Published in final edited form as:

Plast Reconstr Surg. 2012 April ; 129(4): 850–856. doi:10.1097/PRS.0b013e31824422f2.

Deferoxamine Enhances the Vascular Response of Bone Regeneration in Mandibular Distraction Osteogenesis

Alexis Donneys, MD, MS, Aaron S. Farberg, BS [MD candidate], Catherine N. Tchanque-Fossuo, MD, MS, Sagar S. Deshpande, BS, and Steven R. Buchman, MD

University of Michigan Craniofacial Research Laboratory, Plastic Surgery Section, Ann Arbor, MI

Abstract

Background—Bone regeneration during Distraction Osteogenesis (DO) is intricately associated with an enhanced vascular response. Augmenting this response may offer considerable clinical advantages such as optimizing the quality of regenerate formation, decreasing lengthy consolidation periods or increasing regenerate size and distance. Using Deferoxamine (DFO), an angiogenic transcriptional activator, we posit that substantial increases in vascular volume beyond the normal response to mechanical distraction can be quantified with Micro-Computed Tomography (μ CT) after vessel perfusion during mandibular DO.

Methods—Two groups of Sprague-Dawley rats ($n=12$) underwent external fixator placement, mandibular osteotomy, and a 5.1 mm distraction. During distraction, the experimental group ($n=6$) was treated with DFO injections into the distraction gap. After consolidation, the animals were perfused and imaged with μ CT. Vascular radiomorphometrics were calculated and statistical comparison was conducted with the independent samples t -test. $p < 0.05$ was considered statistically significant.

Results—A 40% statistically significant increase in the number of vessels (0.82 vessels/mm vs. 1.15 vessels/mm, $p = .012$), and a complimentary decrease in the space between vessels (1.18 mm vs. 0.86 mm, $p = .012$) was calculated in the experimental regenerate when compared to control. This robust increase in vascularity could also be readily observed with μ CT image reconstruction. Gross examination revealed a denser regenerate in the DFO injected group that is clearly illustrated with faxitron radiography.

Conclusion—Our study quantifies the ability of DFO to augment the vascular response of mandibular DO and establishes correlations between this therapeutic enrichment and enhanced regenerate formation.

Introduction

Recent enhanced tissue-engineering strategies offer an opportunity for the optimization of bone regeneration and repair. Unique challenges in craniofacial reconstruction such as large

Financial Disclosure: No commercial association or financial interests exist by any author.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

bony deficits due to trauma and oncologic resection typify scenarios where successful formation of a robust regenerate would be highly desirable. Unfortunately, these scenarios are often associated with substantial damage to vascular anatomy precluding the use of many forms of either endogenous or exogenous tissue engineering strategies¹⁻³. The current clinical application of Distraction Osteogenesis (DO), an endogenous form of tissue engineering, in the craniofacial skeleton is limited largely to the pediatric population. Presumably, its success in the reconstruction of congenital craniofacial deformities can be largely attributed to the patients' ability to mount an adequate vascular response capable of supplying the rigorous metabolic demands required for successful bone regeneration.

Efforts to enhance bone regeneration have largely focused on optimizing the critical timing for latency, and the appropriate rate and rhythm of distraction^{4,5,6}. Novel approaches have also been explored with varying degrees of success including the addition of extracorporeal shock wave therapy, cyclic mechanical lengthening and compression, and the addition of several osteogenic factors⁷⁻¹⁰.

With the development of adequate experimental animal models, our understanding of the angiogenic sequelae and the vascular responses surrounding mandibular DO has advanced. Innovative strategies, improved quantitative vascular outcome metrics, and molecular analysis of angiogenic factors have offered considerable insights into the physiology of the vascular response to DO¹¹⁻¹⁴. Using anti-angiogenic therapies designed to interfere with endothelial cell cycle progression, investigators have demonstrated the critical importance of angiogenesis during bone regeneration by evidencing the reproducible formation of therapeutically induced non-unions in rodent models of DO¹⁵. Imaging studies such as μ CT after vessel perfusion have added efficient means for quantifying changes in vascular density during DO, and have corroborated the early assertions of an enhanced vascular response beyond that of fracture repair in mandibular DO¹⁶.

Along with these developments, current advancements in tissue engineering strategies involving the use of angiogenic therapies have also evolved. These therapies may offer exploitable avenues for the optimization of bone regeneration. The introduction of angiogenic therapies may fill a void needed to diversify the current applications of DO to scenarios where the vascular environment is sub-optimal due to chronic co-morbidities, trauma, radiation injury, oncologic resection, or burn injuries. These angiogenic therapies may also enhance the capabilities of bone regeneration in congenital craniofacial anomalies, potentially allowing for shorter consolidation periods, more rapid rates of distraction, and the formation of larger regenerates of better bone quality.

Interestingly, advancements in long bone studies have found valid correlations between enhancing vascularity and enhancing bone healing. Investigators have demonstrated increases in vascular density and the quality of bone repair demonstrated by radiomorphometric analysis and biomechanical testing¹⁷⁻¹⁹. Deferoxamine (DFO), an iron-chelating agent has been shown to increase angiogenesis via the hypoxia inducible factor pathway in long bones. Hypoxia inducible factor-1 alpha (HIF-1 α) is part of a transcriptional activator complex involved in the transcription of gene programs essential in angiogenesis. In the presence of oxygen, HIF-1 α undergoes prolyl hydroxylation and

subsequent degradation by a proteasome. In hypoxic conditions, prolyl hydroxylation is inhibited, and HIF-1 α accumulates in the nucleus where it forms a dimer responsible for the transactivation of gene programs such as VEGF, and angioproteins integral in angiogenesis. This mechanism essentially triggers the body's oxygen sensing mechanism by delivering a signal otherwise triggered by hypoxia and thus begins an angiogenic response even in normoxic conditions¹⁷⁻¹⁹.

We posit that DFO therapy will function to enhance vascularity and improve bone regeneration in the mandible during DO. Here we examine the therapeutic potential of this exciting pharmacologic preparation to engineer a potent angiogenic response and offer conclusive evidence of enhanced vascularity even beyond that of the normal regenerative response in a rat model of mandibular DO.

Materials and Methods

Adult male Sprague-Dawley rats weighing approximately 400g were paired in cages and maintained in a pathogen-free environment on a 12-hour light/dark schedule. Rats were fed standard hard chow and water ad libitum during a seven-day acclimation period prior to surgery. All animal procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the University of Michigan Animal Care and Use Committee.

Surgical Procedure and Device Placement

Preoperative subcutaneous injections of Gentamycin (5 mg/kg), Buprenorphine (0.03 mg/kg) and Lactated Ringer's solution (25cc/kg) were administered prior to surgery. Anesthesia was achieved with the inhalation of an oxygen/isoflurane mixture. The animals were prepared for surgery, and underwent placement of custom titanium external mandibular fixator devices followed by surgical osteotomy as previously described¹¹.

The critical size defect utilized in our DO model is predicated on our previous experimental experience demonstrating that gradual distraction to 5.1 mm with a 28 day consolidation period consistently resulted in bony healing across the entire regenerate. Comparatively, defects created using acute separation to 5.1 mm or greater consistently formed fibrous union²⁰.

Postoperative Procedures

Both groups underwent distraction after 4 days of latency. One 180-degree clockwise turn of the distraction screw corresponded to a 0.3-mm separation of the osteotomy fronts. Active distraction began on the evening of post-operative day 4 and went through the evening of post-operative day 12. A total of 17 half-turns were performed on an every-12-hour interval, resulting in a maximum 5.1-mm distraction gap. The experimental DFO group (n=6) was treated with a 200 μ M/300 μ L (0.9% Sodium Chloride Solution) DFO injection into the distraction gap every other day during the active distraction period. This concentration was derived from the long bone literature and modified to encompass the larger volumetric dimensions of the mandibular region of interest¹⁷. Local injection was conducted by feeling for the callus site, which at this early stage is a readily palpable defect

in the bone. 150 μ L was administered inferiorly, then superiorly to ensure filling of the callus volume (Figure 2a). Methylene blue dye was added to the treatment and used in a test specimen to verify localization of the injection at the callus site. The specimen was injected as described and resected 30 minutes after injection to coincide with the short half-life of DFO (Figure 2b). Animals were anesthetized during DFO injection with an inhalational mixture of oxygen and isoflurane until lightly sedated. No analgesic or sedation was required during the distraction.

Postoperative Animal Care

Animals were housed one per cage and fed moist chow with Hill's high-calorie diet (Columbus Serum, Columbus, Ohio) and water ad libitum. Two postoperative doses of Gentamycin (5 mg/kg subcutaneously every 12 hours) were given as well as continuation of Buprenorphine (0.03 mg/kg) and Lactated Ringer's solution (5cc) subcutaneously every 12 hours through postoperative day four, and as needed thereafter. Weights were monitored daily and diets adjusted as needed. Pin care was performed with Silvadene (Monarch Pharmaceuticals, Inc., Bristol, Tenn.) every other day. Maxillary incisors were clipped weekly due to overgrowth from cross bite and staples were removed by postoperative day 10. Animals were allowed to complete a 40-day recovery period prior to perfusion and sacrifice.

Perfusion Protocol

All rats were anesthetized prior to thoracotomy and underwent left ventricular catheterization. Perfusion with heparinized normal saline followed by pressure fixation with normal buffered formalin solution ensured euthanasia. After fixation, the vasculature was injected with Microfil (MV-122, Flow Tech, Carver, MA). Mandibles were harvested and demineralized using Cal-Ex II (Fisher Scientifics; Fairlawn, NJ), a formic acid solution. Leeching of mineral was confirmed with serial radiographs to ensure adequate demineralization prior to scanning.

Micro-Computed Tomography

Specimens were scanned at 18 μ m voxel size with μ CT. We have previously utilized this voxel size and have found it optimal to adequately resolve small murine mandibular vessel networks. The region of interest was defined as a distance measuring 5.1 mm corresponding to the surgical site of the osteotomy and gap distance. Analysis of the left hemimandible region of interest (ROI) was then performed with MicroView 2.2 software (GE Healthcare, Milwaukee, WI.). Contours were defined highlighting the regions of interest using the spline tool in MicroView. Analysis of vascularity in this region is accomplished by setting a global grayscale threshold of 1000 to differentiate vessels from surrounding tissue as previously described¹². Using this information, MicroView allocates only the number of voxels above this threshold and uses stereologic algorithms to assign relative densities to the voxels based on the contrast content within the vessels. The ROI was analyzed for quantitative radiomorphometrics for vascularity. In addition for qualitative comparison, 3-dimensional visualization of vascular anatomy was accomplished utilizing the maximal intensity projection (MIP) feature. This enables instant volume rendering of a volumetric data set yielding a 3-dimensional representation of the scanned specimen.

Statistical Analysis

The independent samples *t*-test was used to analyze differences between group means (SPSS version 16.0; SPSS, Inc., Chicago, IL.). Vascular metrics were compared between groups and reported as their respective means with significance indicated at $p = .05$.

Results

All animals tolerated the osteotomy procedure without incident. Gross union of the left hemi-mandibles was verified upon harvest prior to μ CT scanning. (Figures 1-5)

Gross Assessments

The DFO/DO group grossly demonstrated a thicker and better-developed regenerate in comparison to control. Verification of DFO injection localization to the callus site was conducted. As demonstrated, most of the injection remained in the callus site 30 minutes after injection (Figure 2b). Although some seepage out of the callus is expected, it is important to consider that the half-life of DFO is extremely short (about 30 min) therefore any effects the drug may elicit outside of the callus would have to occur within this very short time period. Further, DFO is currently used on formulary for the treatment of iron overload in exponentially larger doses than the one being used here, and even at those doses, the side effect profile is limited and well known. Although both groups evidenced radio-opacity consistent with bone bridging, faxitron radiographs demonstrated enhanced radio-opacity in the regenerate area with a diminished line of lucency near the centermost region of distraction (Figure 3). Appraisal of mandibular vascular networks in the DFO/DO group characteristically revealed augmented vascularity throughout the distracted regenerate. Contributions of robust, well-developed branches stemming from the inferior alveolar artery characteristically appear to supply the bulk of the distracted regenerate (Figure 4).

Quantitative Assessments

Stereological analysis demonstrated a greater than 40%, statistically significant increase in vessel number (0.82 vessels/mm vs. 1.15 vessels/mm, $p = .012$) and a 27% statistically significant corresponding decrease in vessel separation (1.18 mm vs. 0.86 mm, $p = .012$) in the experimental DFO/DO group when compared to DO alone (Figures 5,6). Vessel Number is a representation of the number of vessels per mm within the ROI, whereas Vessel Separation is a representation of the distance between vessels per mm within the ROI.

Discussion

There is emerging evidence that the therapeutic augmentation of angiogenesis leads to quantifiable enhancements in fracture repair in long bones¹⁷⁻¹⁹. This finding may have a beneficial application in more rigorous forms of bone healing such as craniofacial DO. The widespread utility of DO for the correction of craniofacial anomalies such as micrognathia may directly benefit from this therapeutic exploitation. Furthermore, finding mechanisms to trigger, augment and maintain vascularity may offer insights for extending the reach of this procedure to more difficult clinical scenarios where vascularity has been compromised beyond a level needed to heal a critical sized bony defect. Consider the devastating toll of

ballistic injuries sustained in modern combat to the head and neck, or the complex critical sized defects created with resection of Head and Neck Cancer²¹⁻²³.

In this study we utilized a dose and scheduled delivery of Deferoxamine that has previously been reported^{17,18,24}. Other investigators have demonstrated enhancements in callous size formation and biomechanical strength in long bone models¹⁹. This dose was injected locally into the regenerate site and given every other day during distraction. It is important to consider that during bone regeneration, angiogenesis is triggered as a result of the mechanical separation, and thus delivery of this angiogenic potentiating therapy during this time period coincides with normal physiologic angiogenic events¹³. It is also worth reiterating that bone regeneration as a result of DO normally augments vascularity beyond that of a normal fracture repair^{16,25}. Thus, it was not readily apparent that the addition of this therapy would predictably demonstrate the capacity to augment an already enhanced vascular response in mandibular DO. We have previously utilized μ CT after vessel perfusion to demonstrate and calculate the normal augmentation of vascular density in mandibular DO and have found it to be an exceedingly valuable method for efficiently quantifying vascular responses¹². We observed a near doubling of vascular density within our regenerate area at the time of full consolidation when comparing distraction to fracture repair¹⁶. Given our previous findings, the use of angiogenic therapies would purportedly have to increase vascularity even further than the already hypervascular state induced by DO itself without impeding osteogenesis. Interestingly, we found that this enhanced augmentation was not only possible, but established a grossly more developed and radiographically denser distracted regenerate.

In this study our results demonstrate a statistically significant 40% increase in the number of vessels imaged per millimeter of regenerate area due to the administration of DFO angiogenic therapy in mandibular DO. This result was substantiated with a corresponding 27% decrease in the space between vessels. These findings demonstrate our ability to develop and utilize a pharmacologic trigger to increase and maintain an already enhanced vascular response in mandibular bone regeneration. In addition, we also observed a grossly and radiographically denser regenerate in the experimental group. These compelling findings foster the potential utility of vascular enhancement as a means of optimizing bone regeneration in DO. The most clinically relevant consideration is what implication this increased vascular response may have on bone quality and biomechanical strength. Although we observed a more robust regenerate, further analysis is needed to test the overall mineral density and strength of the regenerate. Such findings could be enormously helpful in the development of strategies to enhance and extend DO beyond its current clinical capabilities.

Practicality, cost effectiveness, and the ability to translate our findings from the bench to the bedside make DFO therapy particularly well suited for enhancing DO. DFO is widely used for the treatment of iron-overload due to repeated transfusions, thus, its clinical limitations and side effect profiles are well known²⁶. We did not observe any clinically adverse events beyond irritation at the injection site, and conclude that for the purposes of augmenting the vascular response and enhancing the bone regeneration capabilities of mandibular DO this will be a relatively safe and inexpensive prospect.

Acknowledgments

Funding was provided by National Institutes of Health grant RO1 CA 12587-01 to Steven R. Buchman. We would also like to thank Salman Ahsan and Noah S. Nelson for assistance with manuscript preparation, Jacklyn Kreider for μ CT assistance and Charles Roehm for mechanical engineering.

Funding supported by: NIH RO1 #CA12587-01 "Optimization of Bone Regeneration in the Irradiated Mandible" to PI: Steven R. Buchman, MD.

References

1. Jacobsson M, Albrektsson T, Turesson I. Dynamics of irradiation injury to bone tissue. A vital microscopic investigation. *Acta Radiol Oncol*. 1985; 24(4):343–50. [PubMed: 2994392]
2. Jacobsson MG, et al. Short- and long-term effects of irradiation on bone regeneration. *Plast Reconstr Surg*. 1985; 76(6):841–50. [PubMed: 4070452]
3. Markbreiter LA, et al. The effect of radiation on the fracture repair process. A biomechanical evaluation of a closed fracture in a rat model. *J Orthop Res*. 1989; 7(2):178–83. [PubMed: 2918417]
4. King G. Effect of distraction rate and consolidation period on bone density following mandibular osteodistraction in rats. *Archives of Oral Biology*. 2003; 48(4):299–308. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0003996903000049>. [PubMed: 12663075]
5. Troulis MJ, Glowacki J, Perrott DH, Kaban LB. Effects of latency and rate on bone formation in a porcine mandibular distraction model. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons*. 2000; 58(5):507–13. discussion 514.
6. Park JW, et al. *Innovations and Techniques Journal of Craniofacial Surgery*. 2001
7. Lai JP, Wang FS, Hung CM, et al. Extracorporeal shock wave accelerates consolidation in distraction osteogenesis of the rat mandible. *The Journal of trauma*. 2010; 69(5):1252–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/20404761>. [PubMed: 20404761]
8. Cillo JE, Gassner R, Koepsel RR, Buckley MJ. Growth factor and cytokine gene expression in mechanically strained human osteoblast-like cells: implications for distraction osteogenesis. *Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics*. 2000; 90(2):147–54.
9. Ashinoff RL, Cetrulo CL, Galiano RD, et al. Bone Morphogenetic Protein-2 Gene Therapy for Mandibular Distraction Osteogenesis. *Annals of Plastic Surgery*. 2004; 52(6):585–590. [PubMed: 15166991]
10. Kontaxis, a; Abu-Serriah, M.; Ayoub, aF; Barbenel, JC. Mechanical testing of recombinant human bone morphogenetic protein-7 regenerated bone in sheep mandibles. *Proceedings of the Institution of Mechanical Engineers. Part H, Journal of engineering in medicine*. 2004; 218(6):381–8.
11. Buchman SR, Ignelzi MA, Radu C, Wilensky J, Rosenthal AH, Tong L, Rhee ST, Goldstein SA. A Unique Rodent Model of Distraction Osteogenesis of the Mandible. *Annals of Plastic Surgery*. 2002 Nov; 49(5):511–519. [PubMed: 12439020]
12. Jing XL, Farberg AS, Monson LA, Buchman SR. Radiomorphometric Quantitative Analysis of Vasculature in Rat Mandible Utilizing Microcomputed Tomography. *Plast Reconstr Surg*. 2009 Oct; 124(4S):26–27.
13. Al-Aql ZS, Alagl aS, Graves DT, Gerstenfeld LC, Einhorn Ta. Molecular Mechanisms Controlling Bone Formation during Fracture Healing and Distraction Osteogenesis. *Journal of Dental Research*. 2008; 87(2):107–118. [PubMed: 18218835]
14. Glowacki J. Angiogenesis in fracture repair. *Clin Orthop Suppl*. 1998; 355:S82–S89.
15. Fang TD, Salim A, Xia W, et al. Angiogenesis is required for successful bone induction during distraction osteogenesis. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*. 2005; 20(7):1114–24.
16. Donneys A, Tchanque-Fossuo CN, Farberg AS, Deshpande SS, Buchman SR. Bone Regeneration in Distraction Osteogenesis Demonstrates Significantly Increased Vascularity in Comparison to Fracture Repair in the Murine Mandible. *Plast Reconstr Surg*. 2011; 127(5):27. [PubMed: 21200196]

17. Wan, et al. Activation of the hypoxia-inducible factor-1alpha pathway accelerates bone regeneration. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105(2):686–91. [PubMed: 18184809]
18. Street, et al. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proceedings of the National Academy of Sciences of the United States of America. 2008; 99(15):9656–61. [PubMed: 12118119]
19. Shen X, Wan C, Ramaswamy G, et al. Prolyl hydroxylase inhibitors increase neoangiogenesis and callus formation following femur fracture in mice. Journal of orthopaedic research : official publication of the Orthopaedic Research Society. 2009; 27(10):1298–305. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19338032>. [PubMed: 19338032]
20. Tong L, Buchman SR, Ignelzi Ma, Rhee S, Goldstein Sa. Focal adhesion kinase expression during mandibular distraction osteogenesis: evidence for mechanotransduction. Plastic and reconstructive surgery. 2003; 111(1):211–22. discussion 223–4. [PubMed: 12496582]
21. Kumta, P.; Sfeir, C. [Accessed July 6, 2011] Signaling and Cellular Strategies of Injectable Biomimetic Matrices for Craniofacial Bone Tissue Engineering. Pittsburg Tissue Engineer Initiative. Web site. <http://www.ptei.org/interior.php?pageID=108>. Updated 2011
22. Behnia H, Motamedi MH. Reconstruction and rehabilitation of short-range, high-velocity gunshot injury to the lower face: a case report. Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery. 1997; 25(4):220–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9268901>. [PubMed: 9268901]
23. Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics , 2010 Both sexes female both sexes estimated deaths. Health Policy. 2010; 60(5):277–300.
24. Farberg AS, Jing XL, Monson LA, Tchanque-Fossuo CN, Donneys A, Deshpande SS, Buchman SR. Deferoxamine Treatment Restores Vascularity in Irradiated Mandibular Distraction Osteogenesis. Plast Reconstr Surg. 2010 Jun.125(6):39.
25. Aronson J. Temporal and spatial increases in blood flow during distraction osteogenesis. Clinical orthopaedics and related research. 1994; (301):124–31. [PubMed: 8156663]
26. Brittenham GM. Iron-chelating therapy for transfusional iron overload. New England Journal of Medicine. Jan 13; 364(2):146–156. [PubMed: 21226580]

Figure 1. Schematic outlining the figure of the normal left hemi-mandible with the region of interest highlighted for visual comparison

Figure 2.

a) Drawing depicting the placement of DFO injection into the callus. The rat is placed on its dorsum and then 150 μ L of DFO solution is injected inferiorly then superiorly into the palpated callus as shown. **b)** Methylene blue dye verification of localization of the injection at the callus site.

Figure 3.

Radiographs demonstrating an enhancement in radio-opacity near the bony fronts (arrows) in the experimental group that received DFO prior to DO (Right) in comparison to the non-treated group (Left). This difference is better appreciated in the corresponding enlarged images focused on the regenerate area (Bottom). Faxitrons were taken uniformly at 3× magnification with 27 kV/5 second exposure.

Figure 4. Representative MIPs of DO (top) vs. DFO/DO showing a significant increase in vascularity with the addition of DFO (bottom). The inferior alveolar artery and its robust branching network are shown in the superior aspect of the highlighted region.

Figure 5. Statistically significant increase in Vessel Number with the addition of DFO

Figure 6. Statistically significant corresponding decrease in Vessel Separation with the addition of DFO