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 servicemembers 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 use by the first responder, nor do they address the question of hemorrhage control, which is 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 after 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 a pathogen profile compared to mammalian fibrinogens.

Introduction

Blast injuries have become increasingly 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 Servicemembers 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.

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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 with 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[7] deep into the wound at the site of the bleeding. Second, fragmented bone must be stabilized if the limb will be saved. 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 and bovine collagen and commercial bone filler (BF). A combination of salmon fibrinogen and thrombin (SFT) had been tested as a hemostatic agent in other projects and was shown to be an effective hemostatic reagent with a commercially viable cost.[10-12]

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 were 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, MO) or SFT (Sea Run Holdings, Freeport, ME) preparations were used to coat the surface of slides or wells to induce differentiation for microscopy and reverse transcription (RT)–polymerase chain reaction (PCR) analysis. Concentrations of fibrinogen and thrombin tested were 5mg/mL fibrinogen–10U/mL thrombin, 5mg/mL fibrinogen–100U/mL thrombin, 30mg/mL–0U/mL thrombin, 30mg/mL fibrinogen–100U/mL thrombin, 50mg/mL fibrinogen–10U/mL thrombin, and 50mg/mL fibrinogen–100U/mL thrombin. For comparison, Greiner Bio-One collagen (Type 1)–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 upregulation 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 via centrifugation at 300*g* for 5 minutes. RNA was prepared using the Qiagen RNeasy spin column procedure (Qiagen, Valencia, CA) and evaluated for quality via electrophoresis. RT-PCR was performed using SABiosciences Human Osteogenesis 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 42°C for 15 minutes before stopping the reaction by incubation at 95°C. 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 system (Roche Applied Science, Indianapolis, IN), and the results analyzed were using the Roche LightCycler software package.

Surgical Preparation of Animals

Animal Care Standards

All animal procedures were conducted according to a Uniformed Services University of the Health Sciences (USU) 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 (50µg/hr; Watson Laboratories, Inc., Corona, CA) was placed on each animal 18 hours before 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, IA). The animals were then intubated, and anesthesia was 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 noncompressible 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 a general veterinary surgery text.[15] A nonadherent 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 (0.3mg buprenorphine, Hospira, Inc., Lake Forest, IL) were administered as needed for pain control and sedation during the first 3 days postsurgery. 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 were used to estimate blood flow, tissue oxygenation, and edema formation (M. Bodo, manuscript in preparation). At day 21, animals were euthanized by intravenous 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) SFT (Sea Run Holdings, Inc., Freeport, ME, (2) porcine fibrinogen and thrombin (PFT) (Enzyme Research, Inc. South Bend, IN), (3) bovine collagen (Sigma-Aldrich, St. Louis, MO), and (4) CopiOS Bone Void Filler (BF) (distributed by Zimmer, Inc., Warsaw, IN; manufactured by Kensey Nash Corp, Exton, PA). Eight animals were assigned to each treatment group. SFT [salmon fibrinogen (20mg/mL)–thrombin (450U)] and PFT [porcine fibrinogen (18.8mg/mL)–thrombin (287.2U)] solutions were filter sterilized separately, and the fibrinogen and thrombin were combined during instillation into the wound. Before use, CopiOS was mixed with 5mL citrated autologous blood. Bovine collagen was sterilized by irradiation (5 kGy; JL Shepherd Mark 109 cobalt source, San Fernando, CA), suspended in 5mL sterile phosphate-buffered saline, and instilled into the wound. Placement of a treatment into the wound site was made possible by a 3mL 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 (Siemens 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 (E.L.) 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/Hamamatsu Nanozoomer (Hamamatsu Corporation, Bridgewater, NJ), and images were collected with use of 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 (S.R. and T.S.), blinded to treatments, analyzed the CT scans for bone alignment, inflammation, bone filling, and calcification. Numerical scores from 1 to 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.

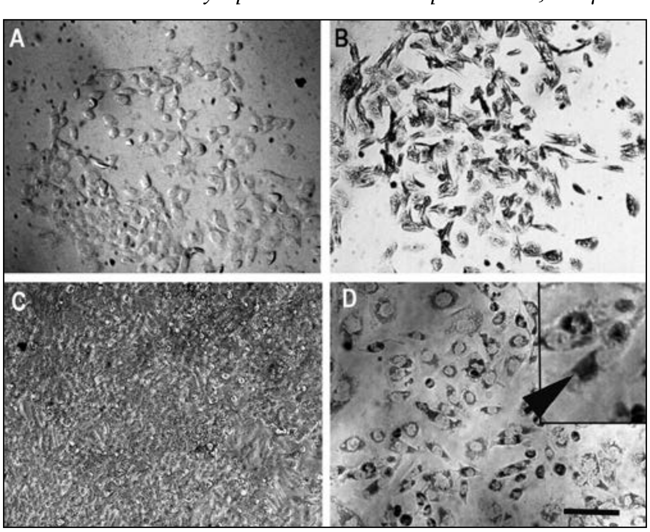
Results

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 an SFT matrix could induce a similar transformation, cells were cultured for 1 week in maintenance flasks, flasks coated with collagen 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 nondifferentiated morphology and stained negatively in the von Kossa assay (Figure 1A). When cultured in differentiation media that had the required growth factors for osteogenic differentiation, the cells changed their appearance and deposited calcium phosphate as indicated by the dark precipitates (Figure 1B) 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 1C). In contrast, cells grown on a salmon fibrin matrix (50mg/mL fibrinogen with 10U salmon thrombin) displayed rounded osteoblastic morphology with darkened Golgi regions and intense extracellular mineral deposits (Figure 1D). This would indicate that components of the SFT matrix have the capacity to alter the normal status of the cells.

RNA expression was quantitated using 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 7 days. Even at this early time point, changes could be detected (Table 1). When cells were grown on coated plates for 21 days, more robust changes in the RNA profile could be observed (Table 2). Cells were grown in (1) undifferentiating media on SFT [salmon fibrinogen (5mg/mL)–thrombin (10U)] for 21 days, (2) undifferentiating media on SFT [salmon fibrinogen (50mg/mL)–thrombin (100U)] 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 compared with 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 upregulated fall into several major groups. One group is composed of structural proteins such as the collagens, and several different types of collagens were upregulated. Type 1 is a collagen variety expected to be increased because it is found in bone, but types IV and XI were also increased. Type IV is found in the basal lamina of epithelial cells, whereas 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 (TGF) and mediate the TGF-ß 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.

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 10U 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 D indicates the cytoplasmic mineral deposits. Bar, 100µm.*



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 1cm by 6cm was created that passed through the skin, muscle, and bone. The bone was reproducibly perforated by the bolt (Figure 2A). 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 2B). Instillation of the treatment matrix filled the injury tract and, in the case of salmon

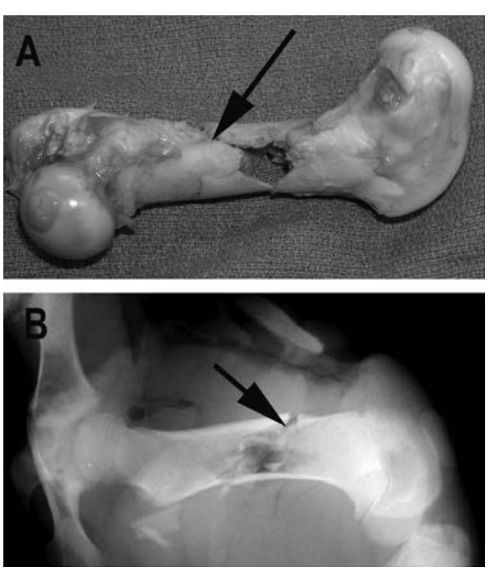
Table 1 *Changes in RNA Expression Following 7 days Incubation on Fibrin Matrices (fold changes).*

Gene Symbol	Protein Name	7-day Diff	Fibrin 1	Fibrin 2	Fibrin 3
BMP1	Bone morphogenic protein 1	0.76	1.43	1.44	2.06
BMP5	Bone morphogenic protein 5	0.43	0.48	0.76	0.48
COL1A1	Collagen 1A1	1.37	1.48	1.44	2.06
FGF2	Fibroblast growth factor2	0.97	1.15	1.43	1.61
FN1	Fibronectin 1	0.93	1.13	1.60	2.12
ITGA1	Integrin, alpha 1	1.40	1.69	1.75	1.64
MINPP1	Multiple inositol polyphosphate histidine phosphatase, 1	1.31	1.48	2.02	1.06
SMAD1	SMAD family member 1	1.04	1.60	2.31	1.03
TUFT1	Tuftelin 1	1.04	2.38	3.15	1.89
TWIST1	Twist homolog 1 (Drosophila)	0.75	1.89	1.53	0.94

Notes: 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 50mg/mL; salmon thrombin 100U/mL.

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 20mL). Healing occurred uneventfully at the injury site (Figure 3B). Injuries treated with salmon fibrinogen routinely reepithelialized with hair growth reoccurring (Figure 3C).

Figure 2 *Perforating bone injury was produced in the mid-shaft of the femur. (A) Isolated bone removed immediately after injury without treatment or surgical fixation. (B) Plain radiograph of the injured bone in situ.*



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, showing the uninjured bone (Figure 5A) and the bone immediately after injury (Figure 5B). The arrow indicates the point of impact of the penetrating bolt. Figure 5C shows femur at 10 days postinjury. 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 Figure 5D and 5E (days 16 and 21), the lesions in the bone are healing (arrow 1). Figure 5F and 5G 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

Table 2 *Changes in Protein Expression Following 21 days Incubation on Fibrin Matrices (fold changes).*

Gene Symbol	Protein name	7 day diff	Fibrin 1 undiff	Fibrin 3 undiff	Collagen diff
COL11A1	Collagen, type XI, alpha 1	NS	2.93	4.24	4.20
COL1A1	Collagen, type I, alpha 1	NS	2.93	1.87	1.66
COL4A3	Collagen, type IV, alpha 3 (Goodpasture antigen)	NS	2.93	4.64	1.66
EGF	Epidermal growth factor (beta-urogastrone)	NS	2.93	3.93	2.62
FGF2	Fibroblast growth factor 2 (basic)	NS	2.60	2.56	4.96
FN1	Fibronectin 1	NS	1.39	1.85	1.19
ITGA1	Integrin, alpha 1	1.41	2.41	1.64	1.33
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	NS	2.85	1.82	1.61
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	NS	2.93	4.24	2.36
MINPP1	Multiple inositol polyphosphate histidine phosphatase, 1	NS	2.51	2.11	1.51
MMP10	Matrix metallopeptidase 10 (stromelysin 2)	NS	2.93	4.07	2.13
PHEX	Phosphate regulating endopeptidase homolog, X-linked	NS	2.93	8.43	4.20
RUNX2	Runt-related transcription factor 2	NS	2.93	6.39	1.66
SMAD1	SMAD family member 1	NS	2.41	–17.09	3.20
SMAD3	SMAD family member 2	NS	2.93	6.52	5.31
SOX9	SRY (sex determining region Y)-box 9	NS	2.93	2.26	1.66
TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	NS	2.93	2.40	1.66
TUFT1	Tuftelin 1	NS	3.07	3.15	5.31
TWIST1	Twist homolog 1 (Drosophila)	NS	1.92	2.14	1.84
VEGFB	Vascular endothelial growth factor B	NS	2.93	2.72	1.96
RPL13A	Ribosomal protein L13a	NS	2.93	3.99	3.20

Notes: 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 = 7 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. Diff. = Celprogen differentiation media; Undiff = Celprogen Maintenance media.

Figure 5F, 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 3 weeks had eroded the stability of the pins.

CT of the Isolated Femurs Permits Qualitative and Quantitative Analyses of Bone Healing

At the 3-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. After computer reconstruction, the images were presented as three-dimensional images and as planar sectional images (see Figure 5). These images were analyzed independently by two investigators (S.R., T.L.) 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 PFT and SFT treatments were slightly better on the callus score, and the PFT treatment was slightly better than the other treatment groups in the bone-filling category. However, none of the treatments was substantially different to reach statistical significance in the numbers of animals observed (n = 7 or 8/group).

Analysis of the CT scans by use of the Vivoquant software package permits quantitation of the bone density within the healing region. Bone density was hypothesized

Figure 3 *Progression of wound healing at the wound site. (A) Wound at time of time of injury. Application of salmon fibrin treatment results in an instantaneous clot. (B) Scab formation as the wound site healed (7 days). By 21 days, the wound site had typically reepithelialized with hair regrowth (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, 1cm.*

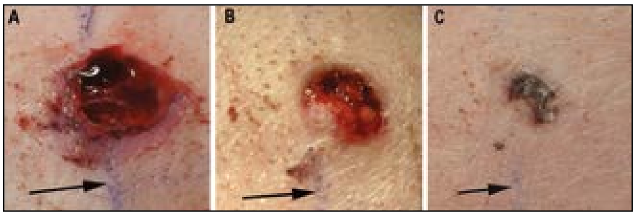
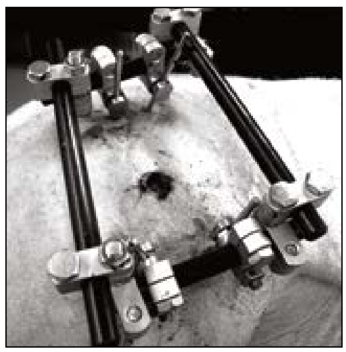


Figure 4 *Stabilization of femur injury by external fixation. The anatomy of the swine is not conducive to good stabilization of a fractured femur solely by a cast. Therefore, the bone was stabilized by placement of four surgical pins and fixed by an external apparatus. The frame was protected with a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



to equate to calcification and regeneration of bone. The three-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 SFT- 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 use of the Hamamatsu Nanozoomer scanner. As was performed in the analysis of the CT scans, the histology slides were assessed by both expert human judgment and computer-assisted analysis. Histopathological diagnosis was conducted by a trained veterinary pathologist (E.L.) blinded to the treatments and scored based on an inflammation scale of 1 to 5 (minimal, mild, moderate, marked,

Figure 5 *Progression of healing of the femur. (A) Uninjured bone. (B) Bone directly after injury. The arrow indicates the point of impact of the penetrator bolt. (C) 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. (D and E [days 16 and 21]) Lesions in the bone are healing (arrow 1). (F and G) Views from the post-mortem CT scan. Arrow 3 indicates the lesion in the cortex that is filling in. In contrast, the lesion site in the medulla (arrow 2), shown in the slice view (F), is still low density and is not equivalent to the uninjured regions of the marrow space.*

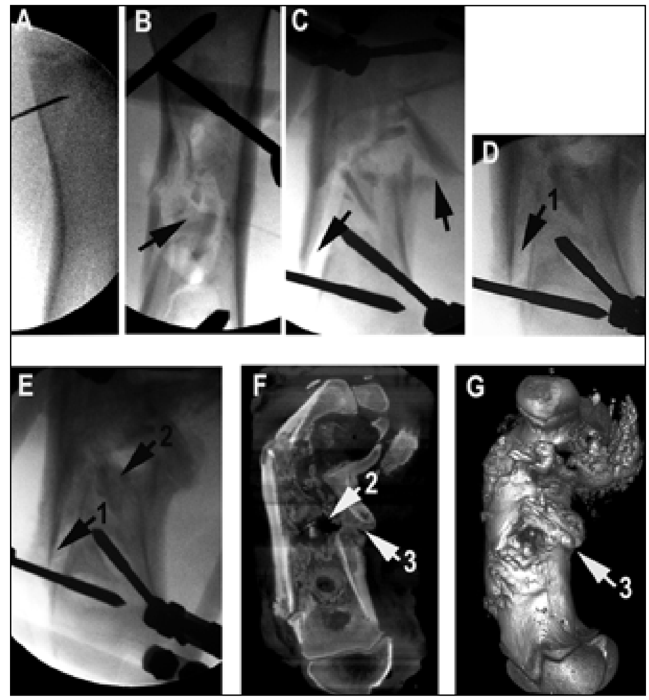
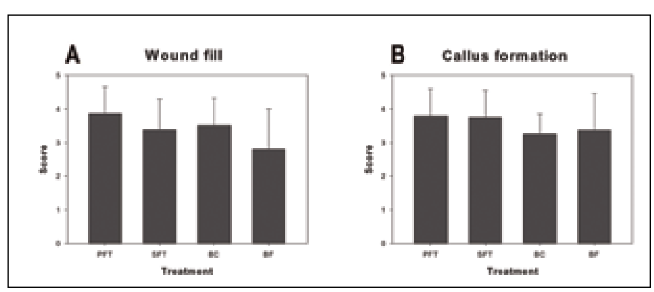


Figure 6 *Relative callus and bone filling scores as assessed by investigator CT analysis. The CT scans for each animal were examined by the investigators separately and then in consultation to reach a consensus score for callus formation and bone filling. Scores from 1 to 5 were assigned based on wound filling, bridging of the break, and calcification. BC = bovine collagen.*



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

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 were calculated using VivoQuant analysis. BC = bovine collagen.*

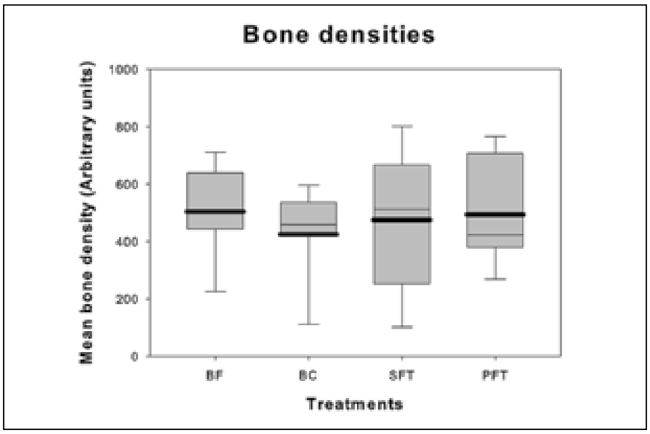
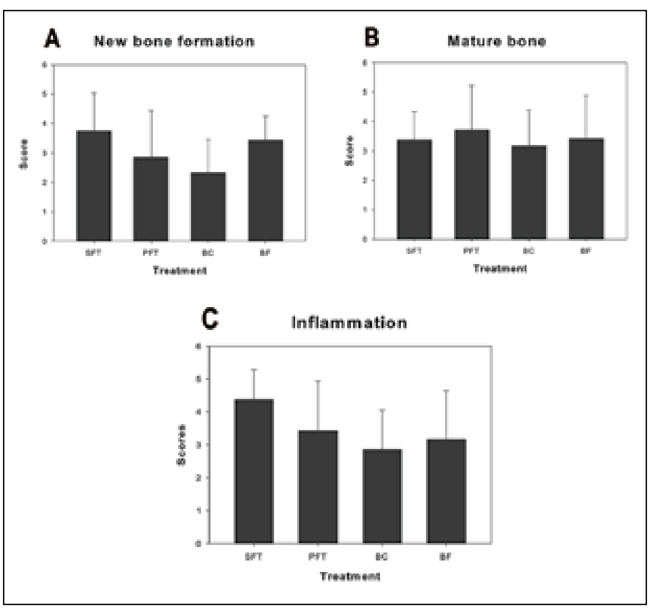


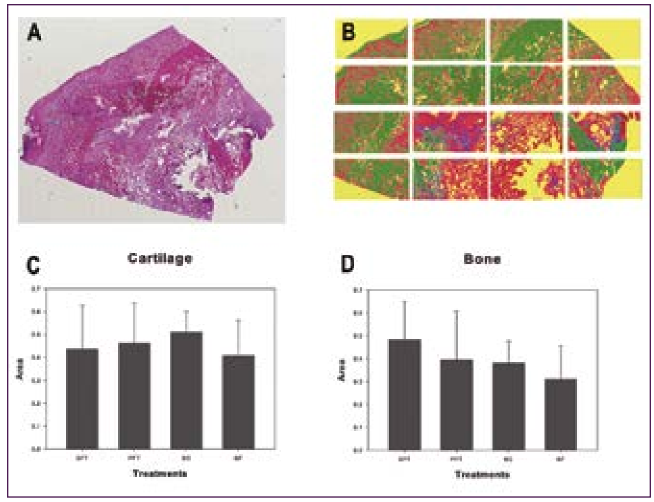
Figure 8 *Analysis of histology slides by a board-certified veterinary pathologist. Scores were based on an inflammation scale of 1 to 5 (minimal, mild, moderate, marked, severe); maturation of bone (based on fibroblast infiltration, collagen deposition, and myelofibrosis with 1 = most immature to 5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



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 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,

Figure 9 *Analysis of histology slides by image recognition software. Each histology slide (A) was digitized and divided into 16 regions. Visopharm software was set to recognize bone (green), cartilage (blue), and fibrous material (red) (B). Mean ± SD values for cartilage (C) and bone (D) are plotted. BC = bovine collagen.*



but, as before, none of the values achieved statistical significance. All of the cartilage values were 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 after injury by the first responder.

Many factors may contribute to nonunion of fractures, including nutritional or hormonal status, age of the patient, and presence of bacterial contamination. Atrophic nonunions may be due to inadequate blood supply or failure of callus formation at the fracture site. More severe instances of nonunion 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 nonunion. In addition, while nonunion is estimated to occur in approximately 2.5% of all tibia fracture repairs,[16,17] if vascular injury is involved, the frequency increases 5- to 6-fold.[18,19] The phenomenon of nonunion 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 on (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 researchers in tissue engineering 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 because 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 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 more than 20 years ago.[32] Fibrin-based gels have been used in conjunction with hydroxyapatite-coated beads or mesenchymal stem cells and were shown to 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 SFT matrices suggests that the fish protein is unique compared with 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, the 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. Two very different methodologies, radiography/CT scanning and histology, were used to examine the samples. 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 were also analyzed in two different ways. The first was a calculation of density values measured by the software interpretation of radiographic or histological 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 group seemed to rank consistently lower, and the 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, 3mL 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 nonunions can increase.[39] At 3 weeks time postinjury, 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 proved because the porcine 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 positron emission tomography. Although this model may not mimic human anatomy and physiology as closely as the swine model presented here, we expect that the scientific payoff of increased sample size and improved resolution of the biology of healing will more than compensate for this tradeoff.

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Disclaimer

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