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Rabbit model of a biofilm-contaminated, percutaneous orthopaedic endoprosthesis

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Abstract Preclinical models of osseointegrated orthopaedic implants tend to focus on implant stability, surface modifications to enhance integration with host tissue, and reduction in iatrogenic contamination through antibiotic-eluting/bacteria-resistant coatings. While these studies are imperative to early success in osseointegration, continued success of percutaneous devices throughout the lifespan of the patient is also critically important. A perpetual challenge to the implant is formation of bacterial biofilm on the abutment. Once adhered, biofilm-based bacteria are recalcitrant and readily contaminate the subcutaneous soft tissue of the stoma. To this end, the rabbit model reported herein replicates the clinical scenario of a patient with a biofilm-contaminated abutment. This model enables preclinical testing of advanced therapeutics beyond the traditional antibiotic-based approach, potentially increasing the longevity of the device.

Keywords: osseointegration, rabbit model, biofilm

1. Introduction

An osseointegrated implant and prosthesis system for lower extremity amputation presents a clear advantage over the traditional socket-based approach in that it permits direct transmission of the weight load to the residual bone, generating the essential biochemical stress signals to maintain structural integrity.[1] Socket-based prosthetic limbs do not directly transmit the load to the residual bone, which leads to a regional osteoporosis-like condition.[2] Furthermore, applying the load through the skin, which is not a load-bearing tissue, increases secondary complications such as the generation of pressure ulcers, sweating, difficulty with fit, and pain.[3]

Patients who are best suited to receive osseointegrated implants are those who are otherwise healthy (e.g. traumatic accident, tumor resection) as the patient's innate potential for bone regeneration is imperative for a strong interface.[4] Often, these patients' desire to return to an active lifestyle, in combination with the effectiveness of the prosthesis, presents challenges to maintaining the integrity of the implant; the patient may push the boundaries of what the implant can withstand from a physical and biological standpoint. Excessive range of motion and exposure to natural environments with bountiful bacterial communities, such as those experienced during endurance competitions, increase the probability of contamination. For example, it is reported that up to 20% of patients with transfemoral amputations develop implant-associated osteomyelitis within 10 years of implantation.[5] This is due to the fact that the endoprosthesis protrudes through the physical skin barrier, generating an access point to bacterially vulnerable, subcutaneous tissue, even in cases with exceptional healing. While there have been many attempts to improve soft-tissue integration through implant surface modification to enhance fibroblast attachment, there is still no clear method to replicate the keratinized tissue found at the eponychium, for example, which prevents bacteria from entering at the skin–nail interface.[6]

The formation of a bacterial biofilm on the percutaneous stem is an inevitable outcome, as biofilms develop on all surfaces, and may closely resemble the dry-surface biofilms that form on clinical surfaces.[7] Various bacterial species are capable of producing a biofilm, including those that routinely populate the skin, such as *Staphylococcus epidermidis*.[8] While some bacterial populations are beneficial, others can be detrimental, and over time, populations fluctuate.[9] Contamination may remain localized and asymptomatic, but in some cases, it can progress to a

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deep infection requiring implant removal or revision of the residual limb.

Routine cleansing of the implant surface helps manage planktonic bacterial populations; however, biofilm-based communities present a more challenging issue. Sessile bacteria within biofilms are protected by an extracellular polymeric substance, which serves as a nutrient reservoir and barrier against antibacterial agents.[10] Furthermore, the development of antibiotic resistance, such as in the case of methicillin-resistant *Staphylococcus aureus* (MRSA), presents additional challenges, expected to increase over time.

Preclinical animal models designed to assess antimicrobial therapeutics at the implant interface in osseointegrated prostheses are not well reported. The most commonly used animal models in studies investigating osseointegrated implants are ovine,[11–13] rabbits,[14,15] and canines.[16] Ovine models, although suitable because of their large bone size accommodating full-sized endoprostheses, pose financial challenges because animal husbandry requirements often demand in-house resources. Canines, typically enrolled in studies when terminal diseases such as lower extremity malignant neoplasia are diagnosed, offer a more economical option.[16] However, the variety in breeds and body conditions adds variability, which can mimic the diversity in patient populations but also complicates study parameters. Rabbits are most frequently used in studies modeling pin tract infections because of the similarity in their immune system to humans.[14,15]

The aim of this article was to describe a rabbit model of a percutaneous endoprosthesis for transtibial amputation to evaluate antimicrobial therapeutic strategies against biofilm-based bacterial communities in situ. The rabbit model offers a balance between key factors such as size, stability, cost-effectiveness, and practicality. While rabbit skin may be quite different from human skin for superficial inflammatory assessment,[17] the immune system is quite similar,[18] providing an advantageous model for studying bacterial contamination.

2. Materials and Methods

2.1. Surgical Procedure

New Zealand White rabbits (n = 2, 3–3.5 kg, Charles River Laboratories, Wilmington, MA) were housed and used in accordance with local IACUC (Dartmouth College) guidelines and an approved protocol (#00002320). The protocol was further approved by the DOD's Animal Care and Use Review Office (ACURO). On arrival, all animals underwent a minimum of 5 days of acclimation before surgery. On the day of surgery, rabbits were first sedated with meloxicam 1 mg/kg subcutaneous (SC) and buprenorphine 0.03 mg/kg intramuscular (IM) injections 30 minutes before surgery. Famotidine 2 mg/kg was delivered orally for gastric ulcer prevention. Cefazolin 20 mg/kg was administered intravenously (IV) every 90 minutes during the operation. Mirtazapine was applied topically to the pinna to stimulate appetite. The rabbits were then intubated, and anesthesia was maintained by isoflurane. Mechanical ventilation was used to maintain $PaCO_2$ of approximately 40 mm Hg with a mixture of air and oxygen.

Hair of the right hind limb was removed using clippers and scrubbed with chlorhexidine gluconate, and the incision site was prepared by circumferential subcutaneous injections of lidocaine 1 mg/kg. An initial incision was created near the distal portion of the tibia, and an electric cautery knife was used to expose the bone. The muscle and periosteum were pushed proximally to expose the tibia–fibula junction using a periosteal elevator. An oscillating bone saw with saline irrigation was then used to transect the tibia 2 cm below the fibula junction.

A 3.5-mm drill bit was used to ream the medullary canal and remove bone marrow. At this time, medium-viscosity bone cement (DePuy Orthopaedics, Warsaw, IN) was mixed according to the package insert and instantly injected into the medullary canal using a 3-mL syringe and 18 G needle. A #8 stainless-steel threaded rod (McMaster-Carr, Princeton, NJ), cut to 82-mm length, was then driven into the bone cement–loaded medullary canal. A cerclage wire was placed if fracturing of the tibia was observed on the distal end. The residual limb is then sutured closed by building a muscle layer over the transected bone and followed by the skin. A prosthetic limb was threaded onto the exposed rod and secured in place with a stainless-steel #8-32 jam nut and lock washer (McMaster-Carr).

2.2. 3D-Printed Prosthetic Limb

A prosthetic limb was designed to securely attach to the implant while allowing for removal at a later time point. The main part of the prosthesis was 3D printed on a Prusa MK4 3D printer (Prusa Research, Prague, Czech Republic) using a polylactic acid (PLA) filament (Hatchbox, Rowland Heights, CA). The snap-fit bottom was printed using a FiberFlex 40D filament (Fiberlogy, Brzezie, Poland). The prosthesis was attached to the implant using a #8-32 stainless-steel heat-set insert for plastic (McMaster-Carr).

2.3. Postoperative Care

The pain management strategy is provided in Table 1. In brief, buprenorphine 0.02 mg/kg SC injections were given every 8 hours for the first 3 days following surgery. Meloxicam 1 mg/kg subcutaneously, famotidine 2 mg/kg subcutaneously, and mirtazapine 1 mg/kg topically applied to pinna were administered daily for the first 3 days. On day 4, buprenorphine was removed, and on day 5, the remaining pharmaceuticals were removed. Signs of pain were monitored at each injection interval using a combination of the grimace scale and a behavioral scale, creating a total pain score, which was used to guide when further medication was needed. All animals were weighed daily during the first 2 weeks

Table 1

Pain management strategy to ensure animal welfare.

Perioperative phase		
Meloxicam	Buprenorphine	Famotidine
1 mg/kg	0.02–0.1 mg/kg	1–5 mg/kg
Cefazolin 15-35 mg/kg (IV) every 30 min during the surgical procedure		
Early postoperative phase (day 0–day 3)		
Meloxicam	Buprenorphine	Famotidine
1 mg/kg	0.02–0.1 mg/kg T.I.D.	1–5 mg/kg S.I.D.
SC mirtazapine (1 mg/kg) will be given transdermal (once per day)		
Middle postoperative phase (day 4–day 5)		
Meloxicam SI.D.	Famotidine SI.D.	Mirtazapine SI.D. (transdermal)
0.6–1 mg/kg	1–5 mg/kg	1 mg/kg
If signs of pain continue when NSAID is in effect, administer 0.01–0.03 mg/kg buprenorphine up to 3x/day and 1–5 mg/kg famotidine SI.D.		
Late postoperative phase (day 5 and beyond)		
Meloxicam SI.D.	Famotidine SI.D.	
0.6 mg/kg	1–5 mg/kg	

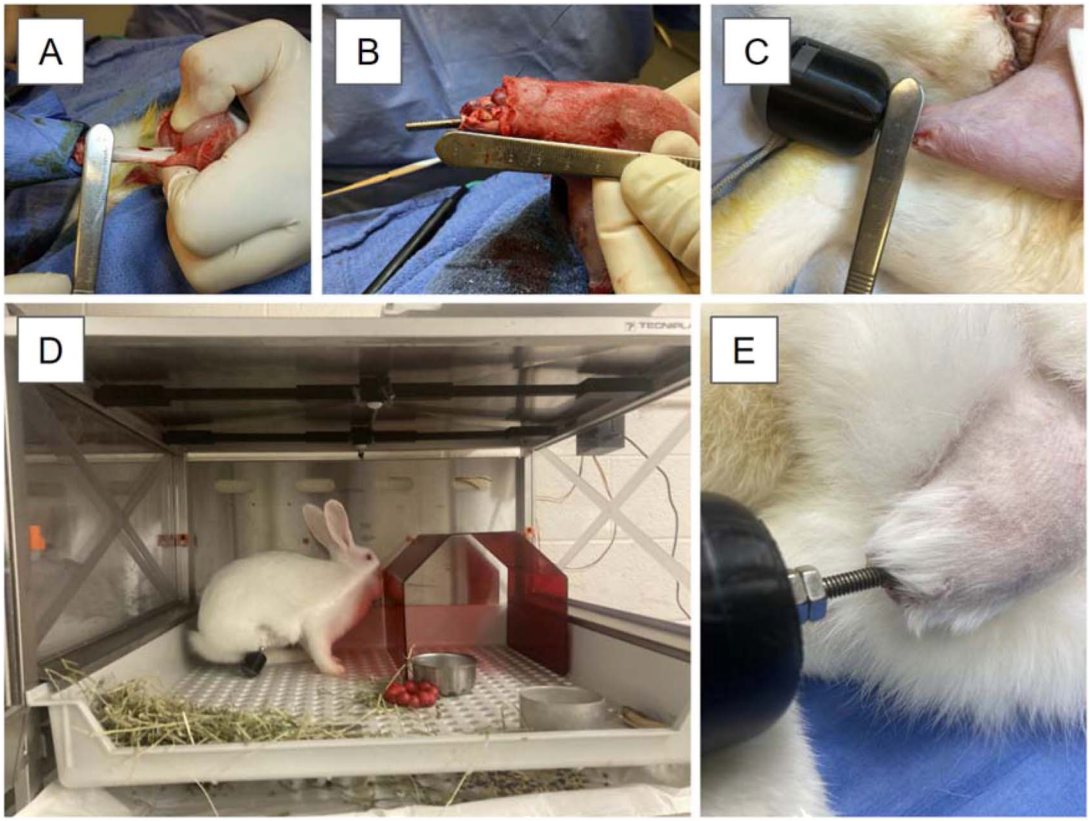

Figure 1. Surgical procedure. A, A circumferential incision proximal from the tarsocrural joint was made to expose the tibia, and the muscle was pushed back until the fibula was visible. B, A complete transection of the tibia was performed 2 cm distal from this point, and a #8 stainless-steel threaded rod, 82 mm in length, was driven into the medullary canal after it was filled with bone cement. C, The prosthetic foot was threaded onto the implant and secured in place using a jam nut. D, Ten days postoperatively, the rabbit was using the prosthetic foot without pain. E, A well-healed residual limb 23 days after operation.

postoperatively. Excessive weight loss was defined as a loss of 15% or greater of initial postoperative weight.

2.4. Biofilm

Bioluminescent MRSA (SAP231) was obtained from Dr. Richard Plaut, FDA. Bacteria were grown overnight in tryptic soy broth to reach log phase growth. Bacteria colony-forming units (CFUs) were calculated by measuring the optical density at 600-nm wavelength. 1×10^8 bacteria were added to each well of a 3D-printed macrofluidic flow chamber system constructed as previously reported.[19] Each well of the device contained a #8-32 stainless-steel T-nut (McMaster-Carr). A MRSA biofilm was well formed on the T-nut after 3 days in the macrofluidic device, as indicated by the presence of bioluminescent signal using a Perkin-Elmer IVIS Spectrum CT system. Biofilm thickness was also visualized using optical coherence tomography to validate its presence.

2.5. Inoculation

Postoperative healing was deemed complete if weight loss over a 5-day window was absent and pain scale scores were in the low range. Midazolam, 1 mg/kg, was injected intramuscularly to sedate the rabbit, and after 5 minutes, isoflurane was introduced to anesthetize for imaging and inoculation. The IVIS was used to capture a baseline bioluminescent image of the residual limb. The prosthetic foot was removed, and then the inoculated nut was threaded onto the implant and it was imaged again. The prosthesis was replaced, temperature was taken rectally, and the animal was monitored until regaining consciousness. Three days a week, the rabbit was anesthetized and imaged using the IVIS to check for bioluminescent signal.

3. Results

3.1. Surgery

The steps of the surgical procedure, as shown in Figure 1, were performed in an operating room with a sterile field. The procedure was performed in under 1 hour using common orthopaedic surgery instruments (oscillating bone saw). The prosthetic foot, as shown in Figure 1D, was 3D printed and weighed 39 g compared with the 37-g weight of the amputated limb. The bottom portion of the prosthetic limb was printed using the Flex printing filament to increase traction and provide a cushioning effect as to reduce the impact of its use (Fig. 2). The prosthesis was designed as a hollow shell, providing space for incorporation of sensors.

Pain associated with transtibial amputation was found to be well controlled by the pharmacological regimen (Fig. 3). In all surgeries, the total pain score never went above a value of 5, which was well within the low range of the total pain scale (Supplemental Figure 1, http://links.lww.com/OTAI/A109). The most persistent pain scale criterion expressed by rabbits was not continuously using the amputated limb, which was anticipated. Rabbit weight decreased over the first 10 days but then remained stable for the duration of the study. Implant stability was also a

Figure 2. Prosthetic limb: The prosthetic limb was designed to be easily removable from the implant to allow the introduction of bacteria at a later time point. Other key factors of the design were to (1) match the weight of the amputated limb, (2) offer a cushioning support to the limb, (3) provide traction on the cage floor, and (4) provide the opportunity for incorporation of electronic sensors in future studies. The upper portion of the prosthetic limb was 3D printed with PLA, and the bottom gray portion was printed with a Flex filament. A stainless-steel heat-set insert was used as a fixation point in the top of the prosthesis.

concern, and the implant remained in place for more than 50 days after operation.

3.2. Inoculation

A MRSA infection was introduced to 1 rabbit by threading a biofilm-coated nut onto the implant, as shown in Figure 4, once healing was deemed sufficient by a veterinarian. The biofilm grew on the nut for 3 days in a macrofluidic device that continuously replenishes the culture broth. The presence of bacteria was verified by the presence of a bioluminescent signal (Fig. 5A). There was no detectable signal 3 days after inoculation, and the residual limb appeared normal, similar to what was found on day 0 (Fig. 5B). On day 10, the residual limb appeared swollen and inflamed (Fig. 5C) and the bioluminescent signal was robust (Fig. 5D). The rabbit appeared otherwise healthy as there were no signs of distress nor an elevated temperature.

On day 18, the bioluminescent signal was not spreading in size, but the temperature of the animal was elevated to 104.7°F, which was well into the fever range for a rabbit. Overall motion in the cage increased significantly when the infection had a systemic effect (Fig. 6). Meloxicam was administered in an attempt to mitigate the severity of the fever; however, within 24 hours, the fever had not subsided and the rabbit was euthanized on day 19 after infection. The affected hind limb was removed for imaging (Fig. 7). On closer inspection, it was documented that the soft tissue had receded, as shown in Figure 7A, exposing the entirety of the previously embedded nut. An incision was made to dissect the muscle, exposing the distal portion of the tibia. The bioluminescent signal was found deep within the tissue (Fig. 7B).

4. Discussion

Therapeutics to treat infections that arise from metallic, percutaneous devices in orthopaedics are of interest to those treating both patients with amputations and patients with fixation pins. Animal models of this condition are quite varied and tend to focus on antibiotic coatings or surface modifications[20] to prevent planktonic bacteria adhesion and growth, most often replicating an iatrogenic contamination.[21] However, neither of these therapeutic approaches replicates what happens to patients with lower extremity amputations and osseointegrated implants; they present an endoprosthesis with a percutaneous stem that may over time develop a sessile, biofilm-based contamination. This is a critically important distinction in that biofilm bacteria are known to withstand 1000X the inhibitory dosage compared with their planktonic counterparts.[22] Generating a model of this scenario is difficult in that it would require a substantial amount of time for the biofilm to develop and the inherent variability of resulting biofilm may be sufficiently significant to confound therapeutic assessment.

The choice of animal models to use for a biofilm-based investigation is dependent on a number of factors, but given the timescale of biofilm development, large animal models may not be

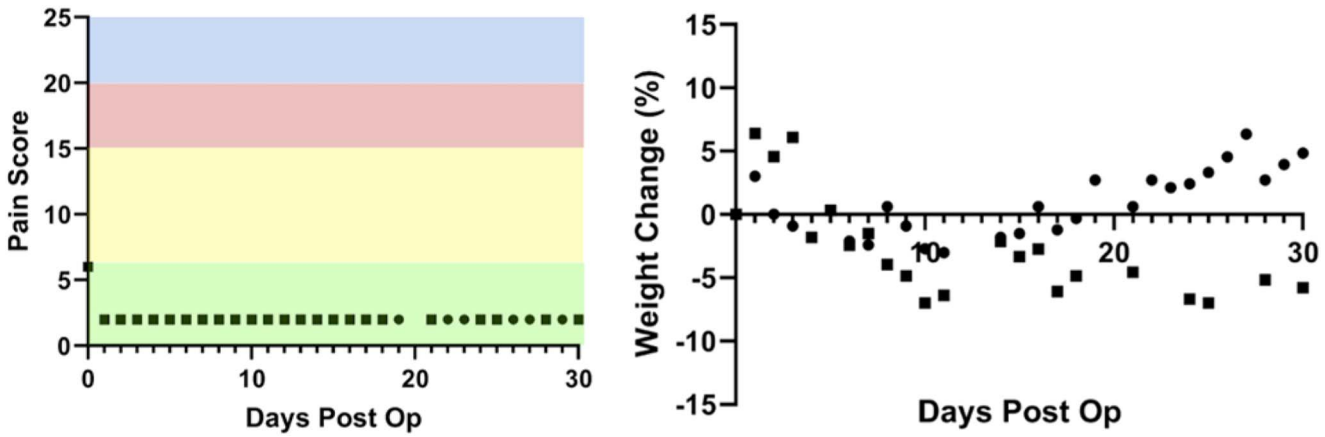

Figure 3. Postoperative care: Left: Total pain was recorded each day and was never found to be above the threshold of the low, green domain (n = 2). The yellow and red zones require pharmacological intervention and increased monitoring to alleviate pain. The blue zone requires immediate euthanasia. Right: Weight tends to drop for the first 10 days after the operation. A weight loss of greater than 15% requires immediate euthanasia.

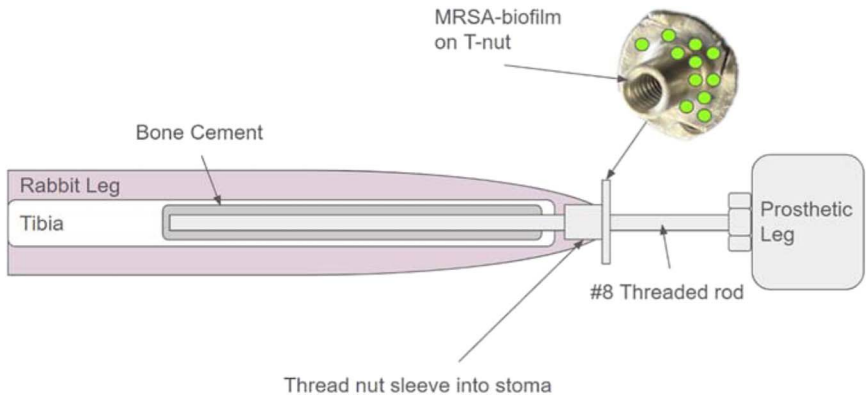

Figure 4. Inoculation strategy: A schematic of the implanted rod and bone cement within the medullary canal is shown as follows: MRSA biofilm was grown on a stainless-steel T-nut that was modified by flattening the securing prongs. The nut was threaded onto the rod, sliding the sleeve of the nut into the stoma of the residual limb to ensure direct soft-tissue contact with the biofilm.

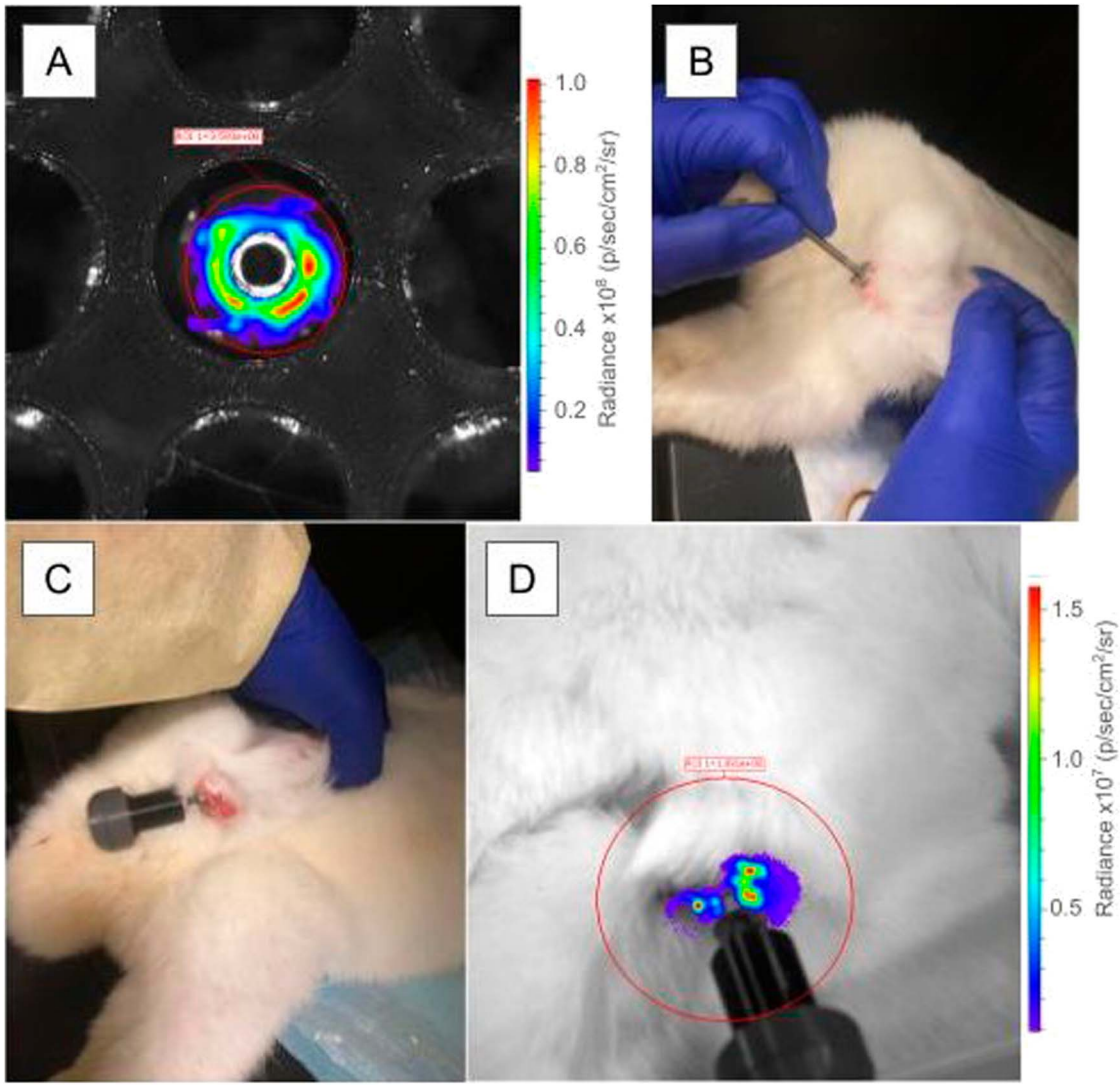

Figure 5. Inoculation of the implant. A, Bioluminescent MRSA bacteria on the T-nut were visualized using a Perkin-Elmer IVIS before placement. B, The nut was threaded onto the implant to insert the sleeve of the nut into the stoma of the residual limb. C, After 10 days, there were visible signs of contamination of the residual limb. D, The residual limb displayed the bioluminescent signal, indicating that the infection was a result of the bioluminescent MRSA.

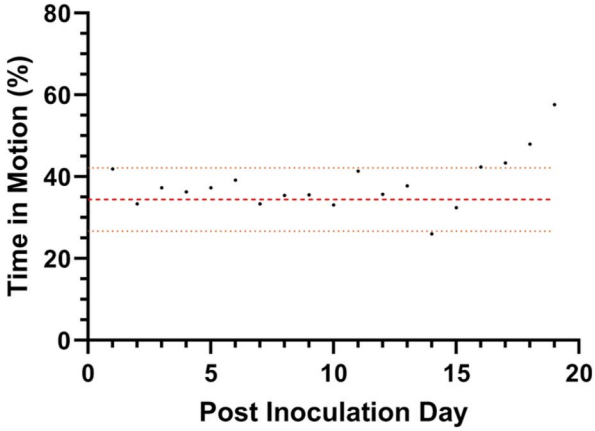

Figure 6. Assessment of cage activity: Motion within the cage was continuously monitored using passive infrared sensors. The average amount of daily movement for 24 days before inoculation was calculated and plotted over the daily movement values (dashed line) along with 3 times the standard deviation above and below the mean (dotted lines). On postinoculation day 18, the movement was above normal, which corresponded with an increase in temperature on that day (104.7°F, baseline 102.7°F). On day 19, the movement was elevated again and the animal showed no signs of recovery from the infection; thus, the animal was euthanized.

favorable because of economics of such a study, although they would allow the use of commercially available implants. Conversely, small animals such as rodents may not present a readily manipulable implant. Rabbits are a middle ground; the implant can be handled using conventional orthopaedic tools while also providing an economically viable platform that permits studies to be performed with statistically significant populations. Furthermore, rabbits are widely known to provide an immune system markedly similar to humans, which is especially beneficial in instances such as being confronted with a bacterial contamination.[18]

Early attempts to develop this model within the laboratory unveiled 3 impediments: (1) implant instability, (2) postoperative weight loss, and (3) inoculation strategy. Initially, a 3.5 × 80-mm cortical bone screw was selected as the endoprosthesis as the diameter and length mated well with the medullary canal diameter and length. The screw was driven into the medullary canal, but over time, the purchase of the screw lessened and it would back out of the tibia under normal movement. It was discovered that the diameter of the medullary canal increased from the distal to proximal end, and thus, only the most distal aspect was in contact with the implant.

Perhaps concomitantly, weight loss trajectories were concerning but never reached the 15% cutoff. Weight loss may also be a result of opioid use, buprenorphine in this case, as it is shown to reduce gastrointestinal transit time in rabbits[23] and is widely reported in rabbit surgeries.[24,25] Unfortunately, there are no approved sustained release formulations of buprenorphine for use in rabbits[26]; thus, injections were administered 3 times daily for the first 4 days, which may impart an effect on the digestive tract that recovers approximately 10–14 days postoperatively.

The third impediment, inoculation strategy, was challenging in that when a planktonic suspension of bacteria was topically applied to the well-healed residual limb, insufficient bacteria remained attached to induce a contamination, and it did not replicate the true nature of the clinical scenario to the desired extent. Changing the endoprosthesis design to a threaded rod with the addition of bone cement created an individualized implant that not only remained stable for the duration of the experiment but also would allow for introduction of the desired biofilm-based inoculation strategy.

5. Conclusion

Percutaneous osseointegrated implants are particularly advantageous to patients as they increase patients' quality of life through improved mobility and function. However, the percutaneous nature of these implants presents a challenge that is not present with traditional socket-based prosthesis where the physical skin barrier remains continuous across the residual limb. All implants, regardless of their anatomical location and application, present an abiotic surface on which bacteria find refuge as it is safe harbor

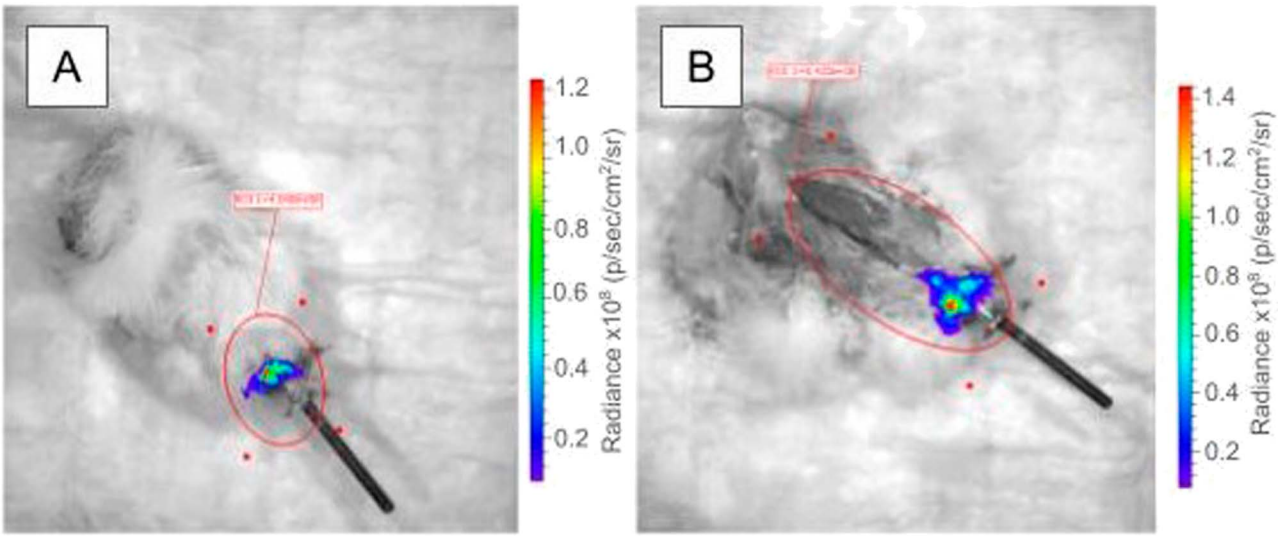

Figure 7. Terminal infection: On postinoculation day 18, the rabbit had an elevated temperature but no signs of discomfort. The temperature was not mitigated by meloxicam, and therefore, the animal had to be euthanized on day 19. A, The infection caused the soft tissue to retract, exposing the implanted nut. B, An incision was made to visualize the extent of the infection, which migrated proximally, following the tibia surface.

from circulating immune cells and vascularly delivered antibiotics. Percutaneous endoprostheses demand alternate therapeutic strategies to account for their unique position; not only do they provide an abiotic surface but they are also exposed to the outside world. Development of those strategies requires an animal model for preclinical testing that replicates the paramount variables: a well-healed residual limb and the introduction of sessile, biofilm-based bacteria. The results presented herein are the development of a rabbit model that meets those specific requirements, paving the way for preclinical testing of advanced therapeutics to mitigate the condition this patient population endures.

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