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Original Article

Evaluation of zoledronate, cytochalasin-D, and desferrioxamine on osseointegration in an intra-medullary femoral implant model

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Abstract

Objective: The rise in primary and revision surgeries utilizing joint replacement implants suggest the need for more reliable means of promoting implant fixation. Zoledronate-(Zol), cytochalasin-D-(cytoD), and desferrioxamine-(DFO) have been shown to enhance mesenchymal stem cell (MSC) differentiation into osteoblasts promoting bone formation. The objective was to determine whether Zol, cytoD, and DFO can improve fixation strength and enhance peri-implant bone volume about intra-medullary femoral implants. **Methods**: 48 Sprague-Dawley female rats were randomized into four treatments, saline-control or experimental: Zol-(0.8 μg/μL), cytoD-(0.05 μg/μL), DFO-(0.4 μg/μL). Implants were placed bilaterally in the femoral canals following injection of treatment solution and followed for 28 days. Mechanical push-out testing and micro-CT were our primary evaluations, measuring load to failure and bone volume. Qualitative evaluation included histological assessment. Data was analyzed with a one-way ANOVA with Holm-Sidak mean comparison testing. **Results**: Significant results included pushout tests showing an increase in maximum energy for Zol (124%) and cytoD (82%); Zol showed an increase in maximum load by 48%; Zol micro-CT showed increase in BV/TV by 35%. **Conclusions**: Our findings suggest that locally applied Zol and cytoD enhance implant mechanical stability. Bisphosphonates and actin regulators, like cytoD, might be further investigated as a new strategy for improving osseointegration.

Keywords: Bisphosphonates, Cytochalasin-D, Implants, Osseointegration, Rats

Introduction

Intra-medullary implants are commonly used for a variety of orthopaedic procedures, such as joint replacements and transcutaneous prosthetic attachments. Over recent years, there has been a substantial rise in these common surgeries followed by a rise in aseptic loosening and hardware failure, suggesting the need for additional therapies to improve osseointegration and implant stability.

It is predicted that total knee replacements (TKR) and revisions will increase by 673% and 601%, respectively, between the years 2005 and 2030[1]. The predicted rise in the need for primary and revision surgeries is largely due to the aging population, which is associated with arthritis and metabolic bone diseases like osteoporosis. Age-related skeletal changes such as these lead to poor quality bone and a reduction in capacity of the bone to regenerate and properly integrate onto the implant[2]. Failure is often associated with early micro-motion of the implant-bone interface and osteolysis associated with peri-implant bone loss[3,4]. Utilization of medications to promote bone ingrowth around cementless implants thereby improving implant stability and reducing access of wear particles to the bone-implant interface represents an attractive option to prevent implant loosening. In this regard, bisphosphonates have been shown to positively enhance fixation in implant studies[5]. Additional therapies are needed to enhance

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peri-implant bone formation to promote early fixation of cementless implants.

Current methods shown to improve bone formation include bone morphogenetic proteins (BMPs) and bisphosphonates. BMPs are known to stimulate osteogenesis and are clinically used, but are very expensive and are associated with ectopic ossification[6-8]. Bisphosphonates are used clinically to combat metabolic bone diseases and are relatively safe. Bisphosphonates' mechanism of improving bone quality has historically been attributed to its ability to inhibit osteoclasts, but recently they have been shown to stimulate mesenchymal stem cell (MSC) differentiation into osteoblasts[9]. While many bisphosphonate studies have shown positive results, the majority utilize systemic administration which may be associated with some risk for adverse events, such as gastrointestinal upset and mandibular osteonecrosis[10]. Local delivery is an attractive option that may provide local benefit at the administration site without the risks of systemic medications which is particularly appropriate in joint replacement surgery. In a rat intramedullary osseointegration model, zoledronate exhibited a robust improvement in implant fixation strength when administered by a single injection in the femoral intra-medullary canal[11,12]. A limitation to this study was the large injection volume (approaching the volume of the canal) used which may not be feasible clinically.

A promising approach to enhance osseointegration of intra-medullary implants and improve surgical outcomes is local delivery of compounds that stimulate MSCs to differentiate into osteoblasts. In addition to zoledronate (Zol), desferrioxamine (DFO) and cytochalasin D (cytoD) have also recently been shown to induce MSCs into the osteogenic lineage through different mechanisms[13,14]. DFO works through β-catenin signaling cascades, and CytoD's mechanism involves cytoskeleton disruption/intra-nuclear actin transport[13-15]. DFO is currently FDA approved as an iron chelating agent for treatment of iron overload, but has been shown in bone fracture studies to improve bone density and healing of segmental defects[16-18]. CytoD has only recently been discovered to induce osteogenesis, and in an in-vivo study showed significant new bone formation one week after intramedullary administration into mice tibias[13]. Even though there is evidence that DFO and cytoD increase bone formation, they have not been evaluated with respect to osseointegration of implants within the medullary canal. Of note, the medullary canal is rich in MSCs that may provide a robust environment for osteogenesis. Additionally, local injection of Zol, DFO, or cytoD would be significantly more cost effective than current therapies and could provide the improved osseointegration necessary to ensure fixation of intra-medullary implants. Therefore, the objective of this study was to evaluate the ability of a single local intramedullary dose of solutions containing Zol, cytoD, or DFO to improve fixation strength and enhance peri-implant bone formation about intra-medullary femoral implants in a rat model.

Materials and methods

All experimental procedures were approved by the University of North Carolina (UNC) Institutional Animal Care and Use Committee (IACUC) prior to initiation of the study. 48 retired breeder (mean age=24 weeks) female Sprague Dawley rats (Envigo Inc., Dublin, VA) were randomly assigned to a control group or one of three experimental groups (N=12/group) based on weight to give equivalent mean body weights. The control injection solution consisted of 25μL of phosphate-buffered saline and the three experimental treatments consisted 25 μL of Zol (0.8 μg/μL), cytoD (0.05 μg/μL), or DFO (0.4 μg/μL). The concentrations of DFO and Zol were based on previous studies showing a positive result on bone formation while limiting side effects[11,16]. CytoD has only recently been found to induce osteogenesis and previous concentrations were not found in the literature for this type of study, thus we based our CytoD concentration on a previous study showing the stimulation of intramedullary bone formation[13]. Via a medial arthrotomy of the knee implants were placed in the bilateral femoral canals following local injection of 25 μL of the treatment solution into the reamed femoral canal. X-rays were taken immediately post-operation to ensure correct placement of implants. Rats were housed in pairs in a registered and accredited USDA Animal Research facility and given *ad libitum* access to food and water with a 12-hr light/ dark cycle (7 am to 7 pm) throughout the study. Animals were followed for 28 days before being euthanized.

Implants

The implants were 20 mm in length, 1.5 mm in diameter, and made of a titanium alloy (Ti-6Al-4V) with dimples on each end to allow mechanical push-out testing. They were produced by electron beam melting manufacturing. Implants produced by this method have an "as built" 23μm arithmetic surface roughness. Implants were ultrasonically cleaned in a 1% Alconox 10 gm/L solution at 65°C for 15 minutes. The implants were rinsed twice with 65°C deionized water for ten minutes under ultrasonic agitation. All implants were textured by acid etching in a 48% sulfuric acid (H2SO4) bath at 60°C and were agitated with a stir bar for 30 minutes[19]. The implants were rinsed in deionized water, dehydrated in a 70% ethanol solution, and allowed to air dry before packaging for sterilization by autoclave.

Surgical method

Under isoflurane anesthesia, the bilateral lower extremities were shaved, prepped, and draped in sterile fashion. Initially, an approximately 1.5 cm longitudinal incision was made through the skin lateral to the knee, the skin reflected medially, then an approximately 0.5 cm incision was made just medial to the patella and the patellar tendon. The patella was reflected laterally to expose the femoral condyles. An 18 gauge (1.16 mm) needle was used to start a hole between the condyles and into the medullary canal. The final hole was

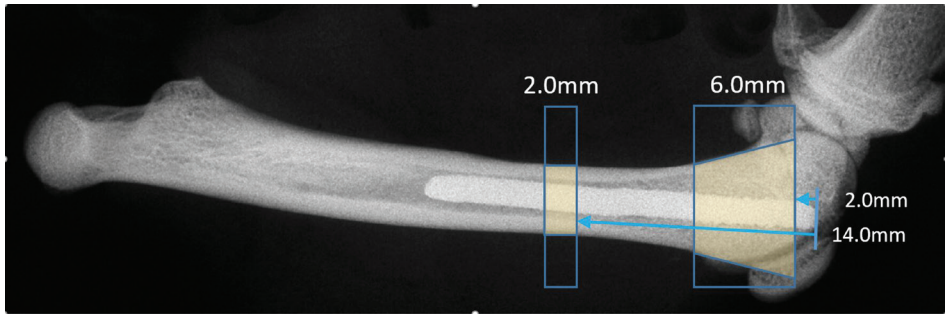

Figure 1. Radiographic image of femur demonstrating the scanned regions utilized for micro-CT evaluations.

reamed and extended to 27 mm manually with a 1.3 mm twist bit secured in a Jacob's chuck. One of our four solutions at the above specified concentration was drawn up in a 0.5 ml tuberculin (TB) syringe with permanently attached needle and then administered via single injection into the canal followed by insertion of the implant until it was flush with the bone surface of the trochlea. The patella was reduced, the joint capsule closed with a 4-0 Vicryl suture (Ethicon Inc., Cornelia, GA), and the skin closed with wound clips (Autoclips, MikRon Precisioin Inc, Gardena, Ca) and tissue adhesive (TA5, Med Vet International, Mettawa, IL). Wound clips were removed 12 days post-surgery. All animals were given prophylactic ceftriaxone and a 0.03 mg/kg injection of buprenorphine at time of surgery and twice per day for 48 hours. They were given *ad libitum* access to acetaminophen-doped drinking water (1.6 mg/ml) for seven days after surgery.

The rats' masses were recorded upon arrival to the facility, immediately before surgery, and weekly to monitor weight loss/gain. The rats were followed for 28 days after surgical placement of implants before being humanely euthanized by CO_2 inhalation. Following euthanasia, femurs were carefully dissected. The right femurs were wrapped in saline soaked gauze and stored at -20°C until mechanical pushout testing, the left femurs were placed in 10% neutral buffered formalin for 48 hours, then rinsed in deionized water and placed in 70% ethanol for holding before micro-CT scanning. Following micro-CT scanning, three left femurs from each group were randomly selected and transferred from the ethanol and placed in Immunocal solution (StatLab, McKinney, TX), a mild formic acid decalcifying agent, for ten days for decalcification with the solution changed every three days. Once the bone became soft, it was rinsed in deionized water and placed into Cal arrest solution (StatLab, McKinney, TX), a neutralizing agent, before being removed from solution, rinsed in deionized water again and submitted in 70% ethanol to the histology service for hematoxylin and eosin (H&E) staining.

Biomechanical testing

Prior to mechanical testing, the femur was cut to implant length using a fine-toothed rotary bone saw. Specimens were potted in a custom tapered mold using a self-curing acrylic resin (Ortho Jet BCA, Lang Dental, Wheeling, IL) and allowed to cure for 30 minutes. Specimens were kept hydrated by submerging the potting fixture into 250 ml of 27°C saline while the resin cured for 20 minutes and then wrapped in saline soaked gauze for the remaining 10 minutes. The axis of the implant was aligned in the direction of the push-out with two opposing tapered pins resting in the dimple at each end of the implant while the resin set. A 3 mm diameter, 2 mm thick silicon disk was used during potting to ensure consistent support of the femoral condyles during mechanical testing while allowing an opening for the implant to be pressed through.

Mechanical testing was carried out with a material testing system (MTS) (8500 Plus, Instron Corp.,Norwood, MA). The uniaxial servohydraulic motion of the MTS was transferred to the implant through a tapered stainless steel pin secured in a Jacob's chuck. Linear load was measured with a 500 N load cell. The potted specimen was allowed to sit squarely on a platform with a hole in the center for implant pushout. Specimens were preloaded with 5 N and pushed out at a constant rate of 2 mm/min until failure of the bone-implant interface was reached. Maximum energy, load, displacement, and stiffness prior to failure were determined from the resulting data using the system software.

Micro-CT

For micro CT analysis, specimens were sent to UNC Biomedical Research Imaging Center for assessment of 3-dimensional peri-implant bone volume/total volume (BV/TV) with the implant still in place. A 6 mm region, 2 mm proximal to the distal end of the implant and a 2 mm region, 14 mm proximal to the distal end of the implant were scanned (Figure 1). Evaluation was performed in the distal metaphyseal region of the femur in a 3mm long zone that started 1 mm proximal to the most proximal aspect of the epiphyseal plate. The entire scanned region of the diaphysis was evaluated. The implant was dilated by five pixels (60 µm) to exclude the metal-induced artifact as determined by a prior study[20]. The bone was segmented out using a low and high threshold of

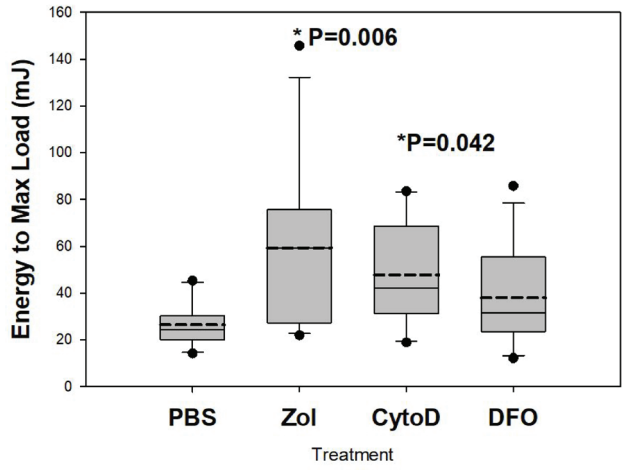

Figure 2. Box plot (mean=dashed, median=solid, boxed ends=25th and 75th %, whiskers=10th and 90th %) of energy to maximum load values demonstrating significant increases in Zol and cytoD.

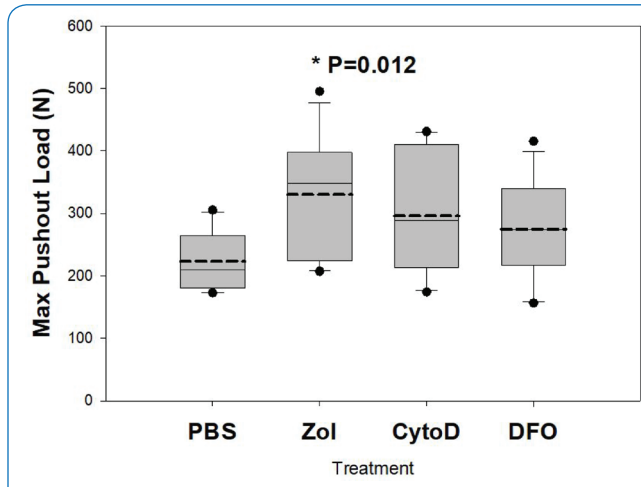

Figure 3. Box plot of maximum pushout load values showing a significant increase in Zol.

529 and 1615 mg HA/cm^3 respectively. The implants were segmented using a threshold ≥2249 mg HA/cm^3. The BV/TV within 500 µm of the implant was calculated. A cylinder was constructed in the femur that had a diameter 1000 µm larger than the mean diameter of the implant. The volume of bone within this region was divided by the volume of the cylinder minus the dilated implant volume within the cylinder. The resulting percentage represented the medullary BV/TV.

All left femurs were scanned using a specimen µCT system (Model µCT 40, Scanco Medical, Brüttisellen, Switzerland) with a 10 mm field of view on medium resolution with a voxel size of 12 µm. The X-ray power setting was 70 kVp, 114 µA, and 8 W. The scans had an integration time of 300 ms and were averaged once. The resulting µCT scans were analyzed using software developed for processing medical images (Image J, National Institutes of Health, Bethesda, Maryland).

Histology: qualitative evaluation

Following micro-CT scanning, implants were removed from three randomly selected left femurs. The bone was demineralized, as described above in materials and methods, and stained with H & E for qualitative assessment of bone morphology and bone cell activity.

Data analysis

A pre-power analysis determined that a sample size of 11 rats per group would be required to detect a 45% improvement in fixation strength assuming a standard deviation of 30% of the control mean for a power of 0.8 and a significance level of 0.05.

Results were analyzed with a one-way analysis of variance (ANOVA) with Holm-Sidak mean comparison testing between the experimental and control groups using a statistical analysis program (SigmaPlot v11.0, Systat Software Inc, San Jose, CA).

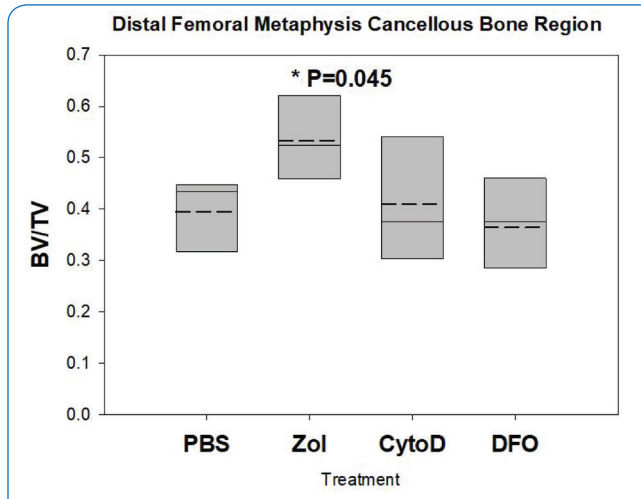

Figure 4. Box plot of BV/TV in the distal femoral metaphysis region showing a significant increase in BV/TV for Zol.

Results

Two rats were euthanized prematurely and excluded from analysis; one rat due to incorrectly placed implants (cytoD group) found on post-op radiographs and one rat due to an illness (saline).

Mean weight and standard deviation for each group on the day of surgery, along with percent lost/gained at the end of the study were: saline: 420g±60g, 0.83% weight gained

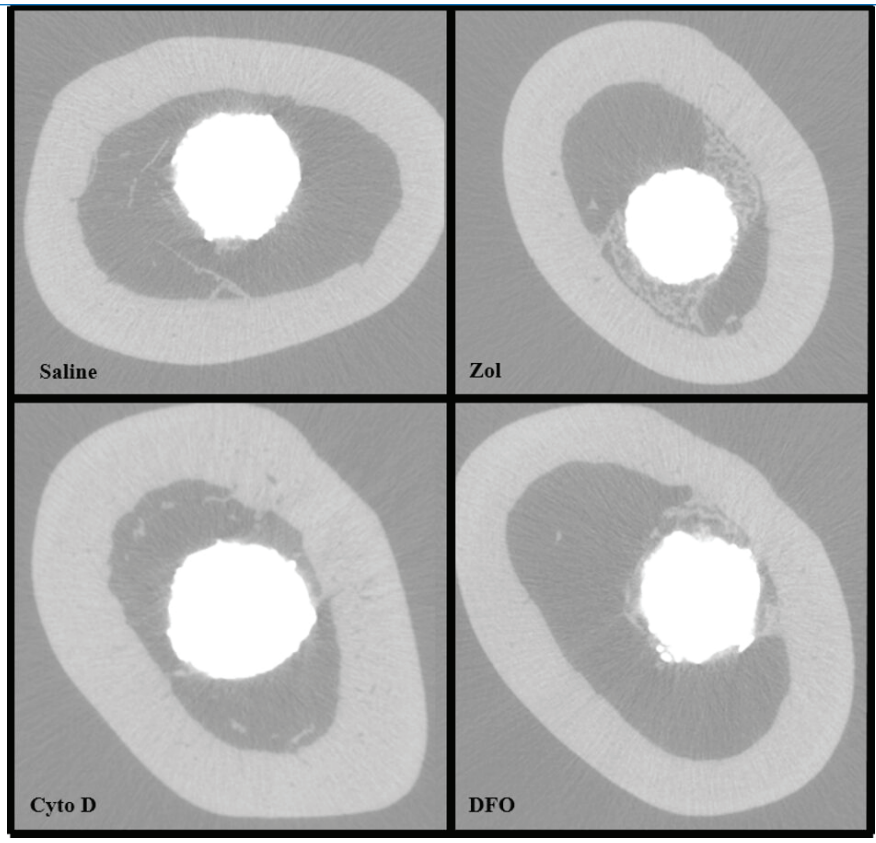

Figure 5. *Micro*-CT images at a similar mid-diaphyseal level for the median specimen in all four groups. There appears to be increased cancellous bone in the representative specimen from the Zol group compared to the other groups.

on average, Zol: 425g±51g, 1.45% lost, cytoD: 413g±37g, 0.83% lost, and DFO: 424g±45g, 0.12% lost. The change in body weight across the study did not differ significantly among the groups.

Biomechanical testing

Evaluation of mechanical pushout tests reported as mean±SD prior to implant failure showed a maximum energy of: saline: 26.5mJ±9.2mJ, Zol: 59.3mJ±34.9mJ, cytoD: 47mJ±22.2mJ, and DFO: 38.1mJ±21.3mJ. This corresponds to a significant increase in maximum energy to failure of 124% for Zol (p=0.006) and and 80% for cytoD (p=0.042), as compared to the control (Figure 2). Maximum load prior to failure showed saline: 223.3N±46.5N, Zol: 330.3N±90.3N, cytoD: 296.1N±95.4N, and DFO: 274.7N±78.0N, which corresponds to a significant increase in maximum load of 48% (p=0.012) for Zol compared to saline. CytoD had a 33% increase in maximum load relative to the saline control but this was not significant (p=0.09) (Figure 3). No significant differences in stiffness or displacement at implant failure were detected among the groups. Stiffness values were: saline: 1164N/mm±305N/mm, Zol: 1161N/mm±170N/mm, cytoD: 1092N/mm±285N/mm, and DFO: 1275N/mm±193N/mm. Displacement values were: saline: 0.253mm±0.061mm, Zol: 0.355mm±0.113mm, cytoD: 0.333mm±0.081mm, and DFO: 0.288mm±0.106mm.

Micro-CT

BV/TV for the distal metaphyseal evaluated region was: saline: 0.395±0.082, Zol: 0.534±0.086, cytoD: 0.410±0.137, and DFO: 0.364±0.095, corresponding to a significant increase of 35% for Zol (p=0.045) (Figure 4). BV/TV for the mid-diaphyseal evaluated region showed: saline: 0.047±0.025, Zol: 0.140±0.139, cytoD: 0.063±0.063, and DFO 0.039±0.014, demonstrating a trend for an increase with Zol (p=0.064). Qualitatively assessing cross sections of the mid-diaphyseal regions at a similar level, there appeared to be increased bone formation in the Zol group compared to the control (Figure 5).

Histology

On qualitative histological examination of each group there appeared to be increased peri-implant bone surrounding the removed implant in all three treatment groups, most noticeably in the Zol group (Figure 6).

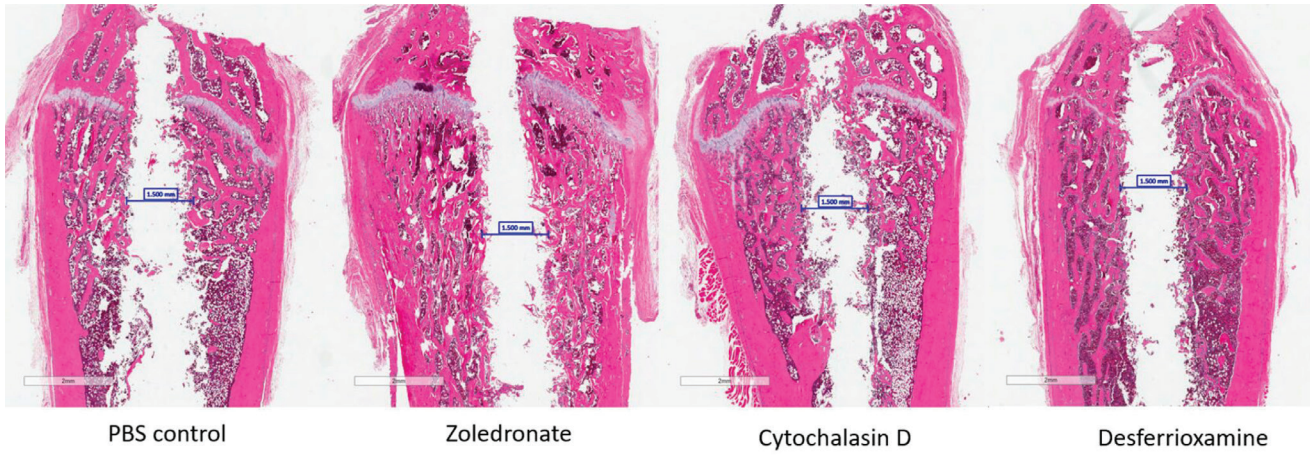

Figure 6. Histologic H&E representations of each group. There appears to be increased bone formation in all treatments, most significant in the Zol group.

Discussion

In investigating ways to improve success rates of intramedullary implant osseointegration, our findings show promise in that both Zol and cytoD enhanced implant mechanical stability. The positive effect of Zol could be due its ability to inhibit bone resorption as well as through increased bone formation through MSC differentiation into osteoblasts[9]. Given there was cancellous bone in the mid-diaphyseal region of the Zol group, a region typically void of cancellous bone, it is likely Zol did stimulate some bone formation (Figure 5). CytoD's positive results were likely related to increasing MSC osteoblast formation, although we cannot say definitively since we only conducted mechanical tests and imaging. Though recent reports from this laboratory have demonstrated improvements in osseointegration with local delivery of Zol as an injectable solution in the medullary canal; the current study extends these findings by demonstrating that improvements in osseointegration can be achieved using much smaller volumes of treatment solution (which would be easier to apply clinically)[11].

While all the agents investigated were believed to have potential to accelerate osseointegration due to their established role in driving marrow-derived mesenchymal stem cells toward an osteogenic lineage, the mechanism by which each achieves this varies. Cytochalasin D has been shown to initiate and accelerate development of osteoblast lineage from murine and human mesenchymal stem cells, an effect that requires dissolution of cytoplasmic F-actin and mass transfer into the nucleus[13,21,22]. The efficacy of this strategy would require that MSC are available to take up the drug. The modest improvement in mechanical stability with cytochalasin D injection may indicate that the marrow derived MSCs were not sufficiently exposed to the drug. Past work has demonstrated improved fracture healing and bone defect healing with local delivery of DFO; however, in the current study no significant improvements were found with the evaluated concentration of DFO used in this study. It is possible that a higher concentration of DFO or different administration method (larger volume, injected with tourniquet,) may have produced improvements in osseointegration as blood was observed to flow from the intramedullary space in some animals during intramedullary injection.

While other studies have utilized a local delivery method, they utilized much higher volumes that would be unrealistic. A unique feature of our study was delivery of a smaller volume of treatment solution that would be more easily applied clinically while continuing to minimize the risks of systemic effects with larger volume administration. This local administration method is also attractive due to its cost-effectiveness and ease of delivery at the time of operation. However, a limitation of our intramedullary injection administration method is that we were not able to ensure total drug delivery to target cells; in some instances, there was leakage from the femoral canal prior to implant insertion. Delivery of the agents via a coating may give more consistent results. While the dosage of each agent was selected based on past studies showing enhanced bone formation effects, our single dosage approach represents a limitation of the study and further work of different dosage regimens utilizing a larger sample size would be appropriate.

Conclusion

Local delivery of Zol and cytoD at the time surgery resulted in an increase in the energy required to pushout implants after 4 weeks of healing in rats. Local delivery of DFO did not improve osseointegration at the dosage utilized. Overall, this study supports the further investigation of local delivery of Zol and cyto D as strategies to improve osseointegration of intra-medullary implants.

Acknowledgements

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