High and Low Compressive Loading on Tissue Engineered Cartilage

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Thesis

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INTRODUCTION

Media change is an essential step in cell culture as it provides a continuous supply of necessary nutrients and growth factors. Although media change is a standard step in cell culture, a universal procedure for media change has yet to be established. The amount and frequency of media changes varies between laboratories and experiments. The laboratory of K.A. Athanasiou performed two experiments both on self-assembling cartilage. In their first experiment, they changed 50% of the media in each well twice a day whereas the second experiment changed media 100% every 24 hours (Hu & Athanasiou, 2006; Athanasiou et al., 2008). Mauck et al., (2003) performed a different experiment using the same type of cells but instead changed 100% of the media 5 times a week. In addition to changing daily, there are also labs that change media 100% every two days and others that change media 50% three times a week (Mauck et al., 2002; Lien, et al., 2009; Kahn & Bilgen, 2016; Nims et al., 2014). The lack of a universal procedure for media change indicates a gap in cell culture. Understanding the optimal amount and frequency of media changes would provide researchers the ability to save resources and time.

The constituents of the media are also crucial to the success of the experiment. A commonly used supplement for growing cartilage is transforming growth factor (TGF-β1). This growth factor has shown to help in transforming mesenchymal stem cells into chondrocytes and maintaining chondrocytes differentiated (Trippel, 1995; Davidson et al., 2007; Blunk et al., 2002; Huang et al., 2009; Sheehy, Buckley & Kelly, 2011). Another emerging supplement that could potentially provide the similar benefits to TGF-β1 is a small biocompound Kartogenin

(KGN). Johnson *et al.* (2012) showed that KGN promotes chondrocyte differentiation and is effective at a concentration of 100 nM. Researchers have provided evidence that KGN could promote chondrogenic differentiation in bone-derived mesenchymal stem cells (BMSCs) (Liu *et al.* 2015). Figure 1 shows the effect of KGN in rabbit BMSCs and patellar tendon stem/progenitor cells (PTSCs) at concentrations ranging from 1 nmol/L to 5 µmol/L. This study aims to compare Kartogenin to TGF-β1 in its ability to promote chondrocyte differentiation, as it has not been done before.

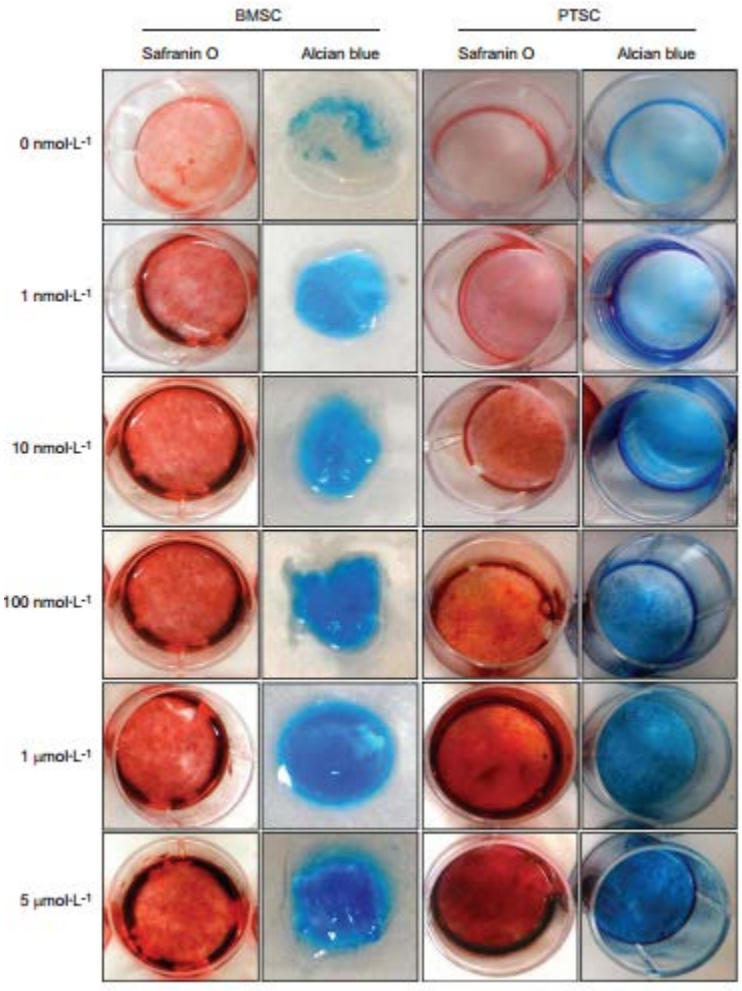


Figure 1 The effect of KGN in rabbit BMSCs and PTSCs at concentrations ranging from 1 nmol/L to 5µmol/L. The samples were stained with both safranin-O and alcian blue without counterstains (Liu et al., 2015).

This chapter discusses two studies for optimizing cell feeding for chondrocytes and synovial stem cells used to grow tissue engineered cartilage. The first study tests four media change procedures commonly used in cartilage growth research. The samples used for the experiment was tissue engineered cartilage made from agarose and chondrocytes. The media conditions were evaluated by analyzing the chondrogenic properties exhibited by chondrocytes; including glycosaminoglycan (GAG) disposition and the amount of DNA produced. We hypothesize that the procedure for media change would not significantly affect the amount of DNA and GAG produced by chondrocytes after four weeks of cell culture.

The second study investigates the effects of KGN on chondrocytes and synovial-derived stem cells(SDSC) in comparison to TGF-β1. This study was performed on a monolayer of chondrocytes and a monolayer of synovium-derived stem cells. The two cells types were visually evaluated using safranin-O with hematoxylin and fast green counterstain. We hypothesized that the staining for GAG will be similar or superior for the groups with 100 nM KGN than the groups with 10ng/ml TGF-β1.

METHODS: Study 1

Sample Preparation

Primary chondrocytes were isolated from the cartilage of three to six month Yorkshire porcine knees. The cartilage was harvested and digested the same day of sacrifice to optimize cell viability. The dissected samples were cut into $1mm^3$ sections and digested in 0.15% Collagenase Type II (Worthington #LS004176) overnight at 37°C in a sterile spinner flask. After digestion, the solution was filtered to remove undigested tissue using 100µm cell strainer and then 70µm cell strainers. The cells were then washed with PBS-PS-EDTA and counted using a

hemocytometer. The average cell yield varied depending on the age of the cartilage and the size of the cut samples during digestion.

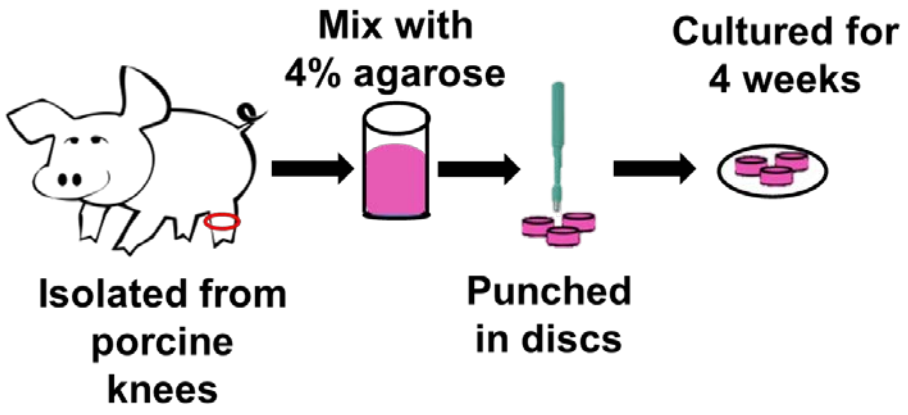


Figure 2 Experimental Plan for Study 1.

The cells were suspended in agarose to provide a scaffold. The agarose allows the chondrocytes to remain in the spherical shape found in native tissue while allowing nutrients to flow. The cell suspension was combined in a 1:1 ratio with 4% agarose that resulted in a 2% agarose sample. The mix as cooled for 20 minutes at room temperature to allow the gel to set and then punched into discs with final dimensions of 4 mm in diameter and 1.5 mm thick.

Experimental Plan

The discs were placed in a 24-well plate with 2 ml of media in each well containing: High glucose DMEM with 1% ITS+ Premix, 100 U/mL penicillin, 100 µg/mL streptomycin, 2mM L-glutamine, 2.5 µg/mL amphotericin B, 0.1 mM nonessential amino acids, 0.4 mM proline, 50 µg/mL L-ascorbic acid 2- phosphate. Media was changed per Table 1. The samples were cultured for four weeks changing the media per Table 1 and then analyzed for biochemical properties. These samples were cultured statically to limit variables. Culture time was increase from two weeks to four weeks to increase the possibility of seeing a difference in the groups.

Table 1 Media Change groups for Study 1.

Group	Frequency	Amount of Media
A	3 days a week	50%
B		100%
C	2 days a week	50%
D		100%

After four weeks, the samples were lyophilized and digested using 125µg/mL papain enzyme in PBE/ cysteine at 60°C overnight. DNA was quantified by using the Quant-iT PicoGreen dsDNA Kit by Molecular Probes and GAG was analyzed using the Dimethylmethylene Blue (DMMB) assay. The data was analyzed using a one-way ANOVA with a confidence of 95% in Minitab 17.

RESULTS: Study 1

There were no statistically significant differences between any of the four groups ($p>0.05$) for either DNA or GAG. As shown by Figure 3, the average weights for groups A, B, C, and D were 29.95 ± 2.15mg, 30.98 ± 1.26mg, 30.23 ± 2.65mg and 31.27 ± 0.56mg, respectively. The average DNA per sample for groups A, B, C, and D were 1.13 ± 0.21, 1.22 ± 0.05, 1.24 ± 0.16, 1.26 ± 0.17, respectively.

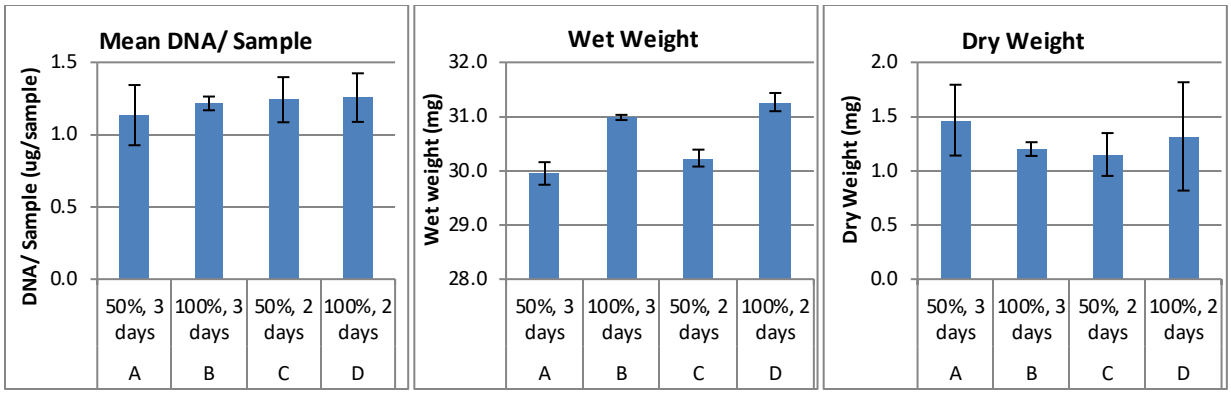


Figure 3 DNA and weights measured for study 1.

Although there appears to be a slight increase in GAG in groups that were given media every two days, the results are not statistically significant, see Figure 4. The GAG per DNA for groups A, B, C, and D were 2.91 ± 0.49, 3.10 ± 0.73, 3.48 ± 1.06, and 3.39 ± 0.98, respectively.

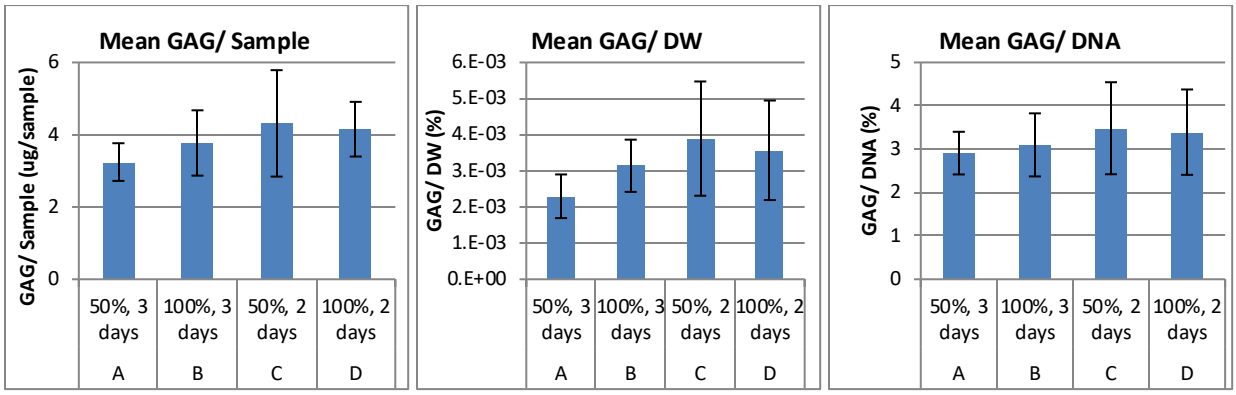


Figure 4 GAG measurements for study 1. GAG is analyzed per sample, wet weight, and DNA.

DISCUSSION: Study 1

The aim of study 1 was to determine the effect of media change on the production of DNA and GAG in chondrocytes. There were no statistically significant differences between the four procedures, suggesting that any of the procedures can be used without sacrificing GAG and DNA production. This would allow researchers to choose the procedure based on resources. However, the conclusions that can be drawn from this experiment are limited by the length of culture time. It could be possible that the effect of media availability does not affect the cells until later stages of experiments. Experiments that involve tissue engineered cartilage are often

longer studies as samples take more than 4 weeks to show significant improvement in GAG and strength (Kisiday et al., 2004; Waldman et al., 2006; Cigan et al., 2016). Nevertheless, the results of this study could be beneficial for experiments that are intended to run for short periods.

METHODS: Study 2 KGN vs. TGF-β1

Study 1 determined the dosing and frequency of cell feeding. The next study investigates KGN as a substitute for the growth factor TGF-β1, typically used in growing tissue engineered cartilage.

Sample Preparation

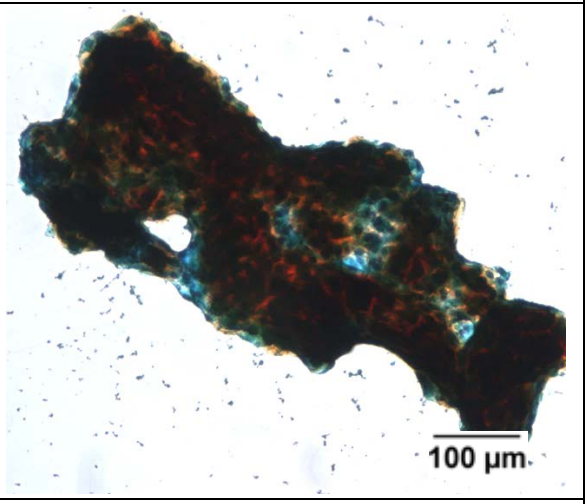
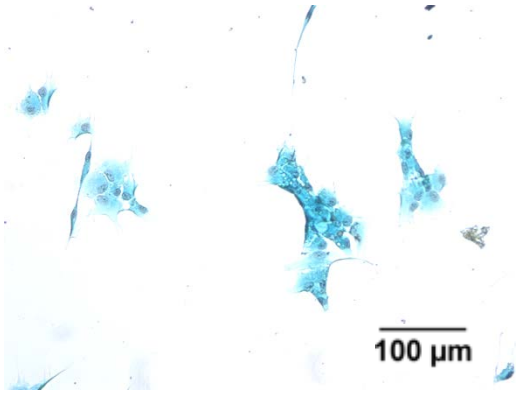
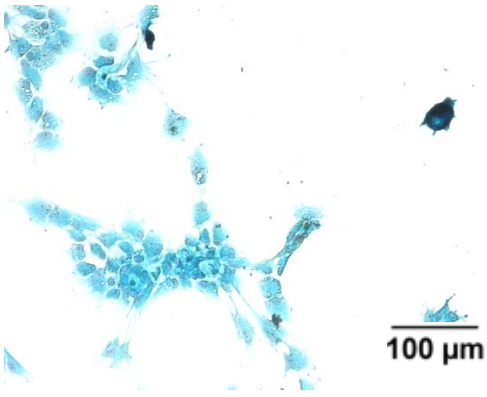
Chondrocytes (P0) and Synovium-derived stem cells (SDSC) (P2) were thawed and expanded. Chondrocytes and SDSC were seeded at one million cells/ mL in T-75 flasks and cultured until confluent, approximately one week. The cells were then trypsinized and then counted using trypan blue and a hemocytometer. Two 48-well plates for each cell type was seeded. Each well contained approximately 24,000 cells and 200µl of serum-free media, containing: High glucose DMEM with 1% ITS+ Premix, 100 U/mL penicillin, 100 µg/mL streptomycin, 2mM L-glutamine, 2.5 µg/mL amphotericin B, 0.1 mM nonessential amino acids, 0.4 mM proline, 50 µg/mL L-ascorbic acid 2- phosphate, and 100 mM dexamethasone. KGN (Sigma-Aldrich) and rhTGF-β1 (R&D Systems) was added to the wells in different concentrations as shown in Table 2.

Table 2 KGN and TGF- β1 dosing for Study 2.

Condition*	Kartogenin (KGN)	Transformation Growth factor – beta 1 (TGF-β1)
1 (KGN-1)	1 nmol/L	0
2 (KGN-10)	10 nmol/L	0
3 (KGN-100)	100 nmol/L	0
4 (TGF-β1)	0	10ng/ml

RESULTS: Study 2

The purpose of this study was to analyze the effects of Kartogenin on chondrocytes and synovial stem cells in comparison to TGF-β1. Unlike TGF-β1, Kartogenin did not exhibit red staining in the chondrocytes, indicating that it did not help in maintaining chondrogenic differentiation, refer to Figure 5. The elongated shape of the cells in the KGN groups also indicate dedifferentiation of the cells since chondrocytes are characteristically spherical and tend bind together tightly as exhibited by the TGF-β1 group.

	Once a week	Three times a week, MWF
TGF-β1	N/A Note: TGF dose was always MWF	
KGN 1		

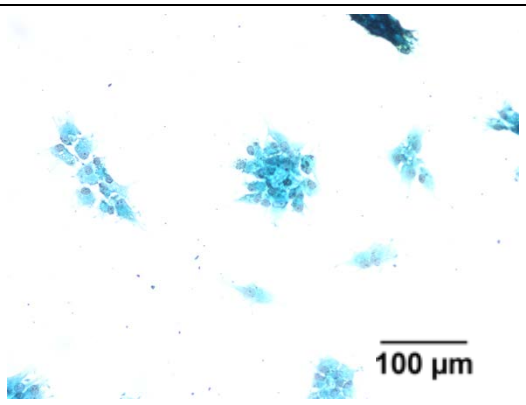
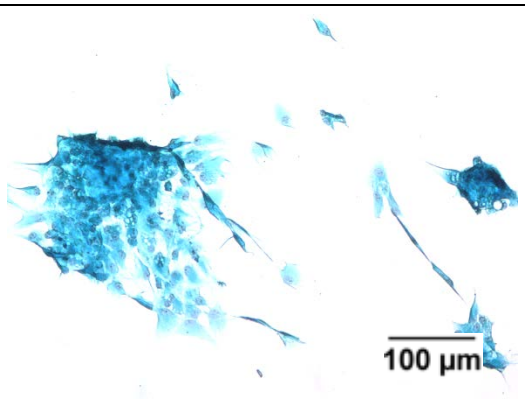
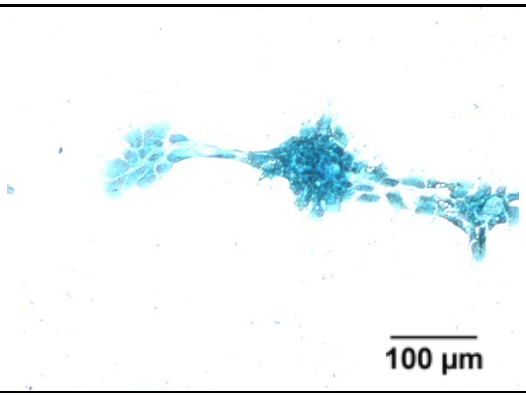
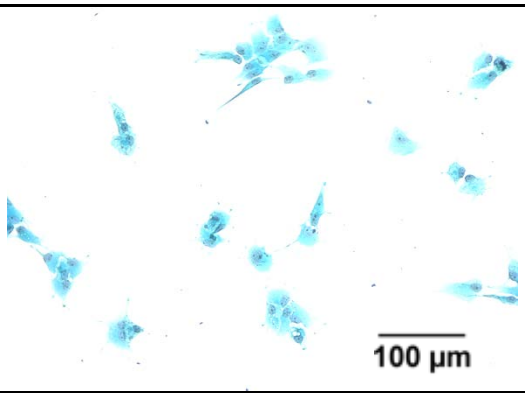
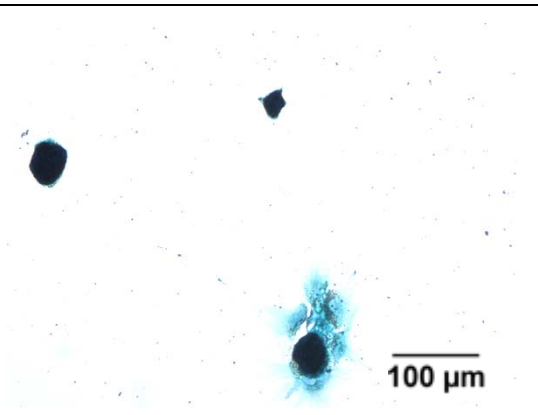
KGN 10		
KGN 100		

Figure 5 Chondrocytes stained with Safranin-O, hematoxylin and fast green counterstain with 20x magnification.

As seen with chondrocytes, Kartogenin did not induce chondrogenic differentiation in SDSCs, see Figure 6. The dispersed elongated cells in the KGN groups indicate that they are still exhibited stem cell characteristics whereas the rounded groups of cells in the TGF-β1 group indicates chondrogenic characteristics.

	Once a week	Three times a week, MWF
TGF-β1	N/A Note: TGF dose was always MWF	

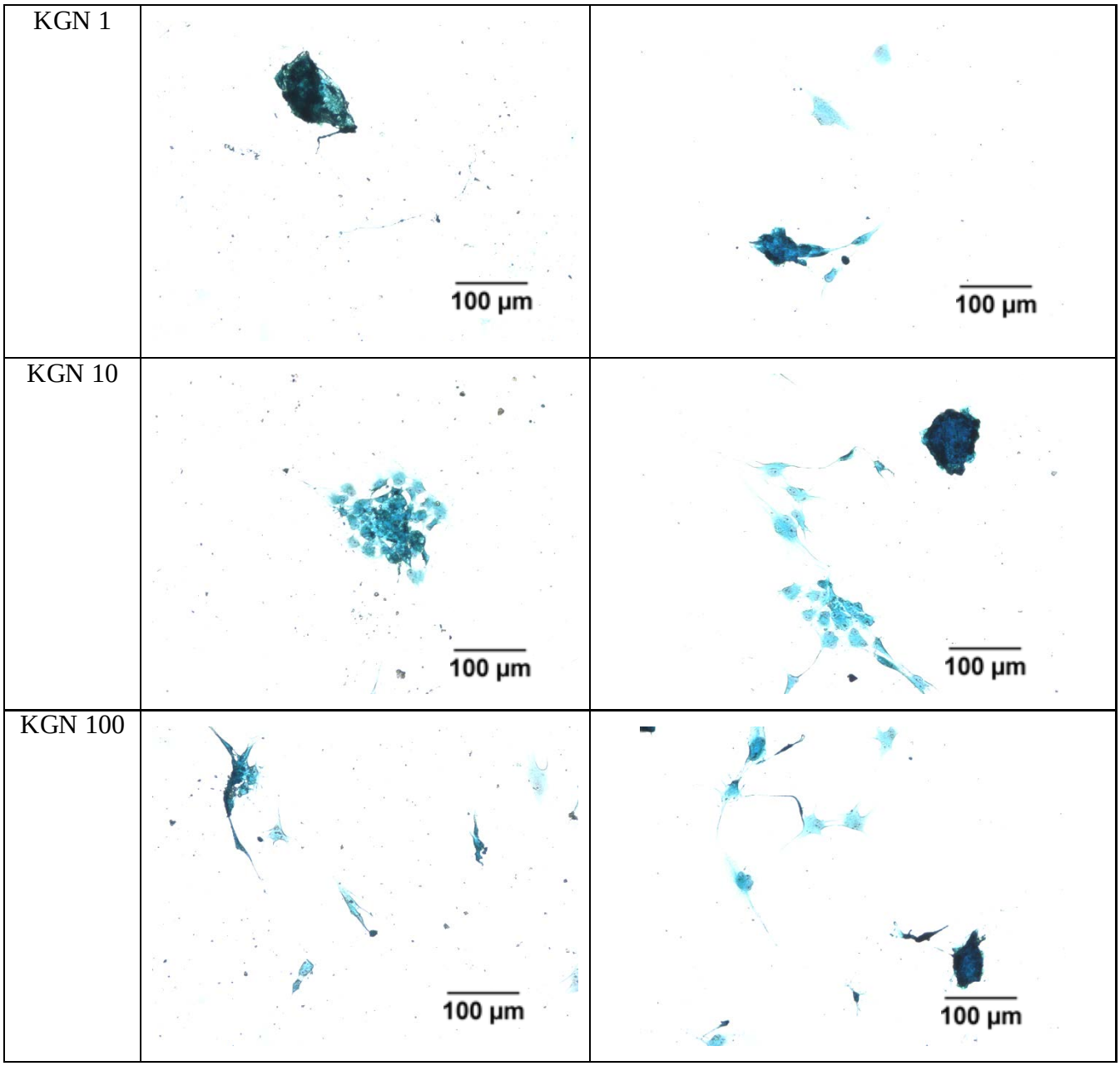


Figure 6 SDSCs stained with Safranin-O, hematoxylin and fast green counterstain with 20x magnification.

DISCUSSION: Study 2

The specific aim of study 2 was to compare the effects of Kartogenin for chondrogenic cells and synovial stem cells in comparison to TGF-β1. The study was not able to replicate the results seen in previous studies. KGN did not appear to induce chondrogenic differentiation in SDSCs or maintain it in chondrocytes. During cell culture, KGN was dissolved in Dimethyl sulfoxide

(DMSO) and then added to the media. It could be possible that the mixture did not fully distribute and may not have accessed all the cells. The concentration of KGN may have also been incorrect for these cell types. Future work should consider adjusting the concentration and delivery method.

CONCLUSION

The purpose of the studies presented in this chapter was to optimize the feeding for tissue engineered cartilage seeded with chondrocytes. Study one suggests that in the first two weeks of culture the amount and frequency of the media used does not significantly change the amount of GAG and DNA produced. Futures study should determine if media availability has an effect for longer studies as tissue develops and matures. The second study investigated the effectiveness of KGN on chondrocytes and synovial derived stem cells in comparison to TGF-β1. Although this study was not successful in using KGN, future work should not be discouraged in investigating further as this molecule is new in this application and researchers have seen some success in using this biocompound.

CHAPTER 2: COMPRESSIVE LOADING ON TISSUE ENGINEERED CARTILAGE

INTRODUCTION

Osteoarthritis is a debilitating joint disease that affects over 27 million Americans. The limited ability of articular cartilage to heal is due to its low cell density and avascular nature. Tissue engineered cartilage could be a viable option for patients that have lost cartilage due to injury or osteoarthritis. However, reaching optimal mechanical and biochemical properties in tissue engineered cartilage is still a challenge. Loading tissue engineered cartilage with dynamic compression has been shown to increase strength, GAG disposition and cell proliferation in tissue engineered cartilage. Dynamic loading tissue engineered cartilage at 10% to 15% strain with a 0 to 5% tare strain is widely used to improve the characteristics of tissue (Lee & Bader, 1997; Mauck et al., 2000; Demarteau et al., 2003; Bian et al., 2010; Thorpe et al., 2010).

In addition, our previous study showed that loading the samples with 30% strain during the first weeks of culture had significantly higher GAG disposition and mechanical strength than groups loaded with 10% strain (Kahn & Bilgen, 2016). This chapter describes two studies that continue the investigation on the use of 30% strain on chondrocyte and SDSCs seeded agarose hydrogels.

Study 3 investigates the effects of loading chondrocyte seeded agarose hydrogels with a combination of 30% strain and 10% strain. Study 4 builds on the results of study 3 with the use of co-culture samples of chondrocytes and SDSCs. This study includes the use of SDSCs as its availability and reproducibility is much greater than chondrocytes (Giovanni et al, 2010; Cals et al., 2012). However, our previous study has shown that SDSCs alone does not reproduce the biochemical and mechanical strength as shown in the chondrocyte seeded hydrogels (Kahn &

Bilgen, 2016). Our hypothesis is that early high compressive loading will enhance GAG deposition in both groups.

METHODS: STUDY 3 AND 4

Sample Preparation

Samples for study 3 were prepared using the same procedure used to prepare study 1 samples described in chapter 1. The sample properties are summarized in Table 3.

Table 3 Study 3 sample properties.

Cell density (M cells/ml)	20
Cell type	Chondrocytes
Construct Dimensions	4 mm in diameter x 1.5 mm thick
Construct Volume (ul)	~18.85
Total Volume (ml)	~3.02
Total Culture Time (wks)	6
Agarose %	2

The samples for study 4 were also prepared using the same procedure as study 1 however the discs contained a 1:1 co-culture of chondrocytes and SDSCs. The sample properties are summarized in Table 4.

Table 4 Study 4 sample properties.

Cell density (M cells/ml)	20
Cell type	50% Chondrocytes and 50% SDSCs
Construct Dimensions	4 mm in diameter x 1.5 mm thick
Construct Volume (ul)	~18.85
Total Volume (ml)	~3.02
Total Culture Time (wks)	6
Agarose %	2

The discs were cultured in non-treated petri dishes. The discs were not confined to allow for growth vertically and horizontally. The discs were cultured statically for six weeks in Serum-free chondrogenic media containing: High glucose DMEM with 1% ITS+ Premix, 100 U/mL penicillin, 100 µg/mL streptomycin, 2mM L-glutamine, 2.5 µg/mL amphotericin B, 0.1 mM nonessential amino acids, 0.4 mM proline, 50 µg/mL L-ascorbic acid 2- phosphate. The first two weeks of culture also had 100 mM dexamethasone and 10 ng/mL TGF-β1, to promote chondrogenic differentiation.

After two weeks, the discs were then introduced to mechanical loading. Study three samples were separated into five loading groups as shown in Figure 7a. Study four samples were loaded per Figure 7b. The groups were dynamically loaded either at 10% strain, 30% strain, or no loading using a custom bioreactor. A tare strain of 5% was used to ensure that the platen remained on the samples during cyclic loading. The samples were loaded at 1 Hz for 3hrs/day and 5 days/week.

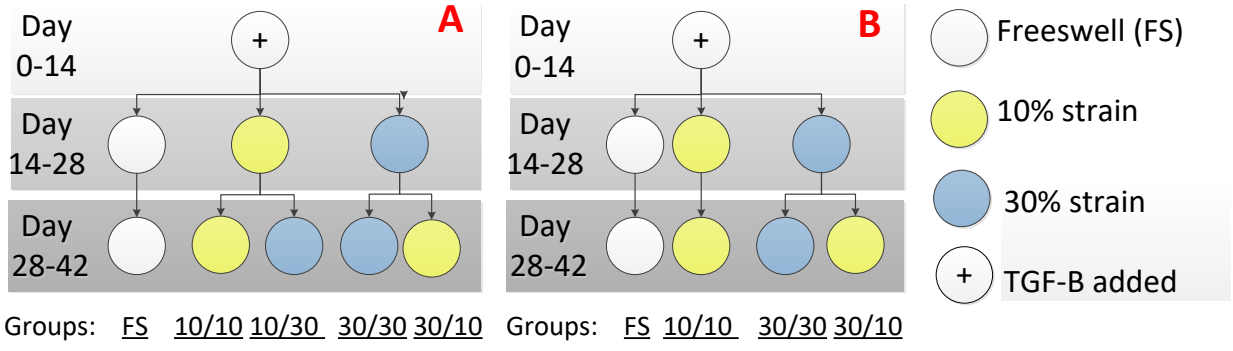


Figure 7 Experimental Plan for a) study 3 and b) study 4

The samples were analyzed at four time points; day 0, day 14, day 28 and day 42. At each time point the samples were weighed and measured for thickness and diameter. The mechanical properties were measured by using Instron Electropuls and Wavematrix software. The samples were first equilibrated by applying a load of 1.5 gf. Then, to calculate the equilibrium modulus a strain of 10% was applied for 30 minutes. The equilibrium modulus was calculated by using the relax load at the end of the 30 minutes using Equation 1.

$$Equilibrium\ Modulus = \frac{Equlibrium\ Stress}{Strain} = \frac{\sigma_{eq}}{\varepsilon}$$

Where equilibrium stress, σ_{eq} [N/m^2] $= \frac{Final\ Equilibrium\ Load\ [gf] \cdot \left(\frac{1N}{101.9716gf}\right)}{Sample\ Area\ [mm^2] \cdot \left(\frac{1m^2}{1x10^6\ mm^2}\right)}$

And strain, $\varepsilon = \frac{Change\ in\ Thickness}{Initial\ Thickness} = \frac{L}{L_0} = \frac{L_0 \cdot 0.1}{L_0} = 0.1$

Combining equations, the Equil. Modulus [N/m^2] or [Pa] $= \frac{Final\ Load\ [gf] \cdot \left(\frac{1N}{101.9716gf}\right)}{Sample\ Area\ [mm^2] \cdot \left(\frac{1m^2}{1x10^6\ mm^2}\right)} \cdot \frac{1}{0.1}$

Equation 1 Equilibrium Modulus

Lastly, a cyclical load oscillating between 9% strain and 11% strain was applied at frequency of 1Hz/minute to obtain the dynamic modulus. The dynamic modulus is calculated using the load response of the sample at -1% strain and +1% strain along with Equation 2.

$$Dynamic\ Modulus = \frac{\Delta Stress}{\Delta Strain} = \frac{\Delta\sigma_{eq}}{\Delta\varepsilon}$$

$$\Delta\sigma_{eq} = \frac{(Load\ at -1\%\ strain\ [gf] - Load\ at +1\%\ strain\ [gf] \cdot \left(\frac{1N}{101.9716gf}\right)}{Sample\ area\ [mm^2] \cdot \left(\frac{1m^2}{1x10^6\ mm^2}\right)}$$

$$\varepsilon = \frac{Change\ in\ Thickness}{Initial\ Thickness} = \frac{L}{L_0} = 0.11 - 0.09 = 0.02$$

Combining equations, the Dynamic Modulus [N/m^2] or [Pa]

$$= \frac{(Load\ at{-}1\%\ strain\ [gf] - Load\ at{+}1\%\ strain\ [gf]) \cdot (\frac{1N}{101.9716gf})}{Sample\ area\ [mm^2] \cdot (\frac{1m^2}{1x10^6\ mm^2})} \cdot \frac{1}{0.02}$$

Equation 2 Dynamic Modulus

The samples were then lyophilized and digested using 125µg/mL papain enzyme in PBE/ cysteine at 60°C overnight. DNA was quantified by using the Quant-iT PicoGreen dsDNA Kit by Molecular Probes. GAG was analyzed using the Dimethylmethylene Blue (DMMB) assay. Collagen was measured using the hydroxyproline (OHP) assay after hydrochloric acid hydrolysis. Samples from each time point and group were stained for GAG with Safranin-O, hematoxylin, and fast green and for collagen with Picrosirius Red.

The data was analyzed using ANOVA with Tukey Method with a confidence of 95% using Minitab 17.

RESULTS: STUDY 3 AND 4

Study 3

Group 30/10 had significantly higher DNA/ dry weight than any group on day 42. DNA did not significantly change between groups in the initial 2 weeks of mechanical loading ($p<0.05$, day 28 of culture), see Figure 8.

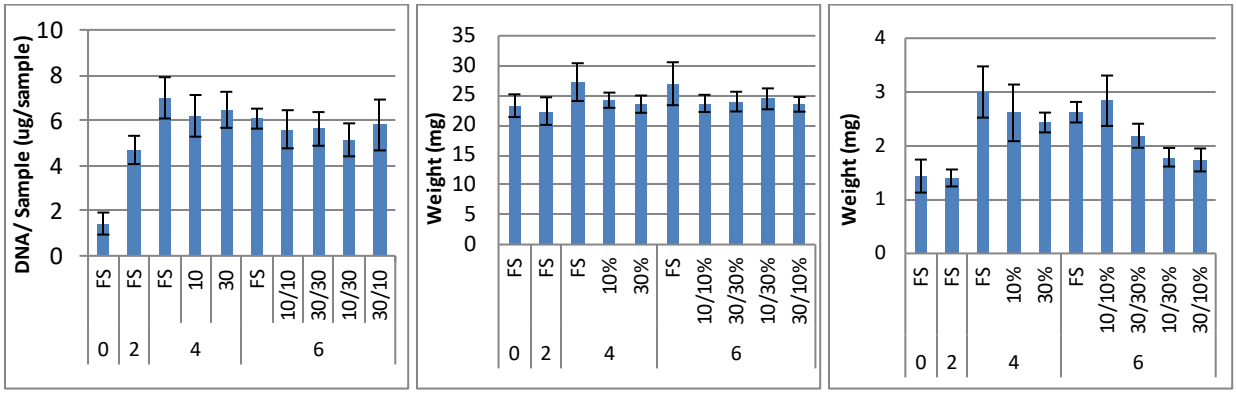


Figure 8 Study 3 DNA and sample weight summary. Data presented as mean ± Std. dev,* = p ≤ 0.05, compared to FS of that week

On day 28, samples loaded with 30% strain showed significantly higher GAG/ dry weight than samples that were cultured in free-swell ($p<0.05$). On day 42, there was no statistically significant difference between groups and there was a statistically significant decrease in GAG when compared to day 28, see Figure 9.

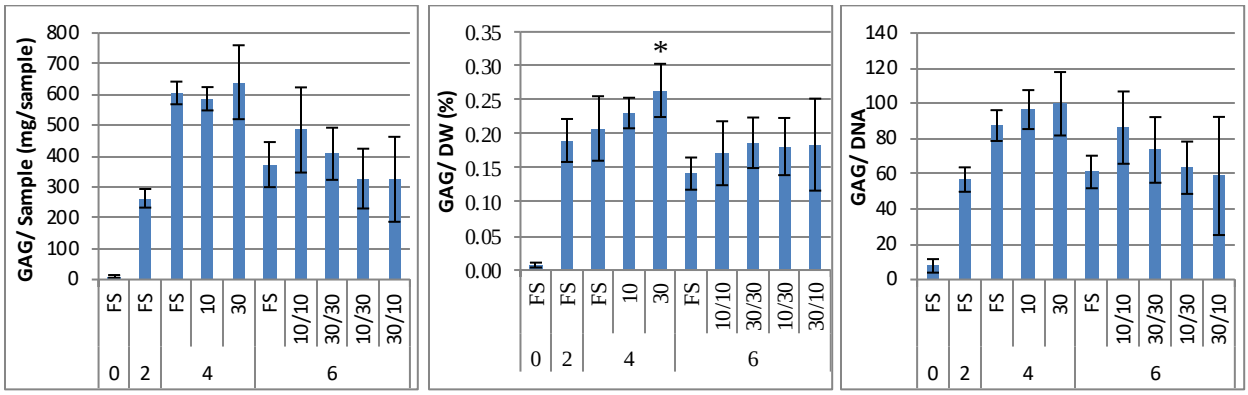


Figure 9 Study 3 GAG summary. Data presented as mean ± Std. dev,* = $p \leq 0.05$, compared to FS of that week

Group 30/10 also had significantly higher collagen per dry weight than the free-swell group ($p<0.05$), however as seen with GAG, there was also a decrease in collagen for all groups when compared to day 28. There was no statistically significant difference in collagen between groups after 2 weeks of mechanical loading (day 28 of culture), see Figure 10.

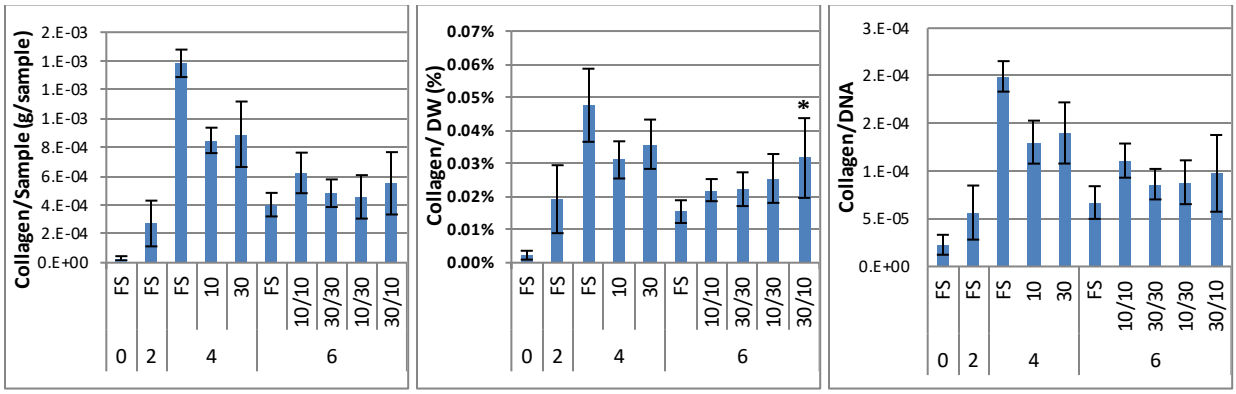


Figure 10 Study 3 Collagen summary. Data presented as mean ± Std. dev,* = $p \leq 0.05$, compared to FS of that week

The samples did exhibit equilibrium modulus values consistent with native cartilage (60-250kPa) in the first 28 days, indicated by the red box in Figure 11. Equilibrium modulus increased for all groups between day 14 and day 28 of culture but decreased during day 42. On day 28, samples

that received no loading and samples that received 10% strain had significantly higher equilibrium modulus (EM) than groups loaded with 30% strain. On day 42, group 10/10 had significantly higher EM than the free-swell group. The data necessary to calculate the dynamic modulus was not collected and therefore was not analyzed for this study.

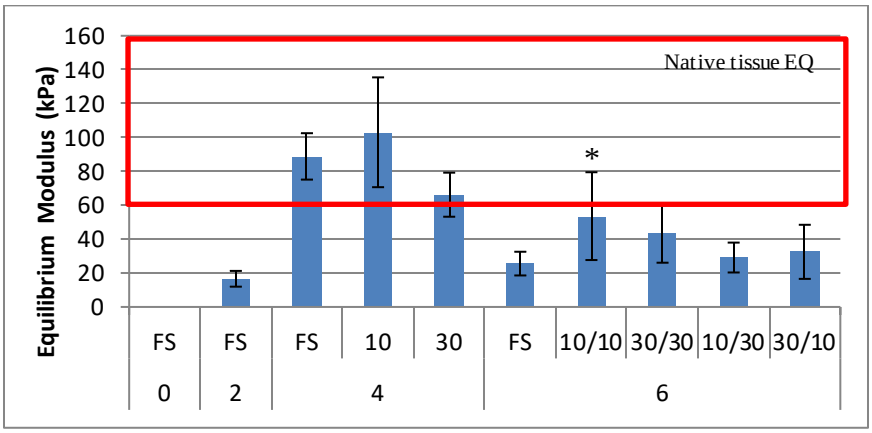


Figure 11 Study 3 equilibrium summary. Data presented as mean ± Std. dev,* = p ≤ 0.05, compared to FS of that week

Samples loaded with 30% strain at 28 days showed the most intense safranin-O staining. In agreement with Figure 12, less intense staining was observed in all groups at 42 days, indicating possible loss of GAG in the periphery of the samples.

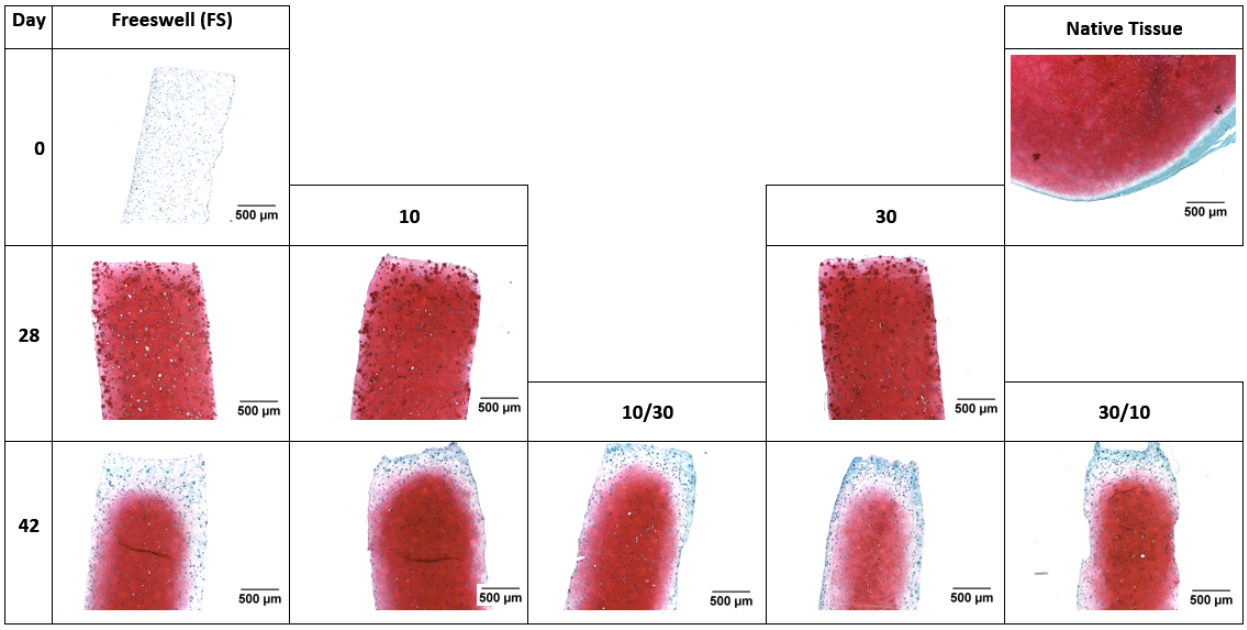


Figure 12 Study 3 safranin-O/Fast Green Staining for Glycosaminoglycan at 4x (scale bar = 500µm).

Polarized light images show the collagen orientation in Figure 13. Native tissue section shows that collagen fibers are aligned in parallel to the cartilage surface. Each sample exhibited a thin border of collagen along edge. The 30/10 loading group appears to have more aligned collagen fibers near the edge at 42 days.

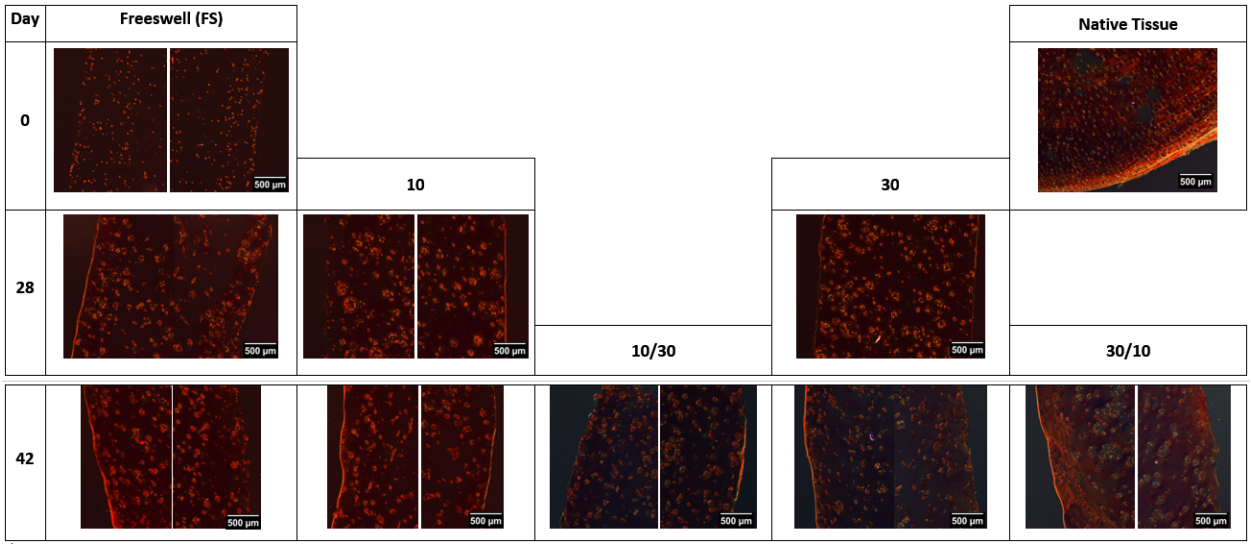


Figure 13 Study 3 Picrosirius Red Staining for Collagen at 10X (scale bar = 500µm)

The picrosirius staining shown in Figure 13 stains the collagen in the sample and does not differentiate between types. To determine the amount of collagen 1 and collagen 2 separately, the samples were stained using Immunohistochemistry (IHC). This type of staining uses antibodies that target either collagen type 1 or collagen type 2. The collagen is stained brown using diaminobenzidine (DAB) and the nucleus is stained blue using hematoxylin. Figure 14 and Figure 15 show the staining for collagen 1 and 2, respectively. The native tissue shows light staining for collagen 1 and an intense staining for collagen 2, as expected for cartilage. Each sample reflected the native tissue by having a stronger stain for collagen 2 in comparison to collagen 1. However, the samples showed stronger staining for collagen 1 than the native tissue. Lastly the samples seem to loss some intensity for collagen type 2 during day 42 (week 6).

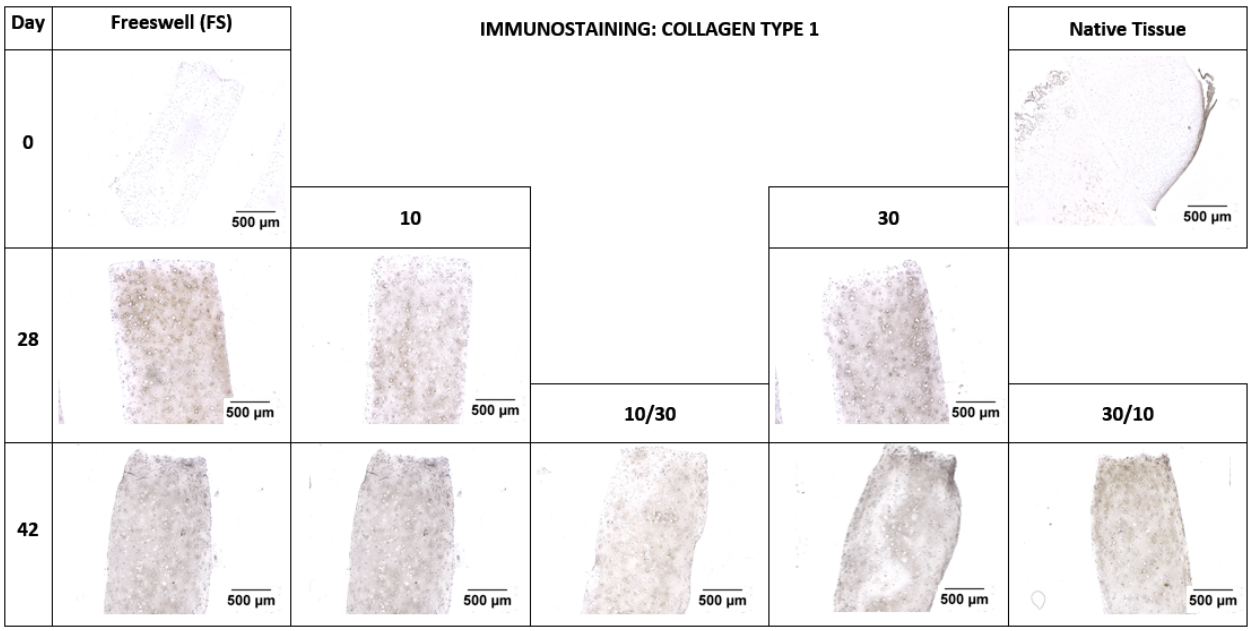


Figure 14 Study 3 IHC for Collagen Type 1 at 4X.

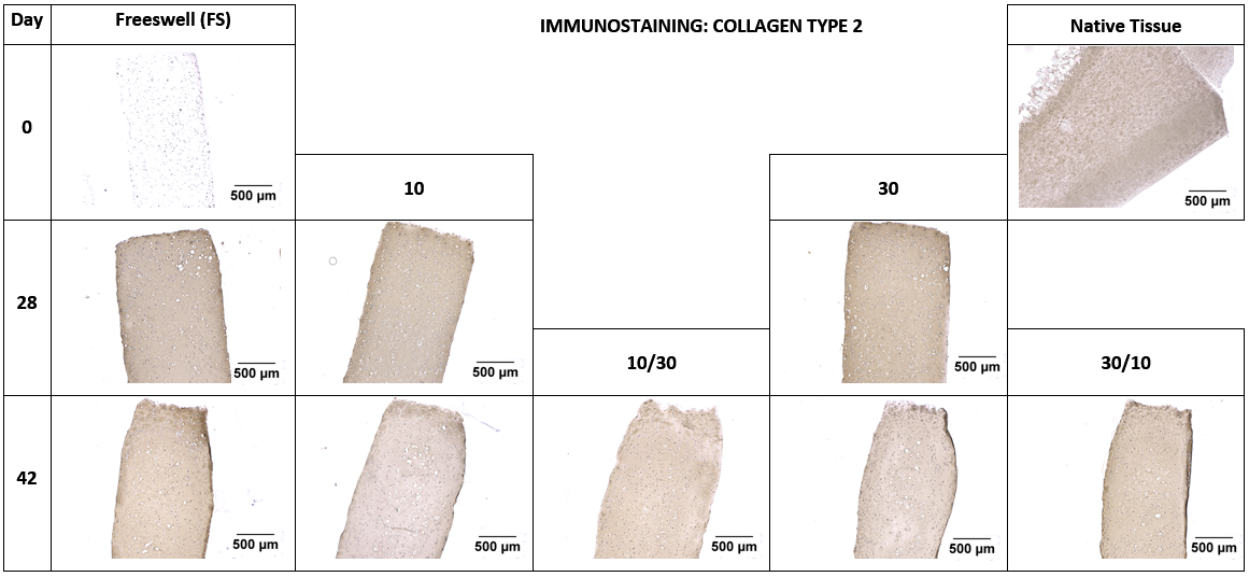


Figure 15 Study 3 IHC for Collagen Type 2 at 4X.

Study 4

Group 10% had significantly higher DNA/ sample than freeswell and 30% group during week 4. However, there was no statistically significant difference between any of the groups during week 6 and there was significant decrease in DNA in all groups from week 4 to week 6, see Figure 16.

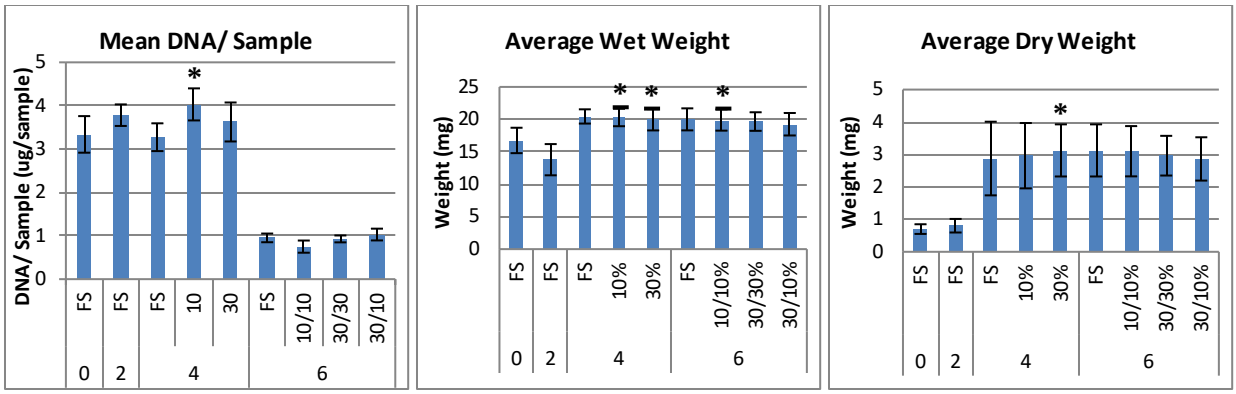


Figure 16 DNA and sample weight summary for study 4. Data presented as mean ± Std. dev,* = $p \leq 0.05$, compared to FS of that week.

Group 10% had a significantly higher GAG/ sample content than any of the groups during week 4 and group 30/30 had a significantly higher GAG/ sample amount during week 6. There was also a statistically significant increase in GAG/DW from week 4 to week 6. However, there was a significant decrease in GAG/sample in all groups after 4 weeks of culture and a decrease in all loaded groups after 6 weeks. The GAG content in all the groups was also 10-fold lower than study 3 where the sample consisted of just chondrocytes.

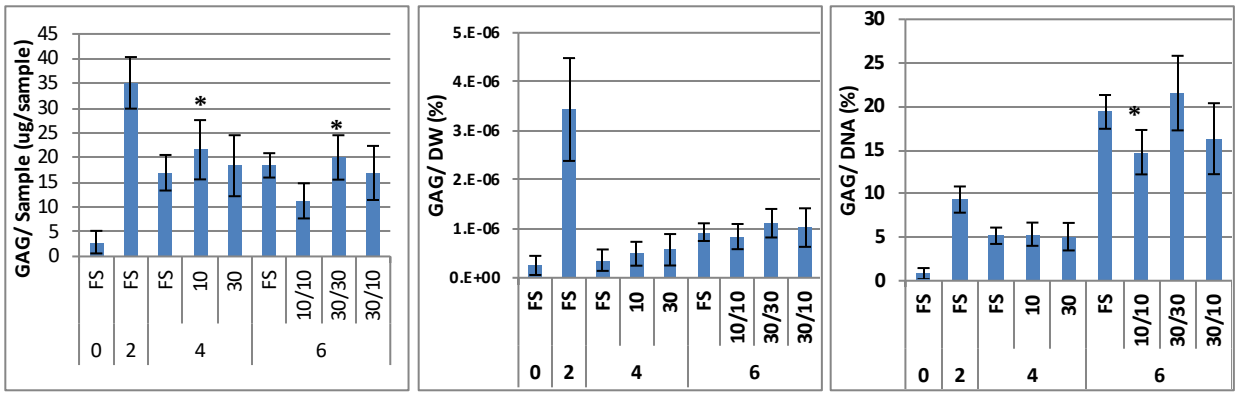


Figure 17 GAG measurements for study 4. GAG is analyzed per sample, wet weight, and DNA. Data presented as mean ± Std. dev,* = $p \leq 0.05$, compared to FS of that week.

The collagen/sample and collagen/ DW in all groups increased significantly from week 2 to 4. Group 10/10 also had a significantly higher GAG/sample and GAG/ DNA than both groups in week 4 and freeswell in week 6. However, all groups had significantly lower GAG/ DW from week 4 to week 6.

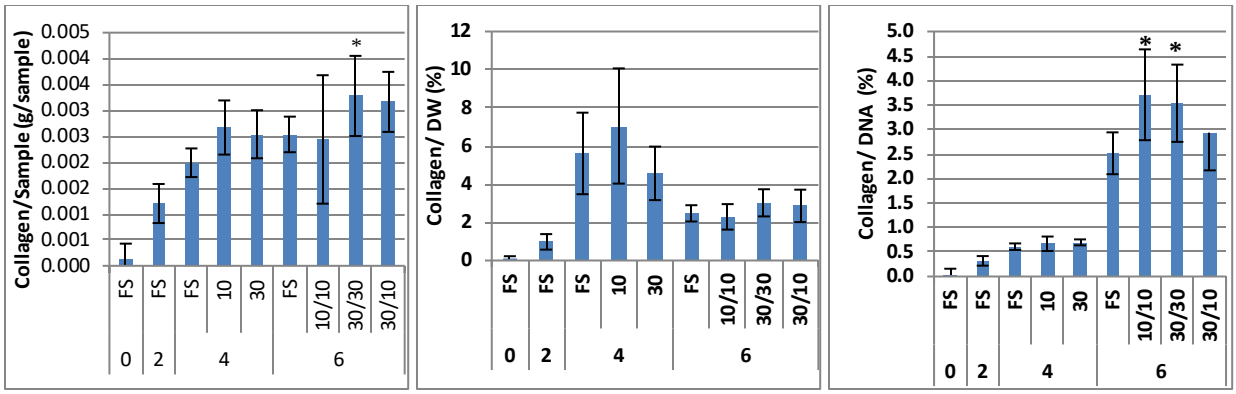


Figure 18 Collagen measurements for study 4. Collagen was analyzed per sample, wet weight, and DNA. Data presented as mean ± Std. dev,* = $p \leq 0.05$, compared to FS of that week.

Samples were not able to obtain equilibrium modulus in the range of native tissue (60 to 250kPa). There were also no statistically significant differences between any of the groups during any time point. The data necessary to calculate the dynamic modulus was collected and therefore was not analyzed for this study.

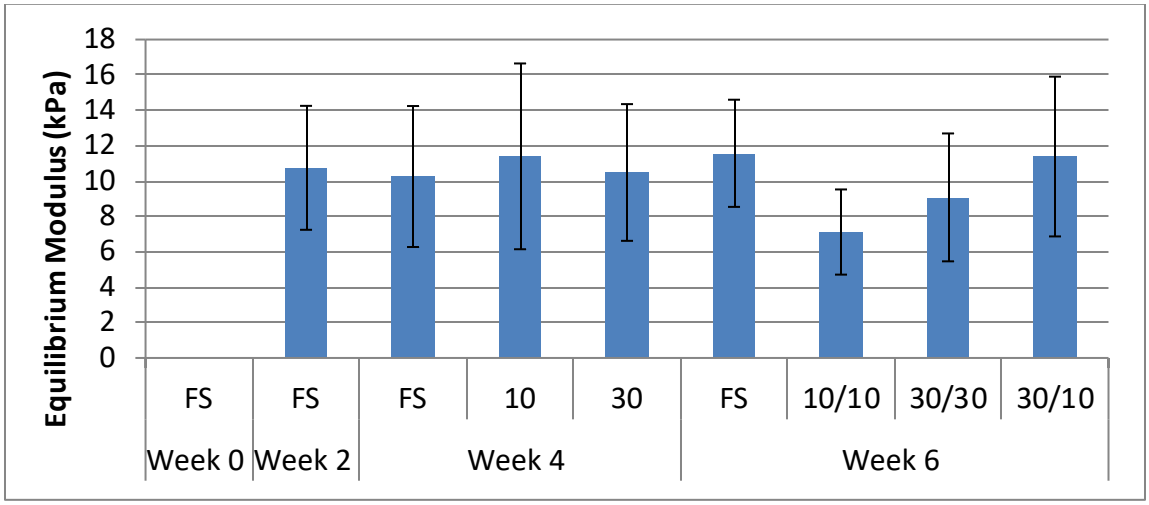


Figure 19 Equilibrium Modulus measurements for study 4. Data presented as mean ± Std. dev,* = $p \leq 0.05$, compared to FS of that week.

The images in Figure 20 show the safranin-O staining for all groups. The samples show no red indicating that GAG production was very limited. This stain reflects the small GAG amount measured in the DMMB assay.

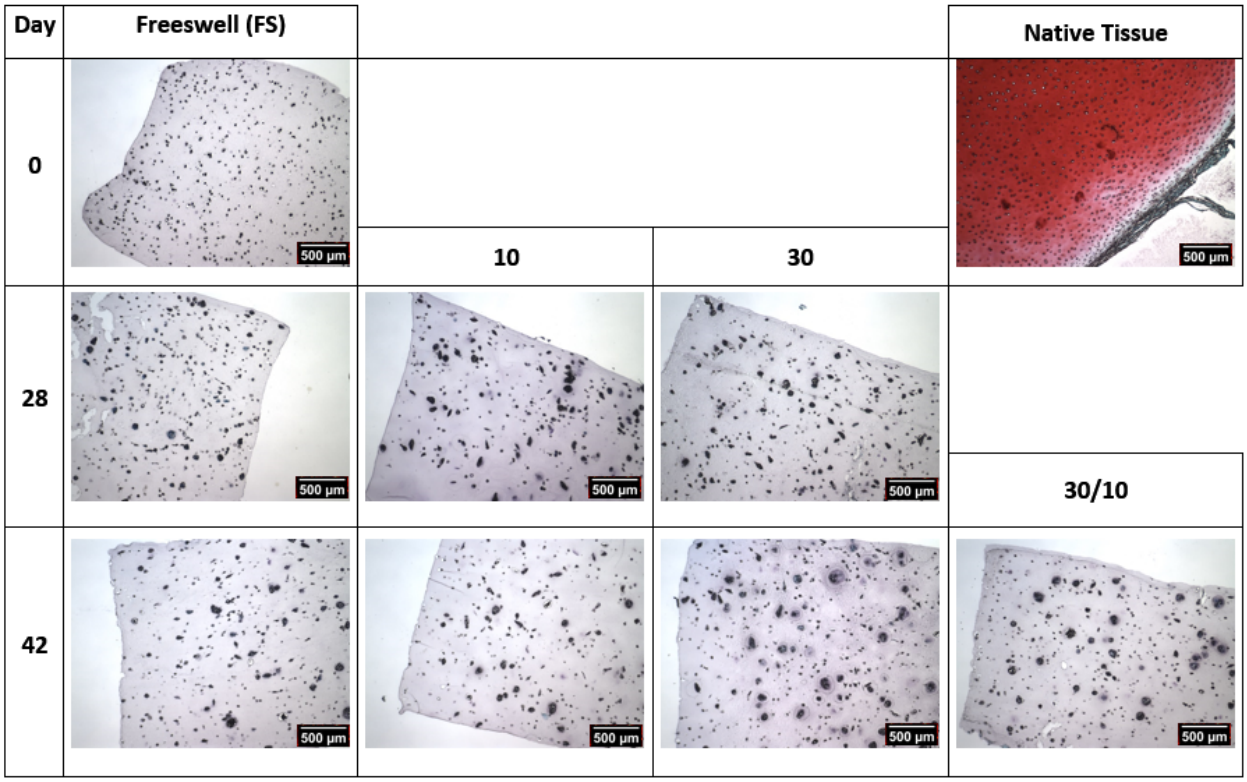


Figure 20 Study 4 safranin-O/Fast Green Staining for Glycosaminoglycan at 10x (scale bar = 500µm).

Polarized light images show the collagen orientation in Figure 21. Native tissue section shows that collagen fibers are aligned in parallel to the cartilage surface. Each sample exhibited light staining for collagen. It seems that the collagen fibers are starting to align along the edge after day 42 (week 6).

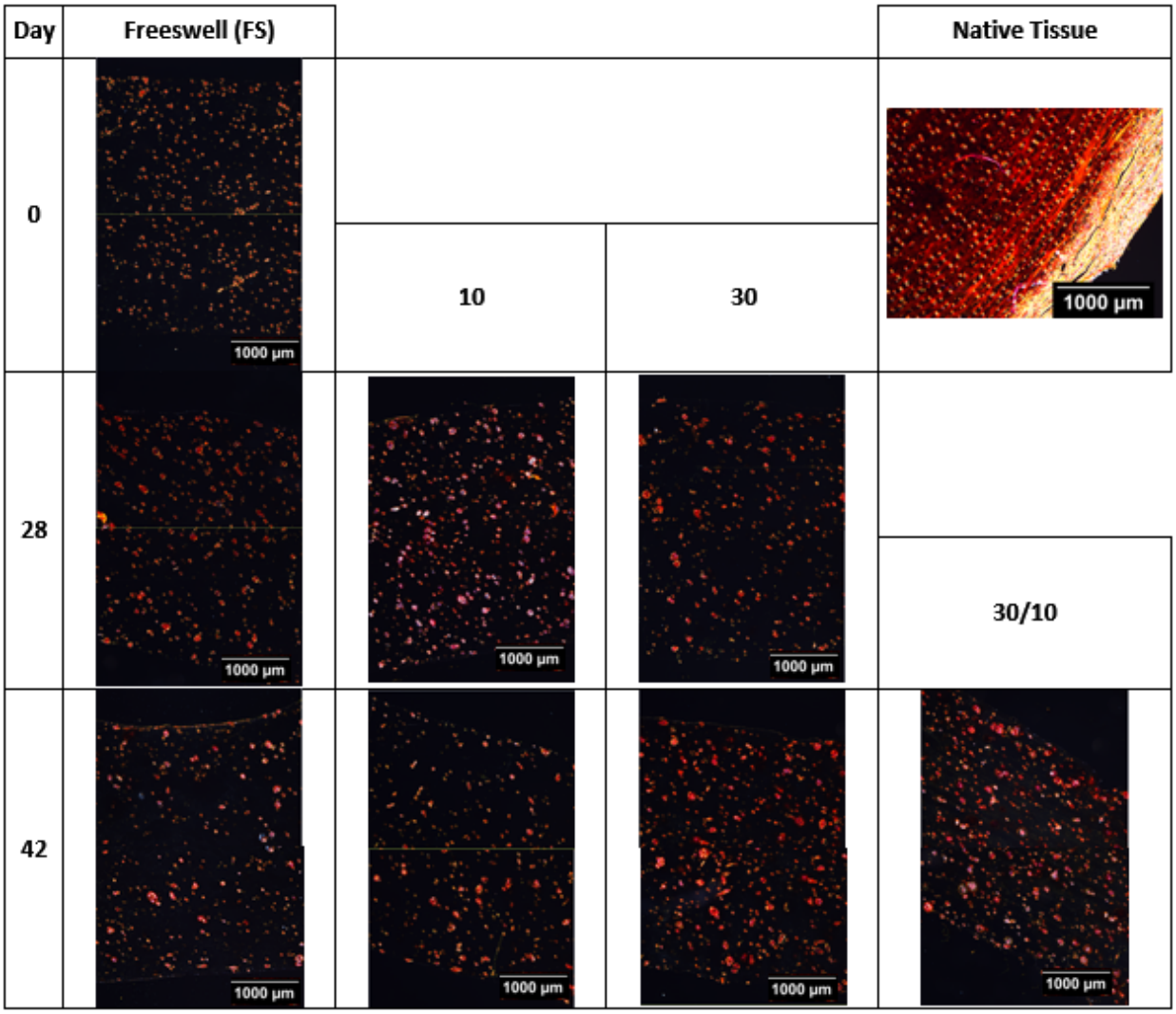


Figure 21 Study 4 Picrosirius Red Staining for Collagen at 10X (scale bar = 1000µm).

DISCUSSION: STUDY 3 AND 4

In study 3, dynamic loading significantly enhanced GAG production by chondrocytes in the first 28 days of culture. The samples were able to reach equilibrium modulus values consistent with native cartilage (60-250kPa) in the first 28 days. After 28 days, there was a decrease in GAG, collagen, and equilibrium modulus in all the groups. DNA production increased after 28 days indicating cell death did not contribute to the decrease in GAG, collagen, and mechanical

properties. The decrease in these properties could indicate dedifferentiation of the cells to more fibroblastic cell types. The decrease staining in safranin-O for week 6 also indicates dedifferentiation could have begun in the cells at the periphery of the samples. The staining for total collagen seen in the picrosirius staining did not visually decrease in samples during week six also suggesting that cells might have been producing collagen that is not chondrogenic in nature. The IHC staining confirms that the production of the more chondrogenic type 2 collagen decreased after week 4 whereas the production of collagen type 1 slightly increased.

A similar study performed by Mauck *et al.*(2002), also showed a decrease in GAG and mechanical strength in chondrocyte seeded agarose samples after 28 days of culture. They hypothesized that the decrease in these properties could be due to rapid matrix elaboration and thus the samples are subjected to limited nutrient supply (Mauck et al., 2002). The decrease in GAG at the edges of the samples could also indicate a loss of GAG into the media. Chondrocytes and MSCs have shown to release GAG into the media and mechanically loading chondrocytes has shown to increase that GAG release into the media (Babalola & Bonassar. 2010; Sheehy, Buckley, & Kelly, 2011). Although the study results failed to prove our hypothesis that early high compressive loading would enhance GAG deposition, the data suggests that subjecting the samples with a strain of 30% and then 10% could lead to higher DNA and collagen deposition than freeswell. Future studies should investigate the amount of GAG lost in the media during cell culture and possible prevention of dedifferentiation in the chondrocytes.

Study 4 did not reflect the results shown in study 3. The only difference between these two experiments was that study three only had chondrocytes and study four had a co-culture of chondrocytes and SDSCs. The samples were not able to replicate the biochemical, mechanical, or histological properties exhibited by the samples containing chondrocytes alone. The results of

study reflected our previous study that used SDSCs only (Kahn & Bilgen, 2016). Both studies showed a decrease in DNA, GAG, and collagen over time. As described in the study performed by Kahn and Bilgen (2016), the low GAG could be due to the ability of the SDSCs to differentiate into chondrocytes. The SDSCs might have needed more culture time and TGF-β1to increase chondrogenic properties. The current study included a co-culture of chondrocytes and SDSCs because previous studies have shown that SDSC cultures with the presence of chondrocytes significantly improved and controlled chondrogenesis (Varshney et al, 2010). However, the improvement of the co-culture could have been due to the production of biochemical properties by the chondrocytes alone. Giovannini et al (2010) conducted an experiment that included pellets with human articular chondrocytes and human bone marrow mesenchymal stem cells (hbMSC). They concluded that the presence of HAC in the samples only influenced early signs of neochondrogenesis on the MSC and that the proteoglycans produced where created by the HAC only (Giovannini et al., 2010). Kelly et al. (2010) found that allowing mesenchymal stem cells at least 3 weeks of freeswell with TGF-β1 increased their response to mechanical loading. Future studies could investigate a method of differentiating SDSC into chondrocytes before any mechanical loading is applied by allowing more time for freeswell and prolonging the use of TGF-β1.

CONCLUSION

The purpose of the studies presented in this chapter was to improve the properties of tissue engineered cartilage. Dynamic loading significantly enhanced GAG production by chondrocytes in the first 28 days of culture. The samples were able to reach equilibrium modulus values consistent with native cartilage (60-250kPa) in the first 28 days. After 28 days, there was a decrease in GAG, collagen, and equilibrium modulus in all the groups. However, DNA

production increased after 28 days indicating cell death did not contribute to the decrease in GAG, collagen, and mechanical properties. The results could indicate a decrease in chondrogenic characteristics or loss of GAG into the media. Although the studies presented herein were not able to consistently improve GAG production, data suggest that subjecting the samples with a strain of 30% and then 10% could lead to higher DNA and collagen deposition than freeswell.

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