ASSESSING PERIIMPLANT TISSUE INFECTION PREVENTION IN A PERCUTANEOUS MODEL

by

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THE UNIVERSITY OF UTAH GRADUATE SCHOOL

SUPERVISORY COMMITTEE APPROVAL

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This thesis has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

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ABSTRACT

Prevention of infection remains a challenge to the implementation of the percutaneous osseointegrated implant technology of prosthetic limb attachment in amputees. The purpose of this investigation was to determine if a broad spectrum antimicrobial, Ceragenins^{TM} (CSA - 13), could prevent pin track infections in a percutaneous pin wound site in a sheep model.

The pin was inserted through both cortices of the proximal tibia and protruded through the skin on the medial side of the hind leg. All twenty sheep received the smooth titanium alloy pin. Ten sheep were treated with a polyurethane foam pad coated with a CSA-13–polyurethane polymer conjugate, and ten sheep served as controls receiving an uncoated, sterile foam pad. The sheep were euthanized at the end of the 24-week trial or when they presented with clinical signs of pin track infection. After euthanasia, cultures were obtained of muscle, blood, and bone, and muscle and bone were harvested for histology. In addition to the clinical signs of infection, the sheep was considered infected if at least one tissue culture was positive and/or one histologically stained sample was found positive.

Statistical analysis included Kaplan-Meier survivorship curves to display time to pin track infection rates and the Wilcoxon-Mann-Whitney test to compare the Appositional Bone Index between the groups.

The data demonstrated that, when compared to the control pads, the CSA-13-polyurethane conjugate coated pads did not prevent pin track infection ($p=0.88$). All sheep were euthanized during the first 40 days after implantation. Large gaps around the pin indicated a lack of skin-pin adhesion. Radiographic implant loosening was found in both CSA-13 and untreated control groups. Fibrous tissue formation was found in the gaps between the implant and bone. Skin and soft-tissue motion may have led to micromotion along the implant, which in turn led to pin track infection and/or implant loosening.

CSA-13 was not effective in preventing pin track infections in a percutaneous sheep model in the application used. The data suggest that maintaining skin attachment to the implant surface is essential and that antimicrobials may only be used as secondary barriers following skin attachment.

To Justin.

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CHAPTER 1

BACKGROUND AND INTRODUCTION

Source of Amputations

Sixty-four percent of those injured in World War I survived their injuries while 72% of those injured in the Vietnam War survived. About 87% of those injured in Operation Iraqi Freedom and 68% of those injured in Operation Enduring Freedom are surviving.[1,2] Of this current greater percentage of surviving wounded warriors, 2% from Operation Iraqi Freedom have one or multiple amputations compared with 3% in Vietnam and 1% in WWI.[1]

A closer examination of one airman demonstrates the type of injuries a warrior can sustain and survive currently. This patient lost one lower extremity above the knee, the other in a hip disarticulation, as well as his right hand, and a portion of his face.[2] This airman survived these injuries because of the vastly more advanced medical care found closer to combat zones than the care available in previous wars. A functional hospital can now be located within 60 minutes of a battlefield.[2] Surgeons and medical professionals are now taught to immediately resuscitate, stabilize and debride all wounds to keep the wounded warrior alive and maintain a progressive wound care protocol while the patient is transported to successively more

sophisticated levels of surgical care and final wound closure. These treatment tactics are saving lives that would have been lost in previous wars.

Another life-saving system that has been implemented on the current battlefield is the proper training and use of tourniquets. Once thought of as a "danger to life and limb"[3] in 1918, views and studies on tourniquets have shown them to have great potential as life-saving devices without jeopardizing limb survival. All military personnel are now trained in the use of and carry tourniquets. Tourniquets have become standard issue to each warrior on the battlefields of Iraq and Afghanistan.[4]

Current Treatment of Amputees

With survival there are now new challenges in the aftercare and treatment of these wounded warriors, especially those with high level, multiple amputations. Conventional socket prosthesis attachment is extremely difficult on a short residual limb. Limited residual limb length requires complex docking mechanisms. Also, sockets are difficult to attach to a short residual limb and may impinge on the proximal joint (pelvis or shoulder girdle) when the artificial limb is moved.[5] Other problems associated with socket technology include heat and sweating in the prosthetic socket, sores/chafing, and skin breakdown as well as the more complex skin problems of infection and skin lesions. Sockets limit the ability to walk quickly or adequately power a prosthetic hand.[6,7] There are also problems maintaining a good fit due to weight fluctuations and pain.[7] Trans-femoral socket technology can cause discomfort when sitting and reduce the range of motion of the hip.[8]

Osseointegration

To increase range of motion, give an increased level of comfort when sitting, and even allow the possibility of osseoperception (sensory input through the prosthesis),[8,9] osseointegrating implants directly with the bone of the residual limbs has been introduced and found to increase the quality of life [6] of amputees, especially patients with short residual limbs.

Dr. Rickard Brånemark has developed a two-stage implant procedure for osseointegration.[10] This procedure involves screwing the implant into the bone, closing the wound site, and allowing healing for 6 months. A percutaneous abutment is then screwed into the implant during the second operation. A year of progressive loading under the supervision of a physical therapist then follows.[10,11]

Infection was initially the most common complication encountered by Branemark, in a 3-year follow-up of the first 16 transfemoral patients to receive the percutaneous implant. Fourteen superficial infections and seven deep infections were reported. Other complications included abutment, abutment screw, and fixture (implant) failure.[11]

The infection control strategy in the Branemark model includes his surgical technique where the skin is adhered operatively to the distal region of the bone. Strict daily hygiene is mandated that involves the use of a cotton flossing cloth, moistened with sterile saline, which is spun around the skin-implant interface.[12] This flossing may reduce the bacteria burden, debride cellular residue, and possibly removc and prevent biofilm formation.

The Branemark osseointegration two stage procedure to treat transfemoral amputees has also been performed in the United Kingdom. After 1 year, 2 of the 11 patients (11%) had their abutment and internal fixture removed because of infection.[13]

Limitations to current applications of osseointegration technology include the risk of infection, implant loosening, and implant breakage. Two types of infection can develop: infection at the implant–skin interface (superficial) or deep infection. Deep infection can lead to life-altering consequences including chronic infection, bone resorption leading to bone fracture or implant loosening, and possible eventual reamputation at a higher level.[11,13,14] The development of an effective infection-prevention strategy is crucial to combat these destructive, debilitating, and unacceptable infections.

Staphylococcus aureus has been found as a source responsible for biomaterial, device-related, and/or pin track infections[15,16] *Staphylococcus aureus* can tolerate high saline levels as shown by the Mannitol Salt Agar *S. aureus* detection method where *S. aureus* grows on plates containing 7.5% saline.[17,18] *S. aureus* as a prominent infectious agent in pin track infections thriving in high concentrations of saline leaves suspect the Branemark method of infection prevention through flossing with a cloth moistened with saline. Thus, an alternative barrier to saline should be explored.

Options for Treatment of Infection in Osseointegrated Prosthetics

Antimicrobials are molecules that can kill or slow the growth of an infection-causing microbe. Resistance has developed to many traditional antibiotics.[19,20] An example is *Staphylococcus aureus,* which has in a step-wise fashion developed resistance to penicillin (via beta-lactamase production), resistance to methicillin

(MRSA via penicillin-binding protein, PBP2a production coded by the *mec*A gene),[21] and now is in the early stages of developing resistance to vancomycin.[22] Changes in the bacterial cell wall chemistry are important in the resistance-development process.[21] Two events can cause antibiotic resistance. The first is horizontal gene transfer between individuals in a bacterial population. The second is mutations encoded in the chromosome of the bacteria.[20] These horizontally transferred genes are transferred by episomes, which are genetic particles within certain cells that can exist either autonomously in the cytoplasm or as part of a chromosome.[23]

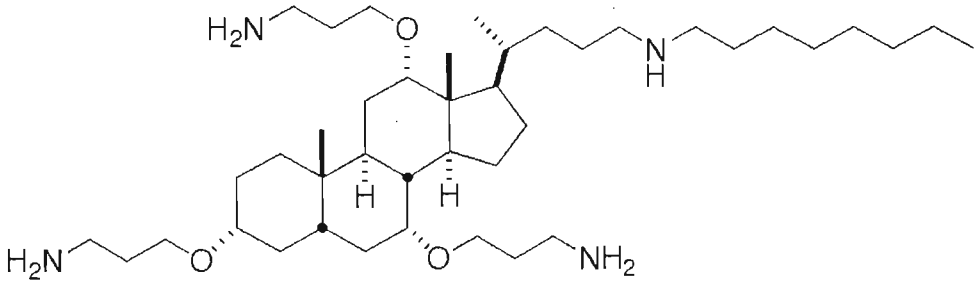
To combat these resistance-developing bacteria, novel antimicrobials are currently being developed and studied. Antibacterial peptides have been isolated from diverse organisms[24,25] and these peptides are, in general, cationic and facially amphiphilic molecules. They display broad-spectrum antibacterial activity, rapid killing times, and are less likely to induce the formation of resistant stains of bacteria. The mechanism for their lethal action against bacteria is permeabilization or disruption of the bacterial cell membrane.[25] At high concentration these cationic peptides can be toxic to mammalian cells which are enveloped by a cell membrane which is similar to the cell membrane found in bacteria. Pexiganan is an example of an antibacterial peptide that has been studied *in vitro* and in a transcutaneous bone/pin rabbit model and found to have bactericidal properties.[26] Although pexiganan, isolated from the African clawed frog, demonstrated bactericidal properties, the company producing the product abandoned the pursuit of FDA approval because of difficulties in maintaining manufacturing consistency.

Pexiganan illustrates a limitation to the use of naturally derived antibacterial peptides. Because of their relatively large size (> 20 amino acids), manufacturing antibacterial peptides can be technically difficult and costly. For example, at the current production efficiency with the available manufacturing techniques, for each 100 kg of recombinant peptide, one million liters of fermentation mixture is necessary. This creates a cost that is 5 to 20 times as high as that of conventional antibiotics.[27] Many of these peptides also have short lifetimes in the presence of proteases.[28] Because of these limitations, antimicrobial focus has shifted to developing mimics of these naturally effective cationic antimicrobials.

One such mimic was developed by the synthesis of steroids with amine groups. This novel series of antimicrobial compounds, termed Ceragenins, is based on derivatives of bile acids with covalently attached amines (Figure 1.1).[29] CSA-13 is the most potent of the Ceragenin compounds tested to date[29] and has been shown *in vitro* to effectively kill both gram-negative and gram-positive bacteria.[30] Although Ceragenins have been found to have weak hemolytic activity,[29] they display broad-spectrum bactericidal activity,[31] which made them an optimal antimicrobial choice for this study.

Model to Test Infection Prevention

A model was needed that would provide a challenging environment for testing the chosen antimicrobial, an environment that would preferably mimic the possibly contaminated situations encountered by an active warrior amputee. The model also needed to mimic the large amount of soft tissue motion observed clinically in the distal ends of residual limbs of the local veteran amputee population. The test model



CSA-13

Figure 1.1: CSA-13 structure displaying steroid backbone and attached amine groups giving the structure its characteristic amphipathic (both hydrophilic and hydrophobic groups) morphology[32]

should not mimic the Branemark operative procedure, which removes the soft tissue aspect of the residual limb to create a skin seal around the bone.[10] This is because the use of that operative procedure would cause difficulty in distinguishing between the advantages of skin immobilization which alone has been shown to reduce percutaneous infection[33] from a mobile skin site and the chosen primary infection barrier CSA-13. The test model was also not meant to mimic the goat model chosen by Pendegrass to facilitate dermal attachment into flanged implants placed in the relatively stable distal end of the medial aspect of the tibia. Those implants were not subject to large mechanical forces from soft tissue impacting the implant.[34]

A sheep model was chosen because of previously successful studies on bone and fracture healing using this animal.[35,36] The chosen model was also a modification of a previously used rabbit model which had a highly mobile skin site at the Bone and Joint Research Lab (SLC, UT) that proved ineffective at keeping the antimicrobial at the wound site due to animal behavior.[26] That rabbit model failed because the rabbit was able to access the tibial pin site notwithstanding the use of Elizabethan collars, plastic caps, and conforming bandages. This implant site access was in contrast to an

effective wound healing rabbit model that implanted the percutaneous device into the back of the animal.[37] That model, although effective at preventing animal disturbance of the implant site, did not allow the desired highly mobile skin site.

The chosen model for this study also needed to show successful infection in the control animals to demonstrate the type of infections currently seen in the osseointegrated amputees[11] as well as provide a challenge for the CSA-13 to act as the primary barrier to infection. In a previously tested rabbit percutaneous model with the implant inserted into the tibia near the ankle joint, only 1 in 10 of the test rabbits experienced an exit-site infection.[38] This low infection rate was improved in the Bone and Joint Research Lab rabbit model, but animal behavior was a detriment which led to the use of the sheep model. The proximal end of the tibia was kept as the surgical implantation site in the chosen sheep model to allow for maximal soft tissue motion around the implant site.

Hypothesis Tested and Rationale

The sheep animal model and medial, proximal tibial site of surgical implantation were chosen based on animal behavior, soft tissue motion, and the ability to test an antimicrobial as a primary barrier to infection. This animal model allowed testing of the following hypothesis:

> A cationic steroidal antimicrobial (CSA-13) as a medical device will prevent pin track infections at a mobile, soft tissue, percutaneous implant site in a sheep model.

CSA-13 was chosen to act as a primary barrier to pin track infection at a percutaneous implant site with surrounding highly mobile soft tissue.

CHAPTER 2

MATERIALS AND METHODS

Implant Design

All implants were titanium alloy (Ti-6Al-4V) and manufactured by the University of Utah School of Medicine Machine Shop.

A pilot study was conducted in two sheep with an implant 120 mm long with 28 mm of threading that transversed both the medial and lateral aspects of the tibia. This model was found to be too aggressive because the skin on the lateral side was excessively mobile causing difficulty in keeping the pads at the wound site. Another problem with the model was that there was wool on the lateral aspect and hair on the medial aspect of the tibia making the comparison unequal. Because of these findings from this pilot study, the implants were shortened and the surgical procedure slightly altered to make the implants protrude from the skin on the medial side only (Figures 2.1 and 2.2). Modified implants were made 95 mm in length with a 5 mm diameter. A 10 mm long, 1 mm deep notch was cut into the metal 5 mm away from the exposed portion of the implant (Figures 2.1 and 2.2). At the opposite end a 28 mm length of threading was located 5 mm from the tip. Implants were passivated using citric and nitric acid, and autoclaved in separate autoclave pouches for sterilization

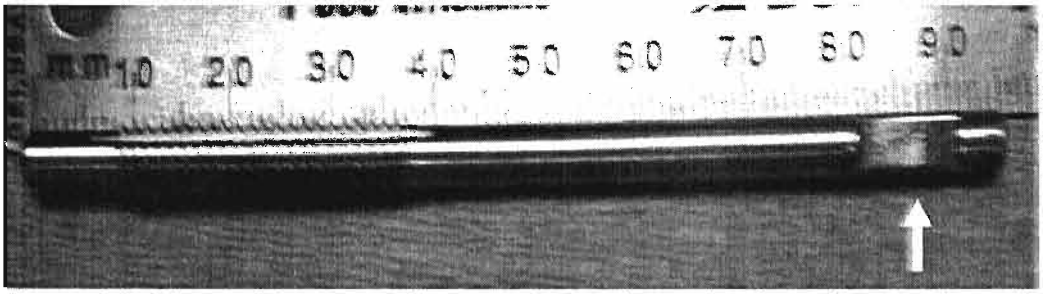


Figure 2.1: Photo of the 5 mm diameter, 95 mm long, titanium alloy implant. The arrow indicates the notch preventing the Jurgen ball from being displaced. The Jurgen ball assured the pad was in contact with the skin-implant interface.

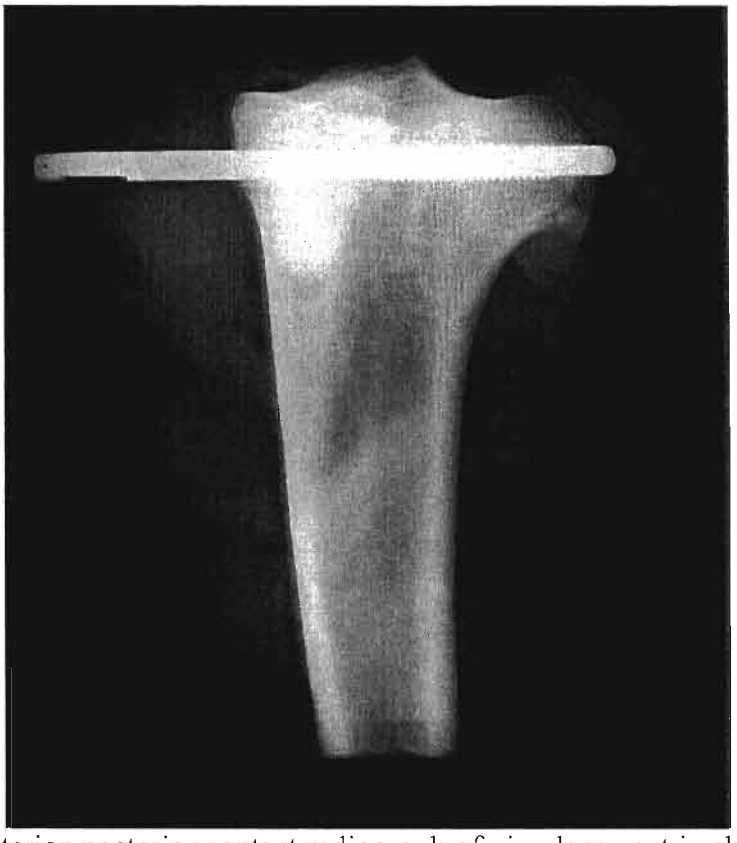


Figure 2.2: Anterior-posterior contact radiograph of pin placement in sheep tibia. Notice the medial tibial entry point at the medial-coronal portion and approximately one cm distal to the knee joint line.

Experimental Groups and Infection Prevention

All animal protocols and amendments were reviewed and approved by the Salt Lake City, Utah, Veterans Affairs Institutional Animal Care and Use Committee (IACUC) and the University of Utah IACUC in accordance with NIH guidelines.

Twenty nonpregnant adult Rambouillet ewe sheep, ranging from 2.5 – 6 years old with weights of 75-90 kg, were used in this study. The sheep were randomized into two groups, one consisting of 10 untreated baseline controls and the other the 10 test sheep. Approximately 25 mm outer diameter, 4 mm inner diameter, 2-5 mm width polyurethane foam pads (Rynel, Inc., Wiscasset, ME, USA) were prepared as a vehicle for the cationic steroidal antimicrobial (CSA-13)-polyurethane polymer conjugate (Cerashield™, Ceragenix Pharmaceuticals, Inc., Denver, CO). The polyurethane used to conjugate with the CSA-13 had a high acid number (19) (AST Products, Inc., Billerica, MA) in contrast with the polyurethane foam pads used in both groups which were not acidic. These pads had a central circular hole that allowed the pad to fit tightly over the implant and cover the skin-implant interface. Ten sheep were treated with the CSA-13-polymer conjugate that was coated onto the polyurethane foam pads. Ten control baseline sheep received autoclaved polyurethane foam pads without the CSA-13-polymer conjugate. CSA-polyurethane polymer coated pads and the control pads remained at the implant site for the length of the study and were changed at least weekly or more frequently in the first days of the study if purulent discharge, blood and/or exudate were present.

Surgical Preparation

The animals were fasted for approximately 12 hours prior to surgery and given an oral bolus of Tetracycline (0.5 gram-1.5 gram 12 hours preoperatively and 0.5 gram – 1 gram 1 hour before surgery) to reduce rumen bacterial activity. An IV catheter was placed in the jugular vein and secured. The anesthesia induction dose was calculated using the animals' receipt weight.

Baseline nose, throat and skin swabs were obtained for culture using standard microbiological procedures. This was to determine the normal flora of the sheep at time zero. Animals were initially anesthetized with an intravenous injection of diazepam (0.1-0.5 mg/kg) and an IM injection of ketamine hydrochloride (4.4-7.5 mg/kg to effect). They were intubated and maintained under anesthesia with isoflurane (0.5-5% to effect) in oxygen delivered with a rebreathing anesthesia circuit. A rumen tube was placed as needed to control regurgitation. Lactated Ringer's solution was administered via the IV catheter throughout the procedure at a rate of approximately 15 ml/kg/hr.

The hind leg and area over the sacrum and the dorsal aspect of the neck was close-shaved. A 50 μg Fentanyl patch was placed on the neck and a bandage placed over it to protect the patch. The animal was positioned in ventral recumbence and the area over the sacrum was prepped with betadine scrub, alcohol and betadine solution. Morphine (0.1 mg/kg not to exceed 10 mg total dose) was given as an epidural to provide post operative analgesia. The animal was then positioned on a warm water recirculating blanket in lateral recumbence with the operative limb to up. Anesthesia monitoring included: respiratory rate, tidal volume, end tidal CO_2, heart rate, and

oxygen saturation. The animal was prepped for surgery by scrubbing from the surgical site outward with a betadine scrub followed by a 70% alcohol wipe. This was repeated three times with a brief dry time and final spray of betadine solution.

Surgical Technique

Once the animal reached a surgical level of anesthesia, the surgical procedure began. Because the skin at the level of the sheep's knee is highly mobile, the site of the medial skin wound was determined to allow the skin tension on the implant, once inserted, not to be excessive when the limb was in full flexion or extension and minimal when the limb was in a normal standing at rest position. An appropriate medial tibial entry point at the medial-coronal portion and approximately one cm distal to the knee joint line was determined (Figure 2.2). A 2.5 mm guide wire was then driven across the tibia and pierced the lateral cortex in the coronal plane. Care was taken to avoid the *digital extensor foramen*. Accurate implant placement was verified with a mobile C-Arm image intensifier (Series 9800^{TM} Mobile C-Arm, 1k x 1k Mobile Workstation, OEC Medical Systems, Inc., Salt Lake City, UT).

A #15 blade scalpel was then used to enlarge the medial incision to accept a 5-mm cannulated reamer which reamed across both cortices. During reaming the skin surrounding the incision was secured using a drill sleeve (Figure 2.3).

The implant was then drilled across both cortices with the lateral blunt end of the implant extending only 3 or 4 mm beyond the bone and buried in the lateral soft tissues. The medial end of the implant protruded approximately 1.5 – 3 cm outside the skin and allowed for attachment of the control and antimicrobial pads.

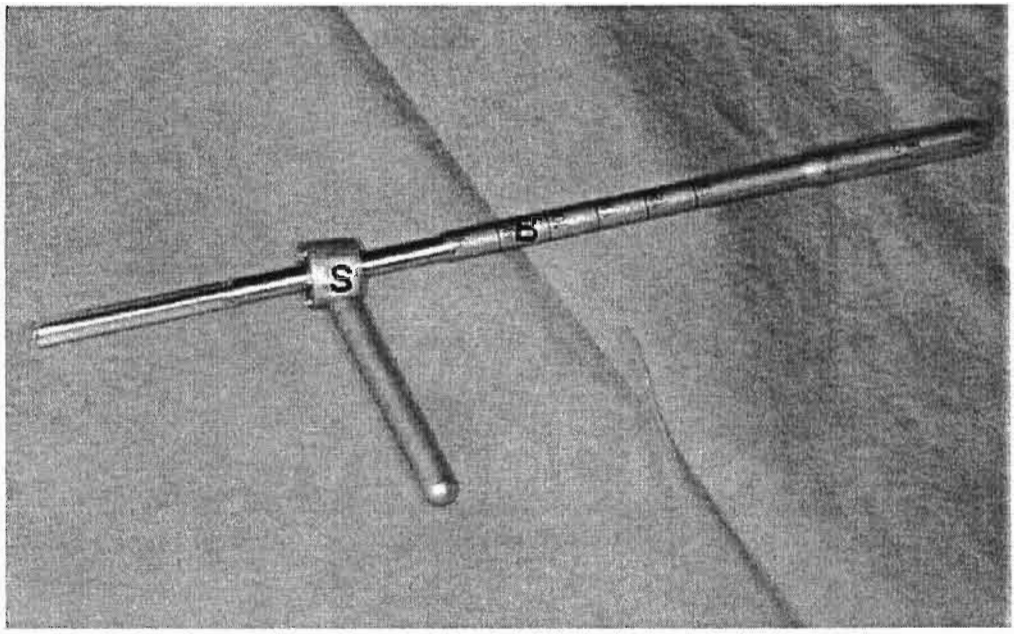


Figure 2.3: Drill sleeve (S) and cannulated drill bit (B) used during surgery.

Depending on the group to which the sheep was randomly assigned, CSA-polyurethane polymer coated or uncoated control, sterile blank polyurethane foam pads were placed at the medial implant-skin interface. Two CSA-13-polyurethane conjugate or two control pads stacked on top of each other were placed with one pad being in direct contact with the skin-implant interface (Figure 2.4). The initial removal torque was measured using a torque indicator (Dillon Quantrol^{TM} AFTI Advanced Force/Torque Indicator, Meldrom Scale Co., UT). The torque indicator was set to measure data in a counterclockwise fashion. A silicon/Teflon washer was placed on top of the pads. To prevent irritation of the sheep apposing flank by the implant rubbing against it, a modified Jurgen ball was placed and tightened into the notch machined into the pin with its top flush with the tip of the implant (Figure 2.4).

Anesthesia was discontinued and the animal was recovered from anesthesia. The animal was placed in its cage after it was determined that it was breathing on its

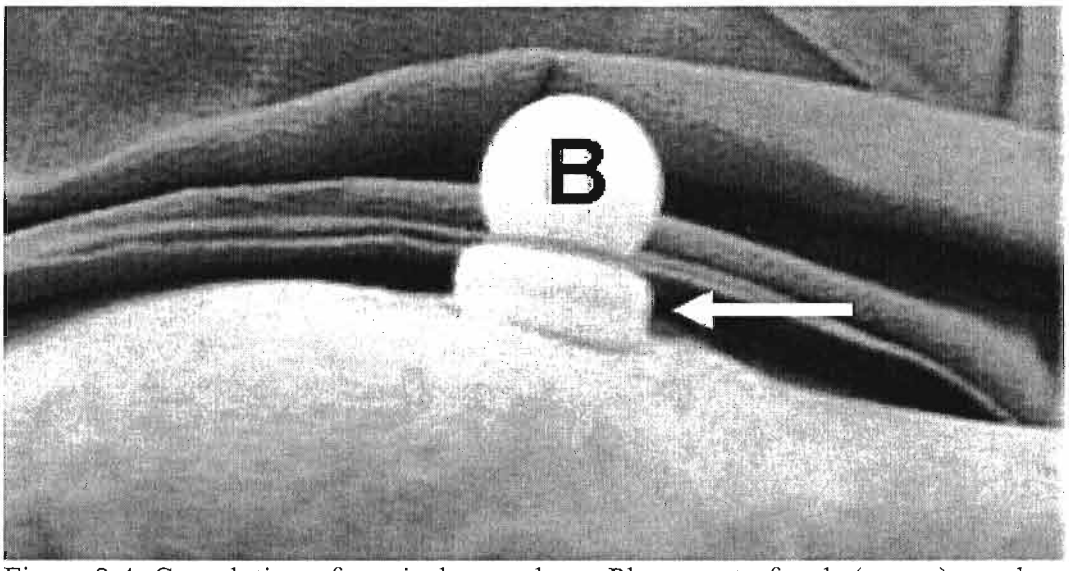


Figure 2.4: Completion of surgical procedure. Placement of pads (arrow), washer, and modified Jurgen ball (B) for maintaining pad contact at the skin-implant interface.

own. Recovery was monitored until the animal was able to stand unassisted. Feed was returned at this time.

Flunixin (1.1-2.2 mg/kg) and Ceftifur (Excenel) (1.1 – 2.2 mg/kg) were administered daily IM for 48-96 hours postoperatively.

Pad Exchange Protocol

During pad exchange, the modified Jurgen ball and silicon/Teflon washer were removed and wiped with isopropyl alcohol. To reduce the risk of contamination, when a new pad was placed at the skin-implant interface, the implant was wiped with Betadine followed by alcohol. Care was taken not to allow the Betadine or alcohol to contact the wound site, preventing an additional variable of antimicrobial treatment. Pads were aseptically removed and a swab was taken of the wound site for bacterial growth determination. The pads were placed and the swabs streaked onto Columbia blood agar (Hardy Diagnostics, Santa Maria, CA) and left overnight at 37° C for

observation the following day. The wound site was gently irrigated with 20-30 ml of sterile saline and dabbed with sterile gauze which removed varying quantities of dried exudates. Two new pads (CSA-13-polymer conjugate coated or sterile, depending on the group) were placed over the implant and lightly pressed against the wound site followed by a clean silicon/Teflon ring and Jurgen ball.

Euthanasia Criteria

The study team observed the sheep daily for general health and signs of infection. Euthanasia was performed when the implant site had Grade II clinical signs of infection as described by Checketts et al.:[39] 1. Redness of skin, 2. Discharge from the implant site, and 3. Pain and tenderness in soft tissues or showed Grade I[39] clinical signs of infection as demonstrated by slight redness around the implant with slight discharge with one or more of the following: 1. Appetite suppression, 2. Limited water consumption, 3. Lethargy, 4. Distress/limping, and/or 5. Pain and tenderness at the implant site.

If the animal had one or more of the previous and/or unforeseen complications with the model design such as implant loosening, the animal was also euthanized.

Microbiologic Cultures

Immediately prior to euthanasia the sheep was calmed by an intravenous injection of 5 ml of Ketamine. Betadine and alcohol were applied liberally at the jugular vein site. Approximately 5 cc of blood were drawn aseptically and transferred to an aerobic blood culture bottle (BD BACTEC™ Plus Aerobic/F Medium, 50/sp, catalog # 442192, Franklin Lakes, NJ). Euthanasia was then performed by an IV

injection of Beuthanasia D at approximately 1 mL per 10 lbs of body weight. The pads, either control or CSA-13, were removed from the implant site and a swab for culture was taken. A swab distal to the implant site was likewise taken to determine if normal flora bacteria were contributors to the infection. The implant site and surrounding areas were then clipped and sterilized using Betadine/alcohol.

A 7 mm biopsy punch was used to take a tissue sample 5 mm away from the implant insertion site (to keep the tissue-implant interface intact for light microscope analysis and histology) and through sterile skin. This allowed evaluation of the tissues deep into the implant insertion site for infection. Tissue was placed in Fastidious Broth (Hardy Diagnostics, catalog #K31, item #15923, Santa Maria, CA). The pin was wiped clean with alcohol and an extraction torque measurement was obtained using the torque indicator. A bone marrow sample was obtained for culture after aseptically removing the lower half of the tibia and swabbing through the medullary canal nearest to the implant insertion site. The limb was disarticulated at the knee joint and placed in formalin for further histological processing. Pads and swabs were cultured on Columbia blood agar overnight at 37° C whereas the blood culture bottle was incubated for 48 hours and subsequently plated on Columbia blood agar overnight at 37° C. The tissue sample in broth was incubated overnight at 37° C then streaked onto Columbia blood agar and incubated overnight at 37° C. The presence or absence of bacteria was observed qualitatively indicating a positive or negative infection. Isolates were permsaved for future identification.

Imaging

Gross photos were taken of all implant-containing specimens. Radiographs were then taken at 70 kV for 90 seconds on AGFA Scopix CR5B Electronic Imaging Film (AGFA HealthCare, Branchburg, NJ), using the Faxitron Cabinet X-Ray System Model 43855A (Faxitron X-RAY LLC, Wheeling, IL), and AGFA CP 1000 X-ray Film Processor (AGFA HealthCare, Branchburg, NJ) (Figure 2.2).

Histology

Tissue samples were obtained using a scalpel from an area directly superior and inferior to the implant site. These samples were dehydrated using a Vacuum Infiltration Processor (Tissue Tek Vacuum Infiltration Process, Miles Scientific, Elkhart, IN) and embedded in paraffin (Surgipath Medical Industries, Inc., Richmond, IL) using the Histocentre 2 embedding center (Thermo Shandon, UK). They were then sliced to 5 μm using a Reichert-Jung (Leica) 2050 Microtome (Leica Microsystems Inc., Bannockburn, IL) with Accu-Edge® Low Profile Blades (Sakura Finetek U.S.A., Inc., Torrance, CA). At least three slices were obtained from each sample. The slices were then placed on slides and cover slipped for staining.

Bone samples were obtained by using an 8 mm outside diameter (6 mm inside diameter) screw extracting bit in conjunction with a United Heavy Duty Drill Press Model No. 810 (United by New Corp, Las Vegas, NV). Two samples were taken on the superior side on the medial and lateral aspects and one sample was taken on the inferior side.

The bone samples were decalcified, dehydrated, and embedded in paraffin. They were then cut to 5 μm widths using a microtome and cover slipped for staining.

Stains performed on both bone and tissue samples were the Brown-Brenn modified gram stain, Peroidic Acid-Schiff (PAS), and Hematoxylin and Eosin.[26] The Brown-Brenn stain was used to detect the presence of bacteria. The periodic acid-Schiff stain was used to detect the presence of fungus and the H&E stain was used for observing inflammation and fibrosis. The H&E stain was performed using a Microm DS 50 Slide Stainer (Richard-Allan Scientific, Kalamazoo, MI). The Brown-Brenn stain and PAS stain were performed at ARUP Laboratories (Salt Lake City, UT). All histological analyses were performed by a board-certified pathologist at ARUP, while blinded to the study groups.

Methyl Methacrylate Embedment and Contact Radiography

Specimens containing implants were cut superior and inferior to the implant approximately 2-5 cm near the implant using a water saw (Marmed Inc., Cleveland, OH). These cut specimens were then dehydrated using a Vacuum Infiltration Processor (Tissue Tek Vacuum Infiltration Process, Miles Scientific, Elkhart, IN) set on a 72-hour cycle that consecutively soaked the specimens in 70% Ethanol, 80% Ethanol, 95% Ethanol, 100% Ethanol, and the clearing reagent Xylene (Richard-Allan Scientific, Kalamazoo, MI). These specimens were then placed in a container of a freshly prepared solution (Solution A) containing 800 mL methyl methacrylate (MMA) (Sigma-Aldrich Co., St. Louis, MO) and 200 mL n-Butyl Thalate (Sigma-Aldrich Co., St. Louis, MO) with constant stirring at room temperature. Specimens were left in Solution A for at least 5 and no more than 10 days. Specimens were then transferred to a freshly prepared Solution B (800 mL MMA, 200 mL n-Butyl Thalate, 2.5 gm Perkadox 16 (Akzo Nobel, Dayton, OH). These specimens in a container

containing Solution B were then placed in a vacuum desiccator refrigerated at approximately 4°C and left for at least 7 and not more than 10 days. The specimens were then removed from refrigerations and allowed to equilibrate to room temperature. They were then immersed in a container containing Solution C (800 mL MMA, 200 mL N-Butyl Thalate, 5 gm Perkadox 16). The specimens were then placed back into the vacuum desiccator at 4°C for at least nine but not more than 10 days. The specimens were then removed from the vacuum desiccator and refrigeration and allowed to equilibrate to room temperature. Solution C was prepared and specimens were placed in a polypropylene container with a pre-polymerized layer. Fresh Solution C was poured into the container to create a 1-3 cm layer which was allowed to polymerize under UV light at room temperature under a fume for 1-2 days. One to three cm layers were then consistently added until the specimen was completely embedded with a 1-3 cm layer covering the top of the specimen.[40]

Individual specimens were then cut with an industrial vertical band saw (Model 20, Rockwell International, Pittsburg, PA) to remove excess MMA and reduce the size of the specimen for grinding.

Specimens were then ground on an 8-inch Buelher Polimet 1 Polisher (Buehler Ltd, Lake Bluff, IL) using a 60 grit grinding paper (Silicon Carbide Wet/Dry C Weight, Leco Corp., St. Joseph, MI) and grinding until the implant width was fully visible. A slow, continuous drip of tap water onto the grinding wheel was maintained during grinding.

These sections (now between 3-5 mm thick) were then radiographed at 70 kV for 35 seconds on AGFA Scopix CR5B Electronic Imaging Film (AGFA HealthCare, Branchburg, NJ), using the Faxitron Cabinet X-Ray System Model 43855A (Faxitron X-RAY LLC, Wheeling, IL), and AGFA CP 1000 X-ray Film Processor (AGFA HealthCare, Branchburg, NJ).

Scanning Electron Microscope Imaging and Analysis

Three untreated control specimens and four CSA-13 treated specimens were ground on the 8 inch grinding wheel with a slow continuous drip of tap water. Sixty, 240, 400, and 600 grit papers were used sequentially to achieve a fine finish. The specimens were then polished using a polished cloth attached to the polishing wheel sprayed with 1μm alpha alumina. The slow, continuous drip of tap water was also maintained during polishing. The specimen was polished until the fine grain from the final grit paper was completely removed. The specimen was then rinsed under tap water and dried using a clean cloth (Kimwipe, Kimberly-Clark, Tucson, AZ) resulting in a mirrored finish.

These polished specimens were then sputter coated with carbon for 15 seconds using a carbon coater (Model Number 208, Cressington Scientific Instruments Ltd., Watford, England). The carbon coated specimens were individually placed in a JSM-6100 Scanning Electron Microscope (SEM) (JOEL USA, Inc., Peabody, MA) equipped with a backscattered electron detector (Tetra, Oxford Instruments Ltd, Buckinghamshire, UK) and attached image capture software (Noran System Six, Thermo Scientific, Madison, WI). SEM settings were set at the following: voltage: 20kV, working distance: 15 mm, probe current: -0.9 nA. The

probe current was measured with a SM-16100 probe current detector (JOEL USA, Inc., Peabody, MA) attached to an external picoammeter (Keithley Instruments, Cleveland, OH). Fine alterations in probe current were made frequently throughout image acquisition. To obtain images of a larger portion of the pin-implant interface, probe current was changed to approximately -3.0 nA and working distance was altered to 34 mm at 12x magnification or 39 mm at 10x magnification.

Microscope Analysis

Six 3-5 mm sections (two untreated control and four CSA-13 treated) were glued to plastic slides and ground to 50-70 μm thick specimens. These specimens were then stained with Sanderson's Rapid Bone Stain using an Acid Fuchsin counterstain (Surgipath Medical Industries, Inc., Richmond, IL) and examined under light microscope (Nikon Eclipse E600, Nikon, Japan) with associated camera (Optronics, Goleta, CA) and image capture and processing software (Optronics MagnaFIRE™ SP version 1.0x5, Optronics, Goleta, California).

Appositional Bone Index Measurements

Appositional Bone Index (ABI) measurements[41] were taken to quantify percent of bone in contact with implant upon euthanasia using Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). Measurements of the areas of radiolucencies were also taken and divided by the length of possible bone-implant contact to provide an average width of radiolucency. These average widths of radiolucencies were compared between the untreated control and CSA-13 treated groups also using the Image Pro Plus software.

Statistics

A log-rank test for equality of survivor functions was used to compare infection rates between the two study groups. A p-value of ≤ 0.05 was considered statistically significant. Sheep were classified as infected if they demonstrated two or all three of the following: 1) Clinical signs greater than a Grade I infection as demonstrated by slight redness around the implant with slight discharge.[39] 2) Positive culture results for blood, soft tissue, and/or bone samples taken at euthanasia, and/or 3) Positive bone and/or soft tissue histology results of samples processed after euthanasia. Kaplan-Meier survivorship curves were used to display these time to infection rates. The Fisher's exact test was used to compare clinical implant loosening and the Wilcoxon-Mann-Whitney test was used to compare the Appositional Bone Index (ABI) between the groups. A student t test was used to compare the average widths of radiolucencies between the groups as well as initial fixation torque. An analysis of covariance (ANCOVA) was used to compare final fixation torque.

CHAPTER 3

RESULTS

Clinical Observations

The model was observed to be highly aggressive. The muscle and skin motion of the medial side of the tibia was observed to tear the skin surface and expose the underlying torn proximal ends of the *fibularis tertius* and *extensor digitorum longus* muscles. This led to gaps in the skin tissue ranging from approximately 15 mm to 30 mm around the implant-skin interface, which indicated a lack of skin-implant adhesion (Figure 3.1).

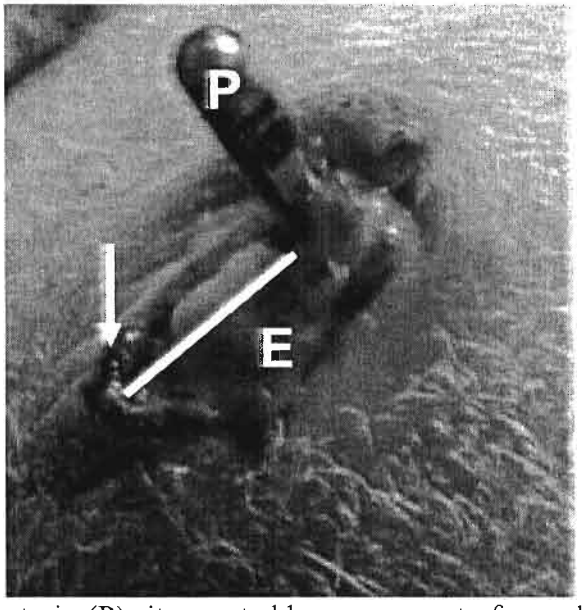


Figure 3.1: Skin gap at pin (P) site created by movement of muscle and skin. Notice the skin boundary (arrow) and exudates (E) surrounding the pin/skin interface. The size of the gap is approximately 24 mm (solid white line).

The wound site was observed to expand approximately 1-7 mm beyond the diameter of the pads in 10% (1 of 10) of CSA-13 treated sheep and 30% (3 of 10) of untreated control sheep. Clinical signs of infection in addition to other signs of distress and model complications observed in the groups included high rates of redness, discharge, necrotic tissue, limping, the pad not covering the wound site, and swelling found in both the CSA-13 treated sheep and the untreated control sheep (Table 3.1).

Infection Rates

Eighty-five percent (17 out of 20) of the sheep had bacteria cultured in the blood, bone, and/or soft tissue samples taken at euthanasia. Of these 17, nine were CSA-13 treated and 8 were untreated controls. The CSA-13 treated group had one positive blood culture, nine positive soft tissue cultures, and zero positive bone cultures. The untreated controls had two positive blood cultures, eight positive soft tissue cultures, and three positive bone cultures.

Eighty-five percent (17 out of 20) of the sheep had positive histology results showing small, gram positive rods indicative of infection. From these positive results, eight CSA-13 treated and nine untreated controls had positive histological results in soft-tissue specimens taken adjacent to the implant sites. One CSA-13 treated and two untreated controls had positive bone histological results from bone samples removed either proximal or distal to the implant.

Upon combining culture, histology, and clinical signs of infection results, it was determined that 95% (19 of 20) of the sheep were infected at euthanasia. Five percent of the sheep (1 of 20, CSA-13 treated) were euthanized due to clinical signs

Table 3.1: Summary of Clinical Observations

Treatment: CSA-13 treated or untreated control	Redness	Discharge / Necrotic tissue	Appetite suppression	Limited water consumption	Swelling / Induration around implant site	Lethargy	Distress / limping	Pain and Tenderness	Pad not cover wound site	Gap in inner circle of pads	Lateral swelling	Implant loose	Bad odor
control	X	X			X								
control	X	X			X		X						X
control	X	X			X		X					X	
control	X	X	X		X	X	X		X		X	X	
control	X	X			X		X		X		X	X	
control	X	X			X							X	
control	X	X	X		X		X				X		
control	X	X			X		X		X				
control							X						
control	X	X			X	X	X						
CSA	X	X			X		X				X	X	X
CSA	X	X					X	X				X	
CSA	X	X			X		X	X			X	X	X
CSA	X	X			X		X			X	X	X	X
CSA	X	X			X		X				X	X	
CSA	X	X			X		X					X	X
CSA	X	X			X				X			X	
CSA	X	X			X		X					X	X
CSA	X	X	X		X		X					X	
CSA	X	X	X	X	X		X				X		X

of infection but were not classified as infected because of negative culture and histology results.

The infection data demonstrated that, when compared to the untreated control pads, the CSA-13 did not prevent pin track infection (p=0.88 with euthanasia as endpoint, p=0.16 with the endpoint being weekly observations conforming to the predefined definition of infection, Figure 3.2).

All sheep were euthanized between 8 and 40 days after surgical implantation due to clinical signs of infection or animal distress. All sacrifices occurred prior to the intended 6-month endpoint.

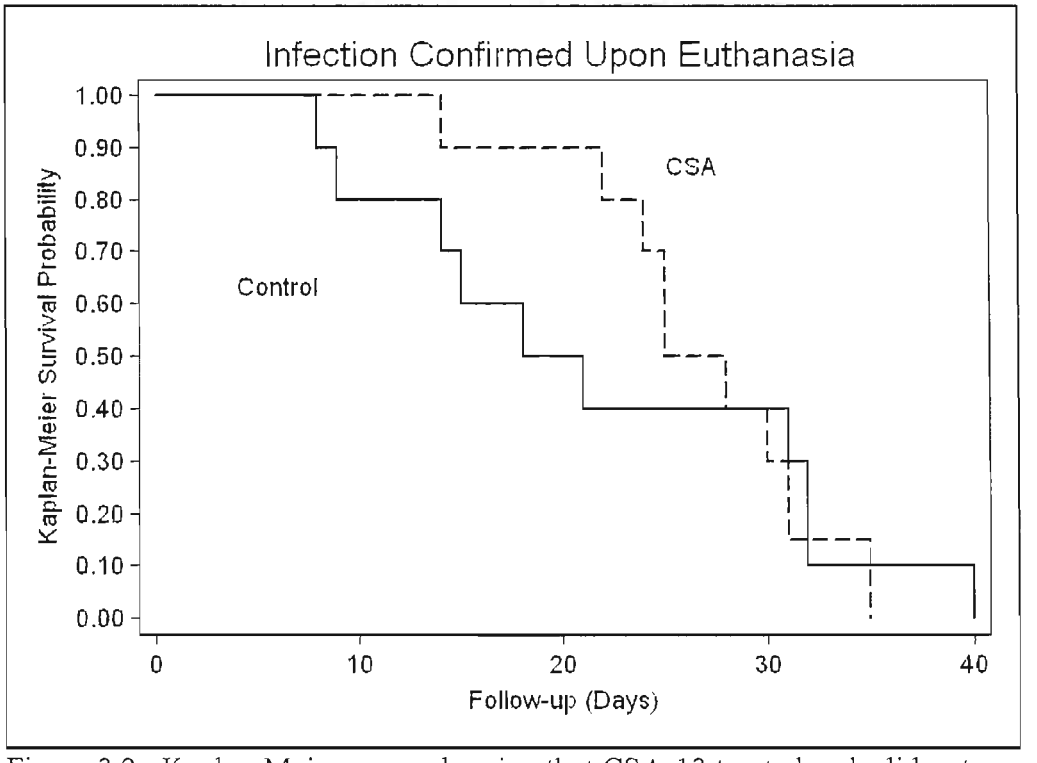


Figure 3.2: Kaplan-Meier curve showing that CSA-13 treated pads did not significantly prevent infection compared to untreated control pads (p=0.88).

Clinical Loosening

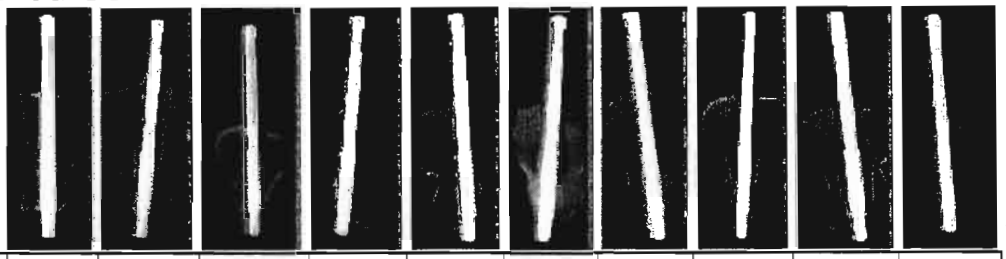
Large skin gaps around the implant indicated a lack of skin-implant contact and extreme soft tissue mobility. Movement of muscle and contact with the apposing flank may have caused loading on the implant and micromotion leading to increased infection rates and implant loosening. The CSA-13 treated group had a higher rate of clinical implant loosening when compared with untreated controls (Fisher's exact test p=0.005). Nine of 10 sheep treated with CSA-13 had clinically loose pins at the time of sacrifice. In contrast, 2 of 10 untreated control sheep had clinically loose pins at time of sacrifice.

Radiographic Loosening

In addition to clinical implant loosening, radiographically determined implant loosening was examined. It was found that the CSA-13 treated group [median (IQR), 1.4 (0, 11.2)] had a higher percentage of radiographic lucency and a lower ABI than the untreated control group [8.6 (0, 41.7)]. The variation in percent bone contact between the CSA-13 treated sheep and the untreated controls was not statistically significant (Wilcoxon-Mann-Whitney test, p = 0.53). This radiographic analysis suggested that both groups had clinically unacceptable radiographic lucencies, indicative of early implant loosening (Figure 3.3).

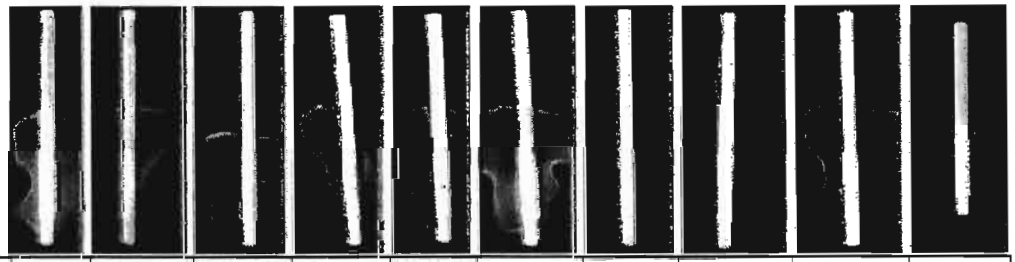
Average width of radiographic lucencies was compared between the two groups and not found to be statistically significant (cranial side radiographic lucency widths, p=0.57; caudal side radiolucency widths, p=0.09). The average radiographic lucency widths from the control group were 1.00 mm (cranial side) and 1.11 mm

Untreated Control



Days Out	8	9	14	15	18	21	31	32	32	40
% Bone Contact	96	0	10	0	7	78	42	0	0	12

CSA-13 Treated



Days Out	14	22	24	25	25	28	30	30	31	35
% Bone Contact	100	0	0	11	0	66	6	0	3	0

Figure 3.3: Contact radiographs of implant-bone interfaces for the 20 sheep. "Days out" signifies days from surgery to euthanasia. "% Bone Contact" is the length of the bone to implant interface along the threaded portion of the pin divided by the total length of the threaded portion of the pin after euthanasia (ABI). The radiographs clearly demonstrate that there was extensive radiographic loosening in both groups.

(caudal side). The average radiographic lucency widths for the CSA-13 treated group were 0.93 mm (cranial side) and 0.87 mm (caudal side). Fibrous tissue was visually observed within these gaps between bone and tissue.

Histological Review

Implant-bone interfaces as well as the area of bone immediately surrounding the interface site were histologically examined. Even after 25 days *in situ*, it was found that bone fragments did not incorporate with the host bone tissue in either the CSA-13 treated group or the untreated control groups at the bone-implant interface. The lack of healing response was in contrast to woven bone formation and remodeling, which were occurring approximately 1-4 millimeters away from the implant site (Figure 3.4).

Further histological examination of the pin-implant interface revealed viable osteocytes within microns of the interface which suggests that the bone was viable but that remodeling at the interface was arrested (Figure 3.5). These viable osteocytes are evidence that lack of bone remodeling at the interface was not due to necrosis of the bone caused by possible bone overheating during implantation.

Fibroblasts and fibrous tissue were observed at the pin-implant interface which demonstrated a foreign body response to the implant (Figure 3.6).

Lack of bone healing response at the implant-bone interface as well as woven bone formation millimeters away from this interface was also found using a scanning electron microscope (SEM) in backscatter electron imaging mode (Figure 3.7).

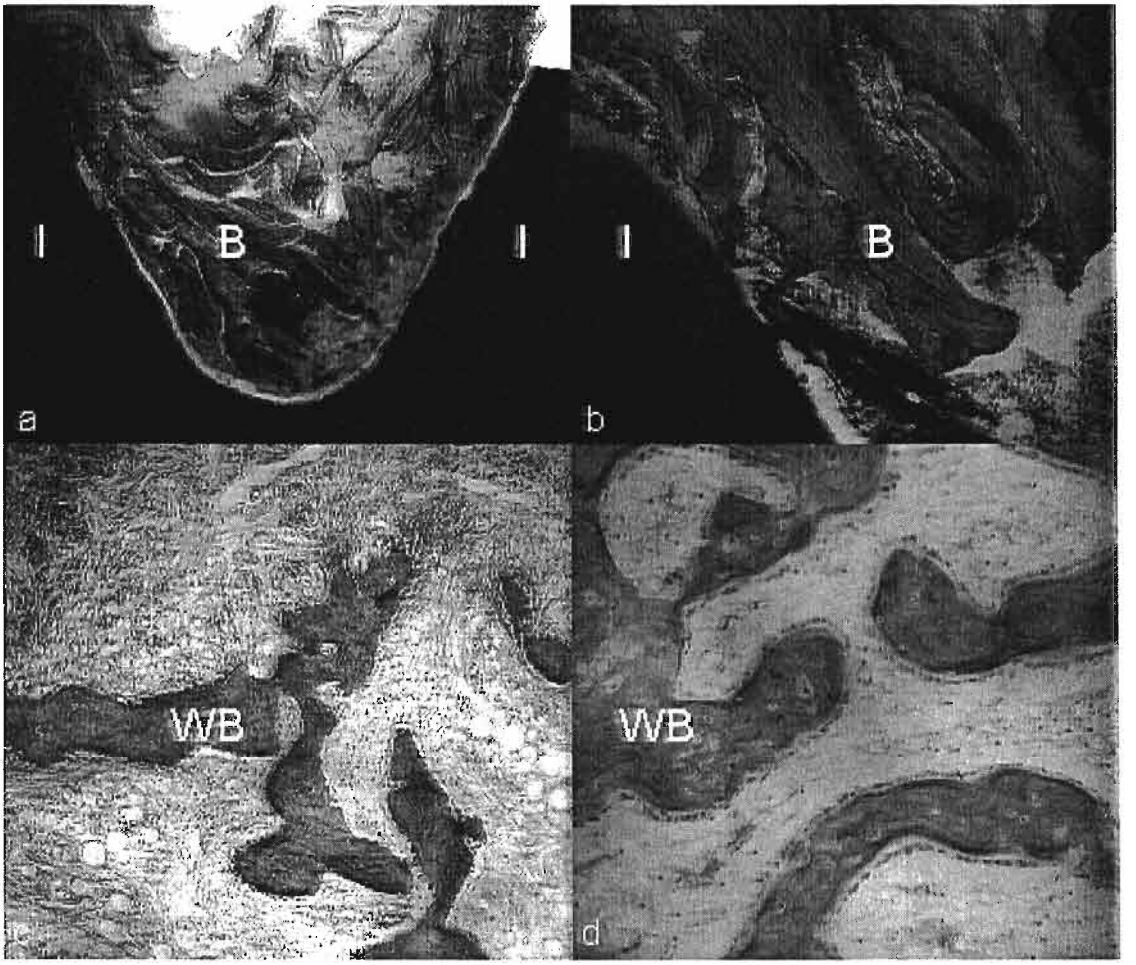


Figure 3.4: Demonstration of lack of healing response at bone-implant interface with healing response approximately 1-4 millimeters away from interface. a) Untreated control, 32 days: Bone(B)-implant(I) interface showing unincorporated bone fragments without remodeling activity. b) CSA-13 treated, 25 days: Bone(B)-implant(I) interface with bone fragments demonstrating no healing or remodeling activity. c) Untreated control, 32 days: Woven bone (WB) formation identified by osteoblast lining around the perimeter of the bone. d) CSA-13 treated, 25 days: Woven bone (WB) being created with osteoid secretion from osteoblasts lining the bone. Slides stained using Sanderson's Rapid Bone Stain. a, b, d original, uncropped images taken at magnification of 200x. c original image taken at magnification of 100x. c and d taken approximately 1-4 mm away from bone-implant interface.

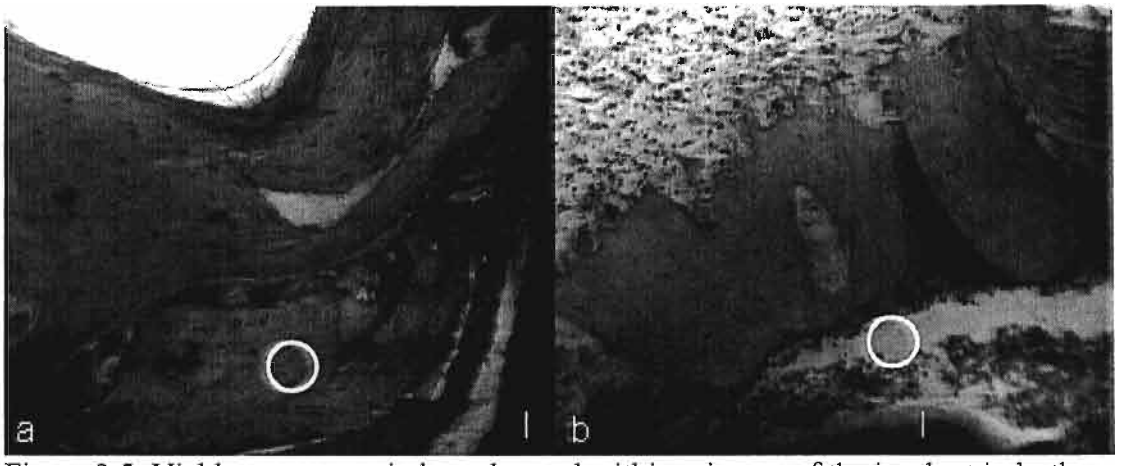


Figure 3.5: Viable osteocytes in bone located within microns of the implant in both untreated control and CSA-13 treated specimens. a) Untreated control, 8 days: Viable osteocytes (example circled) in bone within microns of the implant (I) demonstrating healthy bone at interface near the time of surgical implantation. b) CSA-13 treated, 25 days: Viable osteocytes (example circled) also found within microns of the implant demonstrating that the bone remained healthy. These viable osteocytes demonstrate that the bone near the implant was not excessively heated causing bone necrosis during the surgical procedure. Slides stained using Sanderson's Rapid Bone Stain. Original images taken at magnification of 200x.

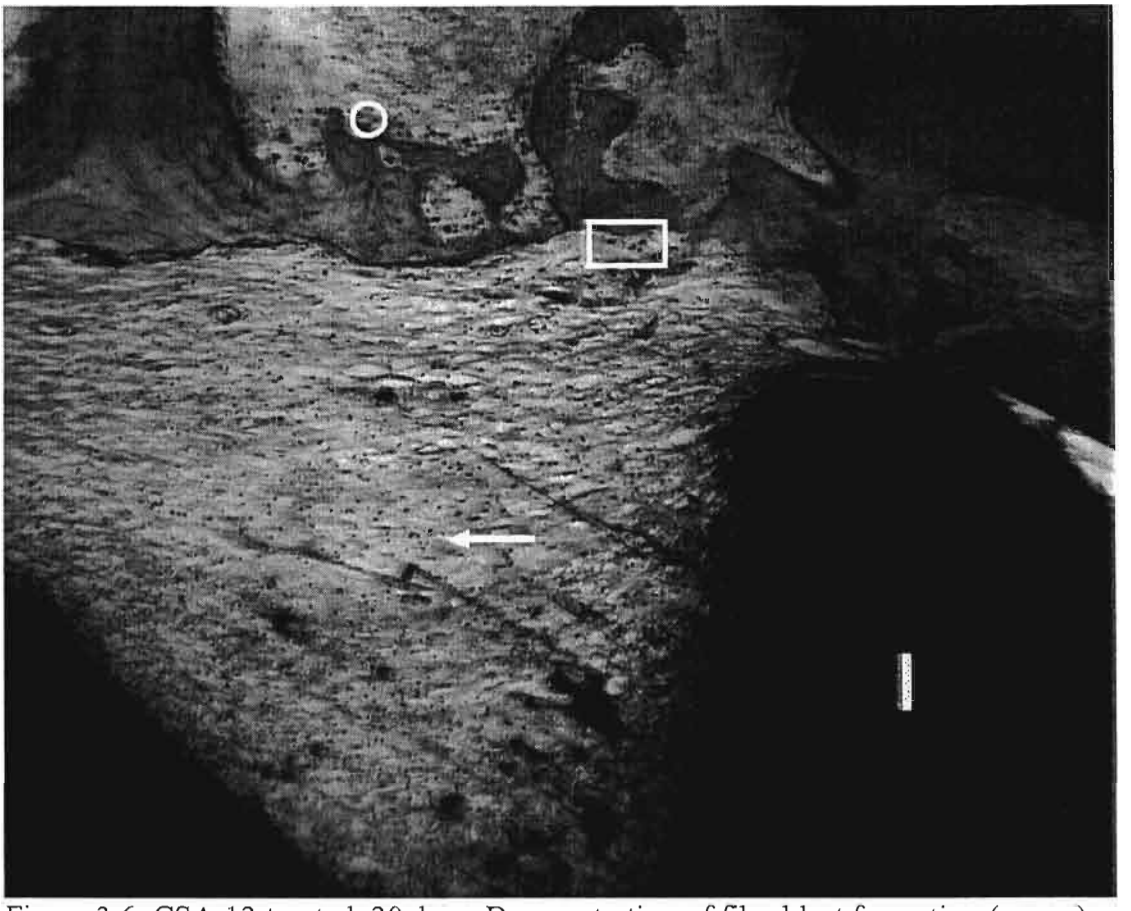


Figure 3.6: CSA-13 treated, 30 days: Demonstration of fibroblast formation (arrow) of fibrous tissue in foreign body response surrounding implant (I). Osteoblasts (examples within circle) and osteoclasts (examples within rectangle) activity also found within one millimeter of the implant but not directly at the interface. Slides stained using Sanderson's Rapid Bone Stain. Original image taken at magnification of 100x.

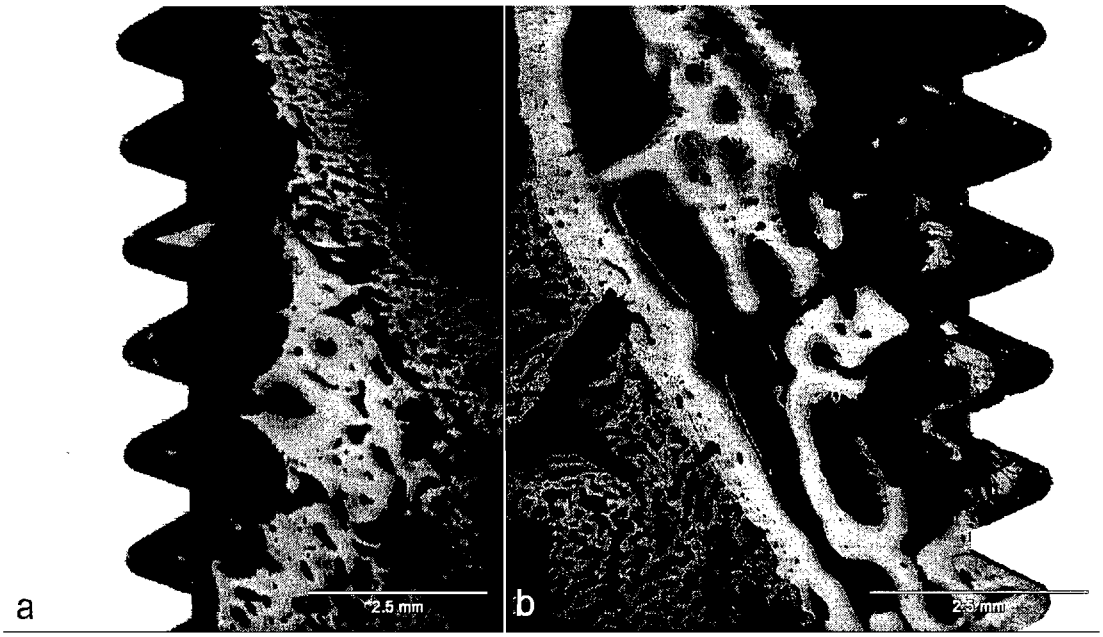


Figure 3.7: Backscatter electron images of bone tissue showing lack of bone healing and/or lack of ongrowth on implant surface. a) CSA-13 treated, 30 days: showing lack of bone contact at bone-implant interface and woven bone formation approximately 2 - 4 mm away from the interface. b) Untreated control, 40 days: showing bone-implant interface with bone fragments with no healing or remodeling activity and woven bone formation approximately 2.5 – 4 mm away.

Torque

The time zero torque values measured for the CSA-13 treated group had a mean of 0.60 ± 0.47 N-m and a median of 0.43 N-m. The time zero torque values measured for the untreated control group had a mean of 1.29 ± 0.60 N-m and a median of 1.37 N-m. There was a statistically significant difference found in torque at time zero between the groups ($p=0.02$, student t test) showing that the untreated control group had a tighter initial fixation immediately following surgery. Controlling for this baseline difference, using an analysis of covariance, there was not a significant difference found in endpoint torque between the groups ($p=0.80$).

CHAPTER 4

DISCUSSION

CSA-13

CSA-13 was not effective in preventing pin track infections along percutaneous implants in this study's sheep model. Because of other factors involved, such as excess amounts of skin and soft tissue motion at the foam pad-implant site interface, this result does not conclusively exclude CSA-13 as a preventative barrier to pin track infections if a stable skin-implant interface could be established.

Goals of Study

The first goal of the study was to develop an animal model that would accurately represent the mobile soft tissue conditions at the distal end of the residual limb of active warrior amputees, unlike the Pendegrass et al. goat model.[34] The second goal was to examine the use of a broad-spectrum antimicrobial as a primary barrier to infection rather than skin immobilization followed by a saline flossing treatment as seen currently in the Branemark model.[10] A third goal was to develop an animal model that would have a strong infection signal, contrasting with the Gerritsen rabbit model which only had a 1 in 10 infection rate,[38] in the control animals to

provide a signal that would possibly model an active amputee with retained residual limb distal soft tissue.

The first goal was achieved as a highly mobile soft tissue interface at the implant site was observed around the percutaneous implant at the proximal end and medial region of the tibia.

The second goal was not achieved, but the results support the idea that antimicrobials could only be used as secondary rather than primary barriers to infection in osseointegration. This leads to a need for further exploration of antimicrobial usage as a secondary barrier to infection. The goal of infection prevention using an antimicrobial as a primary barrier appeared to have been significantly hindered by the challenging animal model. The lack of success of preventing infection without skin immobilization supports the conclusion that soft-tissue motion around the implant, although similar to that seen at the distal end of residual limbs in amputees, must be controlled and/or eliminated as a major operative and implant design component of the infection-prevention strategy before osseointegrated implant technology is introduced as a standard of care.

The third goal was achieved as 10 of 10 untreated control sheep were infected prior to the 6-month predetermined endpoint of the study demonstrating that a strong infection signal was present in this model.

Implant Loosening

The time zero torque values measured were significantly different between the untreated control group and the CSA-13 treated group. The untreated control group had higher time zero torques and thus stronger initial fixation. This stronger initial

fixation could have been a key factor in the delay of clinical pin loosening observed in the untreated control group when compared with the observed clinical loosening in the CSA-13 treated group.

Implant loosening was statistically more clinically apparent in the CSA-13 treated sheep. This could have been caused, along with looser initial fixation in CSA-13 treated group as stated previously, by CSA-13 causing a higher rate of hemolysis along the length of the implant. This increased amount of hemolysis and possible lysis of other cell types could have contributed to possible earlier tissue fibrous capsule formation along the length of the implant that limited bone healing and implant osseointegration. Further investigations are required to confirm this conjecture. This earlier clinical implant loosening initiated sheep blood agar hemolysis studies that demonstrated the α-hemolytic properties of CSA-13 when used at the concentration and in the delivery method chosen for this study. This α-hemolytic response was in comparison with no hemolytic response caused by untreated control polyurethane polymer pads.

Because this earlier loosening in the CSA-13 treated group was observed clinically but was not confirmed radiographically (as shown by radiographic lucencies in both groups and ABI) than the untreated control group, it is proposed that clinical loosening would have occurred with more time in the untreated control group. This concurrent radiographic loosening points to a factor other than CSA-13 as the cause of the observed detrimental effect of the extensive fibrous tissue formation limiting bone healing around the implant and osseointegration.

Another factor that could have contributed to both clinical and radiographic pin loosening was the initial implantation technique which allowed for a 0.5 mm circular gap in the bone on the medial aspect. This was because the threading in this investigational implant design was raised 0.5 mm (Figure 2.1). This raised threading design was unlike the threading chosen by Pendegrass et al. in a goat model which tested for implant fixation through dermal tissue ingrowth which did not report any clinical loosening.[34,42] Because this study's threading was limited to a combination of cancellous bone and cortical fixation on the lateral aspect of the bone (approximate unthreaded portion of pin in tibia: 15-30 mm, length of threading in each pin: 28 mm), 15-30 mm of bone was not in apposition to the implant surface, but allowed for a 0.5 mm gap at the medial cortical bone region (Figure 2.2). This gap could have allowed fluid transport and mechanical loosening from micromotion of the implant when the soft tissue loads were applied during activity. Pressure was also applied to the implant site by the apposing flank when the sheep was resting in both a standing and lying down position. The resulting micromotion of the implant may have also contributed to initial bone resorption, fibrous tissue formation, and implant loosening.[43]

The gap along with skin and soft-tissue motion left a region around the implants where bacteria could track along the implant surface and cause infection. Excessive pin site tissue motion has been stated as one of the main clinical factors contributing to pin track infections.[44] Infection observed in this investigation could have contributed to bone resorption and increased implant loosening. This agrees with findings from a study performed by Mahan et al., which found a direct positive

correlation between loose pins and positive cultures for infectious organisms.[16] In conclusion, the muscle and skin motion observed clinically in the sheep model prevented a strong skin seal from being maintained in either group, could have caused micromotion, and possibly led to increased infection and increased implant loosening.

Skin is an important physical barrier between an organism and its external environment. It also contains a specialized cutaneous immune system that consists of lymphocytes and antigen presenting cells that will generate and support localized immune and inflammatory reactions.[45] Because of these properties, creating and maintaining a strong and viable skin seal around osseointegrated devices is crucial to infection prevention success.

Fibrous tissue formation surrounding the implant that was visually observed for both treatment groups in the sections embedded in methyl methacrylate, as has been previously mentioned, was a possible factor in implant loosening. Hofmann et. al. found that when fibrous tissue was present at the interface between porous-coated devices and cancellous bone in humans, bone remodeling was prevented.[46] Human cancellous bone ingrowth has been found to have a similar growth pattern as ovine cancellous bone formation.[47]

Bone Viability Around Implant

A factor examined histologically was determining the viability of the bone found at the implant-bone interface. Because viable osteocytes in the bone tissue were located within microns of some regions of the implant surface, initial concerns of possible overheating of the bone during the cannulated reaming part of the implantation surgical procedure was not considered a factor in preventing implant

incorporation with the host bone. The bone was found viable near the implant-bone interface where present (Figure 3.5). Because active bone remodeling was not found at these interfaces (Figure 3.4), other factors than bone viability were suspected such as early fibrous tissue formation. Factors in absence of bone remodeling at the bone-implant interface could also include infection and/or tissue motion leading to micromotion along the implant surface.[14,48]

Study Limitations

A previously mentioned study limitation was the raised threading design on the implant.

Sheep Behavior

The animals used were range animals not accustomed to human interaction and being housed indoors. This lack of previous human interaction was a probable cause of aggressive behavior in the animals when inspecting their implantation sites. Aggressive behavior led to animal handling challenges when performing weekly (or more frequent) pad exchanges which appeared to disturb the sheep. The sheep disturbance could have led to more aggressive sheep activity leading to more mechanical loads from the apposing flank of the sheep being placed on the Jurgen ball covering the implant. More mechanical disturbance meant more micromotion along the length of the implant possibly contributing to implant loosening.[48]

This aggressive behavior was not observed in a similar goat study done by Pendegrass et al.[42] A possible explanation for the differences in animal behavior was the Pendegrass et al. model placing the implants more distally at a location containing

a significantly smaller amount of soft tissue between the bone and skin. That placement encountered less mechanical forces being placed on the implants as well as no apposition between the animal flank and the implants. The current investigation's implant placement caused pain and distress that could have been caused by soft tissue shearing against the implant, another possible cause of animal aggressive behavior.

CSA-13 Delivery Device

Another study limitation could have been the delivery method chosen for the CSA-13. As mentioned, this device was a polyurethane foam pad coated with CSA-13 conjugated to a hydrophobic, average MW 80,000, polyurethane with a high acid number (19).[49] This device was tested *in vitro* by immersing the CSA-13-polymer-containing foam disk in 45 mL of phosphate buffered saline (PBS) for 24 hours. The disk was removed and the resulting solution was inoculated with either methicillin-resistant Staphylococcus aureaus or tobramycin-resistant Pseudomonas aeruginosa to give ca. 10^6 CFU/mL. The bactericidal activity was determined at 2, 4, and 6 hours. The ceragenin-polymer releasing foam was placed into a fresh solution of PBS each day for 18 days. Even on the 18th day of resoaking the original ceragenin-polymer releasing foam, the solution inocula were eradicated by 6 hours.[30]

Alone, this *in vitro* testing did not completely depict the type of conditions experienced in the sheep model tested as the CSA-13 treated pad was not immersed in any solution before placement at the wound site. Thus the CSA-13 device was placed on blood agar, having been spread with a 0.5 McFarland standard of *Staphylococcus aureus* ATCC 49230, in a Kirby-Bauer-like fashion to perhaps more closely model the experimental situation. The CSA-13 device created a kill zone of ~5mm from the

outer edge of the pad. These results combined with the published *in vitro* results indicated that CSA-13 is effective against *S. aureus* and other common clinical pathogens.

A condition observed *in vivo* that did not exist *in vitro* was exudate leaving the fresh implantation site. The exudate included blood and serous discharge in the first few days post-surgery, followed by necrotic tissue and pus as infection developed. This exudate soaked into the CSA-13 treated pad. The exudate hardened and formed a crust. This may have precluded the CSA-13 from diffusing into the wound site, when the intended delivery method, according to the previously stated *in vitro* testing, would deliver CSA-13 in the presence of moisture for proper treatment. Thus, effective delivery of the antimicrobial may have been compromised and efficacy not accurately represented. In addition, the motion of the soft tissues in the proximal ends of the *fibularis tertius* and *extensor digitorum longus* muscles at the implant site pressed and withdrew against the skin-pad interface. This may have also led to prevention of constant and complete contact between the delivery device and the skin-implant interface.

These delivery device limitations, probably compounded by the rigorous *in vivo* model, suggest that CSA-13 should not be discounted as an effective bactericidal treatment until tested in a less rigorous *in vivo* model. Such experimentation could allow for a more complete understanding of CSA-13's infection prevention qualities in osseointegrated implants.

Conclusions

The data suggests that implant-skin attachment was essential before antimicrobials could be determined efficacious. Aggressive soft tissue motion and animal behavior may result in an over-challenging environment. This study reinforced the importance of limiting soft tissue motion around percutaneous devices in order to prevent infection and micromotion leading to implant loosening. This conclusion is in agreement with that made by Pendegrass et al. as determined in a goat model[34] as well as Branemark et al., as shown in human studies.[33] Following this dermal attachment to the implant to limit soft tissue motion around osseointegrated implants, antimicrobials may be used as secondary barriers to prevent infection.

Future Studies

Possible future studies include testing for percutaneous implant dermal attachment in a less-rigorous animal model. Following this dermal attachment success, CSA-13 in alternative delivery forms as well as other antimicrobials may be tested as secondary barriers to infection in osseointegrated implants. This will be used to determine possible antimicrobial effects on the skin seal around percutaneous implants as well as sound human skin. A one stage osseointegration procedure could be examined and multiple implant designs could be compared including microscopic porous coating, threaded, and macroscopic porous coating.

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