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14. ABSTRACT

Long bone fractures resulting from high impact trauma can result in delayed healing. In spite of currently available treatments to enhance bone healing, these fractures can take up to a year to fully heal. Photodynamic therapy (PDT) is a minimally invasive local treatment currently used to treat cancer and skin diseases. Surprisingly, recent findings from studying the effect of PDT on vertebrae when treating spinal metastases have shown that PDT improved the strength and stiffness of the vertebrae. A critical size defect fracture of the femur was generated in 20 adult female Sprague-Dawley rats. PDT treatment was applied either 1d or 7d after fracture generation. µCT analyses of the non-infection rats showed a bone mineral density (BMD (gHA/cm2) slightly higher in the PDT treated groups (1d PDT: 0.86 ± 0.02; 7d PDT: 0.85 ± 0.08) compared to controls (0.79 ± 0.09), however the differences were not statistically significant. A smaller gap and more bone formation in the PDT treated groups could only be seen when the fracture gap at surgery was ≥6 mm. In these 6 animals the 1dPDT rats had the most bone formation (4.18±1.68 mm) compared to control (2.26±0.7 mm) and 7d PDT (3.80±0.98 mm). Further studies with longer time-points and a defect ≥6 mm are necessary to validate these observations.

15. SUBJECT TERMS
Long bone fractures, Photodynamic therapy, Fracture healing, Animal model;

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Table of Contents

	Page
Introduction	**2**
Body	**2**
Key Research Accomplishments	**5**
Reportable Outcomes	**6**
Conclusion	**7**
References	**8**
Appendices	**8**

Photodynamic Therapy Treatment to Enhance Fracture Healing

Introduction

The majority of long bone fractures heal successfully without complications, however compound and/or comminuted fractures resulting from high impact trauma can result in delayed healing or non-union. Early intervention in high risk fractures could decrease patient morbidity and reduce health care system costs [1]. Photodynamic therapy (PDT) is a non-surgical, non-ionizing minimally invasive local treatment, which has been successfully applied to treat multiple types of cancer, skin diseases and age related macular degeneration. This treatment involves the local or systemic administration of a photosensitizing drug, which is activated by non-thermal laser light at a photosensitizer specific wavelength. This light activation leads, in the presence of oxygen, to the generation of cytotoxic species (e.g. singlet oxygen) [2], which can induce apoptosis and/or necrosis of targeted cells and tissue and also influence immune responses [3]. PDT treatment of metastatically involved vertebrae resulted in improved vertebral bone strength, stiffness and architecture, motivating the investigation of PDT as an approach to augment bone healing [4]. Furthermore, PDT has been shown to upregulate vascular endothelial growth factor (VEGF); VEGF recruits new vasculature leading to enhanced fracture healing [5-7].

Body

Specific Aim # 1: To determine the optimal timing of PDT administration post injury to enhance fracture healing

Task 1:

As previously reported closed tibial fractures were created in a total of 17 rats using the drop weight system following intramedullary pinning(Figure 1). While modifications to the drop weight apparatus improved the consistency of the generated fractures, additional analyses have demonstrated that the inherent variability in the closed model may mask the treatment effects of PDT being studied. To date µCT analyses and histologic assessment have been conducted on 17 tibiae. The increase in bone formation after PDT treatment observed in the first group of 11 rats (Figure 2) (see annual report October 2011) in the µCT analyses were not present in the second group of 6 rats (n=2 control; n=2 1d-PDT; n=2 7d-PDT). However, the slight increase in

local VEGF protein expression after PDT treatment as determined through positive pixel count (Positivity = Npositive/NTotal) remained (control (n=6) 51±5 %; 1dPDT (n=5) 55±5 %; and 7dPDT (n=6) 56±7 %). In contrast, VEGF levels in the serum were found to be lower in the PDT treated groups (control (n=2) 57±5.5 pg/ml; 1dPDT (n=2) 35.2±14.8 pg/ml and 7dPDT (n=2) 33.0±13.7 pg/ml). A systemic elevated and prolonged VEGF level has been found as an indication of impaired bone healing in patients with long bone fractures [7]. Based on these relatively inconclusive results found in the closed fracture model the focus was shifted to the more repeatable open critically sized defect fracture model.

Task 2: Development of a critical size defect fracture model in rats

As previously reported the gap model was accomplished using the RatFix plating system (RISystem, Davos, Switzerland). The 6-mm gap distance is considered to be a critical size defect in rats which will not heal [8]. The defect was generated as follows: Under general inhalation anaesthesia the rat was placed in lateral position and the hind limb was prepared for surgery. A 2.5 cm skin incision along the femur was followed by separation of the m. vastus lateralis and m. biceps femoris to expose the femur. After positioning of the Gigly wire saw underneath the femur, a 23 mm PEEK plate was secured to the femur with 6 screws. The 6 mm osteotomy was achieved by positioning the drill- and saw guide to the plate using screw shafts and using the the Gigli saw. (Figures 3 a-e).

Femoral fractures with critically sized defects were generated in 20 adult female Sprague-Dawley (SD) rats which were randomly allocated to 3 groups: control (no treatment); PDT applied 1 day (1d) post fracture and PDT applied 7 days (7d) post fracture (Figure 4). Three animals had to be euthanized earlier due to plate displacement. The femur fracture of n= 5 (control group); n= 5 (1d PDT group) and n=7 (7d PDT group) animals were available for analyzes. µCT images at an isotropic 13.3 µm/voxel resolution (Inveon MicroCT, Siemens, Erlangen, Germany) were acquired of the fracture site and callus for 3D architectural analysis (AmiraDev 5.2, FEI Visualization Science Group, Burlington, USA)) (Figure 5). Original gap distance was measured on µCT images obtained after sacrificing (control group: 5.89 ± 0.92 mm; 1d PDT-group: 5.96 ± 0.55 mm; 7d PDT-group: 5.77 ± 0.55 mm). Variations in gap distance at surgery were present despite using the drill and saw guide.

After analyzing the CT data, we observed that it is very important to create a ≥ 6 mm defect which was achieved in 2 rats in each group. When looking at the new bone formation (smaller gap at day of sacrifice) in these 6 rats, the PDT treated rats showed more bone formation compared to the control rats (1d PDT group: 29 %; 7d PDT group: 23 %) (Figure 6). However, due to the small number further experiments are necessary to verify this observation. All other rats started with a fracture gap slightly below 6 mm and differences in new bone formation were diminished. The total bone volume (TV) was evaluated from µCT images in a region of interest extending incorporating the fracture gap and extending to the first screw placements on either side of the gap (9mm). Statistical analysis of the bone volume measurements was performed using a 1-way analysis of variance. No significant differences were found in TV (control: 58 ± 29 mm^3; 1d PDT: 49 ± 17 mm^3; and 7d PDT: 38 ± 9 mm^3). In contrast, BMD (gHA/cm^2) trended toward higher values in the PDT treated groups (1d PDT: 0.86 ± 0.02; 7d PDT: 0.85 ± 0.08) compared to controls (0.79 ± 0.09).

Vascular endothelial growth factor (VEGF) serum concentration was determined using Quantikine® RatVEGF Immunoassay (R&D Systems, Minneapolis, MN, USA), the C-terminal telopeptide of type I collagen (PICP) using the RatLaps™ EIA and osteocalcin using the RatMID™ Osteocalcin EIA (Immunodiagnostic Systems Ltd., Fountain Hills, AZ, USA).

The PICP concentration in the control group was generally stable, decreasing slightly after surgery and returning to pre-surgical levels after 4-5 weeks. In the PDT treated groups, the PICP concentration fluctuated greatly at both day 14 and day 42 with half of the specimens demonstrating increases in concentration compared to pre-op values and half reductions. There were no differences found in the serum osteocalcin levels or the VEGF serum level concentrations between groups. The high variance seen in the serum measurements of bone markers is not uncommon and has previously reported within the literature[1].

Specific Aim # 2: Evaluation of the ability of PDT to improve fracture healing in the presence of infection

Task 3: Creating the infected critical size fracture model and PDT treatment

A 6 mm defect was created in four female SD rats as described under Task 2. Bioluminescent bacteria at a concentration of 10^5 colony forming units (CFU) (St. aureus, XEN29, Perkin Elmer, Waltham, MA) were incubated with a gelatin sponge (Surgifoam®, Ferrosan, Soborg, Denmark) and placed into the defect (Figure 7). Four sponges were prepared per rat; 1 used for surgery and 3 control sponges incubated overnight (Fig 8a and b). The gelatin sponge was chosen, because it could be cut to the size of the defect and has shown in preliminary in-vitro studies to carry bacteria. The sponge allowed for the delivery of the bacteria suspension into the fracture gap preventing unintended contamination of the surrounding tissue. Two rats were treated with PDT 7 days after surgery and 2 rats served as control. The rats tolerated the surgery well and did not show any signs of a general infection. Three days after surgery a weak bioluminescent signal (Figure 9) could be detected in all rats, however 8 days after surgery the signal intensity started to go down independent from the treatment and was negative on the day of sacrifice (week 7). The bacteria dose was chosen according to the literature [9, 10], however the virulence may change overtime and in our case the bacteria may have been eliminated by the host immune system.

Key Research Accomplishments

AIM 1: Task 1:

- Establishment of a repeatable rat tibia fracture model using a custom drop weight impact apparatus
    - A local increase in VEGF expression (immunohistochemistry; Annual report October 2011) was found in the 7d-PDT group in the closed tibia fracture model with a concurrent decrease in systemic VEGF serum levels (positive indication for fracture healing).

- **Task 2:**
- Establishment of a repeatable rat femur critical size defect fracture model
    - Recognizing the importance of achieving a defect of > 6mm. PDT treated rats with a defect > 6 mm showed more bone formation compared to the control rats (1d PDT group: 29 %; 7d PDT group: 23 %).

Aim 2: Task 1

- Developing a system to transfer the bacteria suspension into the fracture gap without causing an undesired contamination of the surrounding tissue and general infection

Reportable Outcomes

- Poster presentation at the 58. Annual Orthopaedic Research Society Meeting, 4. – 7. February 2012, San Francisco, CA, USA
- Podium presentation at the Canadian Orthopaedic Research Society (CORS) Annual Meeting; 08. June 2012 in Ottawa, ON, Canada
- Poster presentation at the Military Health System Research Symposium (MHSRS); 13.-16. August 2012 in Fort Lauderdale, FL, USA

The Effect of Photodynamic Therapy (PDT) on Long Bone Fracture Healing

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ORS 2012 Annual Meeting
Poster No: 1407

- Poster Presentation at the 60. Annual Orthopaedic Research Society Meeting, 15. – 18. March 2014, New Orleans, LA, USA

The Effect of Photodynamic Therapy (PDT) on Femur Fractures with Critically Sized Defects

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ORS 2014 Annual Meeting

Poster No: 0635

Conclusion

As previously reported the variability of the closed tibia model leads to inconclusive results with respect to bone healing following PDT. Local application of VEGF has been shown to accelerate fracture bone healing. Interestingly, PDT has been reported to locally induce VEGF and enhance angiogenesis. The increase in local VEGF and concurrent decrease of serum VEGF levels in the closed tibia model indicates a potential positive role of PDT in fracture healing.

PDT treatment of rat femur critical size defect fractures led to lower overall formation of bone, but the bone had higher density with a decrease in the size of the fracture gap. The increase in bone density in the PDT treated groups may suggest formation of better quality bone (vs. quantity of bone). Histologically, with more cartilage and woven bone present in the control group in contrast to more mature bone and in the PDT group, the fracture healing seems to follow different pattern, which requires further investigation. In future experiments it is critical to achieve a minimum defect from > 6 mm. Additionally, further extension of the time course of the study (3 to 6 month) is needed to determine if PDT can reduce the incidence of non- or mal-unions in critically sized defects.

The balance between creating a local infection without a high risk of causing a general infection needs further investigation before it is possible to apply the PDT treatment.

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[10] Bisland SK, Chien C, Wilson BC, Burch S. Pre-clinical in vitro and in vivo studies to examine the potential use of photodynamic therapy in the treatment of osteomyelitis. Photochem Photobiol Sci. 2006;5:31-8.

Appendices

1. Figures
2. MHSRS abstract
3. ORS abstracts and posters

No personnel is receiving pay from the research effort.

Figures

Photodynamic Therapy Treatment to Enhance Fracture Healing

Specific Aim # 1: To determine the optimal timing of PDT administration post injury to enhance fracture healing

Task 1: Closed Fracture Model

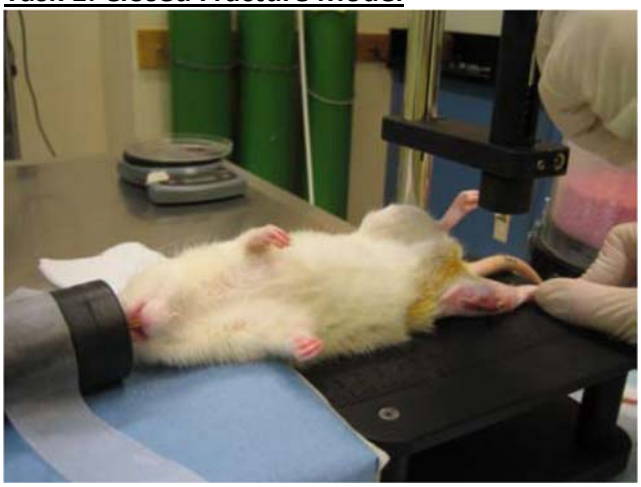


Figure 1: Fracture generation after insertion of a K-wire into the medullary canal of the tibia.

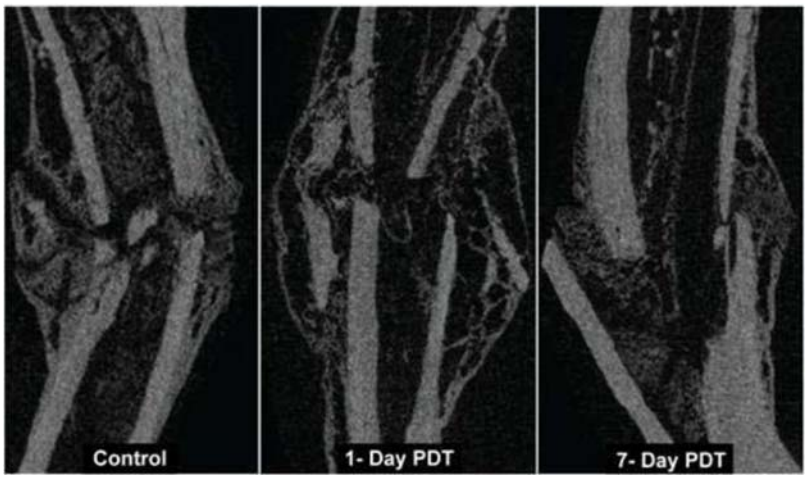
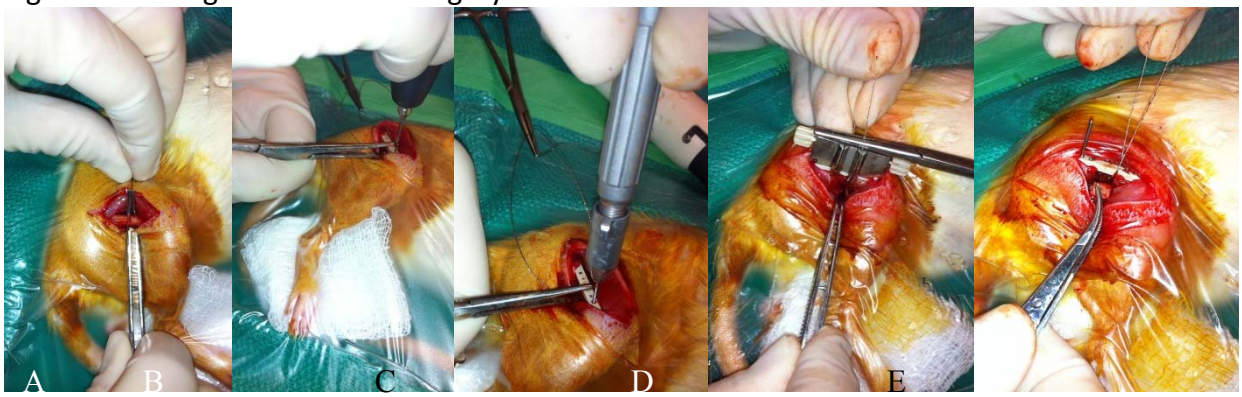


Figure 2: µCt images of the rat tibiae 28 days after fracture generation. The fracture line is clearly evident in the control specimen with minimal bridging bone formation. At 1d PDT and 7d PDT post fracture bone is seen to be forming within the fracture gap. This remains true despite the increased severity of the 7d PDT comminuted fracture shown here.

Task 2: Critical Size Defect Fracture Model

Figure 3 a-e: Segmental defect surgery in the rat femur



A) Positioning of the Gigly wire saw underneath the femur. B) Drilling the hole and C) placing the screw to secure the PEEK plate to the femur. D) Using the drill- and saw guide to guide the Gigly wire. E) Removing of the femur segment.

Figure 4: Photodynamic Therapy of a gap fracture under general anaesthesia

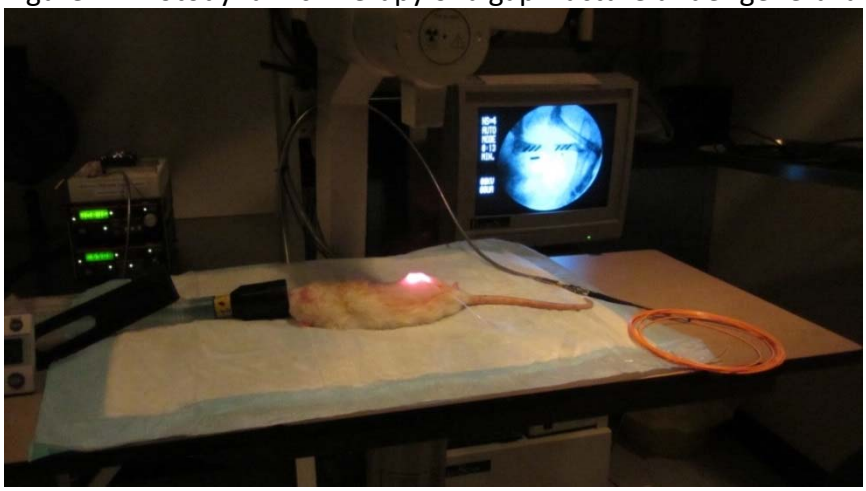


Figure 5: CT-images, H&E and VEGF immunhisto stain of mid-sagittal sections of the rat femur.

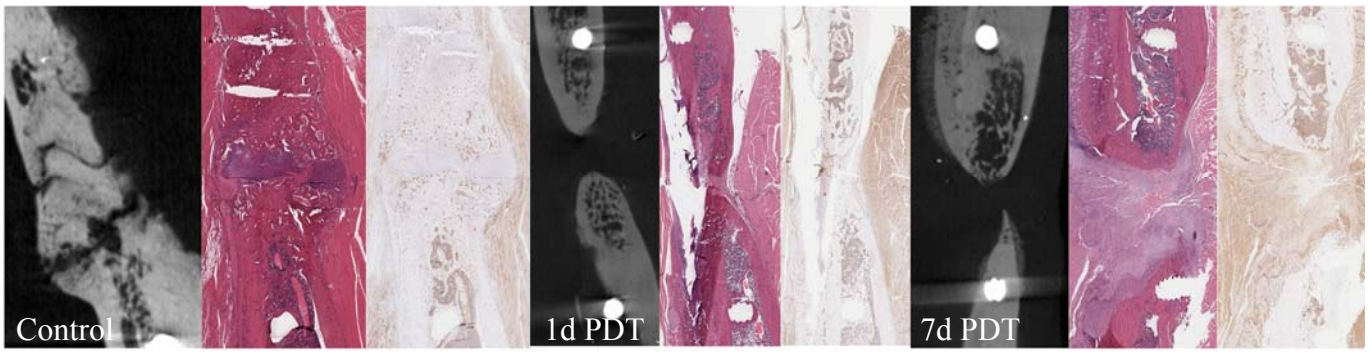
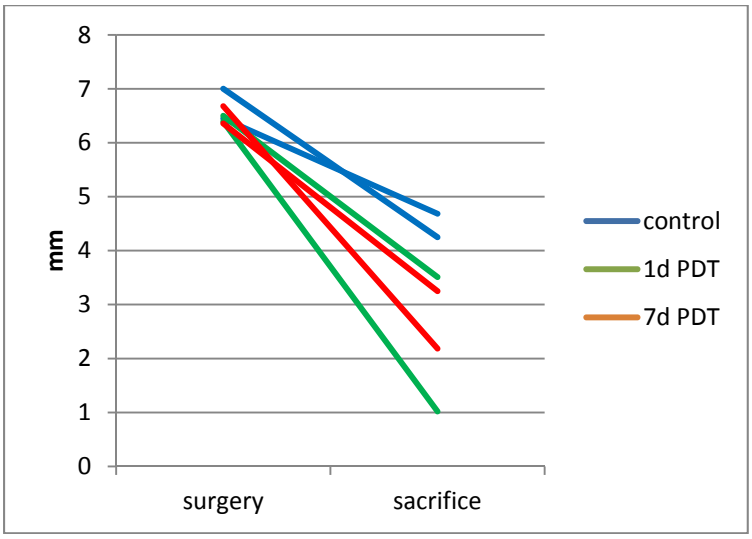


Figure 6: Gap difference of the 6 rats with a defect ≥ 6mm at day of surgery



Specific Aim # 2: Evaluation of the ability of PDT to improve fracture healing in the presence of infection

Task 3: Creating the infected critical size fracture model and PDT treatment

Figure 7: Implantation of bacteria sponge into fracture gap

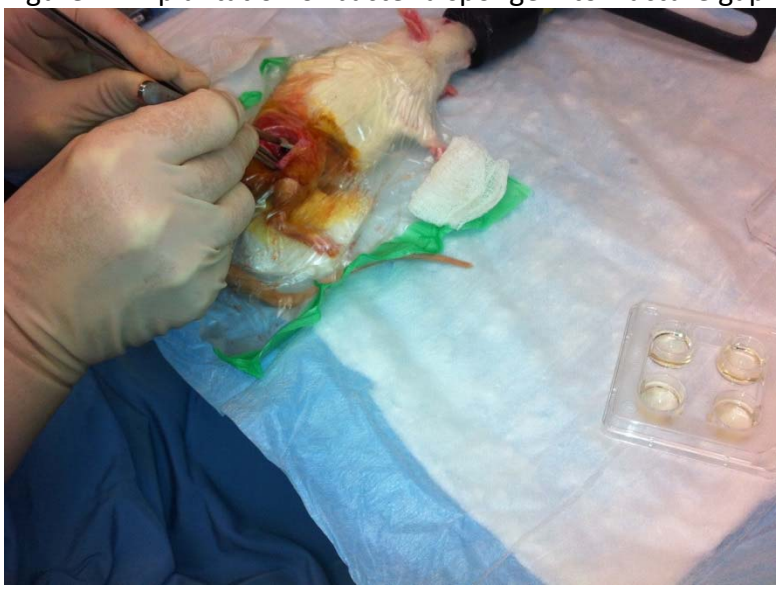


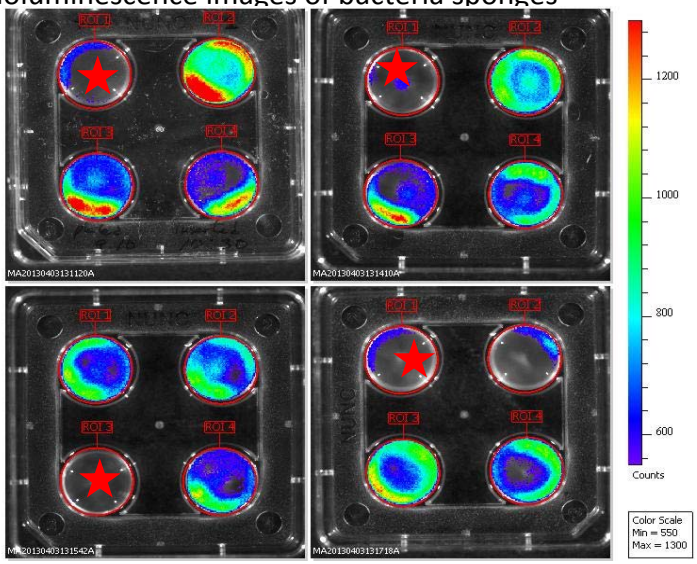
Figure 8 a: Bioluminescence images of bacteria sponges

Rat # 38

Rat # 39

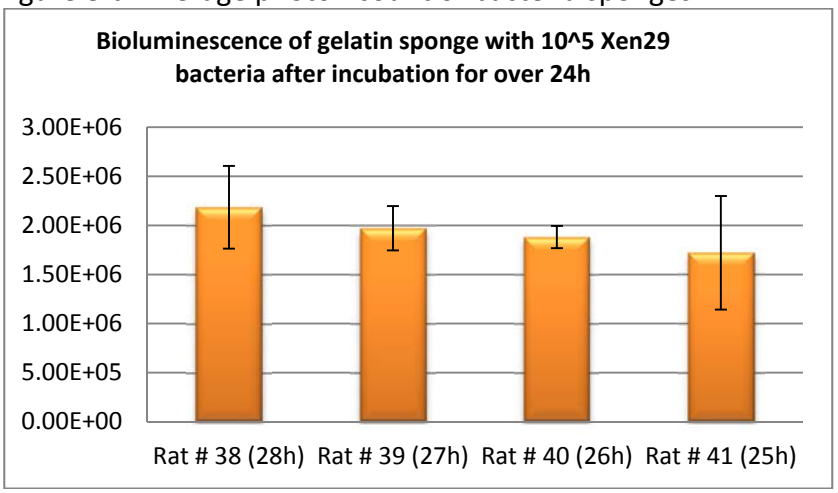
Rat # 40

Rat # 41



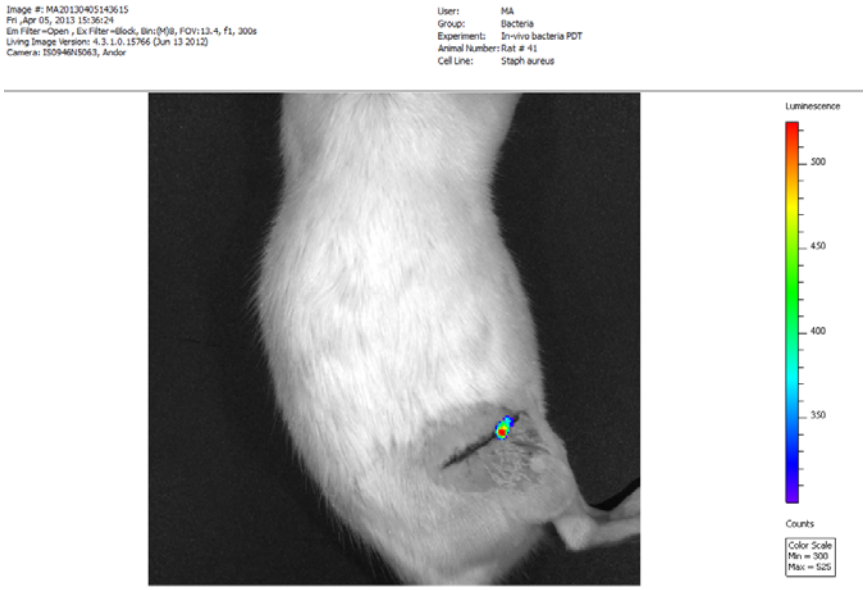
4 gelatin sponges were incubated with 10^5 CFU St. aureus (Xen29) for 1h prior to implantation of 1 sponge into the fracture gap, removed from well marked with a ★. 3 control gelatin sponge were incubated for over 24 hours and bacteria growth was evaluated by measuring bioluminescence

Figure 8 b: Average photon count of bacteria sponges



The fracture gap surgery takes 1 h including preparation and the start of the gelatin sponge incubation with bacteria was staggered accordingly. The bioluminescence of all 4 plates was measured the next day at the same time. The differences in bioluminescence signals are due to the different incubation times of each plate.

Figure 9: Bioluminescence image of control rat



Rat # 41: Three days after surgery a weak signal was visible but disappeared within the next week independent of treatment.

ENCLOSURE 4
ABSTRACT COVER PAGE

Title of Study: _Photodynamic Therapy to Enhance Fracture Healing_____

This abstract is being submitted for (check one):
( ) Oral presentation or poster display
( ) Oral presentation only
(X) Poster display only

This presentation represents:
(X) Quantitative research ( ) Qualitative research
( ) Research utilization ( ) Combined methods
( ) Clinical innovation

Consideration for Young Investigators' Forum (check one): ( ) YES (X) NO

Research Topic (from abstract call or describe): _Combat casualty care_____

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PHOTODYNAMIC THERAPY TO ENHANCE FRACTURE HEALING

Margarete K. Akens, PhD, David Wright, MSc., Sadiya Yousef, RLAT, Diane Nam, MD, MSc., Albert J. Yee, MD, MSc., Brian C. Wilson, PhD, Cari M. Whyne, PhD

PURPOSE/AIMS: The purpose of this research was to explore the potential of photodynamic therapy (PDT) to enhance healing in traumatic long bone fractures. PDT is a non-surgical, non-ionizing minimally invasive local treatment that utilizes a photosensitizing agent activated by light of a specific wavelength. This treatment has been successfully applied to treat multiple types of cancer and skin diseases. Surprisingly, recent findings from studies aimed at understanding the impact of PDT on vertebral metastases have shown that PDT rapidly improved vertebral bone strength, stiffness and architecture. These observations form the basis of the current study.

DESIGN: A comminuted tibia fracture was generated in 17 adult female Sprague-Dawley (SD) rats. The rats were randomly allocated to 3 groups: control (no treatment), PDT applied 1 day (1d) post-fracture or PDT applied 7d post-fracture.

MATERIALS AND METHODS: Under general inhalation anaesthesia a 0.8 mm Kirschner wire was placed inside the tibial medullary canal. After skin closure a unilateral fracture was generated in the mid-tibia with a custom drop weight impact apparatus (500g). The tibial and fibular fracture was confirmed using high resolution x-ray imaging. Prior to the application of light, a photosensitising drug (Visudyne, Novartis, Dorval, QC) was injected intravenously at a concentration of 1mg/kg. Fifteen minutes later, light energy of 75J was delivered at 690 nm using a 1 cm diffuser fibre placed subcutaneously parallel to the fracture under fluoroscopic guidance. Four weeks after fracture induction the tibiae were harvested and fixed in 10 % buffered formalin.

DATA ANALYSIS: Vascular endothelial growth factor (VEGF) serum concentration was determined an immunoassay. μCT images at an isotropic 14 μm/voxel resolution were acquired of the fracture site and callus for 3D architectural analysis. Histology sections were stained with haematoxylin and eosin and anti-VEGF antibody.

FINDINGS: To date, structural data from the first 11 rats have been analyzed. The total bone volume (TV) including callus formation, of the fracture site increased from $148\pm43mm^3$ in the control group, to $157\pm59mm^3$ in the PDT 1d group (6%) and to $175\pm25mm^3$ in the PDT 7d group (18%). Similarly, the bone volume within the callus (BV) increased from $75\pm8mm^3$ (control), to $78\pm19mm^3$ (1d-PDT) and $85\pm11mm^3$ (7d-PDT). The average VEGF serum level concentration was higher in the control group (n=2) compared to the treatments groups (n=4), however local VEGF staining revealed an opposite trend (n=17). Local application of VEGF has been shown to accelerate bone healing, yet a systemic more elevated and prolonged VEGF level has been found in patients after long bone fractures as an indication of impaired bone healing

PRELIMINARY CONCLUSIONS: PDT may have a beneficial effect on local VEGF expression and bone structure within the healing fracture callus.

IMPLICATIONS: If ongoing studies confirm the benefits of PDT are enhanced during the secondary stage of fracture healing, PDT could be applied to patients expected to encounter impaired healing, even without access to immediate medical care.

FROM/TO TIME PERIOD OF STUDY: October 2010 - Present.

FUNDING: PRORP CDMRP grant # OR090260.

The Effect of Photodynamic Therapy (PDT) on Long Bone Fracture Healing

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Introduction:
The majority of long bone fractures heal successfully without complications, however compound and/or comminuted fractures resulting from high impact trauma can result in delayed healing or non-union. Despite currently available treatments to reduce the risk of infection and/or enhance bone healing, such as the use of antibiotic-impregnated beads or bone-morphogenetic proteins, high impact traumatic fractures can take up to a year to fully heal. Some injuries progress to non-union, potentially leading to repetitive surgeries and long-term disability of the injured patient.
Photodynamic therapy (PDT) is a non-surgical, non-ionizing minimally invasive local treatment, which has been successfully applied to treat multiple types of cancer, skin diseases and age related macular degeneration. This treatment involves the local or systemic administration of a photosensitizing drug, which is activated by non-thermal laser light at a photosensitizer specific wavelength. This light activation leads, in the presence of oxygen, to the generation of cytotoxic species (e.g. singlet oxygen) [1], which can induce apoptosis and/or necrosis of targeted cells and tissue and also influence immune responses [2]. Unexpectedly, recent findings from studies aimed at understanding the impact of PDT on skeletal metastases secondary to breast cancer have shown that PDT rapidly improved vertebral bone strength, stiffness and architecture [3]. Based on these observations, the aim of this study is to explore the potential of PDT to enhance healing in traumatic long bone fractures.

Materials and Methods:
A comminuted tibia fracture was generated in 11 adult female Sprague-Dawley (SD) rats. Institutional animal care committee approval was obtained for all procedures (University Health Network, Toronto). General anaesthesia in the rat was induced with 4% isoflurane in oxygen and maintained at 2 % isoflurane in oxygen. Under sterile conditions a small lateral skin incision was made at the knee joint. Bending the knee, a 23g needle was used to enter and ream the medullary canal at the tibial plateau. Thereafter, a 0.8 mm Kirschner wire was placed inside the medullary canal. The skin incision was closed and a unilateral fracture was generated in the mid-tibia with a custom drop weight impact apparatus (500g). The tibial and fibular fracture was confirmed using high resolution x-ray imaging (Faxitron X-Ray LLC, Lincolnshire, IL). The rats received antibiotics and analgesics (0.05 mg/kg buprenorphine). The rats were randomly allocated to 3 groups: control (no treatment), PDT applied 1 day (1d) post-fracture or PDT applied 7d post-fracture. Prior to the application of light, a photosensitising drug (Visudyne, Novartis, Dorval, QC) was injected intravenously at a concentration of 1mg/kg. Fifteen minutes later, light energy of 75J was delivered at 690 nm using a 1 cm diffuser fibre placed subcutaneously parallel to the fracture under fluoroscopic guidance [4]. Weekly faxitron images and blood samples were acquired. Vascular endothelial growth factor (VEGF) serum concentration was determined using Quantikine® RatVEGF Immunoassay (R&D Systems, Minneapolis, MN). The rats were euthanized 4 weeks after induction of the fracture and their tibiae were harvested. µCT images at an isotropic 14 µm/voxel resolution (SkyScan 1172 High Resolution MicroCT System, Skyscan, Belgium) were acquired of the fracture site and callus for 3D architectural analysis (CTAn, Skyscan, Belgium).

Results:
All rats recovered well from the fracture generation and PDT treatments, with the exception of one animal, which had to be euthanized early due to large displacement of the K-wire. The total bone volume (TV) including callus formation, of the fracture site increased from 148±43mm^3 in the control group, to 157±59mm^3 in the PDT 1d group (6%) and to 175±25mm^3 in the PDT 7d group (18%). Similarly, the bone volume within the callus (BV) increased from 75±8mm^3 (control), to 78±19mm^3 (1d-PDT) and 85±11mm^3 (7d-PDT). The average VEGF serum level concentration was higher in the control group compared to the treatments groups.

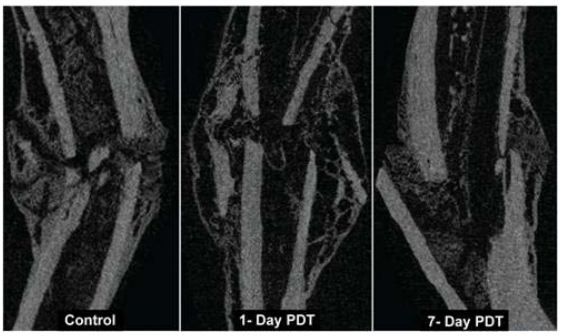

Figure 1: µCt images of the rat tibia fracture 28 days after fracture generation. The fracture line is clearly evident in the control specimen with minimal bridging bone formation. At 1d PDT and 7d PDT post fracture bone is seen to be forming within the fracture gap. This remains true despite the increased severity of the 7d PDT comminuted fracture shown here.

Discussion:
PDT treatment of the fractured rat tibiae resulted in an increase of bone formation after 4 weeks as compared to control untreated fractures, despite the high variability in the generation of the comminuted fractures. This observation of increased bone concurs with previous findings of the impact of PDT in the bony spine [3]. Further, the relative increases (compared to control) in both bone and callus volume in the 7d PDT group was found to be ~3 times higher than in the 1d PDT group. This indicates that the tissue response to PDT stimulation is dependent on the fracture healing remodeling stage. As such, PDT may be less effective during inflammation, the first stage of fracture healing at 1d post treatment, vs. during the formulation of granulation tissue, the second stage of fracture healing at 7d post-fracture.
Local application of VEGF has been shown to accelerate bone healing [5]. Interestingly, PDT has been reported to locally induce VEGF and enhance angiogenesis in the retinal pigment. Yet, a systemic more elevated and prolonged VEGF level has been found in patients after long bone fractures as an indication of impaired bone healing [5]. Histology and immunostaining of the samples will give more information about the local expression of VEGF, angiogenesis and the progress of bone remodeling and fracture healing.

Significance:
If PDT is able to enhance fracture healing in complex fractures it may provide a cost-effective local minimally invasive treatment for long bone fractures at risk for impaired bone healing. Further, if ongoing studies confirm the benefits of PDT are enhanced during the secondary stage of fracture healing, PDT could be applied to patients expected to encounter impaired healing, even without access to immediate medical care.

Acknowledgements:
Funding for this project was provided by the Orthopaedic Research Program (PRORP) of the Office of the Congressionally Directed Medical Research programs (CDMRP), grant # OR090260.

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5. Sarahrudi, K. et al. (2009) J Orthop Res. 2009 (10):1293-7.

The Effect of Photodynamic Therapy (PDT) on Long Bone Fracture Healing

Margarete K. Akens*, David Wright*, Sadiya Yousef#, Diane Nam*, Albert J.M. Yee* Brian C. Wilson# and Cari M. Whyne*

* Orthopaedic Biomechanics Laboratory, Sunnybrook Research Institute, University of Toronto, Toronto, ON, Canada, #Division of Biophysics and Bioimaging, Ontario Cancer Institute, Toronto, ON, Canada

Introduction

✓ The majority of long bone fractures heal successfully without complications, however compound and/or comminuted fractures resulting from high impact trauma can result in delayed healing. Despite currently available treatments to reduce the risk of infection and/or enhance bone healing, such as the use of antibiotic-impregnated beads or bone-morphogenetic proteins, high impact traumatic fractures can take up to a year to fully heal. Some injuries progress to non-union, potentially leading to repetitive surgeries and long-term disability of the injured patient.

✓ Photodynamic therapy (PDT) has been applied successfully as a non-radiative treatment for numerous malignancies and skin diseases. This treatment involves a local or systemic administration of a photosensitizing drug, which is activated by laser light at a photosensitizer specific wavelength. The light activation leads, in the presence of oxygen, to the generation of singlet oxygen, which can induce apoptosis and/or necrosis of targeted cells or tissue and also influences immune responses.

✓ Unexpectedly, recent findings from studies aimed at understanding the impact of PDT on spinal metastases have shown that PDT rapidly improved vertebral bone strength, stiffness and architecture[1].

✓ The aim of this study was to explore the potential of PDT to enhance healing in traumatic long bone fractures.

Materials and Methods

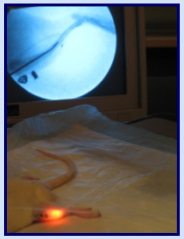
✓ A comminuted tibia fracture was generated in 17 adult female Sprague Dawley (SD) rats. Institutional animal care committee approval was obtained and acceptable tumor endpoints in animal care adhered to. Under general inhalation anaesthesia a 0.8 mm K-wire was inserted into the medullary canal of the tibia. The fracture was generated in the mid-tibia with a custom drop weight impact apparatus (500g)(Figure 1a).

✓ Rats were randomly allocated to 3 different groups: **A**: control, no treatment; **B**: PDT treatment 1 day after fracture generation; **C**: PDT treatment 7 days after fracture generation. For the PDT treatment, the photosensitizer verteporfin (Visudyne®, Novartis, Dorval, QC) was injected (1.0 mg/kg i.v.) and 15 minutes later light (75J) was delivered using a 1 mm diffuser fibre placed subcutaneously parallel to the fracture (Figure 1b)[2].

✓ Four weeks after fracture generation the tibiae were harvested, fixed in 10% buffered formalin. Prior to processing specimen for histology, µCT images at an isotropic 14 µm voxel resolution were acquired of the fracture site and callus for 3D architectural analysis. Thereafter, the tibiae were decalcified and stained with H&E (Figure 3) and vascular endothelial growth factor (VEGF)-antibody. Immunhistochemistry staining was used to histo-morphometrically quantify VEGF expression within the callus (ImageScope™, Aperio Technologies, Inc., Vista, CA, USA) on midsagital sections (Figure 4). VEGF serum concentration was determined using Quantikine® RatVEGF Immunoassay (R & D Systems, Minneapolis, MN).

✓ Statistical analyses were performed using a one-way ANOVA with a Tukey post hoc test. A p-value of $p<.05$ was considered to be statistically significant.

Figure 1a: Fracture generation in the mid-tibia



1b: PDT treatment of a rat tibia fracture

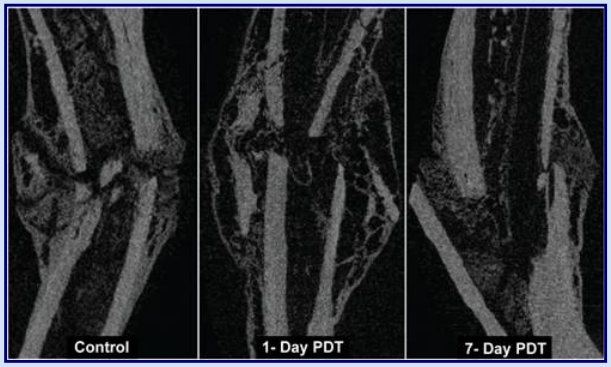


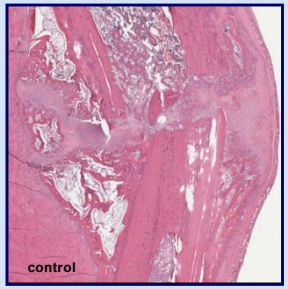
Figure 2: µCT images of the rat tibia fracture 28 days after fracture generation. The fracture line is clearly evident in the control specimen with minimal bridging bone formation. Following PDT treatment 1d or 7d post fracture bone is seen to be forming within the fracture gap. This remains true despite the increased severity of the 7d PDT comminuted fracture shown here.

Results

✓ All rats recovered well from the fracture generation and PDT treatments, with the exception of one animal, which had to be euthanized early due to large displacement of the K-wire. The total bone volume (TV) including callus formation, of the fracture site increased from 148±43 mm^3 in the control group, to 157±59 mm^3 in the PDT 1d group (6 %) and to 175±25 mm^3 in the PDT 7d group (18%). Similarly, the bone volume within the callus (BV) increased from 75±8 mm^3 (control), to 78±19 mm3 (1d-PDT) and 85±11 mm3 (7d-PDT).

✓ The average VEGF serum level concentration 28 days after fracture generation was higher in the control group (57.3 ± 5.5 pg/ml) compared to 1d-PDT (35.2 ± 14.8 pg/ml) and 7d-PDT (33.0 ± 13.7 pg/ml)($p > .05$).

✓ In contrast to the VEGF serum level, the local VEGF expression in the callus was slightly higher in the 7d-PDT group (57 ± 5 %) compared to 1d-PDT (54 ± 5 %) and control (53 ± 4%) ($p> .05$).



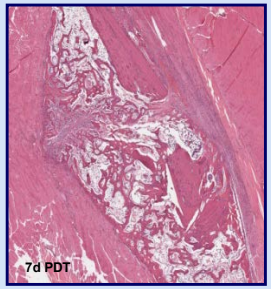


Figure 3: H & E staining of a midsagital sections of a rat tibiae four weeks after fracture generation

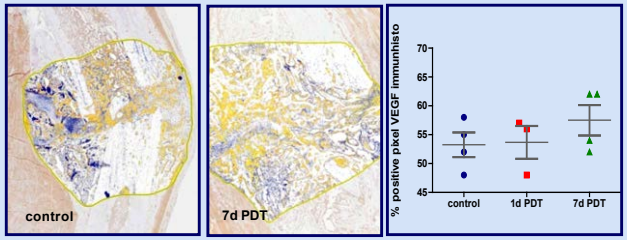


Figure 4: VEGF-antibody staining of a midsagital sections of a rat tibiae four weeks after fracture generation. The VEGF staining intensity was analyzed using positive pixel count (orange-yellow) (n=11).

Discussion

✓ PDT treatment of the fractured rat tibiae resulted in an increase of bone formation after 4 weeks as compared to control untreated fractures, despite the high variability in the generation of the comminuted fractures. This observation of increased bone concurs with previous findings of the impact of PDT on the bony spine[1]. Both bone and callus volume in the 7d PDT group was found to be ~ 3 times higher than in the 1d PDT group. This indicates that the tissue response to PDT stimulation is dependent on the fracture healing remodeling stage.

✓ Local application of VEGF has been shown to accelerate bone healing. Interestingly, PDT has been reported to locally induce VEGF and enhance angiogenesis in the retinal pigment. We found a slightly increase in local VEGF expression in these tibiae fractures, which may have contributed to increased bone formation in the treated groups. Systemically, an increased VEGF serum level was found in the control, untreated group compared to the PDT treated groups. A systemic elevated and prolonged VEGF level has been found as an indication of impaired bone healing in patients with long bone fractures [3].

Significance

✓ If PDT is able to enhance fracture healing in complex fractures it may provide a cost-effective local minimally invasive treatment for long bone fractures at risk for impaired bone healing. Further, if ongoing studies confirm the benefits of PDT are enhanced during the secondary stage of fracture healing, PDT could be applied to patients expected to encounter impaired healing, even without access to immediate medical care.

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2. Akens, M. K., M. R. Hardisty, et al. (2009). Breast Cancer Research Treatment 119(2): 325-333.
3. Sarahrudi, K., A. Thomas, et al. (2009). J Orthop Res.

Acknowledgements

Funding for this project was provided by the Orthopaedic Research Program (PRORP) of the Office of the Congressionally Directed Medical Research programs (CDMRP), grant # OR090260.

The Effect of Photodynamic Therapy (PDT) on Femur Fractures with Critically Sized Defects

+[1,4]Akens, M.K.; [2,4]Bernick, J.; [3]Yousef, S.; [2,4]Nam, D., [2,4]Yee, A.J.M, [3,4]Wilson, B.C., [2,4]Whyne, C.M.
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Significance: PDT may provide a cost-effective local minimally invasive treatment to enhance long bone fracture healing, by improving bone quality and reducing the development of non-unions in fractures with critically sized defects.

Introduction: The majority of long bone fractures heal successfully without complications, however compound and/or comminuted fractures resulting from high impact trauma can result in delayed healing or non-union. Early intervention in high risk fractures could decrease patient morbidity and reduce health care system costs(1). Photodynamic therapy (PDT) is a non-surgical, non-ionizing minimally invasive local treatment, which has been successfully applied to treat multiple types of cancer, skin diseases and age related macular degeneration. This treatment involves the local or systemic administration of a photosensitizing drug, which is activated by non-thermal laser light at a photosensitizer specific wavelength. This light activation leads, in the presence of oxygen, to the generation of cytotoxic species (e.g. singlet oxygen) (2), which can induce apoptosis and/or necrosis of targeted cells and tissue and also influence immune responses (3). PDT treatment of metastatically involved vertebrae resulted in improved vertebral bone strength, stiffness and architecture, motivating the investigation of PDT as an approach to augment bone healing(4). In our previous work, applying PDT treatment to comminuted rat tibia fractures yielded an increase of bone formation after 4 weeks(5). The aim of this study was to evaluate the ability of PDT treatment to enhance healing and/or prevent the development of non-union in femoral fractures exhibiting critically size defects.

Materials and Methods: Femoral fractures with critically sized defects (6 mm in length) were generated in 20 adult female Sprague-Dawley (SD) rats. Under general anaesthesia the femur was exposed and an 8-hole PEEK plate attached laterally with 6 screws. Using a Gigly saw, a 6-mm mid-shaft bone piece was then removed beneath the middle of the plate. The musculature and skin incision was then closed. The positioning of the plate and screws was confirmed using high resolution x-ray imaging (Faxitron X-Ray LLC, Lincolnshire, IL). Rats were randomly allocated to 3 groups: control (no treatment); PDT applied 1 day (1d) post fracture and PDT applied 7 days (7d) post fracture. For PDT treatment prior to the application of light, a photosensitising drug (Visudyne, Novartis, Dorval, QC, Canada) was injected intravenously at a concentration of 1mg/kg. Fifteen minutes later, light energy of 75J was delivered at 690 nm using a 1 cm diffuser fibre placed subcutaneously parallel to the fracture under fluoroscopic guidance(6). Weekly faxitron images and blood samples were taken and serum stored for further analyses. Institutional animal care committee approval was obtained for all procedures. Vascular endothelial growth factor (VEGF) serum concentration was determined using Quantikine® RatVEGF Immunoassay (R&D Systems, Minneapolis, MN, USA), the C-terminal telopeptide of type I collagen (PICP) using the RatLaps™ EIA and osteocalcin using the RatMID™ Osteocalcein EIA (Immunodiagnostic Systems Ltd., Fountain Hills, AZ, USA). The rats were euthanized 7 weeks after induction of the fracture and their femora harvested. µCT images at an isotropic 13.3 µm/voxel resolution (Inveon MicroCT, Siemens, Erlangen, Germany) were acquired of the fracture site and callus for 3D architectural analysis (AmiraDev 5.2, FEI Visualization Science Group, Burlington, USA)). Thereafter, the bone was decalcified and processed for histology. Statistical analysis of the bone volume measurements was performed using a 1-way analysis of variance.

Results: All rats recovered well from the fracture generation and PDT treatments; however three animals were euthanized early due to plate displacement. The PICP concentration in the control group was generally stable, decreasing slightly after surgery and returning to pre-surgical levels after 4-5 weeks. In the PDT treated groups, the PICP concentration fluctuated greatly at both day14 and day 42 with half of the specimens demonstrating increases in concentration compared to pre-op values and half reductions. There were no differences found in the serum osteocalcein levels or the VEGF serum level concentrations between groups. The total bone volume (TV) was evaluated from µCT images in a region of interest extending incorporating the fracture gap and extending to the first screw placements on either side of the gap (9mm). No significant differences were found in TV, however a trend toward lower volumes was seen with PDT treatment and longer time until treatment (control: 58 ± 29 mm^3; 1d PDT: 49 ± 17 mm^3; and 7d PDT: 38 ± 9 mm^3). In contrast, BMD (gHA/cm^2) trended toward higher values in the PDT treated groups (1d PDT: 0.86 ± 0.02; 7d PDT: 0.85 ± 0.08) compared to controls (0.79 ± 0.09). The fracture gap measured on histology slides/ µCT images demonstrated a trend toward smaller gaps in the PDT treated groups (control: 3.68 ± 0.94 mm; 1d PDT 2.81 ± 1.17 mm; 7d PDT 3.43± 0.59 mm). The histology slides of the control group showed more cartilage and woven bone formation in contrast to the PDT treated groups which exhibited more structured and mature bone (Fig 1). Two of the control rats showed histological signs of the development of non-unions, which was not seen in the PDT treated groups.

Discussion: PDT treatment of rat femur fractures led to lower overall formation of bone, but the bone had higher density with a decrease in the size of the fracture gap. The increase in bone density in the PDT treated groups may suggest formation of better quality bone (vs. quantity of bone). Histologically, with more cartilage and woven bone present in the control group in contrast to more mature bone and in the PDT group, the fracture healing seems to follow different pattern, which requires further investigation. The high variance seen in the serum measurements of bone markers is not uncommon and has previously reported within the literature(1). Further extension of the time course of the study is needed to determine if PDT can reduce the incidence of non or mal unions in critically sized defects.

Acknowledgements: Funding for this project was provided by the Orthopaedic Research Program (PRORP) of the Office of the Congressionally directed Medical Research programs (CDMRP), grant # OR090260.

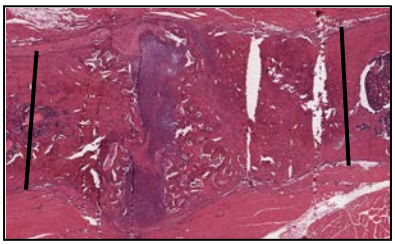
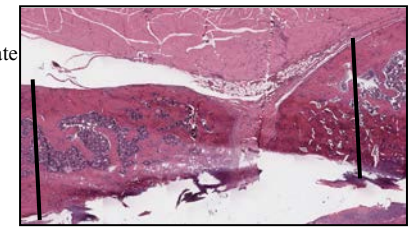


Figure 1: Sagital histology section (H&E) of a femur fracture gap 7 weeks after fracture generation. Lines indicate the original gap size



Control group

1d PDT group

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The Effect of Photodynamic Therapy (PDT) on Femur Fractures with Critically Sized Defects

Margarete K. Akens*¶∞, Joshua Bernick*, Sadiya Yousef#, Diane Nam*¶, Albert J.M. Yee*¶, Brian C. Wilson# and Cari M. Whyne*¶

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Introduction

- The majority of long bone fractures heal successfully without complications, however compound and/or comminuted fractures resulting from high impact trauma can result in delayed healing or non-union. Early intervention in high risk fractures could decrease patient morbidity and reduce health care system costs(1).
- Photodynamic therapy (PDT) is a non-surgical, non-ionizing minimally invasive local treatment, which has been successfully applied to treat multiple types of cancer, skin diseases and age related macular degeneration. This treatment involves the local or systemic administration of a photosensitizing drug, which is activated by non-thermal laser light at a photosensitizer specific wavelength. This light activation leads, in the presence of oxygen, to the generation of cytotoxic species (e.g. singlet oxygen) (2), which can induce apoptosis and/or necrosis of targeted cells and tissue and also influence immune responses (3).
- PDT treatment of metastatically involved vertebrae resulted in improved vertebral bone strength, stiffness and architecture, motivating the investigation of PDT as an approach to augment bone healing(4). In our previous work, applying PDT treatment to comminuted rat tibia fractures yielded an increase of bone formation after 4 weeks(5).
- **The aim of this study was to evaluate the ability of PDT treatment to enhance healing and/or prevent the development of non-union in femoral fractures exhibiting critically size defects**.

Materials and Methods

- Femoral fractures with critically sized defects (6 mm in length) were generated in 20 adult female Sprague-Dawley (SD) rats. Under general anaesthesia the femur was exposed and an 8-hole PEEK plate attached laterally with 6 screws (Fig 1). Using a Gigly saw, a 6-mm mid-shaft bone piece was then removed beneath the middle of the plate (RISystem, Davos, Switzerland). The musculature and skin incision was then closed. The positioning of the plate and screws was confirmed using high resolution x-ray imaging (Faxitron X-Ray LLC, Lincolnshire, IL) (Fig 2a).
- Rats were randomly allocated to 3 groups: control (no treatment) (n=5); PDT applied 1 day (1d) post fracture (n=5) and PDT applied 7 days (7d) (n=7) post fracture. For PDT treatment prior to the application of light, a photosensitising drug (Visudyne, Novartis, Dorval, QC, Canada) was injected intravenously at a concentration of 1mg/kg. Fifteen minutes later, light energy of 75J was delivered at 690 nm using a 1 cm diffuser fibre placed subcutaneously parallel to the fracture under fluoroscopic guidance(6) (Fig 2b). Weekly faxitron images and blood samples were taken and serum stored for further analyses. Institutional animal care committee approval was obtained for all procedures.
- Vascular endothelial growth factor (VEGF) serum concentration was determined using Quantikine® RatVEGF Immunoassay (R&D Systems, Minneapolis, MN, USA), the C-terminal telopeptide of type I collagen (PICP) using the RatLaps™ EIA and osteocalcin using the RatMID™ Osteocalcein EIA (Immunodiagnostic Systems Ltd., Fountain Hills, AZ, USA). The rats were euthanized 7 weeks after induction of the fracture and their femora harvested. µCT images at an isotropic 13.3 µm/voxel resolution (Inveon MicroCT, Siemens, Erlangen, Germany) were acquired of the fracture site and callus for 3D architectural analysis (AmiraDev 5.2, FEI Visualization Science Group, Burlington, USA)). Thereafter, the bone was decalcified and processed for histology. Statistical analysis of the bone volume measurements was performed using a 1-way analysis of variance.

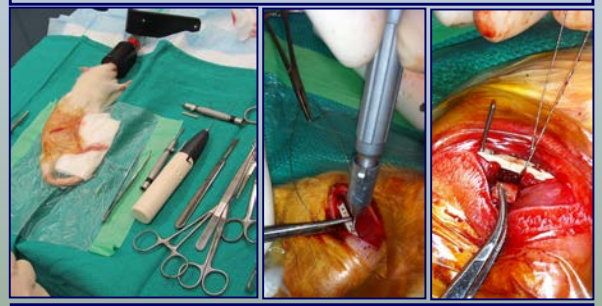

Figure 1: Surgical creation of a 6 mm critical size defect rat femur fracture.

Figure 2a: Faxitron image post surgery. 2b: PDT treatment of a critical size defect in the rat femur.

Results

- All rats recovered well from the fracture generation and PDT treatments; however three animals were euthanized early due to plate displacement.
- The PICP concentration in the control group was generally stable, decreasing slightly after surgery and returning to pre-surgical levels after 4-5 weeks. In the PDT treated groups, the PICP concentration fluctuated greatly at both day 14 and day 42 with half of the specimens demonstrating increases in concentration compared to pre-op values and half reductions. There were no differences found in the serum osteocalcein levels or the VEGF serum level concentrations between groups.
- The total bone volume (TV) was evaluated from µCT images in a region of interest extending incorporating the fracture gap and extending to the first screw placements on either side of the gap (9mm). No significant differences were found in TV, however a tendency toward lower volumes was seen with PDT treatment and longer time until treatment (control: 58 ± 29 mm^3; 1d PDT: 49 ± 17 mm^3; and 7d PDT: 38 ± 9 mm^3). In contrast, BMD (gHA/cm^2) trended toward higher values in the PDT treated groups (1d PDT: 0.86 ± 0.02; 7d PDT: 0.85 ± 0.08) compared to controls (0.79 ± 0.09). The fracture gap measured on histology slides (control: 3.68 ± 0.94 mm; 1d PDT 2.81 ± 1.17 mm; 7d PDT 3.43± 0.59 mm) and µCT images (control: 2.24 ± 2.06 mm; 1d PDT 1.80 ± 0.85 mm; 7d PDT 2.64 ± 0.87 mm) demonstrated slightly smaller gaps in more rats in the 1d PDT group compared to control. The histology slides of the control group showed more cartilage and woven bone formation in contrast to the PDT treated groups which exhibited more structured and mature bone (Fig 3 and 4). Two of the control rats showed histological signs of the development of non-unions, which was not seen in the PDT treated groups.

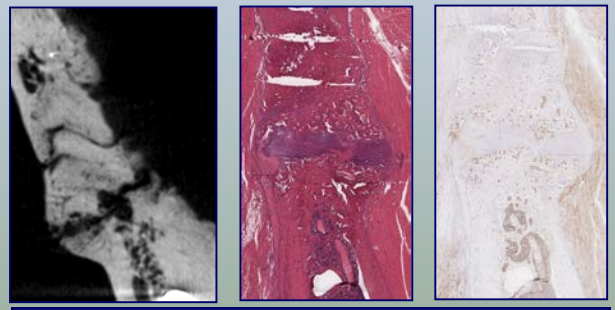

Figure 3: µCT and histology images (H&E; VEGF immunhistostain) of the femur of a control rat.

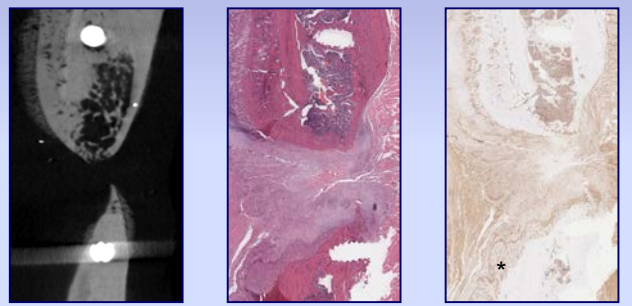

Figure 4: µCT and histology images (H&E; VEGF immunhistostain) of the femur of a 1d PDT group rat. Intensive VEGF staining can be seen in the newly formed bone *.

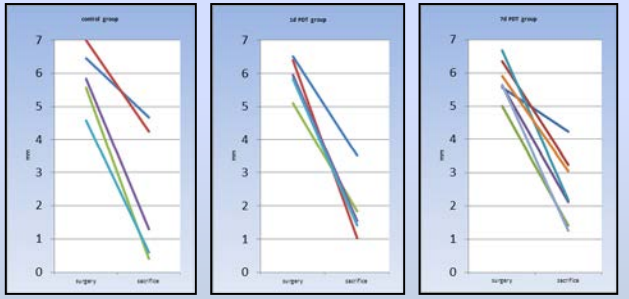

Figure 5: Fracture gap sizes (µCT images) difference between the time of surgery and sacrifice.

Discussion and Conclusion

- PDT treatment of rat femur fractures led to lower overall formation of bone, but the bone had higher density with a decrease in the size of the fracture gap. The increase in bone density in the PDT treated groups may suggest formation of better quality bone (vs. quantity of bone).
- Histologically, more cartilage and woven bone are present in the control group in contrast to more mature bone and in the PDT group. The possibility that fracture healing may follow a different pattern with PDT treatment requires further investigation
- The high variance seen in the serum measurements of bone markers is not uncommon and has previously reported within the literature(1).
- Further extension of the time course of the study and larger sample sizes are needed to determine if PDT can reduce the incidence of non or mal unions in critically sized defects.

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