

ARTICLE

Contaminated open fracture and crush injury: a murine model

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Modern warfare has caused a large number of severe extremity injuries, many of which become infected. In more recent conflicts, a pattern of co-infection with *Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus* has emerged. We attempted to recreate this pattern in an animal model to evaluate the role of vascularity in contaminated open fractures. Historically, it has been observed that infected bones frequently appear hypovascular, but vascularity in association with bone infection has not been examined in animal models. Adult rats underwent femur fracture and muscle crush injury followed by stabilization and bacterial contamination with *A. baumannii* complex and methicillin-resistant *Staphylococcus aureus*. Vascularity and perfusion were assessed by microCT angiography and SPECT scanning, respectively, at 1, 2 and 4 weeks after injury. Quantitative bacterial cultures were also obtained. Multi-bacterial infections were successfully created, with methicillin-resistant *S. aureus* predominating. There was overall increase in blood flow to injured limbs that was markedly greater in bacteria-inoculated limbs. Vessel volume was greater in the infected group. Quadriceps atrophy was seen in both groups, but was greater in the infected group. In this animal model, infected open fractures had greater perfusion and vascularity than non-infected limbs.

Bone Research (2015) 3, 14050; doi:10.1038/boneres.2014.50; Published online: 27 January 2015

INTRODUCTION

The United States sustained more than 52 000 wounded-in-action casualties in operations Iraqi Freedom, New Dawn and Enduring Freedom (<http://www.defenselink.mil/news/casualty.pdf>). A large proportion of these were severe extremity injuries (often as a result of improvised explosive devices) involving extensive soft tissue damage with open fractures.¹ A difficult and frequent complication of these injuries is deep infection, which may occur in as many as 15% of wounds overall and as many as half of severe open tibial fracture wounds.^{2–3} New patterns of infection of extremity wounds have emerged in recent military conflicts. Initial wound cultures usually show predominantly skin bacteria.⁴ Early in the course, Gram-negative pathogens such as *Acinetobacter baumannii* complex (ABC) and *Pseudomonas aeruginosa* are present.³ These pathogens seem to be of comparatively low virulence and are later replaced by Gram-positive organisms such as *Staphylococcus aureus*, including methicillin-resistant strains (MRSA).² In a report of combat-associated open tibial fractures, bone healing was delayed in one-third of

wounds that were ultimately infected with *Staphylococcus* species.³ ABC is a common group of bacteria found in soil and was a frequent wound contaminant during the Vietnam War. These organisms have also become concerning pathogens in nosocomial infection outbreaks.^{5–7} ABC is characterized by a high rate of mutation leading to development of multi-drug resistance and persistence on surfaces despite standard antiseptic treatment.^{8–9} Additionally, ABC is known to form biofilms, which could facilitate bone and implant infection, although this feature was not observed in an animal model of implant-associated ABC osteomyelitis.^{10–12} Nevertheless, it has been speculated that early wound contamination with ABC facilitates persistence or development of infection with *S. aureus*.

In this study, we used a rat model that mimics the combat-related extremity injury described above, attempting to reproduce the five basic components of infected open fractures: a comminuted, high-energy fracture pattern; soft-tissue necrosis; bone loss; periosteal stripping; and bacterial contamination.^{1–4,13–14} This model was originally

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Received: 18 November 2014; Revised: 16 December 2014; Accepted: 27 December 2014

developed with a mixed Gram-positive/Gram-negative contamination and was modified in this study to include ABC and MRSA to more accurately reflect the clinical condition outlined above.¹³

Despite clinical observations that infected bones often lose their blood supply, no studies have attempted to quantify vascularity and perfusion in the setting of contaminated open fractures. We hypothesized that contaminated open fractures would have diminished bone vascularity, but that inflammation associated with infection would increase overall limb perfusion. We tested our hypothesis using a rat infected open fracture model.

MATERIALS AND METHODS

Approval for this study was granted by our institutional animal care and use committee and the animal care and use office. Adult male brown Norway rats were anesthetized by isoflurane inhalation.

Surgical procedures

A modified drop-weight apparatus with an instrumented crushing arm was used to create a mid-shaft, comminuted fracture of the right femur by dropping a 500-g weight from a height of 25 cm. Quadriceps muscle crush was performed by applying 7 psi pressure for 5 min using a load sensor to monitor the pressure. The leg was prepared for surgery, and the fracture site was exposed via a lateral approach. A rotary saw was used to create an 8-mm gap. Two millimeters of periosteum were then removed from the remaining bone ends using electrocautery. The femur was stabilized by retrograde intramedullary insertion of a fully threaded 1.6-mm Kirschner wire, leaving an 8-mm gap. The injury site was then inoculated with bacteria (described below) or an equal volume of sterile saline (control). Buprenorphine was administered pre-emptively and for postoperative analgesia. Animals were euthanized at 1, 2 and 4 weeks after injury. Quadriceps were harvested from the injured legs at 1, 2 and 4 weeks and weighed to assess muscle atrophy (total $n=61$ with bacteria, $n=28$ controls).

Microbiological procedures

The injury site was inoculated with 10 μL of a saline dilution prepared from an actively growing overnight culture containing 1×10^5 colony forming units (CFU) of AC4795, a strain of *A. baumannii* complex obtained from the US Department of Defense Multidrug-Resistant Organism Repository and Surveillance Network (clinically isolated from a bone infection) and a second 10 μL saline dilution containing 1×10^4 CFU of UAB 05-197, a low passage clinical isolate of MRSA originally isolated from bone in a patient with osteomyelitis.¹⁵

Bacterial burden was assessed using quantitative microbiologic cultures of contaminated bone. At the indicated

time points, rats were euthanized and thighs were harvested using aseptic technique. Femurs were stripped of tissue, placed in sterile 5 mL cryovials, snap frozen in liquid nitrogen and stored at -80°C until processed for culture. Bone specimens were thawed upon receipt in the microbiology laboratory, weighed and pulverized in 3 mL of LB broth in a tissue grinder under sterile conditions. Six serial 10-fold dilutions were performed and then 100 μL of the original tube and each dilution was inoculated onto Columbia nalidixic acid and MacConkey agars and spread over the plates with a flamed and cooled glass spreader. The original specimen was also plated onto sheep blood agar in order to determine if other bacterial species were present. Agar plates were incubated overnight at 37°C under atmospheric conditions. Blood cultures were obtained from rats that died or appeared septic (lethargy, fever). Rats that appeared septic were euthanized before their designated endpoints by isoflurane overdose and bilateral thoracotomy. Blood was obtained by open cardiac puncture using aseptic technique. A volume of 1 mL blood was inoculated into 6.5 mL trypticase soy broth blood culture media and incubated for up to 5 days at 37°C under atmospheric conditions. If turbidity was evident, a 10 μL loop of broth was streaked for isolation on sheep blood agar, Columbia nalidixic acid and MacConkey agars and incubated. Colony counts from dilutions of bone cultures were determined for MRSA and ABC grown on agar plates that contained 30–300 colonies per plate. Results were expressed as CFU per gram of bone tissue. MRSA was identified on the basis of Gram stain morphology, positive catalase and coagulase tests and growth on Mueller-Hinton agar plates containing $6 \mu\text{g}\cdot\text{mL}^{-1}$ oxacillin. ABC was identified by Gram stain morphology, negative oxidase, spot indole and lactose fermentation tests and characteristic morphology on selective MDR *Acinetobacter* media (Hardy Diagnostics, Santa Maria, CA, USA).

Assessment of vascularity and perfusion

To evaluate vascularity, we used microCT angiography as previously described.^{16–18} Briefly, animals were anesthetized and the left ventricle was cannulated. The vasculature was flushed, fixed and then perfused with a silicone lead contrast agent (Microfil MV-122; Flow Tech, Inc., Carver, MA, USA). The femurs were then harvested and decalcified. The intramedullary wire was removed to prevent imaging interference and replaced with a small wooden dowel to maintain alignment. The bone segments were allowed to approximate, as there was no bone healing in the segmental defect. MicroCT analysis was performed using a Scanco 40 μCT (ScanCo Medical). A 16- μm isotropic voxel size was used and the volume of interest was defined as 500 slices centered on the bone defect.

A segmentation threshold of 270 was used and direct calculation of bone parameters was performed. Three-dimensional histomorphometric values of vessel volume, connectivity, number, thickness, thickness distribution, separation and degree of anisotropy were calculated with the accompanying software. Vessel volume of the femur was the primary endpoint assessed.

An *in vivo* SPECT nuclear imaging technique was used to assess perfusion.¹⁹ Rats underwent anesthesia and were injected with technetium (Tc)-99m labeled bovine serum albumin (100–200 MBq) via penile vein. We used a small-animal SPECT imager (X-SPECT; Gamma Medica-Ideas,

Inc., Northridge, CA, USA) with each animal placed in a supine position. The data matrix size of each projection was 56×56 , the final three-dimensional reconstructed image was $56 \times 56 \times 56$ and the axial field of view was 12.3 cm. Sixty-four projections were acquired with a 10-s acquisition time per projection using a parallel-hole collimator and a radius of rotation of 3.8 cm. Images were reconstructed using a filtered back projection algorithm. The expected spatial resolution of the images was approximately 3 mm in full width at half maximum, as specified in the operating manual, and each pixel size was 2.2 mm. The rat body temperature was maintained at 32–33 °C

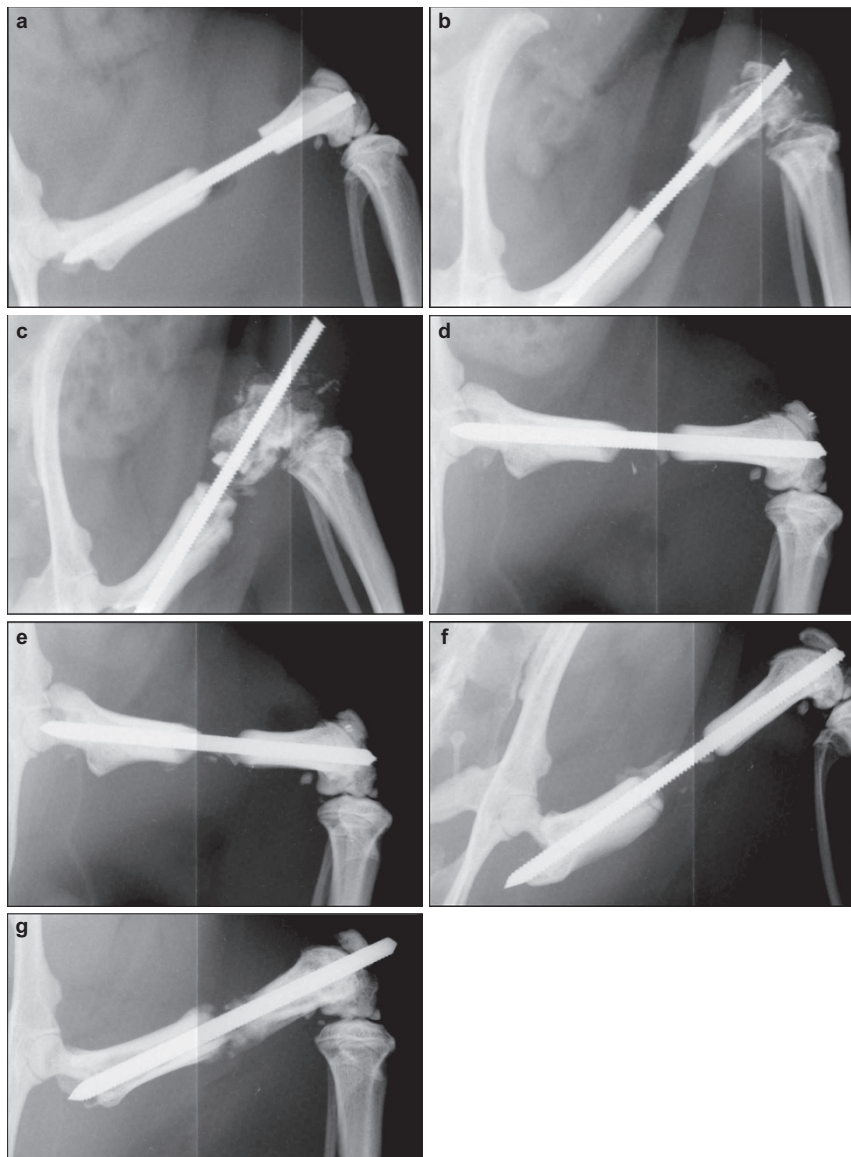


Figure 1. Radiographs of right rat hind-legs in the intervention group show intramedullary threaded Kirschner wires and gaps after repair of traumatic fracture and crush injury, 1 day after surgery (a), 2 weeks after surgery (b) and 4 weeks after surgery (c). Note the osteolysis, reactive bone, and loss of fixation by 2 and 4 weeks. Radiographs from the control group were taken 1 day after surgery (d), 2 weeks after surgery (e) and 4 weeks after surgery (f). No radiographic signs of infection are present. Another image taken 4 weeks after injury in a rat in the control group shows loss of fixation, but no osteolysis (g).

during the procedure. Image analysis was performed using ImageJ, version 1.37v (National Institutes of Health, Bethesda, MD, USA). The total uptake of Tc-99m bovine serum albumin into the right thigh (injured side) was quantified and compared with that into the left thigh (control side). The maximal signal values in the regions of right and left thighs were also compared.

Statistical analysis

Student's *t*-test was used to compare groups and time points within groups for muscle atrophy, bacterial counts,

and microCT parameters. One-way analysis of variance was used to analyze perfusion data using SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

We observed changes consistent with development of osteomyelitis in the infected group, including osteolysis, reactive bone and loss of fixation (Figure 1). Similar changes were not observed in the control group, with the exception of occasional loss of fixation because the

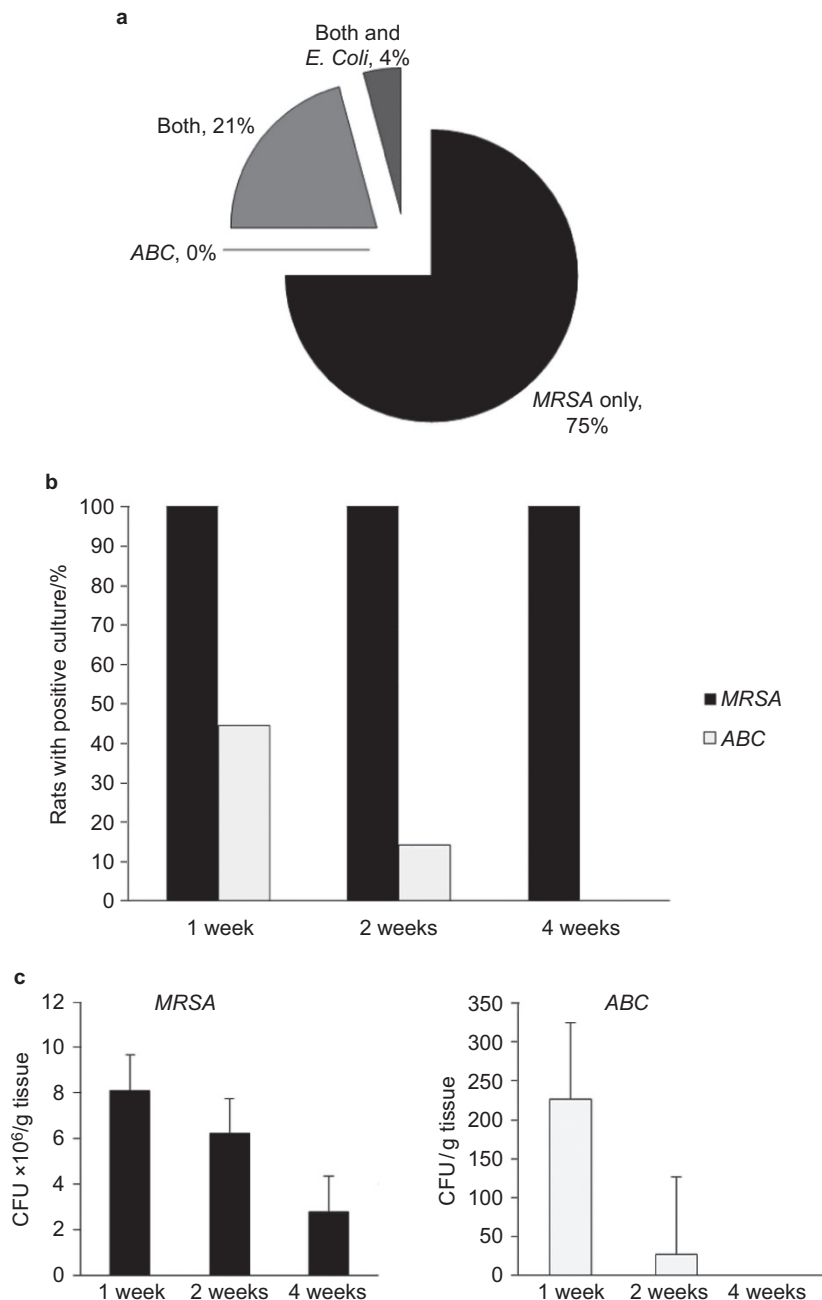


Figure 2. Proportion of samples with positive bacterial cultures at all time points (a). Percent of samples positive for MRSA and ABC are shown in (b). Quantitative cultures were performed and CFU of MRSA and ABC per gram of bone are shown in (c).

bone defect was critically sized and so did not spontaneously heal.

Cultures were obtained from 23 rats ($n=9$ at 1 week, $n=7$ at 2 weeks, $n=7$ at 4 weeks.) MRSA was recovered from all femur cultures at all time points, whereas ABC was detectable by culture in less than half of cases at 1 week, fewer at 2 weeks and no cases by 4 weeks (Figure 2a and 2b). ABC was never present in bone in the absence of MRSA infection (Figure 2a). Bone cultures from one rat grew *Escherichia coli* in addition to MRSA and ABC. Four rats died or were euthanized with presumed sepsis within the first week after surgery. All had positive blood cultures for ABC and negative blood cultures for MRSA. With respect to quantification, MRSA was found at the highest concentrations in the first week (mean: 8.11×10^6 CFU/gram of tissue) and declined thereafter (Figure 2c). ABC was present

in much lower concentrations (mean 226 CFU/gram of tissue at 1 week).

Muscle atrophy was noted at all time points in the infected group ($n=10$ at each time point) and at 2 and 4 weeks in the control group ($n=2$ at 1 week, $n=4$ at 2 weeks, $n=4$ at 4 weeks) (Figure 3). At 2 and 4 weeks, the quadriceps of the injured legs weighed only 60% as much as those of the uninjured legs in the infected group. There was less atrophy in the control group, with injured legs weighing 75% and 80% of uninjured legs at 2 and 4 weeks, respectively. Muscle weights in the uninjured legs did not vary significantly between time points within the groups.

Vessel volume at 1 week was noted to be low in the bone adjacent to the defects in both groups, but was found to be greater in the infected femurs. At 2 and 4 weeks, substantial increases in vessel volume were noted in the infected femurs, but not in the controls (bacteria: $n=7$ at 1 week and 2 weeks, $n=6$ at 4 weeks; controls: $n=2$ at 1 week, $n=4$ at 2 weeks and 4 weeks). Representative images and graphs of the quantification of microCT angiography are shown in Figure 4.

SPECT scanning at 1, 2 and 4 weeks showed that perfusion was greater in injured hind-limbs compared with the contralateral limbs in both the infected and control groups (Figure 5) ($n=8$ in both groups at 1, 2 and 4 weeks). This difference was much more marked in the infected group than the control group. In the infected group, the maximum perfusion values were increased by as much as 60% compared with those in uninjured limbs, and the total limb perfusion was increased more than 200% compared with the uninjured limbs. In the control group, maximum perfusion values were 10%–20% higher in injured limbs than in uninjured limbs, and the total limb perfusion was less than 50% higher in injured limbs compared with the uninjured limbs.

DISCUSSION

Using this model of contaminated open fractures, we reproduced many of the sequelae of severe extremity injury caused by combat, including polymicrobial bacterial infection with MRSA and ABC, systemic and local inflammation, and muscle atrophy. In agreement with our hypothesis, we found that limb perfusion is greater with infection. Contrary to our expectations, however, recovery of bone vascularity was greater in infected injuries. To our knowledge, no studies have evaluated vascularity and perfusion in an infected open fracture model.

Bacterial burdens of both MRSA and ABC in the infection site decreased with time after surgery. MRSA achieved higher concentrations and persisted longer in the infection sites. These findings suggest that, in the setting of co-infection with ABC, MRSA contributes to the bulk of bacterial burden in traumatic open fracture and compression injur-

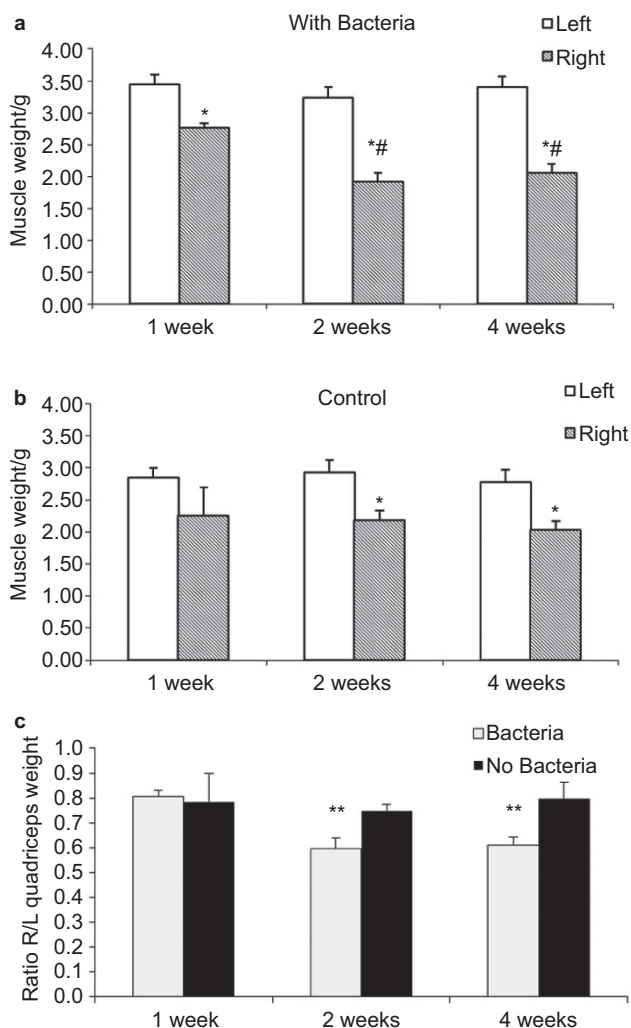


Figure 3. Weight of right and left quadriceps for rats with bacterial contamination (a) and controls (b) at 1, 2, and 4 weeks after surgery. Weight ratios for right and left quadriceps with bacterial contamination or controls are shown at 1, 2 and 4 weeks (c). * $P < 0.05$ vs. left, # $P < 0.05$ vs. 1 week, ** $P < 0.05$ vs. no bacteria.

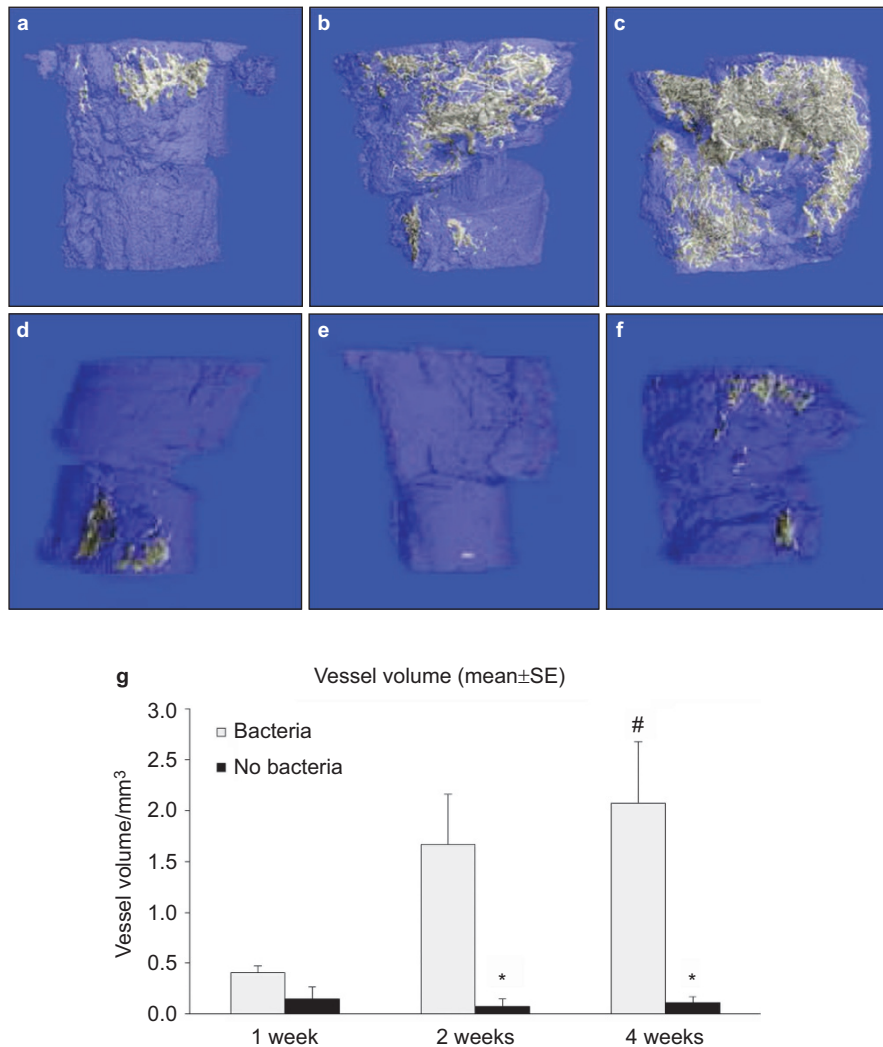


Figure 4. Bones from injured limbs were studied via Microfil perfusion followed by decalcification and microCT scanning. Reconstructed images of vascular casts within the blue shadow of bone from limbs with bacteria at 1-week (a), 2-week (b) and 4-week (c) time points are shown in the top row. The proximal femur segment is toward the bottom of each image. The gap between the femur segments has been allowed to compress for imaging purposes. Representative reconstructive images from injured legs without bacteria are shown in the bottom row at the same time points (d–f). Total vessel volume was quantified and is represented graphically (g) (* $P < 0.05$ vs. bacteria, # $P < 0.05$ vs. 1 week).

ies, especially beyond the first week after injury. ABC did not persist in the local wound in the absence of MRSA, indicating that infection with ABC may not have substantial virulence beyond the first week. The role of ABC in combat-related infections has come into question, and despite using an ABC isolate from a clinically obtained bone infection, the results of our model suggest that ABC does not play a major role in local wound infection as an isolated pathogen.^{1,15,20–22} Conversely, in the small fraction of rats that died in the first week before their scheduled euthanasia, ABC sepsis was likely the cause of death. These results suggest that although the local effect of ABC may be modest, the systemic effects of ABC, especially early in the post-operative period, can cause substantial mortality. Our findings agree with those of the recent literature describing the association of ABC in the

incidence of sepsis and nosocomial infections in military hospitals.^{23–24} Given that our primary interest is in the local bone infection and healing, however, one could question whether eliminating ABC from our model and using MRSA alone would simplify the procedure and analysis without substantially altering the local effects.

With respect to vascularity, blood vessel volume within the femur measured by microCT angiography was low but gradually increased over time. The increase was greatest between the first and second weeks after surgery. This was expected and consistent with findings of a previous study of segmental defects in a similar model.²⁵ Contrary to our hypothesis, we found that infection was associated with improved recovery of bone vascularity, particularly in the distal segments, which are adjacent to the metaphysis. We were unable to find any previous studies evaluating

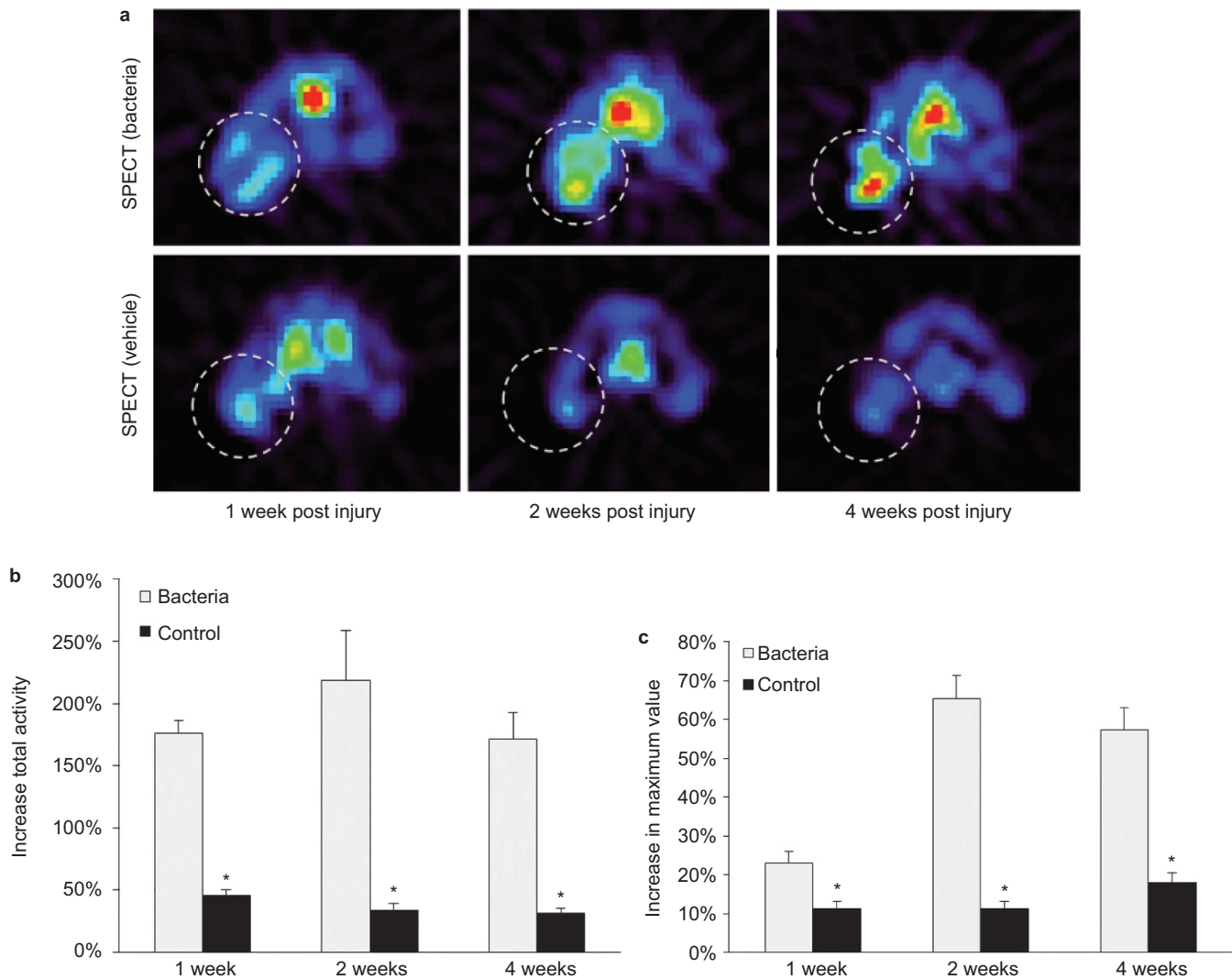


Figure 5. Representative scout axial SPECT images from a rat with bacteria top or controls with vehicle (saline) bottom at 1, 2 and 4 weeks post injury are shown (a). The dashed circle indicates the injured right leg. Perfusion in the injured right leg was compared with that in the uninjured left leg. Quantitative results are shown as percent increase in total activity (b) and percent increase of maximum activity (c) in the injured compared with uninjured limbs ($*P < 0.05$ vs. bacteria) (a reproduced from Ref. 19).

the effects of infection on bone vascularity. A possible mechanism could be the influence of inflammatory pathways, which are known to impinge upon angiogenic pathways. Bacterial infections in other studies have been shown to both increase or decrease VEGF signaling.^{26–32}

Our study showed that, in both the infected and control groups, the injured limb had greater perfusion relative to the uninjured limbs, and in the setting of infection, the difference was much more pronounced. Additionally, infection was associated with a longer period of greater perfusion following open fracture creation. Increased perfusion following traumatic fracture and crush injury has been established in the literature.^{33–34} The relationship between vascularity and perfusion did not seem proportional in these experiments.

The local muscle atrophy seen in our study is intriguing. We observed no statistically significant decline in quadriceps weight in the contralateral limbs, whereas there was weight decline in the operative quadriceps, indicating that the direct injury (and presumed disuse) was likely more responsible for the atrophy. The rats were observed, however, to be ambulatory and bear weight on the injured limbs almost immediately after surgery. There was greater muscle atrophy in the infected group compared with the control group, suggesting a possible direct effect of infection. Many studies have demonstrated systemic muscle wasting in response to sepsis (common models are cecal ligation and puncture or quadriceps injection with *S. aureus*). Several previous studies have reported increased muscle degradation via ubi-

quitin proteasome proteolysis, which may be activated through a variety of pathways including forkhead box protein O1.^{35–36} Additionally, decreased protein production has been noted with sepsis. For example, tumor necrosis factor- α may inhibit calmodulin-dependent protein kinase II.³⁷ Similarly, inhibition of rapamycin treatment to inhibit mTOR, which is known to be upregulated in sepsis, leads to decreased muscle protein synthesis.³⁸ Despite these studies examining the effects of sepsis on systemic muscle wasting, little has been published on the local effects of infection on muscle.³⁹

Limitations of the current study include the complexity of the model. A simpler model would potentially decrease variation between animals and facilitate efficient animal use. However, a simplified model may be less relevant to and representative of the clinical condition we intended to study.

In summary, the rat model described herein combined fracture, crush injury and infection with MRSA and ABC, resulting in greater limb perfusion but less bone vascularity in injured limbs than in uninjured limbs. Contrary to our expectations, recovery of bone vascularity was actually better in the infected fractures. We noted significant loss of quadriceps muscle mass, consistent with impairment of limb function, which was more pronounced in infected limbs. Further studies are needed to understand the underlying mechanisms and determine the effect on ultimate healing.

Competing interests

The authors declare no conflict of interest.

Acknowledgements

Rena L. Stewart participated in study design. Surgical support and animal care was provided by Liqun Tian, Mary Patricia George and Drew Gunnells. Maria Johnson provided assistance with microCT and faxitron imaging. Technical assistance with preparation of bacterial inocula and performance of bone cultures was provided by Donna Crabb, Amy Ratliff, and Marlene Whitehart. The contributions of these individuals are gratefully acknowledged. Grant support is given from Department of Defense, Congressionally Directed Medical Research Program OR 090206 to SG. The Small Animal Phenotyping Core provided faxitron and microCT imaging (P30DK056336 and P30DK079626).

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