

Evaluating an Interleukin-1 β Injection to Induce Degeneration in the Rat Lumbar Intervertebral Disc

NSF Summer Undergraduate Fellowship in Sensor Technologies
Alta Berger (BME) – George Washington University
Advisors: Amy Orlansky, MS, and Dawn Elliot, PhD

ABSTRACT

Early signs of degenerate intervertebral discs include a decrease in the glycosaminoglycan (GAG) content within the nucleus pulposus and the annulus fibrosus, which has been linked to an alteration in the mechanics of the disc. However, the mechanisms for this reduction in GAG are currently unknown. An animal model that demonstrates disc degeneration would be useful not only for understanding the process of degeneration, but also for exploring methods of treatment or regeneration. Specifically, this study aimed to develop an *in-vivo* murine model resembling early intervertebral disc degeneration by introducing Interleukin-1 β (IL-1) into the lumbar spine. IL-1 was injected into the nucleus pulposus of the lumbar intervertebral discs of rats, and these discs were evaluated using mechanical testing, biochemical assays, and histology at one and four weeks post-injection. A control group and a sham injection of PBS were used to evaluate the significance of the IL-1 injection. At one week, a reduction was seen in nucleus GAG content in the IL-1 discs. At four weeks, glycosaminoglycan restoration was seen in the IL-1 β treated discs, suggesting the possibility of recovery of the disc.

TABLE OF CONTENTS

1. INTRODUCTION	2
2. BACKGROUND	2
2.1 The Intervertebral Disc: Composition	3
Figure 1. An intervertebral disc. [5].....	3
2.2 The Intervertebral Disc: Degeneration.....	3
2.3 Glycosaminoglycan.....	4
2.4 Chondroitinase-ABC.....	4
2.5 Interleukin-1.....	4
2.6 Histology.....	4
3. MATERIALS AND METHODS.....	4
3.1 Study Design.....	4
3.2 Surgery.....	5
3.3 Mechanical Testing.....	5
3.4 Biochemistry	6

3.5 Histology.....	6
4. RESULTS.....	7
4.1 Disc Mechanics.....	7
4.2 Disc Biochemistry.....	9
4.3 Disc Histology.....	11
5. DISCUSSION.....	14
6. CONCLUSION.....	15
7. RECOMMENDATIONS.....	16
8. ACKNOWLEDGEMENTS.....	16
REFERENCES.....	16

1. INTRODUCTION

Intervertebral disc degeneration is currently recognized as a major cause of debilitating lower back pain, affecting millions of people worldwide. Despite the prevalence of this health concern, little is known about the cause of this degeneration. To date, treatments exist to cope with degenerated discs (such as disc removal and spinal fusion), but no methods have been developed to stop or reverse the process of degeneration. With age, biological alterations in the intervertebral disc (IVD) occur which lead to a decreased ability to absorb force. Degeneration begins in the nucleus pulposus (NP) of the disc [1], and therefore an animal model that mimics early NP alterations would be useful to understand progression and to develop treatments. Previous studies have shown that a loss of glycosaminoglycan (GAG) content in the intervertebral discs correlates to a decrease in neutral zone stiffness, and to an increase in neutral zone displacement and range of motion [2]. The decrease of GAG content in the nucleus pulposus significantly affects the mechanical function of the intervertebral disc and therefore may contribute to degeneration of the disc.

Although a decrease in GAG content has been linked to disc degeneration, the reason for this reduction still remains unclear. Interleukin-1 (IL-1), a naturally occurring cytokine, has been shown to play a role in cartilage degradation [3]. Specifically, it has been associated with the destruction of aggrecan. A connection between IL-1 and the decline of GAG would suggest the possibility of inhibitors capable of preventing or reversing degeneration and thereby alleviating lower back pain.

The objective of this study was to develop an *in-vivo* model in the rat lumbar spine mimicking disc degeneration possibly brought on by interleukin-1. Specifically, GAG content and mechanical properties were analyzed. This data helped to describe the relationship between IL-1 and disc degeneration.

2. BACKGROUND

2.1 *The Intervertebral Disc: Composition*

In the spine, the soft tissue structures located between the bony vertebral bodies are known as intervertebral discs. These discs are made up of two components: the nucleus pulposus and the annulus fibrosus (AF). The nucleus pulposus, located at the center of the disc, has a gelatinous consistency. Surrounding the NP is the annulus fibrosus, which consists of highly organized fibers. Figure 1 demonstrates this organization of the IVD. The disc has visco-elastic properties, with the NP responsible for the viscous behavior and the AF responsible for the elastic response.

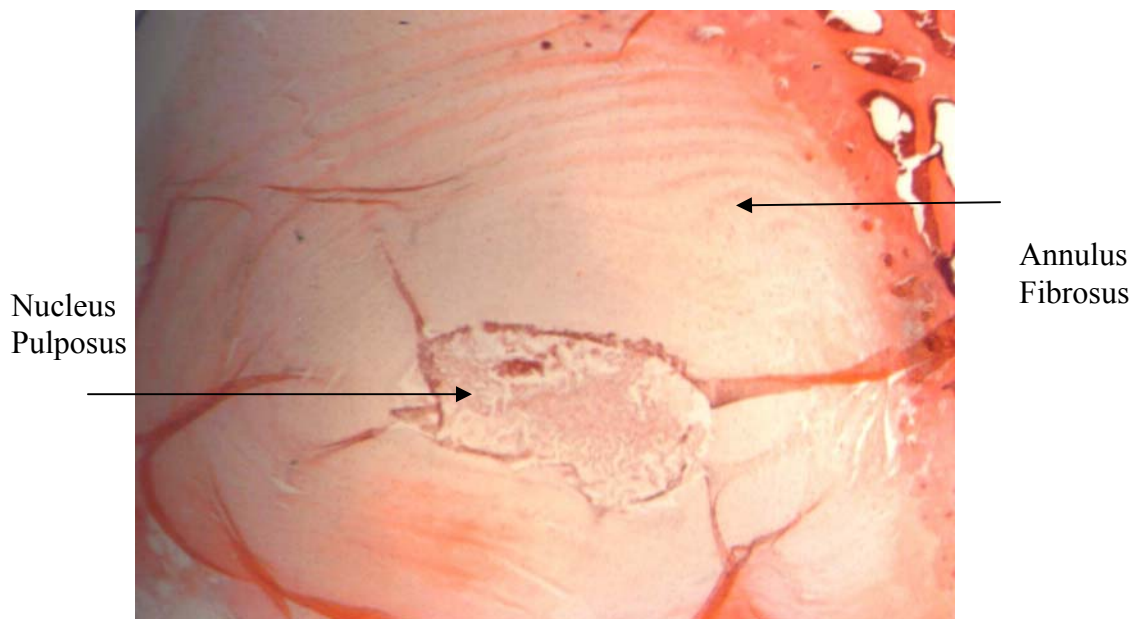


Figure 1. An intervertebral disc.

2.2 *The Intervertebral Disc: Degeneration*

The clinical relevance of disc degeneration is paramount; at least 70% of the population will suffer from lower back pain during their lives [1]. Degeneration typically begins in the NP, with alterations in the types and proportions of aggrecans and collagens. Specifically, there is a shift from the production of type II collagen to type I collagen. Also, large aggregating proteoglycans are broken down, which results in a reduction of GAG content in the NP. These changes reduce the water content of the NP, which causes the NP to lose some of its gelatinous consistency. With age, the fibers of the AF become disorganized and are vulnerable to tears. As the NP loses its ability to dissipate force, the AF becomes responsible for absorbing a greater amount of force. With degeneration come a loss of mechanical function and a reduced ability to withstand load in the disc.

2.3 Glycosaminoglycan

Glycosaminoglycans are long, unbranched polysaccharides with a high affinity for water. A reduction in the GAG content of the nucleus pulposus is an early marker of intervertebral disc degeneration. A decrease in GAG content correlates to a loss of water content in the NP, which will lower the osmotic pressure of the disc [5]. This results in a decreased ability to withstand load, shown by a decrease in neutral zone stiffness and an increase in both range of motion and in neutral zone displacement.

2.4 Chondroitinase-ABC

Aggrecan, which is the major proteoglycan in the IVD, contains chondroitin sulfate side chains [5]. Chondroitinase-ABC (ChABC) is an enzyme that digests chondroitin sulfate isomers, and therefore has previously been used to induce degeneration in the disc [11]. Injecting discs with ChABC significantly reduced GAG content of the NP, which corresponded to reduced mechanical ability [5, 13]. While ChABC aptly stimulates the changes associated with disc degeneration, it is not naturally occurring in animals and therefore is not a possible cause of degeneration.

2.5 Interleukin-1

Le Maitre and coworkers showed that both degenerate and non-degenerate IVDs produce interleukin-1 [4]. The presence of IL-1 has been linked to the presence of matrix degrading enzymes as well as a decrease in the production of proteoglycans and type II collagen. An *in-vivo* model of reduced GAG content due to IL-1 could be used for future studies of treatment involving inhibiting IL-1 in the disc.

2.6 Histology

Numerous histological grading systems have been developed to classify the degree of degradation of intervertebral discs. These scoring systems often consider the annulus fibrosus, the border between the AF and NP, the matrix of the NP, and the cellularity of the NP [9, 10, 11, 12]. With degeneration, the fibers of the annulus become disorganized, and the border between the AF and the NP becomes less clearly defined. In addition to this, the NP becomes more populated with chondrocytes while the abundance of notochordal cells decreases with degeneration.

3. MATERIALS AND METHODS

3.1 Study Design

This study included ten skeletally mature Sprague Dawley rats (age 7-9 months). Animals were assigned to either a 1-week or 4-week time point. Each group had rats assigned to receive a 1 μ L injection of rh-IL-1 β (n=4), a one μ L injection of 1X phosphate-buffered saline (PBS) (n=4), or no injection (n=2). Within each animal, the L3L4, L4L5, and L5L6 discs were injected.

3.2 *Surgery*

The rats were operated on using aseptic technique. Via inhalation, the rats were anesthetized and placed in a supine position. The lumbar spine was reached using an anterior approach. A custom made 33-gauge needle attached to a microsyringe was inserted anteriorly to a controlled depth of 2 mm for the injections. This specific depth positioned the needle tip approximately in the center of the nucleus pulposus. The abdominal wall of the rat was closed and the rats were monitored for 45 minutes while they recovered. After this time period, the rats were returned to normal housing. Animals were euthanized at 1 week or 4 weeks after surgery and motion segments were harvested. The motion segments were then stored until testing in PBS at -20°C.

3.3 *Mechanical Testing*

The motion segments were thawed for one hour in room temperature PBS. The mechanical testing consisted of axial compression-tension cyclic testing followed by a compressive creep test. In order to ensure tissue hydration, testing was done in a 37°C PBS bath. The motion segments were gripped using customized microvises attached to an Instron 5542 testing system (Instron, Canton, MA). Compression-tension testing involved 20 cycles of loading from 4.5 N compression to 3 N tension at a frequency of 0.1 Hz. Following the cyclic loading was a ramp from 0 to 4.5 N compression and a compressive creep test with an applied load of 4.5 N for 45 minutes. After the testing samples were rehydrated in a room temperature PBS bath for 1 hour and then returned to -20°C for storage until biochemical analysis.

Data from the final cycle of compression-tension were analyzed using a trilinear fit model [2, 6, 7, 8] to obtain values for compressive, tensile, and neutral zone stiffness along with cyclic range of motion and neutral zone displacement. The final cycle was analyzed to ensure that the sample had gone through adequate preconditioning, eliminating the effects of super hydration that could have occurred from the free swelling in PBS during the treatment protocol [2, 6, 7, 8].

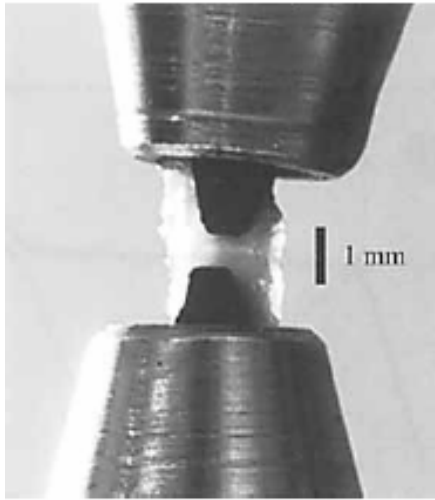


Figure 2: Customized microvises gripping bone-disc-bone motion segment. [8]

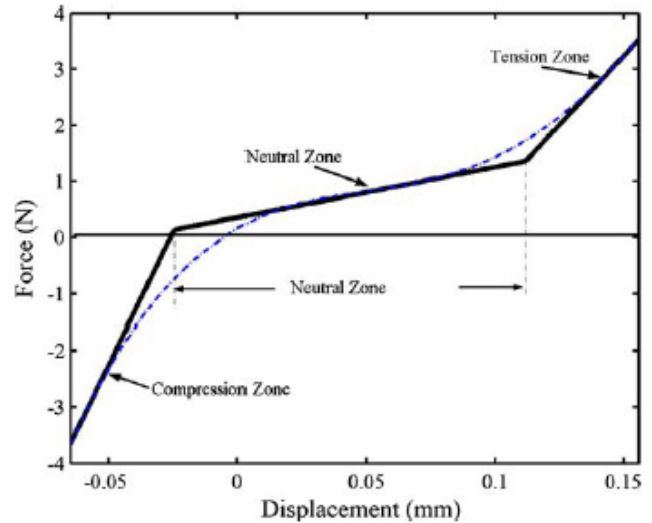


Figure 3: Representative force displacement curve showing the three loading zones: tension, compression, and the neutral zone. [8]

3.4 Biochemistry

Samples were analyzed for glycosaminoglycan content using the 1-9 dimethylmethylene blue (DMMB) binding assay. The discs were isolated from the vertebral body using a scalpel immediately upon removal from -20°C . The separated discs were put onto a freezing stage for 2 minutes to prevent the loss of nucleus pulposus as well as to maintain a frozen state [2]. A hollow 1.5-mm-diameter dermal biopsy punch (Miltex Instrument Comp., Bethpage, NY) was used to remove the entire nucleus pulposus from the isolated disc. A hollow 3.6 mm X 2.5 mm elliptical biopsy punch was used to isolate the inner annulus fibrosus, and the remaining disc tissue was considered to be the outer annulus fibrosus. The wet weights of the tissues were measured and the samples were then dehydrated at 65°C for 24 hours. Dry weight was then obtained and water content was calculated as $\%H_2O = (WW - DW) / WW$. This was followed by tissue digestion using a 5 mg/mL proteinase K solution at 65°C for 18 hours. Finally, the spectrophotometer was used to determine the GAG content based on the color reaction with the DMMB assay.

3.5 Histology

After fixation and decalcification the discs were embedded in paraffin for histological analysis. Axial sections of the disc were taken at a thickness of $7\ \mu\text{m}$. Slides were then stained with either alcian blue and picosirius red or hematoxylin and eosin and were

analyzed for disc organization and cellularity. The specific stains used, picosirius red and alcian blue, stain red for collagen and blue for glycosaminoglycan.

4. RESULTS

4.1 Disc Mechanics

A significant portion of the one week mechanical data was compromised due to difficulties encountered during mechanical testing. The 4 week data showed that the IL-1 injected discs had a smaller neutral zone length and range of motion and greater neutral zone stiffness as compared to the PBS injected discs.

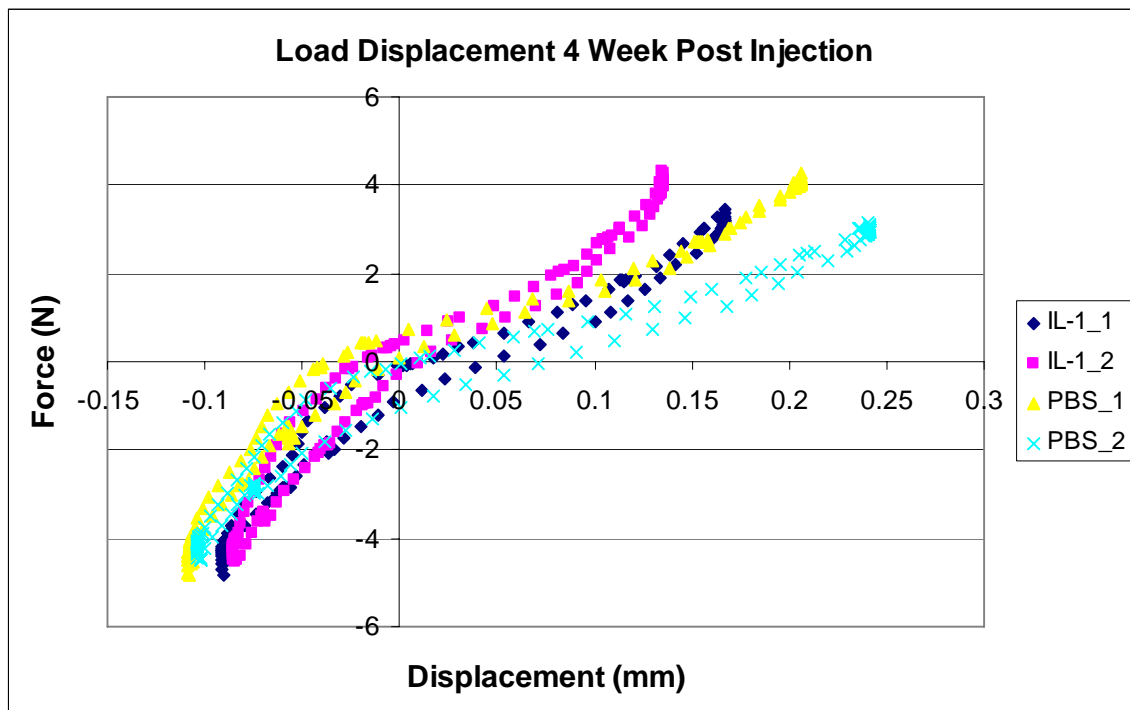


Figure 4: Load displacement curves from mechanical testing

<i>Rat Intervertebral Disc</i>	<i>Neutral Zone Length (mm)</i>	<i>Neutral Zone Stiffness (N/mm)</i>	<i>Range of Motion (mm)</i>
IL-1_1 L3L4	0.161	18.56	0.258
IL-1_2 L3L4	0.112	19.33	0.220
IL-1 Average	0.1365	18.94	0.239
PBS_1 L3L4	0.179	12.40	0.315
PBS_2 L3L4	0.213	11.62	0.345

PBS Average	0.196	12.01	0.330
--------------------	--------------	--------------	--------------

Table 1: 4 week mechanical data.

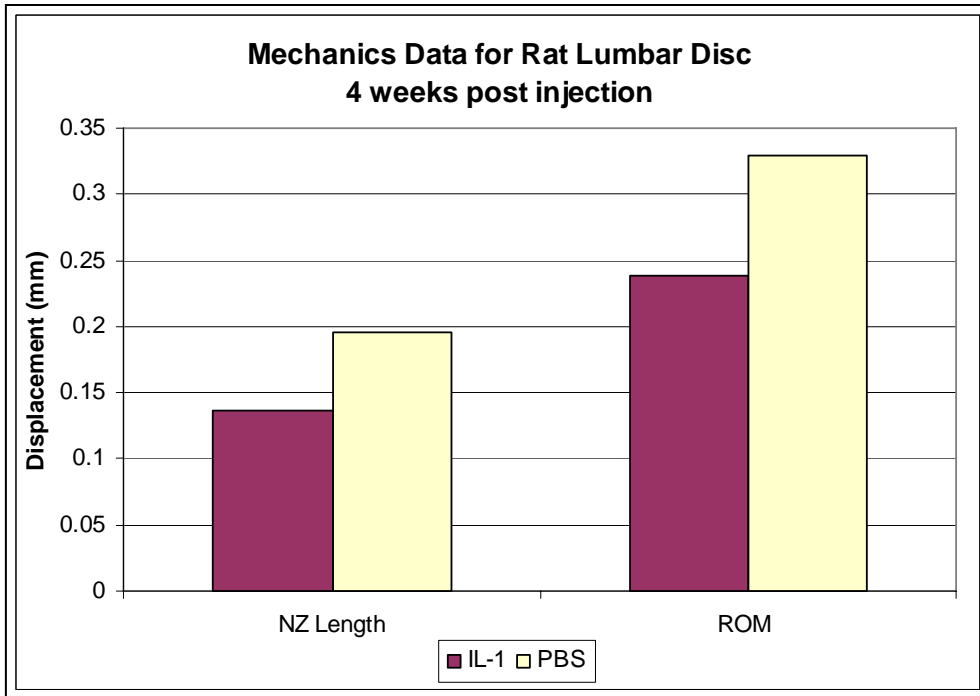
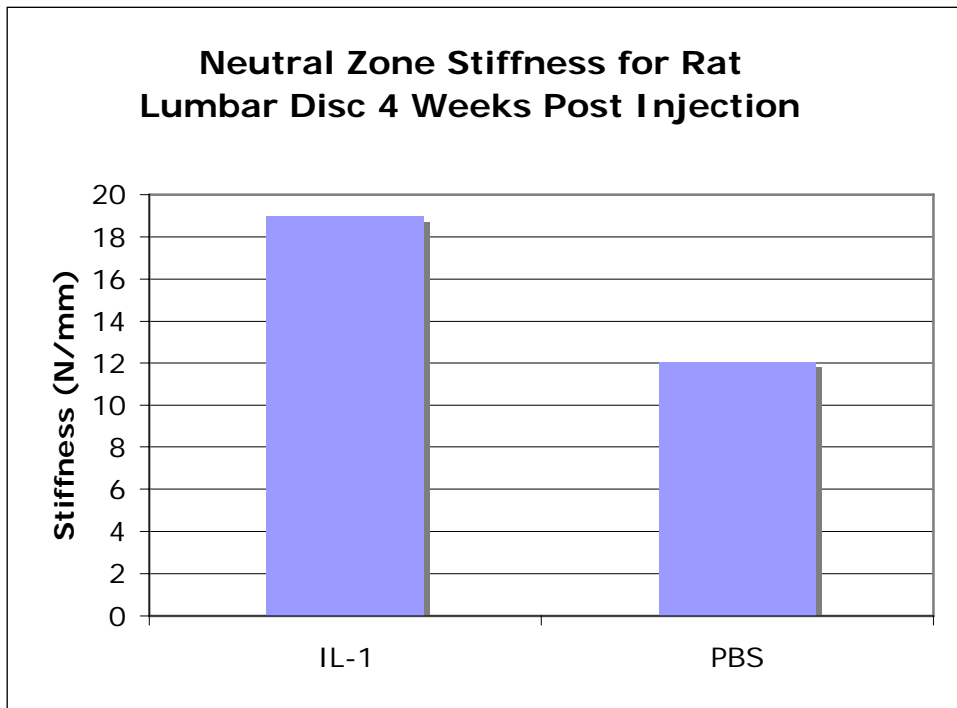
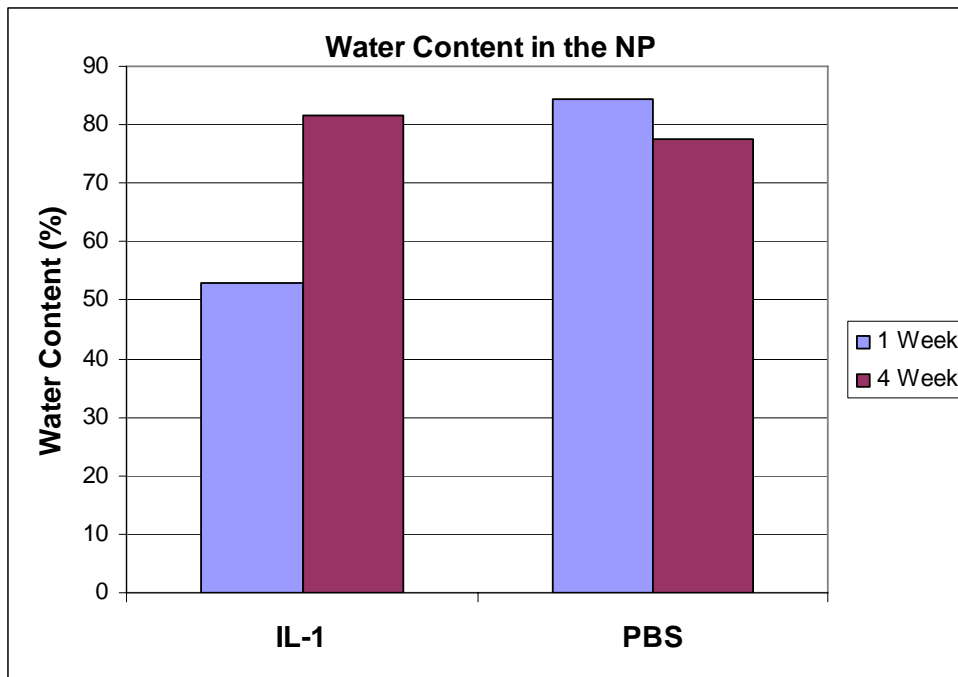


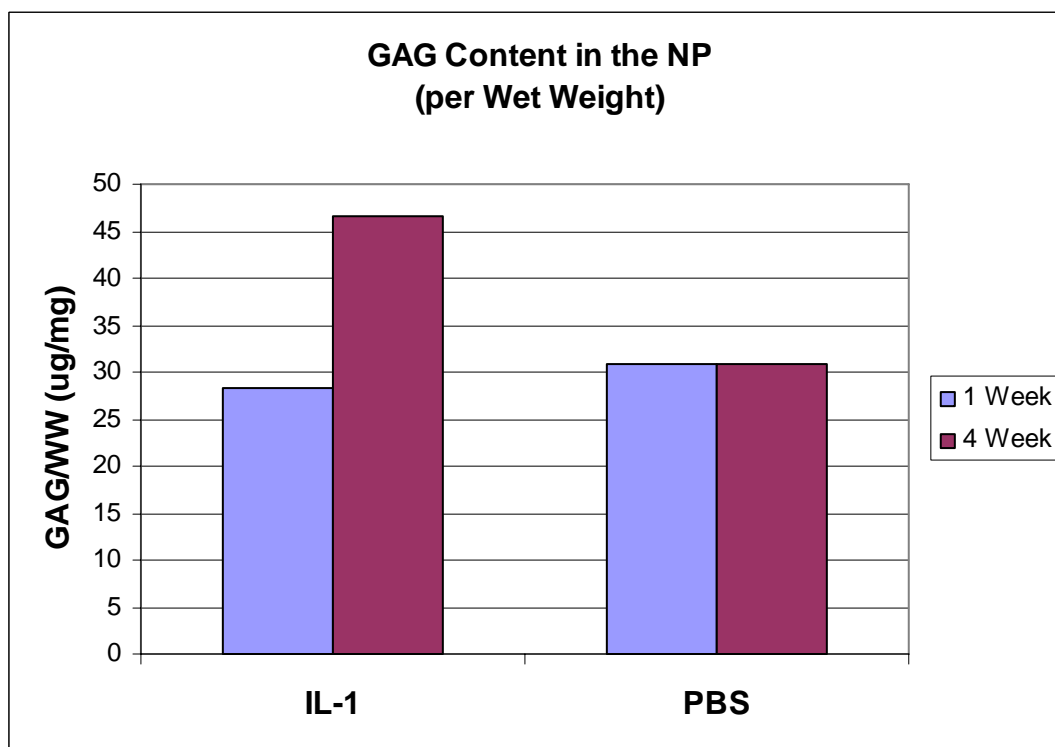
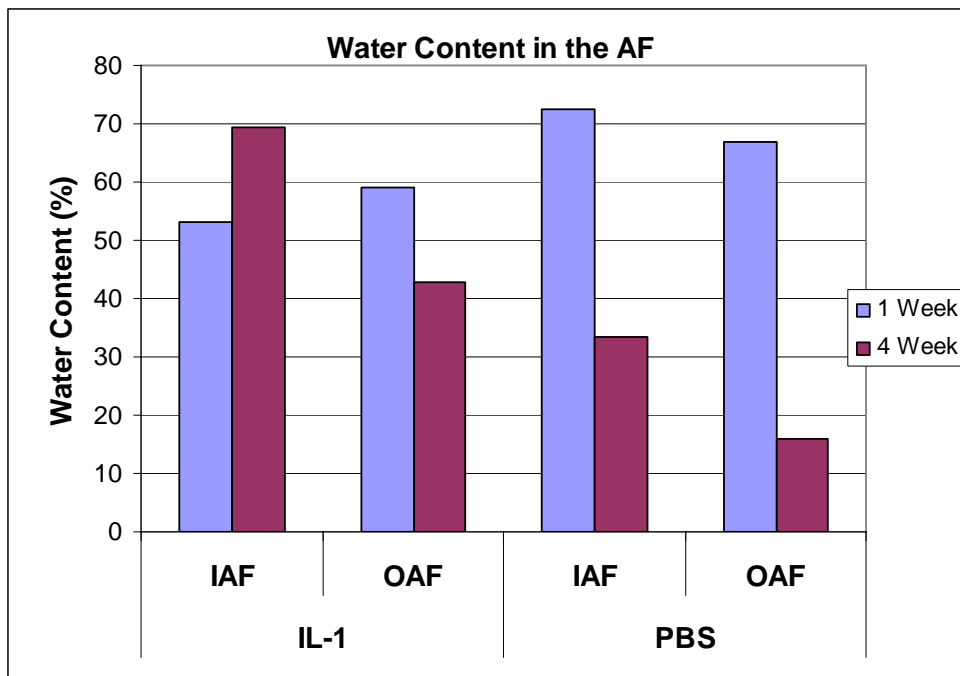
Figure 5: Mechanics results for 4 week rats.



4.2 *Disc Biochemistry*

After 1 week, there was a significant drop in the water content in the NP of the IL-1 injected discs as compared to the PBS injected discs. In comparing GAG content, there was an 8.63% reduction in the IL-1 NP versus the PBS NP. At four weeks, the IL-1 discs had higher water content and a 51.23% increase in GAG content compared to the PBS discs.





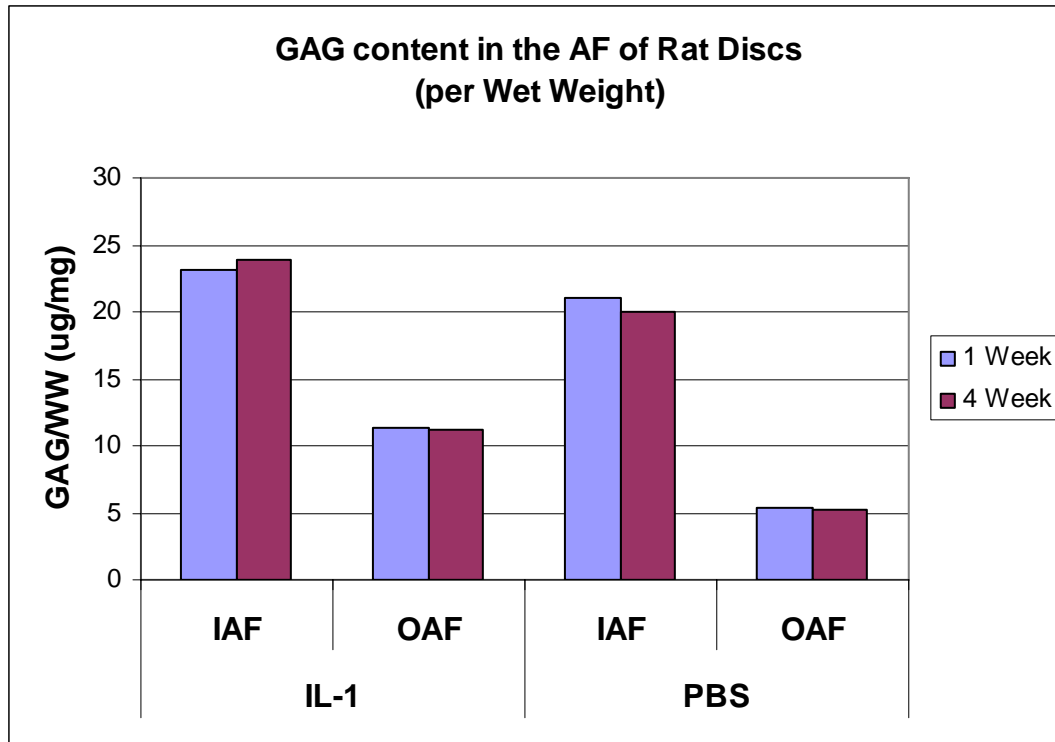


Figure 6: Biochemical Data: Water and GAG content 1 and 4 weeks post injection.

4.3 Disc Histology

The following slides were stained with alcian blue and picosirius red. The nucleus pulposus, composed mostly of glycosaminoglycan, stained a bright blue while the annulus fibrosus, which is comprised of collagen, stained red. The slides were analyzed for signs of degradation.

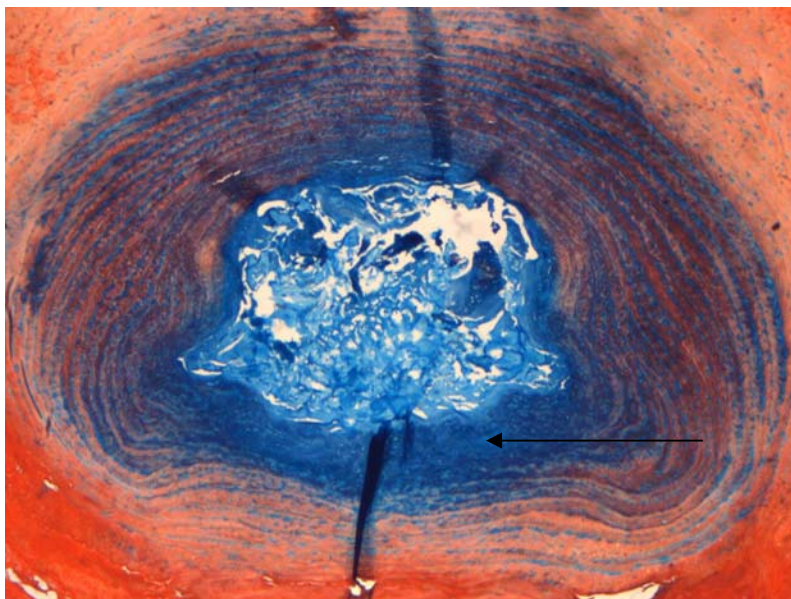


Figure 7: IL-1 injected, 1 week. The black arrow indicates the decreased ability to distinguish the NP-AF border that comes with degeneration.



Figure 8: PBS injected, 1 week. The white area seen is due to tearing which occurred while sectioning the disc for histology and is not a result of degeneration.

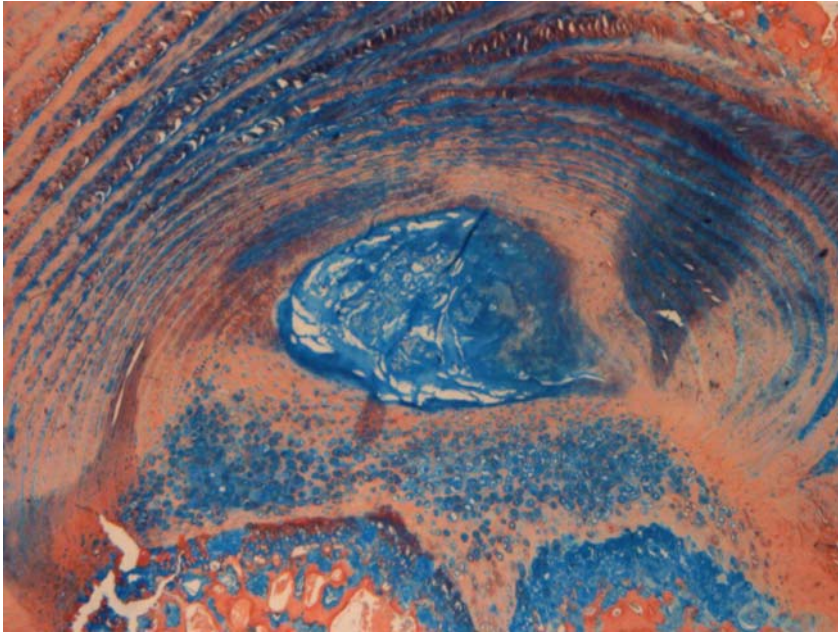


Figure 9: No injection, 1 week. The spotted area at the bottom of the disc is the endplate.

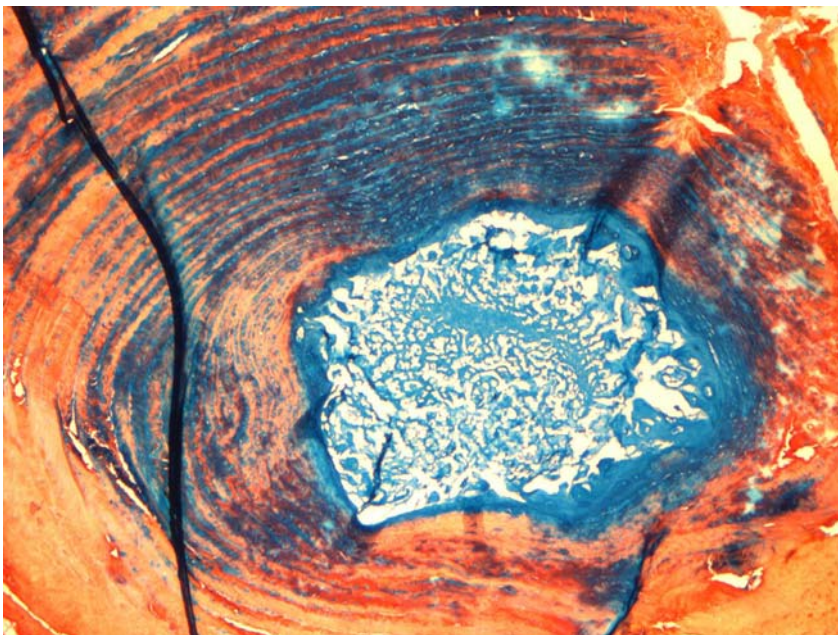
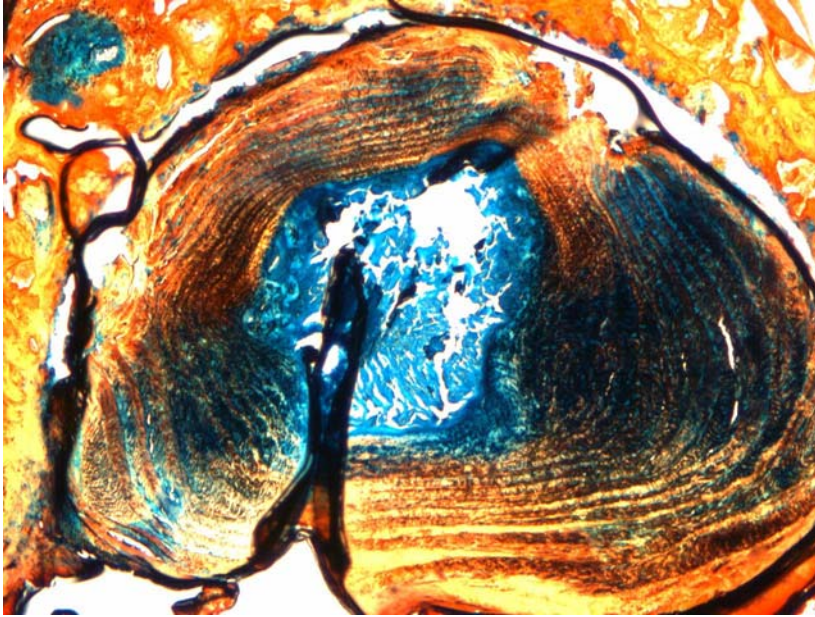
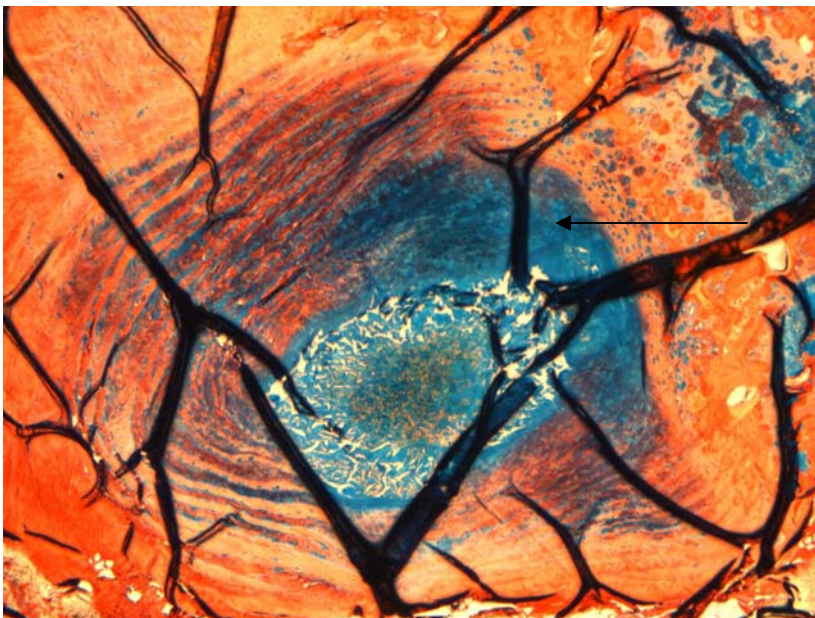


Figure 10: PBS injected, 4 week.



*Figure 11:
No injection,
4 week.*



*Figure 12: IL-1
injected, 4 week.
The dark lines seen
are folds in the
tissue due to the
process of creating
the slides. The black
arrow is used to
highlight the
decreased ability to
determine the border
between the NP and
the AF.*

5. DISCUSSION

The 8.63% change in GAG content seen in the IL-1 discs as compared to the PBS discs supported the hypothesis that this cytokine would affect the composition of the NP. As predicted, the water content was connected to the GAG content in the discs. While the mechanical data from the 1 week group was lost due to problems with testing, previous studies have shown a correlation between GAG content and mechanical function [2, 13]. Therefore, one would expect that the IL-1 injected discs would show an increase in the

range of motion and neutral zone length as well as a decrease in the neutral zone stiffness. Histology from the 1 week discs indicate signs of degeneration in the IL-1 injected discs. Specifically, the IL-1 disc exhibits a blue area that does not appear to be part of the nucleus, but is also not considered annulus (indicated by the black arrow in figure 7). This corresponds to the decreased ability to distinguish the border between the NP and the AF seen in degenerate discs. In addition to this, while the fibers of the AF in the control and PBS injected discs are organized around the NP, the AF of the IL-1 disc contains portions with a serpentine pattern. This disorganization is characteristic of degenerate discs.

The drastic increase in GAG content seen in the 4 week IL-1 injected discs suggests the possibility of recovery from the matrix degrading effects of the cytokine. One possible explanation of this increase is that the presence of IL-1 may be causing an increase in IL-1 receptor antagonist, which would allow it to further regulate IL-1. If IL-1 acts as a foreign substance when it is injected, cells may produce antibodies against it in order to prevent further attack. This increase in antibodies, or IL-1 RA, could be responsible for the increase in GAG by inhibiting the effect of IL-1 on the proteoglycans. This reaction could be tested by checking for the presence of IL-1 and IL-1 RA in the discs. For future studies, it will be important to distinguish between the presence of IL-1 and the activity of IL-1. It is possible that, although the IL-1 remained in the discs 4 weeks post injection, the cytokine was no longer active after this period of time. Because the IL-1 did not produce a lasting effect on the discs, a different method needs to be developed to ensure that the discs are experiencing a constant process of degeneration. This could be achieved by using beads that will release a certain amount of the cytokine over time.

The mechanical data corresponds to the biochemical results; an increase in GAG content in the IL-1 discs led to an increase in neutral zone stiffness and a decrease in range of motion and neutral zone length compared to the PBS discs. In the histology from the week 4 discs, the border between the NP and AF was somewhat difficult to establish in the IL-1 injected disc, shown by the arrow in figure 12. The 4 week PBS injected disc also contains an area where it is hard to distinguish between the NP and AF. All three of the discs show organized fibers in the AF.

6. CONCLUSION

There was a decrease in the levels of GAG seen in the 1 week IL-1 injected rats, supporting the idea that IL-1 plays a role in disc degeneration. While the 4 week mechanical data corresponded to the biochemical data, with $n=2$ the need for a larger study is great in order to draw significant conclusions. The recovery of GAG levels in the IL-1 injected discs of the 4 week rats indicates that a method needs to be developed that can release the IL-1 into the NP over time. Although histology remains a somewhat subjective area, certain markers of degeneration can be identified. These include the decreased ability to distinguish the border between the NP and the AF as well as the disorganization of the AF.

7. RECOMMENDATIONS

A necessary continuation for this study would be to include a greater number of subjects. In addition to this, a method needs to be developed to ensure that the IL-1 produces a lasting effect on the discs. A possible way to achieve this would be to encapsulate the IL-1 in such a way that it would be released periodically. In addition, the activity of IL-1 should be monitored to distinguish between remaining in the disc and staying active in the disc.

8. ACKNOWLEDGEMENTS

I would like to thank my advisors, Amy Orlansky and Dawn Elliot, for their advice and helpfulness. The process used in this study was developed through the McKay lab and is detailed in previous studies [2, 6, 7, 8, 13]. I would also like to thank the National Science Foundation for providing continued support to the SUNFEST program, which has provided me with a great experience and exposure to interesting research.

REFERENCES

- [1] A.J. Freemont, A. Watkins, C. Le Maitre, M. Jeziorska, J.A. Hoyland. (2002) Current Understanding of Cellular and Molecular Events in Intervertebral Disc Degeneration: Implications for Therapy. *Journal of Pathology* [Online]. 196, 374-379. Available: 10.1002/path.1050
- [2] John I. Boxberger, Sounok Sen, Chandra S. Yerramailli, Dawn M. Elliot. (2006, Sep.) Nucleus Pulposus Glycosaminoglycan Content Is Correlated with Axial Mechanics in Rat Lumbar Motion Segments. *Journal of Orthopaedic Research* [Online]. 24, 1906-15. Available: 10.1002/jor.20221
- [3] Jacques Claire, Marjolaine Gosset, Francis Berenbaum, Cem Gabay. (2006) The Role of IL-1 and IL-1 RA in Joint Inflammation and Cartilage Degradation. *Interleukins* [Online]. 74,371-403. Available: 10.1016/S0083-6729(06)74016-X
- [4] Christine Lyn Le Maitre, Anthony J. Freemont, Judith Alison Hoyland. (2005, Apr.) The Role of Interleukin-1 in the Pathogenesis of Human Intervertebral Disc Degeneration. *Arthritis Research & Therapy* [Online]. 7, R732-R745. Available: 10.1186/ar1732

- [5] Alessandra Colombini, Giovanni Lombardia, Massimiliano Marco Corsi, Giuseppe Banfi. (2008) Pathophysiology of the Human Intervertebral Disc. *The International Journal of Biochemistry & Cell Biology* [Online]. 40, 837-842. Available: 10.1016/j.biocel.2007.12.011
- [6] C.S. Yerramalli, A.I. Chou, G.J. Miller, S.B. Nicoll, K.R. Chin, D.M. Elliot. (2007) The Effect of Nucleus Pulposus Crosslinking and Glycosaminoglycan Degradation on Disc Mechanical Function. *Biomechanics and Modeling in Mechanobiology*. [Online]. 6, 13-20. Available: 10.1007/s10237-006-0043-0
- [7] Wade Johannessen, Jordan M. Cloyd, Grace D. O'Connell, Edward J. Vresilovic, Dawn M. Elliot. (2006, Apr.) Trans-endplate Nucleotomy Increases Deformation and Creep Response in Axial Loading. *Annals of Biomedical Engineering* [Online]. 34, 687-696. Available: 10.1007/s10439-005-9070-8
- [8] Joseph J. Sarver and Dawn M. Elliot. (2005) Mechanical Differences Between Lumbar and Tail Discs in the Mouse. *Journal of Orthopaedic Research* [Online]. 23, 150-155. Available: 10. 101 6/j.orthres.2004.04.010
- [9] Koichi Masuda, Yoichi aota, Carol Muehleman, Yoshiyuki Imai, Masahiko Okuma, Eugene J. Thonar, Gunnar B. Andersson, Howard S. An. (2004) A Novel Rabbit Model of Mild, Reproducible Disc Degeneration by an Anulus Needle Puncture: Correlation Between the Degree of Disc Injury and Radiological and Histological Appearances of Disc Degeneration. *Spine* [Online]. 30, 5-14.
- [10] T. Wang, L. Zhang, C. Huang, A. G. Cheng, G. T. Dang. (2004) Relationship Between Osteopenia and Lumbar Intervertebral Disc Degeneration in Ovariectomized Rats. *Calcified Tissue International* [Online]. 75, 205-213. Available: 10.1007/s00223-004-0240-8
- [11] Roel J. Hoogendoorn, Paul I. Wuisman, Theo H. Smit, Vincent E. Everts, Marco N. Helder. (2007) Experimental Intervertebral Disc Degeneration Induced by Chondroitinase ABC in the Goat. *Spine* [Online]. 32, 1816-1825.
- [12] Andreas G. Nerlich, Erwin D. Schleicher, Norbert Boos. (1997, Dec.) 1997 Volvo Award Winner in Basic Science Studies: Immunohistologic Markers for Age-Related Changes of Human Lumbar Intervertebral Discs. *Spine* [Online]. 22, 2781-2795.
- [13] John I. Boxberger, Joshua D. Auerbach, Sounok Sen, Dawn M. Elliott. (2008) An *In Vivo* Model of Reduced Nucleus Pulposus Glycosaminoglycan Content in the Rat Lumbar Intervertebral Disc. *Spine* [Online]. 33, 146-154.