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An Isogenic Model of Murine Mandibular Distraction Osteogenesis

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Introduction

Mandibular reconstruction or regeneration is a routine task for the modern-day craniofacial plastic surgeon. A reconstructive need can arise for a variety of reasons, from congenital—such as hemifacial microsomia and Treacher-Collins syndrome—to acquired, such as that resulting from physical trauma, such as a gunshot wound, or bone loss secondary to oncological resection and segmental mandibulectomy.^{1, 2} Currently, most clinical practice dictates that such deficiencies be remediated utilizing rigid fixation in coordination with bone grafting and free tissue transfer.

However, bone grafts and rigid fixation both have drawbacks that make the techniques suboptimal in a variety of situations. First, rigid fixation can compromise developing dentition, leading to a spectrum of subsequent craniofacial issues.³ Second, fixative hardware may migrate within the soft bone of the immature mandible, mitigating whatever curative effect is imparted by the graft.⁴ Furthermore, relapse and reoperation are significant concerns with rigid fixation, especially in large anterior advancements of the mandible, as the surrounding functional matrix imparts a significant recoil force on the graft.⁴ Finally, bone grafts are associated with donor site morbidity and a lack of vascularity, which can diminish the chance of operative success and may be a true hindrance in elderly patients or those with other significant infirmity.⁵

Distraction Osteogenesis (DO) is the gradual separation of two osteogenic fronts, which stimulates new bone formation. Its use has been documented in medical literature for over a century, beginning with Codivilla in 1905, and has been used to remediate a number of orthopaedic diseases, such as clubfoot, congenital leg length discrepancies, and traumatic or oncological bone loss.^{6,7,8} Despite our increasingly ubiquitous use of the procedure, its clinical use was not initially predicated on scientific evidence. This issue has illuminated the need for development of various animal models in recent years to quantify the biological nature of distraction and further to try and optimize or advance the procedure in the laboratory. Our laboratory has previously developed and published a murine model of

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mandibular distraction osteogenesis where we successfully reproduced bony union with a distraction of 5.1 mm.⁹ Furthermore, we showed that 5.1 mm represented a critical-size defect, as acute lengthening to 5.1 mm invariably resulted in fibrous union. Acute lengthening to distances as short as 3 mm was associated with sporadic fibrous and non-union.

The quest for advancement of bone regeneration and the eminent success of stem cell technology has reached a crossroad where quantifiable enhancements may be gained if our previous models could circumvent the issues of immunity and lack of appropriate donor sites. Our previous model utilized the Sprague-Dawley rat, an outbred strain purchased from Harlan Laboratories. Recently, our laboratory has attempted to integrate stem cell therapies with distraction osteogenesis, such as mesenchymal stem cell administration to the distraction site. For this purpose, the Sprague-Dawley model is insufficient, as we required a means to culture and place stem cells within the distraction gap without an immune response. Autologous stem cell therapies are difficult in a murine model, as the volume of bone marrow that can successfully be harvested from a single rat without major detriment and morbidity is insufficient. Utilizing allograft in an outbred, immune-competent model would almost certainly result in a substantial immune response at the site of healing--a non-ideal outcome. Using xenograft in an immune-compromised model is also an option, however, as infection is a major concern with many surgical procedures. As such, utilizing an athymic / xenograft model carries a significant risk of infection and animal attrition, wasting valuable research time, effort, and resources. Furthermore, it is known that athymic rats have altered inflammatory cytokine production and expression^{10, 11, 12} and that these cytokines, such as FGF-2, TGF- β , TNF- α , INF- γ (to name a few) play central roles in regulating bone healing and osteogenesis. An isogenic strain of rodent that would avoid the problems of immunogenicity and allow for the easy harvest and free transfer of components, would have an intact immune system preventing infection and altered cytokine and healing profiles, and would be large enough to perform this surgical procedure would be an ideal model system for studying craniofacial regeneration and stem cell therapies in concern. We found the isogenic Lewis rats to fit these criteria, however, no previous studies to our knowledge had been performed that would validate the ability of an isogenic model to work and demonstrate equivalence in radiomorphometry and biomechanical response parameters. Therefore, we chose to utilize the isogenic Lewis rat strain to attempt to replicate our previous distraction osteogenesis model in the Sprague-Dawley rat.

It is our global hypothesis that mandibular DO will proceed in a similar manner between Lewis and Sprague-Dawley rats, and posit that the subtle differences between these two species of rats will be of far less consequence than the mechanisms involved in bone regeneration. Our specific aim is to compare radiomorphometric parameters as well as biomechanical response to test this hypothesis and ascertain the viability of Lewis strain rats as a suitable alternative to Sprague Dawley strain rats for the purpose of an isogeneic murine mandibular distraction osteogenesis model.

Once validated this model should serve the surgical scientist well in order to explore the limits of DO, and aid the ability to examine mechanism as well as explore molecular manifestations of tissue engineering strategies.

Materials and Methods

Experimental Groups and Model

A total of 19 male rats were utilized for this study. Rats were assigned to the following experimental groups based on their strain. Group 1, Sprague-Dawley mandibular distraction osteogenesis (n = 10); group 2, Lewis mandibular distraction osteogenesis (n =9). Both

surgical groups underwent 28 days of consolidation postoperatively, excluding 4 days of latency and 8 days of distraction to a 5.1-mm total gap width. Our experimental protocol for distraction osteogenesis and subsequent testing has been published previously,⁹ but will be briefly described below.

Animals

Both male Sprague-Dawley rats, approximately 400 g, and male Lewis rats, approximately 350 g, were housed three per cage in a pathogen-free, restricted-access facility on arrival to our laboratory during a 4-day acclimation before surgery. The differences in their weights are based upon the independent growth charts and reflect animals of approximately the same age. The diet was changed to moist chow 48 hours preoperatively along with Hill's high-calorie diet (Columbus Serum, Columbus, Ohio) and then continued postoperatively in individual cages. All animal procedures were performed according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and approved by the University of Michigan Animal Care and Use Committee.

Surgical Procedure

Preoperatively, animals were given gentamycin (5 mg/kg subcutaneously) prophylactically, and buprenorphine (0.15 mg/kg subcutaneously) along with 25 cc/kg lactated Ringer's solution was administered subcutaneously. Then, the surgical procedure was performed under sterile conditions and general anesthesia using an isoflurane/oxygen mixture. A modified Illazarov External Fixator is affixed to each animal. The design of this fixator has been extensively published previously (Buchman 2002). After appropriate pre-operative surgical preparation, a 2–3 cm midline incision is placed ventrally from the inferior aspect of the chin to the cervical crease. Skin flaps are secured laterally with four silk sutures and hemostats. The anterior-lateral mandible is exposed by the mental region, and using a drill (1.1 mm bit) with irrigation we create a through-and-through defect. A 1.5-inch, no. 0–80, stainless steel, threaded rod was inserted horizontally across the anterior mandible. Both ends are brought externally through the skin, creating the forward portion of our modified external fixator. Then, an incision in the midportion of the masseter muscle, directly over the inferior border of the mandible exposes the angle of the mandible, which is dissected laterally and superiorly towards the sigmoid notch and coronoid process. Drilling from lateral to medial, a small defect is placed for the posterior stainless steel threaded pin, bilaterally. This is secured medially with a titanium threaded washer-nut combo, and laterally with a washer and nut. The ends of the pins are brought externally through the skin for fixator placement.

The fixator caps are placed on the ends of all threaded pins, and the Distraction device is secured. Sterile saline irrigation is maintained during drilling, and after each pin is secured. A vertical osteotomy was created approximately 2 mm anterior to the titanium washer on the left hemimandible using a 10-mm micro-reciprocating blade (Stryker, Portage, Mich.) attached to a power saw (Stryker, Portage, Mich.). The osteotomy extended from the inferior mandible border superiorly to the sigmoid notch along the anterior aspect of the coronoid process. The osteotomy edges were reduced and then re-secured with the fixator. The wound was irrigated, hemostasis was verified, tissues were approximated using 4–0 Vicryl suture, and the midline incision was closed with staples. The distractor device design and orientation has been previously explained at length.⁹

Postoperative Animal Care

Buprenorphine was continued (0.15 to 0.5 mg/kg) with 5 cc of lactated Ringer's solution subcutaneously every 12 hours through postoperative day 7 and as needed thereafter. Animals were fed moist chow/Hill's high-calorie diet and water ad libitum. Pin sites were

cleaned daily, maxillary incisors were clipped weekly because of overgrowth from crossbite, and staples were removed by postoperative day 14. Weights were monitored daily and diets adjusted as needed.

Distraction Protocol

Mandibular distraction osteogenesis was performed 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. A total of 17 half-turns were performed on an every-12-hour schedule, resulting in a maximum 5.1-mm distraction gap over 8 days. No analgesic or sedation was required during the distraction.

Micro-Computed Tomography

Mandibles were harvested and then scanned at 45- μ m resolution with micro-computed tomography. Analysis was then performed with Microview 2.2 (GE Healthcare, Milwaukee, Wis.) within the left posterior hemimandible. Microview assigns each voxel a grayscale value; to calculate densities, it uses an algorithm that converts the grayscale value of each voxel to mineral content.

Contours were created on every tenth serial coronal section within the regenerate gap. Only bone within each section was selected. In cases where a part of the incisor or canal was present; the tooth, the canal and the periodontal tissue were excluded for uniformity. The region of interest was set with its anterior frontier at the section where the root of the third molar was no longer visible. This region was then extended for 113 coronal sections, corresponding to a 5.1 mm region of interest—the length of the distraction gap.

Bone volume fraction (BVF) was defined as the volume of newly remodeled bone divided by the total volume of the gap, which included air and soft tissue. Bone mineral density (BMD) was defined as the mineral content of bone (bone mineral content, BMC) divided by the total volume (TV) of the gap. Tissue mineral density (TMD) was defined as the mineral content of the tissue (tissue mineral content, TMC) that was greater than 1250 HU, the cutoff we have previously defined as the lower bound of bone. BV was defined as the volume which fell above the bone threshold of 1250 HU divided by the total volume. Microdensitometric analysis of bone mineral density, our microdensitometric measure, was defined as the mineral content of newly remodeled bone divided by the volume of newly remodeled bone alone (not including air and soft tissue). Microdensitometric analysis of bone mineral density represents the average density of all bone voxels.

Mechanical Testing

Animals that underwent mandibular distraction osteogenesis were humanely sacrificed on postoperative day 40. The mandibles were harvested en bloc, split between the incisors, and frozen at -20°C until testing. The potting medium was a bismuth alloy (Cerrobend; Cerro Products, Bellefont, Pa.). The posterior aspect of the mandible was potted first, to a depth that consistently exposed only the regenerate callus, followed by the anterior aspect being potted. These were placed as close to the healed regenerate as possible, without crossing into the gap, to ensure that mechanical forces were limited to the regenerate and not other regions of the mandible.

Potted mandibles were tension tested to failure at 0.5 mm/second in the anteroposterior direction using a servohydraulic testing machine (858 Mini Bionix II; MTS Systems, Eden Prairie, Minn.). Load head displacement was monitored using an external linear variable differential transducer (Lucas Schavitts, Hampton, Va.), and load data were collected with a 10- and 50-lb load cell (Sensotec, Columbus, Ohio). Load and displacement data were

acquired using the TestStar IIs system (version 2.4; MTS Systems) at a sampling frequency of 2000 Hz. Results were expressed in a load-displacement curve, and numeric data were transferred to Microsoft Excel (Microsoft Corp., Redmond, Wash.) for further analysis to determine breaking load and yield. Three independent reviewers calculated both parameters.

Union Assessment

Dissected mandibles were gently flexed by hand to assess union. If any bony resistance was observed, a union was noted. If the mandible flexed freely, it was graded as a nonunion.

Statistical Analysis

A two-tailed independent samples Student's T-test was used for analyzing all metric of mandibular distraction osteogenesis quality between the Sprague-Dawley and Lewis groups (SPSS version 19.0; SPSS, Inc., Chicago, Ill.). Results were accepted as statistically significant at a value of $p < 0.05$.

Results

Following mandibular distraction osteogenesis in the rat mandible in outbred Sprague-Dawley rats and inbred Lewis rats we noticed some qualitative differences between the two rat types. Lewis rats tended to be lighter at delivery (12 weeks of age)—whereas the average weight of Sprague-Dawley rats were 400 g at 12 weeks, Lewis rats weighed only 350 g at the same time point. Sprague-Dawley rats typically rebounded to their preoperative weight within two weeks of surgery, whereas Lewis rats rarely recovered to their preoperative weight at post-operative day 40, the conclusion of the experiment. Lewis rats were also much more responsive to anesthesia—using a combination of isoflurane and oxygen, 1.5% isoflurane / 1 L/min oxygen was ample enough to achieve sufficiently deep anesthesia for surgery. However, Sprague-Dawley rats required a higher dose of 2.5% isoflurane / 1 L/min oxygen for sufficiently deep anesthesia for surgery. As a corollary, in our pilot studies, we discovered that Lewis rats were more prone to overdose due to anesthesia, and prolonged exposure to the Sprague-Dawley anesthesia dose in the Lewis rat is fatal. However, the increased apparent frailty of the Lewis rat did not translate to a higher infection rate.

Despite these gross, qualitative differences between the two rat strains, there was an equivalent 100% union rate between the two groups. Furthermore, there were not statistically significant differences between volume-normalized radiomorphometric parameters (BMD and TMD). However, there were trending differences in some non-volume-normalized radiomorphometric parameters (TV and BV) and significant differences in biomechanical response parameters between Sprague-Dawley and Lewis rats (yield and failure load) (Table 1.). When comparing the means for the yield value of mechanical testing the p-value was 0.002 and for failure load the p value was 0.000, indicating significant differences (Figure 3). The TV ($p = 0.097$), BV ($p = 0.097$), BMC ($p = 0.170$), BMD ($p = 0.948$), TMC ($p = 0.310$), TMD ($p = 0.160$), and BVF ($p = 0.265$) showed no statistically significant differences (Figure 3).

Discussion

Rat models, in general, have several advantages to other models of distraction osteogenesis. As has been seen previously, large animal models, such as canine models, are often prohibitively expensive and are difficult to use for mechanistic studies, as few molecular markers have been developed for these types of experiments. However, such markers are readily available for rats. The reduced cost and animal care burden of these animals allows for larger experimental groups. Our bilateral external frame fixator system with unilateral

distraction allows for the use of the same fixation system for both distraction and rigid fixation experiments, allowing for easy comparison between the two and eliminating the potentially confounding factor of variable fixation methods.

In 2002, our laboratory published the first model of murine distraction osteogenesis that utilized a bilateral external fixator with unilateral distraction device, which produced superior results when compared to jackscrew/methyl methacrylate designs.⁹ Prior to this, mandibular distraction osteogenesis was not typically attempted in small animal models (such as the rat) as the diminished size made this technically impractical, given established methodology. Here, we demonstrate success in murine mandibular distraction osteogenesis in an even smaller and less robust rat strain that has been previously reported.

However, the primary advantage of this particular model is the isogeneic nature of the rat. This allows for the free transfer of components from one rat to another without fear of immunologic response while simultaneously protecting the inflammatory pathways necessary to mediating healing. This includes, but is not limited to, stem cells, bone grafts, and muscle constructs, and is essential for future experimentation, as biomedical science increasingly focuses attention on cellular therapies. Furthermore, it allows for the common preparation and culture of components, greatly reducing the burden to the researcher.

We have found the Lewis rat strain to be an optimal, isogeneic replica of our Sprague-Dawley model. A major consideration for the selection of Lewis rats was body weight. Lewis rats had the steepest growth curve of all the isogenic strains available from our supplier. We have found this factor significant for the health and survival of animals who receive surgical treatment. Subsequent to the distraction osteogenesis procedure, rats typically experience significant swelling and edema. As such, the rats' ability to eat is temporarily compromised. Therefore, it was essential that the rats we selected for utilization for the study possessed sufficient fat reserves to prevent illness or death. However, while Lewis rats were the largest available strain, they are smaller than Sprague-Dawley rats, and as such, additional care must be taken.

We did not notice a significant difference in infection rates between the two groups. However, while undergoing the DO procedure, Lewis rats were considerably more sensitive to anesthesia. Furthermore, morphologically, the Lewis mandibles were slightly thinner and smaller. This difference was noticed most prominently with a narrower inferior ramus of the mandible. This made incidental fracture of the mandible during posterior pin installation a greater concern than in Sprague-Dawley rats. Finally, Lewis rats tended to take longer to recover to their post-operative weight when compared to Sprague-Dawley rats.

We observed significant differences in our biomechanical response parameters, and trending differences in the non-volume normalized radiomorphometric parameters. As volume-normalized parameters (such as BMD and TMD), which control for the thickness of the mandible, were not significantly different, we can attribute differences in yield and failure load to the decreased size of the Lewis rat mandible. This was both observed grossly and implied by the trending differences in TV and BV, as our ROI's length was defined as the 5.1mm section posterior to the third molar, but the width and height was dependent on the contours of the morphology of the mandible.

Given these results, we propose that Lewis rats an acceptable isogenic substitution for Sprague-Dawley rats for the purpose of mandibular distraction osteogenesis, so long as additional care is taken to compensate for these rats' additional needs. The altered size and biomechanical response results between Sprague Dawley rats and Lewis rats indicate that data produced in one model may not be directly comparable to the other. In our study we found internal consistency within each rat strain as well as comparability in volume-

normalized metric. As would be expected, however, those data that depend on size and weight would vary accordingly. With these conditions in mind, we find that where experimental burden can be lessened by utilizing an isogenic model, Lewis rats demonstrate success and validity as a model for DO and provide an excellent substitute for Sprague-Dawley rats.

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Figure 1.
Left: Modified Ilizarov Distraction Device. Right: Rat with device attached, showing characteristic “cross-bite” following distraction.

Figure 2.
Gross photo and Faxitron of distracted rat mandible, with the approximate region of interest (ROI) highlighted in red

Figure 3. Radiomorphometric and Biomechanical Response parameters for Sprague-Dawley and Lewis Rats following mandibular distraction osteogenesis. * indicates significance at $p < 0.05$. Only biomechanical response parameters were significant.

Table 1

Radiomorphometric and Biomechanical Response parameters for Sprague-Dawley and Lewis Rats following mandibular distraction osteogenesis.

	<u>Sprague Dawley</u>	<u>Lewis</u>
Total volume (mm ³)	97.6 ± 22.0	81.7 ± 14.3
Bone Volume (mm ³)	97.6 ± 22.0	81.7 ± 14.3
BMC (mg)	53.6 ± 11.7	45.6 ± 11.8
BMD (mg/cc)	554.0 ± 64.8	552.9 ± 71.9
TMC (mg)	46.0 ± 10.9	40.3 ± 12.4
TMD (mg/cc)	710.5 ± 61.9	673.4 ± 40.0
BVF (unitless)	0.665 ± .080	0.716 ± 0.107
Yield (N)	162.8 ± 26.3	92.9 ± 23.6*
Failure Load (N)	107.6 ± 18.5	71.0 ± 21.8*