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I am submitting herewith a thesis written by William David Merryman, II entitled "Mechanical Stimulation of Intervertebral Disc Cells by Cyclic Hydrostatic Pressure". I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Engineering Science.

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Vice Provost and Dean of Graduate Studies

MECHANICAL STIMULATION OF INTERVERTEBRAL DISC CELLS BY CYCLIC HYDROSTATIC PRESSURE

A Thesis Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

William David Merryman, II
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Dedication

This thesis is dedicated to my wife, Erin, and my mother, Cindy, who have supported me through so much. Thank you for believing in me and always helping me to reach higher and further than I ever thought I could. Also, I would like to thank all my family and friends for their constant encouragement that has had such an impact on my life.

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Finally, I thank my family and friends for the suggestions and encouragement that made this work possible.

Abstract

Objective. To examine the effects of hydrodynamic cyclic loading on intervertebral disc cell biosynthesis.

Design. With the use of a piston-chamber pressure vessel, hydrodynamic pressure was applied to intervertebral disc cells in monolayer culture.

Background. The influence of compression on intervertebral disc cells has been examined in a number of previous studies. However, in most cases, hydrostatic pressure was used at low levels, and in none were high, hydrodynamic pressures at varying frequencies applied to intervertebral disc cells.

Methods. Cells were harvested from porcine intervertebral discs. After digestion, the cells were plated and grown to confluence in culture flasks, and then trypsinized and re-attached to 35mm culture dishes. By the means of cyclic, hydrodynamic loading, the cells were exposed to varied pressures and frequencies for 20 minutes a day for 3 and 7 days. Following the 2^{nd} and 6^{th} day of loading, [^{3}H]-proline was used to label the cells and they were incubated overnight to allow incorporation of the isotope. After loading on the 3^{rd} and 7^{th} day, the medium was removed and both cells and medium were frozen separately waiting analysis. By scintillation, the incorporated and released [^{3}H]-proline into and by the cells could be determined, and these values were normalized by DNA(ug).

Results. As reported previously, mechanical stimulation inhibited disc degeneration versus the Control. The treatment of 1Hz and 5MPa for both the 3 and 7 day trial caused the least ratio of collagen released, and for the 3 day trial, this treatment had the highest average of incorporated collagen, though for the 7 day trial this was not the case. Also, there appears to be a possible shift in biosynthesis near the frequency of 8Hz as this is where the cells began to increase degeneration after decreasing from the Control to 1Hz. It is believed that a detailed examination of frequencies from 1 to 15Hz should be conducted at constant pressure to determine the point of singularity.

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Chapter 1: Introduction

Low back pain is the one of the primary causes of disability under the age of 40 and plays a major role in the medical, social, and economic structure of industrialized countries. The etiology of low back pain is still unclear, and therapeutic approaches are often ineffective [18,19]. Human intervertebral discs (IVDs) undergo age-related degenerative changes that contribute to some of the most common causes of impairment and disability for middle aged and older persons such as spine stiffness, neck pain, and back pain [20]. Potential causes of the age-related degeneration of IVDs include declining nutrition, loss of viable cells, cell senescence, post-translational modification of matrix proteins, accumulation of degraded matrix molecules, and fatigue failure of the matrix. It has long been recognized that the healthy disc contains a small cell population, even smaller than that seen in articular cartilage. With arthritic changes and aging, this population declines even further [20].

Before understanding the problems concerning disc degeneration, it is necessary to understand the makeup of the IVD. There are three types of cartilage: hyaline, fibrocartilage, and elastic. Hyaline cartilage refers to articular cartilage, which is found in the articulating joints of the body and tracheal cartilage, fibrocartilage is found in the meniscus and IVD, and elastic cartilage is found in the ear. Hyaline cartilage and fibrocartilage are frequently studied in tissue engineering research, as they are more prominent and functional. Hyaline cartilage lends itself more readily to research because of accessibility, homogenous cell content, and clinical relevance. Fibrocartilage, however, is more difficult to study due to distinct cell types; there is much debate over the classification of some of these heterogeneous cell mixtures. Because they cannot be termed as fibroblasts or chondrocytes, they are often called fibrochondrocytes. Furthermore, the IVD cells very in type radially and with age. Fibrocartilage is largely studied *in vitro* [16].

The IVD is found between each vertebral column in the spine (Figure A.1; all figures and tables denoted by A are found in Appendix) and consists of two distinct regions (Figures A.2 and A.3) containing a complex mix of heterogeneous cells [1,2,3,7]. The two regions are the nucleus pulposus (NP), which is a gel-like substance found in the center, the anulus fibrosis (AF) which is an organized lamellar structure surrounding the center [1], and between these two is the transition zone (TZ) that is made up as a combination of the two. The composition of collagen changes from nearly all type I to nearly all type II from the AF to the TZ. The NP contains mostly type II collagen and has large amounts of aggregating and non-aggregating proteoglycans [1,2]. Primarily, the cells of the anulus originate from the mesenchyme and are chondrocyte-like or fibrochondrocyte-like with additional notochord originated cells found in the NP [2,3] that disappear in humans as they age and are replaced with fibrochondrocyte-like cells [1,7]. The cells obtained from the TZ represent the disc as a whole since it combines the two distinct regions.

Disc degeneration is a multifactor phenomenon. It has been found that IVD cells respond to such factors as pH, osmotic pressure, genetic factors, and mechanical loading [3]. Both biomechanical and biological factors have been implicated in cases of accelerated degeneration [24]. Mechanical factors seem to play an important role, as IVD cell death is dependent on the magnitude and duration of spinal loading [22]. Static compression *in vivo* has been demonstrated to induce cell death. Cell death, in turn, has been associated with disc degeneration in humans.

The process of disc degeneration may be monitored in a variety of ways. One of the most common methods is the incorporation of radio labeled proline prior to treatment. By allowing the isotope to incorporate into the cells via the media overnight and then removing the media, the cells contain a collagen matrix that cannot escape unless the cell layer is disturbed by a physical force or degraded by a matrix protease. When a force is applied to the cell wall, the cell produces an excess of matrix protease, which disturbs the cell layer and allows collagen to be released into the media, hence degrading the cell. It is not clearly known whether the ECM is available to absorb this excess released collagen,

or if this released collagen is of use to the ECM. We believe that the release of collagen into the media is a response of the cells to unfavorable loading conditions that cause degeneration.

Using an *in vivo* mouse model, it was found that static compressive loading initiates a number of harmful responses that are dose-dependent: disorganization of the AF, an increase in apoptosis and associated loss of cellularity, and down regulation of collagen II and aggrecan gene expression [21]. Compression-induced changes in IVD properties in a rat-tail model have also been found [23]. It was discovered that chronically applied compressive forces and immobilization cause changes in the biomechanical behavior and biochemical composition of rat-tail IVDs. Another study used coil springs stretched and attached to produce a compressive force across the lumbar IVD of dogs *in vivo* [25]. It was found that a high compressive force on the disc over a period of time initiates changes in proteoglycans and collagen produced by the intervertebral disc cells. A continuation to the previous research applied compressive forces to canine lumbar IVD cells *in vitro* and found that 0.35MPa inhibited proteoglycan synthesis in the AF and NP cells while stimulating collagen synthesis in the NP but inhibiting synthesis the AF [4]. Therefore, maintenance of appropriate stress or hydrostatic pressure within the disc may be an important basis for strategies to mitigate disc degeneration and initiate disc repair.

It has been reported that high magnitudes of hydrostatic pressure (10MPa) inhibit synthesis, while more moderate pressures (2.5MPa) have stimulated synthesis of proteoglycans or collagen in the TZ and NP [11,13,14]. Unfortunately, these results were found only in quasi-static environments, which are not representative of actual *in vivo* situations. For instance, while lifting a mass of 20kg, the resulting hydrostatic pressure measured *in vivo* has been found to be ~2.3MPa with the pressure proportional to the weight [4].

The effects of hydrostatic pressure on matrix synthesis in different regions of the bovine IVD have also been studied. They reported that applications of hydrostatic pressure in the range of 1-7.5MPa for only 20 seconds stimulated matrix synthesis while a long (2 hours)

continuous exposure to the same levels of pressure caused no stimulation in the NP of bovine discs and significant stimulation was only observed at 5MPa in the TZ. Exposure to 10MPa for either 20 seconds or 2 hours inhibited proteoglycan synthesis in these regions of the disc. In contrast, in the AF, where loading does not lead to a rise in hydrostatic pressure *in vivo*, there was no significant response to hydrostatic pressure over the range of 1-10 MPa in bovine or human discs [11].

Another study reported that cyclic mechanical tensile stress increases the growth rate and collagen synthesis of rabbit NP cells *in vi*tro [26]. Although the physiological loading on the NP is hydrostatic pressure rather than tensile, this was a good attempt in considering the application of loading in a cyclic manner. The IVD is routinely subjected to compressive loads that alter with posture and muscle activity and can produce pressures >2 MPa in human lumbar discs *in vivo* [27,28]. Pressures have been measured in the NP and within the AF of the human spinal disc due to extreme loading, and results showed that for normal discs, pressures in the NP and in the AF were linearly related to each other and to the applied compressive loads. In this case, IVD pressure varies from 0.19 at rest up to 34MPa at failure [29].

Previous trials indicated that articular cartilage under oscillatory compression *in vitro* at frequencies ~0.01-1Hz stimulated biosynthetic response but lower frequencies near 0.001 had no effect and these effects varied by region. The regional variation in the cartilage was dependent on both frequency and pressure [6,8-10]. The effect of pressure on disc cells in a cyclic manner is not fully known.

During daily occupational activities the intervertebral disc is exposed to dynamic oscillatory hydrostatic loads, and long-term occupational exposure to whole-body vibration increases the risk of disc degeneration [15]. However, a physiologic level of frequency and hydrostatic pressure may be essential for maintaining the matrix of the disc, while abnormal levels may accelerate disc degeneration. The pathogenesis of vibration-induced disorders is still not completely clear and there is no effective treatment. Although the potential effects of vibrational stress on extracellular matrix

(ECM) assembly and degradation are particularly relevant to the clinical findings of the vibration induced disorders, the effects of vibrational loads on disc cells are largely unknown.

We aim to develop a system capable of delivering a wide range of loading frequencies and dynamic pressure to cultures of disc cells to gain a better understanding of cyclic, high-compression environments and their constructive and destructive effects on intervertebral disc cells. Our purpose is to investigate these effects of various hydrodynamic loading doses (pressure, frequency, and duration) on cell synthesis.

Chapter 2: Methods and Materials

Tissue Dissection and Cell Isolation

Porcine intervertebral discs (IVDs) from the same specimen were obtained from a local abattoir[1] within two hours of euthanasia. Discs were submerged in Hank's Balanced Salt Solution (HBSS) and stored at 4°C until fine dissection, which took place approximately three hours post mortem. Under sterile conditions, the nucleus pulposus (NP) and transition zone (TZ) were separated and collected in 10cm culture dishes filled with HBSS; the NP portions were discarded since previous tests indicate they do not culture well in monolayer. Tissue was minced into 1-3mm^3 pieces to aid in digestion. The contents of the dishes were then transferred to four, 50mL conical tubes. Excess HBSS was removed and 40mL of complete media (DMEM "c")[2] with Bovine Testicular Hyaluronidase (2mg/mL) added to each tube. Hyaluronidase enzyme catalyzes the hydrolysis of hyaluronic acid, which is the cement substance of tissues.[3] Digestion continued for one hour in a 37°C, 5% CO^2 incubator with gentle agitation. After digestion, the cells were pelleted by centrifuging at 2000rpm for 5 minutes, and the hyaluronidase solution was removed.

40 mL of sterile DMEM"c" with 0.25mg/mL Collagenase and 0.25mg/mL of Pronase E was added to each conical tube. Collagenase enzyme catalyzes the degradation of collagen, and Pronase E or Protease, a peptidase, catalyzes the hydrolysis of peptide bonds.[4] The tubes were emptied into 150mm dishes and incubated overnight.

The following day, digests were pipetted up and down 10 times with a 25mL serological pipette at a flow rate of ~1mL/sec. Digests were then incubated for 2½ more hours to

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[1] Wampler's Farm Sausage Co. Inc., Lenoir City, TN. Special thanks to Mike Marney.

[2] Dulbecco's Modified Eagle Medium (Sigma)
10% Fetal Bovine Serum (all from GIBCO unless stated)
2% Penicillin Streptomycin Solution
0.5% Fungizone

[3] Definition taken from Dorland's Illustrated Medical Dictionary. Twenty-sixth edition, 1985.

[4] Ibid.

eliminate cell clumps. After the 15-hour total digestion time, the dishes contained a single cell suspension.

The cells were then strained through a 70μm nylon mesh into 50mL conical tubes. Cells were then pelleted at 1500 rpm for 5 min. Collagenase/Pronase E solution was removed and the cells were resuspended in 5 mL of DMEM"c". Using a hemacytometer, an aliquot of the cell suspension was stained with 0.4% Trypan Blue to determine cell viability. The four tubes revealed a cell count of ~200,000 cells/mL which corresponded to a total of ~1e6 cells per tube. A stain of Trypan Blue revealed that there was ≥95% cell viability. Cells were stored in a 75cm² flask in 45mL of DMEM"c" (Figures A.4 and A.5). After three days, the media was changed in the flasks (Figure A.6) once the cells had attached and was furthermore changed every two days.

Cells were cultured for 17 days to reach confluence and were then transferred to culture dishes to begin loading after an additional 10 days of growth. The cells were removed from the flasks by incubating with 5mL of 0.25% Trypsin at 37°C for 5 minutes after removal of the media and washing with 25mL of HBSS. The cells rounded up and were placed in a 50 mL tube for pelleting at 1500 rpm for 5 min. Solution was diluted with DMEM"c" to prevent cell damage by trypsin. After pelleting, trypsin was poured off and cells were resuspended in 28mL of fresh media. The suspension was divided into 14, 35mm culture dishes with 2mL of suspension per dish; this was done with each flask. The dishes (Figure A.7) were covered and placed into 150mm covered dishes (four 35mm/150mm, one 35mm from each of the four flasks). These were then incubated at 37°C and 5%CO2.

After 48 hours, the cells were attached to the culture dishes so the media was changed and was also changed every two days afterwards. At this point, the dishes contained large, isolated cell populations of both chondrocyte-like and fibrochondrocyte-like cells.

Comments on Cell Culture and Loading

For hydrodynamic loading of IVD cells, it is advantageous to culture the cells in monolayer. However, as the cells are cultured in monolayer, there tends to be dominance by the fibrochondrocyte-like cells most likely due to the fact that chondrocytes-like cells have poor attachment ability and are therefore typically cultured in alginate. The other major advantage of culturing IVD cells in monolayer is due to their high level of proliferation [7].

It should be noted that cells cultured in monolayer could possibly dedifferentiate as the culture time increases. In order for cells to best maintain their phenotype characteristics and exhibit significant biosynthesis, they should be cultured in alginate, but this limits cell number and proliferation [1,7], and would not lend itself to the design of this loading procedure.

Cell Loading

A piston-chamber system (drawing of design specifications, Figure A.8) was used to apply loads to the cells (photo, Figure A.9). It was made from 316 stainless steel by the Mechanical, Aerospace, and Biomedical Engineering Machine Shop at The University of Tennessee. The piston head and inner walls of the chamber were finely machined to prevent debris from forming due to excessive friction. No bleeding valves were used for fear of leakage. A 8-tiered, stainless steel shelf (Figure A.10) was made to hold 8 culture dishes that would fit in the chamber and allow fluid to easily reach the entire volume. Because of the desired high pressure to be generated by the piston, a tight seal was imperative, and an o-ring (from Parker, #2-325) with high toughness was chosen.

The bottom of the chamber was bored out and threaded to match the load cell attachment in order to effectively mate the chamber with the load cell of the Instron Mechanical Testing Machine (model 8511.20) that was used for all loading. In order to insure a moment-free contact between the piston head and the inner walls of the chamber, it was

necessary to machine the tail of the piston to accommodate a ball bearing that would translate the load from the actuator only in the vertical direction (Figure A.11).

Under sterile conditions, the 35mm culture dishes were loaded on the 8-tiered shelf (Figure A.12), and the piston was filled with DMEM without growth factors and antibiotics. The reason for not "completing" the medium was to conserve materials and there was little need since the cells were being exposed to loading for only 20 minutes a day. While still in the biological cabinet, the piston was engaged by hand to seal the vessel. The chamber was then transported to the mechanical testing machine (Figure A.13). Again with a female-threaded bottom, the chamber was mated to the load cell and a ball bearing was used as the contact between the piston and the actuator.

The cells were then loaded cyclically for 20-minute intervals subsequently every 24 hours. The chosen pressures, calculated from the piston head geometry, were $\leq$3MPa (pressure did not exceed 3MPa, but was kept between 2.5 and 3MPa) and $\geq$5MPa (pressure did not fall below 5MPa and was allowed to climb to ~6MPa), and the chosen frequencies were 1, 8, and 15Hz. Each pressure was paired with each frequency for a total of 6 scenarios plus a control that received no loading. A preload of 0.5MPa was used in each loading case. This loading was done for three and seven days, respectively to study variation in cell synthesis and degeneration.

Following each loading, the entire chamber was moved to the biological cabinet, and the piston was removed from the chamber. Using sterile tweezers, the shelf was lifted and the dishes were carefully removed one at a time. The test medium was aspirated and the dishes were filled with 3mL of DMEM"c". The lids were replaced and the 35mm dishes were housed in 150mm covered, culture dishes while incubating (Figure A.14) to allow for ease of maneuvering and to prevent contamination. The possibilities of contamination were increased due to the fact that the 35mm dishes were wet with medium when they came out of the loading chamber. Drying the dishes was necessary to prevent the pooled medium from developing fungus or bacteria; this was done simply by sitting the dishes on absorbent material while changing the media.

Radioisotope Labeling

After loading on the 2nd and 6th day, the 3-day and 7-day specimens were label with [^{3}H]-proline[5] (Figure A.15). Due to experimenter error, an excessive amount of proline (~80uCi) was injected into the 3-day dishes when only 20uCi (10uCi/mL) was intended. To compensate for this excess, an additional 3mL of media was added to reduce the concentration to 16uCi/mL. After the error was recognized, special care was taken not to replicate the episode not only because it altered the experiment but also because it depleted the remaining isotopes. When trying to obtain more isotopes from the supplier, it became apparent that they would not be able to deliver any by the 6th day of loading for the 7-day samples due to back orders. Out of necessity, more isotopes were ordered from another company[6]; however, this company does not make their entire isotope concentration in a 1mCi-to-1mL ratio as the previous company does. Without knowing, 5mCi of isotope was ordered in a volume of 1mL. Approximately 32uL of proline were added to the 7-day dishes assuming that this corresponded to 32uCi or 16uCi/mL, but because of the different concentration of the isotope, each dish was actually given 160uCi. This was not noticed until the 1mL volume was used up unexpectedly. Since both sets were labeled inaccurately but consistently, no further adjustments were made. The following day, the medium in each dish, containing isotope, was removed and discarded in radioactive liquid waste, and the cells were washed three times with warm (37°C) HBSS to remove any excess isotope that may be attached to the exterior of the cells. Then, after loading, fresh DMEM"c" was given to each dish and the dishes were incubated overnight. The following day, the media was aspirated and frozen down, and the cells were frozen down on their dishes to await extraction.

[5] 100Ci/mmol, 3.7TBq/mmol (ICN)
[6] Moravek

Size Exclusion Chromatography

Because the cells have many, many proteins that may have incorporated the proline, it was necessary to distinguish the large proteins, such as collagen, by size exclusion. By running the extracted proteins through columns containing Sephadex with certain size exclusion, it was possible to capture the larger proteins of interest since they were too large to enter the pores that are present in the slurry and simply run out of the bottom. A schematic drawing (Figure A.16) explains this process more clearly.

The frozen cells were extracted by adding 1mL (0.5mL for extraction and 0.5mL for DNA quantification) of Cell Extraction Solvent (CES)[7] to each 35mm dish and incubating at 4°C overnight. The frozen medium was extracted by thawing and transferring 0.25mL to a 5mL test tube and adding 0.25mL of Media Extraction Solvent (MES)[8] and incubating for at least 2 hours at 4°C.

The columns were prepared with a slurry of 20g Sephadex G-50 (fine) in 300mL of water. The slurry was boiled and allowed to cool to room temperature. 10mL serological pipettes were broken to allow a volume of 6mL and steel pellets used in carbon dioxide air guns were gently rolled into the pipettes (1 pellet/pipette) to provide a seal that allows water but not slurry to escape. For each extracted sample, a different pipette was used.

While gently stirring the slurry to keep the particles suspended, 3mL were transferred into the pipettes. As the slurry began to settle and the water ran out of the column, additional slurry was added to achieve a flat surface level of 2mL in each column. Then, 2mL of CES were run completely into the column without disturbing the slurry surface. Finally, the 0.5mL extracts were loading into their respective columns, and were then

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[7] 4M Ultra Pure, Guanidine-HCl (Life Technologies); 50mM Sodium Acetate, pH 6; 1% Triton-X-100; 50mM 6-Amino Hexanoic Acid; 10mM EDTA; 0.8mg/mL Benzamidine; 10mM N-Ethyl Maleimide; 1mM Phenylmethylsulfonyl Fluoride

[8] 8M Ultra Pure, Guanidine-HCl; 100mM Sodium Acetate, pH 6; 2% Triton-X-100; 100mM 6-Amino Hexanoic Acid; 20mM EDTA; 1.6mg/mL Benzamidine; 10mM N-Ethyl Maleimide; 1mM Phenylmethylsulfonyl Fluoride

eluted by adding 0.75mL of CES to each column. The flow was collected in 7mL scintillation vials. Since the columns were subjected to cells and medium that contained radioisotopes, they were discarded in radioactive waste containers.

3mL of scintillation cocktail[9] was added to each vial, mixed thoroughly, and counted on the tritium channel in a scintillation counter.

DNA Assay

In order to normalize each dish, it is necessary to have some way of counting the number of cells present. Because of the radioactivity and quantity of the cells it was determined that the use of a hemacytometer would not be very safe or efficient. An alternate method of DNA quantification by fluorescence was chosen because of its ease of use and rapid results. Theoretically, each cell should have the exact same amount of genomic DNA that when extracted would bind to bisbenzimide dye, a DNA intercalator, and could be excited in a fluorometer and quantified. Before quantifying the cells, a standard curve was generated with Calf Thymus DNA (Figure A.17), a common reference for plant and animal DNA, since it is double-stranded, highly polymerized, and is ~58% AT [17]. Beginning with a concentration of 5000ng/mL, the DNA was diluted serially as follows: 2500, 1000, 500, 250, 100, 50, 25, 10, 5, 1, 0.5 ng/mL. The standard curve yielded an R^2=0.9743 with the linear regression equation, y = 15.695x + 50686.

From each dish of extracted cells in 1mL of CES, 100uL was drawn and loaded into a well of a 96-well plate. To each well, 100uL of High Range Assay Solution[10] were also added. The well plate was placed in a multi-channel Victor counter and set on fluorometry with excitation at 350nm and emission at 450nm. The quantities were then calculated from the standard curve equation. Since the volume of Calf Thymus DNA and

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[9] ScintiSafe Econo 1 (Fisher Scientific)

[10] 100uL Hoechst 33258 Stock Dye (10mg Hoechst Dye, 10mL Sterile Water), 10mL 10xTNE Buffer Stock (0.1M Trizma Base, 10mM EDTA, 2M NaCl, adjust pH to 7.4 with concentrated HCl), 90mL Sterile Water

Chapter 3: Results

Data

All data is located in the Appendix. Tables A.1 & A.2 contain the counts from the scintillation counter for the 3 Day and 7 Day loading, respectively. Table A.3 contains the DNA assay results from each dish. Table A.4 has the calculated values for the Incorporated Collagen from the cells of each dish, and Table A.5 is the calculated Ratio of Collagen Released for each media sample.

Incorporated Collagen (IC)

Figure 1 (on the following page) represents the Incorporated Collagen (IC) for the 3 day loading scenarios, and Figure 2 represents the IC for the 7 day loading scenarios. The values represent the mean±standard deviation of the four samples (n=4) from each group. Scenario 1Hz/5MPa/3Days only had three samples as one was lost because the cells did not successfully run the size exclusion column, and scenario 15Hz/3MPa/3Days also had only three samples because the culture dish was broken during loading and could not retain media. The IC is reported as CPM/DNA(ug)/1000 or counts per million from the scintillation counter results divided by the ug of DNA from the fluorescence counter divided by 1000; this format of reporting was used in previous unpublished research by Dr. Kasra. All treatments are reported as Hz/MPa/Days unless otherwise stated. The Student t-test was used to test for significance between treatments, and $p<0.05$ level of significance was used in all cases.

There is significant increase in IC between the 15/5/3 scenario and scenarios Control, 8/3/3, 8/5/3, and 15/3/3. No other treatments are significantly different for IC for 3 day loading (Figure 1).

There is no significant difference between any of the 7 day treatments for IC (Figure 2).

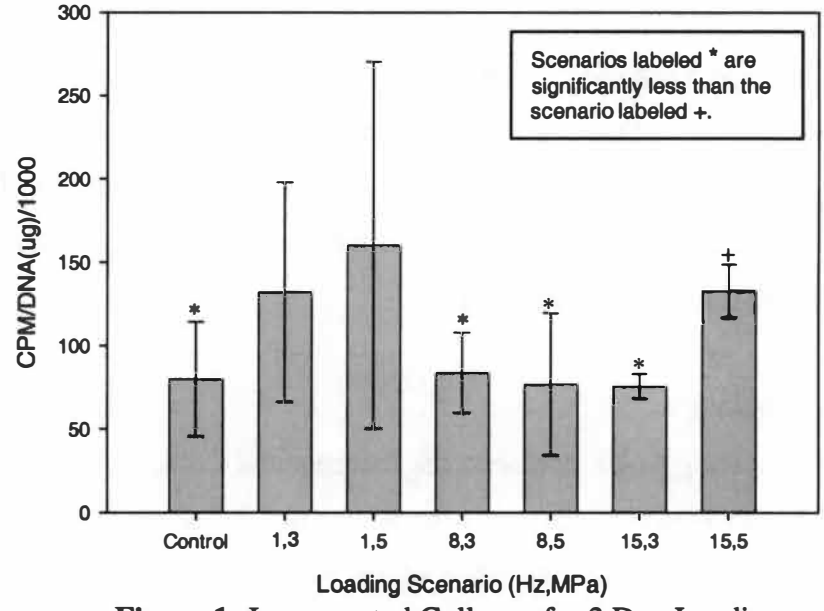


Figure 1: Incorporated Collagen for 3 Day Loading.

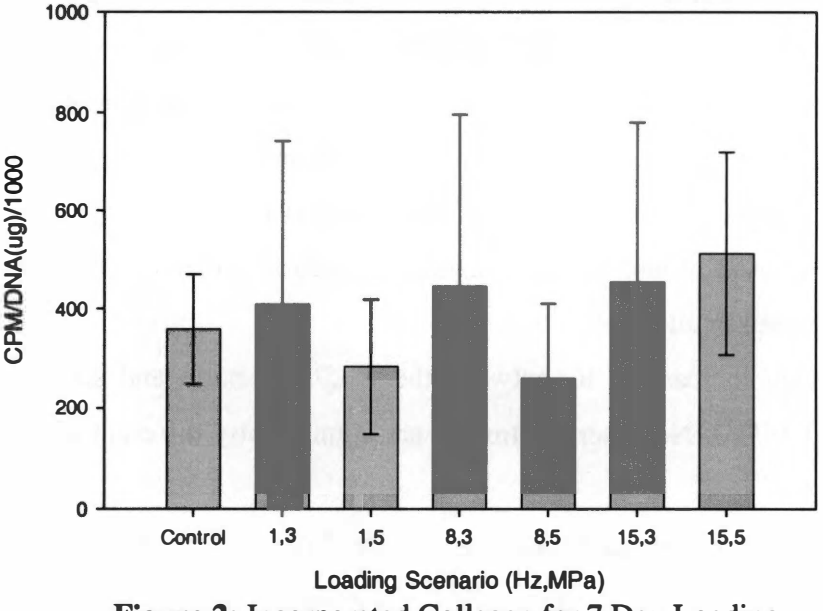


Figure 2: Incorporated Collagen for 7 Day Loading.

Released Collagen (RC)

The quantity of Released Collagen was determined by scintillation counts of the media. This number, RC, could then be used to determine what ratio of collagen was released by the cells into the media in the 24 hours following the final day of loading for both the 3 and 7 day treatments. This ratio, the Ratio of Collagen Released (RCR=RC/(IC+RC)), gives some indication of how the cells are thriving; as the ratio increases, it is believed that the cells are becoming less healthy and this leads to tissue degeneration.

Figure 3 represents the RCR into the media from the cells for the 3 day loading, and Figure 4 represents the RCR into the media for the 7 day loading.

There is significant increase of RCR from 1/5/3 to 15/5/3, and an increase nearing significance between 1/5/3 and both 8/5/3 and 15/3/3 with values p=0.0648 and 0.0616, respectively. There are no other significant differences between 3 day treatments.

There are significant decreases of RCR from Control to 1/5/7, 8/3/7, 15/5/7 and a decrease nearing significance (p=0.056) from Control to 1/3/7. A decrease nearing significance was found from 1/3/7 to 1/5/7 (p=0.07). There is significant increase in RCR from 1/5/7 to 8/5/7, and increases nearing significance from 1/5/7 to 8/3/7 (p=0.061) and 15/3/7 (p=0.0735). There are no other significant differences between treatments for 7 day treatments.

Collagen Released by Pressure, Frequency

Figure 5 represents RCR by loading pressure for both 3 and 7 day treatments while ignoring loading frequency. There are significant decreases from Control to 3 and 5MPa for the 7 day treatments. There are no other significant differences between loading pressures.

Figure 6 represents RCR by loading frequency for both 3 and 7 day treatments while ignoring loading pressure. There are significant decreases from Control to 1, 8, and 15Hz for the 7 day treatments. There are significant increases between 1Hz and 8 and 15Hz for the 3 day trial. There are no other significant differences between loading frequencies.

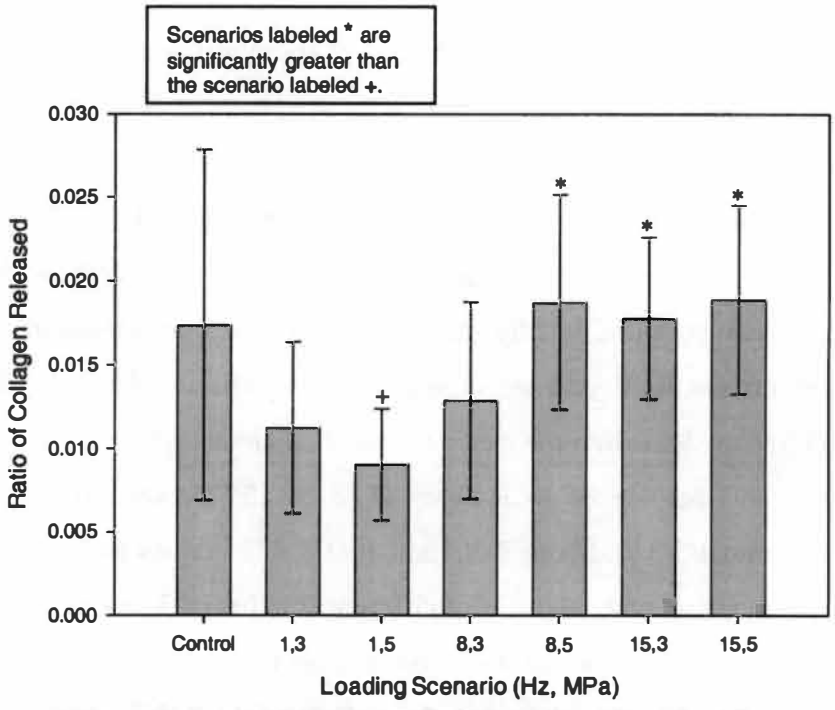


Figure 3: Ratio of Collagen Released for 3 Day Loading.

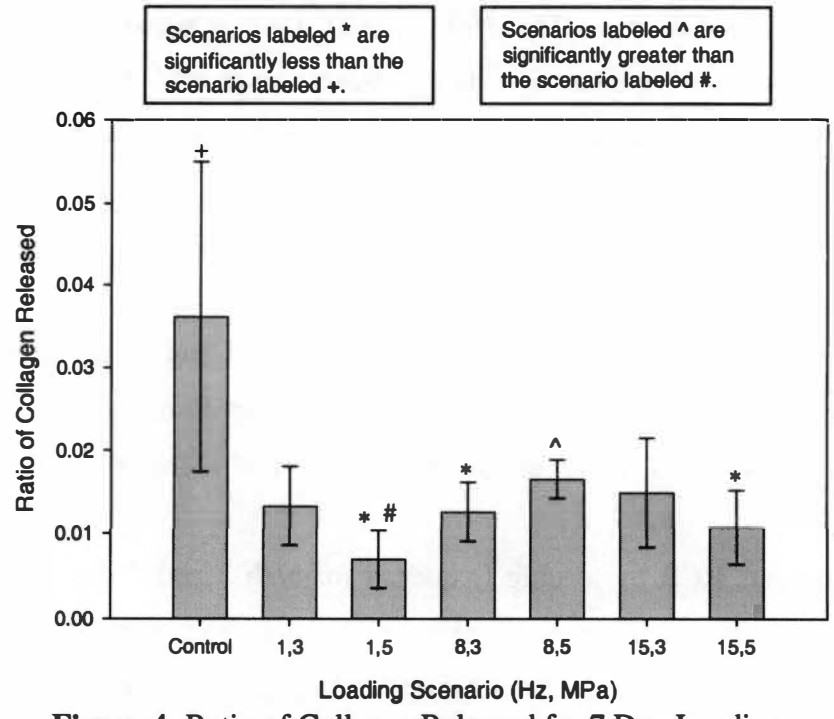


Figure 4: Ratio of Collagen Released for 7 Day Loading.

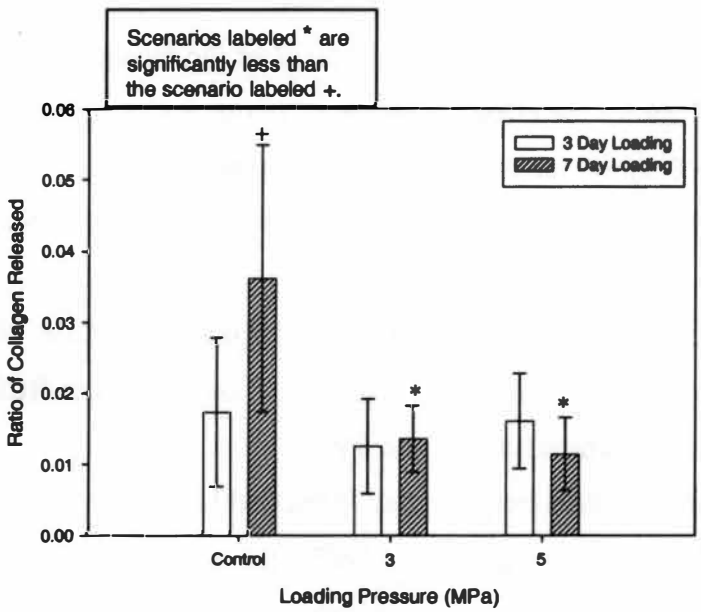


Figure 5: Ratio of Collagen Released vs. Loading Pressure

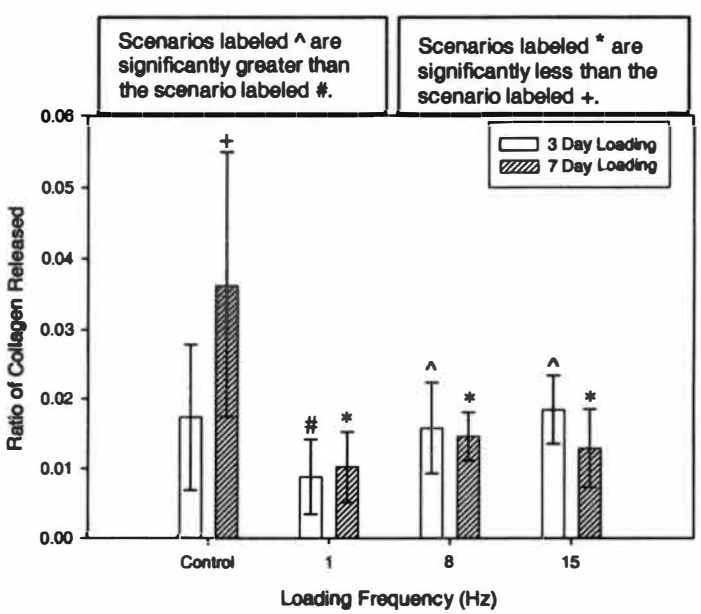


Figure 6: Ratio of Collagen Released vs. Loading Frequency

Collagen Released, Pressure and Frequency Trends

Figures 7 & 8 represent the overall effects on RCR of the treatments of both the 3 and 7 day trials. Figure 7 shows the effect of increased frequency at constant pressures (3 and 5MPa), and Figure 8 shows the effect of increased pressure at constant frequencies (1,8, and 15Hz). These figures are merely the non-aggregated data that is shown in Figures 5 & 6.

Figure 7 shows that increased pressure at 1Hz and 15Hz decreased the RCR, while at 8Hz it had the opposite effect. Figure 7 also shows that the maximum RCR for both trials, except for the Control, occurred at 8Hz and 5MPa.

Figure 8 shows that increased frequency at 3MPa had little effect for the 7 day trial but did increase the RCR for the 3 day trial. At 5MPa, the effect of frequency above 1Hz had a dominant effect in increasing the RCR for both 3 and 7 day trials.

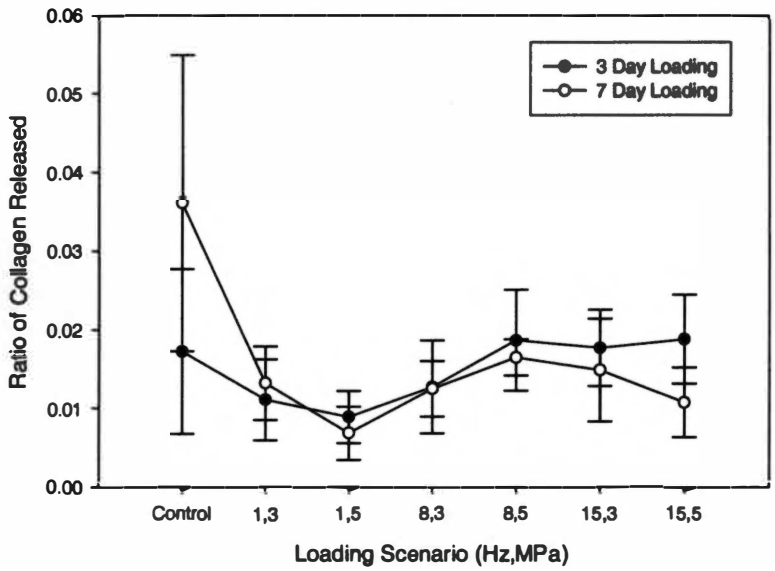


Figure 7: Ratio of Collagen Released, Pressure Trend

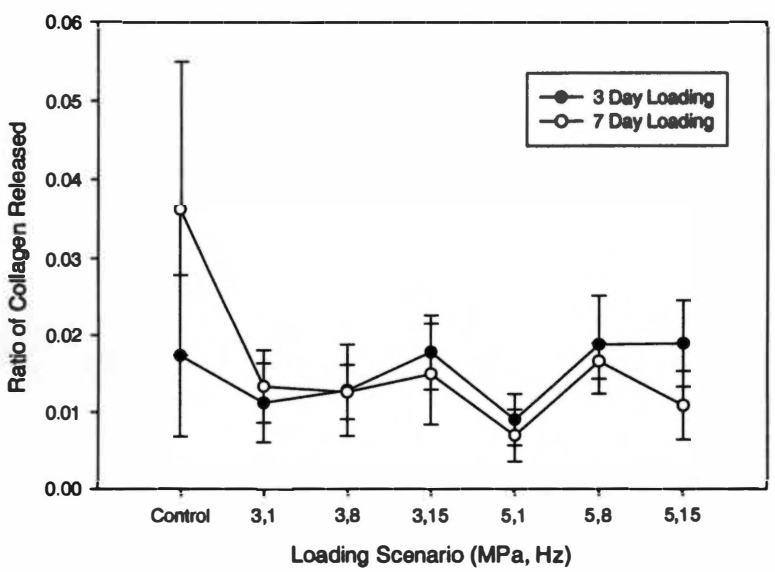


Figure 8: Ratio of Collagen Released, Frequency Trend

Chapter 4: Discussion

In this study we mainly focused on introducing a method of investigating the effect of dynamic hydrostatic pressure on intervertebral disc cells over a wide rage of pressure and frequency spectrums. Using a hydraulic chamber to transfer a load through a fluid media, the stiffness of the system was much higher than that of the traditional way of load transfer through the air in a pressure vessel. Therefore, we could have a wider range of loading pressures and frequencies to high levels simulating different low and severe *in vivo* dynamic loading conditions.

The purpose of this research is two-fold but closely related: to investigate different mechanical stimulation environments and their effect on cell synthesis, and to determine which unfavorable cell environments lead to tissue degeneration. The amount of incorporated [^{3}H]-proline (which is assumed to be equivalent to collagen) is believed to be consistent with healthy cells that are thriving in a given environment while the ratio of collagen released is an indication that cells are unhappy and this is the beginning of tissue degeneration.

Application of load to connective tissues affects the mechanical, electrical and chemical behavior of cells, including changes in shape and volume, expulsion of fluid, changes in PH and osmotic pressure, among others [14,33-35]. Several studies have previously shown that application of improper loads can lead to cellular changes and even loss of cells in the intervertebral disc and surrounding tissues. For example, noticeable cell loss has been observed in the deformed discs of kyphoscoliotic mice [36]. It appears that long-term load-induced changes to the environment of the disc's cells can lead to cell death. Such loading would lead to fluid loss, compaction of matrix, changes in osmotic and hydrostatic pressure and cell shape, and a fall in transport of nutrients and other solutes to and from the cells. All these factors have been shown to have a strong influence on cellular metabolism in vitro [11]. In other work with canine IVDs cultured in alginate, it was seen that hydrostatic pressure (0.35MPa) applied constantly (9 days) stimulated

synthesis of collagen in NP cells but was inhibited in AF cells versus atmospheric pressure [4]. This 0.35MPa is equivalent to a human standing relaxed. This work was undertaken after previous experiments of constant hydrostatic pressure for up to 2 hours [11,13] and 24 hours with little effect [14]. Also previously investigated was the influence of hydrostatic pressure on the expression of cytokines and heat shock protein 70 in a chondrocyte-like cell line. The incorporation of [35-S] sulfate into the cultured cells increased following exposure to 1-5 MPa of hydrostatic pressure and decreased following 10-50 MPa of pressure. These results suggest that hydrostatic pressure at physiologic levels enhances the expression of transforming growth factor-beta 1 mRNA in addition to increasing proteoglycan synthesis in chondrocytes and that excessively high hydrostatic pressure reduces the expression of transforming growth factor-beta 1 mRNA and increases the expression of heat shock protein 70 mRNA while decreasing proteoglycan synthesis. These studies investigated only the extent of the effect of quasi-static hydrostatic loads [32].

Kasra, et al. found that there was an increase in RCR for rabbit IVDs versus the control for both 3 and 9 day loading under similar conditions [37]. These results differ from the ones found here, but the cause is not known. Obviously, the choice of different animals may have a great impact on the results. For instance, the pressures used in the rabbit model could be out of the normal physiological range for such a small animal where the pressures used for the porcine disc were within the animal's range. The rabbit model was the precursor for the current work. Porcine discs were used here due to their size and ease of dissection. It is believed that the current research gives more understandable results that show the effect of mechanical stimulation.

There was no conclusive evidence concerning IC for either treatment durations, although for the 3 day trial, the 15Hz/5MPa loading gave a significant increase over every other scenario except the two at 1Hz. The two 1Hz treatments gave a wide array of IC values that need to be investigated further. All IC values for the 7 day trial were so inconsistent that no trend could be made; no differences were significant.

Every loading scenario used produced less degeneration than the Control for the 7 day trial while three treatments (8Hz/5MPa, 15Hz/3MPa, and 15Hz/5MPa) for the 3 day trial were nearly equal to the control. This indicates that, over a longer period of time, the effect of mechanical stimulation can be largely seen.

There appears to be some combination of interaction between frequency and pressure for the rate of synthesis (degeneration) of cells cultured in monolayer. This effect is seen when either pressure or frequency is ignored and the other is focused upon (Figures 5 & 6). There are significant decreases in RCR when mechanical stimulation is applied. When both parameters are studied simultaneously, a trend arises that leads to the opinion that a phenomenon is occurring between 1 and 8Hz that does not appear to be dependent upon the pressures used in the study. Of course, this was just a small sampling of pressures that may be applied and the 5MPa value may not be of great interest since it is not necessarily in the physiological range.

In this study, we only investigated changes in synthesis rate and degradation. Other parameters such as changes in cell shape, age, proliferation, gene expression, etc. are still largely unknown. The effect of loading conditions with higher pressures and frequencies and different durations of loading cycles than those used here should also be investigated. As degeneration is judged solely on the amount of collagen released into the media, a better understanding of the proteases expressed due to applied compression is essential to understand why the cell layer is disturbed. The fact that the ECM is composed largely of collagen raises the question; would not the collagen released into the media correspond to cells supplying more collagen to the ECM in order to counteract the effect of the loading since the ECM absorbs the majority of compression that is applied to tissue? It is uncertain whether released collagen is released for this exact purpose or if it is simply lost by the cells, thereby making it useless.

Loading stimulation may also be used in conjunction with other treatments such as exogenous growth factors as a therapeutic approach to degenerative disc disease involving enhanced tissue repair [30,31]. There are likely different combinations of

pressure, frequency, and duration with optimum levels below which loading is constructive and can be used for tissue generation, prevention, and rehabilitation of disc degeneration; however, above this optimum levels disc degeneration will occur. Also, to what extent porcine disc cells show similar response to those of human cells should also be investigated.

It must also be mentioned that in an earlier sturdy [7], TZ cells underwent a reversible phenotype shift while cultured in monolayer (with no external loading), and cells also decreased in Type II collagen as articular chondrocytes have done while cultured in monolayer [38-40]. Because of this phenomenon, it would difficult to compare a replicate of this work for cells cultured in alginate. This is unfortunate since this is the next step that will likely be pursued for investing mechanical stimulation of IVD cells.

Considering the ranges of amplitude, frequency, and duration of the loading used in this study, short term application of high loading pressures and frequencies is likely beneficial in stimulation of collagen synthesis and reduction of collagen degradation. This study is expected to shed new light on the mechanism of vibration induced intervertebral disc disorders at a cellular level. It also proposes that vibrational loading can likely be used as a preventive or a treatment tool if applied in a controlled manner.

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Appendix

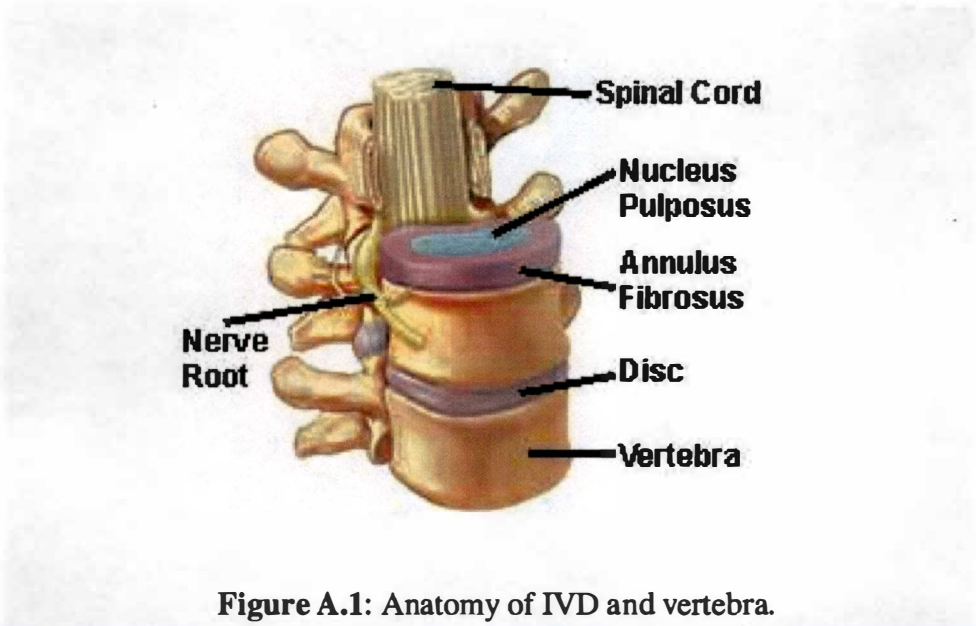


Figure A.1: Anatomy of IVD and vertebra.
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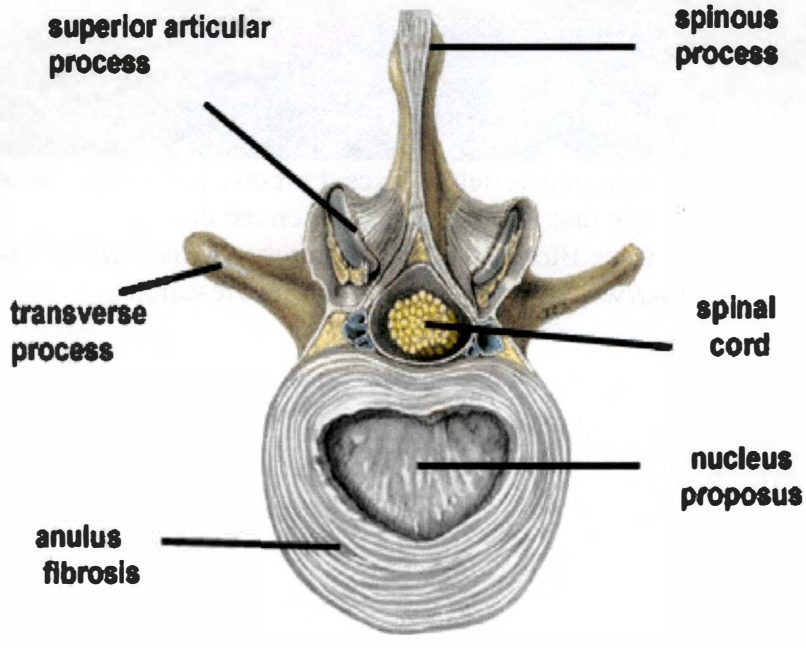


Figure A.2: Top view spinal column.
Taken from http://www.spineuniverse.com/displayarticle.php/article1682.html

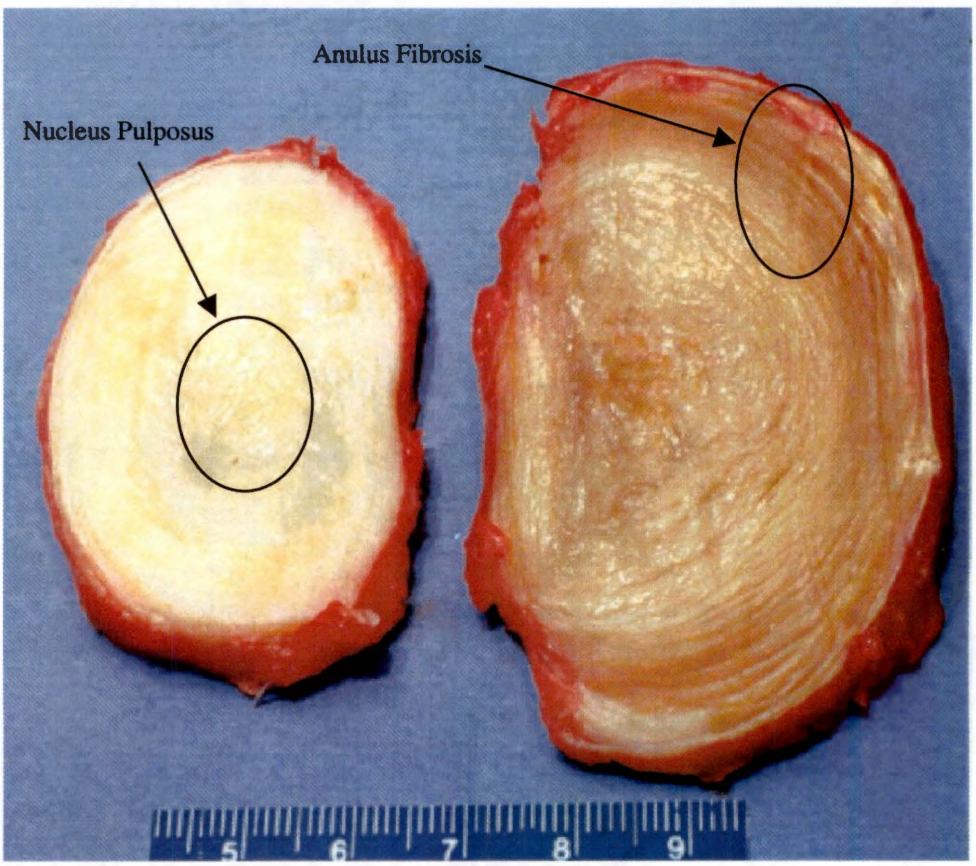


Figure A.3: Dissected human intervertebral discs. Left disc represents a healthy disc and right disc represents a degenerated disc.
Picture courtesy of Spine Biomechanics Laboratory. (University of Vermont).
http://www.emba.uvm.edu/~iatridis/research/

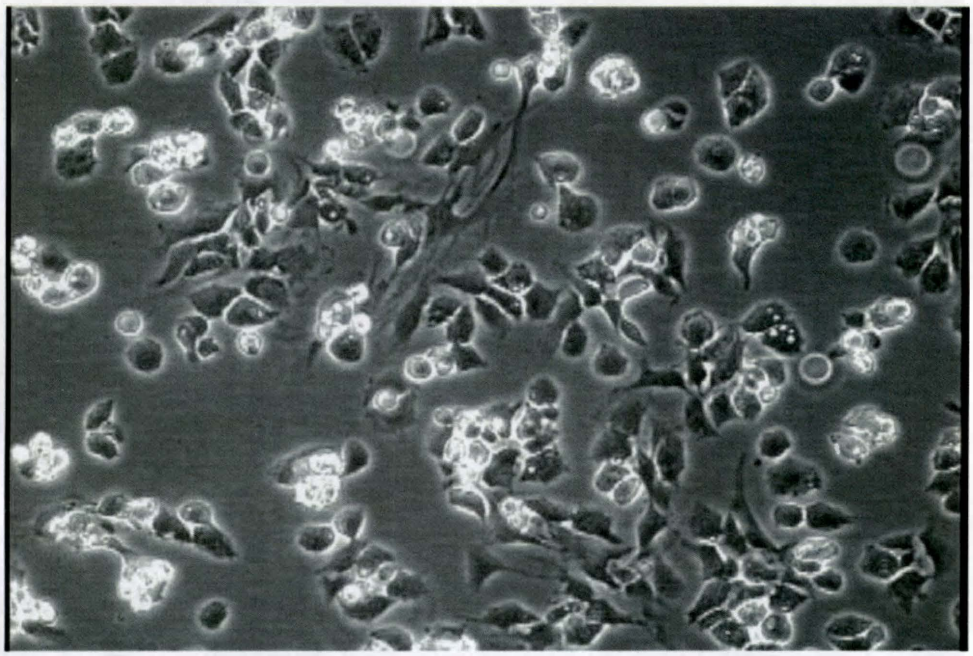


Figure A.4: Microscopic view (10x) of attached IVD cells.

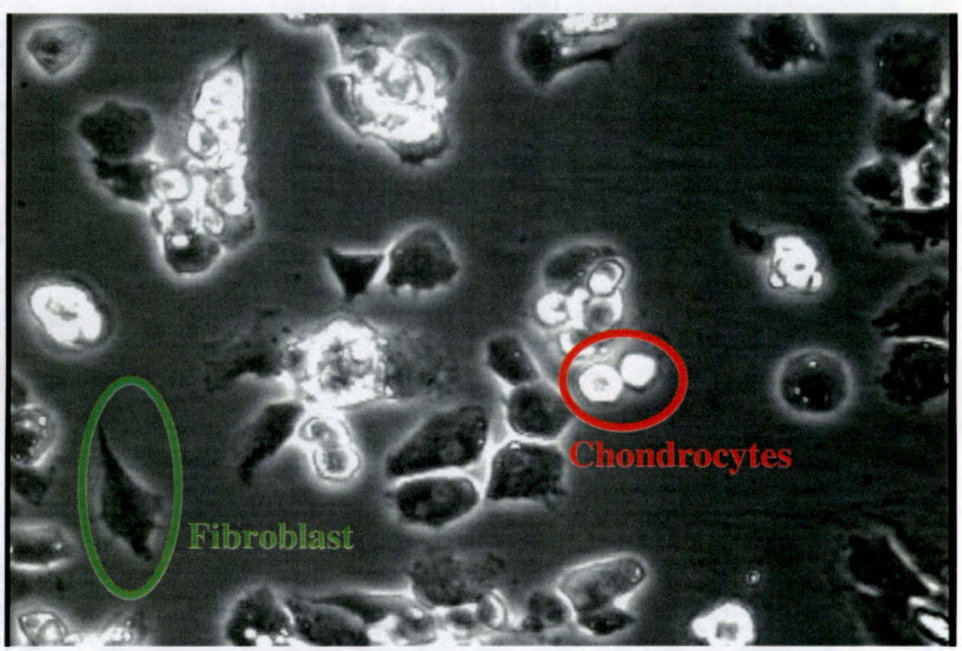


Figure A.5: Microscopic view (15x) of attached IVD cells.

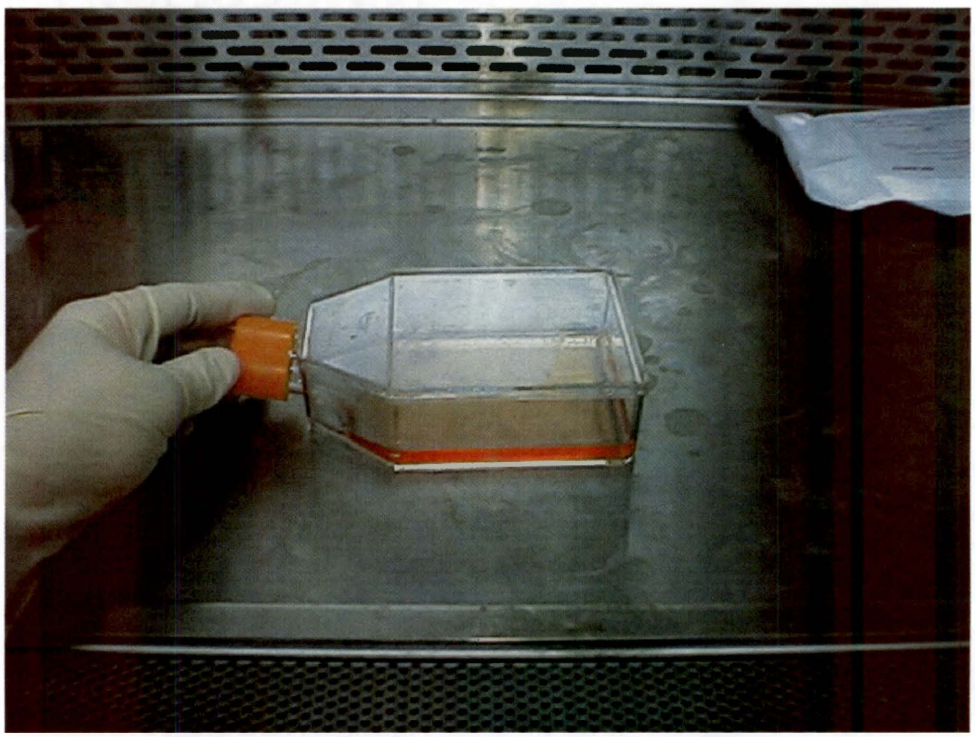


Figure A.6: Flask with attached cells and media.



Figure A.7: Cells and media in 35mm culture dishes in 150mm dish.

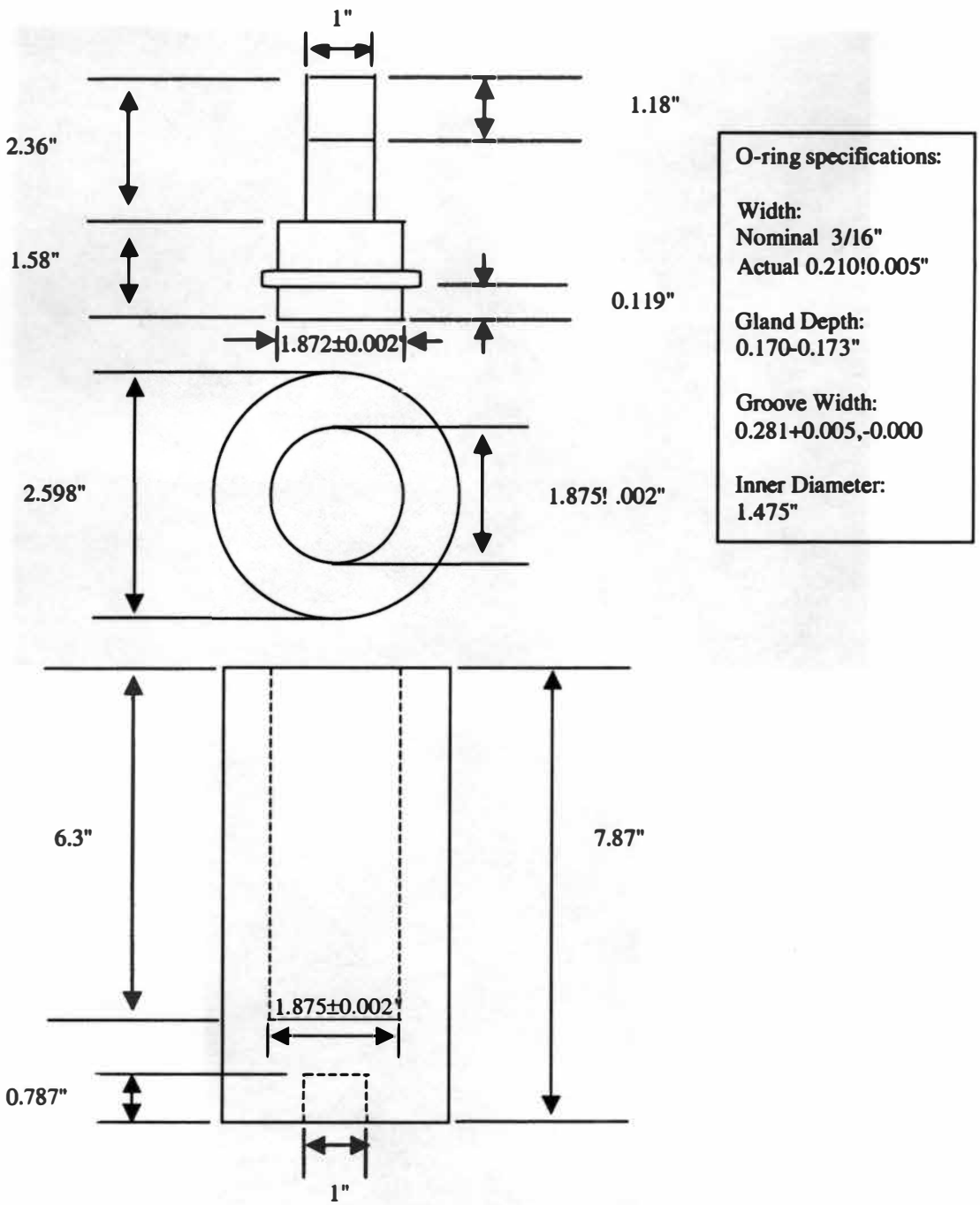


Figure A.8: Piston-Chamber Design Specifications



Figure A.9: Piston-chamber used for cell loading.

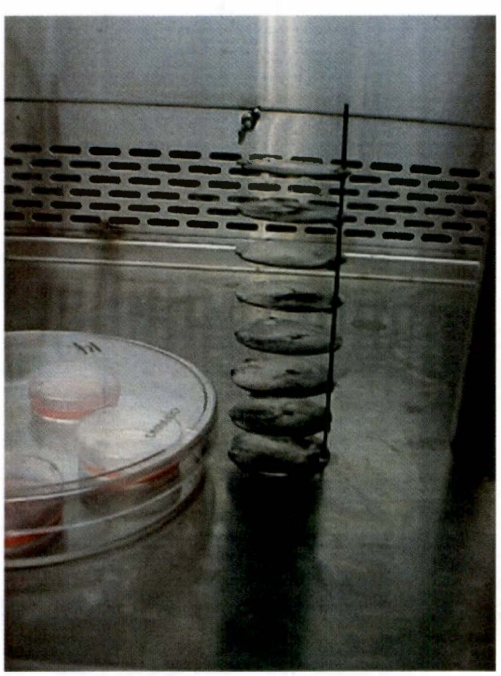


Figure A.10: 8-tiered shelf.

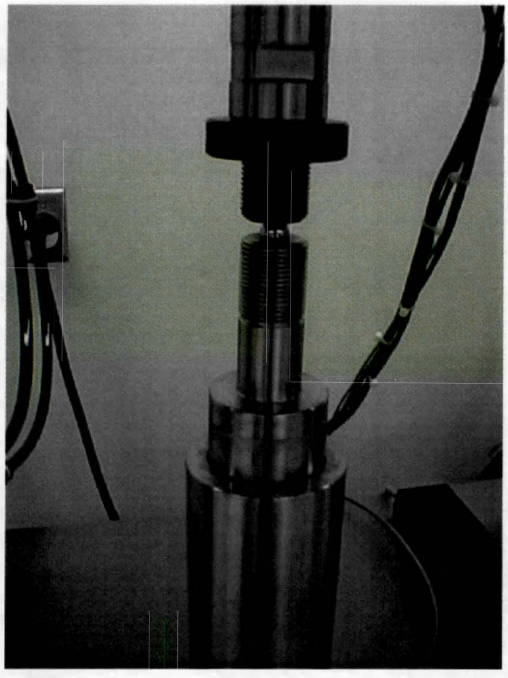


Figure A.11: Piston head with ball bearing and actuator.

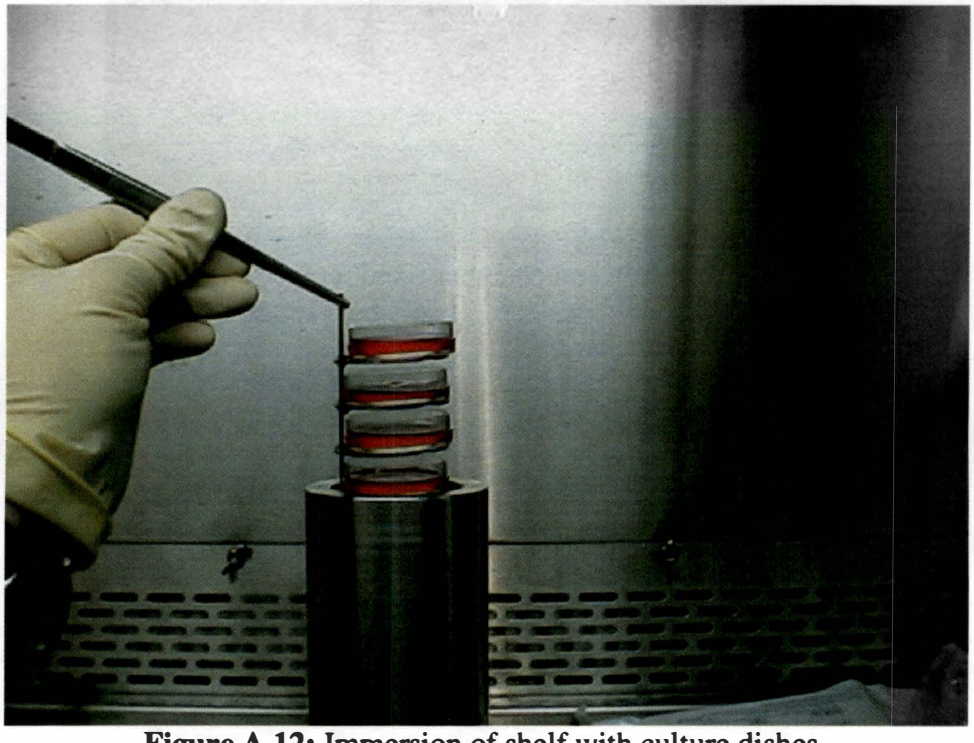


Figure A.12: Immersion of shelf with culture dishes.

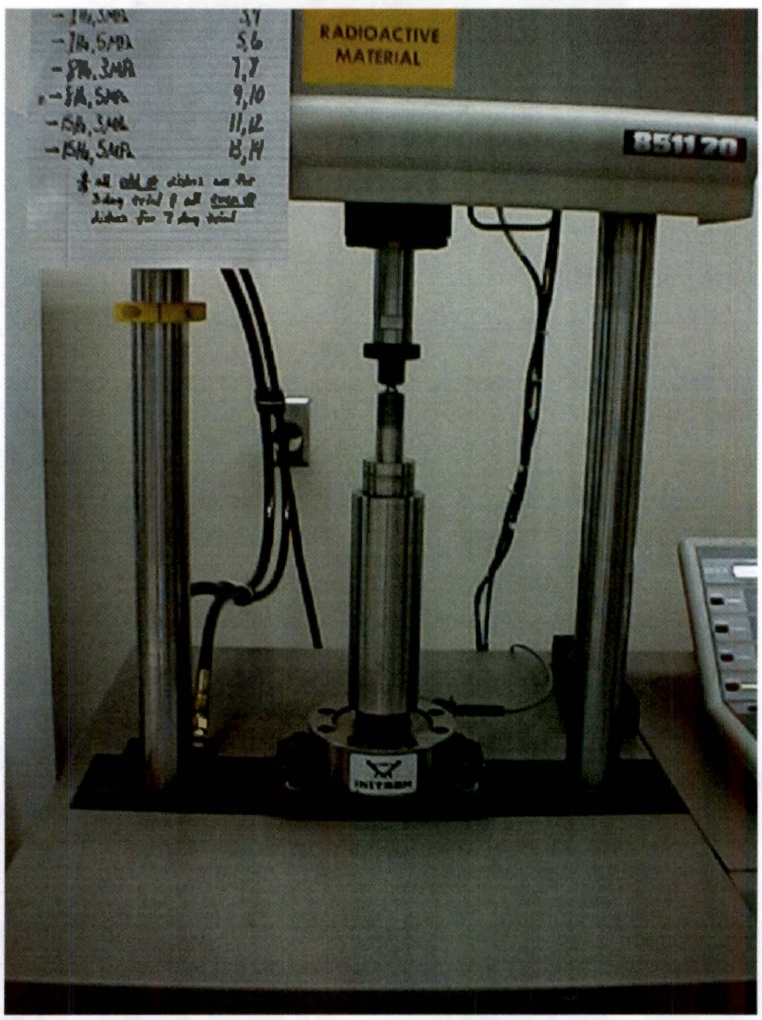


Figure A.13: Piston and chamber fixed on Instron Machine.

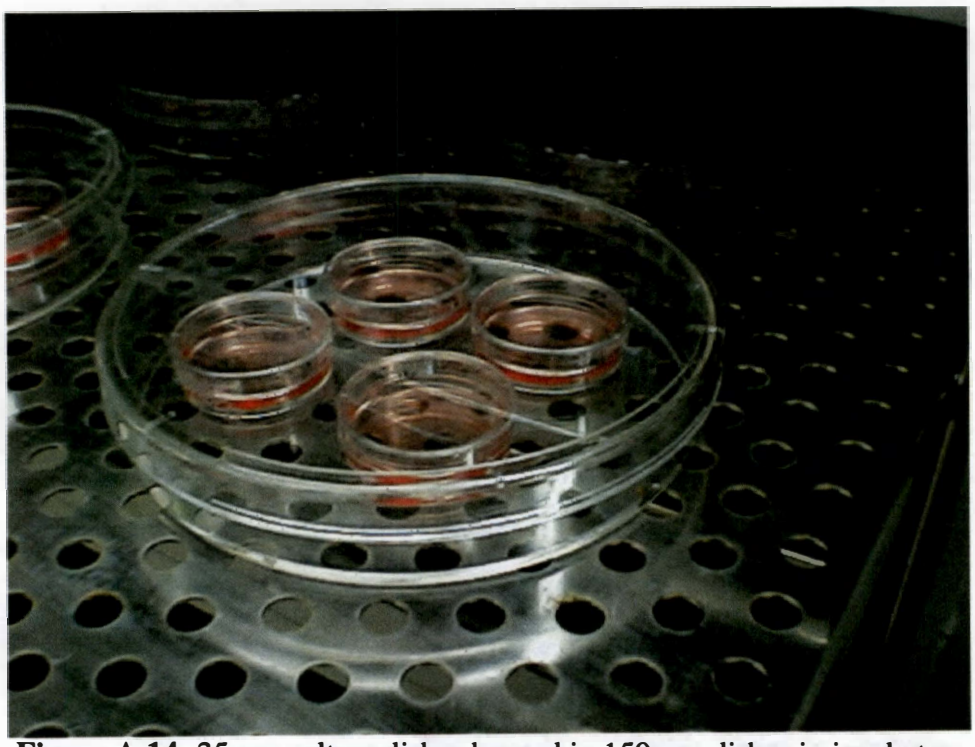


Figure A.14: 35mm culture dishes housed in 150mm dishes in incubator.

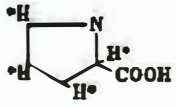


Figure A.15: [^{3}H]-proline.

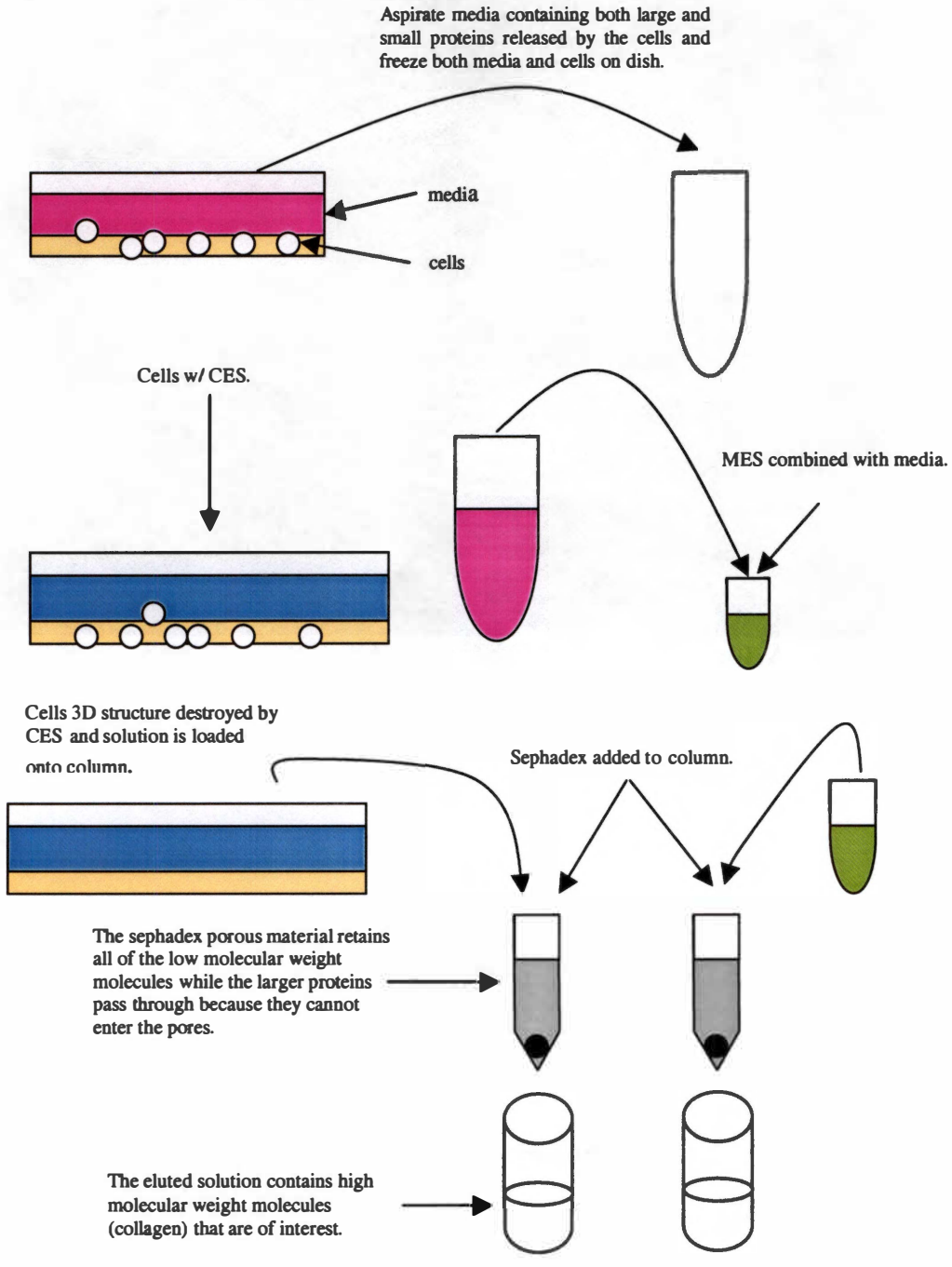


Figure A.16: Schematic of Column Chromatography.

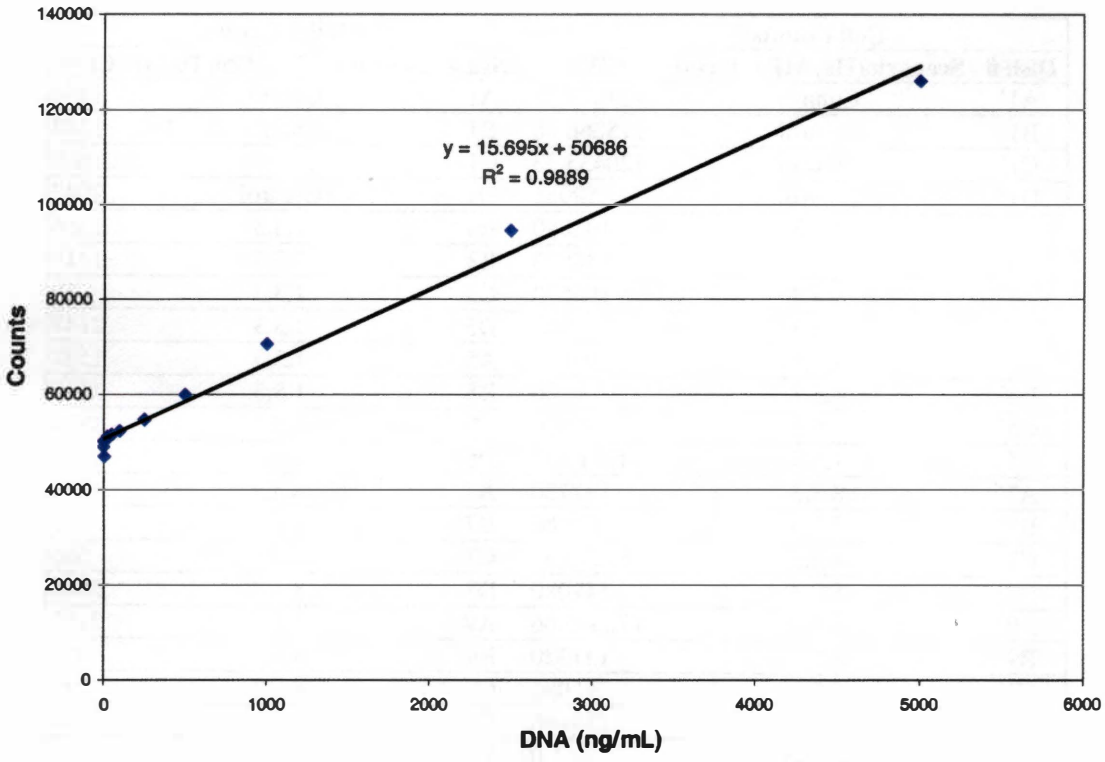


Figure A.17: Standard curve for Calf Thymus DNA.

Table A.1: Scintillation Counts for Cells and Media for 3 Day Loading.

Cell Counts			Media Counts		
Dish #	**Scenario(Hz, MPa, Days)**	**CPM**	**Dish #**	**Scenario(Hz, MPa, Days)**	**CPM**
A1'	Control	127973.33	A1	Control	1458
B1'	Control	115266.66	B1	Control	1344
C1'	Control	120433.33	C1	Control	1669
D1'	Control	59955	D1	Control	2047
A3'	1,3,3	191050	A3	1,3,3	1366
B3'	1,3,3	146073	B3	1,3,3	1419
C3'	1,3,3	195573	C3	1,3,3	19
D3'	1,3,3	124360	D3	1,3,3	2148
A5'	1,5,3	293620	A5	1,5,3	1532
B5'	1,5,3	0	B5	1,5,3	1948
C5'	1,5,3	177560	C5	1,5,3	1964
D5'	1,5,3	149513.33	D5	1,5,3	1660
A7'	8,3,3	177730	A7	8,3,3	1854
B7'	8,3,3	170226.66	B7	8,3,3	3225
C7'	8,3,3	87273.33	C7	8,3,3	505
D7'	8,3,3	147080	D7	8,3,3	2502
A9'	8,5,3	172946.66	A9	8,5,3	2152
B9'	8,5,3	131320	B9	8,5,3	1948
C9'	8,5,3	65095	C9	8,5,3	1742
D9'	8,5,3	135160	D9	8,5,3	3046
A11'	15,3,3	0	A11	15,3,3	0
B11'	15,3,3	118050	B11	15,3,3	1655
C11'	15,3,3	100860	C11	15,3,3	1674
D11'	15,3,3	115390	D11	15,3,3	2737
A13'	15,5,3	208710	A13	15,5,3	3120
B13'	15,5,3	149446.66	B13	15,5,3	2138
C13'	15,5,3	133233.33	C13	15,5,3	3574
D13'	15,5,3	175206.66	D13	15,5,3	3670

Table A.2: Scintillation Counts for Cells and Media for 7 Day Loading.

Cell Counts			Media Counts		
Dish #	**Scenario(Hz, MPa, Days)**	**CPM**	**Dish #**	**Scenario(Hz, MPa, Days)**	**CPM**
A2'	Control	466733.3	A2	Control	8232
B2'	Control	492780	B2	Control	28725.7
C2'	Control	537520	C2	Control	12682.5
D2'	Control	377590	D2	Control	19552.7
A4'	1,3,7	670753	A4	1,3,7	7542
B4'	1,3,7	491419	B4	1,3,7	7314
C4'	1,3,7	327753.3	C4	1,3,7	2764
D4'	1,3,7	293226.7	D4	1,3,7	5750
A6'	1,5,7	528900	A6	1,5,7	5943
B6'	1,5,7	325460	B6	1,5,7	1344
C6'	1,5,7	262460	C6	1,5,7	1138
D6'	1,5,7	329890	D6	1,5,7	2789
A8'	8,3,7	1031927	A8	8,3,7	13168
B8'	8,3,7	407493.3	B8	8,3,7	3778
C8'	8,3,7	546493.3	C8	8,3,7	9733
D8'	8,3,7	356400	D8	8,3,7	4042
A10'	8,5,7	343226.7	A10	8,5,7	5362
B10'	8,5,7	244033.3	B10	8,5,7	3902
C10'	8,5,7	220633.3	C10	8,5,7	3396
D10'	8,5,7	586526.6	D10	8,5,7	11982.3
A12'	15,3,7	964719.9	A12	15,3,7	7322
B12'	15,3,7	451040	B12	15,3,7	6215
C12'	15,3,7	250460	C12	15,3,7	6000
D12'	15,3,7	494293.3	D12	15,3,7	7680
A14'	15,5,7	588926.6	A14	15,5,7	5543
B14'	15,5,7	413640	B14	15,5,7	6589
C14'	15,5,7	377773.3	C14	15,5,7	4927
D14'	15,5,7	474940	D14	15,5,7	2604

Table A.3: DNA(ug) for 3 and 7 Day Loading.

3 Day Loading			7 Day Loading		
Dish #	**Scenario(Hz, MPa, Days)**	**DNA(ug)**	**Dish #**	**Scenario(Hz, MPa, Days)**	**DNA(ug)**
A1'	Control	2.17470532	A2'	Control	1.087225231
B1'	Control	1.148391207	B2'	Control	1.142656897
C1'	Control	1.030264415	C2'	Control	1.415801211
D1'	Control	1.374195604	D2'	Control	1.929722842
A3'	1,3,3	0.848550494	A4'	1,3,7	0.740618031
B3'	1,3,3	1.408028034	B4'	1,3,7	1.675119465
C3'	1,3,3	1.545842625	C4'	1,3,7	1.362726983
D3'	1,3,3	1.721121376	D4'	1,3,7	1.494042689
A5'	1,5,3	1.024211532	A6'	1,5,7	1.213889774
B5'	1,5,3	1.588722523	B6'	1,5,7	0.918763938
C5'	1,5,3	1.626377827	C6'	1,5,7	1.835680153
D5'	1,5,3	1.755208665	D6'	1,5,7	1.633705002
A7'	8,3,3	1.89741956	A8'	8,3,7	1.068875438
B7'	8,3,3	1.861102262	B8'	8,3,7	1.227588404
C7'	8,3,3	1.827843262	C8'	8,3,7	1.926409685
D7'	8,3,3	1.44695763	D8'	8,3,7	1.763491558
A9'	8,5,3	1.340426888	A10'	8,5,7	1.330614845
B9'	8,5,3	2.10971647	B10'	8,5,7	1.309843899
C9'	8,5,3	2.329404269	C10'	8,5,7	1.797132845
D9'	8,5,3	1.527938834	D10'	8,5,7	1.248486779
A11'	15,3,3	0	A12'	15,3,7	1.028352979
B11'	15,3,3	1.409111182	B12'	15,3,7	1.426823829
C11'	15,3,3	1.44906021	C12'	15,3,7	1.097483275
D11'	15,3,3	1.575278751	D12'	15,3,7	1.467155145
A13'	15,5,3	1.474800892	A14'	15,5,7	0.721376234
B13'	15,5,3	1.218604651	B14'	15,5,7	1.169034724
C13'	15,5,3	1.146989487	C14'	15,5,7	0.852628226
D13'	15,5,3	1.164447276	D14'	15,5,7	1.082128066

Table A.4: CPM/DNA(ug)/1000 for each 3 and 7 Day Loading.

3 Day Loading			7 Day Loading		
Dish #	**(Hz, MPa, Days)**	**CPM/DNA(ug)/1000**	**Dish #**	**(Hz, MPa, Days)**	**CPM/DNA(ug)/1000**
A1'	Control	58.84628543	A2'	Control	429.2885197
B1'	Control	100.3722941	B2'	Control	431.2580629
C1'	Control	116.8955544	C2'	Control	379.6578192
D1'	Control	43.6291601	D2'	Control	195.6705864
A3'	1,3,3	225.1486522	A4'	1,3,7	905.6665808
B3'	1,3,3	103.7429628	B4'	1,3,7	293.3635543
C3'	1,3,3	126.515466	C4'	1,3,7	240.5128203
D3'	1,3,3	72.25521786	D4'	1,3,7	196.2639101
A5'	1,5,3	286.6790607	A6'	1,5,7	435.7067762
B5'	1,5,3	0.012588731	B6'	1,5,7	354.2368031
C5'	1,5,3	109.1751234	C6'	1,5,7	142.9769775
D5'	1,5,3	85.18265262	D6'	1,5,7	201.9275204
A7'	8,3,3	93.66932001	A8'	8,3,7	965.4320637
B7'	8,3,3	91.46550595	B8'	8,3,7	331.9462034
C7'	8,3,3	47.74661581	C8'	8,3,7	283.6848851
D7'	8,3,3	101.6477587	D8'	8,3,7	202.0990679
A9'	8,5,3	129.0235682	A10'	8,5,7	257.9459121
B9'	8,5,3	62.245331	B10'	8,5,7	186.3071852
C9'	8,5,3	27.94491316	C10'	8,5,7	122.7696275
D9'	8,5,3	88.45903841	D10'	8,5,7	469.7900128
A11'	15,3,3	0	A12'	15,3,7	938.1214039
B11'	15,3,3	83.77621405	B12'	15,3,7	316.1147093
C11'	15,3,3	69.60373302	C12'	15,3,7	228.213045
D11'	15,3,3	73.25052783	D12'	15,3,7	336.9059582
A13'	15,5,3	141.5174083	A14'	15,5,7	816.393155
B13'	15,5,3	122.6375263	B14'	15,5,7	353.8303537
C13'	15,5,3	116.1591553	C14'	15,5,7	443.0692049
D13'	15,5,3	150.4633688	D14'	15,5,7	438.89442

Table A.5: Ratio of Collagen Released.

Scenario	Sample	Ratio of Collagen Released
Control	A1	0.011264661
Control	B1	0.011525533
Control	C1	0.013668863
Control	D1	0.033015064
Control	A2	0.017331792
Control	B2	0.055082254
Control	C2	0.023050604
Control	D2	0.049233509
1,3,3	A3	0.007099202
1,3,3	B3	0.009620861
1,3,3	C3	9.7141E-05
1,3,3	D3	0.016979163
1,3,7	A4	0.011119056
1,3,7	B4	0.014665162
1,3,7	C4	0.008362648
1,3,7	D4	0.019232271
1,5,3	A5	0.005190546
1,5,3	B5	0
1,5,3	C5	0.010940041
1,5,3	D5	0.010980773
1,5,7	A6	0.011111672
1,5,7	B6	0.004112557
1,5,7	C6	0.00431718
1,5,7	D6	0.008383457
8,3,3	A7	0.01032386
8,3,3	B7	0.018593077
8,3,3	C7	0.005753128
8,3,3	D7	0.016726611
8,3,7	A8	0.012599816
8,3,7	B8	0.00918615
8,3,7	C8	0.017498273
8,3,7	D8	0.011214009
8,5,3	A9	0.012290214
8,5,3	B9	0.014617162
8,5,3	C9	0.026063408
8,5,3	D9	0.022039564
8,5,7	A10	0.015382026
8,5,7	B10	0.015737975
8,5,7	C10	0.015158729
8,5,7	D10	0.020020335
15,3,3	A11	0
15,3,3	B11	0.013825655
15,3,3	C11	0.016326292
15,3,3	D11	0.023169978
15,3,7	A12	0.007532597
15,3,7	B12	0.013591978
15,3,7	C12	0.023395461
15,3,7	D12	0.015299618
15,5,3	A13	0.014728792
15,5,3	B13	0.01410433
15,5,3	C13	0.026124331
15,5,3	D13	0.02051693
15,5,7	A14	0.009324278
15,5,7	B14	0.015679547
15,5,7	C14	0.012874304
15,5,7	D14	0.005452901

Vita

William David Merryman, II was born in Nashville, TN on December 10, 1977. He graduated from Ezell-Harding Christian School in 1996. From there, he went to the University of Tennessee, Knoxville where he received both B.S. and M.S. degrees in Engineering Science with Biomedical concentration in May 2001 and December 2002, respectively. He also studied philosophy and history at the University of Wales, Swansea in 1999-2000. Currently, David is working as a research associate/lab engineer while pursuing a doctorate in Bioengineering at the University of Pittsburgh in Pennsylvania.

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