Investigation of the Effects of Cryopreservation on Cellular Viability and Mechanical Integrity of the Intervertebral Disc

by
Ryan James Forcier

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

Presented August 26, 2019
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AN ABSTRACT OF THE THESIS OF

Ryan James Forcier for the degree of Honors Baccalaureate of Science in Bioengineering presented on August 26, 2019. Title: Investigation of the Effects of Cryopreservation on Cellular Viability and Mechanical Integrity of the Intervertebral Disc.

Abstract approved:_____

Morgan Giers

Degeneration of the intervertebral disc (IVD) is a phenomenon that has yet to be adequately addressed. Treatment options remain limited and incomplete. The ability to effectively cryopreserve IVDs would allow for better allograft transplantations and storage of samples for research purposes. This study aimed to assess how cryopreservation with different pre-freeze incubation times and the inclusion of PrimeGrowth in the protocol would affect the cellular viability and mechanical integrity of the IVD. Whole bovine caudal IVDs (n = 18) were harvested and processed using various pre-freeze incubation times of 2-hours, 12-hours, and 20-hours. After storage in a -80ºC freezer, the cryopreserved IVDs were quickly thawed for mechanical compression testing and cellular viability imaging. The low-strain elastic modulus, high-strain elastic modulus, and viability were calculated and compared to fresh control, negative control, and non-PrimeGrowth treated groups. There was no statistical difference between treatment groups for the low-strain or high-strain elastic modulus. Groups with 2-hour and 12-hour incubation times showed no difference from the negative control in viability. The 20-hour incubation group showed the highest cell viability among the treated groups. The non-PrimeGrowth treated group showed lower viability than the 20-hour group but higher than 2-hour and 12-hour groups.

Key Words: Intervertebral Disc (IVD), Cryopreservation, Biomechanics, Back pain, PrimeGrowth

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Ryan James Forcier, Author

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To Dr. Morgan Giers: Thank you for all your help, knowledge, and encouragement. I have learned an incredible amount through this, and it would not have been possible without you.

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INTRODUCTION

Back Pain & IVD Degeneration

Back pain is a debilitating condition with significant socioeconomic consequences[1]. Not only is it one of the most common health problems worldwide with a reported prevalence in the USA of 31%[2], but the total cost of lower back pain in the USA far exceeds $100 billion per year[3]. While direct health costs are certainly important, the indirect costs of lost wages due to an inability to work and reduced productivity in the workplace account for two-thirds of this total cost. A recent study demonstrated that adults experiencing back pain reported more concurrent conditions, exhibited more psychological distress, and engaged in more risky health behaviors such as smoking and heavy drinking[2].

While it is not always the case, the cause of back pain has been strongly correlated to the degeneration of the intervertebral disc (IVD)[4]. IVD degeneration is a phenomenon that is not completely understood but years of research suggest that degeneration can be influenced by several factors including mechanical[5,6], nutrition[7], and genetics[8], but is most often a consequence of aging[9]. Though the cascade of events leading to degeneration may vary, the result is an imbalance between the synthesis and degradation of the extracellular matrix (ECM). This failure to maintain the ECM composition leads to weakened tissue strength and altered cell metabolism[10,11]. IVD degeneration is thus characterized by changes in disc hydration, cellular concentrations due to apoptosis and senescence, cell type, and cell phenotype[12,13].

Treatments for IVD degeneration and back pain can be grouped broadly into the following strategies: 1) conservative, 2) surgical, and 3) biological. Conservative strategies include

pharmacological interventions (NSAIDs, corticosteroids, etc.), and therapies such as physical therapy, chiropractic care, or yoga[13,14]. Surgical strategies include more invasive options when conservative strategies fail to provide relief. These include a spinal fusion, discectomy, and artificial or allogenic disc replacement[10]. Although both conservative and surgical strategies can, at some level, offer care for IVD degeneration and back pain, they are primarily aimed at treating the symptoms and not the cause of the degeneration. Biological strategies, while not necessarily approved yet for clinical use, have shown promise in research settings. These include approaches such as injections of cells or proteins[11], gene or tissue engineering[12], and seeded hydrogels[13].

Of more relevance to the work addressed here are the surgical strategies listed above: spinal fusions, discectomies, artificial disc replacements, and allogenic disc replacements. The first three strategies offer their own unique shortcomings for truly long-term relief. A discectomy is a surgery limited to removal of herniated disc material and is simply not intended for many of the instances of disc degeneration. Spinal fusions result in a loss of spinal motion which can increase the stress on adjoining segments, thereby contributing to the degradation of the neighboring levels as well[14]. Artificial disc replacement has shown potential in the short-term, however it may eventually fail, and the success of these implants is not guaranteed. Replacement relies upon the design and exact positioning of the implant to achieve optimal restoration of spinal kinematics to avoid further damage[15,16]. In contrast, allogenic disc transplantation has been shown to be a feasible solution allowing for self-integration through natural remodeling[17]. However, for this transplantation to be successful, the preservation and storage of allograft IVDs is essential to allow for proper disc size matching and immunocompatibility.

IVD Structure & Function

The IVD lies between adjoining vertebral bodies and is composed of three main substructures: the cartilaginous endplates (CEP), the interior nucleus pulposus (NP), and the exterior annulus fibrosus (AF). The external structures include the vertebral bodies, which are comprised of cancellous bone surrounded with a shell of cortical bone, and the bony endplate (BEP), which provides an interface with the CEP[18]. The CEP is a thin horizontal layer of hyaline cartilage. The AF is comprised of concentric lamellae mostly composed of collagen type I fibers[11]. In contrast, the ECM of the NP is often described as the gelatinous center of the IVD and is composed mainly of collagen type II and aggrecan, a proteoglycan largely responsible for the high water content[10,12]. The IVD itself is the largest avascular structure in the body and relies on diffusion from the blood supply at the endplates and the outer AF to meet oxygen and nutritional demands as well as to remove undesired metabolites[11,13]. A sectioned diagram of these structures by Smith et al can be found in Appendix B.

The function of the IVD is to absorb biomechanical forces placed on the spinal column and allow a range of motion along the spinal column. It transfers loads between the vertebral bodies, dissipates energy, and facilitates joint mobility in a variety of motions including bending and rotation[10,13,14]. The complexity of biomechanical forces placed on the disc are one of the reasons artificial disc replacement has yet to become a relevant option[12]. It is this complex function that makes the IVD so vital to daily life and why IVD degeneration has a substantial socioeconomic impact.

Cryopreservation

Cryopreservation is the maintenance of biological material in a state at which cellular function has stopped, or at least slowed down, while preserving physiochemical and biomechanical properties at cryogenic temperatures[20]. This is done with the addition of a cryoprotective agent (CPA) to reduce the occurrence of cryoinjury that may occur during the freezing and thawing process. In this state, cells and tissues can be kept in storage for a very long time and have the potential to be recovered with little to no damage. The major variables to consider include CPA type and concentration, incubation time at different temperatures, cooling and warming rate, and storage duration and temperature[21].

Crystallization plays an important role in cryopreservation and damage done during the process. The specifics of cryoinjury are not clearly established, though it can be divided generally into two mechanisms: mechanical injury and cellular dehydration. Cellular dehydration is a result of extracellular crystallization causing an ion concentration gradient to form across the cell membrane. This gradient causes solutes to enter the cell and water to leave it. Consequently, the variations in salt concentration can lead to denaturation of certain proteins. Extracellular crystallization also leads to mechanical injury by deformation of the cell. As extracellular crystallization progresses, cells experience greater deformation. Intracellular crystallization is also of concern as it can cause mechanical damage to the cell from within[20].

The CPA, or combination of CPAs, reduces the damage from the cryopreservation process by reducing the formation of ice crystals. CPAs are divided into two categories: 1) intracellular cryoprotectants and 2) extracellular cryoprotectants. Intracellular CPAs, such as dimethyl

sulfoxide (DMSO), glycerol, and propylene glycol, have low molecular weights and are able to permeate into the cells. Extracellular CPAs have relatively high molecular weights and are unable to permeate the cell membrane; these include sugars like trehalose and sucrose and other compounds like hydroxyethyl starch and polyvinylpyrrolidone[20,21].

Cryopreservation of the whole IVD offers a potentially feasible solution for long-term storage if conducted in a way that maintains both mechanical properties and cellular activity. This is advantageous as it would allow for better allograft transplantations and storage of samples for research purposes. Luk et al (2008) reported that the use of a cryopreserved IVD allograft represents an "attractive possible alternative" for the treatment of disc degeneration, but preservation of the allograft should be improved to increase storage time and cell viability[18]. The objective of the present study was to investigate variations of a cryopreservation protocol by manipulating the pre-freeze incubation time and adding the use of a recently developed media system named PrimeGrowth™. PrimeGrowth was developed to extend and maintain long-term viability of whole IVD organ models. During IVD isolation, it is suspected that the blood vessels within the BEP become obstructed and thereby limit the nutrient supply to disc cells eventually leading to cell death. Current whole IVD organ culture models often are limited due to the inability to achieve long-term cell viability due to this hindrance in nutrient diffusion. PrimeGrowth has been shown to increase nutrient diffusion throughout the whole disc allowing for a 5-month organ culture, far exceeding the previous 21-day culture period[22]. It is a three-step media system with isolation, neutralization, and culture solutions. Within this project, the goal of using the isolation and neutralization solutions of PrimeGrowth was to encourage quicker

penetration of the CPAs into the IVD model leading to improved cell viability over non-PrimeGrowth treated models after IVDs are subjected to the cryopreservation protocol.

Several groups have investigated the effects of different factors important to whole IVD cryopreservation. Chan et al (2010) examined the effects that cooling rate, CPA concentration, and pre-freeze incubation time had on the mechanical properties, cell metabolic activity, and morphology of the cryopreserved IVD. However, their variation in pre-freeze incubation time was relatively small, only comparing 2 hours and 4 hours, and no difference was found between the times. Additionally, a single elastic modulus was used to compare the mechanical properties of the IVD[23]. Lam et al (2011) examined the effects of differing CPA concentrations on the complex, elastic, and viscous modulus, loss tangent, and stiffness of the disc. These were compared against a fresh control and results suggested that CPAs had the ability to help retain the mechanical properties of the IVD following cryopreservation[24]. It is important to note that Chan did not report any comparison to a negative control, in which no CPA was used, and are unable to show the difference in mechanical properties and cellular viability with the use of CPAs.

In this investigation, the pre-freeze time is further explored with a larger variation in time by using 2-hour, 12-hour, and 20-hour incubation times to allow for increased CPA diffusion through the IVD. The elastic deformation of the disc was modeled with the use of two elastic moduli, one when under low-strain and one for high-strain, allowing for increased characterization of a non-linear material which is typical of a biological material. The inclusion of a negative control allows for an additional comparison to show the benefits in viability of

using a CPA over simply freezing in culture media. Finally, to the best of knowledge, this investigation examines the novel use of PrimeGrowth for the cryopreservation of the whole IVD.

MATERIALS AND METHODS

Bovine Disc Harvest & Isolation

Tails from six cows were obtained within four hours of slaughter from the local slaughterhouse. After removal of surrounding muscle and spinous and transverse processes, the three most proximal discs of each tail, giving a total of 18 discs, were prepared by parallel cuts through the adjacent vertebral bodies, ~2-3 mm from the endplates, using a mallet (VWR, Radnor, Pennsylvania, USA) and osteotome (VWR, Radnor, Pennsylvania, USA). The endplate surface was flattened using a high-speed burr (Fine Scientific Tools, Foster City, California, USA). The IVDs were then washed in sterile, 1X phosphate-buffered saline (PBS) (VWR, Radnor, Pennsylvania, USA) four times for five minutes each time with 1% Penicillin/Streptomycin (P/S) (MilliporeSigma, Burlington, Massachusetts, USA).

Treatment with PrimeGrowth™

Rinsed discs (n=9) were incubated in 30 mL PrimeGrowth Isolation Medium (Wisent Inc., Quebec, Canada) for one hour. Though the specifics of PrimeGrowth are proprietary, it is thought to dissolve blood clots and marrow from the bony endplates, thereby increasing transport. To neutralize the reaction, IVDs were washed thrice for two minutes with PrimeGrowth Neutralization Medium (Wisent Inc., Quebec, Canada).

Disc Cryopreservation

Processed discs were placed into sterile specimen cups containing 50 mL of the CPA at the following concentrations: 78% Dulbecco's Modified Eagle Medium (DMEM) (MilliporeSigma, Burlington, Massachusetts, USA), 10% propylene glycol (MilliporeSigma, Burlington,

Massachusetts, USA), 10% DMSO (VWR, Radnor, Pennsylvania, USA), and 2% P/S. These were then incubated for a pre-determined length of time at 4ºC before being placed in -80ºC freezer. After at least a period of one week, disc specimens were removed from the freezer and thawed quickly in a 37ºC water batch immediately prior to biomechanical testing.

Measuring Freezing Rate Profile

The freezing rate was estimated by measuring the temperature of the CPA solution, beginning when it was placed in the -80ºC freezer, every five minutes for two hours using a thermometer. The freezing rate was taken to be the slope of the resulting temperature plot (Figure 1).

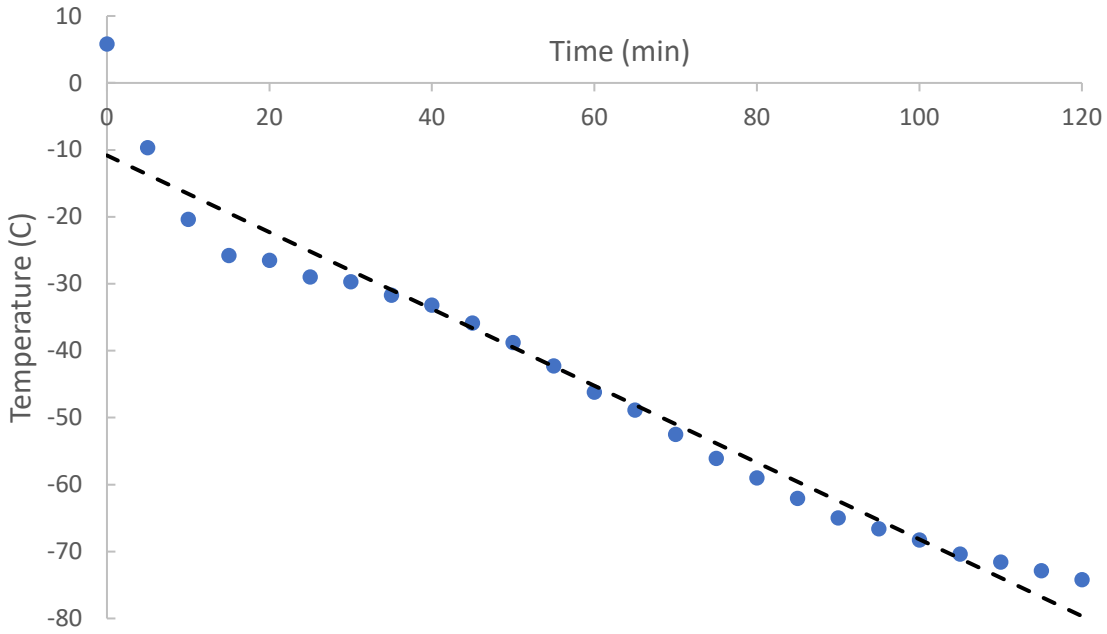

Figure 1. Freezing rate profile. The dashed line represents the fitted slope.

Modifying Pre-Freeze Incubation Time

The effect of pre-freezing incubation time was tested by altering the incubation time at 4ºC of the IVDs treated with PrimeGrowth and CPA. Incubation times included 2 hours (n=3), 12 hours

(n=3), and 20 hours (n=3). The incubation time for the positive control (fresh) (n=3), negative control (n=3), and the non-PrimeGrowth treated (n=3) IVDs was 12 hours.

Mechanical Testing

Non-destructive uniaxial compression testing was performed using an Instron 5576 Universal Testing Machine (Instron, Norwood, Massachusetts, USA). Samples were placed between two porous platens inside a PBS bath (Figure 2). Samples were loaded with a basal compressive load of 10 N to maintain contact between pucks and samples during testing. Preconditioning was performed by applying 10 cycles of 50 N compressions at a rate of 0.05 mm/sec. After a three-minute break, a compression ramp to 0.5 MPa was applied at a rate of 0.05 mm/sec (Figure 3). To determine the necessary load required to achieve 0.5 MPa, the cross-sectional area of each sample was determined using ImageJ image processing software (https://imagej.nih.gov/ij/). A low-strain and high-strain elastic modulus for each sample were estimated as the slope of the stress-strain curve from a strain value of 0 to 0.01 and from a strain value 0.01 below the highest value to the highest value, respectively (Figure 4).

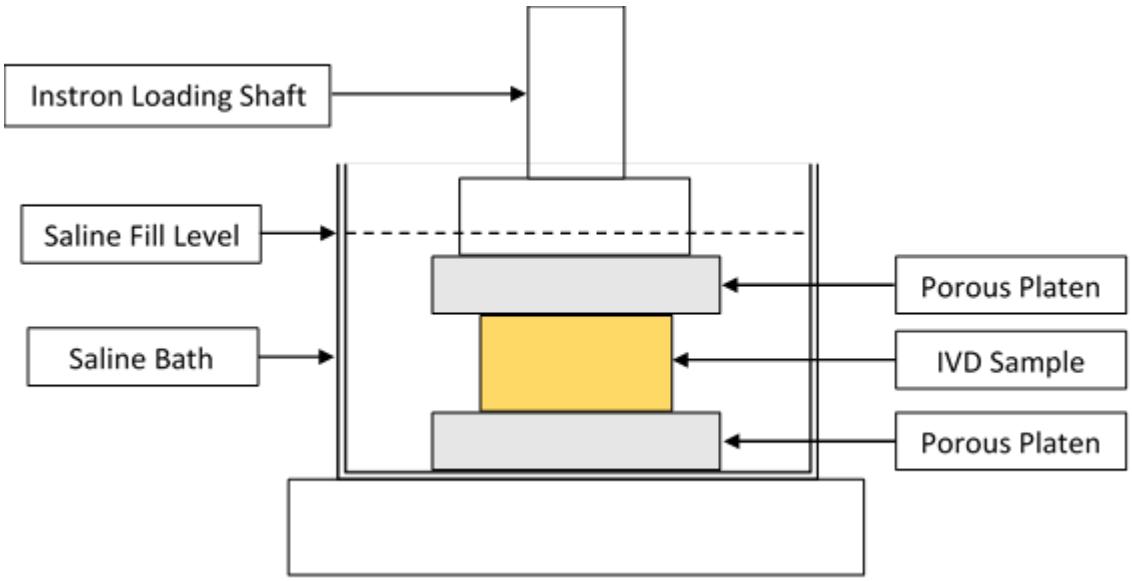

Figure 2. Mechanical testing setup for intervertebral disc.

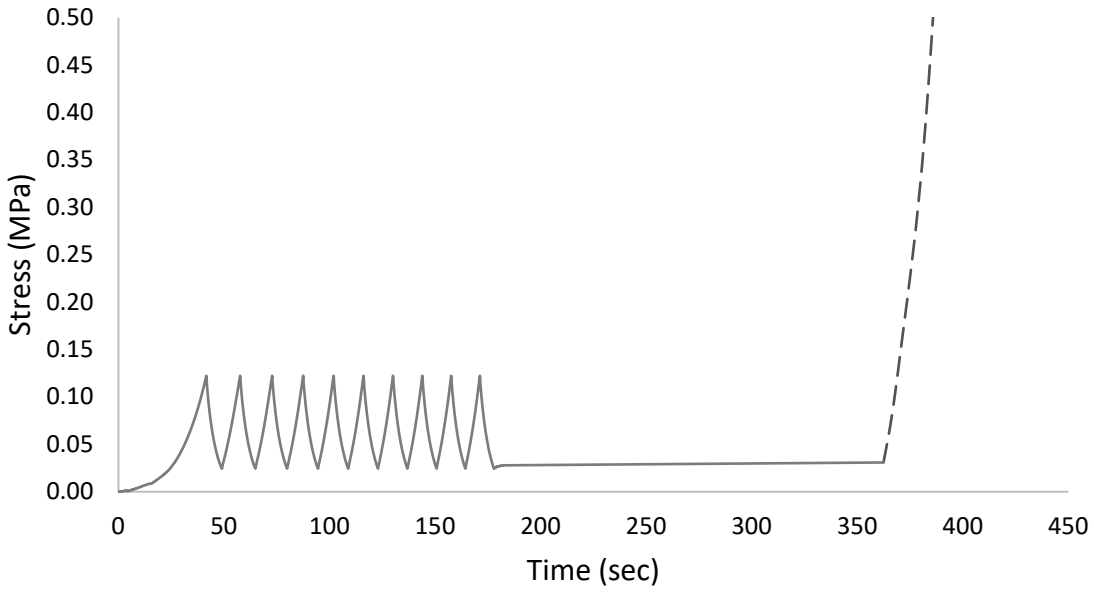

Figure 3. Example of the preconditioning (solid) and loading (dashed) scheme from a disc in the negative treatment group. The area between vertical lines represents the 3-minute break between preconditioning and loading.

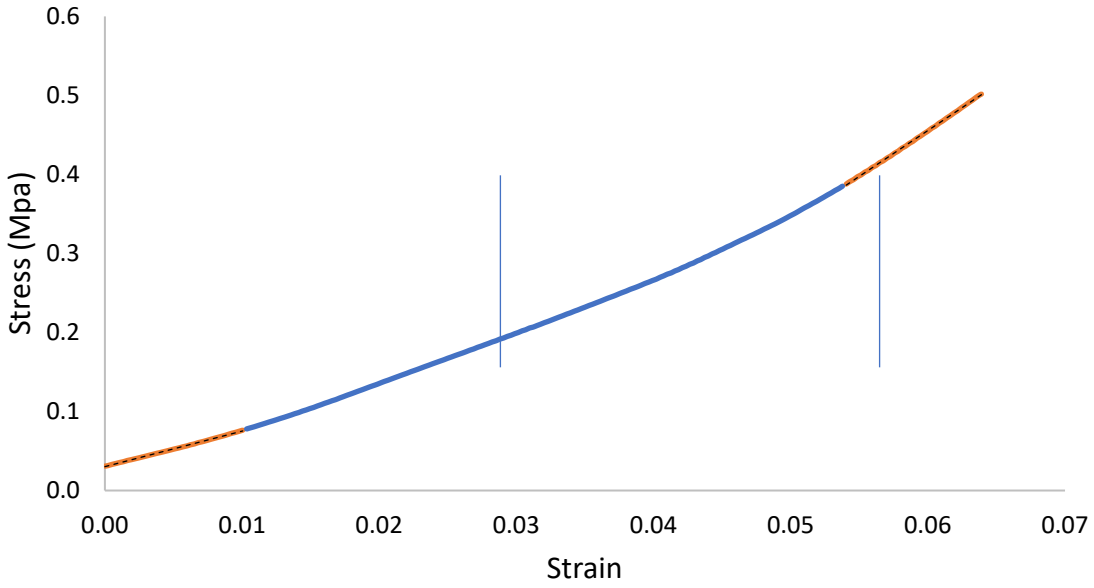

Figure 4. Example of mechanical testing results from a disc in the negative treatment group. The low-strain modulus was taken as the slope of the stress-strain curve from a strain value of 0 to 0.01. The high-strain modulus was taken as the slope of the stress-strain curve from a strain value 0.01 below the highest value to the highest value.

Evaluation of Cell Viability

The cellular viability of the cryopreserved discs was evaluated using a live/dead fluorescence assay (LIVE/DEAD® Viability/Cytotoxicity Kit, Thermo Fisher Scientific, Waltham, Massachusetts, USA). IVD sections were prepared by removing the bony endplate along the centerline using a high-speed burr and then cutting the disc into two, half-circle pieces. From one piece, a thin (1 mm) slice of tissue was taken from one-half of the newly exposed face. The other piece was cut transversely, and a thin, quarter-circle slice was taken from the exposed surface (Figure 5). Tissue samples were then placed in individual wells of a 6-well plate (VWR, Radnor, Pennsylvania, USA) containing DMEM and 1% P/S and were incubated for 72 hours at 37°C and 5% CO_2. The live/dead stain was prepared according to the manufacturer's protocol, with final concentrations of 5 µM ethidium homodimer-1 (EthD-1) and 8 µM calcein AM (CaAM), immediately prior to use. Samples were washed thrice, for five minutes each, in PBS before incubation with the live/dead stain for two hours at room temperature while kept in the dark. Prior to imaging, samples were washed once in PBS for three minutes to remove excess stain. Tissue samples were placed on a slide and visualized by fluorescent microscopy using a Leica DM2500 optical microscope (Leica Microsystems, Wetzlar, Germany) (Figure 6). Three locations of the sagittal tissue slices were imaged: top, middle, and bottom. Four locations of the transverse tissue slices were imaged: inner, outer, and two more spaced evenly between. Percent viability was determined by counting the total number of green (live) and red (dead) cells from merged images using the analyze particles function in ImageJ and calculating the live/total cell ratio (live/live+dead). Figure 7 summarizes the process from disc harvest to testing.

Statistical Analysis

One-way ANOVA followed by the least significant difference (LSD) *post-hoc* test was used to evaluate the statistical difference among different treatment groups for both the cell viability as measured by the calcein-AM/EthD-1 staining and the elastic moduli, both low-strain and high-strain. One-way ANOVA was also used to evaluate the statistical difference of the moduli between tails and disc levels. Linear regression was used to evaluate the statistical effect of disc height and cross-sectional area on cell viability and elastic moduli. For all analysis, a p-value < 0.05 was considered statistically significant. All statistical analysis was done using Statgraphics Centurion 18 (http://www.statgraphics.com/centurion-xviii).

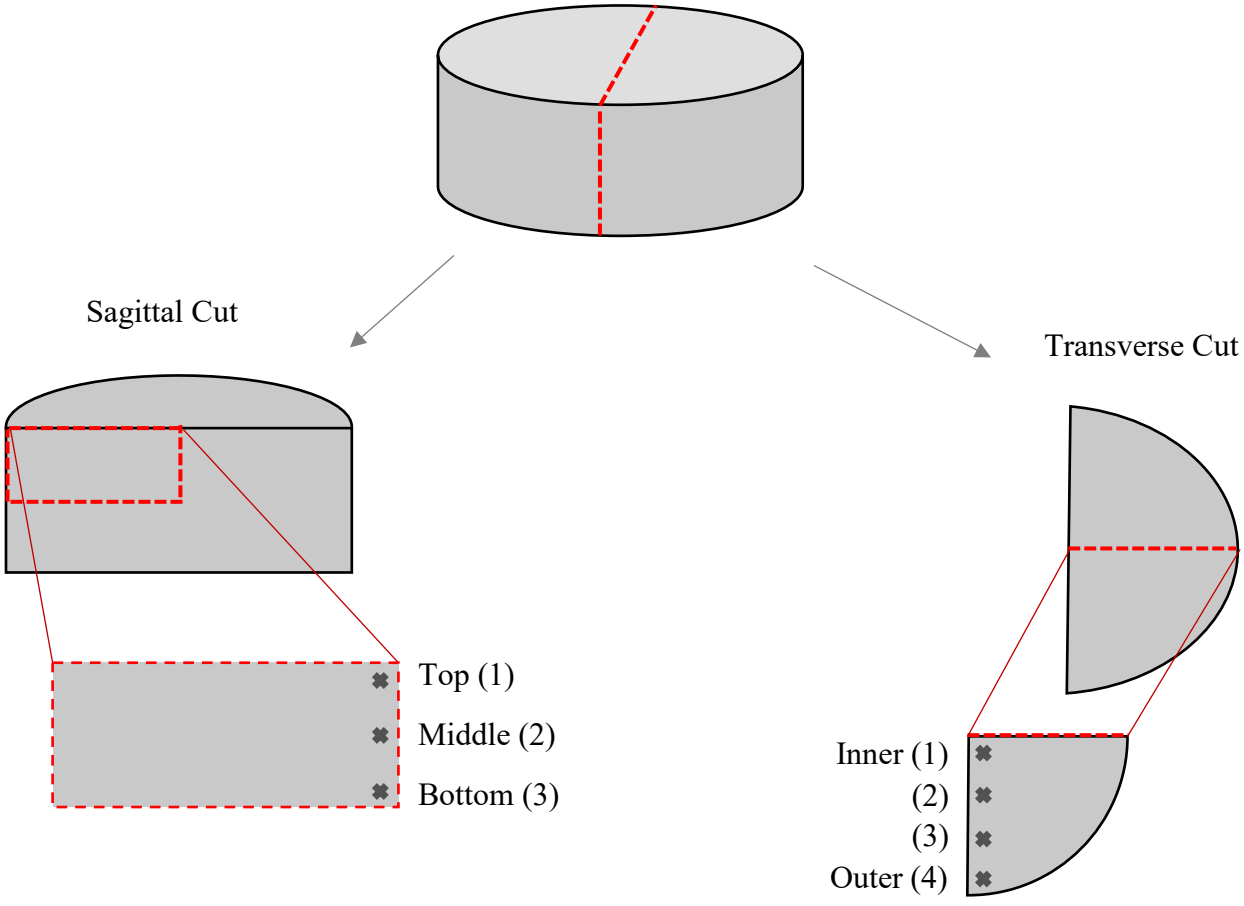

Figure 5. Diagram of sagittal and transverse cuts depicting cuts made to achieve tissue samples. The locations of imaging are marked with X's.

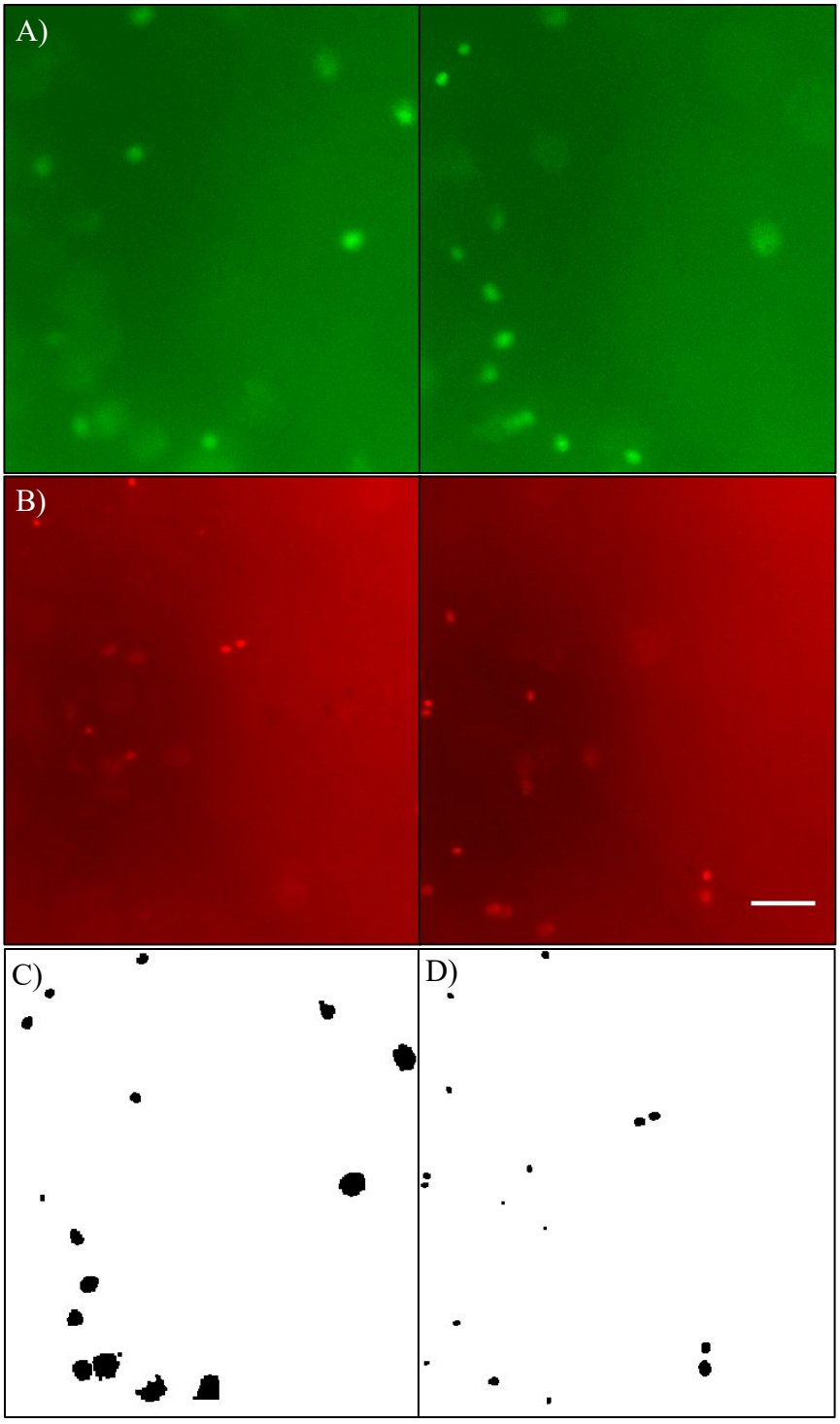

Figure 6. Images taken from the sagittal slice of a fresh control disc for evaluating cell viability. (A and B) The images demonstrate cells that are A) alive and B) dead. Images were all taken at the same location. (C and D) Resulting merged images from combining C) live images and D) dead images. Scale bar indicates 50 μm.

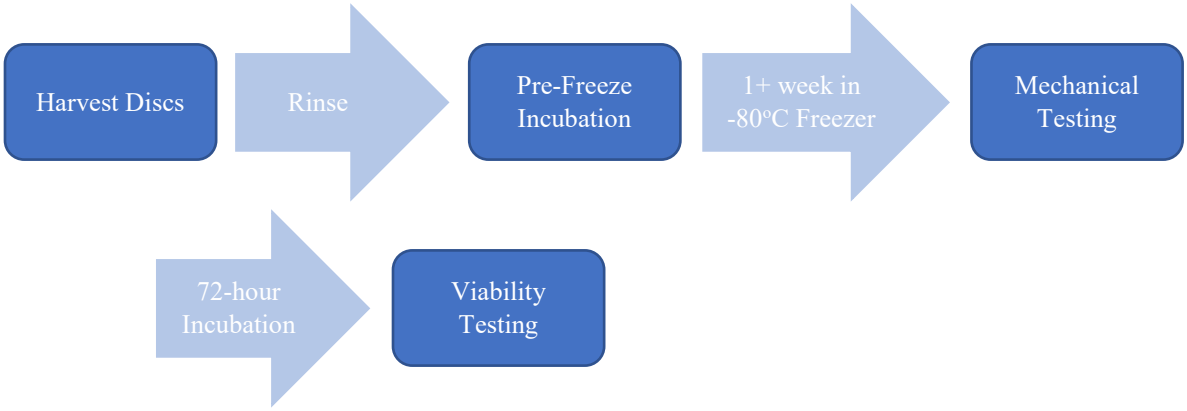

Figure 7. Flowchart depicting the process taken from harvesting discs to testing.

RESULTS AND DISCUSSION

Estimation of Freezing Rate

The freezing rate was estimated to be -0.6 ºC/min. This rate is similar to the rates seen in literature for the preservation of cells, including NP cells, which vary between -0.5 and -1.0 ºC/min[23–25].

Effect on Mechanical Integrity

The low-strain and high-strain elastic modulus of the fresh control and the different treatment groups are presented in Figure 8. The low-strain elastic modulus (mean ± SEM) of the fresh control group was 11.55 ± 4.21 MPa, of the 2-hour group was 10.79 ± 1.62 MPa, of the 12-hour group was 16.57 ± 3.93 MPa, of the 20-hour group was 8.06 ± 2.91 MPa, of the non-PrimeGrowth group was 12.06 ± 2.82 MPa, and that of the negative control group was 3.42 ± 0.58 MPa. The high-strain elastic modulus (mean ± SEM) of the fresh control group was 16.86 ± 3.74 MPa, of the 2-hour group was 16.23 ± 1.09 MPa, of the 12-hour group was 25.00 ± 5.96 MPa, of the 20-hour group was 12.49 ± 2.36 MPa, of the non-PrimeGrowth group was 15.15 ± 3.99 MPa, and that of the negative control group was 12.22 ± 0.31 MPa. No significant difference was found between any of the treatment groups for low-strain ($p = 0.12$) or high-strain ($p = 0.19$) elastic modulus.

Previous work by Chan et al (2010) reported a single elastic modulus value for each disc rather than accounting for the non-linear nature of the stress-strain curve. They reported average values of 14.5 ± 2.4 MPa, 14.5 ± 0.9 MPa, and 14.8 ± 4.4 MPa for the different treatment groups with no difference between groups and no comparison to a negative control[23]. Despite using a single

value, it is still useful to compare the low-strain and high-strain moduli calculated. The overall high-strain modulus averaged across all treatments is 16.3 ± 1.6 MPa and the overall low-strain modulus is 10.4 ± 1.4 MPa. While not identical to the previous reported values, they do surround the values and are not unreasonably far given the error reported. Additionally, it is reasonable, given the methods, that the high-strain value is greater while the low-strain value is lower than the reported values as they are not an entire average of the stress-strain curve but rather the upper and lower ends. The observation of no significant difference between the elastic moduli of treatment groups in this study is important as it indicates that cryopreservation had no effect on the mechanical properties of the IVD as previously observed[23,24]. This preservation of mechanical properties is encouraging for both transplantation and research purposes.

The effects of tail and disc level on the strain moduli was also investigated (Figure 9). The tail that the disc originated from had no significant effect on the low-strain modulus ($p = 0.08$) or the high-strain modulus ($p = 0.06$). The disc level also had no significant effect on the low-strain ($p = 0.61$) or high-strain ($p = 0.60$) modulus.

To determine if there was a correlation between the disc area or disc height and the elastic modulus, linear regression was used (Figure 10). The height of the disc had no significant effect on the low-strain ($p = 0.14$) or high-strain ($p = 0.14$) modulus. The cross-sectional area of the disc did have a significant effect on the low-strain ($p = 0.002$) and high-strain ($p = 0.02$) moduli. While this relationship was unexpected as the elastic modulus of a material should not, in theory, be affected by the geometry of the object, there are other factors associated with biological materials such as origin. In this case, larger cross-sectional areas could be associated with older

animals. Physiological changes due to aging could indicate why larger disc areas had lower elastic moduli.

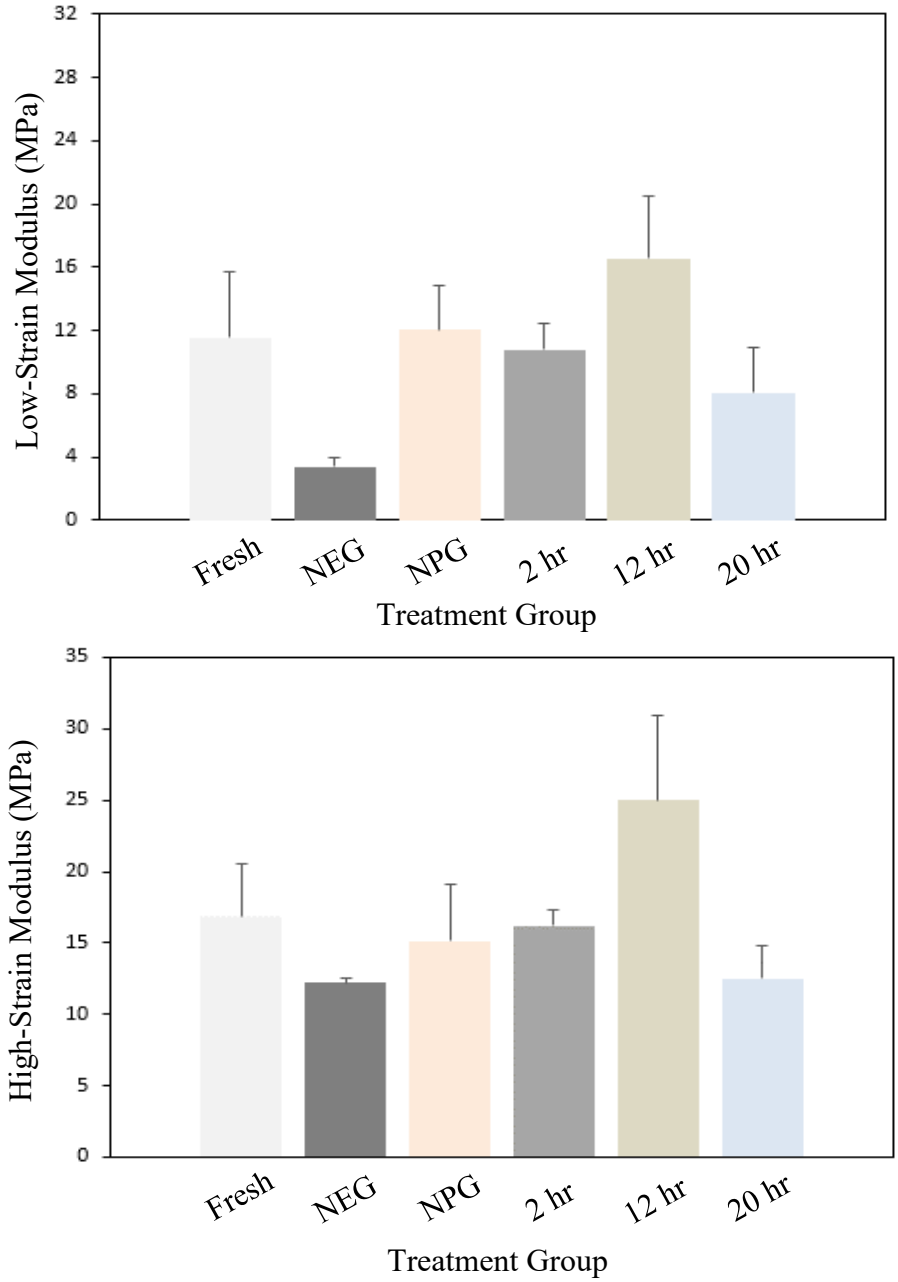

Figure 8. Effects of treatment on the low-strain and high-strain modulus of bovine IVD (Mean ± SEM; n = 3). No statistically significant difference was found between low-strain moduli ($p = 0.12$) or between high-strain moduli ($p = 0.19$). NEG = negative; NPG = non-PrimeGrowth.

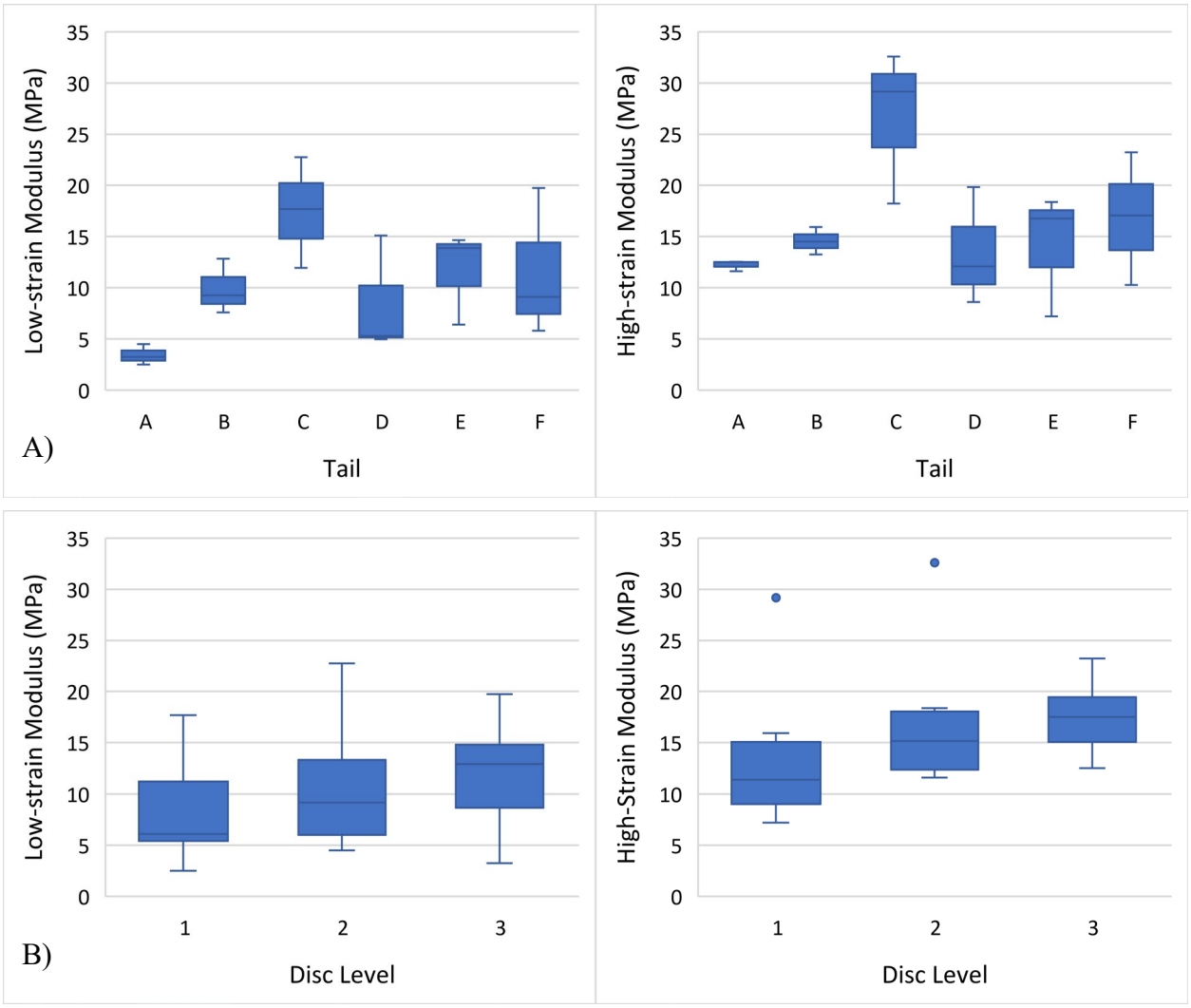

Figure 9. Box-and-whisker plots of low-strain and high-strain elastic modulus grouped by A) tail and B) disc level. No significant difference between low-strain moduli was found between tails ($p = 0.08$) or disc level ($p = 0.61$). No significant difference between high-strain moduli was found between tails ($p = 0.06$) or disc level ($p = 0.60$).

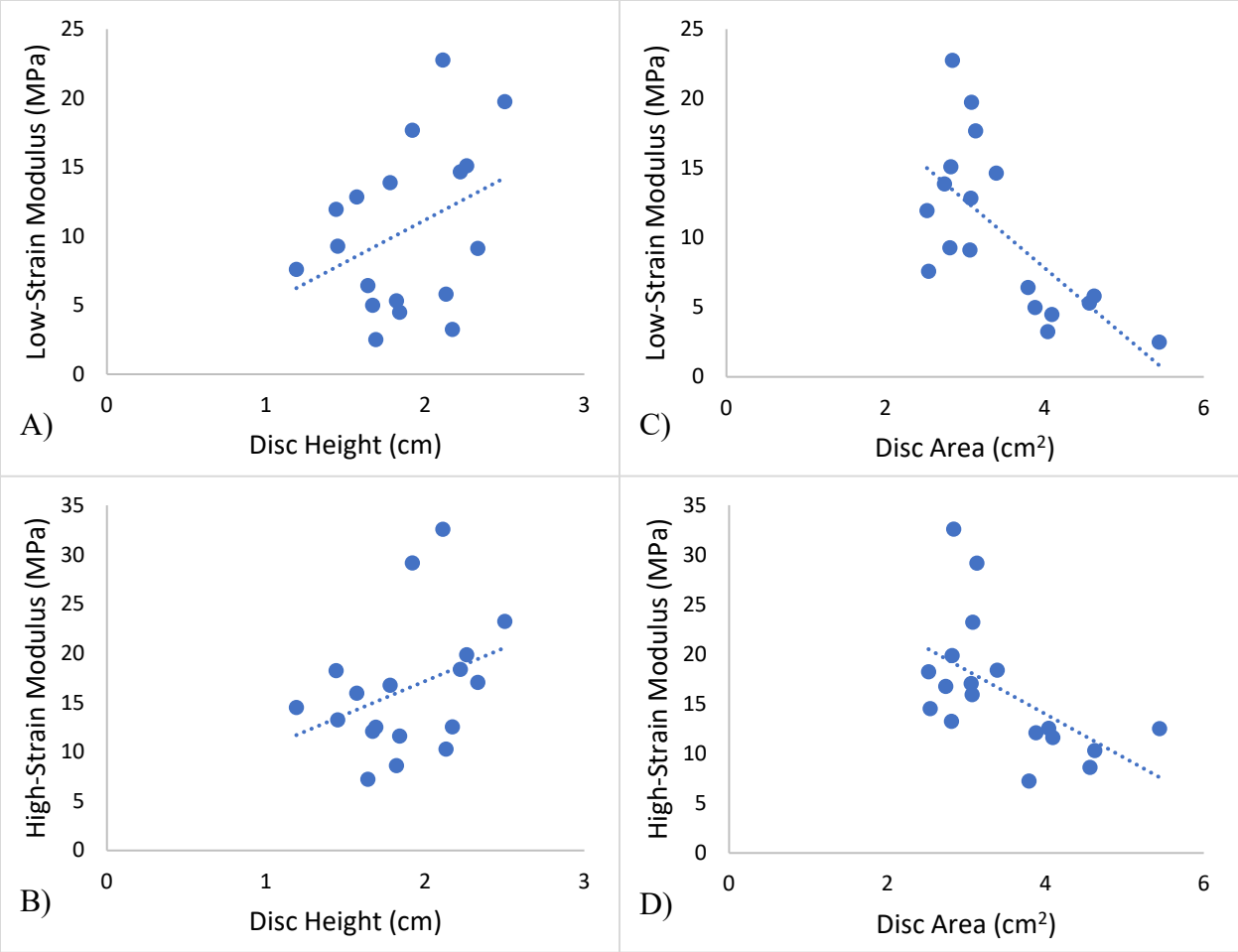

Figure 10. Low-strain and high-strain elastic modulus against height and area. Height had no effect on the A) low-strain (*p*-value = 0.14) or B) high-strain (*p*-value = 0.14) moduli. Area did have a significant effect on the C) low-strain (*p*-value = 0.002) and D) high-strain (*p*-value = 0.02) moduli. The dashed lines represent the fitted model; n = 18.

Effect on Cellular Viability

In the sagittal cut, the negative, 2-hour, and 12-hour treatment groups had the lowest cellular viability with mean viability ranging from 0% to 20%. The fresh treatment group maintained a viability above 60% with a maximum of 72 ± 15%, while the non-PrimeGrowth treatment group

viability fluctuated between 54% and 68%. The 20-hour treatment group ranged from 34% to 52% viability (Figure 11A). There was no significant difference between positions when the average viability of each position for all treatments was compared ($p = 0.83$) (Figure 12A).

In the transverse cut, the negative, 2-hour, and 12-hour treatment groups maintained below 5% viability except for one instance when the 12-hour treatment spiked to 19 ± 13%. The fresh control group decreased from a viability of 90 ± 8% at the center to 22 ± 11% at the exterior edge. The non-PrimeGrowth treatment group increased from 29 ± 15% at the center to 42 ± 14% at position 3 before dropping to 0% viability at the outer edge. The 20-hour treatment group decreased from a viability of 50 ± 20% in the center to 0% at the outer edge. All treatment groups, except for the fresh control, were below 2% viability at the outer edge (Figure 11B). The outer edge had a significantly lower viability when compared to the other positions ($p = 0.04$) (Figure 12B).

The results indicate that viability is uniform from top to bottom within the NP. The inner regions of the IVD also showed increased viability compared to the outer regions of the AF. Previous work by Chan et al (2010) observed the effects of cryopreservation on the metabolic activity of NP and AF cells. Much like in this investigation, regions in the NP consistently exhibited higher activity compared to the AF regions[23]. This supports the observation of decreased viability in the outer regions of the IVD while NP showed higher viability. The two treatments resulting in the highest cell viability were the non-PrimeGrowth treated and 20-hour treatment groups. There was no significant difference between the two treatments at any sagittal position and only at

position 3 of the transverse cut was the non-PrimeGrowth treated group higher than the 20-hour group as determined by the *post-hoc* comparison.

To determine if there was a correlation between the disc area or disc height and the cellular viability, linear regression was used (Figure 13). The cross-sectional area of the disc did not have a significant effect on the viability of the sagittal ($p = 0.66$) or transverse ($p = 0.96$) cuts. The height of the disc did have a significant positive effect on the viability of the sagittal ($p = 0.003$) and transverse ($p = 0.03$) cuts. It was originally expected that an increase in disc height means a longer diffusion path for nutrients to travel, leading to a decrease in viability but that is not what was seen. A possible explanation for this occurrence is the health of the discs. Healthier discs tend to be taller and this would explain the increased viability with an increase in disc height.

This study was limited by its use of bovine tissue rather than human tissues. With the goal of disc replacement, the difference in effects of cryopreservation should be examined using human tissues. Bovine tails were more conveniently available, but mechanics and composition vary from human tissues. Despite this, bovine tissue is often used as an IVD model[28]. Future work should focus on generating a protocol that increases the survival of cells within the AF as this region has exhibited consistently lower viability.

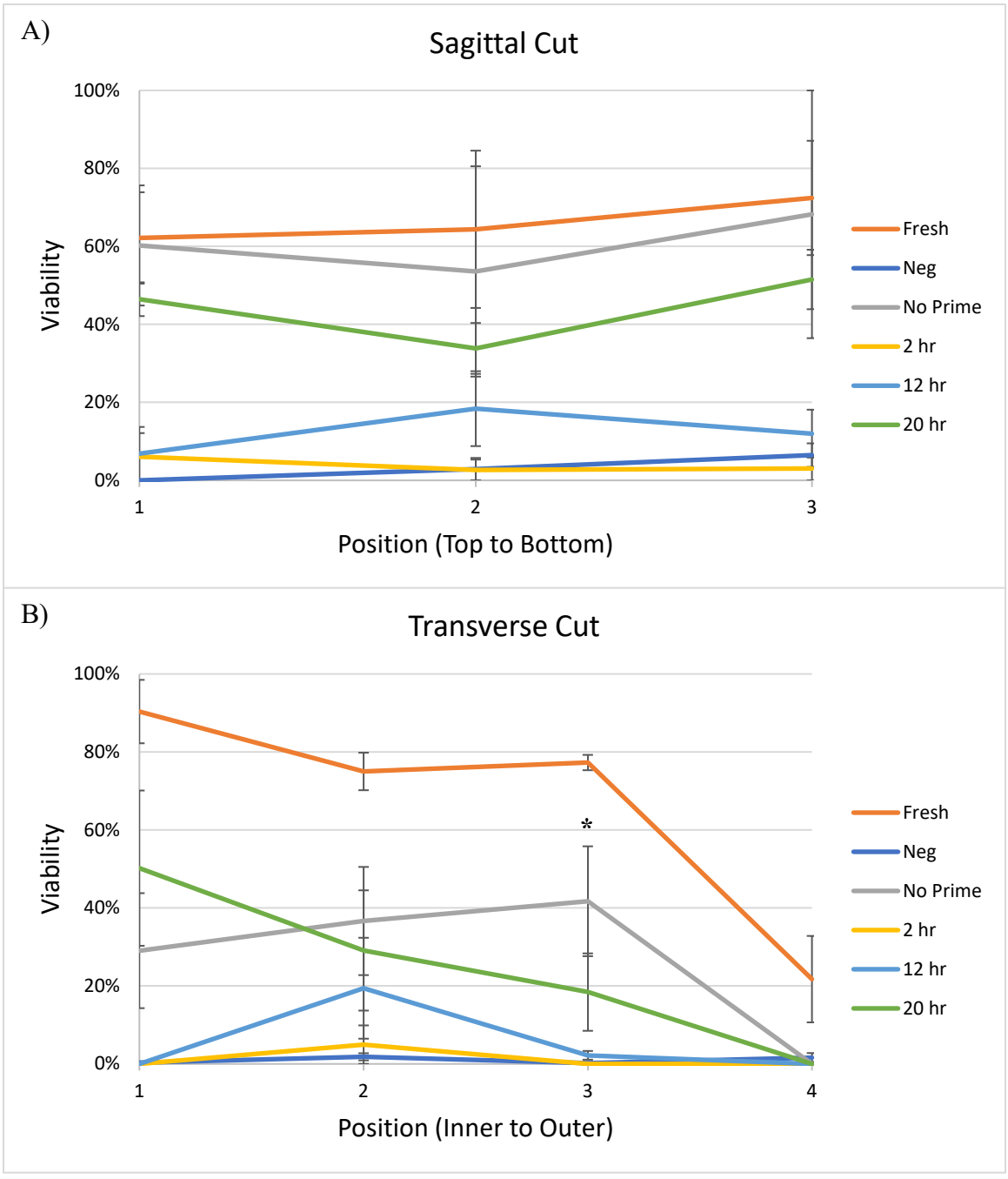

Figure 11. Cellular viability for A) sagittal and B) transverse cuts for each treatment. Error bars represent ± SEM; n=3. * indicates significant difference between 20 hr and No Prime groups. Neg = negative; No Prime = non-PrimeGrowth.

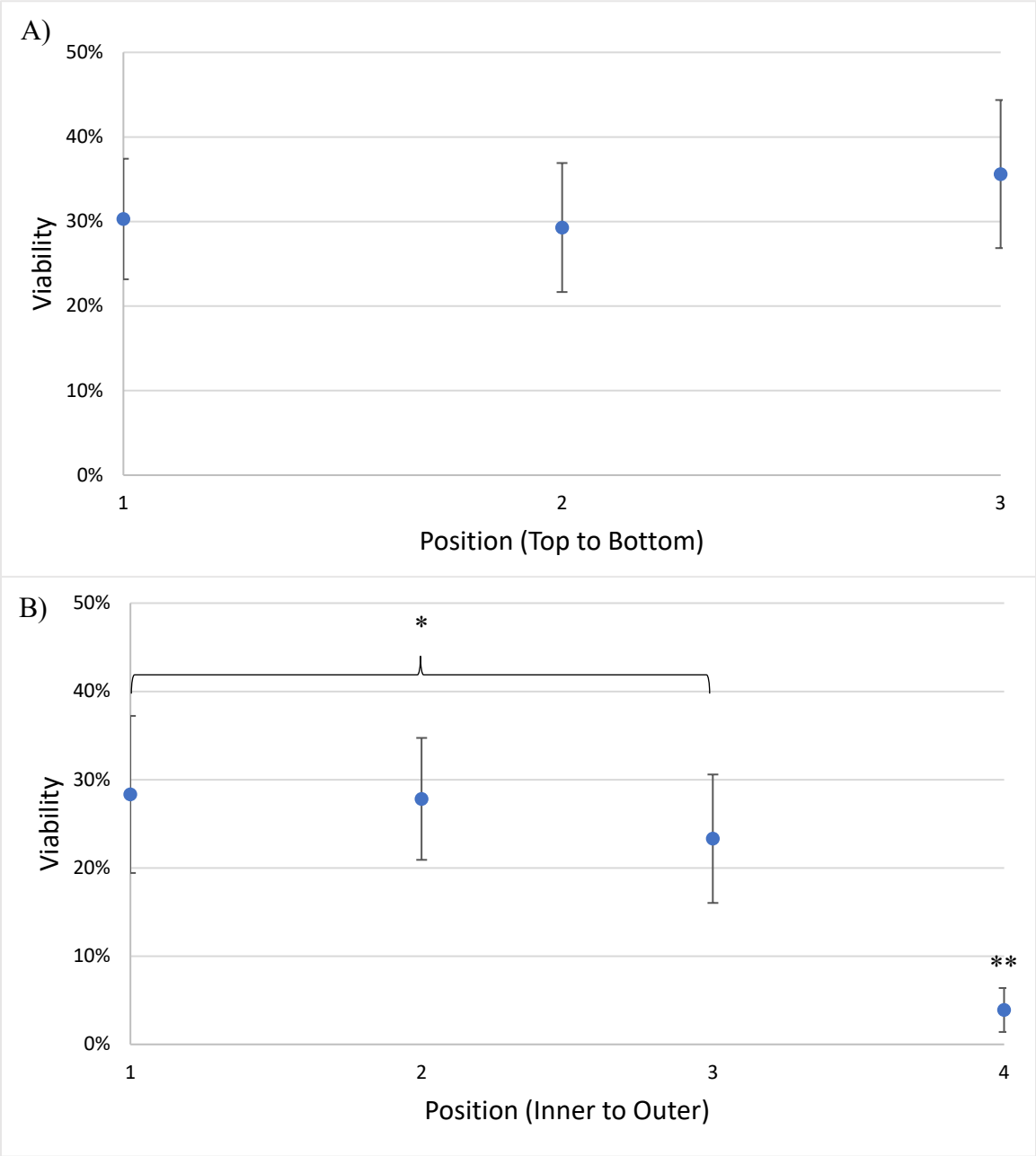

Figure 12. Cellular viability for A) sagittal and B) transverse cuts averaged across all treatments. Error bars represent ± SEM; n=18. There was no significant difference between positions of the sagittal cut ($p = 0.83$). There was a significant difference between positions of the transverse cut ($p = 0.04$). Positions with different number of asterisks (* or **) indicate a significant difference between those positions ($p < 0.05$).

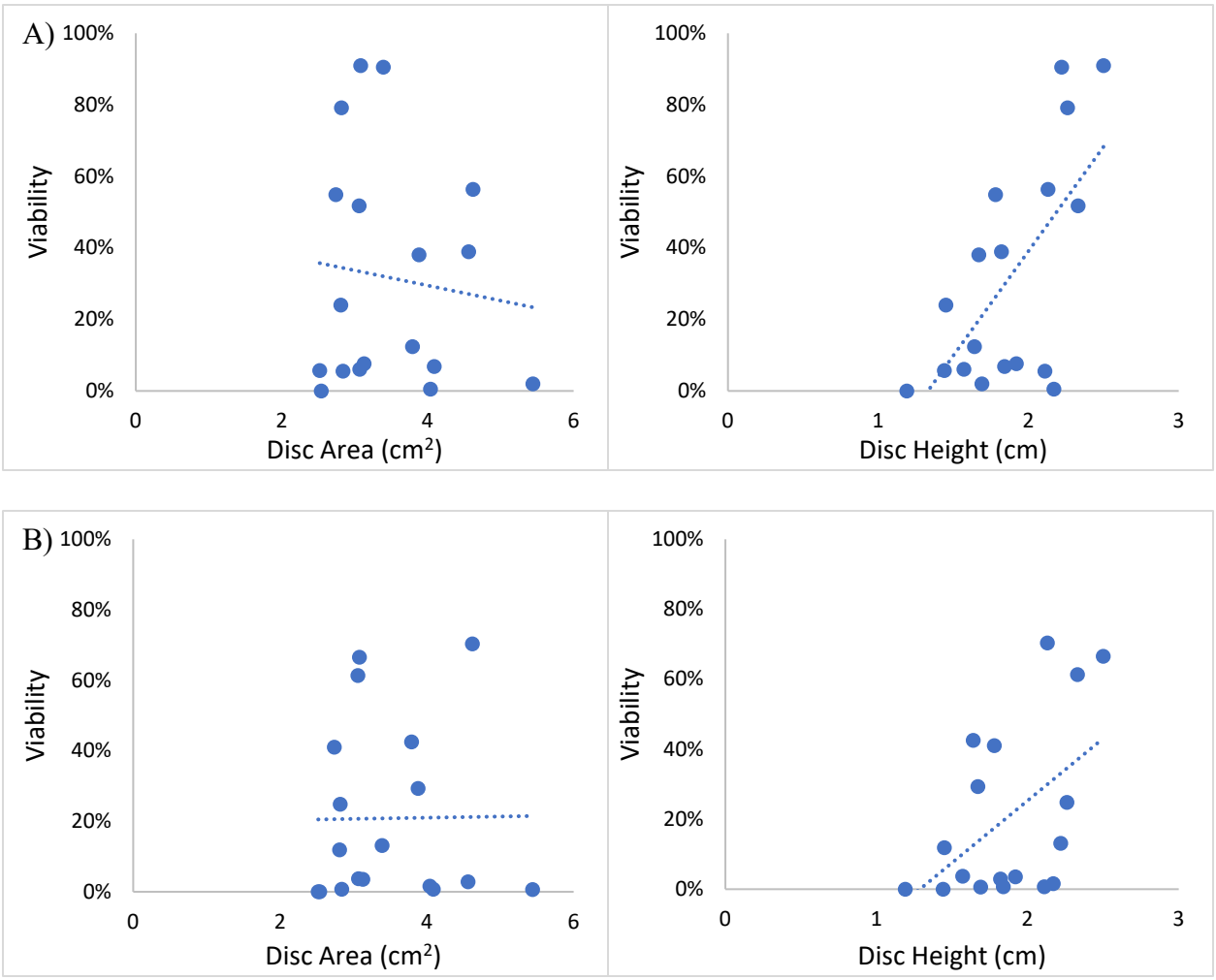

Figure 13. Cellular viability against disc cross-sectional area and height for the A) sagittal and B) transverse cuts. Cross-sectional area had no effect on the viability of the sagittal (*p*-value = 0.66) or transverse (*p*-value = 0.96) cuts. Disc height did have a significant effect on the viability of the sagittal (*p*-value = 0.003) and transverse (*p*-value = 0.03) cuts. The dashed lines represent the fitted model; n = 18.

CONCLUSION

This work describes the effects of cryopreservation on the cellular viability and mechanical integrity of the IVD. Results from mechanical compression testing indicate that treatment had no significant impact on the elastic modulus of the IVD. Rather, the cross-sectional area is of importance when considering the mechanical properties. Results from cell staining indicate that treatment did have a significant effect on the cellular viability within the disc. Of the treatments, the non-PrimeGrowth treated IVDs with a pre-freeze incubation time of 12 hours demonstrated the highest cellular viability overall beating the 20-hour treatment group at one position. There was no difference in viability from top to middle of the disc, though there was a strong decrease in viability as the position moved from the middle towards the outer radial edge of the disc. Finally, the area of the disc had no effect on viability, but the height was positively correlated to overall viability of the IVD. This indicates that the use of a 12-hour pre-freeze incubation time without the use of PrimeGrowth may offer the best cryopreservation protocol of those examined, though there are still areas for improvement.

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APPENDICES

Appendix A: Abbreviations

Abbreviation	Meaning
IVD	Intervertebral Disc
ECM	Extracellular Matrix
CEP	Cartilaginous Endplate
NP	Nucleus Pulposus
AF	Annulus Fibrosus
BEP	Bony Endplate
CPA	Cryoprotectant Agent
DMSO	Dimethyl Sulfoxide
PBS	Phosphate Buffered Saline
DMEM	Dulbecco's Modified Eagle Medium
P/S	Penicillin/Streptomycin
EthD-1	Ethidium Homodimer-1
CaAM	Calcein AM

Appendix B: Supplemental Figures

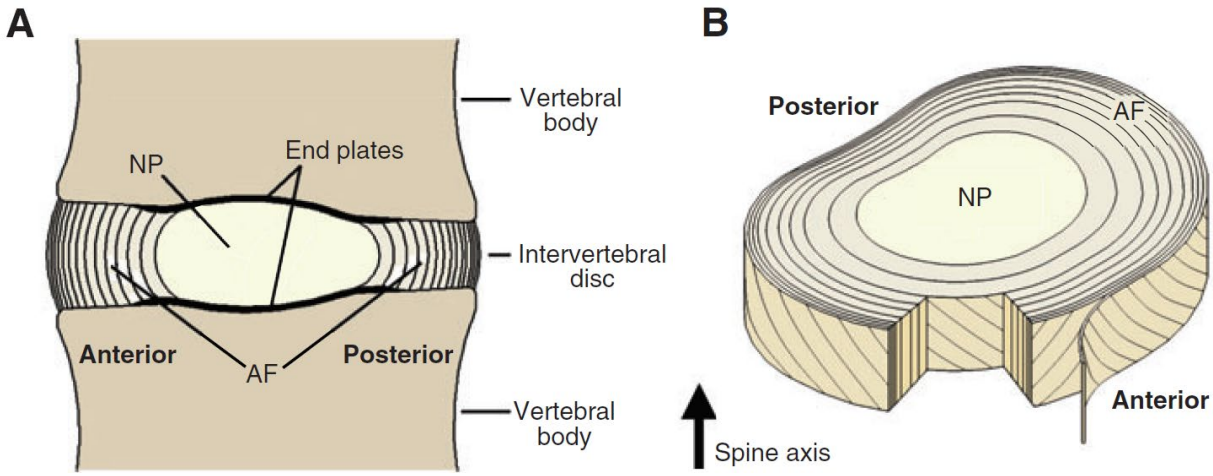

Figure B.1. From Smith et al (2011): Schematic representations of the adult intervertebral disc. (A) Mid-sagittal cross-section showing anatomical regions. (B) Three-dimensional view illustrating AF lamellar structure.