Standard Review Article

OPEN

Development of a small animal bone-anchored limb replacement model for infection interventions

Bailey V. Fearing, PhD[a,b], Sarah M. Romereim, PhD[a], Kerry Danelson, PhD[c], Matthew Smykowski, BS[a], Marina Barankevich, BS[a], Ryan Serbin, MD[a], Nainisha Chintalapudi, MD[a], Jana Davis, MD[d], Susan Appt, DVM[e], Heather Burkart, DVM[e], Rachel B. Seymour, PhD[a,b], Joseph R. Hsu, MD[a,b]

Abstract

Purpose: Osseointegration-associated infections are a critical barrier to widespread implementation of osseointegrated (OI) prosthetics. To address this challenge, a preclinical animal model must exist of the human model to test potential interventions. In this article, we describe a novel rabbit model of OI implant-related infection that can act as a platform for rapid translation and development of therapeutic approaches to combat these uniquely challenging infections.

Methods: A single-stage amputation was performed by exposure, transection, reaming, and tapping of the tibia, followed by placement of a 75-mm Ti-6Al-4V cortical screw implant. Muscle and skin were closed, and a prosthetic was attached to the screw. Hematology, clinical chemistry, and imaging were performed up to 8 weeks. High-resolution microCT and histology were conducted at terminal end points. Intraosseous vancomycin delivery was compared with intravenous delivery. Serum and bone marrow collection was conducted across a period of 5 hours.

Results: Rabbits maintained normal ambulation, mobility, diet, and weight throughout the study period. Clinical chemistry results indicate normal ranges over the study course. microCT and histology demonstrate osseointegration between the threads of the implant within the medullary cavity. Pharmacokinetic data determined that intraosseous vancomycin delivery results in significantly lower vancomycin concentrations systemically compared with intravenous delivery and higher peak vancomycin concentration within the tibial canal.

Conclusion: This preclinical translational model represents a reproducible small animal model of OI transtibial amputation that successfully recreates the bone–skin–implant interface, material–bone interactions to match human OI, and a similar immune response. Preclinical efficacy of infection interventions will be further explored with establishment of this model.

Keywords: OI transtibial amputation, rabbit model, infection, bone-anchored limb replacement, osseointegration

1. Introduction

There are an estimated 185,000 new amputations in the United States every year including military personnel, with more than 500 new amputees on average every day. This includes more than 1650 service members who sustained major limb amputations in US military conflicts since 2000.[1] To improve the functional outcomes and prosthetic experience of these patients, bone-anchored limb prostheses (osseointegrated [OI] prostheses) have been developed, with the FDA authorizing use of these devices under the humanitarian device exemption pathway in the United States. Studies have shown that these patients with OI prostheses have significantly improved daily use of prosthetics, overall mobility, quality of life, and patient satisfaction.[2–4] Infection remains a critical concern in these patients because of the transdermal nature of the implant, remaining a major barrier to widespread implementation of these OI prostheses.

OI prosthetics offer significant promise for improved patient outcomes in proximal amputees who have exhausted all other options for improved function. Plagued by high infection rates in large part due to the soft tissue–implant interface, previous studies have shown that as many as 25% of patients will develop infection. While most infections are superficial and can be treated with oral antibiotics alone, there is approximately 20% cumulative osteomyelitis or deep infection risk and a 9% explant risk at 10 years.[5] Treatment of infection in this group of patients is challenging because of the intimate nature of the

Dr. Hsu reports consultancy for Globus Medical and personal fees from Smith & Nephew speakers' bureau. The remaining authors state they have no conflicts of interest.

[a] Atrium Health—Carolinas Medical Center, Atrium Health Musculoskeletal Institute, Charlotte, NC, [b] Wake Forest University School of Medicine, Charlotte, NC, [c] Atrium Health Wake Forest Baptist, Wake Forest University School of Medicine, Orthopaedic Surgery and Rehabilitation, Winston-Salem, NC, [d] PennState Health, Penn State Bone and Joint Institute, Hershey, PA, [e] Wake Forest University School of Medicine, Comparative Medicine, Winston-Salem, NC.

** Corresponding author. Address: Atrium Health Musculoskeletal Institute, 2001 Vail Ave, Charlotte, NC 28207. E-mail address: bailey.fearing@atriumhealth.org (B. V. Fearing).*

This manuscript received funding from grant #171408-FEL2020 provided by AO Trauma North America.

OTAI (2025) e366

Received: 11 October 2024 / Received in final form: 11 November 2024 / Accepted: 8 December 2024
Published online 7 March 2025

http://dx.doi.org/10.1097/OI9.0000000000000366

bone–implant–skin interface. The potential for implant-associated intracellular bacteria is a challenge particularly pertinent in OI where the implant is, by definition, contiguous with the outside environment.[6,7] Because OI is designed to be a lifelong procedure (as a result of bone–implant integration), consequences of removing the ingrown implant in the case of deep infection include fracture and shortening of the residual limb, leading to increased difficulty wearing a conventional socket. Thus, research aimed at infection prevention and treatment in this patient population is warranted. Much of the existing literature around implant-related infections comes from the periprosthetic joint infection area, where antibiotic regimens focus on prophylaxis, intraosseous (IO) administration, and combinatorial approaches. Use of prophylactic antibiotics, namely intravenous (IV) and oral delivery routes, is widespread. However, evaluation of IV and oral antibiotic effectiveness has indicated that tissue concentrations of systemically administered antibiotics cannot reliably achieve the minimum requirements for effective prophylaxis.[8] Studies have also investigated the IO administration route for antibiotic treatment of established infections, which can allow for 10–20 times higher local concentrations of antibiotics than with systemic antibiotics.[9]

Many years of ground-breaking OI research have been published by Brånemark et al. Most of these studies have been conducted in rabbits as a well-established model for OI implants, although the focus has predominantly remained on characterizing and optimizing implant surface modifications and biomechanical properties toward the goal of improved OI and prosthetic function.[10–13] These models rely on an "internal" amputation approach where multiple holes in each metaphysis are drilled into the tibia or other long bones, thus excluding the characteristic bone–skin–implant interface of bone-anchored transdermal prostheses, which make them particularly susceptible to infection. In addition, rabbits have emerged as a promising infection model, including orthopaedic implant infections,[14–16] because experimental infectious processes are reliable and reproducible and the rabbit immune system is sophisticated and relevant to human physiology.[17] Rabbits have been used extensively in osteomyelitis research, so methods for creating repeatable localized infection have been validated.[18–20] They have also been indispensable in experimental research of basic implant infection susceptibility.[21,22] Despite the foundation being clear, there remains no small animal model to investigate OI implant-associated infection. The small size of mice or rats would be a limitation in developing a similar model as antibiotic delivery vehicles may need to be less than a few millimeters to fit within the bone space. Rabbit models offer the opportunity to use human scale implants (eg, a screw used for fracture fixation in a patient can be used as an OI implant in a rabbit), and interventions may be delivered in more controllable volumes. In this study, we describe a first-of-its-kind small animal model designed to test a variety of OI implant interventions. Procedures, techniques, and methods used to develop and optimize this model are discussed and analyzed toward the goal of rapid translation to human clinical studies to improve patient care.

2. Methods

2.1. Prosthetic Limb Design and Prototyping

A custom prosthetic limb was designed for this study. The intent of the prototypes was to provide the animals with a secure platform for weight bearing and shock absorption during movement. An aluminum sleeve (6-mm radius, 18-mm length) was designed to slide over the end of the implanted hardware using AutoCAD (Autodesk, San Francisco, CA). The engineering drawing was uploaded to a third-party site (Protolabs, Chicago, IL) for fabrication out of aluminum 6061-T651. Small set screws were used to lock the metal sleeve in place. At the end of the sleeve, a spherical rubber cap was also designed using AutoCAD. This rubber was larger than the metal sleeve (15-mm radius, half-sphere shape) to provide shock absorption for the limb during all types of movement. To allow for compression, the rubber component also had 4 vent holes on the flat portion of the part with a radius of 2 mm. The rubber pieces were printed on a PolyJet system using a digital photopolymer (Protolabs). The rubber pieces were printed in variable stiffnesses, 30A and 40A hardness with a wall thickness of 2 mm, to provide more shock absorption for early time points and less shock absorption but more efficient energy transfer for later time points. The rubber was attached to the aluminum sleeve by threading a small screw into the base of the sleeve. To replace the rubber, this single screw attachment was removed and then replaced with the new rubber material.

2.2. Presurgical Planning

To optimize surgical outcomes, all animals were subjected to preoperative CT and radiographic imaging as well as an acclimation period to custom rabbit jackets (Lomir Biomedical, Malone, NY) used to limit weight bearing postoperatively.

2.2.1. Preoperative imaging. Computed tomography (CT) and radiographic imaging were conducted before surgery (1–2 weeks preoperatively) under sedation (25 mg/kg ketamine/0.025 mg/kg dexmedetomidine) and maintained using isoflurane (1%–3%). CT images were taken of each animal on a Vimago CT scanner (Epica Animal Health, San Clemente, CA), with a slice thickness of 0.09 mm, detector coverage of 20 mm, pitch factor of 0.531:1, voltage of 100 kVp, current of 80 mA, rotation time of 1 second, scan field of view (FOV) "large body," and display FOV 50. Measurements of medullary canal diameter and width were analyzed using RadiAnt DICOM Viewer (Medixant, Poznań, Poland) and SkyScan NRecon (Bruker Corporation, Billerica, MA) (Fig. 1A). Blood draws were collected for assessment of baseline levels for CBC hematology and clinical chemistries (IDEXX Laboratories, Westbrook, ME) while animals were still anesthetized.

2.2.2. Acclimation. A jacket acclimation period was also initiated to allow greater ease of use for minimizing weight bearing postoperatively (Fig. 1B). Flexible plastic e-collars were also placed during the acclimation period. Acclimation was conducted in the following phases: (1) jackets were hung outside the cage to acclimate to sight/smell (1–2 days); (2) jackets were placed over the rabbit with zipping for a few short sessions while supervised (1–2 days); (3) jackets were placed fully on the rabbit and left in place for a day but removed before night (1–2 days); and (4) if no signs of stress were noted, the jacket was left in place during the day and overnight period. Observations were taken during this acclimation period to ensure minimization of stress, normal fecal output, and normal appetite.

2.3. Animal Surgical Procedure

All animal procedures were approved under Atrium Health Wake Forest School of Medicine's Institutional Animal Care and Use

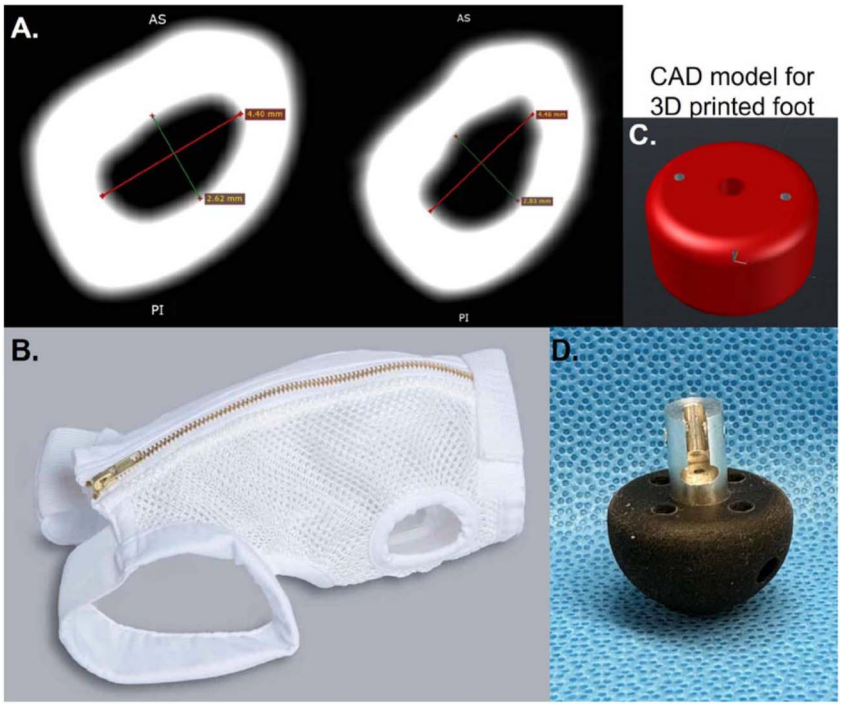


Figure 1. Preoperative CT scans were obtained to measure tibial canal width for appropriate choice of screw size (A). A custom rabbit jacket designed to limit weight bearing in the immediate postoperative period (B). Example of a CAD model (C) for designing the rabbit prosthetic foot attachment (D).

Committee (IACUC; protocols A21-042 and A24-041, PI BV Fearing). Figure 2 displays images detailing key surgical steps. New Zealand White male and female rabbits at skeletal maturity (≥7 months old) were sedated (25 mg/kg ketamine/0.025 mg/kg dexmedetomidine) and intubated for delivery of isoflurane (1%–3% maintenance) and oxygen during the surgical procedure. Eye lubricant was placed in both eyes. An intravenous catheter was placed in an ear vein for delivery of fluids and drugs. An epidural was placed at the lumbosacral space for additional anesthesia according to standard procedures. In brief, the lumbosacral area was shaved and aseptically prepared. A 22 G short bevel needle was inserted into the space and location identified by the ability to aspirate air or cerebrospinal fluid. Once localized, the medication was administered (0.75 mg/kg bupivacaine). The right hindlimb undergoing the procedure was shaved and aseptically prepared.

A sterile tourniquet was placed on the right hindlimb, and a circumferential incision through the skin and muscles was made slightly distal to the planned tibial transection point. The distal tibia was exposed by sharp blunt dissection of the soft tissues. Hemostasis was achieved by ligation or direct pressure. The bone was examined and the site of amputation identified, just distal from the fibula fusion point on the tibia. Using a Stryker Total Performance System surgical power tool console (Stryker, Portage, MI), the tibia was transected with the handheld oscillating saw attachment and the medullary cavity was reamed using the drill attachment. The outer 1.5–2 mm of the medullary canal was then tapped to aid in screw placement.

A Ti-6Al-4V (3.5 mm × 75 mm) or Ti-6Al-7Nb (4.0 mm × 75 mm) self-tapping cortical screw (DePuy Synthes, Raynham, MA) was selected based on the presurgical CT measurements of the medullary canal diameter. The screw surface was roughened with a Bovie scratch pad (Aspen Surgical, Caledonia, MI), rinsed with sterile normal saline, and then implanted into the tibial canal. Any simple peri-implant fractures were stabilized with a suture-based cerclage (4-0 Vicryl). The tourniquet was released, and minimal-to-no bleeding was noted in all animals.

A muscle platform was built around the distal bone by approximating the surrounding soft tissues together using simple interrupted, simple continuous, and/or ford interlocking sutures (3-0 polydioxanone (PDS) II) until a stable stump was achieved. The skin was then closed using a simple interrupted or continuous pattern with absorbable suture (3-0 PDS II Plus), and Dermabond skin glue applied. A cold compress was applied to help prevent postoperative swelling or bleeding. The prosthetic limb replacement was then attached to the screw abutment. A bandage including Telfa pad, cast padding, and Coban wrap was placed to protect the site.

Postoperative CT scans and radiographs were obtained (as described for preoperative imaging) to confirm correct implant placement while animals were still under sedation. Animals were allowed to recover on heating pads while monitored until fully maintaining an upright position.

2.4. Postoperative Care and Monitoring

The rabbit jacket was placed to minimize weight bearing on the operated limb while cushioning and protecting the surgical site in the custom pocket plus support strap. Jackets remained in place for 1–2 weeks postoperatively to simulate clinical non–weight-bearing recommendations. Rabbits were closely monitored for signs of discomfort or stress from the jackets. A flexible plastic e-collar was placed as needed to prevent the animal from manipulating the jacket, bandage, and/or surgical site. Bandage changes were performed weekly. If needed, more frequent bandage changes occurred in the early postoperative period to monitor healing and less frequently once incisions were healed.

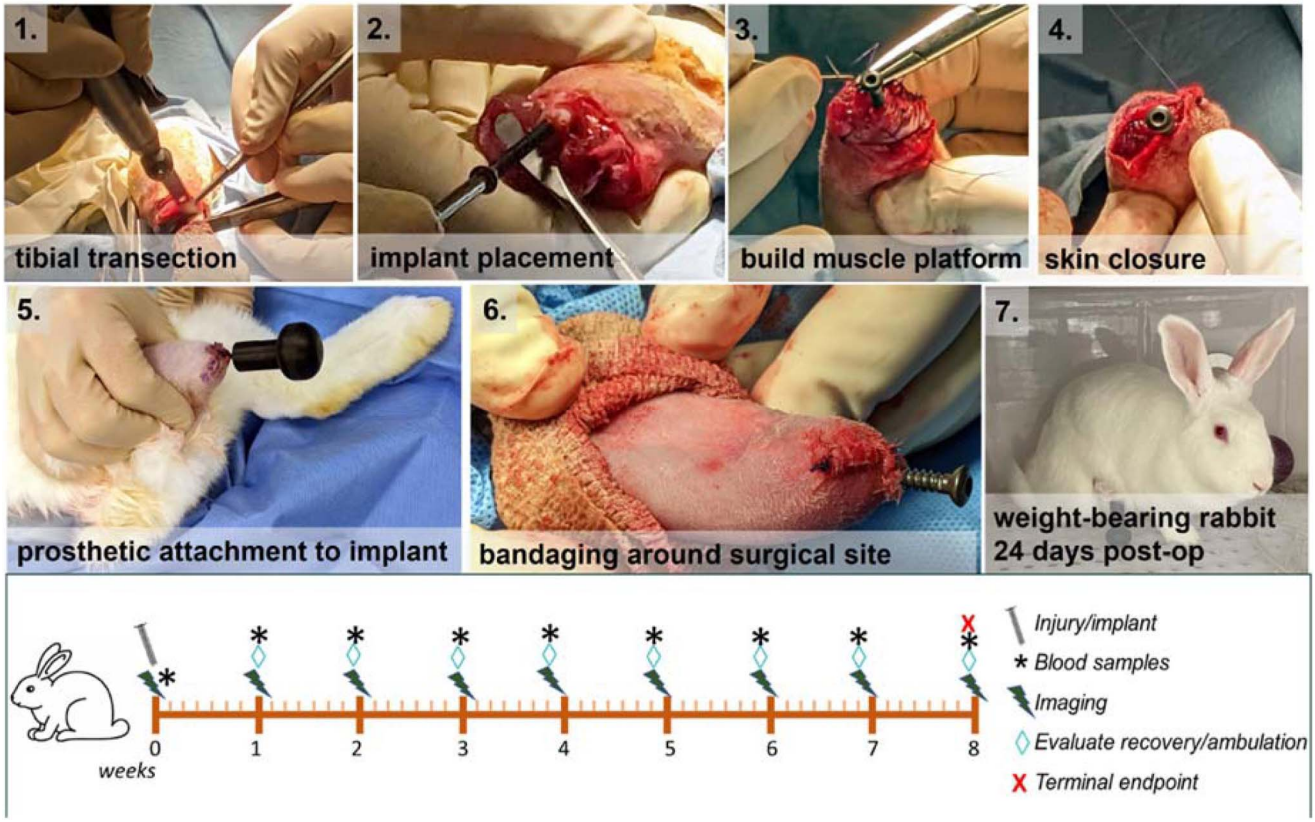


Figure 2. Rabbit tibial amputation procedural schema (1–7), and time line indicating outcomes throughout the study period (bottom).

Rabbits were given postoperative analgesics (0.02 mg/kg buprenorphine day of surgery and every 6–12 hours for 3 days; 0.5 mg/kg meloxicam day of surgery and every 24 hours for 3 days) and as indicated by the attending veterinarian.

CT and radiographic imaging were conducted as described above once weekly under sedation (25 mg/kg ketamine/0.025 mg/kg dexmedetomidine) and maintained using isoflurane (1%–3%). Blood draws were also collected during these periods for assessment of complete blood count (CBC) hematology and clinical chemistries (IDEXX Laboratories). Animals were maintained under protocol for 8 weeks.

2.5. Pharmacokinetics

To compare in vivo concentrations of vancomycin, 6 rabbits underwent a nonsurvival procedure to compare intravenous (IV) delivery with IO delivery. Animals were sedated, anesthetized, and prepared as previously described above. Rabbits were randomly assigned to IV or IO groups. A small cortical "window" was created in the distal aspect of the tibia for collection of medullary canal samples from animals in both delivery groups. For IO administration, a 1.1-mm K-wire was used to prepare a hole before insertion of an 18 G needle into the cortical bone of the proximal tibia for administration of 30 mg/kg vancomycin in 5 mL into the intraosseous space (ie, medullary canal) of the tibia. Correct placement was verified by slow, careful aspiration of the blood. Vancomycin was delivered over a 30-minute period using an infusion pump. For IV administration, an IV catheter was placed in the ear vein. 30 mg/kg vancomycin was delivered over a 30-minute period. Blood and medullary bone samples were collected from all animals before vancomycin (T0) administration, followed by immediately after the 30-minute delivery period (T1) and hourly from the beginning of the delivery period (T2-T6) for a total of 6 hours. Rabbits were humanely euthanized at the end of the collection period. Samples were frozen at −80°C before transportation to the Proteomics and Metabolomics Shared Resource at Atrium Health Wake Forest School of Medicine where sample extraction with methanol was conducted and liquid chromatography-mass spectrometry (LC-MS/MS) quantification of vancomycin was performed with an internal standard of aminopterin.

2.6. microCT

At the terminal end point, microCT was conducted on the isolated tibia with the intact implant. Samples were scanned using Bruker SkyScan 1275 with phantoms of known densities (250 g/cm^3 and 750 g/cm^3) scanned as a reference. The x-ray source was set at 70–80 kV with a current of 125–130 μA and a 1-mm aluminum filter. Rotation step and frame averaging were balanced to optimize scan time and image quality. Three-dimensional images were reconstructed using Bruker SkyScan NRecon software restricted to regions of interest around the bone–implant interface. RadiAnt software (as described previously) was used to gather representative images of microCT reconstructions.

2.7. Histological Analyses

After microCT scanning, the implants and surrounding bone were removed and the tibia was immersed in formalin for at least

72 hours. Decalcification was then performed using 5% formic acid, with the end point determined by microCT at regular intervals to ensure complete decalcification. Specimens were subsequently processed and embedded in paraffin. Samples were sectioned at a thickness of 4 μm and stained with hematoxylin & eosin (H&E; for general bone structure), Gamori trichrome, and Picrosirius Red/Fast Green/Alcian Blue (RGB; for visualization of osteoid and mineralized bone tissue). Osseointegration was determined by the thickness of mineralized bone around the space where the implant and its threads were located.

2.8. Microscopy

After histological staining, image acquisition was performed using a Leica Aperio VERSA digital slide scanner (Leica Microsystems, Wetzlar, Germany). For all visible implant thread spaces within the intramedullary canal, the area of bone within space was quantified as the percentage of total tissue that was ossified using FIJI software (NIH).

2.9. Statistical Analyses

Statistical analysis was performed in Prism software version 10.3.1 (GraphPad, Boston, MA). Pharmacokinetic results were analyzed by 2-way ANOVA (serum) or mixed-effects analysis (bone) with repeated measures. Histomorphometric results were compared using two-way ANOVA with the Bonferroni post hoc test where appropriate. Statistical differences considered significant when $P < 0.05$.

3. Results

3.1. Optimization and Mitigation of Adverse Events

For optimization of amputation location, 4 separate carcass tissues were used to test implant material and anatomic location variances. Completion of this phase determined other long bones (femur, humerus) to be less compatible with both human-size implants (a criterion of this model development for clinical relevance and translatability) and rabbit ambulation and mobility. Considerations for prosthetic design also concluded the tibia to be the ideal amputation transection location. Following cadaveric testing, nonsurvival surgeries were conducted to determine optimal veterinary clinical drug delivery and dosage. The amputation procedure was performed in sedated and anesthetized animals to further characterize steps to maintain skin integrity around the bone–implant interface, building the muscle platform around the transection site and suturing techniques to optimize recovery.

The next model development phase included survival surgeries and careful observations continued to improve surgical outcomes. The authors note the adverse events (AEs) that occurred here to assist future implementation of this model by different research groups toward the goal of promoting ethical and transparent preclinical animal research. *Fracture (incidental)*, rate of AE up to 100%: During nonsurvival studies, 2 of the 4 limbs experienced nondisplaced fractures. These fractures were successfully supported by a cerclage. Extensive postmortem evaluation showed that they were stable following this repair. Among the first 6 pilot survival animals, nondisplaced fractures occurred at the distal aspect in 5 of 6 animals. These were successfully stabilized during surgery with cerclage sutures. Follow-up CT scans showed fracture healing in the postoperative period. As the rabbit tibia naturally narrows from proximal to distal aspects and a tight screw fit is critical, it is possible that up to 100% of animals may experience minor perioperative fractures at the very distal aspect. *Fracture (resulting in instability)*, rate of AE 10%–15%: There is a small risk of fractures resulting in instability of the implant. In the 5 pilot animals that had incidental fractures at surgery (stabilized with suture cerclage), 1 rabbit's fracture worsened in the postoperative period, which coincided with the animal being allowed to return to its environmental enrichment (EE) pen while weight bearing. In response, several precautions were taken to decrease the chance of fracture worsening in the postoperative period and causing instability, such as improved bandaging of the leg for support and exempting rabbits from EE pen to avoid jumping with forceful impacts. *Implant loosening*, rate of AE 20%–30%: Based on pilot study animals, some implants became loose (ie, no bony ingrowth/osseointegration) in a rotational manner. This loosening did not improve over time and is indicated as a humane end point. In efforts to reduce this AE, presurgical planning (in Methods) was implemented to measure tibial canal diameters to account for anatomic variances between rabbits and ensure that the best-fit implant screw was used. Addition of a Bovie scratch pad to roughen the surface of the screw was also implemented to improve surface adhesion of endogenous cells and facilitate osseointegration. A custom jacket was also introduced to reduce weight bearing and offer protection (additional padding) of the injury site for the initial postoperative period. *Vancomycin reaction*, rate of AE 20%: Similar to vancomycin flushing syndrome in humans, a rapid administration of vancomycin in rabbits can cause a hypersensitivity or anaphylactic reaction. By slowly administering the drug over 30 minutes with an infusion pump, this will greatly reduce chances of this reaction. Signs of this reaction in rabbits include changes in respiratory rate or effort, changes in heart rate, increased temperature, and skin color changes. Diphenhydramine or dexamethasone should be available for administration.

3.2. Feasibility and Animal Recovery

By using a 75-mm screw and allowing an approximately 5-mm exposed length of screw head, we have replicated the critical and characteristic bone–skin–implant interface (Fig. 3A). Consistent and repeatable healing has been observed at the transection site (Fig. 3B). Overall, rabbits tolerate the surgery well and recovery is uneventful with animals maintaining good appetite and normal body weight (Fig. 3C). Rabbits have consistently been off pain medications by 5 days after surgery, and their mobility and ambulation are normal. Advanced versions of the prosthetic foot provided reduced, softer loading on the surgical leg (Figs. 1C, D and 3D), while allowing secure bandaging around the transection site during healing phases (Fig. 3E).

CT (Fig. 4A) and radiographic (Fig. 4B–D) imaging across study time points indicate the screw fit and angle to be optimized to allow stable and consistent contact within the medullary canal, where the proximal end of the screw butts up against the tibial plateau (Fig. 4B–D). Model development phases demonstrated this to be a key component for overall stability and success of the implant.

Hematology and clinical chemistry analyses reveal that health of the rabbits is within normal and expected ranges over the course of the study period (Fig. 5). Some early fluctuations were noted in creatine kinase (Fig. 5A) and calcium (Fig. 5B), which may be related to the initial musculoskeletal injury.

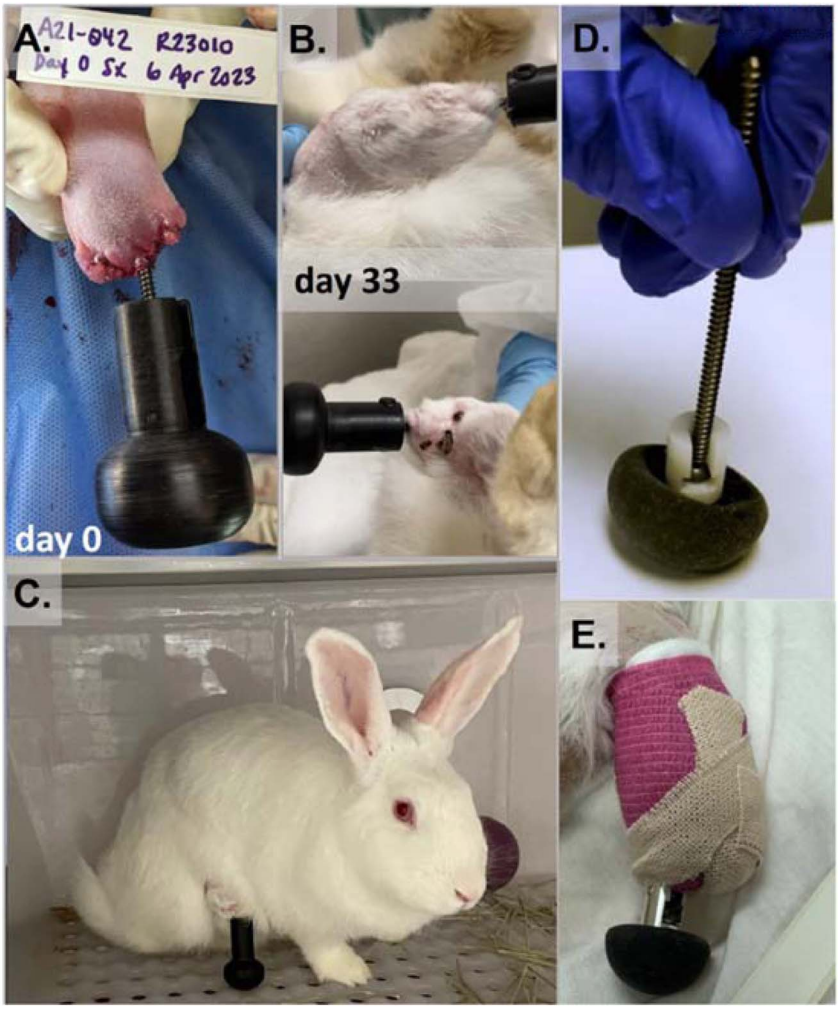


Figure 3. The muscle platform built around the amputation site to mimic the skin–bone–implant interface of human OI procedures (A). Consistent and repeatable healing is observed around the amputation site (B). Rabbits maintain normal mobility and ambulation (C). Improvements to the prosthetic foot allow for decreased loading and improved screw fit (D), as well as optimal bandaging around the amputation site (E).

3.3. Implant Osseointegration and Histomorphometry

High-resolution microCT imaging indicated bony ingrowth and osseointegration around the screw at 8 weeks (Fig. 6A). Increased stability of the implant was noted when this osseointegration occurred in response to the screw. Histology revealed remodeling within the tibial canal occurring along the thread lines of the screw (Fig. 6B–D). Histomorphometric analyses demonstrated that the calcified area within individual threads had significantly higher bone area in rabbits reaching the full 8-week time point compared with earlier time points (Fig. 6D and E). A significant difference was also noted in thread location, with distal threads containing significantly greater bone area compared with proximal threads.

3.4. Pharmacokinetics

Comparing IO vancomycin delivery with IV vancomycin delivery revealed differences in serum and medullary canal concentrations. IO delivery resulted in significantly lower serum vancomycin concentrations compared with IV administration (Fig. 7A, $P < 0.005$). IV delivery showed a high systemic peak soon after bolus delivery with a steady decline across time. By contrast, IO delivery had a slight peak but remained at relatively consistent serum levels across time points. Medullary canal concentrations peaked at a higher level (1900 ng/μL at T1, the end of vancomycin delivery) following IO delivery compared with IV delivery (163.9 ng/μL at T2, 30 minutes after vancomycin delivery ended) (Fig. 7B).

4. Discussion

Bone-anchored limb replacements offer significant promise for improved patient outcomes for proximal amputees who have exhausted all other options for improved function. These OI prostheses represent the highest probability for return to duty. However, infection remains a large area of concern for these patients, given the implant's contact and reliance on the residual bone and proximity to an incomplete skin interface. Weight-bearing progression and overall success of the OI implant with prosthetic are severely compromised in the presence of an infection.[23] Treatment of deep infections in this amputee population has advanced in recent years, but irrigation and debridement of the infected tissues and removal of the implant

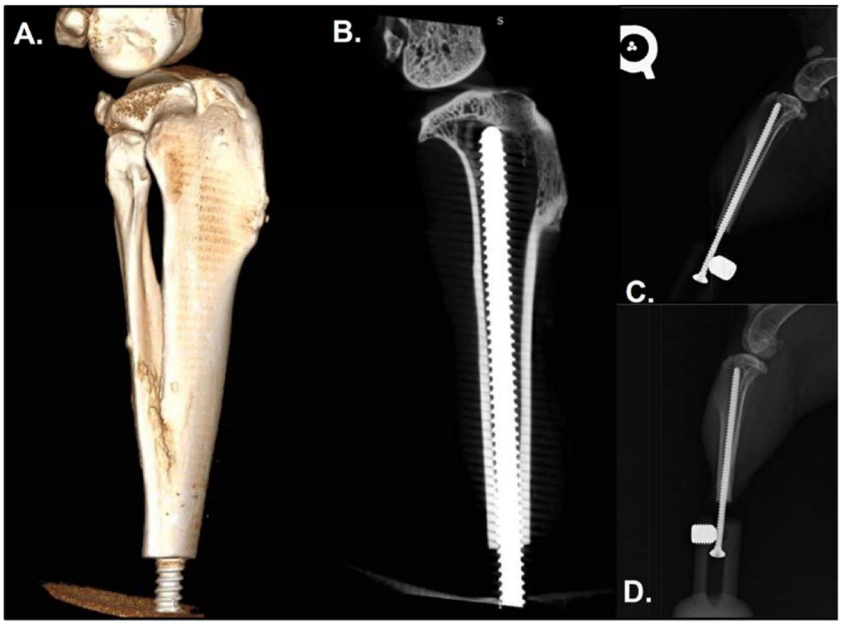


Figure 4. Representative terminal microCT reconstruction (A) showing stable screw fit against the tibial plateau (B). CT imaging was conducted across time points to ensure no screw loosening (C, D).

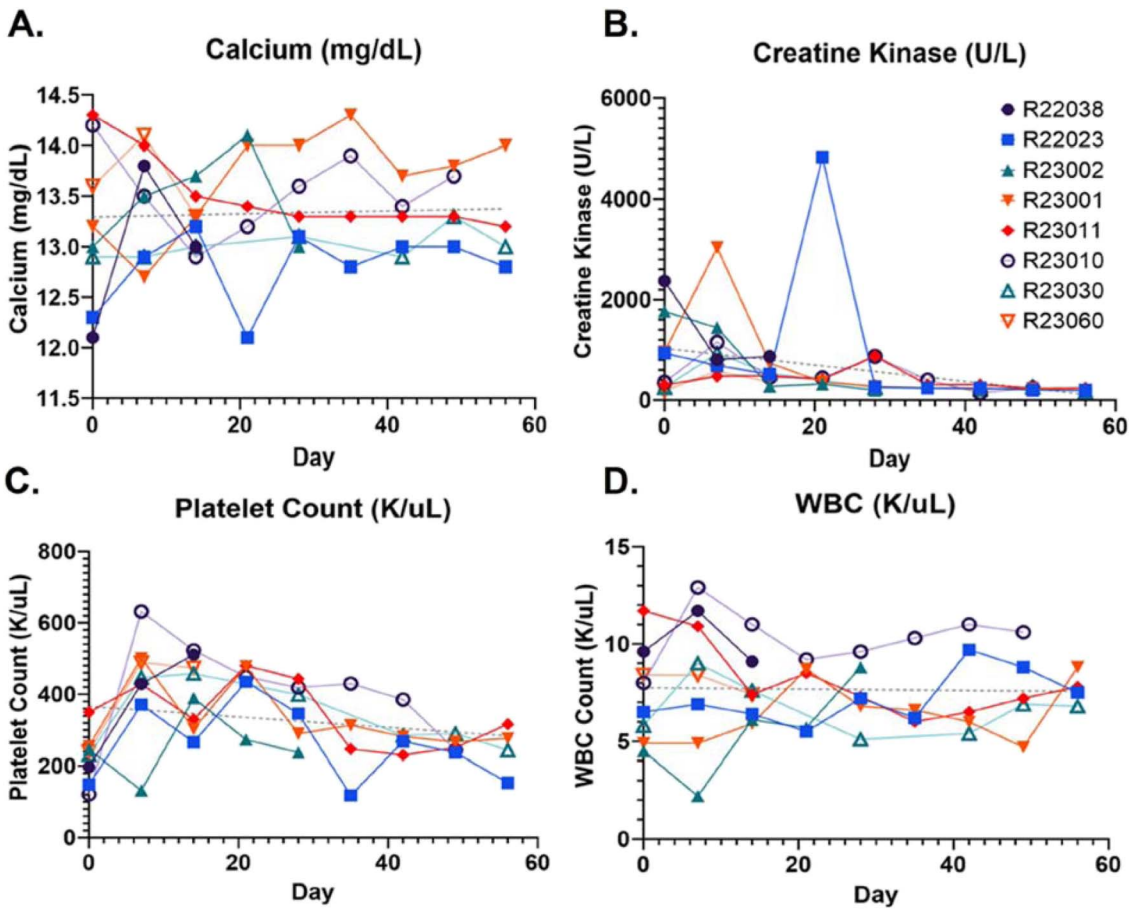
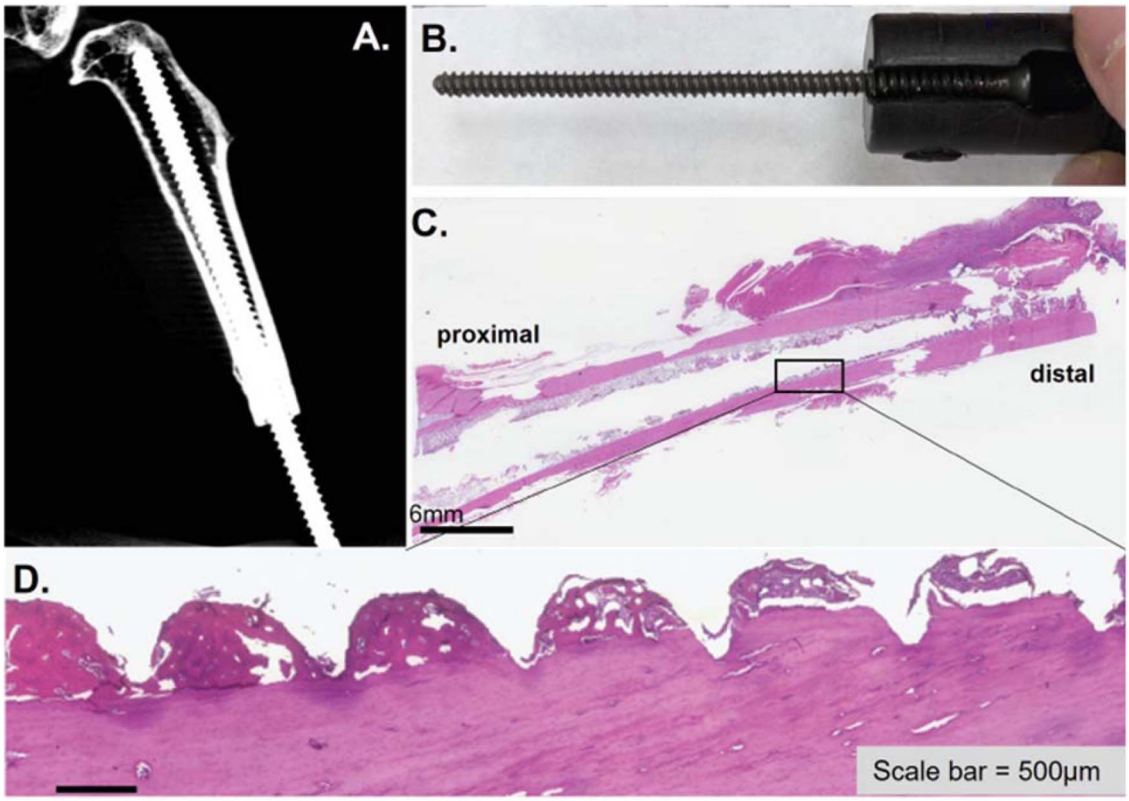
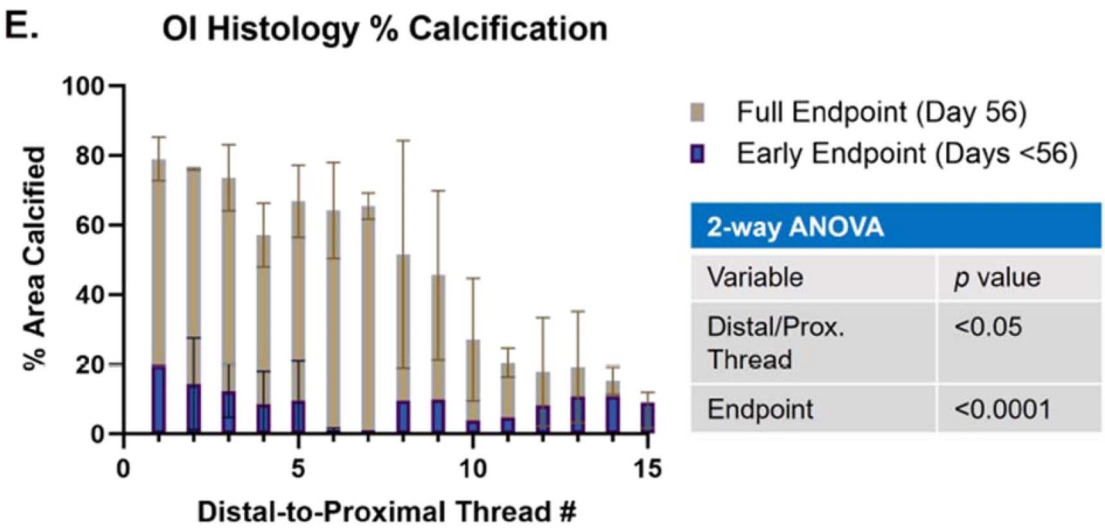


Figure 5. Clinical chemistries were conducted across the study period for calcium (A), creatine kinase (B), platelet count (C), and WBCs (D) to monitor the overall health of the animals.





2-way ANOVA	
Variable	*p* value
Distal/Prox. Thread	<0.05
Endpoint	<0.0001

Figure 6. microCT imaging indicates stable osseointegration (A). Histological analyses reveal bony ingrowth in response to the screw (B, C) with osseointegration occurring around the screw threads (D). Quantification of this bone growth shows significantly higher calcified areas in animals reaching the full 8-week time points compared with earlier time points (E).

remain the primary treatment of infections, with antibiotics playing a secondary role.[23] Contributing to this challenge is that there is currently no preclinical animal model to investigate various interventions and improve patient care. OI implant-associated infections represent unique trials that require a dedicate model.

It is well established that bacteria will adhere to and colonize metal implants almost immediately following placement. This can lead to an extensive implant-associated infection, a difficult-to-eradicate and often recalcitrant infection particularly if the infection is deep. Deep bone infections represent a challenging problem as diffusion of a drug or treatment to locally effective concentrations is challenging, if not impossible, through traditional antibiotic administration routes. If classified as a deep infection, operative irrigation and debridement, deep cultures, and IV antibiotics are typically the only treatment options. Conventional management of infections with systemic antibiotics raises several challenges, including an inability to achieve locally effective doses while avoiding toxicity and improper use that can lead to drug-resistant bacterial strains. Although implant

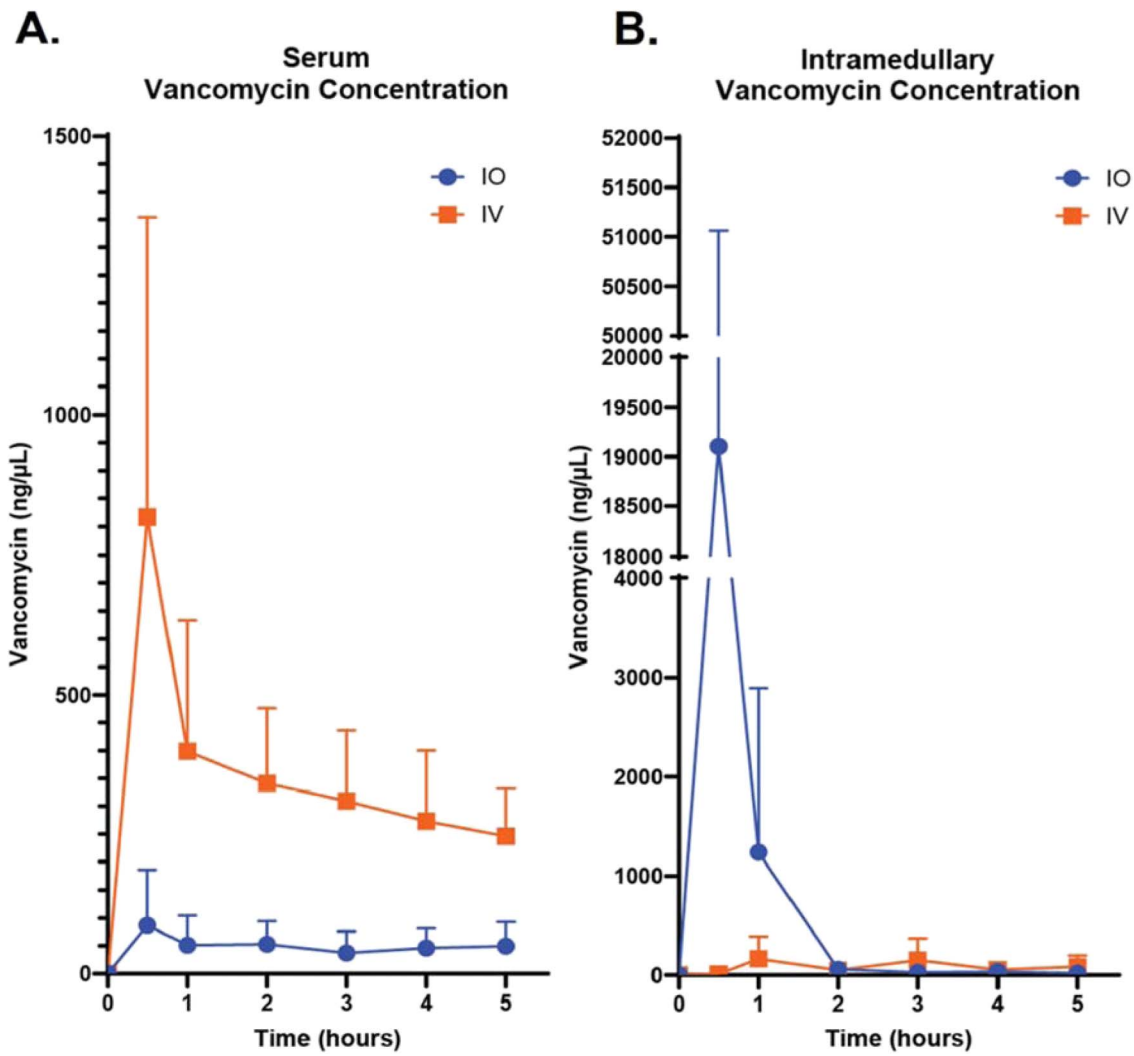


Figure 7. Concentrations of vancomycin measured in the serum (A) and tibial canal (B) show that IO delivery allows locally higher concentrations of vancomycin while minimizing systemic concentrations compared with IV delivery.

retention is the ultimate goal, it may not be feasible if the infection remains unmanageable or causes implant loosening at the bone–implant interface. In addition, because OI is designed to be a lifelong procedure (as a result of bone–implant integration), consequences of removing the ingrown implant in the case of deep infection include shortening of the residual limb (with increased difficulty wearing a conventional socket) and fracture. Thus, OI implant-associated infections remain a major driver of increased disability. IO antibiotic delivery represents an alternative solution, and the small animal model developed and characterized here will both provide preclinical support for IO antibiotic use in amputee patients and will allow investigation of future novel interventions.

IO delivery of drugs and other fluids has been in practice for almost 100 years[24] and is routinely used in battlefield care and acute civilian trauma resuscitation where venous access is compromised or not readily available. IO delivery can lead to 10–20X higher local antibiotic concentrations compared with IV antibiotics while avoiding the toxicity associated with IV delivery. This is an especially effective method in combating established infections because of the high antibiotic concentrations required to eradicate biofilms. Once adherent bacteria proliferate to the degree of forming an implant-associated infection or biofilm, it is presumably an antibiotic-tolerant phenotype where antibiotics are rendered nearly ineffective at targeting the now impermeable biofilm cell wall. Higher levels of antibiotics, called supra-minimal inhibitory concentrations (supra-MICs), are needed to effectively kill the bacteria, concentrations impossible to achieve with systemic antibiotics due to organ toxicity.[25] Other antibiotic delivery methods, such as beads and spacers, are severely limited for OI prosthesis use where the infection may be deep and implant retainment is the end goal. These local depot delivery methods are also associated with stimulation of antibiotic resistance as local concentrations taper to subtherapeutic levels.[26] IO delivery offers a practical alternative strategy for combating OI implant-associated infections, yet there remains no antibiotic with FDA clearance for IO administration despite current safety documentation and long historical use. In periprosthetic implant infection animal model, IO delivery demonstrated greater efficacy than systemic IV antibiotics in reducing bacterial counts in total knee implants, although this study used vancomycin prophylactically rather than against an established implant-associated infection.[27]

While this is a promising result for OI infection, the complex microenvironment of the diaphyseal intraosseous interface found in OI is vastly different from the intra-articular environment of knee joints and must be considered for further applications.

A broad-spectrum antibiotic, vancomycin is one of the more effective antibiotics at targeting methicillin-resistant strains of *Staphylococcus aureus* and is generally reserved for serious biofilm-associated and/or drug-resistant gram-positive infections. It is not well absorbed orally and, for systemic infections, is given intravenously. For localized or nonsystemic infections, other routes of administration include oral, topical, intrathecal, and intraperitoneal. Although vancomycin is largely well tolerated, it is associated with side effects such as infusion reactions, nephrotoxicity, and hepatotoxicity. Despite these systemic adverse effects, studies have indicated that vancomycin must be present in very high concentrations in the infected tissue before local, nonbacterial cells are damaged and has been shown to elicit low systemic levels when delivered by IO administration in a porcine model.[28,29] Findings here corroborate such previous studies, indicating low serum concentrations suggesting a marked reduction in adverse systemic effects. Methods to achieve safe, hypertherapeutic concentrations of a robust antibiotic, such as vancomycin, that could overwhelm bacterial resistance mechanisms and penetrate biofilms could rapidly translate to improving patient care.

OI is a promising treatment, but refinements are still necessary. Knowledge gaps remain in the science of OI prosthetic interface, which has traditionally lagged behind other prosthetic advancements. However, future areas of development (such as mechatronics and sensory feedback) will remain limited if prosthetic retainment is not improved by addressing OI implant-associated infections, which represents one of the more chronic and significant challenges facing OI prostheses.

5. Conclusions

A repeatable, clinically relevant small animal model of bone-anchored limb replacement is described. Careful consideration was given to specific model phases of development to ensure animal welfare and integrity of the model to remain as close to the clinical problem as possible. Rabbits were chosen as an experimental model because of their established track record as both orthopaedic and infection animal models. Their size allows for use of human-scale implants and more controllable volumes for drug delivery. Studies showed animals tolerated the transtibial amputation well under veterinary care, while maintaining normal appetite, mobility, and body weight. Analgesics and dietary supplements were not necessary past 5 days after surgery. Bony ingrowth around the screw implant threads confirmed osseointegration occurring in response to the implant. Early pharmacokinetic studies demonstrate significantly higher levels of vancomycin in medullary tissue compared with serum with IO delivery, suggesting that locally effective doses are feasible while avoiding adverse side effects associated with high levels of systemic vancomycin. Studies are ongoing, and the present model described is positioned for application of OI implant interventions for the treatment and prevention of infections.

Acknowledgments

The authors thank the entire Animal Resource Program at Atrium Health Wake Forest School of Medicine, Erin Mitchell, DVM, and Cara Clouse, DVM, as well as Jingyun Lee, PhD, and Anderson Cox. The authors acknowledge the support of the Wake Forest Baptist Proteomics and Metabolomics Shared Resource, supported by the National Cancer Institute's Cancer Center Support Grant award number P30CA012197. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors also thank our funding source OTA/AO Trauma Foundation.

References

1. Fischer H. *A Guide to U.S. Military Casualty Statistics: Operation Freedom's Sentinel, Operation Inherent Resolve, Operation New Dawn, Operation Iraqi Freedom, and Operation Enduring Freedom*. Washington, DC: Congressional Research Service; 2015.
2. Branemark R, Berlin O, Hagberg K, et al. A novel osseointegrated percutaneous prosthetic system for the treatment of patients with transfemoral amputation: a prospective study of 51 patients. *Bone Joint J*. 2014;96-B:106–113.
3. Frossard L, Hagberg K, Häggström E, et al. Functional outcome of transfemoral amputees fitted with an osseointegrated fixation: temporal gait characteristics. *J Prosthetics Orthot*. 2010;22:11–20.
4. Hagberg K, Hansson E, Branemark R, et al. Outcome of percutaneous osseointegrated prostheses for patients with unilateral transfemoral amputation at two-year follow-up. *Arch Phys Med Rehabil*. 2014;95:2120–2127.
5. Tillander J, Hagberg K, Hagberg L, et al. Osseointegrated titanium implants for limb prostheses attachments: infectious complications. *Clin Orthop Relat Res*. 2010;468:2781–2788.
6. Ellington JK, Harris M, Hudson MC, et al. Intracellular Staphylococcus aureus and antibiotic resistance: implications for treatment of staphylococcal osteomyelitis. *J Orthop Res*. 2006;24:87–93.
7. Gristina AG, Costerton JW. Bacterial adherence to biomaterials and tissue. The significance of its role in clinical sepsis. *J Bone Joint Surg Am*. 1985;67:264–273.
8. Yamada K, Matsumoto K, Tokimura F, et al. Are bone and serum cefazolin concentrations adequate for antimicrobial prophylaxis? *Clin Orthop Relat Res*. 2011;469:3486–3494.
9. Chin SJ, Moore GA, Zhang M, et al. The AAHKS clinical research award: intraosseous regional prophylaxis provides higher tissue concentrations in high BMI patients in total knee arthroplasty: a randomized trial. *J Arthroplasty*. 2018;33:S13–S18.
10. Branemark R, Emanuelsson L, Palmquist A, et al. Bone response to laser-induced micro- and nano-size titanium surface features. *Nanomedicine*. 2011;7:220–227.
11. Palmquist A, Emanuelsson L, Branemark R, et al. Biomechanical, histological and ultrastructural analyses of laser micro- and nano-structured titanium implant after 6 months in rabbit. *J Biomed Mater Res B Appl Biomater*. 2011;97:289–298.
12. Palmquist A, Lindberg F, Emanuelsson L, et al. Morphological studies on machined implants of commercially pure titanium and titanium alloy (Ti6Al4V) in the rabbit. *J Biomed Mater Res B Appl Biomater*. 2009;91:309–319.
13. Palmquist A, Lindberg F, Emanuelsson L, et al. Biomechanical, histological, and ultrastructural analyses of laser micro- and nano-structured titanium alloy implants: a study in rabbit. *J Biomed Mater Res A*. 2010;92:1476–1486.
14. Ambrose CG, Clyburn TA, Mika J, et al. Evaluation of antibiotic-impregnated microspheres for the prevention of implant-associated orthopaedic infections. *J Bone Joint Surg Am*. 2014;96:128–134.
15. Arens D, Wilke M, Calabro L, et al. A rabbit humerus model of plating and nailing osteosynthesis with and without Staphylococcus aureus osteomyelitis. *Eur Cell Mater*. 2015;30:148–162; discussion 161–162.
16. Gillaspy AF, Hickmon SG, Skinner RA, et al. Role of the accessory gene regulator (agr) in pathogenesis of staphylococcal osteomyelitis. *Infect Immun*. 1995;63:3373–3380.
17. Bottagisio M, Coman C, Lovati AB. Animal models of orthopaedic infections. A review of rabbit models used to induce long bone bacterial infections. *J Med Microbiol*. 2019;68:506–537.
18. Lazzarini L, Overgaard KA, Conti E, et al. Experimental osteomyelitis: what have we learned from animal studies about the systemic treatment of osteomyelitis? *J Chemother*. 2006;18:451–460.
19. Metsemakers WJ, Emanuel N, Cohen O, et al. A doxycycline-loaded polymer-lipid encapsulation matrix coating for the prevention of implant-related osteomyelitis due to doxycycline-resistant methicillin-resistant Staphylococcus aureus. *J Control Release*. 2015;209:47–56.

20. Reizner W, Hunter JG, O'Malley NT, et al. A systematic review of animal models for Staphylococcus aureus osteomyelitis. *Eur Cell Mater*. 2014;27: 196–212.
21. Moriarty TF, Schlegel U, Perren S, et al. Infection in fracture fixation: can we influence infection rates through implant design? *J Mater Sci Mater Med*. 2010;21:1031–1035.
22. Schlegel U, Perren SM. Surgical aspects of infection involving osteosynthesis implants: implant design and resistance to local infection. *Injury*. 2006;37(suppl 2):S67–S73.
23. Goff BJ, McCann TD, Mody RM, et al. Medical issues in the care of the combat amputee. Bethesda, MD: Care of the Combat Amputee. TMM Publications, Borden Institute, Walter Reed Army Medical Center, Office of the Surgeon General; 2009:191–228.
24. Drinker CK, Drinker KR, Lund CC. The circulation in the mammalian bone-marrow. *Am J Physiol Leg Content*. 1922;62:1–92.
25. Costerton JW. Biofilm theory can guide the treatment of device-related orthopaedic infections. *Clin Orthop Relat Res*. 2005;437:7–11.
26. Bayramov DF, Neff JA. Beyond conventional antibiotics - new directions for combination products to combat biofilm. *Adv Drug Deliv Rev*. 2017; 112:48–60.
27. Young SW, Roberts T, Johnson S, et al. Regional intraosseous administration of prophylactic antibiotics is more effective than systemic administration in a mouse model of TKA. *Clin Orthop Relat Res*. 2015;473:3573–3584.
28. Jaimovich DG, Kumar A, Francom S. Evaluation of intraosseous vs intravenous antibiotic levels in a porcine model. *Am J Dis Child*. 1991; 145:946–949.
29. Rathbone CR, Cross JD, Brown KV, et al. Effect of various concentrations of antibiotics on osteogenic cell viability and activity. *J Orthop Res*. 2011; 29:1070–1074.