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APPLYING IMMUNOHISTOCHEMISTRY & REVERSE TRANSCRIPTION PCR TO INTERVERTEBRAL DISC DEGENERATION IN AN ANIMAL MODEL

NSF Summer Undergraduate Fellowship in Sensor Technologies
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ABSTRACT

STUDY DESIGN: Devise a set of protocols for immunohistochemical (IHC) and gene expression analysis that will permit the measurement of changes that occur during degeneration in the lumbar intervertebral disc of a rat model. OBJECTIVES: Quantify how the composition of the disc changes with degeneration in the animal model and prove that these changes correspond to those within a degenerate human disc, supporting the rat as a valid model of spinal disc degeneration. In the future, use the model to determine mechanisms for the degenerative process. SUMMARY OF BACKGROUND DATA: Rodents have been widely used as models to study disc degeneration[1,2,3,4]. The chemical changes within the human disc have also been extensively studied by various methods[5,6,7,8,9]. However, the research to understand the same chemical changes in the rat has been limited. METHODS: IHC staining of the lumbar intervertebral discs of healthy adult rats (as a basis) began with fixing thin slices of paraffin-embedded tissue onto glass slides. The samples were then treated with an antibody specific to the chosen antigen that was then coupled to a biotin-labeled secondary antibody. Finally, the antigens were localized using colorimetric staining. Reverse transcription-polymerase chain reaction (RT-PCR) was also employed to understand gene expression changes within the disc. An RNA extraction was first performed, then RT-PCR to create first-strand cDNA, and finally, standard PCR to amplify a desired gene. RESULTS: The nucleus pulposus on the slide experienced some degradation during the fixing and staining procedures. The collagen I staining proved the most problematic, with staining occurring within the inner annulus and nucleus, a result contradictory with

literature[7]. Collagen II primarily stained within the inner annulus, as hypothesized. Finally, aggrecan stained as expected, but there was evidence of significant background effects. The gene expression work produced results for aggrecan, collagen I, and fibronectin, proving that the protocol employed for the RNA extraction and RT-PCR was effective. CONCLUSIONS: A number of techniques were established for IHC and RT-PCR, but further development is needed. There is significant evidence that the staining was problematic due to background effects. Thus, the continuation of the study will focus on perfecting the fixation and sectioning procedure and doing regular histological staining to insure a sample which does not disintegrate and stains evenly. The preliminary gene expression work's success leads to the conclusion that more specific results may be achieved by splitting the nucleus from the annulus. These results can be quantified and compared by normalizing them to a standard gene.

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1. INTRODUCTION

The study of the intervertebral disc has been motivated by the high incidence of back pain. Over 100 billion dollars are spent annually in connection to back pain. This includes health care, disability payments, and other related costs. On the United States alone, over 5 million people are permanently disabled by back pain[10,11]. Previous research has associated back pain with the degeneration of the intervertebral disc, and although much work has gone into understanding that degeneration, little is known about the mechanisms by which the process occurs. By improving the understanding of how degeneration takes place, research can move forward to develop improved treatments to alleviate and cure many forms of back pain that currently debilitate people.

2. GOAL OF STUDY AND BACKGROUND

2.1 Goal of Study

The goal of the work conducted was to devise a set of protocols for immunohistochemical (IHC) and gene expression analysis which would permit the study of changes that occur during degeneration in the lumbar intervertebral disc of a rat model. These protocols were then to be tested for the quality of results obtained. This work initially focused on healthy discs in order to acquire a set of baseline data against which further tests could be compared.

2.2 Intervertebral Disc Background

2.2.1 Anatomy

The intervertebral disc is a soft tissue which exists between the vertebral bodies of the spine. The disc permits motion of the spine and dissipates energy, acting as a cushion and a support. As Fig. 1 shows, it is composed of two major sections. The first is the annulus fibrosis, and the second is the nucleus pulposus. Located between these two regions is the inner annulus, which exhibits some properties of both sections. Finally, above and below the disc there are endplates.

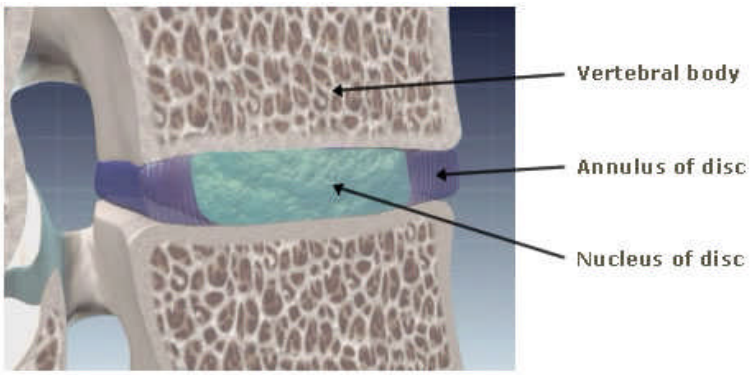


Fig. 1: Anatomy of the intervertebral disc.

The nucleus pulposus is a gel-like structure, while the annulus fibrosis is fibrous, as its name suggests. These fibers are arranged in an organized fashion within the outer annulus, but are more disorganized within the inner. Overall, the concentration of cells within the disc is low, and thus the extracellular matrix within the disc is believed to control disc function[12].

2.2.2 Chemical Composition

The chemical composition of this extracellular matrix is highly significant. Several important proteins which exist in the matrix have been examined within this study. These include: collagens type I and II, the proteoglycan aggrecan, and the fibrous protein fibronectin. Both types of collagens form fibers, although collagen I is known to be primarily present in bone and the outer annulus while collagen two is present in the inner annulus and the nucleus. Aggrecan is a proteoglycan and is known to exist in high concentration in the nucleus, the inner annulus, and at the endplates[12]. It is a relatively short protein which is negatively charged. It binds to long chains of carbon and creates a negatively charged network. This then leads to osmosis of water into the disc, which gives the disc its gel-like properties[12]. Finally, fibronectin is a protein which is associated with tissue repair and can be expected to be found both within the annulus and the nucleus[13]. Samples were stained for collagen II, but its gene expression was not analyzed. Also, the fibronectin genes were looked at with PCR, but the protein itself was not stained immunohistochemically. There are also a number of enzymes and inhibitors that play major roles within the disc and warrant future study.

2.2.3 Degeneration

The human intervertebral disc is known to undergo irreversible degeneration with age. Almost immediately after birth, the disc begins to alter. Degeneration changes the mechanical properties of the disc, its composition, and its structure. Disc degeneration has also been linked to pain[14], which is the reason it has been studied so extensively. Although much research has been done in the field, the mechanisms by which early degeneration occurs remain unknown[14,15].

It is known, however, that a drop in proteoglycan content occurs early in degeneration. There is also evidence that aggrecan is increasingly downregulated as degeneration proceeds[16,17], while collagen localization is changed[7,18] along with the regulation of significant enzymes and their inhibitors[6,9,18]. Fibronectin has also been to shown to be upregulated with increasing degeneration[13].

Due to the reasons discussed earlier, understanding these changes in detail is important. The work discussed here attempts to lay a foundation for studies which will enrich the current standard of knowledge and ultimately lead to the understanding of the mechanisms of degeneration. This may ultimately allow for the prevention, cessation, or even reversal of the process.

2.3 Staining Background

Histologic staining is generally very straightforward. This type of staining utilizes reagents that mark certain types of tissues. Cell nuclei may stain, or for example, collagen. The reason that histological staining is not sufficient to localize a desired protein is because it is not specific enough. Although histologic staining can mark collagen or proteoglycan, the stain cannot distinguish between different types of these proteins. Thus, immunohistochemistry is required for a more detailed picture of the sample. Immunohistochemical, staining, however, is a more complex process. The goal of the stain is to identify and locate some desired entity, such as the antigen, within a sample. IHC does this by labeling the desired entity with a probe. This probe must bind to the antigen in question irreversibly, avoid binding to anything else, and be detectable. Antibodies are very specific proteins, and will bind irreversibly and highly selectively to their target. Thus, antibodies have been employed for IHC. Primary antibodies are typically raised in an animal that is a different species from the sample being stained.

The sample is exposed to the primary antibody which binds to the antigen. This combined structure must be visualized. It is possible to attach some marker to the primary antibody before exposing the sample to it, but since these are so specific, it would not be practical to do so. Thus, the primary antibody-antigen complex is further exposed to a secondary antibody which is specific against the antibodies in the animal used to produce the primary antibody. This secondary antibody can be much less specific, as it only needs to recognize tissue from the primary antibody animal. It is this secondary antibody which comes conjugated to some marker which can be easily visualized by light or fluorescence microscopy.

2.4 Gene Expression Analysis and RT-PCR Background

The general procedure for RT-PCR can be split into four main sections: 1) extracting genetic information from a sample in the for of RNA, 2) converting this information into first-strand cDNA, 3) amplifying a desired gene to make a billion copies of that gene's cDNA, and 4) finally viewing the product of the previous four steps by using gel electrophoresis. Gel electrophoresis separates DNA fragments by size. The larger segments (those with a higher molecular weight) do not travel as far down the agarose gel as smaller fragments when a potential is induced over the length of the gel. This allows for the identification of the size, and thus the identity, of DNA present in the sample.

2.5 Previous Research Done on Animal Models and Disc Degeneration

Rodents have been widely used as models to study mechanical effects of disc degeneration. The extracellular chemical changes within the human disc have also been extensively studied by various methods. However, the research to understand the same chemical changes in an animal model has been limited. The obstacle has been the difficulty in developing a good enough animal model of degeneration[1]. Animals, specifically rats, do not experience degeneration, and it must therefore be induced. The rat is a desirable model

for a number of reasons: it is cheaper, easier to obtain, and most importantly, as any good model must be, the rat model is easier to control than a set of human samples. In order for this to be true, however, the degeneration induced must mirror the degeneration which naturally occurs within the human.

Earlier studies have used chemonucleolysis to model degeneration in ovine and canine samples[2], but were expensive, degraded the nucleus aggressively, and did not allow for the extraction of genetic information from the discs. Other work has shown that puncture models demonstrate degeneration, but this mechanism does not follow the natural progression of degeneration.

Our laboratory uses a moderate chemonucleolysis model in the rat which resembles human disc degeneration[20,21]. This work has aimed at developing the methods by which this model may be evaluated and applied to understanding the mechanisms and changes of degeneration.

3. METHODS

3.1 Sample Preparation

The animals used to obtain specimens were adult Sprague Dawley rats. These were sacrificed, and the spines were surgically removed. The intervertebral discs were appropriately separated into individual samples. The bone above and below each disc was partially left intact for the samples to be sectioned and stained, while those samples which would undergo the gene expression study were removed completely.

Those discs which were to be stained were fixed and then decalcified for 54 hours in a 10% formalin and formic acid solution. Some excess bone was then trimmed, and the sampled were imbedded in paraffin. After cooling, sagittal and axial sections of these were made on a microtome at a thickness setting of approximately 7 μm. The staining study primarily utilized the sagittal sections, although axial cross-sections should also be stained at a later point. These sections were then placed on glass slides and heated to assure fixation.

The discs used for the gene expression work were, as stated earlier, completely separated from bone. They were then placed into RNAlater (Ambion; Austin, TX) to prevent degradation.

3.2 Sample Staining

The sections were stained in two ways. First, two different histologic stains were performed. Then, the sections underwent IHC staining for several proteins of interest.

3.2.1 Histological

Hematoxylin & Eosin: The samples were first immersed in CitriSolv (Fisher Scientific; Pittsburgh, PA) to dissolve the paraffin, and then rehydrated using a series of baths with decreasing alcohol concentration, from 100% to 0%. These were then immersed in a hematoxylin solution (Hematoxylin Gill no. 2, Sigma; St. Louis, MO) for 10-15 minutes, and then washed in tap water. The next wash was with acid alcohol for 20 seconds, followed with a tap water wash, a wash in Scott's buffer, and another tap water wash. They were then immersed in the eosin solution (Eosin Y alcoholic, Sigma; St. Louis, MO) for 1-2.5 minutes, again water washed, and finally dehydrated by a reverse version of the rehydration process. Finally, the samples were dehydrated in CitriSolv, set under an acrylic sealant, dried, and viewed under three different magnifications on a light microscope. All stained samples were viewed at: 2.5 X, 10X, and 40X magnifications.

Alcian Blue & Picrosirius Red: The samples were rehydrated by the same procedure as described above. They were then stained in an acidic alcian blue solution (Alcian Blue 8GX, Sigma; St. Louis, MO) for 30 minutes, washed with tap water, stained with picrosirius red (Sirius Red, Aldrich; St. Louis, MO) (Picric Acid, Fisher Scientific; Hampton, NH) for 45 minutes, washed with acidified water, dehydrated, and sealed. The samples were imaged by the method described above.

3.2.2 Immunohistochemical

Step	Purpose
1. De-wax	Remove paraffin from tissue to allow rehydration
2. Rehydrate Specimen	Condition the fixed specimen to aqueous reagent penetration
3. Antigen Retrieval	Improve epitope exposure from fixed tissues
4. Endogenous Enzyme Block	Inhibit any endogenous enzyme activity that could non-specifically develop a colored reaction
5. Protein Block	Limit any non-specific protein binding to specimen
6. Primary Antibody	Specific binding to antigen
7. Secondary Antibody	Amplify the antibody-antigen reaction
8. Enzyme Complex	Label the immune complex with an enzyme
9. Color Development	Visualize antigen expression by a colored precipitate
10. Dehydrate Specimen	Set stain and prepare for storage
11. Seal	Set sample permanently

Table 1: General steps for immunohistochemical staining.

Each immunohistochemical stain performed followed the same general steps, which are presented in Table 1.

The three proteins studied in this way were collagen I, collagen II, and the proteoglycan aggrecan. The primary antibodies used were as follows: a 1:100 dilution of a polyclonal rabbit antibody against collagen I (Chemicon; Temecula, CA), a 1:4 dilution of a monoclonal mouse antibody for collagen II (DSHB; Iowa City, IA), and a 1:100 dilution of a polyclonal rabbit antibody against aggrecan (Abcam; Cambridge, UK). Control slides were included in every run. These were treated with everything except the primary antibody, and were instead exposed to non-immune horse serum. The secondary, biotin-labeled, antibodies used were 1:100 rat anti-mouse monoclonal and 1:200 goat anti-rabbit monoclonal (BD Pharmigen; San Diego, CA), choice depending on the primary antibody used. The enzyme complex used was a Vestastain ABC and the colored precipitate agent was DAB kit (Vector Laboratories; Burlingame, CA). Once sealed, the slides were viewed in the same way as those in basic histology.

3.3 Understanding Gene Expression

All materials, containers, and solutions used were assured to be RNAse and DNAse free. The samples were removed from RNAlater, and homogenized. (Two complete discs were included in each tube.) This was done by adding Trizol (Invitrogen; Carlsbad, CA) and grinding the sample with a mortar and pestle. Once the sample had completely disintegrated, the solution was homogenized with a power homogenizer for 1 minute. The sample was then allowed to separate before being homogenized again. This was repeated until complete blending was achieved. The sample was then centrifuged to remove RNA from the heavier components of the solution. The supernatant was removed and chloroform was added, then centrifuged. This formed several layers, with the top layer containing the RNA. This layer was drawn off and transferred to another container. Finally, isopropyl alcohol was added, the tube centrifuged, and the supernatant drawn off. Left in the tube was a pellet of RNA. This was then dissolved in DEPC water and stored at -80º C.

The concentration of RNA in the stored solution was determined by a spectrophotometer. RT-PCR was performed following the guidelines presented in the Background section. Everything was kept RNAse and DNAse free. Enough RNA solution was added to assure 5 micrograms of RNA in each PCR tube. Water and OligoDT primer were added. The samples were exposed to 70°C for 10 minutes. To this, buffer, DTT, dNTPs, and Superscript II were added and the mixture was incubated at 42°C for fifty minutes and then at 70°C for fifteen minutes. This was now a solution of first strand cDNA.

This cDNA was then used in PCR to amplify a gene of interest. The cDNA was combined with buffer, dNTPs, forward primer, reverse primer, water, and Taq polymerase. PCR was performed for three genes: aggrecan, fibronectin, and collagen I. Each required a different annealing temperature and number of cycles: aggrecan required 56° C and 36 cycles, fibronectin required 60° C and 32 cycles, and collagen I required 60° C and 32 cycles.

Finally, gel electrophoresis was performed to test for PCR products.

4. RESULTS AND DISCUSSION

4.1 Section Staining

4.1.1 Histological

Basic histology was performed on the healthy disc samples as described within the methods section. The staining by Hematoxylin and Eosin (H&E) produced expected results. The hematoxylin stained nuclei a dark blue while the eosin stained the background tissues red. Fig. 2 presents this staining at three different magnifications.

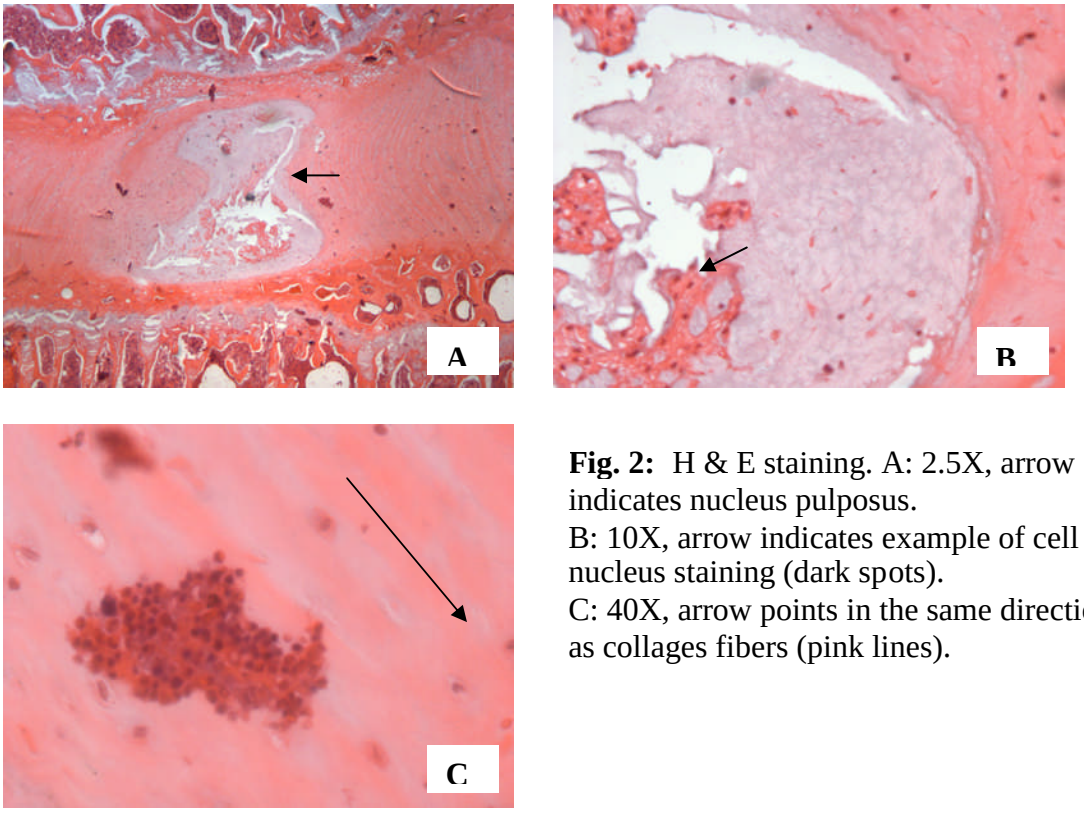


Fig. 2: H & E staining. A: 2.5X, arrow indicates nucleus pulposus.
B: 10X, arrow indicates example of cell nucleus staining (dark spots).
C: 40X, arrow points in the same direction as collages fibers (pink lines).

The make-up of the disc is clearly visible in the first image. The porous sections above and below the disc are bone, while the disc itself is composed of the nucleus, which stained lightly, and the annulus, which stained more heavily. The second image, taken within the nucleus, shows more detail. Specifically, the cell nuclei are visible as dark dots. They are scattered throughout the disc, at a relatively low concentration, as expected. The third image reveals the fibrous structure of the annulus fibrosis. The pink lines are these fibers, believed primarily to be made up of collagen I in the outer annulus (shown). Located between the outer annulus and nucleus is a less organized inner annulus, which is somewhat difficult to see in these images but is known to contain a high concentration of collagen II.

An Alcian Blue & Picrosirius Red stain was also performed, again, as described in the methods section. This stain labels proteoglycan blue, and collagen red. This protocol also produced expected results (see Fig. 3).

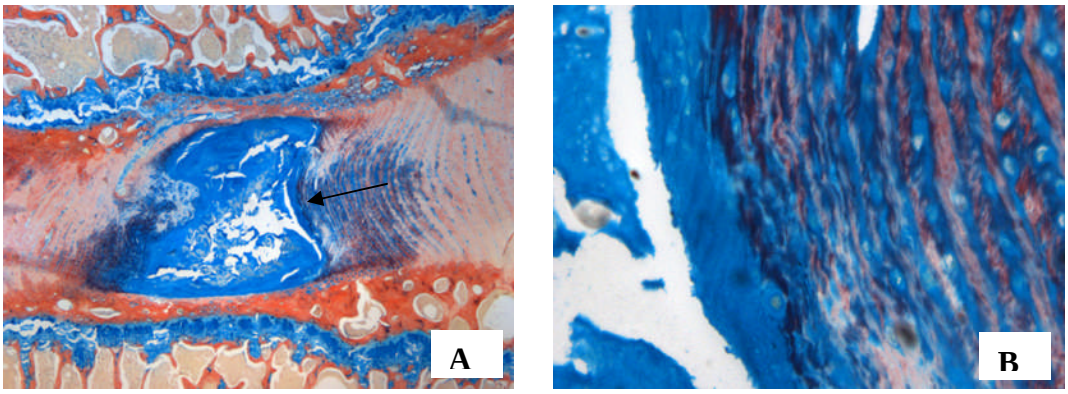


Fig. 3: Alcian Blue & Picrosirius Red staining. A: 2.5X B: 10X arrow in A points to location of magnification.

As expected, the nucleus pulposus stained a solid blue, supporting the fact that healthy discs are primarily made up of proteoglycan. A significant amount of proteoglycan is also visible at the endplates, along with some collagen. The annulus appears to have stained correctly as well. The inner annulus has a high concentration of proteoglycan and some collagen, in a somewhat disorganized structure. The structure becomes more organized further away from the nucleus, and much of the blue staining is replaced by red. This transition in color and structure supports the expected transition from the inner to outer annulus. This transition is accompanied by a more organized structure and an increase in the ratio of collagen to proteoglycan.

Thus, the basic histological stains were overall successful. They could, however, be improved. Due to some loss of detail and solidity of color, it is possible that these tissues were overstained (see also aggrecan IHC stain). The proposed solution to this problem is presented below. It is likely that the tissue sample was too thick. The nucleus pulposus also experienced disintegration on the slide. This problem occurs due to the original high concentration of water in the nucleus, which results in a relative weakness and fragility of this tissue.

4.1.2 Immunohistochemical

Immunohistochemical staining was performed as presented in the methods section for collagen I, collagen II, and the proteoglycan aggrecan. Fig. 4, 5, and 6 present the results.

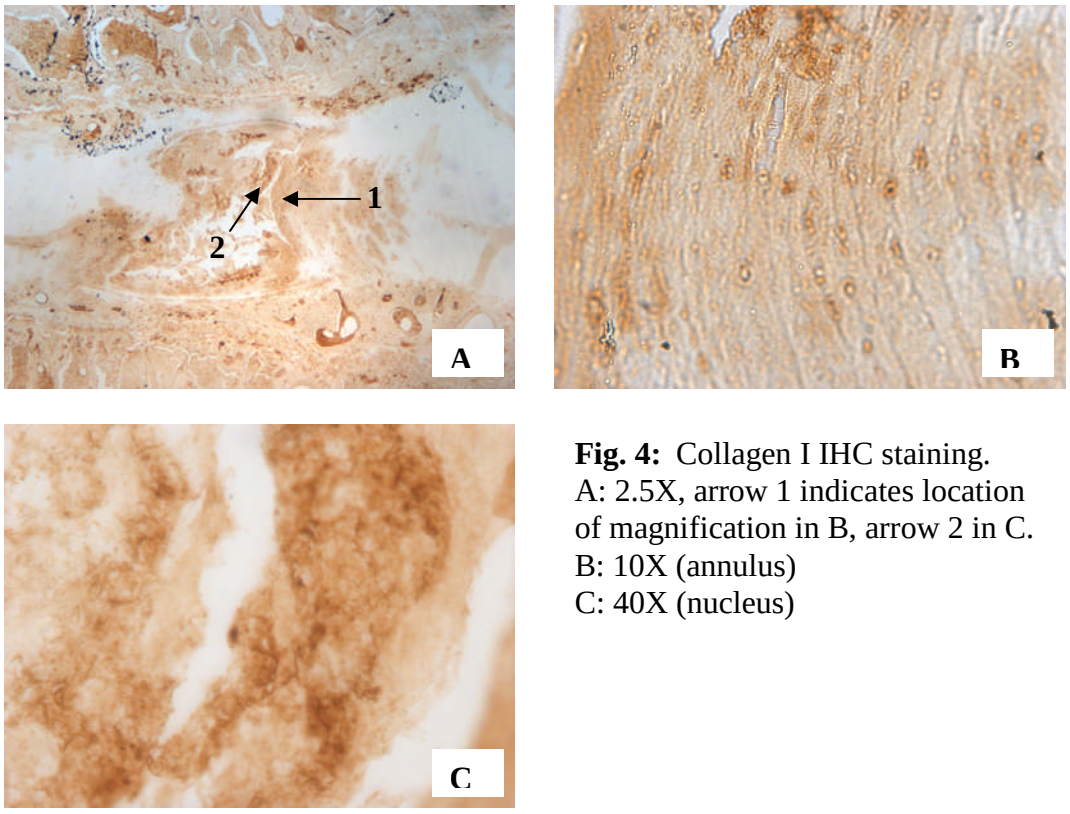
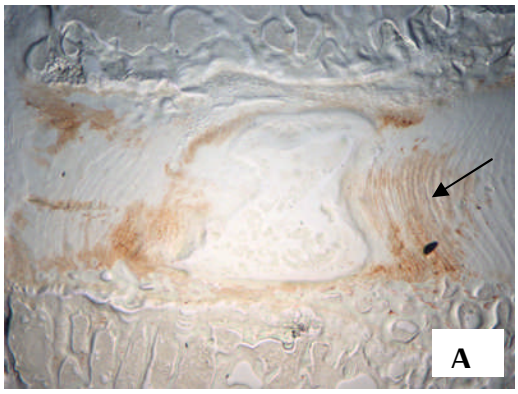


Fig. 4: Collagen I IHC staining.
A: 2.5X, arrow 1 indicates location of magnification in B, arrow 2 in C.
B: 10X (annulus)
C: 40X (nucleus)

Collagen I staining proved to be the most problematic. The bone, endplates, and some of the outer annulus stained for this protein, as expected. The nucleus and inner annulus, however, also stained, in some regions significantly more than the outer annulus. By comparison to previous work done in both humans and animals, these results were deemed incorrect. Although the second image in Fig. 4 contains the expected fibrous structure on the right (outer annulus region) the amount of staining within the more irregular inner annulus (left) is comparable. The third image is a high magnification of the nucleus, which reveals highly irregular staining.

The presence of staining in the inner annulus and nuclear region, and the overall brightness of the stain in all locations suggests the presence of background staining. Published data suggests that staining for collagen I in the disc should be very faint overall. The material on the slide is therefore believed to be trapping the reagents and causing a false positive colorimetric response. There is also a possibility of some cross reactivity within the disc of the antibodies and something other than the target antigen.



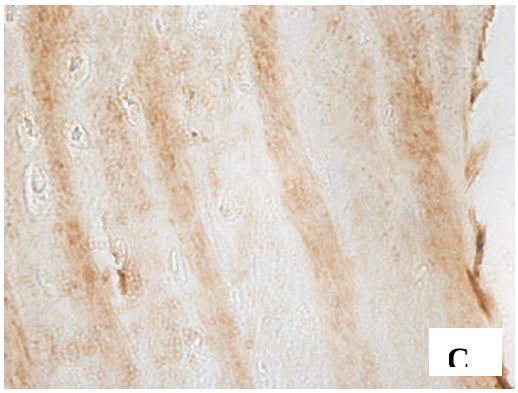


Fig. 5: Collagen II IHC staining.
A: 2.5X, arrow indicates location of magnification in B and C.
B: 10X (annulus)
C: 40X (annulus)

The stain for collagen II proved more successful (see Fig. 5), A. Although the nucleus is thought to contain this protein, the concentration of it is low in a healthy disc and thus little staining was expected there. The samples obtained from this stain support that fact. Unlike the nucleus, the inner annulus did stain for collagen II, as expected. All three images also show some staining of the outer annulus. Although collagen I is known to dominate the outer annulus in a healthy disc, this stain proves that some collagen II exists in this region as well.

Aggrecan, which is a proteoglycan (see Fig. 6), stained successfully when compared to the Alcian Blue stain from routine histology. The locations of staining by IHC correspond to the blue regions in the Alcian Blue stain. The end plate, included here as an example in the second two images, stained quite dramatically.

The drawback to such vivid staining is the high probability of significant background effects. Reagents may become trapped within the tissue and stain more than is actually there. Since the staining is so dark in this case, it is quite probable that this phenomenon explains the results. The goal of IHC staining is to localize proteins on a cellular level. General background noise in the data must be minimized to make that possible.

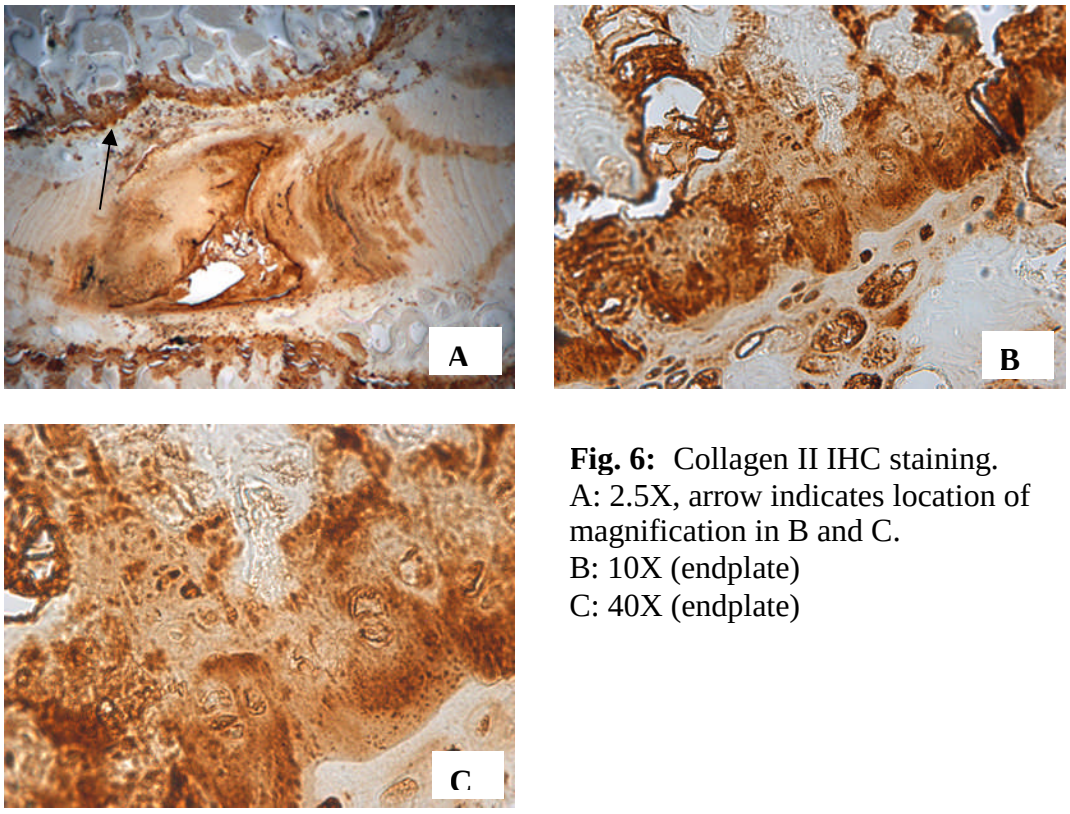


Fig. 6: Collagen II IHC staining.
A: 2.5X, arrow indicates location of magnification in B and C.
B: 10X (endplate)
C: 40X (endplate)

In all three cases the immunohistochemical staining would improve significantly if background staining and cross-reactivity effects were minimized. To assure such a minimization, the protocols for staining could be adjusted by: 1) lowering the concentration of reagents- such as the antibodies, 2) increasing the number of washes between each reagent, 3) changing the amount of time a reagent spends on the sample, and 4) any combination of these.

The most effective way to minimize background staining, however, is to minimize the amount of tissue that is being stained. In order to do this, the thickness of the sample on the slide must be minimized. In theory, samples can be sliced to a size as thin as 4 or 5 µm (on the particular equipment available). Currently, the thinnest slices accomplished have been 6 µm. The goal is to be able to slice 5 µm sections which have an intact nucleus. This can be done by adjusting the fixation procedure and the physical slicing methods. When the appropriate thickness is achieved, basic histology can provide information about how well that sample actually stains. Only when fixation and sectioning, tested by basic histology, have been optimized should immunohistochemistry be attempted again.

4.2 Understanding Gene Expression

As described in the methods section, RNA was extracted from healthy rat lumbar discs. The RNA was then converted to cDNA by RT-PCR, and finally, appropriate primers were added and PCR performed for three proteins: aggrecan, fibronectin, and collagen I. Fig. 7 shows the results. The leftmost column in each image is the ladder

which specifies the size of the product. The brightest band visible (closest to the major bands) is at 600 base pairs. Each band is 100 base pairs away from its neighbor. The next column to the right is the product from the L2 level of the spine, while the one to the right of that is from the L3 level.

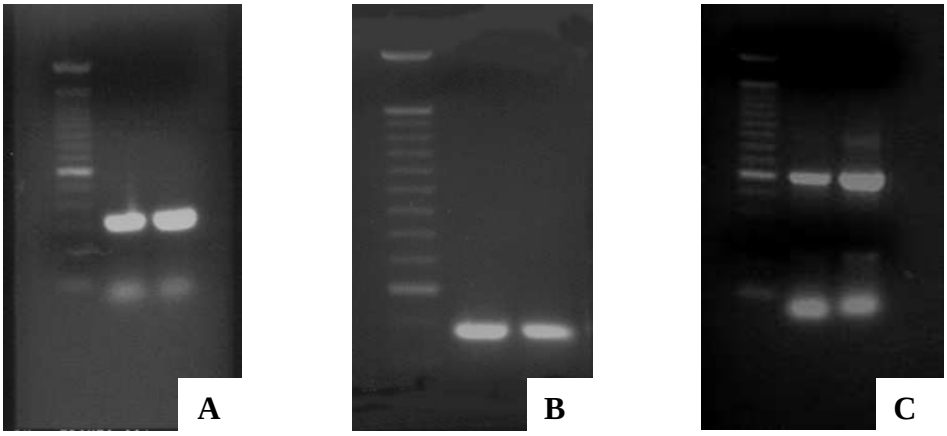


Fig. 7: A: Agarose gel of product from RT-PCR for aggrecan (322bp) **B:** Agarose gel of product from RT-PCR for fibronectin (481bp) **C:** Agarose gel of product from RT-PCR for collagen I (599bp)

Fig. 7, A is the product of the PCR for aggrecan. The presence of bands clearly means that the RNA extraction was successful. These bands are also in the location we expect, at 322 base pairs. Thus, the gene for the correct protein has been amplified. The only real problem with the bands lies in their brightness. Such brightness suggests over-amplification which could be fixed by reducing the number of cycles during PCR.

Fig. 7, B is the product of the PCR for fibronectin. Again, the presence of bands in the correct locations suggests a successful PCR. It is known that fibronectin content increases with degeneration of the disc in humans, so the presence and detection of it in a healthy rat disc is significant.

Fig. 7, C is the product of the PCR for collagen I. Bands for collagen I are clearly present, but so are a number of other bright bands which we do not expect. This suggests that the primer designed for collagen I was not as successful as it should have been and fragments of cDNA which were not coding for the protein of interest were amplified along with the collagen I fragments. A different primer must be tried to assure that the dominant bands are those for collagen I only.

Future work will split the nucleus from the annulus to study the two separately. It will also apply this protocol to more proteins and enzymes. Finally, and most importantly, the PCR will be quantified. This must be done by including a control sample for each sample of interest when doing PCR. This control sample would amplify a gene that is always present in the disc and is not affected by degeneration. Thus, by measuring

the intensity of the band for the desired gene and normalizing it by the intensity of the band for the control, comparable information can be obtained for each sample.

Another possibility for a more quantitative result is real time PCR. In the future, the gene expression work will move to incorporate this tool. In real time PCR, the concentration of DNA is measured by the instrument as the reaction progresses. This process removes the need to perform gel electrophoresis- other than to check on the identity of the product. Real time PCR, however, requires a different set of primers than standard PCR. Consequently these will have to be redesigned.

5. CONCLUSIONS

The work conducted this summer has shown that immunohistochemistry and RT-PCR are powerful tools that can be applied to understanding the intervertebral disc. Collagen II and aggrecan stained as expected under the application of IHC. All three proteins studied by RT-PCR supported the success of the protocol. There is, however, still a significant amount of development that must be done before these techniques can be applied to an animal study. Each staining process showed some results, suggesting that the general protocol used is a good first step, but most had background staining which must be minimized in order to make the results meaningful. The nucleus must, for the most part, remain intact. Accomplishing this will require significant effort to optimize the fixation and slicing protocol of the samples. The gene expression work was successful, but must be quantified before a study can be attempted.

Once the protocols have been perfected, a study can be devised. A number of rat samples should be used with each animal at a different time point and at a different level of degeneration. IHC and RT-PCR can then monitor the expression and localization of various proteins, enzymes, and inhibitors of interest within the disc. By understanding these changes, we may understand the mechanisms of degeneration and ultimately be able to better diagnose, prevent, halt, and even reverse this process. This work may ultimately lead to back pain relief for the millions who suffer with this ailment.

6. RECOMMENDATIONS

As noted previously, the immunohistochemical staining would improve significantly if background staining and cross-reactivity effects were minimized. To assure such a minimization, the protocols for staining should be adjusted by lowering the concentration of reagents, such as the antibodies, increasing the number of washes between each reagent, changing the amount of time a reagent spends on the sample, and most importantly, minimizing the thickness of the tissue by optimizing the fixation and slicing protocols. Basic histologic stains should be used to test the quality of the sections, and immunohistochemistry only attempted when no background staining is apparent.

Future work in understanding gene expression should split the nucleus from the annulus to study the two separately. It should also apply this protocol to more proteins and enzymes. Finally, and most importantly, the PCR must be quantified. This must be

done by including a control sample for each sample of interest when doing PCR. This control sample would amplify a gene that is always present in the disc and is not affected by degeneration. Thus, by measuring the intensity of the band for the desired gene, and normalizing it by the intensity of the band for the control, comparable information can be obtained for each sample.

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