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TITLE: Desferrioxamine for Stimulation of Fracture Healing and Revascularization in a Bone Defect Model

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14. ABSTRACT

Our study explored local delivery of deferroxamine with and without bone allograft as a means to accelerate fracture healing in a tibia defect model of the rat. Experimental groups in the first study included: control, CS: calcium sulfate annulus around spacer at defect, CS/DBA: CS implant and demineralized bone allograft (DBA); CS/DFO: DFO (300µg) loaded CS implant, CS/DFO/DBA: DFO loaded CS implant with DBA allograft. Experimental groups in the second study included: control, DBA, L-DFO/DBA: DBA soaked with DFO (10µg), H-DFO/DBA: DBA soaked with DFO (100ug). Fractures were evaluated at 6 weeks postinjury for radiographic cortical bridging, µCT mineralized callus volume and torsional properties. The bulk CS annulus was found to impair healing compared to the control in the 1st study. In the 2nd study L-DFO/DBA was found to increase cortical bridging and torsional strength relative to the control. Bulk form CS is not an effective method of delivery of DFO to stimulate fracture healing. Low dosage DFO treatment directly to DBA was found to enhance the ability of DBM to stimulate fracture healing, while higher dosage DFO treatment of DBA was not effective.

15. SUBJECT TERMS
Desferrioxamine, fracture healing, revascularization, bone defect,angiogenesis

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INTRODUCTION:

A compromised vascular supply occurring during fracture healing often results in a delayed union or a non-union. Deferoxamine (DFO) is an FDA approved iron chelating agent that has been found to stimulate several angiogenic mechanisms by altering the metabolism of hypoxia inducible factor-1α (Wan et al., 2008; Wang et al., 2007; Yamakwa et al., 2003). Recently, it has been demonstrated in a segmental femoral defect model that local delivery of DFO at the injury site can increase vascularity and stiffness of the healing fracture (Stewart et al. 2011). Demineralized bone (DMB) allograft is frequently used to stimulate healing in segmental defects, however its effectiveness might be improved by enhancing the vascularity of the fracture site. The objective of this study was to evaluate local delivery of DFO in a calcium sulfate (CS) implant to accelerate fracture healing in an impaired healing tibia defect model. It was hypothesized that DFO given alone or in combination with DMB would lead to enhanced torsional strength and radiographic bridging of the healed fractures compared to the CS implant alone or DMB with a CS implant.

BODY:

The first task of the project is listed below.

Task 1. Conduct Rat Tibial Defect Model Study of Aim 1 with 2mm Defect Size
Aim1. To evaluate the ability of a DFO-CS implant to accelerate fracture healing in a rat tibial defect (2mm) impaired healing model when given with and without bone allograft. Healing will be evaluated at 6 weeks after injury by torsional testing, radiographic evaluation of cortical bridging, mineralized fracture callus volume by micro-CT, and qualitative histology for callus tissue quality and vascularity in 5 groups (No implant, CS implant, DFO+CS implant, bone allograft + CS implant, bone allograft + DFO+CS implant). This 2mm defect markedly impairs/delays fracture healing. It is hypothesized that the DFO+CS implant will accelerate fracture healing beyond the response seen with the CS implant alone and that this improvement will be similar to, or better than the response observed with bone allograft. Allograft+DFO may be even better.

The research accomplishments associated with this task have been reported in the most recent annual report for the grant which is included in this final report as Appendix A.

A crucial finding of this initial task was that the use of bulk form calcium sulfate at the defect site as a drug delivery agent was ineffective as the bulk form calcium sulfate alone inhibited the fracture healing process relative to the control.

Despite the negative influence of the bulk form calcium sulfate in this first task are findings were suggestive that DFO may have some ability to accelerate fracture healing however we were unable to detect any statistical effect due to the increased variability in our study due to the animals growing to too large of a size that was nonoptimal for our spacer defect model. A remaining subtask of task 1 is to draft a manuscript from the findings of task 1. Because of the issues with the variability with the animal model in

this first study we are uncertain if we will be able to publish the work in a higher impact journal. We are still contemplating if we should publish the results of the bulk form calcium sulfate relative to the control as a separate article or lump the DFO portion of the study with a previous pilot study we have carried out using DFO in particulate calcium sulfate.

The second task of our project was the following:

Task 2. Conduct Rat Tibial Defect Model Study of Aim 2 with 3mm Defect Size

Aim2. To evaluate the ability of a DFO-loaded calcium sulfate carrier to promote/achieve fracture healing in a rat tibial defect (3mm) nonunion model when given with and without bone allograft. Healing will be evaluated at 8 weeks after injury using the same evaluations as Aim 1 in the same 5 Groups. This 3mm defect consistently results in nonunion. It is hypothesized that the DFO-CS implant will result in a higher union rate with respect to the CS implant alone similar to or better than bone allograft alone, and that adding the DFO-CS implant to the bone allograft will further promote healing.

For task 2 we returned to using the retired breeder female rats for our spacer defect model to eliminate the issue with variability in a faster growing animal model. We again used the 2mm defect model given the equivocal results of the first study. We also decided to eliminate use of the calcium sulfate in the model based on the evidence of the first study that it was impairing the fracture healing process. Instead, we delivered the DFO directly on the DMB allograft. In our previous evaluation of potential delivery agents for DFO we found DMB to be effective in stimulating capillary sprouting in an *in vitro* model (Hertzberg et al. 2010).

We are pleased to report that the task2 study went much smoother than the task 1 study with a number of significant findings. The research findings are detailed in a manuscript that we have drafted for submission to the Journal of Orthopaedic Trauma and is included as Appendix B.

KEY RESEARCH ACCOMPLISHMENTS:

- Bulk form calcium sulfate placed at the fracture defect was found to inhibit the fracture healing process and was an ineffective delivery method for DFO.
- When delivered in bulk form calcium sulfate desferoxamine showed some ability to stimulate fracture healing though statistical significant effects could not be detected due to variability in our animal model.
- Lower dosage DFO (10μg) administered directly to DMB was found to enhance the stimulatory effects of DBM on fracture healing as demonstrated by the improved torsional properties and radiographic bridging of this group relative to the control.
- Higher dosage DFO (100 μg) administered directly to DMB was not found to enhance the stimulatory effects of DBM on fracture healing as demonstrated by by equivalent torsional properties and radiographic bridging of this group relative to the control.

REPORTABLE OUTCOMES:

The following abstracts were submitted from this research:

- Bulk Calcium Sulfate Impairs Fracture Healing in a Tibia Defect Model Exploring the Stimulatory Effects of Desferroxamine, Keller, B.; Turvey, B.; Davis, M.; Dahners, L.; Weinhold, P. North Carolina Tissue Engineering and Regenerative Medicine Meeting, November 4, 2011; Winston Salem, NC. (presented)

- Desferroxamine with Demineralized Bone Allograft to Promote Fracture Healing in a Segmental Tibia Defect Model. Keller, B.; Turvey, B.; Davis, M.; Dahners, L.; Weinhold, P. Orthopaedic Research Society Meeting, February 4-7, 2012; San Francisco, CA. (was not accepted)

- Desferrioxamine Combined With Demineralized Bone Matrix Improves Fracture Healing in a Tibial Segmental Defect Model, Weinhold, P.; Keller, B.; Turvey, B.; Jones, T.; Dahners, L. Orthopaedic Trauma Association 2012 Annual Meeting, October 3-6 , 2012; Minneapolis, MN. (submitted)

- Desferrioxamine Combined With Demineralized Bone Matrix Improves Fracture Healing in a Tibial Segmental Defect Model, Weinhold, P.; Keller, B.; Turvey, B.; Jones, T.; Dahners, L. (preparing for submission to Journal of Orthopaedic Trauma)

We will be investigating if direct administration of DFO on DBM could be a reportable invention.

Ben Keller has been pursuing his M.S. in Biomedical Engineering while he has been working on this study.

CONCLUSION:

Bulk form calcium sulfate placed in at a tibia segmental defect in the rat was found to inhibit the fracture healing process as demonstrated by reduced cortical bridging across the fracture and reduced torsional properties of the healed fracture. As such it does not appear to an effective delivery method for DFO to a fracture site. It is unclear if this effect of bulk form calcium sulfate is specific to the rat due to the faster healing response of this species. These findings imply that a bone defect in the tibia should not be completely filled with calcium sulfate as this may inhibit the fracture healing process.

Desferoxamine when delivered in calcium sulfate to a tibia segmental defect in the rat with and without demineralized bone allograft was found to have a limited ability to enhance cortical bridging and the torsional strength of the healing fracture as statistically significant differences were not found. However, the nonoptimal size of the animals used for our spacer defect model introduced greater variability in our model making it more challenging to detect statistical differences with the treatments. Furthermore, the inhibitory effect of the bulk form calcium sulfate on the fracture healing process may have also curtailed the ability of desferoxamine to stimulate healing in this model.

Low dosage DFO treatment of DBM was found to enhance the ability of DBM to stimulate fracture healing, while higher dosage DFO treatment of DBM was not effective suggesting that high dosages may impair the effect.

In summary, we feel these findings are important as they are initial steps towards the identification of the optimal method of delivering desferoxamine to a fracture site to improve healing.

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Appendix A:

1st Annual Report

INTRODUCTION:

A compromised vascular supply occurring during fracture healing often results in a delayed union or a non-union. Deferoxamine (DFO) is an FDA approved iron chelating agent that has been found to stimulate several angiogenic mechanisms by altering the metabolism of hypoxia inducible factor-1α (1-3). Recently, it has been demonstrated in a segmental femoral defect model that local delivery of DFO at the injury site can increase vascularity and stiffness of the healing fracture (4). Demineralized bone (DMB) allograft is frequently used to stimulate healing in segmental defects, however its effectiveness might be improved by enhancing the vascularity of the fracture site. The objective of this study was to evaluate local delivery of DFO in a calcium sulfate (CS) implant to accelerate fracture healing in an impaired healing tibia defect model. It was hypothesized that DFO given alone or in combination with DMB would lead to enhanced torsional strength and radiographic bridging of the healed fractures compared to the CS implant alone or DMB with a CS implant.

BODY:

A brief summary of the specific tasks of the statement of work that have been completed and are uncompleted will be given followed by a more detailed presentation of the research data that has been generated.

Tasks Completed

Task 1. Conduct Rat Tibial Defect Model Study of Aim 1 with 2mm Defect Size (months 1-9)

1a. **Milestone #1*:*** Acquire Approval of Study from Animal Care and Use Committee (months1-2)
1b. USAMRMC Office of Research Protections review and approval of animal regulatory documents(months2-4)
1c. Construct Implant Preparation Fixtures, Assess Proper Dimension In Situ, & prepare CS implants (months2-3)
1d. Prepare Bone Allograft Samples (months2-3)
1e. Order Rats for 5 groups (145 animals), start surgeries with initial radiographs, and follow animals for 6 weeks. (months 5-7)
1f. **Milestone #2:** Sacrifice animals and take final radiographs (months7-8)
1g. Perform Torsional Testing on 120 specimens.(months7-8)
1k. Grade radiographs (145 animals, 2 views) for cortical bridging. (month8)
1l. Perform statistical analysis of torsional testing, micro-CT, and radiographic evaluations. (months 8-9)

Task 2. Conduct Rat Tibial Defect Model Study of Aim 2 with 3mm Defect Size (months 1-12)

2a. Acquire Approval of Study from Animal Care and Use Committee (months1-2)

2b. USAMRMC Office of Research Protections review and approval of animal regulatory documents(months2-4)
2c Construct Implant Preparation Fixtures, Assess Proper Dimension In Situ, & prepare CS implants (months2-3)
2d Prepare Bone Allograft Samples (months2-3)
2e Order Rats for 5 groups (145 animals), start surgeries with initial radiographs, and follow animals for 8 weeks. (months 9-10)

Tasks to be Completed

Task 1. Conduct Rat Tibial Defect Model Study of Aim 1 with 2mm Defect Size (months 1-9)

1h. Fix 25 specimens in buffered formalin and perform micro-CT scanning and analysis of mineralized callus volume.(months 7-8)
1i. Subsequently send 25 specimens for histological processing and sectioning (months 7-8)
1j. Qualitatively evaluate histological sections for cellularity, tissue types, vascularity, and tissue integration. (months 8-9)
1m. **Milestone #3:** Draft manuscript from results of Aim 1 study (months 9)

Task 2. Conduct Rat Tibial Defect Model Study of Aim 2 with 3mm Defect Size (months 1-12)

2f **Milestone #4:**Sacrifice animals and take final radiographs (months10-11)
2g Perform Torsional Testing on 120 specimens.(months10-11)
2h Fix 25 specimens in buffered formalin and perform micro-CT scanning and analysis of mineralized callus volume.(months 10-11)
2i Subsequently send 25 specimens for histological processing and sectioning (months 10-11)
2j Qualitatively evaluate histological sections for cellularity, tissue types, vascularity, and tissue integration. (month11-12)
2k Grade radiographs (145 animals, 2 views) for cortical bridging. (month 9)
2l Perform statistical analysis of torsional testing, micro-CT, and radiographic evaluations. (months 11-12)
2m **Milestone #5:**Draft manuscript from results of Aim 2 study (month 12)

Task1 Methods Description

After approval from the local IACUC, 12 week-old male Sprague Dawley rats were obtained, weighed and divided into 5 groups. All animals were surgically subjected to a transverse osteotomy 14 mm distal to the tibial tubercle with stabilization by an intramedullary stainless steel pin. A 2mm bone defect was created by placing a 2mm cylindrical PEEK (polyether ether ketone) spacer about the pin at the fracture gap as described (5). The experimental groups consisted of the following: Control, Calcium

Sulfate Implant (CS, n=25): CS was circumferentially set-up around the PEEK spacer in the form of a cylinder, Calcium Sulfate Implant with DMB Allograft (CS/DMB, n=24): 50 mg of DMB allograft was added around the CS implant and PEEK spacer, Deferoxamine in CS implant (CS/DFO; n=24): CS implant surrounding the PEEK spacer was loaded with DFO (1mg/kg BW), DFO in CS implant with DMB Allograft (CS/DFO/DMB n=24): 50 mg of DMB allograft was added circumferentially around the DFO loaded CS implant & PEEK spacer. All rats had lateral radiographs taken at 4 weeks to determine the efficacy of the PEEK spacer in holding the gap. These radiographs were graded qualitatively by 3 independent examiners blinded to the treatment groups concerning the maintenance of the fracture gap: 0 (no gap), 1 (partial gap), 2 (full maintenance of gap). The rats were euthanized 6 weeks postoperatively and weighed. Radiographic examination was carried out in both the AP plane and the lateral plane. Biplanar radiographs were used to calculate the total bridging of the defect site at all 4 cortices (2 on AP view and 2 on lateral view) by 3 independent examiners blinded to the treatment groups. Bridging scores varied from 0 to 4 cortices bridged.

Tibias from the operated legs had the surrounding soft tissues removed before potting specimens for torsional testing. To assess the mechanical competence of the healed fracture, the stiffness, ultimate torque to failure, and energy to ultimate load of the fracture callus were determined by torsional testing in external rotation on a uniaxial servohydraulic material testing machine fitted with fixtures to convert the axial motion to rotary motion. Specimens were torqued at a constant rate of six degrees/second until failure or 60 degrees of rotation. The torque and deflection angle were recorded and torsional properties computed. The ultimate torque was recorded as the maximum torque prior to 35 degrees of angulation. Displacement > 35 degrees without reaching a peak torque was felt to be indicative of tensioning of fibrous callus rather than bony union. Initially, the control and CS implant were compared by an unpaired t-test to determine if these groups could be lumped together and to determine which was the more appropriate control for the other groups. A two-way ANOVA (DFO and DMB status were the two factors) followed by multiple comparison testing was used to determine statistical differences between the groups for the evaluation variables ($P<0.05$).

Task1 Results

Surgeries were performed on 121 animals. Difficulties post-surgery required exclusion of 43 animals from evaluation by torsional testing and radiographic assessment. The exclusion categories and the number of animals in each category were the following: loss of fixation (12), immediate loss of fracture gap (8), infection (11), wound self-mutilation (7), experimental error (4), premature death (1). We believe the high number of post-surgical difficulties that arose during our study were related to the gender and age of the animals selected and a shortage of postsurgical analgesic (buprenorphine) that caused us to have to delay our surgeries once the animals arrived in our facility. In our previous studies using this osteotomy model we had used female retired breeder animals with a mean weight of 300grams. However, in our most recent study we experienced some difficulty with the vendors providing sufficient quantities of these animals in the desired weight range. We decided to use a younger male Sprague-Dawley rat within the desired weight range instead. At the onset of our study the research DEA license to use the

postsurgical analgesic buprenorphine of one our co-investigators was being renewed. The renewal process had typically taken only 3 months or less in the past, but in the present instance it lasted 10 months until July of 2011. As a result of this delay in our DEA license renewal we became dependent on our Division of Laboratory Animal Medicine (DLAM) at UNC for providing buprenorphine for our studies. Unfortunately, our DLAM was experiencing a shortage of buprenorphine as all the approved vendors on their DEA license had the drug on backorder for an extended period of time. This situation resulted in sporadic access to buprenorphine such that once animals arrived in our facility we had to delay surgeries until we were assured we would have sufficient quantities for postsurgical analgesia. During this extended delay the tibias of our younger animals grew to a size that was larger than the ideal conditions for our PEEK spacer gap healing model (average weight of animals = 537g compared to ideal weight of 300g). This resulted in loss of our fracture gap in some animals due to the PEEK spacer entering into the medullary canal. It also resulted in excessive bending of the fixation pin or fracture of the fixation pin because of the size of the animals. Also, the younger male rats seemed to have a greater tendency to bite at their wounds and open them causing a higher incidence of self-mutilation of the wound beyond repair and greater problems with infection. Regrettably these circumstances caused us delay in completing the tasks of our statement of work. In July of 2011 our research DEA license was approved and we added a compounding facility to our list of approved vendors so that we would be immune to regional shortages of buprenorphine in the future.

(Control vs. CS comparison):

Animals in the control and CS groups displayed similar body weights at the end of the study (Table 1). In addition, the two groups displayed a similar ability to maintain the fracture gap based on the qualitative grading of the 4 week lateral radiograph (Table 1). Surprisingly, radiographic grading at the 6 week sacrifice time point revealed improved cortical bridging in the control group compared to the CS group (Table 1). Corresponding with the radiographic results, the torsional testing revealed improved stiffness, ultimate torque and energy absorption in the control group compared to the CS group (Table 2). While CS is often described as an osteoconductive material and has been used as a drug delivery carrier in bone applications, there have been some studies (6,7,8) to suggest that when CS is used in bulk form in a defect that it can retard some aspects of bone healing. Of particular importance to the current work, one of these studies (7) has observed decreased early vascularization when using CS compared with other bone graft substitutes. Such an effect may curtail the angiogenic stimulus of the DFO treatment. Our findings also seem to suggest that when CS is used in bulk form to fill a defect that some aspect of its degradation may retard the bone healing process. In our past studies (9) where we have used calcium sulfate in particulate form at the fracture site, we have observed no inhibitory effect of CS on the fracture healing process. As a result of the evidence for delayed fracture healing of the CS group relative to the control group we felt it was inappropriate to compare the other treatments which all possessed a CS implant to the control group. As such only the 4 groups that possessed a CS implant were compared in the remaining analyses.

Table 1. Mean (SD) body weight and radiographic grades of the control and CS alone groups.

	Control	CS
Final Body Weight (g)	555 (33)	531 (62)
Radiographic Gap Maintenance Grade (0-2)	1.08 (0.60)	1.33(0.72)
Radiographic Bridging Grade (0-4)	3.64 (0.55)	2.11 (1.65)*

* Significant difference from Control ($P < 0.05$)

Table 2. Mean (SD) torsional properties of the control and CS alone groups.

	Control	CS
Ultimate Torque (N*mm)	254 (111)	159 (92)*
Stiffness (N*mm/deg)	13.9 (5.3)	7.8(4.5)*
Energy to Ultimate Torque (N*mm*deg)	3029 (1016)	2034 (1229)*

* Significant difference from Control ($P < 0.05$)

(4 Groups Containing CS Comparison):

All 4 groups displayed similar body weights at the end of the study (Table 3). In addition, the 4 groups displayed a similar ability to maintain the fracture gap based on the qualitative grading of the 4 week lateral radiograph (Table 3). While the CS/DFO, CS/DMB, and CS/DFO/DMB groups appeared to enhance cortical bridging compared to the CS alone group, no statistically significant effects of DFO status (P=0.30)and DMB status(P=0.26) were detected and no differences were detected between any of the groups (Table 3). Similarly, while the CS/DFO, CS/DMB, and CS/DFO/DMB groups appeared to enhance the torsional strength of the healed fractures, no statistically significant effects of DFO status (P=0.32) and DMB status (P=0.20) were detected and no differences were detected between any of the groups (Figure 1). This lack of differences was despite a 50% improvement in strength in the CS/DFO/DMB group relative to the CS alone group suggesting the lack of differences was more due to the variability of the data. The energy to ultimate torque (Table 4) measure also showed a similar pattern of the means to that of the ultimate torque, however no statistical differences were detected. No differences in stiffness were found between the groups (Table 4). Overall, our results are suggestive that DFO may have some ability to accelerate fracture healing however we were unable to detect any statistical effect due to the increased variability in our study due to the animals growing to too large of a size that was nonoptimal for our spacer defect model. In addition, the negative impact of the bulk CS on the fracture healing process may have also curtailed the stimulatory effects of DFO on fracture healing. A recommended change for future work is to return to using the retired breeder female rat for our spacer defect model even though it may be more difficult to get a narrow body weight range with this population. In addition, we recommend not using the CS in bulk form for local delivery of the DFO at the defect site in future studies.

Table 3. Mean (SD) body weight and radiographic grades of the 4 groups containing CS.

	CS	CS/DMB	CS/DFO	CS/DMB/DFO
Final Body Weight (g)	531 (62)	546(50)	535(47)	537 (47)
Radiographic Gap Maintenance Grade (0-2)	1.33(0.72)	1.17(0.74)	1.04(0.82)	1.00(0.82)
Radiographic Bridging Grade (0-4)	2.11 (1.65)	2.87(1.24)	2.83(1.47)	3.0 (1.26)

* Significant difference from Control ($P < 0.05$)

Table 4. Mean (SD) torsional properties of the 4 groups containing CS.

	CS	CS/DMB	CS/DFO	CS/DMB/DFO
Stiffness (N*mm/deg)	7.8 (4.5)	12.2(7.6)	11.5(7.1)	9.7(4.6)
Energy to Ultimate Torque (N*mm*deg)	2034(1229)	2540(1384)	2451(1106)	3031(2055)

* Significant difference from Control ($P < 0.05$)

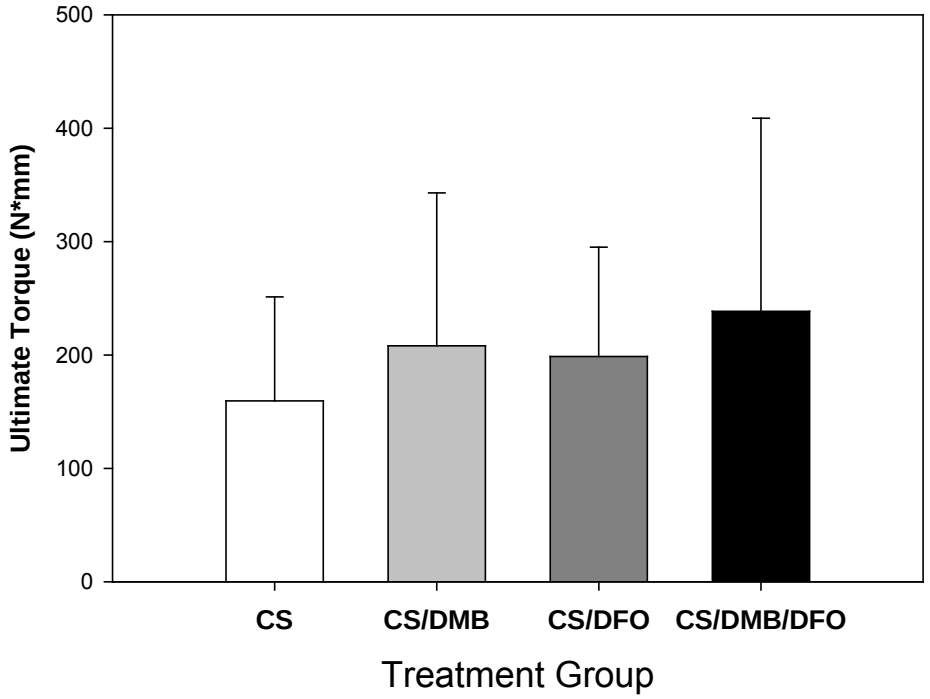

Fig. 1. Ultimate torque of the 4 treatment groups receiving the CS implant. No differences were detected ($P>0.05$) despite a 50% improvement in torque between the CS/DMB/DFO and CS groups.

The task items that were not completed according to our planned schedule will be addressed next. The histology of Task 1 is still being processed by a histology service and we will proceed with qualitative assessment of the sections when they are available. In considering the expense of the micro-CT assessment and the variability in how well

the gap defect was maintained in the current study because of the animal size issue, we decided it may be wiser to forgo completing the micro-CT evaluation on the task 1 study and use these funds to do additional micro-CT evaluations on the specimens from the task 2 study. In addition, we have decided to delay the writing the manuscript for task 1 as we believe it may be more beneficial to publish these results together with the results from task 2 in considering the experimental difficulties with animal size that were encountered in the task 1 study.

Task 2 Summary

For task 2 we are using retired breeder female rats for our spacer defect model and we are again using a 2mm defect model given the equivocal results of the first study. We have also decided to eliminate use of the CS in the model based on the evidence of the first study that it was impairing the fracture healing process. Instead, we are delivering the DFO directly on the DMB allograft. In our previous evaluation of potential delivery agents for DFO we found DMB to be effective in stimulating capillary sprouting in an *in vitro* model (10). We have completed all of the animal surgeries of task 2 and are following the animals currently. We have observed a marked reduction in the postsurgical difficulties upon returning to the female retired breeder rats. All of the animals will be sacrificed by the end of November and we anticipate completing the radiographic, torsional, micro-CT, and histological evaluations of these animals in December and January. We will begin drafting a manuscript for both the results of the task 1 and task 2 studies once the findings of the task 2 study are complete.

KEY RESEARCH ACCOMPLISHMENTS:

- Bulk form calcium sulfate placed at the fracture defect was found to inhibit the fracture healing process.
- Desferoxamine showed some ability to stimulate fracture healing though statistical significant effects could not be detected.

REPORTABLE OUTCOMES:

The following abstracts were submitted from this research:

- Bulk Calcium Sulfate Impairs Fracture Healing in a Tibia Defect Model Exploring the Stimulatory Effects of Desferroxamine, Keller, B.; Turvey, B.; Davis, M.; Dahners, L.; Weinhold, P. North Carolina Tissue Engineering and Regenerative Medicine Meeting, November 4, 2011; Winston Salem, NC.

- Deferoxamine with Demineralized Bone Allograft to Promote Fracture Healing in a Segmental Tibia Defect Model. Keller, B.; Turvey, B.; Davis, M.; Dahners, L.; Weinhold, P. Orthopaedic Research Society Meeting, February 4-7, 2012; San Francisco, CA.

Ben Keller has been pursuing his M.S. in Biomedical Engineering while he has been working on this study.

CONCLUSION:

Bulk form calcium sulfate placed in at a tibia segmental defect in the rat was found to inhibit the fracture healing process as demonstrated by reduced cortical bridging across the fracture and reduced torsional properties of the healed fracture. It is unclear if this effect of bulk form calcium sulfate is specific to the rat due to the faster healing response of this species. These findings imply that a bone defect in the tibia should not be completely filled with calcium sulfate as this may inhibit the fracture healing process.

Desferoxamine when delivered in calcium sulfate to a tibia segmental defect in the rat with and without demineralized bone allograft was found to have a limited ability to enhance cortical bridging and the torsional strength of the healing fracture as statistically significant differences were not found. However, the nonoptimal size of the animals used for our spacer defect model introduced greater variability in our model making it more challenging to detect statistical differences with the treatments. Furthermore, the inhibitory effect of the bulk form calcium sulfate on the fracture healing process may have also curtailed the ability of desferoxamine to stimulate healing. Future work should consider delivering the desferoxamine to the defect by other means. In addition, future studies with the rat tibia space defect model should be performed in older animals with a slower growth rate to reduce variability with the model.

In summary, we feel these findings are important as they are an initial step towards the identification of the optimal method of delivering desferoxamine to a fracture site to improve healing.

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Appendix B:

Draft of Manuscript of Findings of Task 2

Desferrioxamine Combined With Demineralized Bone Matrix Improves Fracture Healing in a Tibial Segmental Defect Model

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INTRODUCTION

Approximately 10% of fractures will have some difficulty in healing (Tzioupis & Giannoudis, 2007). These numbers highlight the need to identify cost-effective means for stimulating fracture healing. Demineralized bone matrix (DBM) has seen wide use as a bone defect filler and agent for stimulating fracture healing. Animal model studies have demonstrated the ability of DBM to stimulate healing in various fracture models (Bolander et al., 1986; Einhorn et al., 1984). However, well-designed clinical studies for demonstrating the effectiveness of DBM in stimulating healing are lacking (Kinney et al., 2010; Veillette & McKee, 2007). While the bone morphogenic proteins present in DBM provide a stimulus for accelerating fracture healing (Urist, 1965), others have noted the importance of an adequate vascular environment in order to realize the stimulatory potential of DBM to fracture healing (Kinney et al., 2010). The importance of a sufficient vascular supply to effective fracture healing is well recognized (Eckardt et al., 2005; Street et al., 2002).

Desferroxamine (DFO) is an FDA approved iron chelating agent that has been shown to increase the expression of vascular endothelial growth factor (VEGF) in mesenchymal stem cells (Shen et al. 2009) and other angiogenic agents in other cell types (Yamakawa et al. 2003) through the stabilization of HIF-1α . Past animal studies have shown DFO can stimulate angiogenesis and increase mineralization and mechanical properties of the healing fracture callus when given locally at the fracture site (Shen et al. 2009; Stewart et al. 2011). However, no studies have given DFO in concert with DBM to see if improving the vascular environment for DBM may enhance the stimulatory effects of DBM.

Our hypothesis was that combining DFO with DBM would further enhance fracture healing relative to a control group or DBM alone group. As demonstrated by the range of DFO dosages that have been used in animal studies evaluating the effects of DFO on fracture healing (Shen et al. 2009; Stewart et al. 2011), the optimal dosage to stimulate fracture healing is not known. A secondary objective of this study was to investigate how DFO dosage would influence the fracture healing process. Our hypothesis was that a lower dosage of DFO may be more effective in stimulating fracture healing than a higher dosage of DFO.

METHODS

Research Design:

After approval from the local IACUC, 120 retired breeder female Sprague Dawley rats were obtained, weighed and divided into 4 groups. All animals were surgically subjected to a transverse osteotomy 14 mm distal to the tibial tubercle with stabilization by an intramedullary stainless steel pin. A 2mm bone defect was created by placing a 2mm long cylindrical PEEK (polyether ether ketone) spacer about the pin at the fracture gap as described (Miles et al 2011). The experimental groups consisted of the following: Control (n=27), allogenic demineralized bone matrix (DBM, n=30): 50 mg of DBM placed circumferentially around the PEEK spacer , DBM with low dosage DFO (DBM + L-DFO, n=36): 50mg of DBM was soaked in 50 µL of saline containing 10 µg of DFO 30 minutes prior to surgery, DBM with high dosage DFO (DBM + H-DFO, n=27): 50mg of DBM was soaked in 50 µL of saline containing 100 µg of DFO 30 minutes prior to surgery. Buprenorphine was administered at surgery and for 3 days postoperatively for

analgesia. In addition, acetaminophen (250mg/kg) was administered in the drinking water for 21days postoperatively for extended analgesia. All animals were sacrificed at 6weeks.

DBM Preparation:

DBM was harvested from femurs and humeri of adult male Sprague Dawley rats. The bones were harvested, crushed, ground, and cleaned with a waterpik prior to demineralization. Demineralization occurred over 3 hours in two changes of 0.6N hydrochloric acid (HCl). The bone was then rinsed with sterile saline and titrated to a pH between 7 and 7.4 using concentrated sodium hydroxide (NaOH). The DBM was then placed in 70% ethanol for 12 hours. The wet DBM was weighed and allotted into sterile micro-centrifuge tubes. Finally, the DBM was lyophilized leaving dry, sterile DBM in individual tubes.

Radiographic Evaluation:

All rats had lateral radiographs taken at 1 and 4 weeks and biplanar radiographs at 6 weeks. Week 1 radiographs were used to determine the efficacy of the PEEK spacer in holding the gap. The 4 week radiographs were graded qualitatively by 3 independent blinded examiners to determine early gap bridging. Bridging scores varied from 0 to 2, representing the number of cortices bridged the defect. Radiographic examination at 6weeks was carried out in both the AP plane and the lateral plane. These radiographs were used to calculate the total bridging of the defect site at all 4 cortices (2 on AP view and 2 on lateral view) by 3 independent examiners blinded to the treatment groups. Bridging scores varied from 0 to 4 cortices bridged.

Biomechanical Evaluation:

Tibias from the operated and nonoperated limb were potted in molds for torsional testing with the intramedullary pin or tibial axis coinincident with the torsional axis. To assess the mechanical competence of the healed fractures and intact tibias, the stiffness, ultimate torque to failure, and energy to 35 degrees of deformation of the fracture callus were determined by torsional testing in external rotation on a uniaxial servohydraulic material testing machine fitted with fixtures to convert the axial motion to rotary motion. Specimens were torqued at a constant rate of six degrees/second until failure or 60 degrees of rotation. The torque and deflection angle were recorded and torsional properties computed. The ultimate torque was recorded as the maximum torque prior to 35 degrees of angulation. Displacement > 35 degrees without reaching a peak torque was felt to be indicative of tensioning of fibrous callus rather than bony union. The properties of the injured limb were normalized by the properties of the contralateral intact tibia.

Micro-CT Evaluation of Mineralized Callus Volume:

Subsequent to torsional testing ten specimens from each group were randomly chosen and scanned on a micro-CT system (μCT-40 Scanco Medical) for evaluation of the mineralized tissue volume at the 2mm defect site using a segmentation threshold of 642mg HA/cm^3.

Histology:

Three specimens from each group were prepared for histological examination. Specimens were fixed in 10% neutral buffered formalin, dehydrated in ethanol, decalcified in Immunocal, and embedded in paraffin. Five micrometer frontal sections were cut longitudinally from the anterior portion of the callus from 2 depths separated by 100μm.

Sections were stained with hemotoxylin-eosin and reviewed qualitatively for the tissue type, tissue organization, and cellularity.

RESULTS

Animals excluded from evaluation for surgical reasons are shown in Table 1. There was no statistical difference in the frequency of specific exclusions between the groups (P=0.133), however there was a trend for a higher number in the DBM+ L-DFO group. Corresponding with these exclusions, qualitatively it was observed that there appeared to be more pronounced swelling at the injury site within the initial 48hours postoperatively within some of the animals in the DBM+ L-DFO group compared to the control. In addition, a lesser trend for such swelling was observed in animals from the DBM and DBM+H-DFO groups compared to the control. In addition to the noted surgical exclusions, one animal entered the study with poor health and died (DBM group) and two other animals (Control, DBM+ L-DFO group) were excluded from biomechanical evaluation due to dissection or potting errors. Preoperative body weights and weight changes across the study were not found to differ between the groups.

Radiographic Evaluation:

Radiographic evaluation of the healing fracture at 4and 6 weeks demonstrated significantly more cortical bridging in the DBM+ L-DFO group compared to the control (Table 2). The remaining groups were not found to differ from the control, though they were also not found to differ from the DBM+ L-DFO group.

Biomechanical Evaluation:

Biomechanical evaluation of the healing fracture at 6 weeks demonstrated the DBM+ L-DFO group to have a 69% increased ultimate torque compared to the control ($P<0.01$) and a 42% increased ultimate torque relative to the DBM+ H-DFO group ($P<0.01$) (Figure 1). The DBM group was not found to differ in ultimate torque from the control or either of the DFO groups. The ultimate torque of the DBM+ H-DFO group was not found to differ from the control. The energy of the DBM+ L-DFO group was found to be increased 89% relative to the control ($P<0.01$) and 62% relative to either the DBM or DBM+ H-DFO group ($P<0.01$) (Figure 2). None of the other groups were found to differ relative to each other. The torsional stiffness was not found to differ between any of the groups (Figure 3).

Micro-CT Evaluation of Mineralized Callus:

Micro-CT evaluation of the healing fracture at 6 weeks did not reveal any significant differences in mineralized callus volume at the defect site. Three-dimensional renderings of mineralized callus volume at the defect site demonstrated less bridging in the control group (Figure 5). The bone bridging the defect region in the DBM + H-DFO group appeared to be more porous with larger pores than the DBM and DBM+ L_DFO groups (Figure 5).

Histological Evaluation:

Representative images from the histology sections are shown (Figure 6). The fracture gap was more evident in the control group sections with significant cartilage tissue present and minimal bone bridging the fracture gap. In the DBM group the fracture gap was less evident with some cartilage tissue present and some woven bone bridging the gap. In the DBM+L-DFO group more limited cartilage tissue was present

and the gap was bridged by a more dense trabecular bone. In the DBM+H-DFO group there was minimal cartilage tissue and the gap was bridged by a more porous trabecular bone.

DISCUSSION

Our study investigated the potential of administering DFO locally with DMB to enhance the effectiveness of DMB in stimulating fracture healing in a challenged healing scenario. Our findings of improved torsional properties and radiographic union with low dosage DFO partially supported our hypothesis of DFO enabling improved healing relative to DBM alone or control. While the low dosage DFO did not differ from the DBM alone for many of the evaluation measures, the DBM alone measures were also not found to differ from that of the control indicating an intermediate response.

While the lack of an effect of DBM alone relative to the control in our study may seem contrary to the past animal studies investigating the effect of DBM (Bolander et al., 1986; Einhorn et al., 1984) , these results may be related to the greater difficulty in achieving healing in the tibia and also the multiple group comparisons conducted in this study. While all of the groups using DBM displayed larger diameter calluses, the extent of bridging and the quality of bone across the bridge appeared to be improved in the L-DFO group.

Our study confirmed previous work done with DFO in other fracture models that may have had less of a vascular deficit than the current tibial defect model. In one study

where a rat femoral defect model was used (Stewart et al., 2011), local application of DFO to a synthetic bone graft material improved the vascular network at the fracture site and also improved the bending mechanical properties of the healing fractures. In this work a dosage of 8µg per rat was used. In other work using a femoral distraction osteogenesis model in the mouse a rat equivalent dosage of DFO of 100µg per rat was used and was found to increase mineralization of the callus and the vascular network of the callus. The differing dosage of these studies lead us to the secondary objective of our study which was to determine how the dosage of DFO would influence healing.

Our findings of no difference in radiographic bridging, callus mineralization, and torsional properties for the DMB+H-DFO group relative to the control seem to support our hypothesis that a low dosage of DFO may be more effective in stimulating fracture healing. It is unclear from our results what was the mechanism of the dosage effect. It might be that high dosage DFO causes too much vessel ingrowth which then delays formation of denser trabecular bone and lamellar bone. Alternatively, it may be that at higher dosages the chelation ability of DFO begins to interfere with other enzymatic processes of osteogenesis. Past studies have demonstrated evidence that DFO may inhibit collagen synthesis in cultured fibroblasts at higher concentrations (Hunt et al., 1979).

The potential of low dosage DFO to further improve the ability of DBM to stimulate fracture healing is appealing clinically due to the vast number of DBM products available (Veillette & McKee, 2007) and the potential ease that it might be applied. However, one aspect that may need to be looked at more carefully before pursuing clinical use is trend for increased swelling and wound problems observed with low

dosage DFO in the current fracture model. It is unclear to what extent these wound problems were dependent on the rats natural tendency to bite at their wounds.

There are several limitations associated with the current study. Additional vascular assessments may have provided additional information for our study, however these assessments have been a strong focus of past fracture healing studies investigating DFO and thus we elected to focus on more functional assessments of clinical relevance. In addition, the DBM used in this study was not a particular product or formulation that is used clinically, but is a more generic recipe for producing DBM. Using a specific product would have required the use on an athymic rat model and may have narrowed the applicability of the findings of the study. It is possible that the DFO treatment may be more or less effective with specific DBM products depending upon their formulation and processing. The current study evaluated most outcome measures at a single time point. However, this time point was effective in demonstrating that the low dosage DFO group had progressed to complete union with torsional properties comparable to the intact tibia in many of the animals. In addition, this study has only examined one mechanism by which a vascular deficiency may be caused, a noncritical defect. It will remain for further study to determine if DFO might accelerate fracture healing in other clinically relevant conditions were vascular deficits exist such as diabetes, nicotine exposure, atherosclerosis, and other conditions.

In conclusion, low dosage DFO treatment of DBM was found to enhance the ability of DBM to stimulate fracture healing, while higher dosage DFO treatment of DBM was not effective suggesting that higher dosages may impair the effect.

Acknowledgements:

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Table 1. Animal surgical exclusions for injured limb.

	Treatment Group			
Exclusion Criteria	Control	DBM	DBM+L-DFO	DBM+H-DFO
Wound Mutilation	0	2	5	0
Infection	2	2	2	0
Loss Fixation/Gap	2	2	4	0

Table 2. Radiographic grades of cortical bridging across the fracture gap for the treatment groups.(Mean±SD)

	Evaluation Measure	
Treatment	4 week xray (0-2)	6week xray (0-4)
Control	0.54 ± 0.63	2.03 ± 1.29
DBA	0.88 ± 0.76	2.56 ± 1.19
DBM+ L-DFO	1.08 ± 0.75*	3.13 ± 1.04*
DBM+ H-DFO	0.90 ± 0.67	2.67± 1.16

*Significant difference from control ($P<0.05$)

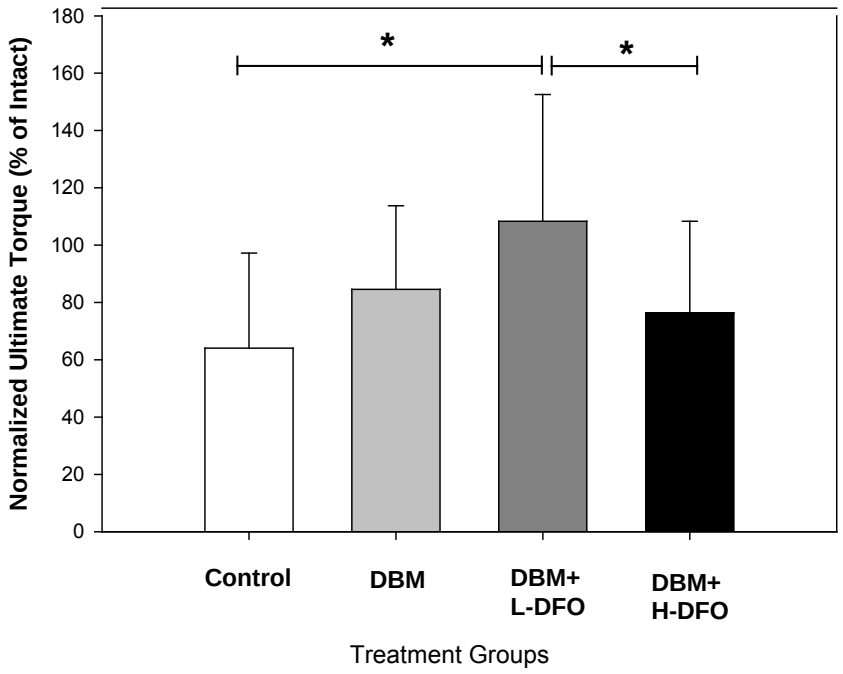

Figure 1. Ultimate torque of the injured tibia differed among the groups at 6 weeks of healing. Properties normalized by the contralateral intact tibia properties. (Mean±SD) Groups at ends of horizontal bar differed ($P<0.05$).

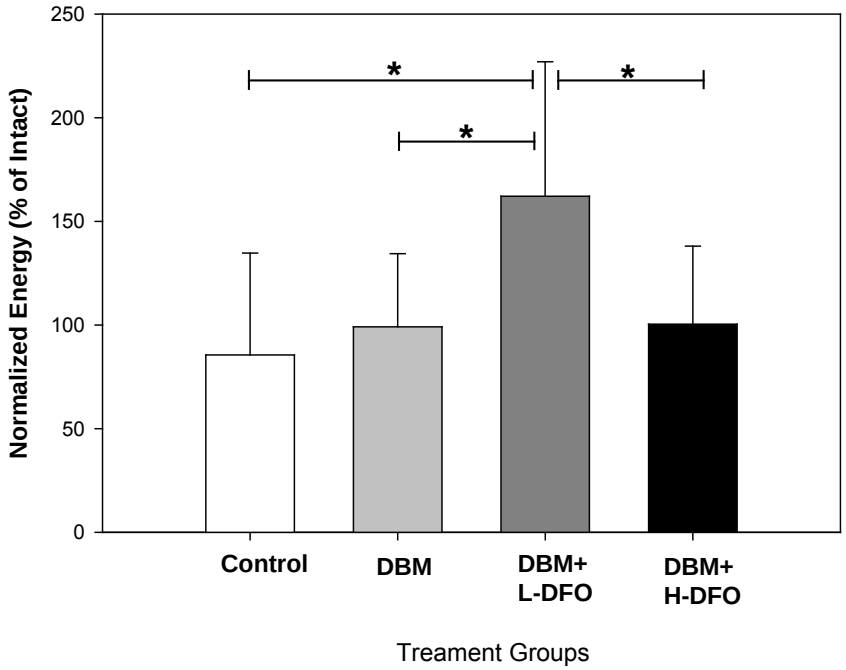

Figure 2. Torsional energy of the injured tibia differed among the groups at 6 weeks of healing. Properties normalized by the contralateral intact tibia properties. (Mean±SD) Groups at ends of horizontal bar differed ($P<0.05$).

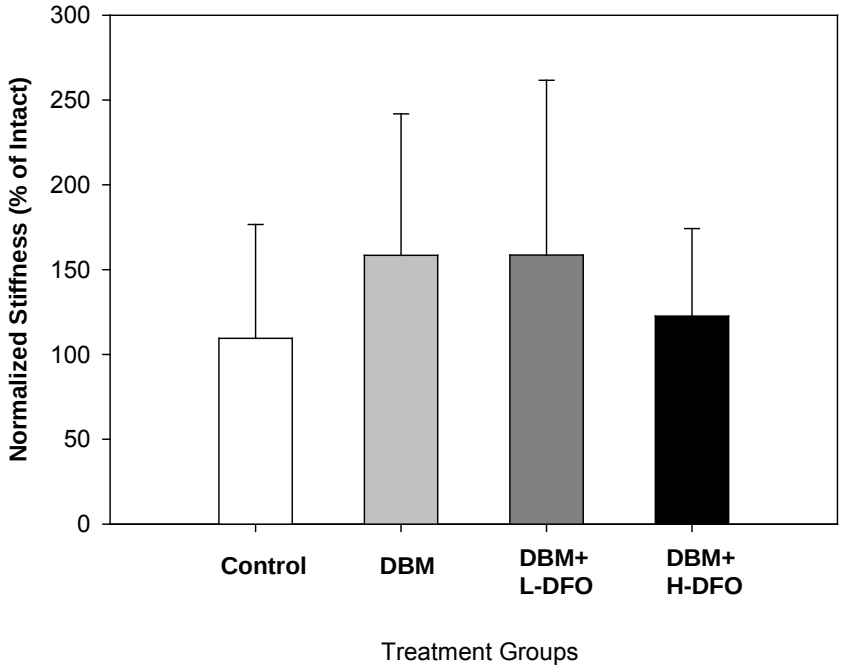

Figure 3. Torsional stiffness of the injured tibia did not differ among the groups at 6 weeks of healing. Properties normalized by the contralateral intact tibia properties. (Mean±SD) Groups at ends of horizontal bar differed ($P<0.05$).

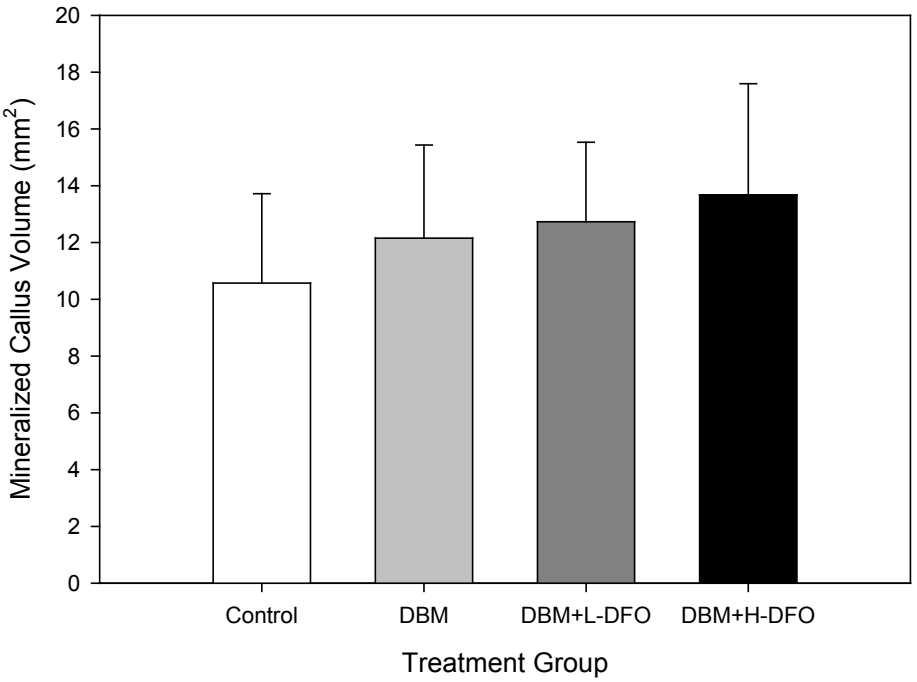

Figure 4. Mineralized callus volume did not differ among the groups at 6 weeks of healing. (Mean±SD)

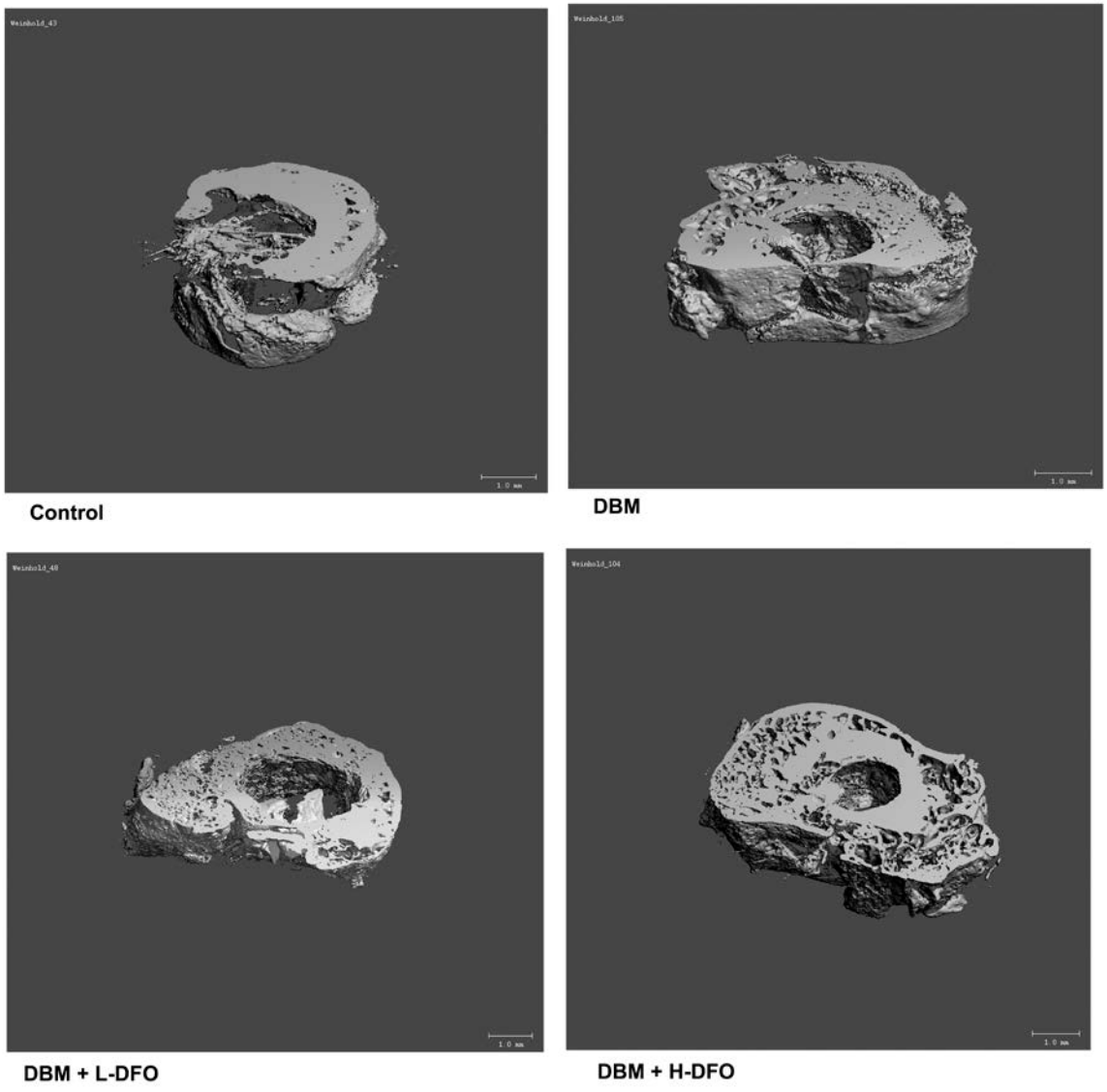

Figure 5. Representative three dimensional renderings of the mineralized callus volume at the defect site at 6 weeks of healing for each of the groups.

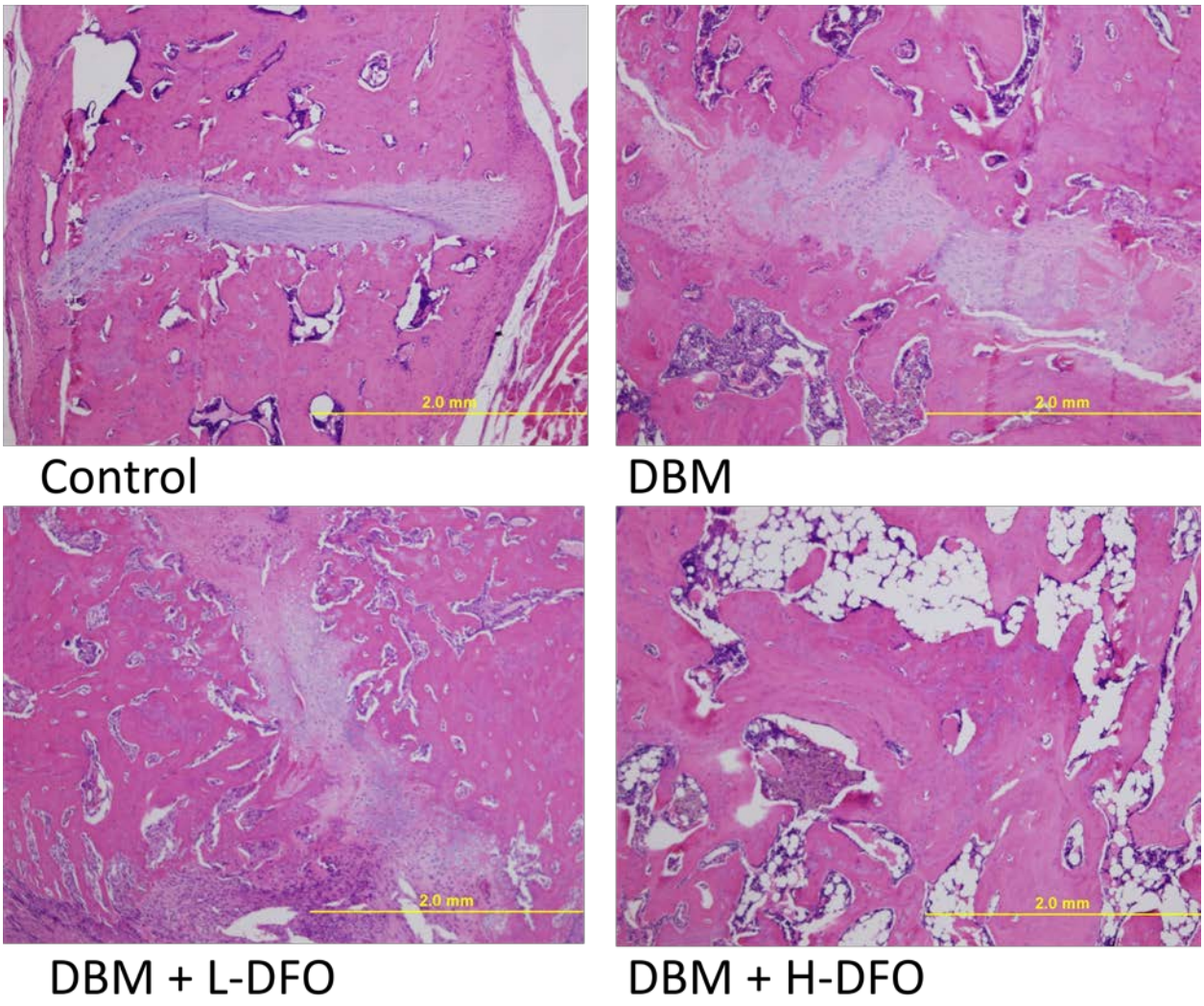

Figure 6. Representative H& E longitudinal sections of the anterior region of the fracture site for each group. Control: fracture gap was more evident with little bone bridging the gap and significant cartilage tissue still present. DBM: Fracture gap less evident with some bone bridging the gap though cartilaginous tissue still present. DBM + L-DFO: Fracture gap less evident with more complete bone bridging with denser trabecular bone and less cartilage tissue present. DBM +H-DFO: Fracture gap difficult to discern and no cartilage present with highly porous trabecular bone with greater vascularity bridging the gap.