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14. ABSTRACT

BACKGROUND: Intervertebral disc (IVD) degeneration is a major source of lower back pain. Current treatment strategies aim to alleviate the associated symptoms and not on restoring the biological function of the intervertebral disc.

PURPOSE: The objective of this study is to construct an injectable scaffold in vitro as a therapeutic delivery system to improve the mechanical properties of the nucleus pulposus (NP) of IVD to withstand physiologic loading.

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INTRODUCTION:

Subject: Intervertebral disc (IVD) degeneration is a major source of lower back pain and accounts for billions of dollars in healthcare expenditures. Mechanical properties of the nucleus pulposus (NP), the center cartilage-like tissue of the IVD, play a significant role in maintaining the function of the IVD. The extracellular matrix (ECM) is the functional aspect of the NP allowing frictionless movement. With a decrease in production of the ECM, the NP degenerates and loses essential mechanical function.

Purpose: The objective of this study is to create a unique tissue-engineered injectable scaffold *in vitro* to provide a functional alternative to restore the biological and biomechanical function of degenerated IVD.

Scope: Tissue engineered NP constructs were fabricated using injectable alginate hydrogels supplemented with chondroprotective agents. Biochemical and biomechemical properties of these NP constructs were evaluated.

BODY: We have successfully accomplished the two proposed objectives:

The proposed objective 1: Optimize growth factor conditions for nucleus pulposus (NP) cell expansion. 1)We accomplished this objective by evaluating growth factors that enable the maintenance of chondrogenic phenotypes of NP cells during the expansion process. Growth conditions for human NP cells was optimized (See Appendix 2, page 17-18); and 2) Fluorescence microscopy protocol has been established for cell viability (See Appendix 2, page 18-19).

The proposed objective 2: engineer NP constructs with hyaluronan scaffolds. The overall purpose of this objective is to fabricate a tissue engineered injectable scaffold *in vitro* to improve the mechanical properties of a degenerative nucleus pulposus. We first test the hypothesis that biochemically functional three-dimensional IVD constructs can be engineered by using injectable hyaluronan scaffolds for chondrogenically-expanded NP cells. To these, hyaluronan scaffolds were applied individually at three different concentrations each, to determine the one concentration treatment that would yield constructs with the highest ECM production. Passaged NP cells using conditions determined from objective 1 were used to form tissue engineered constructs. However, the cells were not able to proliferate efficiently, when cultured in the three-dimensional hyaluronan scaffolds (data not shown). Since the (morphological, histological, biochemical and

immunohistochemical) results showed that the hyaluronan approach is not sufficient, as a contingency plan, we have successfully developed alternative methods (to optimize other natural derived injectable biodegradable materials, such as alginate, for the proposed objective 2)(See Appendix 2, page 14-15). The testing hypothesis of the alternative methods is that biochemically functional three-dimensional IVD constructs can be engineered by using injectable alginate scaffolds for chondrogenically-expanded NP cells. The mechanical properties of the hydrogel were focused upon immensely. This was done by optimizing the concentration of alginate (See Appendix 2, page 14-15), divalent cations)(See Appendix 2, page 15), and supplementation in the gel (See Appendix 2, page 15-16) based on the mechanical effect these chemicals had. Results show that an increase in alginate and divalent cations both had a direct effect on increasing the confined compression modulus (See Appendix 2, page 21-22).Biochemical properties, including total collagen, DNA, and GAG contents, of the tissue engineered alginate NP constructs were also evaluated (See Appendix 2, page 18-19). In summary, we have successfully engineered a set of functional three-dimensional NP constructs by using supplemented alginate hydrogel to biochemically and biomechanically mimick the native NP tissue.

Statistical Analysis: Statistical analysis was performed on measures of cell viability, compressive moduli, collagen type II content, and proteoglycan content. Origin 8 software was used to perform one way analysis of variance (ANOVA) to determine significant differences between the different groups. The differences were considered statistically significant if $p<0.05$.

KEY RESEARCH ACCOMPLISHMENTS:

- NP cell expending conditions (passage 1-5), as determined by chondrogenic cell number and cartilage specific extracellular matrix production, has been optimized for NP tissue engineering research.
- A customized mechanical testing apparatus was developed (See Appendix 2, Figure 1).
- In this study, nucleus pulposus tissue-engineered scaffolds were fabricated using calcium-cross-linked alginate hydrogels to mimic the structure of the native tissue environment(See Appendix 2, Figure 2a and Figure 3a).
- Chondroprotective supplementations (the combination of chondroitin sulfate and glucosamine) in the alginate scaffolds increase the confined compression modulus of the

supplemented tissue-engineered constructs (See Appendix 1).

- Chondroprotective supplementations (the combination of chondroitin sulfate and glucosamine) in the alginate scaffolds helps in maintaining NP cell phenotype (See Appendix 2, Figure 6).
- Chondroprotective supplementations (the combination of chondroitin sulfate and glucosamine) in the alginate scaffolds promote NP cell biochemical (See Appendix 2, Figure 8 and Figure 11)and biomechanical (See Appendix 2, Figure 10) properties.

REPORTABLE OUTCOMES:

A list of reportable outcomes that have resulted from this research:

1. Foss, B., T. Maxwell, and Y. Deng, Chondroprotective Supplementation Promotes the Mechanical Properties of Injectable Scaffold for Human Nucleus Pulposus Tissue Engineering. Acta biomaterialia., Submitted.(**APPENDIX 2**)
2. Deng, Y., The in vitro effect of chondroitin sulfate and glucosamine on tissue-engineered nucleus pulposus. 47th ACS Midwest Regional Meeting (Symposium: Advances in Drug Delivery I). October 24 - October 27, 2012., 2012.
3. Foss, B., Y. Deng, and T. Maxwell, Chondroitin Sulfate and Glucosamine Promote the Mechanical Properties of Alginate Hydrogel for Nucleus Pulposus Tissue Engineering Application. ASME 2012 International Mechanical Engineering Congress & Exposition (Symposium: Biomedical & Biotechnology). Houston, Texas. November 09 - November 15, 2012., 2012. **(APPENDIX 1)**
4. Foss, B., Y. Deng, and T. Maxwell, Enhancement of Mechanical Properties for Tissue-Engineered Nucleus Pulposus Through Chondroprotective Supplementation. University of South Dakota Ideafest. April 10-11, 2013., 2013.

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Foss, B., Enhancement of Swelling Pressure for Tissue-engineered Nucleus Pulposus through Chondroprotective Supplementation. University of South Dakota Graduate Student Research and Creative Activity Grant, (January 2013-August 2013), 2013.

CONCLUSION:

This study demonstrated that the combination of chondroprotective supplementation and alginate hydrogel plays a significant role in increasing the biomechanical properties of the NP tissue engineered construct through the production of a mechanically

functional matrix. This research shows potential to develop a minimally invasive strategy to restore natural biochemical and biomechanical function to a degenerative disc.
Future work will evaluate different mechanical properties of the scaffold relevant to the biomechanics of the nucleus pulposus, such as shear modulus and swelling pressure.

REFERENCES: Please see Appendix 2 from page 43-44 for references.

APPENDICES: We have attached Appendix 1 and 2 to support the text.

APPENDIX 1

IMECE2012-93798 *Poster Presentation Only*

Chondroitin Sulfate and Glucosamine Promote the Mechanical Properties of Alginate Hydrogel for Nucleus Pulposus Tissue Engineering Application

Authors

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Thomas Maxwell, *University of South Dakota*

Abstract

Intervertebral disc (IVD) degeneration is a major source of lower back pain and accounts for billions of dollars in healthcare expenditures. Mechanical properties of the nucleus pulposus (NP), the center cartilage-like tissue of the IVD, play a significant role in maintaining the function of the IVD. The extracellular matrix (ECM) is the functional aspect of the NP allowing frictionless movement. With a decrease in production of the ECM, the NP degenerates and loses essential mechanical function. The objective of this study is to create a unique tissue-engineered injectable scaffold in vitro to provide a functional alternative to restore the biological function of degenerated discs. In this study, cells were encapsulated in a 2%-w/v alginate hydrogel using 0.025 M calcium chloride to fabricate cell-alginate constructs. Chondroitin sulfate (CS) and glucosamine (GCSN), two chondroprotective supplements, were applied to examine their possible role in increasing the mechanical function of the tissue-engineered cell-alginate constructs. Negative controls were alginate hydrogels without supplementation or cells and alginate hydrogels without supplementation with cells. Based on reported results in articular cartilage and the similarity of NP to articular cartilage, alginate hydrogels were supplemented with GCSN and CS in a 5:4 ratio at concentrations of 125:100 µg/mL, 250:200 µg/mL, and 500:400 µg/mL, respectively. Mechanical analysis was performed using an MTS Insight Electromechanical Testing System. Confined compression results show supplementation increased the modulus of the supplemented hydrogel with no cells (67.03±8.62 kPa) when compared to the control (33.63±3.86 kPa). It is worth to note that addition of cells decreased the modulus of the supplemented hydrogel initially, but showed a greater increase in modulus over time compared to the control with cells. From Day 1 to 7, the 125 µg/mL GCSN/100 µg/mL CS treated cell-alginate construct had a 36.6% increase whereas the cell-alginate-control only had a 7.7% increase. Over a 14 day time-period, cell distribution and viability were observed through confocal microscopy showing cells uniformly distributed through each gel and equal viability between the samples. MTT assay was employed to quantify the cell proliferation and viability in the hydrogels. There was no significant difference in proliferation or viability among each tested group. The results show that GCSN and CS increase the confined compression modulus of the supplemented tissue-engineered constructs. The unique construct with the GCSN/CS supplementation may serve as an injectable tissue engineered application to restore mechanical function of the degenerated disc in vivo.

Keywords: Nucleus pulposus, confined compression, alginate, hydrogel

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APPENDIX 2

Chondroprotective Supplementation Promotes the Mechanical Properties of Injectable Scaffold for Human Nucleus Pulposus Tissue Engineering

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Abstract:

The nucleus pulposus (NP) plays an essential role in the mechanical properties of the intervertebral disc. As a result of intervertebral disc degeneration, the NP is no longer able to withstand applied load ultimately leading to pain and disability. Current treatment strategies can only alleviate the associated symptoms such as pain, but do not restore the biological function of the intervertebral disc (IVD). The objective of this study is to fabricate a tissue engineered injectable scaffold with chondroprotective supplementation *in vitro* to improve the mechanical properties of a degenerative nucleus pulposus. Tissue-engineered scaffolds were fabricated using different concentrations of alginate and calcium chloride and mechanically evaluated. Scaffold fabrication conditions were chosen based on the structural and mechanical resemblance to the native NP as well as ability to be injected through an 18 gauge needle. Chondroprotective supplementation, glucosamine and chondroitin sulfate, were added to the selected scaffolds to create supplemented scaffolds and were mechanically analyzed. Supplemented scaffolds were then used to fabricate tissue engineered constructs for further through encapsulation of human nucleus pulposus cells (HNPCs). A 2% w/v alginate solution was prepared with chondroprotective supplementation at concentrations of 0:0 µg/mL (0:0-S), 125:100 µg/mL (125:100-S), 250:200 µg/mL (250:200-S), and 500:400 µg/mL (500:400-S), glucosamine and chondroitin sulfate respectively, to mimic the native NP structural environment. Human nucleus pulposus cells (HNPCs) were encapsulated in the alginate solution. The alginate-cell solution was then crosslinked with 0.025 M calcium chloride solution to fabricate the tissue engineered construct. The tissue engineered constructs were then cultured up to 28 days. Samples were collected at days 1, 14, and 28 for biochemical and biomechanical evaluations. Confocal microscopy showed HNPC viability and that the HNPCs maintained their native rounded morphology over the 28 day period. MTT analysis showed increasing cell proliferation with a significant difference between Day 1 and Day 28 for each group. Collagen type II ELISA quantification on Day 28 showed the 125:100-S and 250:200-S were significantly greater than the 0:0-S. Mechanical analysis showed the compressive moduli of the 250:200-S and the 500:400-S groups were significantly greater than the 0:0-S and the lower supplemented 125:100-S on Day 28. Water content data showed a significant decrease in the 250:200-S and the 500:400-S groups between Days 1 and Day 28. The water content and collagen type II quantification result implies the increased mechanical properties of the 250:200-S and the 500:400-S were due to production of a functional matrix. This study demonstrated that chondroprotective supplementation plays a role in increasing the compressive modulus of the NP tissue engineered construct through the production of a mechanically functional matrix. This research shows potential to develop a minimally invasive strategy to restore natural biomechanical function to a degenerative disc.

KEYWORDS: Nucleus Pulposus, Hydrogel, Confined Compression, Tissue Engineering

1. Introduction:

The primary role of the nucleus pulposus (NP) in the intervertebral disc (IVD) of the spine is mechanical [1]. The NP mechanical properties allow load transfer, shock absorption, and flexibility throughout the spine [2]. Degeneration of the IVD has been attributed to lower back pain which is a major health concern in the United States with an estimated 90 billion dollars in healthcare expenditures [3]. As the IVD degenerates, the NP can no longer perform its essential biomechanical function leading to disability and chronic pain. Disc degeneration occurs as a cell-mediated response to progressive structural failure by which changes in the NP structure and composition give rise to mechanical dysfunction [2].

The composition of the NP extracellular matrix (ECM) determines its mechanical function [4]. The mechanical function of the IVD is primarily dictated by the NP and the NP extracellular components: proteoglycans and collagen type II [2, 3, 5]. Proteoglycans in the NP contain highly anionic glycosaminoglycans (GAG) that provides the osmotic properties which enable the NP to resist compression [6]. Proteoglycans are embedded into a fine network of collagen type II, an essential protein with uniform distribution in the NP ECM [1, 7]. Collagen type II governs the mechanical properties of the NP [1]. In combination, proteoglycans (containing GAG molecules) and collagen type II create a mechanically functional NP enabling the IVD to undergo compressive and shear forces [1, 2, 4, 8]. ECM turnover is a natural process which involves synthesis and catabolism. With IVD degeneration, this turnover is no longer balanced and matrix degradation exceeds matrix synthesis leading to degeneration of the tissue. Chondroitin sulfate is a GAG that is naturally occurring in the ECM of the NP and has a strong negative charge allowing for water retention within tissues [9]. Glucosamine is an amino

monosaccharide naturally synthesized and present in other cartilage tissues which provides strength, flexibility and elasticity [10]. Both chondroitin sulfate and glucosamine are termed chondroprotective supplements and have been shown to inhibit enzymes that catabolize the extracellular matrix [11-15].

Current strategies aim to decrease the pain associated with disc degeneration beginning with conservative methods, such as physical therapy, followed by highly invasive methods such as interbody fusion surgery significantly changing the biomechanics of the spine [16, 17]. These highly invasive methods aim to treat the symptoms of degeneration, but do not preserve the natural biological or biomechanical function of the intervertebral disc or improve disc degeneration [17-19]. Tissue engineering strategies provide an alternative capability to restoring the mechanical properties of degenerated discs [18]. Different types of biomaterials have been tested *in vitro* and experimentally *in vivo*, in order to promote and assist in the repair of NP and IVD cartilage. For the gelatinous NP, hydrogels have been investigated because of their 3D, hydrated macromolecular structure mimicking the natural NP extracellular environment [7, 16-18, 20-22]. Alginate, a naturally derived polysaccharide from brown algae, has been used in cell encapsulation and tissue engineering due to its biocompatibility, low toxicity, low cost, and spontaneous gelation [8, 22, 23]. Alginate has been investigated and established as a potential tissue engineering biomaterial for cartilage tissue, IVD, and NP repair [22, 24-26].

The objective of this study is to fabricate a tissue engineered injectable scaffold *in vitro* to improve the mechanical properties of a degenerative nucleus pulposus. In this present study, a tissue-engineered scaffold was fabricated using alginate and chondroprotective supplementation. We theorize that the injectable, chondroprotective scaffold may be able to restore the

compressive modulus within the native nucleus pulposus to improve and further delay intervertebral disc degeneration thus decreasing lower back pain.

2. Materials and Methods

2.1 Materials and reagents:

The materials and reagents used in this study are from Sigma unless otherwise noted: Alginic acid sodium salt, Dulbecco's Phosphate Buffered Saline modified without $CaCl_2$ and $MgCl_2$ (DPBS), calcium chloride, sodium citrate, sodium chloride, papain, L-cysteine, Na_2HPO_4, EDTA, chondroitin sulfate, glucosamine hydrochloride, trypan blue, fluorescein diacetate (FDA), propidium iodide (PI), acetone, thiazolyl blue tetrazolium bromide, and isopropanol. Human nucleus pulposus cells (HNPCs) were purchased from ScienCell Laboratories. Dulbecco's Modified Eagle Medium (DMEM) was purchased from Fisher Scientific. Collagen Type II enzyme linked immunosorbent assay (ELISA) kit was purchased from Chondrex, Inc.

2.1 Tissue Engineered Scaffold Fabrication and Mechanical Analyses:

Tissue engineered scaffolds were prepared based on reported methods with modifications [21, 23].

2.1.1 Effect of alginate concentration on scaffold mechanical properties

In order to mimic the native mechanical properties of the NP and to optimize the concentration of alginate used for the scaffold, hydrogels were prepared using different concentrations of alginate. A 0.5% w/v, 1.0% w/v, 2% w/v, and 4% w/v alginate solutions were prepared dissolving the alginate in DPBS. The alginate solutions were crosslinked with a 0.025

M $CaCl_2$ solution at a 1:1 ratio. Samples were washed twice with DPBS. The hydrogels (n=4) of varying alginate concentrations were then mechanically analyzed.

2.1.2 Effect of calcium chloride concentration on scaffold mechanical properties

To further characterize the mechanical properties of the scaffold, $CaCl_2$ concentration was analyzed using serial dilution. Hydrogels were prepared using $CaCl_2$ concentrations of 0.0125 M, 0.025 M, 0.05 M, 0.1 M, and 0.2 M. At a 1:1 ratio, a 2% w/v alginate solution was crosslinked with each of the $CaCl_2$ concentrations. These hydrogels (n=4) were then analyzed mechanically. The 0.2 M, 0.1 M, and 0.05 M CaCl2 concentrations examined were not able to be injected using the 18 gauge needle and were therefore not chosen for further analysis.

2.1.3 Alginate and calcium chloride concentration combinations on scaffold mechanical properties

Based on the mechanical results from the alginate and $CaCl_2$, a range of concentrations were chosen based on resemblance in structure and mechanical properties of the native NP. The alginate concentration was focused to 1.0% w/v, 1.5% w/v, 2% w/v, and 2.5% w/v. Sterile alginate solutions were dissolved and crosslinked at a 1:1 ratio with $CaCl_2$ at ranges of 0.025 M, 0.030 M, 0.040 M, and 0.050 M. Mechanical analyses were performed on the scaffolds (n=4).

2.1.4 Chondroprotective supplementation concentration effect on scaffold mechanical properties

Chondroprotective supplements, Glucosamine hydrochloride (GCSN) and chondroitin sulfate (CS), were used to supplement the scaffolds. GCSN and CS solutions were prepared in a 5:4 ratio at concentrations of 125:100 µg/mL, 250:200 µg/mL, and 500:400 µg/mL, respectively, and dissolved in DPBS. The concentrations were chosen to mimic the concentrations in the

native human NP tissue based on literature values [4]. The supplemented solutions were sterile filtered using a 0.22 µm syringe filter. Sterile alginate at a concentration of 2% w/v was dissolved in the supplemented DPBS solution. Scaffolds were prepared by crosslinking the alginate with 0.025 M $CaCl_2$ at a 1:1 ratio. Controls were alginate hydrogels without supplementation. Mechanical analyses were performed on the scaffolds (n=4). Table 1 describes supplementation concentrations and experimental groups for the scaffolds for *in vitro* analysis.

2.1.5 Confined compression determination

Mechanical testing of the hydrogels was performed using an MTS Insight Electromechanical apparatus (MTS Systems Corp., Eden Prairie, MN) connected in series with a 100 N uniaxial load cell interfaced to a computer equipped with TestWorks 4 software. Cylindrical specimens were extracted from the well plate and placed into a custom-built confined compression chamber to eliminate lateral expansion [2, 4, 27]. A diagram of the apparatus can be found in Figure 1. The mechanical testing apparatus was built specifically for the size of the scaffold examined with a 1.5 cm diameter. A stainless steel platen was fastened to the top of the testing system and the machine was calibrated. The platen was then lowered until even contact was made with the surface of the hydrogel. The height of the gel was recorded from this separation using a digital caliper and entered into the testing program. Samples were compressed axially at a speed of 1 mm/min while reaction force and cross-head displacement data were collected by the load cell and extensometer at a rate of 10 Hz. Software analysis provided a graph of normalized compressive stress vs. axial strain from this data. The linear

portion of this graph was used to calculate the compressive elastic modulus according to the following derivation of Hooke's Law:

$$\delta = \frac{PL}{AE} \qquad (1)$$

where δ is the axial deflection, L is the height of the sample and separation distance between the platens, A is the cross-sectional area of the hydrogel, and E is the modulus of elasticity. Knowing that load and deflection are linearly related and that L and A are constants, the modulus can be calculated as:

$$E = \left(\frac{\Delta P}{\Delta \delta}\right)\left(\frac{L}{A}\right) \qquad (2)$$

Where $\frac{\Delta P}{\Delta \delta}$ is taken to be the slope of the linear region of the graph. Average compressive moduli values of each hydrogel concentration were graphed with standard deviation using the units mega Pascal (MPa), as cited in literature, to describe confined compression of native NP tissue.

2.1.6 Injectable analysis:

For both alginate and calcium concentrations prepared in 2.1.1 and 2.1.2, each scaffold was injected based on a previously reported protocol with modification using an 18-gauge needle in order to determine whether the scaffold is injectable [28, 29].

2.2 Cell Culture:

The HPNCs were thawed and cultured at a density 5,000 cells/cm^2. The cells were incubated at 37˚C at 5% CO_2 in culture media (DMEM/High Glucose containing 1% non-essential amino acids (Fisher, MA), 1% antibiotics (Fisher), and fetal bovine serum (Sigma)). Medium was changed the next day and then every three days until 70% confluency. Medium was then changed every other day until confluency reached 90%. Cells were then subcultured

using 0.05% trypsin with EDTA. Cell count and viability were quantified using trypan blue and a hemocytometer.

2.3 Tissue Engineered Construct Fabrication

Approximately $6x10^5$ human nucleus pulposus cells were suspended in sterile 2% w/v alginate solutions containing supplementation of 125:100 µg/mL, 250:200 µg/mL, and 500:400 µg/mL as described previously. Controls were scaffolds with no supplementation. A 0.025 M $CaCl_2$ solution was then added to polymerize the alginate/cell solution at a 1:1 ratio to form the tissue engineered construct (TE-construct) [30]. TE-constructs were washed twice with DPBS and immersed in culture media. The TE-constructs were incubated at 37˚C at 5% CO_2 for 28 days. At different time points, Day 1, 14 and 28, scaffolds were examined for confocal, MTT viability, mechanical, and biochemical analyses. Table 1 describes the experimental groups. For each experimental group, a TE-construct and a scaffold were analyzed to determine biochemical effects of supplementation on HNPCs. Abbreviations for the scaffolds are also listed in Table 1.

2.5 Cell Morphology, Viability, Distribution, and Proliferation Determination:

2.5.1 Confocal Microscopy for Cell Morphology, Viability and Distribution:

Confocal microscopy was performed on Days 1, 14 and 28. Propidium iodide (2 mg/mL) in DPBS was used to view apoptotic cells and fluorescein diacetate (1 mg/mL) was used to observe viable cells [31]. Using an inverted Olympus FV1000 confocal microscope employing a 3D Z-series, cell morphology, viability, and distribution were observed. Imaris scientific software was used to obtain 3D videos of the hydrogels to observe distribution.

2.5.2 MTT Assay for Cell Proliferation:

Cell proliferation by MTT method was assessed on Days 1, 14 and 28. In a 96 well plate, TE-constructs were incubated in 100 µL of medium supplemented with 10 µL of yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) at a concentration of 5 mg/mL in PBS (Sigma, St. Louis, MO). Cell number was normalized by standards using increasing cell population. After 18 hours of incubation, formazan crystals were extracted using isopropanol for 2 hours at 37°C [32]. Absorbance was quantified using a microplate reader at 570 nm with reference at 630 nm.

2.6 Biochemical Analysis of Type II Collagen:

Collagen Type II content was assessed at Day 0, 1, 7, 14, and 28 through enzyme-linked immunosorbent assay (ELISA) (Chondrex Inc., Redmond, USA) [33]. ELISA was carried out according to manufacturer protocol with modification of the solubilization protocol. Collagen was digested by dissolving the TE-construct in 0.055 M sodium citrate buffer [30]. Pepsin, 0.10 mg/ml, in 0.05 M acetic acid was added to the sample and digested overnight at 4˚C. ELISA was carried out according to manufacturer protocol.

2.7 Mechanical Testing of Tissue Engineered Constructs:

Mechanical testing of the TE-constructs was performed as previously described in Section 2.2.5. Mechanical testing of the TE-constructs was performed at 1, 14, and 28 days. Average compressive moduli values of each experimental group were graphed over this time period.

2.8 Water Content

Samples were collected on Days 1, 14, and 28. After recording the wet weight of each sample (n=4), samples were incubated at 64°C for 24 hours in order to dry the weight [4, 8]. The dry weight was measured and the water content (%) was determined using Equation (3).

$$H_2O\ \% = \frac{(Wet\ Weight - Dry\ Weight)}{Weight\ Wet}\ x\ 100\% \qquad (3)$$

2.8 Statistical Analysis

Statistical analysis was performed on measures of cell viability, compressive moduli, collagen type II content, and proteoglycan content. Origin 8 software was used to perform one way analysis of variance (ANOVA) to determine significant differences between the different groups. The differences were considered statistically significant if $p<0.05$.

3 Results:

3.1 Tissue Engineered Scaffold Fabrication and Mechanical Analyses:

The custom-built confined compression chamber was specifically designed for the scaffold size to eliminate the lateral expansion of the scaffold as *in vivo* the NP is confined by the annulus fibrosis (Figure 1). For both alginate and calcium chloride, there is a direct relationship between concentration and confined compressive modulus. As the concentration of alginate was increased, the compressive modulus also increased (Figure 2b). As the alginate concentration increased, the compressive moduli of the 2% and 4% w/v groups were both significantly greater in compressive moduli compared to the 0.5% and 1% w/v groups. As the modulus and alginate concentration increased, it was observed that the groups were able to maintain the cylindrical shape of the mold (Figure 2a).

The increase in calcium chloride concentration significantly increased the compressive modulus of each group (Figure 3b). The results showed that as the concentration of the calcium chloride for the scaffolds was increased, the compressive modulus increased. The 0.1 M and 0.2 M were significantly higher than the 0.05 M, 0.025, and 0.0125 M $CaCl_2$ concentrations. Significant differences were observed between each of the 0.05 M, 0.025 M, and 0.0125 M $CaCl_2$ (Figure 3b). As the $CaCl_2$ concentration was increased, the samples were able to maintain the cylindrical mold shape as well as becoming increasingly opaque (Figure 3a).

To further optimize the alginate and calcium chloride concentrations, more defined concentrations of both alginate and $CaCl_2$ results were mechanically examined. Results showed an increasing trend with increasing concentration of both alginate and $CaCl_2$ as similar results showed (Figure 4). Based on these results and to minimize the calcium concentration based on research results for damaged IVDs, the combination of 2% w/v alginate solution with the 0.025 M $CaCl_2$ concentrations were chosen for further analysis with cell encapsulation [34].

After the determination of the alginate, 2% w/v, and calcium chloride, 0.025 M, concentrations, the effect on mechanical properties through the addition of the different concentrations of glucosamine and chondroitin sulfate was analyzed. There were no significant differences in mechanical properties between the scaffolds upon the addition of increasing concentrations of supplementation (Figure 5). This indicated that chondroprotective supplementation alone does not affect the compressive modulus of the scaffolds. The effect of supplementation was examined further biochemically and mechanically after HNPC encapsulation.

3.2 Cell Morphology, Distribution and Viability

The results for confocal microscopy showed cell viability for each time point over the 28 day period (Figure 6). Fluorescein diacetate (FDA) stained viable cells green. Propidium iodide (PI) stained non-viable cells red. Confocal microscopy showed cells viability over the 28 day period. Observations showed the presence of non-viable cells on Day 1. The amount of non-viable cells was observed through confocal microscopy to decrease over the 28 day period. Mimicking the *in vivo* nucleus pulposus, the hydrated environment of the TE-construct allowed the HNPCs to maintain a rounded morphology within each TE-construct group throughout the whole experimental period. Over time, the cells were observed to form clusters of isogenous groups (small nests of chondrocytes indicated by a red arrow in Day 28 images in Figure 6). Uniform distribution of HNPCs was observed for each group on Day 1. Over the 28 day time period, HNPCs were observed to remain distributed throughout the TE-constructs over the 28 day time period.

3.3 Cell Proliferation

Due to the presence of non-viable cells, MTT analysis was performed to quantify viable cells and proliferation (Figure 7). MTT analysis displayed a significant increase in proliferation over the 28 day period, from Day 1 to Day 28 for each group. There were no significant differences between groups as was expected.

3.4 Biochemical Analysis

Collagen Type II was minimally detected in each group on Day 1 (Figure 8). From Day 14 to Day 28, the 0:0-S showed a decreasing trend, from 6.13±1.73 ng/mL to 3.64 ±2.5 ng/mL, indicating a loss of the produced matrix. The 125:100-S significantly decreased from Day 14,

15.66±0.80 ng/mL, to Day 28, 7.98±0.43 ng/mL. Day 28 for the 125:100 μg/mL-S was significantly higher than the 0:0-S.

The 250:200-S showed an increasing trend over the 28 day period and the Day 28 collagen type II concentration was the highest of all groups with a production of 7.98±0.43 ng/mL of collagen type II. The 250:200-S group was significantly higher than the 0:0-S production, 3.64±2.5 ng/mL, and the 500:400-S production, 3.33±0.21 ng/mL. On Day 14, the 500:400-S group was significantly lower, 1.26±0.21 ng/mL, than the other TE-construct groups. By Day 28 there was no significant difference in collagen type II content between the 0:0-S and the 500:400-S.

3.5 Mechanical Analysis of Tissue Engineered Constructs:

The confined compressive moduli for the TE-constructs are shown in Figure 9 for Days 1, 14 and 28. It is important to note that the alginate used to form the tissue engineered scaffold is biodegradable and the mechanical properties of the biomaterial are expected to decrease overtime. As the HNPCs form a functional extracellular matrix within the TE-construct, the confined compressive modulus is expected to increase [35]. The 0:0-S decreased in modulus over the 28 day period from Day 1, 0.0452±0.0100 MPa, to Day 28, 0.0410±0.0038 MPa. The 125:100-S maintained the compressive moduli between Day 1, 0.0422±0.008 MPa, and Day 28, 0.0423±0.001 MPa. The 250:200-S had an increased in modulus over the 28 day period with an overall from a Day 1, 0.0502±0.019 MPa, to Day 28, 0.0586±0.006 MPa. The 500:400-S significant increased between Day 1, 0.030±0.006 MPa, and Day 28, 0.0566±0.008 MPa with a 46.2% increase.

Statistical analysis for Day 1 shows no significant difference in compressive moduli between the groups. However, on Day 28, the 250:200-S and the 500:400-S compressive moduli were significantly greater than both the 0:0-S TE-construct and the lower supplemented 125:100-S (Figure 9).

3.6 Water Content (%)

On Day 1 the 250:200-S had significantly greater water content, 16.87±2.45%, than the 0:0-S, 11.74±3.09%. Over the 28 day period both the 250:200-S and 500:400-S had a significant decrease in water content from Day 1 to Day 28. The water content of the 0:0-S and the 125:100-S did not change over the 28 day period. There was no significant difference between the TE-constructs at Day 14 and Day 28 (Figure 10).

4 Discussion:

In this study, nucleus pulposus tissue-engineered scaffolds were fabricated using calcium-cross-linked alginate hydrogels to mimic the structure of the native tissue environment. Scaffolds were analyzed mechanically to define the selected concentrations of alginate and calcium chloride that would be used for further analysis prior to cell encapsulation. The concentrations of calcium chloride were chosen based on the native calcium ion concentration range in the center of a normal IVD: 0.080–0.1 M [1]. Studies have shown that when cartilage is damaged, calcium is an important regulator of chondrocyte death: an increase in calcium concentration increases cell death [34]. Because of the application of the tissue engineered scaffold for degenerative NP cartilage, a lower concentration of calcium for this experiment was preferred to prevent further cell death. The 0.025 M $CaCl_2$ concentration enabled the hydrogel to

maintain the cylindrical shape and had no significant differences in mechanical properties compared to the higher 0.030 M and 0.040 M $CaCl_2$. The 0.025 M concentration was also able to be injected through an 18 gauge needle and was thus chosen for the concentration for fabrication of the tissue engineering scaffold for further analysis. The 2% w/v alginate was selected in combination with the 0.025 M CaCl2 as the scaffold's viscoelastic and mechanical properties mimicked the gelatinous nucleus pulposus native environment [4, 26].

The effect of supplementation on the mechanical properties of the tissue-engineered scaffold was examined using confined compression. As Figure 5 shows, there were no significant differences in the mechanical properties between the 0:0-S and the supplemented scaffolds, 125:100-S, 250:200-S, and 500:400-S. When cells were encapsulated in the scaffold to form the TE-construct, Day 1 mechanical analysis, again, showed no significant differences between the compressive moduli (Figure 8). However, over the experimental time period, the higher supplemented TE-constructs, the 250:200-S and 500:400-S groups, had significantly greater compressive moduli than the 0:0-S and 125:100-S groups on Day 28. This indicates the production of a functional matrix that is able to withstand a greater load influenced by the chondroprotective supplementation. It is worth noting that immersion in culture medium and incubation conditions of 37˚C decreased the modulus of the TE-constructs significantly. This is similar to previously reported data [36].

The increase in the mechanical properties of the 250:200-S and 500:400-S was explained through biochemical analysis of collagen type II production. The collagen network in the native tissue forms a functional matrix that governs its mechanical behavior [1]. Extracellular matrix turnover is naturally occurring; however, when catabolism is higher than production, the matrix

begins to break down. The use of chondroprotective supplementation is expected to maintain balance of the produced ECM in the TE-construct. As the ELISA results show for Day 1, the mechanical properties are provided by the alginate scaffold as no collagen type II was detected all groups. On Day 14, as each TE-construct produced collagen type II, the compressive modulus between each group for Day 14 increased with the production of collagen as shown in Figure 8. On Day 28 however, the collagen type II concentration decreases for the 0:0-S and the 125:100-S groups. As a result of this, the compressive moduli of the 0:0-S and the 125:100-S groups decreased. In contrast, both the 250:200-S and 500:400-S, collagen type II content increased significantly between Day 14 and Day 28 resulting in significantly greater mechanical properties when compared to the 0:0-S and 125:100-S on Day 28. As the functional collagen network is formed, the increase in collagen content begins to govern the mechanical properties of the TE-constructs. Collagen type II forms an irregular network to create a firm, gel matrix increasing the mechanical properties of the scaffold [37].

As age increases, HNPCs have shown an increase in catabolic activity displaying more of a diseased state [35]. The breakdown of the matrix is caused by an increase in catabolism, but production remains the same. As collagen matures it forms intra- and inter-molecular cross-linkages forming a collagen network. The results of the ELISA collagen type II quantification method show a significant decrease in collagen concentration between Day 14 and Day 28 of the 0:0-S and the lowest supplemented 125:100-S, whereas both the 250:200-S and 500:400-S significantly increased. The ELISA kit is limited in that the more crosslinked the collagen, the lower yield will be produced as digestion does not occur at the helical conformation region of the collagen and the intra and inter-molecular cross linkages [38]. The collagen type II

quantification of the 250:200-S and 500:400-S produced a lower yield than the 0:0-S and 125:100-S TE-constructs on yet had an increasing production trend and had significantly greater mechanical properties on Day 28. The comparison of the mechanical and biochemical quantification of collagen type II results suggest that the collagen type II produced in the 250:200-S and 500:400-S TE-constructs created intra- and inter-molecular cross linkages which ultimately supported an increased compressive load. This indicates that the supplementation, GCSN and CS, maintains the produced chondrocyte matrix within the TE-construct allowing for the formation of a mechanically functional collagen type II network.

A low cell-seeding density was used to mimic the low cell density in the native NP and to determine the biochemical effects of chondroprotective supplementation on the HPNCs. Confocal microscopy showed uniform distribution on Day 1 and the hydrated hydrogel environment allowed cells to maintain a rounded morphology. The rounded morphology is characteristic of the nucleus pulposus cells in the native tissue which can assist in cell proliferation and phenotype retention allowing for the assembly of a functional extracellular matrix [8]. In this study, by Day 14 the cells begin to form clusters of cells which are characteristic of chondrocytes (Figure 6). This phenomenon has been demonstrated by previous researchers as these clusters, isogenous groups, form because of the last mitotic division of a chondrocyte [39]. As MTT analysis has shown, the HNPCs proliferated over the 28 day period with a significant difference between Day 1 and Day 28 (Figure 7). On Day 28, confocal observation showed cell distribution less uniform as the cells begin to agglomerate (Figure 6-Day 28). This observation is indicative of the degradation of the TE-construct [35]. Degradation is a key prerequisite in tissue engineering strategies and as the HNPCs slowly form a functional

extracellular matrix, the scaffold degrades. Degradation of the TE-construct is observed on Day 28 through confocal images of agglomeration of cells and the presence of flattened HNPCs as they become un-encapsulated within the TE-construct (Figure 6, Day 28-white arrows) [35]. As the confocal results show evidence of degradation and results from collagen type II production, the increase in the mechanical properties of the 250:200-S and 500:400-S groups is largely indicated by the production of a mechanically functional matrix.

Correlations between the compressive modulus (Figure 9), collagen type II concentration (Figure 8) and the water content (Figure 10) reveal that the best prediction of increased mechanical function was the production of a functional extracellular matrix. Both the 250:200-S and 500:400-S showed a significant loss in water content over the 28 day period, but had significantly greater mechanical properties. This is related to the maintenance and production of the collagen type II produced between Day 14 and Day 28 for the 250:200-S and 500:400-S. This indicates chondroprotective supplementation decreased the catabolism of the matrix when compared to the 0:0-S.

The intervertebral disc is one of the largest avascular tissues in the body with limited repair and self-healing mechanisms. This study demonstrates that chondroprotective supplementation increases the compressive modulus of the NP tissue engineered scaffolds while maintaining biocompatibility and native cellular phenotype. This research shows potential to develop a minimally invasive strategy using an injectable scaffold to restore the natural function of a degenerative disc. Future work will evaluate different mechanical properties of the scaffold relevant to the biomechanics of the nucleus pulposus, such as shear modulus and swelling pressure.

This work demonstrates that although it is possible to maintain functional disc cells in a biomatrix, it will be necessary to optimize proteoglycan synthesis and retention if any resulting tissue is to be of value in the biologic repair of the degenerate disc.

6 Acknowledgments

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Table 1. Scaffold Groups and Supplementation Detail

Scaffold Groups	**Supplementation**[#]

Scaffold	Glucosamine (μg/mL)	Chondroitin Sulfate (μg/mL)
0:0-S (control)	0	0
125:100-S	125	100
250:200-S	250	200
500:400-S	500	400

[#]Supplementation concentrations were chosen based on biochemical analysis of normal human NP tissue [4].

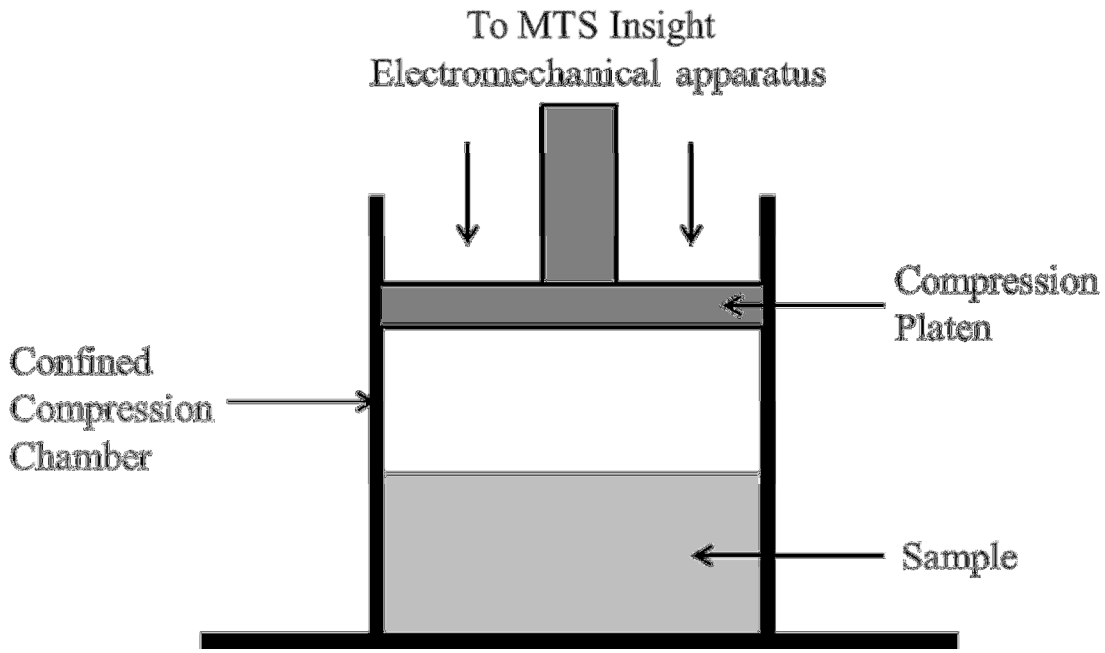
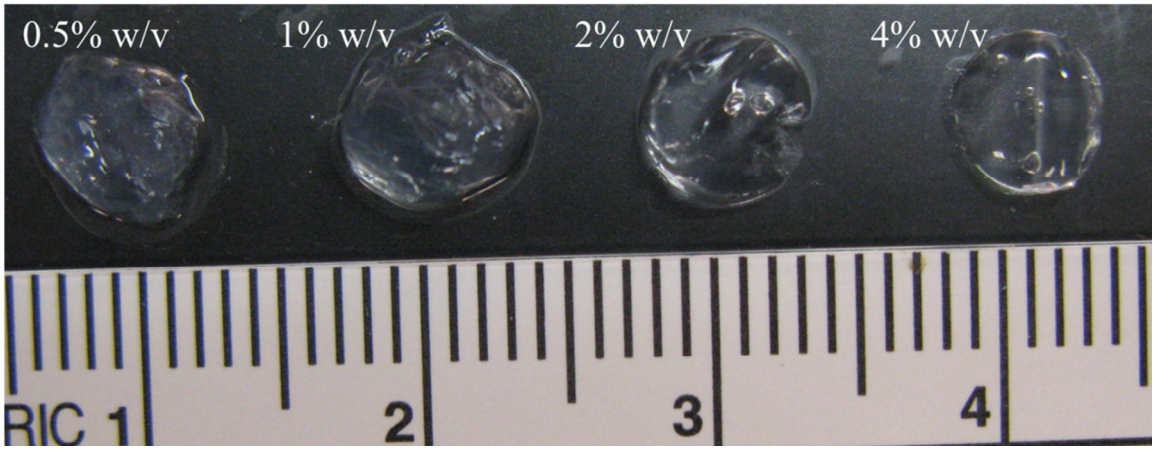


Figure 1: Schematic diagram of the mechanical testing apparatus. The sample was compressed with a stainless steel metal platen. Confined compression was performed due to physiological relevance.

a)



b)

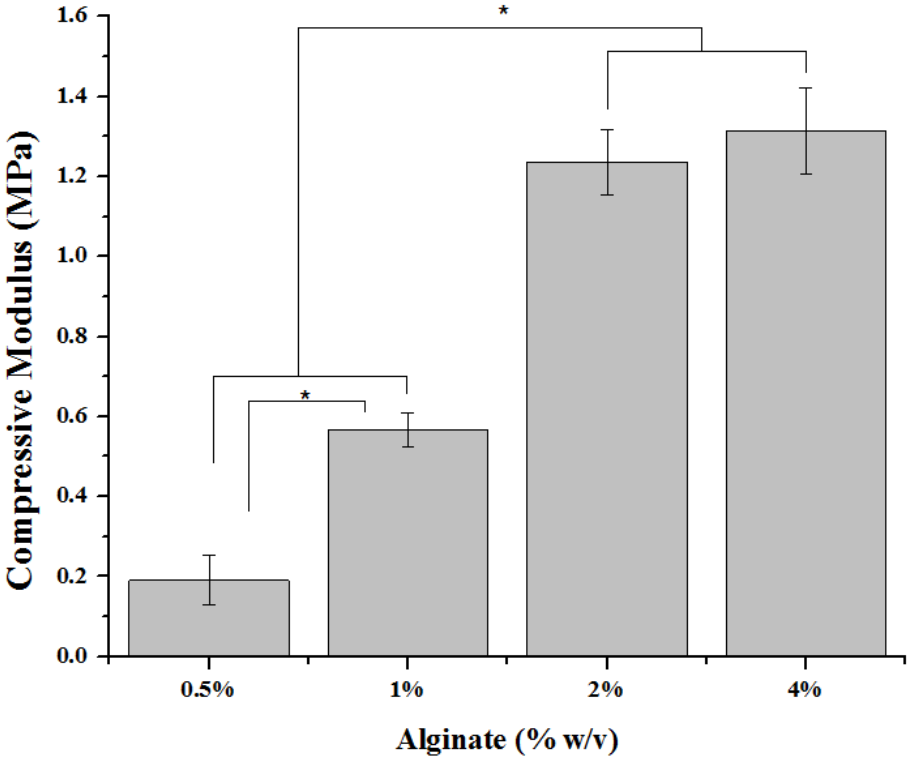
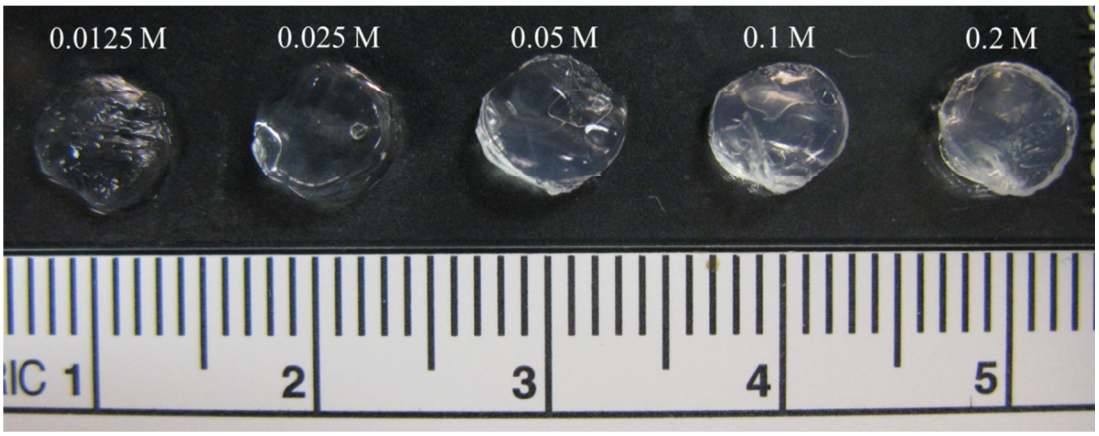


Figure 2: The effect of alginate concentration on scaffold mechanical properties. Alginate hydrogels with increasing concentration of alginate observed physical changes (a). Confined compression results for the scaffolds examined for different alginate concentrations (b).

Increasing alginate concentration significantly (*) increased the compressive modulus of the scaffold ($p<0.05$) (n=4). A 0.025 M $CaCl_2$ concentration was used in this analysis.

a)



b)

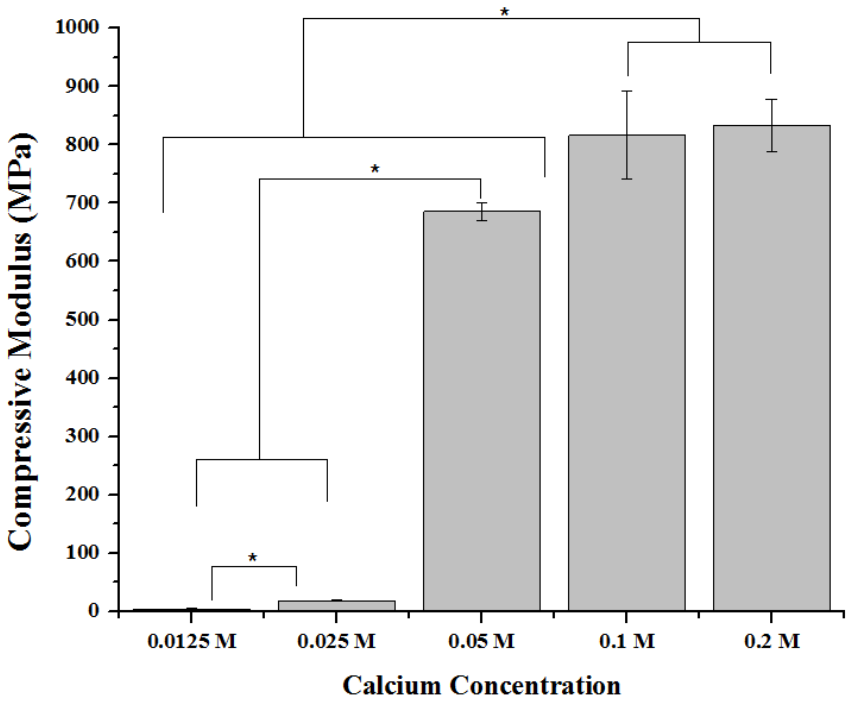


Figure 3: The effect of $CaCl_2$ concentration on scaffold mechanical properties. Scaffolds with increasing calcium chloride concentrations showed visible physical changes (a). Confined

compression results for the scaffolds examined for different $CaCl_2$ concentrations (b). Increasing $CaCl_2$ concentration significantly (*) increased the compressive modulus of the scaffold ($p<0.05$) (n=4). A 2% w/v concentration was used for this analysis.

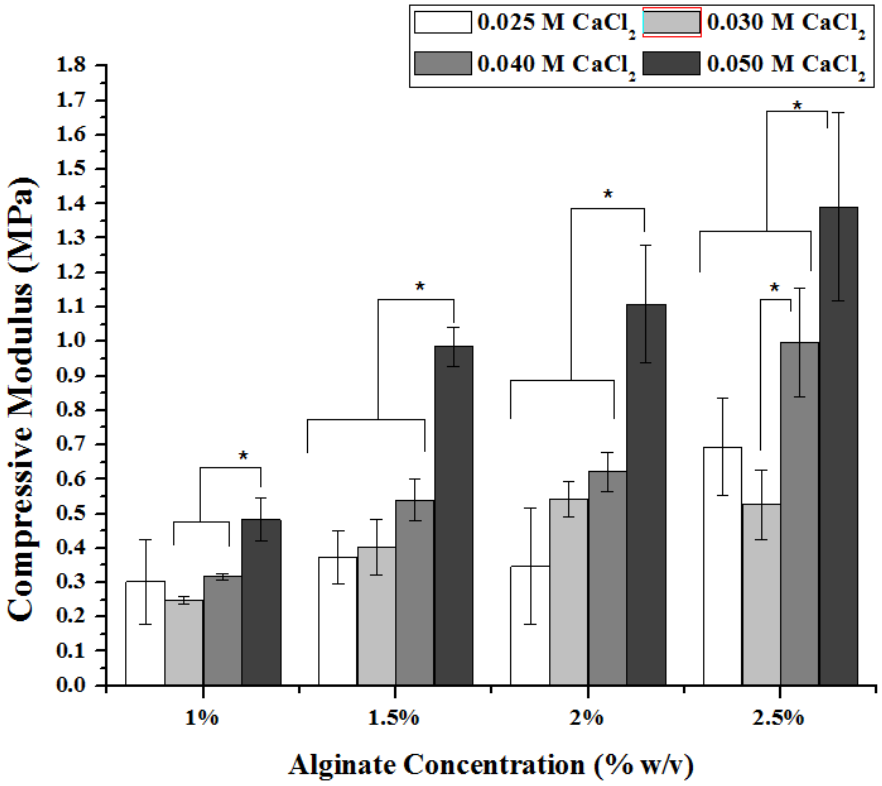


Figure 4: Confined compression moduli for the scaffolds examined using different combinations of alginate and CaCl2 concentrations. Alginate and $CaCl_2$ concentrations showed a direct relationship in compressive modulus as the concentration of each reagent increased, the compressive modulus of the scaffold increased as well. Statistical significant is denoted by * ($p<0.05$) (n=4).

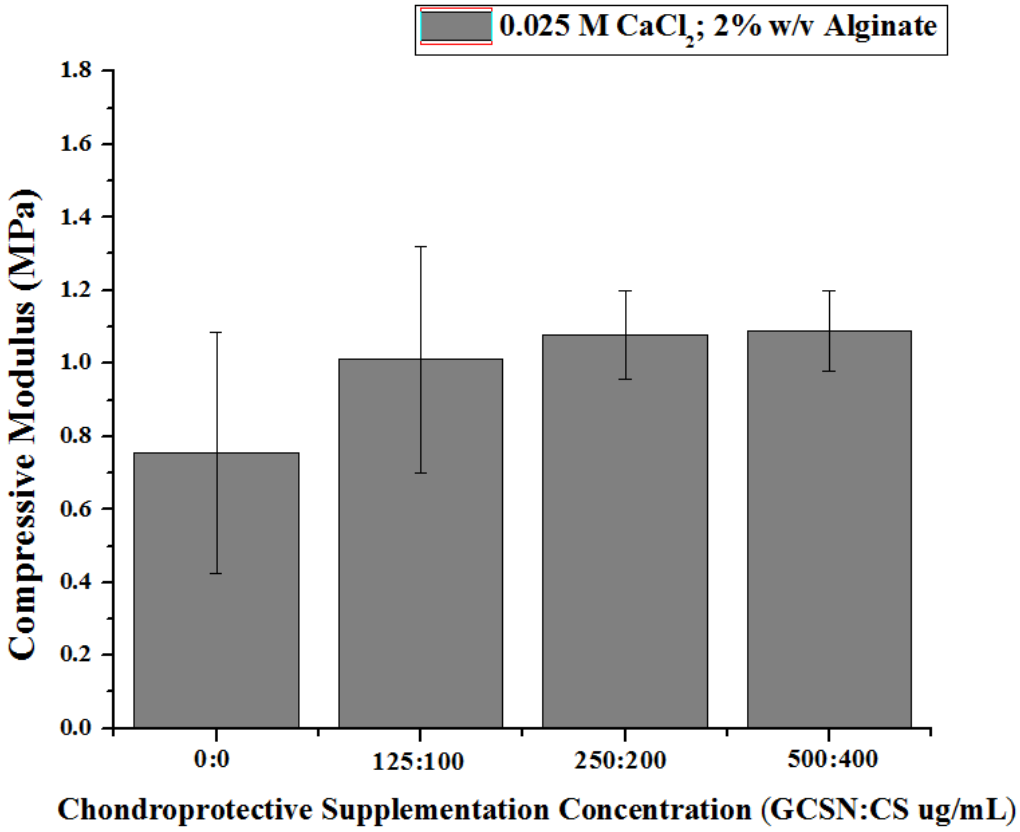


Figure 5: The effect of supplementation on the scaffold confined compression moduli. No significant differences were found between the different groups indicating that supplementation alone does not improve the mechanical properties of the scaffolds ($p<0.05$) ($n=4$).

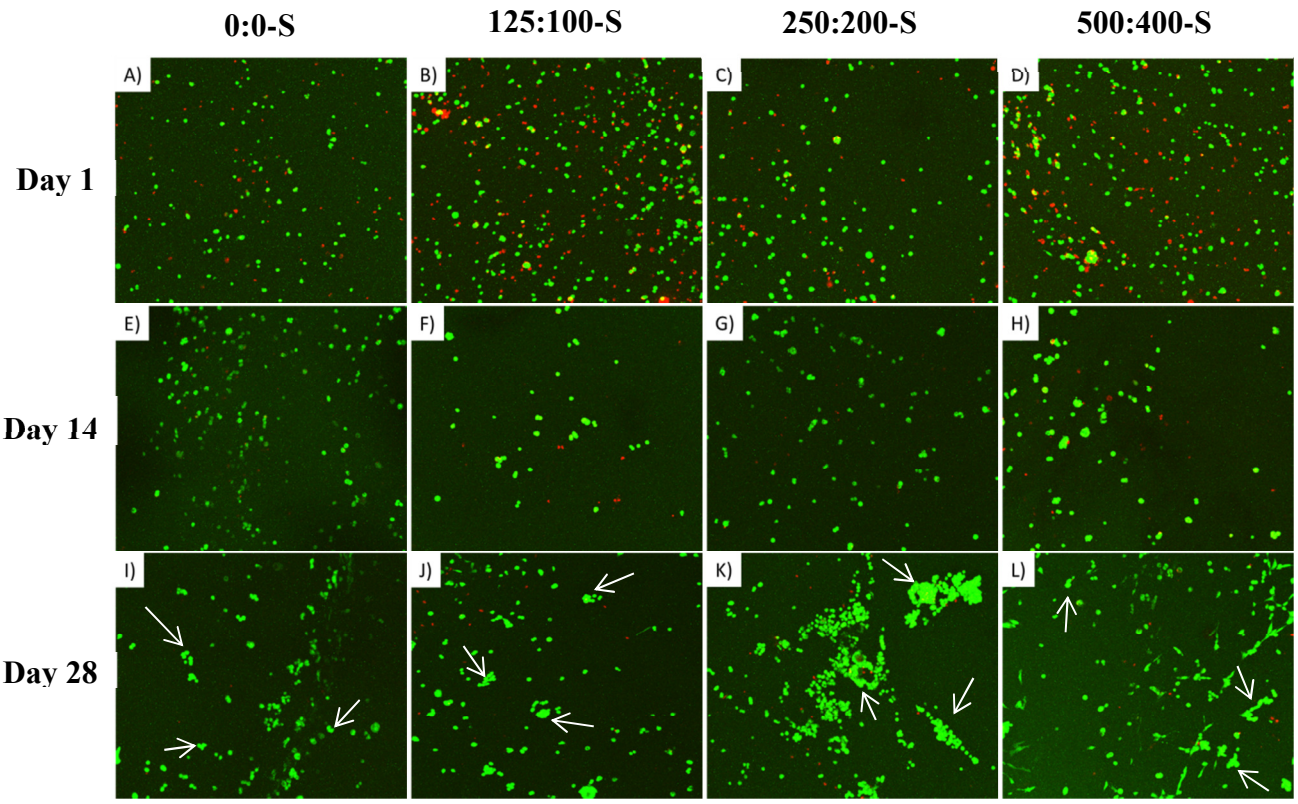


Figure 6: Confocal images displaying viability, morphology, and distribution of the HNPCs in the TE-constructs over a 28 day period. Viable HNPCs are in green and the nonviable cells are in red. The rounded morphology, which is the native cell morphology of the NP, is maintained throughout the 28 day period in each group. The 0:0-S group is A, E, and I. The 125:100-S groups are found in images B, F, and J. The 250:200-S groups are found in C, G and K. The 500:400-S groups are found in images D, H, and L. Arrows for Day 28 (I, J, K, L) indicate isogenous groups (clusters).

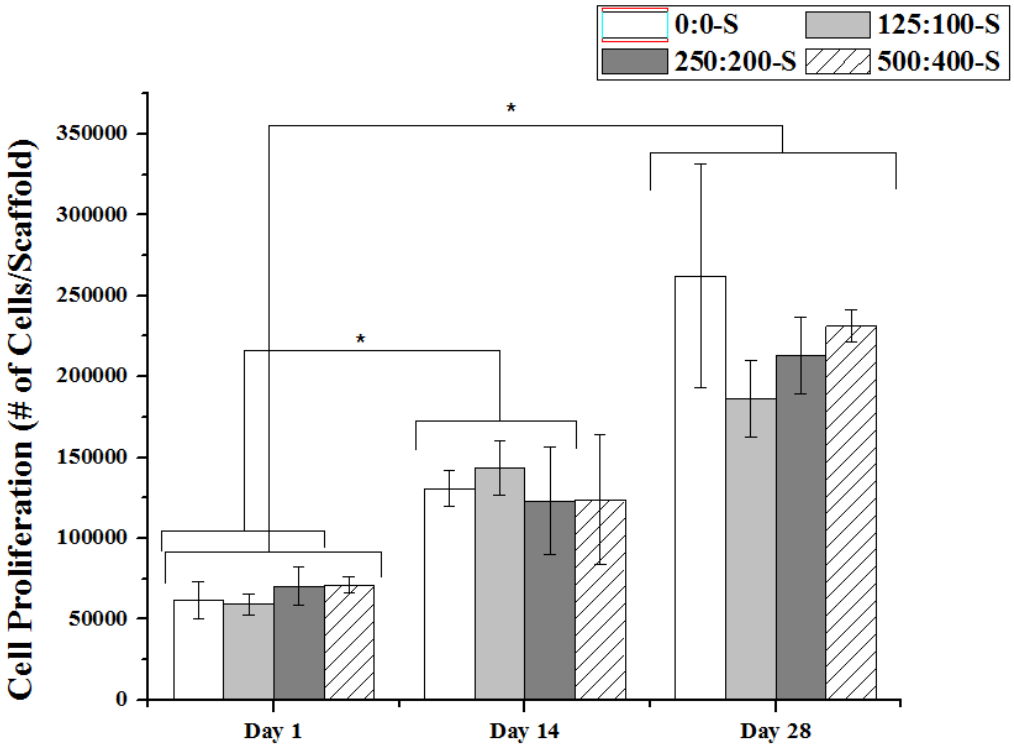


Figure 7: Cell proliferation analysis using MTT assay for the tissue engineered constructs over a 28 day period (n=3). The results show an increasing trend with significant differences denoted by (*) ($p<0.05$). No significant differences in proliferation between the groups.

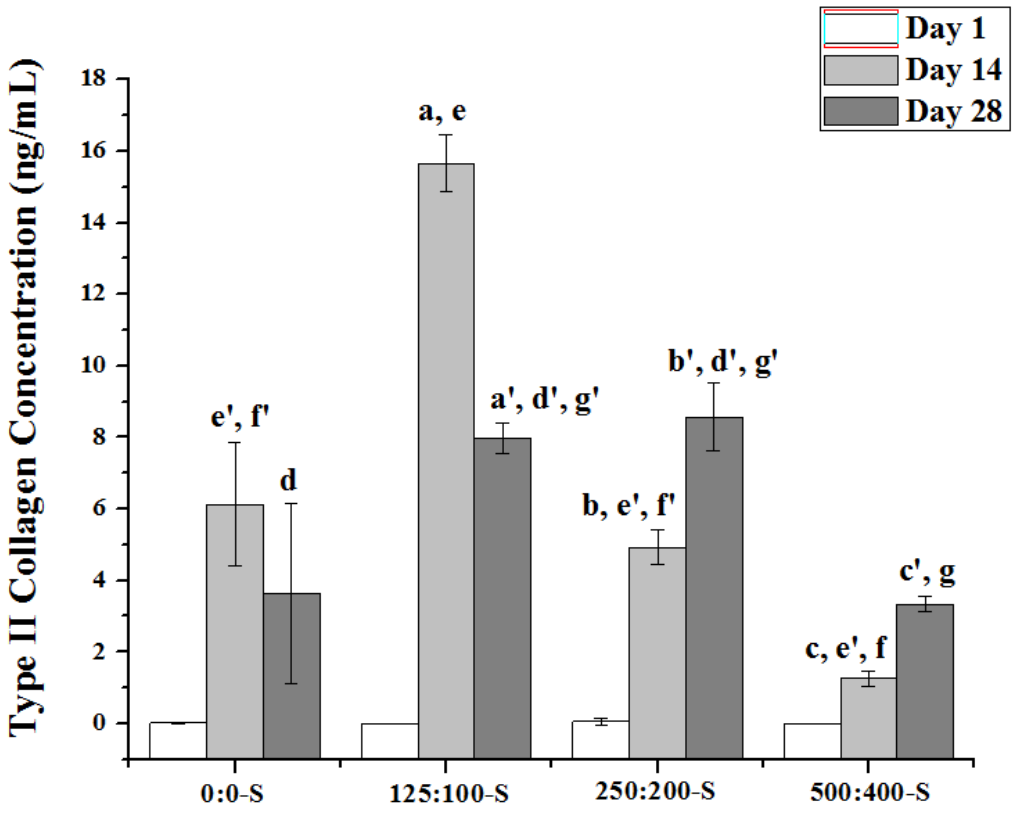


Figure 8: ELISA results for Collagen Type II quantification for each group. The 500:400-S and 250:200-S groups both showed significant increases in Type II Collagen Concentration between Day 14 and Day 28. Both the 125:100-S and 250:200-S had significantly greater Type II collagen concentration than the 0:0-S on Day 28. Similar letter denotes statistical difference to letter (a-a',b-b', c-c', d-d', e-e', f-f', g-g') ($p<0.05$) (n=3).

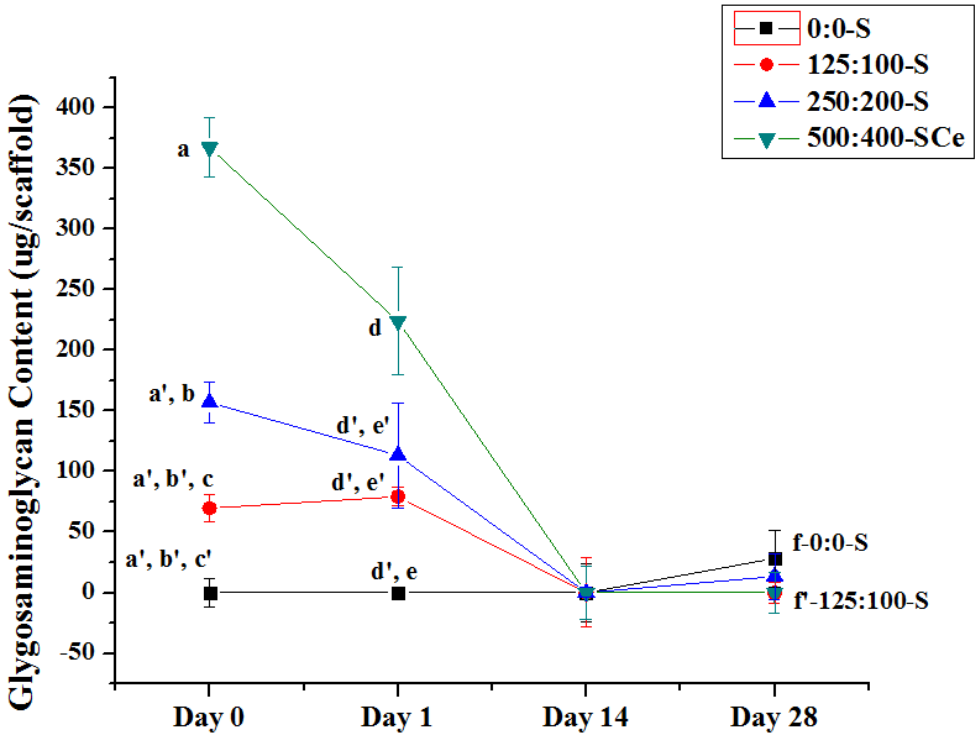


Figure 9: Sulfated glycosaminoglycan (GAG) content in the scaffold over the 28 day time period. The GAG content on Day 0 and Day 1 is due to the supplementation as the chondroitin sulfate is a GAG molecule naturally occurring in NP tissue. Similar letter denotes statistical difference to letter (a-a',b-b', c-c', d-d', e-e', f-f') ($p<0.05$) (n=3).

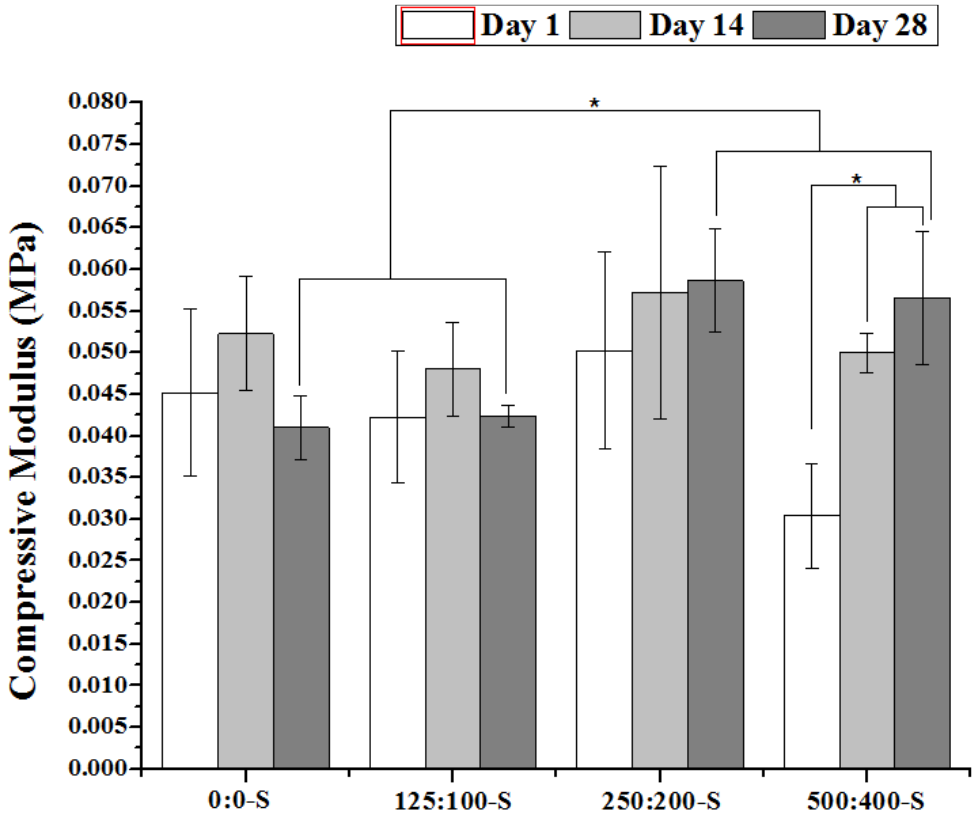


Figure 10: Confined compression results for the TE-construct between Day 1 and Day 28. The compressive moduli results for Day 28 of the 500:400-S and 250:200-S groups are significantly (*) greater than the 0:0-S group. The 500:400-S group increased significantly from Day 1 to Days 14 and Day 28 ($p<0.05$) (n=3).

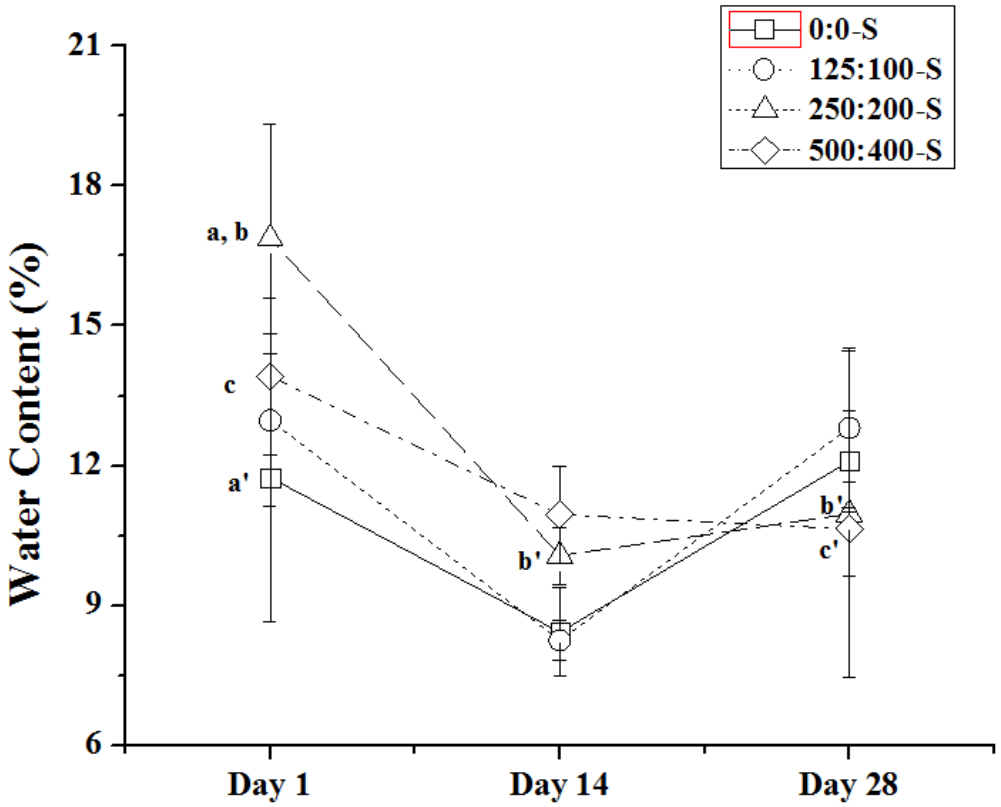


Figure 11: Water content (%) trend over the 28 Day time period. Data for Days 1, 14, and 28 are shown. The water content of the 250:200-S on Days 14 and 28 was significantly different than Day 1. The water content of the 500:400-S also showed a significant difference between Day 1 and Day 28. The 0:0-S and 125:100-S showed no significant changes in water content (%) over the 28 day period. Similar letters denotes statistical difference (a-a', b-b', c-c') ($p<0.05$) (n=4).

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