OPTIMIZATION OF MEDIA TO ENHANCE THE GROWTH OF TISSUE ENGINEERED CARTILAGE

By

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Optimization of Media to Enhance the Growth of Tissue Engineered Cartilage, Andjela Bajic, Master of Applied Science, Biomedical Engineering, Ryerson University, 2019

Abstract

Unlike other self-repairing tissues, cartilage has a very low regenerative capacity, thus, giving reason to examine different approaches to potential reparative therapies such as tissue engineering. Although once chondrocytes are placed *in vitro* they start to synthesize less cartilaginous extracellular matrix (ECM). A promising method used to upregulate the synthesis of ECM constituents is new media formulations. Thus, the objective of this study was to explore different media formulations to upregulate the accumulation of cartilaginous extracellular matrix (specifically, proteoglycans and collagen) by providing the cells with different availability of nutrients (e.g. glucose, glutamine) as well as examining the influence of different basal media formulations. The accumulation of GAG and collagen had two different media formulations which showed a significant increase in upregulation of each constituent in the ECM; highlighting the importance of having new media formulations specifically geared to each constituent.

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Chapter 1: Introduction

1.1 Articular Cartilage, Damage and Degradation

Articular cartilage is a thin layer of tissue that covers the ends of articulating bones in order to provide a frictionless surface for joint motion. Articular cartilage can be damaged in degenerative osteoarthritis or trauma, causing pain, inflammation, and reduced range of motion [1]. Osteoarthritis is a chronic degenerative disease and is known as a classic age-related disorder; which involves the entire synovial joint, such as the cartilage, synovium, and the underlying bone [2]. In osteoarthritis, continuous destruction of the cartilage extracellular matrix takes place, leading to a progressive loss in joint function [2,3]. Additionally, destabilization of the collagen network and changes in the expression profile of the matrix molecules also takes place [3]. Osteoarthritis is one of the leading causes of disability in adults in the United States, with more than 10% of the entire population suffering from osteoarthritis [4].

1.2 Strategies for Potential Repair

Repairing damaged cartilage therapeutically is also challenging due to the lack of appropriate donor tissue. However, strategies that currently exist to repair cartilage include: (i) arthroscopic lavage and debridement, (ii) tissue grafts, and (iii) autologous chondrocyte transplantation [5]. The primary use of arthroscopic lavage and debridement is to alleviate joint pain [5]. Lavage is a saline solution that is introduced into the knee joint and its removal of fluid and inflammatory agents [6]. The removal of these inflammatory agents allows the inflammation in the

osteoarthritis area to subside which in turn alleviates the pain from the joint. Debridement involves enlarging the joint with saline and washing out the particles of articular cartilage debris [7]. After debridement, some patients report relief of pain in specific areas of the joint [8]. Numerous studies report that the combination of lavage and debridement has the potential to alleviate some of the symptoms, particularly pain, of early osteoarthritis [8]. Combining both procedures allows the alleviation of pain in earlier stages of osteoarthritis [9]. Furthermore, tissue grafting, involves fitting in osteochondral grafts (one or several cylindrical plugs of cartilage attached to bone) into the defect site [10]. Grafts are obtained from either the patient's own cartilage (autograft), in non-load-bearing regions, or from cadavers (allografts) [1,5]. Potential drawbacks with autogenic grafts would be the limited sources for harvesting autografts, as well as the potential of other healthy joint areas to be damaged [11]. Furthermore, drawbacks with allogenic grafts have the potential to elicit an immunological response, possible transmission of diseases can occur and potential cell death as a result of cryopreservation [11]. Autologous chondrocyte transplantation involves directly implanting cultured chondrocytes, obtained from a biopsy of the patient's non-load bearing region of articular cartilage, into the defect site [5]. As chondrocytes are grown in culture, a periosteal graft is taken from the medial tibia, and sutured over the defect site, and the chondrocytes are then injected under the periosteal flap [5]. Potential drawbacks of autologous chondrocyte transplantation are the additional causes of pain due to the surgical procedure need to obtain the periosteal flap as well as, damage to health cartilage due to the suture of the flap [12]. Further challenges include low mechanical stability since there is minimal physical stress, as well as the chondrocytes could disperse unequally in the lesion zone [12]. Even with several

methods of repair, there is currently no treatment that effectively restores the functionality of articular cartilage.

1.3 Cartilage Tissue Engineering

Tissue engineering is the development of biological replacements to restore, maintain or improve tissue function [1]. It is a strategy that has the capability to provide permanent solutions related to cartilage damage and repair. Furthermore, there are many potential areas of focus when it comes to tissue engineering, it is based upon three fundamental components: cell source, scaffolds and signaling [13].

In order to have successful cartilage tissue engineering, it requires the cells to be capable of experiencing chondrogenic differentiation upon treatment with specific biochemical factors [14]. Different cell sources for cartilage engineering models include mesenchymal stem cells, differentiated chondrocytes, and pluripotent stem cells [15]. Mesenchymal stem cells that are present in the joint environment infiltrate the damaged cartilage area which may regulate the regenerative capacity of the therapeutic cells [15]. Chondrocytes, however, are a more plentiful cell source, but have a limited proliferative potential [15]. Pluripotent stem cells (e.g. embryonic stem cells) represent a potentially unlimited source of chondrocytes, however, no specific protocol has been developed to provide a constant source of chondrocytes for tissue repair [14]. Scaffolds are of equal importance to the cell source, since they provide the structural framework which immobilizes cells in a 3D environment in order to maintain the differentiated phenotype of chondrocytes [14]. Scaffolds can be made from both synthetic and

natural polymers that are biocompatible [16]. Signaling factors have been used to accelerate and/or enhance cartilage formation [17,18]. Signaling factors includes cytokines, growth factors, and other regulatory molecules that regulate cellular metabolism [18]. Additionally, physical forces are important signaling factors that play a key role in load-bearing tissue to help develop and maintain the function of tissue [18].

Moreover, a hurdle that needs to be overcome for cartilage tissue engineering to be a viable repair strategy is to improve the growth of tissue *in vitro*, in the sense of extracellular matrix (ECM) accumulation. One of the major limitations for obtaining high-quality constructs is the low production of cartilage-specific ECM by chondrocytes [19,20].

1.4 Research Outline and Objectives

The purpose of this study was to explore different media formulations to upregulate the accumulation of cartilaginous extracellular matrix (specifically, proteoglycans and collagen) by providing the cells with different availability of nutrients (e.g. glucose, glutamine) as well as examining the influence of different basal media formulations.

Using response surface methodology, the first study explored nine different media formulations and their effects on the biosynthetic response of chondrocytes seeded in 3D culture. The objective was to understand how each media type influenced the accumulation of proteoglycans and collagen; the two major constituents of the cartilage extracellular matrix [21]. After analysis, the potential optimal values of each design factor (glucose concentration, glutamine concentration, media volume and basal media) were determined. The second study explored the previously determined optimal values with the objective of validating the specific media formulations to elicit maximal cartilaginous tissue growth.

Chapter 2: Literature Review

2.1 Structure and Function of Articular Cartilage

Articular cartilage is the tissue covering the surface of contacting joints (Figure 2.1), which provides a gliding surface for the bones to articulate on. Even though the structure of articular cartilage is complex, it is primarily composed of a dense extracellular matrix (ECM). Encapsulated within the ECM are a population of specialized cells called chondrocytes [21,23]. The ECM is primarily composed of water, collagen, proteoglycans and other non-collagenous proteins [23]. Furthermore, articular cartilage has four distinct zones, each with a unique composition and organization allowing for a complex functional role [24]. Articular cartilage is also an avascular tissue such that the resident chondrocytes rely primarily on diffusion/convection from synovial fluid for nutrition [21]. Synovial fluid functions both as a biological lubricant of the joint as well as a biochemical pool which allows nutrients to support cartilage homeostasis [25].

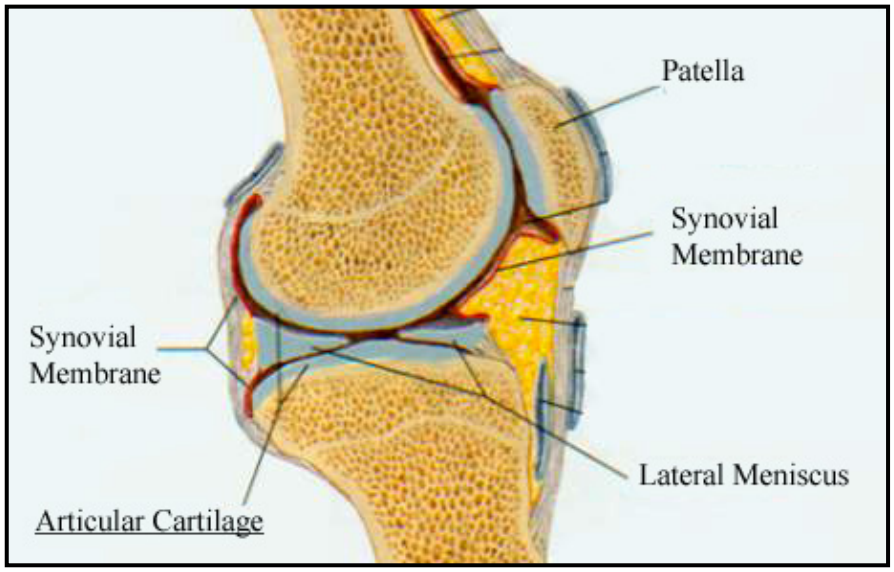

Figure 2.1 Cross-section of a knee joint [22]

2.1.1 Extracellular Matrix

The ECM is composed primarily of type II collagen and very large networks of proteoglycans, collagens and water [21,26], with a collagen to GAG ratio in the ECM of approximately 3:1 [27]. Other non-collagenous structural proteins, lipids, phospholipids and glycoproteins are present in the ECM at lesser amounts [21]. Due to the ECM having a diverse nature, it can have many functions, such as providing support and regulating intercellular communications. Articular cartilage ECM plays a vital role in regulating chondrocyte functions with cell-matrix interaction, and providing nutrients for the tissue [27].

2.1.1.1 Water

Water is the major component of articular cartilage, accounting for 70-80% of the total wet weight [23,28,29]. Additionally, the superficial zone contains about 80% water, which declines to 65% water in the deep zone of cartilage [21]. Therefore, water within the matrix could potentially influence the mechanical properties of the tissue [23]. Frictional resistance against interstitial fluid release is very high because the ECM is dense and composed of charged molecules [21]. Interfibrillar water, as it moves through the ECM, applies a pressure gradient across the tissue [21]. The combination of frictional resistance to water flow and the pressurization of water within the matrix allows articular cartilage to withstand significant compressive loads [21,23]. Water also serves as a vehicle for transporting dissolved ions such as sodium, calcium, chloride and potassium to chondrocytes, which are required for cellular homeostasis [21].

2.1.1.2 Collagen

Collagen is the most abundant structural macromolecule in the ECM, and it makes up about 60% of the dry weight of cartilage [21,30]. Type II collagen is the predominant collagen type, which accounts for 90-95% of the total collagen in articular cartilage [21,23,30,31]. Collagen consists of three polypeptide chains (α-chains) wound into a triple helix [21,31]. The specific roles of collagen type II fibrils is that they provide tensile strength and maintain the integrity of articular cartilage by forming a network that resists swelling pressure resulting from the hydration of proteoglycan aggregates in the extracellular matrix [32]. Type II collagen typically organizes itself by aligning head to tail, and side by side to form fibrils that contain overlaps and holes. Collagen II fibrils have different orientations with the distinct zones of the tissue [31]. In the superficial zone the fibrils are parallel to the surface, the middle zone is less organized, whereas the deep zone is perpendicular to the surface of the joint [31]. It should be noted that other collagen types (e.g. I, IV, V, VI, IX, XI and X) are also present but to a much lesser extent compared to collagen II in the cartilaginous ECM [21,23,30].

2.1.1.3 Proteoglycans

Proteoglycans represent the second largest group of macromolecules in the ECM and account for 10-15% of the wet weight [21]. They consist of a protein core and one or more linear glycosaminoglycan chains (long unbranched polysaccharide chains) (Figure 2.2) [21,23]**.** Proteoglycans contain repeating sulfate and carboxylate groups down the length of the chains, which become negatively charged when placed in aqueous solutions [33]. This further creates a high fixed density charge [31]. Therefore, the dense concentration of negatively charged

proteoglycans apply a large swelling pressure, which is resisted by the tensile stress of the surrounding collagen network [23,33]. Thus, the balance between the swelling pressure and the tensile stress determines the degree of hydration in cartilage [23]. This provides cartilage with its osmotic properties, which aid in its ability to resist compressive loads [21]. Nonetheless, if there is a potential disruption in this balance, it could potentially alter the ability of the cartilage to bear compressive loads [33].

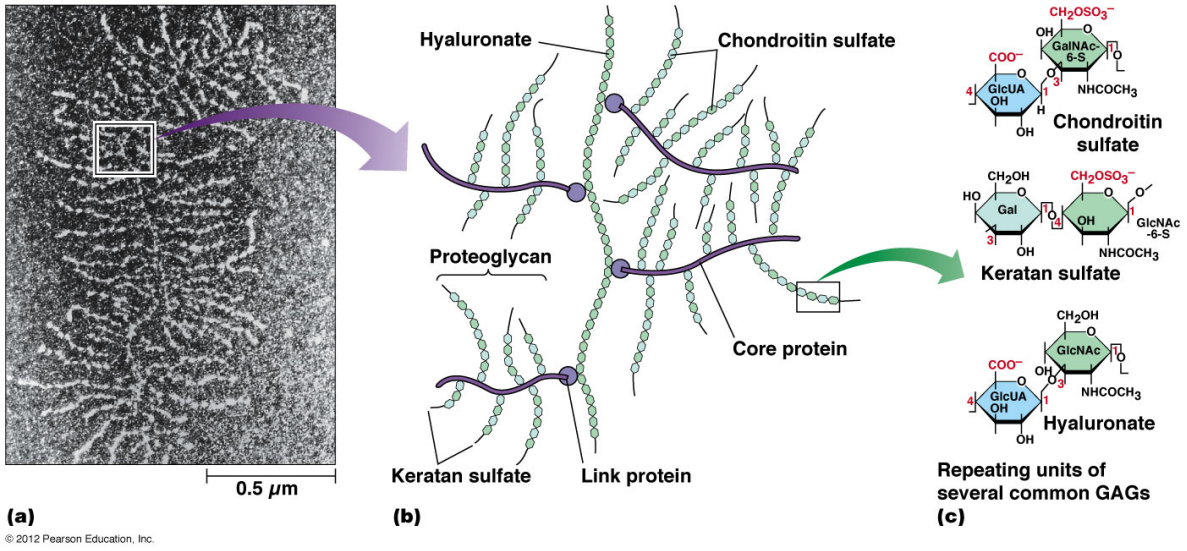

Figure 2.2 Structure of a PG macromolecule. The PG is composed of multiple GAG units covalently bound to a protein core [14].

Furthermore, chondroitin sulfate, keratin sulfate, dermatan sulfate and hyaluronan are the four main glycosaminoglycans (GAGs) that are found in articular cartilage [23]. The majority of proteoglycans that are found in articular cartilage are known as aggrecans, which bind to hyaluronan via link proteins [23, 31]. Link proteins appear to have a direct link to assembling aggregates (large amounts of aggrecan molecules) [23]. Aggrecan occupies the interfibrillar space of the cartilage ECM, contributing 90% of the total cartilage matrix proteoglycan mass

[21,23]. Each aggrecan contains about 100 chondroitin sulfate chains, and fewer keratin chains [34]. As a structural proteoglycan, aggrecan performs an important role in facilitating chondrocyte to chondrocyte interactions and chondrocyte to matrix interactions [34].

2.1.2 Chondrocytes

The cells that are responsible for the formation, maintenance and repair of cartilage tissue are known as chondrocytes [21]. Chondrocytes originate from mesenchymal stem cells and represent about 1-5% of the total volume of tissue cartilage [21,35,36]. They are spherical in shape and are responsible for secreting proteoglycans and collagens that make up the ECM. A unique aspect of chondrocytes is that they rarely form cell to cell contacts for direct communication between cells [21]. However, they do respond to a wide variety of stimuli, such as growth factors and mechanical loads [21]. Chondrocytes have a very limited potential for replicating, which causes the healing process of damaged cartilage to be very difficult and slow [21]. Chondrocytes are very metabolically active cells, that produce and turnover a large volume of extracellular matrix components (collagens and proteoglycans) [23,36]. Their metabolic activities may be altered due to certain factors in their chemical and mechanical environments that elicit anabolic and catabolic effects [36]. Therefore, it is vital to provide the cells with the correct type of environment to maintain cartilage homeostasis.

2.1.3 Zones

Articular cartilage has four different zones, each having a unique organization and composition. The 4 different zones are: i) superficial zone; ii) middle zone; iii) deep zone and; iv) calcified zone (Figure 2.3).

The superficial zone provides the highest tensile properties found in articular cartilage, although, the amount of aggrecan present within this zone is the lowest [24]. The chondrocytes in this zone arrange themselves so that they are parallel to the articular surface [23,24]. Furthermore, they synthesize a matrix that has a higher amount of collagen and a relatively lower level of proteoglycans due to the fact that they degrade faster [23]. The middle zone has rounded cells and has a very high concentration of proteoglycan aggrecan and the lowest concentration of water when compared to the superficial zone [23,24]. The chondrocytes in this zone arrange themselves in columns perpendicular to the joint surface [23]. The deep zone contains aggrecan at its maximal peak, although the collagen content is at its minimal peak [24]. This zone has a thin layer of calcified cartilage; therefore, the chondrocytes usually express a hypertonic phenotype [24]. The cells in this zone are usually surrounded by calcified cartilage [23].

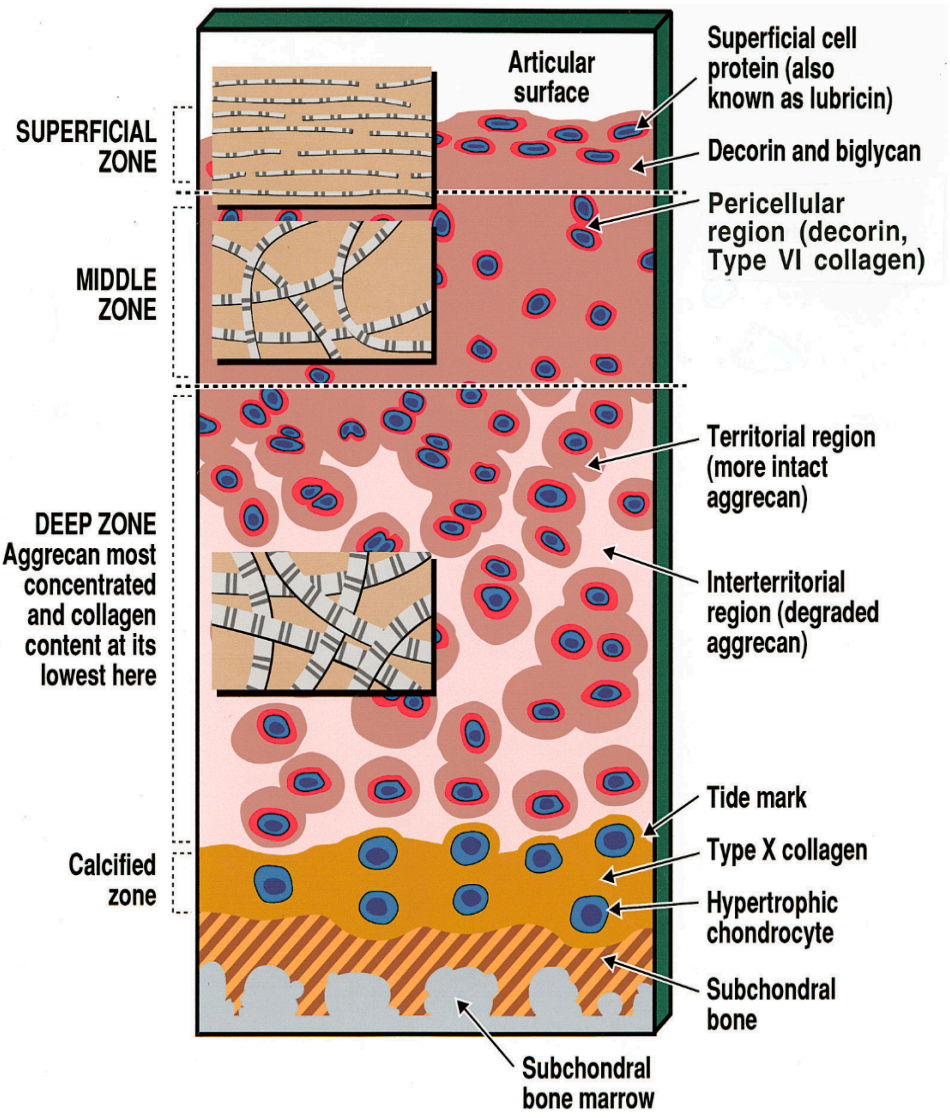

Figure 2.3 Representation of articular cartilage, with specified zones [5].

2.2 Chondrocyte Metabolism

The avascular environment of articular cartilage restricts chondrocyte metabolism to primarily anaerobic glycolysis due to its limited rate of oxygen and nutrient diffusion from the synovial fluid [37]. For example, chondrocytes consume glucose as a primary substrate for both ATP and amino acid production in glycolysis [38]. These processes are dependent on hexose uptake and delivery to metabolic and biosynthetic pools [38]. Thus, the importance of glycolysis and other pathways that branch off from glycolysis (e.g. hexosamine biosynthesis pathway) are crucial for the survival and homeostasis of chondrocytes.

2.2.1 Glycolysis and Fermentation Pathways

Glucose is a primary substrate for adenosine triphosphate (ATP) production in glycolysis [38], which is consumed by chondrocytes in order to survive avascular environments. Chondrocytes of articular cartilage have very low O_2 consumption rates [39-41]. Therefore, they rely heavily on anaerobic glycolysis to provide the energy that they need to maintain proper function. The anaerobic glycolysis (also known as the Embden-Meyerhof-Parnas) pathway is a reaction of converting glucose to lactate to produce energy for cells (Figure 2.4). Suppression of glycolysis has been shown restrict matrix synthesis by chondrocytes [39].

One of the most important steps in the glycolytic pathway is the transportation of glucose from the extracellular space into the cell [39]. Glucose enters chondrocytes via GLUT transporters (e.g. GLUT1, GLUT3) by passive diffusion [38,39]. Each glucose molecule is then broken down through the glycolytic pathway to form two (2) lactic acid molecules which are then converted

to lactate and removed from the cell into the ECM [38,39]. As the two lactic acid molecules are formed, the production of two (2) ATP molecules occurs [39].

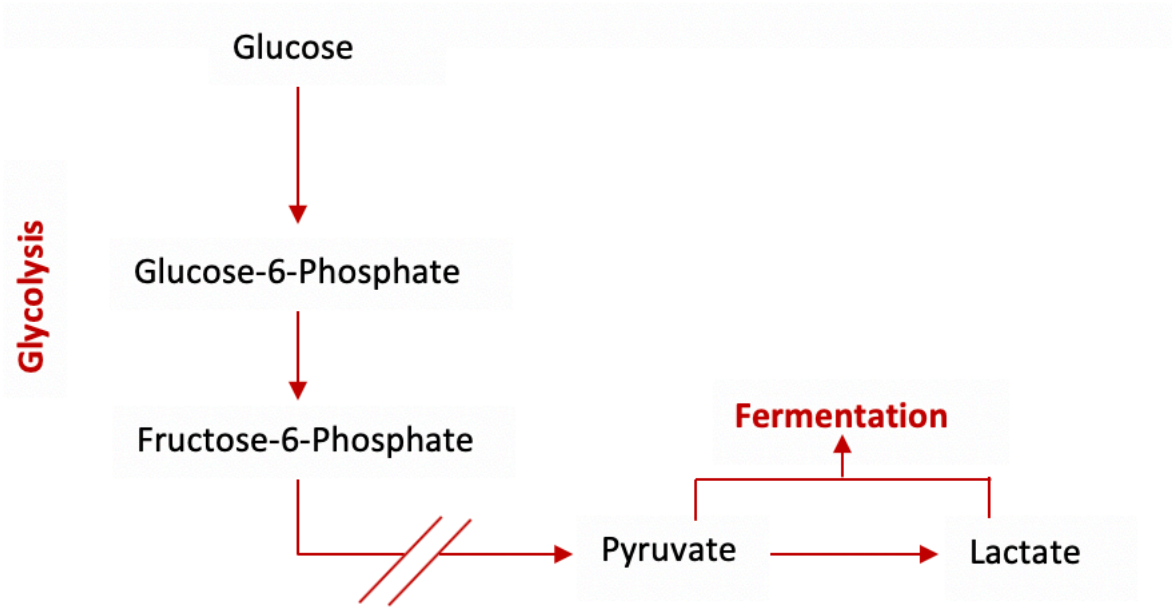

Figure 2.4 An overview of the Glycolysis and Fermentation pathway

2.2.2 Hexosamine Biosynthetic Pathway

Although the majority of glucose molecules taken up by cells is metabolized through the glycolysis pathway, 2-5% of metabolites enter the hexosamine biosynthesis pathway, which branches off of glycolysis at fructose-6-phosphate [21]. The hexosamine biosynthesis pathway starts by converting fructose-6-phosphate to glucosamine-6-phosphate (GlucN-6-P) by glutamine:fructose-6-P amidotransferase (GFAT) [42] (Figure 2.5). Glutamine is required for this process as it is used as an amino donor during the creation of GlucN-6-P [21]. GlucN-6-P is converted further to uridine-5-diphosphate-N-acetylglucosamine (UDP-GlucNAc), which in turn is the primary building block for the synthesis of proteoglycans, glycosaminoglycans and other glycoproteins [21].

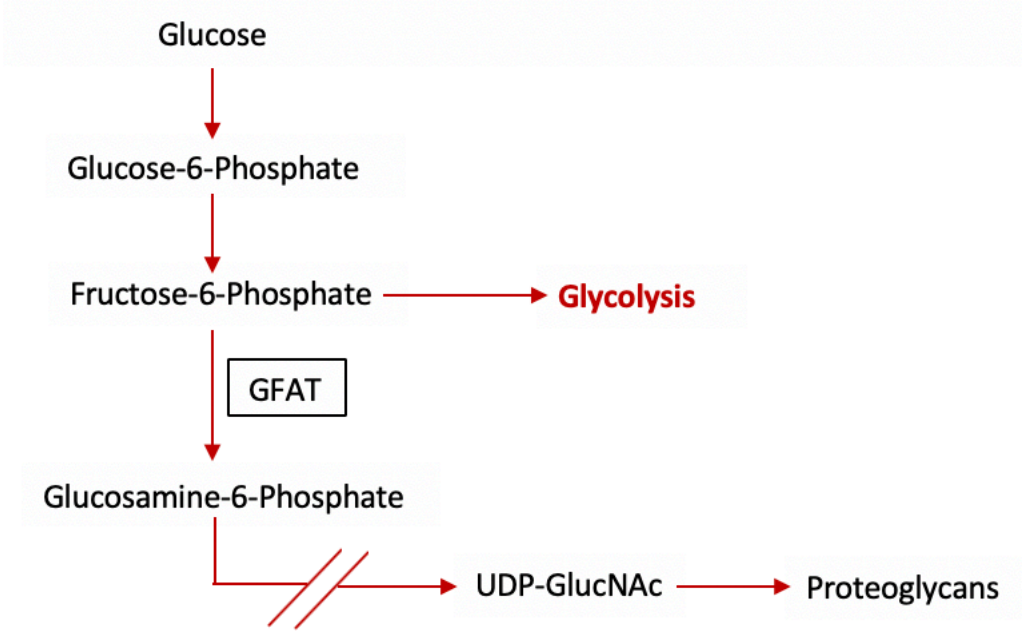

Figure 2.5 The Hexosamine Biosynthesis pathway in the production of proteoglycans

2.2.3 TCA Cycle

As mentioned previously, chondrocytes do not typically utilize the citric acid cycle (also known as the TCA or Krebs cycle) and thus high flux through the TCA cycle is unusual [43,44]. However, this pathway is important in chondrocytes for the synthesis of collagen [45]. Glucose metabolites enter the TCA cycle as acetyl-coenzyme A (Acetyl-CoA) [39]. Acetyl-CoA is converted into citrate, which is further converted into isocitrate [45,46]. Isocitrate is then oxidized into alpha-ketoglutarate (AKG). AKG is a required co-factor to synthesize hydroxyproline; one of the abundant amino acids present in collagen molecules [45]. Alternatively, AKG can be created from the transamination of glutamine [45,46].

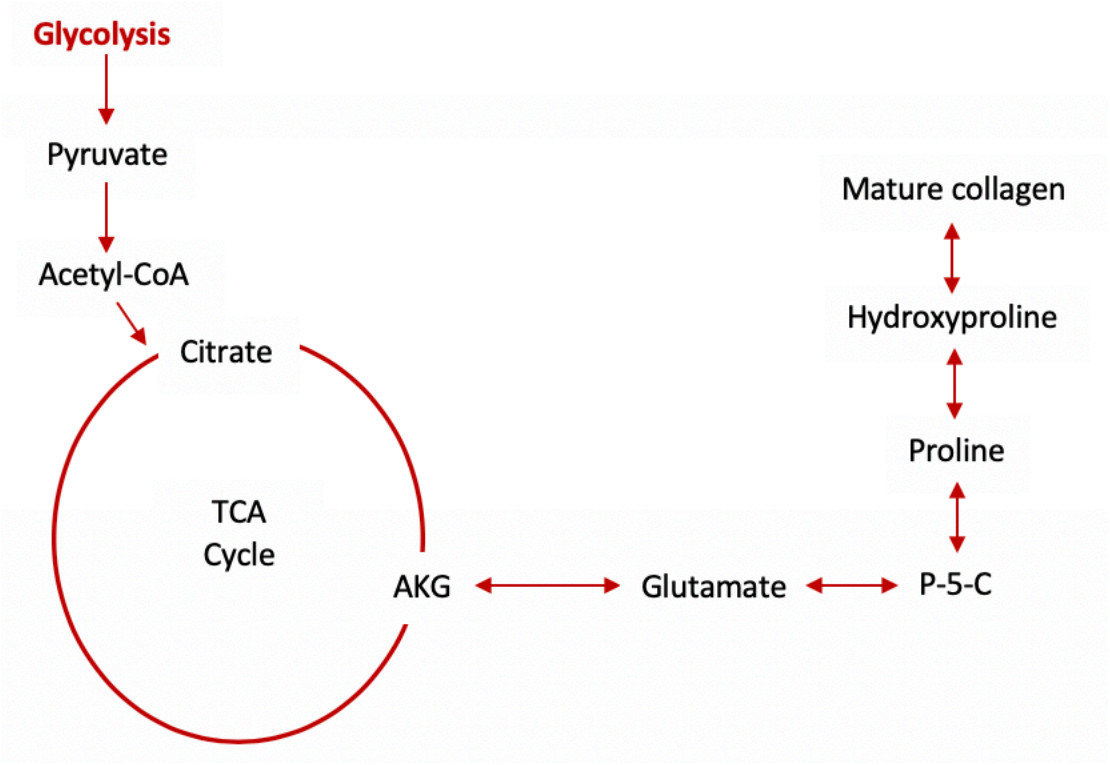

Figure 2.6 The TCA Cycle pathway

2.3 Chondrocyte Cell Culture

Cell culture varies between different basal medias, glucose and glutamine concentrations as well as different levels of fetal bovine serum (FBS) provided at different levels.

2.3.1 Basal Media

Common basal medias pertaining to cell culture are Dulbecco's Modified Eagle's Medium (DMEM) and Ham's nutrient mixture F12 (F12). A few studies have utilized a 50:50 (v/v) mixture of DMEM and Ham's nutrient mixture F-12 for cell growth [47]. All synthetic media require further supplementation with both organic and inorganic nutrients, vitamins, amino acids,

carbohydrates, and serum to aid in cell survival and growth [48]. DMEM medium is an amino acid rich formulation supplemented with inorganic salts and vitamins to allow cell survival and function [49]. Ham's F12 media is known as the first chemically defined medium [49] that is rich in B vitamins and supplemented with inorganic salts and amino acids. In recent years, studies have shown that the combination of DMEM and F12 media supported growth of high density cultures [47, 50]. This media formulation combines DMEM's high amino acid content with Ham's F12 enriched media [47]. DMEM/F12 media has been the most widely utilized basal media for chondrocyte culture [47].

2.3.2 Glucose

Glucose concentrations are very important in cell culture and need to be maintained within specific ranges. High glucose concentrations potentially induce non-responsiveness of chondrocytes to important growth factors such as insulin-like growth factor-1 (IGF-1) [51]. Additionally, high concentrations of glucose lead to high concentrations of lactate, which could potentially cause inhibitory effects on cell viability and matrix synthesis [51]. The upper end of glucose concentrations is typically around 25 mM to accommodate cells with high nutritional demands [49]. Low glucose concentrations are also inhibitory for chondrocytes, and previous studies have shown that low concentrations of glucose (i.e. 2.5 mM) can directly impair the accumulation of proteoglycans [52]. The use of low glucose concentrations in culture would most likely result in inhibition of the glycolysis pathway, which in turn can prevent ECM synthesis. Therefore, the range of normal glucose concentrations in culture is between 5 mM and 17 mM [53, 54].

2.3.3 Glutamine

Glutamine is a precursor for the synthesis of proteins and is important in dividing cells and cells that use glucose inefficiently [47, 55]. Additionally, deficient quantities of glutamine can decrease hexosamine biosynthesis pathway activity which will ultimately affect glycosaminoglycan and proteoglycan synthesis [56]. Studies have shown that supplementing cell culture media with 1 mM of glutamine stimulates protein and glycosaminoglycan synthesis [56].

2.3.4 Media Volume

Higher media volumes can maintain cellularity and glycosaminoglycan synthesis [51]. Furthermore, cells cultured in greater media volumes may demonstrate improved efficacy for use in functional tissue-engineering applications when compared with constructs maintained in smaller volumes of media [52]. Changes in nutrient supply of tissue cultures by altering their culture media volumes could potentially influence their biochemistry [57].

2.3.5 Fetal Bovine Serum

Fetal bovine serum (FBS) is commonly used to substitute for the growth factors needed to enhance cell culture survival and function. In terms of chondrocyte culture, the total amount of proteoglycan synthesized by chondrocytes was lower in lower concentrations of FBS when compared to higher concentrations [58]. Similarly, chondrocyte cultures grown in media

containing 20% FBS increased proteoglycan synthesis by three times compared to cultures grown in the presence of 2.5% FBS [59].

2.4 Response Surface Methodology

Response surface methodology (RSM) is a term applied to multivariate methods that can generate response surfaces and provide optimal solutions for specific processes [60]. RSM is a collection of statistical and mathematical techniques useful in improving, optimizing and developing processes [61-63]. Applications involving the use of RSM tend to revolve around situations where several input variables potentially influence some type of performance measure or the quality of certain products or processes [61]. Two classic designs of RSM are Box-Behnken design and central composite design.

2.4.1 Box-Behnken design

Box and Behnken (1960) developed efficient three-level incomplete factorial designs for fitting second-order response surfaces [61,64]. This design takes the midpoints of the edges of the processing space and the center point into consideration [60] (Figure 2.6). Therefore, in order to use this design, one must use more than two variables. In many scientific studies that utilize the RSM approach, researchers are prone to require three evenly spaced levels [61]. This design is usually an alternative design to the central composite design.

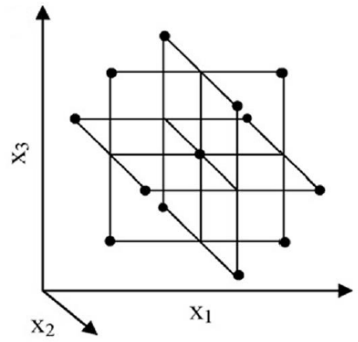

Figure 2.7 Three factor Box-Behnken

2.4.2 Central Composite Design

The central composite design was introduced by Box and Wilson [65]. This design consists of two parts: (i) a full factorial or fractional factorial design; (ii) an additional design, frequently including a star design, which experimental points are at a distance of α from the center point [66]. Central composite design is widely used for fitting a second-order response surface [61]. It involves the use of two or three level factorial combined with star points (axial points) [61, 67]. Three main central composite designs are circumscribed, inscribed and face centered.

2.4.2.1 Central Composite Circumscribed

Central composite circumscribed (CCC) designs, are the original form of the central composite design. CCC involves factorial points, center points and star points [60] (Figure 2.7a). The star points in this case represent the extreme values of the independent variables [60].

2.4.2.2 Central Composite Inscribed

Central composite inscribed (CCI) designs, are designed specifically for those situations in which the limits specified for certain factors are truly limits [60]. CCI involves factorial points, center points and star points, in which, the star points take specific limit values of the independent variables [60] (Figure 2.7b).

2.4.2.3 Central Composite Face Centered

Central composite face centered (CCF) design involves factorial points, center points and star points, in which, the star points are at the center of each face of the factorial space [60] (Figure 2.7c).

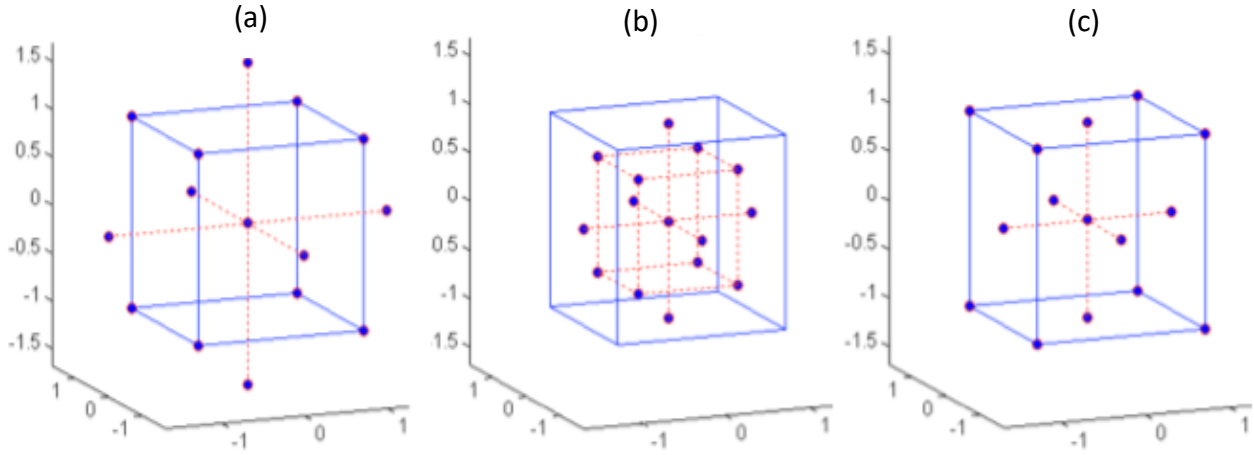

Figure 2.8 (a) circumscribed, (b) inscribed, and (c) faced designs

2.4.3 Response Surface Design and Analyses

RSM has a first-order model, which includes only the main effects and their interactions, as well as, a second-order model, which includes the curvature of the effects [61].

The mathematical model described;

$$\text{ŋ} = f(x_1, x_2, \ldots, x_n) \quad (1)$$

Where ŋ is the response, *f* is the unknown function of response, $x_1, x_2, \ldots, x_n$ indicate the independent variables, and, *n* is the number of the independent variables [68].

First-order polynomial;

$$\text{ŋ} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_n x_n \quad (2)$$

Second-order polynomial;

$$\text{ŋ} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots + \beta_n x_n + \beta_n x_1 x_2 + \ldots. + \beta_n x_n x_n + \beta_1 x_1^2 + \beta_2 x_2^2 + \ldots + \beta_n x_n^2 \quad (3)$$

Where the coefficients β_n represent the parameters to be estimated from the data [63]. Although, the second-order model is widely used in RSM due to its flexibility, it is easy to estimate the parameters and lastly, it indicates that it works in solving real response surface problems [61].

Once a second order model is fitted to its corresponding response, the next step is to locate the stationary points (critical points) which includes, the maximum, the minimum and the saddle point of a function [61, 63]. In order to find the stationary points, the first partial derivative test is used. The first partial derivatives are simultaneously set to equal zero which results in a linear system of equations [69]. Solving the pair of equations provides you with the critical points.

Using the second-order partial derivatives, a Hessian matrix is generated to give the nature of the stationary points using eigenvalues [61]. For example, if all eigenvalues are negative, the stationary point is at a maximum response [61]. Similarly, if all eigenvalues are positive, the stationary points are at a minimum response [61]. Lastly, if the eigenvalues are mixed, the stationary points are a saddle point [61]. Eventually, a set of optimal operating conditions can be determined and used for future work.

Chapter 3: Methods

3.1 Experimental Design

3.1.1 Response Surface Design

The response surface method design (face centered central composite design) was used for this work, which consisted of a three-level factorial (parameter) with 8 corner points, a centre point and lastly, 6-star points all within the ranges of the constituents. A face centered central composite design is used to find the best values, for a set of factors, providing an optimal response [70]. This mathematical approach helps in exploring quadratic surface responses where each experimental response can be represented [70]. The following four parameters were investigated: glucose, glutamine, media volume and basal media formulation.

3.1.2 Ranges of constituents

The ranges for each constituent were predetermined. According to section 2.3.2, it was stated that the normal range for glucose concentration was 5 mM to 17 mM. To not overload the cells with too much glucose, a range of 5 mM to 10 mM was chosen. Similarly, section 2.3.3 stated that 1 mM of glutamine proved effective for GAG synthesis, therefore, the range for glutamine chosen was 1 mM to 3 mM. However, section 2.3.4 stated that growth at higher volumes stimulated GAG synthesis compared to constructs in lower volumes, thus, media volume was set between 3 mL and 5 mL. Lastly, section 2.3.1 stated how both DMEM and F12 media are the most commonly used basal media types and how DMEM/F12 is being used in more recently now for chondrocyte culture. Additional components were added to the media formulations,

specifically, sodium pyruvate (1 mM) to help cells metabolize glucose in the media and HEPES (25 mM) to provide buffering.

3.2 Cartilage Tissue Culture

3.2.1 Cell Isolation

Articular cartilage was dissected from skeletally mature cow (20-24 months old) metacarpal-phalangeal joints provided by Millgrove Packers Ltd. (Toronto, ON). Cartilage slices were dissected from the upper and lower condyles found within the joint capsule. Dissections were performed aseptically in a UV sterilized laminar flow hood the following day that the cows were sacrificed. Cartilage sections were then digested in a Petri dish containing 0.5% protease (w/v) (Sigma-Aldrich), in 20 mL of Ham's F12 media (Gibco) supplemented with 25 mM HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) for one hour in an incubator at 37°C with 95% relative humidity and 5% CO_2 [71]. Following protease digestion, cartilage sections were washed twice with Ham's F12 media and was replaced with 20 mL of 0.15% collagenase A (w/v) (Sigma-Aldrich) in Ham's F12 media. After an 18-hour incubation period with collagenase A, the cartilage digest was passed through a 70 µm cell strainer (Sigma-Aldrich) in order to remove undigested tissue and bone fragments. Isolated chondrocytes were centrifuged at 700 RCF for 7 minutes to obtain a cell pellet, which was washed twice with Ham's F12 media and once with serum free custom media. Once the cells were re-suspended with the serum free custom media, 20 µL of re-suspended cells were taken for cell viability. Cell viability was assessed using a hemocytometer and trypan blue dye exclusion method (Sigma-Aldrich) [71].

3.2.2 Cell Culture

Isolated chondrocytes were seeded in 3D culture on Millicell Teflon inserts (Millipore), which promote the development of cartilaginous tissue [72]. Inserts were prepared for chondrocyte seeding by coating them with 100 µL of 0.5 mg/mL type II collagen (from chicken sterna) (Sigma-Aldrich) dissolved in 0.1 N glacial acetic acid in a 24-well culture plate. The 24-well culture plate was left overnight in the laminar flow hood uncovered to let the liquid evaporate. The inserts were UV sterilized for one hour and washed twice with the serum free custom media to remove residual acid. The prepared inserts were then seeded at 2 million viable cells/mm^2 and maintained in 1 mL of specific custom basal medias, supplemented with low glucose (5 mM) and low glutamine (1 mM) concentrations, and 20% fetal bovine serum (FBS) (v/v) (Sigma-Aldrich). After 72 hours, inserts were placed in their specific media groups, and the culture media was changed every 2-3 days. All cultures were maintained for a duration of 4 weeks.

3.3 Media Formulation

3.3.1 Media Screening Formulations

F12 and DMEM custom media had no glucose, glutamine, sodium pyruvate and HEPES. Each media formulation was made with different concentrations of glucose, glutamine and fed at different volumes, supplemented with 20% fetal bovine serum. Culture media was further supplemented with 1% v/v antibiotics/antimycotics, and 0.2% ascorbic acid. Specific media formulations used can be found in Appendix 1.

3.4 Cell Culture Feeding

3.4.1 Media Screening Media Formulations

DMEM, DMEM/F12 and F12 cultures were fed with 9 different media types (Table 3.1).

MEDIA TYPE	GLUCOSE (mM)	GLUTAMINE (mM)	VOLUME (mL)
A (low glucose-low glutamine)	5	1	3mL/5mL
B (high glucose-low glutamine)	10	1	3mL/5mL
C (low glucose-high glutamine)	5	3	3mL/5mL
D (high glucose-high glutamine)	10	3	3mL/5mL
E (mid glucose-mid glutamine)	7.5	2	3mL/4mL/5mL
F (low glucose-mid glutamine)	5	2	4mL
G (high glucose-mid glutamine)	10	2	4mL
H (mid glucose-low glutamine)	7.5	1	4mL
I (mid glucose-high glutamine)	7.5	3	4mL

Table 3.1 Media Formulations

3.5 Tissue Assessment

3.5.1 Tissue Harvest

Immediately after the culture period, cartilaginous tissues (along with the attached Teflon filter membrane) were removed from culture and cut out from the inserts using a No. 11 scalpel blade. Samples were rinsed in phosphate buffered saline (PBS) and blotted dry with Kimtech wipes, before being placed into 1.5 mL microcentrifuge tubes.

3.5.2 Tissue Wet/Dry Weights

Tissue wet and dry weights were measured using a model (Mettler Toledo ME104E). Tissue wet weight was determined by weighing the microcentrifuge tube containing the tissue sample. The total measurement was subtracted from the empty microcentrifuge tube weight and the filter membrane weight. After wet weight was taken, each microcentrifuge tube was covered in parafilm, three holes were poked at the top and the samples were lyophilized (-47°C under vacuum) overnight. After lyophilization the parafilm was removed and the tubes were weighed once more to get the overall tissue dry weight. The total measurement was subtracted from the empty microcentrifuge tube weight and the filter weight. The average mass of the filter membrane was 1.783 mg.

3.5.3 Tissue Digestion

Tissue cultures were first digested by papain (40 µg/mL) (Sigma-Aldrich) in 20 mM ammonium acetate, 1 mM ethylenediaminetetraacetic acid, and 2 mM dithiothreitol (Sigma-Aldrich) for 72 hours at 65°C [72]. Digested tissues were stored at -20°C until needed for analysis.

3.6 Extracellular Matrix Accumulation

The total amount of DNA, glycosaminoglycans (GAG) and collagen that accumulated in the cartilage tissues was measured from the tissue digest (section 3.4.3) using biochemical assays.

3.6.1 DNA Assay

DNA content was measured from papain digested cartilage using the Hoechst 33258 (Sigma-Aldrich) dye binding assay and fluorometry as described by Kim et al. [73]. Tissue aliquots were diluted with PBS, prior to performing the assay. Fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 450 nm. Standard curves were generated using calf thymus DNA (Sigma-Aldrich) and used to correlate fluorescence measurements to DNA content. This assay was performed in standard 96-well fluorescence plates (Thermo-Fisher) and all standards and samples were measured in triplicates.

3.6.2 GAG Assay

Glycosaminoglycan content was measured from papain digested cartilage using the dimethyl-methylene blue dye (Sigma-Aldrich) binding assay and spectrophometry, which measures the sulphated GAG content [74]. Tissue aliquots were diluted with 1% (w/v) bovine serum albumin (Sigma-Aldrich) in PBS, prior to performing the assay. Absorbance was measured at a wavelength of 525 nm. Standard curves were generated using bovine cartilage chondroitin sulphate A (Sigma-Aldrich) and used to correlate absorbance to GAG content. This assay was performed in standard 96-well plates (Thermo-Fisher), and all standards and samples were measured in triplicates.

3.6.3 Hydroxyproline Assay

Collagen content was estimated by measuring hydroxyproline content, which is assumed to account for 10% of the total collagen mass. Hydroxyproline content was measured using the chloramine-T/Ehrlich's reagent assay described by Woessner et al. [75] and later modified by Lin et al. [76]. Papain digests were hydrolyzed in 6 N HCl for 18 hours on a heating block at 110°C. Samples were neutralized with 5.7 N NaOH and diluted with distilled water to get the right dilution rate. Aliquots of the samples were then reacted with chloramine-T and Ehrlich's reagent (Sigma-Aldrich). Absorbance was measured at a wavelength of 560 nm. Standard curves were generated using L-hydroxyproline (Sigma-Aldrich) and used to correlate absorbance to hydroxyproline content. This assay was performed in standard 96-well plates (Thermo-Fisher), and all standards and samples were measured in triplicates.

3.7 Histology and Immunohistochemical Evaluation

Histological analysis was performed to provide a visual depiction of the developed tissue. Directly after harvest, tissue samples attached to filter membranes were rinsed in PBS for 10 minutes, and fixed in 4% paraformaldehyde (v/v) (Sigma-Aldrich) overnight at 4°C. The following day, they were transferred to 70% ethanol, and tissues were placed in the tissue processor to dehydrate and later embedded in paraffin. Thin sections (5 μm) were cut and bound to glass slides.

For histological staining, tissue sections were placed in the oven at 65°C to remove the paraffin, followed by immersion into xylene, and a series of graded alcohols (100%, and 95%), and water solutions to rehydrate the tissue sample. Tissue sections were stained with hematoxylin and eosin (general connective tissue stain used to stain nuclei dark blue and proteins pink, respectively). Tissue sections were also stained with Safranin O (nuclei stained black, and cartilage stained orange to red) and Picrosirius Red (collagen stained red on a pale-yellow background).

Immunohistochemical methods were used to identify type I collagen and type II collagen in the developed tissues. Following paraffin embedding and sectioning, tissue sections were placed in the oven at 65°C to remove the paraffin, followed by immersion into xylene, and a series of graded alcohols (100%, and 95%), and water solutions to rehydrate the tissue sample. A humidified chamber was created to keep the samples moist. Samples were then circled with a histology marker to prevent any of the liquid from spreading. Prior to antigen staining, tissue sections were subjected to enzymatic pretreatment in order to facilitate primary antibody binding. For sections stained for type I collagen and type II collagen, sections were digested in 0.5% pepsin (w/v) (Sigma-Aldrich) in acidified water for 30 minutes at 37 ºC. Samples where then washed twice with PBS for 5 minutes each. To reduce non-specific protein binding, type I and type II collagen sections were blocked with 1% (w/v) bovine serum albumin (Sigma-Aldrich) in PBS for 30 minutes at room temperature.

Mouse primary antibodies (Abcam) were added to type I collagen sections (at a 1:100 dilution in 1% (w/v) bovine serum albumin in PBS) and mouse primary antibodies (Developmental studies Hybridoma Bank) were added to type II collagen sections (at a 1:6 dilution in 1% (w/v) bovine serum albumin in PBS) overnight at 4 ºC. Sections where then washed twice with PBS for 5 minutes each. Goat anti-mouse (Texas Red) secondary antibodies (Abcam) were added to sections at a 1:200 dilution in 1% (w/v) bovine serum albumin in PBS for 2 hours at room temperature. Sections where then washed three times with PBS for 5 minutes each, and mounted with DAPU mounting media. To ensure there was no background staining, the primary antibody was omitted from selected slides for negative control samples.

3.8 Statistical Analyses

First, bovine articular chondrocytes were obtained from a pooled source of at least two dissected legs for all experiments. Second, each experiment was performed at least two times with chondrocytes obtained from different donors (~20-24 months) and the combined data was used for analysis. In total, each experimental group had a sample size of at least 4. All numerical results were normalized to DMEM control media for each experiment, and expressed as the mean ± standard error of the mean (SEM). Second-order response surfaces (quadratic including all first level interactions) were generated by least-squares regression (SAS) for each outcome variable. Stationary points (min, max or saddle points) were then determined by analyzing the partial derivatives of the specified function. Once the stationary points were determined, the Hessian matrix was generated through MATLAB, to determine the associated eigenvalues and eigenvectors.

Additionally, collected data were analyzed statistically using a one-way of variance (ANOVA) and the Bonferoni's and Tukey's post-hoc test (SPSS version 25, SPSS Inc) to determine the comparison of specific media to the control group. Significance was associated with p-values less than 0.05. Trends were noted with p-values between 0.05 and 0.1.

Chapter 4: Results

4.1 Study 1: Media Screening

Media screening was conducted over 9 different media formulations and observations were made primarily in terms of the accumulation of cartilage ECM constituents (i.e. GAG accumulation, and collagen accumulation). Tissue growth varied based on the different ranges of basal media, glucose, glutamine, and media volume with differential effects on specific cartilage constituents. The specific outcome variables that were analyzed were: GAG, collagen, DNA, and their normalizations to wet weight and dry weight (e.g., GAG/dry weight).

4.1.1 Effects of media formulation on GAG accumulation

GAG accumulation (GAG accumulation and its normalizations to wet and dry weights) was primarily influenced by basal media, media volume and glucose concentrations determined from the surface response curve fits. (Table 4.1). Specifically, in terms of total GAG accumulation, the response surface indicated that accumulation appeared to be primarily influenced by basal media and media volume (Table 4.1; Figure 4.1a). While the overall fit was typically poor (R^2=0.062) and critical points for this response surface were inconclusive, the maximal curvatures of the response surface were in the basal media (eigenvalue of 0.3 in the x_1 direction) and media volume (eigenvalue of 0 in the x_2 direction) directions. Therefore, the optimal media formulation to elicit total GAG accumulation was deemed to be a basal media of DMEM/F12 mixture (x_1=0), and a media volume of 4.8 mL or higher.

Additionally, in terms of GAG accumulation normalized to wet weight (GAG/WW), the response surface indicated that accumulation appeared to be influenced by glucose concentration and media volume (Table 4.1; Figure 4.1b). While the overall fit was also typically poor (R^2=0.052) and a global minimum was observed at (0,0), the maximal curvatures of the response surface were in the media volume (eigenvalue of 0.12 in the x_2 direction) and glucose concentration (eigenvalue of 0 in the x_3 direction) directions. Therefore, the optimal media formulation to elicit GAG accumulation with respect to wet weight was deemed to be at a media volume of 4.7mL or higher and a lower glucose concentration of 5 mM or lower.

Analysis of GAG accumulation normalized to dry weight (GAG/DW) revealed a more complex response surface that appeared to be influenced by glutamine, basal media and media volume (Table 4.1). While the overall fit was slightly improved (R^2=0.147), a saddle point on the response surface at (0,0,2.26) was observed with a minimum accumulation in the glutamine direction. The maximal curvature of the response surface was in the glutamine concentration (eigenvalue of 0.38 in the x_4 direction) direction and smaller curvatures were observed in both the media volume (eigenvalue of 0.06 in the x_2 direction) and basal media (eigenvalue of 0.06 in the x_1 direction) directions. Nonetheless, lower glutamine concentrations (<2.26 mM) appeared to be more optimal based on the curvature of the response (Figure 4.1c). Therefore, the optimal media formulation to elicit GAG accumulation with respect to dry weight was deemed to be at a glutamine concentration of 1 mM.

Therefore, the optimal media formulation for future experiments to elicit GAG accumulation was selected as following: basal media of DMEM/F12 mixture (1:1), a media volume of 4.8 mL or higher, glucose concentration of 5 mM and a glutamine concentration of 1 mM.

Response	$y = f(x_1, x_2, x_3, x_4)$	R^2	Critical Points	Maximum Eigenvalues and Associated Eigenvectors
GAG	$y = -0.15\ x_1^2 + 0.12\ x_2 + 0.59$	0.062	Inconclusive	$0.3 – x_1$ direction
GAG/WW	$y = 0.05\ x_2^2 + 0.01\ x_3^2 - 0.04\ x_2x_3 + 0.83$	0.052	(x_2,x_3) - (0,0) (minimum)	$0.12 – x_2$ direction
GAG/DW	$y = 0.19\ x_4^2 + 0.06\ x_1x_2 - 0.86\ x_4 + 2.09$	0.147	(x_1,x_2,x_4) – (0,0,2.26) (saddle point)	$0.38 – x_4$ direction

Table 4.1 Representation of the response surface equations of GAG accumulation. Response surfaces were fit to a general model (second-order response including all first-level interactions) with only significant terms included ($p<0.05$). Where y is the outcome variable, x_1 is basal media (range: -1 (DMEM) to 1 (F12)), x_2 is media volume (mL), x_3 is initial glucose concentration (mM), and x_4 is initial glutamine concentration (mM).

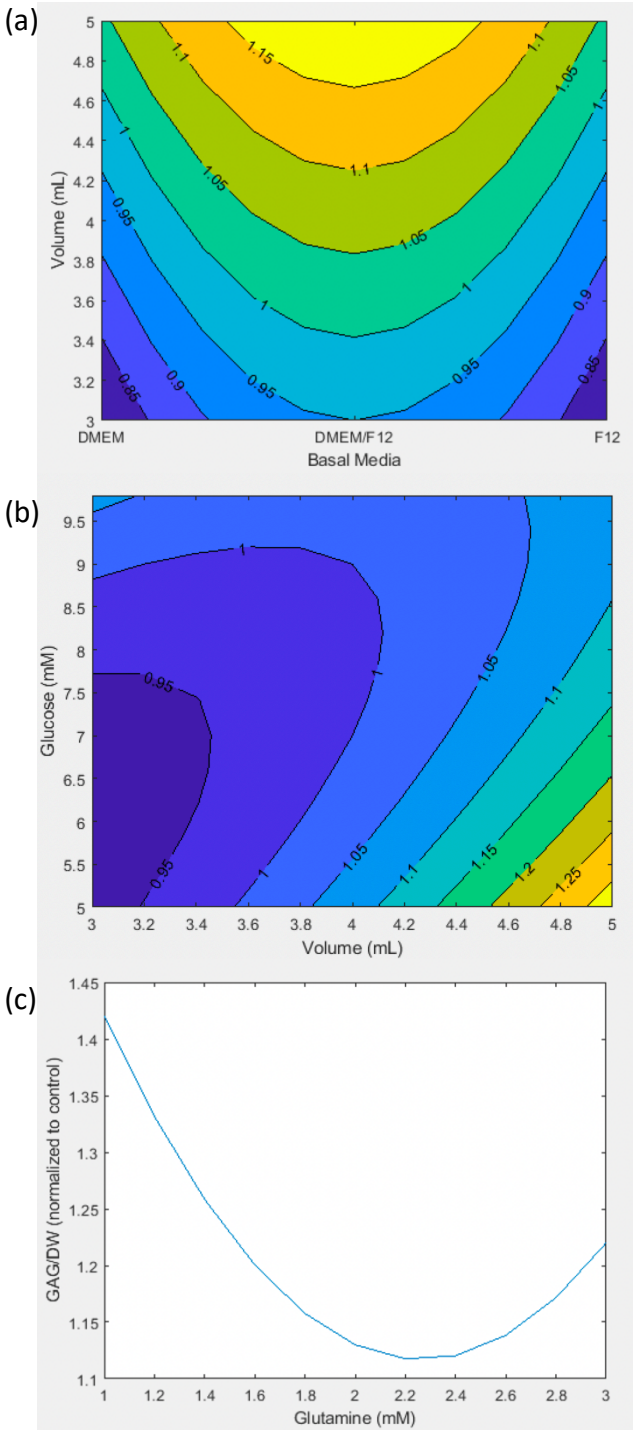

Figure 4.1 The Effect of glucose (mM), glutamine (mM), basal media and volume (mL) on the accumulation of GAG normalized to wet weight and dry weight: (a) GAG (b) GAG/WW, and (c) GAG/DW: basal media and media volume were fixed ($x_1 = 0$; $x_2 = 5$).

4.1.2 Effects of media formulation on collagen accumulation

Collagen accumulation (collagen accumulation and its normalizations to wet and dry weights) was primarily influenced by basal media, media volume, glucose concentration and glutamine concentration determined from the surface response curve fits (Table 4.2). Specifically, in terms of total collagen accumulation, the response surface indicated that accumulation appeared to be influenced by basal media, media volume, glucose and glutamine concentrations (Table 4.2). The overall fit was fair (R^2=0.385) and the critical point (0,4.46,0,0) for this response surface was inconclusive. However, maximal curvatures of the response surface were observed in the basal media (eigenvalue of 0.9 in the x_1 direction) and media volume (eigenvalue of 0.2 in the x_2 direction) directions (Figure 4.2a). However, the smaller curvatures of the response surface were also observed in the glutamine (eigenvalue of 0.2 in the x_4 direction) and glucose (eigenvalue of 0 in the x_3 direction) directions. Further analysis of the response with respect to glucose concentration and media volume (Figure 4.2b) was also conducted to determine optimal glucose concentrations. Therefore, the optimal media formulation to elicit total collagen accumulation was deemed to be a basal media of F12, a media volume of 4.6 mL or higher, and a glucose concentration of 7.5 mM or higher.

Similar responses were observed with collagen accumulation normalized to wet weight (Col/WW), with the response surface influenced by basal media, media volume, glucose and glutamine concentrations (Table 4.2). Since the overall magnitude of the response was at control levels, the analysis of this parameter should not be considered at optimal values. The overall fit was fair (R^2=0.395) with a saddle point at (0.087, 0.05, 8, 0.025). The maximal curvatures of the response surface were in the media volume and glucose concentration (eigenvalue of 10. 1 in the x_2 and x_3 direction) directions (Figure 4.2c). The smaller curvatures of the response were observed in the glutamine (eigenvalue of 9.9 in the x_4 direction) and basal media (eigenvalue of 0.04 in the x_1 direction) directions. Therefore, the optimal media formulation to elicit collagen accumulation with respect to wet weight was deemed to be at a glucose concentration of 5 mM with a volume of 3.8 mL or higher.

Similarly, collagen accumulation normalized to dry weight (Col/DW) also had a response surface influenced by basal media, media volume, glucose and glutamine concentrations (Table 4.2). The overall fit was fair (R^2=0.348) with a saddle point at (0, 0, 0, 0). The maximal curvatures of the response surface were in the media volume (eigenvalues of 0.8 in the x_2 direction) and glutamine concentration (eigenvalue of 0.1 in the x_4 direction) directions (Figure 4.2d). Smaller curvatures of the response surface were observed in the basal media (eigenvalue of 0.05 in the x_1 direction), and glucose concentration (eigenvalue of 0.003 in the x_3 direction) directions Therefore, the optimal media formulation to elicit collagen accumulation with respect to dry weight was deemed to be at a glutamine concentration of 1 mM and a volume of 4.2 mL or higher.

Therefore, the optimal media formulation for future experiments to elicit collagen accumulation was selected as follows: a basal media of F12, media volume of 4.2 mL or higher, glucose concentration of 7.5 mM or higher and a glutamine concentration of 1 mM.

Response	$y = f(x_1, x_2, x_3, x_4)$	R^2	Critical Points	Maximum Eigenvalues and Associated Eigenvectors
Collagen	$y = 0.17\,x_1^2 - 0.14\,x_2^2 + 0.02\,x_1x_3 - 0.08\,x_1x_4 + 1.25\,x_2 - 2.03$	0.385	(x_1, x_2, x_3, x_4) – (0,4.46,0,0) (Inconclusive)	0.0455 – x_1 direction
Col/WW	$y = 0.23\,x_1^2 - 0.1\,x_2^2 - 0.1\,x_1x_2 - 0.02\,x_2x_3 + 0.04\,x_3x_4 + 0.47\,x_1 + 1.04\,x_2 - 0.32\,x_4 - 1.37$	0.395	(x_1, x_2, x_3, x_4) – (0.087, 0.05, 8, 0.025) (saddle point)	10.136 – in x_2 and x_3 direction
Col/DW	$y = 0.37\,x_1^2 - 0.05\,x_2^2 - 0.01\,x_3^2 + 0.09\,x_1x_3 - 0.19\,x_1x_4 + 0.05\,x_2x_3 + 0.91$	0.348	(x_1, x_2, x_3, x_4) – (0, 0, 0, 0) (saddle point)	0.795 – in x_2 direction

Table 4.2 Representation of the response surface equations of collagen accumulation. Response surfaces were fit to a general model (second-order response including all first-level interactions) with only significant terms included ($p<0.05$). Where y is the outcome variable, x_1 is basal media (range: -1 (DMEM) to 1 (F12)), x_2 is media volume (mL), x_3 is initial glucose concentration (mM), and x_4 is initial glutamine concentration (mM).

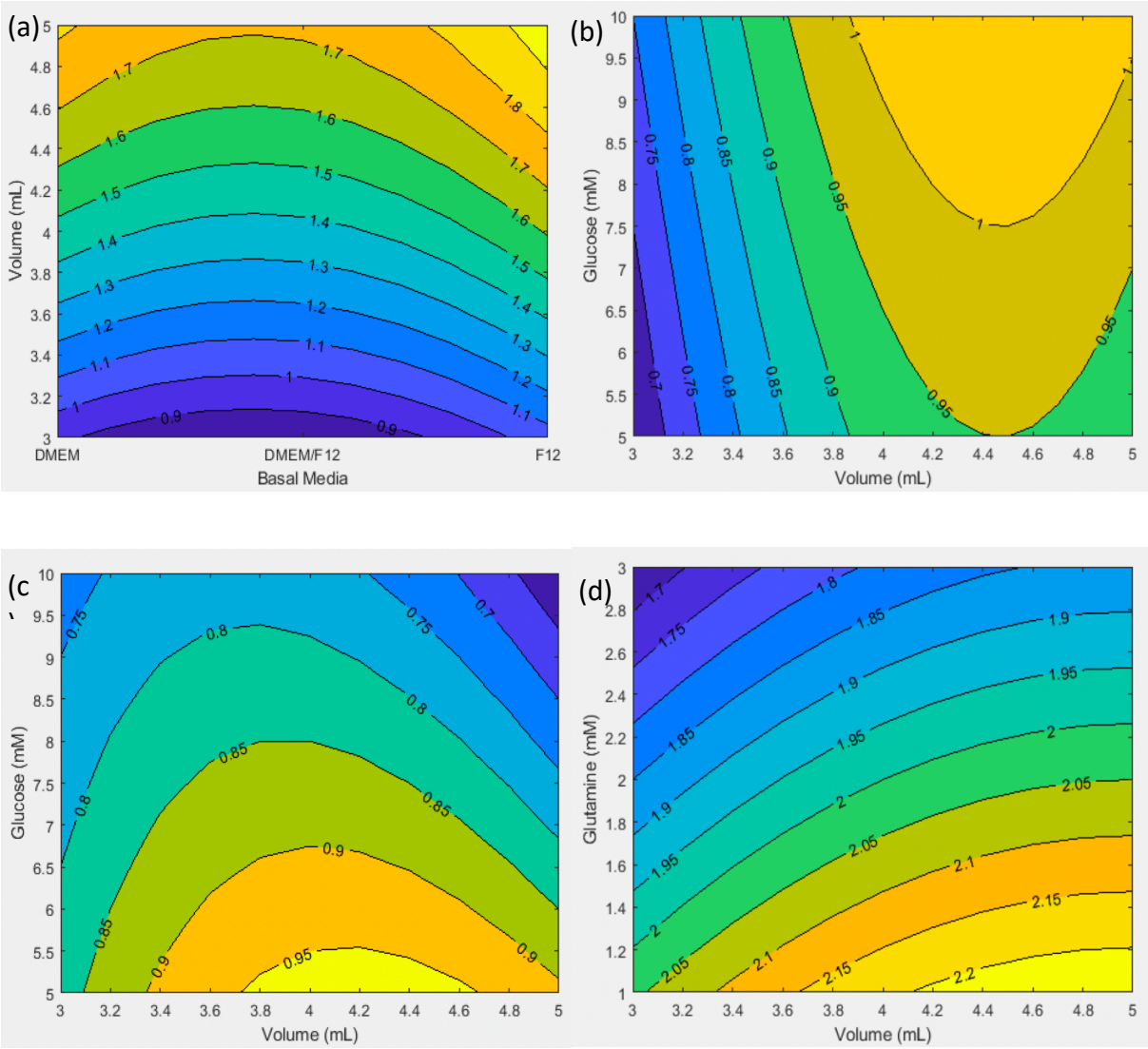

Figure 4.2 The effect of glucose (mM), glutamine (mM), basal media and volume (mL) on the accumulation of collagen normalized to wet weight and dry weight: (a&b) Collagen: (a) glucose and glutamine concentrations were fixed (x_3 =7.5; x_4=1), (b) basal media and glutamine concentration were fixed (x_1 =1; x_4=1), (c) Col/WW: basal media and glutamine concentration were fixed (x_1 = 1; x_4 = 1), and (d) Col/DW: basal media and glucose concentrations were fixed (x_1= 1; x_3=10).

4.1.3 Effects of media formulation on DNA accumulation

DNA accumulation (DNA accumulation and its normalizations to wet and dry weights) was primarily influenced by basal media, glucose and glutamine concentrations (Table 4.3). Specifically, in terms of total DNA accumulation, the response surface indicated that accumulation appeared to be influenced by basal media and glucose concentration (Table 4.3; Figure 4.3a). The overall fit was fair (R^2=0.235) and a global minimum was observed at (0, 0). The maximal curvatures of the response surface were in the basal media (eigenvalue of 0.98 in the x_1 direction) and glucose concentration (eigenvalue of 0 in the x_3 direction) directions. Therefore, the optimal media formulation to elicit total DNA accumulation was deemed to be a basal media of DMEM/F12 mixture (1:1), and a glucose concentration ranging from 5 mM to 10 mM.

Similarly, in terms of DNA accumulation normalized to wet weight (DNA/WW), the response surface also indicated that accumulation appeared to be influenced by basal media and glucose concentration (Table 4.3; Figure 4.3b). The overall fit was poor (R^2=0.193), with a global minimum at (0, 0). The maximal curvatures of the response surface were in the basal media (eigenvalue of 0.7 in the x_1 direction) and glucose concentration (eigenvalue of 0 in the x_3 direction) directions. Therefore, the optimal media formulation to elicit DNA accumulation with respect to wet weight was deemed to be a basal media of DMEM/F12 mixture (1:1), and a glucose concentration ranging from 5 mM to 10 mM.

Analysis of DNA normalized to dry weight (DNA/DW) accumulation, revealed a slightly more complex response surface that appeared to be influenced by basal media, media volume and glutamine concentration (Table 4.3). The overall fit was fair (R^2=0.341) with a saddle point at (0.96, 0, 0) that was maximal in the basal media direction. The maximal curvatures of the response surface were in the basal media (eigenvalue of 0.5 in the x_1 direction) and glutamine concentration (eigenvalue of 0.2 in the x_4 direction) directions (Figure 4.3c). However, the smaller curvature was observed in the media volume (eigenvalue of 0.2 in the x_2 direction) Therefore, the optimal media formulation to elicit total DNA accumulation with respect to dry weight was deemed to be at a basal media of F12 and a glutamine concentration of 3 mM.

Therefore, the optimal media formulation to elicit DNA accumulation as deemed as follows: basal media between DMEM/F12 mixture (1:1), media volume of 3 mL, glucose concentrations between 5 mM and 10 mM and a glutamine concentration of 3 mM.

Response	$y = f(x_1, x_2, x_3, x_4)$	R^2	Critical Points	Maximum Eigenvalues and Associated Eigenvectors
DNA	$y = -0.49\ x_1^2 + 0.01\ x_1x_3 + 1.54$	0.235	(x_1, x_3) – (0, 0) (global minimum)	$0.98 – x_1$ direction
DNA/WW	$y = -0.35\ x_1^2 + 0.02\ x_1x_3 + 1.46$	0.193	(x_1, x_3) – (0, 0) (global minimum)	$0.7 – x_1$ direction
DNA/DW	$y = -0.27\ x_1^2 + 0.11\ x_4^2 - 0.1\ x_2x_4 + 0.52\ x_1 + 2.13$	0.341	(x_1, x_2, x_4) – (0.96,0,0) (saddle point with a max at basal media)	$0.54 – x_1$ direction

Table 4.3 Representation of the response surface equations of DNA accumulation. Response surfaces were fit to a general model (second-order response including all first-level interactions) with only significant terms included ($p<0.05$). Where y is the outcome variable, x_1 is media (range: -1 (DMEM) to 1 (F12)), x_2 is media volume (mL), x_3 is initial glucose concentration (mM), and x_4 is initial glutamine concentration (mM).

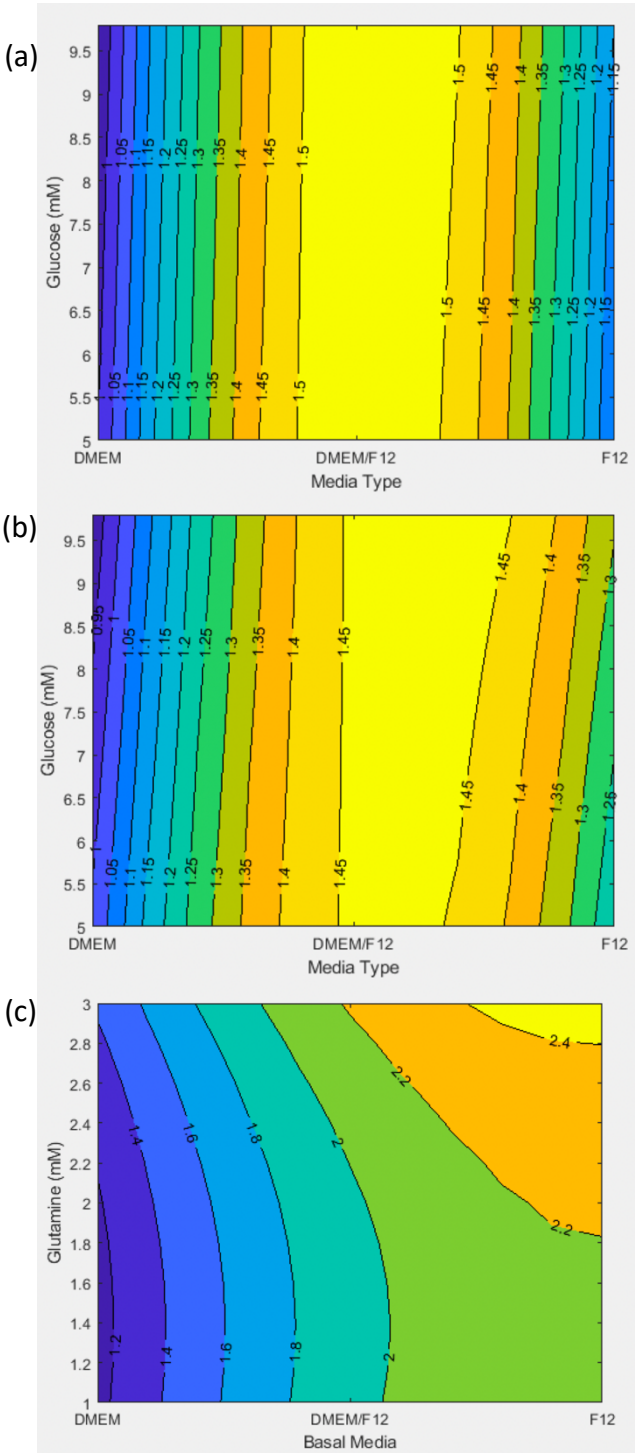

Figure 4.3 The effect of glucose (mM), glutamine (mM), basal media and volume (mL) on the accumulation of DNA normalized to wet weight and dry weight: (a) DNA, (b) DNA/WW, and (c) DNA/DW: media volume was fixed ($x_2 = 3$).

4.2 Study 2: Validation Study

The next series of experiments was a validation study conducted using the optimal media formulations to elicit GAG and collagen deposition determined from the previous media formulation screen (section 4.1). Specific media formulations used were as follows:

	Basal Media	Media Volume (mL)	Glucose Concentration (mM)	Glutamine Concentration (mM)
Optimal GAG Media	DMEM/F12	5	5	1
Optimal Collagen Media	F12	4.5	9.5	1

Table 4.4 Optimal media formulations for GAG and collagen accumulation.

Two additional groups were included in this study to investigate whether GAG and collagen accumulation could be upregulated simultaneously. The culture period for these samples were split into two-week segments with the samples maintained in one optimal media formulation followed by the other optimal media formulation, specifically: optimal GAG media followed by optimal collagen media (group G1) and optimal collagen media followed by optimal GAG media (group G2). In this study, the same outcome measures were utilized (section 4.1) in addition to analyzing the histological and immunohistochemical appearances of the cultures.

It should also be noted that the optimal media formulation to elicit DNA accumulation (section 4.1.3) was not investigated further as the primary goal of this work was determine optimal media conditions to elicit the accumulation of cartilaginous extracellular matrix constituents.

4.2.1 Effects of optimal media formulations on GAG Accumulation

The total GAG accumulation of developed cartilaginous tissues maintained in the optimal media formulations did not change significantly, but there was a trend with respect to optimal GAG media when compared to control ($p<0.1$) which elicited a 1.2-fold increase in total GAG accumulation (Figure 4.4). Interestingly, the switch medias (G1 and G2) has no apparent effect on total GAG accumulation, and as expected, the optimal media to elicit collagen accumulation also had no observable effect on total GAG accumulation. GAG accumulation normalized to either wet weight (GAG/WW) or dry weight (GAG/DW) was also not affected by any of the optimal media formations (including the switch medias) (Figures 4.5 and 4.6).

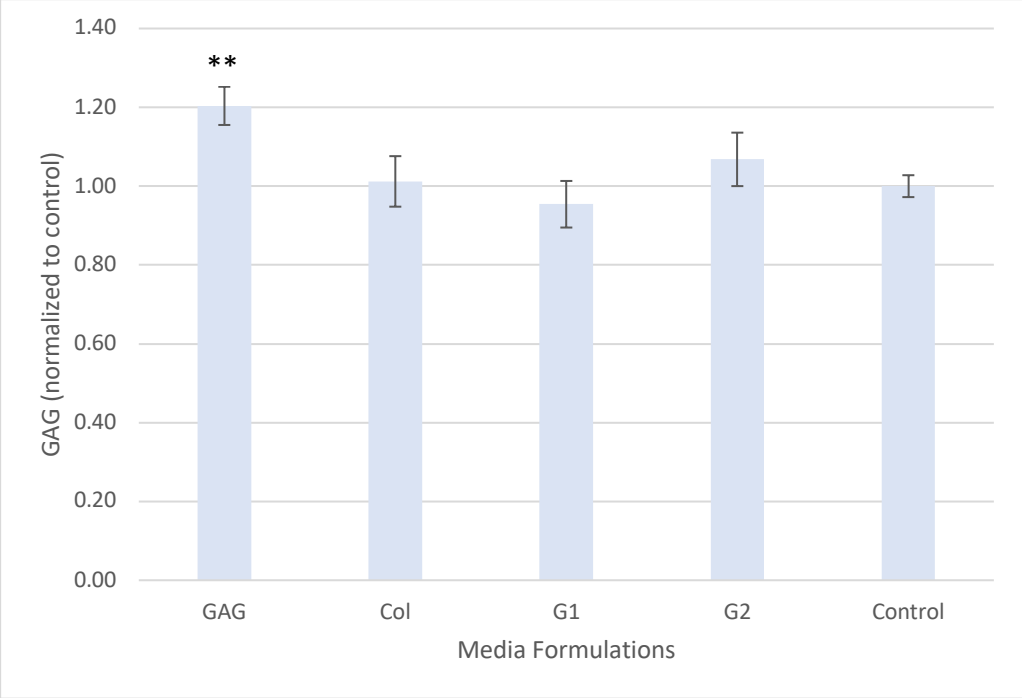

Figure 4.4 Effect of media on GAG accumulation. Data is presented as the mean ± SEM normalized to control, n = 4-7 samples per group. ** Denotes a trend from control ($p<0.1$).

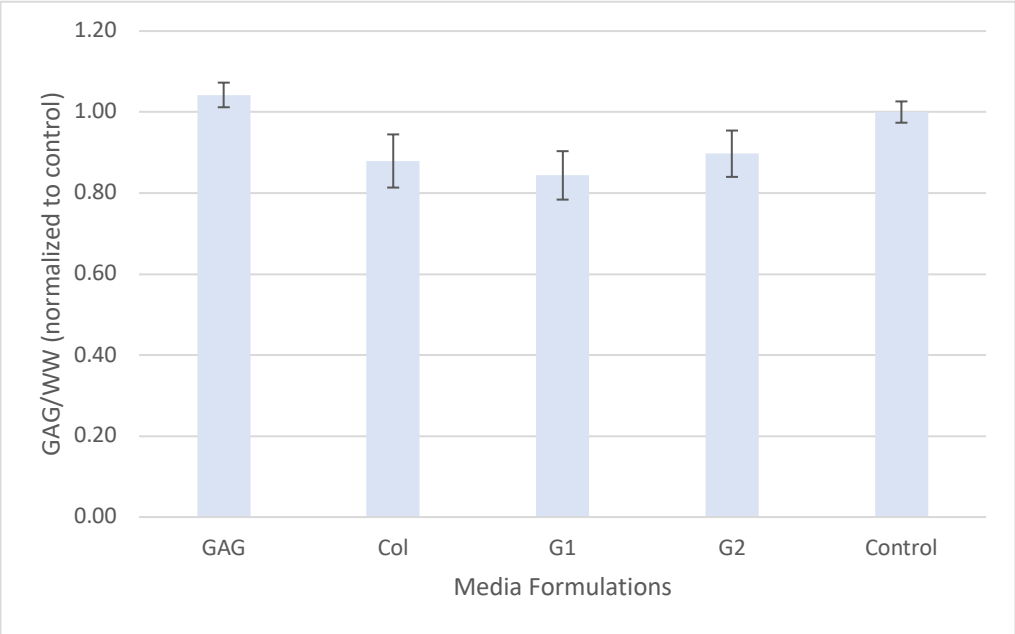

Figure 4.5 Effect of media on GAG/WW accumulation. Data is presented as the mean ± SEM normalized to control, n = 4-7 samples per group. None of the groups were statistically different from control **(p>0.1).**

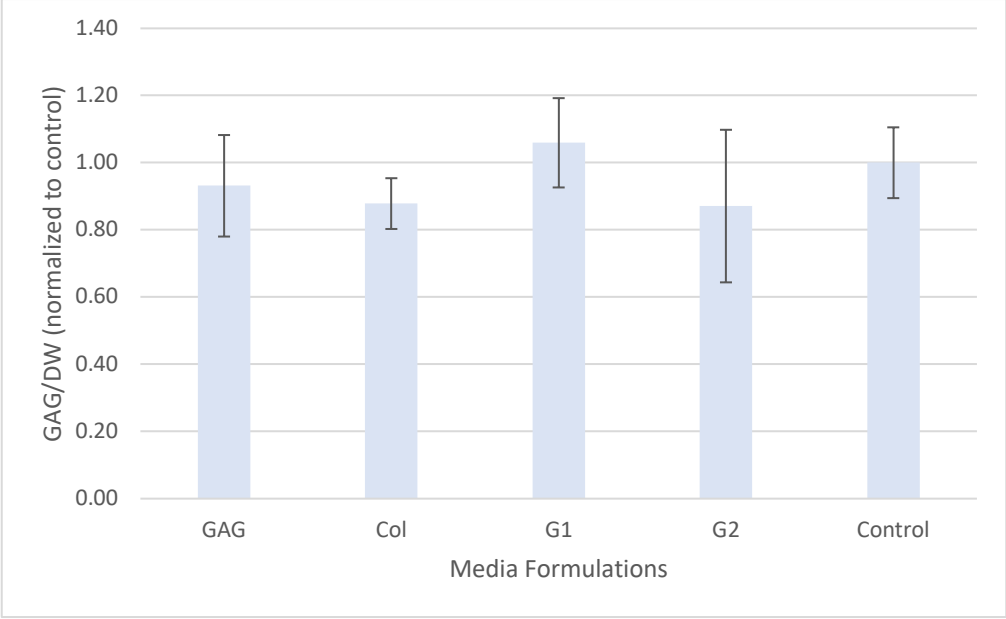

Figure 4.6 Effect of media on GAG/DW accumulation. Data is presented as the mean ± SEM normalized to control, n = 4-7 samples per group. None of the groups were statistically different from control **(p>0.1).**

4.2.2 Effects of optimal media formulation on collagen accumulation

The total collagen accumulation of developed cartilaginous tissues was significantly affected by both the optimal collagen media and switch media G1 (optimal GAG media followed by optimal collagen media) (Figure 4.7). Optimal collagen media, and switch media G1, elicited an increase in total collagen accumulation by 1.44-fold ($p=<0.01$) and 1.38-fold ($p<0.02$), respectively compared to the control. The other optimal media formulations (optimal GAG media and switch media G2) did not have any apparent effect on total collagen accumulation. Similar trends (although less significant) were observed with collagen accumulation normalized to either wet or dry weight (Col/WW and Col/DW) (Figures 4.8 and 4.9). Optimal collagen media elicited a 1.25-fold increase ($p<0.04$) increase in Col/WW compared to the control. Similarly, all other optimal media formulation had no apparent affect on Col/WW or Col/DW accumulation.

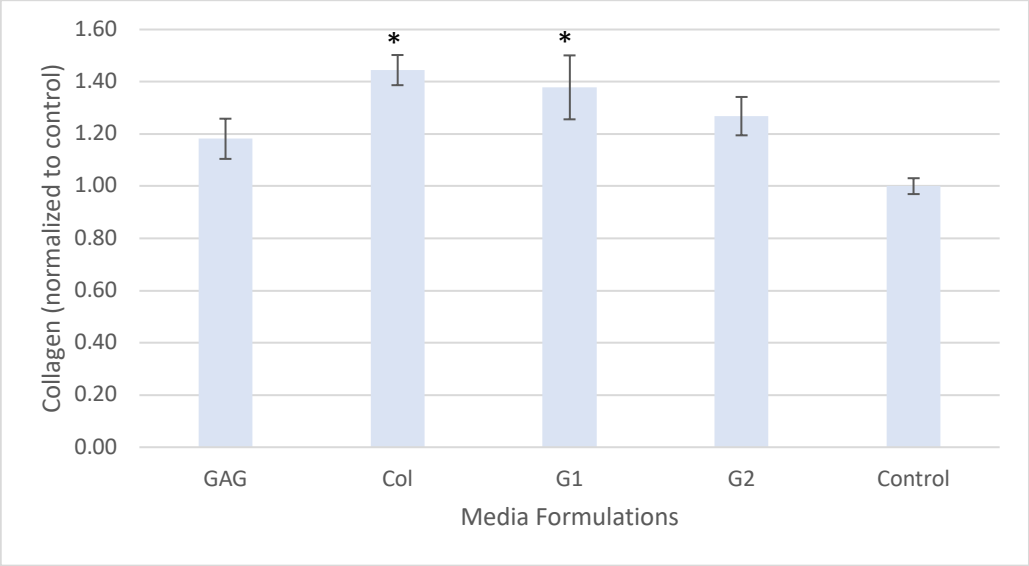

Figure 4.7 Effect of media on collagen accumulation. Data is presented as the mean ± SEM normalized to control, n = 4-7 samples per group. * Denotes a significant difference from control ($p<0.05$).

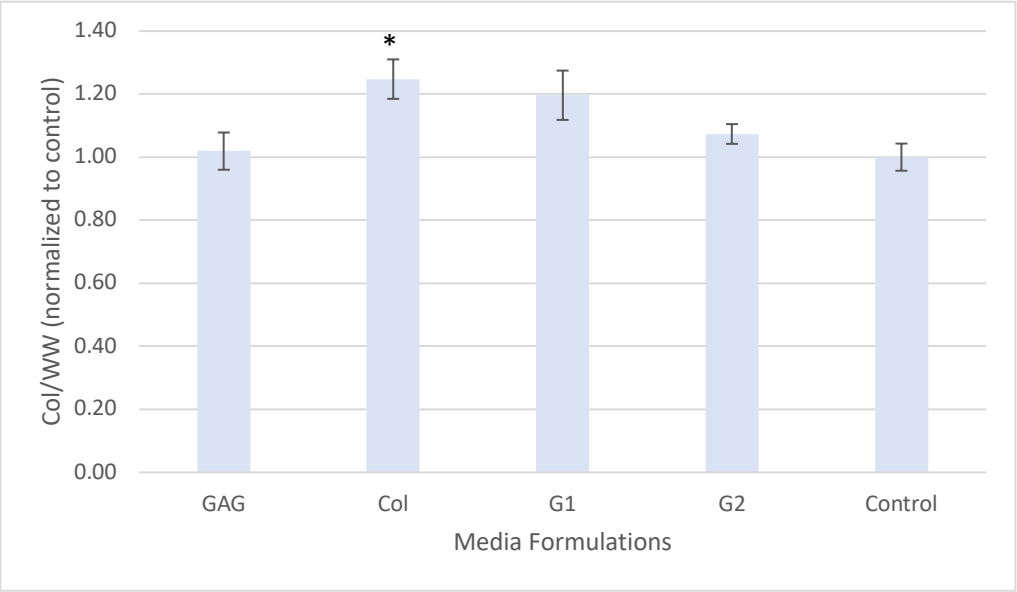

Figure 4.8 Effect of media on Col/WW accumulation. Data is presented as the mean ± SEM normalized to control, n = 4-7 samples per group. * Denotes a significant difference from control ($p<0.05$).

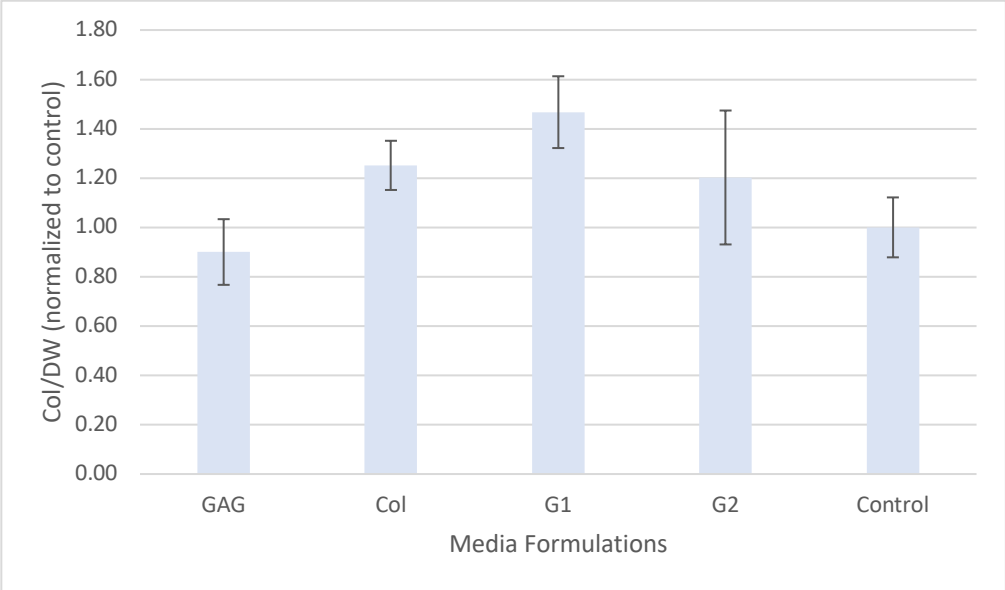

Figure 4.9 Effect of media on Col/DW accumulation. Data is presented as the mean ± SEM normalized to control, n = 4-7 samples per group. None of the groups were statistically different from control ($p>0.1$).

4.2.3 Effects of optimal media formulation on DNA accumulation

Total DNA accumulation of developed cartilaginous tissues was significantly affected by all optimal media formulations (Figure 4.11). Each media upregulated DNA accumulation in a similar fashion with increases observed between 1.13-fold to 1.38-fold ($p<0.001$) compared to control. However, when normalized to tissue weight (DNA/WW or DNA/DW), all optimal media formations had no apparent effect DNA accumulation (Figures 4.12 and 4.13).

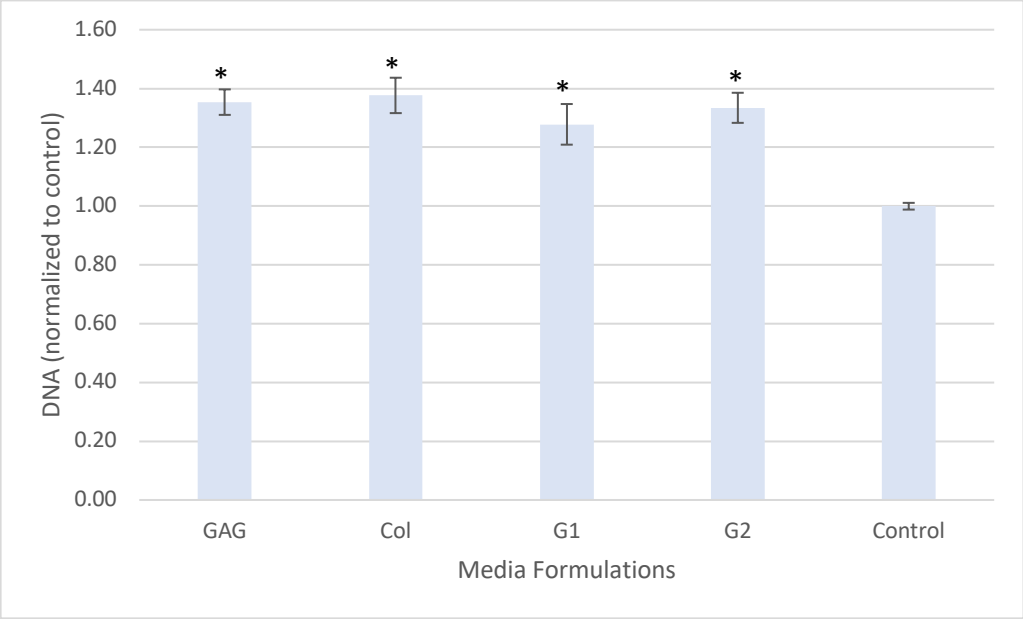

Figure 4.10 Effect of media on DNA accumulation. Data is presented as the mean ± SEM normalized to control, n = 4-7 samples per group. * Denotes a significant difference from control ($p<0.05$).

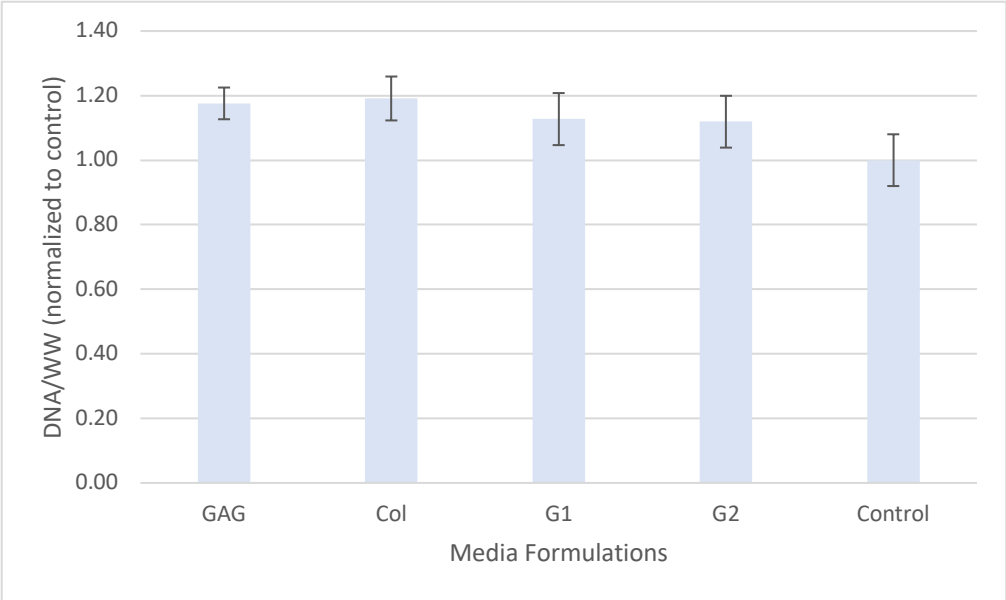

Figure 4.11 Effect of media on DNA/WW accumulation. Data is presented as the mean ± SEM normalized to control, n = 4-7 samples per group. None of the groups were statistically different from control **(p>0.1).**

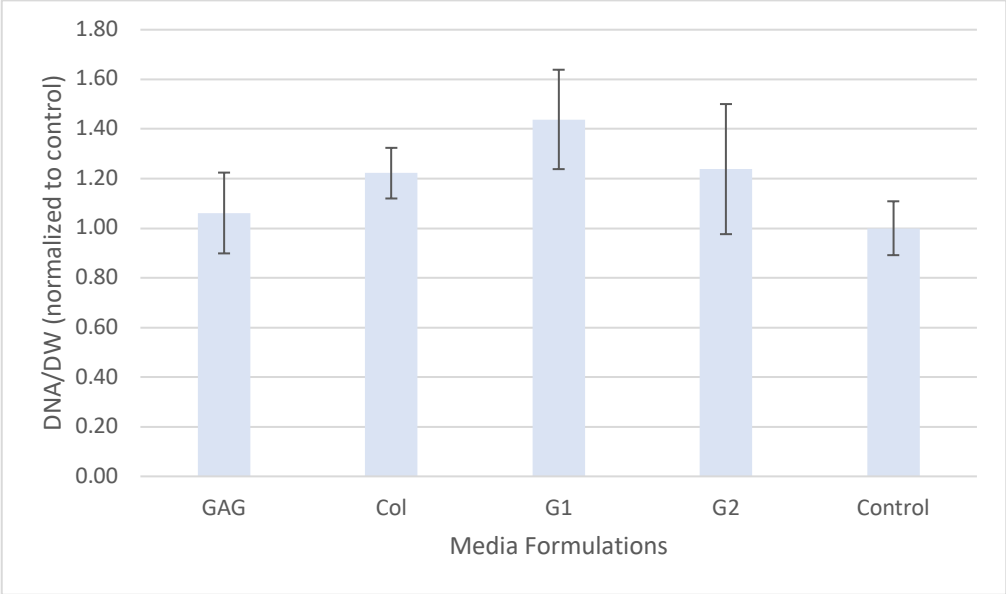

Figure 4.12 Effect of media on DNA/WW accumulation. Data is presented as the mean ± SEM normalized to control, n = 4-7 samples per group. None of the groups were statistically different from control (p>0.1).

4.2.4 Histological and immunohistochemical evaluation

Histological staining for proteoglycans (safranin-O; Figure 4.13b) displayed similar results compared to the biochemical accumulation of GAG. Compared to the control media, constructs cultivated in all optimal media formulations (with the exception of switch media G1) generally displayed more intense staining. In addition, constructs cultured in the optimal GAG media displayed the greatest intensity of staining compared to all other groups. Collagen staining (picrosirius red; Figure 4.13a) also displayed similar results compared to the biochemical accumulation of collagen. The optimal collagen media as well as switch media G1 appeared to display the greatest staining compared to the other media formulations and control media.

Immunohistochemical (IHC) staining for collagen type I (dedifferentiation marker; Figure 4.14a) generally appeared to be minimal throughout the constructs cultivated in all media formulations. However, constructs maintained in either the optimal GAG or collagen medias expressed some staining which was predominantly intracellular in nature (not expressed extracellularly). Alternatively, collagen type II staining in the developed constructs (Figure 4.14b) was predominantly extracellular in nature and the most abundant in both the optimal GAG and collagen media formulations compared to the control media.

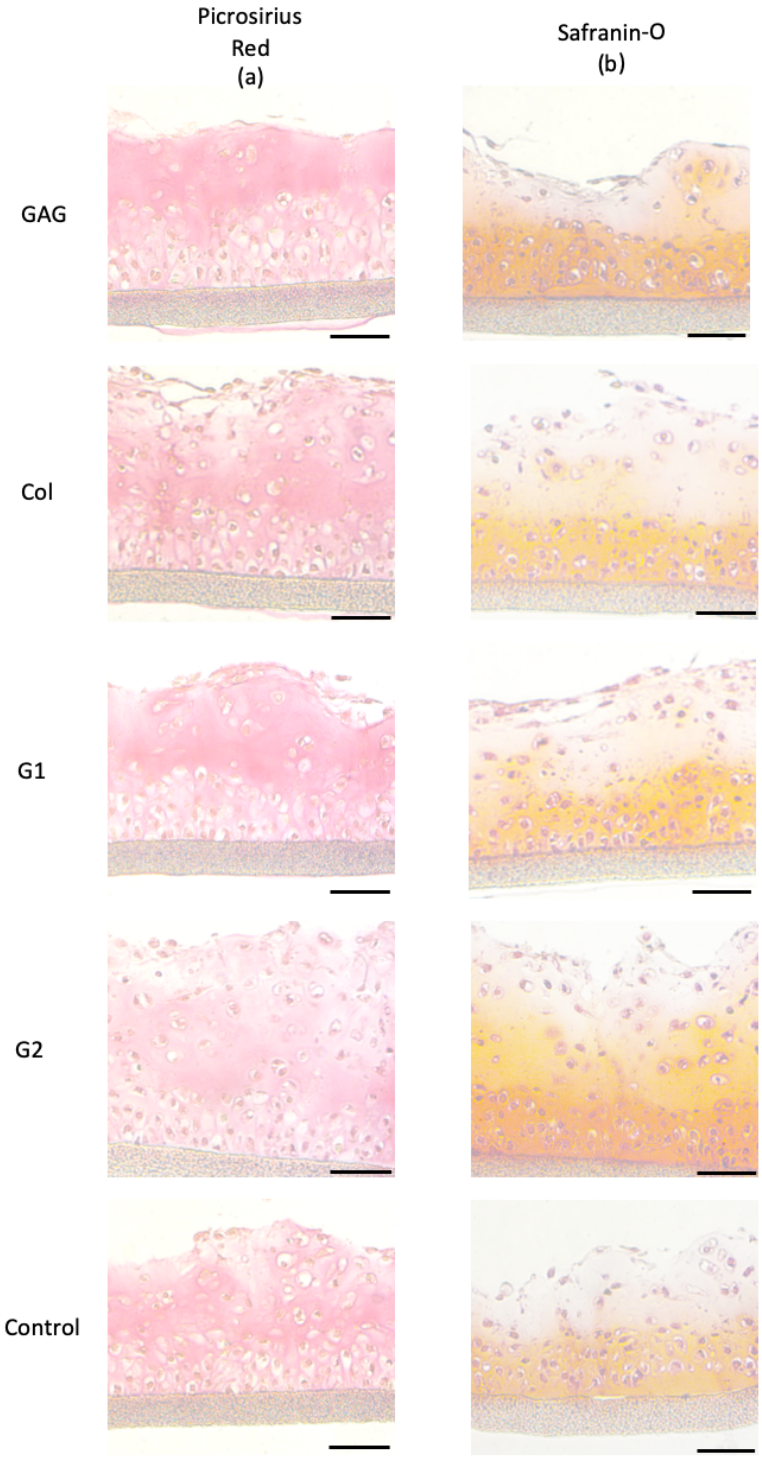

Figure 4.13 Histological staining for samples cultured in different media with Picrosirius red staining for general collagens and Safranin-O staining for proteoglycans. Scale bar: 100µm.

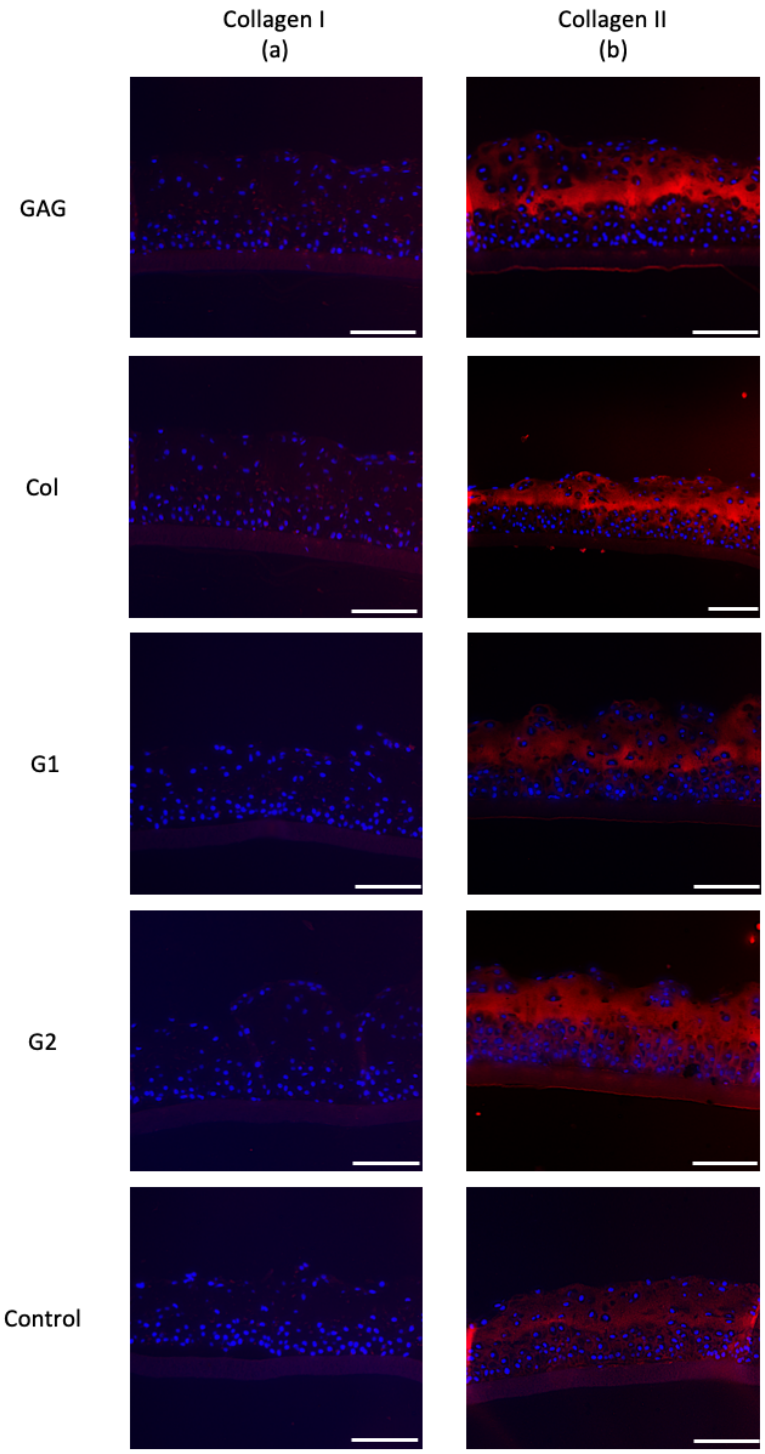

Figure 4.14 Collagen type I and II immunofluorescent staining of samples cultured in different media types. Scale bar: 100µm.

Chapter 5: Discussion

The purpose of this thesis was to explore different media formulations to upregulate the accumulation of cartilaginous extracellular matrix (specifically, proteoglycans and collagen) by providing the cells with different availability of nutrients (e.g. glucose, glutamine, basal media, media volume) as previous studies have demonstrated that nutrient availability can affect the accumulation and growth of tissue engineered cartilage [49,51-56].

This thesis was separated into two objectives. The first objective investigated nine different media formulations with the aim of finding optimal formulations to maximize the accumulation of proteoglycans and collagen. The second objective was to validate the previously identified media formulations that elicited maximal tissue growth. Each objective was accomplished and the results are discussed below.

5.1 Media Screening

5.1.1 Effect of media formulation on GAG accumulation

The results indicated that total GAG accumulation was primarily affected by the basal media formulation and media volume as determined by analysis of the eigenvalues of the response surface. Specifically, the eigenvalue that was the highest for total GAG accumulation stated that the maximal curvature was in the basal media direction. Based on the contour plot, it showed that the optimal value in terms of basal media was a mixture of DMEM/F12 media, fed at higher volumes (5 mL). A previous study has shown that supplementing cells with equal

volumes of DMEM/F12 with FBS has shown to increase GAG accumulation [77]. Combining DMEM and Ham's F12 is very important because DMEM has a high amino acid content, whereas, F12 is a vitamin rich formulation [47]. Samples that are cultured only in Ham's F12 show a lower amount of GAG production and cell growth when compared to samples that are cultured in DMEM/F12 mixture [78]. Therefore, in order to increase GAG accumulation, the optimal basal media formulation would be DMEM/F12 (1:1) mixture.

Additionally, in terms of GAG/WW, the results indicated that GAG accumulation was primarily affected by glucose concentration and media volume. The eigenvalue that was the highest for GAG/WW stated that the maximal curvature was in the media volume direction. Based on the contour plot, it showed that the optimal value in terms of media volume was at higher volumes. Generally, chondrocytes cultured in low glucose concentrations display significantly greater GAG accumulation [79]. However, glucose concentrations lower than 5 mM or higher than 25 mM can affect chondrocyte function [80]. Previous studies have shown that increasing the media volume, as well as having lower glucose concentrations, increases the GAG accumulation of tissue engineered constructs [51,81]. Moreover, constructs that are maintained in smaller volumes of media demonstrated a decrease in GAG accumulation when compared with constructs that are maintained in higher volumes [81]. Also, GAG accumulation has been shown to be directly proportional to media volume, therefore having higher volumes increases the rate of synthesis of GAG [51,52,57,81].

However, in terms of GAG/DW, the results indicated that GAG accumulation was primarily affected by glutamine concentration, basal media and media volume. The eigenvalue that was the highest for GAG/DW stated that the maximal curvature was in the glutamine direction. Previously observing total GAG and GAG/WW accumulation, basal media and media volume were fixed at DMEM/F12 and higher volumes (5 mL) for GAG/DW to depict the influence of glutamine concentrations. The results state that as the glutamine concentration is decreased, the effect on GAG accumulation increases. While it is known in the literature that glutamine is an essential nutrient required for cell culture; GAG synthesis decreases by at least 50% when glutamine is absent from culture media compared to media with 1 mM of glutamine [55,56,82,83]. Thus, it is critical to have glutamine in media cultures to allow for ECM synthesis.

Thus, it is expected that feeding chondrocytes with a media formulation that has lower glucose (5 mM) and glutamine (1 mM) concentrations that are fed at higher volumes (5 mL) should prove effective in maximizing GAG accumulation.

5.1.2 Effect of media formulation on collagen accumulation

The results indicated that total collagen accumulation was primarily affected by the basal media, media volume, glucose and glutamine concentrations. The eigenvalue that was the highest for total collagen accumulation stated that the maximal curvature was in the basal media direction. Collagen accumulation was least affected by glucose concentration and was maximal under low concentrations of glutamine and large media volumes. Glucose was fixed at a higher concentration (9.5 mM) because chondrocytes need glucose in order to sustain ECM

synthesis [52]. The use of low glucose concentrations in culture can inhibit the glycolysis pathway, which in turn prevents ECM synthesis [52]. Based on the contour plot, it showed that the optimal in terms of basal media was F12 media, fed at higher volumes. DMEM media is a medium that is very high in amino acids, which are also important for collagen synthesis [84]. However, F12 media is rich in vitamin B12, which previous studied have shown to support collagen synthesis by chondrocytes as well as osteoblasts [85-87].

Additionally, in terms of Col/WW and Col/DW, the results indicated that collagen accumulation was also primarily affected by the basal media, media volume, glucose and glutamine concentrations. The eigenvalue that was the highest for Col/WW stated that the maximal curvature was in the media volume and glucose concentration direction. Furthermore, the eigenvalue that was the highest for Col/DW stated that the maximal curvature was in the media volume direction. Chondrocytes fed with higher volumes tended to display higher collagen accumulation when compared to lower media volumes (section 4.1). Maintaining constructs in higher volumes successfully increased the rate of synthesis of collagen [51,52,57,81], than when comparing constructs that are grown in smaller volumes [81,82]. Collagen accumulation was less affected by basal media and a glutamine concentration; therefore, they were fixed at F12 media and a lower glutamine concentration to be able to visualize the effects of Col/WW. Whereas, for Col/DW, collagen accumulation was less affected by basal media and glucose concentration; therefore, they were fixed at F12 media and higher glucose concentrations. Based on the contour plots, the optimal in terms of media volume were at higher volumes and

lower glucose concentrations for Col/WW, but for Col/DW the optimal in terms of media volume were at higher volumes with lower glutamine concentrations.

Glutamine is also a very important factor for collagen accumulation [82]. Elevated glutamine concentrations in the media decreased the specific activity of collagen synthesis [83,89]. Adding lower concentrations of glutamine had a stimulating effect on collagen synthesis by directly impacting it at a transcriptional level [89-90]. Glutamine increases mRNA levels at exact concentrations that were needed for optimal collagen production [89-90].

Thus, it is expected that feeding chondrocytes with a media formulation that has higher glucose (10 mM) and glutamine (1 mM) concentration that are fed at higher volumes (4.5 mL) should prove effective in maximizing collagen accumulation.

5.1.3 Effect of media formulation on DNA accumulation

The results indicated that total DNA accumulation was primarily affected by the basal media, glucose and glutamine concentrations. The eigenvalue that was the highest for total DNA accumulation stated that the maximal curvature was in the basal media direction. Based on the contour plot, the range that was established for glucose had little effect on DNA accumulation, but the basal media was determined to be DMEM/F12 mixture. Additionally, in terms of DNA/WW, the results state the total DNA accumulation was primarily affected by the basal media and glucose concentration. The highest eigenvalue for DNA/WW accumulation was also in the basal media direction. As previously mentioned, the range that was established for

glucose had little effect on DNA accumulation, but the basal media was between DMEM/F12 mixture and F12. Furthermore, in terms of DNA/DW, the results show that DNA accumulation was affected by basal media, media volume and glutamine. Having higher concentrations of glutamine allowed for the cell culture medium to have an excess of amino acids, which in turn increased the rate of DNA synthesis [89-90]. In addition, having a combination of DMEM and Ham's F12 media (DMEM/F12) is important to balance amino acid and vitamin concentrations, which have been shown to stimulate DNA synthesis [47]. The highest eigenvalue for DNA/DW accumulation was also in the basal media direction. Since media volume affected DNA accumulation the least, it was fixed at a mid-point of the volume range. Based on the contour plots, having a basal media of F12 with a higher glutamine concentration enhanced DNA accumulation.

Optimal media formulation to stimulate DNA accumulation was then identified as follows: basal media between DMEM/F12 mixture (1:1), media volume of 3 mL, glucose concentration between 5 mM and 10 mM and a glutamine concentration of 3 mM.

5.2 Validation Study

In the validation study, tissue engineered constructs were grown in two different media formulations, specifically geared towards increasing GAG accumulation and collagen accumulation. Additionally, two switching medias groups were added: group 1 (G1) and group 2 (G2). G1 was fed with the optimal GAG media formulation for two weeks and then switched to the optimal collagen media formulation; whereas, G2 was fed with the optimal collagen media

formulation for two weeks and then switched to the optimal GAG media formulation. The reason for investigating switching media formulations was primarily to determine the effect of having both optimal media formulations influenced the growth of tissue engineered constituents more so than separate optimal media formulations. Tissue samples were evaluated for changes in collagen and proteoglycan accumulation, as well as DNA content and histological and immunohistochemical evaluations.

5.2.1 Effects of optimal media formulation on GAG accumulation

With respect to total GAG and its normalizations (to wet and dry weights), the rate of GAG accumulation appeared to increase with the optimal GAG media formulation when compared to control. GAG optimal media consisted of DMEM/F12 mixture, with lower glucose (5 mM) and glutamine (1 mM) concentrations, fed at higher volumes (5 mL). Whereas, optimal collagen media consisted of F12 basal media, with higher glucose (9.5 mM) and lower glutamine (1 mM) concentrations, fed at higher volumes (4.5mL). Expectedly, the rate of GAG accumulation appeared to decrease when cultured in the optimal collagen media formulation, compared to the optimal GAG media formulation. GAG accumulation decreased in the optimal collagen media formulation primarily due to the basal media and glucose concentrations. When Ham's F12 media is used, it lowered the amount of GAG accumulation since it is not as rich with other components as DMEM/F12 media [85]. However, culturing samples in DMEM/F12 has been proven to increase GAG accumulation due to the rich amino acid content of both basal media [47,85,88]. Additionally, when cultures were grown in a higher glucose concentration, they decreased the amount of GAG accumulation but increased the amount of collagen

accumulation [52,79]. Generally, low glucose concentrations tended to favour GAG accumulation [79].

Interestingly, the switching media (G1 and G2) had no apparent effect on total GAG accumulation. In G1, cultures were fed specifically with a low glucose concentration and DMEM/F12 basal media mixture for two weeks and a high glucose concentration (9.5 mM) and F12 basal media for the following two weeks, which inhibited GAG accumulation. In G2, cultures were fed specifically with a high glucose concentration and F12 basal media for two weeks and a low glucose concentration and DMEM/F12 basal media mixture for the following two weeks, which also inhibited GAG accumulation. At lower glucose concentrations, chondrocytes are being stimulated to produce more GAG, but once they are introduced to higher glucose concentrations, it impairs the formation of GAG [51,79,81]. Furthermore, in the case of G2, the chondrocytes ability to produce GAG is decreased by the higher glucose concentration [51], thus, when introduced to the lower glucose concentrations, they might not have the ability to increase GAG accumulation. Overall, the glutamine concentration and media volume had no negative effect on GAG accumulation. Glutamine helped maintain cell viability and increase GAG accumulation [82,83,89,90]. Whereas, media volume was maintained at higher volumes to also increase GAG accumulation [51,52,81].

5.2.2 Effects of optimal media formulation on collagen accumulation

With respect to total collagen and its normalizations to wet weight and dry weight, the rate of collagen accumulation appeared to significantly increase with the optimal collagen media

formulation when compared to the control. The optimal collagen media consisted of F12 basal media, with higher glucose (9.5 mM) and lower glutamine (1 mM) concentrations, fed at higher volumes (4.5 mL), whereas, the optimal GAG media differed in basal media (DMEM/F12 mixture), glucose concentrations (5 mM) and media volume (5 mL). Expectedly, the rate of collagen accumulation appeared to decrease with the optimal GAG media formulation when compared to the optimal collagen media formulation. The primary difference in this case between the media formulations is the basal media and glucose concentration. F12 media is important for collagen synthesis, since it is rich in vitamins, specifically vitamin B12 [85-87]. Vitamin B12 is an important factor because it increases hydroxyproline content which is needed in order to stimulate collagen synthesis [88]. Chondrocytes that are exposed to higher concentrations of glucose (10 mM) tend to accumulate more collagen and favour the formation of the ECM than when cultured in lower glucose (5 mM) containing media [79,80].

Interestingly, the switching media G1 had a significant effect on total collagen accumulation. G2 had no apparent effect on total collagen accumulation. In G1, cultures were fed specifically with a low glucose concentration and a DMEM/F12 basal media mixture for two weeks and a high glucose concentration and F12 basal media for the following two weeks, which increased collagen accumulation. In G2, cultures were fed specifically with a high glucose concentration and F12 basal media for two weeks and a low glucose concentration and DMEM/F12 basal media mixture for the following two weeks, which based on observations also inhibited collagen accumulation. In the case of G1, the chondrocytes could have potentially had the time to lay down GAG so that once they were switched to higher glucose concentrations, it allowed

them to successfully start producing even more collagen [79,80]. Furthermore, in the case of G2, the chondrocytes' ability to produce collagen was decreased by the lower glucose concentration [52]. At higher glucose concentrations, chondrocytes were being stimulated to produce more collagen, but once they were introduced to lower glucose concentrations, it impaired the formation of collagen and increased GAG accumulation [51,52,79-81]. Overall, the glutamine concentration and media volume had no negative effect on collagen accumulation. Glutamine helped maintain cell viability, whereas, media volume was maintained at higher volumes to increase collagen accumulation.

5.2.3 Effects of optimal media formulation on DNA accumulation

While an optimal DNA media formulation was not created due to the fact that the primary objective was to increase ECM accumulation and for this reason, we did not investigate the effect of an optimal media formation. With respect to total DNA, the rate of DNA accumulation appeared to be significantly increased with all the optimal media formulations compared to control. Interestingly, the rate of DNA accumulation appeared to be significantly increased in the optimal collagen and optimal GAG media formulations; with the only difference between the two media formulations being the basal media and the glucose concentration. Although, the concentration of glutamine in this case was an important factor in increasing DNA content.

A DMEM/F12 basal media is rich in amino acids since it combines the two most common media that are both full of amino acids and vitamins [47]. Having a basal media that is rich in amino acids aids in DNA synthesis [47]. DMEM on its own has fewer vitamins and amino acids and the

basic components needed for cell culture when compared to F12 and DMEM/F12 [47]. Higher concentrations of glutamine allowed for cell culture media to have an increased amount of amino acids which in turn stimulates the rate of DNA synthesis [89,90]. Glutamine is an important direct or indirect precursor to other amino acids and nucleotides which stimulates DNA synthesis [91]. Therefore, depletion of glutamine could be detrimental to the upregulation of DNA synthesis [91].

5.2.4 Histological and immunohistochemical evaluation for GAG and collagen accumulation

Histological images appeared to display similar results when compared to both biochemical accumulation of GAG and collagen. As mentioned above, constructs that were cultured in optimal GAG media had the greatest intensity of staining in all groups when compared to the control. The accumulation of GAG in the Safranin-O staining appeared to be towards the bottom of the tissue, which is an indication that GAG was at its maximal peak in the deep zone of cartilage tissue [24]. Expectedly, the staining showed that the optimal collagen media formulation decreased the rate of GAG accumulation. Additionally, constructs that were cultured in optimal collagen media had the greatest intensity of staining in the collagen and G1 groups. The accumulation of collagen in the picrosirius red staining appears to be towards the top of the tissue, which is an indication that collagen was at its maximal peak in the superficial zone of cartilage tissue [24]. Expectedly, the staining showed that the optimal GAG media formulation decreased with the rate of collagen accumulation when compared to the other groups.

Furthermore, staining for collagen type I appeared to be minimal throughout the constructs that were cultured in all media formulations. Normal articular cartilage has a collagen content that is composed of 90-95% type II collagen with little to no type I collagen [21]. Collagen type II staining in the constructs was the most abundant in both the optimal GAG and collagen media formulations when compared to the control media. Both optimal GAG and collagen media formulations stimulated an increase of collagen type II due to the composition of their media. As stated previously, both DMEM/F12 and F12 media are essentially basal media types in order to increase collagen type II. Both basal medias are enriched with amino acids and vitamins that are necessary for the production of collagen in general [47,85-88]. Another important factor is also the concentration of glucose. At higher concentrations of glucose, the chondrocytes were stimulated to increase the production of collagen [52]. The immunohistochemical analysis appears to be similar when compared to the biochemical accumulation of collagen for all groups.

5.3 Potential Limitations

A potential limitation is that the age range was set from 20-24 months. Widening the age range could potentially increase the statistical analysis of the data to provide more accurate results. However, conducting experiments with skeletally mature chondrocytes is more representative of the aged chondrocytes that would be encountered clinically. Another potential limitation was the lower R^2 values of the curve fits determined during the media screening experiment. The lower R^2 value might lead to misinterpretations when determining the optimal media parameters; however, review of the literature and the validation experiment confirmed the optimal parameters chosen in media screening study.

Chapter 6: Conclusions and Recommendations

6.1 Conclusions

In conclusion, the optimization of media for chondrocytes was explored in 3D culture. The major objective of the work presented was to explore the application of different media formulations to enhance extracellular constituents in tissue engineered cartilage. It has been shown that the accumulation of proteoglycans and collagen by chondrocytes can be significantly affected by nutrient availability (basal media, media volume, glucose, and glutamine)**.**

6.1.1 Glucose and glutamine concentrations

When ranges of glucose concentrations were added to the different basal media, it influenced accumulation of proteoglycans and collagen differently. There was an upregulation of proteoglycan accumulation with lower glucose concentrations. However, there was an upregulation of collagen accumulation with higher glucose concentrations. Ultimately, upregulation of constituents relies heavily on different glucose concentrations. This study showed that the same media formulation for both constituents was not possible since it required two very distinct formulations for upregulation. When ranges of glutamine concentrations were added to the different basal media, it did not influence the accumulation of proteoglycans and collagen differently. In regards to media screening, to showed that having lower concentrations proved effective in the accumulation of proteoglycans and collagen. This was also supported by previous studies that have noted that higher concentrations of glutamine could potentially be detrimental to chondrocyte survival [29].

6.1.2 Basal media and media volume

Specific basal medias influenced the accumulation of proteoglycans and collagen differently. Proteoglycan accumulation was heavily influenced by having a DMEM/F12 (1:1) mixture. Whereas, collagen accumulation was heavily influenced on having only F12 basal media. Basal media plays an important role due to the nutrients available from each specific medium. Similarly, media volume is also an important factor in proteoglycan and collagen accumulation. Based on media screening, to upregulate both constituents' accumulation, a higher media volume is needed. In the validation study, it was confirmed that feeding at higher volumes increased accumulation.

6.2 Recommendations and future work

The ultimate goal of tissue engineering is to re-create human tissue for eventual implantation into patients. A potential recommendation is to ensure that the age of the cells from which the chondrocytes are harvested is the same. The age range presented in this thesis was from 20-24 months, therefore, narrowing the range could provide a stronger data set. A potential source of error is human error as it always plays a role in research. Another potential source of error is random error, as there might have been some variation with the measuring scale.

6.2.1 Future work

Future work must be carried out to understand how these optimal media formulations will work without fetal bovine serum; since it would be challenging for a regulatory agency to approve products for clinical use that contain non-human proteins. Supplementing the media directly with different growth factors that could get rid of fetal bovine serum shows promising results for future work and further optimization provided in this work. Investigation of specific growth factors for the development of human cartilaginous tissue *in vitro* would be beneficial, as bovine articular chondrocytes could exhibit different responses compared to articular chondrocytes from different species.

Appendix 1

DMEM

Low Glucose, Low Glutamine Media (Media 1)					
		mol/L	MW	g/L	Total (g)
5 mM	Glucose	0.005	118.16	0.5908	0.23632
1 mM	Glutamine	0.001	146.14	0.14614	0.058456
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.1 DMEM media formulation with low glucose and low glutamine concentrations.

High Glucose, Low Glutamine Media (Media 2)					
		mol/L	MW	g/L	Total (g)
10 mM	Glucose	0.01	118.16	1.1816	0.47264
1 mM	Glutamine	0.001	146.14	0.14614	0.058456
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.2 DMEM media formulation with high glucose and low glutamine concentrations.

Low Glucose, High Glutamine Media (Media 3)					
		mol/L	MW	g/L	Total (g)
5 mM	Glucose	0.005	118.16	0.5908	0.23632
3 mM	Glutamine	0.003	146.14	0.43842	0.175368
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.3 DMEM media formulation with low glucose and high glutamine concentrations.

High Glucose, High Glutamine Media (Media 4)					
		mol/L	MW	g/L	Total (g)
10 mM	Glucose	0.01	118.16	1.1816	0.47264
3 mM	Glutamine	0.003	146.14	0.43842	0.175368
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.4 DMEM media formulation with high glucose and high glutamine concentrations.

DMEM/F12

Low Glucose, Low Glutamine Media (Media 1)					
		mol/L	MW	g/L	Total (g)
5 mM	Glucose	0.005	118.16	0.5908	0.23632
1 mM	Glutamine	0.001	146.14	0.14614	0.058456
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.5 DMEM/F12 media formulation with low glucose, and low glutamine concentrations.

High Glucose, Low Glutamine Media (Media 2)					
		mol/L	MW	g/L	Total (g)
10 mM	Glucose	0.01	118.16	1.1816	0.47264
1 mM	Glutamine	0.001	146.14	0.14614	0.058456
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.6 DMEM/F12 media formulation with high glucose, and low glutamine concentrations.

Low Glucose, High Glutamine Media (Media 3)					
		mol/L	MW	g/L	Total (g)
5 mM	Glucose	0.005	118.16	0.5908	0.23632
3 mM	Glutamine	0.003	146.14	0.43842	0.175368
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.7 DMEM/F12 media formulation with low glucose and high glutamine concentrations.

High Glucose, High Glutamine Media (Media 4)					
		mol/L	MW	g/L	Total (g)
10 mM	Glucose	0.01	118.16	1.1816	0.47264
3 mM	Glutamine	0.003	146.14	0.43842	0.175368
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.8 DMEM/F12 media formulation with high glucose and high glutamine concentrations.

Mid Glucose, Mid Glutamine Media – Control Media					
		mol/L	MW	g/L	Total (g)
7.5 mM	Glucose	0.0075	118.16	0.8862	0.17724
2 mM	Glutamine	0.002	146.14	0.29228	0.058456
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.022008
25 mM	HEPES	0.025	238.31	5.95775	1.19155

Table A1.9 DMEM media formulation with mid glucose and mid glutamine concentrations (media control).

F12

Low Glucose, Low Glutamine Media (Media 1)					
		mol/L	MW	g/L	Total (g)
5 mM	Glucose	0.005	118.16	0.5908	0.23632
1 mM	Glutamine	0.001	146.14	0.14614	0.058456
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.10 F12 media formulation with low glucose, and low glutamine concentrations.

High Glucose, Low Glutamine Media (Media 2)					
		mol/L	MW	g/L	Total (g)
10 mM	Glucose	0.01	118.16	1.1816	0.47264
1 mM	Glutamine	0.001	146.14	0.14614	0.058456
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.11 F12 media formulation with high glucose, and low glutamine concentrations.

Low Glucose, High Glutamine Media (Media 3)					
		mol/L	MW	g/L	Total (g)
5 mM	Glucose	0.005	118.16	0.5908	0.23632
3 mM	Glutamine	0.003	146.14	0.43842	0.175368
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.12 F12 media formulation with low glucose, and high glutamine concentrations.

High Glucose, High Glutamine Media (Media 4)					
		mol/L	MW	g/L	Total (g)
10 mM	Glucose	0.01	118.16	1.1816	0.47264
3 mM	Glutamine	0.003	146.14	0.43842	0.175368
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.13 F12 media formulation with high glucose, and high glutamine concentrations.

Mid Glucose, Mid Glutamine Media – Control DMEM and DMEM/F12					
		mol/L	MW	g/L	Total (g)
7.5 mM	Glucose	0.0075	118.16	0.8862	0.35448
2 mM	Glutamine	0.002	146.14	0.29228	0.116912
1 mM	Sodium Pyruvate	0.001	110.04	0.11004	0.044016
25 mM	HEPES	0.025	238.31	5.95775	2.3831

Table A1.14 DMEM and DMEM/F12 media formulation with mid glucose and mid glutamine concentrations (media control).

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