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EFFECTS OF CROSS-LINKING ON MECHANICAL FUNCTION IN THE DEGENERATE NUCLEUS PULPOSUS

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ABSTRACT

Background Context: In the United States, the most prevalent cause of disability of workers under 45 years of age is low back pain. Past research suggests that there is a link between low back pain and intervertebral disc degeneration. One of the earliest known components of disc degeneration is a decrease in proteoglycan content in the nucleus pulposus, which in turn leads to changes in mechanical properties. In this study, the effects of proteoglycan content and collagen cross-linking on swelling behavior were investigated.

Study Design: Swelling pressure of the nucleus pulposus was measured in a confined compression experiment. The effects of two injectable agents were studied. The first was Chondroitinase ABC (ChABC), which is an enzyme that breaks down proteoglycan. ChABC was used to model disc degeneration. In addition, genipin -- a cross-linking agent that promotes the cross-linking of fibers in the disc's collagen network -- was also used.

Objectives: The main objective of this study was to determine the effects of cross-linking on the mechanical properties of degenerate nucleus pulposus. The first step was to determine baseline swelling pressure for control or normal sheep discs. The second was to determine the concentration of ChABC needed to mimic the degeneration found in human discs. The final step of the study was to investigate the potential of genipin required to restore the mechanical function in degenerate discs.

Results: The average swelling pressure measured for normal sheep discs was 0.21 ± 0.13 MPa. The optimal ChABC dose was 0.5 U with an average swelling pressure of 0.082 ± 0.04 MPa. The average swelling pressure for the 0.5% genipin group and 0.5% genipin + 0.5 U ChABC were 0.15 ± 0.07 MPa and 0.083 MPa, respectively.

Conclusion: Though findings from this study were inconclusive, they do not eliminate the potential of genipin as a treatment for disc degeneration. This warrants further investigation into effects of cross-linking using genipin and the mechanisms by which genipin increases cross-linking.

TABLE OF CONTENTS

1. INTRODUCTION 3

2. MATERIAL AND METHODS 5

 2.1 Study Design 5

 2.2 Methods 6

3. RESULTS 8

 3.1 ChABC Dose Finding Study 8

 3.2 Preliminary Genipin Study 9

 3.3 Additional Genipin Study 11

4. DISCUSSION AND CONCLUSIONS 11

5. RECOMMENDATIONS 14

6. ACKNOWLEDGMENTS 14

7. REFERENCES 14

1. INTRODUCTION

Low back pain is a widespread health problem. Between 70% and 85% of the population will experience back pain at some point in their lives [1-3]. In the United States, it is the most prevalent cause of activity limitation in people under 45 years of age [1, 4]. Among the reasons cited for people losing time at work and seeking medical care, low back pain is second only to the common cold [5]. Further, low back pain results in at least \$50 billion dollars in annual health care costs in the United States [3, 6].

Though the causes of low back pain remain unclear, approximately 75% of low back pain cases are linked to intervertebral disc degeneration [6, 7]. The intervertebral disc consists of three parts, the endplate, the nucleus pulposus and the annulus fibrosus (Figure 1). The nucleus pulposus has a high proteoglycan content. Proteoglycan is a core protein linked to glycosaminoglycans (GAGs) [8]. GAGs are chains of polysaccharide, the majority of which have a negative charge [8]. This leads to an overall negative charge in the proteoglycan and attracts cations, such as Na^+ . This then results in an intake of water in order to equilibrate the ion concentrations and forms a hydrated gel [9]. Hence, the nucleus pulposus has a high water content that ranges from 60% to 80% [9]. This hydrated gel is the structure that supports the axial loads in the disc. The annulus fibrosus, unlike the nucleus pulposus, is highly organized into a fibrous structure and consists mainly of collagen. In tension, it supports load due to the swelling behavior of the nucleus pulposus. In compression, the annulus fibrosus supports load due to bulging and deformation [10].

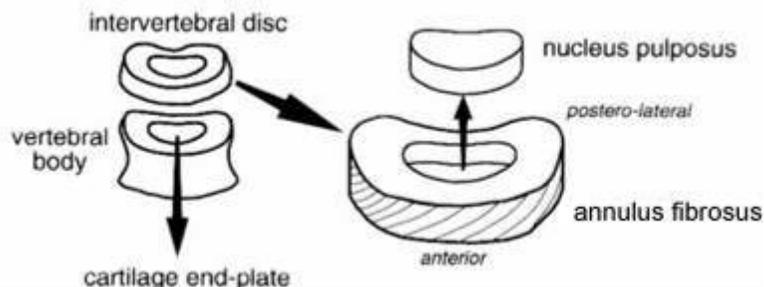


Figure 1. The structure of the intervertebral disc, which consists of the nucleus pulposus surrounded by the annulus fibrosus [11]

Disc degeneration consists of a complex process of interacting mechanisms that include chemical, biological and mechanical changes [12, 13]. The early stages of disc degeneration are characterized by a loss of proteoglycan and a decrease in water [12-16]. This leads to several changes in mechanical properties, including a significant decrease in swelling pressure [13, 17, 18].

Past research has looked at the swelling pressure of the nucleus pulposus in humans with the average pressure ranging from 0.05 to 3 MPa [11]. The swelling pressure is dependent on the loading conditions. It was also found that the nucleus pulposus is capable of swelling to more than 2 times its original volume [11].

Some studies have investigated the effects of cross-linking on the mechanical function of the nucleus pulposus. Preliminary studies done in the McKay Orthopaedic Research Laboratory

looked at the effects of genipin, a natural cross-linking agent (Figure 2). In one of these studies, it was hypothesized that genipin could be used to model degeneration in sheep intervertebral discs. However, it was observed that genipin increased the swelling pressure of the nucleus pulposus. This was unexpected. It was previously believed that genipin would cause increased stiffness and decrease the swelling pressure due to the increased number of cross-links. These preliminary results suggested that genipin could be used as a treatment for disc degeneration.

Genipin is used in traditional Chinese medicine and is obtained from geniposide, its parent compound. Geniposide can be found in the fruits of *Gardenia jasminoides* ELLIS [19-21]. Previous research has focused on genipin effectiveness as a cross-linking agent in biological tissue fixation [19]. Genipin promotes cross-links by reacting with free amino groups, including lysine, hydroxylysine and arginine residues. A blue pigment forms at the site of these reactions as shown in Figure 4 [20, 21]. Past studies suggest that genipin forms intramolecular and intermolecular cross-links within collagen fibers in biological tissue (Figure 5) [20]. Collagen fibers are present in both the annulus fibrosus and the nucleus pulposus, with type II being the predominant collagen type found in the nucleus pulposus [22].

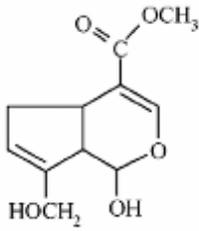


Figure 2. The chemical structure of genipin used in this study[21]



Figure 3. Isolated control intervertebral disc



Figure 4. Isolated genipin-treated intervertebral disc

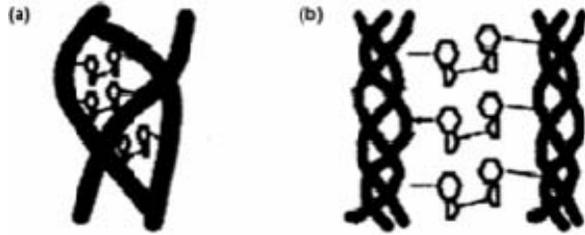


Figure 5. Illustration of (a) intramolecular and (b) intermolecular cross-links [23]

The objective of this study was to first to determine the baseline swelling pressure in the normal intervertebral disc and then to model degeneration in the nucleus pulposus using Chondroitinase ABC (ChABC), an enzyme that breaks down proteoglycan. The next objective was to determine the effect of increased cross-linking on the swelling pressure of the nucleus pulposus measured in confined compression using an in vitro model of degeneration. It was hypothesized that increasing cross-linking would increase the nucleus pulposus swelling pressure.

2. MATERIAL AND METHODS

2.1 Study Design

Studies have assessed the sheep as a model for human spine and have shown the sheep spine is a viable model due to the similarities in anatomy, size and material characteristics [24, 25]. Several studies have documented the similarities between the sheep and human lumbar discs in water content and proteoglycan content in both the nucleus pulposus and annulus fibrosus [22, 25]. However, due to the difference in mechanical loading in quadrupedal animals, intervertebral discs in these types of animals do not undergo degeneration to the same extent that human discs do [26]. Chondroitinase ABC (ChABC) (Seikagaku America, USA) is an enzyme that depolymerizes proteoglycans and has been used in previous studies to induce degeneration in animals discs [27-30]. To determine the effects of cross-linking, ChABC and/or genipin was injected into sheep intervertebral discs.

A confined compression experiment was used to determine the mechanical effects of ChABC injection in non-degenerate nucleus pulposus tissue. Because little research of this kind has been done, this study first focused on determining the average swelling pressure of the sheep nucleus pulposus. The second part of the study focused on optimizing the concentration of ChABC in non-degenerative nucleus pulposus to best mimic the changes in degenerative nucleus pulposus mechanics. In particular, this study looked at the proportional changes in swelling pressure. In a previous study of human nucleus pulposus, the degenerate swelling pressure was 27% of the non-degenerate swelling pressure [31]. A dose finding study was performed using 4 different ChABC doses (0.1, 0.5, 1.0, and 2.5 U / 0.1 ml). Additional samples were tested to find the average swelling pressure of the optimal ChABC dose.

With these preliminary results, a preliminary genipin study was then performed. One disc was tested with 0.5% genipin and a second disc was tested with 0.5 U ChABC and 0.5% genipin. Finally, a preliminary study was performed using genipin. Four discs were treated with 0.5% genipin and four discs were treated with PBS. These eight discs were harvested from sheep

spines with an approximate age of 6 months. All previous discs were harvested from sheep spines with an approximate age of 2.5 years.

2.2 Methods

Genipin (Challenge Bioproducts Inc., Taiwan) was prepared as a 0.5% concentration solution in 0.15 M PBS solution. The 0.5% genipin concentration was selected based on previous work [32, 33]. The combination solution of ChABC and genipin was prepared by using an aliquot of the previously prepared 0.5 U ChABC solution and adding genipin to reach the 0.5% concentration.

The cadaveric sheep discs were obtained in motion segments, where a motion segment is the intervertebral disc surrounded by the vertebrae on each side. The motion segments were thawed to room temperature and injected with 0.1 mL of the treatment solution using a 27-gauge needle. Following injection, the discs were incubated at 37° C for 18 hours in PBS and returned to a freezer at -20° C to prevent swelling of the nucleus pulposus. ChABC enzymatic breakdown of proteoglycan is optimal at 37°C and requires between 1 to 5 hours of reaction time [34]. Genipin cross-linking requires 12 hours at 37°C [23]. Hence, all discs were incubated at the same temperature of 37°C for the same duration of 18 hours in order to avoid differences in mechanical testing due to variations in incubation. Discs were then dissected by removing the surrounding vertebrae (Figure 6). A sledge microtome (Model SM2400; Leica, Nussloch, Germany) with a freezing stage (Model BFS-30; Physitemp, Clifton, NF) was then used obtain a uniform thickness of 2.5 mm, measured using a non-contact micro laser sensor (LM10 Laser, Aromat, New providence NJ) (Figures 7 and 8). Average thickness was 2.50 ± 0.20 mm (n = 25). Samples were frozen at -20°C until testing.



Figure 6. Intervertebral disc in the process of being dissected from the surrounding vertebrae

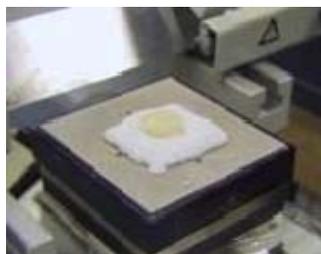


Figure 7. Intervertebral disc being microtomed to a uniform height of 2.5 mm



Figure 8. Micro laser sensor used to measure the thickness of samples

A 4.37 mm diameter circular punch was used to remove cylindrical plugs from the nucleus pulposus of each disc (Figure 9). These plugs were mechanically tested in a custom-built load and displacement controlled compression testing device (Figure 10). The device consists of a 10lb uniaxial load cell (Model 31; Honeywell Sensotec, Columbus, OH), LVDT (Model PR812-200, Macro Sensors, Pennsauken, NJ), linear stepper motor (Model 18512; Spectra Physics Oriel, Stratford, CT) and porous platen (diameter = 4.76, 50% porosity, 45-53 μm pore size). Displacement and load data were acquired through a LabView interface. The plug was placed into the chamber of the device (Figure 11). The porous platen was lowered at 10 $\mu\text{m}/\text{sec}$ by the linear stepper motor until a contact load of 0.045 lbs was measured. The chamber was then filled with 0.15 M phosphate buffered solution (PBS). After a 5 minute wait period, a 1% compressive strain was applied followed by a 3 hour hold to measure the equilibrium swelling pressure. Swelling pressure was calculated as the reported load divided by the cross-sectional area of the device.

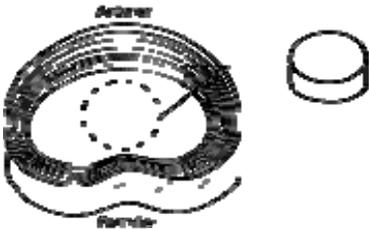


Figure 9. Schematic of a plug removed from the nucleus pulposus



Figure 10. Custom-built confined compression testing device

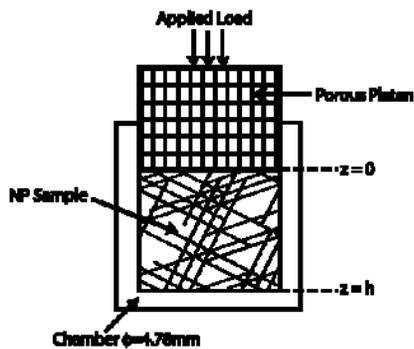


Figure 11. Schematic of chamber where the nucleus pulposus sample is placed with the porous platen applying the load from above

Data analysis consisted of two parts. First, a one-way ANOVA with Bonferroni post hoc test were used to determine if the average swelling pressure of each ChABC treatment group differed significantly between treatment groups. Second, a one-way ANOVA with the Bonferroni post hoc test were used to determine any significance between the control, ChABC, genipin and ChABC + genipin groups.

3. RESULTS

3.1 ChABC Dose Finding Study

The control discs with PBS injections were first evaluated. Three control discs were tested. An average swelling pressure of 0.35 ± 0.02 MPa was measured. A previous study measured the swelling pressure of non-degenerate and degenerate human nucleus pulposus in confined compression [31]. Degeneration led to about a 63% decrease in swelling pressure. A similar decrease was targeted in a ChABC dose finding study. The target swelling pressure for induced degeneration in the sheep nucleus pulposus was then 0.095 MPa. One disc was tested with 2.5 U ChABC with a swelling pressure of 0.051 MPa. A second disc was tested with 1.0 U ChABC

(0.040 MPa). Four discs were tested at 0.5 U ChABC with an average swelling pressure of 0.082 ± 0.051 MPa. Seven discs were tested at 0.1 U ChABC with an average swelling pressure of 0.17 ± 0.11 MPa. Therefore, 0.5 U ChABC was selected as the optimal dose of ChABC to induce degeneration in the sheep nucleus pulposus (Figure 12). The 0.5 U ChABC group's average swelling pressure was significantly different from the control group ($p < 0.05$).

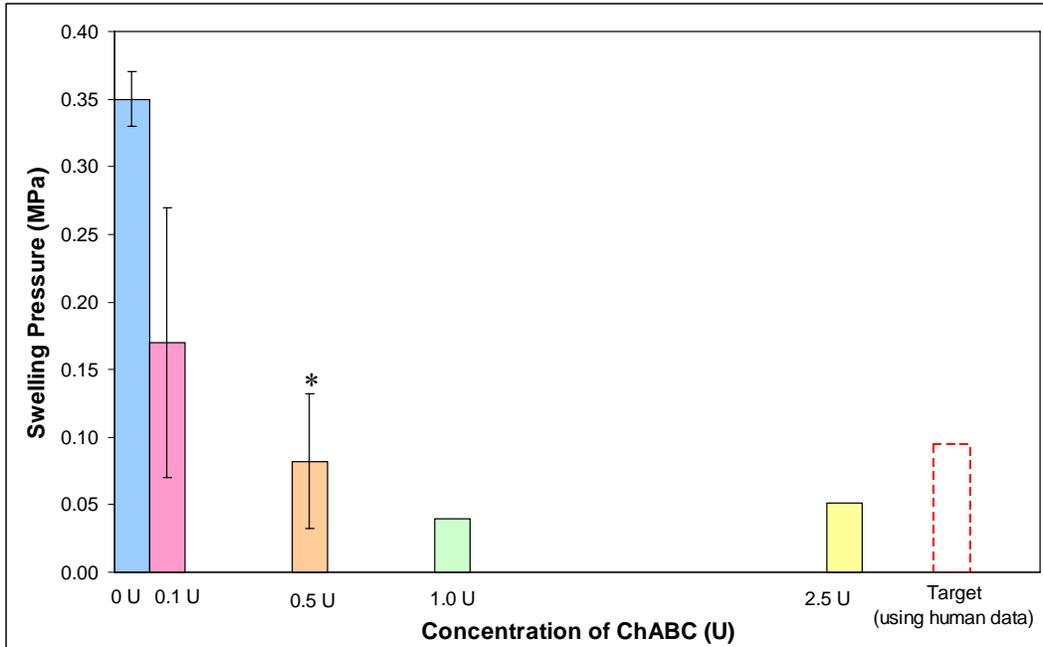


Figure 12. Confined compression results for ChABC dose finding study. Significant differences between the control and the 0.5 U ChABC group are indicated by *.

3.2 Preliminary Genipin Study

One disc treated with 0.5% genipin had a swelling pressure of 0.27 MPa. One disc treated with 0.5% genipin and 0.5 U ChABC had a swelling pressure of 0.083 MPa (Figure 13, Table 1). Representative swelling curves of the different treatments are shown in Figure 14. The control group's average swelling pressure was significantly different from both the ChABC and ChABC + genipin group. The ChABC was not significantly different from the ChABC + genipin group.

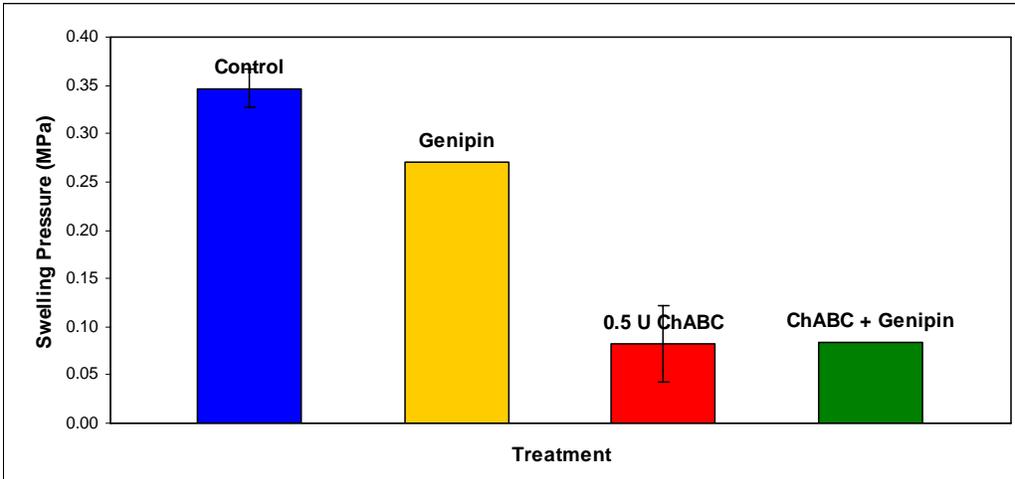


Figure 13. Comparison of swelling pressure across different treatment groups.

	Swelling Pressure (MPa)
Control (<i>n</i> = 3)	0.35 ± 0.02
0.5 U ChABC (<i>n</i> = 4)	0.082 ± 0.051
0.5% Genipin (<i>n</i> = 1)	0.27
0.5 U ChABC + 0.5% Genipin (<i>n</i> = 1)	0.083

Table 1. Summary of swelling pressure across four treatment groups

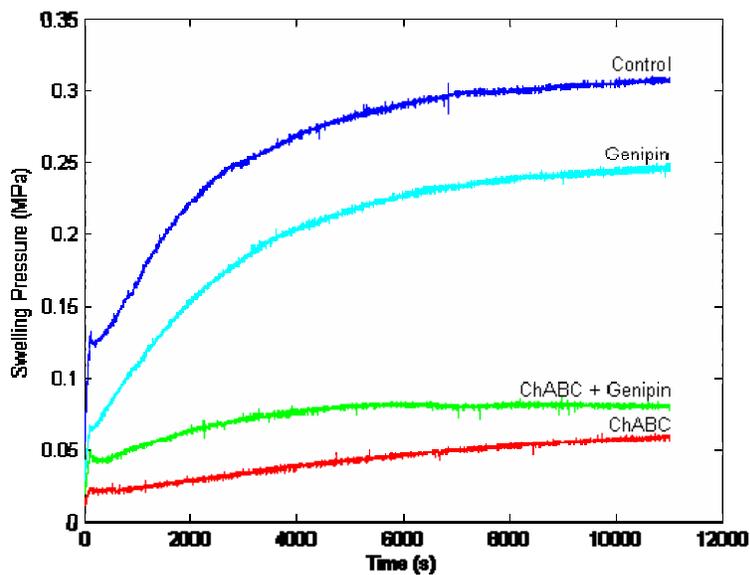


Figure 14. Representative swelling curves of 4 different groups

3.3 Additional Genipin Study

Four additional genipin treated discs and sham treated discs were tested. The average swelling pressure for the 0.5% genipin discs was 0.12 ± 0.01 MPa and the average for the control discs was 0.086 ± 0.02 . There was a large difference between the swelling pressure measured for the preliminary genipin-treated disc and the swelling pressures measured for the genipin-treated discs of the young sheep (Figure 15). This difference was also observed in the control discs of the mature sheep and the control discs of the young sheep (Figure 16). A second plug was tested from the control group with mature sheep discs and was within the range of the preliminary data. Data collected using the young sheep discs showed that the average swelling pressure measured for the genipin group was significantly different from the average swelling pressure of the control group ($p < 0.05$).

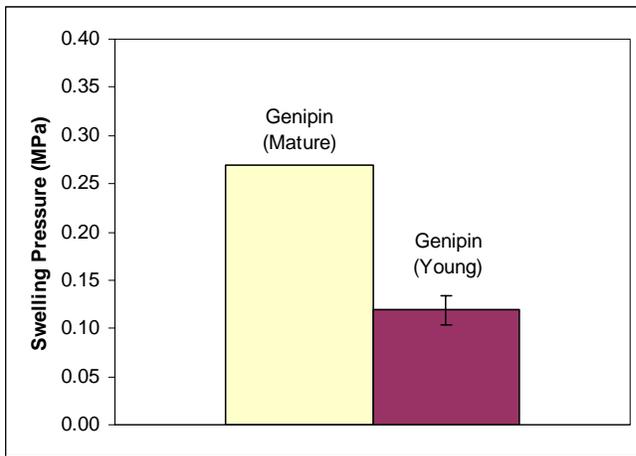


Figure 15. Comparison of average swelling pressure for 0.5% genipin groups

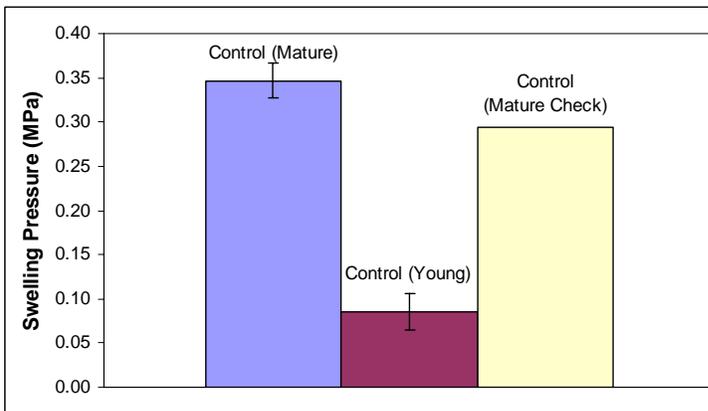


Figure 16. Comparison of average swelling pressure for control groups

4. DISCUSSION AND CONCLUSIONS

The average swelling pressure was measured in confined compression experiments in a ChABC dose finding study followed by a study of genipin cross-linking. Swelling pressure has been

studied in human nucleus pulposus but not in sheep nucleus pulposus [31]. With the preliminary data on genipin, the effect of cross-linking on swelling pressure was inconclusive.

In the first part of this experiment, the ChABC dose finding study was performed. As expected, the swelling pressure decreased as the concentration of ChABC increased. With the average swelling pressure for the control group being 0.35 ± 0.02 MPa, the target swelling pressure using ChABC was 0.095 MPa. Looking at figure 12, the 2.5 U and 1.0 U ChABC doses led to average swelling pressures lower than the target swelling pressure. The target swelling pressure was within one standard deviation of both average swelling pressures measured for 0.1 U or 0.5 U ChABC groups. Due to the large variability in the 0.1 U ChABC group, 0.5 U ChABC was selected for the preliminary study with genipin.

The second part of the experiment involving genipin yielded inconclusive results. Using the mature discs, a genipin treated sample resulted in a decrease in swelling pressure when compared to the control group. Testing a sample with both genipin and ChABC yielded no difference with the ChABC only group. However, using the young discs, the genipin group had a significantly higher swelling pressure than the control group. It was expected that increasing cross-linking using genipin would increase the swelling pressure.

The variability observed in the 0.1 U and 0.5 U ChABC groups may be explained by the proteoglycan content of the cylindrical test samples measured. The changes in swelling pressure are highly correlated to the proteoglycan content. Further, delivery via injection may be a variable leading to non-uniform change in proteoglycan within each ChABC dosage group. Hence, biochemical analysis to measure proteoglycan content of the discs treated with ChABC will allow for a more accurate understanding of the changes in swelling pressure. Future work in this study includes using biochemistry to quantify the proteoglycan content.

The overall trend in the ChABC dose finding study was that as concentration of ChABC increased, the swelling pressure measured decreased. However, when comparing the 2.5 U ChABC treated disc with the 1.0 U ChABC treated disc, there was a slight decrease. This discrepancy can be explained by the variability of the data and a small sample size of only one sample per treatment. Further, data showed small changes in swelling pressure from 2.5 U to 0.5 U of ChABC. A large increase between the average swelling pressures of the 0.5 U and 0.1 U ChABC groups was observed. This suggests that there may be a threshold effect for ChABC treatment. Above this threshold, ChABC affects the tissue to such an extent that very low swelling pressures are measured. Data suggests that this threshold is between 0.5 U and 0.1 U ChABC. Further, biochemistry may reveal that there is no significant difference in proteoglycan content in the 2.5 U and 1.0 U ChABC groups. It is possible that above the threshold, the ChABC has depolymerized the majority of proteoglycan in the sample; using a concentration of ChABC higher than the threshold would not increase depolymerization of proteoglycan.

The main objective of this study was to determine the effect of cross-linking on swelling pressure in sheep nucleus pulposus. Though the average swelling pressure measured for the genipin + ChABC group was not significantly different from the ChABC group, this may be explained by the small sample size and the variability in mechanical testing. Further, there was a significant difference between the genipin and control groups (Figures 15 and 16). A second plug

from the preliminary discs was tested to check for a device malfunction. However, this second plug was within range of the preliminary data and the difference between the young and old sheep data was not believed to be the result of a device malfunction. The significant differences between the young and old sheep data may be attributed to the different sheep spines used. The approximate age of the sheep spines used in gathering the preliminary data was 2.5 years whereas the approximate age of the sheep spines used in the secondary genipin study was 6 months. Hence, the differences in skeletal maturation may have led to the difference in swelling pressures measured. This large difference between the young and mature discs was not expected because these animals do not experience disc degeneration.

A past study investigated collagen cross-linking in the intervertebral disc and correlated two different types of cross-linking with aging and degeneration [35]. A decrease in pyridinoline and an increase in pentosidine cross-links were found with disc aging. Pyridinoline cross-links are thought to be the most predominant cross-link in adult intervertebral discs (Figure 16) [36]. Pyridinoline cross-link levels increase from birth until skeletal maturation. Afterwards, pyridinoline cross-link levels tend to decrease slightly. Unlike pyridinoline, pentosidine cross-link levels have been observed to increase with age. Little is known about the specific type of cross-links that genipin promotes. It is possible that genipin increases the level of pyridinoline cross-links and thus counteracts the effects found with disc aging and degeneration.

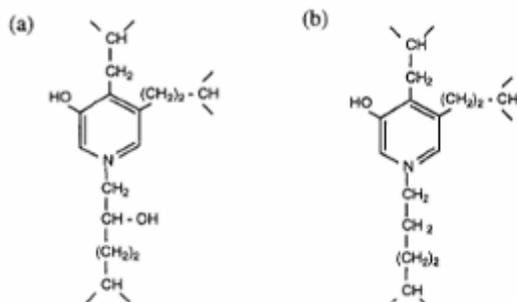


Figure 16. The structure of two different types of pyridinoline cross-links: (a) hydroxylysyl pyridinoline and (b) lysyl pyridinoline.[36]

In conclusion, data from this study showed that ChABC treatments lead to a degenerative change in the mechanical function of nucleus pulposus. The effects of genipin on this degenerative change were not fully determined because of the inconclusive data.

There were several limitations associated with this preliminary study. First, confined compression experiments are a more accurate measure of nucleus pulposus mechanics than unconfined compression experiments. *In situ* the nucleus pulposus is confined by the endplates and the annulus fibrosus but this is neither fully confined nor fully unconfined [31]. In addition, the swelling pressure was indirectly measured through a uniaxial load cell. A more direct method is to measure the interstitial fluid pressure, the pressure of the fluid within the nucleus pulposus [9]. The interstitial fluid is the fluid which the nucleus pulposus uses in pressurization under loading. Consequently it plays a key role in supporting loads in the intervertebral disc. Interstitial fluid pressure has previously been measured in articular cartilage [37].

The second study limitation involves using ChABC as model for degeneration. Disc degeneration is a complex process that is not limited to just proteoglycan depolymerization. Using ChABC, only one of the early changes in disc degeneration is modeled. The final study limitation involves genipin. Little is known about genipin's cross-linking mechanism and cross-linking was not quantified in this study. Genipin treatment may not have equally affected each sample. Correlating amount of cross-linking to swelling pressure would provide a better understanding of cross-linking and its effect on mechanical function. Moreover, a study has not been done to investigate any joint action between genipin and ChABC. There may have been interaction between the genipin and ChABC that further affected mechanical function.

In conclusion, results from this study, though inconclusive, indicate that further investigation into the effects of cross-linking is worthwhile. While various treatments for disc degeneration exist, the majority require invasive surgery. By understanding the effects of cross-linking on the mechanical function in the degenerate nucleus pulposus, an alternative, less invasive treatment for disc degeneration and low back pain may be developed. Finally, the ChABC dose finding data reported for sheep nucleus pulposus are especially valuable to other studies aiming to study degeneration using in vitro animal models.

5. RECOMMENDATIONS

This study was the first step in evaluating genipin's effectiveness as a treatment for disc degeneration. It would be useful to perform a dose response study of genipin both to gauge genipin's effect as it relates to concentration and to determine any interaction with ChABC. Genipin's cross-linking mechanism can be investigated using high-performance liquid chromatography and a detector [38]. Cross-linking can also be quantified using a ninhydrin assay [23]. In addition, a more in-depth dose response study of ChABC would be useful for further work in modeling disc degeneration. Finally, adding a pressure sensor to the current confined compression testing device would allow measurement of interstitial fluid pressure [37, 39, 40].

6. ACKNOWLEDGMENTS

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