Deferoxamine Expedites Consolidation during Mandibular Distraction Osteogenesis

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Abstract

Background—A limitation of mandibular Distraction Osteogenesis (DO) is the length of time required for consolidation. This drawback subjects patients to possible pin-site infections, as well as a prolonged return to activities of normal daily living. Developing innovative techniques to abridge consolidation periods could be immensely effective in preventing these problematic morbidities. Deferoxamine (DFO) is an angiogenic activator that triggers the HIF-1α pathway through localized iron depletion. We previously established the effectiveness of DFO in enhancing regenerate vascularity at a full consolidation period (28 days) in a murine mandibular DO model. To investigate whether this augmentation in vascularity would function to accelerate consolidation, we progressively shortened consolidation periods prior to μCT imaging and biomechanical testing (BMT).

Materials and Methods—Three time points (14d, 21d and 28d) were selected and six groups of Sprague-Dawley rats (n=60) were equally divided into control (C) and experimental (E) groups for each time period. Each group underwent external fixator placement, mandibular osteotomy, and a 5.1mm distraction. During distraction, the experimental groups were treated with DFO injections into the regenerate gap. After consolidation, mandibles were imaged and tension tested to failure. ANOVA was conducted between groups, and $p < 0.05$ was considered statistically significant.

Results—At 14 days of consolidation the experimental group demonstrated significant increases in Bone Volume Fraction (BVF), Bone Mineral Density (BMD) and Ultimate Load (UL) in comparison to non-treated controls. The benefit of treatment was further substantiated by a striking 100% increase in the number of bony unions at this early time-period (C:4/10 vs. E:8/10).

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Furthermore, metrics of BVF, BMD, Yield and UL at 14 days with treatment demonstrated comparable metrics to those of the fully consolidated 28d control group.

Conclusion—Based on these findings, we contend that augmentation of vascular density through localized DFO injection delivers an efficient means for accelerating bone regeneration without significantly impacting bone quality or strength.

Keywords

Mandibular Distraction Osteogenesis; Consolidation; Angiogenesis; Deferoxamine

(1.1) Background

Mandibular distraction osteogenesis (DO) has emerged as an efficacious surgical technique for the treatment of congenital retrognathia, micrognathia and mandibular hypoplasia. Increasing surgeon experience and device innovation has allowed for the expansion of this attractive technique throughout the craniofacial skeleton to allow for the correction of maxillary hypoplasia, midface hypoplasia and craniosynostosis [1-4]. Despite these clinical advancements, the length of time required for bone consolidation continues to be a significant limitation to the widespread use of this procedure [5].

In contrast to fracture healing in which bone takes six to eight weeks to heal, DO requires device manipulation during lengthening and a subsequent consolidation period that is proportional to the amount of bone lengthening. This consolidation period ranges from approximately two to six months depending on the desired length and anatomic location of distraction [3,6].

Numerous factors play a role in the success of DO, with blood flow as paramount to the formation of a healthy union. The importance of sufficient blood flow to the fractured ends during consolidation is related to osteoblast survival [7-9]. Osteoblast survival, in turn, relies on the close proximity of these cells to nutrient vessels (<100μm) [10,11]. Studies of non-unions demonstrate ischemic, fibrous tissue; whereas healthy unions often demonstrate high levels of neovascularization and active osteogenesis [12]. The dense vascular network in the craniofacial skeleton allows for a high success rate of DO; however, improved angiogenesis would reasonably lead to faster consolidation. Improving vascularity would not only enhance osteogenesis through improved blood flow to osteoblasts, but vascular tissues themselves also secrete osteogenic cytokines such as BMP-2 and vasculogenic cytokines such as VEGFA, which have also been shown to enhance osteogenesis [13].

Other novel treatment methods of distraction optimization have been reported; however, considerable regulatory hurdles have prevented translation of these methods to the clinical arena [14-17]. A therapeutic paradigm focused on the optimization of vascularity with an established, FDA approved therapy may be a strategy for distraction optimization with real translational potential.

Recent tissue engineering strategies have shed light on the use of angiogenesis and vascular augmentation as mechanisms to enhance bone regeneration in long bone murine models. Investigators have utilized deferoxamine, an iron chelator with angiogenic properties, to

trigger angiogenesis and report quantifiable enhancements in vascularity at 14 days and bone quality at 28 days–a widely accepted normal time-point for expected full consolidation [9,18]. Deferoxamine exerts its angiogenic function through the stimulation of the HIF-1 α pathway. Iron is a co-factor in a reaction that leads to the degradation of HIF-1 α. In the absence of iron, the production of HIF-1 α becomes constitutive resulting in an accumulation of the factor in the nucleus. HIF-1 α then dimerizes and begins a transcriptional cascade of events leading to the production of VEGF, as well as other downstream angiogenic factors. Deferoxamine simply removes iron from this equation, thereby triggering angiogenesis and subsequently, new blood vessel formation [18-20].

We previously reported on the ability of deferoxamine to successfully augment vascularity and bone cellularity even beyond that of the normal regenerative response in our rat model of mandibular DO at a full 28d consolidation period. Our results demonstrated a significant 40% increase in vessel number and a 45% increase in osteocytes within the regenerate site [21,22].

In order to expand on our findings, we aim to investigate the therapeutic potential of deferoxamine to substantially shorten consolidation periods. Here we examine its utility in our model at three consecutive time-points (14d, 21d and 28d). We posit that the addition of deferoxamine will allow for successful consolidation at substantially shortened time-points (14 and 21 days) without compromising regenerate quality or mechanical strength when compared to a normal, fully consolidated regenerate (28 days).

(1.2) Materials and Methods

(1.2.1) Experimental Design

Twelve-week-old male Sprague Dawley rats weighing approximately 400 g were paired in cages and maintained in a pathogen-free environment on a 12-hour light/dark schedule. Six groups of animals (n=60) were equally divided into control (C) and experimental (E) groups for each time-period (n=10 per group). Three time-points of consolidation were chosen (14d, 21d and 28d). The 28d time-point was selected as a full, standard consolidation period based on what is widely accepted in the literature and our previous experimentation with this animal model [9,18,21,23,24]. During a 7-day acclimation period before surgery, rats were fed standard hard chow and water ad libitum. Animals underwent mandibular osteotomy and placement of an external distraction device. Experimental groups received deferoxamine injections during the distraction period. Subsequently, all groups were subjected to a variable consolidation period prior to outcome analysis (Figure 1 top). All animal experimentation was conducted in accordance with the guidelines published in the *Guide for the Care and Use of Laboratory Animals: Eighth Edition*, ISBN-10: 0-309-15396-4 and approved by the University of Michigan Animal Care and Use Committee.

(1.2.2) Mandibular Osteotomy

The techniques for mandibular osteotomy and DO were previously described. Briefly, pre-operative subcutaneous injections of gentamycin (5 mg/kg), buprenorphine (0.03 mg/kg) and lactated Ringer's solution (25 cc/kg) were administered. Animals were then anesthetized with an inhalational oxygen/isoflurane mixture. The animals were prepared for surgery and

an osteotomy was created behind the third molar of the left hemi-mandible. This was subsequently secured with a custom-made titanium external distractor device as previously described [23]. Post-operatively, animals were housed one per cage and fed moist chow with Hill's high-calorie diet (Columbus Serum, Columbus, Ohio). Incisors were clipped when needed due to unopposed lengthening. Incisor growth is accelerated due to the soft diet and induced cross-bite from the unilateral distraction. Animals underwent a group-specific consolidation period (14d, 1d or 28d) before euthanasia and outcome analysis.

(1.2.3) Mandibular Distraction

Animals underwent active distraction from the evening of postoperative day 4 through the evening of postoperative day 12. One 180-degree clockwise turn of the distraction screw resulted in a 0.3 mm separation of the osteotomy fronts. Turning of the screw was done every 12 hours for a total of 17 half turns, which produced a cumulative final gap distance of 5.1mm. No analgesic or sedation was required during distraction. The selection of our gap distance was based on findings from our previous experimentation with this model over the years. A 5.1 mm acute gap in the rat mandible consistently produces non-union (critical size defect), whereas gradual separation to this gap distance reliably heals by a predominantly intramembranous mechanism characteristic of DO [25].

(1.2.4) Deferoxamine Injection

For the experimental groups, deferoxamine (200 μM in 300 μL NS) was locally injected directly into the distraction gap every other day during the active distraction period starting on post-operative day 4 and continuing through post-operative day 12. This dose was derived from an extensive literature search regarding the use of deferoxamine in long bone animal models, as well as our experimentation with this drug in recent years [18-21,26,27]. We implemented the injection of deferoxamine therapy during the active distraction period to coincide with the known augmentation of angiogenesis naturally occurring in response to the mechanical stretching of the callus [7,8,10]. Animals were briefly anesthetized (using an isoflurane bell jar technique) during injection to avoid pain and prevention of motion. The injection was administered directly into the clinically palpable regenerate gap. One hundred fifty micro-liters were administered inferiorly and then superiorly to fill the regenerate gap volume. Methylene blue dye was injected in a subset of animals to ensure drug delivery into the regenerate site as previously described [21].

(1.2.5) Bony Union Analysis

En-bloc dissection of mandibles allowed for assessment of bony union. This was clinically defined as absence of movement across the regenerate site on manual manipulation of the regenerate gap after removal of the fixator device. Union status was subsequently verified with Micro-Computed Tomography (μCT) imaging [28].

(1.2.6) μCT

Images were obtained using 80 kVp, 80 mA and 1100 ms exposures. Three hundred ninety-two projections were taken at a 45-micron voxel size for bone analysis. General Electric's Microview 2.2 software was utilized to define our region of interest (ROI) volume and

derive radiomorphometrics. The ROI spans a distance of 5.1mm posterior to the third molar corresponding to the surgical site of osteotomy (Figure 1 bottom). Utilizing the splining tool, only bone within the ROI was selected, ensuring exclusion of surrounding spaces and the incisors. Metrics for bone volume fraction (BVF), bone mineral density (BMD) and tissue mineral density (TMD) were obtained.

(1.2.7) Biomechanical Tension Testing

Mandibles were potted after imaging and loaded to failure in uniaxial monotonic tension at 0.5 mm/s using a servohydraulic 858 Minibionix II testing machine (MTS Systems Corporation; Eden Prairie, MN). Crosshead displacement was recorded by using an external linear variable differential transducer (LVDT; Lucas Schavitts, Hampton, VA), and load data were collected with a 100-lb load cell (Sensotec, Columbus, OH). Data were sampled at 200 Hz on a TestStar system (TestStar IIs System version 2.4; MTS Systems Corporation). Load displacement curves were analyzed for yield load (Y), stiffness (S) and ultimate load (UL), using custom computational code (MATLAB 7.11; Mathworks Inc., Natick, MA).

(1.2.8) Statistical Analysis

Utilizing SPSS v. 20 software, an analysis of variance (ANOVA) with post-hoc Tukey's test was used to determine significance between group means with $p < 0.05$ considered statistically significant.

(1.3) Results

(1.3.1) µCT Results

Radiomorphometrics collected through µCT imaging revealed the clear effects of therapy at each time point (Figures 2 and 3a-c; Tables 1 and 2).

In comparison to the standard 28d fully consolidated control (28d C), radiomorphometric analyses of the non-treated group at 14 days (14d C) demonstrated an expected significant disparity in BVF and BMD (both were 77% of 28d C). This difference was not apparent with deferoxamine therapy, indicating a benefit of treatment at the earliest time-point (14d E BVF = 101% of 28d C; 14d E BMD = 96% of 28d C). At 21 days, a similar effect was observed for BMD, indicating a continued beneficial effect of deferoxamine therapy (21d C = 89% of 28dC vs. 21d E = 109% of 28d C). BVF, however, was no longer significantly decreased between 21d C and 28d C, reflecting the normal effect of increases in regenerate volume and mineralization over time (21d C = 89% of 28d C). Further, when comparing E to C groups within each respective time-point, a significant increase in BMD and BVF was observed at 14 and 21 days. Lastly, throughout the study TMD showed no differences between any of the groups, indicating that neither time nor therapy affected the mineralization of new bone.

In summary:

- The effect of therapy was most evident on increases in BVF and BMD at the earliest (14d) time-point, and in BMD at the second (21d) time-point. With therapy, these increases were normalized to fully consolidated levels (28d C).

(1.3.2) Biomechanical Testing Results

Tension testing the mandibles to failure further indicated the effects of therapy at each time point (Figure 4a-c; Tables 3 and 4).

In comparison to the standard 28d fully consolidated control, biomechanical analyses demonstrated significantly lower values for Y, S and UL in the non-treated group at 14 days (28%, 17% and 29% of 28d C respectively). This difference was not apparent for Y and UL with therapy in the 14d E group (61% and 76% of 28d C respectively). Despite treatment, S in the 14d E group exhibited a significant disparity from control levels at 28d (39% of 28d C). Twenty-one day control Y, S and UL were still significantly less than 28d C levels, as expected (41%, 35% and 35% of 28d C respectively). These differences were no longer apparent with the addition of therapy to the 21d groups (103%, 80% and 94% of 28dC respectively). When comparing C to E groups within each time-point, treatment groups exhibited increased UL at 14 days and both UL and Yield at 21 days. There were no significant differences between groups within each time-point for S, nor were there any differences observed for Y, S or UL at 28d between C and E groups.

When examining group means over time, it was apparent that control Y, S and UL continued to increase throughout the study. In contrast, the experimental group Y and S reached fully consolidated levels by 21 days and largely plateaued thereafter. Both groups continued to increase in UL over time, although the relative rise was much higher in the control group, particularly between 21 and 28 days. With the exception of S at 28d, experimental means were higher than control means for each metric at each time-point in the study.

In summary:

• The effect of therapy was most evident on increases in UL at 14 and 21 days. These values were normalized to fully consolidated levels (28d C) in the experimental group, whereas the non-treated group exhibited a significant disparity.

(1.3.3) Bony Union Results

At the earliest time-point (14d), only 4/10 control mandibles exhibited bony union compared to 8/10 experimental mandibles (Figure 5). Further, unions in the 14d control group exhibited unions due to sparse bony bridging in the regenerate gap when compared to experimental mandibles (Figure 6).

(1.4) Discussion

(1.4.1)

In the present study we investigated the therapeutic potential of deferoxamine to accelerate regenerate consolidation after mandibular DO. While other methods to accelerate consolidation have been attempted, we specifically aimed to optimize an ongoing and well-established phenomenon occurring during distraction. Angiogenesis and vascular augmentation in response to the mechanical stretching of a callus are critical mechanisms integral to the successful regeneration of bone [10,29-32]. Here we sought to enhance the natural vascular augmentation achieved in distraction by utilizing an angiogenic therapy

during the distraction period. Ultimately, we hypothesized that the addition of deferoxamine would enable successful consolidation at substantially shortened time-points without compromising regenerate quality or mechanical strength. In this experiment, quantitative metrics consistently demonstrated the beneficial effects of therapy at abridged time-points.

(1.4.2) Radiomorphometric Data

By 21 days, control BVF had reached a plateau consistent with fully consolidated levels; however, a disparity still existed in the control BMD. It was apparent that therapy continued to impact positive changes in BMD at this time-point, allowing for a beneficial increase in BMD to fully consolidated levels. By 28d of consolidation, statistical differences between groups no longer existed. We posit that the untreated bone finally "caught up" to the accelerated process resulting from DFO treatment.

In summary:

- Deferoxamine therapy impacted an acceleration of mineral deposition leading to increased mineral volume within the regenerate at the earlier time-periods when compared to control.

(1.4.3) Biomechanical Data

The greatest effect of therapy was observed at the second time-period where all metrics (Y, S and UL) reached fully consolidated levels in the experimental groups. This was not observed in the control groups. The time between the second and third time-points (day 21-28) also appeared to be the most critical period for normal regenerate development from a biomechanical perspective. While control mandibles only marginally increased in Y, S and UL between the first and second time-periods (days 14-21; = +9N, + 211N/mm and + 5N respectively), the biggest gains were observed between the second and third time-periods (= +36N, + 782N/mm and + 58N respectively). This pattern was not observed in the experimental group. Y and S increased notably between the first and second time period, but achieved control levels and plateaued thereafter. Experimental UL steadily increased throughout the study, presumably highlighting the effect of continued regenerate organization as a dominant factor, as opposed to increases in UL due to mineral deposition and callus enlargement over time as observed in the control group. These results reflect that the biomechanical integrity of the treated regenerate is largely established by the 21d time-point, whereas substantial increases in regenerate mineralization and associated mechanical integrity are ongoing in the control groups throughout the experiment.

In summary:

- Without therapy, the regenerate was still experiencing large gains in biomechanical strength throughout the study, while treated mandibles achieved the bulk of biomechanical integrity by 21 days.

(1.4.4) Bony Union Assessment

On gross inspection of the dissected mandibles, we observed a natural progression of bony union formation in the control group over time. At each time-point the number of unions

increased, ultimately reaching a 100% bony union rate by 28 days (4/10, 6/10, 10/10 respectively). Conversely, the experimental group achieved a higher union rate than the control group at both earlier time periods (8/10 at 14 and 21 days). By 28 days, both groups exhibited 100% union rate (10/10). Taken together, these findings highlight the effect of therapy in the early formation of a clinically relevant benchmark–the formation of bony union.

In summary:

- Treated mandibles exhibited twice as many unions at the earliest time period when compared to untreated controls.

(1.4.5)

All of our findings amalgamate to our conclusion that deferoxamine functions to expedite consolidation and healing during mandibular DO.

Despite our promising findings, several clinically relevant limitations should be addressed. While we saw increases in bony union at the earlier time-periods, it is important to note that 100% union rate was not achieved with therapy at 14 and 21 days. Further, when regarding biomechanical data, statistical significance does not directly translate to clinical relevance. For example, while our 14d experimental ultimate load was not statistically different than fully consolidated controls, there was in fact a disparity of 63N. This difference, while not statistically significant, may have considerable clinical implications. Due to these limitations, future directions may consider optimization of early consolidation with the addition of synergistic therapies. Finally, while our results are promising with regards to the acceleration of consolidation, it is important to note the potential to over-accelerate distraction and cause premature consolidation. With regards to this potential complication, future investigations may consider the controllability of deferoxamine by examining dose-response relationships between the amount of injected deferoxamine and the rate of consolidation in this model.

(1.4.6) Conclusions

Our results substantiate the assertion that angiogenic and vascular enhancements during DO serve to stimulate powerful mechanisms that can function to hasten bone regeneration and shorten lengthy consolidation periods. This study demonstrates quantifiable increases in radiomorphometric and biomechanical parameters of regenerate healing at significantly abridged time-points during consolidation due to vascular augmentation with deferoxamine therapy. While further studies are needed to address the aforementioned limitations of this work, based on our findings, we contend that this therapeutic strategy should be considered as a translational option for the acceleration of bone healing in patients undergoing DO.

Acknowledgments

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Highlights

We demonstrate the degree of acceleration of consolidation during distraction osteogenesis with the utility of angiogenic deferoxamine therapy. > We report quantifiably enhanced radiomorphometrics of bone quality and metrics of biomechanical strength at abridged time-points that are comparable to fully consolidated controls. > Our findings suggest that augmentation of vascular density through localized DFO injection delivers an efficient means for accelerating bone regeneration without significantly impacting bone quality or strength.
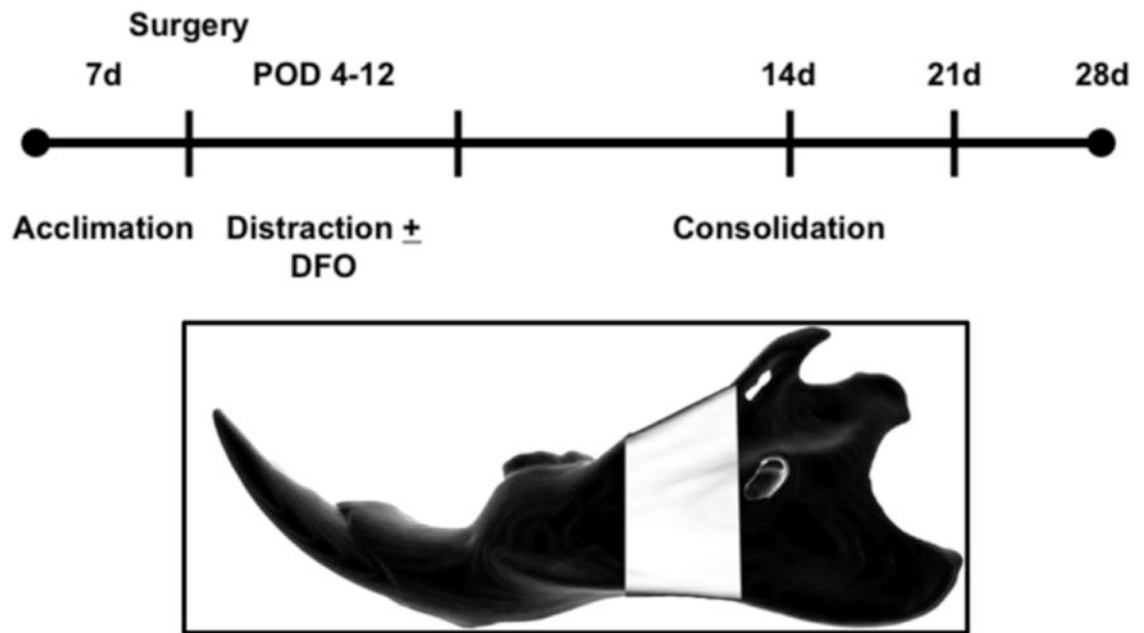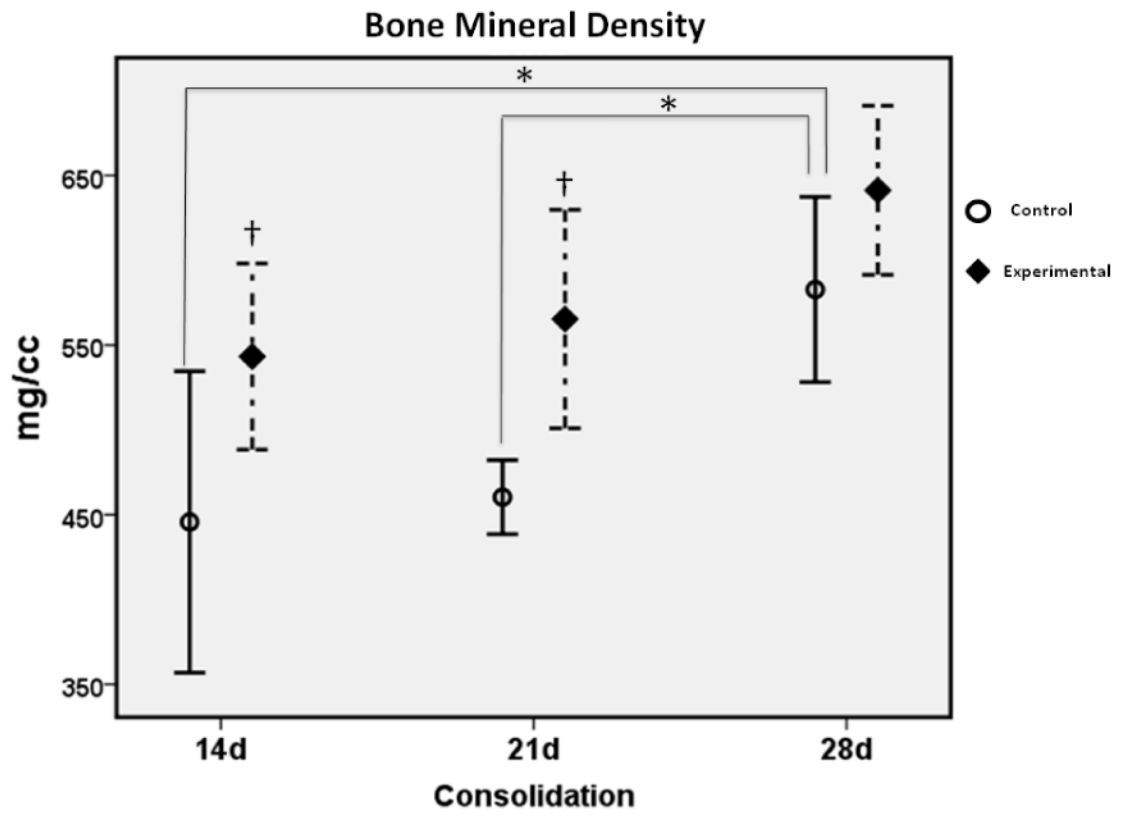
Figure 1.
(Top): Experimental timeline illustrating seven days of acclimation, osteotomy, distraction over an eight day period, and three variable consolidation periods (14, 21 and 28d). Note that deferoxamine is given to animals in the experimental group after surgery on POD 4-12. (Bottom): Schematic left hemi-mandible demonstrating the region of interest highlighted in white.
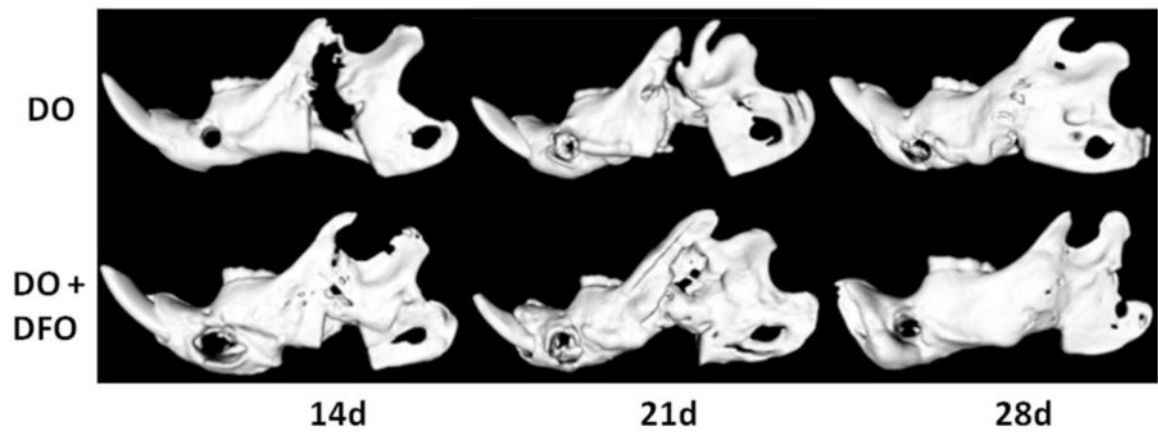
Figure 2.
Select µCT images demonstrating the DO group (top row) and the DO + DFO group (bottom row) at each time point. At the earlier 14 and 21d time-points, note the visible augmentation in regenerate radiodensity in the treatment group indicative of accelerated consolidation with therapy.
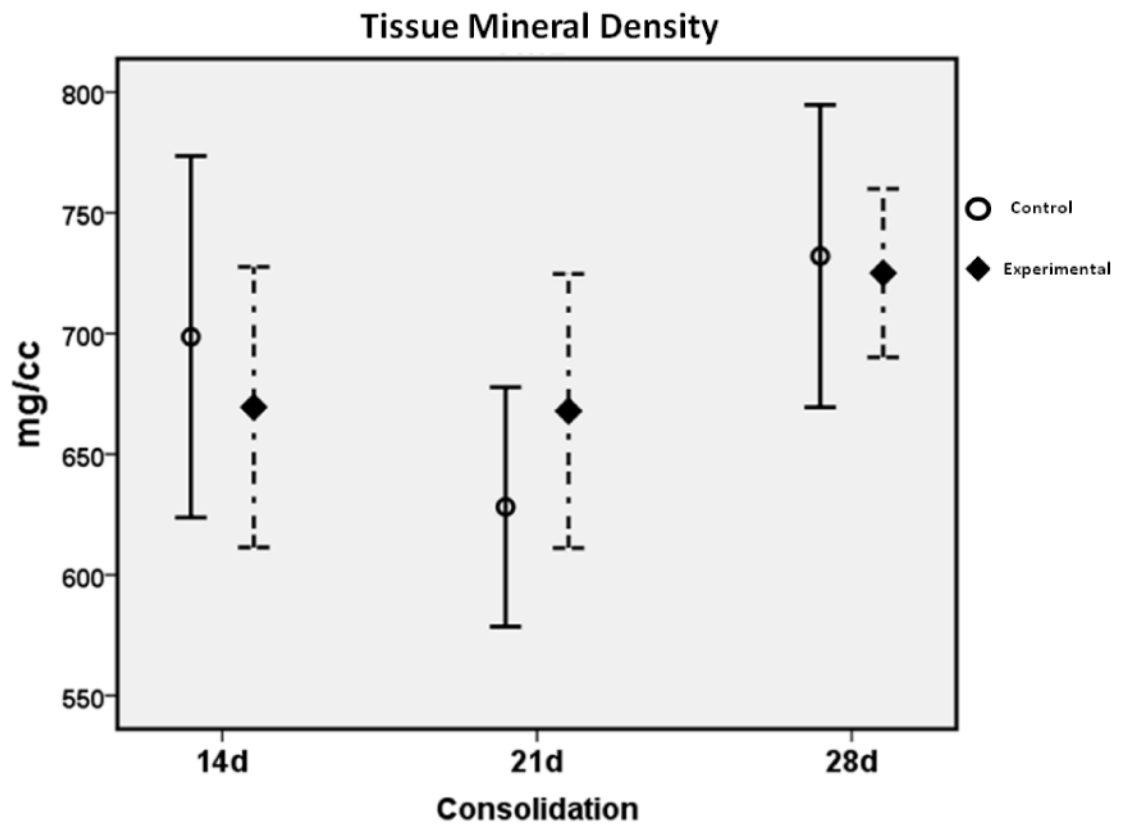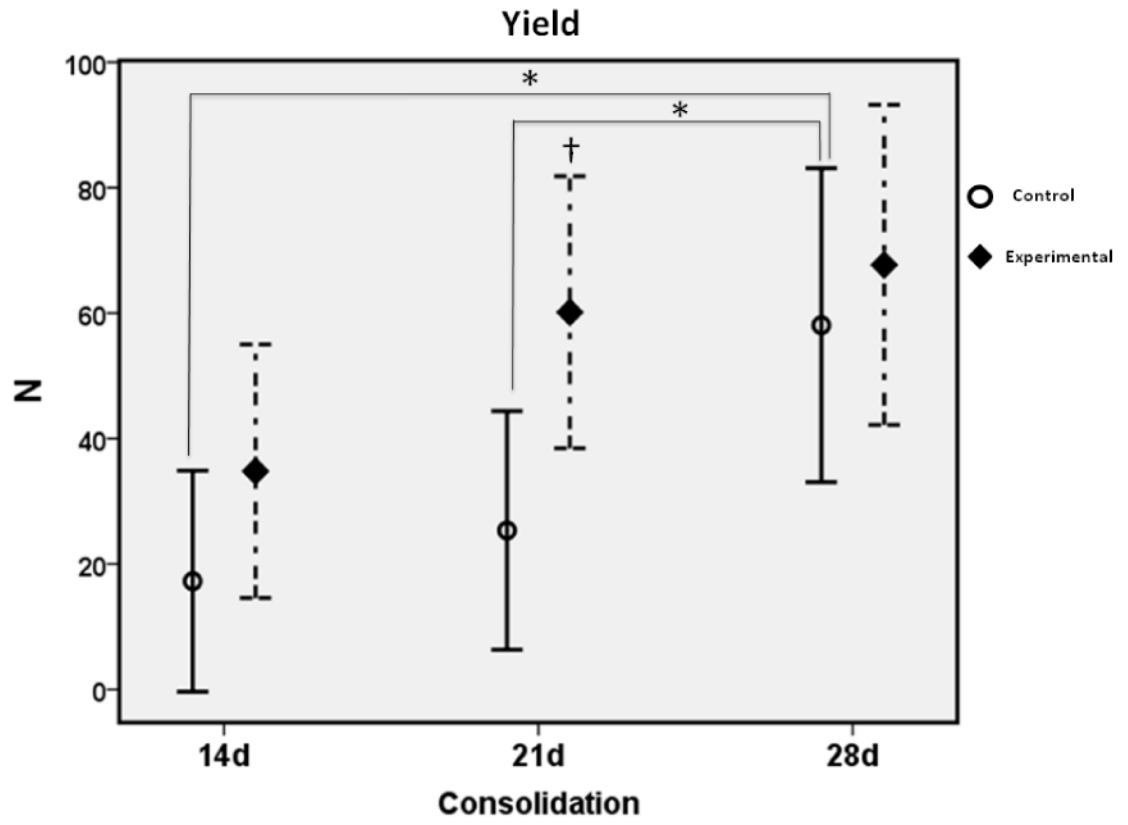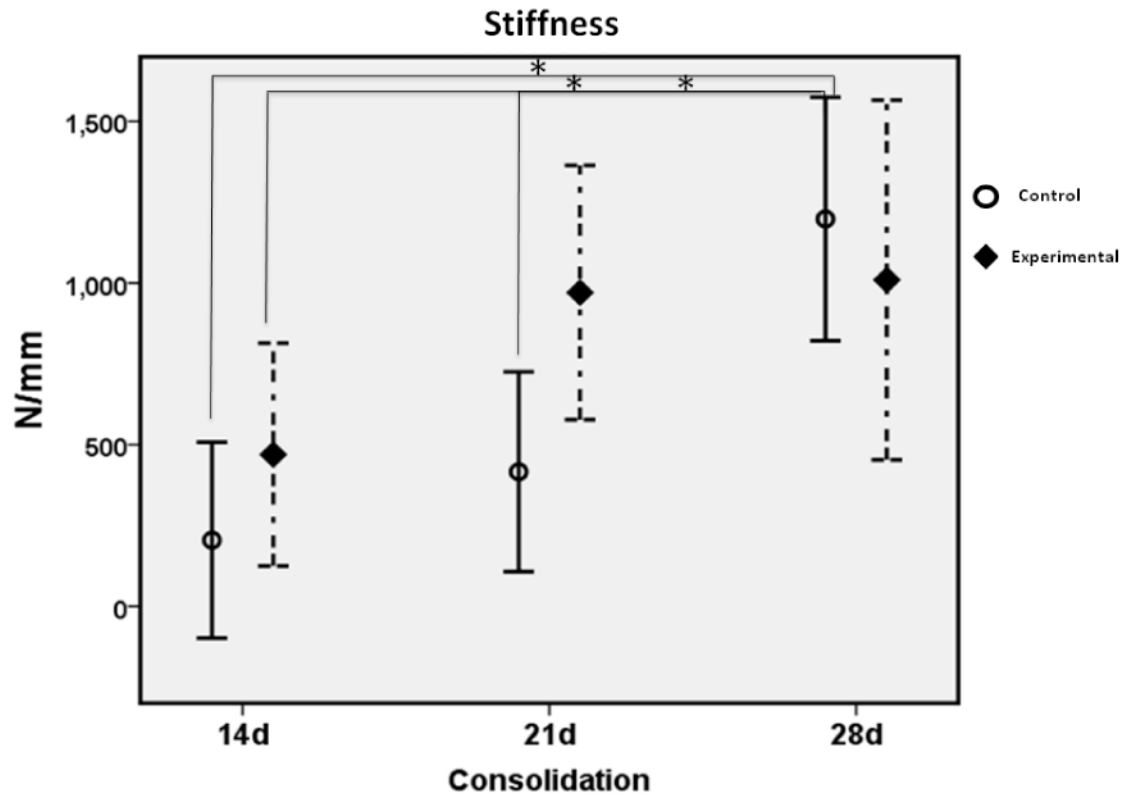
Bone Volume Fraction

3a

Bone Mineral Density

3b

Tissue Mineral Density

3c
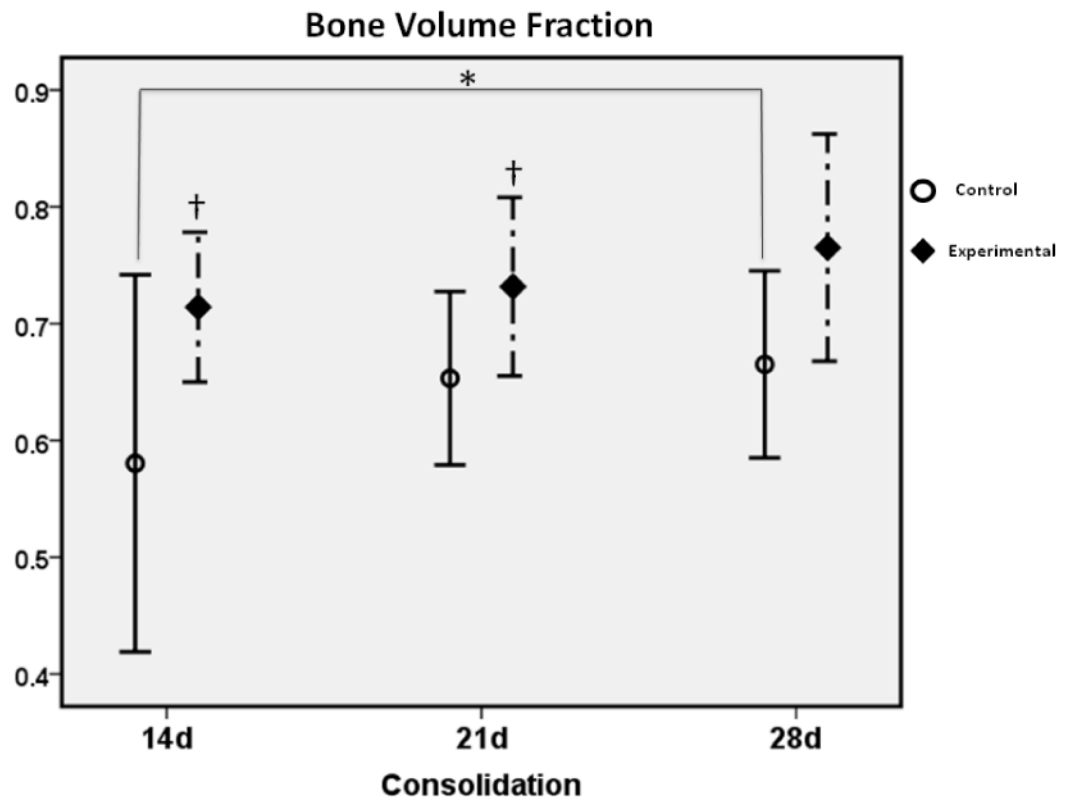
Figure 3a-c.
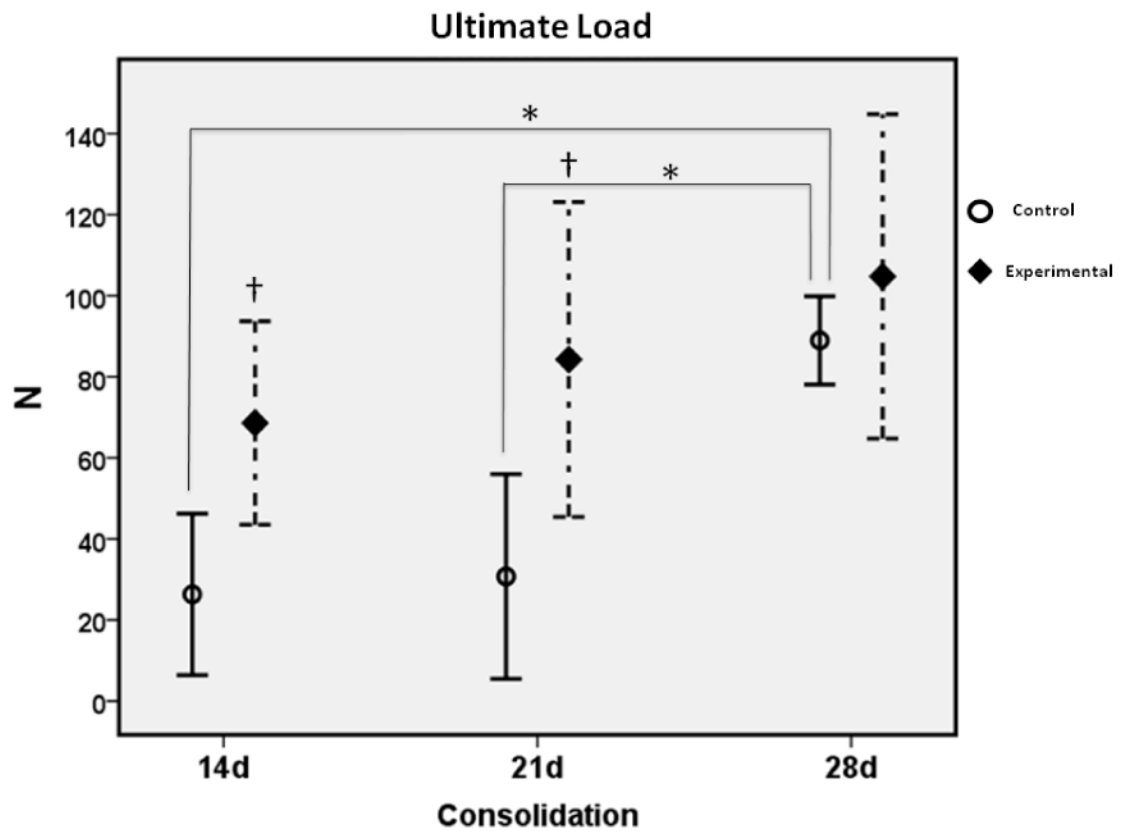μCT graphs depicting the means ± standard deviations for each group. * denotes statistical significance in comparison to 28d C ($p < 0.05$). † denotes statistical significance between E and C groups within a single time-point ($p < 0.05$).

4a

4b

4c

Figure 4.
a-c. Biomechanical graphs depicting the means ± standard deviations for each group. *
denotes statistical significance in comparison to 28d C ($p < 0.05$). † denotes statistical
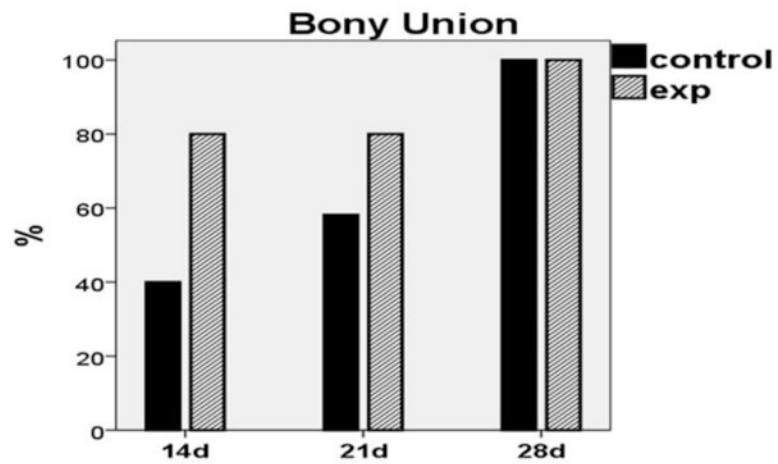significance between E and C groups within a single time-point ($p < 0.05$).

Figure 5.
Graph demonstrating bony union rates within groups. Note the doubling of bony unions in the experimental group at 14d when compared to non-treated controls. At 21d, control union rate increased by 20%; however, experimental union rate remained increased by comparison. By 28 days, both groups exhibited 100% bony union rate.

Figure 6.
Control (L) and Experimental (R) hemimandibles after 14 days of consolidation. Visible gross enhancements in regenerate quality are apparent at this early time-period.

Table 1

μCT radiomorphometric values as a percentage of the standard full consolidation 28d control values.

Radiomorphometric Comparison to 28d C				
%Of 28dC	14d C vs. 28d C	14d E vs. 28d C	21d C vs. 28d C	21d E vs. 28d C
BVF	77%	101%	89%	109%
BMD (mg/cc)	77%	96%	81%	99%
TMD (mg/cc)	95%	92%	87%	93%

Table 2

μCT radiomorphometric values as a percent change from control.

Radiomorphometric Comparison between Groups within a Time-point			
%diff	14d C vs. 14d E	21d C vs. 21d E	28d C vs. 28d E
BVF	+17%	+10%	+11%
BMD (mg/cc)	+25%	+22%	+10%
TMD (mg/cc)	-3%	+7%	-1%

Table 3

Biomechanical testing metric values as a percentage of the standard full consolidation 28d control values.

	Biomechanical Metric Comparison to 28d C			
	14d C vs. 28d C	**14d E vs. 28d C**	**21d C vs. 28d C**	**21d E vs. 28d C**
Y (N)	28%	61%	41%	103%
S (N/mm)	17%	39%	35%	80%
UL (N)	29%	76%	35%	94%

Table 4

Biomechanical testing metric values as a percent change from control.

Biomechanical Metric Comparison between Groups within a Time-point			
	14d C vs. 14d E	**21d C vs. 21d E**	**28d C vs. 28d E**
Y (N)	+118%	+152%	+16%
S (N/mm)	+129%	+129%	-16%
UL (N)	+165%	+171%	+18%