ISSLS Prize Winner: Adaptations to the Multifidus Muscle in Response to Experimentally Induced Intervertebral Disc Degeneration

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Study Design. Basic science study of the rabbit multifidus muscle response to intervertebral disc degeneration.

Objective. To assess changes in passive mechanical properties, associated protein structure, and histology of multifidus in response to disc degeneration produced by experimental needle puncture.

Summary of Background Data. Relationships between muscle dysfunction and low back injury; however, little is known about the cause and effect of such relationships.

Methods. Twelve rabbits were studied; 4 in each of 3 groups: control, 4-weeks postintervertebral disc injury (4-week disc degeneration), and 12-weeks postintervertebral disc injury (12-week disc degeneration). Single multifidus fibers and bundles of fibers were isolated and tested for slack sarcomere length and elastic modulus. Titin isoform mass, myosin heavy chain distribution, and muscle histology were also examined.

Results. Compared to control, individual muscle fibers were 34% stiffer and fiber bundles 107% stiffer in the 12-week disc degeneration group. No changes were detected at 4-week disc degeneration. No statistically significant change was found for MHC distribution in the 12-week disc degeneration group when compared to control, whereas titin isoforms were larger (P < 0.05) in the 12-week disc degeneration group. Histology revealed select regions of multifidus, at 12-week disc degeneration, with increased space between bundles of fibers, which in some instances was partly occupied by adipose tissue.

Conclusion. Multifidus becomes stiffer, both in individual fibers and fiber bundles, in response to experimentally induced intervertebral disc degeneration. This cannot be explained by change in fiber-type due to reduced muscle use, nor by the increased size of the protein titin (which would reduce stiffness). We hypothesize that fiber bundles become stiffer by proliferation and/or reorganization of collagen content within the muscle but the basis for fiber stiffening is not known.

Key words: adaptation, connective tissue, extracellular matrix, intervertebral disc, muscle, spine, stiffness. Spine 2011;36:1728–1736

Numerous studies have linked chronic low back pain (LBP) with abnormal muscle use and abnormal muscle structure. However, cause-effect relationships among LBP, injury, and muscle dysfunction remain elusive. Defining these relationships is essential for understanding as well as treating LBP, and is the goal of this study.

To date, two studies by Hodges et al have elucidated some cause-effect relationships between back injury and muscle function. In this porcine model, experimentally induced intervertebral disc injuries were used to demonstrate multifidus muscle atrophy and fatty infiltration 3 days postinjury, and increased multifidus motor excitability 15 minutes postinjury, respectively. A third, prospective study, by Cholewicki et al demonstrated in a population of varsity athletes that slower onset of abdominal muscle electrical activity, in response to spinal perturbations, increased the likelihood of sustaining a low back injury. Thus, it appears that muscle and the motor system have the ability to both adapt in response, and predispose to, spine injury. Although each of these studies provides important information regarding the relationship between spine injury and muscle control and structure, a number of questions remain.

Experimentally induced intervertebral disc injury has been used in animal models to approximate degenerative changes that naturally occur in the human lumbar spine with age. These structural and biochemical degenerative changes are related, in humans, to pain and dysfunction. Despite the relatively routine use of such models to study changes in the cellular and mechanical components of the disc and spine, only the aforementioned Hodges et al study used this model to examine...
structural changes in the spine musculature. Specifically, Hodges et al. employed ultrasound imaging of the multifidus muscle preinjury and postinjury, and reported reduced muscle size by 3 days postinjury, along with histological evidence of increased fatty infiltration within the muscle. However, these are rough estimates of muscle morphology and, at such short time points postinjury, it is still not known how these changes will progress or regress over longer periods of time. Furthermore, previous research has hypothesized that muscles can adapt to surrounding injury by undergoing what appears to be atrophy, yet maintain their physiological cross-sectional area (thus force producing capability) through structural remodeling. Thus, it is still uncertain whether multifidus undergoes remodeling, in response to disc injury, which could alter or compromise its functional capabilities. Passive mechanical properties help define such functional capabilities in muscle, and have been shown to differentiate between healthy and dysfunctional skeletal muscle in some populations. Thus, the purpose of this study was to assess changes in passive mechanical properties, associated protein structure, and histology of multifidus in response to experimentally induced intervertebral disc degeneration in the rabbit.

MATERIALS AND METHODS

Twelve female New Zealand White rabbits (9 months old at sacrifice) were separated into three groups of four. The first group (Control) received no intervertebral disc injury prior to sacrifice. The second and third groups incurred experimentally induced disc injury and recovered for 4 weeks (4-week disc degeneration) and 12 weeks (12-week disc degeneration), respectively. Procedures were approved by the local IACUC.

Experimentally Induced Intervertebral Disc Degeneration

Rabbits were anaesthetized via subcutaneous injection of ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg). Rabbits were positioned in a lateral prone position, and a posterolateral incision was made to enter the retroperitoneum. After palpating and exposing the anterior surface of the L2–L3 and L4–L5 intervertebral discs, an 18-gauge needle was guided linearly through the right-anterior annulus fibrosus into the nucleus pulposus of each disc. After removing the needle the wound was thoroughly rinsed with sterile saline and closed with layered sutures. Animals were monitored during recovery and allowed to carry out their normal routines under daily observation.

Harvesting Multifidus Muscle

Rabbits were sacrificed and immediately afterwards two multifidus biopsies were taken at each of two locations (four biopsies total). The first two were taken at the approximate level of the L3 vertebral body (just caudal to the most cranial disc injury site) (Figure 1); the second two were taken at the approximate L7 level (two vertebral levels caudal to the most caudal disc injury site). One biopsy from each site was immediately placed in a storage solution to prevent hyperpolarization and destruction, and stored at −20°C; this tissue was used for passive mechanical testing. The second biopsy was pinned to cork and frozen with liquid nitrogen and stored at −80°C; this tissue was used for histological and protein analyses.

Passive Mechanical Testing

All muscle was tested within 2 weeks of harvest. Testing was performed in a relaxing solution to ensure muscle properties were assessed in a completely passive state. Single fibers and small fiber bundles (composed of approximately 4–8 fibers ensheathed in their connective tissue matrix) were isolated and tied with 10–0 silk thread to pins secured on one side to a microlevel force transducer (Model-405A, Aurora Scientific, Aurora, Ontario, Canada), and on the other side to a high-speed motor (Model-318B, Aurora Scientific, Aurora,
Ontario, Canada) to manipulate specimen length (Figure 2). A 7mW diode laser was used to generate a diffraction pattern in each sample, enabling measurement of sarcomere length throughout testing. Specimens were initially lengthened until they started to resist passive force; further tests were initiated from this length (termed slack sarcomere length). The motor was controlled to provide rapid (100 fiber lengths/second) stretch to the specimens in increments of approximately 0.25 μm/sarcomere. After each stretch, the fiber or fiber bundle was held at constant length for 3 minutes to allow stress relaxation to occur. A minimum of seven stretches were conducted on each specimen. Changes in sarcomere length relative to initial length were computed as a measure of specimen strain. Force at the end of each 3-minute relaxation period was normalized to fiber or fiber bundle cross-sectional area to provide a measure of stress. Stress-strain profiles were assessed for each specimen and the slope of the linear portion of each curve was defined as the relaxed modulus (stiffness).

**Histology**

Cross sections (5-μm thick) of the frozen muscle samples were placed on glass slides for staining. Hematoxalin and eosin (H&E) stain was used to assess gross muscle structure and morphology, and Oil Red O stain was used to assess the presence of adipose in the muscle tissue. Stained sections were photographed through a microscope and visually assessed.

**Protein Analysis**

Titin molecular mass was analyzed in single fibers that had been tested mechanically from the control and 12-week disc degeneration groups. Briefly, single fibers were boiled in sample buffer solution and SDS-VAGE analysis was used to quantify titin migration relative to the migration of titin standards of known size (human soleus and rat cardiac).

Myosin heavy chain (MHC) isoforms were analyzed in cross sections of the frozen muscle sample from the control and 12-week disc degeneration groups. Briefly, muscle cross
sections were homogenized and boiled in sample buffer, and SDS-PAGE procedures were utilized to assess the relative intensity of MHC bands corresponding to different fiber types.

**Radiologic Analysis of Disc Degeneration**
Radiographs were obtained at preoperative, 4-week and 12-week postoperative time-points, all with rabbits in the lateral decubitus position. All radiographs were assessed by an experienced blinded orthopedic researcher. Disc height index (DHI) was calculated by averaging measures of intervertebral disc height at the anterior, middle and posterior disc, and dividing this by the average of the height of the anterior, middle and posterior of the two adjacent vertebral bodies. This was done for both punctured discs (L2–L3 and L4–L5) as well as one nonpunctured disc (L3–L4). Percentage change in DHI was calculated for each postoperative disc as a ratio to its preoperative DHI and further normalized to the DHI of its adjacent nonpunctured (L3–L4) disc:

\[
\text{Normalized } \% \text{DHI}_D = \left( \frac{\text{postoperative DHI}_D}{\text{preoperative DHI}_D} \right) \times 100
\]

where the subscript D represents the degenerated L2–L3 or L4–L5 disc.

**Statistical Analysis**
Slack sarcomere length and relaxed modulus were assessed via three-way ANOVA (factors; 1. Group: Control, 4-week disc degeneration, 12-week disc degeneration, 2. Muscle location- L3 level, L7 level, 3. Muscle size- single fiber or fiber bundle). Titin molecular mass and percentage of type I MHC isoforms were assessed via two-way ANOVA (factors; 1. Group, 2. Muscle location). Type I MHC percentages were arcsine transformed to satisfy the assumption of normality prior to statistical testing. Where appropriate, Tukey HSD test was used for post hoc analysis. Statistical significance was \( P < 0.05 \).

**RESULTS**

**Radiographic Analysis of Degeneration**
Mean ± SEM normalized % DHI scores were 84.4 ± 7.9 and 79.5 ± 7.9 for L2–L3 and L4–L5 discs 4 weeks postinjury, respectively, and 77.0 ± 0.9 and 81.6 ± 3.0 for L2–L3 and L4–L5 discs 12 weeks postinjury, respectively. These %DHI scores represent loss of intervertebral disc height, demonstrating that disc degeneration was achieved by 4-weeks postinjury.

**Passive Mechanical Properties of Multifidus**
The multifidus muscle in the 12-week disc degeneration rabbits was significantly stiffer (\( P < 0.0001 \)) than in both the 4-week disc degeneration and control rabbits (Figure 3). This result was modified by a statistically significant interaction with muscle size (\( P = 0.0405 \)); the increase in stiffness being greater in fiber bundles (107% increase compared to control) than in individual fibers (34% increase compared to control) (Figure 3). Increased multifidus stiffness occurred at both the L3 and L7 levels. No significant main effects were found for muscle size (\( P = 0.3339 \)) nor spinal level (\( P = 0.0633 \)).

For slack sarcomere lengths, main effects were found for both muscle size (\( P < 0.0001 \); bundles shorter slack length than fibers) and spinal level (\( P < 0.0001 \); multifidus at L7 shorter slack length than multifidus at L3) (Figure 4). A significant interaction (\( P = 0.0455 \)) existed between experimental...
No obvious gross fiber degeneration was observed in any of the muscles. It appeared that regions of multifidus in the 12-week disc degeneration group had increased extracellular matrix space between fiber bundles compared to control (Figure 7A); however, this was not universal across all regions of muscle nor obviously pathological in nature.

Oil Red O staining uncovered the presence of isolated adipose occupying space between fibers in all multifidus muscles (control and both disc injury groups) (Figure 7B); this adipose occupied very small areas of the muscle. No obvious increase in adipose staining was present in the 4- or 12-week disc degeneration groups; however, it did appear that in select regions of the 12-week disc degeneration multifidus, adipose tissue between fiber bundles increased.

**DISCUSSION**

The most striking result of the current work is the significant increase in stiffness that the multifidus muscle manifested group and spinal level, whereby a decline in slack length occurred in the 4-week disc degeneration group at L7, but not at L3. No significant main effect was found between control and disc degeneration groups (P = 0.1502).

**Titin Isoform Sizes**

Primary titin isoforms in control multifidus muscles had a very small but significantly different (P = 0.0492) mean molecular mass (3405 ± 6 kD) compared to the 12-week disc degeneration group (3488 ± 35 kD); this change primarily manifested itself at the L3 spinal level (Figure 5A). Titin molecular mass was also significantly different (P = 0.0377) between L3 and L7 levels in the disc-degenerated animals.

**Myosin Heavy Chain Distribution**

No statistically significant differences were found in percentage of type I multifidus fibers between the control and 12-week disc degeneration groups, nor between L3 and L7 levels (Table 1; Figure 6).

**Histology**

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represent the first direct evidence of changes in mechanical properties of back muscles in response to intervertebral disc degeneration and indicate a cause and effect between low back injury/pain and back muscle dysfunction.

Previous work\(^9\) demonstrated atrophy and cellular changes in porcine multifidus as early as 3 days postexperimentally induced disc injury. Conversely, in the current work no histological changes or fatty infiltration were identified 4 weeks after disc injury, but some minor changes were seen by 12 weeks. Primarily, an increase in extracellular matrix space, likely collagen-based, between fiber bundles appeared in some regions of multifidus. This would help explain the large increase in stiffness of fiber bundles (that are composed of both fibers and connective tissue). A small increase in adipose also seemed to be present in the 12-week disc degeneration muscles, which was confined to the space between fiber bundles (rather than within individual fibers). Rapid onset of fatty infiltration has been documented in directly injured muscle, but this accumulation of fat returned to control levels by 10 days postinjury.\(^{22}\) Such a time-course might explain why, in contradiction to Hodges et al\(^9\) at 3 days postdisc injury, multifidus muscles in the current study showed no elevated fat at 4 weeks postdisc injury. However, by 12 weeks of disc degeneration, muscle remodeling including proliferation and/or reorganization of its connective tissue matrix may have

<table>
<thead>
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<th>Group</th>
<th>Type I</th>
<th>Type 2B</th>
<th>Type A/X</th>
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<tr>
<td>Control L3</td>
<td>11.4 ± 6.2</td>
<td>11.9 ± 2.1</td>
<td>76.7 ± 4.1</td>
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<tr>
<td>12-week disc injury L3</td>
<td>17.2 ± 6.5</td>
<td>20.9 ± 4.4</td>
<td>62.0 ± 4.5</td>
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<td>Control L7</td>
<td>7.6 ± 0.9</td>
<td>14.8 ± 2.2</td>
<td>77.6 ± 1.8</td>
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<tr>
<td>12-week disc injury L7</td>
<td>6.6 ± 0.9</td>
<td>20.1 ± 6.0</td>
<td>73.3 ± 5.4</td>
</tr>
</tbody>
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**Figure 6.** Mean percentage type I myosin heavy chain (MHC) isoform for control and 12-week disc degeneration groups at each of the L3 and L7 spinal levels. No statistically significant differences were found. Data are plotted as mean ± SEM.

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been accompanied by minor amounts of adipose occupying the space between bundles of muscle fibers. It is possible that over time, disc injury and muscle remodeling will progress leading ultimately to dysfunctional muscle occupied by more significant amounts of fat. Greater amounts of this type of adipose occupation of multifidus have been reported for patients suffering from chronic LBP.\textsuperscript{7,8}

In the current work, both individual fibers and fiber bundles became stiffer in response to intervertebral disc injury. This suggests a remodeling process that is intended to reduce intervertebral joint motion by increasing the passive mechanical stiffness of the deepest large spine muscle, the multifidus. Previous work examining the mechanical properties of back muscles from patients undergoing spine surgery documented higher passive modulus values for multifidus in comparison with both longissimus and iliocostalis.\textsuperscript{19} It is unclear whether this resulted from a natural phenomenon, or as an adaptation to low back injury. These data demonstrate that multifidus can become stiffer in response to intervertebral disc degeneration. However, we did not perform experiments on the rabbit longissimus or iliocostalis muscles, and therefore cannot comment as to whether the multifidus alone demonstrates an increase in stiffness.

As fiber bundle stiffness is thought to be predominantly determined by the amount and quality of connective tissue,\textsuperscript{23,24} we hypothesize that the increased bundle stiffness documented here is due to proliferation and/or reorganization of the connective tissue matrix within the muscles. Conversely, individual muscle fiber stiffness has been most often attributed to the giant muscle protein titin.\textsuperscript{24,25} As sarcomeres are lengthened, titin is stretched to bear passive force and provide stiffness to the fiber. Prado \textit{et al}\textsuperscript{24} identified titin isoforms of different sizes in different skeletal muscles, and found a rough negative correlation between titin size and fiber stiffness.\textsuperscript{19} In the rabbit multifidus muscles studied here, mean larger titin isoforms were identified in the 12-week disc degeneration group, mainly at

\begin{figure}
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\caption{(A) Cross-sectional micrographs of H&E stained multifidus from control and 12-week disc degeneration rabbits at the L7 spinal level. The region shown for the 12-week disc degeneration muscle has increased extracellular matrix between bundles of fibers; however, this was not uniform across all regions of the muscle. (B) Images of Oil Red O stained multifidus from control and 12-week disc degeneration rabbits at the L7 spinal level. Arrows indicate areas positively stained for fat. Scale bars = 100 \textmu m.}
\end{figure}
the L3 level (Figure 5). This, therefore, does not explain the increased stiffness demonstrated in these muscles. A second expected result of increased titin size is longer slack sarcomere lengths. Interestingly, although not statistically significant, a trend existed of slack sarcomere lengths being longer in the 12-week disc degeneration multifidus compared to control multifidus (Figure 4). It would seem that this increased slack length might parallel the increase in titin size seen at 12 weeks at the L3 level, although most likely cannot be explained by the smaller titin size increase at L7.

The mechanism for the rapid multifidus atrophy reported in the Hodges et al study was hypothesized to stem from a reduction in muscle usage postdisc injury. To assess disuse as a cause of the mechanical muscle changes found in the current study, MHC distribution was measured. Previous work has demonstrated that muscle fiber type and MHC distribution transform toward a faster (type 2) isoform with decreased use.26,27 Thus, MHC distribution can be used as an indirect indicator of relative level of use. Clinical relevance for back injury has been demonstrated by Mannion et al,28 who found a significantly lower percentage of type I fibers and a significantly greater percentage of type 2X (identified as 2B in their paper) fibers in back pain patients compared to matched controls. In the current study, no significant difference in multifidus MHC isoform distribution was found between the control and disc degeneration groups, nor between the L3 and L7 levels (Table 1, Figure 6). Thus, it does not appear that multifidus atrophy, over the 12-week postdisc injury period, was a dramatic factor initiating the observed changes in muscle mechanical properties.

It cannot be ruled out that multifidus adaptations demonstrated in the current study occurred in response to the surgical procedure rather than the actual disc injury. Despite this, a number of factors make it unlikely that the invasive surgical procedure produced the observed effects. First, Hodges et al performed sham surgeries in which all surgical procedures aimed to repair and rehabilitate the intervertebral disc and spine.

Possible that increased stiffness acts to stabilize regions of the spine that are compromised by degenerated discs. In this case, treatment to reduce muscular-based stiffness could prove detrimental and lead to instability-type injuries. Conservative rehabilitation approaches should carefully retrain the back and abdominal muscles by considering such mechanical adaptations and modifying movement patterns to suit. Surgical approaches to disc degeneration should also consider the importance of maintaining intact musculature, as the ability of the muscle to adapt likely signals its importance in preserving a functional spine. Future studies will examine the ability of multifidus to recover its original mechanical properties in response to biological and conservative treatments designed to repair and rehabilitate the intervertebral disc and spine.

Key Points

- Intervertebral disc degeneration was induced in rabbits and changes in the mechanical properties and associated protein structure of the multifidus muscle were assessed.
- Individual muscle fibers (34% increase) and fiber bundles (107% increase) were stiffer, as compared to controls, after 12-weeks of disc degeneration.
- Histology revealed select regions of multifidus, after 12-week disc degeneration, with increased space between bundles of fibers.
- If it is hypothesized that fiber bundles become stiffer by proliferation and/or reorganization of collagen content within the muscle but the basis for fiber stiffening is not known.
- These results represent the first direct evidence of changes in the mechanical properties of back muscles in response to intervertebral disc degeneration and indicate a cause and effect between low back injury/pain and back muscle dysfunction.

References

Adaptations to the Multifidus Muscle • Brown et al