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Enzymatic Digestion of the Intervertebral Disc Alters Intradiscal Injection and Leakage Mechanics

Zachary Appel
Department of Chemical and Biomolecular Engineering,
Clarkson University,
Potsdam, NY 13699

Arthur J. Michalek[1]
Department of Mechanical and Aerospace Engineering,
Clarkson University,
8 Clarkson Avenue, Box 5725,
Potsdam, NY 13699

Intradiscal injection is required to deliver therapeutic agents to the intervertebral disc (IVD) nucleus pulposus (NP). However, injectate leakage following needle retraction may result in decreased treatment efficacy and adverse side effects. While enzymatic digestion is a common research approach for simulating degeneration in healthy animal IVDs, contributions to the leakage phenomenon are unknown. In this study, bovine caudal discs were treated with injection into the NP of either a tris buffer control, collagenase (to primarily target collagen), or trypsin (to primarily target proteoglycans) and then injected with fluorescent saline using a through-puncture defect protocol. Pressure–volume records during injection were used to determine volume and pressure at leakage. Discs were then frozen, transected, and photographed to visualize injectate dispersion. Collagenase treatment resulted in a large increase in injectate dispersion, along with a decrease in injection pressure relative to control. Trypsin treatment resulted in a moderate increase in dispersion, with no associated effect on pressure. This study concludes that care should be taken when employing enzymatic digestion to simulate IVD degeneration, as NP tissue disruption may affect both retention and dispersion of subsequent therapeutic injections.
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Keywords: enzymatic digestion, intervertebral disc, injectate retention

Introduction

Degeneration of the intervertebral disc (IVD) is a multifactorial condition characterized by regional changes in collagen matrix composition along with loss of proteoglycans and subsequently hydration [1]. In laboratory models of disc degeneration, these effects are frequently simulated or induced via enzymatic digestion [2]. Two commonly utilized enzymes are collagenase and trypsin [3]. Collagenase, which acts by cleaving collagen, is used to disrupt tissue structural integrity [4–8]. Similarly, trypsin acts by cleaving the proteoglycan core protein and is used to reduce tissue swelling pressure [9–11].

As the IVD is the largest avascular organ in the body, needle injection is the most practical means of delivering therapeutic agents, such as stem cells and growth factors. However, it has recently been shown that following injection into the inner nucleus pulposus (NP) injected fluid remains at a high pressure for a significant length of time [12]. This pressurized fluid may then readily leak out of the disc upon retraction of the needle by following the needle track through the anulus fibrosus (AF) [13,14]. The maximum volume which may be delivered without leakage is affected by a number of parameters, including needle diameter, axial compression, and subject movement [15]. In addition to decreased therapeutic efficacy, postinjection leakage has been implicated in adverse events [16–19].

Prior studies on postinjection leakage have utilized healthy bovine caudal intervertebral discs due to their ready availability and low interspecimen variability [12,15]. The inability to inject large volumes of liquid into these discs has been variously attributed to both low permeability [12] and high osmotic swelling pressure [20]. In degenerate IVDs, NP permeability is increased [21], and NP swelling pressure is reduced [22]. As a result, intradiscal injection compliance (defined as volume injected per unit of applied pressure) has been shown to positively correlate with degree of disc degeneration in live humans [23]. However, the effects of enzymatically simulated disc degeneration on injectate retention and leakage remain unstudied.

This study hypothesized that enzymatic digestion of either collagen or proteoglycans would increase both maximum volume and dispersion of intradiscal injectate. This hypothesis was tested by utilizing a through-puncture model of postinjection leakage using bovine caudal IVDs treated with either collagenase or trypsin. Recordings of injectate pressure and volume were used to calculate maximum injectate volume prior to leakage, and fluorescently dyed injectate was used to visualize dispersion.

Methods

A total of 15 specimens were prepared from three adult bovine tails obtained from a local abattoir. Following removal of skin and

[1]Corresponding author.

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muscle, levels cc1-cc2 through cc5-cc6 were isolated by transecting the vertebral bodies transversely at midheight using a hand saw.

Specimens were divided into three groups of five specimens each, such that tails and disc levels were evenly distributed between groups, and each subjected to one of three enzymatic treatment protocols previously used to model degeneration in bovine caudal discs [3]. The first group (control) was injected with 50 mM tris buffer, *p*H7.0, with 1 mmol/L $CaCl_2$. The second group (collagenase) was injected with the same buffer with the addition of 125 mg/mL collagenase (C9891, Sigma-Aldrich, St. Louis, MO). The third group (trypsin) was injected with the buffer with the addition of 125 mg/mL trypsin (T8003, Sigma-Aldrich, St. Louis, MO). All injections were performed from the lateral aspect of the disc, using a 26 g needle into the approximate center of the NP. After approximately 5 s, the plunger on the syringe was retracted to aspirate any unabsorbed injectate. The syringe was then weighed to estimate delivered injectate volume, which averaged 30 ± 7.1 μL with no difference between groups. Following injection, the specimens were wrapped in saline soaked gauze, placed in sealed bags, incubated at 40 °C for 8 h, and then frozen to −20 °C in order to stop further enzyme activity while waiting for injection testing. The incubation time and temperature were found by a pilot study to be sufficient for both enzymes to produce visible cavities in the NP.

Prior to injection testing, specimens were slowly warmed to room temperature. Testing was performed in an order such that average time between removal from the freezer and testing was equal across groups. Injectate retention was tested using a through-puncture model as previously described [15]. The goal of this procedure is to simulate the moment of postinjection needle retraction by first creating a needle track defect through the AF by puncturing all the way through the disc from anterolateral to posterolateral at midheight and then retracting the needle to place its tip at approximately the center of the NP. Needle tip placement is determined to within 1 mm by using a dial caliper to measure disc diameter and retracting the needle until the distance from the needle hub to disc surface equals the needle length minus the disc radius. Fluid is then injected into the center of the NP, while pressure and volume are recorded. Initially pressure increases monotonically with volume, suggesting that a needle retracted below that pressure will not result in leakage. Once the opening pressure of the needle track defect has been reached, pressure drops indicating that needle retraction at or above that volume would be followed by leakage. While this procedure was previously developed [15] using fresh discs, a pilot study indicated no effect of freezing on leakage behavior.

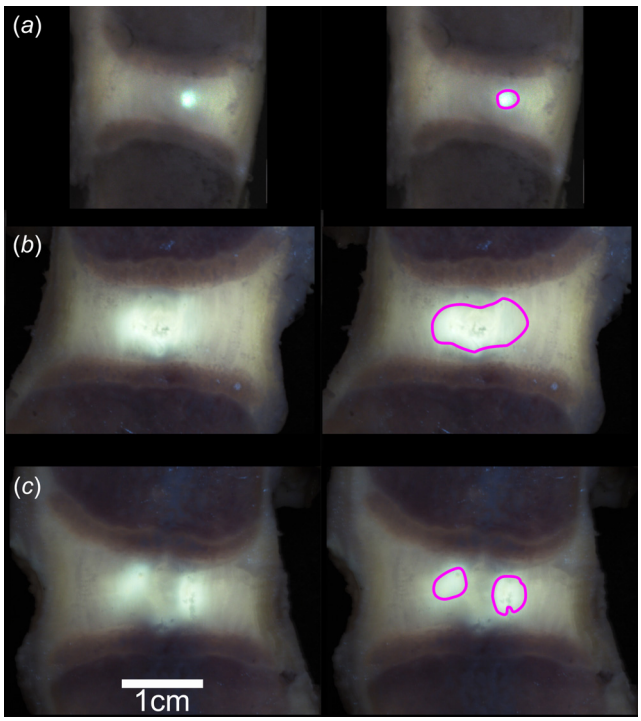

Fig. 1 Representative photographs of control (*a*), collagenase (*b*), and trypsin (*c*) treated discs showing distribution of fluorescent saline. Raw images are shown in the left column, while the right column highlights areas where fluorescence intensity is above background.

In this study, injection was performed using a 21 g needle (38 mm length), and the injectate was 0.15 M phosphate buffered saline with the addition of 0.05 mg/mL 5-DTAF (Sigma-Aldrich, St. Louis, MO) for visualization. The injectate was manually delivered in steps of approximately 5 μL followed by 3-s dwells. The step-dwell procedure was used to ensure that pressure measured in the injector was equal to fluid pressure in the NP, without the confounding effect of Poiseuille drag in the needle. This dwell time was previously shown to be sufficient for needles that were both smaller (30 G) and longer (60 mm) than used here [15]. Both pressure and volume of delivered fluid were sampled at 20 Hz, and pressure–volume traces were analyzed as previously described. Briefly, pressure measured during the last points of each dwell period was extracted, and an adaptive linear fit was used to identify pressure drops or deviations from monotonic increase which indicated leakage events [15]. From these events, the maximum pressure (P_{max}) and volume (V_{max}) delivered to the prior to leakage through the needle track were obtained. As the pressure–volume behavior prior to leakage was linear, injection compliance was defined as V_{max}/P_{max}. Maximum pressure, maximum volume, and injection compliance were compared between groups using a Wilcoxon rank sum test. Maximum volume was further analyzed by fitting with a log-normal cumulative distribution function to predict likelihood of injectate retention.

In order to qualitatively visualize the dispersion of injectate, specimens were frozen to −20 °C immediately following testing. Frozen specimens were transected in the midsagittal plane using a hand saw, and the cut faces were then photographed using a digital camera (E-M5 III, OM Digital Solutions, Tokyo, Japan) with a 50 mm lens. Specimens were illuminated by a 24 W light emitting diode lamp with peak emission between 450 and 460 nm (ABI, Indianapolis, IN), and the camera lens was filtered with a peak wavelength of 550 nm. Exposure time was kept constant for all photographs. Additionally, average disc height and disc diameter were obtained by digitizing these images and used to calculate approximate disc volume.

Effectiveness of enzyme treatment was assessed histologically. After separating one side of each transected disc from its flanking vertebrae, cryosections with 14 μm thickness were taken in the midsagittal plane and placed on coated glass slides (MAS-GP, Masunomi, Osaka, Japan). Sections were stained with for collagen using methyl blue (5 mg/mL; Sigma-Aldrich, St. Louis, MO) and for proteoglycans using safranin O (1 mg/mL; Sigma-Aldrich) as previously described [24]. Following staining, the slides were dried and mounted (Polyglass—Polysciences, Warrington, PA). Slides were then placed on an light emitting diode light panel and photographed using the camera above with a 100 mm lens. Care was taken to maintain exposure and white balance across all images. Images of the NP were analyzed using MATLAB (Mathworks, Inc., Natick, MA) as follows. First, the images were converted into hue-saturation-value space. Then, pixels in which the value channel was less than 0.98 were identified in order to exclude the white background. Histograms of the hue values of the included pixels were then produced and normalized by dividing by the total number of included pixels.

Results

Representative photographs of fluorescent injectate are shown in Fig. 1. Enzymatic treatment had a marked effect on injectate distribution. In control discs (Fig. 1(*a*)), injectate was localized to an area approximately 2 mm across at the point of injection, as

Table 1 Specimen details and pressurization results (median and interquartile range (IQR))

Group	Tail No.	Disc level	P_{max} (kPa)	V_{max} (μL)	Disc volume (μL)	Stiffness (kPa/μL)
Control	1	cc1cc2	26.06	109.13	2838	0.2388
	1	cc4cc5	2.70	310.34	1867	0.0087
	2	cc2cc3	8.10	64.99	2066	0.1246
	2	cc5cc6	20.66	77.32	1226	0.2672
	3	cc3cc4	3.06	264.93	6720	0.0116
		Median (IQR)	8.10 (17.6)	109 (188)	2066 (971)	0.125 (0.227)
Collagenase	1	cc2cc3	0.10	302.61	2311	0.0003
	1	cc5cc6	6.07	212.38	1436	0.0286
*	2	cc3cc4	1.19	181.96	1636	0.0066
*	3	cc1cc2	1.56	200.05	6857	0.0078
	3	cc4cc5	2.23	294.55	5891	0.0076
		Median (IQR)	**1.56 (1.04)**	212 (94.5)	2311 (4255)	**0.00758 (0.00122)**
Trypsin	1	cc3cc4	5.09	262.05	1890	0.0194
	2	cc1cc2	5.97	90.35	2503	0.0661
	2	cc4cc5	8.10	47.71	1228	0.1697
	3	cc2cc3	12.25	268.16	6566	0.0457
	3	cc5cc6	19.31	98.87	4344	0.1953
		Median (IQR)	8.10 (6.28)	98.9 (182)	2503 (2455)	0.0661 (0.124)

*indicates that injectate leakage was observed at the enzyme delivery site. Bold text indicates median significantly ($p < 0.05$) different from control.

demonstrated previously [15]. Collagenase treatment resulted in uniform dispersion throughout the disc (Fig. 1(*b*)), while trypsin treatment (Fig. 1(*c*)) resulted in dispersion throughout large, though still sharply demarcated areas of the NP. These effects were consistent across all five replicates in the three groups.

Specimen details and pressurization test results are provided in Table 1. No trends with tail number or disc level were observed. In 13 specimens, injectate leakage was observed only through the through-puncture defect site, while leakage was also observed through the enzyme delivery site in the other two. Median volume at leakage (V_{max}) was not significantly affected by either enzyme treatment. However, the minimum volume at leakage for collagenase treated discs was approximately three times higher than control and trypsin. Additionally, the lowest V_{max} values in the collagenase group were recorded in two specimens where injectate was seen leaking from the enzyme delivery site. Maximum volume at leakage was consistent across all groups. Leakage pressure (P_{max}) was significantly decreased by collagenase treatment, but not by trypsin. As a result, injection stiffness was significantly decreased by collagenase treatment, but not by trypsin treatment.

While V_{max} was not significantly different between groups, the log-normal fit (Fig. 2) predicted approximately a twofold increase in injectable volume above a 60% likelihood of retention for collagenase treatment relative to control. There was no difference with trypsin treatment.

Representative histological staining of NP tissue is shown in Fig. 3. Control NPs (Fig. 3(*a*)) were largely intact with strong

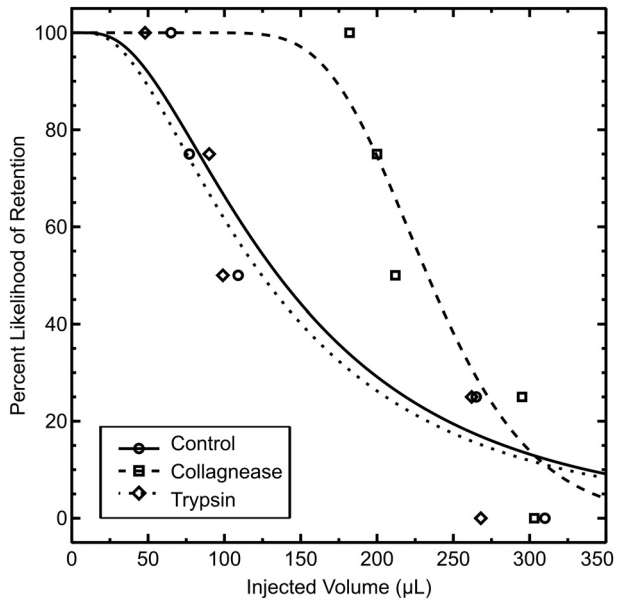

Fig. 2 Likelihood of injectate retention by injected volume

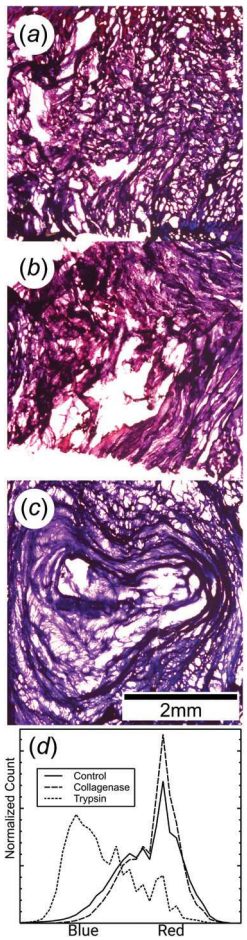

Fig. 3 Representative histological staining of control (*a*), collagenase (*b*), and trypsin (*c*) treated NPs. Red color (safranin O) indicates proteoglycans, and blue (methyl blue) indicates collagen. Normalized hue-space histograms (*d*) show changes in red and blue staining relative to control from both treatments.

staining for both collagen (blue) and proteoglycans (red). Collagenase (Fig. 3(*b*)) treated NPs showed less collagen staining along with significant fissuring. Trypsin treated NPs (Fig. 3(*c*)) showed weak to no safranin O staining along with cavity formation. Relative to control (Fig. 3(*d*)), collagenase treatment resulted in a reduction in blue and an increase in red, while trypsin treatment resulted in an increase in blue and a decrease in red.

Discussion

The results of this study support the hypothesis that NP tissue integrity plays an important role in the mechanics of intradiscal injection. While proteoglycan-driven swelling pressure had previously been suggested as a factor limiting maximum injection volume [20], our results showed that digestion of proteoglycans via trypsin injection had some effect on injectate dispersion, but not on safe injectable volume. Additionally, injection compliance was not increased by trypsin treatment, suggesting that injection pressure is not limited by the need to overcome swelling pressure. It should be noted that even in the control group, maximum pressure before leakage was less than a tenth of the previously reported 300 kPa swelling pressure of the bovine NP [25].

Similarly, low permeability of the healthy NP has been implicated in maintaining high injectate pressure following needle retraction, resulting in leakage [12]. In support of this hypothesis, increasing tissue permeability via collagenase treatment greatly increased injection compliance and resulted in more widespread dispersion of injectate. While median volume at leakage was not affected by collagenase, the increase in minimum observed volume at leakage indicates that a larger volume of fluid may be injected into a more permeable disc while maintaining a low probability of leakage. The lack of an observed effect of enzyme treatment on maximum volume at leakage suggests that this is limited by the AF.

This study used a sequential injection protocol meant to simulate a typical organ culture experiment in which an enzyme is injected to induce degeneration, and a therapeutic agent is injected at a later time point. While this process results in two needle track defects, it was previously shown that the permeability of the healthy NP is so low that even a small distance between the point of fluid injection and an AF defect makes the injection insensitive to the presence of the defect [26]. However, with enzymatic digestion this may not be the case. Enzymatic treatments were delivered using a 26 g needle, which has previously been shown to have minimal leakage risk below 40 μL [15], and care was taken to aspirate excess enzyme solution before needle retraction. While the enzyme delivery sites in the control and trypsin discs were not visible following incubation, those in the collagenase group were visible and large suggesting that enzyme had diffused to the needle track and widened it. As a result of these two factors (decreased permeability and widening of the enzyme delivery track) fluorescent saline was visually observed leaking out at the enzyme delivery site in two of the five collagenase treated specimens during the injection protocol. As this phenomenon occurred in the specimens with the two lowest V_{max} values in the treatment group, a downward bias in median V_{max} may be attributed to it. Leakage through enzyme delivery sites was not observed in control or trypsin treated specimens.

While bulk permeability of NP tissue is increased with degeneration [21], natural degeneration results in a far more heterogenous tissue [27] than results from enzymatic treatment. Additionally, severely degenerated discs may contain annular tears providing secondary leakage pathways similar to those seen in collagenase treated discs in this study [28]. Both heterogeneity of NP permeability and AF tearing likely contributed to the increased variability seen in the pressure response to injection into degenerated human IVDs [23].

Bovine caudal discs were utilized in this study due to their low interspecimen variability and common use as a healthy IVD analogue. When compared to total disc volume, the V_{max} values ranged from 2.3% to almost 15%. Scaling to an average human lumbar disc volume [29], this corresponds to a range of 0.4–2.4 mL, which contains the range of 1–2 mL used in clinical injections [30]. This study was also conducted in the absence of axial compressive load, which was previously shown to increase both P_{max} and V_{max} by effectively pinching the needle track closed [15]. However, as digestion of the NP with either collagenase or trypsin would be expected to reduce disc height, the effect on V_{max} may be offset by reduced NP volume. Interaction between enzymatic digestion and axial load remains an important avenue of future study.

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Data Availability Statement

The datasets generated and supporting the findings of this article are obtainable from the corresponding author upon reasonable request.

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