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Development of a Novel Biological Intervertebral Disc Scaffold

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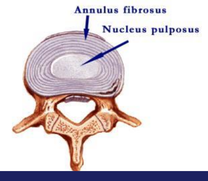
Introduction

Lower back pain (LBP) is a significant burden affecting the majority of our society. Although LBP is multifactorial; it has been shown that LBP can be caused by degenerating intervertebral discs (IVDs), which provide flexibility and shock absorbance for the vertebral column. Twenty percent of teens have mild IVD degeneration, which progresses with age and sixty percent of those over age 70 have severely degenerated IVDs.[1] Current solutions are conservative or surgical, and there is significant room for improvement. The first goal of our project is to create a fully decellularized bovine caudal IVD to be used as a scaffold on which to seed adult human stem cells in an attempt to engineer a healthy, replacement IVD for patients suffering from IVD degeneration and lower back pain.

Figure 1: Schematic illustrating the anatomic location of the IVD between the vertebral bodies of the spine from a top view and the two primary components of the IVD: the *nucleus pulposus* (NP) *and annulus fibrosus* (AF).[2]



Materials & Methods

IVD Extraction Method

1. Betadine solution was used to coat the tails
2. Caudal Discs (C_{4-5} to C_{7-8}) were targeted
3. Fascia and muscle were then cut away with scalpels, vertebrae were separated heavy duty clippers, and scalpels were used to cut out IVDs.
4. IVDs were immediately placed in empty 120mL containers before starting decellurization.

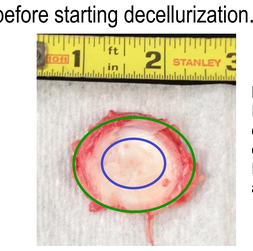


Figure 2: Freshly isolated bovine caudal IVD immediately prior to the initiation of decellularization. Area inside the blue ellipse indicates the NP region of the IVD while the area between the green and blue ellipses illustrate the AF region.

Decell Solution 1

- 0.1% decell solution was composed of 2.21mM ethylene diamine tetraacetic acid (EDTA) and, 0.1% sodium dodecyl sulfate (SDS) dissolved into phosphate buffered saline (PBS).

Decell Solution 2

- 0.05% decell solution was comprised of was 2.21mM EDTA, and 0.05% SDS dissolved into PBS.

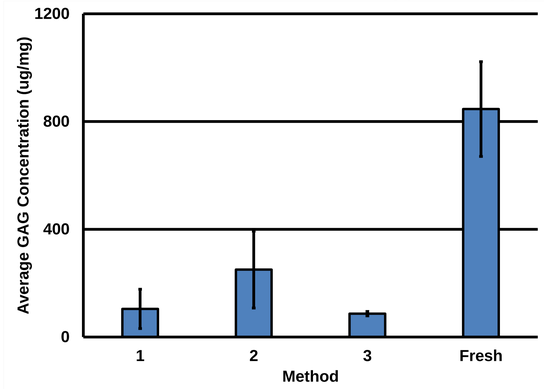
Decellurization Method

1. Two IVDs IVDs were kept at -20° C for use as fresh, control samples. The other 9 IVDs were thawed and they were divided into three groups of three.
2. Three IVDs were put in 100 mL of 0.1% SDS decell solution and six were put in 100 mL 0.05% decell solution. These containers were left on an orbital shaker at 150rpms at room temperature for 24 hours.
3. IVDs were subjected to ultrasonication for 10 minutes at 40 kHz. Agitation and sonication cycles repeated every 24 hours for a total of 4 cycles for three of the IVDs in 0.05% SDS and 6 cycles for the remaining 6 IVDs.
4. The respective decell solutions were refreshed for during each sonication cycle.
5. The IVDs were then removed from their containers, and were thoroughly rinsed in 1X PBS on an orbital shaker at 150rpms at room temperature for 24 hours.
6. After 24 hours, each IVD was removed from its container and cut in half using a scalpel. One half of each IVD was placed in a tissue cassette and placed in10% neutral buffered formalin in preparation for be fixed for histological analysis, and the other half was frozen at -20° C for use in biochemical analysis (DMMB and PicoGreen assay).

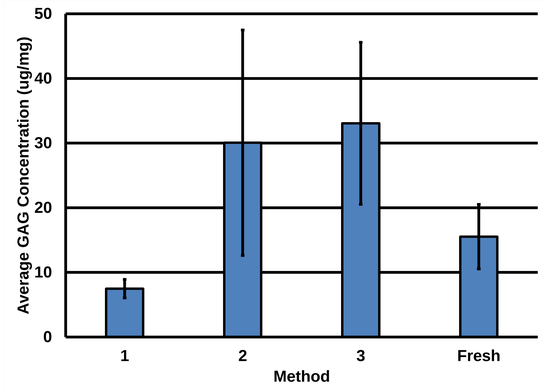
Results

DMMB Assay:

Assay completed in order to determine total glycosaminoglycan content of fresh and decellularized IVD samples



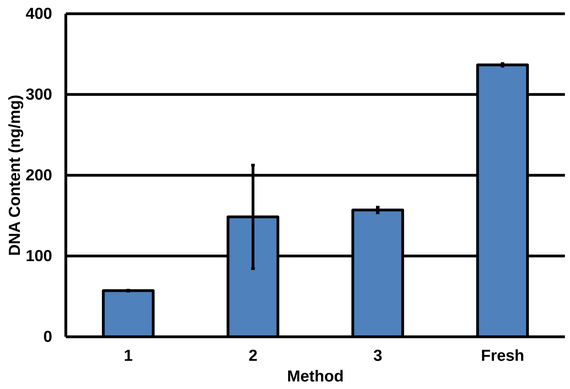
(Figure 3): Nucleus Pulposus GAG Content. Comparison of average GAG content in the NP of decelled IVDs to the NP of fresh IVDs. Results indicate a dramatic reduction in GAG content in decelled IVDs compared to fresh. (n=3/group)



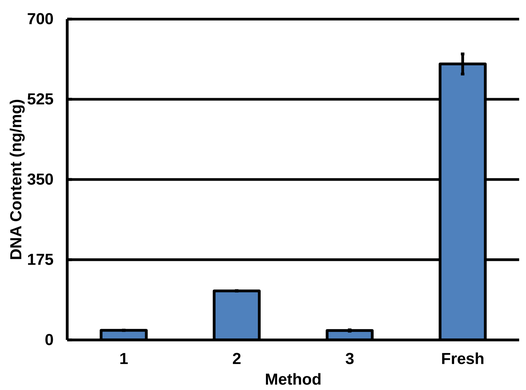
(Figure 4): Annulus Fibrosus GAG Content. Comparison of average GAG content in the AF of decelled IVDs to the NP of fresh IVDs. *Data point missing method 3. (n=3/group)

PicoGreen Assay

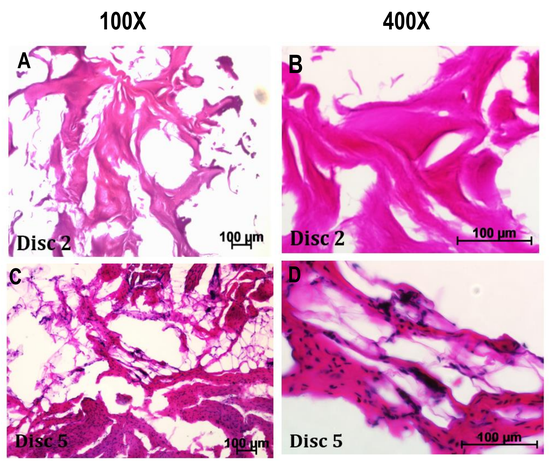
Assay completed in order to determine DNA content in fresh and decellularized IVD samples



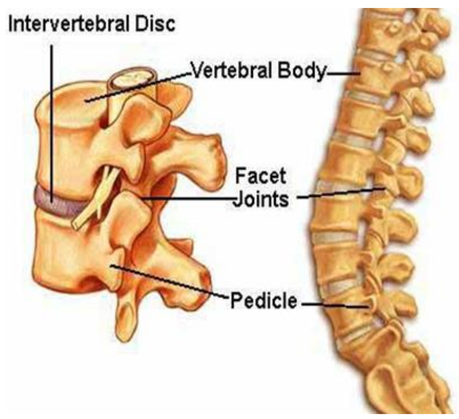
(Figure 5): Nucleus Pulposus DNA Content. Comparison of average DNA content in the NP of decelled IVDs to the NP of fresh IVDs. Results indicate a dramatic reduction in bovine DNA content in decelled IVD NPs compared to fresh. *Data point in method 2 missing. (n=3/group)



(Figure 6): Annulus Fibrosus DNA Content. Comparison of average DNA content in the AF of decelled IVDs to the NP of fresh IVDs. Results indicate a dramatic reduction in bovine DNA content in decelled IVD AFs compared to fresh. *Data points in methods 1 and 3 missing. (n=3/group)



(Figure 7): IVD Histology Staining. Representative H&E staining of A-B) decellularized and C-D) fresh bovine IVDs. The black/dark purple regions are cell nuclei and the pink/light purple is extracellular matrix (ECM). Results indicate a reduction in cell nuclei, but minimal loss in ECM staining.



(Figure 8): Spinal Anatomy. Diagram showing the location of the IVD between vertebral bodies from a side view and demonstrating how it provides shock absorbance and flexibility in the spine.[3]

Decell Method Key:

Method 1: 0.1% SDS solution, 4 cycles (IVDs 1-3)
Method 2: 0.05% SDS solution, 4 cycles (IVDs 4-6)
Method 3: 0.05% SDS solution, 6 cycles (IVDs 7-9)

Discussion

- Decellularization of a targeted tissue is becoming a popular method by which to generate a tissue-specific scaffold for use in tissue regeneration applications. When using this approach to generate a scaffold, it is imperative to completely decellularize the tissue while maintaining the tissue-specific extracellular matrix (ECM) components / micro-architecture.
- Herein, we evaluated two different decellularization solutions and assessed their ability to fully remove bovine cells from the NP and AF regions of cow tail IVDs, while attempting to maintain as much GAG (an ECM component which contributes significantly to the mechanical function of the IVD) as possible.
- We also observed the effect of altering decellularization time on treatment efficacy. We had 3 IVDs go through 6 cycles of sonication and decell in 0.05% SDS solution and 3 IVDs go through 4 cycles of sonication and decell in 0.05% SDS solution (the 3 IVDs in 0.1% SDS solution went through 4 cycles).
- Our results indicate:
  - Decell method #1 resulted in a 82% reduction of DNA content in the NP and a 96% reduction in the AF as compared to fresh tissue. Decell method #2 resulted in a 56% reduction of DNA content in the NP and a 92% reduction in the AF as compared to fresh tissue. Decell method #3 resulted in a 62% reduction of DNA content in the NP and a 96% reduction in the AF as compared to fresh tissue. From these results we concluded that Decell method #1 was the most efficient at removing DNA from both NP and AF regions of the bovine caudal IVD. Overall, these decell methods appeared to work much better for AF than for NP for removing of DNA.
- The data from the DMMB assay illustrate a reduction in NP GAG content but wide variability for GAG content in the AF. We will repeat GAG content analysis from the AF.

Conclusions and Future Work

- Looking forward, various steps of the decell process should be observed to see how much each step helps in clearing DNA from the IVDs. This would help to determine the most effective step in the current decellularization process. We also would be more careful with the IVDs to try to avoid outliers and low dry weights. More variables must be tested to pinpoint exactly what removes DNA in the IVDs. Once that chemical / method is found, it can be optimized in order to effectively remove DNA while maintaining GAG content.
- Currently, our lab is comparing 0.05% SDS decell solution to a new formulation of decell solution. Also, use of a snap-freeze method with liquid nitrogen is being compared to a longer, slower freeze at -80°C. Overall, 4 IVDs are being analyzed and a fifth IVD is being used as a control. The intention is to perform a DMMB assay, a PicoGreen assay, and a histological analysis before the end of the semester.
- In the future, we will fine tune procedural errors and be more careful when conducting assays so meaningful results can be obtained. These results should provide more definite conclusions that show success in decellularizing the IVDs to our goals for DNA content and GAG content. We will also work towards adding human stem cells to our scaffolds.

Acknowledgements

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[1]Urban, Jill, and Sally Roberts. "Degeneration of the Intervertebral Disc." Arthritis and Research Therapy. 5.3 (2003): 120-130. Web. 26 Mar. 2014

[2]http://www.porcpotlas.hu/en/porckorong.html

[3]spineuniverse.com