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Original Research

Four-Point Bending as a Method for Quantitatively Evaluating Spinal Arthrodesis in a Rat Model

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The most common method of evaluating the success (or failure) of rat spinal fusion procedures is manual palpation testing. Whereas manual palpation provides only a subjective binary answer (fused or not fused) regarding the success of a fusion surgery, mechanical testing can provide more quantitative data by assessing variations in strength among treatment groups. We here describe a mechanical testing method to quantitatively assess single-level spinal fusion in a rat model, to improve on the binary and subjective nature of manual palpation as an end point for fusion-related studies. We tested explanted lumbar segments from Sprague–Dawley rat spines after single-level posterolateral fusion procedures at L4–L5. Segments were classified as 'not fused,' 'restricted motion,' or 'fused' by using manual palpation testing. After thorough dissection and potting of the spine, 4-point bending in flexion then was applied to the L4–L5 motion segment, and stiffness was measured as the slope of the moment–displacement curve. Results demonstrated statistically significant differences in stiffness among all groups, which were consistent with preliminary grading according to manual palpation. In addition, the 4-point bending results provided quantitative information regarding the quality of the bony union formed and therefore enabled the comparison of fused specimens. Our results demonstrate that 4-point bending is a simple, reliable, and effective way to describe and compare results among rat spines after fusion surgery.

Lower back pain has several etiologies and affects approximately 70% to 80% of American adults at some point in their lives.[10] Spinal fusion and its clinical goal of reducing or eliminating motion remains the surgical 'gold standard' of care for patients, with rates of surgery increasing dramatically in recent years.[6,22] Although successful fusion can greatly benefit patients, unsuccessful fusion (pseudoarthrosis) can result in significant morbidity and costly reoperation procedures.[20] Consequently, research regarding fusion procedures and associated grafting technology is ongoing. According to a 2013 systematic review of bone-graft alternatives, approximately 1400 products are available on the international market, with rates of successful bony union ranging from 45% to 100% depending on the grafting material, spinal instrumentation, patient population, and operative procedure used.[9] In addition, biologics such as bone morphogenetic protein,[3,14,23] demineralized bone-matrix-based products,[11] parathyroid hormone,[13,18,21] stem cells,[1,8,16] and vitamin D[15] are under investigation to determine each compound's ability to enhance bone formation after a spinal fusion procedure. Fusion procedures typically are performed in rat models to evaluate the preclinical efficacy, safety, and rate of bony union among these various bone-forming adjuvants.[1-3,5,7,8,13-19,21,23-25]

The most common method of evaluating the success (or failure) of rat spinal fusion procedures is manual palpation testing. However, the resultant data are subjective, binary, and do not provide any measurable information on the strength of the subsequent union (fusion). In an effort to provide quantitative data, previous studies have used a variety of mechanical testing methods in addition to manual palpation. The approaches used in these studies vary and are either inappropriate, difficult to replicate, or require an intricate experimental setup.[7,19,25] One such method is 3-point bending, a common and simple means of mechanically testing the strength of materials. By definition, 3-point bending creates combined bending and significant shear stress at the midpoint of specimens with high thickness-to-span ratios. For this reason, a specimen length-to-thickness ratio of at least 20:1 has been suggested to ensure that shear stresses are relatively insignificant when compared with the bending stresses.[4] Conforming to this stipulation is possible for protocols testing long bones, such as the femur, but becomes impractical when examining the small span of a single-level (that is, L4–L5) fusion segment of a rat spine.

The goal of this study, therefore, was to develop a mechanical testing method to quantitatively assess single-level spinal fusion in a rat model, thereby improving on the binary and subjective nature of manual palpation as an end point for fusion-related studies. We hypothesized that the resistance generated during 4-point bending would confirm the results obtained through manual palpation and, more importantly, would provide additional insight into the overall strength of the fusion formed.

Materials and Methods

Preparation of specimens. Lumbar spinal segments were collected from Sprague–Dawley rats that participated in previous IACUC-approved studies in our lab (Spine Tissue Engineering, Cedars–Sinai Medical Center) analyzing grafting materials for spinal fusion at the L4–L5 motion segment using a posterolateral intertransverse process surgical procedure. This procedure has

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previously been described in detail.[3] For use as nonoperated controls, 3 additional lumbar segments from Sprague–Dawley rats with no history of spinal surgery were obtained from the comparative medicine staff after sentinel rats were euthanized. All rats were obtained from Charles River Laboratories (San Diego, CA).

Prior to mechanical testing, lumbar spines underwent manual palpation testing by bending in the sagittal and coronal planes by 2 researchers who were trained in this technique and who were blinded to the experimental grafting material used. No motion at the L4–L5 segment on manual palpation was determined as 'fusion success.' A detailed explanation of this technique has previously been described.[3] Typically, when using manual palpation, surgeries yielding 'slight motion,' 'restricted motion,' or 'full motion' are considered failed fusions and are all classified together as 'not fused.' For this particular study, however, specimens were graded and classified into 1 of 3 categories according to the degree of motion as 'not fused,' 'restricted motion,' or 'fused.'

After manual palpation testing, specimens were meticulously cleaned of nonstructural soft tissue, such as muscle and fat, leaving the joint capsule, ligaments, and intervertebral disc intact. This step was of particular importance to ensure a rigid bond between the vertebral bodies and the pots that hold them in place; otherwise the testing procedure can be compromised. Each specimen then was potted to approximately half-axial height on both the L4 and L5 vertebral bodies in a 2-part polymer resin (Smooth-Cast 300, Smooth On, Easton, PA) to isolate each L4–L5 motion segment. Sections of square plastic tubing (2 in. each) were used as the pots to hold the spines and the resin. Specimens were positioned carefully such that the anterior midline of the L4–L5 motion segment was aligned with the midline of one of the faces of the square tubing for both the inferior and superior pots. After the L5 vertebral body was fixed, both pots were rigidly attached to a metal 90°-angle bar and spaced such that the material could be poured to reach the appropriate height of the L4 vertebral body and the pots would be well-aligned vertically (Figure 1). This procedure allowed specimens to easily rest in positions aligned with the coronal or sagittal planes and to maintain this position throughout testing. In addition, it prevented movement at all motion segments other than the one being tested (L4–L5).

Mechanical testing of rat spinal fusion. After each specimen was potted, it was mounted onto a servo-hydraulic actuator (MTS Bionix 370.02, MTS, Eden Prairie, MN) equipped with a miniload cell (MINI45 Transducer, API, Apex, NC) and a 4-point bending apparatus (MTS 642.001A-02, 3-, and 4-Point Bend Fixture, MTS). The inner and outer spans of the apparatus were held constant at 25 mm and 60 mm, respectively, for all specimens, with 5-mm-diameter steel rollers serving as the contact points (Figure 2). All specimens were destructively tested at a loading rate of 3 mm/min to approximately quasi-static conditions. Flexion was chosen as the imposed pure-bending moment in light of its ubiquity in rodent and human motion and its regular use as the direction for bending during manual palpation tests.

Vertical load versus deflection curves were produced for each specimen, which were then converted to moment–deflection curves according to the following relation for 4-point bending: moment = force × (outer span – inner span) / 4. The slope of the first linear region was taken as the measure of stiffness in units of N-mm/mm. If there was a prolonged initial linear region, as was often the case for restricted and fused specimens, regression between 175 and 350 N-mm was used to determine stiffness, representing a reliable linear source early in the loading phase.

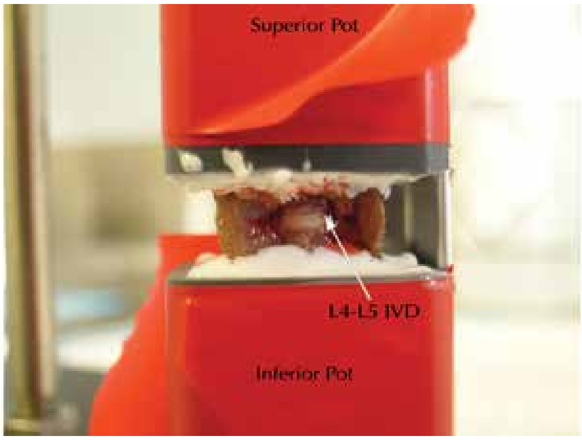

Figure 1. Potted specimen shown with correct vertical alignment and appropriate height to expose the L4–L5 motion segment. The arrow indicates the L4–L5 intervertebral disc space (IVD).

Statistical analysis. In this analysis, surgical specimens were coded according to their manual palpation testing result (fused, restricted motion, or not fused). Nonoperated sentinels composed the fourth and final group. ANOVA was performed by using SAS statistical software (SAS Institute, Cary, NC) to determine statistical differences in stiffness among the specimens across the manual testing result groups. Statistical tests for significance between groups were performed by using 2-tailed Student t and Tukey tests. A P value of less than 0.05 was considered significant in all analyses.

Results

Manual palpation results confirmed that all spines from nonoperated sentinel rats ($n = 3$) were not fused. Of the 17 operated specimens, 5 were classified as not fused, 6 were classified as restricted motion, and 6 were classified as fused. A posthoc power analysis indicated that a total of 15 specimens was necessary to allow at least 80% power with an α of 0.05, demonstrating that our study was sufficiently powered.

In 4-point bending analysis, spines determined to be fused according to manual palpation had a stiffness (mean ± SEM) of 502.9 ± 82.1 N-mm/mm and were mechanically stiffer ($P < 0.05$) than were all other groups. Specimens in the restricted motion group had an average stiffness of 306.1 ± 54.1 N-mm/mm, which was stiffer than both of the not fused groups (operated and not operated), which yielded stiffnesses of 224.5 ± 56.2 and 122.1 ± 5.1 N-mm/mm, respectively (Figure 3). In addition, the operated spines that were not fused were somewhat stiffer, demonstrating some bone remodeling (Figure 4) than were the nonoperated spines. This feature was not detected with manual palpation alone.

Analysis of the operated subset of spines demonstrated a clear distinction ($P < 0.0001$) between all unfused ($n = 11$; restricted motion + not fused, 269.0 ± 67.4 N-mm/mm) and fused (502.9 ± 82.1 N-mm/mm) rat spines. Overall, there was 72% common explained variability between 4-point bending analysis and traditional manual palpation testing.

Discussion

The objective of this study was to develop and validate a reproducible method to quantitatively assess the quality of a fusion mass in a rat model. Our results demonstrate that 4-point bending

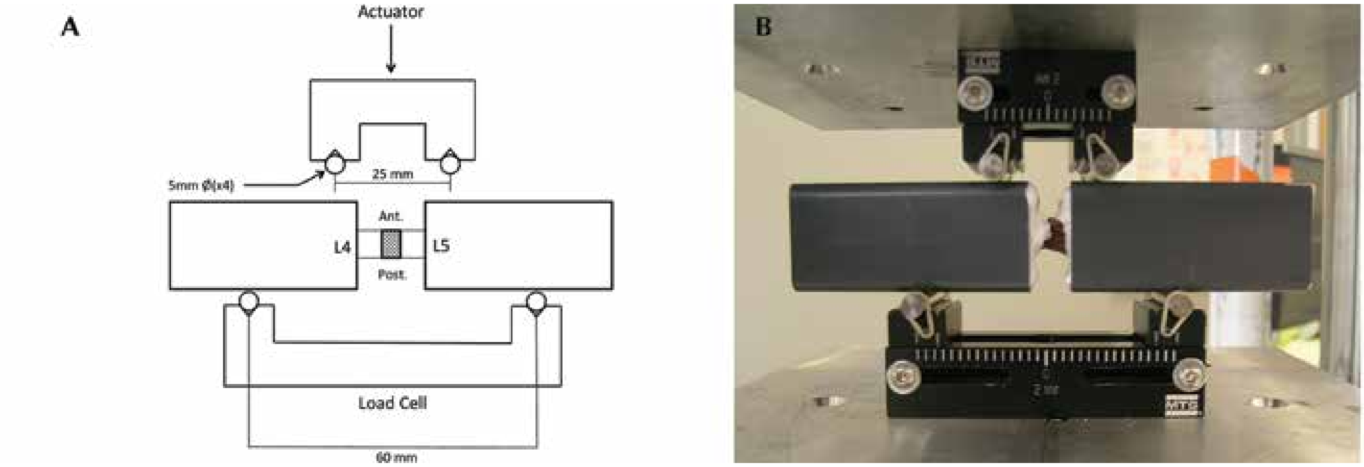

Figure 2. (A) Diagram and (B) image of 4-point bending setup. The inner and outer spans were 25 mm and 60 mm, respectively, and the contact points were 5-mm-diameter steel rollers.

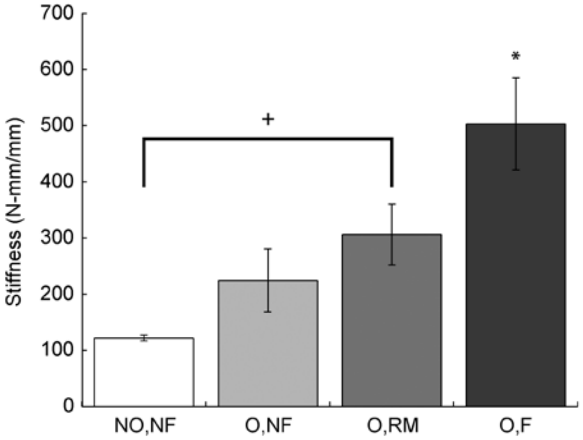

Figure 3. Stiffness (mean; bar, SEM) defined by resistance to bending in 4-point flexion for all experimental groups, generated by using the averages of the slopes of the moment–deflection curves. F, fused; NF, not fused; O, operated; NO, not operated; RM, restricted motion. *, F group is significantly ($P < 0.05$) stiffer than all 3 nonfused groups; +, RM group is significantly ($P < 0.05$) stiffer than the NO group.

analysis successfully provides more comprehensive data than does traditional binary manual palpation in the evaluation of rat spinal fusions. Fused specimens were significantly stiffer than those that were classified as having restricted motion. Likewise, specimens in the not fused group were significantly less stiff than those in the restricted motion group, representing a clear and significant numerical distinction that would otherwise be lost if manual palpation were the only outcome measure reported. This finding demonstrates how the addition of a mechanical outcome measure, such as 4-point bending, can be advantageous when assessing different fusion adjuvants or the maturity of the consolidated bony mass. Another advantage can be seen when making comparisons within the same group. For instance, a restricted motion specimen with a stiffness value closer to that of the fused group could be classified separately from another with a stiffness value closer to that of the unfused specimens, thus allowing better differentiation within a group.

Another potential gain of adding biomechanics to the traditional manual palpation testing method is the data analysis and statistical techniques. Compared with the nonparametric statistical test (for example, the Fisher Exact test) that is applied to the binary outcome from the manual palpation testing, parametric ANOVA with a grouping factor for type of grafting material (treatment) can be used. Statistical tests using data of continuous-type measures reveal quantitative distinction among multiple groups within the same analysis.

When testing rat spinal fusions, 4-point bending analysis is superior to other mechanical testing methods because it imparts a pure moment on the section of the specimen between the 2 inner loading points. Other methods can induce shear or other unwanted forces, making the data more difficult to interpret. In addition, when using 4-point bending, the point of contact is always between the steel rollers and the pots, unlike 3-point bending or other methods, for which the specimen is in direct contact with the actuator, thus making these techniques more prone to error.

In 4-point bending tests of more conventional materials (that is, not tissue), mechanical properties such as elastic modulus can be derived from beam bending equations. However, because these equations rely on several assumptions (including constant specimen cross-section and minimal vertical deflection[4,12]) that are challenged in the analysis of rat motion segments, we refrained from making definitive statements regarding the material properties of the bone. The value of our analysis instead lies in the quantification of the fusion mass as a whole. The minimal deflection assumption is better approximated for fused specimens and early in the loading cycle (when we took stiffness measurements),[4] making this testing method well-suited for distinguishing subtle differences between fused specimens for which bone material properties might also be reasonably assessed.

Although the experimental setup was shown to be effective for our purposes, assumptions and simplifications regarding the mechanics of the system should be considered. One limitation of the described 4-point bending method is the uncertainty regarding the deflection of the center of the specimen. Ideally, deflection measurements should be taken from the beam itself, at the midpoint of the section subjected to the pure moment.[12] Given the small and irregular specimens we evaluated, we used the displacement of the superior actuator to estimate this deflection. An accurate midpoint deflection measurement is only critical when implementing beam bending equations to determine material

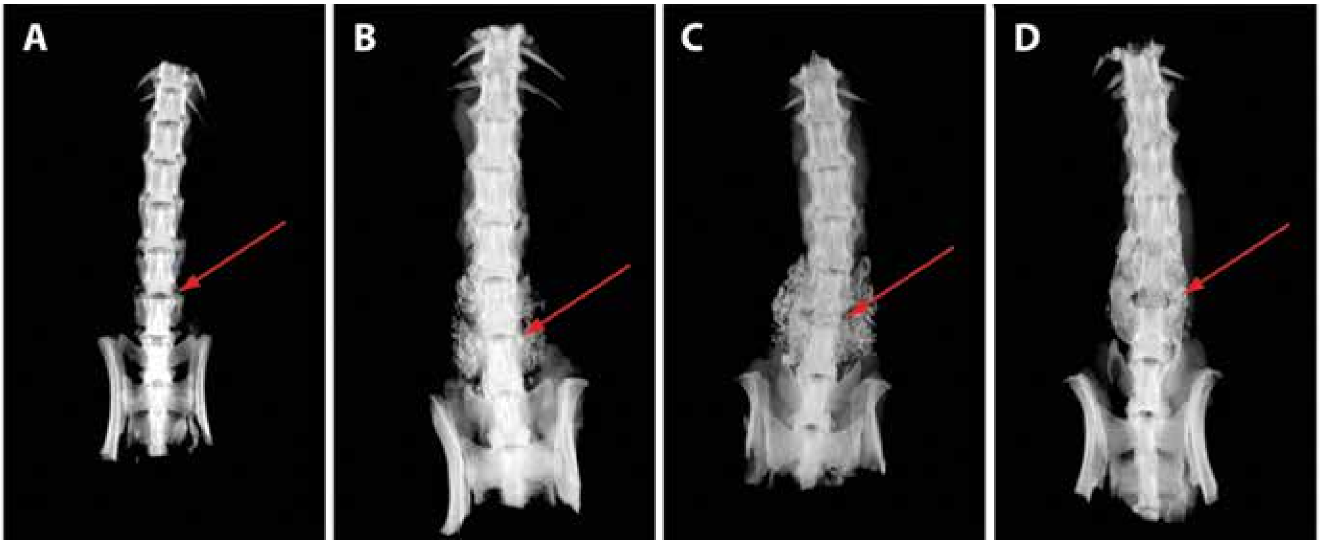

Figure 4. Example radiographic images of (A) nonoperated, not fused; (B) operated, not fused; (C) operated, restricted motion; and (D) operated, completely fused rat spines with continuity of bone between the transverse processes on both the right and left sides. The arrows mark the L4–L5 intervertebral disc space (IVD), across which the fusion surgery was performed.

properties. Because we instead focused our analysis on the moment–deflection curve, we do not consider this feature to be a significant drawback to our study. Another potential limitation was the single, centralized load cell beneath the lower supports. Specimens were rigidly aligned during the potting procedure, but because we had only a single centralized load cell, we were unable to verify that the force was distributed evenly between the supports, a characteristic that is necessary to ensure the accuracy of the force-to-moment conversion described previously. However, by using considerable precision during the potting and alignment procedures and by taking measurements early in the loading cycles, we believe these deviations were minimized.

In conclusion, as the rate of spinal fusion operations continues to increase with our aging population so will the need for a well-validated, simple, and affordable preclinical small animal model. Our current study demonstrates that 4-point bending analysis provides structural information on the fusion mass formed after grafting in a rat spinal fusion model. In addition, our 4-point bending method validates the outcome measures of traditional manual palpation testing. Additional work—such as correlating these or similar results with other quantitative measures such as bone density, bone volume, or cross-sectional moment of inertia—may provide useful and supplement information regarding the main endpoint of fused compared with not fused.

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