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14. ABSTRACT

Four treatments were compared in an acute femur fracture model in swine for their capacity to improve the progress of bone healing and regeneration. The treatments examined were 1) salmon fibrinogen and thrombin, 2) porcine fibrinogen and thrombin, 3) bovine collagen and a commercial FDA approved treatment called CopiOs. The treatments were instilled into the injury site and the bone was stabilized by surgical external fixation. The subject animals were maintained for 3 weeks and examined biweekly using fluoroscopy to visualize the bone healing and Near Infrared Spectroscopy, electric impedance measurement and Doppler ultrasound to assess the healing of the sound tissue. Wound severity was consistent in 29 animals. Cellularity and bone regeneration varied by treatment with CopiOS being the least effective in inducing a growth response and salmon fibrinogen and porcine fibrinogen being the most inductive. Computerized analysis of the CT and histology were consistent with human interpretation. A rat survival mode has been developed to permit longitudinal PET studies.

15. SUBJECT TERMS
Fracture, bone healing, osteogenesis, salmon thrombin, fibrinogen, porcine, rodent, PET, CT

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INTRODUCTION

Penetrating injuries to extremities caused by blast fragmentation include extensive tissue damage accompanied by uncontrolled bleeding and high grade open fractures (Holcomb, Stansbury et al. 2006; Holcomb, McMullin et al. 2007). Bleeding at distal sites is controlled by tourniquet application but proximal wounds (ex. femoral) may be inaccessible. Serious bone injuries can be later treated by autologous bone grafts but bone grafts are subject to the possibility of serious side effects, such as morbidity and infection at the secondary site, pain and limitations for the supply of bone graft material. To attempt to overcome these limitations, new directions in the first response to penetrating wounds are required. Bleeding must be controlled by the direct application of a hemostatic agent deep into the wound at the site of the bleeding. Second, fragmented bone must be stabilized if the limb will be saved (Brandoff, Silber et al. 2008). The approach of this project is to inject fibrin sealants directly into the wound to stop the bleeding and into the bone lesion to provide an environment that will sustain the cellular component of bone and encourage bone regeneration. The objective of this project is to develop a new injectable reagent that can form a matrix at the site of a bone fracture and enhance the preservation of bone tissue following injury. This treatment should permit successful surgical repair and accelerate subsequent healing. Specific parameters that will be examined will be 1) cell viability of bone fragments at the site of injury as well as those embedded in the injected matrix but removed from contact with the primary bone, 2) resistance of the injured bone to necrosis, 3) induction of new bone growth at the site of injury and 4) comparison of rate of bone regeneration in animals treated with different implantable matrix. The material to be tested in this project is a salmon fibrin matrix derived from salmon fibrinogen and thrombin (Wang, Gorlin et al. 2000; Michaud, Wang et al. 2002; Rothwell, Reid et al. 2005).

BODY

The methodology of this project is to create a fracture in the femur of pigs that will replicate the injuries caused by high velocity penetrating projectiles. The fracture is then stabilized by external fixation according to standard practice and the treatment agent is injected into the wound site. The limb is then further stabilized by the application of a fiberglass cast. The animals receive Fentanyl patches the day before surgery to initiate analgesia and continue to wear the patches for 2 weeks following surgery. Buprenorphine and metacam (Meloxicam) are also administered at the time of surgery for short term pain relief. Fluoroscopy is performed before and after injury to ascertain the degree of injury and to assess the reproducibility of the injury. Blood is drawn to permit assays for immune reaction and infection. Physiological monitoring of the animal to measure vital signs, blood flow and edema in the hind legs is conducted to

track changes following injury. Monitoring is continued every three days to include bandage change, fluoroscopy and physiological parameters. At three weeks the animals are sacrificed and the femurs are recovered for analysis. The bones are imaged using X-ray computerized tomography. Following imaging, the specimens are fixed in formaldehyde and processed for histological examination. Expert analysis of the histological specimens is provided by MAJ Eric Lombardini, veterinary pathologist.

In vitro studies are conducted using mesenchymal stem cells in culture. The cells are cultured in media with and without the treatment agents and assessed for cell viability and induction of osteogenesis. The osteogenesis is measured by cell staining and examination for morphological changes in the cells and by RNA analysis. Using real time PCR, the RNA expression is analyzed to determine if the different treatments have up-regulated the expression of bone-specific genes.

Changes to the methodology for **year 3**:

1. Computerized analysis of the bone sections and the CT scans were conducted minimize human bias in the analysis of the samples. The histology bone slides were digitally scanned with an Olympus/Hammatsu Nanozoomer and images were collected with the NDP Nanoviewer software. Files were then analyzed using Visiopharm/ Visiomorph software package (Medicon Valley, Denmark) to quantify bone, cartilage and fibrous tissue in the section.
2. CT scans and plain radiographs were examined visually and by software analysis. Co-investigators, blinded to treatments, analyzed the CT scans for bone alignment, inflammation, bone filling and calcification. Numerical scores from 1-5 were assigned for each parameter and mean scores for each group were calculated. Computer analysis of the CT scans was accomplished using VivoQuant (Invicro-Imaging Services, Boston MA).
3. Development of a rat femur injury that can be followed longitudinally by CT and PET scanning. Because of the size of the porcine femur, CT scanning could only be accomplished after euthanasia of the animal and isolation of the bone. This precludes longitudinal studies that follow healing over an extended period. To work around this limitation, we have developed a rat model of the femur injury. Animals are treated with fibrinogen/thrombin preparations (salmon and rat), Copios bone filler or no treatment. Animals are then assessed by CT and NaF^{18} Positron Emission Tomography (PET). PET permits localization and quantitation of the uptake of the fluoride to the bone injury site.

Progress in the project

In the **first year** of this project we have made progress on both the cell culture aspects of the project and the animal studies. By establishing stem cell cultures that

have the capacity to differentiate into bone-producing cells, we have demonstrated that, with controlled culture conditions, we can manipulate the expression of bone-specific proteins. The expression of these proteins can be visualized by microscopic examination of the cells and by biochemical detection of bone-specific RNA. The expression levels can be modulated by culturing the cells with different matrices. We found that the salmon fibrinogen and thrombin matrix induced increases in 21 proteins associated with osteogenesis. Furthermore, these changes were dependent on the concentration of the salmon proteins in matrix. For the *in vivo* aspects of the project, we have established the basic femur fracture model and determined an efficient and humane method for caring for the animals post-operatively. We have currently treated nine animals with either salmon fibrinogen (3), porcine fibrinogen (3), unmodified collagen (2) and TR Matrix (1). The bones have been CT scanned and prepared for histological examination. One set of salmon fibrinogen-treated bone fracture slides have been prepared and examined. Blood samples have been collected and basic blood parameters measured and evaluated for the production of adverse immune reaction.

In the **second year**, 4 different treatments were tested. These treatments were 1) salmon fibrinogen and salmon thrombin, 2) porcine fibrinogen and porcine thrombin, 3) a commercially available, FDA approved bone matrix called CopiOS and 4) bovine collagen. Our plan was to have four groups of eight animals. To date we have collected data from 30 animals. Several animals have been excluded from the data group due to termination prior to the completion of the trial period. Data collected included biological samples for immunology testing, physiological parameters to assess wound healing, fluorography (X-rays) to assess the bone formation and growth and CT (computed tomography) for 3-D reconstruction of the bone. In addition, all bones were preserved for histological examination by Dr. Eric Lombardini, veterinary pathologist at AFRII. Our final results are still being analyzed because the last of the animals was only recently completed. However, preliminary results show that there are definite differences between animals, both at the level of radiographic examination and histological examination. The analysis of the radiographs is quantifying the degree of displacement of the bone, severity of injury and degree of fibrosis and calcification of the injury site. Sepsis at the bone fixation site and the injury site is also evaluated since this may have an impact on the healing process. Numerical scores are assigned to each parameter to permit comparison between samples and groups. The histological slides are assessed in a similar fashion with numerical score assigned to infection, bone formation and osteoclast frequency. It will not be possible to make conclusions on the virtues of one treatment group versus another until we have analyzed all of the histological data.

A final aspect of the project was a subproject concerning the effectiveness of the pain alleviation protocol that was used. Dr. Joseph Royal, veterinary fellow, has conducted this project in which he administered femoral and sciatic nerve blockades in addition to the fentanyl patches that were the standard of care for the animal. These data are currently being analyzed for heart rate variability and activity levels which have been used as criteria for pain in other studies.

In the **third year,** we completed the analysis of the swine data concerning the bone healing process, non-invasive measures of tissue damage and healing and the effectiveness of nerve blockades as a method of alleviating extremity pain in swine. Manuscripts have been prepared and submitted on these topics (manuscripts are attached). In addition, a CRADA has been implemented with Dr. Ki Chon of Worchester Polytechnic Institute, MA to collaborate on analysis of the ECG data and how it relates to subjective measurements of pain.

A new bone healing protocol has been developed in rats to permit the study of bone healing over time. In this protocol, an injury is produced in the mid-femur with a motorized drill, resulting in a uniform, regularly shaped lesion that can be reproducibly made. The animals are treated with fibrinogen/thrombin preparations made from rat or salmon protein preparations, the commercial bone filler CopiOS or recovered without any treatment. At 2 days, 7 days, 14 days and 28 days the animals are injected with radioactive fluoride and scanned for deposition of radioactivity in the injury site. The radioactivity can be visualized (Figure 1) and quantitated. Changes in radioactivity will be normalized to the uninjured femur and compared between treatments. CT and PET scans will be collected.

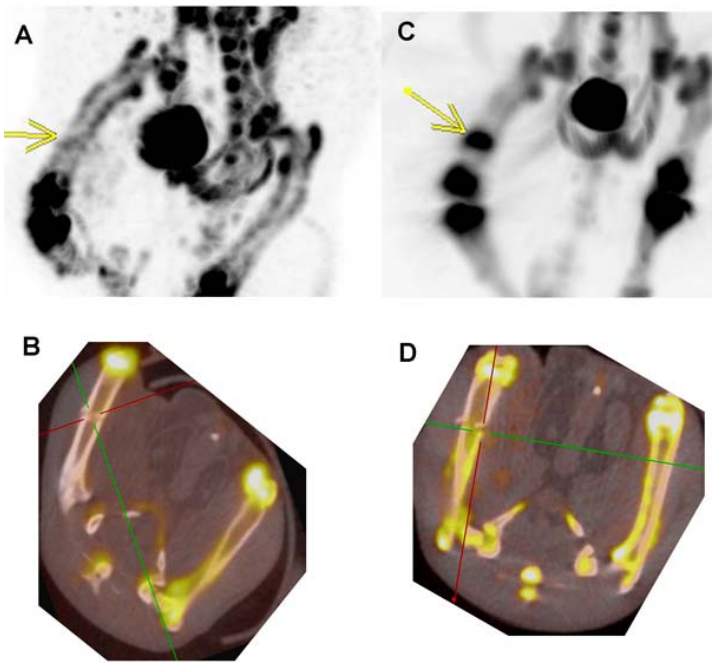

Figure 1. PET and CT scans from a control rat with a femur injury without treatment. Panels A and B show the PET and CT scans, respectively, at Day 2 following the injury. Panels C and D show the PET and CT scans of same animal at Day 7 following injury. The yellow arrow indicate the site of injury and the uptake of NaF^{18} following 30 minute incubation with the tracer. Rate of uptake is greater at Day 7 compared to Day 2.

KEY RESEARCH ACCOMPLISHMENTS

- **Completed** the full analysis of the swine femur CT scans with computer analysis.
- **Completed** the full analysis of the swine femur histology slides with computer analysis.
- **Wrote and submitted** manuscript on the bone healing results.
- **Completed** initial analysis of cardiac tracings from twenty animals treated with regional nerve blocks.
- **Wrote and submitted** manuscript on the efficacy of nerve blockades in swine in comparison to conventional Fentanyl patch analgesia.
- **Completed** the full analysis of the swine impedance, Doppler flow and near Infrared measurements.
- **Wrote manuscript** on the use of surrogate measures of bone and tissue healing in preparation for submission
- **Developed** rat bone healing protocol with PET/CT scanning.
- **Wrote and submitted** application for in-house pilot grant for rat PET/CT imaging project.

Personnel receiving salary from this research project
Dr. Michael Bodo, research physiologist

REPORTABLE OUTCOMES

Presented the following posters on the therapeutic qualities of salmon fibrinogen and thrombin:

- Bodo M., S. W. Rothwell, T. Settle, J. Royal, E. Lombardini and E. Sawyer. 2012. Surrogate Markers of Healing from Penetrating Traumatic Compound Femur Fracture in Swine. USU Research Week, Bethesda, MD.
- Bodo M., S. W. Rothwell, T. Settle, J. Royal, E. Lombardini and E. Sawyer. 2012. Surrogate Markers of Healing from Penetrating Traumatic Compound Femur Fracture in Swine. USU Research Week, Bethesda, MD.
- Jiang, G., S.W. Rothwell, Jacobowitz, D., Mueller, G., Pollard, H. 2012. A rational approach for protein sequencing. USU Research Week, Bethesda, MD.
- Rothwell, S.W., Eric Lombardini, Michael Bodo, Evelyn Sawyer, Joseph Royal and Timothy Settle. 2012. Enhancing the Healing of Penetrating Traumatic Compound Femoral Fractures in Swine with Injectable Fibrinogen Preparations. USU Research Week, Bethesda, MD.
- Royal, J.M., T. L Settle, M. Bodo, E. Lombardini, M. L Kent, J. Upp, and S. W. Rothwell. 2012. Ultrasound-guided Regional Anesthesia in a Swine Femur Fracture Model. USU Research Week, Bethesda, MD.

Submitted the following manuscripts:

- Rothwell, S.W., E. Sawyer, E, Lombardini, J. Royal, H. Tang, M. Bodo, T. L. Settle 2012. Comparison of fibrinogen and collagen-based treatments for penetrating wounds with comminuted femur fractures in a swine model. J Special Oper Med.

- Royal, J.M., T. L Settle, M. Bodo, E. Lombardini, M. L. Kent, J. Upp, and S.W. Rothwell. 2012. A Comparison of Postoperative Analgesic Effects of Ultrasound-guided Regional Anesthesia in a Swine Femur Fracture Model. J Am Assoc Lab Animal Sci.

Prepared the following manuscript for submission:

- Bodo, M., T.L. Settle, J.M. Royal, E. Lombardini, E. Sawyer, S.W. Rothwell. 2012. Noninvasive monitoring of healing from penetrating traumatic compound femur fracture in swine. J Clinical Monitoring and Computing

Published manuscript:

- Floyd, C.T., S.W. Rothwell, R. Martin, J. Risdahl and N. Rose. 2012. A salmon thrombin-fibrinogen dressing allows greater survival and preserves distal blood flow compared to standard kaolin gauze in coagulopathic swine with lethal injury. J Special Operations Medicine. J Special Operations Medicine. 12(2): 16-26.

CONCLUSIONS

In summary, we have made substantial progress in achieving the goals outlined in our initial project proposal. We have collected the data from all of our animals and have made complete analysis and conclusions. We completed the computerized analysis that has quantitated the area of bone calcification and validate the observational measurements of the Dr. Lombardini. We also completed a computerized analysis of the CT scans of the healing bone. We have also used the Infrared spectroscopy, Doppler and impedance measurements to demonstrate that these could useful, non-invasive measurements of healing.

Our conclusions are that fibrinogen-based treatments showed a trend towards better healing based on cellular and mineralization patterns than did the collagen or mineral-blood preparations. However, the limited numbers of animals in this trial did not allow these measurements to reach statistical significance in the measured parameters, even with the computerized methods.

To counter this problem, we have developed a rat injury model that will permit larger numbers of animals and multiple scanning sessions/animal over time. With the preliminary data acquired on the limited animals done on the small animal protocol, we have applied for a small pilot grant and will use the additional data from that study to apply for a grant that will be fully powered to achieve statistical significance.

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Comparison of fibrinogen and collagen-based treatments for penetrating wounds with comminuted femur fractures in a swine model

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Abstract

Introduction: Military service members in combat operations often sustain injuries to the extremities from high speed projectiles resulting in bleeding and comminuted open fractures. Severe injury with bone fragmentation can result in limb amputation. Surgical treatment options include materials that promote osteogenesis and bone proliferation such as growth hormones, stem cells or mineralized matrix adjuncts. However, none of these are amenable to usage by the first responder and nor do they address the question of hemorrhage control which is often a common problem in traumatic injuries.

Hypothesis: Our hypothesis was that treatment with a fibrinogen-based protein mixture at the time of the bone injury will provide both hemostasis and a supportive environment for preservation of injured bone.

Methods: A comminuted femur fracture was produced in 28 female Yorkshire swine and one of four treatments was instilled into the wound immediately following injury. Each animal was evaluated for the following parameters: inflammation, new bone growth, osteoclast proliferation, callus formation and femur wound cavity fill, using post-mortem Computed Tomography and analysis of histological sections.

Results: Overall, salmon fibrinogen-thrombin and porcine fibrinogen-thrombin showed a trend for improved healing based on bone filling and calcification. However, statistically significant differences could not be established between treatment groups.

Conclusions: These findings indicate that a fibrinogen-thrombin matrix may be a useful as an immediate response product to enhance fracture healing. Salmon fibrinogen/thrombin has the advantages of cost and pathogen profile compared to mammalian fibrinogens.

Introduction

Blast injuries have become increasingly more frequent and severe in recent military and civilian incidents as terrorists become more sophisticated in their application of bomb-making technology. An additional escalation of the number of casualties has been caused by the inclusion of metal fragments placed in the explosive charge, resulting in the widespread discharge of shrapnel. In a study published by Weil et al. [1] that examined terrorist bombings in Israel, penetrating injuries to the extremities caused by high explosive attacks were associated with extensive tissue damage that was usually accompanied by high grade open fractures.

For American military members, operational and logistical operations in Iraq and Afghanistan often rely on ground convoy movements that expose service members to risk of injury or death from roadside explosives. Vehicle-borne explosives have often been deployed against fixed sites. The force of the explosive devices, the accompanying shock wave ("wind of explosion") and the storm of shrapnel and debris carried by the explosion can cause massive soft tissue and orthopedic injuries. Extensive deployment of Kevlar helmets and body armor has mitigated the incidence of penetrating head and body wounds, but extremity injuries with concomitant bone fragmentation have continued to be a source of concern [2, 3]. It is estimated that 60-70% of injuries now occur in the extremities[4]. A treatment is needed that can be directly inserted into the wound site to stop bleeding and stabilize the shattered fragments.

Penetrating injuries to extremities caused by blast fragmentation often involve extensive tissue damage accompanied by uncontrolled bleeding and high grade open fractures [5, 6]. Bleeding at distal sites may be controlled by tourniquet application but wounds in more proximal sites, such as the proximal femoral area, may be inaccessible to tourniquets. Serious bone injuries can be later treated by autologous bone grafts but bone grafts can have serious side effects, such as infection at the donor site and pain. Furthermore, the quantity of bone graft material that can be obtained by this method is limited. To attempt to overcome these limitations, new directions in the first response to penetrating wounds are required. First, bleeding must be controlled by the direct application of a hemostatic agent deep into the wound at the site of the bleeding. Second, fragmented bone must be stabilized if the limb will be saved [7]. Third, any intervention must be relatively inexpensive and environmentally stable enough to be backpack compatible.

The hypothesis of this project was that lyophilized fibrinogen/thrombin preparations rehydrated and instilled into the wound shortly after injury would control hemorrhage and stabilize bone fragments. The approach was to inject fibrin sealants directly into the wound and the bone lesion to provide an environment that will sustain the cellular component of bone and encourage bone regeneration. This technique can also provide a medium to support the growth of cells, either native or exogenous mesenchymal stem cells, that may be implanted into the site [8, 9]. This treatment should permit successful surgical repair and accelerate subsequent healing. The materials tested in this project were two fibrinogen preparations, swine and salmon fibrinogen, bovine collagen and commercial bone filler. A combination of salmon thrombin and fibrinogen [10-12] has been tested as a hemostatic agent in other projects and has been shown to be an effective hemostatic reagent with a commercially viable cost.

Materials and Methods

Mesenchymal stem cell cultures

Human mesenchymal stem cells were purchased from Celprogen, Inc. (San Pedro, CA) and grown either on coverslips in 6-well cell culture plates or directly in 6-well culture plates with or without surface modification. Media designated by the company as "maintenance" or "differentiation" media was used to either permit the cells to proliferate in the undifferentiated state or induce differentiation along the osteogenic pathway. Bovine collagen (Sigma-Aldrich, St Louis, MI) or salmon fibrinogen/thrombin (Sea Run Holdings, Eastport, ME) preparations were used to coat the surface of slides or wells to induce differentiation for microscopy and RT-PCR analysis. Concentrations of fibrinogen and thrombin tested were: 5 mg/mL fibrinogen 10u/mL thrombin, 5 mg/ml fibrinogen 100u/ml thrombin, 30 mg/ mL fibrinogen 10u/ mL thrombin, 30 mg/ mL fibrinogen 100u/ mL thrombin, 50 mg/ mL fibrinogen 10u/ mL thrombin and 50 mg /mL fibrinogen 100u/ mL thrombin. For comparison, Greiner Bio-one collagen (Type1) coated plates (Greiner Bio-One North America, Inc., Monroe, NC) were used with complete or differentiation media.

Differentiation was assessed morphologically by the ability to deposit calcium phosphate and metabolically by the up-regulation of osteogenic RNA. Morphological assessment was performed using two different staining techniques to identify the mineral deposition, 1) Von Kossa staining which detects the presence of phosphate ions and 2) Alizarin Red which detects calcium deposits.

RT-PCT

Mesenchymal cells were harvested from 6-well culture plates digested with trypsin and collected by centrifugation at 300xg for 5 min. RNA was prepared using the Qiagen RNeasy spin column procedure (Qiagen, Valencia, CA) and evaluated for quality by electrophoresis. RT-PCR was performed using SABiosciences Human Osteogenic pathway kit and reagents (Frederick, MD). Briefly, genomic DNA was removed using the SABiosciences Genomic DNA elimination kit, the reverse transcriptase reagents were added to the genomic elimination sample and incubated at 42C for 15 min before stopping the reaction by incubation at 95C. The completed First Strand cDNA solution was mixed with SABiosciences RT^2 qPCR Master Mix and pipetted onto the 96 well pathway plate. The plates were developed on a Roche LightCycler 480 PCR cycler (Roche Applied Science, Indianapolis, IN) and the results analyzed using the Roche Light cycler software package.

Surgical preparation of animals

Animal care standards

All animal procedures were conducted according to a Uniformed Services University Institutional Animal Care and Use Committee approved protocol. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals*, [NRC Publication, 2011 edition].

Animal surgical model

Female Yorkshire swine (*Sus scrofa Domestica)* (weighing 35-40kg) were prepared for surgery and monitored during the procedure as described previously [11]. A transdermal fentanyl patch (50 ug/hr; Watson Laboratories, Inc., Corona, CA) was placed on each animal 18 hours prior to surgery to provide postoperative analgesia. Anesthetic induction was accomplished by intramuscular injection of tiletamine-zolazepam (Telazol©) (4.4mg/kg) (Fort Dodge Animal Health, Fort Dodge, Iowa). The animals were then intubated and anesthesia maintained using isoflurane (2-3%). The injury was produced at the right femoral midshaft using a Schermer KS self-retracting penetrating captive bolt gun (QC Supply, Schuyler, NE). This resulted in tissue and muscle damage at the point of entry and full penetration of the femoral bone, producing a non-compressible bleeding wound and a compound fracture following the method of Majetschak et al. [13, 14]. A biplanar type I fixation device with threaded half pins was placed proximal and distal to the fracture in a bilateral configuration with interconnecting bars as previously described in general veterinary surgery texts [15]. A non-adherent dressing (Telfa ,Tyco Healthcare, Mansfield, MA) was applied to the wound and the entire leg was immobilized and encased in a cast (Vetcast Plus, 3M Health Care, Neuss, Germany). This dressing/cast was secured to the pig using Elastikon elastic tape (Johnson & Johnson Consumer Products, Skilman, NJ). Analgesics (0.3 mg, buprenorphine; Hospira, Inc., Lake Forest, IL) were administered as needed for pain control and sedation during the first 3 days post-surgery. The animals were sedated twice a week to permit cleansing of the pin sites and blood collection. At days 7, 14 and 21 radiographs were taken to assess union at the fracture. Bioimpedance measurement and Doppler ultrasound was used to estimate blood flow, tissue oxygenation and edema formation (Bodo, manuscript in preparation). At day 21, animals were euthanized by IV injection of pentobarbital and the femur was dissected for computed tomography (CT) and collection of tissues for histology.

Bone injury treatments

Four different preparations were tested for their effects on bone formation: (1) salmon fibrinogen and thrombin (Sea Run Holdings, Inc., Eastport, ME, (2) porcine fibrinogen and thrombin (Enzyme Research, Inc. South Bend, IN), (3) bovine collagen (Sigma-Aldrich, St. Louis, MO) and (4)CopiOS^{R} Bone Void Filler (Distributed by Zimmer, Inc., Warsaw, IN; manufactured by Kensey Nash Corp, Exton,PA)(BF) . Eight animals were assigned to each treatment group. Salmon fibrinogen (20 mg/mL) and thrombin (450 U) (SFT) and porcine fibrinogen (18.8 mg/mL) and thrombin (287.2 U) (PFT) solutions were filter sterilized separately and the fibrinogen and thrombin

were combined during instillation into the wound. Prior to use, CopiOS^{R} was mixed with 5 mL citrated autologous blood. Bovine collagen was sterilized by irradiation (5kGy; JL Shepherd Mark 109 Cobalt source, San Fernando, CA), suspended in 5 mL sterile PBS, and instilled into the wound. Placement of a treatment into the wound site was made possible by a 3 mL syringe with the tip cut off to produce an open-ended tube, which was then inserted into the injury track down to the level of bone lesion. The treatment material was deposited into the syringe barrel and the syringe plunger was inserted to expel the treatment material as the syringe barrel was withdrawn. Two inert plastic radiopaque beads (1mm diameter) were included with the treatment in five animals to verify correct placement of the treatment material into the fracture site. Plain radiographs were obtained with an Insight Fluorscan C-arm fluoroscopy machine (Hologic, Inc. Bedford, MA). CT scans were performed on a Siemens Inveon Multimodal system (Seimens USA, Malvern PA).

Analysis of bone slides and CT scans

Following euthanasia, each femur was isolated and used for CT imaging. The femoral head and the lateral condyle were then detached to permit infiltration of paraformaldehyde, and the tissues were decalcified, sectioned, and stained with hematoxylin and eosin. Images were analyzed in a blinded fashion by a veterinary pathologist (EL) and coded according to morphology, inflammation, new bone growth, presence of cartilaginous islands and the absence or presence of osteoclasts. The slides were digitally scanned with an Olympus/Hammatsu Nanozoomer and images were collected with the NDP Nanoviewer software. Files were then analyzed using Visiopharm/ Visiomorph software package (Medicon Valley, Denmark) to quantify bone, cartilage and fibrous tissue in the section.

CT scans and plain radiographs were examined visually. Co-investigators (SR and TS), blinded to treatments, analyzed the CT scans for bone alignment, inflammation, bone filling and calcification. Numerical scores from 1-5 were assigned for each parameter and mean scores for each group were calculated. Computer analysis of the CT scans was accomplished using VivoQuant (Invicro-Imaging Services, Boston MA).

Data analysis

Simple summary statistics and graphical displays were used to describe the results of this study. Summary statistics included means, standard deviations, and confidence intervals. Analysis of variance was conducted for multiple group comparison. Graphical displays included bar and box plots.

Results

Human mesenchymal cells grown on a salmon fibrin matrix are induced to express osteogenic proteins

Human bone marrow-derived mesenchymal stem cells obtained from Celprogen, Inc. can be maintained as undifferentiated stem cells or induced to attain differentiated morphology based on the media and surface treatment of the culture flasks. To determine if a salmon fibrinogen/thrombin matrix could induce a similar transformation, cells were cultured for one week in maintenance flasks, flasks coated with collage type I or coated with protein matrices formed by the clotting of salmon fibrinogen with salmon thrombin. The cellular response to the different conditions was assessed histologically. As shown in **Figure 1**, cells grown in the Celprogen maintenance media on

maintenance cell culture plates retained a non-differentiated morphology and stained negatively in the Von Kossa assay (**Figure 1**, panel A). When cultured in differentiation media which had the required growth factors for osteogenic differentiation, the cells changed their appearance and deposited calcium phosphate as indicated by the dark precipitates (**Figure 1**, panel B) resulting from alkaline phosphatase as detected by the Von Kossa assay. Cells grown on bovine collagen lost their contact inhibition and became confluent but did not show significant mineral deposits (**Figure 1**, panel C). In contrast, cells grown on a salmon fibrin matrix (50mg/mL fibrinogen with 10 units salmon thrombin) displayed rounded osteoblastic morphology with darkened Golgi regions and intense extracellular mineral deposits (**Figure 1**, panel D). This would indicate that components of the salmon fibrinogen/thrombin matrix have the capacity to alter the normal status of the cells.

RNA expression was quantitated using reverse transcriptase polymerase chain reaction RT-PCR and the osteogenic panel from SA Biosciences. Assays were run on undifferentiated cells to establish a baseline of RNA expression and cells grown on maintenance plates in differentiation buffer or fibrinogen coated plates for seven days. Even at this early time point changes could be detected (**Table1**). When cells were grown on coated plates for 21 days, more robust changes in the RNA profile could be observed. Cells were grown in (1) undifferentiating media on salmon fibrinogen (5 mg/mL) /thrombin (10 U) for 21 days, (2) undifferentiating media on salmon fibrinogen (50 mg/mL) /thrombin (100 U) for 21 days and (3) collagen coated plates in differentiating media for 21 days. As shown in **Table 2**, the cells grown on salmon matrices showed increases in 22 of the 86 proteins on the array when compared to the baseline values of the undifferentiated cells. This compares favorably with the cells grown in differentiating media on a collagen substrate. The proteins that are up-regulated fall into several major groups. One group is comprised of structural proteins such as the collagens and several different types of collagens were up-regulated. Type 1 is a collagen variety expected to be increased since it is found in bone, but type IV and type XI were also increased. Type IV is found in the basal lamina of epithelial cells while type XI is secreted by chondrocytes. This may reflect the natural progression of bone healing which often proceeds through a cartilage stage on its way to transforming into bone [11, 12]. Transcription factors comprise another group of proteins that play an important regulatory role in the expression of proteins and the subsequent phenotype that results from their expression. In this experiment, two SMAD proteins which interact with transforming growth factor and mediate the TGF-β protein expression response were increased by the differentiation process. An interesting protein that was increased was the protein tuftelin which has been identified as a nucleating protein for tooth enamel. Teeth and bones are related structures but have distinct components and cellular components

Surgical results- animal responses and results of surgical care

The injury created by the bolt penetrator was a realistic reprisal of the type of injuries caused by penetrating projectiles. A path approximately 1 cm by 6 cm was created that passed through the skin, muscle and bone. The bone was reproducibly perforated by the bolt (**Figure 2**A). In addition, there was a variable element introduced by the unpredictable degree of fracturing surrounding the hole produced by the bolt. Sometimes, fragments of bone would be displaced from the shaft. Frequently, fracture lines would spiral out from the impact point. Fracture displacement was not always readily visible at the initial surgery and external fixation, but became evident several

days later as the bone would separate along the fracture lines (**Figure 2**B). Instillation of the treatment matrix filled the injury tract and, in the case of salmon and porcine fibrin treatments, effectively stopped any bleeding. Severe bleeding was not produced in this model because the femoral artery and vein were avoided, but there was slow bleeding from soft tissue that was quickly stopped by fibrinogen treatment (**Figure 3A**). This slow bleeding continued for some time after application of the collagen and CopIOS preparations, but volume of blood loss was minimal (less than 20 mL). Healing occurred uneventfully at the injury site (**Figure 3B**). Injuries treated with salmon fibrinogen routinely re-epithelialized with hair growth reoccurring (**Figure 3C**).

Bone healing measured by radiography showed fibrous deposition and some calcification in all treatments

Radiography was conducted weekly to monitor the healing progress in each animal. It can be appreciated from the radiographs that the animal movement caused a displacement of the bone fragments despite the insertion of four pins (two proximal and two distal) and stabilization with a box arrangement of four bars and fiber cast (**Figure 4**). With plane radiography, we could follow the deposition of a fibrous callous and the beginning of the calcification process. **Figure 5** shows the progression of healing of the femur. Panel A is a view of the uninjured bone and Panel B shows the bone immediately following injury. The arrow indicates the point of impact of the penetrating bolt. Panel C shows femur at 10 day post-injury. Note that the animal has put weight on the limb and, despite the fixation, has caused detraction of the bone. Fractures in the bone have widened and are indicated by the arrows. In Panels D and E (Days 16 and 21) the lesions in the bone are healing (arrow 1). Panels F and G are views from the post-mortem CT scan. Arrow 3 indicates filling of the lesion in the cortex. In contrast, the lesion site in the medulla, shown in the slice view in panel F, is still low density and is not equivalent to the uninjured regions of the marrow space. An unanticipated consequent of the placement of external fixation pins was the onset of infection at the pin sites. Although the sites were cleaned twice weekly, this was not sufficient to keep the sites infection free. In some cases, the infection migrated down the pins and by three weeks eroded the stability of the pins.

Computed tomography of the isolated femurs permits qualitative and quantitative analysis of bone healing

At the three week time point, the animals were euthanized and the hind legs were removed for analysis. The uninjured leg was used as a control limb for comparison to the injured bone. Following computer reconstruction, the images were presented as three-dimensional images and as planar sectional images (**Figure 5**). These images were analyzed independently by two investigators (SR, TL) for bone alignment, inflammation, bone filling and calcification (callus score) without prior knowledge of the treatment group. Callus and bone filling scores are shown in **Figure 6**. The pig (PFT) and the salmon (SFT) fibrinogen/thrombin treatments were slightly better in the callus score and the PFT was slightly better than the other treatment groups in the bone filling category. However, none of the treatments were substantially different to reach statistical significance in the numbers of animals observed (n=7 or 8/group).

Analysis of the CT scans by the Vivoquant software package permits quantitation of the bone density within the healing region. Bone density was hypothesized to equate to calcification and regeneration of bone. 3-

dimensional regions of interests were mapped out that included only the damaged and healing region and did not include the original bony cortex which would skew the density analysis. Using this measurement, bone densities were calculated. (**Figure 7**). Box plots show the range of mean density (measured in arbitrary voxel units) for each animal in the treatment groups. The mean density was similar but the range of density varied widely. The SFI and PFT – treated animals usually had the highest density healing but both groups also had one animal with lower than average density repair which increased the range of values.

Histological analysis of the injured sites identifies different tissue responses to the treatments

Slides for each animal were prepared from the fixed and decalcified femurs and transformed into digital files with Hammasutu Nanozoomer scanner. As was performed in the analysis for the CT scans, the histology slides were assessed by both expert human judgment and by computer-assisted analysis. Histopathologic diagnosis was conducted by a trained veterinary pathologist (EL) blinded to the treatments and scored based on inflammation scale 1-5 (minimal, mild, moderate, marked, severe); maturation (based on fibroblast infiltration, collagen deposition and myelofibrosis with 1=most immature to 5=most mature) and the presence of new bone growth (scored qualitatively on a 1-5 scale**) Figure 8**. The results are variable, but the scores between the new bone growth (**Figure 8A**) and inflammation (**Figure 8C**) show the same patterns. In both categories, the SFT had the highest scores, PFT and CopiOS bone filler (BF) were intermediate and the collagen was lowest.

The computer analysis of the slides was set up to distinguish three tissue types (bone, cartilage and fibrous tissue) and open space on the slides. **Figure 9** shows the scores for the percentage of each region of interest that was coded as either cartilage or bone. SFT appeared slightly higher for the presence of bone and BF was lowest, but as before, but none of the values achieved statistical significance. All of the cartilage values were very similar.

Discussion

In this study, we compared the ability of four different treatments to stabilize a fragmented femur replicating the type of injury often encountered in traumatic accidents and military operations. These injuries present major difficulties in initial treatment and stabilization of the site and long-term survival of the limb. It is our goal to develop a field stable, transportable, easy-to-use bone stabilizer that can be applied following injury by the first responder.

Many factors may contribute to non-union of fractures, including nutritional or hormonal status, age of the patient, and presence of bacterial contamination. Atrophic non-unions may be due to inadequate blood supply or failure of callus formation at the fracture site. More severe instances of non-union may arise when pieces of the bone are totally missing. Mechanical stability at the fracture site must be achieved and failure to attain this may be a leading reason for non-union. In addition, while non-union is estimated to occur in approximately 2.5% of all tibia fracture repairs [16, 17], if vascular injury is involved, the frequency increases 5-6 fold [18, 19]. The phenomenon of non-union of fracture has led to the development of substances that can bridge the gap where the bone is absent. These substances typically seek to cause the migration of bone producing cells into the lesion or cause the differentiation and proliferation of osteogenic cells from precursors and stem cell populations [20].

The most commonly used substance for bone grafting is bone itself [21], preferably an autologous graft from the iliac crest of the patient. This material is still considered the ”gold standard” of bone grafting [22, 23]. It is osteogenic (can induce new bone formation), osteoconductive (provides a surface for bone formation), and osteoinductive (can stimulate the differentiation of bone precursor cells). It is the only graft material that has been tested that fulfills all three of these criteria. Trabecular bone is preferred over cortical bone because of the larger numbers of cells contained within the trabecular bone. While the bone graft itself does not confer mechanical stability, if the fracture is stabilized by the surgical procedure, high rates of repair are common [24]. In contrast, bone marrow aspirate from the bone contains osteoprogenitor cells but is not osteoconductive and the numbers of stem cells, especially in elderly patients, may be low.

Because autologous bone grafting is not always feasible, other alternatives have been put forth. Allograft bone, stripped of organic material, will serve as a framework for vascularization and bone formation. In a similar fashion, mineralized beads composed of calcium sulfate, calcium phosphate or hydroxyapatite have been used. These materials can be effective in aiding bone union form by working as volume expanders. However, they only can work by osteoconduction, not by osteogenesis or osteoinduction [25] so better artificial alternatives are required.

The recognition that true bone regeneration relies upon (1) having the proper progenitor cells at the injury site, (2) the molecular signals that can drive these cells down the proper differentiation pathways, (3) the adequate vascular systems to nourish the developing bone and (4) a sturdy system of mechanical support, has led members of the tissue engineering field to propose alternative artificial systems that incorporate as many facets of the natural system as possible. The starting point for most of the systems is the fabrication of a matrix that will provide support and mechanical stability. The matrix may be inert and act solely as a carrier for cells and bioactive molecules or it may have osteoinductive properties of its own. Collagen has been a logical starting point for many of the engineered matrices since this family of proteins forms the natural ground substance of bone [26-29]. Collagen has been used in combination with hydroxyapatite crystals in various forms in a number of studies to support the growth of human bone marrow stromal cells [30, 31]. A second protein matrix that has been evaluated in different systems is the fibrin gel. Fibrin is a major component in blood clotting and plays a major role in wound healing. It has been proposed to have an intrinsic osteogenic influence of its own [32], but there is no clear consensus on whether or not fibrin matrices by themselves can promote bone growth. A report from Meyers et al. using mammalian fibrin reported osteogenic effects in bone fractures in dogs over 20 years ago [32]. Fibrin-based gels have been used in conjunction with hydroxyapatite-coated beads or mesenchymal stem cells, and were shown support bone regeneration [33-35]. Fibrin may also prevent resorption of bone grafts, making grafts more efficient [36]. Fibrin has also been used in conjunction with collagen pads with fibrinogen injected as a platelet-rich plasma solution into the graft site and was found to increase the formation of bone [27]. Preliminary work with salmon fibrin/thrombin matrices suggests that the fish protein is unique when compared to human or bovine fibrin in supporting cell growth. Cell culture studies growing primary neurons demonstrated that the cells grew better and extended longer axonal processes on the salmon protein substrate [37]. However, not all studies of the effect of fibrin on bone regeneration have been favorable. Zarate-Kalfopulos et al, in a surgical repair model of a rabbit lumbar injury, reported a negative effect of autologous fibrin on bone fusion [38].

The four treatments tested in this study encompassed the major structural approaches to bone stabilization with two treatments based on fibrinogen, one treatment using collagen and the final treatment based on a combination of collagen and calcium phosphate that was mixed with the patient's own blood. The injury used in this study was produced by a penetrating bolt that caused soft tissue injury and penetrated completely through the bone, causing extensive fragmentation of the bone shaft. The healing injury was assessed for stability of the fracture site, the amount of filling of the bone void, degree of calcification and the level of inflammation

Analysis of the results makes it clear that given the variability of the injury and the differences in the individual animal's course of recovery, a study of this sort will require larger numbers of animals and a longer course of development to generate data with statistical significance. The samples were examined by two very different methodologies; x-ray/CT scanning and histology. The first technology examines hard tissue and the latter examines the cellular components of the limb. So, using these two approaches, it is possible to appreciate the different aspects of healing. The data was also analyzed in two different ways. The first was a calculation of density values measured by the software interpretation of X-ray or histology data. Although this method still requires an investigator to select the region of interest, the program calculates different densities of bone formation. The second analysis relied on human interpretation of healing. Although it may be considered to be more subjective, this analysis was based on years of collective expertise of three experienced investigators.

From these various analyses, several trends emerged. The fibrinogen treatments (SFT, PFT) displayed a tendency for improved development in terms of new growth, callous formation and bone filling. The unmodified collagen (BC) group seemed to rank consistently lower and the bone filler (BF) treatment was usually intermediate. These numbers suggest that a larger sample size could produce results with significant results.

Soft tissue and skin regeneration were also assessed. From a hemostatic perspective, both of the fibrinogen preparations proved effective at stopping the bleeding that resulted from the injury. Although this was not a hemorrhage model and there was not a large amount of bleeding, 3 mL fibrinogen/thrombin solution injected into the wound site instantly sealed the wound. This was not the case with either of the other two treatments. Subsequent healing of the tissue proceeded similarly in the four treatments as judged from a gross anatomical sense. In comparison, histological analysis showed that the fibrinogen treatments seemed to be more active from an inflammatory perspective, with a higher score for the presence of neutrophils, reactive fibroblasts, lymphohistiocytes and active osteoclasts. The presence of the inflammatory response could have a contradictory influence on the bone healing process, although it is thought that the initial immune response has a positive effect in triggering osteogenic activity [39]. Treatment of mice with fractures with the anti-inflammatory drug, indomethacin, has been found to inhibit fracture repair [40]. However, when inflammation progresses into the chronic stages, as observed with rheumatoid arthritis, diabetes mellitus and sepsis, fracture healing time and complications such as non-unions can increase [39]. At three weeks of time post injury, the immune response may still be a positive stimulus for tissue repair.

Our hypothesis that the salmon fibrinogen would have a particular advantage in this setting was not conclusively proven as the pig fibrinogen also seemed to give good response in our bone healing model. This study does suggest that a fibrinogen based agent could form the basis for a practical application for the treatment of

penetrating injuries with major fractures. Our future studies will be focused on longitudinal studies in rats. This will permit us to follow the progression of the healing process through time at multiple times points by CT and PET scanning. Although this model may not mimic human anatomy and physiology as closely as the swine model presented in this paper, we expect that the scientific payoff of increased sample size and improved resolution of the biology of healing will more than compensate for this trade-off.

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Figure legends

Figure 1 Differentiation of human mesenchymal stem cells on salmon fibrinogen matrices. Cells were grown in Celprogen maintenance media on maintenance plates (A), in Celprogen differentiation media (B), on collagen matrix (C) or on a salmon fibrinogen matrix, 50mg/mL fibrinogen with 10 units salmon thrombin (D). Cells were stained with the Von Kossa stain to detect calcium phosphate precipitates deposited caused by the induction of alkaline phosphatase activity. Inset in Panel D indicates the cytoplasmic mineral deposits. Bar, 100um.

Figure 2. Perforating bone injury was produced in the midshaft of the femur. Panel A shows the isolated bone removed immediately after injury without treatment or surgical fixation. Panel B a plane X-ray of the injured bone in situ.

Figure 3. Progression of wound healing at the wound site. Panel A shows the wound at time of time of injury. Application of salmon fibrin treatment results in an instantaneous clot. Panel B shows scab formation as the wound site healed (7 days). By 21 days, the wound site had typically re-epithelialized with hair regrowth (Panel C). Arrows show ink mark used for orientation during surgery. The ink marks can be used for comparison of the wound status at the different times during healing. Bar, 1 cm.

Figure 4. Stabilization of femur injury by external fixation. The anatomy of the swine is not conducive to good stabilization of a fractured femur solely by a cast. Therefore, the bone was stabilized by placement of four surgical pins and fixed by an external apparatus. The frame was protected with a fiberglass cast and the entire dressing was secured with Elasticon tape (Johnson and Johnson, Inc.).

Figure 5. Progression of healing of the femur. Panel A, uninjured bone and Panel B, bone directly following injury. The arrow indicates the point of impact of the penetrator bolt. Panel C shows femur at the 10 day point. There has been movement in the bone. Cracks in the bone have widened and are indicated by the arrows. In Panels D and E (Days 16 and 21) the lesions in the bone are healing (arrow 1). Panels F and G are views from the post-mortem CT scan. Arrow 3 indicates the lesion in the cortex which is filling in. In contrast, the lesion site in the medulla (arrow 2), shown in the slice view in panel F, is still low density and is not equivalent to the uninjured regions of the marrow space.

Figure 6. Relative callus and bone filling scores as assessed by investigator CT analysis. Examination of the CT scan for each animal was examined by the investigators separately and then in consultation to reach a consensus score for callus formation and bone filling. Scores from 1-5 were assigned based on wound filling, bridging of the break and calcification. SFT = Salmon fibrinogen/thrombin, PFT = porcine fibrinogen/thrombin, BC = Bovine collagen and BF = CopiOS bone filler.

Figure 7. Analysis of bone density by VivoQuant Imaging software. The range (box plot), mean (dark line) and median (light line) of the repaired bone was calculated using vivoQuant analysis. SFT = Salmon fibrinogen/thrombin, PFT = porcine fibrinogen/thrombin, BC = Bovine collagen and BF = CopiOS bone filler.

Figure 8. Analysis of histology slides by a board-certified veterinary pathologist. Scored were based on an inflammation scale 1-5 (minimal, mild, moderate, marked, severe); maturation of bone (based on fibroblast infiltration, collagen deposition and myelofibrosis with 1=most immature to 5=most mature) and the presence of new bone growth (scored qualitatively on a 1-5 scale**)**

Figure 9. Analysis of histology slides by image recognition software. Each histology slide (panel A) has digitized and divided into 16 regions. Visopharm software was trained to recognize bone (green), cartilage (blue) and fibrous material (red) (Panel B). Mean values (±SD) for cartilage (C) and bone (D) are plotted. SFT = Salmon fibrinogen/thrombin, PFT = porcine fibrinogen/thrombin, BC = Bovine collagen and BF = CopiOS bone filler.

Figure 10. Prothrombin times for 19 animals sampled from Day 0 throughout the treatment period. Asterisks mark two samples from animals treated with PFT with lower than average values. Both animals had normal values on subsequent assays.

Table legends

Table 1 Changes in RNA expression for bone-associated proteins in human mesenchymal stem cells at 7-day incubation. Cells were grown in differentiation medium or maintenance medium in the presence of salmon fibrinogen/thrombin. RNA was recovered from the cell samples and RNA expression was quantitated by RT-PCR. Values are expressed as the ratio of baseline expression in undifferentiated cells grown in maintenance media compared to cells grown in the specified condition. 7-Day diff = 7 day incubation with Celprogen differentiation media; Fibrin 1 = salmon fibrinogen 5mg/mL, salmon thrombin 10U/mL; Fibrin 2 = salmon fibrinogen 50mg/mL, salmon thrombin 10U/mL and Fibrin 3 = salmon fibrinogen 3 = 50mg/mL, salmon thrombin 100U/mL

Table 2. Proteins showing increased RNA association during growth on various substrates with different media after 21 days. Cells were grown in differentiation medium or maintenance medium in the presence of salmon fibrinogen/thrombin. RNA was recovered from the cell samples and RNA expression was quantitated by RT-PCR. Values are expressed as the ratio of baseline expression in undifferentiated cells grown in maintenance media compared to cells grown in the specified condition. Dif. = 21 day incubation with Celprogen differentiation media; Fibrin 1 = salmon fibrinogen 5mg/mL, salmon thrombin 10U/mL; Fibrin 3 = salmon fibrinogen 50mg/mL, salmon thrombin 100U/mL. Collagen = Greiner Bio-One collagen coated flasks.

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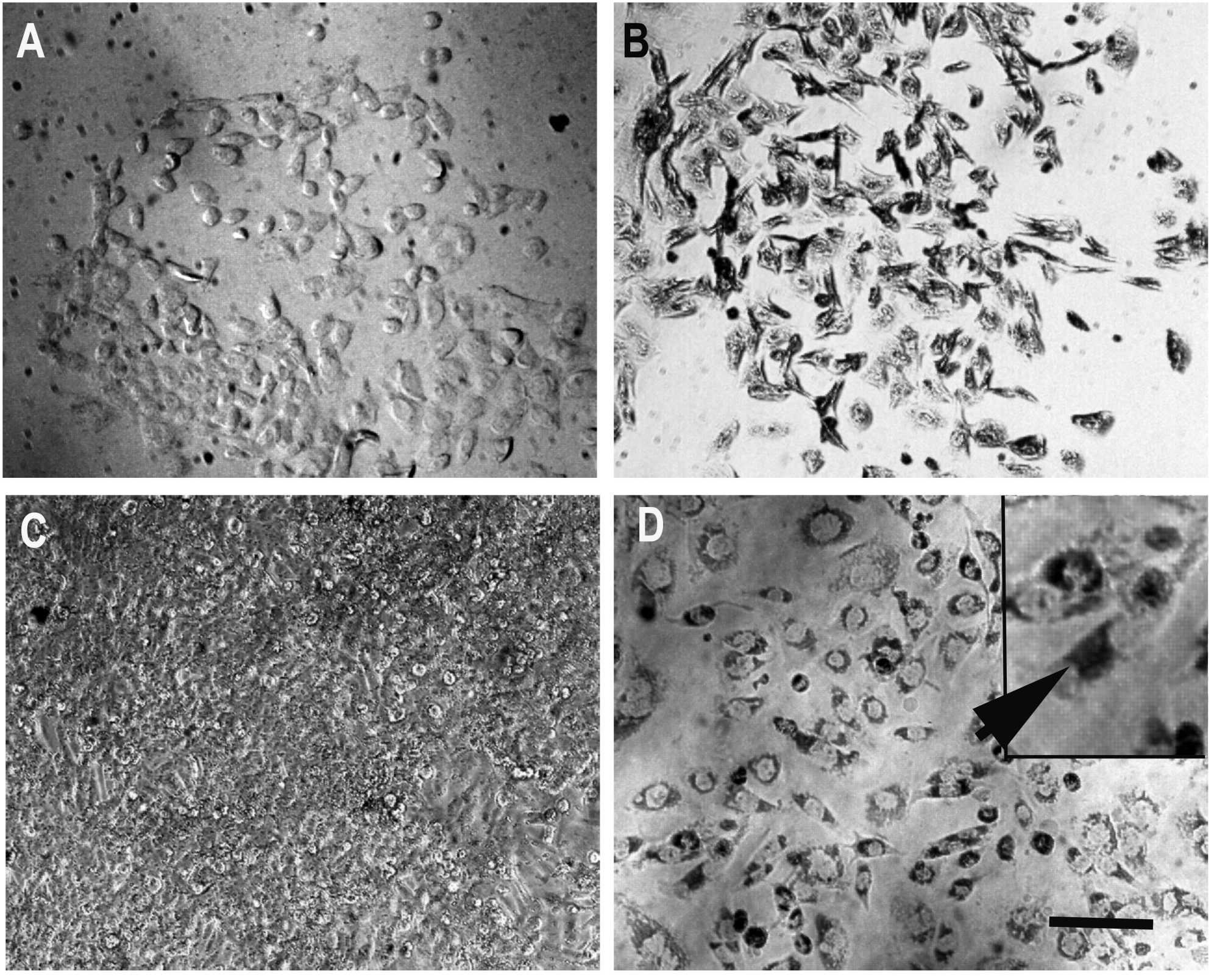
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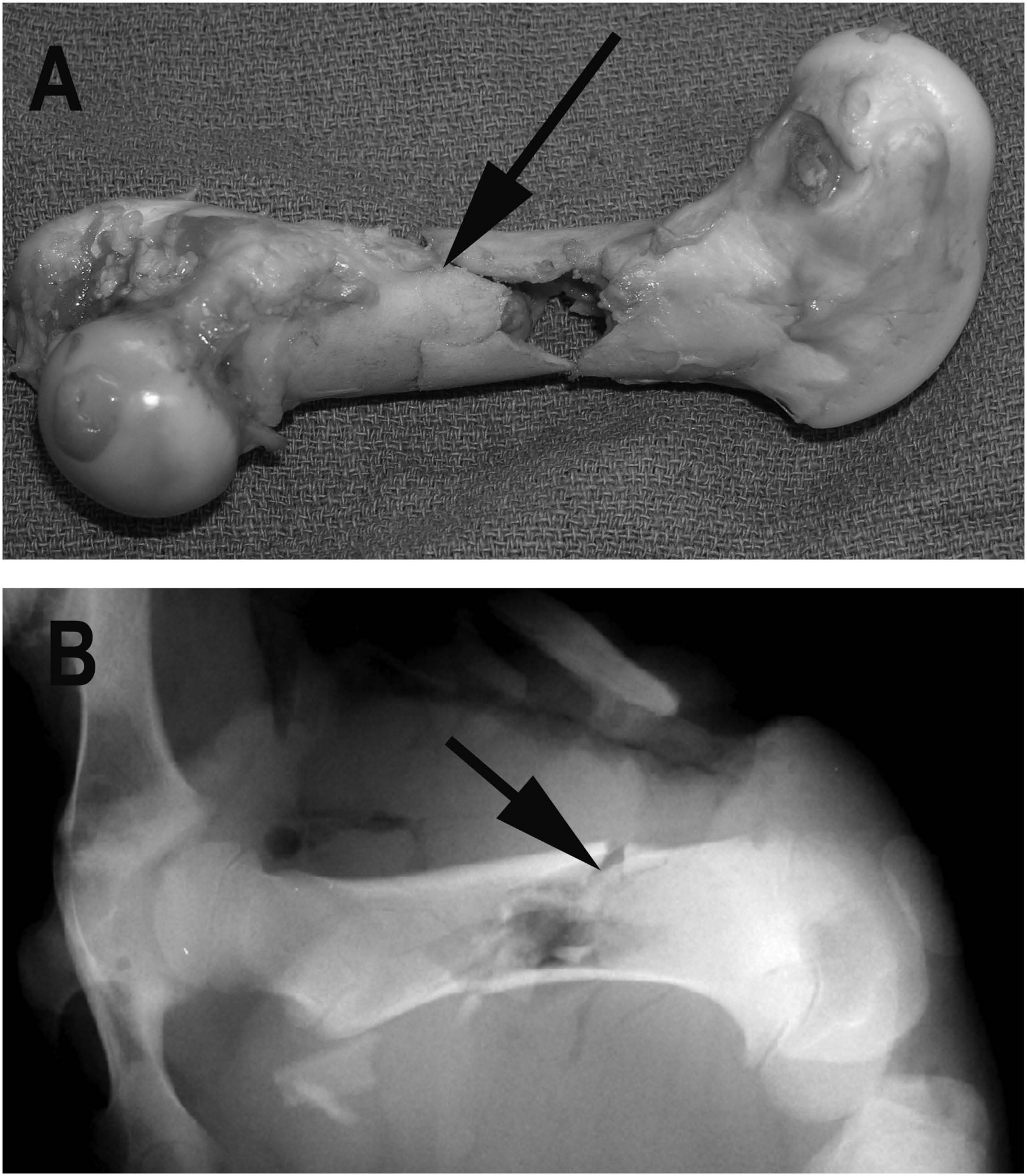
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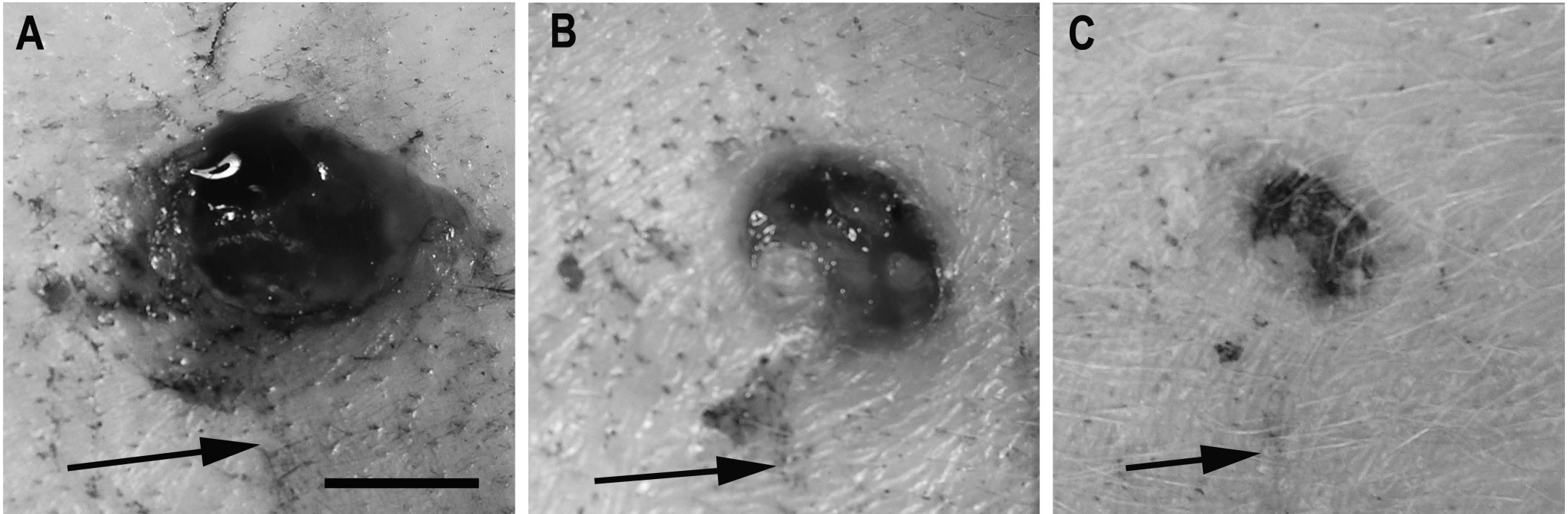
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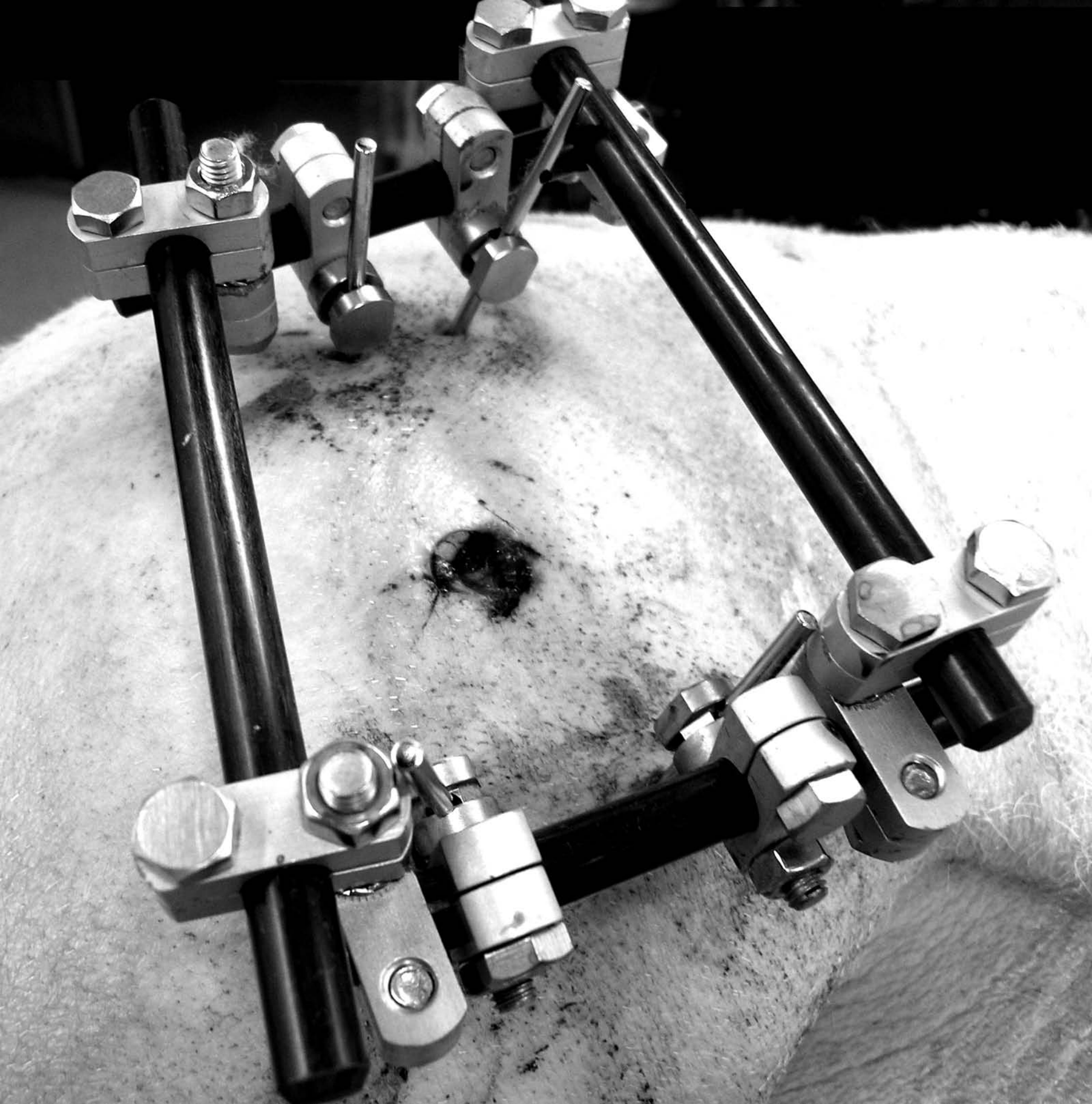

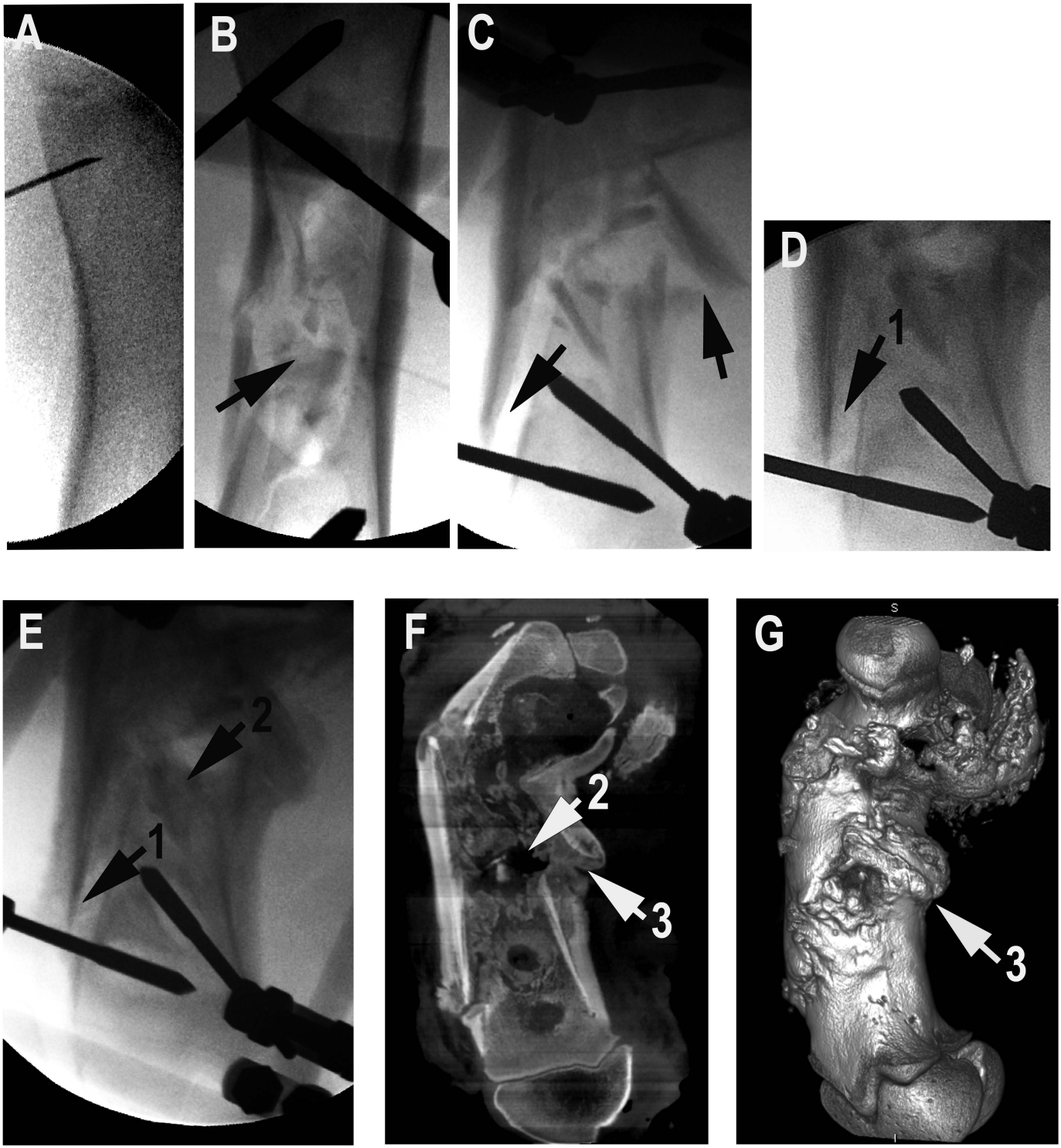
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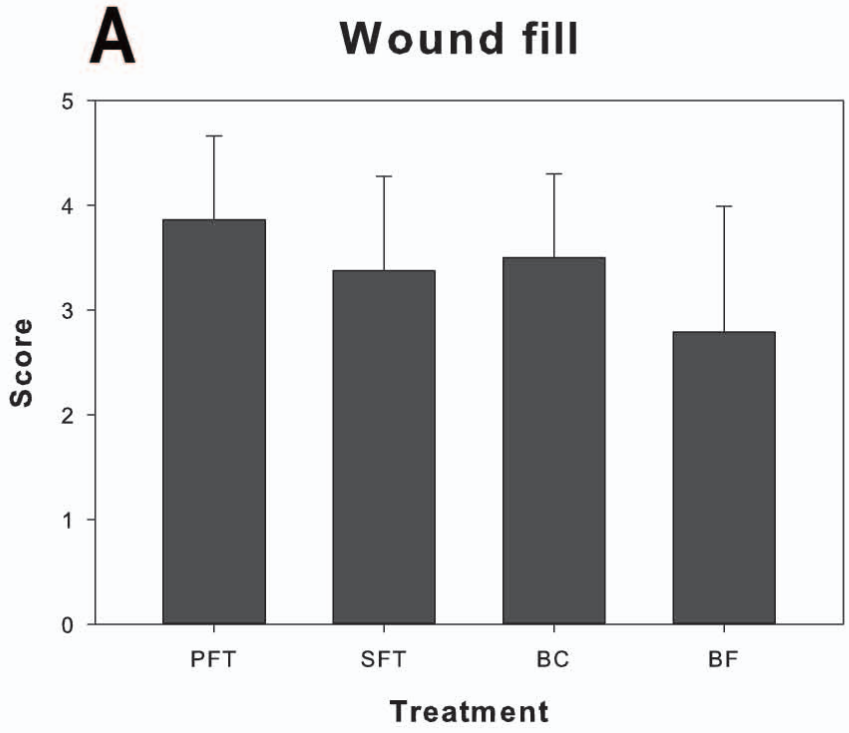
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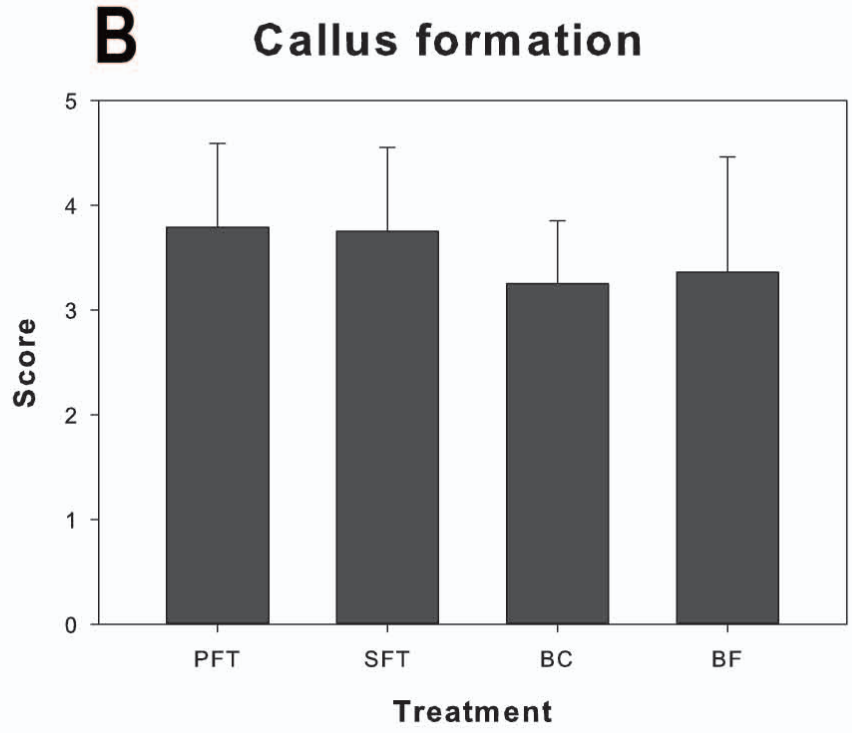
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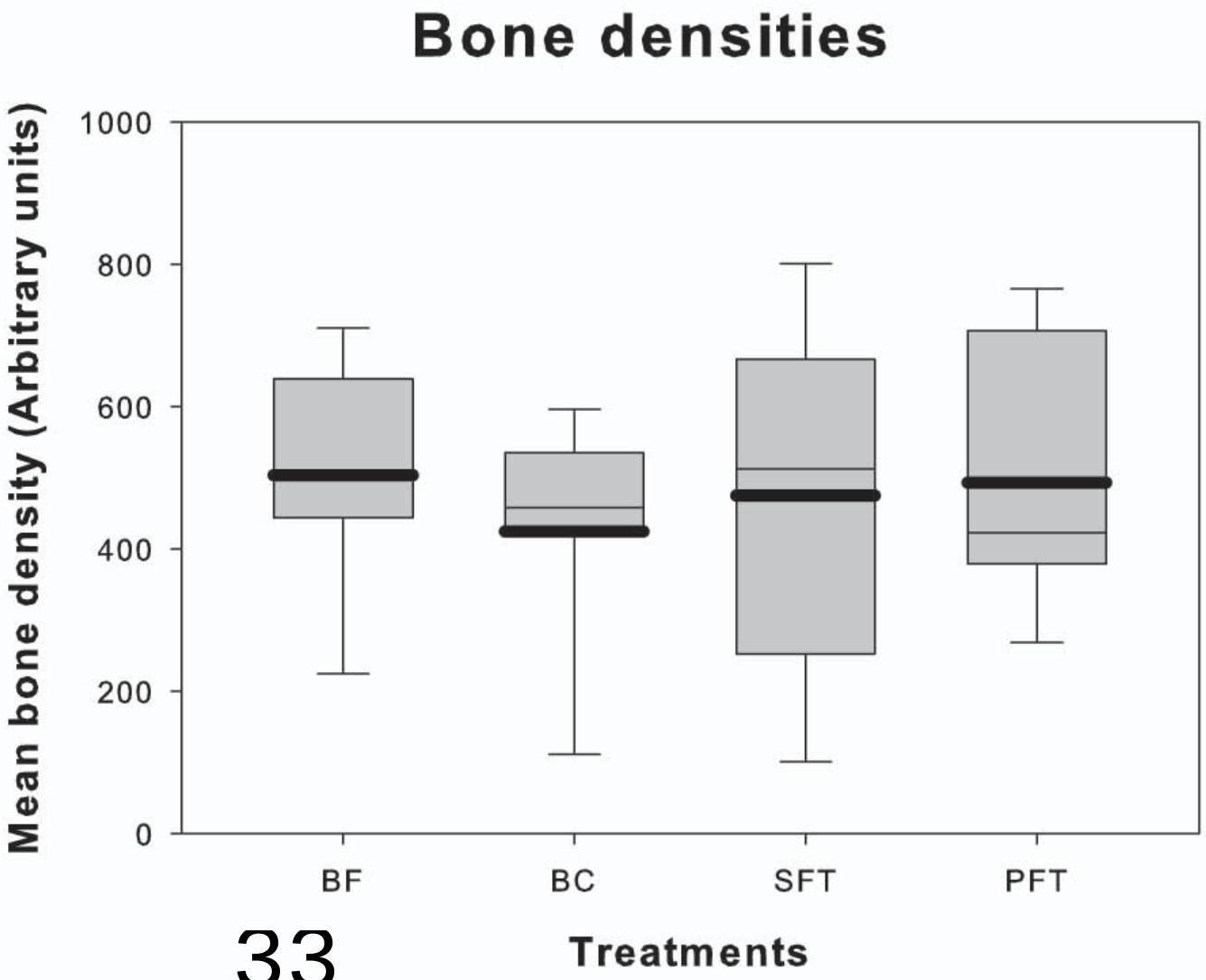
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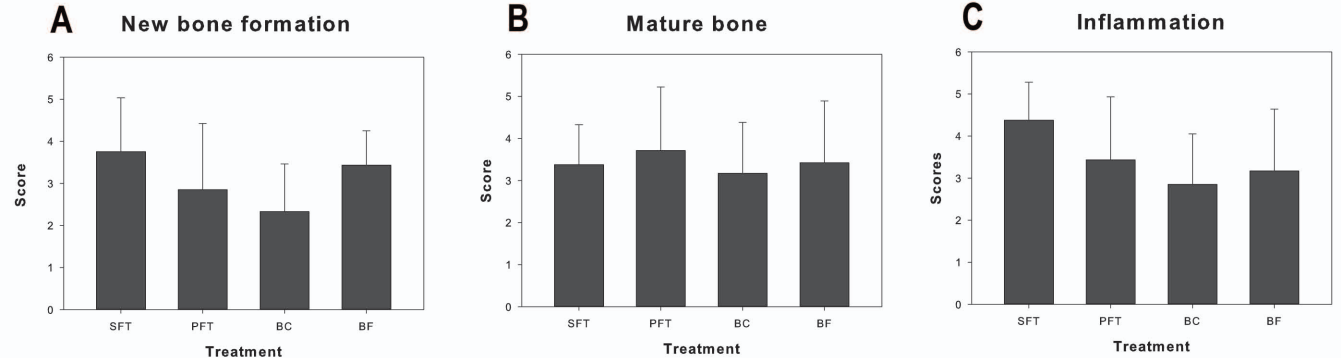
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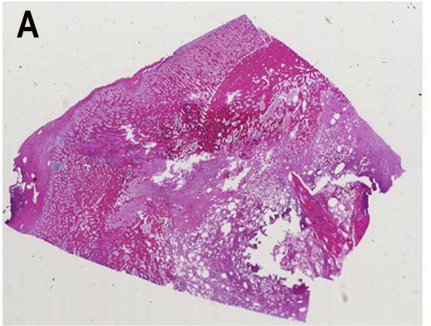
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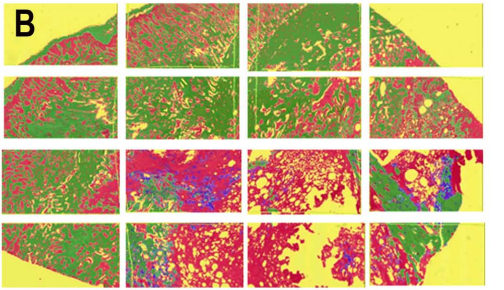
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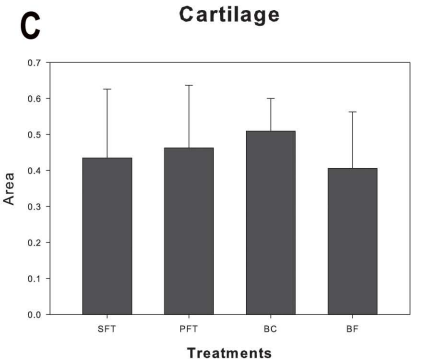
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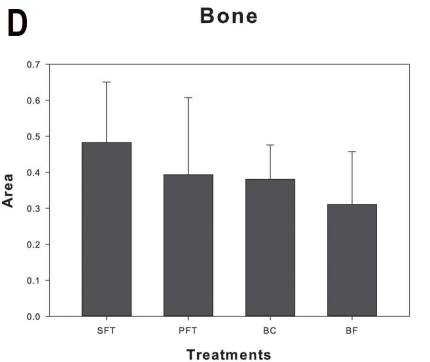
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A Comparison of Postoperative Analgesic Effects of Ultrasound-guided Regional Anesthesia in a Swine Femur Fracture Model

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Title: **A Comparison of Postoperative Analgesic Effects of Ultrasound-guided Regional Anesthesia in a Swine Femur Fracture Model**

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Running head: **Ultrasound-guided regional anesthesia in swine**

Abbreviation: **VAS, visual analog scale**

Abstract

Management of pain in research swine used for studies involving painful procedures is a significant challenge. This study assessed whether a regional anesthesia method is effective for pain control of hindlimb injuries in pigs used for research in bone fracture healing. For this randomized controlled study, we administered regional anesthesia before an experimental femur injury was produced. Using ultrasound guidance, we placed sterile infusion catheters near the sciatic and femoral nerves for infusion of local anesthetic for the first 24 hours following surgery. We evaluated various behavioral and physiologic parameters to test the hypothesis that this method of regional anesthesia would provide superior analgesia compared to systemic analgesic medications alone. We also collected blood samples to evaluate serum levels of cortisol and fentanyl postoperatively. At the end of the study period, we collected sciatic and femoral nerves and surrounding soft tissues for histopathologic evaluation. Treatment animals had lower subjective pain scores compared to control animals. Control animals had an increased time to first feed consumption and required rescue analgesia earlier in the postoperative period than treatment animals. Ultrasound-guided regional anesthesia emerges as a viable and effective adjunct to systemic analgesics for providing pain control in swine with experimental femur fractures.

Introduction

Experimentally-induced injuries in research animals may cause pain that requires the use of analgesia to ensure the welfare of the animal and to avoid the profound physiologic effects of unalleviated pain that can confound research. A study at our institution used swine for an induced femur fracture model to study bone healing. Because of the potentially painful nature of such injuries and the extended follow up required, pain control was a significant concern.

Pain in research swine is often alleviated using systemic opioids. The most common opioid used in research swine is buprenorphine, a centrally-acting mixed opioid receptor agonist/antagonist, which derives most of its analgesic effect from its partial agonistic activity at the mu opioid receptor.[16] However, to achieve continuous analgesia, it is necessary to give multiple parenteral injections over the course of a day, which can be distressing to pigs. Another opioid analgesic that has been used in swine is fentanyl, a centrally-acting pure mu opioid receptor agonist, which can be administered transdermally. However, transdermal fentanyl absorption in the pig is highly variable and may not reliably achieve effective levels in the blood.[20,32] Non-steroidal anti-inflammatory drugs, such as meloxicam, act by inhibiting the cyclooxygenase enzymes, which are key components of inflammatory pain pathways.[16] These drugs may be delivered parenterally or orally. However, oral analgesics may be difficult to deliver reliably in swine and non-steroidal anti-inflammatory drugs alone may not provide sufficient analgesia for moderate to severe pain.[16] Furthermore, in addition to their analgesic and anti-inflammatory effects, NSAIDs can also affect renal function, platelet function, and the integrity of gastrointestinal mucosa, potentially affecting patient welfare and altering the outcomes of research.[16]

An alternative option for providing effective analgesia that can be administered with minimal stress to the animal is perineural infusion of a local anesthetic. Local anesthetics such as lidocaine or bupivacaine produce anesthesia by blocking the function of voltage-gated sodium channels in the neuronal cell membrane, thus inhibiting neuronal signal conduction.[16] In this way, they can produce different degrees of anesthesia, analgesia, and motor blockade in a localized area with minimal systemic effects. Perineural catheters allow administration of regional anesthesia over extended periods of time with minimal stress to the patient. These methods have seen much use in human patients, and constitute an effective option for postoperative pain control.[28]

Successful delivery of regional anesthetic treatments can be facilitated by the use of ultrasound imaging. Regional anesthesia and ultrasound-guided regional anesthesia have been extensively described in the biomedical literature, and many reviews of these techniques are available.[8,9,28,33,40,42-44] Ultrasound guided methods of regional anesthesia have demonstrated distinct advantages over methods such as blind injection or nerve stimulation. Reported advantages include increased success of the nerve block, less local anesthetic usage, faster procedure time, and faster onset.[9,33,40]

Regional anesthesia is commonly used for surgical procedures in veterinary patients.[11,12,31,35,47] Continuous or intermittent infusion of local anesthetics for postoperative or persistent pain in animals has also been reported.[10,48,50]. Techniques for pre-procedural single injection ultrasound-guided and nerve stimulation-assisted regional anesthesia have been described in dogs.[3,4,7,14] Swine have been used as a model for evaluating complications of regional anesthesia, such as intraneural injection and systemic and localized toxicity due to local anesthetics.[1,5,6,30,51,52] However, there are no published studies in the literature on the use of

ultrasound for placement of peripheral perineural infusion catheters for treatment of postoperative pain in animals.

Anesthesia-related studies in swine are scarce. Peripheral nerve regional anesthesia with or without ultrasound guidance for analgesia of painful injuries has not been reported in research pigs. There are few clinical trials in the literature describing pain assessment and control in pigs.[17] This is problematic given the common use of pigs as research models involving surgical procedures and the standards and legal requirements for alleviation of pain in laboratory animals. The development of advanced anesthetic techniques, such as the method we present here, can support advances in medical research while satisfying accepted animal welfare standards.

All animals in our study received systemic opioids, including parenteral buprenorphine and transdermal fentanyl, and non-steroidal anti-inflammatory medications for postoperative analgesia. However, because of the nature of the injury, we recognized the need for highly effective analgesia in the postoperative period and considered regional anesthesia as a viable option. We decided to test the hypothesis that ultrasound-guided regional anesthesia as an adjunct to systemic analgesics would reduce the need for systemic analgesics and improve subjective pain scores compared to systemic analgesics alone for postoperative management of pain associated with femur fracture in research swine.

Materials and Methods

This study was reviewed and approved by the Uniformed Services University of the Health Sciences IACUC and performed in accordance with the Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals*.[21] Our study evaluated 19 female Yorkshire swine (*Sus scrofa domestica*) that were being used in a study on bone healing.

Animals. The pigs were obtained from a commercial source (Animal Biotech Industries, Inc., Danboro, PA) from a herd that is free of pseudorabies, brucellosis, and *Salmonella*. All pigs receive parenteral iron dextran supplementation at 3 days of age. The pigs were been vaccinated for *Mycoplasma hyopneumoniae*, porcine circovirus type 2, swine influenza virus, *Bordetella bronchiseptica*, *Erysipelothrix rhusiopathiae*, and *Pasteurella multocida*, and all received anthelmintic treatment (ivermectin) prior to shipment. Pigs were shipped to our institution in pairs and underwent physical examination by a veterinarian upon arrival at the facility. These entry examinations included weight measurement, recording of vital signs (temperature, heart rate, respiratory rate), and fecal flotation for endoparasites. All parasite examinations were negative.

Housing and Husbandry. Study animals were housed individually in pens in the large animal section of an AAALAC-accredited animal facility. Each pen measured approximately 2.3 X 1.9 meters. Primary enclosures consisted of epoxy coated floors with hard rubber floor mats to provide a non-slip surface within each pen. Walls of primary enclosures consisted of epoxy coated concrete, chain link fencing, and stainless steel panels. Animals were provided water via an automatic watering system and fed a standard swine diet ad libitum. Primary enclosures were cleaned and sanitized twice daily by animal care staff. Each animal was allowed a minimum of 5 days acclimation to the facility before use in the study.

Operating Room. A dedicated operating room was used for all surgical procedures. A Datex/Ohmeda S/5 Anesthesia Delivery Unit and vital sign monitoring system (Datex/Ohmeda; Bromma, Sweden) and an InSight Fluoroscan fluoroscopy machine (Hologic, Inc; Bedford, MA) were used for anesthetic monitoring and radiographic imaging of the injured limb, respectively.

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Anesthesia and monitoring. For analgesia, all pigs received a transdermal fentanyl patch (50 µg/h) approximately one day prior to surgery (mean 21.3 h, SD 3.1 h). Hair was removed from the skin over the dorsal trunk using a commercial depilatory agent (Nair®, Church & Dwight Co., Inc, Princeton, NH). The site was then rinsed, dried, and wiped with isopropyl alcohol before placing the fentanyl patch and applying a transparent film dressing (Tegaderm Film™, 3M, St Paul, MN) over the patch. On the day of surgery, all animals were premedicated for anesthesia with tiletamine-zolazepam (Telazol©) 6 mg/kg IM in the neck. They were then intubated and maintained on isoflurane anesthesia for the duration of the procedure. All animals were maintained on an IV infusion of lactated ringer's solution at a rate of approximately 10 mL/kg/h, and a closed urine collection system was used for the duration of the procedure. A trained anesthesia technician monitored and recorded intraoperative parameters, including electrocardiography, pulse oximetry, capnography, bispectral index, inspired isoflurane concentration, respiratory rate, body temperature, non-invasive blood pressure, and anesthetic depth.

Regional anesthesia administration. Prior to surgery, one animal from each pair was randomly assigned to the treatment (bupivacaine) group and the other to the control (saline) group in a blinded randomized controlled trial design. Once the animals were anesthetized, we used ultrasound guidance to locate the sciatic nerve as it exits the pelvis thru the greater sciatic foramen in the parasacral region and the femoral nerve in the proximal inguinal region. The skin over each site was marked for reference.

After antiseptic preparation and sterile draping of the right dorsal parasacral area, the ultrasound probe was placed in a sterile sleeve and applied to the injection site with sterile ultrasound gel. After identifying the sciatic nerve, a 17 gauge 9 cm Tuohy needle was inserted in

plane with the probe with a short axis view of the nerve. With ultrasound visualization, the needle was advanced until it was immediately adjacent to the nerve. A syringe containing 10 mL of either 0.5% bupivacaine (treatment group) or 0.9% sodium chloride (control group) was attached to the hub of the needle, and after aspirating and confirming the absence of blood, 1 mL was injected. If the ultrasound image showed proper placement of the injected fluid, the remaining volume was infused while observing the ultrasound image. A 19 gauge closed tip multi-orifice polyamide infusion catheter (Perifix® or Contiplex® Tuohy Set, B. Braun, Germany) was then threaded through the Touhy needle, and held in place while the needle was retracted. The catheter was secured in place using tissue adhesive and transparent adhesive dressings with an accessible injection port.

For the femoral nerve, a short axis view of the nerve was obtained in the inguinal area as far cranial as possible. After antiseptic preparation and draping of the lateral flank, the Tuohy needle was introduced through the skin of the lateral ventral flank area cranial to the thigh and directed medially and caudally in plane with the ultrasound probe towards the site of interest, where the femoral nerve exits the inguinal canal. Local anesthetic (0.5% bupivacaine) or saline (10 mL) was then infused and the infusion catheter was placed and secured, as described for the sciatic nerve.

For ultrasound guidance of catheter placement, we used a model DP-6600Vet Universal UMS 700 Digital Ultrasonic Diagnostic Imaging System with a 7.5 megahertz linear transducer (Universal Medical Systems, Inc., New York). We also used a Stimuplex HNS12 peripheral nerve stimulator (B Braun, Germany) with the first four subjects to confirm accuracy of ultrasound in localization of the nerves of interest.

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Injury. Following application of regional anesthesia and aseptic preparation of the right lateral thigh, femurs were pre-stabilized using an external fixation device. A right-side midshaft femoral fracture was then produced using a captive bolt device on the lateral aspect of the thigh. All fractures were evaluated radiographically immediately following injury. The degree of injury was generally consistent across groups with penetration of both cortices and fragmentation around the fracture site. After evaluation, the limb and fixation device were covered with a protective cast.

Postoperative monitoring and analgesia. Following surgery and prior to recovery from anesthesia, all pigs received one dose of buprenorphine 0.05 mg/kg IM and one dose of meloxicam 0.4 mg/kg SC. Repeat doses of bupivacaine (0.25%, 10 mL at each site) or the same volume of sterile saline were then administered at 6-8 hour intervals for the first 24 hours postoperatively, after which the pig was sedated with a combination of ketamine 10 mg/kg and xylazine 2 mg/kg given IM, and the perineural catheters were removed. In all, 4 doses of local anesthesia/saline control were administered: one dose preoperatively and 3 doses postoperatively.

Following full recovery from anesthesia on the day of surgery, pigs were monitored frequently with full evaluations at least every 6-8 hours in the first 24 hours post-surgery. Pigs were subjectively assessed by a clinical veterinarian to determine if pain levels merited rescue analgesia (buprenorphine 0.05-0.1 mg/kg IM). Feed was offered immediately following full recovery from anesthesia, and time to first consumption of any feed was recorded. We also evaluated several pain indicators pre- and postoperatively, including heart rate as measured by direct cardiac auscultation, respiratory rate by observation, and subjective pain assessment.[16,17,22] Activity levels were estimated using a remote telemetry monitoring system (Data Sciences

International, St Paul, MN), which counted physical movements by the subjects starting the day prior to surgery through the fourth postoperative day.

To obtain baseline measurements, all evaluations were performed at two time points preoperatively: once before and once after placement of a transdermal fentanyl patch (Figure 1). Evaluations were then repeated at 3 time points on the day of surgery: 2 and 4 hours after recovery from anesthesia and again the night following surgery (approximately 10-11 hours post-surgery). Each pig was then evaluated twice daily for the next four days: once each morning (AM) and once each afternoon (PM). Additionally, each pig was weighed prior to surgery and weekly thereafter.

Five categories of subjective pain indicators were scored using a modified visual analog scale (VAS): Passive observation of the animal from outside its enclosure before entering (VAS1, Observation), strength and character of response to physical contact when placing a hand on the side of abdomen (VAS2, Contact), impairment of ability or of willingness to ambulate (VAS3, Ambulation), nature and intensity of vocalization (VAS4, Vocalization), and an overall subjective assessment of pain level after completing the full evaluation (VAS5, Overall). The VAS chart consisted of a 100 mm horizontal line for each category. After assessing the animal, the evaluator placed a mark on the line according to the assessed level of pain, with pain scores increasing from left to right on the scale (Figure 2). The VAS score was then derived by measuring the distance of the mark in millimeters from the left side of the scale. In all cases, a higher score indicated more severe impairment or pain.

All personnel involved in the study were blinded to treatment. Ninety percent of evaluations were performed by one individual. In cases where other evaluators were used, each pair of animals was evaluated by the same individual.

Blood sampling and analyses. Blood samples were collected from the left femoral vein under anesthesia intraoperatively and under heavy sedation at approximately 24 hours post-surgery to measure serum levels of cortisol and fentanyl. Blood samples were also taken under sedation one week after surgery to measure serum fentanyl levels.

Serum cortisol levels were measured using a commercially available porcine cortisol ELISA kit (Novatein Biosciences, Cambridge, MA). Serum fentanyl was measured using a competitive direct ELISA kit (BQ Kits, San Diego, CA). All assays were conducted according to manufacturer instructions.

Hematology and serum biochemistry data obtained at a commercial reference laboratory were analyzed for changes consistent with pain or distress, such as hyperglycemia or altered neutrophil to lymphocyte ratios. We also analyzed serum chemistry data for indirect signs of pain, such as physiologic indicators of dehydration (azotemia, hyperalbuminemia, increased hematocrit, hyperosmolality) due to decreased water intake. Total serum protein was measured using a clinical refractometer.

Histopathology. At three weeks post-surgery, all animals were euthanized and necropsied for collection of peripheral nerves and surrounding soft tissue for histopathological analysis. Bilaterally, 5 cm portions of both the femoral and sciatic nerves were dissected away along with surrounding skeletal muscle for evaluation. Tissue samples were fixed in 10% buffered formalin, paraffin wax-embedded, sectioned at approximately 5 μm, and submitted to the Armed Forces Radiobiology Research Institute Division of Comparative Pathology for evaluation. Sections were routinely stained with hematoxylin and eosin and examined by light microscopy in a blinded manner for histopathological evaluation by a board-certified veterinary pathologist. Sections were evaluated for any pathological change within the peripheral nervous

system and surrounding soft tissues, with specific emphasis on degenerative or inflammatory changes within nerves.

Statistical analysis. We evaluated data graphically for normality and used Student's t-test to compare means or the Mann-Whitney U test to compare medians, as appropriate, for continuous independent variables. For data with repeated measures, we used Student's t-test with no adjustment for multiple comparison to compare preoperative time points and postoperative time points outside the expected period of analgesia (i.e., day 2 and beyond). We used a linear mixed effects model to compare overall differences between treatment groups and differences between treatment groups over time during the treatment period (i.e., day 0 and day 1). When the main effect of group and/or the group by time interaction were significant in the mixed model, individual time points were compared using Student's t-test with no adjustment for multiple comparisons. We used the log rank test and Kaplan-Meier curves to compare data measuring time to an event, and estimated hazard ratios using Cox proportional hazards regression.

Physical activity data were collected and processed using the DSI Ponema Physiology Platform (DSI Ponema, Valley View, OH). Telemetry data containing counts of physical movements were then divided into 12 hour segments corresponding to the light (7:00 AM to 7:00 PM) and dark (7:00 PM to 7:00 AM) phase of the room light schedule. Total counts per hour for each segment were calculated and then compared between treatment and control groups using a linear mixed effects model as described above.

Statistical software packages were used to perform all statistical analyses (IBM SPSS Statistics Version 19, Chicago, IL) and to create graphs of study data (GraphPad Prism 6, San Diego, CA). Unless otherwise indicated, data are expressed as mean (+/-SEM). All differences

were considered significant if the two-tailed p value was less than 0.05. As this study was appended to an ongoing study, sample size was predetermined by the needs of the parent study.

Results

Three pigs were removed from the study early. Two of these (one treatment and one control) were euthanized early due to inadequate stabilization of the femur fracture. Another pig in the treatment group expired in the immediate post-anesthetic period due to apparent laryngospasm following endotracheal tube removal. The control group had a significantly higher heart rate at the time of pre-study physical examination (Table 1). Weight, temperature, and respiratory rate did not differ significantly. No difference was observed in pain control between the initial 4 subjects in which nerve electrostimulation was used and the remaining subjects in which ultrasound alone was used for catheter placement.

Each animal was evaluated on how soon after surgery they began eating and defecating. The median (+/-standard error of the median) time at which the animals in each group ate their first meal was 4 (+/-0.37) hours for the treatment group and 11 (+/-5.0) hours for the control group (Figure 3). Median time to first defecation was 4.0 (+/-1.4) hours for the treatment group and 19 (+/-3.3) hours for the control group (Figure 4).

At each evaluation, the evaluator determined whether or not the animal was in sufficient pain to merit rescue analgesia. The median time of first intervention was 28 (+/-22.6) hours for the treatment group and 11 (+/-10.4) for the control group. (Figure 5) When comparing total quantity of buprenorphine administered postoperatively, control subjects tended to receive greater quantities on average, although the difference did not achieve statistical significance (Figure 6).

No significant differences were observed in any of the VAS data collected preoperatively or beyond day 1 postoperatively. In the evaluation of day 0 and day 1 VAS scores, mean passive observation (VAS1) score differences were not statistically significant between groups (p=0.55) in the mixed effects model (Figure 7). Mean scores for response to contact (VAS2) were higher at all time points for the control group, and there was a significant difference in overall means between treatment groups (p=0.009) in the mixed effects model. In the pairwise comparison, the difference achieved significance at 3 time points within the first 24 hours of surgery (Figure 8). Mean ambulation scores (VAS3) did not differ significantly between treatment groups (p=0.45) in the mixed effects model (Figure 9). Mean scores for vocalization (VAS4) were higher in the control group at most time points in the first 24 hours after surgery (Figure 10). However, the mixed model analysis did not show a statistically significant effect of treatment between groups (p=0.065). In the overall pain level assessment score (VAS5), there was a significant difference between treatment groups in the mixed effects model (p=0.008), and two time points differed significantly in the pairwise comparison (Figure 11). None of the VAS parameters showed a significant interaction between treatment and time in the mixed effects model.

Physical examination parameters. At each evaluation, heart rate was measured by the evaluator by cardiac auscultation with a stethoscope, and respiratory rate was measured by visual observation of thoracic movement. Heart rate decreased in the treatment group during the first 24 hours after treatment while staying relatively constant in the control group (Figure 12). However, the average heart rate did not differ statistically between treatment groups overall (p=0.061) or between groups over time (p=0.64) in the mixed effects model.

The mean respiratory rate was higher in the control group during the first 24 hours post-surgery (Figure 13). However, in the mixed effects model, the differences between treatment groups (p=0.18) and between groups over time (p=0.25) were not significant.

Activity levels, estimated by frequency of physical movements or motion measured telemetrically in the postoperative period, dropped sharply following surgery relative to preoperative levels. Activity levels then fluctuated on a regular circadian basis, with higher activity levels during the day and lower levels at night (Figure 14). While activity levels tended to be slightly higher in the control group, we did not observe a significant effect of treatment (p=0.84) or a significant interaction between treatment and time (p=0.99) in the mixed effects model.

Clinical pathology. Serum biochemistry values did not differ between treatment groups (Table 2). Of the hematology parameters measured, RBC count, hemoglobin, and hematocrit measured at 24 hours were all significantly elevated in the control group (Table 3). Blood samples were collected for serum fentanyl measurement at 3 approximate time points: 24, 48, and 72 hours following application of a transdermal fentanyl patch (Figure 15). The mean values (+/-SEM) at each respective time point were 0.31 (+/-0.070) ng/mL, 0.21 (+/-0.045) ng/mL, and 0.12 (+/-0.023) ng/mL. There were no significant differences between treatment and control groups in mean fentanyl levels at any of the time points.

Histopathology. Histopathological examination of all submitted tissues revealed an interesting mix of lesions in both saline and bupivacaine cohorts. These included examples of degenerative lesions of peripheral nerves (Figure 16) and lesions of skeletal muscle (Figure 17).

Within the saline cohort, 89% of the animals showed histopathological evidence of pathology in at least one of the sampled nerves. Of these animals, 75% had right sided pathology, 75% had left sided pathology, and 62.5% showed bilateral lesions. Within the bupivacaine cohort, 62.5% of the animals displayed nerve fiber pathology, of which 100% had right sided lesions and 25% had bilateral lesions. However, none of these differences achieved statistical significance.

Discussion

This study provides substantial evidence for a beneficial effect of regional anesthesia in addition to traditional systemic analgesics for hindlimb pain in swine. Most notably, untreated animals required rescue analgesia earlier following surgery than did treated animals. Furthermore, treated animals ate food and defecated earlier in the postoperative period than their untreated counterparts. As anorexia and decreased bowel function may occur with pain in animals, these differences provide evidence for an analgesic effect of the treatment. The observed effect on time to defecation may also be a result of greater inhibition of bowel function in control animals because they received more postoperative opioid analgesics, which are known to decrease motility and increase transit time in the gastrointestinal tract.[16]

Subjective pain assessment results in some categories also suggest a beneficial effect of the treatment. Control animals had higher pain scores despite the fact they tended to receive rescue analgesia sooner than treatment animals. The response to physical contact (VAS2) and the overall assessment of pain levels following examination (VAS5) appeared to best discriminate between treatment groups. In addition to demonstrating a positive effect of the regional

anesthetic treatment, this shows that a subjective assessment that includes the response to physical contact may be helpful in assessing pain in swine.

VAS score trends over time are also consistent with the expected time course of the regional anesthesia effects. Scores tended to be very similar between treatment groups at the first postoperative evaluation (2 hours post-surgery). At that time point, it might be expected that the residual effects of anesthesia and postoperative analgesics either controlled pain adequately or resulted in sufficient sedation to mask the effects of pain as assessed by the subjective observer. However, starting at the next time point (4 hours post-surgery), VAS scores in the control group tend to be higher than in the treatment group, suggesting better pain control in the treatment group. This difference persists thru the treatment period and then disappears somewhere between 8 and 24 hours after the last treatment was given, as would be expected given the typical duration of effect of bupivacaine. This time trend is readily apparent for response to contact, vocalization, and overall pain score. However, it is much less pronounced for simple observation from outside the cage, and is completely absent for ambulation scores.

It is noteworthy that simple observation of the animal from outside the pen—a common practice for pain assessment in laboratory animals—failed to detect any difference between treatment groups. Furthermore, while ability to ambulate improved significantly in both groups over time, it did not differ between treatment groups, suggesting this parameter may not be a reliable indicator of pain control in this model. It may also be that cast material on the legs impeded ambulation sufficiently in both groups to obscure any pain-related differences in ambulation. It is also possible that ambulation was inhibited by impairment of motor function in the treated limb due to the regional anesthesia, though this was not assessed in the study.

At the time of initial entry into the facility prior to any experimental manipulation, average heart rate on physical examination was higher in the pigs that would form the control group than those in the treatment group. However, there were no other indications of baseline differences between treatment and control groups. Furthermore, after acclimation to the facility, subsequent pre- and postoperative examinations showed no significant difference in heart rate between groups. In light of this, we believe this initial difference represents random variation in conditions and not any true baseline difference between groups.

Postoperative heart rates and respiratory rates measured during physical examination did not differ significantly between treatment groups. However, a trend for decreased heart rate was noted in the treatment group for about the first 24h post-surgery. This decrease in heart rate may be a result of decreased activity due to immobility, a direct effect of bupivacaine on heart function, or decreased pain.

We also analyzed various clinical laboratory parameters as indirect indicators of postoperative pain. Erythrocyte measures (RBC count, hematocrit, and hemoglobin) differed between groups at the 24-hour postoperative time point, with slightly lower indices in the treatment group. Changes in erythrocyte indices may occur with changes in hydration, which may be occur if water intake is decreased due to pain. While the small difference observed in our study could be explained by hemoconcentration in the control group due to unapparent dehydration, evaluation of blood chemistry values (e.g., serum Na, Cl, K, BUN, creatinine, and total protein) and clinical status revealed no other indications of a difference in hydration between groups. Furthermore, no significant difference between groups appeared in hematocrits measured intraoperatively or at 1 week following surgery. This difference at 24 hours may

represent random variation in RBC mass between the two groups, though we cannot rule out the possibility of a direct effect of the drug (bupivacaine) on red blood cells in these animals.

We measured serum cortisol as an indirect measure of pain-induced sympathetic nervous system stimulation.[18,39] Cortisol levels did not differ significantly between the treatment and control groups. This may be due to lack of sensitivity of the assay, effects of handling and sedation for blood collection, or high stress in both groups due to immobility and handling. Furthermore, factors unrelated to pain may also affect these measurements. For example, cortisol may be altered with distress due to handling and is subject to circadian variation.[26] Samples were collected at a uniform time of day to avoid any effects of circadian variation, and all animals were sedated in a uniform manner to avoid introducing variation into blood value data. However, no reference range is available for serum cortisol in swine, and levels vary with age, weight, sex, feed consumption, environment, and handling, making absolute levels difficult to interpret.[15,23,38] Furthermore, since we did not measure preoperative baseline cortisol levels, we cannot determine if the cortisol levels observed postoperatively represented stress-induced elevations.

Blood glucose levels did not differ significantly between treatment and control groups in our study. This may suggest that blood glucose is an insensitive measure of pain in swine. However, blood glucose levels can be transiently affected by stress, fear, pain, feed consumption, and the use of sedatives.[13,25,37] Therefore, it is also possible any stress induced alterations in blood glucose could have been obscured by differences in feed consumption. The treatment group tended to have a shorter time to feed consumption post-surgery. However, the amount of feed consumed was not quantified, so the degree to which feeding differences may have altered blood glucose levels cannot be determined.

We measured serum fentanyl levels to ensure that the analgesic effect was similar between groups and to evaluate whether transdermal fentanyl delivery resulted in consistently adequate analgesic levels for 72 hours. Our measured serum fentanyl levels were similar to those of other studies. For example, one study evaluated transdermal fentanyl (50 µg/h) in Yorkshire-Landrace pigs and reported serum fentanyl levels at 24 hours of 0.47 ng/mL (range 0.17-1.0), with peak concentrations occurring at around 12-24 hours.[32] In another study of transdermal fentanyl (100 µg/h) in Yucatan minipigs, plasma fentanyl concentration peaked within 48 hours, with peak concentrations ranging from 0.38 to 0.99 ng/ml.[49] The authors of that study postulated based on extrapolation from other species that therapeutic plasma fentanyl levels in the pig may reasonably be assumed to fall within the approximate range of 0.2 to 3.0 ng/mL. If we assume the conservative end of this estimate (0.2 ng/mL), then 50%, 64%, and 100% of the animals in our study were below therapeutic levels at 24, 48, and 72 hours, respectively. This finding places some doubt on the reliability of transdermal fentanyl delivery in swine.

Some of the histopathologic lesions observed in our study were similar to the findings of others. For example, one study found increased lymphocytic and granulocytic infiltration peri- and intraneurally within 6 hours of bupivacaine instillation in pigs.[46] In another study describing histopathologic changes in pigs 7 and 28 days after a 6 hour perineural infusion of bupivacaine, investigators observed perivascular accumulation of inflammatory cells, myocyte regeneration, calcific myonecrosis, fibrous scar tissue, and degenerative changes.[51] However, we found no statistically significant patterns of distribution between treatment and control groups or between left and right limbs. The prevalence of background lesions in the neural and perineural soft tissue may obscure any potential correlation between lesions and treatments. Consequently, it is not

clear if the lesions were incidental or related to injection of fluid, presence of bupivacaine, the bone injury, or prolonged recumbency.

This study faces several limitations. The small sample size and relatively high variability in some parameters limits the power to detect significant differences. Another limitation involves the difficulty in evaluating pain in animals such as pigs.[2] For example, pigs commonly object strongly to human handling even in the absence of pain. This makes it difficult to discern whether avoidance, guarding, vocalization, or reactions to touch are indicators of pain or simply behavioral idiosyncrasies. Furthermore, depending on the nature and severity of pain, a pig may demonstrate increases or decreases in activity, movement, and responsiveness.[16,17,22] It may also be that some indicators of pain may be more sensitive than others, and some may only be detected in cases of severe or protracted pain or distress. Some analgesics, such as fentanyl and buprenorphine, can cause sedation, which may confound interpretation of behavioral signs of pain. Lastly, control animals received rescue analgesia earlier, which may have abrogated some of the differences in pain-related parameters. Even objective measures of pain are fraught with difficulty, since they may be altered by physiologic processes independent of pain. Heart rate, for example, may increase with painful stimuli, but many other non-painful factors can increase or decrease heart rate, including anesthesia, drugs, excitement, and hemodynamic status. This makes interpretation of such parameters problematic.

Another challenge for this study is confirming continual efficacy of the regional block. It is possible that after placement, the infusion catheter tip may migrate as the animal moves, resulting in some or all of the local anesthetic not reaching the desired site. If catheter migration occurred in some subjects, it could have artificially shortened the apparent duration of analgesia

achieved. Ideally, repeat imaging of the catheter prior to its removal would have confirmed proper location throughout the treatment period.

The relative novelty of this procedure necessitates further research. Future studies may evaluate alternative local anesthetics, such as ropivacaine, or formulations containing vasoconstrictors, as these may reduce toxicity and increase the intensity and duration of local anesthetic blocks.[29,35,45] Other compounds may be investigated for use as adjuncts to local anesthetics in peripheral nerve blocks, including alpha-2 adrenergic agonists, opioids, benzodiazepines, or corticosteroids.[36,41] It may also be preferable to measure different behavioral or physiologic parameters to assess pain, such as quantified intensity and frequency of vocalization or neuroendocrine markers of pain.[19,34] Future studies may examine the use of continuous infusion devices to provide more stable regional anesthesia over time than can be achieved with bolus administration. It would also be useful to evaluate the efficacy of regional anesthesia treatment over a longer period of time and any histopathologic effects of prolonged infusion protocols.

This study makes several significant contributions to laboratory animal welfare. It presents a postoperative pain assessment method for use in swine, which may be adapted and improved for clinical or investigational pain assessment. Another important finding of this study is the potential inability of transdermal fentanyl to adequately and consistently control pain in this model. In light of this, we recommend that transdermal fentanyl not be used as a sole method of analgesia for moderate to severe pain in swine. Most importantly, our data support the hypothesis that ultrasound-guided regional anesthesia provides superior analgesia compared to systemic analgesics alone for management of pain in hindlimb injuries in swine. The temporal patterns of treatment effects fit well with expected values, with maximum differences observed

during the postoperative treatment period and disappearing between 8 and 24 hours after cessation of treatment, which is consistent with the known duration of effect of bupivacaine.[27] In conclusion, ultrasound-guided regional anesthesia represents a significant refinement in pain management in laboratory animals and may realize superior analgesia with fewer potential systemic effects, thus improving both animal welfare and the validity of research outcomes.

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Disclaimers

The authors declare no competing interests. The views of the authors do not purport to reflect the position of the Uniformed Services University or the Department of Defense.

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Figures and tables

Figure 1. Time line of study events. Asterisks (*) indicate where boluses of either bupivacaine or saline were given.

Figure 2. VAS scoring sheet used for subjective pain level assessments. Evaluators were instructed to place a tic mark on the line at the most appropriate position along the line, using the

descriptions in the boxes as guidelines. In general, the left side of the scale represents no pain, and the right side the highest level of pain.

Figure 3. Kaplan-Meier cumulative survival curve showing time in hours to first consumption of feed following surgery. Hazard ratio (95% CI): 4.4 (1.0 - 18.7). The curves differ significantly (p=0.048).

Figure 4. Kaplan-Meier cumulative survival curve showing time in hours to first bowel movement following surgery. Hazard ratio (95% CI): 5.0 (1.3 - 19.6). The curves differ significantly (p=0.021).

Figure 5. Kaplan-Meier cumulative survival curve showing time in hours to first administration of rescue analgesia following surgery. Hazard ratio (95% CI): 3.9 (1.0 - 14.7). The survival curves differ significantly (p=0.044).

Figure 6. Average total daily quantity of buprenorphine administered per animal on the day of surgery (Day 0) and each of the next 3 days post-surgery. Error bars: +/-1 SEM.

Figure 7. Mean VAS1 (Observation) scores by treatment group. Arrow represents last dose of local anesthetic/saline control. d0=day of surgery, d1= first day following surgery, etc. Error bars: +/-1 SEM.

Figure 8. Mean VAS2 (Contact) scores by treatment group. Arrow represents last dose of local anesthetic/saline control. Statistically different time points (Student's t test) are indicated with an asterisk. d0=day of surgery, d1= first day following surgery, etc. Error bars: +/-1 SEM.

Figure 9. Mean VAS3 (Ambulation) scores by treatment group. Arrow represents last dose of local anesthetic/saline control. d0=day of surgery, d1= first day following surgery, etc. Error bars: +/-1 SEM.

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Figure 10. Mean VAS4 (Vocalization) scores by treatment group. Arrow represents last dose of local anesthetic/saline control. d0=day of surgery, d1= first day following surgery, etc. Error bars: +/-1 SEM.

Figure 11. Mean VAS5 (Overall) scores by treatment group. Arrow represents last dose of local anesthetic/saline control. Statistically different time points (Student's t test) are indicated with an asterisk. d0=day of surgery, d1= first day following surgery, etc. Error bars: +/-1 SEM.

Figure 12. Mean heart rate as measured by direct cardiac auscultation during physical examination. Arrow represents last dose of local anesthetic/saline control. Error bars: +/-1 SEM.

Figure 13. Mean respiratory rate as measured by direct observation during physical examination. Arrow represents last dose of local anesthetic/saline control. Error bars: +/-1 SEM.

Figure 14. Mean number of movements counted per hour during the day and night phases. Error bars: +/-1 SEM.

Figure 15. Serum fentanyl levels in ng/mL by elapsed time in hours since placement of a fentanyl patch (50 μg/h). Boxes represent bupivacaine treated subjects and circles are saline controls. Blood sampling occurred at the following times in hours (mean +/- standard deviation) following fentanyl patch placement: first sampling at 21.1 +/- 2.5 hours, second sampling at 47.4 +/- 3.5 hours, and third sampling at 73.2 +/- 2.7 hours.

Figure 16. Right sciatic nerve of a bupivacaine-injected animal showing axonal degeneration and loss characterized by hyperchromatic condensation of the axons (arrows). Hematoxylin and eosin, magnification 600x.

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Figure 17. Right femoral nerve of a bupivacaine-injected animal showing skeletal muscle with rhabdomyocytic atrophy and loss that is focally extensive and severe with abundant fibrosis. Hematoxylin and eosin, magnification 400x.

Table 1. Pre-study physical examination data for all pigs separated by treatment group.

Parameter	Treatment group	Mean +/- SEM	P value
Weight (kg)	Treatment	27.8 +/- 2.3	0.90
	Control	28.2 +/- 2.8	
Temperature (^{o}C)	Treatment	38.9 +/- 0.10	0.088
	Control	39.2 +/- 0.11	
Heart rate (bpm)	Treatment	118.8 +/- 6.9	0.020
	Control	151.3 +/- 11.0	
Respiratory rate (breaths/minute)	Treatment	43.2 +/- 3.3	0.12
	Control	50.7 +/- 3.3	
Age (days)	Treatment	82.2 +/- 4.6	0.97
	Control	82.4 +/- 5.2	

n=10 for treatment, n=9 for control

Table 2. Serum chemistry data at 24 hour post-surgery.

Serum chemistry parameter	Treatment	N	Mean +/-SEM	p	Reference range[a]
Sodium (mmol/L)	Bupivacaine	7	145.2 +/-4.7	0.43	142-149

	Saline	7	145.0 +/-1.0		
Chloride (mmol/L)	Bupivacaine	7	100.3 +/-2.6	0.69	100-109
	Saline	7	101.3 +/-0.92		
Glucose (mg/dL)	Bupivacaine	7	96.3 +/-8.3	0.25	85-160
	Saline	7	113.3 +/-7.2		
BUN (mg/dL)	Bupivacaine	7	7.6 +/-0.48	0.24	6-30
	Saline	7	8.3 +/-0.65		
Creatinine (mg/dL)	Bupivacaine	7	1.1 +/-0.081	0.79	0.5-2.1
	Saline	7	1.2 +/-0.064		
Serum total protein (g/dL)	Bupivacaine	8	5.0 +/-0.43	0.11	6.1-7.5
	Saline	9	5.9 +/-0.33		
Cortisol (ng/mL)	Bupivacaine	8	16.2 +/-0.72	0.42	
	Saline	9	17.1 +/-0.67		

[a]Reference ranges were provided by the commercial laboratory conducting the analyses; no reference range has been established for cortisol in swine.

Table 3. Hematology results at 24 h post-surgery.

Parameter	Treatment	N	Mean+/- SEM	p	Reference range[a]
Hematocrit (%)[b]	Bupivacaine	6	27.4 +/-0.49	0.011	36.9-55
	Saline	6	29.5 +/-0.50		
RBC count (M/uL)	Bupivacaine	6	5.4 +/-0.082	0.024	5.5-8.2

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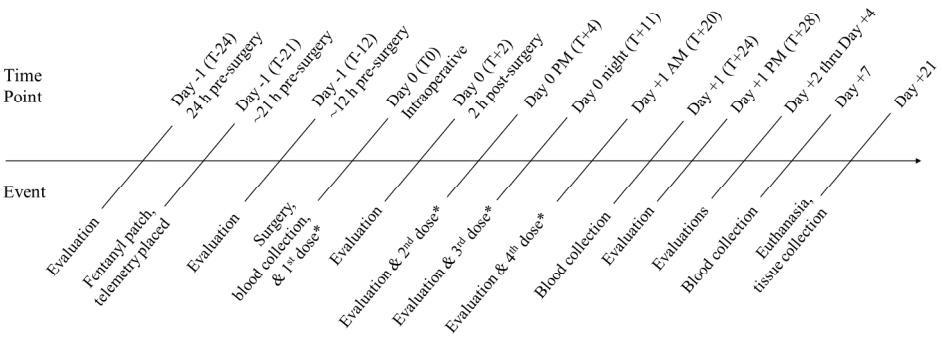
	Saline	6	5.9 +/-0.14		
	Bupivacaine	6	8.9 +/-0.15		
Hemoglobin (g/dL)				0.012	12.6-19.4
	Saline	6	9.6 +/-0.19		
	Bupivacaine	6	1.8 +/-0.84		
N:L ratio [c]				0.91	0.7
	Saline	6	1.9 +/-0.56		

[a]Reference ranges were provided for all erythrocyte indices by the reference laboratory conducting the analyses.

[b]Samples were collected at 24 hours into K_3EDTA tubes from sedated subjects.

[c]N:L (Neutrophil to lymphocyte) ratio reference value was obtained from a reference text.[24]



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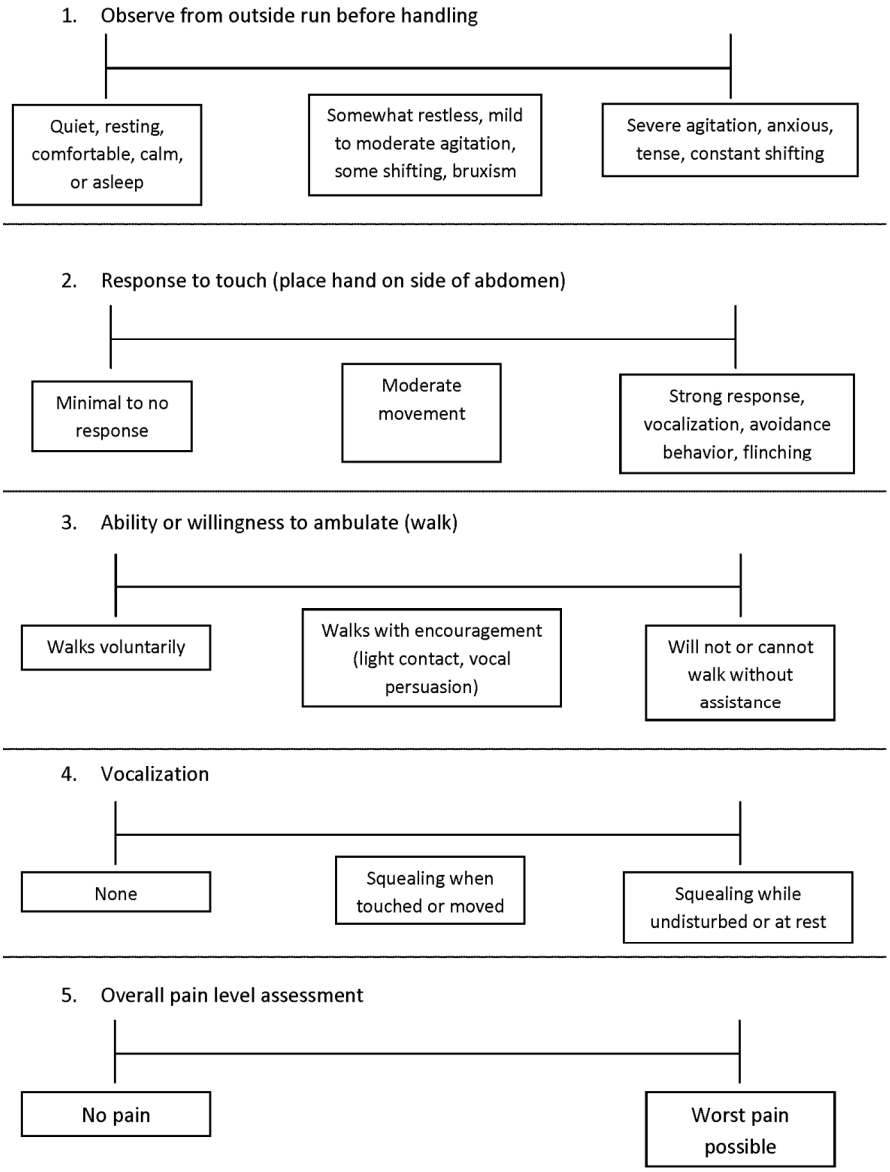

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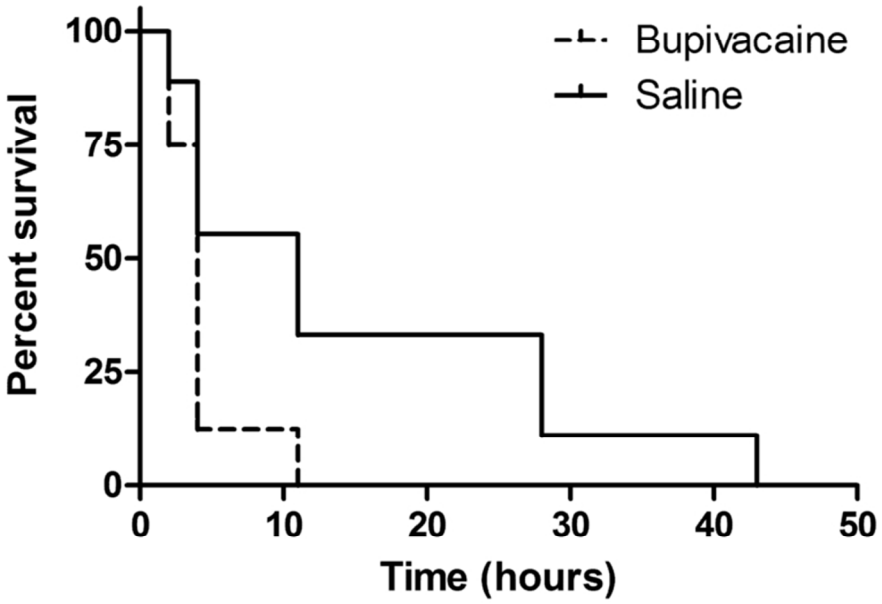

Figure 3. Kaplan-Meier cumulative survival curve showing time in hours to first consumption of feed following surgery. Hazard ratio (95% CI): 4.4 (1.0 - 18.7). The curves differ significantly (p=0.048).
73x50mm (300 x 300 DPI)

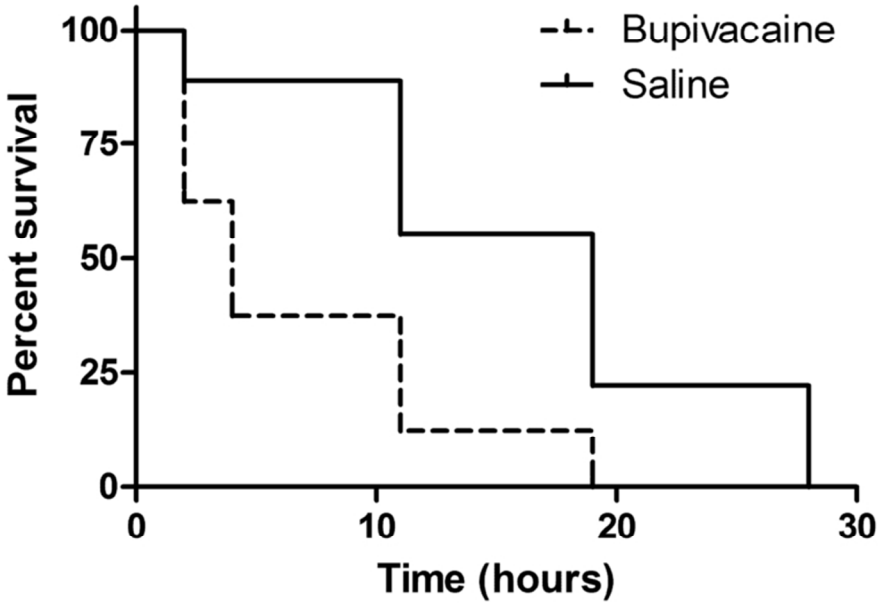

Figure 4. Kaplan-Meier cumulative survival curve showing time in hours to first bowel movement following surgery. Hazard ratio (95% CI): 5.0 (1.3 - 19.6). The curves differ significantly (p=0.021).
73x51mm (300 x 300 DPI)

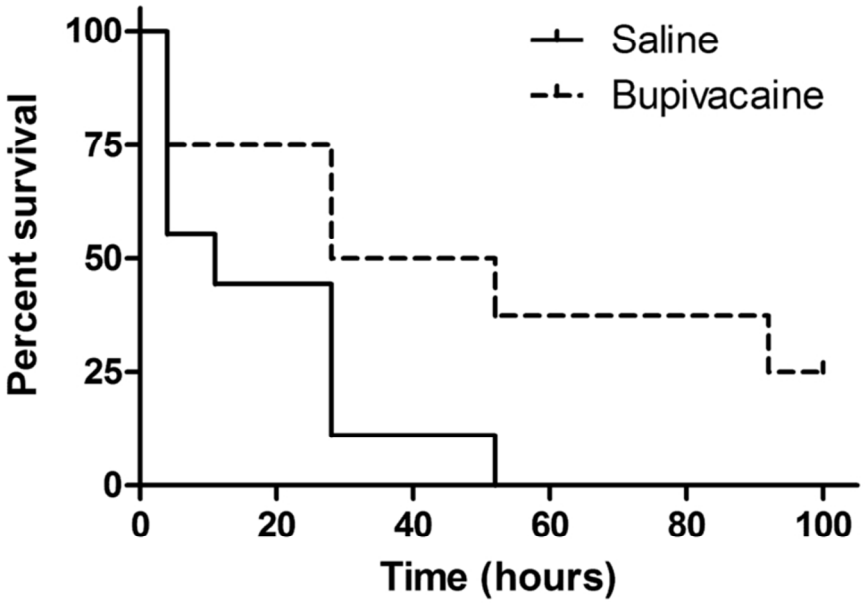

Figure 5. Kaplan-Meier cumulative survival curve showing time in hours to first administration of rescue analgesia following surgery. Hazard ratio (95% CI): 3.9 (1.0 - 14.7). The survival curves differ significantly (p=0.044).
73x50mm (300 x 300 DPI)

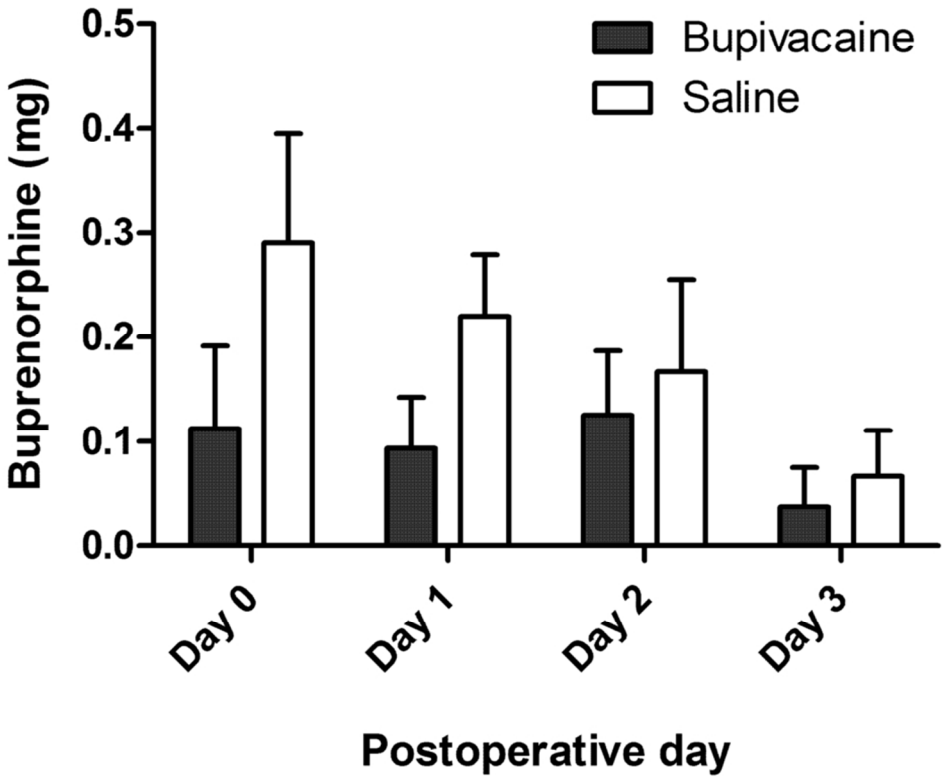

Figure 6. Average total daily quantity of buprenorphine administered per animal on the day of surgery (Day 0) and each of the next 3 days post-surgery. Error bars: +/-1 SEM
83x72mm (300 x 300 DPI)

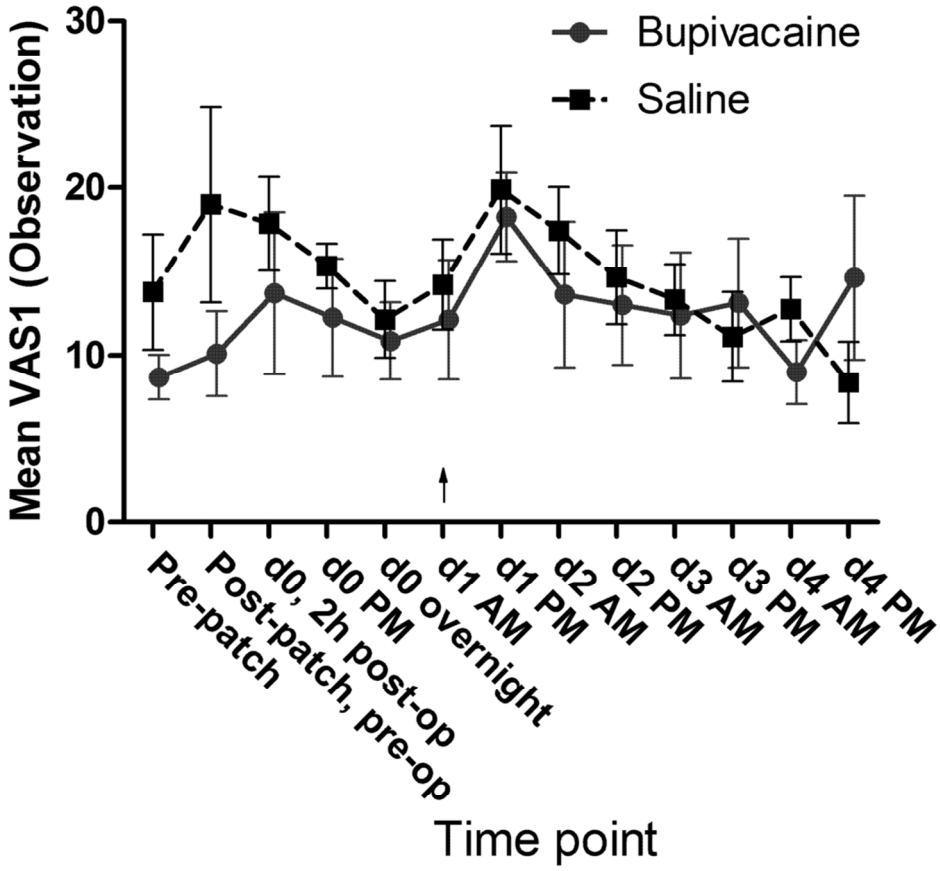

Figure 7. Mean VAS1 (Observation) scores by treatment group. Arrow represents last dose of local anesthetic/saline control. d0=day of surgery, d1= first day following surgery, etc. Error bars: +/-1 SEM.
96x91mm (300 x 300 DPI)

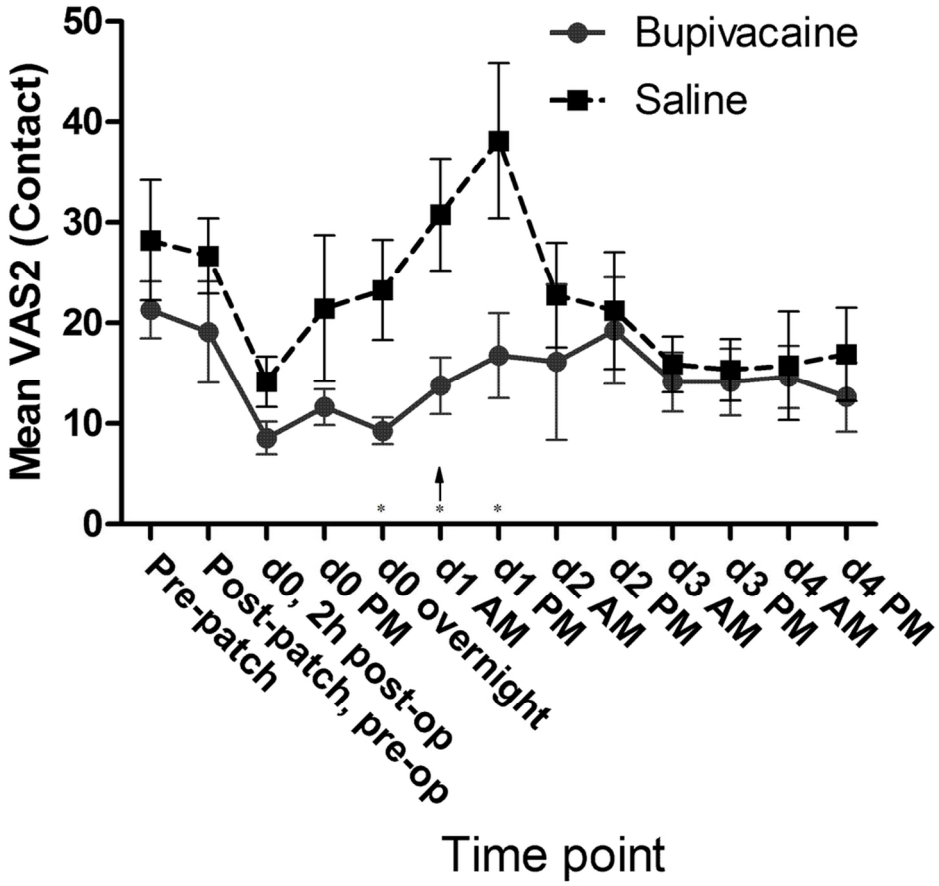

Figure 8. Mean VAS2 (Contact) scores by treatment group. Arrow represents last dose of local anesthetic/saline control. Statistically different time points (Student's t test) are indicated with an asterisk. d0=day of surgery, d1= first day following surgery, etc. Error bars: +/-1 SEM
97x94mm (300 x 300 DPI)

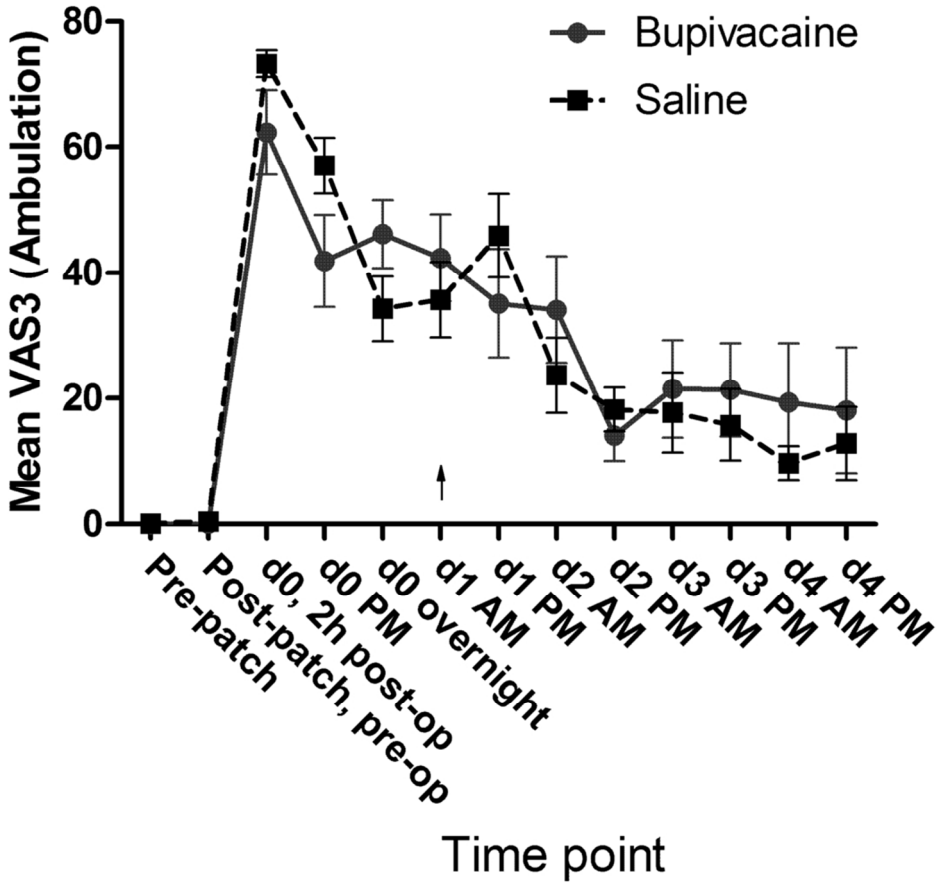

Figure 9. Mean VAS3 (Ambulation) scores by treatment group. Arrow represents last dose of local anesthetic/saline control. d0=day of surgery, d1= first day following surgery, etc. Error bars: +/-1 SEM. 97x94mm (300 x 300 DPI)

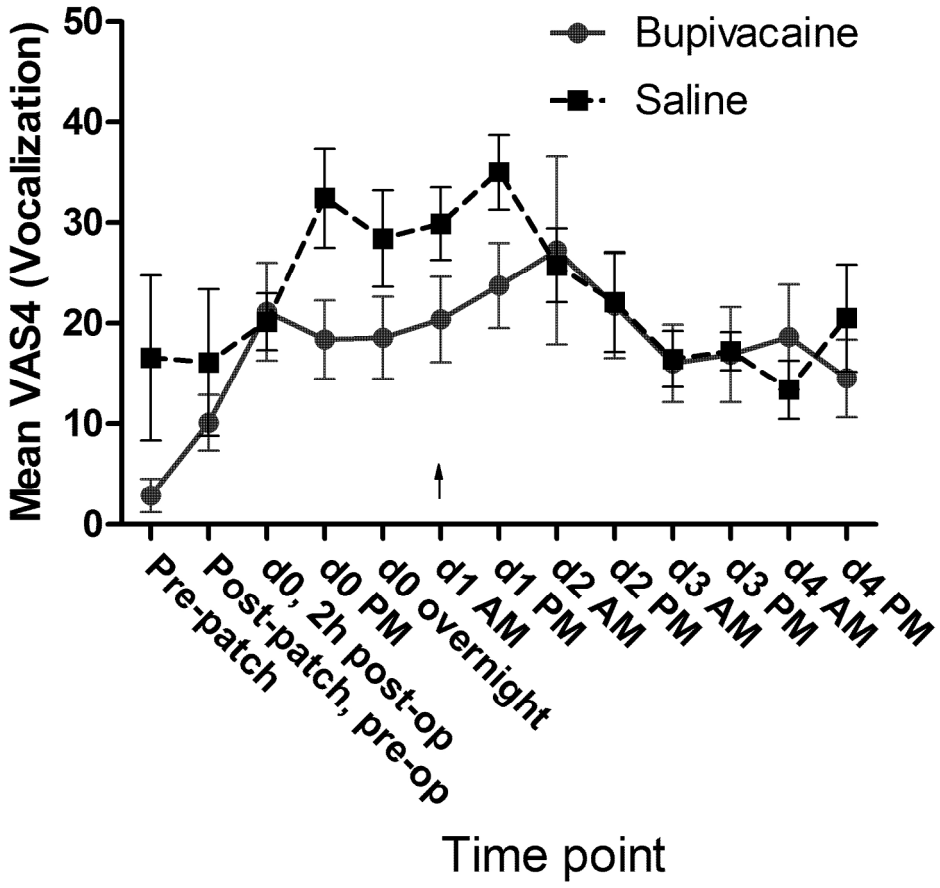

Figure 10. Mean VAS4 (Vocalization) scores by treatment group. Arrow represents last dose of local anesthetic/saline control. d0=day of surgery, d1= first day following surgery, etc. Error bars: +/-1 SEM. 631x609mm (96 x 96 DPI)

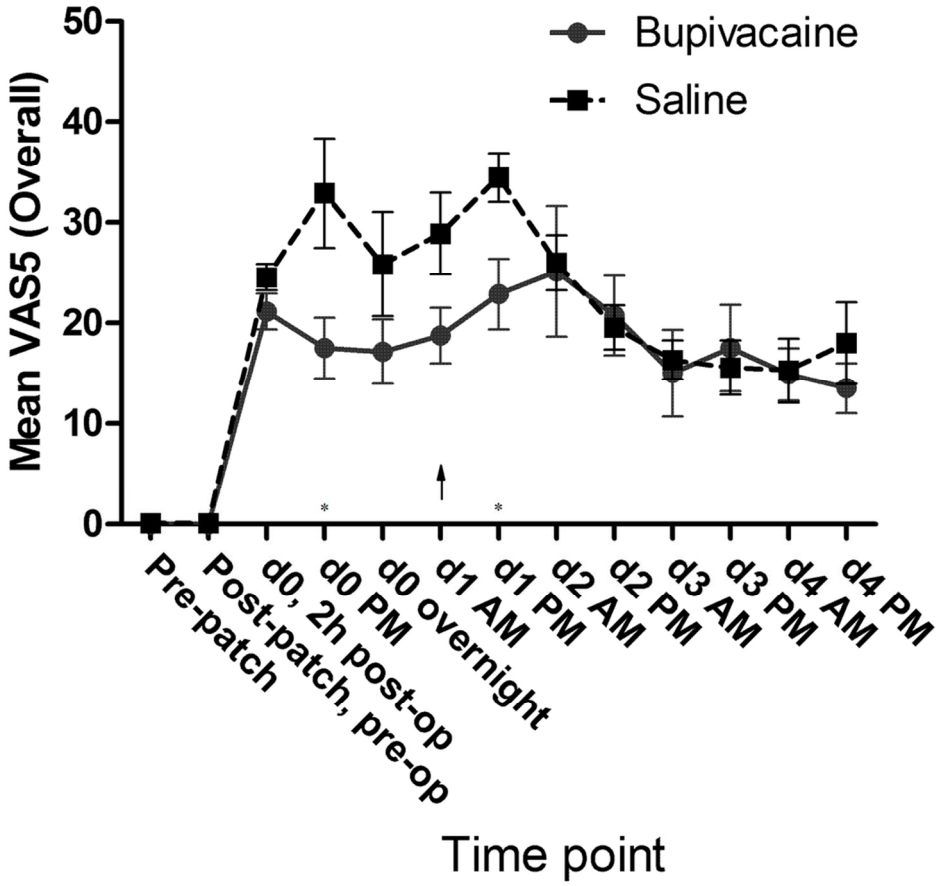

Figure 11. Mean VAS5 (Overall) scores by treatment group. Arrow represents last dose of local anesthetic/saline control. Statistically different time points (Student's t test) are indicated with an asterisk. d0=day of surgery, d1= first day following surgery, etc. Error bars: +/-1 SEM
97x94mm (300 x 300 DPI)

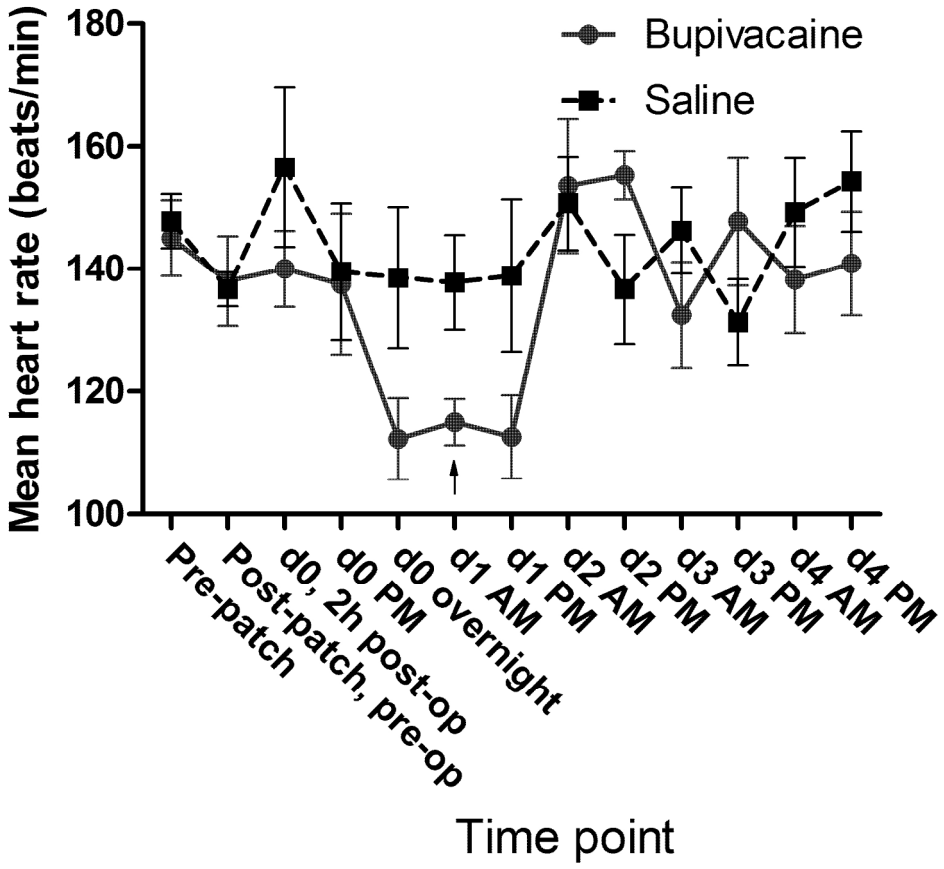

Figure 12. Mean heart rate as measured by direct cardiac auscultation during physical examination. Arrow represents last dose of local anesthetic/saline control. Error bars: +/-1 SEM
647x609mm (96 x 96 DPI)

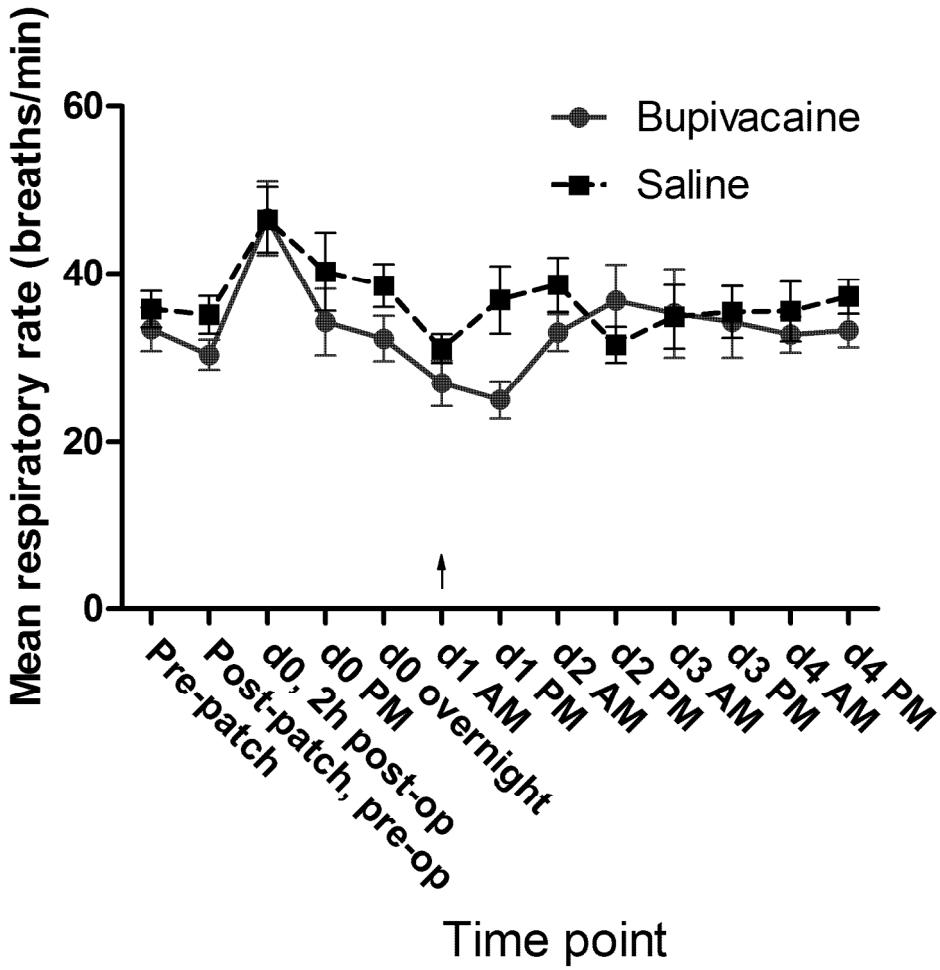

Figure 13. Mean respiratory rate as measured by direct observation during physical examination. Arrow represents last dose of local anesthetic/saline control. Error bars: +/-1 SEM
631x650mm (96 x 96 DPI)

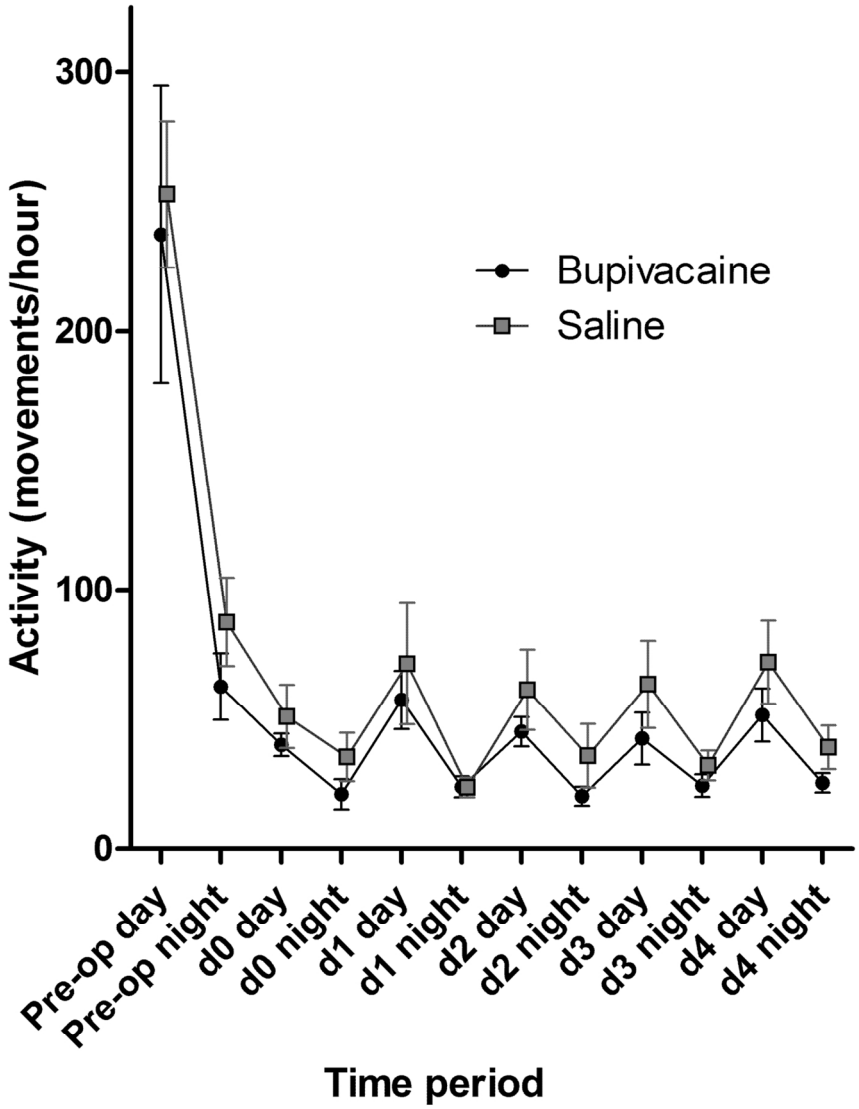

Figure 14. Mean number of movements counted per hour during the day and night phases. Error bars: +/-1 SEM.
126x167mm (300 x 300 DPI)

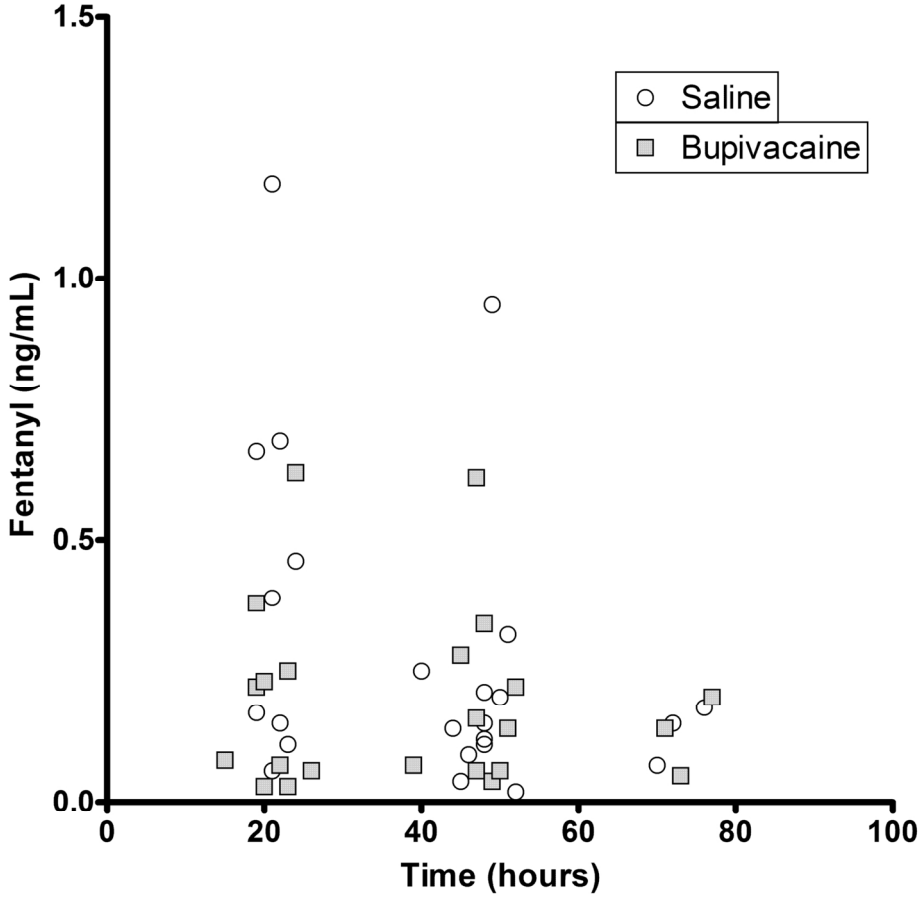

Figure 15. Serum fentanyl levels in ng/mL by elapsed time in hours since placement of a fentanyl patch (50 µg/h). Boxes represent bupivacaine treated subjects and circles are saline controls. Blood sampling occurred at the following times in hours (mean +/- standard deviation) following fentanyl patch placement: first sampling at 21.1 +/- 2.5 hours, second sampling at 47.4 +/- 3.5 hours, and third sampling at 73.2 +/- 2.7 hours.
154x146mm (300 x 300 DPI)

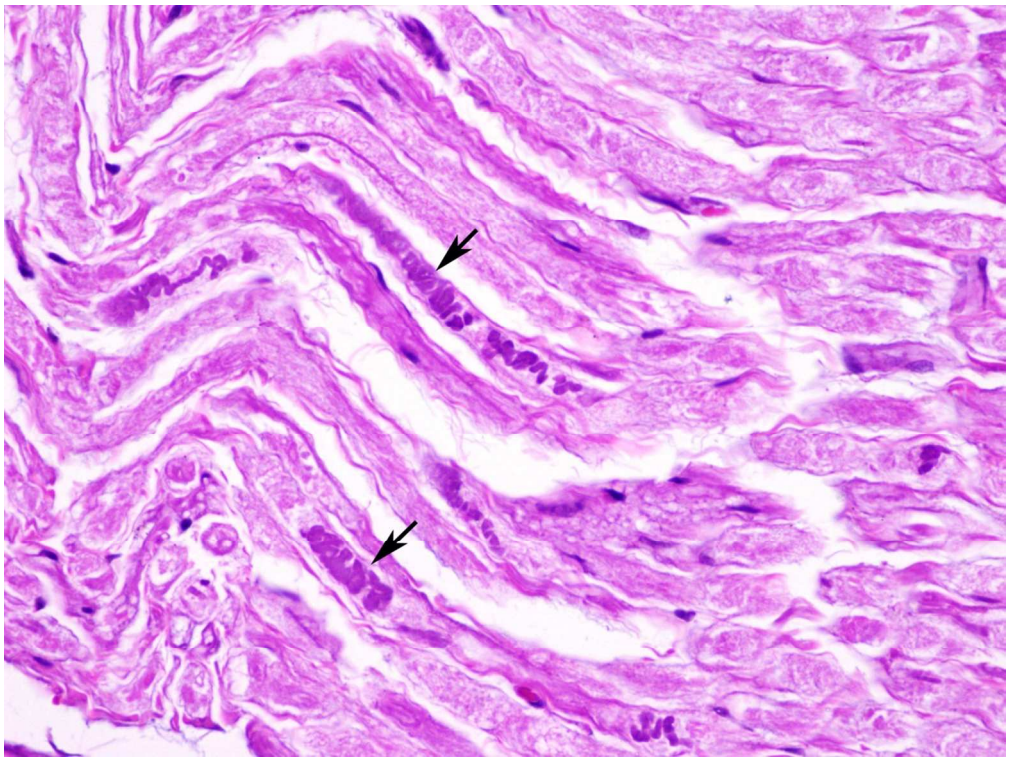

Figure 16. Right sciatic nerve of a bupivacaine-injected animal showing axonal degeneration and loss characterized by hyperchromatic condensation of the axons (arrows). Hematoxylin and eosin, magnification 600x.
164x123mm (220 x 220 DPI)

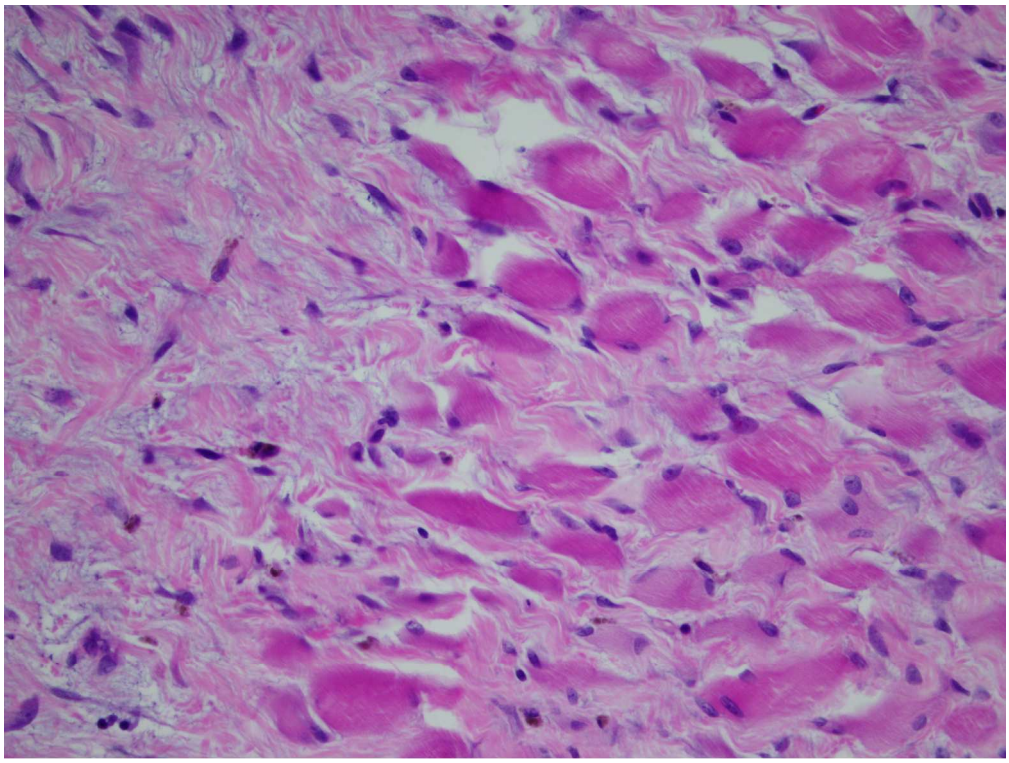

Figure 17. Skeletal muscle in the area of the right femoral nerve of a bupivacaine-injected animal showing rhabdomyocytic atrophy and loss that is focally extensive and severe with abundant fibrosis. Hematoxylin and eosin, magnification 400x.
1439x1083mm (72 x 72 DPI)

Noninvasive monitoring of healing from penetrating traumatic compound femur fracture in swine

Michael Bodo1, , Timothy Settle2 , Joseph Royal2, Eric Lombardini3, Evelyn Sawyer4 Stephen W. Rothwell1

Abstract

The treatment of extremity combat wounds can be optimized with better information on the wound healing process. Here we present results gained by using non-invasive measurement modalities in an animal study. A comminuted femur fracture of the right hind leg was produced under anesthesia in 30 female Yorkshire swine, and one of four treatments was instilled into the wound immediately following injury (salmon fibrinogen/thrombin - n=8; commercial bone filler matrix, CopiOs - n= 7; bovine collagen – n=8, and porcine fibrinogen/thrombin - n=7). The fracture was stabilized with an external fixation device, and the animal was recovered. Blood samples were taken, and radiographic examination and measurement of physiological signals were conducted weekly for three weeks. Analog signals (near infrared spectroscopy, electrical bioimpedance, and blood flow by Doppler ultrasound flowmetry) were recorded on both hind legs at a frequency of 250 Hz and processed off-line. The mean values of ten second periods were used for left – right side comparison. ANOVA was used for statistical analysis. There was no significant difference between treatment groups for body weight, inflammation severity score, regional oxygenation, blood flow, and electrical bio-impedance. However, salmon fibrinogen/thrombin showed different time trends in measures of regional oxygenation compared to the other three treatments. For the salmon fibrinogen/thrombin treatment, the maximal laterality difference was observed after one week. Since all measured modalities reflect both wound healing as well as inflammation, the results need to be correlated with radiographic examination and blood sample analysis for final scoring of measured compounds. Based on our results, these non-invasive techniques may offer convenient quantification of wound healing in humans as well.

Keywords Femur fracture - Noninvasive monitoring - Near infrared spectroscopy - Blood flow – Bioimpedance

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1 Introduction

Accidents or unintentional injuries ranked as the 5th leading cause of death in the United States in 2010 [1]. The nonfatal, construction industry-related injury rate was 410/10,000 [2]. Nearly 25% of adults involved in pedestrian accidents sustained tibia-fibular fractures [3]. Fifty nine percent of trauma center patients came from motor vehicle accidents [4]. Hospital stay, Injury Severity Score, Revised Trauma Score, Glasgow Coma Scale, and the mortality rate all worsen with increasing age [5]. Peak injury period and type of injuries were detailed elsewhere [6].

The epidemiology of military trauma was detailed previously by Bellamy et al, [7], and more recently by Holcomb et al, [8]. Recent U.S. conflicts have advanced the practice of vascular trauma surgery on the battlefield and offer important lessons learned for management of similar types of injury in urban trauma centers. Damage control techniques for complex injuries with associated hemodynamic instability may provide an opportunity to save both life and limb [9, 10].

Infection is a common complication of combat-related injuries [11]. Enhanced medical training of front line medical personnel, personal protective equipment, and the presence of far forward surgical assets have improved the initial survival of casualties in the current wars in Iraq and Afghanistan. As such, more casualties experience infectious complications of injuries including sepsis, which was a noted killer of casualties in previous wars. During the current conflicts, military personnel who develop combat-related injuries are at substantial risk of developing infections with multidrug resistant bacteria [12].

Here we present results of noninvasive monitoring of penetrating traumatic compound femur fracture in swine. Monitoring methods included the following modalities: 1) *Near infrared spectroscopy* (NIRS) which uses *in vivo* optical spectroscopy to provide real-time monitoring of changes in regional oxygen saturation (rSO_2) of blood in the brain or other body tissues beneath the sensor [13]. 2) *Bio-impedance measurement or impedance plethysmography* which is a method for noninvasive measurement of volume and rate of blood flow. This technique relies on the proportional relationship between output signal amplitude (pulse wave) and the fluid content or conductive ion-containing volume within the tissues located between measuring electrodes [14, 15]. 3) *Doppler ultrasound flowmetry* was used to examine the blood flow pulse waves in the adjacent arteries.

Our hypothesis was that the wound, the treatment, and the healing process may alter blood flow, rSO_2, and tissue fluid volume.

2 Methods

Under isoflurane anesthesia, the right femur was stabilized by an external fixation device and a limb injury with soft tissue damage and a comminuted femur fracture was then produced in 3-month-old female Yorkshire swine using a penetrating captive bolt as described by Majetschak , et al [16] (Figure 1). One of four treatments was delivered to the open fracture immediately following injury: (1) salmon fibrinogen and thrombin - SFT (Sea Run Holdings, Eastport, ME); n=8), (2) porcine fibrinogen and thrombin - PFT (Enzyme Research, Inc. South Bend, IN), ; n=7), (3) bovine collagen – BC (Sigma-Aldrich, St Louis, MI); n=7) and (4) CopiOS^R Bone Void Filler (Distributor, Zimmer, Inc., Warsaw, IN; manufacturer, Kensey Nash Corp); n=7). The injury was dressed and the animal was recovered. Radiographic examination and blood sample collection were conducted weekly for three weeks. Post-mortem computed tomography (CT) examination of the femur was conducted following euthanasia to determine the level of bone development and ossification. The following measurements were performed before and immediately after surgery and then weekly thereafter: 1) NIRS using the INVOS® System

(Cerebral/Somatic Oximeter, Somanetics, Troy, MI); 2) *Bioimpedance* (BI) measurement using a Cerberus amplifier (Quintlab, Budapest, Hungary) with a 125 kHz measuring frequency using either 150 X 6 mm 3M M6001 electrode tape and electrode gel with; or SF405 ECG electrodes (Kendall, Medi-Care). 3) *Doppler ultrasound flowmetry* with a Model 812 Doppler flow detector with a 9.5 MHz infant flat probe (head size: 15 x 12 x 4 mm, Parks Medical Electronics, Inc., Aloha, OR) and Aquasonic 100 Ultrasound transmission gel (Parker Laboratories, Fairfield, NJ). Sensor and electrode locations are presented on Figure 2.

2. 1 Data processing

Measurements were made at the following time points: pre-surgery, post-surgery, one, two, and three weeks after surgery under isoflurane anesthesia with the swine in a supine position. Data (analog physiological signals) were digitized at 250 Hz and collected using the Ponemah Physiology Platform (Data Sciences International, St. Paul, MN). Data were processed with DataLyser, a software developed at the Walter Reed Army Institute of Research, Silver Spring, MD, for display, storage, and processing of analog physiological signals. NIRS values were used after stabilization of signal about 10-15 minutes after sensor placement. Bioimpedance and Doppler pulse waveform analysis were performed by measuring the minimum and maximum distance of pulse waves during 10-second periods. For noise reduction, a 0.1 sec smoothing was used. Data were generated in DataLyser and exported into an Excel (Microsoft, Redmond, WA) spreadsheet for further calculations. Additionally, data from anesthetic monitoring of electrocardiography, pulse oximetry, and capnography were also stored as analog waveforms with a 300 Hz sampling rate. Trends were also recorded for pre and post operative heart rate, noninvasive blood pressure, body temperature, inhaled isoflurane concentration, and bispectral index (BIS) value with 10 sec/number. Since inflammation developed after surgery, an inflammation score was created and compared between groups. Weekly body weights were also recorded and compared between groups. The uninjured left limb was used as the negative control, and data are expressed as the difference between the left (uninjured) and right (injured) sides. ANOVA was used to compare data of four groups (Prism, GraphPad Software Inc., La Jolla, CA). Statistical significance was considered as $P< 0.05$.

3 Results

The average body weight of pigs was 37.73 ± 2.34 kg (mean ±SD) at surgery. There was no significant difference among groups.

The left-right side difference was not significantly different among treatment groups in the inflammation severity score, NIRS, Doppler, and BI data. However, some notable trends emerged.

NIRS

The NIRS trend for the SFT group showed a distinct time course compared to the other three treatments (see Figure 3). The NIRS difference increased between the first postoperative measurement and the 1 week postoperative measurement in SFT, PFT and BF (CopiOS) groups but not for BC.

Blood flow

The blood flow difference in tibialis anterior arteries was the smallest in PFT group; Figure 4.

Bioimpedance

BI pulse left – right side difference showed a nearly identical time course in all four groups (Figure 5).

A comparison of the three measured modalities shows that while BI and BF differences were minimal in all 4 groups, NIRS laterality difference showed a time-related alteration with maximal difference at one week in the SFT group and at 2 weeks in the other three groups. Furthermore, the PFT group difference increased further from week 2 to week 3 while the others decreased (Figures 6 and 7).

4 Discussion

In this study we used a swine model of femur fracture treated with one of four compounds and analyzed three surrogate markers of wound healing measured by noninvasive monitoring methods. Biochemical markers of bone and wound healing, inflammation, imaging and histology methods and results published elsewhere [17].

Blast exposure causes more complex and multiple forms of damage than any other wounding agent, are the leading cause of death on the battlefield, and are often used by terrorists [18]. During combat operations, extremities continue to be the most common sites of injury. Despite advances in management of combat wounds, wound infection continues to be a troublesome yet potentially preventable complication of combat-related injuries. Inflammation is an essential component of the adult wound response [19]. The challenge for the surgeon is to prevent sepsis while avoiding amputation, which often requires a hard decision for saving extremities [20]. Traumatology problems are described elsewhere [21].

Extremity vascular injuries predominate on the modern battlefield, representing 50 to 70% of all injuries treated during Operation Iraqi Freedom. Furthermore, exsanguination from extremity wounds is the leading cause of preventable death on the modern battlefield. The management of extremity vascular injuries on the modern battlefield presents many unique and demanding challenges to even the most seasoned of surgeons [22].

The treatment of extremity combat wounds is a complex problem that requires a multidisciplinary approach [23]. The common complications associated with lower extremity trauma and amputations secondary to combat injuries were detailed elsewhere [24]. Plasma and cellular biomarkers of injury as predictors of subsequent septic complications have also been detailed [25]. It would have practical importance if wound healing could be evaluated by noninvasive methods that offer quantitative information on bone and soft-tissue healing [26]. The healing of wounds, including bone fractures, involves factors such as revascularization, tissue oxygenation, inflammation, edema formation, which have been detailed elsewhere [27]. Various categories of drugs have been used in the treatment of healing wounds and fractures, including drugs with anti-inflammatory, antipyretic, analgesic, and ossification stimulant effects [28]. However, changes in these modalities during wound healing may influence all three measured modalities.

While the differences among the four measured treatment modalities were statistically non-significant, possible trends in laterality differences are apparent when the data is analyzed graphically as a function of time.

A sham group was unavailable. Consequently, we did not have a control group to compare laterality difference in normal wound healing to that of wound healing with the applied treatments.. Analysis of additional physiologic data is underway and may shed further light on the effects of these treatment and evaluation modalities.

4.1 NIRS

The understanding of the role of oxygen in wound healing has undergone a major evolution from its long-recognized importance as an essential factor for oxidative metabolism to its recognition as an important cell signaling mediator interacting with growth factors and other signals to regulate signal transduction pathways [29]. Accurate measurements of tissue oxygen tension has led to an understanding of the crucial role of oxygen in wound healing and the degree to which oxygen supply in wounded tissue is often the limiting factor in healing [30]. Since transcutaneous oximetry can be used to measure the peri-wound oxygen tension, a consensus statement was established defining tissue hypoxia as oxygen tension <40 mmHg. Healing was considered measuring above this value referring adequate revascularization [31].

NIRS reflects regional tissue oxygenation, with the light-based signal penetrating to a tissue depth of approximately 25 mm in case of the NIRS device used in this study. The measurement provides an estimate of mixed arterial and venous oxygen levels. The lack of significant laterality difference in our study can be interpreted to mean that re-vascularization in the damaged area proceeded similarly in all cases regardless of the treatment. It is also noted that NIRS can be used to quantify total heme oxidation, but it is unable to distinguish between hemoglobin and myoglobin [32]. This fact may also have contributed to our non-significant NIRS results. An overview of the clinical application of NIRS can be found elsewhere [33].

The pig has been suggested as an excellent potential animal model for human wound healing [34]; however, we have noted that inflammation due to infection present during healing may influence measured modalities, as has been shown in other studies [35, 36].

4.2 Blood flow

Extremity vascular injuries on the battlefield require special attention since hemorrhage is the dominant cause of mortality ([8, 22, 37]. Doppler ultrasound is used frequently in clinical practice to diagnose peripheral arterial disease [38]. Modalities for noninvasive monitoring of peripheral perfusion were detailed elsewhere [39]. Blood flow through the femoral artery was deliberately not compromised because the trial was not designed to evaluate healing in the presence of major arterial damage. This may explain the non-significant difference between healthy and affected areas.

4.3 Bioimpedance

The impedance plethysmography method (also called bioimpedance) measures the electrical resistance to an alternating current (i.e., impedance) in a region of the body [40]. In comparison to other tissues, blood is a very good conductor [41]. Therefore, blood volume changes can be recognized by measuring impedance changes. Impedance plethysmography can be taken on almost any body segment. Measurement typically requires four electrodes to be applied to the body surface. In our study, we used a 2 electrode system with 125 kHz frequency and less than 1 mA amplitude. The current is imperceptible to the patient and does not cause any physiological reaction. The voltage corresponds with the impedance of the body segment and changes with the variation of blood volume variations. On this basis, blood flow can be measured and analyzed [42]. In routine clinical practice mostly used as venous occlusion plethysmography is used for the diagnosis of venous thrombosis and impedance plethysmography measured on the thorax is a special diagnostic application and is usually called impedance cardiography [43, 44]. Bioimpedance measures the conductive ion-containing volume between 2 electrodes. The lack of significant laterality difference suggests that the injury-related edema formation, re-vascularization, and inflammation were similar for all four treatments.

5 **Conclusion**

Hematoma and edema formation, vascularization, infection, bone healing, ion content of treatment and solvent material, as well as its fixation and absorption were potential influencing factors of measured modalities. Because the physiological parameters collected were non-invasive, this process offers the possibility of convenient measurable parameters of wound healing in humans as well. Further studies may incorporate these methods into an injury index, which may provide quantitative assessment of traumatic wound healing over time.

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Disclaimer
The views of the authors do not purport to reflect the position of the Uniformed Services University or the Department of Defense.

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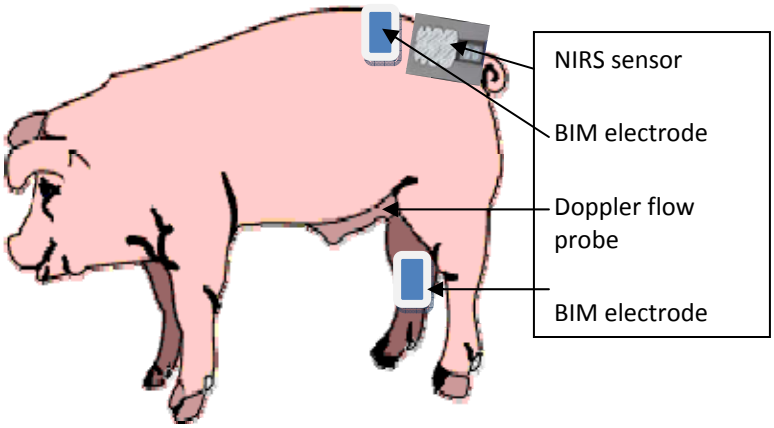

Fig 1. Sensor and electrode placement

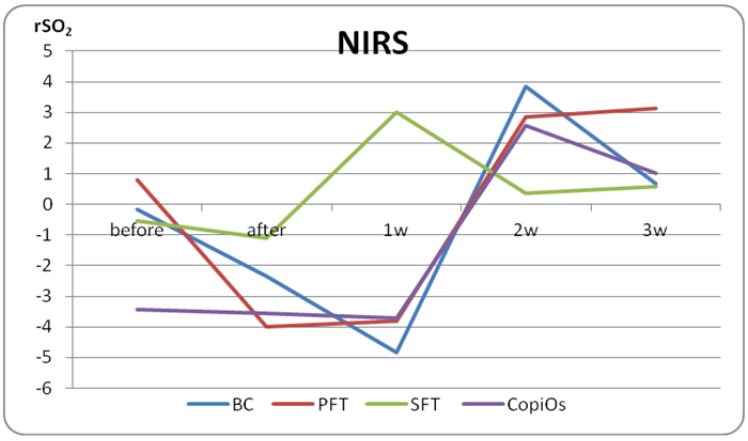

Fig. 2. Left – right differences of NIRS values as a function of time in four treatment groups. .

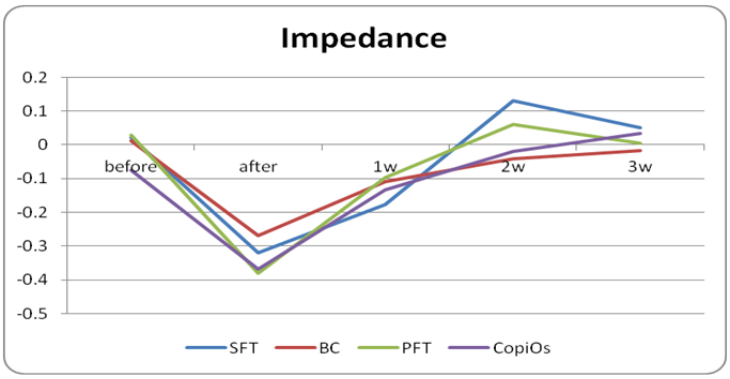

Fig. 3. Left – right differences of impedance pulse values as a function of time.

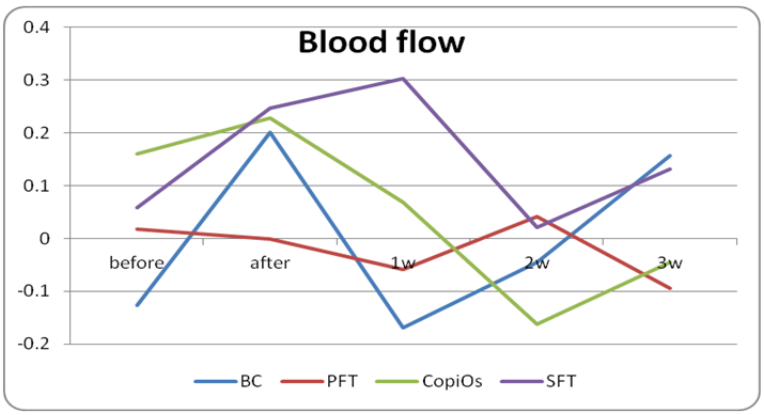

Fig.4. Left – right differences of blood flow values as a function of time

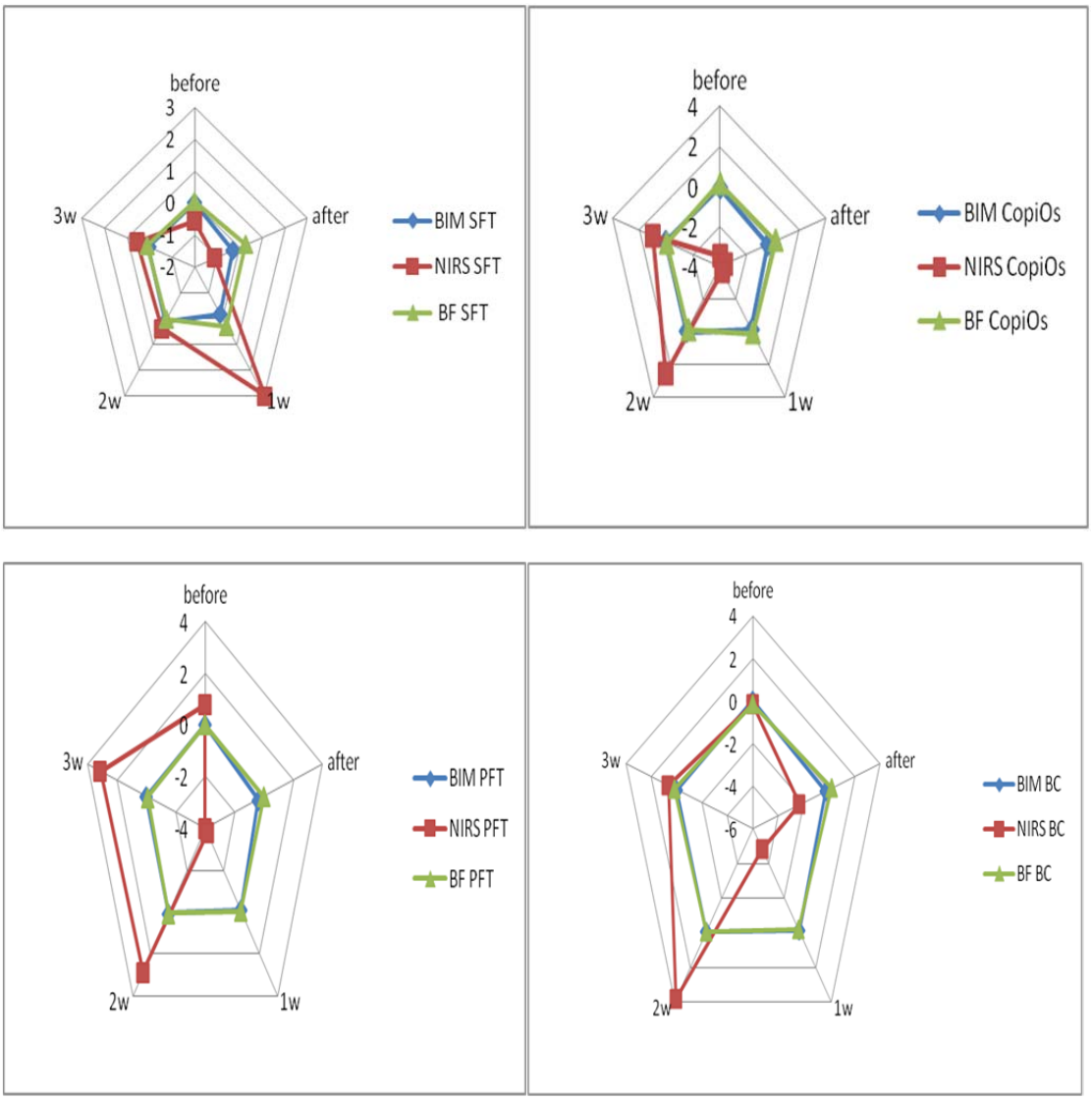

Fig 6. Left – right differences of NIRS, blood flow, and impedance values as a function of time by treatment groups. Legend: before: pre-surgery; after: post-surgery; 1w: one week after surgery; 2w: two weeks after surgery; 3w: three weeks after surgery.