstract

Background Allograft integration in segmental osseous defects is unpredictable. Imaging techniques have not been applied to investigate angiogenesis and bone formation during allograft healing in a large-animal model.

Questions/purposes We used dynamic contrast-enhanced (DCE)-MRI and cone beam (CB)-CT to quantify

vascularity and bone volume in a canine femoral allograft model and determined their relationship with biomechanical testing and histomorphometry.

Methods Femoral osteotomy was performed in three dogs and reconstructed with a 5-cm allograft and compression plate. At 0.5, 3, and 6 months, we performed DCE-MRI to quantify vascular permeability (Ktrans) and perfused fraction and CB-CT to quantify bone volume. We also performed postethanasia torsional testing and dynamic histomorphometry of the grafted and nonoperated femurs.

Results DCE-MRI confirmed the avascular nature of allograft healing (perfused fraction, 2.08%–3.25%). CB-CT demonstrated new bone formation at 3 months (26.2, 3.7, and 2.2 cm³) at the graft-host junctions, which remodeled down at 6 months (14.0, 2.2, and 2.0 cm³). The increased bone volume in one subject was confirmed with elevated Ktrans (0.22) at 3 months. CB-CT-identified remodeled bone at 6 months was corroborated by histomorphometry. Allografted femurs recovered only 40% of their strength at 6 months.

Conclusions CB-CT and DCE-MRI can discriminate differences in angiogenesis and bone formation in the

One or more of the authors (BGS, NE, EMS) have received funding from the Musculoskeletal Transplant Foundation (Edison, NJ, USA). One or more of the authors have received funding from the following grants: NIH PHS AR054041 (EMS), AR056696 (EMS), DE019902 (EMS), AR061307 (EMS), and 1R01EB012048-01A1 (BGS). All ICMJE Conflict of Interest Forms for authors and *Clinical Orthopaedics and Related Research* editors and board members are on file with the publication and can be viewed on request. Each author certifies that his or her institution approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research. All in vivo work and ex vivo cone beam CT and imaging analyses were performed at the University of Rochester, Rochester, NY, USA. The ex vivo biomechanics, histology, and histomorphometry were performed at Colorado State University, Fort Collins, CO, USA.

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canine allograft model, which has potential to detect a small (32%) drug or device effect on biomechanical healing with only five animals per group.

Clinical Relevance These radiographic tools may have the potential to assess adjuvant effects on vascular invasion and new bone formation after segmental allograft transplantation.

Introduction

Massive allografts remain a standard for reconstruction of large bone defects [4]. However, allografts display limited angiogenic and osteogenic potential [25]; thus, healing is slow and limited to cortical-cortical union from internal cancellous-cancellous junctions at 5 years [8, 9]. Additionally, the allograft stimulates a host-mediated fibrotic reaction due to histocompatibility mismatch, which encases the allograft prohibiting vascular invasion and remodeling [3, 22, 23]. These limitations, in addition to graft type (intercalary/osteochondral), preservation technique, and internal fixation approach, are associated with a 25% to 35% failure rate due to nonunion and fracture [2, 14] and a 10-year survival rate of less than 50% due to the accumulation of unremodeled microfractures, which compromise their mechanical properties [29]. To address these shortcomings, investigators have developed biologic adjuvant therapies (ie, BMP-2 [6, 15, 32], teriparatide [19, 24]), stem cell [26, 30, 33], and gene [11, 12, 31]), which stimulate integration of cortical allografts. While these technologies have accelerated healing in small-animal models, their application in humans has been unrealized. This is in part because a large-animal model of allograft transplant has not been established nor have clinically available measures been investigated that may provide quantitative insight into the course of allograft healing. For the latter to occur, noninvasive imaging tools must be validated against current methods of allograft revitalization assessment, including biomechanical testing and histology.

The limitations of traditional radiology, in which two-dimensional radiographs and bone scans are not sufficiently quantitative in longitudinal studies, have long been recognized [13]. Three-dimensional (3D) approaches (MRI and CT) suffer from artifacts due to the orthopaedic metallic hardware required to secure massive allografts [10, 16]. To overcome these issues, we developed dynamic contrast-enhanced (DCE)-MRI as a quantitative assessment tool of intramedullary vascularity in a canine osteotomy model and cone beam (CB)-CT to quantify new bone formation around massive allografts [7]. We found we could quantify cortical bone revascularization by DCE-MRI and new bone formation by effectively suppressing metal artifact using CB-CT.

Building on these findings, we performed a pilot study to evaluate the canine model as a large-animal model to study segmental bone allograft healing and addressed the following questions: (1) Are DCE-MRI and CB-CT able to discriminate subtle differences in angiogenesis and bone formation after femoral allograft transplant? (2) Do DCE-MRI and CB-CT data corroborate traditional biomechanical and histologic measures of massive allograft healing given that these *ex vivo* tests cannot be performed in a clinical trial? And (3) do biomechanical differences (effect size and SDs) between the allografted and contralateral femurs at 6 months support the feasibility of this large-animal model using a relatively small sample size (eg, $n \leq 5$)?

Materials and Methods

We used *in vivo* DCE-MRI and CB-CT in three dogs at 0.5, 3, and 6 months after femoral allografting and post euthanasia CB-CT, biomechanical testing, and histology (Fig. 1). We quantified vascularity, expressed as the Ktrans coefficient and percent perfusion, and bone volume with these techniques to investigate the large-animal model of segmental allograft healing. *Ex vivo* torsional biomechanical testing and dynamic histomorphometric analyses were performed to corroborate the *in vivo* findings regarding angiogenesis and bone formation.

We used three skeletally mature (> 1-year) Walker Hounds (Marshall BioResources, Hamilton, NY, USA): USDA 728373 (Flora), 24 kg; USDA 725510 (Fauna), 23 kg; and USDA 728225 (Merryweather), 20 kg. The dogs underwent a 5-cm femoral middiaphysis osteotomy, which was repaired with a standard 5-cm canine femoral allograft (Veterinary Transplant Services, Inc, Kent, WA, USA) and a 12-hole 3.5-mm dynamic compression plate and screws (Synthes Inc, West Chester, PA, USA) as previously described [7]. All procedures were performed in accordance with NIH guidelines for animal use and were approved by the University of Rochester Committee for Animal Resources. Surgical recovery was monitored every 12 hours for 7 days and examination for lameness, swelling, and pain was performed. Postoperative analgesia was achieved using a combination of the fentanyl patch placed before surgery and adjunctive oral morphine (1–2 mg/kg orally) delivered every 8 hours as needed. Functional and pain assessments over the first week after surgery demonstrated the procedure was well tolerated. No dogs required analgesia after Day 3.

The detailed methodology associated with DCE-MRI and CB-CT scanning is provided in our prior report [7]. We performed DCE-MRI at 0.5, 3, and 6 months postoperatively using a 1.5-T GE LX Signa instrument (GE Medical Systems, Milwaukee, WI, USA) in the coronal plane by

Fig. 1 A flowchart of the experimental design illustrates the in vivo time points at which DCE-MRI and CB-CT were used to quantify vascularity and new bone formation in the allograft-reconstructed

femurs of the three subjects. Ex vivo biomechanical testing and histomorphometry were performed at 6 months to corroborate the in vivo findings.

intravenous (iv) contrast injection of 0.1 mmol/kg gadolinium diethylenetriamine pentaacetic acid (Magnevist®; Berlex Laboratories, Princeton, NJ, USA) using a power injector at 3 mL/seconds, followed by twice that volume of normal physiologic saline. Four slices through the femur and orthopaedic hardware were repeatedly scanned for 40 phases before, during, and after contrast injection. We performed a compartmental-based analysis of DCE-MRI using region-of-interest (ROI) analysis of the allografted femur with 3D geometrically constrained region growth (3D GEORG) (Perfusion Analyzer; VirtualScopics Inc, Rochester, NY, USA) [1]. An arterial region was also identified to guide the derivation of an automated arterial input function from the software program. The volume transfer rate constant between arterial plasma and extracellular extravascular space (Ktrans) was calculated, as an indicator of flow and vascular permeability. The perfusion fraction was also quantified by the Perfusion Analyzer using a vascularized heat map analysis of the allograft in which the saturated (visually red) voxels were divided by the total number of voxels.

CB-CT scanning was performed at 0.5, 3, and 6 months postoperatively. The system produces a volume reconstruction with 3D isotropic resolution ranging from 83 to 194 µm³. Reconstructed images of the object were uploaded in Amira® 5.2.1 (Visage Imaging, Inc, San Diego, CA, USA) where analysis was performed by a single individual (RBB). After isolation from other bone structures, femurs were segmented out using a semiautomated threshold technique in the presence of metal artifact as previously described [7] and the volume of the femur was calculated. Prior work by our group has demonstrated both reproducibility and validity of the CB-CT volumetric measurements [7]. The length of each femur was then measured using the lateral condyle and major trochanter and union ratios were calculated using custom software

(MATLAB®, The Mathworks, Natick, MA, USA) as previously described [18].

Dogs were euthanized at 6 months using an overdose of iv pentobarbital sodium (approximately 1 mL/4.5 kg). The allografted lower limb was harvested for ex vivo CB-CT scans before and after removing the metal hardware, allowing investigation of the error in segmentation due to metal artifacts. Error was less than 5%, with no differences in our in vivo versus ex vivo bone volume measurements with hardware or in the ex vivo bone volume measurements with or without implants, indicating the reproducibility and reliability of CB-CT to suppress metal artifact and to measure longitudinal bone volume.

We performed destructive torsional testing of the allografted and unoperated contralateral femurs to determine torsional rigidity and failure torque as we previously described [17, 20] using a materials testing machine (MTS Systems Corp, Eden Prairie, MN, USA). Two additional unoperated canine femurs from a dog of the same breed and approximate weight that was not part of the in vivo study were included as controls. The femurs were disarticulated and the implants removed and the femoral ends were cemented into potting boxes using polymethylmethacrylate (PMMA) in a custom jig to ensure axial alignment and a consistent gage length. A compressive preload of 20 N was applied to the constructs to establish a reference configuration and the proximal femur was externally rotated at 10°/second (0.17 radians/second) until failure.

For histomorphometry, the dogs received alizarin red (25 mg/kg iv) and calcein green (25 mg/kg iv) 8 days and 1 day, respectively, before sacrifice. We performed histomorphometric analyses on undecalcified sections of the canine femurs, which were processed immediately after biomechanical testing, using methods identical to those previously reported [20, 21]. Briefly, transverse cuts were made at the level of the PMMA to isolate the femoral

diaphysis. The remaining segment was cut in the transverse plane through the middle of the allograft, rendering proximal and distal femoral sections. Each section was subsequently cut in the sagittal plane, producing medial and lateral halves. Tissue sections were infiltrated and embedded in Technovit[®] light-curing polymer (Electron Microscopy Sciences, Hatfield, PA, USA) over 4 weeks. One 80- μ m-thick histologic section/specimen was taken in the sagittal plane. The sections remained unstained until completion of the dynamic analysis and then were stained using Sander-son's rapid bone stain (Surgipath, Richmond, IL, USA) and counterstained with acid fuchsin. A single author (BGS) experienced in histomorphometric analysis [20, 21] measured mineralizing surface (MS), mineral apposition rate (MAR), and bone formation rate (BFR) within eight ROIs/slide, including the host, allograft, periosteal callus, and host-allograft junction. Treating each dog as the experimental unit, global averages were generated for each ROI.

The DCE-MRI-derived volume transfer coefficient (Ktrans) and perfusion fractions (%) are reported as singular values for each dog at the 0.5-, 3-, and 6-month time points, as is the CB-CT-derived bone volume. Union ratio is reported

for each dog at 6 months. Torsional rigidity, failure torque, and rotation at failure are reported as mean $\pm$ SD, as are MS, MAR, and BFR. Given the pilot nature of our experimental design, statistical analysis was not performed. Biomechanical properties of the allografted femurs are reported as a percentage of intact tissue as an indicator of graft healing.

Results

Longitudinal DCE-MRI assessment of allograft vascularity (Ktrans) increased from baseline and peaked at 3 months postoperatively for Flora before decreasing to near 0.5-month values (Table 1, Fig. 2). Concomitant with vascularity increases were CB-CT-derived new bone volume increases at 3 months (26.2 cm³) (Fig. 3). Remodeling of the callus resulted in a 50% decrease in total bone volume at 6 months, paralleled by a decrease in the Ktrans coefficient for this subject and an allograft surface union ratio of greater than 80% (Table 2). Ktrans remained largely unchanged from 0.5 to 3 months in Fauna and from 3 to 6 months in Merryweather, indicating a minimal healing

Table 1. DCE-MRI parameters measured for each animal and visit

Subject	0.5 months		3 months		6 months	
	Regional Ktrans	% Perfused	Regional Ktrans	% Perfused	Regional Ktrans	% Perfused
Flora	0.031	2.77	0.22	2.08	0.015	2.26
Fauna	0.013	3.15	0.011	2.45	NA	NA
Merryweather	NA	NA	0.021	3.25	0.016	2.49

DCE-MRI = dynamic contrast-enhanced MRI; Ktrans = volume transfer rate constant between arterial plasma and extracellular extravascular space; NA = not available.

Fig. 2A–C Longitudinal DCE-MRI of femoral allograft healing is shown. T1-weighted postcontrast coronal MR images of Flora's femurs obtained at (A) 0.5, (B) 3, and (C) 6 months postsurgery are shown, in which a heat map of the contrast signal intensity is superimposed on the allografted femur to illustrate the highly vascularized area (perfused fraction). Note the phantoms (white and

gray circles adjacent to the allografted femur), which were used to validate the contrast enhancement and calculate the Ktrans values. Also of note is that the allograft vascularity peaks at 3 months, coincident with bone remodeling, and leads to an allograft with a small perfused fraction at 6 months.

response toward the allograft. Paralleled by limited angiogenesis in these subjects was minimal bone formation, which remained localized to the host-allograft junctions (Fig. 3) at 3 and 6 months. The unweighted union ratios for these two subjects ranged from 39% to 45%. Within all three subjects, the perfusion fraction of the reconstructed limb was similar throughout and indicative of the largely avascular nature of the allograft. Ktrans values could not be calculated for Merryweather's 0.5-month or Fauna's 6-month DCE-MRI due to failed contrast injection.

Despite the heterogeneity of new bone formation in the three dogs, torsion testing revealed similar biomechanical properties of all three allografted femurs (Table 3), including a common mode of early union failure at the proximal graft-host junction. Histomorphometry of the allografted femurs corroborated the imaging results (Fig. 4). Gross inspection of the tissue revealed the graft was largely avascular with no bone marrow. Evidence of the early union failure was also apparent from the fractured cortical bone at the proximal graft-host junction. Gross inspection of the fluorochrome-labeled tissue sections demonstrated the predicted bone formation and remodeling of the periosteal fracture callus and abutting cortices at the graft-host junction, areas of new vascularity as determined from DCE-MRI analysis. We also noted fluorochrome labeling of the necrotic bone within the medial segment of the allograft in disperse pockets of active graft remodeling. Enhanced metabolic activity in the allografted femur was observed as an increase in MS and BFR of the callus and the graft-host interface compared to the contralateral femurs. Moreover, a trend that this metabolic increase occurred on the allograft surface was also apparent. As expected, there were no differences in MAR at this late stage of healing, nor were there any differences between the host bone on the allografted versus contralateral femur.

Quantitatively, there was less than 20% variability between the allografted femurs in all biomechanical parameters. The three allografted femurs displayed an ultimate torque that was on average only 41.1% of the unoperated control group. Similar differences between the allografted versus control femurs were found for torsional rigidity and failure rotation.

Discussion

Though allograft transplantation is a mainstay for reconstruction of segmental bone defects (> 3 cm), the lack of host-mediated integration is associated with a 25% to 35% failure rate [2, 14]. As a result, investigators have developed preclinical adjuvant therapies [6, 11, 12, 15, 19, 24, 26, 30–33]. As yet, their clinical application has been

Fig. 3A–E Quantitative evaluation of canine femoral allograft healing via longitudinal CB-CT is shown. In vivo CB-CT scans of the grafted femurs were performed at 0.5, 3, and 6 months postoperatively. (A) Representative two-dimensional CB-CT images of AP (left) and mediolateral (right) views of Fauna's allografted femur at 6 months are shown. Volume rendering of the metal-artifact-suppressed data were used to generate 3D images of the callus bone (dark gray) and allograft (light gray) at (B) 3 and (C) 6 months, which illustrate most of the new bone forms on the distal side of the graft by 3 months and consolidates from remodeling by 6 months. (D) This 3D rendering approach was used to calculate the callus, allograft, and host femur bone volumes at each time point for each dog. (E) Ex vivo CB-CT scans of the allografted femurs without the implants were used to generate 3D reconstructed images of the allograft surface with a heat map to illustrate high (red) versus low (blue) areas of connectivity between the allograft and new host bone. These data were used to calculate the unweighted union ratio metric, which is a binary classification of the callus voxels contiguous with cortex voxels of the allograft as either bone or not bone based on bone mineral density. We also calculated the bone mineral density-weighted union ratio, which divides every pixel area of the callus in contact with the underlying allograft by the ratio of the pixel mineral density to that of cortical bone density and is represented by the heat maps, which are shown in medial (left) and lateral (right) views of the specimens from each dog.

unrealized, largely because a large-animal model has not been established, nor have clinically relevant diagnostic tools that can noninvasively quantify graft healing. We previously demonstrated the potential of DCE-MRI and CB-CT with metal artifact suppression to quantify angiogenesis and osteogenesis of massive allograft healing [7]. From this work, we asked the following in a pilot study using a canine model: (1) Are DCE-MRI and CB-CT able to discriminate subtle differences in angiogenesis and bone formation after femoral allograft transplant? (2) Do DCE-MRI and CB-CT data corroborate traditionally implemented ex vivo measures? And (3) do biomechanical differences between the allografted and contralateral femurs at 6 months support the feasibility of this large-animal model using a small sample size?

Our study is subject to limitations. First, the pilot nature of our experiment ($n = 3$ subjects) precludes us from making definitive conclusions regarding the applicability of DCE-MRI and CB-CT in patients with bone allografts. However, our results do provide support using these methods to longitudinally follow graft healing. Secondly, neither radiographic tool is able to quantify, simultaneously, both new bone volume and vascularity, but study results do indicate DCE-MRI-derived vascularity increases were paralleled by increases in CB-CT-derived bone formation. Thirdly, due to the limited number of subjects and the absence of a positive control group, we are unable state the canine model is a definitive large-animal model of allograft transplant. However, our findings indicate allograft healing in dogs is consistent with the established human paradigms. Future studies with longer study end

points will definitively determine whether the canine model recapitulates the healing cascade and time frame of cortical bone allografts in humans.

DCE-MRI and CB-CT were able to quantify the limited vascularity and new bone formation associated with segmental bone allograft healing. Also, both tools were able to

track the disparate angiogenesis and concomitant bone formation patterns in the allograft-reconstructed femurs. Interestingly, DCE-MRI indicated normal live adult canine bone is remarkably avascular (Fig. 2), as we found the intact contralateral femur to be negative for contrast enhancement. While regions of the operated femur that colocalized to new bone formation around the allograft displayed contrast-enhanced signals consistent with angiogenesis, objective thresholding had to be applied to derive an outcome that was largely qualitative (Table 1). Although this low background provides the opportunity to readily see the effects of angiogenic treatments, there are challenges and safety concerns with the iv contrast agent injections. Additionally, a clear advantage of DCE-MRI over traditional ⁹⁹Tc bone scans from a cost-effectiveness standpoint was not apparent from our study.

Longitudinal CB-CT measurements indicated heterogeneous bone formation. While two dogs had the predicted limited osteogenic response restricted to the graft-host junctions, Flora displayed a 10-fold greater anabolic response that covered more than 80% of the allograft (Table 2, Fig. 3). Although this response has been documented after canine limb salvage surgery, we have no formal explanation for this difference, as all dogs received the same surgery and postoperative care. However, this bone formation and increase in union ratio did not translate into superior biomechanical integrity, as all allografted femurs failed as early unions at similar torques (Table 3). The poor predictive value of this callus bone, and our

volumetric and union ratio measurements of it, highlights the major limitations of current in vivo radiology to quantify biomechanical healing and the necessity of improvements to these biomarkers. We also compared our in vivo radiology results to dynamic histomorphometry. Because this ex vivo technique is an important tool in osteoporosis drug trials [27, 28], it also has potential as a allograft healing biomarker. While the results corroborated the CB-CT findings of new bone formation and remodeling in the callus and the graft-host junction, we were surprised to see extensive labeling of the allograft periosteal bone surface compared to the contralateral femur (Fig. 4). Although early histology studies in the 1980s have shown labeling of cortical allograft bone [5], the importance of this finding and its utility as a biomarker of allograft healing are unknown. Thus, additional canine studies are warranted, which would strengthen this approach as a definitive large-animal model of massive allograft healing.

Our study indicates massive femoral allograft healing in dogs over 6 months is characterized by limited vascular invasion, new bone formation, allograft remodeling, and return to normal biomechanical integrity, findings consistent with allograft healing in humans. As the primary function of massive allografting is biomechanical restoration of the limb, our most important finding was the limited recovery of intact strength (only 40%) at 6 months, which was uniform among the subjects (Table 3). From these data, we derived power calculations for a prospective

Table 2. Union ratios from ex vivo CB-CT scans of allografted femurs without the implants at 7 months

Subject	Proximal femur		Distal femur	
	Unweighted union ratio	Weighted union ratio	Unweighted union ratio	Weighted union ratio
Flora	0.8526	0.2294	0.8161	0.2413
Fauna	0.4264	0.0827	0.4503	0.1063
Merryweather	0.4410	0.0259	0.3923	0.0208

CB-CT = cone beam CT; unweighted union ratio = binary classification of the callus voxels contiguous with cortex voxels of the allograft as either bone or not bone based on bone mineral density; weighted union ratio = union ratio weighted for bone mineral density, which divides every pixel area of the callus in contact with the underlying allograft by the ratio of the pixel mineral density to that of cortical bone density.

Table 3. Summary of torsional biomechanical properties of normal (n = 5) and allograft-reconstructed (n = 3) canine femurs

Subject	Torsional rigidity (Nm/rad)		Failure torque (Nm)		Failure angle (°)	
	Allografted femur	Intact femur	Allografted femur	Intact femur	Allografted femur	Intact femur
Flora (24 kg)	128.17	140.03	16.94	36.12	8.18	16.59
Fauna (23 kg)	132.41	137.57	18.84	41.35	8.87	21.69
Merryweather (20 kg)	94.17	146.74	13.72	43.40	8.37	22.07
Unrelated subject (right)		134.99		40.07		22.14
Unrelated subject (left)		143.99		39.42		18.67
Average	118.25	140.66	16.50	40.07	8.47	20.23
SD	20.96	4.75	2.59	2.68	0.36	2.49

Fig. 4A–J Histomorphometry of dynamic labeling of massive femoral allografts at 6 months is shown. Six months after femoral allografting, the dogs ($n = 3$) received alizarin red (25 mg/kg iv) 8 days before sacrifice and calcein green (25 mg/kg iv) 1 day before sacrifice. After the femurs were harvested and used for destructive torsion testing (black arrows indicate the fracture in A and B; dashed line indicates missing bone in A and C), (A) the femurs were cut in half longitudinally and processed for undecalcified histology. Sanderson's rapid bone/acid fuchsin-stained sections from the (B) proximal and (C) distal host-allograft junctions from the median

of the three animals are shown to illustrate the four ROIs analyzed in unstained parallel sections (red arrows) by fluorescent microscopy at $\times 40$: (D) host cortical bone, (E) host-graft interface, (F) allograft cortical bone, and (G) callus bone. Histomorphometry was performed to quantify (H) MS, defined as the ratio of the fluorochrome-labeled bone surface to the total bone surface; (I) MAR, defined as the average distance between double fluorochrome label divided by the time difference between when the labels were administered; and (J) BFR, defined as the product of MS and MAR.

efficacy study using ultimate torque as the primary assessment tool: for $n = 3, 4$, or 5 dogs/group, there will be 80% power at a two-sided significance level of 5% to detect an increase of 48%, 38%, or 32% (from a placebo mean of 16.5 Nm), respectively, in ultimate torque using a two-sample t-test. From these results, we conclude this model fulfills the requirements of a definitive large-animal

model from ethical, scientific, and cost-effectiveness standpoints.

In summary, we found DCE-MRI and CB-CT are clinically relevant radiographic tools that may be used to assess segmental bone allograft revascularization and new bone formation in vivo. The lack of femoral allograft healing in the canine model suggests allograft healing in dogs follows

a clinical course similar to that in humans. We thus believe the canine model holds merit as a definitive large-animal model for bone allograft transplant and healing in which to study therapeutic adjuvant approaches to improve graft integration.

Acknowledgments We thank our collaborative authors from the University of Rochester, Masahiko Takahata, MD, and Chao Xie, MD, for their assistance in the daily observations of the canine subjects and care for the animals during DCE-MRI and CB-CT; David Conover, MS, for his help running the CB-CT scanner; and Hani A. Awad, PhD, for help with the experimental design and data analyses. We also thank Synthes Inc for providing surgical equipment and implants.

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