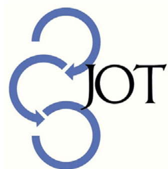
Journal of Orthopaedic Translation (2017) 11, 53—61



Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://ees.elsevier.com/jot

ORIGINAL ARTICLE

# Human and bovine spinal disc mechanics subsequent to trypsin injection

**Jeremy Alsup [a,b], Timothy Bishop [a], Dennis Eggett [c], Anton E. Bowden [a,*]**

[a] *Department of Mechanical Engineering, Brigham Young University, Provo, UT, USA*
[b] *Reliance Medical Systems, Bountiful, UT 84010, USA*
[c] *Department of Statistics, Brigham Young University, Provo, UT 84602, USA*

Received 2 March 2017; received in revised form 6 June 2017; accepted 19 June 2017
Available online 10 July 2017

**KEYWORDS**
mechanics;
protease;
spinal disc;
trypsin

**Abstract** *Objective:* To investigate the biomechanical effects of injections of a protease on the characteristics of bovine coccygeal and human lumbar disc motion segments.
*Methods:* Mechanics of treated tissues were measured immediately after injection and 3 h after injection. Motion segments underwent axial rotation and flexion-extension loading.
*Results:* Stiffness and neutral zone parameters experienced significant changes over time, with bovine tissues more strongly affected than human cadaver tissues. This was true in both axial rotation and flexion-extension. The treatment type significantly affected the neutral zone measurements in axial rotation. Hysteresis parameters were impacted by control injections.
*Conclusion:* The extrapolation of bovine coccygeal motion testing results to human lumbar disc mechanics is not yet practical. The injected treatment may have a smaller impact on disc mechanics than time in testing. Viscoelasticity of human lumbar discs may be impacted by any damage to the annulus fibrosis induced by needlestick.
*The Translational Potential of this Article:* Preclinical testing of novel spinal devices is essential to the design validation and regulatory processes, but current testing techniques rely on cadaveric testing of primarily older spines with essentially random amounts of disc degeneration. The present work investigates the viability of using trypsin injections to create a more uniform preclinical model of disc degeneration from a mechanics perspective, for the purpose of testing spinal devices. Such a model would facilitate translation of new spinal technologies to clinical practice.
© 2017 The Authors. Published by Elsevier (Singapore) Pte Ltd on behalf of Chinese Speaking Orthopaedic Society. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

* Corresponding author. 435 CTB, Department of Mechanical Engineering, Brigham Young University, Provo, UT 84602, USA.
*E-mail address:* abowden@byu.edu (A.E. Bowden).

http://dx.doi.org/10.1016/j.jot.2017.06.003
2214-031X/© 2017 The Authors. Published by Elsevier (Singapore) Pte Ltd on behalf of Chinese Speaking Orthopaedic Society. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

## Introduction

Chronic low back pain (LBP) is a debilitating condition that affects millions every day, and which has no known cure. LBP has several potential sources, such as osteoarthritis, muscle strains, dysfunctional ligaments, and degenerated intervertebral discs (IVD) [1]. As the disc degrades, annulus fibrosis (AF) and nucleus pulposus (NP) tissues alter their cellular and biochemical characteristics. Early degenerative changes in the NP include breakdown of polar aggregating proteoglycans with subsequent loss of hydration and disc height [2–4]. Changes in the NP are closely correlated with compositional and structural changes in the AF including loss of hydration and proteoglycan content, cracks, delamination, a reduced number of layers, collagen fibre reorientation [5], and increased layer thickness [6]. With advanced disc degeneration, the levels of the majority of matrix molecules are decreased, with the exception of biglycan and fibronectin [3]. Degenerated discs have a more abundant nerve supply than normal discs, and the nerves in discs appear to be capable of conducting pain signals [7–9]. Such changes typically cause a shift in mechanical behaviour of the disc, which in turn affects the clinical stability of the spine [10–12].

Despite the lack of strong clinical correlation between the severity of lumbar pain symptoms and the severity of disc degeneration [13,14], over 90% of surgical spinal procedures are performed consequential to the degenerative process [15]. For clinicians, treating disc degeneration is complicated because of the multifactorial traits of this pathology, including changes in morphology, biochemical composition, and mechanical environment of the disc and surrounding tissue.

In severe cases of chronic LBP, surgical operations can be performed to augment or replace the IVD. Orthopaedic spinal procedures may temporarily reduce pain by removing problematic discs, but do so at the expense of normal functional biomechanics. Current surgical treatments for lumbar related damage, namely spinal fusion and total disc arthroplasty, are problematic in the context of disc degeneration, because they alter the mechanical stress fields experienced by adjacent discs [16–18]. Altered stress fields have been linked to accelerated disc degeneration in adjacent levels, which further complicates the long-term well-being of the patient. Surgical treatments designed to function in a healthy environment are often operating in a degenerate environment.

Cadaver testing of spinal devices is an important part of the design validation process, providing key insights into device functionality and interaction with the surrounding tissue. However, availability and cost issues dictate that most cadaver testing is done on spines from a broad cross-section of degenerative states. Thus, it has heretofore been impractical to experimentally quantify the efficacy of spinal treatments confidently due to progressive degeneration of the surrounding tissue.

Research has shown that protease ingestion of bovine coccygeal disc tissue may serve as a potential degeneration model for cadaveric lumbar discs. Protease activity within animal IVDs has been shown to degrade the cellular integrity of the disc [19–23]. Particularly, Roberts et al. [19] subjected bovine tail discs to different protease solutions for up to 3 weeks. In comparison to disc samples in saline-buffered solutions, trypsin- and papain-treated discs showed extensive damage after testing, with most changes taking place in the NP. Papain caused more extensive damage in less time in comparison to trypsin. A second study completed by Mwale et al. [20] subjected bovine tail segments to trypsin treatment and compressive loading for 16 h to determine the effect on MRI parameters, as well as to determine changes in mechanical and biochemical properties. Trypsin caused greater alterations to mechanical properties than the applied loadings. Proteases such as trypsin digest the proteoglycans found within the cellular matrix of the AF. This loss of glycosaminoglycans is a condition of disc degeneration. The simulation of natural degeneration in animal IVDs via proteolysis has been hypothesized to also model the natural degeneration during the advancement of aging in human IVDs. Improved ability to mechanically mimic the onset of natural disc degeneration can also allow for greater precision in testing treatments *in vitro*. Biomechanical behaviours of motion segments adjacent to compromised discs which undergo any number of surgical procedures are of particular interest.

This study explores this proteolysis effect on both bovine coccygeal and cadaver lumbar motion segments, with the hypothesis that controlling the timing of the protease action can cause predictable and similar alterations in disc mechanics that can simulate natural degeneration.

## Materials and methods

### Bovine testing

Twelve bovine coccygeal spines (age 20–25 months) were acquired from a local abattoir and kept frozen at −20 °C until testing. Muscle and adipose tissue were dissected, taking care to preserve each IVD and vertebrae. Discs were screened for testing based on their shape and structural integrity, and irregular or damaged segments were rejected. Twenty functional spinal units (FSUs) [diameter mean = 2.27 cm, standard deviation (SD) = 0.34] were isolated from the spines by cutting through adjacent discs. Hydration was maintained with phosphate-buffered saline (PBS) solution during dissection and a generous coating of petroleum jelly during testing.

Each FSU was potted in custom test fixtures that allowed application of prescribed angular rotations in the flexion-extension (±15°) and axial rotation (±3°) axes. The vertebrae of the FSU were embedded in a two-part polyester resin (Bondo, 3M Corp, St. Paul, MN, USA), and care was taken to align the centreline of the disc horizontally with the fixture. A servo-hydraulic testing machine (Instron model 1321, Instron, Norwood, MA, USA) was equipped with a 20N*m torque transducer (Omega Engineering, Stamford, CT, USA) for angular rotation testing.

Each FSU was tested in two modalities: flexion/extension (FE) and axial rotation (AR). Testing order was randomly selected between specimens, but was consistent between subsequent tests on the same specimen. All

testing was performed at room temperature, and all FSUs were put through 15 cycles of testing at 1 Hz. Torque and rotation data from the final cycle in each testing modality was recorded and analysed.

After testing in both modalities, FSU specimens were heated to 37 °C for 15 min to facilitate the protease action of the trypsin injections. Each FSU was randomly assigned to one of two testing groups: 180 min trypsin or 180 min control. The AF of each FSU was injected with 25 μL of either trypsin (HyClone 2.5% 10x, Thermo Scientific, Waltham, MA, USA) or, in the case of the control group, foetal bovine serum (FBS) in eight equally spaced locations around the disc (a total of 200 μL) with a 27 ga (0.4128 mm outer diameter) needle [24]. Ten FSUs were used as a control with an injection of FBS and incubated for 3 h. FBS was chosen as a control solution similar to previously published work [20,22]. The other 10 FSUs were injected with trypsin and allowed to incubate for 3.0 h at 37 °C. After incubation, the FSU was allowed 15 min to normalize to room temperature before being subjected to the same testing protocol described previously.

## Cadaver testing

Eight cadaveric lumbar spines (4 male and 4 female, aged 16–79 years with an average age of 47.6 years) were dissected of all musculature and adipose tissue, leaving vertebral bodies, IVDs, and spinal ligaments intact. FSUs with damaged anterior longitudinal ligaments, supraspinous ligaments, and interspinous ligaments were excluded. During dissection, the cadaveric spine was spritzed with a PBS solution every 5–10 min to maintain full hydration [25]. Conditions of the spines and the separate FSUs were recorded in a lab notebook and documented visually with a digital camera. After dissection, 18 FSUs (major diameter mean = 5.60 cm, SD = 0.80; minor diameter mean = 3.85 cm, SD = 0.71) were segmented from the spines, deemed eligible for testing, and stored at a temperature of −20 °C until the commencement of testing.

Prior to flexibility testing, the FSU was thawed overnight at room temperature. Afterwards, the FSU was potted to form a rigid gripping surface on each vertebral endplate by using a two-part epoxy resin (Bondo). These potting structures were secured in metal potting fixtures that attached to a custom-built spine simulator. This spine motion simulator was capable of applying pure moment loads via a stepper motor attached to an adjustable motor shaft [26]. The orientation of the potted FSU, as well as the location of the motor, could be altered to test for AR- and FE-based motion tests. The spine simulator had an integrated environmental chamber, which maintained internal conditions of body temperature (37 °C) and near 100% humidity [25]. Once the FSU was secured in the environmental chamber, four high-contrast marker plates were attached to two sides of the superior endplate fixture. A compressive follower load of 440 N (meant to simulate upper body weight and muscle activation forces) was attached to the superior potting fixture through a cable-and-pulley system [27,28]. The placement of the follower load cables was optimized to minimize static moments in both lateral bending and flexion-extension axes. The FSU was then left in the environmental chamber for 30 min to warm the disc up to body temperature.

Each FSU underwent a series of 10 preconditioning motion cycles for each mode of loading (i.e., AR, FE) to ensure greater repeatability in FSU motion. Each test cycle started at the FSU's neutral position (i.e., zero torque), with the torque load increased to 7.5 N*m at a rate of 1°/s. Once the maximum torque was reached, the motor instantaneously switched directions and eventually reached −7.5 N*m of torque while rotating at the same rate [29]. A testing cycle was completed once the neutral position was reached following the movement through the desired maximum and minimum torques.

Following preconditioning, the AF of each of 12 FSUs was injected with 100 μL of either trypsin (HyClone 2.5% 10x, Thermo Scientific) or, in the case of the control group, PBS in six equally spaced locations around the disc (a total of 600 μL) with a 27ga (0.4128 mm outer diameter) needle [24]. Injection depth was controlled at 1 cm using an attached cork spacer on the needle. Prior to injection, the trypsin solution was mixed with a Brilliant Blue FCF food dye (Assorted Food Color & Egg Dye, McCormick & Co., Inc., Hunt Valley, MD, USA), which aided post-test inspection of protease diffusion throughout the disc. During injection, the needle was inserted to the depth of the attached cork spacer and then pulled out approximately 2 mm to allow greater space for solution diffusion. This also minimized trypsin leakage out the injection point by reducing the effect of internal swelling pressure. A total of 27 gauge needles were used to minimize AF tissue damage [24].

The FSU was immediately tested after injection (Time 0) for three cycles in both directions of loading (i.e., AR, FE), with torque-rotation data recorded on the final cycle for each bending direction. Collected data included torque sensor readings from the torque cell and three-dimensional positional data acquired from two cameras positioned outside the environmental chamber angled at 60° from each other. Transparency of the glass separating the cameras and FSU-marker plate system was ensured by using a heat gun to keep the glass warmer than the environmental chamber's internal temperature. This testing was repeated after 3 h. The order of testing directions (i.e., AR, FE) was kept consistent between both flexibility test runs, but randomized between each FSU. Once the final flexibility test was conducted, the FSU was removed from the environmental chamber and transected along the injection points. Preliminary degeneration levels were then visually characterized using the Thompson Scale [30].

This same procedure was followed for the injection of a PBS solution into three FSUs (from 2 cadavers), which was targeted as a control group. A second control group of three FSUs (from 2 cadavers) was subjected to needle penetration at the same six locations, but with no fluid injection, and was also subjected to flexibility testing at the same time points using the same procedure. Previous testing by Stolworthy and Zirbel [29] provided a control scenario for comparison, where FSUs were tested multiple times over an extended period using the same test setup (including the same follower-load and environmental conditions). Their results showed that no differences in segmental flexibility in the flexion-extension mode of loading occurred throughout the duration of their testing [29].

Bovine tail segments were purchased from the local abattoir directly following humane slaughter for the purposes of food production. Human spine samples were obtained from accredited tissue banks after de-identification and the authors' research protocol was classified as exempt and approved by the authors' institutional review board.

## Data analysis

Kinematic data was collected for each FSU using image-based motion analysis based on standard direct linear transformation techniques. Segmental rotation data was synchronized with corresponding torque measurements from the spine simulator. The resulting plots for both bovine and cadaver tests were then fit using a Boltzmann dual-inflection point curve [25] which was used to find the stiffness (K), hysteresis (H), hysteresis area (HA), and neutral zone (NZ) characteristics. The average $R^2$ value of the curve fits was 0.9659.

A two-level mixed-model analysis of variance with a Tukey-Kramer adjustment was applied (SAS University Edition, SAS, Cary, NC, USA) to the data to determine statistically significant trends in test results. Time after injection was the independent within-subjects variable, while injection treatment (i.e., trypsin, saline, fluidless) was the independent between-subjects variable. Analysis was done separately for bovine and cadaver results. Variance was decreased by normalizing the raw differences in the data from the injection baseline time. A $p$ value $< 0.05$ was deemed statistically significant. A previous mixed-models analysis of variance determined that cadaver FSUs initially showing advanced degeneration (i.e., Thompson Grades IV and V) were significantly different from healthier tissue. The four affected FSUs were therefore excluded from final analysis.

# Results

## AR

Flexibility parameters of stiffness, neutral zone, hysteresis, and hysteresis area were calculated from the bovine and cadaver sigmoidal torque-rotation curves. Hysteresis and hysteresis area for cadaveric tissues were significantly higher than bovine tests immediately postinjection (Table 1, Figures 1 and 2). Although the bovine-trypsin group did experience a slight decrease in hysteresis, absolute changes were minimal for bovine segments. Interestingly, fluidless (i.e., needlestick only) cadaver tests experienced the most pronounced change over time for both hysteresis and hysteresis area parameters in axial rotation. Hysteresis area was impacted by trypsin injections, although the changes were opposite in direction between cadaver and bovine samples.

Neutral zone differences were marginal between bovine and cadaver samples immediately postinjection, but increased over time during the 3 h incubation period (Table 1, Figure 3). Trypsin did cause an increase in the neutral zone for both tissues, with bovine seeing a significant increase. This change was significantly different from bovine-saline, with saline having a nominal impact on either tissue.

**Table 1** Posttreatment flexibility parameters.

| | Hysteresis area (HA) (N*m*°) | | Hysteresis (H) (N*m) | | Neutral Zone (NZ) (°) | | Stiffness (K) (N*m/°) | |
|---|---|---|---|---|---|---|---|---|
| | AR | FE | AR | FE | AR | FE | AR | FE |
| Bovine-trypsin | 0.396 (0.0704) | 1.77 (0.226) | 0.0736 (0.0145) | 0.0436 (0.005) | 0.791 (0.0797) | 6.36 (0.714) | 0.144 (0.0271) | 0.0127 (0.0028) |
| Bovine-saline | 0.716 (0.320) | 3.49 (1.268) | 0.13 (0.0687) | 0.0612 (0.0177) | 0.801 (0.116) | 5.41 (1.01) | 0.194 (0.0591) | 0.0155 (0.0044) |
| Cadaver-trypsin | 5.36 (1.113) | 13.17 (3.852) | 2.37 (0.190) | 1.32 (0.166) | 0.552 (0.0963) | 2.39 (0.722) | 7.09 (1.49) | 1.42 (0.462) |
| Cadaver-saline | 5.66 (0.780) | 11.18 (2.975) | 2.57 (0.535) | 1.28 (0.346) | 0.547 (0.0657) | 1.83 (0.913) | 5.49 (1.84) | 1.27 (0.729) |
| Cadaver-fluidless | 3.54 (0.085) | 22.80 (10.575) | 3.13 (0.720) | 1.59 (0.433) | 0.400 (0.0606) | 5.67 (3.01) | 9.73 (0.464) | 0.525 (0.286) |

Values are the mean (standard error) of flexibility parameters in both loading directions for each treatment. Measurements were taken immediately postinjection.
AR = axial rotation; FE = flexion-extension.

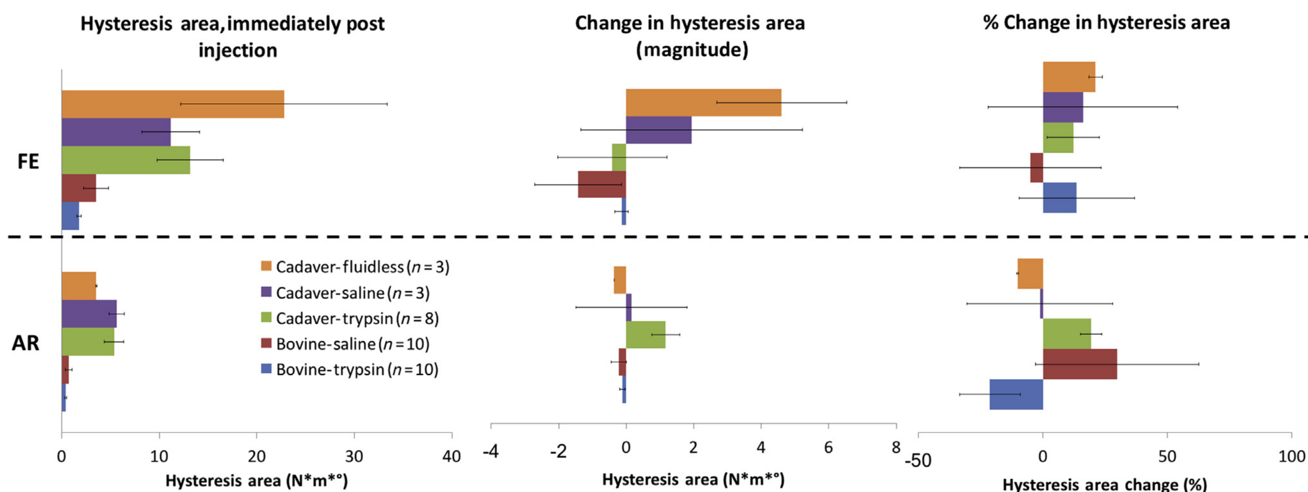



**Figure 1** Hysteresis area effects of each treatment immediately postinjection and then after 3 h incubation at body temperature and 100% humidity (mean with standard error bars). Results showed that fluidless needle stick and foetal bovine serum/saline injections produced significant changes in hysteresis area that were of similar magnitude, but inconsistent direction versus trypsin injections. The testing also demonstrated that cadaveric discs had a different magnitude and even direction of effect consequent to fluid injection as compared with bovine discs. AR = axial rotation, FE = flexion-extension.

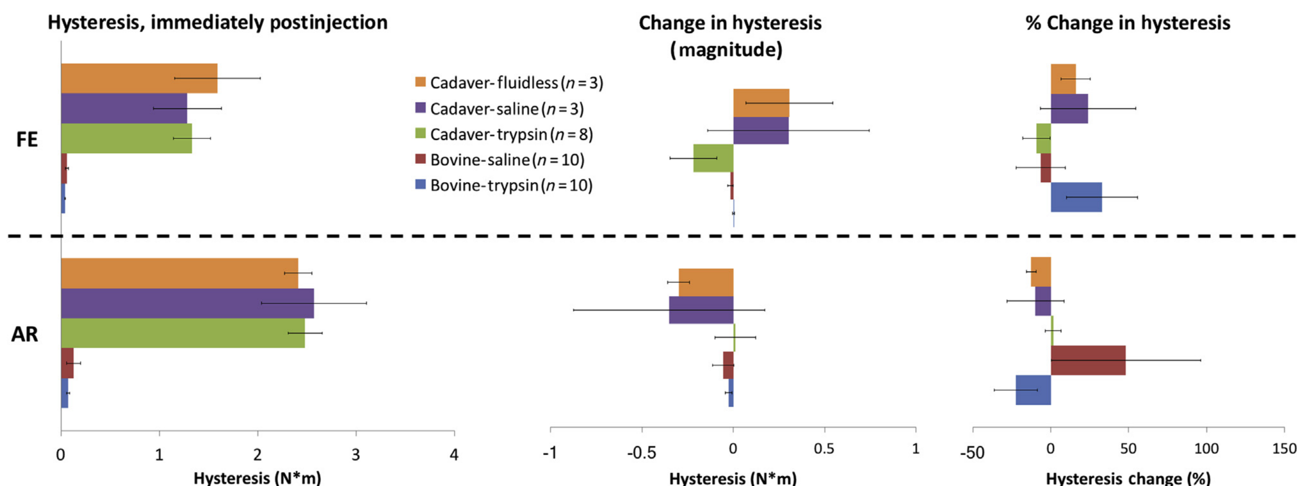



**Figure 2** Hysteresis effects of each treatment immediately postinjection and then after 3 h incubation at body temperature and 100% humidity (mean with standard error bars). Results showed that fluidless needle stick and foetal bovine serum/saline injections produced significant changes in hysteresis that were at least as strong as those from trypsin injections. The testing also demonstrated that cadaveric discs had a different magnitude and even direction of effect consequent to fluid injection as compared with bovine discs. AR = axial rotation, FE = flexion-extension.

Stiffness immediately postinjection was much higher for cadaver samples than bovine tests (Table 1, Figure 4). Cadaver tests underwent a decrease in stiffness during AR over time regardless of injection type, with trypsin causing a significant decrease. The 60.9% decrease for bovine-cadaver stiffness was significantly different from the bovine-saline injections, although neither absolute change was especially large.

## Flexion-extension

As with AR test results, hysteresis and hysteresis area results immediately postinjection were significantly higher for cadaver tissues (Table 1, Figures 1 and 2). Fluidless injections experienced a slight increase over time for hysteresis, and a much more pronounced increase for hysteresis area. Only cadaver-trypsin tests showed any kind of consistent response after 3 h, with a slight decrease in hysteresis but a slight increase in hysteresis area (12.2%).

Bovine neutral zone measurements were much higher than their corresponding cadaver tests, although fluidless injections were approximately equal to bovine results (Table 1 and Figure 3). All injection tests caused an increase in the neutral zone, although only bovine-cadaver changes were statistically significant. Trypsin increases were slightly more pronounced for both tissue types than saline injections.

Stiffness immediately postinjection was much higher for all cadaver tests than for bovine tests (Table 1, Figure 4).

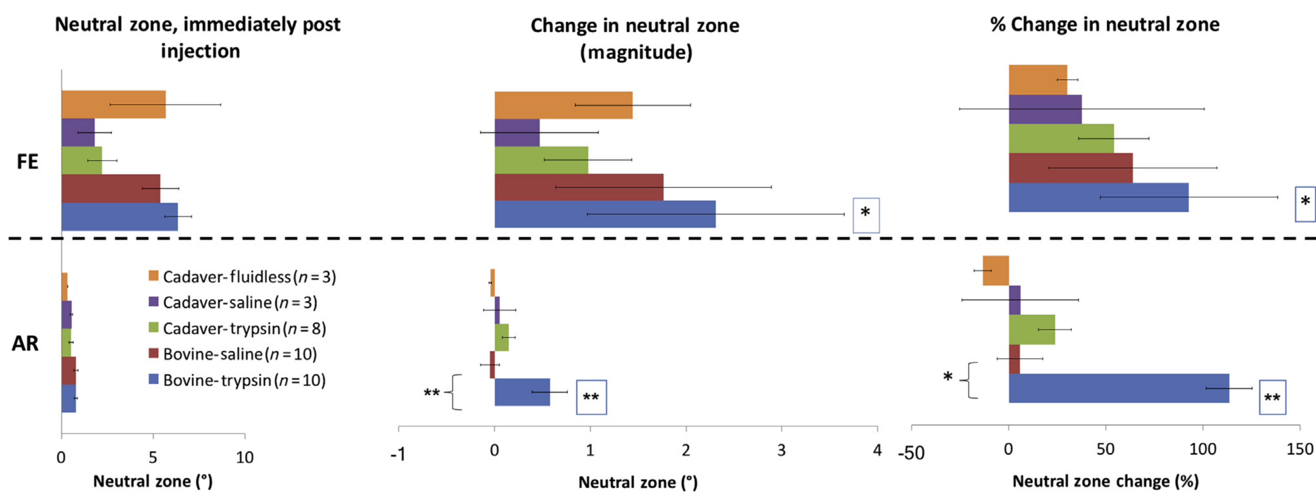



**Figure 3** Neutral zone dimension effects of each treatment immediately postinjection and then after 3 h incubation at body temperature and 100% humidity (mean with standard error bars). Results showed that neutral zone dimensions were consistently changed in a direction consistent with natural disc degeneration in both axial rotation and flexion-extension, and that trypsin had a larger effect than fluidless needle stick or foetal bovine serum/saline injections.* $p < 0.05$. ** $p < 0.001$ (statistically significant symbols in boxes are over time, and symbols with a bracket are between injected treatments). AR = axial rotation, FE = flexion-extension.

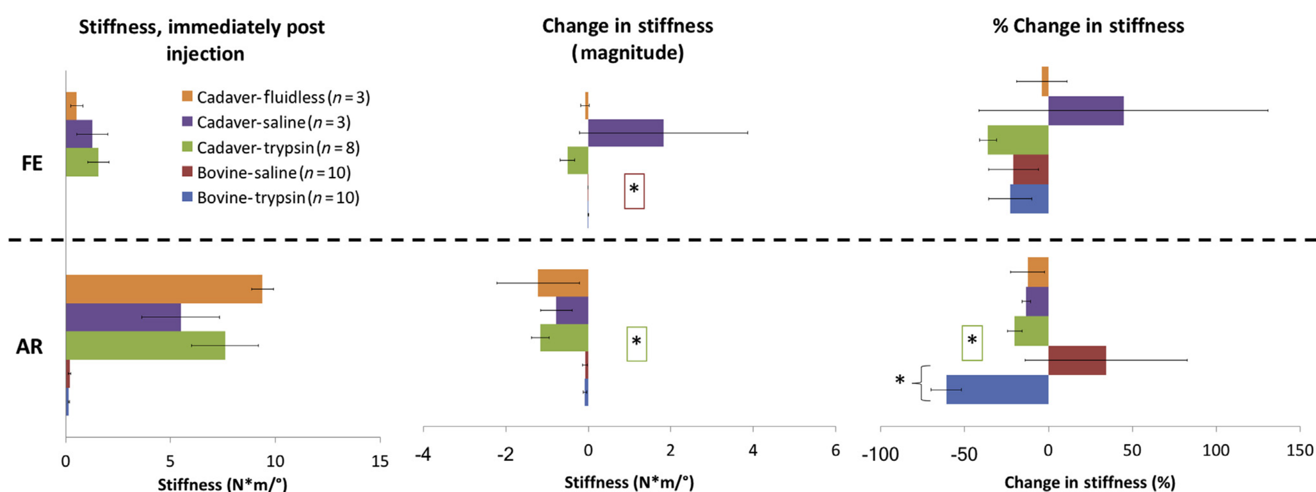



**Figure 4** Segmental stiffness effects of each treatment immediately postinjection and then after 3 h incubation at body temperature and 100% humidity (mean with standard error bars). Results showed that following trypsin injection, segmental stiffness effects were consistently changed in a direction consistent with natural disc degeneration in both axial rotation and flexion-extension. Foetal bovine serum/saline injection results were inconsistent between axial rotation and flexion-extension.* $p < 0.05$ (statistically significant symbols in boxes are over time, and symbols with a bracket are between injected treatments). AR = axial rotation, FE = flexion-extension.

The largest changes in stiffness occurred in the cadaver-trypsin group. Although the absolute change in bovine stiffness results was not large in magnitude, bovine-saline changed enough to be statistically significant. Unlike the two hysteresis parameters and the neutral zone, the stiffness of cadaver FSUs was not truly impacted by the fluidless injections.

Type 3 tests for fixed effects revealed several parameters that underwent statistically significant changes (Table 2). Only stiffness and the neutral zone experienced significant changes. When measuring absolute changes, time caused significant changes in six of the eight affected parameters. Time caused significant changes in percentage in four of the nine affected parameters. Only two of the significant parameters for either absolute or percentage changes were cadaver tests.

## Discussion

As anticipated, basic differences between bovine and cadaver motion characteristics were observed. Bovine coccygeal discs were more flexible, lost less energy to AF matrix fibre friction, and were less viscoelastic than

**Table 2** Statistically significant differences between pretreatment and post-treatment flexibility parameters.

| Type 3 test significant results ($\alpha < 0.05$) for fixed effects (percent changes) | | | | | |
|---|---|---|---|---|---|
| Treatment | | Time | | Time and treatment | |
| AR | FE | AR | FE | AR | FE |
| nz (0.0022) | NZ (0.0496) | nz (0.0019) | nz (0.0025) | nz (0.0041) | |
| k (0.0118) | | K (0.0052) | k (0.0059) | k (0.0164) | |
| **Type 3 test significant results ($\alpha < 0.05$) for fixed effects (absolute changes)** | | | | | |
| Treatment | | Time | | Time and treatment | |
| AR | FE | AR | FE | AR | FE |
| nz (0.0002) | | nz (0.0029) | nz (0.0041) | nz (0.0007) | |
| | | k (0.0059) | k (0.0008) | | |
| | | K (0.0029) | NZ (0.0215) | | |

AR = axial rotation, FE = flexion-extension; H = hysteresis, HA = hysteresis area; K = stiffness; NZ = neutral zone dimension. NZ, K: cadaver results; nz, k: bovine results.
Values are *p* values for fixed effects sorted by loading direction and flexibility parameters. Flexibility parameters listed are statistically significant in the respective loading directions for each effect. Percentage changes are for differences from preconditioning results normalized by percentage, and absolute changes are for differences in magnitude from preconditioning results that are not normalized.

cadaveric lumbar discs. One unexpected observation was the impact of the fluidless injections on cadaver disc mechanics. In both directions for hysteresis, hysteresis area, and the neutral zone, a definitive change was recorded. This suggests that even a small needle puncture into the AF changed the viscoelastic response of the cadaveric disc tissue. Needle diameter choice was based on a study indicating that needles of size 27 ga or smaller do not disrupt mechanical properties in cadaver lumbar discs [24]. However, no hysteresis results were recorded in that study, thus the authors could not comment on changes in hysteresis.

The FSUs underwent more significant changes (i.e., more flexibility parameter changes that reached the level of statistical significance) when undergoing AR motion testing as compared to FE motion testing. One potential reason is the universal torsion experienced by all the AF fibres during motion. This stands in contrast to the sagittal alternation of compression and tension of the AF layers during motion in FE, where only some of the fibres affected by needle puncture, additional fluid pressure, and/or proteolysis were under stress at a particular moment in time. Another possible explanation is the difference in motion curve characteristics, with a much flatter flexibility curve and smaller range of motion allowing for more noticeable changes than the larger, more sigmoidal curve seen during FE.

Our initial hypothesis was that the trypsin injections would induce widespread proteolysis throughout the AF that definitively and predictably altered disc motion mechanics in a manner comparable to that seen in natural degenerative processes. This hypothesis was incorrect. Trypsin and PBS failed to exhibit consistent changes between the different tissues and directions of loading. Differences varied by loading axis, injection treatment, and tissue for all motion parameters. Although a few parameters were significantly altered by the presence of trypsin (neutral zone for bovine in both directions and stiffness for cadaver in AR), the expected widespread change was not observed to be a good match for natural disc degeneration mechanics. The magnitude of change in flexibility parameters was inconsistent across modes of loading as compared to cadaver disc degeneration. Thus, while a protocol could perhaps be developed to produce equivalent changes in some flexibility parameters during flexion-extension mechanics or during axial-rotation mechanics, achieving both at the same time is not easily achievable, and achieving both for all flexibility parameters at the same time is likely impossible.

Another important observation from this study was that great caution is needed when extrapolating a protease disc degeneration model from bovine to cadaver tissues [31,32]. Bovine FSUs appeared to exhibit greater sensitivity to the injections than the cadaver FSUs, suggesting greater robustness of cadaveric discs to needle injections than bovine discs. Additionally, a greater number of flexibility parameters were significantly altered over the course of the incubation time as compared to the cadaveric models.

Separately, we observed that changes in motion response to injection appear to be more dependent on the volume of an injected treatment and the time spent in motion testing, rather than the treatment itself. We also observed that the use of a heated environmental chamber for cadaver tests did not seem to accelerate proteolysis at the protease's optimal temperature [33], likely because the degeneration process was dominated by the rate of fluid diffusion, rather than by the reaction kinetics of the trypsin protease.

A limitation of the present work is that we have specifically examined a single protease (trypsin), over a fairly short protease action window. Previous protease degeneration studies in cadaveric human and animal models [19—23] have varied dramatically in both the choice of protease, as well as the amount of incubation time used to induce degeneration, ranging from several hours for trypsin-based degeneration protocols [19,20] up to 3 weeks for papain-based studies [19]. *In vivo* studies similarly vary widely in incubation time and choice of protease, e.g., [34]. It is possible that the action of some proteases may be a more fidelic degeneration model across multiple modes of loading within a consistent time frame. However, based on our results with trypsin, we find this unlikely, as virtually all reported protease

degeneration protocols result in a similar physical phenomenon observed in the disc tissue at the site of injection post-treatment (i.e., a mechanical defect or hole) [19—23].

Another limitation of the present work is that direct comparison of the bovine versus cadaver results must be done carefully, and is best done qualitatively rather than quantitatively. Geometry and size considerations lead to a slight variation in injection protocol between the bovine and cadaver discs. The bony architecture of the cadaver FSUs prevented direct injections into the posterior portion of the disc. Thus, the most posterior injections into the cadaver discs were angled away from the disc centre in an attempt to more evenly distribute the treatment. This was not the case with bovine discs, where the injections were equally spaced around the entire disc circumference. The amount of injected treatment volume was also dependent on the size of the discs. The cadaver discs received three times the treatment injection volume as compared with the bovine discs. This roughly corresponds to the difference in average disc volume between the specimens. However, normalizing by cross-sectional area (roughly 4×) or not normalizing the volume at all could yield different results.

In summary, the similarities in the size of cadaver lumbar and bovine coccygeal discs do not lead to similarities in their motion characteristics or their response to injected solutions. Thus, despite the numerous published studies that have inferred such a relationship without validating it, we do not recommend the use of a trypsin or protease degeneration model for representing the mechanics of natural human disc degeneration. This recommendation extends to both cadaver and bovine models. Other types of disc degeneration models such as injury models or recently developed natural models for animal disc degeneration [35,36] may provide a better avenue for preclinical evaluation of spinal devices.

## Conflicts of interest

The authors have no conflicts of interest relevant to this article.

## Acknowledgements/Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## References


[1] Adams MA, Roughley PJ. What is intervertebral disc degeneration, and what causes it? Spine 2006;31:2151—61.

[2] Buckwalter JA. Aging and degeneration of the human intervertebral disc. Spine 1995;20:1307—14.

[3] Cs-Szabo G, Ragasa-San Juan D, Turumella V, Masuda K, Thonar EJA, An HS. Changes in mRNA and protein levels of proteoglycans of the anulus fibrosus and nucleus pulposus during intervertebral disc degeneration. Spine 2002;27:2212—9.

[4] Zhao CQ, Wang LM, Jiang LS, Dai LY. The cell biology of intervertebral disc aging and degeneration. Ageing Res Rev 2007;6:247—61.

[5] Guerin HAL, Elliott DM. Degeneration affects the fiber reorientation of human annulus fibrosus under tensile load. J Biomech 2006;39:1410—8.

[6] Marchand F, Ahmed AM. Investigation of the laminate structure of lumbar disc anulus fibrosus. Spine 1990;15:402—10.

[7] Coppes MH, Marani E, Thomeer RT, Groen GJ. Innervation of "painful" lumbar discs. Spine 1997;22:2342—9.

[8] Freemont AJ, Peacock TE, Goupille P, Hoyland JA, O'Brien J, Jayson MIV. Nerve ingrowth into diseased intervertebral disc in chronic back pain. Lancet 1997;350:178—81.

[9] Cavanaugh JM, Ozaktay AC, Yamashita T, Avramov A, Getchell TV, King AI. Mechanisms of low back pain a neurophysiologic and neuroanatomic study. Clin Orthop Relat Res 1997;335:166—80.

[10] Mimura M, Panjabi MM, Oxland TR, Crisco JJ, Yamamoto I, Vasavada A. Disc degeneration affects the multidirectional flexibility of the lumbar spine. Spine 1994;19:1371—80.

[11] Fujiwara A, Lim TH, An HS, Tanaka N, Jeon CH, Andersson GB, et al. The effect of disc degeneration and facet joint osteoarthritis on the segmental flexibility of the lumbar spine. Spine 2000;25:3036—44.

[12] Tanaka N, An HS, Lim TH, Fujiwara A, Jeon CH, Haughton VM. The relationship between disc degeneration and flexibility of the lumbar spine. Spine J 2001;1:47—56.

[13] Boden SD, Davis DO, Dina TS, Patronas NJ, Wiesel SW. Abnormal magnetic-resonance scans of the lumbar spine in asymptomatic subjects. A prospective investigation. J Bone Joint Surg Am 1990;72:403—8.

[14] Jensen MC, Brant-Zawadzki MN, Obuchowski N, Modic MT, Malkasian D, Ross JS. Magnetic resonance imaging of the lumbar spine in people without back pain. N Engl J Med 1994;331:69—73.

[15] An HS, Anderson PA, Haughton VM, Iatridis JC, Kang JD, Lotz JC, et al. Introduction: disc degeneration: summary. Spine 2004;29:2677—8.

[16] Cheh G, Bridwell KH, Lenke LG, Buchowski JM, Daubs MD, Kim Y, et al. Adjacent segment disease following lumbar/-thoracolumbar fusion with pedicle screw instrumentation: a minimum 5-year follow-up. Spine 2007;32:2253—7.

[17] Hilibrand AS, Robbins M. Adjacent segment degeneration and adjacent segment disease: the consequences of spinal fusion? Spine J 2004;4:S190—4.

[18] Schulte TL, Leistra F, Bullmann V, Osada N, Vieth V, Marquardt B, et al. Disc height reduction in adjacent segments and clinical outcome 10 years after lumbar 360 fusion. Eur Spine J 2007;16:2152—8.

[19] Roberts S, Menage J, Sivan S, Urban JP. Bovine explant model of degeneration of the intervertebral disc. BMC Musculoskelet Disord 2008;9:24.

[20] Mwale F, Demers CN, Michalek AJ, Beaudoin G, Goswami T, Beckman L, et al. Evaluation of quantitative magnetic resonance imaging, biochemical and mechanical properties of trypsin-treated intervertebral discs under physiological compression loading. J Magn Reson Imaging 2008;27:563—73.

[21] Chan SC, Bürki A, Bonél HM, Benneker LM, Gantenbein-Ritter B. Papain-induced in vitro disc degeneration model for the study of injectable nucleus pulposus therapy. Spine J 2013;13:273—83.

[22] Gawri R, Moir J, Ouellet J, Beckman L, Steffen T, Roughley P, et al. Physiological loading can restore the proteoglycan content in a model of early IVD degeneration. PLoS One 2014;9:e101233.

[23] Jim B, Steffen T, Moir J, Roughley P, Haglund L. Development of an intact intervertebral disc organ culture system in which degeneration can be induced as a prelude to studying repair potential. Eur Spine J 2011;20:1244—54.

[24] Elliott DM, Yerramalli CS, Beckstein JC, Boxberger JI, Johannessen W, Vresilovic EJ. The effect of relative needle

diameter in puncture and sham injection animal models of degeneration. Spine 2008;33:588—96.

[25] Zirbel SA, Stolworthy DK, Howell LL, Bowden AE. Intervertebral disc degeneration alters lumbar spine segmental stiffness in all modes of loading under a compressive follower load. Spine J 2013;13:1134—47.

[26] Goertzen DJ, Lane C, Oxland TR. Neutral zone and range of motion in the spine are greater with stepwise loading than with a continuous loading protocol. An in vitro porcine investigation. J Biomech 2004;37:257—61.

[27] Patwardhan AG, Havey RM, Meade KP, Lee B, Dunlap B. A follower load increases the load-carrying capacity of the lumbar spine in compression. Spine 2999;24:1003—1009.

[28] Patwardhan AG, Havey RM, Carandang G, Simonds J, Voronov LI, Ghanayem AJ, et al. Effect of compressive follower preload on the flexion—extension response of the human lumbar spine. J Orthop Res 2003;21:540—6.

[29] Stolworthy DK, Zirbel SA, Howell LL, Samuels M, Bowden AE. Characterization and prediction of rate-dependent flexibility in lumbar spine biomechanics at room and body temperature. Spine J 2014;14:789—98.

[30] Thompson JP, Pearce RH, Schechter MT, Adams ME, Tsang IKY, Bishop PB. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. Spine 1990;15:411—5.

[31] Alini M, Eisenstein SM, Ito K, Little C, Kettler AA, Masuda K, et al. Are animal models useful for studying human disc disorders/degeneration? Eur Spine J 2008;17:2—19.

[32] Lotz JC. Animal models of intervertebral disc degeneration: lessons learned. Spine 2004;29:2742—50.

[33] Sriram P, Kalogerakis N, Behie LA. Experimental determination of the rate of autolysis of trypsin at 37 C. Biotechnol Tech 1996;10:601—6.

[34] Norcross JP, Lester GE, Weinhold P, Dahners LE. An in vivo model of degenerative disc disease. J Orthop Res 2003;21:183—8.

[35] Stolworthy DK, Fullwood RA, Merrell TM, Bridgewater LC, Bowden AE. Biomechanical analysis of the camelid cervical intervertebral disc. J Orthop Translat 2015;3:34—43.

[36] Stolworthy DK, Bowden AE, Roeder BL, Robinson TF, Holland JG, Christensen SL, et al. MRI evaluation of spontaneous intervertebral disc degeneration in the alpaca cervical spine. J Orthop Res 2015;33:1776—83.