We address the clinical problem of long-bone fracture healing in the presence of osteomyelitis, where the implant provides necessary stability to the bone and soft tissues, but also is a substrate for bacterial biofilm formation and maintenance of infection. The ability to prevent or lessen the acuity of staphylococcal infections (here, using cost effective silver coating) could advance treatment by reducing the substantial patient morbidity and health system financial burdens. Furthermore, it could increase indications for IM nailing instead of external fixation, reducing the substantial patient morbidity and health system financial burdens. Furthermore, it could increase indications for IM nailing instead of external fixation, reducing morbidity and easing rehabilitation. The primary questions of this proposal are:

1) Does silver coating on a stainless steel nail lessen the acuity of, or prevent acute infection or biofilm? Does systemic antibiotic therapy further lessen the acuity of, or prevent infection?

2) Does the silver coating decrease the number of adhered bacteria on the nail surface?

3) Does the silver coating remain adhered to the nail surface?

In previous work we optimized the silver coating technique, and established the in vivo fracture infection model in the rat. Our model consistently produced osteomyelitis, did not have systemic effects and used a relatively small inoculum (10^4 CFU) of bacteria. Fracture healing is evaluated radiographically, mechanically and histomorphometrically and bacteria/biofilm are quantified via microtiter technique. Here we evaluate silver nails with biofilm producing S. aureus. Non-infected control provides baseline and identifies effects of the silver coating. S. aureus group identifies effects due to bacteria. Systemic antibiotic (current treatment) will identify any synergistic role with the effect of silver coating.
**A) SCIENTIFIC AIMS:** (400 words)

The clinical problem that this study addresses is long-bone fracture healing in the presence of osteomyelitis (e.g. tibia fracture, right). Demographic information and burden of disease are addressed in Section B. Short-term questions (and related hypotheses) posed for this proposal are listed below, along with follow-up long-term questions for further research.

**Short-term questions:**

1. In an animal model of intramedullary fixation, does silver coating on a stainless steel nail lessen the acuity of, or prevent acute infection, based on indices of bone healing (radiographic, mechanical), measures of inflammation (histological, serological), and quantitative bacterial cultures? Does the addition of systemic antibiotic therapy further lessen the acuity of, or prevent infection?

   **Hypothesis 1.1:** Stainless steel (SS) intramedullary nails with silver coating will lessen the acuity of (or prevent) *Staphylococcus* infection, compared to a non-coated SS nail used in the same model.

   **Hypothesis 1.2:** Systemic antibiotic therapy in conjunction with the silver-coated IM nail further lessens acuity of (or prevents) infection.

2. Does the silver coating decrease the number of adhered bacteria present on the nail surface?

   **Hypothesis 2.1:** The number of adhered bacteria is reduced on the surface of a silver-coated SS IM nail, compared to an uncoated SS IM nail.

   **Hypothesis 2.2:** The number of adhered bacteria is further reduced with addition of systemic antibiotic therapy.

3. Does the silver coating remain adhered to the surface of the SS IM nail?

   **Hypothesis 3.1:** Following press-fit insertion, the silver coating will exhibit >10% surface area of partial coating removal.

**Long term questions -- evaluated in future investigations for which this provides preliminary work --**

1. Does silver coating on a Titanium nail have the same effect as on a Stainless Steel nail?

   **Rationale** – Many IM nails used in the US are manufactured from Ti, and decreased bacterial adhesion to Ti has been suggested in research studies. Once we have characterized bacterial adhesion and infection in this model using standard SS nails, it will be possible to expand the same model to the study of different implant substrates in order to identify whether different metals influence infection, as has been suggested by others.

2. Does silver coating have the ability to prevent chronic infection or play a role in the treatment of hematogenous infection?

   **Rationale** – The current model mimics an acute infection. The ability to prevent worsening of a chronic infection and/or cure it, with silver-coating of implants +/- systemic antibiotic therapy will provide more information on the utility of silver coating in other clinically relevant scenarios.
B) BACKGROUND AND SIGNIFICANCE (400 words)

Biomaterial-associated infection following internal fixation of fractures remains a vexing clinical problem. In such a circumstance, the implant not only provides necessary stability to the bone and soft tissues, but also is a substrate for bacterial biofilm formation\cite{1,16,17,19,52} and maintenance of infection. Such infections begin with exposure of the implant’s surface to bacteria capable of adhering to and colonizing the implant surface\cite{17,52}. Any mechanism that disrupts the initial adherence and colonization of implant surfaces by bacteria could potentially reduce these infections, and prevent the problems that arise when a necessary implant has to be removed because of otherwise untreatable infection\cite{6,24,33}.

The SIGN Online Surgical Database\cite{15} (SOSD, signfracturecare.org) catalogs over 46,000 intramedullary nail operations carried out in the developing world since 2003. Young et al.\cite{56} reports follow-up for 10,684 fractures in the SOSD database (of which 17% were open). Infection rates were 3.2% for femoral, 2.9% for humeral, and 6.9% for tibial fractures. Open fractures had a 3.16 times increased adjusted risk of infection. IM nailings for fractures classified as nonunions had a 2.31 times increased risk of infection. This is consistent with recent military fracture findings\cite{31,32}.

The financial burden associated with the treatment of a wound infection is significant in any setting. Current treatment protocols for these infected fractures are extensive and prolonged\cite{14,39,40,41}. Parenteral multi-drug antibiotic regimens are standard\cite{26,27,28}. In civilian and military trauma, multiple surgical I&D procedures and nail replacement are necessary\cite{5,37,38}. Productivity lost from missed work can be significant. In a health care world where finances and resources are limited, the prevention of infection is of paramount importance. A patient (and family) without an economic safety net can seldom compensate for the prolonged disability that accompanies these infections.

In a recent study of proximal femur fractures in the UK\cite{53}, infection was the most common and costly complication (2.9 increase, not including patient costs for disability), and concluded, “…infection is the single most important complication to avoid.”

The ability to prevent\cite{43} or lessen the acuity of staphylococcal infections\cite{48} (here, using a cost effective and clinically available silver coating) could advance treatment of these fractures and lead to a potentially large reduction in patient morbidity and health system financial burdens worldwide. Furthermore, it could potentially increase indications for IM nailing instead of external fixation, reducing morbidity and easing rehabilitation. This could also lead to studies of chronic infections\cite{44}, other pathogens\cite{10,48}, other orthopaedic implants\cite{30,42} or coatings\cite{36,47,54} and to clinical studies.
C) PREVIOUS WORK DONE ON THE PROJECT (400 words and/or one page)

Our previous work includes optimizing and evaluating the silver coating technique, and by establishing the \textit{in vivo} model to evaluate the silver coating. Details are presented below.

**Silver coating on SS IM nail (AgSS).** Our collaborators (Zirkle, DeVasConCellos, et al., 2012\textsuperscript{20}; SIGN nail) have established the feasibility of silver deposition on stainless steel (SS), its antimicrobial activity, and optimized application method. Silver has been long used to prevent bacterial infection, and has been shown to be well tolerated physiologically\textsuperscript{2,3,7,21,49,50,55}. Due to its multiple mechanisms of antimicrobial activity, silver has not been associated with the development of antibiotic resistance, a matter of much public health concern. These factors, in addition to its wide availability and low cost, have motivated coating of IM nails. In initial optimization work by collaborators\textsuperscript{20} (Figure below), smooth stainless steel surfaces were coated with silver, using electrodepositing technique. Optimization was carried out with regards to time of deposition, concentration of silver in solution, voltage applied, and heat treatment temperature (between 400 and 500 °C) and time. Bactericidal properties were evaluated against \textit{Pseudomonas aeruginosa} in a bioreactor, resulting in a 13-fold reduction in bacteria at 24 hours.

\textit{in vivo model}\textsuperscript{34,45}. Our model of implant-associated osteomyelitis following fracture repair (Robinson et al., 2011\textsuperscript{46}) modifies the established Einhorn closed rat femur fracture model\textsuperscript{8}. Thirty male Sprague–Dawley rats were divided into three groups (Control (A), \textit{Staphylococcus aureus} (B), \textit{S. aureus} + ceftriaxone (C)). The closed femur fracture model stabilized with an intramedullary pin, was combined with inoculation of $10^4$ colony-forming units (CFU) of known biofilm forming strain of \textit{S. aureus}. Radiographs were obtained immediately after surgery and weeks 1, 2, and 3. They were evaluated by individuals blinded to treatment group. At necropsy the CFU of \textit{S. aureus} per femur and pin were determined and synovial tissue and blood were cultured. The fractured femur from two rats in each group was evaluated histologically. A statistically significant difference in the CFU/femur and CFU/pin was found across treatment groups, with the highest CFU in the \textit{S. aureus} group and the lowest in the Control group. Cultures of synovial tissue were positive in 11/19 of inoculated limbs. Osteomyelitis was present both radiographically and histopathologically in both \textit{S. aureus} groups but not in the controls. No rats were systemically ill or had positive blood cultures at the study endpoint.

<table>
<thead>
<tr>
<th>Mean ± SE radiographic score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post-operative</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>\textit{S. aureus}</td>
</tr>
<tr>
<td>\textit{S. aureus} + ceftriaxone</td>
</tr>
</tbody>
</table>

3 week histology | 3 week radiographs | Radiographic score post-op and weeks 1,2,3
D) METHOD (1200 words and/or 4 pages)

This OTA proposal intends to provide clinically useful information in a focused protocol adhering to the limits of the available budget. While providing information of direct clinical relevance, we also have designed this study to serve as a solid base for future studies of different treatments and in larger animals, and serve as preliminary data to contribute to a competitive submission.

To this end, here we propose to evaluate a clinically available antibacterial surface modification (silver) with the same biofilm producing *S. aureus* used in our earlier studies. Use of a non-infected control will provide baseline and identify effects of to the silver coating. *S. aureus* will identify effects due to the bacteria. The addition of current treatment (antibiotic) will identify any synergistic role of systemic antibiotic therapy relative to the effect of the silver coating.

**Silver coating (Zirkle, DeVasConCellos et al., Washington State University)**

As discussed above, extensive preliminary work has been conducted to optimize the practical application of particulate silver to a polished stainless steel surface. In addition to the processing parameters (temperature, duration, etc.), cytotoxicity was evaluated for standard 455 MTT assay. Consistent with previous work, no toxicity was seen in presence of silver coating, and cases of cell proliferation were noted. Furthermore, wettability increased with the coating, and surface energy was increased due to lower contact angle with silver coating. Cell attachment and proliferation increased with the silver coating.

Heat treatment optimization was needed in order to balance the competing goals of (a) stronger binding to the substrate to prevent mechanical removal during handling and use, and (b) weaker binding to increase silver release for antibacterial effect. This was achieved by increasing temperature and decreasing time, or decreasing temperature and increasing time.

Since this necessarily results in the situation where some silver will be removed during handling and sterilization, the strategy adopted has been to deposit more silver than will eventually be needed. In this study, we target initial application of 25% more silver than used in the antibacterial studies.

**Stainless steel pin**

Stainless steel pin used to stabilize fractures. The proximal end is ground (tapered) for more proximal and secure seating in the femur. Pin is solid, without hollow core.

These pins will be provided to Washington State University for coating with particulate silver. They will be returned to MMRF for cleaning, sterilization and use in implantation in the closed, infected and non-infected fractures.

**In vivo experimental model**

The advantages of this model are that it consistently produced osteomyelitis, did not have any obvious systemic effects and used a relatively small inoculum (10^4 CFU) of bacteria that did not produce systemic infection. The radiographic changes; bacterial isolation from infected femurs and implants; and histopathological changes document the reliable development of osteomyelitis after the inoculation with 10^4 CFU of *S. aureus* in this model.
Strain of *S. aureus*: A pathogenic strain of *S. aureus* was used to conduct the experiments establishing the model. This strain was initially obtained from a biofilm-forming joint replacement infection, and is readily identified through low-tech means due to its sensitivity to Penicillin (identified in Figure at right as *S. aureus* MORF; dark staining indicates Biofilm production). While we recognize that the use of a standard ATCC strain would enhance generalizability of the model, we prefer to continue at this stage with our characterized and known biofilm-producing bacteria. This will ensure that we will be efficient in our animal studies, and will not need to include animals for piloting the new strain. Further work can evaluate different bacteria; in our laboratory’s open infected femoral defect model we have evaluated MORF *S. aureus, MORF P. aeruginosa* and MORF *A. baumannii* (not shown here), which can also be used in subsequent work if desired.

Description of closed fracture osteomyelitis model stabilized by internal pin:

**Inoculation of bacteria:** Each rat is anesthetized and monitored for depth of anesthesia. The right femur is aseptically prepared and an approach to the distal femur is made via a medial stifle arthrotomy (Figure A, below). An 18-gauge needle is used to create an entry port into the distal aspect of the medullary canal of the femur and ream the canal for placement of the intramedullary pin. An inoculation dose of 50-μl of bacterial suspension is slowly injected into the medullary cavity via an 18-gauge polypropylene catheter left in place for 2-min following inoculation. In the Control group PBS is injected instead of the bacterial suspension.

**Fracture apparatus:** After the bacteria or PBS is injected, the pin is inserted (narrow portion first) into the medullary canal and seated into the cortical bone in the proximal aspect of the femur. The opening in the distal femur is sealed with bonewax (Ethicon, Somerville, NJ, USA) to prevent leakage of the bacterial inoculum from the medullary canal. The surgical site is lavaged with sterile saline and the soft tissues and skin are closed. A mid-shaft closed fracture of the right femur is then created using a specifically designed fracture apparatus (Figure B below). The femur is radiographed to document the fracture and the rat is recovered from anesthesia. Rats in the *S. aureus* + ceftriaxone group receive ceftriaxone (50-mg/kg) every 24-h, starting 4-h after inoculation, via a subcutaneous injection, for the duration of the study.

**Radiographic assessment of osteomyelitis:** Lateral radiographs of the right hind limb will be obtained postoperatively using a digital dental radiographic system (Scan X Digital Imaging System; Air Techniques, Inc., Melville, NY). Two individuals, blinded to study group, will evaluate the radiographs focusing on three regions of interest (ROI): (a) proximal metaphyseal area where the implant was seated in cortical bone; (b) diaphyseal region involving the site of the fracture; and (c) distal metaphyseal area where access to the medullary canal was made. During each evaluation each radiograph will be assessed based on a system used by Lucke et al. The following radiographic changes were evaluated for each ROI: (a) osteolysis; (b) soft tissue swelling; (c) periosteal reaction; (d) general impression; and (e) deformity. The changes were given a score corresponding to the following scale: 0 - absent; 1 - mild; 2 - moderate; or 3 - severe. For the general impression evaluation a 0 represented a normal appearing femur/fracture and a 3 represented severe changes were present overall. In addition, sequestra formation (f) and spontaneous fracture (g) were evaluated for each femur as a whole and were given a score of 0 - absent or 1 - present. The scores were then summed, with highest possible total score being 47. The score from two evaluators was averaged for statistical evaluation.

**Bacterial growth assessment:** After euthanasia, a sample of synovium will be retrieved from both the operated (right) and unoperated (left) stifle joint from each animal for aerobic culture. The tissue samples will be placed in 10-ml of tryptic soy broth (TSB) and incubated at 37°C for 48-h at which time each sample will be classified as culture positive or negative based on the presence of turbidity in the culture vial. Both femurs will be aseptically retrieved and used for bacterial quantification. The SS and SSAg pins will be aseptically retrieved from the operated femurs prior to...
snap freezing. Under sterile conditions, the pins will be placed in 1.0-ml of sterile, chilled PBS, and then sonicated (67kHz), vortexed, and centrifuged (∼11,000-rpm) to dislodge adhered bacteria. Samples will then be collected for microtiter dilution and the results will be used to calculate the CFU/pin (methods described below). With sterile apparatus, each femur will be snap frozen and ground to a powder. The resulting powder will be suspended in 3.0-ml of chilled TSB, which will be kept on ice until sampled (<10-min) for microtiter dilutions and calculation of the CFU/femur (see methods below). Note that sonicated and vortexed bacteria will be assumed to be due to biofilm, and will provide overall amount.

**Microtiter dilution and viable bacterial counts** Microtiter dilutions will be performed using a modification of a previously described technique. The CFU in each tube will be determined in quadruplicate by aseptically collecting a sample. Tenfold dilutions will be made (10−1 to 10−6) using PBS in 96-well round bottom microtiter plates. Twenty microliters will be collected from each well and streaked across a TSA (Beckton Dickinson Diagnostic Systems, Sparks, MD) plate in a uniform manner. The plates will be incubated aerobically at 37oC for 24-h at which time the number of colonies will be counted. Dilutions with up to 30 colonies present will be used to calculate the median CFU/pin or CFU/femur.

**Histopathologic evaluation** After removal of soft tissues, the operated and intact femurs assigned for histopathological evaluation will be fixed in 10% neutral buffered formalin for 48-h, after which they were transferred to 70% ethanol. After decalcification in 10% EDTA, the femurs will be bisected midsagittally and the implant will be removed. The bisected femurs will then be processed for histology and embedded, longitudinally, with the cut surface down, in paraffin. Two 5-μm-thick sections will be obtained from one block from each femur and will be stained with hematoxylin and eosin. The sections will then be evaluated by a veterinary pathologist blinded to treatment group. Histopathologic descriptions will be provided for each section and then combined to provide a summary description for each of the six groups.

**Experimental Design**

The following treatment groups and outcome measures (described in detail in preliminary work and in this section) are proposed in order to answer the primary questions of this proposal, namely:

1. Does silver coating on a SS nail lessen the acuity of, or prevent acute infection or biofilm? Does the addition of systemic antibiotic therapy further lessen the acuity of, or prevent infection?
2. Does the silver coating decrease the number of adhered bacteria present on the nail surface?
3. Does the silver coating remain adhered to the surface of the SS IM nail?

**Treatment groups:**

<table>
<thead>
<tr>
<th>120 rats – 4 week follow up</th>
<th>Non-infection control</th>
<th>S. aureus</th>
<th>S. aureus + antibiotic (cephtriaxone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>SS+silver (SSAg)</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>
Outcome measures (n=20):
1. Radiographic healing (Faxitron X-rays; n=20) and Mechanical healing (torsional strength; n=8)
2. Measures of inflammation (histological; n=5)
3. Bacterial (biofilm) counts (sonication; n=7, both nail and femur)
4. Evaluation of coating continuity on nail (before and after – use 7 bacterial count nails).

Statistical methods: Data will be evaluated for SS vs. SSAg for (a) infected vs. control and (b) infected vs. infected + antibiotic, using ANOVA methods. Due to unknown variance and difference in means for the SS vs. SSAg nail, power cannot be well estimated. The group sizes for outcome measures are similar to what has been reported in previous work, however, and information gained here will help to ensure adequate power in subsequent work, should we encounter large variances or small differences in means.

Mechanical torsional testing – derive strength, stiffness, energy to failure; destructive (MTS Bionix, MTS, MN).
Bacterial/biofilm recovery for femur and pin. Example from SS pin for control, infected, and infected+antibiotic groups, 3 week follow up.
Radiograph showing bony reaction to infection. The radiographic score is outlined above.

Alternate approaches:
Given the mature stage of preliminary work by the collaborators on this project, the silver coating technique and the osteomyelitis fracture model are felt to be well established and present low risk. MMRF has experience with animal handling and staff orthopaedic surgeons have been trained by Dr. Robinson, who developed the model and is included as Investigator on this proposal. The assessment measures are robust, and have been shown to present significant differences with the sample sizes proposed here. The team is experienced in performing and interpreting these studies, and alternative strategies are presented. The group has conducted evaluations of this sample size in a 12-18 month time frame, so the pace is expected to be reasonable as well. The team expects no obstacles to the successful completion of this work.

VERTEBRATE ANIMALS:
1) The use of animals and the number of animals requested for a project must be justified by the institution. If applicable the grantee must provide IACUC approval, regarding use of and number of animals requested for a project. While in vitro evaluation is essential in the development process, eventually in vivo work is required for any device that has potential for use in humans/animals. Additionally, because osteomyelitis is a clinical entity it must be evaluated in a living model. As such, an effective and reproducible animal model is essential to this in vivo work. Given that our ultimate goal and objectives will be applied to human medicine, the use of the rat is appropriate as they are commonly used as models of disease, particularly in the early stages of concept evaluation. A less sentient animal would not be appropriate. The current application is to use the previously established in vivo infection model using the previously described femur fracture model in rats. The infection model was established and published by veterinarian Duane Robinson, with Joan Bechtold, both of whom are investigators on this proposal.

2) All animals used in research supported by OTA grants must be acquired lawfully and be transported, cared for, treated and used in accordance with existing laws, regulations and guidelines. Decisions as to the kind and sources of animals that are most appropriate for particular studies must be made by scientists and institutions. OTA policy requires that such decisions be subject to institutional and peer review for scientific merit and ethical concerns and that appropriate assurances be given that NIH principles governing the use of animals are followed.
These policies are also in effect at AAALAC accredited animal care facility at MMRF, where the proposed work will be conducted. Institutional peer review is routine for MMRF IACUC approval, and will be obtained prior to commencing any work. Pilot Study conducted under this IACUC approved workplan. Full copy of the plan is available.

**Part A**

### 0. Project Identification and Signatures

**0A. Type of Application:** ☑ New Protocol ☐ 3-year Renewal of IACUC #________

(If this is a 3-year renewal, do not use language referring to the previous protocol or grant in this form.)

- **Anticipated Starting Date:** March 01, 2008

**0B. Project Title:** (Project title must match grant title. If different, also provide grant title)

Determining the optimal dose of bacterial inoculum that will produce an infection in an experimentally induced femoral fracture in rats.

**0C. Is this an Agricultural Project?** (Use of agricultural animals in non-biomedical research) ☐ Yes. ☑ No.

**0D. Principal Investigator** (Must be faculty or academic professional administrative staff)

<table>
<thead>
<tr>
<th>Conzemius, Michael G.</th>
<th>Phone Number: 612-625-3147</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mailing Address:</td>
<td>Pager or Cell Phone Number: 612-538-5680</td>
</tr>
<tr>
<td>Room C339</td>
<td>Fax: 612-624-0751</td>
</tr>
<tr>
<td>1352 Boyd Ave</td>
<td>Email: <a href="mailto:conze012@umn.edu">conze012@umn.edu</a></td>
</tr>
<tr>
<td>Saint Paul, MN 55108</td>
<td>University Department (if applicable): Veterinary Clinical Sciences</td>
</tr>
</tbody>
</table>

**U of M Employee ID:** 3728455

**U of M x 500 ID (ex. smith001):** conze012

**Occupational Position:** ☑ Faculty ☐ Staff (must be P & A) Note: students cannot be principal investigator.

**Principal Investigator Certification:** If the IACUC approves my application, I agree to execute this work as described, request approval from the IACUC for changes, comply with the guidelines set forth by the IACUC and Research Animal Resources (RAR), follow Environmental Health and Safety guidelines, and be responsible for the supervision and work of all staff. If appropriate, this application accurately and completely reflects the animal use in the full grant application. The activities described in this study do not unnecessarily duplicate previous experiments.

**0E. Person preparing this document**

**Original Signature of PI**

**Title of PI**

**Date**

*If PI is not a University of Minnesota faculty member, IACUC may notify you that additional signatures will be required.*

DO NOT SUBMIT WITH APPLICATION
E) REFERENCES (not to exceed 2 pages)


50. Stinner et al. Silver dressings augment the ability of negative pressure wound therapy to reduce bacteria in a contaminated open fracture model. J Trauma (2011) vol. 71 (1 Suppl) pp. S147-50


June 25, 2012

Andrew Schmidt, M.D.
Professor, Department of Orthopaedic Surgery,
Hennepin County Medical Center,
701 Park Avenue South,
Minneapolis, MN  55415

RE:  OTA grant application: Infection prevention in long bone fracture osteomyelitis model treated with IM nail

Dear Dr. Schmidt,

I am writing to confirm my interest in your project evaluating the infection reduction potential for an electrodeposited silver coating on a stainless steel IM nail.

As you know, SIGN Fracture Care has successfully developed stainless steel nails for fracture care worldwide. While the infection rate has been very low, we recognize that infection is a devastating complication. Given the low propensity for silver to lead to bacterial resistance, and to its low cost and available application methods, it appears to be a very viable potential option to counteract infection in fracture settings.

I would like to confirm that SIGN Fracture Care will provide the stainless steel (SS) pins for your proposed study. This includes bare pins and silver coated pins. We understand that the study is designed for 120 nails, and we will provide 20% additional nails from the same batch for contingency and analysis.

Please let me know how else I can be of assistance. Good luck with your proposal.

Sincerely,

Lewis Zinkle
President and Founder

Creating Orthopaedic Equality Since 1999   451 Hills St. Suite B, Richland WA, 99354
USA   http://signfracturecare.org
(P) 509.371.1107
(F) 509.371.1316
<table>
<thead>
<tr>
<th>SALARIES AND WAGES</th>
<th>% Of Time on this project</th>
<th>Requested from OTA Funds (Omit Cents)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tony Meglitsch (Animal care technician)</td>
<td>7.5%</td>
<td>$7,834</td>
</tr>
<tr>
<td>Barb Wicklund (bacteria-obtain and handling)</td>
<td>3.5%</td>
<td>$4,082</td>
</tr>
<tr>
<td>Li Zou, MD (Experimental orthopaedic surgeon)</td>
<td>5.0%</td>
<td>$5,220</td>
</tr>
<tr>
<td>Fringe Benefits 13%, 33.5%, 31% of Salaries and Wages</td>
<td></td>
<td>$4,003</td>
</tr>
<tr>
<td>Salaries and Wages plus Fringe Benefits</td>
<td>TOTAL</td>
<td>$21,139</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PERMANENT EQUIPMENT (Justification to be appended)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N/A</td>
<td>Subtotal</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CONSUMABLE SUPPLIES (Exclude animals and animal care)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>120 IM nails (@ $15/nail)</td>
<td>$1,800</td>
</tr>
<tr>
<td>Silver coating on 60 IM nails</td>
<td>$2,893</td>
</tr>
<tr>
<td>Subtotal</td>
<td>$4,693</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ANIMALS AND ANIMAL CARE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>120 Sprague Dawley rats (300-350g) $48.60/animal</td>
<td>$5,832</td>
</tr>
<tr>
<td>General surgical supplies ($25/animal; incl. anesthesia, antibiotic, analgesia)</td>
<td>$4,500</td>
</tr>
<tr>
<td>Per diem (120 rats x 5 weeks; includes 3-7 days pre-op, 5 days post-op care, and 4 weeks housing-$2.02/day)</td>
<td>$9,696</td>
</tr>
<tr>
<td>Subtotal</td>
<td>$20,028</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Outcome measures (n=20):</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Radiographic healing (plain, microCT) and Mechanical healing (torsional strength; n=8)</td>
<td></td>
</tr>
<tr>
<td>2. Measures of inflammation (histological; n=5)</td>
<td></td>
</tr>
<tr>
<td>3. Bacterial counts (sonication; n=7)</td>
<td></td>
</tr>
<tr>
<td>4. Evaluation of coating continuity on nail (before and after – 7 bacterial count nails).</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ALL OTHER EXPENSES</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology (5 bones x 6 groups x $50/femur)</td>
<td>$1,500</td>
</tr>
<tr>
<td>Mechanical testing (8 bones x 6 groups x $20/bone)</td>
<td>$960</td>
</tr>
<tr>
<td>Biofilm (7 nails x 6 groups x $20/nail)</td>
<td>$840</td>
</tr>
<tr>
<td>Bacterial counts (7 nails x 6 groups x $20/nail)</td>
<td>$840</td>
</tr>
<tr>
<td>Subtotal</td>
<td>$4,140</td>
</tr>
</tbody>
</table>

TOTAL DIRECT COSTS $50,000