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Significant Differences in the Bone of an Isogenic Inbred vs. Nonisogenic Outbred Murine Mandible: A Study in Rigor and Reproducibility

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Abstract

Inattention to differences between animal strains is a potential cause of irreproducibility of basic science investigations. Accordingly, our laboratory sought to ensure that cross-comparisons of results generated from studies of mandibular physiology utilizing the Sprague Dawley and Lewis rat strains are valid. We specifically investigated baseline histomorphometrics, bone mineral density, and biomechanical strength of the unaltered endogenous mandibles of the inbred, isogenic Lewis rat and the outbred, nonisogenic Sprague Dawley rat to determine if they are indeed equal. We hypothesized that little difference would be found within these metrics.

Our study utilized 20 male Lewis and Sprague Dawley rats, which underwent no manipulation other than final dissection and analysis. 10 rats from each strain underwent bone mineral density and biomechanical strength analysis. The remaining rats underwent histological analysis. Descriptive and bivariate statistics were computed and the p-value was set at 0.05.

Lewis rats had a significantly greater number of empty lacunae. Sprague Dawley rats exhibited a significantly greater ratio of bone volume-to-total volume, bone mineral density, tissue mineral density, bone volume fraction, and total mineral content. No differences were found during biomechanical testing.

This study demonstrates that differences exist between the Lewis and Sprague Dawley rat within unaltered baseline mandibular tissue. However, these differences appear to have limited functional

impact, as demonstrated by similar biomechanical strength metrics. Other specific differences not addressed in this manuscript may exist. However, we believe that researchers may confidently cross-compare results between the two strains, while taking into account the differences found within this study.

Introduction

The belief that the scientific process is “self-correcting” has for far too long been taken for granted. Concerns regarding the validity and reproducibility of preclinical studies have reached a fevered pitch, with suspicions intoned by all major voices of the scientific community, from the lay press to the National Institutes of Health (NIH) itself.^{1,2} One investigation by Bayer Pharmaceuticals found inconsistencies between published data and the results from in-house validation studies in almost two-thirds of the preclinical drug discovery studies that they analyzed.³ Unbeknownst to many scientific investigators are the impactful variations between animal models that may well play a role in both the soundness and dependability of their research.

While the number of redacted papers has risen steadily in recent years, the aforementioned discrepancies are unlikely to be due to widespread scientific misconduct. Less than one thousand English language scientific papers were redacted from 2000-2010.⁴ This figure is a miniscule fraction of the millions of manuscripts published during that time. Rather, the lack of reproducibility appears to be, at least in part, due to poor experimental design and an absence of meticulousness with regard to the very scientific principles that have led to the success of methodical research over the last few centuries. Work done within animal models is said to be among the areas most susceptible to these basic failures. Among other explanations, the NIH specifically cited “[the use of] different animal strains” as a source of inconsistency.²

In response, the NIH has revised its requirements for both career development awards and grant applications in order to improve “rigor and transparency.” These changes include the requirement of an explanation of how biological variables, such as the specific animal strain, may influence the results of the proposed research.⁵ Furthermore, some of the leading scientific journals have altered their review process in order to assure future reproducibility. For example, Nature Publishing Group recently eliminated their restrictions on the length of the methods sections in order to inspire full methodologic transparency. With this new emphasis in mind, and the call by scientific journals and the NIH for an expanded inquiry and closer scrutiny into key differences in both breed and strain of experimental animals, this investigation was specifically aimed at validating comparisons made between studies using murine models from two different rats: The Sprague Dawley and Lewis strains.

Our laboratory has worked extensively with both of these strains. Much of the research within our laboratory has been dedicated to the investigation of mandibular distraction osteogenesis (MDO) and fracture healing within an irradiated murine mandible.⁶⁻⁸ Throughout these investigations, our approach to augment bone regeneration and healing has generally been pharmacologic, making use of the anabolic effects of deferoxamine and parathyroid hormone, as well as the radioprotective effects of amifostine.⁹⁻¹⁴ For these

studies, the use of the outbred, non-isogenic Sprague Dawley rat strain was ideal due to its large size and relative convenience for housing.

While our investigations of the pharmacologic augmentation of MDO and fracture healing have been successful, we have recently incorporated stem cell-based treatments to improve MDO and post-fracture mandibular healing in irradiated animals. Given the infeasibility of an autologous stem cell transplant within small animals, these studies require isogenicity, immunosuppression, or an athymic strain in order to avoid the immunologic rejection of the stem cell allograft. Because the use of immunosuppression or athymic strains would stifle the inflammatory pathways necessary for bone healing, isogenic strains are the best option for our stem cell-based studies.^{15–17} Accordingly, our laboratory has chosen to utilize the inbred isogenic Lewis strain for our investigations of stem cell therapy within both MDO in radiated animals and fracture healing following radiation. These studies have been successful, illustrating the potential of stem cell-based therapies to augment both MDO and fracture healing.^{18–21}

The efficacy of our stem cell-based therapies in the Lewis rat has been logically benchmarked against our pharmacologic interventions undertaken in the Sprague Dawley rat. However, given the fact that the Lewis and Sprague Dawley rats are indeed different strains with previously characterized differences, we feel that we must follow up on these prior studies and ensure that the control animals used in studies utilizing Lewis and Sprague Dawley strains are intercomparable.^{22,23} The objective of this current study is to apply the goals of the NIH to our own research and validate the assertion that the two rat strains used in our investigations of mandibular physiology have similar baseline cellularity, strength, and bone mineral density. We believe that this will allow laboratories to confidently cross-compare results in those specific metrics from studies utilizing Lewis mandibles to those utilizing Sprague Dawley mandibles.

We posit that baseline mandibular bone quality will not differ drastically between the inbred isogenic Lewis and the outbred nonisogenic Sprague Dawley strains. However, we further postulate that subtle differences may exist between the two models. We do not anticipate these differences to drastically affect radiomorphometry, histomorphometry, or biomechanical metrics. We believe that this study will provide additional evidence that the Lewis rat is an appropriate substitute for the Sprague Dawley rat for studies needing an isogenic model. More importantly, it is our hope that the rigor of this investigation will allow us to detect any differences between the two strains, allowing for valid intercomparison and reproducibility of future investigative studies using these two strains.

Methods

Experimental Groups

A total of 40 male rats were utilized for this study. These rats were divided into two groups: Group 1 was comprised of inbred isogenic Lewis rats (n=20) and Group 2 was comprised of outbred nonisogenic Sprague Dawley rats (n=20). Both rat strains underwent the same experimental procedure and analysis. Upon arrival to our facility, rats were housed and cared for over the course of 47 days.

Animal

Twelve-week-old male Lewis (325 g) and Sprague Dawley (400 g) rats were obtained through the University of Michigan's Unit for Laboratory Animal Medicine (ULAM) in compliance with their subdivision of the University of Michigan's Committee for the Utilization and Care of Animals (UCUCA). All protocols were approved by UCUCA prior to implementation. Rats were weighed and provided water bottles and regular chow *ad libitum* upon arrival to the laboratory.

Tissue Harvest

All animals were sacrificed after acclimation for 40 days via an isoflurane overdose followed by thoracotomy. Mandibles were dissected immediately following euthanasia.

Histology

Half of the mandibular samples underwent histological analysis. Hemimandibles were fixed in 10% neutral buffered formalin for 72 hours and then transferred to 70% ethanol at 4°C. The specimens were cleared of all soft tissue and the teeth were ground, leaving the roots as landmarks, and transferred to a formic acid/sodium citrate decalcification solution (equal parts 40% formic acid and 6.25% sodium citrate), changed every 2 to 3 days, and stored at 4°C. Specimens were vacuum processed (dehydration and paraffin infiltration) under a 48-hour program in the Hypercenter XP tissue processor (Shandon Hypercenter XP, Pittsburgh, PA, USA), and then infiltrated once more for 2 hours in a vacuum bath, followed by embedding using paraffin containing dimethylsulfoxide. Peel-away embedding molds (22 × 40 mm) were used and refrigerated overnight (4°C). Blocks were sectioned from anterior-to-posterior into 7 µm coronal sections and mounted on glass slides. Six to eight sections per slide, chosen approximately 50 to 100 µm apart from each other, from a total of 70 to 100 sections per block, were stained. The prepared slides were deparaffinized in xylene and then dehydrated through graded alcohol. Specimens were then stained with Gomori's "one-step" trichrome and mounted. The Gomori's trichrome stain identifies bone in turquoise and osteoid/nonmineralized matrix in red, providing for easy digital color analysis. Using a computer linked with Nikon scanning software (Nikon, Tokyo, Japan), the slides were scanned, cropped, and saved as TIFF files. Using Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA, USA), the images were oriented with the buccal hemimandible on the right, cropped to 1596 × 1287 pixels, and then adjusted to final dimensions of 640 × 480 pixels. The file was loaded into the imaging analysis software program (Bioquant NOVA Osteo version 7; R&M Biometrics, Nashville, TN, USA) for region of interest (ROI) placement and color thresholding. Using a coronal microcomputed tomographic scan from a prior study for orientation, the appropriate ROI template was loaded onto the TIFF file. Color thresholding was performed as follows: bone volume was obtained by manually selecting a few areas of turquoise bone within the ROI, which the Bioquant program thresholded, and then osteoid volume was similarly obtained by selecting the red-stained regions within the ROI. Bioquant measured the ROI area and then calculated the bone volume/tissue volume (BV/TV) and osteoid volume/tissue volume (OV/TV) ratios. In addition, osteocytes were "point counted" as follows: ROI templates were superimposed onto the digital light microscopy image and then rotated so the bisected base was

landmarked as (0, 0) on an x and y grid and the apex coordinate as (0, 9000). We divided the ROIs into superior, middle, and inferior sub-ROIs, and then randomly selected three high-power fields per each sub-ROI using 16× magnification, for a total of nine high-power fields per ROI. The high-power fields, measuring $295 \times 366 \mu\text{m}$ were stored as TIFF files and printed in color, and the osteocytes were counted inside each high-power field. All analysis was performed by at least three individual reviewers.

Radiomorphometrics

The remaining half of the samples that did not undergo histological analysis underwent Micro-CT (μCT) and biomechanical tension testing analysis. μCT for bone morphology was performed using an eXplore Locus SP μCT machine (GE Healthcare; Fairfield, CT, USA) with the images reconstructed at $45 \mu\text{m}$ voxel size. Our ROI consisted of the region that extended from the third molar to 5.1 mm posterior to that point. Analysis of our ROI was then performed using MicroView 2.2 software. Using the spline tool in MicroView, only bone within our ROI was included for analysis. Rats were analyzed for radiometric bone quality. The specific metrics generated for bone analysis are bone mineral density (BMD), tissue mineral density (TMD), bone volume fraction (BVF), bone mineral content (BMC), and tissue mineral content (TMC).

Biomechanical Tension Testing

Following μCT , rats underwent biomechanical testing. The mandibles were harvested en-bloc, split between the incisors, and frozen at -20°C until testing. The posterior aspect of the mandible was placed into an open cylindrical shaped “pot” using a heated bismuth alloy (Cerrobend, Cerro Products, Bellefont, PA, USA) that, once cooled, secured the mandible in place. The potted mandibles were loaded to failure in uniaxial monotonic tension at 0.5 mm/s using a servohydraulic testing machine (858 Minibiox II; MTS Systems Corporation, Eden Prairie, MN, USA). Crosshead displacement was recorded by using an external variable differential transducer (LVDT; Lucas Schavitts, Hampton, VA, USA), and load data were collected with a 100-lb load cell (Sensotec, Columbus, OH, USA). Data were sampled at 200 Hz on a TestStar system (TestStar IIs System version 2.4; MTS Systems Corporation, Eden Prairie, MN, USA). Load-displacement curves were analyzed for whole bone ultimate load (UL), intrinsic stiffness (S), yield load (Y), failure load (FL), and elastic energy using custom computational code (MATLAB 7.11; Mathworks Inc., Natick, MA, USA).

Statistical Analysis

All metrics were analyzed using SPSS for Windows, version 23.0 (SPSS, Inc., Chicago, IL, USA). The two groups were compared via 2-tailed Student's t-test for statistical analysis.

Results

Histology

No difference was seen between Lewis and Sprague Dawley rats in osteocyte count (Fig. 1a). Lewis rats exhibited a mean osteocyte count of 90.63 ± 8.10 per high power field, while Sprague Dawley rats showed a mean osteocyte count of 94.66 ± 5.32 per high power field ($p=0.181$). Lewis rats had significantly more empty lacunae, however, compared to Sprague

Dawley rats (5.74 ± 1.52 vs. 1.89 ± 0.68 ; $p < 0.01$; Fig. 1b). No difference was seen between Lewis and Sprague Dawley strains within mean OV/TV (0.21 ± 0.09 vs. 0.24 ± 0.07 ; $p = 0.474$; Fig. 1c). Sprague Dawley rats exhibited a greater BV/TV, compared to Lewis rats (0.69 ± 0.11 vs. 0.44 ± 0.09 ; $p < 0.01$; Fig. 1d).

Micro-CT

Significant differences between the two rat strains were seen within all radiomorphometric parameters, aside from BMC. Lewis rats exhibited a mean BMD of 631.01 ± 30.97 mg/cc, compared to 757.98 ± 34.20 mg/cc seen within Sprague Dawley rats (Fig. 2a; $p < 0.01$). Lewis rats displayed a mean TMD of 785.48 ± 17.81 mg/cc, compared to Sprague Dawley rats which displayed a mean TMD of 822.27 ± 25.76 mg/cc (Fig. 2b; $p < 0.01$). Lewis rats demonstrated a mean BVF of 0.72 ± 0.04 cc/cc in comparison to a mean of 0.89 ± 0.03 cc/cc seen in the Sprague Dawley group (Fig. 2c; $p < 0.01$). No difference was seen between Lewis and Sprague Dawley within BMC (Fig. 2d; 40.97 ± 2.05 mg vs. 42.61 ± 2.68 mg; $p = 0.16$). Finally, a mean TMC value of 36.60 ± 1.76 mg was exhibited by Lewis rats, compared to a mean of 40.98 ± 2.59 mg shown in the Sprague Dawley group (Fig. 2e; $p < 0.01$).

Biomechanical Testing

There were no statistically significant differences seen in any of the biomechanical strength parameters. Lewis rats exhibited a mean UL of 154.62 ± 30.53 N, compared to Sprague Dawley rats, which exhibited a mean UL of 163.23 ± 40.89 N (Fig. 3a; $p = .698$). Lewis rats showed a mean FL of 150.58 ± 32.59 N, while Sprague Dawley rats demonstrated a mean FL of 161.42 ± 39.80 N (Fig. 3b; $p = 0.631$). Lewis rats revealed a mean Y of 109.58 ± 48.57 N, compared to 154.60 ± 43.81 N within the Sprague Dawley group (Fig. 3c; $p = 0.144$). Lewis rats exhibited a mean S of 935.10 ± 711.83 N/mm, compared to 1096.54 ± 460.30 N/mm seen in Sprague Dawley rats (Fig. 3d; $p = 0.674$). Lastly, Lewis rats showed a mean elastic energy of 14.41 ± 12.15 J, compared 12.20 ± 4.59 J seen within Sprague Dawley rats (Fig. 3e; $p = 0.695$).

Discussion

Over the course of the past decade, tremendous advancements have been made within the area of stem cell therapy research, and the number of clinical trials utilizing these therapies has risen steeply. As such, current animal models must be updated to accommodate the investigation of stem cell-based therapies. These new animal strains should be cross-checked against previous strains if there is any intention of a longitudinal comparison of former and current results. This extra step will ultimately allow researchers to confidently cross-compare results from these two types of studies, allowing for greater legitimacy and duplicability.

In our study, several differences between the two strains were noted when the animals arrived at our facility. Despite being large in comparison to other isogenic animals, the Lewis rats were substantially smaller than the Sprague Dawley rat. Additionally, gross morphological differences were noticed upon dissection of the Lewis mandibles. Specifically, the Lewis mandibles were slightly smaller along the posterior ramus of the

mandible. As such, researchers must take particular caution during any manipulation of the smaller Lewis mandible.

Additional differences between the Sprague Dawley and Lewis strains were found within radiomorphometric measurements. The greater BMD, TMD, BVF and TMC seen within in the Sprague Dawley strain suggest that, at baseline, Sprague Dawley rats have greater density within the mandible. BMD and TMD both control for the area of the region of interest so an absolute difference in the size of the mandibles is unlikely to be an explanatory factor. Corroborating evidence was seen within histological analysis, as Sprague Dawley rats exhibited fewer EL, along with greater BV/TV.

Despite the fact that the outbred Sprague Dawley rats had denser, more cellular mandibles, with a greater percentage of bone within a given volume, biomechanical measurements suggest that there may be little or no functional difference between the Lewis and the Sprague Dawley mandibles. All biomechanical metrics were found to be indistinguishable between the two strains, which may indicate that decreased absolute cellularity and density may not automatically yield a weaker mandible. This is an important consideration for researchers who have the ultimate goal of producing a functionally capable and strong mandible utilizing stem cell therapies.

These results differ slightly from a single study, which compared radiomorphometric and biomechanical strength metrics between Sprague Dawley and Lewis rats in a murine model of mandibular distraction osteogenesis (MDO).²² In that study, Sprague Dawley rats had slightly stronger mandibles following MDO, but did not differ substantially within radiomorphometrics. That study attributed any differences in strength to a variation in the absolute size of the mandible between the two strains. To the contrary, the results of this present study suggest that absolute size and ultimate strength appear to be at least partially decoupled.

While the rate of advancement in both pharmaceutical and stem cell therapies in recent years has been astounding, ushering in a new era of clinical research, careful consideration must be given to the legitimacy of experimental procedures in order to facilitate a genuine progress. Details that may reduce the validity and reproducibility of experimental results cannot be overlooked. Indeed, this study found surprising differences that, while relatively minute, could influence interpretation of results if gone unnoticed. Accordingly, just as our investigation sought to assess the suitability of the Lewis rat as an appropriate substitute for the Sprague Dawley strain, and therefore confirm the legitimacy of inter-strain comparisons, it is our hope that this practice of meticulous and comprehensive review of experimental procedure becomes widespread, to allow for scientific research to move forward with an authenticity as admired as its rapidity.

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Figure 1.

(a)Graph indicating mean osteocyte count (OC). Lewis and Sprague Dawley rats showed no differences in OC/HPF ($p=0.181$). (b)Graph indicating mean empty lacunae (EL). There was a significantly greater number of empty lacunae within the Lewis group ($p<0.01$). (c)Graph indicating mean osteoid volume divided by total tissue volume (OV/TV). No difference was seen between the two groups ($p=0.474$). (d)Graph indicating mean bone volume divided by total tissue volume (BV/TV). Sprague Dawley rats had a significantly greater ratio of BV/TV, compared to the Lewis group ($p<0.01$).

Figure 2.

(a) Graph illustrating a comparison of mean bone mineral density (BMD). Sprague Dawley mandibles had a significantly greater BMD, compared to the Lewis group ($p < 0.01$). (b) Graph illustrating mean tissue mineral density (TMD). Sprague Dawley rats had a significantly greater mean TMD, compared to the Lewis group ($p < 0.01$). (c) Graph representing mean bone volume fraction (BVF). Sprague Dawley mandibles exhibited a significantly greater BVF, compared to the Lewis group ($p < 0.01$). (d) Graph representing mean bone mineral content (BMC). No difference was found between the two groups ($p = 0.16$). (e) Graph representing mean tissue mineral content (TMC). Sprague Dawley rats had a significantly greater TMC, compared to Lewis rats ($p < 0.01$).

Figure 3.

(a) Graph illustrating mean ultimate load (UL). No difference was found between Lewis and Sprague Dawley rats ($p=0.698$). (b) Graph illustrating mean failure load (FL). No difference was found between the two groups ($p=0.631$). (c) Graph representing mean yield (Y). No difference was found between the two groups ($p=0.144$). (d) Graph representing mean stiffness (S). No difference was found between the two groups ($p=0.674$). (e) Graph representing mean elastic energy. No difference was found between the two groups ($p=0.695$).