THE EFFECT OF DOXYCYCLINE AND COLLAGENASE A ON THE DIFFERENTIATION OF MESENCHYMAL STEM CELLS TOWARDS A NUCLEUS PULPOSUS PHENOTYPE

By

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Abstract

Intervertebral disc (IVD) degeneration costs the healthcare system billions of dollars annually and leads to reduced quality of life. Current treatments are invasive and primarily focus on symptom relief rather than repair. This study aimed to facilitate the development of an injectable therapy using chondrogenically differentiated mesenchymal stem cells (MSCs) in the absence of collagen II deposition. Briefly, pelleted MSCs were cultivated in chondrogenic medium and were supplemented with collagenase A or doxycycline in order to inhibit collagen assembly. Results indicated that collagenase A and doxycycline treatment had no negative effects on DNA or proteoglycan content. Collagenase A at all concentrations affected collagen content, as did doxycycline at low concentrations. Furthermore, preliminary gene expression studies for nucleus pulposus markers showed that collagenase A and doxycycline may have some effect on terminal differentiation of MSCs in chondrogenic medium. Overall, the findings suggest that collagenase A and doxycycline supplementation can be used to inhibit collagen formation, thereby facilitating the further development of an injectable therapy for IVD repair.

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I am especially grateful to my parents and brothers for constantly encouraging me to recognize my potential. I recently read somewhere that “nobody queues for a flat roller coaster” and I am thankful that my mum and dad are the type of adventurous people that always encourage me to go for the ride.

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List of Abbreviations

NP	nucleus pulposus
MSC	mesenchymal stem cells
ECM	extracellular matrix
IVD	intervertebral disc
IVDD	intervertebral disc degeneration
PG	proteoglycan
GAG	glycosaminoglycan
AF	annulus fibrosus
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
cDNA	complementary DNA
TGF	transforming growth factor
BMP	bone morphogenic protein
FGF	fibroblast growth factor
DMEM	Dulbecco's Modified Eagle Media
HIF	hypoxia-inducible factor
MMP	matrix metalloproteinase
FDA	Food and Drug Administration
LG	low glucose
HG	high glucose
EDTA	Ethylenediaminetetraacetic acid
PCR	Polymerase Chain Reaction
BSA	bovine serum albumin
PBS	phosphate buffered saline
bp	base pairs
SEM	standard error of the mean
DMSO	Dimethyl sulfoxide
OH	hydroxyproline

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ACAN	Aggrecan
COL2A1	Collagen, type II, alpha 1
SOX-9	SRY-box 9
COL1A2	Collagen, type I, alpha 2
RUNX2	Runt Related Transcription Factor 2

1.0 Introduction

1.1 Clinical Need for Engineered Intervertebral Disc Nucleus Pulposus

One of the leading causes of back pain is intervertebral disc degeneration (IVDD) which is believed to originate in the nucleus pulposus region of the disc [1, 2]. The medical problems associated with intervertebral disc degeneration are estimated to cost the United States' healthcare system over $90 billion annually [3, 4]. Aside from being expensive to treat, back pain caused by disc degeneration often results in mobility issues and a poor quality of life [3, 5, 6]. Current interventions to deal with those issues related to back pain and intervertebral disc degeneration include surgery, rehabilitation, or lifestyle modification [3, 7]. The typical surgery to treat back pain involves a discectomy, where the intervertebral disc (IVD) is removed, followed by spinal fusion where the vertebrae adjacent to the removed discs are fused together. Although this technique helps to reduce back pain, the procedure leads to areas of immobility as the now excised disc once acted as the "joints" for the spine [8, 9]. After removing the damaged disc, adjacent IVDs experience increased mechanical loading which leads to further degeneration in the previously healthy discs [4, 10]. Prosthetic implants to replace degenerated discs also exist as a potential therapy and are beneficial due to their ability to retain spinal mobility that is lost in vertebral fusion procedures [11-13]. However, prostheses are an expensive therapy and have been shown to migrate from the implantation site [14], lead to ossification of surrounding tissues [12], or to only be recommended in cases where degeneration is not considered to be in an advanced state [13]. Ultimately, current treatments for intervertebral disc degeneration are designed to simply manage pain rather than treat the underlying causes of the disorder [15]. or are associated with unfavourable side effects requiring revision surgeries[14].

1.2 Strategies in Nucleus Pulposus Tissue Engineering

Tissue engineering provides a promising approach to solving the many issues associated with IVDD, specifically of the nucleus pulposus (NP). By providing a means to actually repairing or regenerating damaged nucleus pulposus tissue, tissue engineering allows for degenerated discs to heal rather than continue to degrade and weaken the spine. Engineering methods can restore the function of nucleus pulposus cells to produce extracellular matrix proteins, such as collagen II and proteoglycans, necessary for healthy intervertebral tissue to support mechanical loading and self-repair. Currently, tissue engineering strategies include the use of scaffolds to mimic three-dimensional environments necessary for the cell-to-cell interactions that help produce a nucleus pulposus phenotype. Injectable therapies composed of cells, synthetic extracellular matrices, or a combination of both have also been studied. In addition, research has focused on replenishing unhealthy disc cell populations with *in vitro*-grown viable chondrocytes or stem cells. Through these methods, an *in vivo* environment can be created in which matrix production is not exceeded by matrix degradation [8]. Each approach, however, has its drawbacks. Scaffolds, both injectable and non-injectable, often do not sufficiently meet the biomechanical needs for the NP to endure forces placed on the spine. Cell therapies have failed to produce populations which accordingly match the phenotype and therefore function of native tissues. By restoring the nucleus pulposus tissue using cells capable of replenishing associated extracellular matrix that matches healthy tissue, disc function can be re-established as opposed to simply mitigating back pain as current therapies do.

1.3 Research Objectives

The goal of this research was to review the effect that varying concentrations of two matrix degrading factors, doxycycline and collagenase A, had on stem cells undergoing differentiation

towards a NP-like phenotype capable of producing adequate extracellular matrix proteins without forming a solid tissue. The study aimed to differentiate bone marrow-derived mesenchymal stem cells (MSCs) to a phenotype suitable for a nucleus pulposus injectable therapy by culturing cells in chondrogenic medium. In order to facilitate injectability, collagen formation and assembly was inhibited or degraded in order to create a tissue lacking structural stability and stiffness. . To do so, cultures were supplemented with varying concentrations of doxycycline monohydrate and collagenase A – two agents which inhibit collagen formation and degrade collagen fibers, respectively. By chondrogenic induction, the stem cells would be capable of producing collagen and proteoglycans; however, with a reduced collagen content the resulting culture would have its own scaffold consisting primarily of proteoglycans making it suitable for the future development of an injectable intervertebral disc therapy.

2.0 Literature Review

2.1 Nucleus Pulposus Biology

2.1.1 Intervertebral Disc Structure & Function

Intervertebral disc degeneration affects approximately 97% of adults over the age of 50 [4, 16]. Disc degeneration starts after age 10 and is thought to begin in the nucleus pulposus (NP) region of the disc [3, 15] . The nucleus pulposus is contained in the centre of the intervertebral disc; it is a viscous gelatinous structure surrounded by the annulus fibrosus (AF) which consists of fibroblast-like cells [8, 17]. The annulus fibrosus and nucleus pulposus are "sandwiched" between two cartilaginous end plates [8, 18]. Nucleus pulposus cells secrete an extra cellular matrix rich in collagen type II and proteoglycans such as aggrecan [19, 20]. The proteoglycan to collagen II ratio in the NP is approximately 27:1, which distinguishes it from articular cartilage where this ratio is only 2:1 [20, 21]. The high proteoglycan content of the nucleus pulposus plays a vital role in the mechanical function of the IVD. The proteoglycans carry a negative charge and thus are able to attract water. This water retention causes the disc to swell and gain height [19], allowingfor the intervertebral disc to support the mechanical load placed on the spine [22].

2.1.2 Intervertebral Disc Degeneration

The nucleus pulposus is an avascular region and therefore lacks in sufficient nutrient supply. The environment inside the disc is in a hypoxic state and changes drastically in the first few years after birth [3, 5, 8]. In early human life, the nucleus pulposus is rich in notochordal cells originating from the embryonic notochord [3, 5, 23]. These cells are very metabolically active and are believed to contribute to the maintenance of the nucleus pulposus [23-25]. Over time, as the population of notochordal cells decreases, the nucleus pulposus gains chondrocyte-like cells

surrounded by large amounts of extracellular matrix (ECM) [5, 25, 26]. The nucleus pulposus receives its nutrients primarily through diffusion from the cartilaginous end plates [27]. Although the end plates of the disc are vascularized in early human life, over time, devascularisation occurs and the inside of the disc becomes deprived of nutrients [8, 28]. The causes of disc degeneration vary and are related to aging, devascularisation of the tissue, genetics, the waning population of cells, as well as trauma, mechanical stress from daily activities, and various diseases [1, 8, 27]. Changes occur in the structural and material properties of the nucleus pulposus as it degenerates and so the disc is unable to mechanically function as it should [29]. During degeneration the cells lose viability and are unable to correctly synthesize extracellular matrix. As the rate of matrix degradation begins to exceed the rate of matrix synthesis, the resulting decrease in proteoglycans leads to a loss in water content [9, 15, 19]. This decrease in water content ultimately leads to a loss in disc height [30], as can be seen in Figure 2.1. The water-rich nucleus pulposus is replaced by a more fibrous structure, which is unable to efficiently bear the mechanical loads placed on the spine [8]. The water content of the nucleus pulposus changes from approximately 90% by weight to under 70% after degeneration begins [29, 31]. The nucleus pulposus exhibits fluid-like behaviour before degeneration and a solid-like behaviour with increased degeneration, making the disc less flexible, thus hindering mobility and causing pain[29].

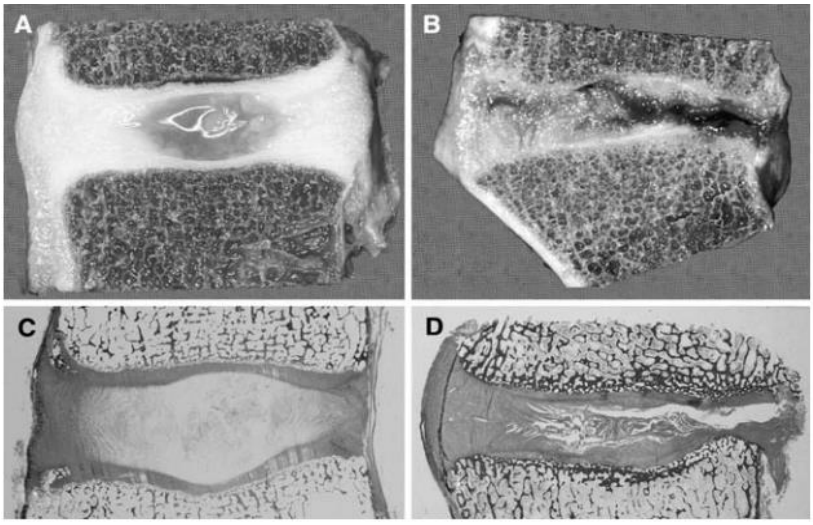


Figure 2.1 Degenerative changes of the IVD. A & C = young and healthy discs, B & D – severely degenerated. [28]

2.2 Nucleus Pulposus Tissue Engineering

2.2.1 Scaffolds

Scaffolds are a useful and necessary tool that can facilitate nucleus pulposus regeneration from a tissue engineering perspective in multiple ways. Scaffolds can be used to provide mechanical strength to cell constructs needed to absorb compressive loads placed on the spine when implanted *in vivo* [32]. They also provide a three dimensional structure that can enhance cell-to-cell and cell-to-matrix interactions, thus aiding in cell proliferation and tissue growth. Furthermore, scaffolds can provide signals necessary to induce differentiation towards specific cell phenotypes or to improve matrix synthesis [8]. Nucleus pulposus cells communicate via gap junctions – due to this arrangement it has been shown that they best maintain their phenotype in 3-D culture [33].

When selecting a scaffold for nucleus pulposus tissue engineering, aside from ensuring biocompatibility of the material, mechanical strength sufficient to support the loads placed on the spine is the most sought after property. Biodegradability is also important to ensure that as the cells begin to proliferate and synthesize extracellular matrix, the scaffold should breakdown and make room for tissue growth [8]. While some research has focused on biphasic scaffolds capable of growing constructs representative of the entire disc [32, 34], many others focus merely on NP repair. Scaffold materials that are already approved-for-medical use and support chondrogenesis have also received attention. Materials such as alginate and collagen are able to yield high accumulation of proteoglycans, however research showed that the viscoelastic properties of these

scaffolds were not sufficient for *in situ* studies[20]. A summary of different scaffolds used in tissue engineering of the nucleus pulposus along with the cells that were seeded into them are listed in Table 2.1.

Table 2.1 Summary of Tissue Engineering of Nucleus Pulposus Research [8]

Collagen type I/Human IVD cells	Cyclic strain and hydrostatic pressure increased expression of collagen II and aggrecan expression collagen I.
Collagen type I and hyaluronan/ Bovine NP and AF cells	NP and AF cells produced PG and collagens.
Atelocollagen type / Bovine NP and AF cells	Glycosaminoglycan synthesis increased by NP cells
Atelocollagen gel/ Mesenchymal Stem Cells	Permitted proliferation, matrix synthesis and differentiation.
Atelocollagen (collagen gel)/ Human NP cells	Cell proliferated and produced proteoglycan.
Alginate hydrogel/ Rat MSC	Hypoxia and TGF-β initiated MSC differentiation into NP-like cells.
Alginate/ Human bone marrow mesenchymal stromal cells (MSC)	Alginate and pellet culture produced chondrogenic -characteristics cells.
Alginate gel/ NP and AF cells	IVD tissue may be engineered in vitro using different cell sources.
Alginate gel/Rabbit IVD cells	Increased synthesis extracellular matrix components.
Alginate/Human IVD cells	Suitable environment for growth of NP and AF cells.
Alginate/Canine IVD cells	Inhibited PG synthesis, and stimulated synthesis of collagen in NP.
Alginate/Rabbit intervertebral disc cells	High amplitude and frequency increased protein synthesis and lowered protein degradation.
Alginate beads/Human NP cells cocultured with MSC cells	MSCs are capable of differentiating into disc cells in vitro.
Agarose/Porcine NP cells	Intermittent hydrostatic pressure enhanced synthesis of ECM.
Chitosan/Adult bovine IVD cells	Feasible to generate a NP with appropriate biochemical and mechanical Properties.
Chitosan gel/Adult bovine IVD cells	Suitable for encapsulation of NP cells and restored function of NP.
Fibrin beads/NP cells	Promoted cell proliferation, differentiation and tissue maturation.
Fibrin/hyaluronic acid (HA)/Porcine NP cells	PG content synthesis was significantly higher in matrix.
Gelatin Demineralised bone (DBM)/ Adult porcine nucleus pulposus cells	Cells were metabolically active and expressed types I and II collagen and aggrecan. Potential to be used as composite injectable scaffold.
Gelatin/Chondroitin-6-sulphate/hyaluranon tri-copolymer/Human NP cells	Increased synthesis of GAG and Collagen type II.
Hyaluranon gel/Adult rat MSC	Hyaluronan gel is not an ideal carrier.
PGA in PLLA) for AF Alginate for NP/NP and AF cells from sheep	Morphology and histology strongly resembled those of native IVD.
Calcium phosphate/Bovine IVD cells	PG content and mechanical properties were similar to native NP.
Bioactive glass/NP cells from adult rabbit	Excellent substrate for cell attachment, proliferation and matrix deposition.
PLLA/Human Mesenchymal stem cells	Human MSC differentiated into NP like cells.
PDLA/Adult porcine nucleus pulposus cells	Nucleus pulpous cells did not attach to polylactide.

Implantation of a solid scaffold construct into the spine would require invasive surgery and undoubtedly damage the annulus fibrosus in the process. More importantly, the nucleus pulposus is gel-like, and matching the mechanical function of the tissue would be better achieved with biomimetic materials capable of water uptake [2]. As a result, researchers have focused on developing injectable materials which more closely resemble native NP tissue and are capable of being injected due to their gelatinous consistency. Injectable scaffolds such as hydrogels have been suggested to be more suitable for nucleus pulposus regeneration due to their hydrophilicity, which allows them to retain water [31]. Injectable hydrogel scaffolds can immediately help to restore disc height with minimal damage to adjacent tissues [2, 8, 27, 35, 36]. Injectables can also be used to carry growth factors which can aid in cell proliferation, gene expression, and protein synthesis [28, 31, 35, 37]. Like other tissue-engineered scaffolds, injectable gels should be

biodegradable, biocompatible, and possess mechanical properties suitable for their application inside the IVD [10]. A study by Wan et al. [2] indicated that the mechanical properties of their self-assembling peptide hydrogel resembled those values of human nucleus pulposus measured in earlier studies [38]. It was found that a dynamic modulus of 7 to 20 kPa and a stiffness of 5.39kPa is similar to native NP tissue values [2, 38], and is indicative of values that should be sought after when creating an injectable NP therapy. Research on injectables is centered on three topics including: (i) materials which mimic properties of extracellular matrix, (ii) cells capable of replenishing disc ECM, (iii) and a combination of the two approaches.

Combination matrix-cell hydrogels have yielded promising results. Sakai et al. [39] injected bone marrow derived mesenchymal stem cell in an atelocollagen gel into degeneration-induced discs of rabbits. The discs were able to regain height to 91% of the control disc height by week 26, and gene expression levels showed upregulation of aggrecan. Histological imaging also indicated that discs injected with MSCs in atelocollagen resembled normal nucleus pulposus tissue controls, however, type II collagen gene expression levels were not different than the sham or normal control groups [39]. Similarly, Wan et al. [2] created a self-assembling peptide hydrogel seeded with bovine NP cells, and found that the relevant gene expression levels increased over the duration of the culture period. Sulfated GAG content – which is a measure of proteoglycan deposition – significantly increased over time, and when subjected to shear stress, the gels flowed and then re-stiffened, indicating that it is a good model for injectability [2].

Regenerative therapies in which cells alone are injected into the spinal discs have also been considered. Vadala et al. [40] injected bone marrow derived mesenchymal stem cells into the intervertebral discs of rabbits and found that the cells completely migrated from the nucleus pulposus to the disc perimeter and instead formed osteophytes [40]. Similarly, allogenic MSCs

injected into the lumbar discs of rabbits showed morphological characteristics similar to native NP cells up to 12 weeks *in vivo*, but by week 24, histology indicated the cells migrated and took on the chatacteristics of annulus fibrosus cells [41]. The studies are representative of common limitations of injecting cells alone into the IVD for NP repair including leakage of cells through the injection site, and the differentiation of cells towards undesired lineages [40, 41].

Injectable hydrogels devoid of cells are often designed to replicate the proteoglycan-rich matrix of the nucleus pulposus as the GAG content of the ECM contributes heavily to maintaining tissue hydration and biomechanical function [42]. Sivan et al. [42] created GAG analogues by crosslinking sodium 2-acrylamido, 2-methylpropane sulfonic acid with 3-sulfopropyl acrylate. They found that the osmotic and rheological properties of the analogue closely resembled that of native tissue, however the study has not yet performed *in situ* tests [42]. Another study utilized hydrogels made of chitosan and hyaluronic acid due to the proteoglycan-like structure of chitosan and the gelation properties of hyaluronic acid. The material showed favourable results as it supported stem cell growth and allowed for the incorporation of signalling molecules which promote NP-like differentiation as well as chondrogenic growth factors. The authors showed that mimicking the *in vivo* tissue environment helps promote and sustain nucleus pulposus-like cells [30].

Similarly, Schmocker et al. [36] created a composite hydrogel from cellulose fibers and poly(ethylene glycol)dimethacrylate, a material which polymerizes upon being exposed to light. The hydrogels were tested using an *ex vivo* bovine model where the hydrogel was injected and illuminated using a custom-designed probe. The hydrogel restored disc height to 91.1% of the healthy disc height over half a million loading cycles. Although compression testing revealed good mechanical performance of the hydrogel, the engineered material extruded from the hole at the

injection site at much lower pressures than native NP tissue [36]. In their study, Nair et al. [43] used a composite hydrogel made of chitosan and polyhydroxybutyrate-co-valerate (PHBV) with chondroitin sulfate nanoparticles. The material binds easily with growth factors and retains large amounts of water, making it an ideal material to be used for NP regeneration *in vivo*. The study found that unseeded hydrogels showed water uptake values similar to human NP tissue and suitable mechanical and viscoelastic properties for the application. The hydrogels were able to support phenotypic changes of adipose derived mesenchymal stem cells to chondrogenic-like cells capable of proteoglycan deposition, however it is unknown how the hydrogel would perform upon injection into the NP [43].

A study aimed to observe the differences between injecting hyaluronan gel with and without bone marrow derived mesenchymal stem cells into the IVDs of rats. The cell population decreased at first, but regained viability by day 28, indicating their ability to survive in the NP. Disc height followed a similar trend, where it decreased from day 0 to day 14 and then regained height by day 28 in those discs injected with the combination gel-cell mixture. The authors note this phenomenon shows that injectable gels with cells outperform those void of cells as the synthesis of extracellular matrix by the stem cells helped contribute to disc height [44].

Injectables are advantageous from a tissue engineering perspective due to their similarities to properties of native extracellular matrix, however, issues with NP injectables remain. Primarily, their inability to match all mechanical properties of native tissue has resulted in a failure to maintain disc height and support the load placed on the spine [36, 42]. Injectable materials also have a tendency to degrade before sufficient amount of ECM can be synthesized by the embedded cells [45]. Commonly, injectables for nucleus pulposus regeneration have shown a tendency for matrix extrusion or cell leakage through the injection site, and many studies require animal

sacrifice early on to access results, thereby posing issues in assessing long-term effects of the therapy [46]. Mechanical properties of hydrogels may be improved through crosslinking of polymeric materials [35, 43]; however, certain reagents used in crosslinking may result in the product becoming cytotoxic [43].

2.2.2 Cell Sourcing

The ideal cell source for most tissue engineering applications is autologous cells as transplanting cells from one's own body eliminates the possibility of a negative immune response [47]. In nucleus pulposus engineering however, there are many challenges associated with using the patient's existing disc cells, leading researchers to explore other cell sources. Among the problems with harvesting NP cells for expansion is that the tissue has a low cell density (~ 4000 cells per mm^3), accounting for only 1% of the disc tissue by volume [22, 35]. Coupled with the low cell availability is the fact that a patient requiring an engineered NP implant likely has degenerated disc cells with low viability [21, 26, 28, 47]. Over time, cells in degenerated discs express matrix degrading enzymes and lose their abilities to proliferate, synthesize proteoglycan-rich matrix, and handle mechanical loads [1, 26, 33, 35, 48]. Often, explanted nucleus pulposus contains fragments of the cartilaginous endplates and annulus fibrosus, making it difficult to isolate NP cells from other IVD cell types [26]. The choice of cells for nucleus pulposus tissue engineering should therefore produce extra cellular matrix proteins in suitable ratios to restore mechanical function of the degenerated disc and have high proliferation capacity to grow into large numbers [48].

The use of stem cells is an increasingly popular method in tissue engineering of the nucleus pulposus. Stem cells are self-renewing, can differentiate down multiple lineages, and are easy to expand for tissue culture purposes [49]. Researchers have used different types of stem cells

including those derived from bone marrow, adipose tissue, synovium, and human umbilical cord [1]. One study compared nucleus pulposus derived stem cells to regular nucleus pulposus cells isolated from the same moderately degenerated discs of humans, and found that the stem cell group was able to yield more favourable results than the degenerated NP cells. By injecting cells into degenerated lumbar discs of New Zealand white rabbits, stem cells showed upregulated mRNA expression and increased content of aggrecan and collagen II. Disc height also increased when compared to regular NP cell groups, signifying that stem cells may be a more viable option than using regular nucleus pulposus cells to regenerate disc tissue. The authors of the study noted that human disc cells were implanted into the intervertebral disc of rabbits and there was no immune response, likely due to the avascular nature of the nucleus pulposus, and therefore xenogeneic transplants could be considered for NP tissue engineering [1].

The notion that stem cells better repair degenerated disc tissue than nucleus pulposus cells is further supported in other studies. A study by Song et al., [15] co-cultured human adipose-derived stem cells with human degenerated nucleus pulposus cells, while the control group included only nucleus pulposus cells. Researchers found that after 1 week of culture, the co-cultured group exhibited significantly greater cell yields and proteoglycan content when compared to the control group. After 21 days of culture, gene expression of NP markers type II collagen, aggrecan, and the master chondrogenic transcription factor *SOX-9* was significantly higher in the co-culture groups versus the control groups. The adipose-derived stem cells also secreted chondrogenic growth factors during co-culture. Based on the experimenters' results, it is suggested that injecting adipose-derived stem cells into discs could also aid in nucleus pulposus repair [15].

In vivo studies have also been performed to determine whether mesenchymal stem cells can be directly injected into the intervertebral disc and still yield tissue repair. Cai et al. [50]

aimed to understand the effects that mesenchymal stem cells have on regeneration of nucleus pulposus by first inducing degeneration in New Zealand white rabbits by annulus fibrosus puncture, and implanting them with bone marrow derived MSCs. Discs implanted with MSCs showed an increase in collagen II and aggrecan gene expression after ten weeks when compared to controls. Magnetic resonance imaging indicated that discs containing MSCs lost height at a significantly slower rate than untreated degenerated discs [50]. Although this study did show promising results in using mesenchymal stem cells to repair degenerated discs, the decrease in disc height suggests the disc could not be completely repaired by stem cells and may not be able to support mechanical loads placed on the spine.

Mesenchymal stem cells (MSCs) are easily obtained from bone marrow or adipose tissues making them an ideal cell source for tissue engineering applications. Other types of stem cells that have been studied for nucleus pulposus regeneration such as embryonic stem cells are more difficult to collect [26]. MSCs can differentiate down several lineages, and like chondrocytes are capable of producing proteoglycans and collagen II [33]. Mesenchymal stem cells present an advantage over the use of cartilage cells as they are found in large quantities in several different tissues and expand easily in culture [20, 33, 51]. Furthermore, the chance of donor site morbidity where MSCs have been explanted from the body is low and MSCs have shown better survival than terminally differentiated cell types. Mesenchymal stem cells are safe for allogenic transplants between siblings as they lack surface antigens that would yield an immune response in other donor pairings [28, 33].

Research also indicates that differentiating stem cells *in vitro* prior to injection into the IVD improves safety and efficacy. Differentiating stem cells *in vitro* helps to lower the tumorigenicity of the cells, and allows the cells to begin to produce necessary ECM proteins [52,

53]. By pre-establishing extracellular matrix proteins, we see an increase in favourable mechanical properties of injectable scaffolds, and reduce the regeneration time of damaged tissue *in vivo* [52, 53]. Furthermore, differentiating stem cells prior to implantation helps them better handle the mechanical forces placed on the spine [54]. The environment inside the intervertebral disc is extremely harsh as there is little nutrient supply and the disc is constantly undergoing stress from the loads placed on the spine [20, 55]. Implanting cells immediately without first allowing them to gain viability *ex vivo* may lead to cell death [20, 28].

Wang et al. [55] aimed to prove that pre-differentiating stem cells contributes to improved cell survival in a nucleus pulposus-like environment. They found that pre-differentiating adipose derived mesenchymal stem cells in chondrogenic medium for 7 to 10 days led to increased cell density and aggrecan and collagen type II gene expression when transferred to a culture environment that mimics *in vivo* tissue conditions. Contrastingly, an undifferentiated control group showed decreased cell density [55], thereby underscoring the importance of differentiating stem cells *in vitro* prior to implantation into the disc, especially for cell survival. Similarly, Colombier et al. [56] showed that human adipose derived stromal cells cultured in specialized differentiation medium outperformed undifferentiated cells when transplanted *in vivo*. After cultivation in differentiation media for 3 weeks, cells were incorporated into a hydrogel and injected subcutaneously into mice. After six weeks, pre-differentiated cells synthesized high levels of collagen and aggrecan-rich matrix and retained their phenotype, while undifferentiated stem cells failed to do so [56].

In their study, Luo et al. [21] aimed to induce the differentiation of MSCs towards a nucleus pulposus-like phenotype by simulated microgravity. After harvesting MSCs from the bone marrow of New Zealand white rabbits, the cells were pelleted and maintained in a rotary cell culture

system. The results showed that those MSC pellets cultured in microgravity had increased cell proliferation, pellet size, and proteoglycan accumulation than control groups that were not simulated in microgravity. Furthermore, gene expression of aggrecan, collagen II, and SOX-9 –all of which are markers of nucleus pulposus cells – was greater in microgravity cultured groups. The authors discussed that culturing MSCs in 3-D environments with a high cell density, as opposed to a monolayer, can lead to differentiation of the stem cells towards a nucleus pulposus-like phenotype due to the culture configuration allowing for greater cell-to-cell interactions. This study yielded results supporting this theory, as the expression of nucleus pulposus markers along with the proteoglycan accumulation indicates differentiation towards a nucleus pulposus like phenotype [21]. The topic of differentiating stem cells is further discussed in section 2.2.3.

2.2.3 Signals and Growth Factors

Growth factors may help to induce the proliferation of nucleus pulposus cells and synthesis of extra cellular matrix rich in proteoglycans [57]. Injecting growth factors into the disc may appear to be a sufficient therapy for IVDD, however, unhealthy discs may not respond well to growth factors. If the cells within the disc have low viability due to their degenerated state, growth factors will have no influence on the ability of the cells to synthesize matrix proteins [8, 45, 57]. Therefore, it may be more useful to focus on growth factors as a route for differentiating mesenchymal stem cells towards a nucleus pulposus-like phenotype, or as a means to stimulate disc cells to proliferate and produce large quantities of extra cellular matrix proteins *in vitro* prior to implantation. A list of growth factors previously proven to be useful in intervertebral disc regeneration is shown in Table 2.2.

As previously mentioned, researchers look to cell sources with high proliferation capacity such as mesenchymal stem cells, which can be differentiated down the desired phenotypic lineage [58]. Mesenchymal stem cells can be induced to express a chondrocyte-like phenotype similar to nucleus pulposus cells with the use of several different growth factors including transforming growth factor-beta (TGF-β) and bone morphogenic proteins (BMP) [20, 33, 43]. Growth factors for nucleus pulposus tissue engineering purposes have been added to chondrogenic medium which includes transforming growth factor-β, ascorbic acid, and dexamethasone – three proven promoters of chondrogenic induction of stem cells and accumulation of matrix proteins like aggrecan and collagen [58-60].

Table 2.2 Growth Factors Applied in Nucleus Pulposus Tissue Engineering [57]

Growth factor	Species	Cell types/tissues	Effect
TGF-β1	Canine	IVD tissues	Increased matrix synthesis and cell proliferation
	E19 rat	AF cells	Pushing cells towards a fibrocartilaginous phenotype
	Rabbit	NP cells	Robust refurbishment of matrix
BMP-2	Human	NP and AF cells	Increased newly synthesised PG and upregulated expression of aggrecan, collagen type I and collagen type II mRNA
	Human	NP and AF cells	Increased matrix protein synthesis
	Rabbit	NP cells	Upregulation of aggrecan, collagen type I, and collagen type II mRNA expression
BMP-12	Human	NP and AF cells	Increased matrix protein synthesis
OP-1	Rabbit	NP and AF cells	Increase in the total DNA, PG and collagen contents
	Rabbit	NP and AF cells	Stimulating matrix repair
	Human	NP and AF cells	Prevention of a decreased cell number during culture, significantly upregulated PG synthesis and accumulation
GDF-5	Bovine	NP and AF cells	Increased PG synthesis and collagen synthesis
	GDF-5 (−/−) mice	NP and AF cells	Dose-dependent upregulation of the aggrecan and type II collagen genes
IGF-1	Mature canine	IVD tissues	Increased matrix synthesis and cell proliferation
	Bovine	NP cells	Increased PG synthesis
	Human	AF cells	Significant reduction in the percentage of apoptotic cells
	Bovine	NP and AF cells	Improved proliferation
	E19 rat	AF cells	Upregulation of ECM
PDGF	Human	AF cells	Significant reduction in the percentage of apoptotic cells
	Bovine	NP and AF cells	Improved proliferation
VEGF	Vegfr-1lacZ/+ and Vegfr-2lacZ/+ mouse	NP cells	Promotion of NP survival
bFGF	Mature canine	IVD tissues	Increased matrix synthesis and cell proliferation
	Bovine	NP and AF cells	Enhanced proliferation
EGF	Mature canine	IVD tissues	Enhanced proliferation
CTGF	Rhesus monkey	NP cells	Enhanced synthesis of PG and collagen II
PRP	Human	NP cells	Stimulation of NP proliferation and chondrogenic differentiation
	Porcine	NP and AF cells	Proliferation of porcine IVD cells
	Porcine	IVD organ	Upregulated levels of mRNAs involved in chondrogenesis and matrix accumulation
	Bovine	AF cells	Increased matrix production and cell number

The use of growth factors to induce differentiation towards a nucleus-pulposus like phenotype is demonstrated by Liu et al. [47]. In their study, induced pluripotent stem cells generated from mouse tail-tip fibroblasts were used to form pellets, and were cultured in medium

containing DMEM and TGF- β1. Pellets cultured with growth factors showed increased proteoglycan content. Moreover, gene expression studied using quantitative PCR showed that collagen II and aggrecan expression was much higher in those pellets supplemented with TGF- β1 than those cultured in the control medium [47]. Although the study did not reveal the ratio of the aggrecan to collagen II content, it did show that culturing stem cells with TGF-β1 can induce differentiation of the cells towards a nucleus pulposus-like phenotype.

Aside from chemical stimulus, the field of tissue engineering of nucleus pulposus may benefit from stimulating cells by placing them in physical environments which mimic *in vivo* conditions. It has been shown that placing MSCs in an environment with less oxygen causes them to differentiate along a nucleus pulposus-like lineage as they express hypoxia-inducing factors (HIF) [33]. *In vivo*, MSCs differentiate along desired lineages due to cues from the surrounding environment [61]. Feng et al. [61] cultured MSCs on a nanofibrous poly(l-lactide) scaffold in chondrogenic media supplemented with TGF-β1, and maintained the constructs in a hypoxia chamber. They were then subcutaneously implanted into mice for 8 weeks. It was found that those constructs cultured under hypoxic conditions retained their phenotype and deposited higher amounts of proteoglycans and collagen II than controls. Gene expression levels of SOX-9 and immunostaining of hypoxia inducing factor-1α, both specific gene markers of nucleus pulposus cells, were also higher in cells cultured in hypoxic conditions. Conclusively, Feng et al. showed that culturing mesenchymal stem cells in a micro environment similar to that of *in situ* NP supplemented with transforming growth factor can help the cells retain their phenotype [61].

2.2.4 Therapeutic Agents for Matrix Remodelling

One strategy for tissue engineering of nucleus pulposus involves combining strategic cell sourcing and signalling, while simultaneously using therapeutic agents to alter matrix composition.

Doxycycline and degenerative enzymes such as chondroitinase and collagenase have been shown to inhibit collagen fibril formation and deplete matrix proteins, respectively. Their use in the field of nucleus pulposus tissue engineering has not been studied, however, their mechanisms of action and therapeutic background is described in the following section.

2.2.4.1 Doxycycline

Doxycycline is part of a group of anti-microbial drugs known as tetracyclines used to treat gram-positive and negative bacterial infections [62]. Early studies indicated that doxycycline is not only an antibiotic, but is also an effective treatment for periodontal disease due to its ability to inhibit extracellular matrix-degrading enzymes known as matrix metalloproteinases (MMPs), which include collagenases, stromelysins, and gelatinases [62-65]. Although all tetracyclines inhibit MMPs to some degree, doxycycline was found to yield better inhibition of collagenase activity than minocycline and regular tetracycline [66, 67]. Doxycycline is currently used as a drug to treat periodontal diseases. It is administered in a low-dose at which its antimicrobial properties no longer exist, but inhibition of MMP-8, the collagenase which contributes most to the degradation of periodontal tissues, is still prevalent [66, 68, 69]. At this dosage, doxycycline does not result in antibacterial resistance, nor does it cause other problems commonly associated with prolonged antibacterial use such as altering candida levels in the gastrointestinal system [66-68]. Doxycycline is the only approved treatment in Canada, the United States, and Europe for the treatment of these gingival diseases due to its efficacy and safety, even for high-risk groups in which periodontal diseases are increasingly common such as diabetics and those suffering from cardiovascular disease [65, 66].

2.2.4.1.1 Doxycycline as an MMP Inhibitor

Studies emerged from the need to find a method to stop the breakdown of collagen I in periodontal tissues due to complications of diabetes, as well as a means to help osteoarthritis patients. Research has found that doxycycline works as an MMP inhibitor in osteoblasts, chondrocytes, tumor cells, and synovial tissue through non-competitive enzymatic inhibition [67, 70]. The method of action by which doxycycline inhibits MMPs is by chelating calcium and zinc ions, which are necessary for maintaining structure and function of these collagenases [64, 69, 71]. This theory was supported by Yu et al. [71] in 1992 when osteoarthritic dogs untreated with doxycycline showed severely worse cartilage degeneration in areas of the knee when compared to those treated with doxycycline, indicating that doxycycline treatment inhibited collagenases and gelatinases responsible for degradation in osteoarthritis patients [71].

2.2.4.1.2 Doxycycline as an Inhibitor of Collagen Synthesis

Some studies of doxycycline indicate that the drug may work in ways other than just inhibiting MMPs. Research indicates that collagen content is often lowered in chondrocytes treated with doxycycline. In a study by TeKoppele et al. [64], it was hypothesized that doxycycline inhibits collagen II synthesis in chondrocytes altered to resemble those found in osteoarthritis patients, which produce higher than normal amounts type II collagen. Bovine chondrocytes were cultured in alginate beads and treated with 0-25 µM of doxycycline. DNA, proteoglycan, and overall collagen content increased over time in all groups. However, at higher concentrations of doxycycline (10-25 µM), collagen synthesis was lower than in untreated groups. The hydroxyproline content in spent medium was also low, showing that collagen components were not released in high amounts from the cells, and therefore doxycycline likely suppresses collagen synthesis and does not degrade existing protein [64].

The findings of TeKoppele et al. are not unique and have been recorded in other studies. Beekman et al. [63] used bovine chondrocytes cultured in alginate beads to determine the effect doxycycline would have on collagen synthesis. Doxycycline was added to culture medium for up to 15 days, at concentrations ranging from 0 to 75 µM. It was found that there was lower collagen synthesis in cultures with doxycycline added versus those cultured without doxycycline, but in general, protein synthesis was only significantly lower in the 75 µM cultures by 20%. Hydroxyproline content of the cultures however was also low, and thus it was hypothesized by the authors as in the previously described study, that the mechanism by which doxycycline works is likely that it inhibits collagen synthesis as opposed to causing degradation. This was supported by the fact that the study also found that after removing doxycycline from the culture after day 11 or 15, the collagen content in doxycycline-dosed groups increased significantly when compared to the control group. Analysis of mRNA levels found that collagen II content in the 25 µM doxycycline group was 30% lower than control levels. The authors believe their study shows that doxycycline supplementation can delay phenotypic changes in osteoarthritis patients that cause an increase in collagen II formation [63].

A later study by Karna & Wolczynski [72] in 2001 revisited the ability of doxycycline to inhibit the synthesis of collagen fibrils. Human fibroblasts were seeded in 24-well plates, and supplemented the medium with 10-200 µg/ml of doxycycline. The results of the study suggested that doxycycline inhibited collagen synthesis in a dose-dependent manner. In this study, the measurement of proline content suggested that doxycycline might reduce prolidase activity, which at normal levels aids in the recycling of collagen degradation products such as proline, which is subsequently used to form new collagen. The authors point out that studies surrounding

osteoarthritic research have found that doxycycline does not degrade collagen, but merely inhibits its synthesis, and the mechanism by which it does so is still widely unknown [72].

2.2.4.2 Degenerative Enzymes (Chondroitinase ABC and Collagenase A)

2.2.4.2.1 Chondroitinase ABC

In cartilage research, it is a common issue that chondrocytes produce proteoglycans in levels similar to native tissue within the time range of an experimental study, but collagen content is often too low [73-77]. Resultantly, mechanical properties of *in vitro* engineered cartilage do not match that of the native tissue [73-77] as researchers fail to achieve the 2:1 proteoglycan-to-collagen ratio of naturally occurring articular cartilage. One method researchers have focused on in order to increase the relative collagen content has been enzymatic digestion of proteoglycans. Chondroitinase ABC works by cleaving chondroitin sulphate, dermatan sulphate, and hyaluronic acid, thereby digesting proteoglycans like aggrecan while collagen content remains unaffected [77, 78]. Some studies have also found that proteoglycans inhibit adhesion, so by removing the glycosaminoglycan chains using chondroitinase the increased collagen content can result in better adherence of engineered cells to cartilage lesions [79].

One study focused on removal of proteoglycans from fibrocartilage, which possesses a higher collagen type I content than collagen II, and is often found in tissues of the knee and jaw. The researchers made fibrocartilage constructs by seeding bovine meniscus cells and articular chondrocytes together in agarose wells. Constructs were fed chondrogenic medium for one week, and were then transferred to well plates for four remaining weeks, during which they were fed 2 units/mL chondroitinase ABC at days 7 and 21. Ultimately, the study found that treating constructs with a combined chondroitinase ABC and TGF-β1 treatment resulted in tensile strength that was

significantly greater than the control group, and an increase in the collagen content per wet weight [75].

Natoli et al. [74] highlighted in their study the need to eliminate scaffolds in cartilage engineering because it is difficult to decide if mechanical testing results of experiments are properties of the cells or the scaffold. The group aimed to prove that if proteoglycan content can be reduced early in the growth period, collagen content will increase and mechanical properties reliant on the collagen growth should also improve. The study treated bovine chondrocytes halfway through the experiment with 2 units/mL of chondroitinase ABC for 4 hours. Results indicated that cells removed from culture after treatment at two weeks had depleted proteoglycan content, but by week four the proteoglycan content recovered. Only the collagen content in the treated group at four weeks was statistically similar to the control, and the Young's modulus and tensile strength were both significantly greater in the four week group than in other groups. The experimenters concluded that increased mechanical properties were due to higher collagen concentrations and potentially greater cross-linking of the collagen network [74].

As chondroitinase ABC treatment has been proven to temporarily deplete proteoglycans thereby allowing collagen content to increase, studies have shifted focus to determining whether continual dosing of the enzyme is more effective than one chondroitinase ABC dose. In a study comparing adding 0.002 units/mL chondroitinase ABC to culture media of agarose encapsulated chondrocytes for four weeks, to adding the same amount of enzyme beginning on part way through the study for only two weeks, the four week cultures did not produce good results in any part of the study. Collagen content in the two week group was greater than the control after day 28, and proteoglycan loss was restored to control levels by day 56 of growth. Mechanical properties of the two week group waned and then recovered by day 56. The second portion of the study added 0.15

units/mL of chondroitinase ABC or agarase at 100 units/mL from day 35 to day 37, and then again to half the groups from day 58 to day 60. The experiment showed that the groups which received two chondroitinase ABC treatments had higher collagen content than the group that only received the enzymatic treatment once, and in all groups proteoglycan content was significantly reduced when compared to agarase treatment, but had recovered by the end of the culture period [73]. It was found that chondroitinase ABC is a more effective treatment in reducing proteoglycan content than other enzymes, and also that multiple or continual treatments of chondroitinase after cells have had time to establish matrix may be advantageous over a single treatment.

A similar study by O'Connell et al. [77] also looked at more frequent dosing. Bovine articular chondrocytes encapsulated in agarose were grown for 14 days in chondrogenic medium and then put in one of two studies. The first study added 0.15 units/mL of chondroitinase ABC to culture medium for two consecutive days either once or four separate times between days 14 and 37 of a 77 day culture period. The second group received 0.15 units/mL, 0.004 units/mL, or 0.015 units/mL of chondroitinase for one week beginning on day 14. In study 1, the group which received only one dose exhibited more favourable mechanical properties and proteoglycan concentration similar to control values compared to the two dose group, however collagen content declined. Results from the second study showed that only the lowest dose group had similar mechanical properties to the control group, and it also yielded the highest collagen and proteoglycan content. The low dose group also resulted in the least cell death, indicating a lower dose of chondroitinase ABC over a longer time period may allow for greater collagen synthesis without reducing DNA content [77].

Although the use of collagenase A in nucleus pulposus tissue engineering is currently limited, by applying the principles of using chondroitinase ABC for proteoglycan depletion to

collagen degradation, collagenase A is a promising nucleus pulposus tissue engineering approach. This study proposes to use collagenase as a means to degrade collagen content in chondrogenic samples to create an injectable therapy. Collagen in articular cartilage is heavily cross-linked, adding stability to the tissue [80]. Even small amounts collagen would create a more solid-like tissue and hence not make for a good injectable. Previous studies have shown that chondrogenic induction of stem cells through the use of pellet culture and chondrogenic medium containing growth factors leads to deposition of an extracellular matrix rich in collagen type II and aggrecan [59, 60]. Stem cells should be differentiated towards a chondrogenic phenotype in order to handle the *in vivo* environment of the nucleus pulposus, and to eventually deposit high amounts of aggrecan and some collagen. Prior to injection however, the collagen content must be degraded to allow for the injectability of the product.

2.2.4.2.2 Collagenase A

Collagenase A is a bacterial collagenase from *Clostridium histolyticum* composed of six different collagenases -α, β, γ, δ, ε, and ζ [81, 82]. Currently, collagenase from *C. hystoliticum* is commonly used in the treatment of dermal ulcers and burns, and is approved under the name XIAFLEX by the FDA for treatment of Dupuytren's contracture – a disease in which collagen deposition in fascia causes immobility and deformation of fingers [81, 83-85]. Bacterial collagenases are collagen degrading enzymes which act to catalyze a hydrolysis reaction. In this reaction, collagen fibres are broken down at multiple sites, thereby distinguishing them from tissue collagenases which only target one part of a collagen fibril [81, 82, 84, 86-89]. Collagenase A is able to target collagen types I, II, and III [88], thereby making it an ideal degenerative enzyme for the purposes of the presented study.

3.0 Materials and Methods

In the following sections, all reagents were obtained from Sigma-Aldrich, Inc. (Oakville, Ontario) unless otherwise noted.

3.1 Harvest, Isolation, and Expansion of Bone Marrow Derived Mesenchymal Stem Cells

Mesenchymal stem cells were isolated immediately following sacrifice from the femoral bone marrow of New Zealand white rabbits obtained from the vivarium of St. Michael's Hospital Li Ka Shing Knowledge Institute. First, the femur was removed from both hind legs by peeling back the fur and skin and scraping the bone clean of fascia. Using a hammer and chisel, the femurs were aseptically broken in the centre of the diaphysis. A sterile spatula was then used to remove bone marrow from the cavity. The cavity was washed using a syringe filled with a solution containing Low Glucose Dulbecco's Modified Eagle Medium (DMEM-LG) and 2% (v/v) antibiotic-antimycotic to remove remaining bone marrow. The bone marrow was then placed in a 50 mL conical tube, centrifuged, and resuspended in erythrocyte lysing buffer containing 8.24 g/L ammonium chloride, 1 g/L potassium bicarbonate, and 37.22 mg/L EDTA in distilled water and allowed to gently mix for 10 minutes. The cells were subsequently centrifuged, resuspended in DMEM-LG, and passed through a 100 µm nylon mesh to remove remaining bone fragments. The cells were then washed, centrifuged, and resuspended in a small amount of media and seeded into two T75 flasks.

The flasks were incubated at 37°C and 5% CO_2 and fed fresh media containing DMEM-LG, 1% (v/v) antibiotics, and 0.001% (v/v) basic fibroblast growth factor (b-FGF) (PeproTech Inc., Rocky Hill, New Jersey, USA) every 2 to 3 days. Cells were passaged at 90% confluence using 0.05% trypsin EDTA solution (Life Technologies) until they reached passage 3.

3.2 Creation of Three Dimensional Pellets

At passage 3 as described above, cells were counted using a trypan blue dye exclusion test [90] and resuspended at a density of 2 million cells per 1 mL in chondrogenic medium formulation as outlined in other studies [59, 91] containing: high glucose DMEM (DMEM-HG), 1% antibiotics, 10^{-7} M dexamethasone, 50 µg/mL L-Ascorbic acid 2-phosphate, 100 µg/mL sodium pyruvate, 40 µg/mL L-proline, 0.01% ITS Liquid Media Supplement (100x), and 10^{-5} mg/mL transforming growth factor - beta 1 (TGF-β1) (PeproTech Inc., Rocky Hill, New Jersey, USA). Pellet culture size and formation protocols were chosen based on previous studies [48, 60, 91]. Two million cells in 1 ml of chondrogenic medium were placed in a 1.5ml Eppendorf tube containing a hole in the lid - created using a 16 gauge needle – in order to allow for gas exchange. To form a pellet, the cells were centrifuged in a microfuge at 500g for 5 minutes, and were placed in a tube rack and covered in an aluminum foil "tent" in order to minimize entrance of contaminants into the Eppendorf tubes, and then placed in the incubator at 37°C and 5% CO_2. The cells were cultured with subsequent media changes occurring every 2-3 days.

3.3 Static Culture Supplementation with Collagenase A and Doxycycline

Following one week of culture in chondrogenic medium, cell pellets were grown in media supplemented with either collagenase A or doxycycline. Pellets were divided into 8 groups containing either 3 or 4 pellets each. Groups were assigned a high, medium, low, or control concentration of collagenase A or doxycycline, and fed with corresponding medium every 2-3 days for the final 21 days of the 4-week culture period. Chondrogenic medium was supplemented with either Collagenase A (Roche Diagnostics, Mannheim, Germany) in DMEM-HG or doxycycline monohydrate in dimethyl sulfoxide (DMSO) at the concentrations shown in Table 3.1.

Table 3.1 Supplementation Dosages of Collagenase A and Doxycycline Monohydrate in Medium

Dose	Collagenase A Concentration	Doxycycline Concentration
Low	100 µU/mL	5 ng/mL
Medium	1 mU/mL	10 ng/mL
High	10 mU/mL	20 ng/mL

Control groups were fed chondrogenic medium containing a "vehicle only" equivalent volume of DMEM-HG or DMSO (no Collagenase A or Doxycycline).

3.4 Tissue Harvest

At the end of the 28 day culture period, cell pellets were removed from incubation and used for either histological, PCR, or biochemical analysis.

3.4.1 Sample Preparation for Biochemical Analysis

Pellets used for biochemical analysis were centrifuged at a speed of 500 g for 5 minutes total in order to collect all DNA content and synthesized proteins at the bottom of the tube and separate these contents from free media. Supernatant was then carefully discarded by pipetting without disturbing the pellet. Due to the inability to remove all free media from the pellet without potentially discarding biological sample, the weights of pellets were not noted. Samples were lyophilized in Eppendorf tubes overnight and then digested in 600 µL of papain working digestion solution (40 µg/mL Papain Stock in digestion buffer containing 2.72 mg/mL ammonium acetate, 0.38 mg/mL disodium EDTA, and 0.31 mg/mL DL-dithiothreitol) at 65ºC for 48 hours. Following digestion, samples were stored at -20ºC until further use.

3.4.2 Sample Preparation for PCR Evaluation

Pellets allocated for PCR evaluation were centrifuged at a speed of 500 g for 5 minutes total to collect biological material at the bottom of the tube. Supernatant was discarded by pipetting without disturbing pellet. Pellets were then covered in approximately 1 mL (10 times their volume as per manufacturer's instructions) of RNAlater® Stabilization Reagent solution and stored at 4ºC overnight. Following overnight refrigeration, pellets were moved to -20ºC and stored until further use.

3.4.3 Sample Preparation for Histological Evaluation

Pellets allotted for histological evaluation were not centrifuged in order to try and retain morphological characteristics of the cultured tissue sample. In order to begin fixation of the tissue samples, 700 µl of culture medium was discarded and replaced with 4% paraformaldehyde solution. After approximately 20 minutes, the liquid covering the pellet was discarded and was replaced with 800 µl of 4% paraformaldehyde solution. Pellets were left, covered, at room temperature for 2 hours. The paraformaldehyde solution was carefully discarded as to try not to disturb the pellet.

Due to their fragility, it was necessary that pellets were first embedded in Richard-Allen Scientific™ Histogel™ Specimen Processing Gel in order to preserve pellets during paraffin embedding. Briefly, Histogel™ was prepared according to manufacturer's directions. 100 µL of liquefied Histogel™ was then carefully added on top of pellet in a 1.5ml Eppendorf tube, and allowed to slowly surround the pellet at room temperature. After a few minutes, tubes were placed on ice to further solidify the gel. The Histogel™-pellet constructs were then carefully removed from the tube one at a time and placed into a single well of a 48-well plate. Additional liquefied Histogel™ was pipetted into the wells until the Histogel™-pellet construct was fully covered. The

48-well plate was then placed on ice in order to allow the construct to fully solidify. Constructs were then carefully removed from the 48-well plate and trimmed to fit into histological cassettes. The Histogel™-pellet construct was then covered in 4% paraformaldehyde for 24 hours before tissue processing for histology.

3.5 Biochemical Analysis

In order to determine the DNA, hydroxyproline, and glycosaminoglycan (GAG) content of each individual pellet, biochemical analysis was performed. First, papain digested samples (Section 3.4.1) were removed from the freezer and thawed at room temperature.

To evaluate the DNA content, the following protocol as outlined by Kim et al. was followed. Briefly, 50 µl aliquots of each sample were placed in triplicate into a black 96-well plate on ice. A DNA standard curve was created using Calf Thymus DNA solution in phosphate buffered saline (PBS), and aliquoted in triplicate into the well plate. The plate was removed from ice, and 200 µL of 1 mg/mL Hoechst 33258 dye solution was added into each well. The assay was performed by reading the excitation at 350 nm and emission at 450 nm of the well plate on a plate reader. Finally, the DNA content was evaluated by finding the concentration from excitation and emission values, and multiplying by the total volume of the sample [92].

In order to quantify the amount of collagen in each pellet, hydroxyproline content was evaluated according to a protocol established by Woessner et al. [93] with some minor adjustments. First, samples underwent hydrolysis by placing 180 µL of papain digested sample with 90 µL of 12 N hydrochloric acid into a glass tube. Samples were heated overnight at 110 °C and then neutralized by adding 90 µL of 11.4 N sodium hydroxide to the hydrolyzed solution. A 96-well plate was prepared by adding 100 µL of each hydrolyzed sample to individual wells in triplicate, along with 100 µL aliquots of standard curve samples made from hydroxyproline stock

solution. The assay was then performed in three parts. First, 0.05 N chloramine-T solution was prepared fresh and added to the well plate in 50 µL aliquots. After waiting 20 minutes, 50 µL of 3.15 N perchloric acid was added into each sample containing well, and the plate was left to stand for 5 minutes. Finally, 50 µL of 200 mg/mL Ehrlich's Reagent in methyl-cellosolve was added to each well, and the 96-well plate was placed in a 60 °C oven for 20 minutes. The well plate was then removed from the oven, placed in 4 °C for 5 minutes, and then the colour was allowed to stabilize at room temperature for 30 minutes. The absorbance of the samples was read on a plate reader at 560 nm, and the hydroxyproline content of each pellet was calculated by multiplying the hydroxyproline concentration of the pellet by the total volume of the digested sample (and corrected for dilution). Negative absorbance values indicated undetectable amounts of hydroxyproline, and therefore hydroxyproline contents for these samples were recorded as 0 µg [93].

To quantify the amount of proteoglycans in each pellet, glycosaminoglycan content was calculated using a dimethylmethylene blue dye-binding assay [94, 95]. Papain digested samples were aliquoted in triplicate at a volume of 10 µL per well into a standard 96-well clear plate. A GAG working solution containing chondroitin sulphate sodium salt in PBS was diluted in 1% bovine serum albumin (BSA) solution and used to create a standard curve. Dimethylmethylene blue dye (16 µg/mL) was added to the plate in 200 µL aliquots and the absorbance of the wells was read at a wavelength of 525 nm. The weight of glycosaminoglycans per pellet was then found by multiplying the total volume of the sample by the GAG concentration evaluated from the assay [94, 95].

3.6 Histological Evaluation

In order to prepare pellets for histological evaluation, the Histogel™-pellet constructs in cassettes were placed in a Leica TP1020 Automatic Tissue Processor (Leica Biosystems Inc., Concord, Ontario). First, the constructs were placed in 10% neutral buffered formalin to ensure proper fixation and then dehydrated in graded ethanol solutions in the following order: 70% ethanol for 1 hour, 80% ethanol for 1 hour, 95% ethanol for 2 hours, and finally 100% ethanol for 3 hours. The alcohol was replaced with pure xylene for 3.5 hours, and the samples were then infiltrated with histological paraffin wax. After embedding the processed constructs in paraffin, blocks were sectioned into 4 or 6 µm thin slices and placed on Superfrost™ Plus microscope slides (Fisher Scientific, Mississauga, Ontario). The tissue slides were de-paraffinized by heating to 65 ^{o}C, rinsed in xylene, and then rehydrated with water. The slides were then stained following standardized protocols for hematoxylin and eosin staining, Safranin-O [96, 97], or Sirius Red [98] staining (one slide per pellet per stain), and were then mounted with Permount™ Mounting Medium (Fisher Scientific, Mississauga, ON). To detect for possible mineralization and therefore ossification, Von Kossa staining was carried out for 1 hour under a UV lamp, and counterstained using hematoxylin & eosin in order to visualize tissue structure. Stained and mounted slides were imaged using a Nikon Upright E800 Microscope (Nikon Instruments Inc., Melville, New York, USA).

3.7 Immunohistochemistry

Immunohistochemistry staining for collagen I and collagen II was performed on 5 µm slices of paraffin embedded tissue as described elsewhere [99]. Slides were first deparaffinized in xylene and graded ethanol solutions as described in Section 3.6, then rehydrated in water. Antigen retrieval to allow for antibody binding to collagen proteins was performed by covering the sections

with a solution of 0.05% Trypsin in phosphate buffered saline, and then placing the slides at 37 °C for 30 minutes. Next, non-specific antibody binding was blocked using a 1% bovine serum albumin in phosphate buffered saline solution at room temperature for 30 minutes. The 1% BSA solution was removed, and the sections were incubated with 1:100 dilution solution of mouse monoclonal anti-collagen I antibody (ab90395; Abcam, Cambridge, Massachusetts, USA) or mouse monoclonal anti-collagen II antibody (II-II6B3; Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) in 1% BSA in PBS at 4 °C overnight. The following day, slides were washed twice with phosphate buffered saline and then treated with a 1:200 dilution solution of Texas Red labeled goat anti-mouse IgG secondary antibody (ab6787; Abcam, Toronto, Ontario) in 1% BSA in PBS for 2 hours at room temperature. Precautions were taken to ensure the secondary antibody treatment was not exposed to light. Slides were then washed in 3 changes of phosphate buffered saline for 5 minutes each. Sections were mounted using Vector Laboratories Vectashield Mounting Medium with DAPI to counterstain and visualize nuclei (Vector Laboratories, Burlingame, California, USA). Slides were imaged using fluorescent microscopy.

3.8 End-Point Polymerase Chain Reaction

Note: All reagents used in subsequent sections are Invitrogen™ brand products supplied by Sigma Aldrich in Oakville, Ontario unless otherwise noted.

End-point polymerase chain reaction was performed to validate primers and assess gene expression in samples. Target genes shown in Table 3.2 were chosen based on either their presence in native nucleus pulposus tissues and cartilage (*ACAN, COL2A1, SOX-9*) [50, 100, 101], or to asses if stem cells may have differentiated down an osteogenic lineage (*RUNX-2, CO*L1A2) [91, 101]. *GAPDH* was chosen as the housekeeping gene [91, 100, 101].

Primer design was provided courtesy of Daniela Herrera using the following method. First, the target gene and species (New Zealand White rabbits/*Oryctolagus cuniculus*) were entered into a gene database in order to obtain the FASTA nucleotide sequence. The nucleotide sequence was then used to find the ideal primer for this study via the PrimerQuest Tool (Integrated DNA Technologies, Inc., Coralville, Iowa, USA). Primer sequences were chosen based on general guidelines for good primer design, including a target amplicon length of approximately 100 bp, a melting temperature near 65°C, and a G-C Content between 35 to 65%.

Table 3.2 Primer Sequences of Target Genes Used in End-Point PCR

Gene	Gene Sequence	Amplicon length (bp)	Melting Temperature (°C)
glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	F: GAAGGTCGGAGTGAACGGATTTG R: TGATGGCGACAACATCCACTTT	89	65
aggrecan (*ACAN*)	F: TCCAGTGAGCTGGACGTTAGT R: AACCCTGATGGCTGTCCTCTAA	110	65
collagen, type II, alpha 1 (*COL2A1*)	F: CAGGCAGAGGCAGGAAACTAAC R: GTTTGACACGGAGTAGCACCATC	118	65
SRY-box 9 (*SOX-9*)	F: GTGTAGAGGACGCATTTGGTAAGC R: TGCAGCAGCTCGGTGTTTAAG	99	65
collagen, type I, alpha 2 (*COL1A2*)	F: TGGTGGCACCCAGTTTGAATAC R: GTGATGTTCTGAGAGGCGTGATTG	108	65
runt related transcription factor 2 (*RUNX2*)	F: TCCGAAATGCCTCTGCTGTTATG R: CAAGGTGAAACTCTTGCCTCGT	95	65

3.8.1 RNA Purification

Total RNA was isolated from individual pellets using reagents from the RNeasy Mini Kit (Qiagen Inc., Toronto, Ontario) and by following the accompanying manufacturer-provided protocol as outlined in the following steps. Pellets in RNAlater Stabilization Reagent were centrifuged and the supernatant was discarded. 350 µL of Buffer RLT (guanidine thiocyanate-

containing cell and tissue lysing buffer) was added to each tube, and the pellet was homogenized in the buffer by vigorous mixing, then centrifuged at 8000 g for 3 minutes. Next, 350 µL of 70% ethanol was added to the sample and the total 700 µL of ethanol and buffer solution was placed into a 2 mL spin column and centrifuged at 8000 g for 15s. The liquid flow through was discarded and 700 µL of Buffer RW1 (washing buffer for membrane-bound RNA) was pipetted into the spin column, and the sample was then centrifuged for 15s at 8000 g. Flow-through was discarded and 500 µL of Buffer RPE (buffer used to remove residual salts from the collection column) was placed in the spin column and again centrifuged at 8000 g for 15 seconds. The previous step was repeated once more, and then the spin column was placed in a new tube and centrifuged to dry the membrane. Next, 30 µL of RNase-free water was placed into the column, and the column was placed into a 1.5 mL Eppendorf tube. After allowing the water to sit on the membrane for 1 minute, the tube was centrifuged for 1 minute at 8000 g. The 30 µL of water that flowed through the spin column membrane was collected and placed back into the spin column, allowed to sit, and then centrifuged for 1 minute at 8000 g one final time. The flow through was used in the following PCR steps.

3.8.2 DNase Treatment

In order to further purify the RNA, single- and double- stranded DNA was digested using deoxyribosenuclease I in buffer by the following protocol. Briefly, 16 µL of each RNA isolated sample from section 3.8.1 was added to a microfuge tube, along with 2 µL of 10X DNase I Reaction Buffer and 2 µL of DNase I, Amp Grade, 1 U/µL, mixed, and allowed to sit at room temperature for 15 minutes. Next, 1 µL of 25 mM EDTA solution was added to the tubes, and the samples were placed to heat at 65 °C for 10 minutes. Following this step, samples that were not immediately used for reverse transcription were kept at -80 °C until further use.

3.8.3 Reverse Transcription – First Strand cDNA Synthesis

Reverse transcription of RNA was performed using a kit from Invitrogen and the following protocol [102]. First, 2 µL of oligo$(dT)_{20}$ and 2 µL of 10mM dNTP Mix were added to the total 21 µL of DNase treated isolated RNA from the previous step (section 3.8.2). The sample was heated at 65 °C for 5 minutes, then incubated on ice for 1 minute. Next 4 µL of 5X First-Strand Buffer, 2 µL of 0.1 M DTT, and 4 µL of SuperScript™ III RT (200 units/µL) were added to the mixture, mixed, and then incubated at 50 °C for 1 hour. Finally, the reaction was inactivated by heating the sample mixture at 70 °C for 15 minutes. Samples that were not used immediately were stored at -20 °C.

3.8.4 Primer Validation and Endpoint PCR

In order to perform endpoint PCR and determine the expression of genes within samples, primer validation was performed in order to choose the optimal primer working concentration and to ensure the primers were specific and uncontaminated with extraneous DNA. The following protocol was adapted from previous work [102]. To do so, cDNA was transcribed from a sample of New Zealand White rabbit articular cartilage obtained from the knee which should have all target genes present. Next, solutions containing PCR MasterMix, cDNA, nuclease free water, and varying concentrations of each primer set were made as shown in Table 3.3. A no template control was included in every primer in order to ensure primer solutions were free of genomic DNA contamination.

Table 3.3 Reagent Volumes Used in Varying Primer Concentrations for Primer Validation & Endpoint PCR

Primer Concentration	150 nM	500 nM	900 nM	No Template Control
	Volumes (µL)			
PCR MasterMix	25	25	25	25
Forward Primer	0.75	2.5	4.5	0.75

Reverse Primer	0.75	2.5	4.5	0.75
cDNA	0.5	0.5	0.5	-
NF-water	23	19.5	15.5	23.5
Total Volume	50	50	50	50

Table 3.4 Reagent Volumes Used in Endpoint PCR for Samples Requiring Higher cDNA Concentration

Primer Concentration	**150 nM**	**500 nM**	**No Template Control**
	Volumes (µL)		
PCR MasterMix	25	25	25
Forward Primer	0.75	2.5	0.75
Reverse Primer	0.75	2.5	0.75
cDNA	2	2	-
NF-water	21.5	18	23.5
Total Volume	**50**	**50**	**50**

The polymerase chain reaction was performed using a thermocycler under conditions shown in Table 3.5. Upon the completion of thermocycling, samples were stored at 4 °C until they were ready for use.

Table 3.5 Thermocycling Conditions Used in Polymerase Chain Reaction

	Temperature (°C)	**Time**	**# Cycles**
Initial denaturation	95	1 minute	1
Denaturation	95	30s	40
Annealing & Extension	60	1 min/kilobp	
Final extension	72	7 minutes	1

To determine gene expression, 30 µL of each sample was mixed with 6 µL of 6X DNA loading dye and pipetted into individual lanes of 10% acrylamide gels for electrophoresis. 9 µL of DNA ladder diluted with loading dye and distilled water was loaded into its respective lane. The acrylamide gels were connected to electrodes and underwent electrophoresis for 45 minutes at 120V, and were then stained with SYBR Safe dye for 30 minutes. The acrylamide gels were rinsed with water and imaged accordingly.

To evaluate gene expression in experimental samples, a 150 nM primer concentration solution was used for all primer pairs with the exception of SOX-9, as it was determined that the

ideal primer concentration of SOX-9 was 500 nM. Solutions were prepared using the volumes listed in Table 3.3 or 3.4 at the ideal primer concentration as determined by validation, and with cDNA synthesized from individual experimental samples. Samples determined to require higher amounts of cDNA in the reaction in order to generate clearer results were prepared as per Table 3.4. Endpoint PCR was performed as described above.

Gene expression levels were assessed from imaged gels in two manners. First, appearance of a dyed band within the acrylamide gels indicated expression of a particular gene, and second, volume analysis was performed in order to quantify band intensity using Image Lab™ V.6 software (Bio-Rad Laboratories Inc., Mississauga, Ontario). The band intensity for each target gene was normalized to the GAPDH expression level for each individual sample.

3.9 Statistical Analysis

Cell culture experiments were performed at least 4 times resulting in total pellet numbers of $n \geq 10$ for each group. Numerical results were first normalized to control values, and then presented as the mean ± standard error of the mean (SEM). Statistical significance of differences between group means were determined by performing a One-Way Analysis of Variance using Microsoft Excel and confirmed using Minitab 16 statistical software (Minitab Inc., Pennsylvania, USA). Tukey's post-hoc tests were performed using Minitab 16 or GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, California, USA) software to further analyze effects of collagenase A or doxycycline concentration. Significant differences were denoted by a p-value of less than 0.05, and p-values between 0.05 and 0.1 were noted as trends.

4.0 Results

4.1 Effect of Collagenase A Dosing on Mesenchymal Stem Cell Differentiation

As described previously, rabbit bone marrow-derived mesenchymal stem cells were formed into pellets at passage 3. Following one week of cultivation in chondrogenic medium, the media was supplemented with a low, medium, or high concentration of collagenase A – 100 µU/mL, 1 mU/mL, or 10 mU/mL, respectively for an additional three weeks of culture. Harvested pellets were analyzed through biochemical assays, histological and immunohistochemical techniques, and polymerase chain reaction (PCR) assays.

4.1.1 Biochemical Evaluation

Harvested pellets were lyophilized and then papain digested. Aliquots of the digest were then biochemically analyzed for DNA, proteoglycan, and hydroxyproline content of samples as described in Section 3.5. Biochemical analysis data was collected from four separate experiments and pooled together. All results were presented as values normalized to the control (no collagenase A) group.

Upon evaluation of DNA content, results showed that there is no significant difference between groups ($p=0.11$; Figure 4.1), indicating that collagenase A concentration had little effect on cell growth. Although it is not statistically significant, the high concentration of collagenase A potentially had some effect on DNA content as indicated by the relatively lower mean compared to the other treated groups and the control. Similarly, glycosaminoglycan content also remained unaffected by collagenase dosing ($p=0.18$; Figure 4.2) with no differences between groups. Hydroxyproline content, however, was affected by collagenase A supplementation ($p < 0.001$; Figure 4.3). Post-hoc testing showed that while all collagenase A groups differed significantly

from the control, there was no apparent effect of collagenase A concentration on hydroxyproline content between treated groups.

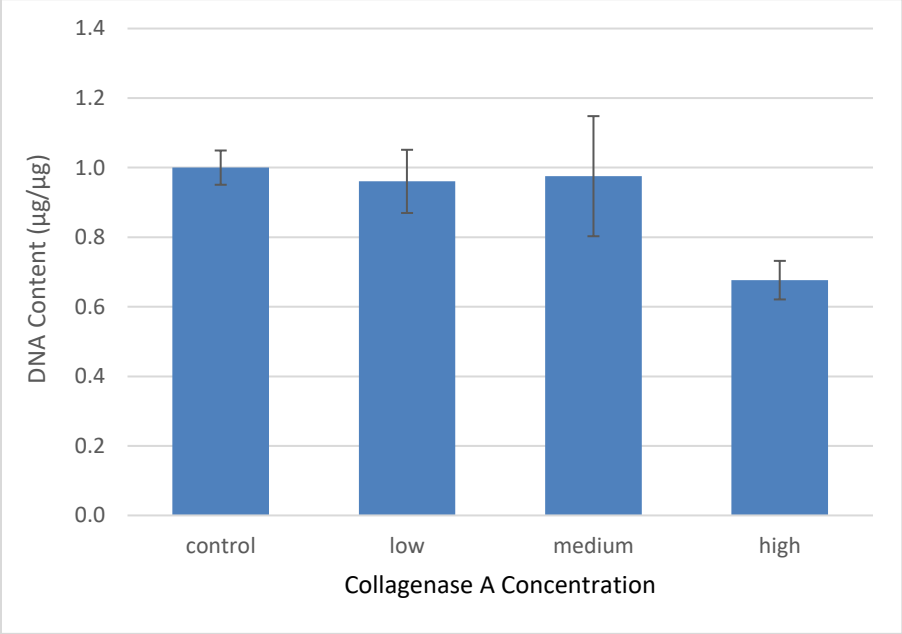


Figure 4.1 Effect of Collagenase A Concentration on DNA Content. Data is presented as means ± SEM, normalized to control (p=0.11), n=11 per group.

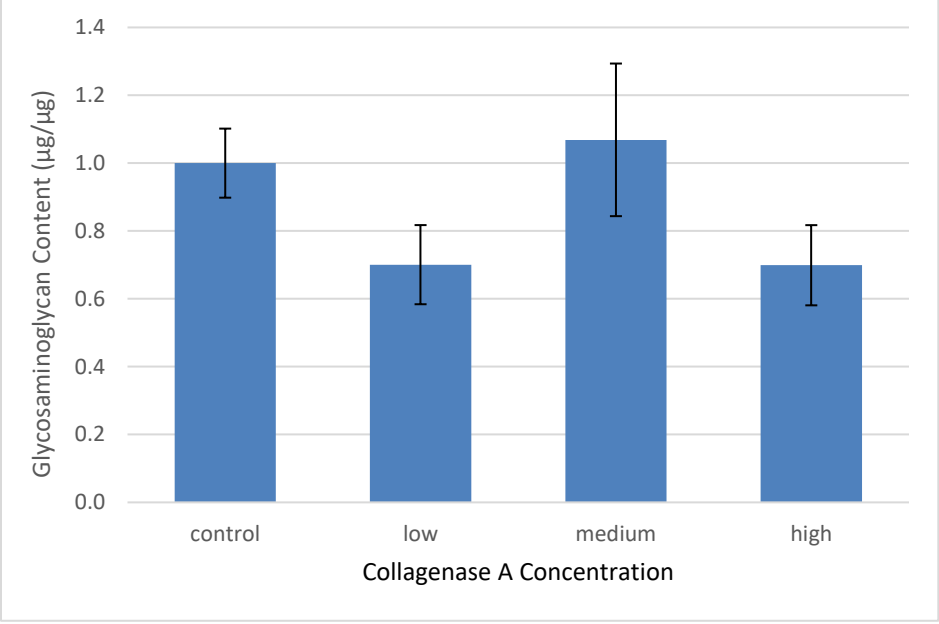


Figure 4.2 Effect of Collagenase A Concentration on Glycosaminoglycan Content. Data is presented as mean ± SEM, normalized to control group (p=0.18), n=11 per group.

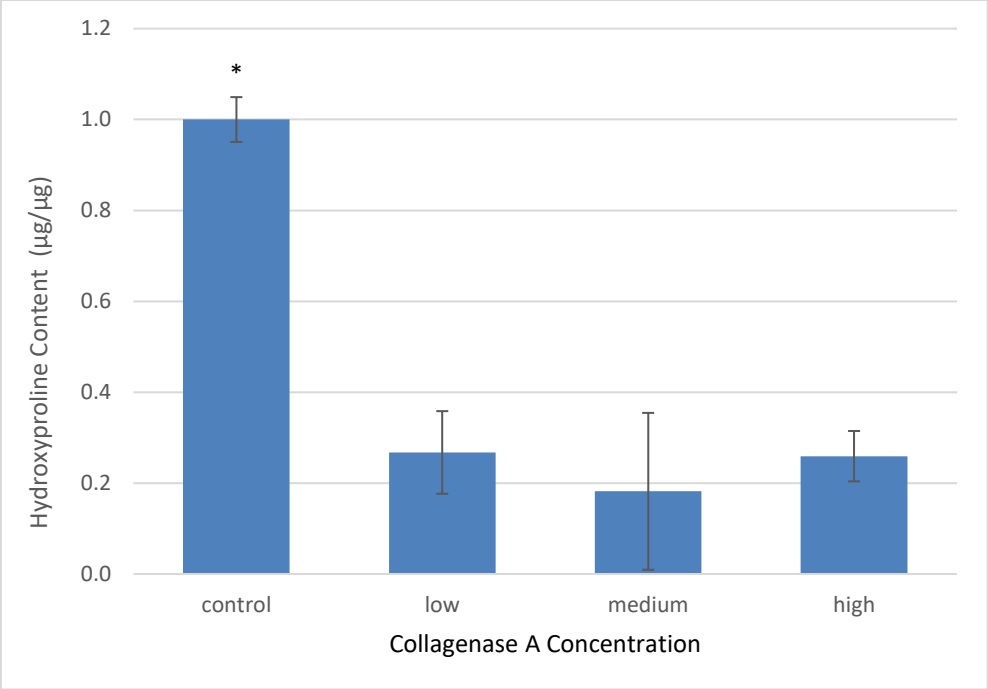


*Figure 4.3 Effect of Collagenase A Concentration on Hydroxyproline Content. Data is presented as mean ± SEM, normalized to control group ($p<0.001$), n=11 per group. * – denotes a significant difference from all other groups ($p<0.002$).*

4.1.2 Histological Evaluation

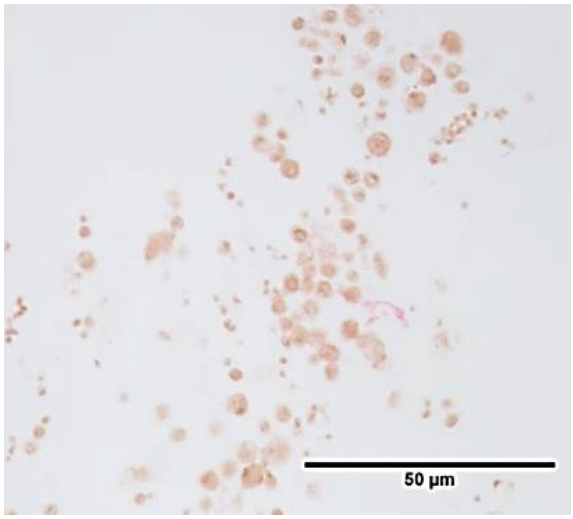
Upon harvest of pellet cultures, individual pellets were briefly fixed in 4% paraformaldehyde prior to encapsulation in Histogel™ Pellets were then fixed for an additional 24 hours, processed for histology, sectioned, and stained with sirius red, safranin O, or Von Kossa stains as described in Section 3.6.

Sirius red sections of collagenase A treated samples can be seen in Figure 4.4. Staining with sirius red indicated low collagen content across all samples, as is detected by the lack of intensity of red on imaged slides. Histology images from sirius red staining, along with safranin O and Von Kossa staining (Figures 4.5 and 4.6, respectively), support biochemical assay results that DNA content does not differ between sample groups, as can be visualized by the nuclei staining. Staining of the control sample indicated slightly more collagen formation then in collagenase A supplemented groups. This is indicated by the areas of intense red staining and red shadows which

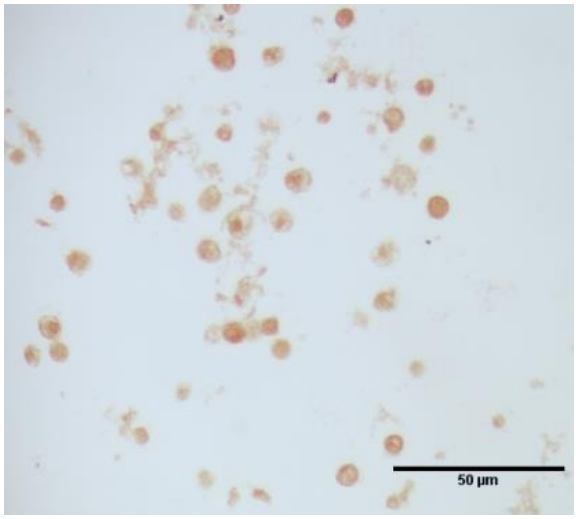
appear in control histological sections but not in the samples treated with collagenase A. This data supports hydroxyproline assay results indicating that hydroxyproline content is higher in the control group than in doxycycline-containing groups.

Safranin O staining (Figure 4.5) similarly supports biochemical evaluation of glycosaminoglycan content, which indicated that there is no significant statistical difference in GAG contents between all collagenase A treated groups and the control. This is evident by the similar staining patterns between sample groups. The lack of red coloured staining indicates low glycosaminoglycan content across all samples.

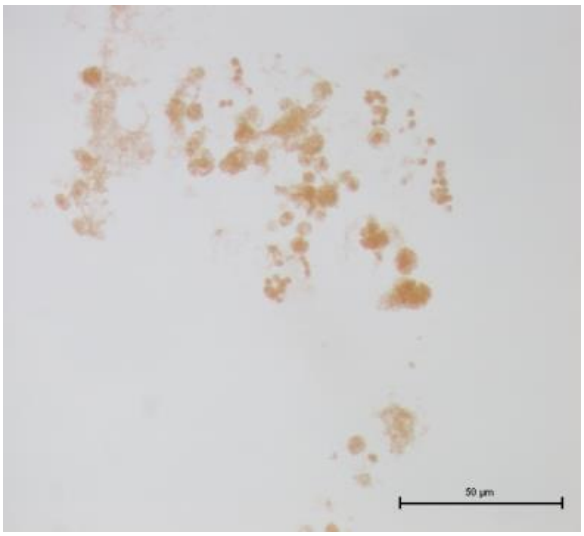
To check for the possibility of mineralization, slides were stained using a Von Kossa staining protocol. No mineralization was detected, which would appear as large black regions. Slides do show areas of light purple washes and small black points (Figure 4.6), however it should be noted that these are likely technical artifacts.



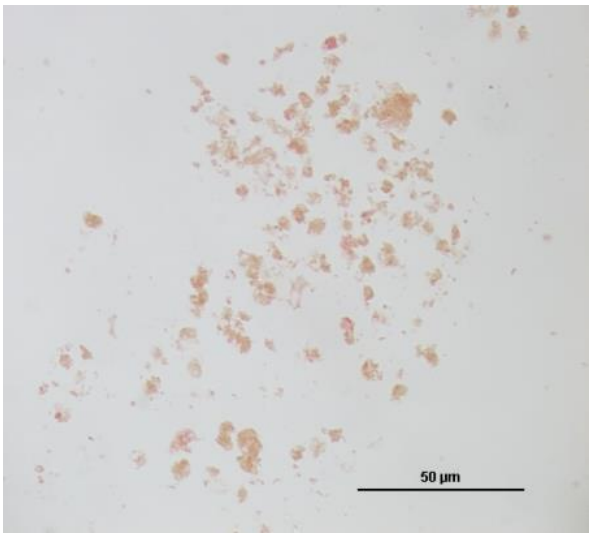
(a) Control - DMEM-HG Vehicle Only



(b) Low

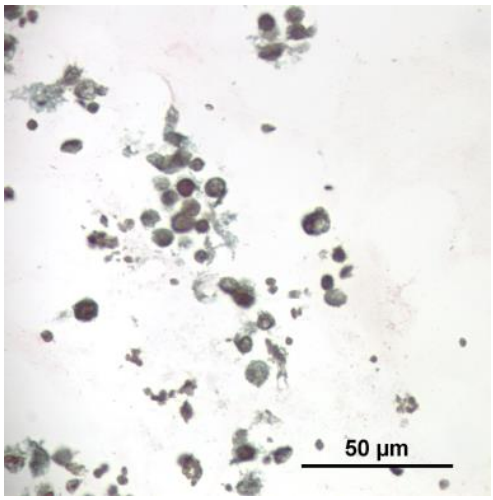


(c) Medium

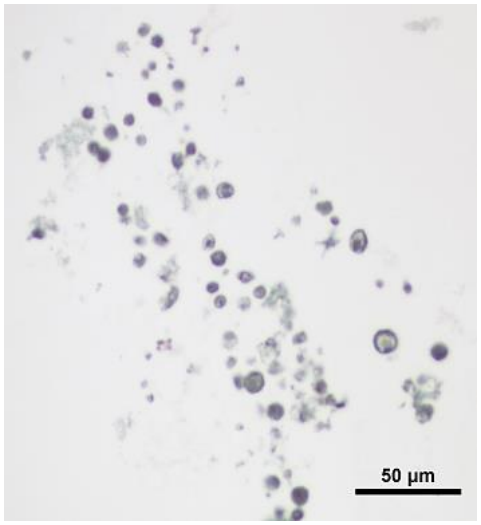


(d) High

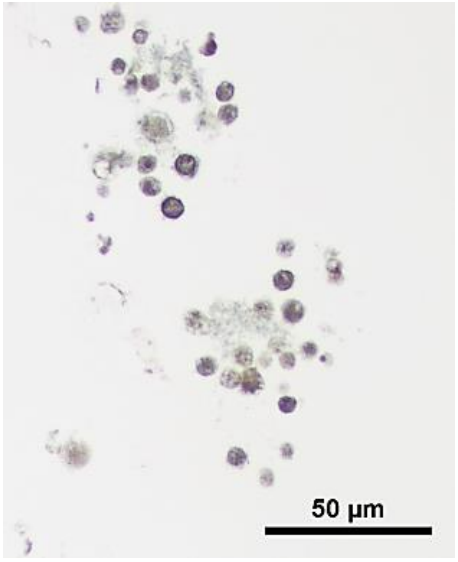
Figure 4.4 Histological Sections of Collagenase A Treated Pellet Samples Stained with Sirius Red to Visualize Collagen Staining



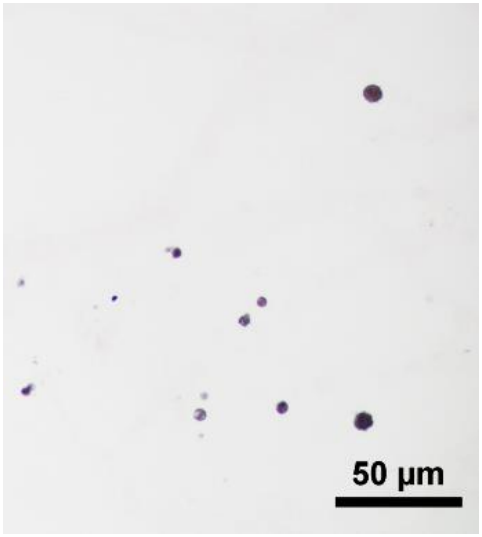
(a) Control - DMEM-HG Vehicle Only



(b) Low Dosage

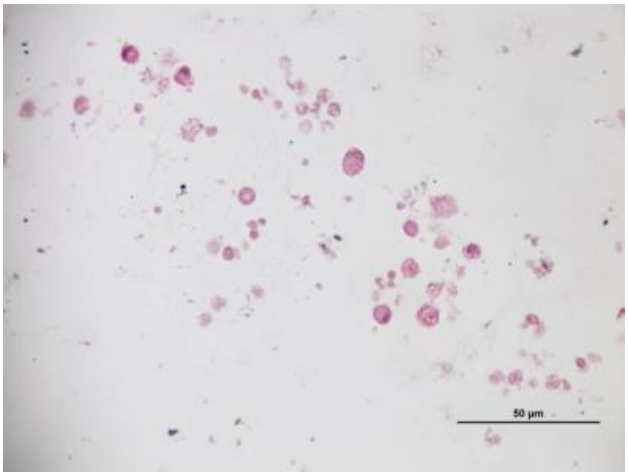


(c) Medium

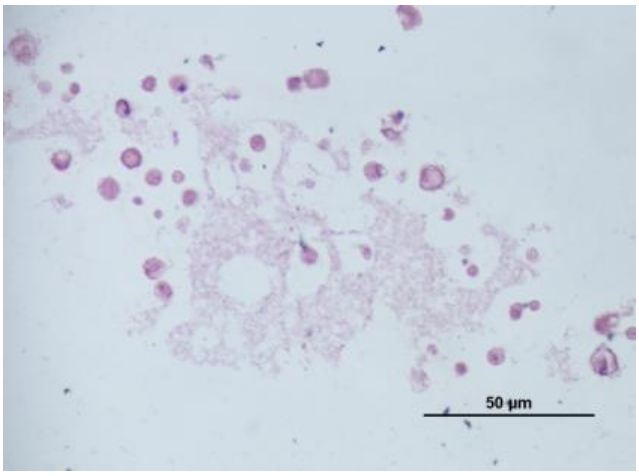


(d) High

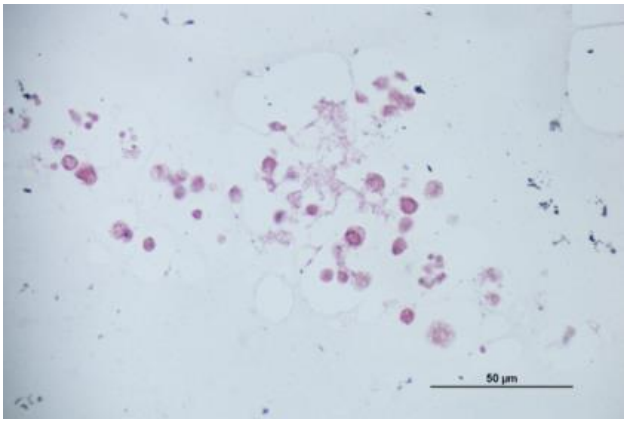
Figure 4.5 Histological Sections of Collagenase A Treated Pellet Samples Stained with Safranin O to Visualize Proteoglycan Staining



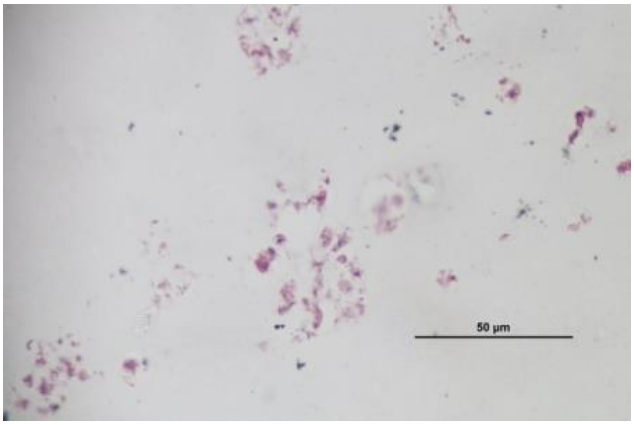
(a) Control - DMEM-HG Vehicle Only



(b) Low



(c) Medium



(d) High

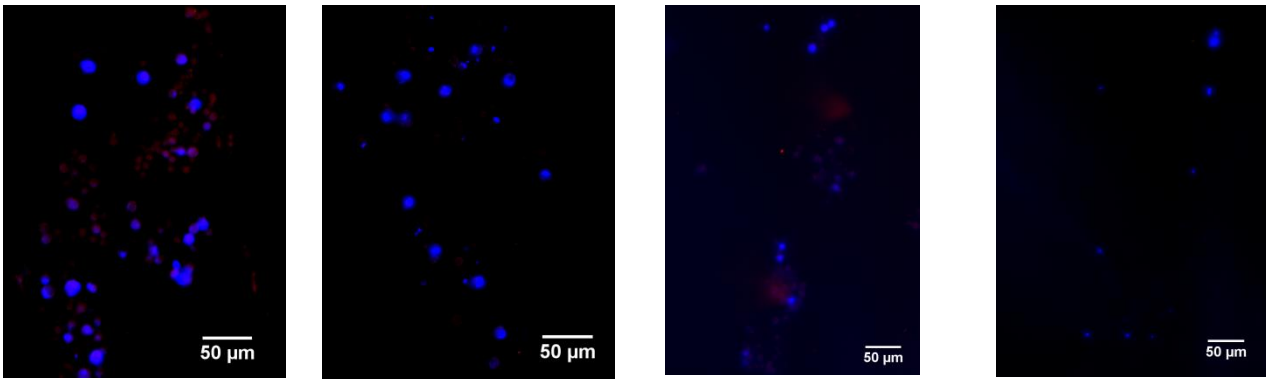
Figure 4.6 Histological Sections of Collagenase A Treated Pellet Samples Stained with Von Kossa Staining and Counterstained with H&E to Visualize Mineralization

4.1.3 Immunohistochemical Analysis

Collagenase A treated samples were prepared for immunohistochemical analysis as described in Section 3.7. Following antigen retrieval, sections were incubated with primary and secondary antibodies for collagen I and collagen II. Finally, slides were counterstained with DAPI to visualize the cell nuclei.

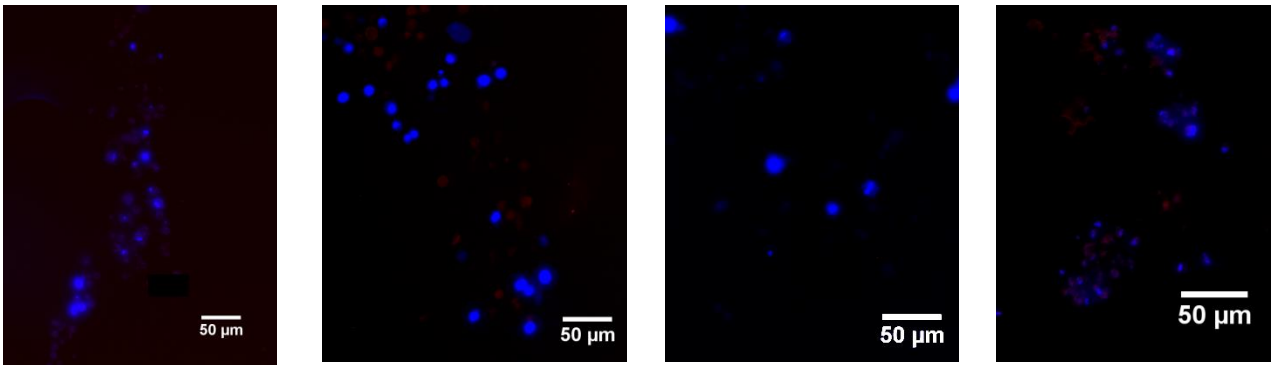
Both collagen I and collagen II immunostaining revealed low collagen content in all samples. It is important to note that collagen normally appears as a network of intense red staining

between blue-coloured nuclei, and any areas of red staining in Figures 4.7 a-d and Figure 4.8 a-d may be indicative only of intracellular collagen staining and not extracellular matrix components. Immunostaining supports histological images of safranin O and sirius red staining which also displayed a lack of collagen networks joining cells.



(a) Control (b) Low (c) Medium (e) High

Figure 4.7 Immunohistochemical Sections of Collagenase A Treated Pellet Samples Stained for Type I Collagen



(a) Control (c) Low (c) Medium (d) High

Figure 4.8 Immunohistochemical Sections of Collagenase A Treated Pellet Samples Stained for Type II Collagen

4.1.4 End Point Polymerase Chain Reaction (PCR) Analysis

RNA was extracted from harvested samples and transcribed into cDNA prior to undergoing doubling in a PCR reaction as described in Section 3.8. Gene expression results were quantified

using volume analysis of bands depicted in Figure 4.9 and are presented in Figure 4.10 as a gene expression level relative to the housekeeping gene.

End point PCR results show that the collagenase A vehicle-only control group expressed similar relative amounts of collagen type I and collagen type II, whereas high and medium concentration groups expressed the collagen II gene in higher amounts than collagen type I. Aggrecan expression was low in all groups. Data for the relative expression of the *RUNX2* gene was only available for the control group and was found to be low but was present. Although relative expression of the *SOX-9* gene was low in all groups, the control group expressed twice as much *SOX-9* gene as the high concentration collagenase A group, whereas in the medium concentration group, *SOX-9* expression was nonexistent.

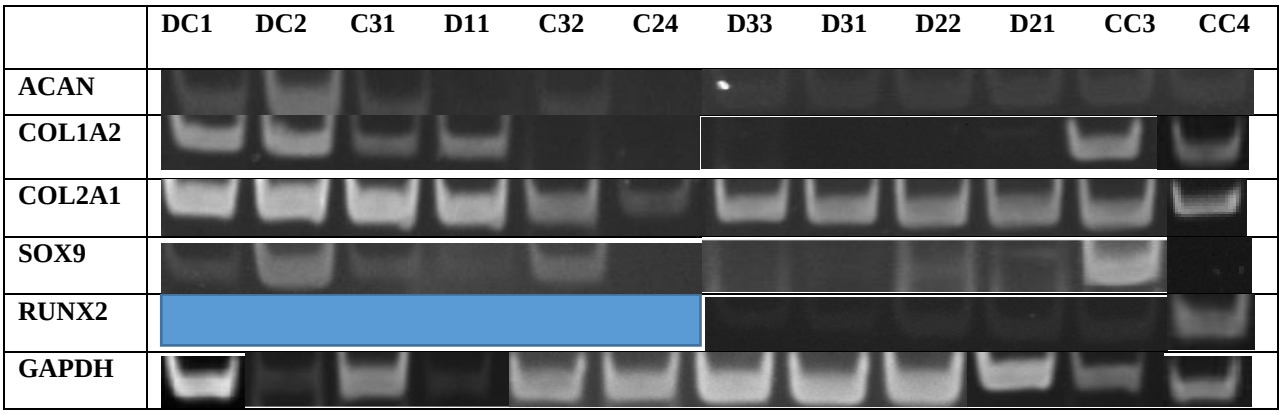


Figure 4.9 End Point PCR Target Gene Expression, Note: Bands appearing under the DC1 & DC2 columns represent doxycycline control group pellets, and D33 & D31, D22 & D21, and D11 represent high, medium, and low concentration doxycycline treated pellets, respectively. Bands appearing under CC3 & CC4 labelled columns represent collagenase A control pellets, and C31 & C32, C24, represent high and medium concentration Collagenase A treated pellets, respectively.

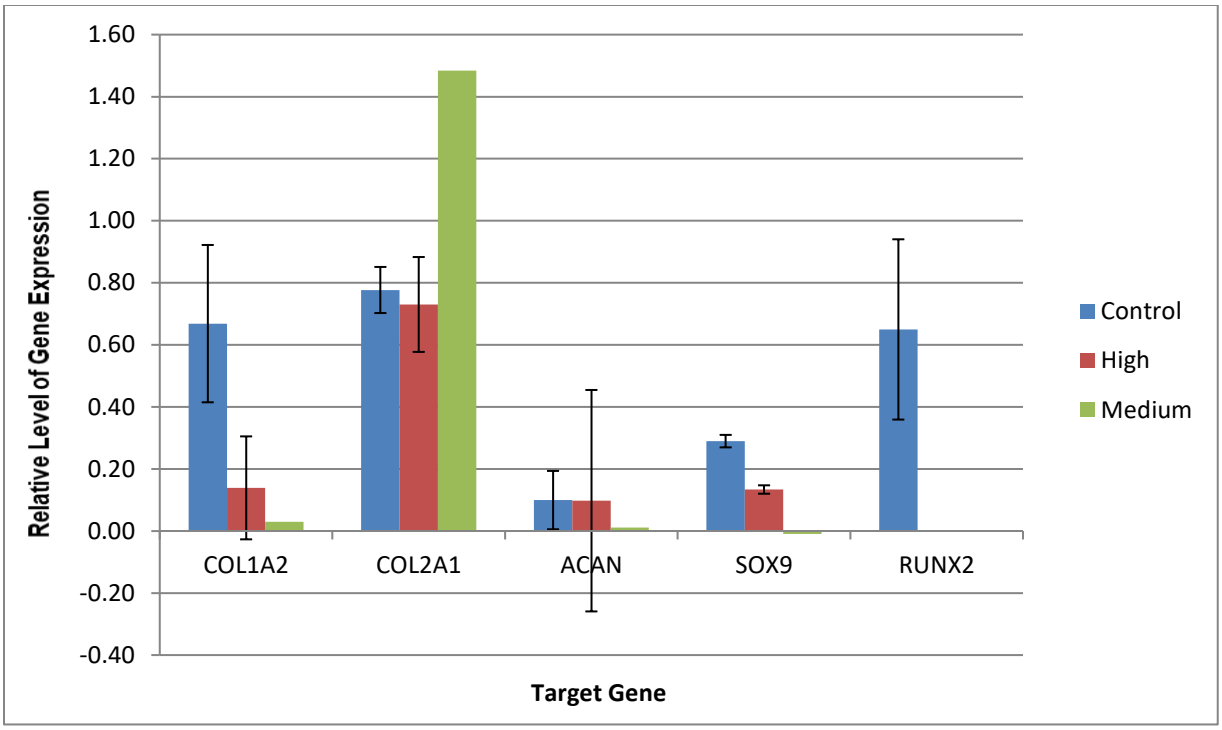


Figure 4.10 End Point PCR Relative Gene Expression Levels of Target Genes of Collagenase A Dosed Samples normalized to GAPDH (housekeeper) Gene Expression Levels. Data is presented as means ± SEM, normalized to GAPDH expression levels.

4. 2 Effect of Doxycycline Dosing on Mesenchymal Stem Cell Differentiation

As per Section 3.1, following one week of cultivation in chondrogenic medium, mesenchymal stem cell pellets were fed chondrogenic medium supplemented with a low, medium, high, (5 ng/mL, 10 ng/mL, or 20 ng/mL, respectively) or DMSO vehicle-only control concentration of doxycycline for the remaining three weeks of the culture period. . The effects of the doxycycline concentrations were also analyzed through biochemical assays, histological and immunohistochemical techniques, and through polymerase chain reaction assays.

4.2.1 Biochemical Evaluation

Samples were prepared for biochemical analysis as described in Section 3.5. DNA, GAG, and hydroxyproline concentrations were converted to weights and presented as a value normalized to the control groups.

DNA content results (Figure 4.11) showed that varying doxycycline concentrations had no significant effect on DNA content ($p=0.62$). Upon evaluation of glycosaminoglycan content (Figure 4.12), proteoglycan content also did not vary significantly between groups ($p=0.54$). Statistical analysis did indicate, however, that hydroxyproline content was significantly affected by doxycycline concentration ($p<0.05$). Post-hoc testing showed that at low concentrations of doxycycline (5 ng/ml), hydroxyproline content was reduced significantly ($p<0.05$) when compared to the control group (Figure 4.13). At medium and high concentrations of doxycycline (10 ng/ml and 20 ng/ml, respectively) however, there was no noticeable effect on hydroxyproline content when compared to control.

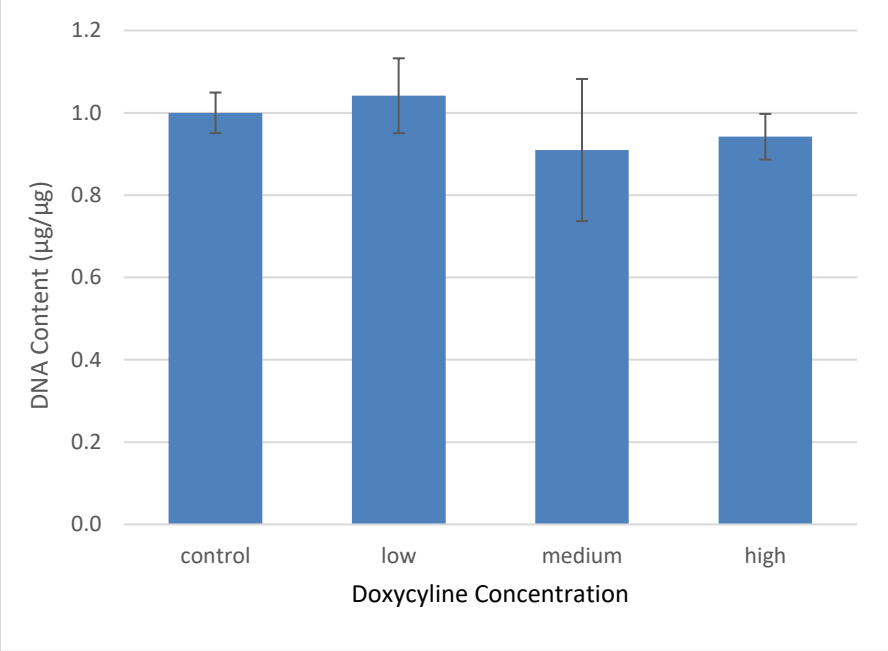


Figure 4.11 Effect of Doxycycline Concentration on DNA Content. Data is presented as means ± SEM, normalized to control ($p=0.62$), n=10-11 per group.

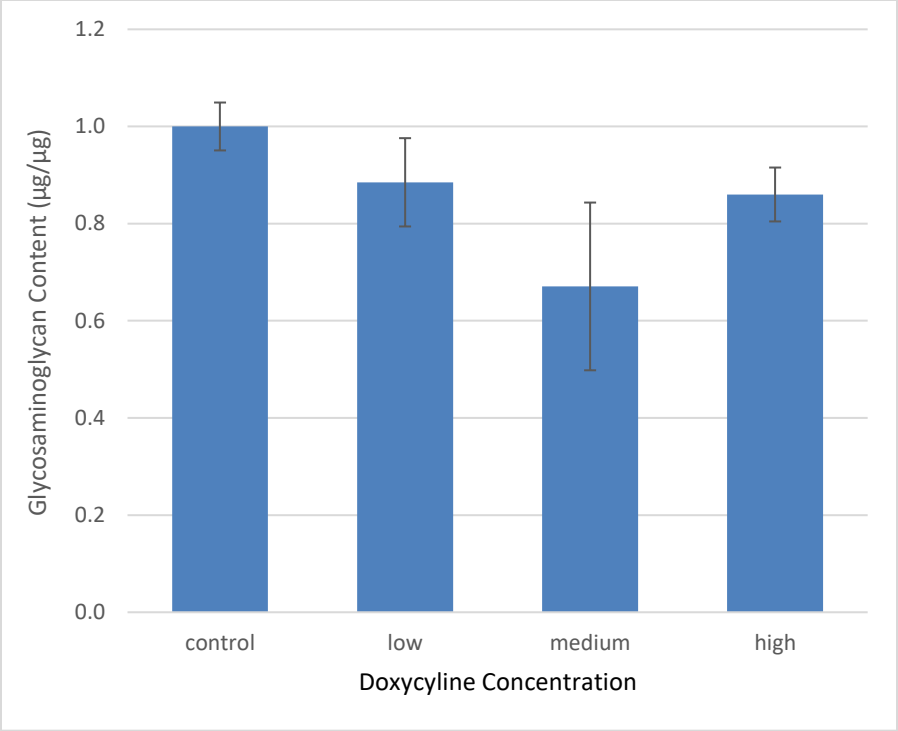


Figure 4.12 Effect of Doxycycline Concentration on Glycosaminoglycan Content. Data is presented as means ± SEM, normalized to control (p=0.54), n=10-11 per group.

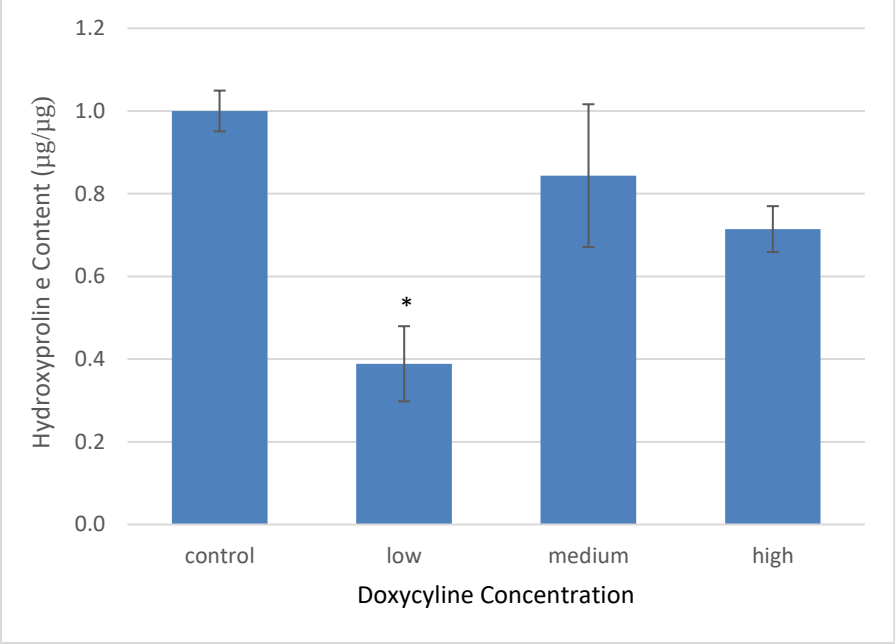


*Figure 4.13 Effect of Doxycycline Concentration on Hydroxyproline Content. Data is presented as means ± SEM, normalized to control (p<0.05), n=10-11 per group. * – denotes a significant difference between the low concentration of Doxycycline and the control (p<0.02).*

4.2.2 Histological Evaluation

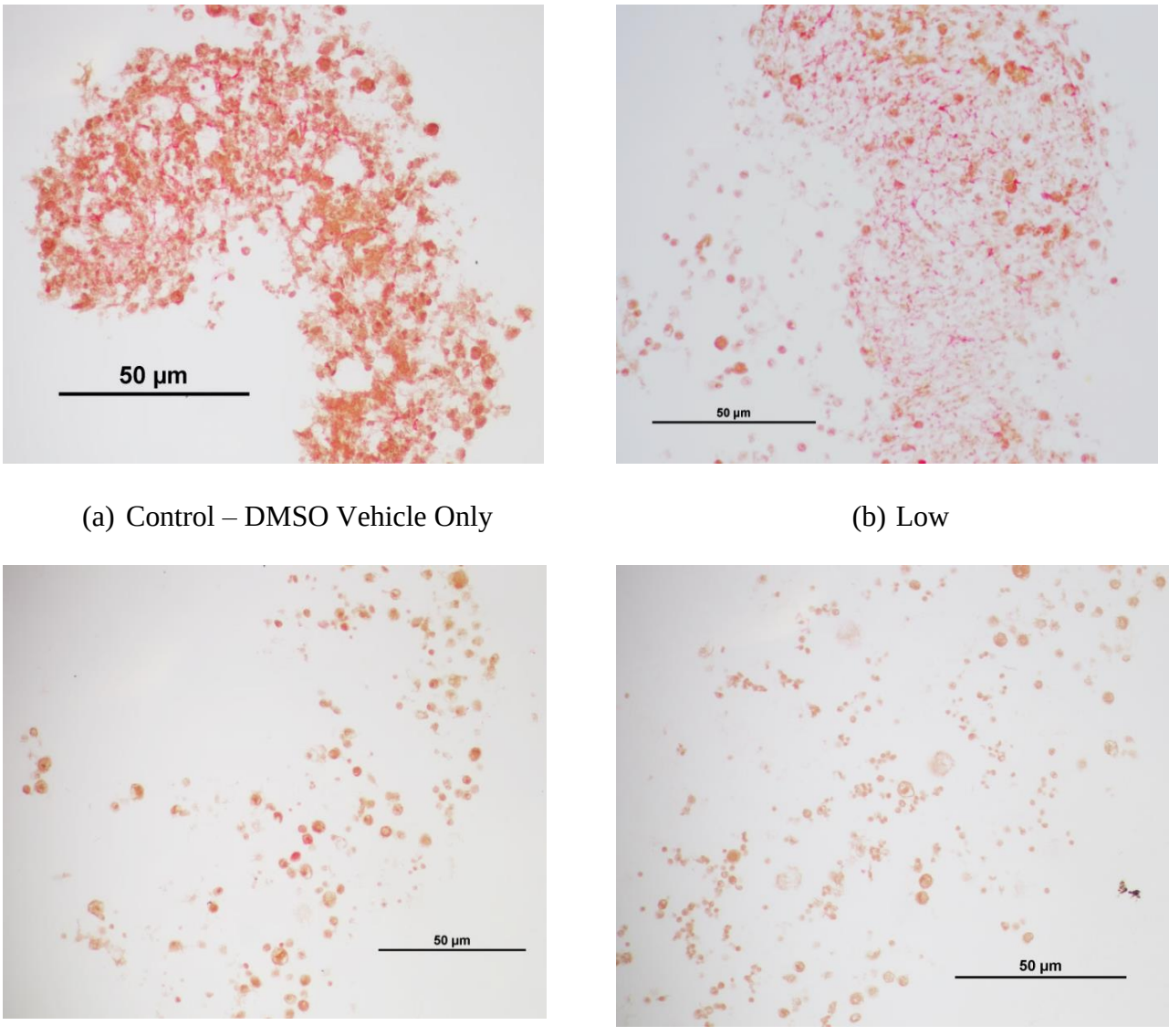
Doxycycline treated samples were fixed and processed for histological evaluation and stained with sirius red, safranin O, or Von Kossa stains as described in Section 3.6.

Sirius red staining (Figure 4.14) indicated present but low collagen content in the control sample. Low doxycycline concentration (5 ng/ml) samples also stained positive for collagen in low amounts, however not as intensely as the control group. Medium and high concentration doxycycline groups (10 ng/ml and 20 ng/ml, respectively) showed no positive staining for collagen content.

When stained with safranin O (Figure 4.15), the doxycycline control group showed moderate intensity blueish-grey staining from the fast green counterstain. This is indicative of background protein contents that are non-glycosaminoglycans (such as collagen). Medium and high doxycycline concentration samples were absent of bluish-grey background staining. The low doxycycline concentration group sample contains one cluster of cells with low intensity positive staining for safranin O, however, the remainder of the sample shows cell nuclei with very little connectivity between adjacent nuclei, with the exception of some mild bluish-grey staining, thus indicating low but present levels of non-GAG protein content. No doxycycline dosed samples, including the control, showed positive safranin O staining for proteoglycans. This pattern of absent glycosaminoglycan staining supports the biochemical data which showed no significant statistical differences in GAG content between groups. Similarly, a lack of bluish green background supports negative presence of collagen by sirius red staining.

In order to check for potential mineralization of samples, Von Kossa staining was also performed (Figure 4.16). Doxycycline sample groups were all negative for mineralization, which would be indicated by intense black staining. The Von Kossa staining was counterstained with a

hematoxylin and eosin nuclear stain. The dispersion of nuclei appears similar between slides in all three staining procedures performed for the purpose of this study, which supports biochemical analysis data that there was no significant statistical difference in DNA content between the low, medium, or high doxycycline dosed groups, nor the control group.



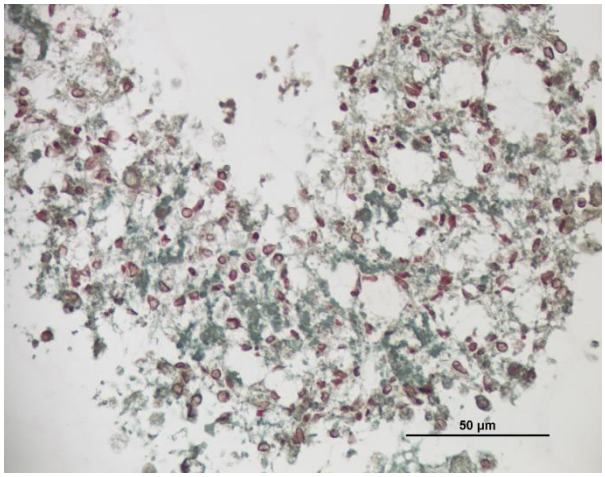
(a) Control – DMSO Vehicle Only

(b) Low

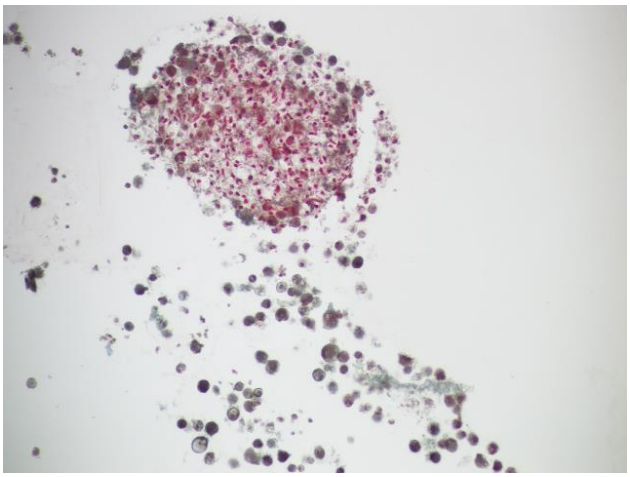
(c) Medium

(d)High

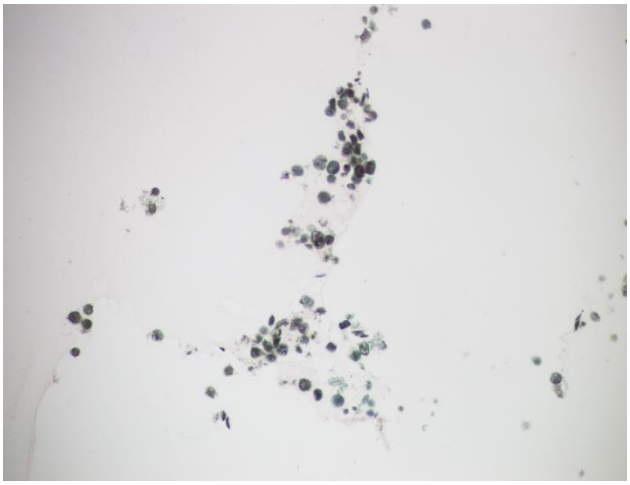
Figure 4.14 Histological Sections of Doxycycline Treated Pellet Samples Stained with Sirius Red to Visualize Collagen Staining



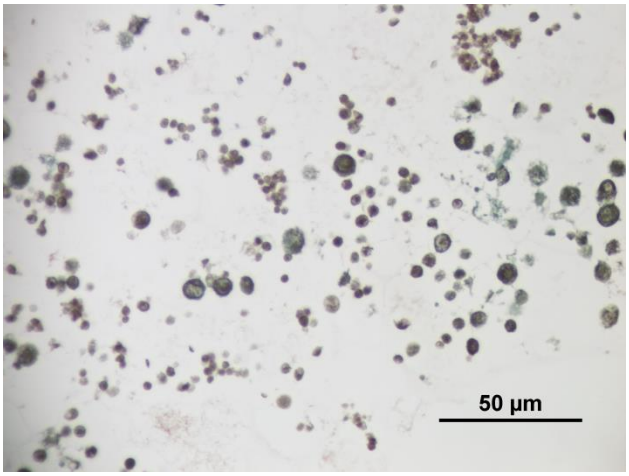
(a) Control – DMSO Vehicle Only



(b) Low

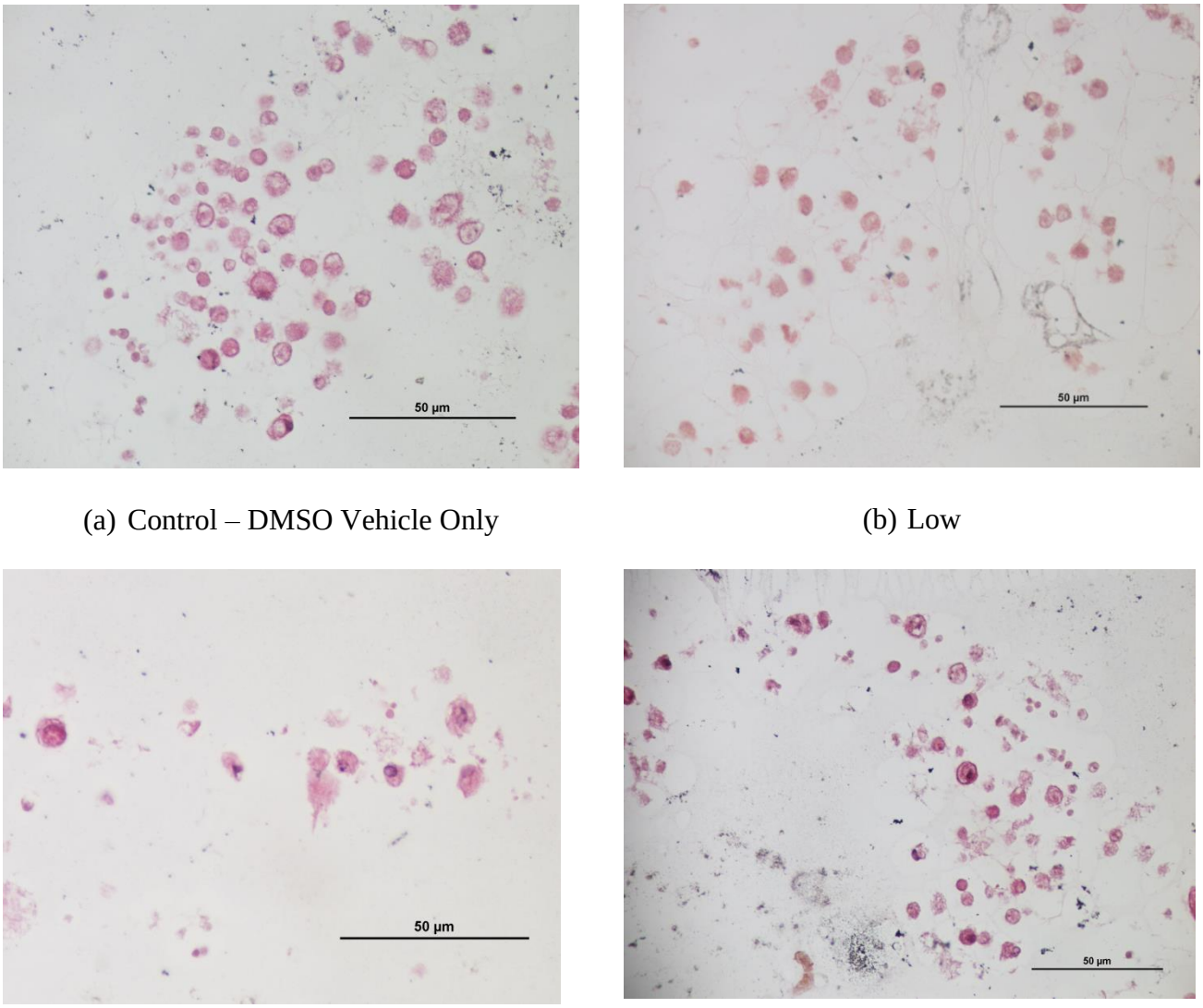


(c) Medium



(d) High

Figure 4.15 Histological Sections of Doxycycline Treated Pellet Samples Stained with Safranin O to Visualize Proteoglycan Staining



(a) Control – DMSO Vehicle Only

(b) Low

(c) Medium

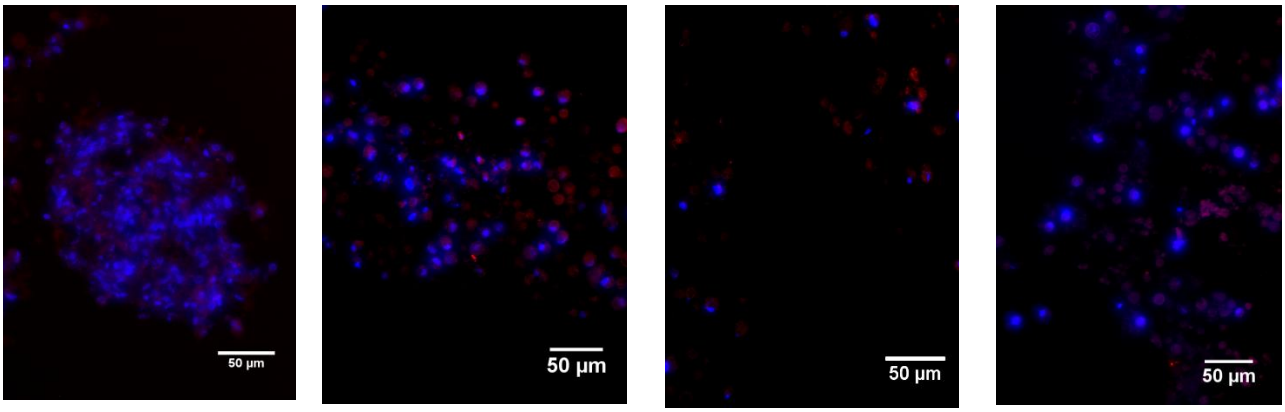
(d) High

Figure 4.16 Histological Sections of Doxycycline Treated Pellet Samples Stained with Von Kossa Staining and Counterstained with H&E to Visualize Mineralization

4.2.3 Immunohistochemical Analysis

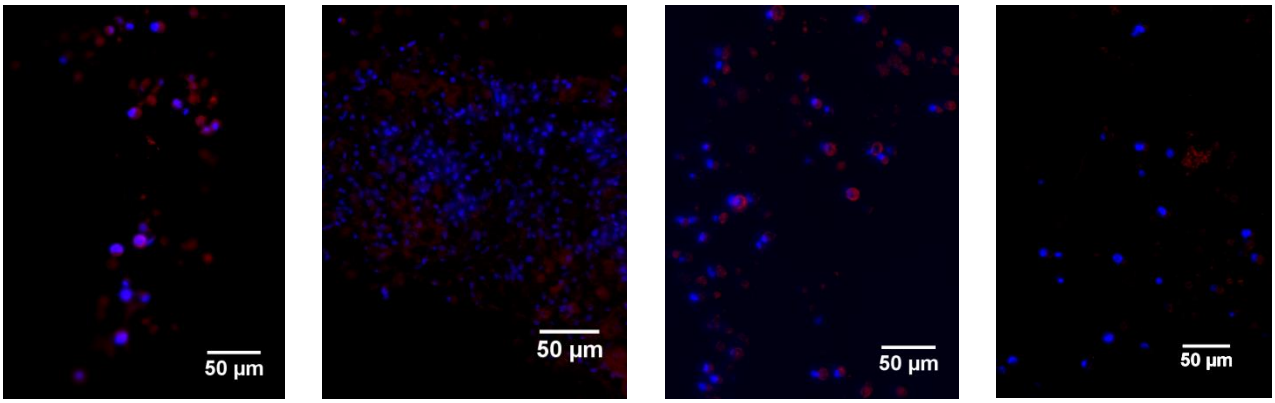
Doxycycline dosed samples were prepared for immunohistochemical evaluation as described in Section 3.7. After incubating histological sections with primary and secondary antibodies for collagen I and collagen II, samples were counterstained with DAPI to visualize cell nuclei.

Both collagen I and collagen II immunostaining revealed low collagen content in all samples. As described in Section 4.1.3, red dots appearing in Figures 4.17 a-d and Figure 4.18 a-d are likely intracellular staining for collagen and do not indicate extracellular collagen-containing matrix. Immunostaining supports histological images of safranin O and sirius red staining, both which indicated low collagen content in most samples.



(a) Control (b) Low (c) Medium (c) High

Figure 4.17 Immunohistochemical Sections of Doxycycline Treated Pellet Samples Stained for Type I Collagen



(a) Control (b) Low (c) Medium (d) High

Figure 4.18 Immunohistochemical Sections of Doxycycline Treated Pellet Samples Stained for Type II Collagen

4.2.4 End Point Polymerase Chain Reaction (PCR) Analysis

Expression of target genes in doxycycline dosed samples was evaluated as described in Section 3.8. As seen in Figure 4.19, volume analysis of gene expression bands (Figure 4.8) was performed using Image Lab™ software, and target gene expression data was normalized to the housekeeper gene. Analysis revealed that in the control group, collagen type I expression was similar to expression of collagen type II. The low concentration sample evaluated expressed high amounts of collagen type I and II, but expressed more than twice the amount of collagen type II then collagen type I. Medium and high concentration groups expressed extremely low amounts of collagen I; however, these groups did express moderate amounts of collagen II. Aggrecan expression was low in all groups with the exception of the low concentration sample, and *SOX-9* and *RUNX2* expression remained low across all groups (Figure 4.19).

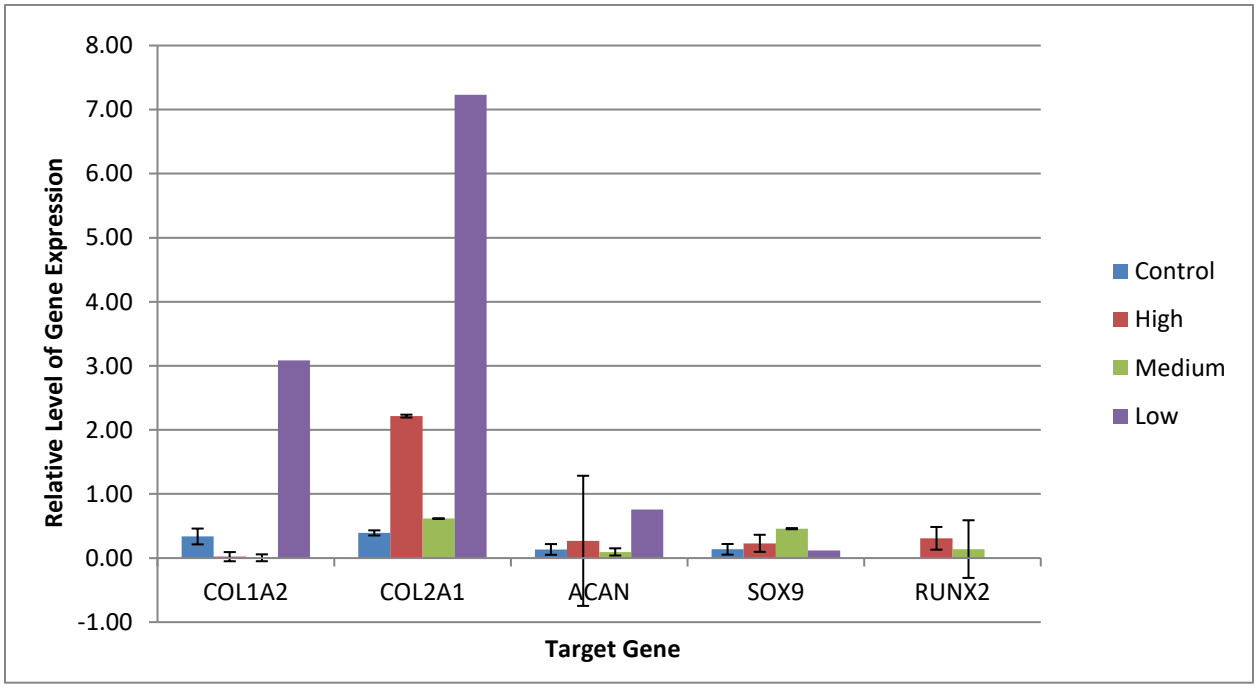


Figure 4.19 End Point PCR Relative Gene Expression Levels of Target Genes of Doxycycline Dosed Samples normalized to GAPDH (housekeeper) Gene Expression Levels. Data is presented as means ± SEM, normalized to GAPDH.

5.0 Discussion

The aim of this research was to differentiate rabbit bone marrow-derived mesenchymal stem cells towards a chondrogenic phenotype in the absence of making tissue. By doing so, the possibility of using this pre-differentiated cell suspension in an injectable IVDD therapy can be investigated in future work. The lack of solid or solid-like tissue is the first step in creating a cell suspension of low viscosity for future exploration of creating an inviscid injectable of chondrogenically differentiated, highly proliferative stem cells capable of producing matrix components. Normally, chondrogenic differentiation of stem cells results in the development of tissue rich in proteoglycans and collagen type II. The accumulation of collagen would lead to solid-like behavior of the tissue construct [80]. For this reason, the effect of doxycycline and collagenase A was investigated to remove or inhibit collagen formation within the cultures during differentiation. This would allow for the removal of extracellular matrix components while the cells remain chondrogenically differentiated.

5.1 Effect of Collagenase A on Extracellular Matrix formation

The collagen content of samples from the low, medium, and high concentration collagenase A treated groups were all significantly lower than the collagen content of the control group (Figure 4.3). This is supported by the absence of positive sirius red staining for collagen in the histological images of collagenase A treated cultures, whereas the control group did show some very mild sirius red staining of collagen fibers (Figure 4.4). Immunohistochemical staining for collagen type I and collagen type II also confirm the lack of collagen in all the collagenase A treated groups (Section 4.1.3). The concentration of collagenase A had little effect on extent of diminishing collagen content in the samples. When normalized to the control data, all concentrations had

similar amounts of collagen, indicating that even the minimum collagenase A concentration of 100 μU/mL was sufficient to significantly decrease the collagen content.

As expected, proteoglycan content was unaffected by collagenase A and remained stable between all groups (Figure 4.2). As collagenase A cleaves collagen fibers, it was not expected that the enzyme would have a profound effect on proteoglycan content. Staining for proteoglycans by Safranin O was negative in all collagenase A treated samples analyzed histologically, including the control group (Figure 4.5). The lack of proteoglycan staining in the collagenase A treated samples is likely due to the low collagen content. Aggrecan is comprised of large sulfated glycosaminoglycan chains consisting of chondroitin sulfate and keratan sulfate, which due to its size is constrained by the system of collagen fibers in the extracellular matrix [103-105]. As the collagen content is diminished by collagenase A, proteoglycans have no means of being retained within the developed ECM and were most likely released to the medium. Furthermore, loose, un-bound proteoglycan chains could have been displaced upon the addition of Histogel™ to the construct during preparation for histological processing.

The total lack of positive safranin O staining in the control sample is not necessarily indicative of an absence of aggrecan within the extracellular matrix, but rather may be a characteristic of low proteoglycan content. A previous study showed that immunostaining for keratan sulfate and chondroitin sulfate yielded positive data in several samples estimated to have low proteoglycan contents, while serial sections of the same specimens were completely devoid of positive safranin O staining [97].

It has also been shown that the use of formalin-saline as a fixative for histology can cause 20-30% of the glycosaminoglycan content to be leached out of intervertebral disc tissues during the fixation process [106]. The composition and salt concentration of buffered formalin-saline is

essentially identical to the 4% paraformaldehyde in phosphate buffered saline solution used in this research. Low proteoglycan content below the sensitivity threshold of safranin O would only be further diminished by proteoglycan extraction by salinated formaldehyde solutions, thereby offering a potential explanation as to why proteoglycan staining was absent from the control (untreated) cultures.

5.2 Effect of Doxycycline on Extracellular Matrix Formation

Biochemical analysis of the doxycycline treated groups and the control group showed no significant differences in proteoglycan content of samples (Figure 4.12). Analysis of hydroxyproline content (Figure 4.13), however, indicated that only the low concentration doxycycline treated group had statistically lower hydroxyproline content than the control group. In addition, type II collagen immunostaining (Figure 4.18) was also mildly positive in the low and control groups, and both samples showed positive sirius red staining for collagen. Safranin O/Fast green histological staining of the low and control group also returned positive results for collagen staining. Those pellets supplemented with medium and high concentrations of doxycycline showed no positive staining for collagen in neither the histological nor the immunohistochemical analysis. It can therefore be concluded that doxycycline did have an effect on collagen content, however the effect was independent of the concentrations used in this research.

From histological evaluation (Section 4.2.2) it is evident that the low doxycycline group contains collagen, albeit in low amounts, and the low concentration of doxycycline is therefore insufficient to fully inhibit collagen synthesis. Sirius red staining is less intense in the low group than it is in the control group indicating that the collagen content is indeed decreased somewhat by the low concentration of doxycycline added into the feeding medium. Positive safranin O

staining is present in one area of the pellet where sirius red collagen staining is similarly intense, suggesting that the region is chondrogenic

It is still unknown how doxycycline inhibits collagen formation, but the inhibitory effect of doxycycline on MMPs has been extensively studied [72, 107]. Due to the low SOX-9 gene expression and the fact that chondrogenesis seemed to be somewhat achieved in the low concentration pellet, the mechanism of action by which doxycycline is inhibiting collagen fibril formation in the higher doxycycline concentration groups may be through it's known ability to inhibit MMPs. A study using MMP inhibitors while chondrogenically differentiating MSCs found that the proteinase inhibitor hydroxamate suppressed chondrogenesis in their cultures. Stem cell pellets undergoing chondrogenic differentiation lacked in proteoglycans and collagen type II. The study determined the results were not due to toxicity of the hydroxamate, but rather that the presence of matrix metalloproteinases is somehow crucial to chondrogenic differentiation [108]. The results of this study are not unlike ours, in which histological collagen and proteoglycan staining was negative in groups cultivated with doxycycline.

Doxycycline has been found in some cases to inhibit matrix metalloproteinases, thereby allowing collagen content to improve. Bedi et al. [109] showed in their study that administration of doxycycline to rats which were surgically given rotator cuff tears lead to better collagen organization and strength upon healing when compared to untreated controls. The researchers found that doxycycline effectively decreased matrix metalloproteinase-13 activity [109]. Histological data (Figures 4.14 and 4.15) showed negative staining for collagen and proteoglycans in the high and medium concentration groups, indicating that collagen content was quite low. In the low concentration group however, expression of collagen II and aggrecan was much higher,

suggesting that at a concentration higher than 5 ng/ml, doxycycline might exhibit an inhibitory effect on chondrogenesis or collagen synthesis.

Low extracellular matrix formation was evident throughout the histological and biochemical analysis of the study. Although several mechanisms by which this may have resulted have thus far been presented, it is important to consider if technical protocol played some factor. During feeding, the pellet cultures were constantly disturbed by fluid turbulence. The inability for the cells to form stable interactions leads to poor chondrogenic differentiation and therefore low extracellular matrix protein synthesis. Pelleting mesenchymal stem cells is believed to promote chondrogenesis as it mimics the *in vivo* process whereby stem cells condense and aggregate prior to differentiating into chondrocytes and forming cartilage tissue [110-112]. Tacchetti et al. [110] highlight that this step allows for direct cell-to-cell contact [110-112]. Studies have shown that growing cells in hydrogels rather than pellet cultures better supports chondrogenesis – and therefore GAG and collagen deposition – as hydrogels help cell cultures to hold their shape, allowing for optimal cell-to-cell and cell-to-matrix interactions [113-115].

5.3 Effect of Collagenase A and Doxycycline on Chondrogenic Gene Expression

While the polymerase chain reaction (PCR) data for the presented study was limited, collected data showed evidence of positive chondrogenic gene expression in the differentiated cultures with relative high expression of both collagen II and aggrecan and relative low expression of collagen I. Treatment with either collagenase A or doxycycline did not appear to overly influence the extent of chondrogenic gene expression (Figure 4.10 and Figure 4.19), as all treated groups regardless of concentration showed similar trends in gene expression levels.

As seen in Figure 4.19, the low, medium, and high doxycycline supplemented groups exhibited a similar trend in gene expression of common markers of differentiation. Each group

showed high collagen II gene expression, lower expression of collagen type I, and low expression of aggrecan. The end point PCR data suggests that varying the concentration of doxycycline did not have an effect on chondrogenic potential as *SOX-9* expression is low in all samples and taking into account error, cannot be concluded at this time to be different between the varying concentrations of doxycycline and the control group.

The analysis also showed that aggrecan gene expression was relatively low in both the collagenase A and doxycycline treated groups. Low gene expression of aggrecan could be a result of the diminished collagen content in the cultures. In a previous study, Bosnakovski et al. [114] compared the effects of culturing MSCs with or without chondrogenic medium in three different hydrogels made of alginate, collagen I, or collagen II. Overall, the study found that the type II collagen hydrogels in combination with chondrogenic medium best supported chondrogenesis of mesenchymal stem cells, including an increase in aggrecan gene expression. The authors explain that the presence of collagen type II in the matrix influences the cells to express chondrogenic genes, as chondrocytes *in vivo* signal each other via collagen II ligands [114].

Interestingly, the expression of *RUNX2* was higher than the *SOX-9* gene expression in the control groups. While these groups were cultured in chondrogenic medium, they did not receive treatment with either collagenase A or doxycycline. *RUNX2* is a transcription factor expressed during bone formation and has also been found to regulate chondrocyte hypertrophy and collagen X deposition, thus making it an indicator of osteogenesis [116, 117]. Mesenchymal stem cells undergoing chondrogenic induction often become hypertrophic [118-120].

In order to ensure these cultures were not undergoing osteogenic differentiation, Von Kossa staining for mineralized tissue was also performed. The staining was negative for calcium deposits in all samples (Figures 4.6 and 4.16). A similar trend was also observed by Pittenger et al. [121],

when human mesenchymal stem cells were cultured in three different media formulations aimed at differentiating the cells towards an adipogenic, osteogenic, or chondrogenic phenotype. Real time-PCR analysis showed that neither the adipogenic nor the osteogenic cultures expressed genes that would mark for the other two phenotypic lineages, however, the cells grown in chondrogenic medium showed positive expression of osteogenic genes without indicating in the histological assessment any signs of osteogenesis [121] .

It is important to consider whether the expression of *RUNX2* alone suggests osteogenic differentiation. Commonly, stem cells in chondrogenic pellet cultures progress into hypertrophic chondrocytes, which subsequently undergo osteogenesis [118-120, 122]. Moreover, type I collagen is also an indicator of osteogenesis as the extracellular matrix secreted during osteoblast differentiation is largely made up of type I collagen [120, 122-124]. While the control cultures expressed similar levels of collagen I and II gene expression, neither the histological or immunohistochemical analyses could support this. Sirius red staining is not a suitable method to distinguish collagen type [125] and immunohistochemical analyses for collagen I tended to have high background staining making it difficult to distinguish the presence of collagen I deposition.

If the cells in this study indeed became hypertrophic, this could also help to explain the lack of positive staining and gene expression for aggrecan. However, without assaying for collagen X deposition or gene expression, it is difficult to know if the MSCs in this study became hypertrophic chondrocytes as *RUNX2* expression and collagen I deposition only indicate if the cells progressed from hypertrophy to osteogenesis.

Interestingly, collagenase A treatment appeared to further decrease the collagen I gene expression compared to the control group which exhibited similar levels of collagen I and II expression. The same effect was observed with doxycycline treatment, where the control group

expressed similar levels of collagen I and II gene expression, yet treated groups expressed higher levels of collagen II than collagen I. Collagenase A and doxycycline treated samples appeared to be more terminally differentiated – that is to say not progressing on to osteogenesis – as evidenced by the lower expression of collagen I and *RUNX2* genes in treated samples versus the control. Immunohistochemistry data (Figures 4.8 and 4.18) further supports that collagen I deposition did not occur in these samples. Chondrogenic differentiation of MSCs dictates that collagen II content increases overtime while collagen I content wanes, unless cells progress to hypertrophy and subsequently to osteogenesis – as they often do – where the collagen I gene will be expressed [126]. This suggests that the doxycycline and collagenase A treatments may have successfully led to terminally differentiated stem cells, and hindered the commonly observed progression from chondrogenic cells to hypertrophic, pre-osteogenic cells.

5.4 Effect of Doxycycline and Collagenase A on DNA Content

The DNA content of cultures treated with either collagenase A or doxycycline did not differ statistically between the control and treated groups (Figures 4.1 and 4.11). This indicates that treatment with doxycycline up to 20 ng/mL will not influence cellular death, which was demonstrated in a previous study [127]. Similarly, it can also be concluded that collagenase A up to a concentration of 10 mU/mL will not cause cell death. Histological data, although the cultures were relatively acellular in general, supports this notion as the cellularity of the samples were relatively consistent amongst all groups (refer to Sections 4.1.1 and 4.2.1). The low DNA content may be due to inherent limitations of pellet culture, which has been shown in the past to result in a reduction in DNA content and can result in cell death, especially in the center of the pellet [111, 128-130].

5.5 Limitations of the study

It is important to note the factors which lead to limited extrapolation of trends from the data. For histological images, only one pellet was used from each concentration and control group in order to obtain serial sections. Variability between donors is possible, leading to differences in the cells' ability to proliferate or express proteins. Similarly, polymerase chain reaction data was based on only one or two pellets, and in the case of the low concentration collagenase A group, gene expression could not be measured at all. The concentration of cDNA in the reaction had to be increased from 1% of the reaction volume to 4% of the reaction volume due to challenges in amplifying sufficient cDNA in order to obtain results in the acrylamide gels. This was due to low quantities of RNA per sample. As a result, the acrylamide gels showed high amplification of noise due to non-specific binding of the primer sequence. It should therefore be noted that the background values obtained for the PCR results were inflated, which in turn lowered the adjusted output volumes of the bands which were reported in the relative gene expression data. The PCR data is therefore indicative of trends but may not reflect the actual gene expression values of the samples. Furthermore, gene expression and histological data is only based on one time point, the end of the 28 day culture period. Harvesting pellets at different time points in the study would allow for comparison in gene expression levels as the culture progresses, as well as visualization of changes in collagen degradation or aggrecan content over the duration of the study. This would be especially helpful in determining changes that occur after the first week, once doxycycline and collagenase A have been added to the culture media for the subsequent 3 weeks.

The feeding method employed in this study often resulted in media turbulence which caused areas of the pellet to detach. Although pellets were left to settle, it is unknown how this detachment might affect the ability of the pellets to undergo chondrogenic differentiation. Studies

utilizing pellet culture to chondrogenically differentiate mesenchymal stem cells describe that the pellet hardens or condenses to a spheroid automatically during the culture period due to the high degree of cross-linking of extracellular matrix proteins [131]. This was not the case in our presented research, likely because the collagenase A and doxycycline led to collagen degradation or inhibition of fibril formation, thereby preventing the extracellular matrix from forming and holding the pellet together. In the pellet treated with the low doxycycline concentration, histological evaluation showed one region that appeared chondrogenic (Figure 4.15 b), so its position in a location that allowed it to be less disturbed may have helped it undergo chondrogenesis.

6.0 Conclusions and Recommendations

6.1 Conclusions

The primary aim of the study was to develop a means to differentiate stem cells towards a nucleus pulposus phenotype while limiting collagen synthesis and accumulation with doxycycline and collagenase A, respectively. Solid-like behavior can be attributed to the accumulation of collagen. Thus, in order to obtain a cell suspension suitable for future application as an injectable therapy, the tissue must be unconstrained by collagen cross-linking or entanglements.

The study showed that collagenase A successfully reduced collagen content in pellet cultures cultivated in chondrogenic medium when compared to the control. Collagen content was unaffected by the concentration of the enzyme, and thereby the minimum collagenase A concentration of 100 µU/mL is sufficient to degrade collagen in the samples. DNA content was stable between collagenase A groups and untreated controls, so it can be concluded that concentrations up to 10 mU/mL will not cause cell death. Proteoglycan content also remained stable between all groups, indicating collagenase A concentration had little effect on proteoglycans, but histological staining showed the proteoglycan concentration in samples was extremely low. Glycosaminoglycan chains are constrained by collagen in healthy tissues, and therefore low proteoglycan content observed in this study can be attributed to the cleavage of the collagen fibres.

Doxycycline treatment at low concentrations (5 ng/mL) was found to be insufficient to fully inhibit collagen formation, as is evidenced by collagen deposits seen in histological images. At higher doxycycline concentrations (10-20 ng/mL), however, histological evaluation showed no evidence of collagen accumulation. Biochemical analysis showed no significant differences between collagen content in the high and medium concentration groups, which appears to be

contradictory to the histological data. The mechanism by which doxycycline works is not yet fully understood, and for the purposes of this study supplemental histological evaluation should be performed. Doxycycline also had no effect on proteoglycan content, and DNA content between the control and treated groups was similar, indicating that up to 20 ng/mL, doxycycline does not result in cell death.

Polymerase chain reaction data showed similar trends between all treated groups when compared to controls. Collagen II gene expression was higher than that of collagen I and aggrecan. Control groups however both showed similar levels of collagen II and collagen I gene expression, and *RUNX2* expression was highest in the collagenase A control group. Collagenase and doxycycline treated samples therefore appear to be more terminally differentiated than controls groups. This suggests that treatment with collagenase A or doxycycline may stop the commonly observed progression of chondrogenic MSC cultures to hypertrophic chondrocytes, and then to osteogenic cells.

In conclusion, this research showed that collagenase A and doxycycline treatment can successfully reduce collagen content in mesenchymal stem cells in chondrogenic induction culture. The results appear to indicate that a cell suspension devoid of typical solid-like tissue formation can be created, thereby making it suitable for use in future development of an nucleus pulposus injectable therapy.

6.2 Recommendations

Although the work presented showed promise, several recommendations to enhance future studies can be made.

First, in order to better understand the mechanism by which doxycycline reduces collagen content, media assays to quantify aggrecan and hydroxyproline content in the media would be

beneficial. By performing these experiments, we can better understand if collagenase indeed just cleaves collagen fibers whereas doxycycline completely inhibits their formation. Studies of aggrecan content in conditioned medium would also confirm if aggrecan is being produced by the cells and released to free medium because of the lack of collagen structure.

In order to confirm speculation that doxycycline and collagenase A led to terminally differentiated cells, cultures should be tested for hypertrophic and osteogenic markers such as collagen X and alkaline phosphatase through gene expression studies or immunohistochemistry.

Moreover, histological and polymerase chain reaction data in this study is limited to only 1 or 2 samples. On such a small sample size it is difficult to be completely confident in observed trends. Sample sizes should be increased, and a PCR method which is more sensitive to lower levels of mRNA, such as Digital Droplet, to counteract the issues ran into in this study should be used. Further, histology and PCR studies are from a single time point in the culture. In order to understand how methods are affecting samples as growth progresses, PCR data would be better presented as a fold increase in relative gene expression levels between various points in the study. A similar notion can be extended to histological data.

It is also recommended that pellet cultures should stop being treated with doxycycline and collagenase A, and growth should be continued afterward to understand if cells are capable of matrix synthesis once treatment has ended. This can be applied *in vivo*, and would further understanding of whether the presented injectable therapy will increase disc height, restore matrix proteins critical for healthy disc function, and if MSCs were properly conditioned to survive the harsh microenvironment of the nucleus pulposus. In order to truly understand if the cell cultures presented in this study,

7.0 References

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