EFFECTS OF ADDITIONAL SODIUM BICARBONATE ON EXTRA/INTRA CELLULAR FACTORS IN A CONTINUOUS FLOW BIOREACTOR FOR THE PRODUCTION OF TISSUE ENGINEERED ARTICULAR CARTILAGE

by

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Abstract

Articular cartilage has a low propensity for self-repair, due to which 27 million people are affected by osteoarthritis every year in North America. The current repair techniques used for cartilage defects possess flaws that reduce long-term clinical success. Tissue engineering carries with it the promise of engineering hyaline-like cartilage with physical and biochemical properties, similar to that of native cartilage.

This being said, the primary objective of my project was to engineer clinically relevant sized articular cartilage constructs. To achieve my objective, first, I investigated the effect of continuous culture on cartilaginous tissue growth. Constructs grown under continuous media flow significantly accumulated more collagen and proteoglycans, and displayed a stratified morphology, similar to that found in native cartilage. The second goal was to further increase chondrocyte proliferation, and extracellular matrix (ECM) accumulation. To achieve this, constructs were grown in a bioreactor with media supplemented with 14 mM sodium bicarbonate ($NaHCO_3$). Constructs cultivated in the bioreactor with $NaHCO_3$ supplementation exhibited a significant *($p<0.05$)* increase in ECM accumulation (a 98-fold increase in glycosaminoglycans and a 25-fold increase in collagen content), cell proliferation (a 13-fold increase), and thickness (a 28-fold increase) compared to all other conditions (static and reactor without $NaHCO_3$ supplementation).

The third goal was to engineer cartilage constructs with as little cells as possible, reducing donor site morbidity. From the results obtained, it was evident that the monolayer constructs outperformed all the other constructs (pellet, biopsy, and minced).

The final goal was to understand the underlying reason for the increased proliferation. First, I investigated if there were any differences present in intracellular pH (pHi) and intracellular

buffering capacity. Second, I determined the role of extracellular pH (pHe) on cell proliferation. In an effort to accurately achieve this, I, for the first time, have reported on measuring pHi of chondrocytes while still in culture (2D and 3D cultures) using a confocal microscope. This study demonstrated the importance of extracellular environments, such as pHe, extracellular buffering capacity, and the presence of carbon dioxide and bicarbonate ions for chondrocyte proliferation.

Dedication

I would like to dedicate this thesis to my parents Nishat Arif Khan and Muhammad Arif Khan who have loved me unconditionally, believed in me, supported me and encouraged me throughout my life. Their multiple sacrifices have made my childhood dream of becoming a scientist, a reality. Thank you Mama and Baba.

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List of Abbreviations

ECM	extracellular matrix
$NaHCO_3$	sodium bicarbonate
pHi	intracellular pH
pHe	extracellular pH
pKa	acid dissociation constant
pHo	solution pH
HEPES	*N*-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
CO_2	carbon dioxide
2D	two dimensional
3D	three dimensional
HCO_3^-	bicarbonate ion
COOH	carboxyl group
SO_4	sulfate group
ACI	autologous chondrocyte implantation
MACI	matrix induced autologous chondrocyte implantation
MMPs	matrix metalloproteinases
TIMPs	tissue inhibitors of metalloproteinases
MSC	mesenchymal stem cells
PDGF	platelet-derived growth factor
bFGF	basic fibroblast growth factor
IGF-I	insulin growth factors
IGF-II	insulin-like growth factors
TGF-β	transforming growth factor-beta
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced
ATP	adenosine-5'-triphosphate
TCA	tricarboxylic acid
GAG	glycosaminoglycan
NHE	sodium-hydrogen exchanger
AE	Cl^-/HCO_3^- anion-exchanger
DNA	deoxyribonucleic acid

SNARF-1-AM	carboxy- seminaphthorhodafluor-1
FBS	Fetal bovine serum
EDTA	Ethylenediaminetetraacetic acid
DDT	Dichlorodiphenyltrichloroethane
H&E	Hematoxylin and eosin stain
TB	Toluidine blue
PAS	Periodic-acid shiff
SEM	Standard error of the mean
H_2CO_3	Carbonic acid
DAPI	4',6-diamidino-2-phenylindole
HBS	HEPES buffered solution
BHBS	Bicarbonate and HEPES buffered solution
NH_4Cl	Ammonium chloride
DMSO	Dimethyl sulfoxide
$CaCl_2$	Calcium chloride
B_I	Intrinsic buffering capacity
B_T	Total buffering capacity
NH_4^+	Ammonium
$NaHCO_3$-CO_2	20 mM HEPES and 14 mM $NaHCO_3$ under 5% CO_2
HEPES-air	35 mM HEPES in air

Chapter 1
Introduction and Literature Review

1.1 Cartilage Anatomy and Function

Articular cartilage is a load bearing soft tissue that lines the surfaces of subchondral bones, like the knee, elbow and shoulder joints. Figure 1.1 is a pictorial representation of a human knee joint. Cartilage provides these surfaces, with low friction and wear resistance essential for repetitive gliding motion and shock absorption, while evenly spreading the applied load on to the underlying bone. Articular cartilage is an acellular and an avascular tissue made up of a sparse population of highly specialized cells known as chondrocytes. Chondrocytes make up only 1-10% of the total cartilage volume depending on the location and thickness of the cartilage [1-7]. The major components of the extracellular matrix (ECM) are water, proteoglycans and collagens (Figure 1.2). Articular cartilage has a high water content (approximately 65-80% wet weight)[8]. Ten to twenty percent of cartilage by wet weight is collagen type II, while 4-7% is aggrecan. Molecules such as proteins, glycoproteins, lipids and phospholipids are also present in the ECM. These molecules account for less than 5% of the wet weight of cartilage [2-5, 8].

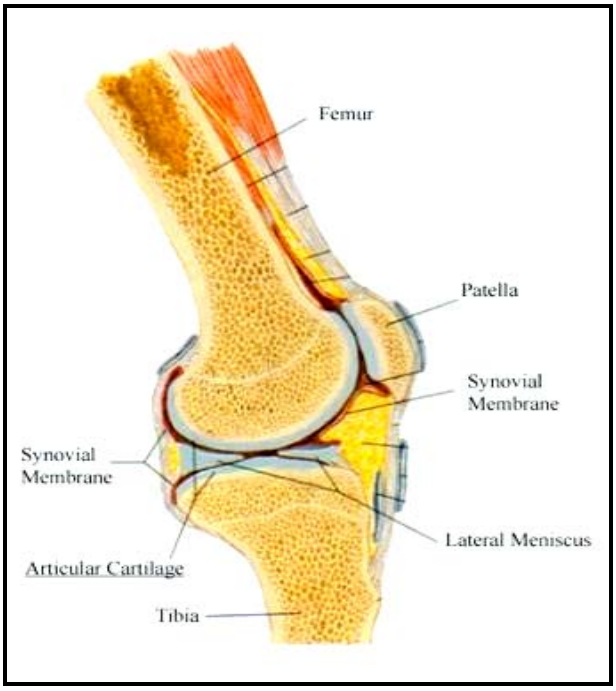

Figure 1.1: Pictorial Representation of the Knee Joint (Unmodified Image)[9].

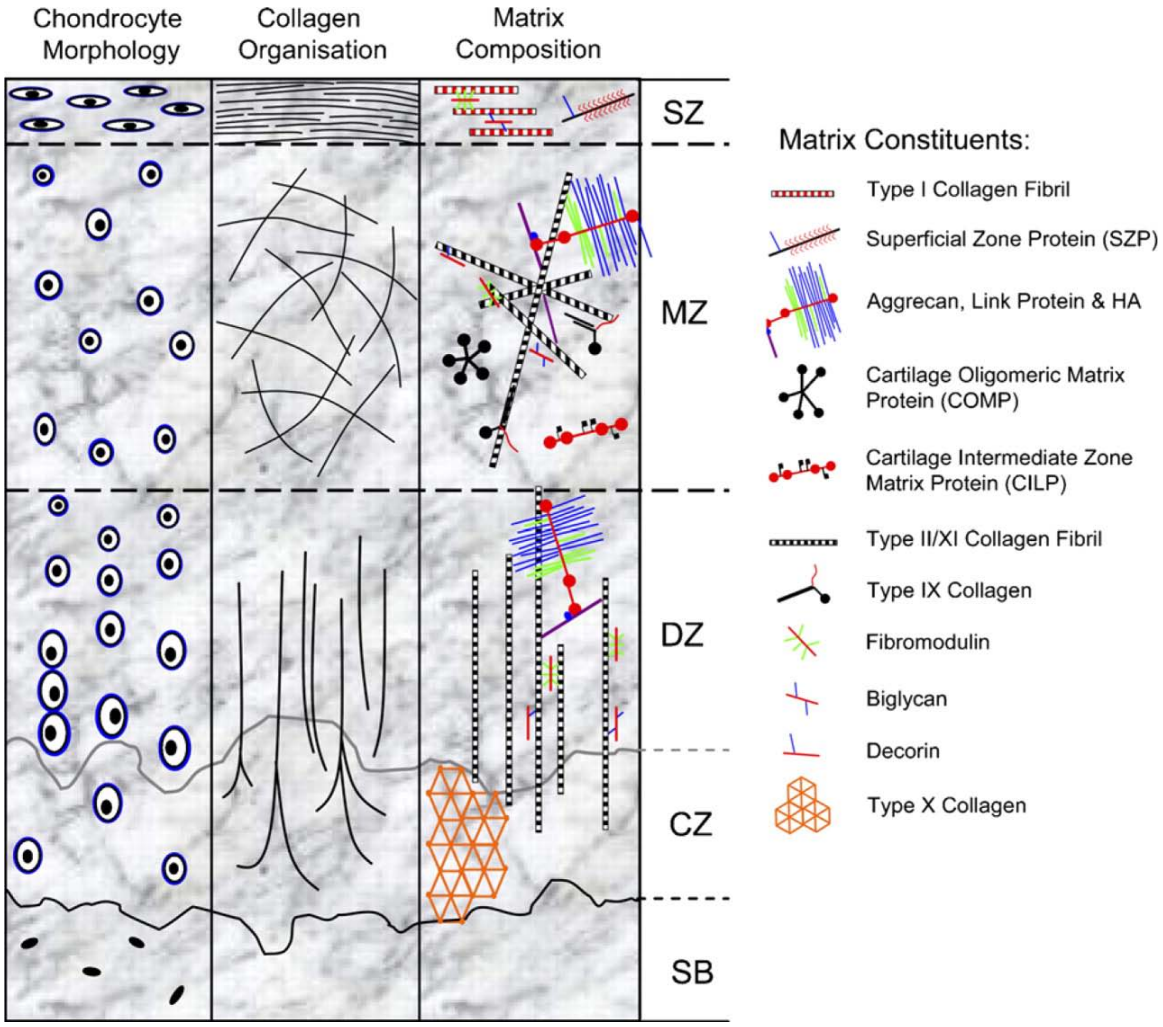

Figure 1.2: Molecular Organization of Articular Cartilage Matrix (Unmodified Image) [10]

1.1.1 Architecture of Articular Cartilage

Articular cartilage is a very specialized tissue made up of four distinctive zones, with the arrangement, structure and composition varying between the individual zones [3, 8, 11-13]. The chondrocytes in each zone respond differently to stimuli and secrete different proteins [14]. Figure 1.3 is a pictorial representation of the architecture of articular cartilage. The uppermost zone, also known as the superficial zone, has the highest amount of water content and the lowest amount of proteoglycan content compared to the other zones. Chondrocytes in the superficial zone are elongated and orientated horizontally [5, 8]. Collagen fibrils found in the superficial zone are thin, closely-knitted and aligned along their horizontal axis [5, 8]. The superficial zone possesses the highest tensile strength, compared to the other zones [15, 16]. The middle zone just below the superficial zone has collagen fibrils with a larger diameter, which are arranged in no particular fashion [8]. The chondrocytes found in the middle zone are more rounded in appearance and are characterized with a low cell density [5]. The deep zone found below the middle zone is characterized with spherical chondrocytes arranged in a columnar fashion, high proteoglycan content, low water content and thick, yet sparse, collagen fibril content when compared to the other zones [8]. The cells in the deep zone have the highest rate of synthesis, approximately ten times higher than the cells in the superficial zone [17, 18]. The calcified zone, found below the deep zone is composed of smaller hypertrophic chondrocytes that produce type X collagen [5]. These cells have the ability to calcify the surrounding matrix. All chondrocytes are surrounded by a pericellular region consisting of collagen type VI and decorin [5].

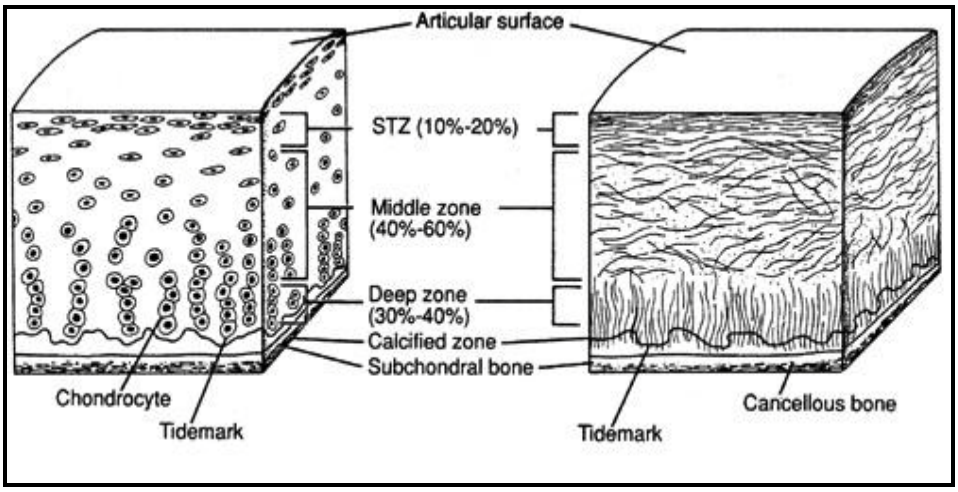

Figure 1.3: Zonal Organization of Articular Cartilage Tissue. Articular cartilage is made up of four zones: superficial, middle, deep and calcified, with the composition and arrangement varying between the individual zones (Unmodified Image) [19].

1.1.2 Extracellular Matrix Molecules

1.1.2.1 Water

Water makes up 65-80% of the tissue's wet weight [20]. Approximately 30% of this water is found in the collagen intrafibrillar space while the rest is found in the extracellular matrices microscopic pores [8]. The percentage of water present varies between the individual zones, with the highest percentage, approximately 80%, being in the superficial zone and the lowest percentage, approximately 65%, being in the deep zone [8]. Inorganic salts, such as calcium, chlorine, sodium, and potassium, are found dissolved in the water present in articular cartilage [8]. Water is moved across the extracellular matrix by either applying a pressure gradient across the tissue or by compression when the tissue is loaded. Articular cartilage possesses low permeability because of the high frictional resistance present; therefore, high pressure is required

to overcome the frictional resistance, thus, allowing movement of water within the tissue [21]. The flow of water through the ECM plays a very important role as it provides lubrication and allows distribution of nutrients within the tissue [21]. Articular cartilage has a high affinity for water due to the hydrophilic nature of proteoglycans [22-26].

1.1.2.2 Collagen

Collagen fibril diameter and orientation change throughout the depth of the four zones in articular cartilage. Collagen provides cartilage with its tensile strength and shear properties while immobilizing the proteoglycans within the ECM. Although collagen is responsible for providing the articular cartilage with tensile strength, it does not contribute much towards compression. This is due to the length-to-diameter ratio of the collagen fibrils, resulting in very slender fibers [15, 27-29]. Fifty percent of articular cartilage by dry weight is collagen of which 90-95% is collagen type II, while the remaining comprises of collagen types I, V, VI, IX, X, and XI [8]. Collagen type II has more carbohydrate groups than any of the other types of collagen; thus, it interacts better with water than the other collagens [17]. Collagen has a characteristic triple helical structure, with the width of each fiber varying from 10 to 100 nm (Figure 1.4) [8]. The collagen fibrils are approximately 20 nm in width (diameter) in the superficial zone and approximately 70 to 120 nm in width (diameter) in the deep zone [5]. Fibers in the triple helix are composed of three α polypeptide chains, each of these chains contain glycine and proline. Due to the presence of proline in these chains, they, on their own, exhibit a left-handed helical configuration, while in the triple helix, they exhibit a right-handed helical configuration giving the collagen molecule the ability to resist tensile forces [30]. Glycine molecules can only be present at the junctions between each of the three α chains [30]. The location of glycine in the α

chain is very crucial for the triple helical form of collagen [8, 30]. Every third residual functional group occupies the interior of the helix, and the sequence is represented by $(Gly\text{-}X\text{-}Y)_n$ where X and Y is generally proline and hydroxyproline [7, 8, 30]. Hydroxyproline, by forming the intramolecular hydrogen bonds along the length of molecule, stabilizes the helical structure of collagen [8]. The distribution of a particular type of collagen in the matrix can be localized, or it can be present throughout the ECM [8]. Collagen type II has uninterrupted triple helical chains aligned head-to-tail and side-by-side, overlapping each other, creating a 3-dimentional structure [30]. The strength and stability of the ECM in articular cartilage is due to the insoluble properties of the collagen fibrils. The insoluble nature of collagen is due to the covalent and non-covalent crosslinks between the collagen molecules and interactions between different collagen fibrils [8].

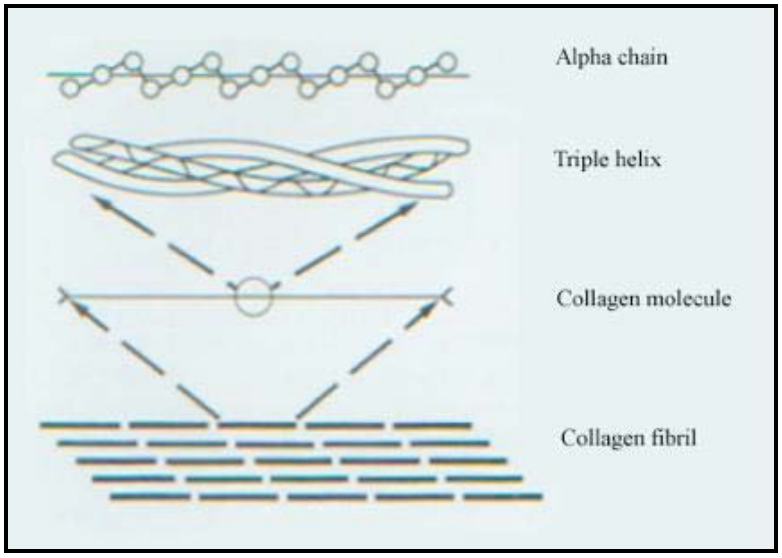

Figure 1.4: Structure and Assembly of Collagen Fiber. Collagens have a characteristic triple helical structure. Collagen type II has uninterrupted triple helical chains aligned head-to-tail and side-by-side, overlapping each other, creating a 3-dimentional structure (Unmodified Image) [8].

1.1.2.3 Proteoglycans

Proteoglycans are composed of a protein core covalently bound to polysaccharide chains. Proteins make up only 5% of the proteoglycans while polysaccharides make up the rest [17]. Four major types of glycosaminoglycans have been found in articular cartilage proteoglycans, namely: (i) chondroitin sulfate 4- and 6-isomers, which account for 55-90% of all glycosaminoglycan; (ii) keratan sulfate, (iii) dermatan sulfate and [31] hyaluronan [8, 32]. Hyaluronan, unlike the other glycosaminoglycans mentioned above, are un-sulfated, plus they are not covalently bound to a protein core; instead, they are present as large un-branched chains. As hyaluronan is not covalently attached to a protein core, it is not a proteoglycan. The size of the proteoglycan aggregates prevent them from leaving the cartilage matrix; there are no covalent bonds formed between the proteoglycan and collagen molecules [33]. The glycosaminoglycan chains in articular cartilage consist of repeating carboxyl (COOH) and sulfate (SO_4) groups. These repeating groups become negatively charged when placed in an aqueous environment; they repel each other and other anions and attract cations, thus allowing better interaction with water [8, 17, 32]. Due to the chains being very closely packed, the charge density is very high; positive counter ions are required to maintain their electro-neutrality [23]. The ions floating freely within the interstitial water cause a Donnan osmotic pressure effect, and their concentration also plays a part in determining the magnitude of the repulsive force [3, 25]. This large charge density causes a swelling pressure [3, 25]. Eighty to ninety percent of all proteoglycans in cartilage are aggrecan. Aggrecan consists of a long protein core with approximately 100 chondroitin sulfate and 50 keratan sulfate attached to it (Figure 1.5) [8]. Aggrecan molecules are stabilized in the ECM by aggregation. Multiple aggrecan molecules bind to the un-branched hyaluronan chain forming a large proteoglycan aggregate. There are other smaller proteoglycans present in articular cartilage,

such as biglycan and decorin [8, 17]. Many of these small proteoglycan molecules present in articular cartilage do not contribute to the physical properties of the articular cartilage, but instead influence cell function and arrangement of collagen in the matrix [8, 17]. From a biomechanical standpoint, aggrecan is important, as it provides articular cartilage with compressive strength [5].

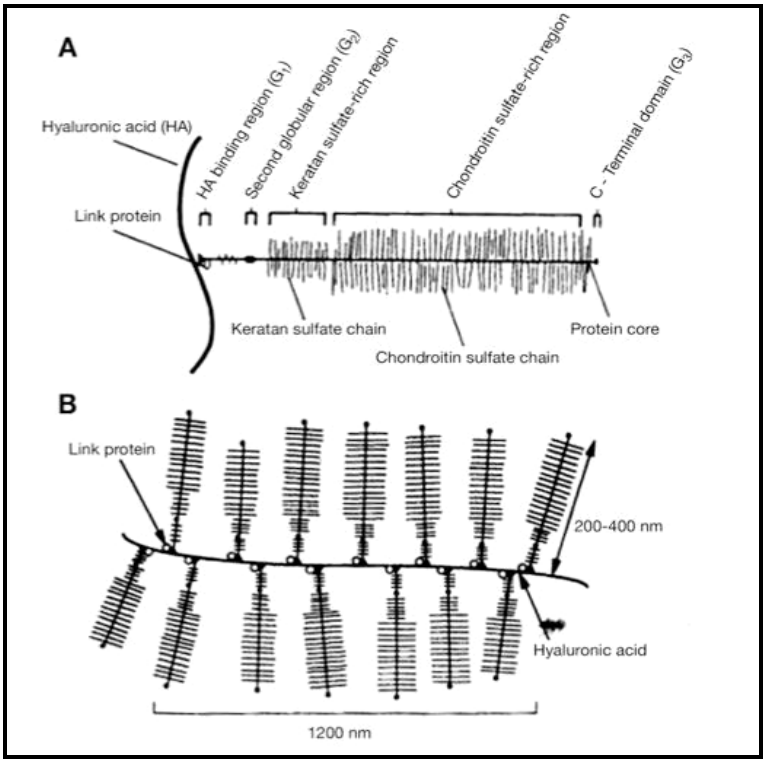

Figure 1.5: Structure and Assembly of Proteoglycans. **(A).** Structure of the proteoglycan monomer consisting of chondroitin sulfate chains, keratan sulfate chains, a hyaluronate chain and a link protein. **(B)** Molecular conformation of a proteoglycan aggregate (Unmodified Image) [34].

1.1.3 Chondrocytes

The cells found in articular cartilage are chondrocytes (1-10% of the entire tissue volume) [6-8]. They are responsible for the maintenance and remodeling of the ECM via catabolic and anabolic processes, with the aid of matrix metalloproteinases (MMPs) and inhibitors of metalloproteinases (TIMPs) [2]. The differentiation of mesenchymal stem cells (MSCs) during skeletal morphogenesis leads to the formation of chondrocytes. The differentiated chondrocytes produce extra-cellular matrix components while they divide [17]. During the final stages of cell division, chondrocytes close to the bone become hypertrophic and produce proteins that will aid in the calcification of the matrix, while chondrocytes in the other zones keep producing the extra cellular matrix [17]. Mature chondrocytes unable to proliferate become rounded and are enclosed within the matrix [17]. Mature chondrocytes examined under a microscope, possess a prominent endoplasmic reticulum and Golgi apparatus [17]. These mature cells generally store glycogen, and some of them even have cilia extending into the ECM allowing them to respond to mechanical stimulus [7, 17]. Chondrocytes isolated from individual zones have shown different growth rates [35, 36], gene expressions [37, 38] and level of biosynthesis [39, 40]. Chondrocytes are sensitive to chemical signals, such as growth factors, interleukins and mechanical signals. The responses to these signals can be either constructive or destructive to the tissue.

1.1.4 Biomechanics

Articular cartilage in the knee experiences extensive stress due to constant loading. The thickness of articular cartilage in the human knee varies between 1mm and 5mm [41]. The structural molecules present in the ECM, arranged in an organized fashion give it strength and prevent its

failure. To understand the mechanical behavior, properties and function, articular cartilage is looked at as a two-phase material with a free flowing fluid phase and a porous-permeable solid phase [24, 42, 43]. The movement of water through the ECM takes place via two mechanisms. The first mechanism involves applied pressure, while the second mechanism involves compressive forces [8, 24, 42, 44]. While there is a direct relationship between permeability and water content there is an inverse relationship between permeability and proteoglycan content thus, the permeability of cartilage decreases non-linearly with increased compression [22]. The non-linear relationship between permeability and compression is to prevent excessive fluid loss from the matrix with compressive loading and to allow the tissue to better distribute stress by increasing interstitial fluid pressure [8, 43].

The compressive modulus of articular cartilage matrix is 0.4 up to 1.5 MPa [8], which is due to its viscoelastic properties. The viscoelasticity of articular cartilage can be explained by: (i) a flow dependant, and (ii) a flow independent mechanism [45-48]. The flow independent mechanism is due to intermolecular friction between the matrix components while the flow dependant mechanism is due to hydrodynamic pressure and the generated drag that contribute to the viscoelastic nature of articular cartilage [43].

The stress, strain and shear properties of articular cartilage can be attributed to the architecture of the collagen fibrils, presence of proteoglycans and the interaction between the collagen fibrils and proteoglycan molecules entrapped in the middle zone of the articular cartilage. The collagen fibrils provide elasticity, while the proteoglycans provide shear. The proteoglycan-collagen interaction provides stiffness and energy dissipation in shear [49-51].

1.2 Metabolism

Chondrocytes control metabolism in articular cartilage, as they are responsible for ECM synthesis (anabolism) and degradation (catabolism) [2, 3]. The anaerobic pathway is the primary means by which chondrocytes produce energy [2]. The ECM is maintained by balancing the rate of synthesis with the incorporation of ECM components to the rate of ECM degradation with the release of ECM components from the articular cartilage [2]. The metabolic activity of chondrocytes is affected by mechanical and chemical signals, which include growth factors, matrix components along with changes in mechanical loads and changes in hydrostatic pressure [17, 52]. Chondrocytes usually respond to these environmental changes by maintaining a stable matrix. However, at times, chondrocytes can alter the matrix composition, which can change the organization of the matrix components leading to degradation of the ECM [8].

1.2.1 Nutrition

Articular cartilage obtains its nutrients from the synovial fluid, which is present in the synovial cavity. Generally, the synovial fluid forms a coating over the articular cartilage approximately 10 to 20 μm thick [8, 17]. The fluid in the tissue contains water, gases, cations and other metabolites [8, 17]. These metabolites in the tissue fluid are constantly exchanged for nutrients and oxygen present in the synovial fluid [8, 17]. Oxygen and nutrients taken up by the tissue fluid are directed towards chondrocytes [8, 17]. The superficial zone of articular cartilage allows only low molecular weight components to diffuse into the tissue [8, 17]. The time required for components to diffuse through the ECM depends on their molecular weight, structure, size and charge.

Polypeptide growth factors influence the synthesis of the ECM by interacting with the cell surface membrane of chondrocytes. The receptors on the cell surface may be highly specific or may be

less specific, thus encouraging competitive binding [8]. The concentration of growth factors present surrounding chondrocytes, along with the quantity of receptors present on the cell surface, determines the cell response [8, 36]. A number of growth factors play an active role in articular cartilage synthesis, namely, Platelet-Derived Growth Factor (PDGF), Basic Fibroblast Growth Factor (bFGF), Insulin and Insulin-Like Growth Factors (IGF-I and IGF-II) and Transforming Growth Factor-Beta (TGF-β) [8, 36].

1.2.2 Proteoglycan and Collagen Synthesis and Catabolism in Articular Cartilage

Proteoglycans are synthesized, assembled and sulfated by chondrocytes. The protein core is synthesized at the ribosome, and the glycosaminoglycan chains are added at the Golgi complex [8]. The carbohydrate region of the glycosaminoglycan chains are added one sugar at a time [8]. These chains are elongated and sulfated, simultaneously and are referred to as the posttranslational enzymatic reaction [7, 8]. The glycosaminoglycan chains are secreted into the ECM, once glycosylation is complete. The protein core makes up approximately 5% of the proteoglycan weight; whereas, the remaining 95% comprises of glycosaminoglycan [7]. The posttranslational enzymatic reaction is not controlled by genes; thus, there exists a variation in glycosaminoglycan chain length [8]. Therefore, any variation in the glycosaminoglycan composition and structure can create a significant variation in the proteoglycan structure.

As people age, they tend to produce less uniform proteoglycan and more aggrecan that varies considerably in size and composition [7, 8]. The mechanism that controls the synthesis of proteoglycan is extremely sensitive to biochemical, mechanical and physical stimuli [5, 7, 8, 53]. In order for articular cartilage to be fully functional, the turnover rate for proteoglycan synthesis should be very rapid [7]. Other environmental changes that effect proteoglycan synthesis include

interstitial hydrostatic pressures, calcium concentration, substrate serum concentration, oxygen tension and pH [8].

Proteoglycans continuously undergo catabolic activity in an effort to remodel or repair [8]. The rate of catabolism varies depending on the event. Soluble mediators such as interleukin-1 and joint immobilization increase the degradation process [8]. One of the sites of proteolytic cleavage is between G1 and G2 protein core domains. G1 is the proteoglycan containing the glycosaminoglycan, and G2 is the hyaluronate and link protein region, which is involved in aggregation [8].

The collagen network in articular cartilage is much more stable than the proteoglycan network. In case of injury or disease, the synthesis of collagen is increased and is much greater when compared to proteoglycan synthesis [7, 8, 19, 53]. Collagen α-chains are synthesized at the rough endoplasmic reticulum [8]. Once the collagen is secreted, the propeptides are cleaved from both ends of the triple helix, and the collagen molecule self-assembles. Finally, the enzyme, lysyl oxidase, catalyses covalent crosslinks [8].

1.2.3 Energy Metabolism

With the absence of blood supply, nerve fibrils and the lymphatic system, chondrocytes form and maintain articular cartilage [41]. They produce energy via carbohydrates (glucose) and amino acids (glutamine) [54-57]. Glucose, via glycolysis, is a major contributor towards energy and glutamine, via oxidative metabolism, is to a lesser extent [56, 57]. Glutamine has multiple functions: it is important for metabolism (Figure 1.6); it is an amino donor during the glycosaminoglycan synthesis; it stimulates the synthesis of glycan [54, 57]; it is required for the

synthesis of hexosamine nucleotides for glycoproteins [58]; and in its deficiency, N-acetylglucosamine 6-phosphate and UDP-N-acetylhexosamines quantities are decreased [56].

Glycolysis is the metabolic pathway occurring in the cytosol of the cell, that converts a 6-carbon sugar such as glucose to a 3-carbon product such as pyruvate, and oxidative phosphorylation is the metabolic pathway occurring in the mitochondria of the cell (Figure 1.7) [59]. NAD^+ is required as a substrate and has to be generated continuously for glycolysis to continue [59]. Once formed, pyruvate may undergo a variety of reactions either in the cytosol or the mitochondria. Chondrocytes for the most part undergo glycolysis. [56]. This is due to chondrocyte's low mitochondria density [60-62], which is approximately 1-2% of the cell volume [60]. Additionally, *in vivo* the mitochondria have been shown to lack certain enzymes, known as cytochromes found on the mitochondrial membrane, involved in oxidative phosphorylation [62, 63]. Hence the rate of oxygen consumption per cell in articular cartilage is low. The oxygen concentration *in situ* is approximately 6% at the surface and 2-3% in the deep zone [64-68]. Although chondrocytes have about the same glycolytic rate per cell as other cells in other tissues, their oxygen consumption per cell is much lower (e.g., approximately 2% less than that consumed by cells in the vascular tissue) [53]. Although chondrocytes hardly utilize any oxygen for energy production oxygen is essential for cellular functions, such as matrix synthesis [62, 69-72].

Glycolysis generates 2 molecules of ATP per glucose molecule (Figure 1.7) [59]. In articular chondrocytes, the pathway of glycolysis, rather than oxidative phosphorylation, predominates as the source of ATP [57, 62, 71, 73] because little of pyruvate is further oxidized to form more ATP molecules [59]. The amount of lactate present is indicative of the amount of glucose that has undergone glycolysis; one mole of glucose produces 2 moles of lactate. The amount of ammonia present can indicate the amount of glutamine utilized and the activity of the tricarboxylic acid

(TCA) cycle [74]. The TCA cycle is active in cartilage to a small degree; however, 80% of glucose is metabolized by glycolysis to lactic acid [53].

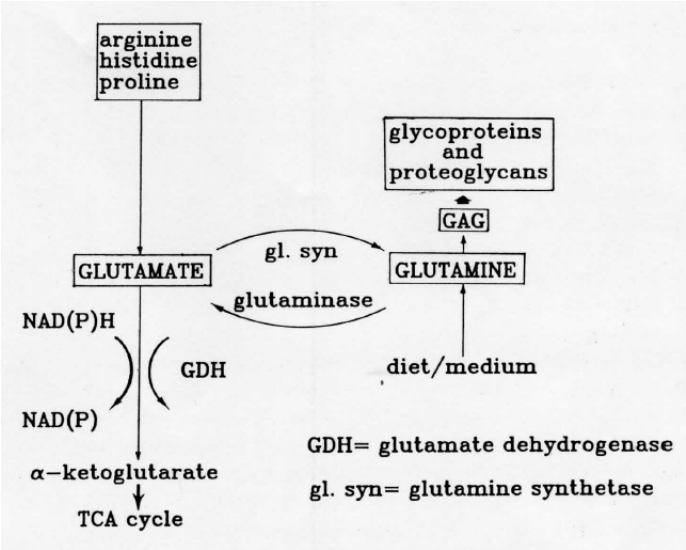

Figure 1.6: Glutamate Metabolism (Unmodified Image) [56].

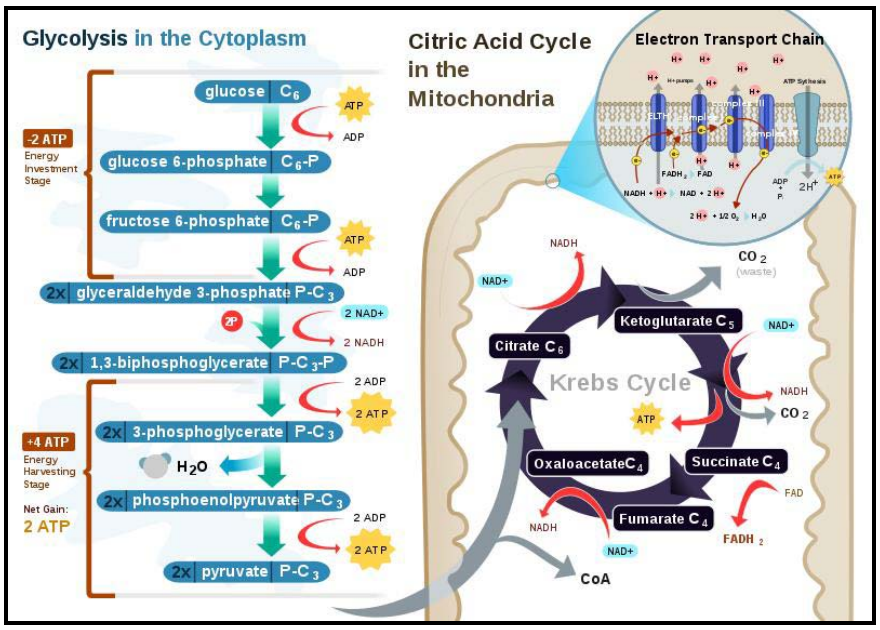

Figure 1.7: Cellular Respiration. One or all of the following are involved in cellular respiration: glycolysis, Krebs cycle, citric acid cycle, and the electron transport chain. Chondrocytes undergo anaerobic respiration; thus, the majority of their cellular respiration takes place in the cytoplasm (Unmodified Image) [59].

1.2.4 pH Regulation

The ECM of articular cartilage has a low pH compared to other tissue matrices found in the body, for several reasons: (i) The high intrinsic fixed negative charge density of glycosaminoglycan (GAG) attracts free cations, such as Na^+, K^+, H^+ and Ca^{2+}. This attraction of H^+ ions causes the pH in the matrix to decrease. Additionally, GAG repels anions, such as Cl^- and HCO_3^-, reducing the anion concentration in the ECM [1, 75-81]. This leads to a higher cation concentration in the ECM than in the synovial fluid and the serum [81]. (ii) Because of the avascular nature of articular cartilage, the low partial pressure of oxygen results in energy production via glycolysis, which leads to the production of large quantities of lactic acid, acidifying the tissue matrix [71, 79]. (iii) Due to the avascular nature of articular cartilage, nutrient and metabolic waste exchange occurs through diffusion across the entire length of the cartilage tissue. This, in turn, lowers the pH, which is caused by the accumulation of waste, as diffusion is a slow process [71, 79].

The external extracellular environment influences the intracellular pH (pHi) of chondrocytes. Changes in ionic concentration and/or osmotic pressure bring about a change in the pHi, which eventually leads to the synthesis and degradation of the macromolecules [81]. Mechanical loading due to normal day activity causes deformation of the cell and affects the permeability of the plasma membrane and the pathways for the transport of ions [79, 82-85]. Short-term changes in ion concentration are regulated and returned to normal physiological levels, while long term ionic concentration changes affect the balance between matrix synthesis and matrix degradation [81]. This results in the reduction of mechanical properties and hence, tissue damage [81]. Chondrocytes are sensitive to extracellular Na^+ concentrations, as changes to the ion concentration influence its metabolic pathway. [86]. Chondrocytes have various mechanisms by which they regulate the intracellular ionic concentration such as ion channels, exchangers and

pumps (e.g., bafilomycin-sensitive H^+-ATPase pump, amiloride-sensitive Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger).

Sodium ions are the most abundantly found monovalent cations in articular cartilage, and their concentration varies zonally. Na^+ concentration varies between 250 and 350 mM in the tissue matrix while chondrocyte intracellular Na^+ concentration is approximately 40 mM [81]. The Na^+/H^+ (NHE) exchanger and the H^+-ATPase pump are the most active in pHi regulation, and they work in unison with each other [79, 87-89]. Although $Na^+/K^+/2Cl^-$ is a major Na^+ co-transporter, when there is an increased concentration of Na^+ in the ECM, this co-transporter is not involved in pHi regulation; instead, it is involved in regulating cell volume. Unlike the mechanisms mentioned above, the Na^+ K^+-ATPase pump removes excessive Na^+ ions from chondrocytes maintaining a low Na^+: K^+ ratio inside the cell [81] for efficient metabolism; a high K^+ concentration, about 150 mM, is required by ribosomes inside the cell for efficient protein synthesis [90-92]. Long term increase in Na^+ concentration can result in the up-regulation of these Na^+ K^+-ATPase pumps [93, 94]. The sensitivity of chondrocytes towards their ionic and osmotic environments have been recorded in explants and isolated cells [93, 94].

Chondrocytes are sensitive to an acidic extracellular environment [95, 96]. The pH of the ECM in healthy cartilage is approximately between pH 6.9 and pH 7.1 [96]. Bovine articular chondrocytes have a resting pHi of 7.1, and any changes to this pHi affects the extracellular matrix synthesis [97]. It has been shown by Ohshima *et al.* [98] and Wilkins *et al.,* [96] that, in regard to extracellular pH (pHe), matrix synthesis follows a bell shaped curve with optimum matrix synthesis occurring between extracellular pH 6.9 and pH 7.1. Effects of acidification, due to low pH within the ECM, has been seen in intervertebral disc explants [98], intervertebral disc slices [98], human chondrocytes [95], bovine chondrocytes [96, 99] and bovine epiphyseal cartilage [100]. Intervertebral discs, like articular cartilage, are avascular tissues that undergo

anaerobic metabolism, producing lactic acid at a high rate and with the pH at the center of the disc being acidic [98, 101].

The reduction in extracellular matrix pH (pH 6.6 and below) reduces energy metabolism (glycolysis) [102, 103] due to the pH sensitivity of the phosphofructokinase enzyme, which is the rate-limiting enzyme during glycolysis [31, 104]. The accumulation of lactic acid within the ECM reduces GAG synthesis and cell proliferation [96, 98, 99, 105-107]. At a pH (pHe) between 6.6 and 7.3, no significant increase in cell proliferation was noticed [106], while an increase in chondrocyte proliferation was observed at pHe values between 7.0 and 8.0 [95]. The optimum GAG synthesis in articular cartilage was found to occur between pHe values of 7.0 and 7.2 [99, 108]. While glycolysis and GAG production are affected by pHe, collagen synthesis remains unaffected [96, 98, 106, 107]. In order to sustain the general articular cartilage health, the physiological pHi of chondrocytes needs to be maintained (Figure 1.8).

Various studies have shown that pHi is primarily regulated by the cation dependent exchangers Na^+/H^+ (NHE) instead of the anion dependent transporter Cl^-/HCO_3^-. NHE is a amiloride-sensative electroneutral exchanger that regulates the influx of one Na^+ ion via NHE protein for one H^+ ion. NHE1 and NHE3 are found in chondrocytes (Figure 1.8) [88, 96]. The expression of NHE3 is increased in a disease state and in the presence of growth factors and serum [109, 110]. A large number of factors can regulate the level of NHE1 expression and activity [111], such as phosphorylation, which stimulates the activity of this protein [112] and a drop in pHi, which increases its activity [81].

The Cl^-/HCO_3^- (AE) transporter regulates the pH of cells by removing membrane-impermeant HCO_3^- from cells. The HCO_3^- transporter protein, more specifically AE2 protein, the only exchanger that has been found in chondrocytes for bicarbonate exchange, is an electroneutral

exchanger, exchanging Cl^- for HCO_3^- [113]. AE2 is found in very low density in the plasma membrane, further suggesting the lack of AE activity in bovine chondrocytes [97]. Even though driven by the gradient of the substrate, this exchanger causes the acidification of the cell by removing HCO_3^- from the cytosol in exchange for Cl^-. The reversal of the gradient may cause an influx of HCO_3^- and an efflux of Cl^- [113]. Bicarbonate, a base, on entering the cell, raises the pH and on leaving it, makes the cell more acidic (Figure 1.9) [114]. Bicarbonate can undergo pH dependent conversion from HCO_3^- to CO_2. At a p*K*a of 6.4 [97], HCO_3^- and CO_2 are both present. Unlike HCO_3^- that moves across the plasma membrane with the aid of transporters, CO_2 has the ability to move freely by diffusion across the plasma membrane and can be eliminated from the cell [114]. Freshly isolated chondrocytes, unlike cultured articular chondrocytes, do not regulate the internal pH of the cell by bicarbonate transporters [79, 87, 97, 115]. This is believed to be due to the low activity of carbonic anhydrase, which catalyses the production of HCO_3^- from hydration of CO_2, thereby slowing the reaction and hence limiting the amount of HCO_3^- available for transport across the membrane [115, 116]. Interestingly, the level of carbonic anhydrase activity has also been reported to be low in human chondrocyte cell lines [115, 116]. In the presence of HCO_3^-/CO_2, the recovery rate of chondrocytes from acidification has been shown to increase, although the recovery was largely dependent on the Na^+ ions present [115].

According to Wilkins *et al.*, [117] bovine chondrocytes show minimal HCO_3^- dependent pHi regulation. A study done by Simpkin *et al.*, [118] showed that the HCO_3^- dependent transporter regulates the pHi of chondrocytes found in the superficial zone but not the pHi of chondrocytes isolated from other regions of bovine articular cartilage. According to Xu *et al.*, [107] bovine chondrocytes grown in media with HCO_3^- had a slightly more basic pHi than those grown in media without bicarbonate. Xu *et al.*, [107] also speculated that the increase in pHi of chondrocytes could be due to the fact that they adapt to increased levels of HCO_3^- ions.

Chondrocytes grown in long-term cultures in the presence of HCO_3^- ions have not only shown an increase in the pHi by 0.2 units, but also an increase in the GAG synthesis, along with cell proliferation [107]. According to Browning *et al.*, [115] in C-20/A4 human chondrocyte cell lines in the absence of HCO_3^- ions, the NHE exchanger regulates the pHi. Dascalu *et al.*, [87] have shown a Na^+-independent HCO_3^- transporter as the primary acid loader of avian chondrocytes, while a NHE exchanger and Na^+ dependent HCO_3^- transporter as the acid extruders. When isolated avian chondrocytes are transferred from a HEPES buffered solution to a medium rich in HCO_3^- ions, an immediate diffusion of CO_2 gas takes place inside the cell causing acid loading. Alongside this process, the CO_2 gas reacts with the water forming HCO_3^- ions. This acidification process activates the transporters to stabilize the pHi to physiological pH levels [87]. When avian chondrocytes were placed in a HCO_3^- rich media, the HCO_3^- transporter was responsible for the majority of the acidification response in comparison to a HEPES-buffered medium in which the NHE exchanger was responsible for the majority of the acidification response (Figure 1.8) [87]. This could be due to the fact that chondrocytes in culture dedifferentiate to a fibroblastic phenotype, at which physiological state all the pH regulatory mechanisms operate [87, 118, 119], or it could also be due to species differences [115]. According to Mobasheri *et al.*, [81] under certain conditions the proton extruding H^+-ATPase pump, HCO_3^- transporter and NHE exchangers, all work together to regulate the pHi of the chondrocytes.

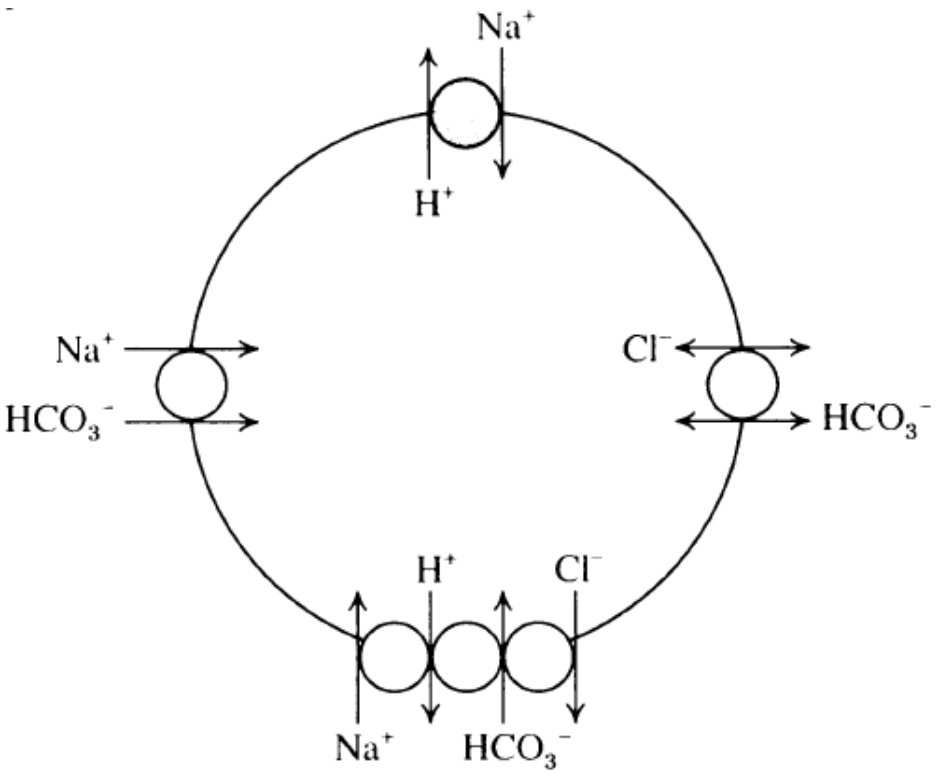

Figure 1.8: Transporters involved in intracellular pH regulation of chondrocytes (Modified Image) [88].

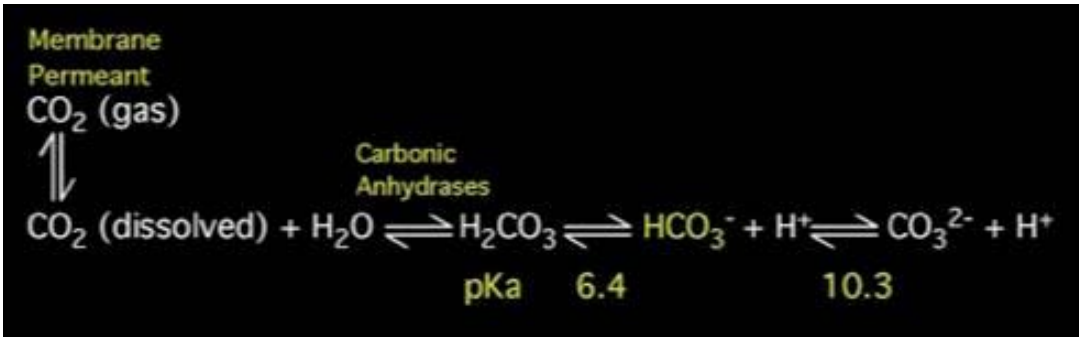

Figure 1.9: pH dependent bicarbonate reaction. Carbonic anhydrases accelerate the rate of conversion of bicarbonate ions to carbon dioxide (Unmodified Image) [114].

1.3 Development and Aging of Articular Cartilage Tissue

Immature articular cartilage differs from adult articular cartilage; it contains more cells per unit volume or per unit mass. On the other hand, adult articular cartilage has fewer cells per unit volume and is well organized into specific zones. In immature articular cartilage, the superficial zone contains larger cells; the middle zone is deeper, and the cells are more randomly arranged; the deep zone cells are arranged in columns [7, 8]. The columns in the deep zone are irregular from the start of the deep zone into the middle of the deep zone, after which the columns get more regular [7, 8]. Further into the deep zone, cells increase in size with larger intracytoplasmic vacuoles that have been shown to contain glycogen [7, 8].

Immature articular cartilage actively undergoes mitosis, which occurs in two distinct zones [7, 8]. The first layer lies just underneath the superficial zone, while the second layer lies under this in the form of a thin layer of cells [7, 8]. As articular cartilage matures, the pattern for cell replication changes; mitosis only occurs at the lowest region of the deep zone, which eventually stops in an adult as that region is replaced with a calcified zone [7, 8]. Along with cellularity, even the chemistry changes from immature to adult articular cartilage. In immature articular cartilage, the water and proteoglycan content is much higher, while the collagen content is much lower [7, 8]. The total chondroitin sulfate concentration decreases with age, while keratan sulfate increases with age, and in adults represent 25-50% of the total glycosaminoglycan [7, 8].

1.4 Current Techniques, their Challenges and the Promise of Tissue Engineering

Articular cartilage is a highly specialized avascular tissue with a low ability to self-repair which makes the treatment of chondral injuries difficult for scientists and surgeons. In the United States alone, 27 million people are affected by osteoarthritis [120, 121]; more than half these people

undergo total joint replacement. The societal cost is more than $15 billion USD per year [121]. Chondrocytes lack the ability to divide and migrate to the injury site [122]. There are two types of chondral defects: partial thickness defects, which are restricted to the superficial layer of the tissue, and full-thickness defects penetrating into the underlying subchondral plate. Partial thickness defects tend to be asymptomatic and are not associated with major clinical problems [123]. Having said this, they deserve attention because they can progress into full thickness chondral defects. These are symptomatic in nature, coupled with pain during movement, and hence restrict motion. Intrinsic repair only occurs in full thickness chondral defects [124], but the MSCs penetrating from the underlying bone form fibrocartilage instead of hyaline cartilage [36, 125-127]. The tissue synthesized by these cells possesses inferior stiffness and resilience [3, 36, 128]. As a result of the above-mentioned limitations, scientists and surgeons have started to work in close cooperation, looking at various strategies to repair chondral defects.

1.4.1 Surgical Techniques

The size and depth of the incurred lesion, patient age, level of activity and the success and failure of previous treatments decide the treatment approach. The treatment can be palliative, reparative or restorative [122].

***Palliative*:** This treatment option includes arthroscopic lavage and debridement. The two mentioned procedures involve the removal of fibrillated surfaces, shaving off osteophytes that hinder motion and extracting inflamed synovium. This is followed by washing away the fragments of tissue from the injury site [129]. This treatment provides temporary relief from pain and is ideal for patients with low physical demands [122, 130].

***Reparative*:** Microfracture is a reparative treatment, and entails making small holes in the underlining subchondral bone plate and causing MSCs to seep out of the underlying bone and into the defect [131, 132]. The body looks at this as an injury and forms a fibrin clot that over a period of 12 to 16 months remodels into fibrocartilage repair tissue, which is largely collagen type I [133]. Smaller lesions (less than 2 cm^2) surrounded by normal articular cartilage intact along the underlying subchondral bone, can be repaired by microfracture. The normal cartilage protects the lesion from shear and compressive force allowing it to develop [122]. This treatment is best for patients with an active life style, who have sustained small lesions [134].

***Restorative*:** Osteochondral grafting, also known as mosaicplasty, is used as a restorative technique. Osteochondral grafting can be done with an autograft or allograft. This treatment is for full-thickness defects when the cartilage along with the underlying bone is damaged. It is a one step procedure; articular cartilage is taken from a low load-bearing region of a joint and is transferred to the lesion site [132]. Autograft procedures are used to treat defects of 2 cm in diameter or less, due to the limited tissue availability, while larger defects are treated by allograft tissue taken from a cadaveric donor [122]. Mosaicplasty is carried out with minimally invasive arthroscopic surgery and without any additional procedures [135, 136]. The graft is held firmly in place and secured to the subchondral bone [135, 136]. Allografts eliminate the problems of donor site morbidity, but it does present the problem of graft availability, cell viability, immune response and disease transmission. Osteochondral autograft treatment is well-suited for patients with small chondral lesions, while osteochondral allografts are ideal for larger chondral lesions involving the subchondral plate [137].

Autologous Chondrocyte Implantation (ACI) is a cell based replacement technique generally used when all of the above fail. The procedure involves two surgeries; in the first, the lesion is examined arthroscopically, taking a biopsy sample approximately 200-300 mg in weight [138]

with about 10,000 cells, from a non-load bearing region of the joint. The chondrocytes are expanded *in vitro* for a period of 6 weeks to 18 months. Once the cell number reaches roughly 10-12 million, the patient undergoes a second surgery, involving suturing a periosteal patch to healthy cartilage around the defect, forming a watertight lid [125]. Cells are then injected into this periosteal patch. Over a period of time, the cells under the patch divide and form tissue [125]. ACI can be used to treat medium to large lesions that have not penetrated into the subchondral plate. This procedure does not result in a homogeneous distribution of cells; the surgery takes a long time, as periosteal grafts have to be harvested and sutured into place [35]. For the most part, this procedure produces fibrocartilage instead of hyaline cartilage [3, 36, 139-141]. This treatment is better for patients with larger chondral lesions [122].

Matrix Induced Autologous Chondrocyte Implantation (MACI) is similar to ACI; the only difference being that instead of injecting cells into a periosteal patch, chondrocytes are seeded in between layers of bilaminate collagen [35]. The collagen used is purified porcine collagen types I and III, resistant to absorption and tear [142, 143]. This construct is held in place by fibrin glue or sutures. This technique gives a homogeneous distribution of cells in the membrane, and the surgery takes a shorter time [35]. So it is recommended for patients with larger chondral lesions [122].

The above mentioned techniques possess flaws that reduce long-term clinical success [144]. These techniques produce fibrocartilage instead of articular cartilage, which is composed of collagen type I instead of collagen type II. Collagen type I lacks the structural arrangement seen in native articular cartilage, and therefore falls short of the biomechanical and biochemical properties in the native tissue. Another major problem is the lack of new tissue integration with the surrounding native tissue [145, 146], causing tissue deterioration. During surgery when the defect site is being prepared for the treatment or when the defect undergoes shaving and

debridement, the cells along the cut surfaces undergo cell apoptosis and necrosis [147-150]. Considering the cellularity of articular cartilage and its low metabolic activity cell death can create a biologically unfavorable environment for the construct implanted [130]. Osteochondral grafts have additional problems which include chondrocyte death at the circumference of the osteochondral graft [151], along with recipient tissue edge. These problems further prevent the integration of the graft with the recipient tissue, increasing degradation and graft failure [152, 153]. In severe cases when an individual is over 60 years of age, the articular surface is replaced with an artificial prosthesis [135].

As all the above-mentioned techniques are associated with problems that reduce the longevity of the repair tissue construct, researchers are focusing their attention on tissue engineering techniques to develop a construct that would improve the quality of tissue being formed along with its longevity.

1.4.2 Tissue Engineering

Tissue Engineering, as defined by the pioneers of the field, Robert Langer and Joseph Philip Vacanti, is "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" [154]. Tissue engineering, unlike the other techniques, presents the possibilities of engineering hyaline-like cartilage tissue with similar mechanical properties and structure, forming tissue with better long-term performance and a construct large enough to fill the entire lesion without causing donor site morbidity and infection. Along with this, it can create good integration with the native tissue and remodel within the body.

Tissue engineering is composed of three components, namely: cells, scaffolds and signals [3, 155]. The tissue can be completely constructed *in vitro* and then transplanted into an individual or partially grown *in vitro*, laying down the ground substance, and then being implanted, with the remaining growth taking place in *vivo* or in *situ* [130].

1.4.2.1 Cells

Most researchers face the challenge of finding an optimal cell source from which cells can be easily isolated. Additionally, the source has to provide enough cell numbers for expansion. The cells should be expanded without loss to their phenotype and should produce appropriate articular cartilage specific molecules. Mesenchymal stem cells (MSCs) from the bone marrow and adipose-derived stem cells from adipose tissue are being explored by various research groups as alternative cell sources for cartilage tissue engineering [156-159]; instead, chondrocytes may be the ideal choice for this, as they are responsible for articular cartilage extracellular matrix synthesis and maintenance in the body [135, 160]. Although, these cells might seem to be the best choice there are a few concerns associated with their use. Firstly, these cells are present in very low quantities in the tissue (approximately 2% of the tissue volume) [1]; harvesting of these cells from the tissue for expansion can cause donor site morbidity. Secondly, chondrocytes, when expanded in monolayer, tend to dedifferentiate [130, 160]. A way around this is to culture chondrocytes in 3D cultures, such as pellets or high-density culture systems, so as to preserve the phenotype. Expanded dedifferentiated chondrocytes, when placed in a 3D culture, system from a 2D culture system, can re-differentiate. This can be accomplished by seeding dedifferentiated cells on a scaffold, in a high-density culture system or in a pellet [161, 162]. Thirdly, after a few passages, they tend to lose the ability to produce the correct extracellular matrix molecules and

lose potency [14, 163-165]. Chondrocyte expansion, proliferation and bioactivity vary with their age; aged chondrocytes lose sensitivity to most chemical and environmental factors [166, 167]. On the other hand, immature chondrocytes proliferate faster with a high rate of bioactivity [38]. Additionally, primary chondrocytes possess proliferation rates and activity that vary not only between the different zones and locations, but also greatly vary between individual cell batches. In spite of having the same trend between different experimental conditions, it could be difficult to average results for different cell batches without getting large standard deviations. Lee *et al*., [71] has also pointed out batch differences in primary chondrocytes.

Stem cells have been researched as an alternate cell source for articular cartilage tissue engineering. The use of stem cells helps overcome the problems of donor site morbidity, limited cell supply and cell expansion; however, stem cells present their own set of challenges. A common problem faced by using stem cells and chondrocytes is that they undergo fibroblastic de-differentiation expressing a hypertrophic phenotype *in vivo* [160, 168, 169]. Another, concern of working with MSC, is to obtain and maintain permanent differentiation into the desired cell type, in this case differentiation into chondrocytes. This is done with the help of growth factors, signaling molecules and physical factors [121, 170, 171]. Although cell lineage has been maintained *in vitro*, there is no research to-date showing proof of cell lineage being maintained *in vivo*. A further concern is that in order to maintain the chondrogenic phenotype *in vivo*, the cells might have to be supplemented with specific growth factors which can result in side effects [172]. MSCs are multipotent cells that possess the ability to differentiate into adipocytes, chondrocytes, fibroblasts and osteoblasts under appropriate signal conditions [160, 173-175]. Signals in the form of growth factors push the cells toward the chondrogenic lineage and prevent them from de-differentiating. Adipose-derived stem cells have lower chondrogenic capabilities than bone marrow-derived stem cells [176-179].

1.4.2.2 Scaffolds for Tissue Engineering

As mentioned previously chondrogenic cells require a 3D environment to produce the correct extra cellular matrix molecules. This can be mimicked by a biomaterial-based scaffold or cells seeded in high-density scaffold free cultures.

Scaffolds: Cells are seeded on a scaffold that possess a defined architecture, helping the cells proliferate and develop tissue after which the cell-scaffold complex is implanted in the defective site [180, 181]. The design criteria required for articular cartilage scaffolds are: (i) biocompatibility, (ii) biodegradability, (iii) architectural similarity of the scaffold to native articular cartilage, [31] (iv) cell-scaffold interaction, coupled with tissue formation, and (v) mechanical properties matching native tissue at the site of implantation [182, 183]. The presence of biodegradability is an added benefit; however, its absence is not a disadvantage so long as it does not trigger an immune response. The architecture of the scaffold should closely mimic the native architecture created by collagen and proteoglycans in articular cartilage [2, 182-184]. Cell attachment and tissue formation depend on scaffold dimensions [182]; the micro-dimensions of the scaffold have to be small enough, to invoke a three-dimensional matrix interaction [182]. The scaffold should be designed to withstand dynamic loading, especially at the implant site [182, 183]. The types of scaffolds used for articular cartilage tissue engineering can be broadly classified into natural and synthetic, based on their source [181, 184]. Polymers (natural and synthetic) and ceramics have been used as scaffolds for articular cartilage tissue engineering. Based on their forms, polymer scaffolds, are classified into: (i) hydrogels, (ii) sponges and (iii) meshes [2, 151, 182, 185]. The most widely used ceramic scaffolds for articular cartilage tissue engineering are hydroxyapatite and hydroxyapatite-based scaffolds [2, 151, 182, 185]. Biomaterials are a vital aspect of articular cartilage tissue engineering [3, 132, 169, 185, 186], however, biomaterials aimed at articular cartilage tissue engineering are not part of this thesis

project, primarily because the focus of my thesis was to generate scaffold free constructs. Furthermore, the chondrocyte-biomaterial interaction aimed at articular cartilage tissue engineering, is a Ph.D. thesis unto itself, and hence beyond the scope of my project.

High-Density Scaffold Free Culture Systems: These systems have also shown to provide a 3D environment. Various researchers have looked into growing articular cartilage tissue from a pellet [187-190] or growing articular cartilage tissue on a membrane seeded with chondrocytes in high density, placed in a culture vessel [191-193]. Like a scaffold, this method of culturing and growing tissue also provides a 3D environment allowing cells to communicate with each other.

1.4.2.3 Environmental Factors

Signaling molecules can inhibit growth or increase growth. The environment in which the tissue is being grown plays a very important part in the type and quality of tissue produced. Chondrocytes are known to be very sensitive to biochemical and mechanical factors.

Various signaling molecules are used to increase tissue growth; they may be in the form of chemical compounds or growth factors. They can bind to the cell surface receptors to activate cell proliferation and extracellular matrix molecule production, or they may be transporter molecules and through channels, exchangers or simply diffusion though the plasma membrane, they may affect the function of the cell.

Bioreactors play an important part in both biochemical and mechanical signaling and are becoming increasingly important in articular cartilage tissue engineering. Bioreactors can be defined as systems in which biological, biochemical or both biological and biochemical processes can develop under carefully monitored and strictly controlled operating conditions, such as pH,

temperature, pressure, nutrient supply and waste removal [194]. Tissue engineers face the challenge of constructing a graft at least a few millimeters in size without causing a hypoxic or necrotic centre. A good nutrient supply and waste removal system is essential in order to mimic native tissue. Lack of oxygen and nutrients can inhibit cell growth and reduce the proliferation and matrix formation *in vitro*. Articular cartilage *in vivo* undergoes shear stress due to the load-bearing bone-on-bone contact in the joint. Bioreactors can increase and improve tissue growth by providing a more efficient nutrient supply, waste removal system and a hydrodynamic environment with a fluid-induced shear stress [2]. Tissue engineers have shown a low shear environment positively affects the tissue-engineered cartilage composition *in vitro* [195, 196]. Bioreactors have also been used to seed chondrogenic cells on a porous scaffold which has been shown to cause faster adhesion and even distribution of cells along with providing a 3D environment [2, 197, 198]. There are a number of bioreactors used for the purpose of articular cartilage tissue engineering, such as a rotating wall bioreactor [199], a spinner flask bioreactor [190, 200], a concentric cylinder bioreactor [201], and a perfusion bioreactor [202-204] (Figure 1.10).

Mechanically Stirred Bioreactor: These bioreactors are essentially spinner flask bioreactors with a magnetic stir bar or impeller to mix the nutrients, which provide constant mixing at various speeds. For articular cartilage, these have not shown much success because there is non-uniform mass transfer as well as non-uniform mechanical stress [205]. This is due to variation in shear force because this force at the surface of the impeller is ten times higher than else where [206].

Perfusion Bioreactor: The nutrients in a perfusion bioreactor are forced through the three-dimensional construct. There is no space between the construct and the walls of the reactor which forces the nutrients to flow through the construct causing a mechanical stress [207]. The fluid flow, set at an optimal velocity or close to optimal velocity, can overcome the problem of mass

transfer and provide mechanical stimulus to the cartilaginous tissue, increasing cellular metabolism, cell proliferation and matrix formation [208]. If the flow rate is high, greater mechanical stress is experienced by the cells that are closer to the inlet causing damage to the cell and non-uniform extracellular matrix deposition. Additionally, some of the matrix products can also be washed out, while if the fluid flow rate is low, the force might not be enough to allow the fluid to reach the cells further from the inlet, causing cell death [207]. Presently no optimal flow rate has been found for a perfusion bioreactor system.

Low-shear Bioreactor: These bioreactors consist of a rotating wall that provides low shear and greater diffusion through the construct. This bioreactor has two concentric cylinders that rotate independent to each other at the same rate or at different rates. Nutrient rich fluid is present between the two cylinders; the rotation of the cylinders creates a shear force on the tissue, and gravity and fluid force keeps the construct suspended in the fluid. For low shear, both the cylinders are rotated at the same slow speed [207]. This bioreactor to-date has shown the best results in articular cartilage tissue engineering, and it also gives a more even distribution of extracellular matrix [199, 209].

On a broader perspective bioreactors used for articular cartilage tissue engineering to-date have produced more extracellular matrix, of better quality, than batch-fed cultures. The reactors mentioned above, that are extensively used for this purpose, rely on convection as the primary means of providing mass transfer. This convective transport imparts uncontrollable mechanical stimuli to the cells resident in the construct. It is important to better understand the factors that cause tissue growth in bioreactors. Mechanical stimulus and increased mass transfer are the major factors contributing to the increased growth. In order to optimize the process, the effects of mechanical stimulus and mass transfer have to be studied individually and in combination. The reliance on diffusive transport may not necessarily be ineffectual, as previous studies have

observed improving cartilaginous tissue growth when the constructs were cultured in elevated volumes of media [210-212]. Hence, a way to determine the effects of increased mass transfer of the culture media is to continuously replenish the media at a specified rate (continuous culture). This can provide an infinite reservoir of media to maximize diffusive transport to the construct.

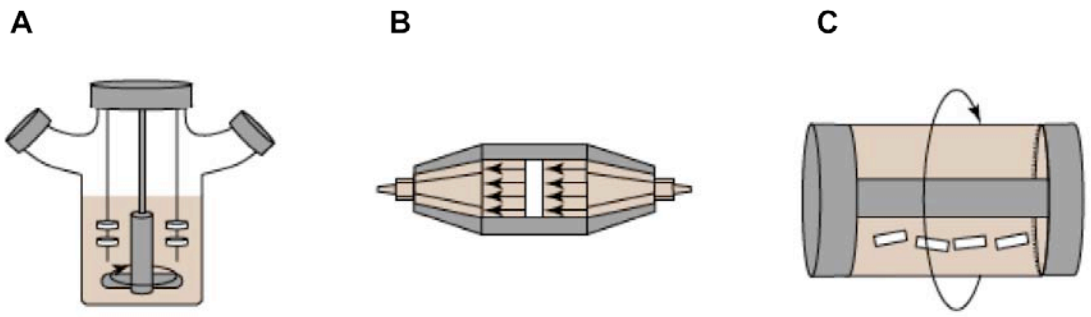

Figure 1.10: Schematic representation of bioreactors for articular cartilage tissue engineering **(A)** Spinner-flask **(B)** Direct-perfusion **(C)** Rotating-wall bioreactor (Modified Image) [194].

Chapter 2
Objectives

The primary objective of this project is to engineer clinically relevant sized (greater than 1 cm^2 [172]**) articular cartilage constructs by increasing chondrocyte proliferation and extracellular matrix (ECM) accumulation.**

This objective will be achieved through the following discrete aims:

1. Optimize a continuous flow bioreactor to support cartilaginous tissue growth *in vitro*
2. Enhance cell proliferation and cartilaginous tissue growth in the bioreactor by supplementing the media with sodium bicarbonate ($NaHCO_3$), and also establish a feasible seeding technique to manufacture cartilage constructs without causing donor site morbidity
3. Determine the underlying mechanism responsible for the increased cell proliferation and cartilaginous tissue growth as a result of bioreactor cultivation and $NaHCO_3$ supplementation

2.1 Development and Optimization of a Continuous Flow Bioreactor

Most tissue engineering bioreactors rely on convection as the primary means to provide mass transfer [194, 203, 208, 213-222]; however, convective transport can also impart potentially unwanted and/or uncontrollable mechanical stimuli to the cells resident in the construct. The reliance on diffusive transport may not necessarily be ineffectual, as previous studies have observed improved cartilaginous tissue growth when the constructs were cultured in elevated volumes of media [193, 210-212]. Additionally, it is not readily possible to maximize the diffusive transport to the tissue construct, as there are size restrictions on the culturing vessels that

can be used. Continuously replenishing the culture media at a specified rate (continuous culture) may potentially be an effective means to approximate an infinite reservoir of media to maximize diffusive transport to the construct.

Hypothesis: Cultivating the tissue construct in a continuous flow bioreactor will up-regulate extracellular matrix (ECM) synthesis and accumulation. This hypothesis will be tested by cultivating the construct with and/or without a continuous media flow of 5 μl/min or 10 μl/min for a period of one week after a two-week pre-culture period.

2.2 Media Supplementation with Sodium Bicarbonate as a Buffering Agent

In our previous study, we found that chondrocytes cultured in the continuous flow bioreactor accumulated considerably more matrix than those cultured under static conditions when both systems were supplemented with only 20 mM HEPES [223]. Hence, a second buffering agent was added (sodium bicarbonate) to help maintain a pHe between pH 6.9 and pH 7.2 in the bioreactor, and thus further enhance cell proliferation and ECM accumulation. Furthermore, various seeding techniques were tested in the continuous flow culture with $NaHCO_3$ supplementation in order to engineer cartilage constructs from as few cells as possible, in an effort to prevent donor site morbidity.

Hypothesis: The combined effects of the continuous flow bioreactor and supplementation of the media with $NaHCO_3$ would further regulate pH, maintaining it between pH 6.9 and pH 7.2 in the bioreactor, and therefore, will increase cellular proliferation and cartilaginous tissue growth, *in vitro*. This hypothesis will be tested by cultivating the constructs in a bioreactor and in static culture, with media supplemented with and without 14 mM $NaHCO_3$ for a period of five weeks. Furthermore, to establish a feasible seeding technique, thereby decreasing donor site morbidity,

the following construct forms will be studied, namely: monolayer, pellet, biopsy and minced. The constructs will be grown in a continuous flow bioreactor with media supplemented with 14 mM $NaHCO_3$ for a period of six weeks. At the end of six weeks, the constructs formed will be assessed for DNA, ECM accumulation (collagen and proteoglycan), and tissue thickness.

2.3 Effects of Extra- and Intracellular Factors on Chondrocyte Proliferation and ECM Synthesis

Chondrocytes are present in low numbers in cartilage, making them inaccessible due to donor site morbidity. For this reason, it is important to determine mechanisms that could increase their proliferation, especially to improve clinical applications of regenerative medicine and tissue engineering of articular cartilage constructs. The constructs I have grown previously in a continuous flow bioreactor supplemented with $NaHCO_3$ increased cell proliferation and extracellular matrix accumulation substantially [224]. In this study, I investigated the underlying reason for the increased proliferation. It is well-established that chondrocytes obtain their energy by anaerobic glycolysis and produce lactic acid at high rates [56], causing the environment to acidify. The rate of acidification not only depends on the rate of lactate production but also on the buffering power. Chondrocyte matrix synthesis rate and rates of cell proliferation are very sensitive to pHe and fall steeply in a pH-dependent manner under acidic conditions [95-99, 106, 107, 225]. Acidic extracellular environments are known to influence the pHi of chondrocytes [96, 97]. The pH of cartilage ECM is between pH 6.9 and pH 7.1 [96]. It is known that bovine articular chondrocytes have a resting pHi of 7.1 and any changes to this pHi have been shown to affect cell metabolism [97].

Hypothesis: Firstly, the increase in matrix production arose mainly from the increase in cell proliferation. Secondly, this increase in cell proliferation and matrix production was due to differences in buffering capacity between cultures containing HEPES and $NaHCO_3$ under CO_2, and HEPES in air, and consequently, influences extracellular pH (pHe) and intracellular pH (pHi).

This hypothesis will be tested by investigating the role of buffering capacity on cell proliferation by studying it in media supplemented with HEPES and $NaHCO_3$ under 5% CO_2 and in media supplemented with HEPES in air. Additionally, here, for the first time, I have reported on measuring pHi of chondrocytes while still in culture (2D monolayer and 3D alginate beads), using a confocal microscope without removal from their attached or encapsulated form to avoid artifacts, as far as possible. Specifically, carboxy SNARF-1 AM (carboxy-seminaphthorhodafluor-1), a fluoroprobe, will be used to measure pHi.

Chapter 3
The Effect of Continuous Culture on the Growth and Structure of Tissue-Engineering Cartilage

3.1 Relation to Overall Project

The results and discussions in Chapter 3 encompass Objective 1 (Section 2.1), which was to develop and optimize a continuous flow bioreactor, aimed at articular cartilage tissue engineering. This section has been published in *Biotechnology Progress* in 2009 (PMID: 19294749). I am the first author of this manuscript and was involved in the design and execution of experiments, data analysis and in drafting the manuscript. Jocelyne Suits and Dr. Stephen Waldman were co-authors of this manuscript.

3.2 Introduction

Articular cartilage is the load-bearing tissue that lines the articulating surfaces of synovial joints. It is relatively simple in composition and is almost entirely composed of extracellular matrix (ECM) interspersed with a small population of specialized cells called chondrocytes [5]. As articular cartilage is not innervated with blood vessels [226, 227], the chondrocytes *in situ* must draw almost all their nutrients from the surrounding synovial fluid. [73, 228]. Because of the low oxygen tension in synovial fluid, chondrocytes rely upon the anaerobic metabolism of glucose (glycolysis) as their primary energy source [71, 228]. During glycolysis, available glucose is rapidly converted to pyruvate with very little entering the citric acid cycle (aerobic pathway) [229]. The majority of pyruvate is then converted to lactic acid by lactate dehydrogenase and

NADH [229]. Isolated chondrocytes grown in 3D culture tend to maintain their phenotype [230, 231] and also primarily consume glucose anaerobically [74]. Traditional culturing methods (such as batch-fed culture) tend to result in a rapid reduction in the supply of nutrients (with the possible exception of oxygen) and accumulation of metabolic waste products in the culture media [73].

As it has been long understood that batch-fed culture is not optimal for cartilaginous tissue growth *in vitro*, a number of different bioreactor systems for cartilage tissue engineering have been investigated [194, 203, 208, 213-222, 232]. The primary focus of these systems has been on using convection to increase the mass transfer of nutrients to the developing tissue. Although these reactors have proven effective at increasing the rate of cartilaginous tissue growth *in vitro*, the effects of increased mass transfer and mechanical stimulation (e.g. fluid-induced shearing) cannot be easily separated. In certain bioreactors (such as through-thickness perfusion bioreactors), if the flow rate is constant, these forces can be applied statically to the construct which may be detrimental to cartilaginous tissue growth [225, 233-235]. However, the reliance on diffusive transport alone may not necessarily be ineffectual. Previous studies have observed improved cartilaginous tissue growth when the constructs were cultured in elevated volumes of media [210-212]. With this approach it is not readily possible to maximize the diffusive transport to the tissue construct, as there are size restrictions on the culturing vessels that can be used. Alternatively, continuously replenishing the culture media at a specified rate (continuous culture) may potentially be an effective means to approximate an infinite reservoir of media to maximize diffusive transport to the construct. Thus, the purpose of this study was to investigate the effect of continuous culture on the growth of tissue engineered cartilage *in vitro*.

3.3 Materials and Methods

3.3.1 Continuous Flow Bioreactor

The continuous flow bioreactor system was composed of a vented 8-chamber polypropylene culture vessel with an inlet port (at the base) and an outlet port (at the top to prevent overflow). Each chamber was 20 mm in diameter and held 2 mL of culture media and housed one tissue construct (approximately the same dimensions as a standard 24-well culture plate used to house the control cultures). Flow through the chambers was provided by two peristaltic pumps (Ismatec, Cole Parmer Canada, Anjou, QC) and vented media reservoirs (fresh and waste) through gas-permeable PharMed® tubing (Cole Parmer Canada). The flow rate of the peristaltic pumps were validated by determining the time needed to collect a known volume of media. The entire apparatus (including the pumps) was housed in an incubator maintained at 37°C and 95% relative humidity supplemented with 5% CO_2: 95% atmospheric air.

3.3.2 Cell Isolation and High-Density 3D Culture (Static and Continuous flow)

Cartilaginous tissue constructs were generated by isolated chondrocytes harvested from calf (12–18 months old) metacarpal-phalangeal articular cartilage by sequential enzymatic digestion described previously [236]. Tissue obtained from several joints (up to 4 per experiment) was pooled to collect enough cells for an experiment. The cells were seeded on the surface of type II collagen-coated Millicell™ filters in high-density, 3D culture (2×10^6 cells/filter or 160,000 cells/mm^2) and maintained in Ham's F-12 media (without $NaHCO_3$ and HEPES and with 10 mM glucose and glutamine) (HyClone, Logan, UT) which was supplemented with 20% fetal bovine serum (FBS), 100 μg/mL ascorbate and 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-

ethanesulfonic acid) (Sigma Aldrich, St. Louis, MI). The cultures were grown in an incubator maintained at 37°C and 95% relative humidity supplemented with 5% CO_2: 95% atmospheric air. The culture medium (2 mL per filter) was changed every 48–72 hr and fresh ascorbic acid was added with each change after day 7 of the culturing. Cultures were grown under static (no-flow) conditions for two weeks to generate a layer of cartilaginous tissue prior to being cultured in the continuous flow bioreactor. Cultures were supplied with a continuous flow of media at a rate of either 5 or 10 μL/min (corresponding to average residence times of 6.7 and 3.3 hr, respectively) or were grown under static (no flow) culture conditions. All experiments were repeated at least three times using separate cell isolations.

3.3.3 Determination of ECM Accumulation and Cellularity

After 7 days of either static or continuous culture, tissue constructs were harvested, lightly patted dry, and weighed (wet weight). Tissues were lyophilized overnight, and the dry weight of the tissue was determined. Tissues were then digested with papain (40 μg/mL in 20 mM ammonia acetate, 1 mM EDTA and 2 mM DTT) for 48 hr at 65°C and stored at -20°C until analysis. Aliquots of the digest were analyzed for glycosaminoglycans, collagen and DNA contents. Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using the dimethylmethylene blue (DMB) dye binding assay (Polysciences, Washington, PA) [237, 238]. The glycosaminoglycan was measured using a plate reader at 525 nm. To determine the collagen content, aliquots of the papain digest were hydrolyzed in 6 N HCl at 110°C for 18 hr. Hydroxyproline content of the hydrolyzate was determined using chloramine T/Ehrlich's reagent assay [239]. Hydroxyproline was measured using a plate reader at 540 nm after reaction with chloramine-T and p-dimethylaminobenzaldehyde. Collagen content was estimated by assuming

that hydroxyproline comprised approximately 10% the weight of collagen [240]. DNA content was determined using the Hoechst dye 33258 assay (Sigma Aldrich Ltd) with calf thymus DNA as the standard [241]. Digest was mixed with Hoechst dye and the fluorescence was measured at excitation of 360 nm and emission of 430 nm.

3.3.4 Determination of Media Metabolites

Medium samples were also collected at the end of the culture period and analyzed for residual glucose, accumulated lactate and pH. Residual glucose and accumulated lactate in the conditioned culture media were detected using colourimetric assays based on the coupled activities of either glucose oxidase/horseradish peroxidase [242] or lactate oxidase/horseradish peroxidase [243] with the product of an oxidative couple between 4-aminoantipyrine and N-ethyl-N-sulfopropyl-m-toluidine as the chromophore. Glucose and lactate levels in the media were expressed both in terms of concentration (mM) and consumption/production (nmol/h). To prevent loss of dissolved CO_2 which will influence media pH, [116] pH measurements were taken immediately after collection using a pH micro-probe.

3.3.5 Histological and Immunohistochemical Evaluation

Selected cultures (continuous culture and no-flow controls) were collected for histological and immunohistochemical evaluation. Upon harvest, tissue constructs were fixed overnight in 4% paraformaldehyde (Sigma Aldrich) and embedded in paraffin. Thin sections (5-μm thick) were cut and stained with hemotoxylin-eosin (H&E) (general connective tissue stain), toluidine blue (TB) (sulfated proteoglycans) and periodic-acid Shiff (PAS) (Sigma Aldrich) (intracellular

glycogen) [244]. For the PAS staining, sections were also pretreated with diastase (α-amylase to preferentially digest glycogen) prior to staining to confirm the presence of glycogen. All sections were examined by light microscopy. Immunohistochemistry with antibodies against types I, II, and X collagen (type X collagen is an early marker of chondrocyte hypertrophy and differentiation towards an osteogenic linage) [245, 246] was performed according to a standard ABC protocol (Vector Laboratories, Burlingame, CA) with diaminobenzidine for color development [247]. Prior to incubation with the primary antibody, sections were predigested with 0.25% trypsin (Sigma Aldrich) and 2.5% hyaluronidase (Sigma Aldrich) to facilitate immunostaining [248]. The following primary antibodies and dilutions were used: rabbit polyclonal type I collagen antibody (T40113R: Biodesign International, Saco, ME), 1:200 dilution; mouse monoclonal type II collagen antibody (II-II6B3: Developmental Studies Hybridoma Bank, University of Iowa, IA), 1:10 dilution; mouse monoclonal collagen type X antibody (C7974: Sigma Aldrich), 1:50 dilution. Nonspecific staining was assessed (for each section) by omission of the primary antibody.

3.3.6 Statistical Analyses

All numerical results were expressed as the mean ± standard error of the mean (SEM). Data were compared between each of the experimental groups using a one-way ANOVA and the Fisher's LSD post-hoc test (SPSS 12.0 for Windows, SPSS, Chicago, IL). Significance was associated with P-values less than 0.05.

3.4 Results

3.4.1 Effect of Continuous Culture on ECM Accumulation and Cellularity

To determine the effect of continuous culture on cartilaginous tissue growth, tissue constructs (after 2 weeks of pre-culture) were cultivated in the reactor for the duration of one week under a media flow rate of either 5 or 10 μL/min. Irrespective of flow rate, cultures grown in the reactor accumulated significantly more ECM which contained more proteoglycans and collagen ($p < 0.001$) (between 50 and 70%) compared to the static (no-flow) controls (Figure 3.1, Table 3.1). There was, however, potentially an effect of media flow rate on tissue cellularity ($p = 0.077$) as cultures grown under a flow rate of 5 μL/min had DNA contents approximately 25% lower than the static (no-flow) controls. This resulted in an apparent two-fold increase in amount of ECM macromolecules accumulated per microgram DNA ($p < 0.05$) in the constructs cultured under the low media flow rate (5 μL/min) (Table 3.1). Under higher media flow rates (10 μL/min), there was no observable effect on DNA content and the amount of ECM macromolecules accumulated per microgram DNA was approximately increased by 1.4-fold compared with the static (no-flow) controls ($p < 0.05$) (Table 3.1). The changes in ECM accumulation of a representative experiment have been presented in Table 3.2.

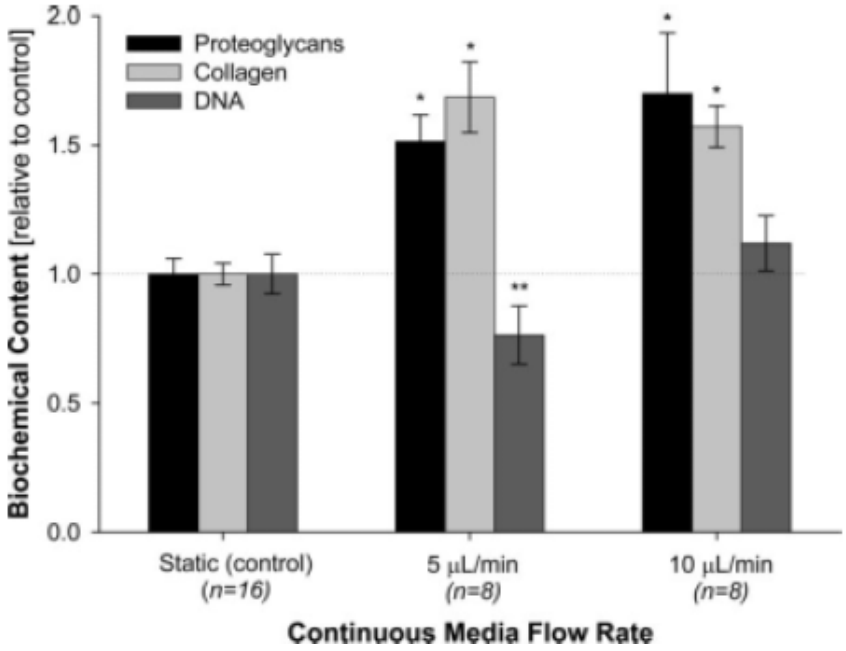

Figure 3.1: The effect of continuous culture on cartilaginous ECM accumulation.

Table 3.1: Relative Change in ECM Accumulation

	Static Culture ***(n = 16)***	**Continuous Culture 5μL/min** ***(n = 8)***	**Continuous Culture 10μL/min** ***(n = 8)***
Wet weight	1.00 ± 0.05	1.4 ± 0.1*	1.13 ± 0.09
Dry weight	1.00 ± 0.04	1.09 ± 0.06	0.96 ± 0.08
DNA/dry weight	1.00 ± 0.05	0.70 ± 0.09*	1.3 ± 0.2
Proteoglycans/dry weight	1.00 ± 0.08*	1.4 ± 0.1	1.9 ± 0.4
Proteoglycans/DNA	1.0 ± 0.1	2.1 ± 0.2*	1.3 ± 0.1
Collagen/dry weight	1.00 ± 0.02*	1.5 ± 0.1	1.7 ± 0.2
Collagen/DNA	1.00 ± 0.07	2.6 ± 0.5*	1.4 ± 0.1*
Collagen-to-proteoglycan ratio	1.00 ± 0.06	1.2 ± 0.2	1.0± 0.2

* Statistically different from all other groups ($p < 0.05$)
n, number of samples per group
Data expressed relative to static culture and presented as mean ± SEM

Table 3.2: Change in ECM Accumulation for a Representative Experiment

	2 Week Preculture (*n = 4*)	**Static Culture (*n = 4*)**	**Continuous Culture 5µL/min *(n = 4)***	**Continuous Culture 10µL/min *(n = 4)***
Collagen (µg)	26 ± 2	39 ± 3	69 ± 8	113 ± 12
Proteoglycans (µg)	30 ± 1	34 ± 4	51 ± 5	67 ± 10
DNA (µg)	2.8 ± 0.4	3.3 ± 0.3	2.0 ± 0.5	5.1 ± 0.3
n, number of samples per group Data presented as mean ± SEM				

3.4.2 Effect of Continuous Culture on Glucose Utilization

Analysis of the conditioned media revealed that the continuous flow reactor was capable of maintaining a stable nutrient environment similar to the prepared media that was provided to the cultures (Table 3.3). Specifically, the reactor maintained pHe close to neutral values and contained both higher levels of glucose and lower levels of lactic acid compared with the static (no-flow) controls ($p < 0.05$). Interestingly, there was an increased consumption of glucose (and associated production of lactate) by the cells when cultured under increasing media flow-rates ($p < 0.05$) (Table 3.3). In addition, the yield of lactate on glucose ($Y_{L/G}$) was approximately 10% lower when the constructs were cultured in the reactor in comparison with the static (no-flow) controls (Table 3.3).

Table 3.3: Effect of Media Flow Rate on Glucose Metabolism

	Static Culture (*n = 16*)	**Continuous Culture 5µL/min *(n = 8)***	**Continuous Culture 10µL/min *(n = 8)***	**Fresh Media (*n = 6*)**
Glucose concentration (mM)	5.0 ± 0.3*	7.8 ± 0.2	8.1 ± 0.1	9.4 ± 0.2*
Lactate concentration (mM)	11.5 ± 0.5*	5.9 ± 0.5	5.4 ± 0.1	3.2 ± 0.2*
Glucose consumption (nmol/h)	186 ± 10*	477 ± 36*	774 ± 87*	n/a
Lactate production (nmol/h)	349 ± 18*	815 ± 63*	1320 ± 133*	n/a
Yield of lactate on glucose ($Y_{L/G}$)	1.9 ± 0.1	1.7 ± 0.1	1.7 ± 0.2	n/a
Extracellular pH	6.66 ± 0.01*	7.09 ± 0.05	7.13 ± 0.04	7.2 ± 0.1

* statistically different from all other groups ($p < 0.005$)
n, number of samples per group
Static culture conditioned media was determined at the end of a 48hr media replacement cycle
Data are presented as mean ± SEM
n/a, not applicable

3.4.3 Histological and Immunohistochemical Assessment

Histological assessment of the developed tissues revealed that the cartilaginous constructs cultured in the continuous flow reactor appeared to be thicker than the static (no-flow) controls (Figure 3.2). Although all of the developed tissues stained positive for sulfated proteoglycans (Figure 3.2, B, D and F), tissues cultured in the reactor displayed depth-dependent staining with increasingly more intense staining at the bottom of the culture and less staining at the tissue surface (Figure 3.2, D and F). This is in contrast to the static controls which displayed uniform toluidine blue (TB) staining under these conditions (Figure 3.2B). Tissues were also immunostained to detect the presence of collagen types II (cartilage-specific), I (dedifferentiation marker), and X (early marker of chondrocyte hypertrophy). Static cultures were stained for collagen type II throughout the ECM, and no type I collagen was observed (Figure 3.3, A and B).

Tissues cultivated in the bioreactor also stained diffusely for type II collagen (Figure 3.3, D and F). However, there was minor localized staining for type I collagen in these tissues (Figure 3.3, C and E). At the tissue surface, type I collagen was observed in the matrix; whereas, deeper within the tissue, type I collagen was primarily in the pericellular region (Figure 3.4). Relatively few cells stained positive for type X collagen which was primarily noted to occur at the intracellular and pericellular regions, and there were no observable differences between the static controls and cultures grown in the reactor (Figure 3.5). Engineered cartilage constructs were also stained with periodic-acid Shiff (PAS) in order to detect glycogen. The presence of intracellular glycogen was not detectable in the static (no-flow) control cultures; whereas, the intracellular glycogen was observed throughout the cultures grown under a continuous flow of media (5 and 10 μL/min) (Figure 3.6).

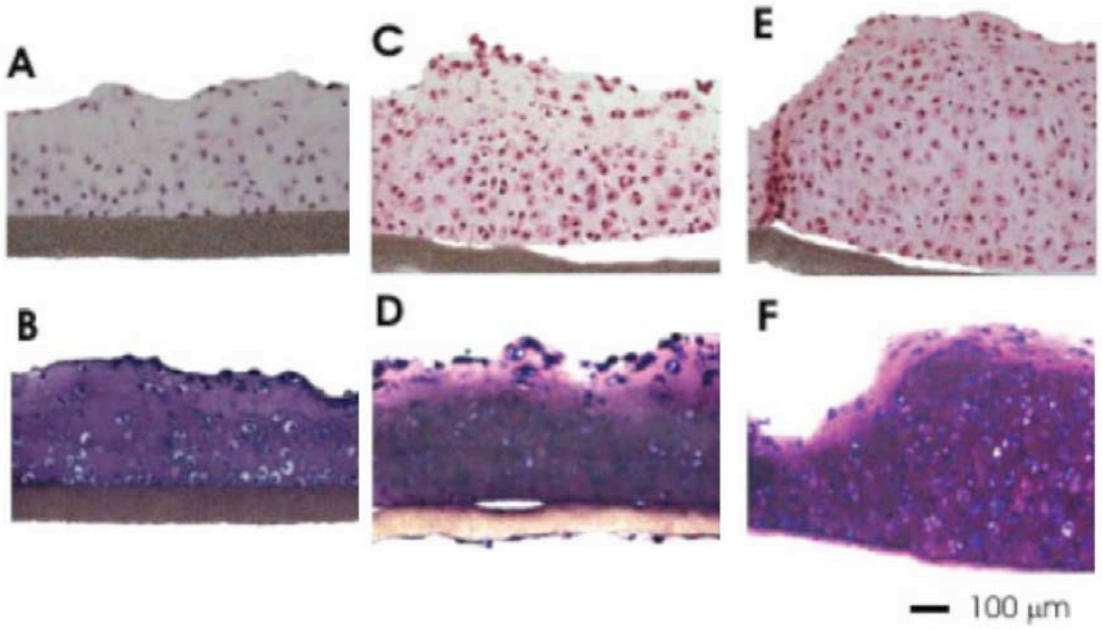

Figure 3.2: Histological assessment of cartilaginous tissue constructs after one week of continuous culture. Tissue sections were stained with hematoxylin and eosin (H&E) (general connective tissue stain) and toluidine blue (TB) (sulfated PG stain). Static culture (**A** and **B**), continuous media flow rate of 5 μL/min (**C** and **D**), and continuous media flow rate of 10 μL/min (**E** and **F**).

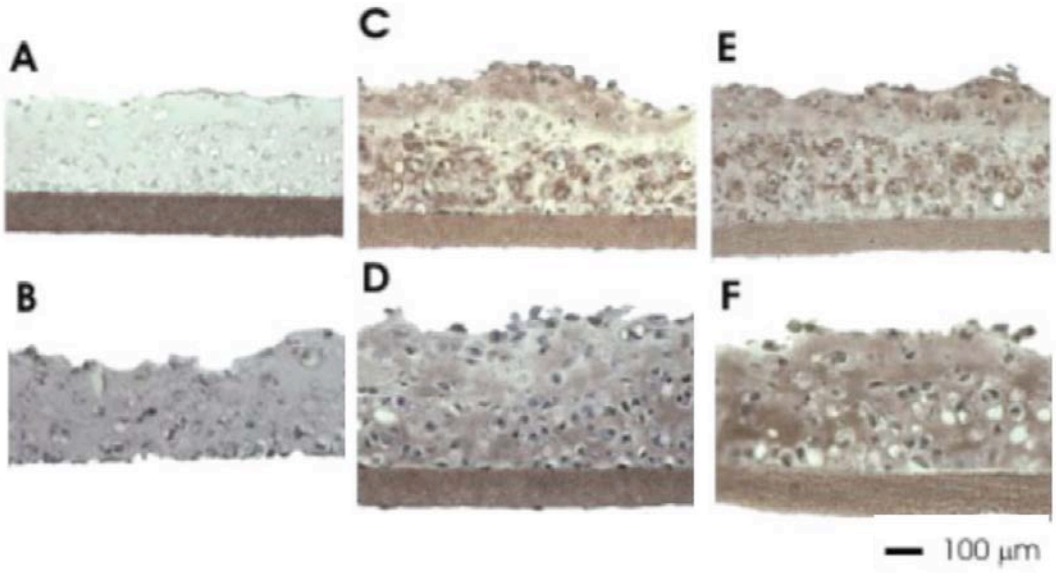

Figure 3.3: Immunolocalization of types I (**A, C** and **E**) and II (**B, D** and **F**) collagen in the cartilaginous tissue constructs after one week of continuous culture. Static culture (**A** and **B**), continuous media flow rate of 5 µL/min (**C** and **D**) and continuous media flow rate of 10 µL/min (**E** and **F**).

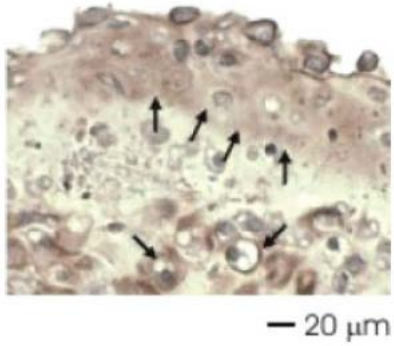

Figure 3.4: Detail of type I collagen immunolocalization in the cartilaginous tissue constructs after one week of continuous culture (sample shown was cultured under a flow rate of 10 µL/min).

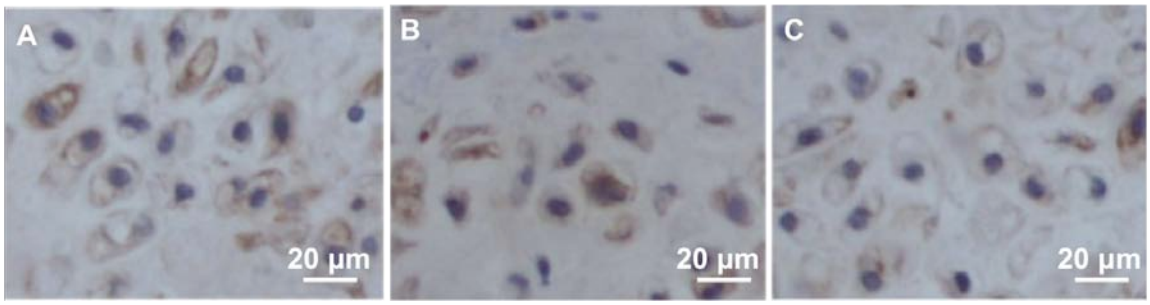

Figure 3.5: Immunolocalization of type X collagen in the cartilaginous tissue constructs after one week of continuous culture. Static culture (panel **A**), continuous media flow rate of 5 µL/min (panel **B**) and continuous media flow rate of 10 µL/min (panel **C**).

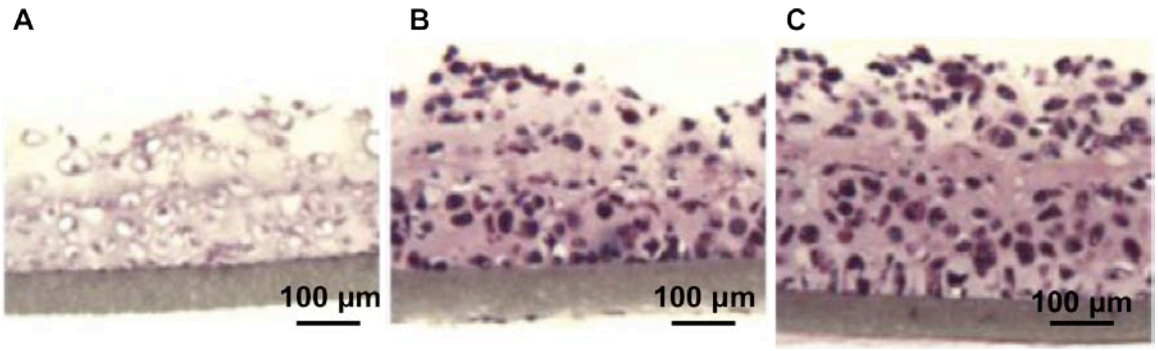

Figure 3.6: Periodic acid-Shiff (PAS) staining for intracellular glycogen in the cartilaginous tissue constructs after one week of continuous culture. Static culture **(A)**, continuous media flow rate of 5 µL/min **(B)**, and continuous media flow rate of 10 µL/min **(C)**.

3.5 Discussion

As traditional batch-fed culture is not optimal for cartilaginous tissue growth *in vitro*, [194, 214, 216, 218, 232] the use of bioreactors has become increasingly more important. However, the convective transport of nutrients, which most bioreactors rely on, can also elicit unwanted and/or uncontrollable mechanical stimuli to the cells resident in the developing tissue construct.

Alternatively, recent studies have demonstrated improved tissue growth in response to an increased diffusive gradient (by culturing in increasingly large volumes of media) [210-212]. To maximize this response, in this study, we have investigated whether continuous culture of engineered cartilage would lead to improved tissue growth. Continuous culture has been extensively used as an effective means to culture anaerobic microorganisms (e.g. fermentation) since the development of the chemostat in 1950 [249, 250]. Although not routinely used for mammalian cell culture (with the exception of hybridoma cultures), continuous culture should also readily apply to the culture of other anaerobic cells, including articular chondrocytes which are known to rapidly deplete the media of its nutrient supply [71, 228].

After one week of continuous culture (at a flow rate of 5-10 μL/min), harvested cartilaginous tissue constructs appeared to be thicker in appearance and contained significantly more ECM marcomolecules (50-70% increase in proteoglycan and collagen content) compared to the static (no-flow) controls. These results were strikingly similar to previous studies that have demonstrated improved tissue growth when the constructs were grown in through-thickness perfusion culture for similar periods of time [214, 216, 217]. In these studies, considerably larger flow rates of media were required to elicit increased biosynthesis compared to continuous culture (240-800 μL/min as opposed to 5-10 μL/min). This suggests that continuous culture is both more efficient and cost-effective than through-thickness perfusion; however, thicker constructs may potentially require larger flow rates due to an increased diffusion distance. Although not explicitly known, the similarity in results between these different types of reactors may be related to the (lack of) shear stresses imparted on the construct. Using computational modeling, Raimondi *et al*., [251] correlated improved proteoglycan accumulation with the application of lower shear stresses generated in the construct during perfusion. In continuous culture, the

constructs were subjected to minimal shear based on the low flow rates used and that the media was not forced through the construct.

Similarly, low shear stress bioreactors (e.g. rotating wall bioreactors) also stimulate ECM biosynthesis [252] and typically impart substantially lower magnitude shearing stresses (approximately 10–100 times lower) than through-thickness perfusion [251]. This could potentially explain why some studies have reported negligible effects of through-thickness perfusion of the growth of cartilaginous tissue *in vitro* [208, 219]. One potential drawback of continuous culture was the observed changes in tissue cellularity. Under low continuous media flow rates (5 μL/min), there was an apparent decline in tissue cellularity (25%) which was not observed under higher flow rates (10 μL/min). In contrast, through-thickness perfusion of media typically results in increased tissue cellularity [194, 214, 216, 218, 251]. Although the exact cause for these differences is presently unknown, Raimondi *et al.*, [218] obtained some experimental evidence to suggest that a slower rate of apoptosis, rather than an increased rate of proliferation, is responsible for the increased cellularity of thickness-perfused cartilage constructs.

One week of continuous culture also led to unanticipated alterations in tissue structure. Bioreactor cultivated cultures displayed depth-dependent staining for sulfated proteoglycans similar to that of native articular cartilage [253]. Although all tissue constructs contained type II collagen (determined by immunohistochemical staining), the tissues grown in continuous culture accumulated some type I collagen in both the superficial and deep aspects of the tissue. The formation of a type I collagen rich region at the exterior of the construct has been observed in both high- and low-shear bioreactors [221, 232]. This suggests that even under the low flow rates used in this study, convection effects cannot entirely be avoided. Interestingly, however, deep within the reactor cultivated tissues, type I collagen staining was primarily pericellular.

Although the expression of type I collagen has been ascribed as a de-differentiation marker for chondrocytes [254, 255], deep-zone chondrocytes have been shown to express type I collagen [256]. Thus, these results may suggest that the developed tissues were differentiating towards the stratified morphology of native articular cartilage. However, the extent of type X collagen staining (an early hypertrophic marker) was unaltered, suggesting that further study is required to confirm whether these changes are indicative of differentiation towards the native articular cartilage phenotype.

Analysis of the conditioned media revealed that continuous culture created a stable nutrient environment similar to that of the prepared media provided to the cultures. The conditioned media contained significantly higher glucose (62% greater), lower lactate (53% lower), and a pH closer to physiological levels compared with static culture. Tissue constructs cultivated in the reactor consumed greater amounts of glucose with increasing media flow rate along with a concomitant decline (10%) in the yield of lactate on glucose ($Y_{L/G}$). The observed changes in glucose utilization may suggest a slight shift towards aerobic metabolism; however, chondrocytes tend to utilize more economical metabolic pathways in response to low nutrient environments [74], which would have been expected in the most extreme conditions (i.e. static culture). An alternative explanation may be the use of glucose for synthesis of glycogen. Under high nutrient availability, glucose can be diverted for the formation of glycogen [257]. *In vivo*, both middle and deep zone chondrocytes store glycogen as a readily available energy reserve [73, 258] and as building blocks for the synthesis of sugar-containing ECM components (e.g. PGs) [73]. This notion was confirmed as the tissue constructs grown in continuous culture stained positive for intracellular glycogen; whereas, no staining was observed in the static (no-flow) controls. Previous work has also demonstrated that the accumulation of intracellular glycogen can be induced by increasing nutrient availability to the tissue during culture [193].

3.6 Conclusions

In this study, we investigated the effect of continuous culture on cartilaginous tissue growth *in vitro*. Engineered tissue constructs cultivated under a continuous flow rate of media for a period of one week accumulated significantly more ECM (between 50 and 70% increase in proteoglycan and collagen content). These increases were similar in magnitude to the reported effect of through-thickness perfusion without the need for large volumetric flow rates. Interestingly, with continuous culture, there was also some evidence that the tissue adopted an appearance closer to that of native articular cartilage. Tissues grown in the presence of continuous media flow displayed some features of the characteristic stratified morphology of native cartilage, as well as, containing stores of intracellular glycogen. Future studies will investigate the effect of long-term continuous culture in terms of ECM accumulation and subsequent changes in mechanical function.

Chapter 4

Importance of Sodium Bicarbonate and Non-Bicarbonate Buffer Systems in Batch and Continuous Flow Bioreactors for Articular Cartilage Tissue Engineering

4.1 Relation to Overall Project

The results and discussions in Chapter 4 encompass Objective 2 (Section 2.2), which was to enhance cell proliferation and cartilaginous tissue growth in the bioreactor by supplementing the media with sodium bicarbonate ($NaHCO_3$). This section has been published in Tissue Engineering, Part C in 2012 (PMID: 22092352). I am the first author of this manuscript and was involved in the design and execution of experiments, data analysis and in drafting the manuscript. Dr. Denver Surrao is a co-author of this manuscript. Furthermore, the results from this study have also been included in a patent application (Publication Number: WO/2010/094128), which was filed with the help of the PARTEQ, the in-house technology transfer company at Queen's University. Please refer to the Appendix for further details on the patent.

4.2 Introduction

Bioreactors are now increasingly used for cartilage tissue engineering, as they stimulate tissue growth more strongly than traditional static cultures [203, 208, 214, 259-262]. Most bioreactors used to date have been batch-fed with media added and removed periodically; continuous flow bioreactors, by supplying the cells with a near-infinite supply of media, appear, however, to improve tissue growth [223]. The beneficial results of such bioreactors in regard to increased

extracellular matrix (ECM) accumulation and cell proliferation could result in part because a continuous flow of fresh media helps maintain a steady extracellular nutrient environment. By contrast, between medium changes in batch bioreactors or culture systems, nutrients are progressively depleted while waste accumulates [208].

One important factor, which could be better controlled in continuous flow than in batch bioreactors, particularly for cartilage tissue engineering, is extracellular pH (pHe). Chondrocytes rely virtually entirely on glycolysis as a source of energy and hence, use glucose and produce lactic acid at a high rate [71, 73]. Lactic acid, if not removed, can rapidly acidify the culture medium [223, 263, 264]. It has long been known that chondrocyte matrix synthesis rates and rates of cell proliferation are very sensitive to pHe and fall steeply in a pH-dependent manner under acidic conditions [95-99, 106, 107, 225]. It has been shown by Ohshima *et al.*, [98] and Wilkins *et al.*, [96] that, in regard to pHe, matrix synthesis follows a bell-shaped curve with optimum matrix synthesis occurring between extracellular pH 6.9 and pH 7.1. Acidic extracellular environments influence the intracellular pH (pHi) of chondrocytes [96, 97]. The pHi of articular chondrocytes is mainly regulated by the amiloride-sensitive Na^+/H^+ exchanger [88, 97]; however, the pHi of chondrocytes within the superficial zone of bovine cartilage is exclusively regulated by HCO_3^-dependent transporter(s) [118]. Bovine articular chondrocytes have a resting pHi of pH 7.1 but can only maintain this level of pHi over a narrow range of external pH values. Since changes to pHi affect ECM synthesis [97], efficient buffering systems are important if pHi is to remain at desirable levels. It is therefore surprising that little attention has been paid to buffering systems and maintenance of an optimal pHe in studies of bioreactors; indeed, in many cartilage bioreactor studies the type of buffering system employed is not even reported in the methods [214, 217, 265-269]. Thus, the effect of changes in pHe on matrix synthesis during chondrocyte culture in bioreactors is still unknown.

In our previous study, we found that chondrocytes cultured in a continuous flow bioreactor accumulated considerably more matrix than those cultured under static conditions when both systems were buffered only with 20 mM HEPES [223]. Therefore, the objective of this study was to determine whether the use of sodium bicarbonate as an additional buffering agent would help maintain pHe between pH 6.9 and pH 7.2 in the bioreactor, and thus further enhance ECM accumulation.

Sodium bicarbonate was added to the media as a second buffering agent for three distinct reasons: (i) bicarbonate (HCO_3^-) is a physiological buffer in the body [114, 270-272]; (ii) $NaHCO_3$ has the capacity to neutralize acids effectively by the carbonic acid (H_2CO_3) dissociation process ($HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O$) [114, 271, 273]; (iii) HCO_3^- ions can actively be transported into the cell to buffer the intracellular space [114, 118] and increase ECM synthesis. So the addition of $NaHCO_3$ to the culturing media is an effective way to maintain extracellular and hence intracellular pH, which in turn helps stimulate ECM synthesis [99, 107, 274, 275]. It is important to note that bicarbonate is a base and the presence of another non-bicarbonate buffering agent (e.g. HEPES) is essential [270, 273, 274].

The media used in this study was Ham's F-12 which was further supplemented with a 14 mM concentration of $NaHCO_3$. The addition of 14 mM $NaHCO_3$ was based on a study conducted in 1965 by Ham *et al*. [276]. Additionally, this concentration of $NaHCO_3$ (14 mM) with Ham's F-12 media is recommended by Sigma Aldrich and Gibco for cell culture under 5% CO_2 [270, 274, 276, 277]. As the addition of bicarbonate also increased the osmolarity of the medium, which could itself perhaps influence cellular activity, we also investigated whether bicarbonate buffering or change in osmolarity was responsible for any changes seen.

4.3 Materials and Methods

4.3.1 Cell Isolation and High-Density 3D Culture (Static and Continuous Flow)

Cartilaginous tissue constructs were generated using isolated chondrocytes harvested from calf (12-18 months old) metacarpal-phalangeal articular cartilage by sequential enzymatic digestion, as described previously [236]. Tissue was obtained from several joints (up to 4 per experiment) and pooled together to get a sufficient cell number. The cells were seeded on the surface of type II collagen-coated Millicell™ filters in high-density, 3D culture (2×10^6 cells/filter or 33,000 cells/mm^2) and maintained in Ham's F-12 media (without $NaHCO_3$ and HEPES and with 10 mM glucose and glutamine) (HyClone, Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS) and 100 mg/mL ascorbate. Ham's F-12 medium without added bicarbonate, but supplemented with 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid) (Sigma Aldrich Ltd., St. Louis, MI, USA), had a pH of 7.4 in air and a pH of 6.95 under 5% CO_2. The cultures were grown in an incubator maintained at 37°C and 95% relative humidity supplied with 5% CO_2 : 95% atmospheric air. The culture medium (1 mL per filter) was changed every 48-72 hours, and fresh ascorbic acid was added with each change. Cultures were grown under static (no-flow) conditions for a week to generate a layer of cartilaginous tissue prior to being cultured in the continuous flow bioreactor, as described previously [223]. In the bioreactor, cultures were supplied with a 10 µL/min continuous flow of medium with or without a supplement of 14 mM $NaHCO_3$. The 10 µL/min flow rate corresponded to an average residence time of approximately 7 hours. The flow rate of the peristaltic pumps were validated by determining the time needed to collect a known volume of media. Additionally, cultures were also grown under static conditions in medium with or without supplementary 14 mM $NaHCO_3$. The above-mentioned culturing

conditions were repeated at least 3 times using bovine chondrocytes isolated at various time points, and all the cultures were grown for five weeks.

Medium samples were also collected at the end of the culture period and analyzed for pH. To prevent loss of dissolved CO_2 which will influence media pH [116], pH measurements were taken immediately after collection using a pH micro-probe.

Osmolarity control experiments were also conducted in the reactor, as described above, for a period of three weeks. In these experiments, the osmolarity of the media without $NaHCO_3$ was adjusted to the same osmolarity as the media containing 14 mM $NaHCO_3$ by the addition of approximately 24 mM mannitol. After the addition of mannitol, the osmolarity of both the solutions was re-measured, by the freezing point depression method, to confirm that it was the same.

4.3.2 Determination of Extracellular Matrix Accumulation and Cellularity

At the end of the culture periods (static and continuous), tissue constructs were harvested, lightly patted dry and weighed (wet weight). Tissues were lyophilized overnight, and the dry weight of the tissue determined. Tissue samples were then digested with papain (80 µg/mL in 20 mM ammonia acetate, 1 mM EDTA and 2 mM DTT) for 72 hours at 65°C and stored at -20°C until analysis. Aliquots of the digest were analyzed for glycosaminoglycans, collagen and DNA content. Proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using the dimethylmethylene blue (DMB) dye binding assay (Polysciences, Washington, PA) [237, 238]. The glycosaminoglycan was measured using a plate reader at 525 nm. To determine the collagen content, aliquots of the papain digest were hydrolyzed in 6 N HCl at 110°C for 18 hr. Hydroxyproline content of the hydrolyzate was determined using chloramine

T/Ehrlich's reagent assay [239]. Hydroxyproline was measured using a plate reader at 540 nm after reaction with chloramine-T and p-dimethylaminobenzaldehyde. Collagen content was estimated by assuming that hydroxyproline comprised approximately 10% the weight of collagen [240]. DNA content was determined using the Hoechst dye 33258 assay (Sigma Aldrich Ltd) with calf thymus DNA as the standard [241]. Digest was mixed with Hoechst dye and the fluorescence was measured at excitation of 360 nm and emission of 430 nm.

4.3.3 Histological and Immunohistochemical Evaluation

Cultures from each of the culturing conditions were subjected to histological and immunohistochemical evaluation. Upon harvest, tissue constructs were fixed overnight in 4% paraformaldehyde (Sigma Aldrich Ltd.) and embedded in paraffin. Thin sections (5 μm thick) were cut and stained with hemotoxylin-eosin (H&E) and toluidine blue (TB). All sections were examined by light microscopy. Cryofixed sections were tested immunohistochemically with antibodies against type I and II collagen [10]. Prior to incubation with the primary antibody, sections were pre-digested with 0.25% trypsin (Sigma Aldrich Ltd.) only for collagen II and 0.5units/mL chondroitinase ABC and 0.5% hyaluronidase (Sigma Aldrich Ltd.) to facilitate immunostaining. The following primary antibodies and dilutions were used: rabbit polyclonal type I collagen antibody (Fitzgerald, MA, USA), 1:200 dilution and mouse monoclonal type II collagen antibody (Developmental Studies Hybridoma Bank, University of Iowa, IA, USA), 1:10 dilution. Fluorescently-labeled secondary antibodies were used with 2% (v/v) FITC-conjugated goat anti-rabbit Fab fragment (Vector Laboratories) for collagen I and FITC-conjugated horse anti-mouse Fab fragment (Vector Laboratories) for collagen II. Sections were washed and mounted with Vectashield hardset mounting medium with DAPI (Vector Laboratories).

4.3.4 Tissue Thickness Measurements

The thicknesses of the tissue constructs were determined via two methods: (i) bright-field imaging of the histology sections and (ii) the needle probe method as reported by Hoch *et al.* [278]. Bright-field images of histological sections for the various culturing conditions were calibrated with an online scale bar, from which the thicknesses of the tissue samples were determined; thickness measurements were taken at nine random locations and the average value recorded. In the needle probe method, a 25-gauge needle (Becton-Dickinson, Franklin Lakes, NJ, USA) was attached to a 1kg load cell of a Mach-1 mechanical tester (Biosyntech) which was then displaced into the tissue at a rate of 5 μm/s. Resistive compressive forces and needle displacement were recorded at a frequency of 10Hz. Abrupt changes in force were interpreted as needle contact with the tissue and the underlying support surface to determine the tissue thickness. Thickness measurements were taken at nine random locations, and the average value was recorded.

4.3.5 Indentation Testing

Elastic moduli of the developed tissue constructs were determined by indentation testing. Harvested tissues were placed flush on the surface of a porous steel stage of a Mach-1™ micromechanical tester (Biosyntech, Canada) equipped with a 1kg load cell and a plane-ended indenter (2.1 mm dia.). Samples were preloaded to 10 mN and then subjected to step indentations of 2% strain to a maximum of 20% strain. At each step, the resulting force decay was recorded until equilibrium was reached ($\leq$2 mN/min). The elastic modulus at 20% strain was then determined using the expression derived by Hayes *et al.* [279] for indentation testing of cartilage.

$$E = \frac{P(1-v^2)}{(2awK)}$$

where: P is equilibrium load, v is Poisson's ratio (v = 0.4 for bovine articular cartilage [8]), a is indenter radius, w is displacement, and K is a correction factor based on v and the aspect ratio of a to tissue thickness h.

4.3.6 Statistical Analyses

All numerical results were expressed as the mean ± standard error of the mean (SEM). Three independent experiments were conducted with three independent cell extractions. Data was compared between each of the experimental groups using a two-way ANOVA and the Fisher's LSD post-hoc test (SPSS 18.0, SPSS Inc., Chicago, IL, USA). Significance was associated with p-values less than 0.05.

4.4 Results

4.4.1 Combined Effect of $NaHCO_3$ and Continuous Flow Bioreactor on ECM Accumulation and Cellularity

To determine the combined effects of $NaHCO_3$ and a continuous flow of medium on cartilaginous tissue growth, tissue constructs after 1 week of pre-culture were cultivated in a continuous-flow bioreactor with a medium flow-rate of 10 µL/min. The medium used in the bioreactor was supplemented with 14 mM $NaHCO_3$, and the constructs were cultured for a duration of five weeks (Figure 4.1). Tissue constructs cultivated in the continuous flow bioreactor with medium supplemented with 14 mM $NaHCO_3$ accumulated significantly more *($p<0.05$)* ECM in comparison to all other conditions (Figure 4.2). Thus, (i) dry weight of tissue: reactor with

$NaHCO_3$: 20 .5 ± 0.98 mg, reactor without $NaHCO_3$: 3.7 ± 0.13 mg, static with $NaHCO_3$: 2.9 ± 0.22 mg, and static without $NaHCO_3$: 2.1 ± 0.1 mg ($p<0.05$) (n = 9), (ii) wet weight of tissue: reactor with $NaHCO_3$: 292.4 ± 16.3 mg, reactor without $NaHCO_3$: 33.2 ± 1.2 mg, static with $NaHCO_3$: 23.1 ± 1.4 mg, and static without $NaHCO_3$: 12.8 ± 1.0 mg ($p<0.05$) (n = 9). For the glycosaminoglycan and collagen results, see (Figure 4.3).

Additionally, the tissue constructs cultured in the bioreactor with $NaHCO_3$-supplemented medium demonstrated a significant increase in cellularity in comparison to all other culturing conditions. Thus, in the reactor, DNA was increased by 6-fold when the medium contained supplementary bicarbonate, while in the static (no flow) controls, the corresponding increase in DNA was 13-fold *($p<0.05$)* (Figure 4). Although, there was a significant increase *($p<0.05$)* in ECM macromolecules accumulated per microgram of DNA in $NaHCO_3$-supplemented static cultures (no flow) in comparison with static controls (no flow) without $NaHCO_3$ supplementation, no significant difference was observed between the two continuous-flow bioreactor conditions (i.e. with $NaHCO_3$, or without $NaHCO_3$) (Table 4.1).

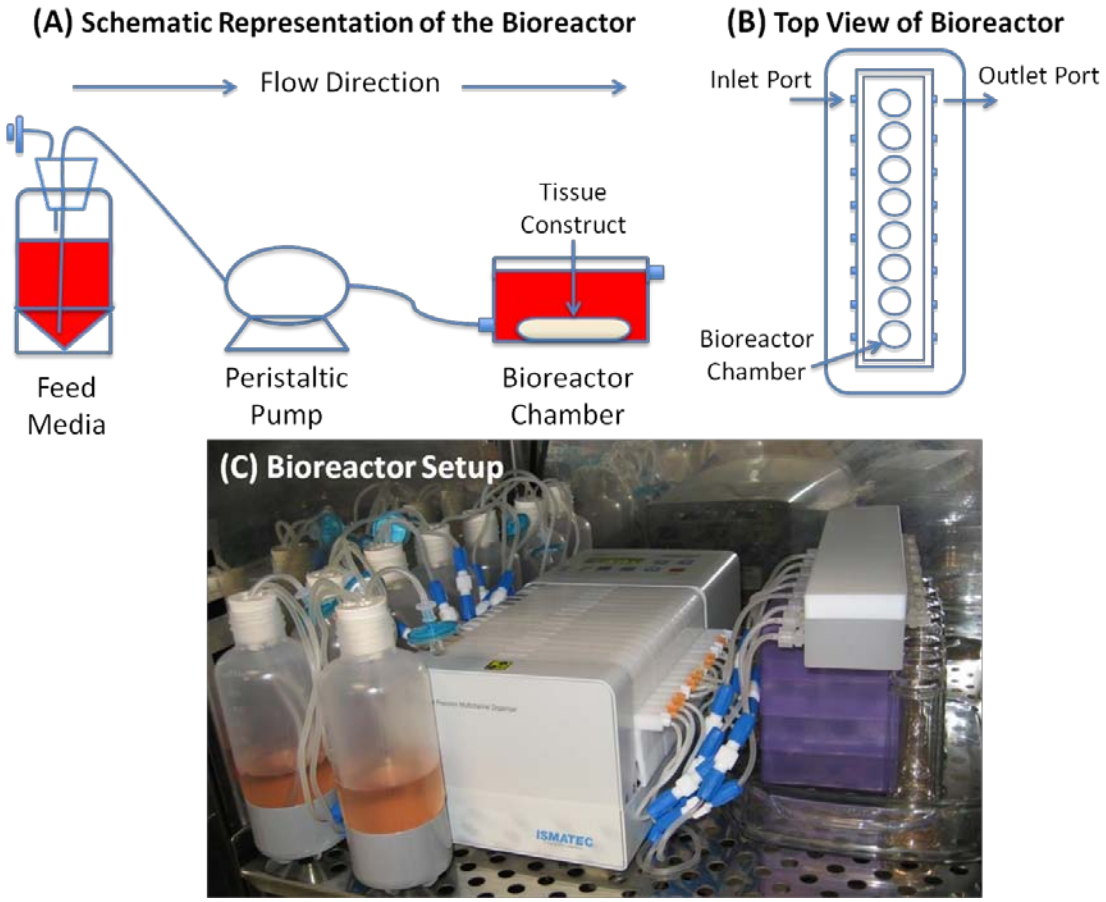

Figure 4.1: (A) Schematic representation of the bioreactor. **(B)** Top View of the Bioreactor vessel **(C)** Pictorial representation of the continuous flow bioreactor.

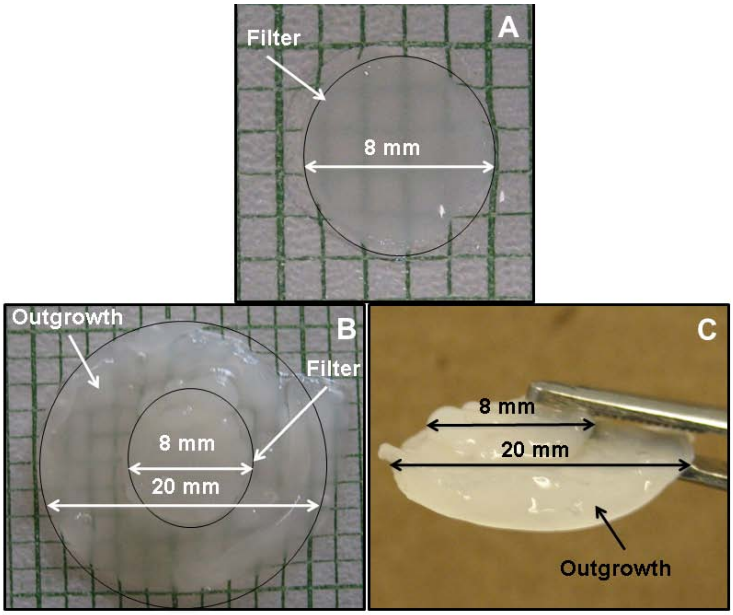

Figure 4.2: Pictorial representation of **(A)** Tissue without $NaHCO_3$ supplementation **(B)** Top view of tissue with $NaHCO_3$ supplementation **(C)** Side view of tissue with $NaHCO_3$ supplementation.

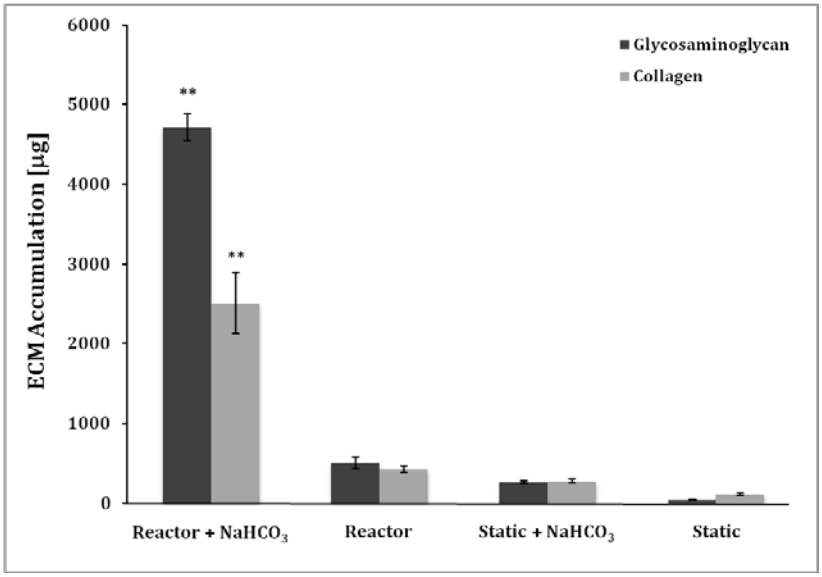

Figure 4.3: Effect of the presence or absence of sodium bicarbonate supplementation on ECM accumulation for constructs grown in a continuous bioreactor and static conditions. Collagen and glycosaminoglycan contents of the engineered tissue constructs were determined biochemically via the dimethylmethylene blue dye binding assay and chloramine-T/Ehrlich's reagent assay, respectively. ** represents significant difference from all other groups *($p<0.05$)*. Data are presented as mean ± SEM.

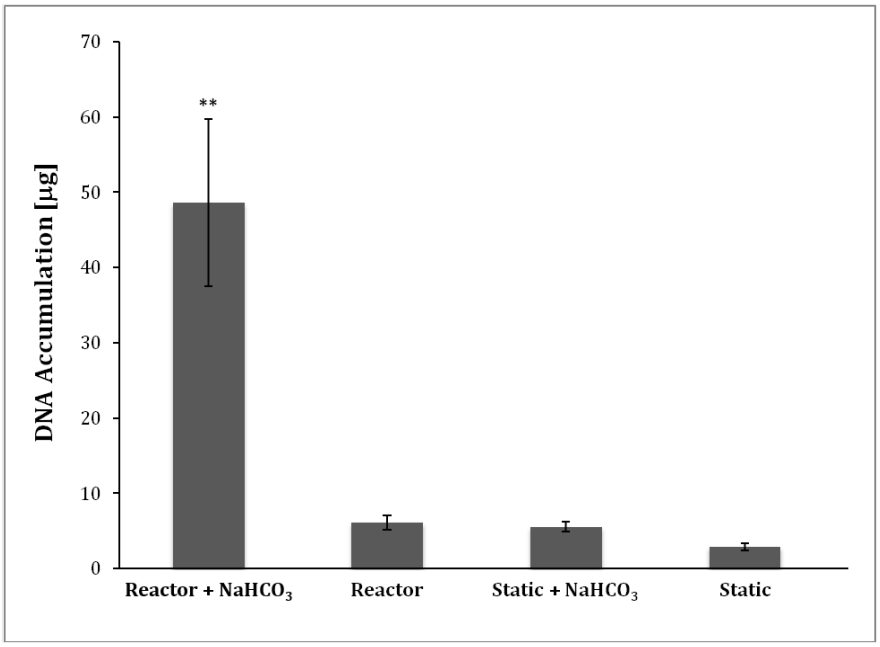

Figure 4.4: Effect of the presence or absence of sodium bicarbonate supplementation on chondrocyte proliferation. DNA contents of the engineered tissue constructs were determined biochemically via Hoechst dye 33258 assay. ** represents significant different from all other groups *($p<0.05$)*. Data are presented as mean ± SEM.

Table 4.1: Relative change in ECM synthesis and cellular proliferation for tissue constructs grown in a continuous flow bioreactor and in static conditions with/without $NaHCO_3$ supplemented medium.

		Bioreactor +/– $NaHCO_3$		**Bioreactor and Static +/– $NaHCO_3$**		
		Bioreactor + $NaHCO_3$	***Bioreactor – $NaHCO_3$***	***Bioreactor – $NaHCO_3$***	***Static + $NaHCO_3$***	***Static – $NaHCO_3$***
Wet Weight		$8.70^{a} \pm 0.68$	1 ± 0.04	$2.67^{c} \pm 0.17$	$2.17^{c} \pm 0.28$	1 ± 0.06
Dry weight		$5.20^{a} \pm 0.27$	1 ± 0.05	$1.97^{b} \pm 0.09$	$1.52^{c} \pm 0.11$	1 ± 0.03
DNA		$6.62^{a} \pm 0.85$	1 ± 0.03	2.24 ± 0.26	1.89 ± 0.26	1 ± 0.03
DNA/Dry Weight		1.21 ± 0.10	1 ± 0.04	1.02 ± 0.10	1.17 ± 0.11	1 ± 0.05
Collagen/Dry Weight		1.26 ± 0.23	1 ± 0.02	$1.92^{c} \pm 0.16$	$1.73^{c} \pm 0.13$	1 ± 0.04
GAG/Dry Weight		$1.94^{a} \pm 0.18$	1 ± 0.03	$4.61^{c} \pm 0.58$	$3.78^{c} \pm 0.56$	1 ± 0.08
DNA/Wet Weight		$0.77^{a} \pm 0.05$	1 ± 0.04	0.99 ± 0.03	0.84 ± 0.07	1 ± 0.06
Collagen/Wet Weight		0.77 ± 0.13	1 ± 0.02	$1.54^{c} \pm 0.11$	1.29 ± 0.13	1 ± 0.06
GAG/Wet Weight		1.15 ± 0.11	1 ± 0.05	$2.62^{c} \pm 0.25$	$2.86^{c} \pm 0.65$	1 ± 0.08
Collagen/DNA		0.99 ± 0.13	1 ± 0.03	$1.73^{c} \pm 0.15$	$1.55^{c} \pm 0.16$	1 ± 0.05
GAG/DNA		1.65 ± 0.11	1 ± 0.04	$4.34^{c} \pm 1.47$	$3.30^{c} \pm 0.55$	1 ± 0.11
Collagen-to-GAG Ratio		$0.61^{a} \pm 0.08$	1 ± 0.02	$0.41^{c} \pm 0.19$	$0.46^{c} \pm 0.04$	1 ± 0.13
Thickness	**Centre**	$11.49^{a} \pm 0.42$	1 ± 0.05	$2.57^{c} \pm 0.12$	0.75 ± 0.04	1 ± 0.04
	Outgrowth	$2.45^{a} \pm 0.13$				
Modulus		$3.60^{a} \pm 0.62$	1 ± 0.18	$0.12^{c} \pm 0.01$	0.34 ± 0.15	1 ± 0.33

[a] represents statistical difference with respect to **bioreactor without $NaHCO_3$** and **static without $NaHCO_3$** ($p<0.05$)
[b] represents statistical difference with respect to **static without $NaHCO_3$** and **static with $NaHCO_3$** ($p<0.05$)
[c] represents statistical difference with respect to **static without $NaHCO_3$** ($p<0.05$)
n = 9 per group
Data is either expressed relative to **bioreactor without $NaHCO_3$ or static without $NaHCO_3$**
Data are presented as mean ± SEM

4.4.2 Combined Effect of $NaHCO_3$ and Continuous Flow Bioreactor on Tissue Thickness and Tissue Modulus

The thicknesses of the tissue constructs were measured via histology, as the results obtained were reliable and consistent. The thickness measurements obtained from histology demonstrated that the constructs cultured in the bioreactor with $NaHCO_3$-supplemented media were significantly thicker (3380 ± 124 μm), in comparison to all other culturing conditions, results from the latter being: reactor: 295 ± 14 μm, static with $NaHCO_3$: 87 ± 4 μm; static control (no-flow): 115 ± 4 μm *($p<0.05$)* (n = 9). Furthermore, the constructs cultured in the bioreactor with $NaHCO_3$-supplemented medium were characterized by tissue outgrowth to average diameter 4 mm and average thickness 720 ± 40 μm. Tissue outgrowth was negligible in all other culturing conditions. The moduli of the tissue constructs cultured in the bioreactor with $NaHCO_3$-supplemented medium were significantly higher *($p<0.05$)* than the bioreactor constructs without $NaHCO_3$-supplemented medium (Table 4.1). The tissue modulus for the constructs cultured in the bioreactor with $NaHCO_3$-supplementated media was significantly higher than without $NaHCO_3$-supplemented media, though neither of them were equivalent to static without $NaHCO_3$-supplementation: reactor: 12 ± 1 KPa; reactor with $NaHCO_3$: 38 ± 6 KPa; static: 112 ± 37 KPa.

4.4.3 Influence of Extracellular Osmolarity on Cell Growth and Matrix Accumulation

To determine if osmolarity was the underlying cause of the increased cell proliferation and ECM accumulation, the osmolarity of the medium without bicarbonate supplementation was adjusted to be equal to the osmolarity of the medium supplemented with bicarbonate. The osmolarity was adjusted by adding mannitol. Results showed that there was considerably more cell proliferation and matrix accumulation in the constructs cultured with bicarbonate than in those cultured

without bicarbonate under iso-osmotic conditions (Table 4.2). Cell proliferation appeared to be the factor that was most stimulated by bicarbonate addition, while GAG and collagen accumulation are mainly a result of an increase in cellular proliferation (Table 4.2). From the results obtained at the end of week 3, it is evident, that osmotic adjustment alone could not explain increased cell proliferation and ECM accumulation in the bicarbonate constructs (Table 4.2).

Table 4.2: Relative change in ECM synthesis and cellular proliferation for tissue constructs grown in a continuous flow bioreactor with/without $NaHCO_3$ supplemented medium under iso-osmotic conditions.

	3 Weeks	
	Bioreactor + $NaHCO_3$	***Bioreactor + $NaHCO_3$***
Wet Weight	2.52 ± 0.09	1.00 ± 0.06
Dry weight	2.29 ± 0.07	1.00 ± 0.04
DNA	2.99 ± 0.37	1.00 ± 0.08
DNA/Dry Weight	1.31 ± 0.16	1.00 ± 0.04
Collagen	2.54 ± 0.02	1.00 ± 0.04
Collagen/Dry Weight	1.11 ± 0.03	1.00 ± 0.02
GAG	3.31 ± 0.24	1.00 ± 0.05
GAG/Dry Weight	1.47 ± 0.10	1.00 ± 0.01
Collagen/Wet Weight	1.01 ± 0.04	1.00 ± 0.03
GAG/Wet Weight	1.34 ± 0.10	1.00 ± 0.05
Collagen/DNA	0.88 ± 0.11	1.00 ± 0.04
GAG/DNA	1.17 ± 0.15	1.00 ± 0.05
Data is either expressed relative to **bioreactor without $NaHCO_3$** at 3 weeks *n = 4* per group Data are presented as mean ± SEM		

4.4.4 Combined Effect of $NaHCO_3$ and Continuous Flow Bioreactor on Extracellular pH

The pH of the medium from inside the wells was measured at the end of a 48 hour media replacement cycle just before harvesting the tissue (Table 4.3 and Table 4.4). A significant difference was observed between the pH of $NaHCO_3$-supplemented medium from static (no-flow) (pH: 6.91) and from static control (no-flow) without $NaHCO_3$ supplementation (pH: 6.72). At the end of week five, there was no significant difference *($p<0.05$)* observed between the bioreactor with $NaHCO_3$-supplemented medium (pH: 6.89) and the reactor with unsupplemented medium (pH: 6.81) (Table 4.3). During the 5 week culture period, the pH of the $NaHCO_3$-supplemented medium in the bioreactor was, for the most part, pH 6.9 or higher, while the media without $NaHCO_3$ was mostly lower than pH 6.9 (Table 4.4). The pH of the medium-only condition with no cells or tissue, 48 hours post the medium replacement cycle, was as follows: (i) medium with $NaHCO_3$ supplementation: pH 7.41 and (ii) medium without $NaHCO_3$ supplementation: pH 6.95 (Table 4.3).

Table 4.3: Change in pH for static (residence time: 48 hours) and bioreactor (residence time: 7 hours) culture systems, in comparison to inlet pH (fresh medium, no cells) (residence time: 48 hours). All are under 5% CO_2.

	Inlet pH (Fresh Medium)		**Bioreactor**		**Static**	
	+ $NaHCO_3$	***– $NaHCO_3$***	***+ $NaHCO_3$***	***– $NaHCO_3$***	***+ $NaHCO_3$***	***– $NaHCO_3$***
pH	7.41* ± 0.00	6.95 ± 0.00	6.89 ± 0.02	6.81 ± 0.01	6.91* ± 0.03	6.72 ± 0.02
* represents statistical differene with respect to without $NaHCO_3$ condition ($p<0.05$) *n = 9* per group Data are presented as mean ± SEM						

Table 4.4: Changes in medium pH for bioreactor (residence time: 7 hours) culture system over a period of 5 weeks.

	2 week	**3 week**	**5 week**
+NaHCO₃	7.00 ± 0.00	6.96 ± 0.01	6.89 ± 0.02
–NaHCO₃	6.83 ± 0.01	6.81 ± 0.01	6.81 ± 0.01
Media collected from each well *n = at least 4* per group Data are presented as mean ± SEM			

4.4.5 Histological and Immunohistochemical Assessment

Histological assessment of the developed tissues via hemotoxylin-eosin and toluidine blue staining revealed that the cartilaginous constructs cultured in the continuous bioreactor with $NaHCO_3$-supplemented media was significantly thicker *($p<0.05$)* in comparison to all the other culturing conditions (Figure 4.5 and Figure 4.6). Tissue sections from all of the culturing conditions stained positive for sulfated proteoglycans (Figure 4.5 and Figure 4.6). All of the culturing conditions, except the static control (no-flow), displayed depth-dependent staining with increasingly more intense staining at the bottom of the tissue section and less staining at the tissue surface (Figure 4.5 and Figure 4.6). Tissues were also immunostained to detect the presence of collagen types II (cartilage-specific) and I (de-differentiation marker). All conditions stained intensely throughout the tissue thickness for collagen type II and less intensely for collagen type I (Figure 4.7).

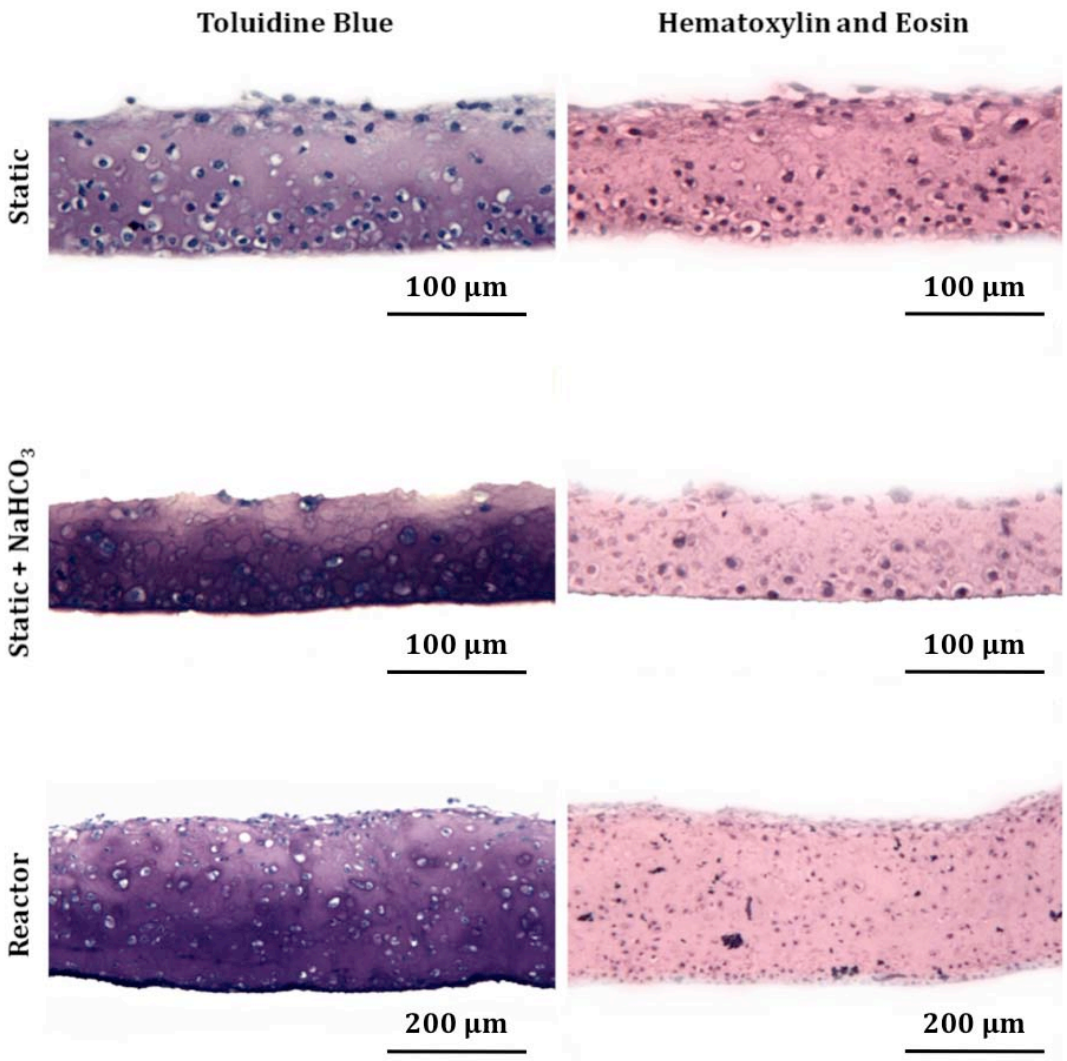

Figure 4.5: Histological assessment of cartilaginous tissue constructs after five weeks of culturing under different culturing conditions (static culture, static with $NaHCO_3$-supplemented medium and continuous-flow culture without $NaHCO_3$ medium supplementation). Tissue sections were stained with toluidine blue (TB) (sulfated proteoglycan stain) and hematoxylin and eosin (H&E) (general connective tissue stain). Scale bar 100 µm and 200 µm.

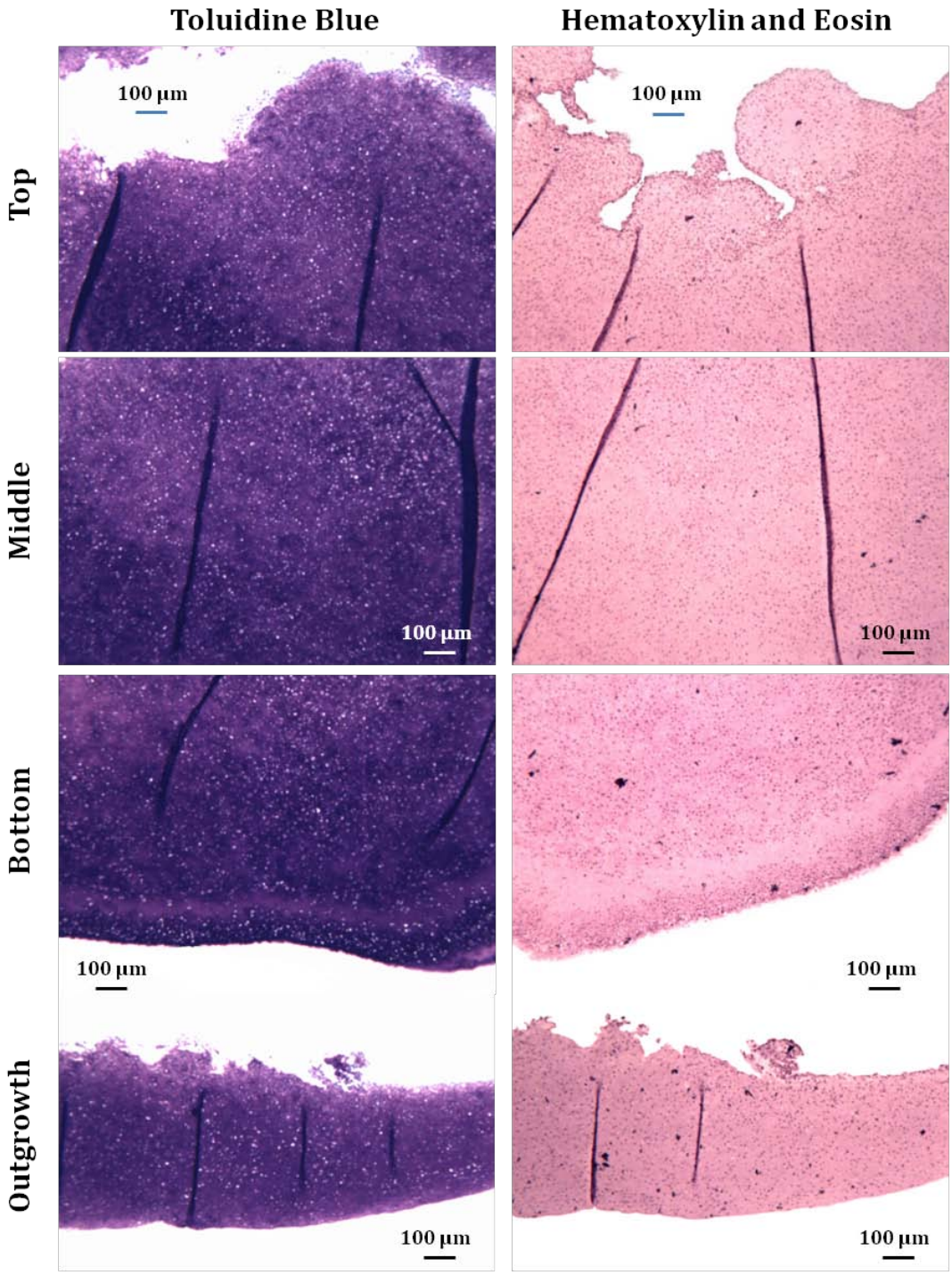

Figure 4.6: Histological assessment of cartilaginous tissue constructs after five weeks of culturing in a continuous flow bioreactor with $NaHCO_3$-supplemented medium. Tissue sections were stained with toluidine blue (TB) (sulfated proteoglycan stain) and hematoxylin and eosin (H&E) (general connective tissue stain). Scale bar 100 µm.

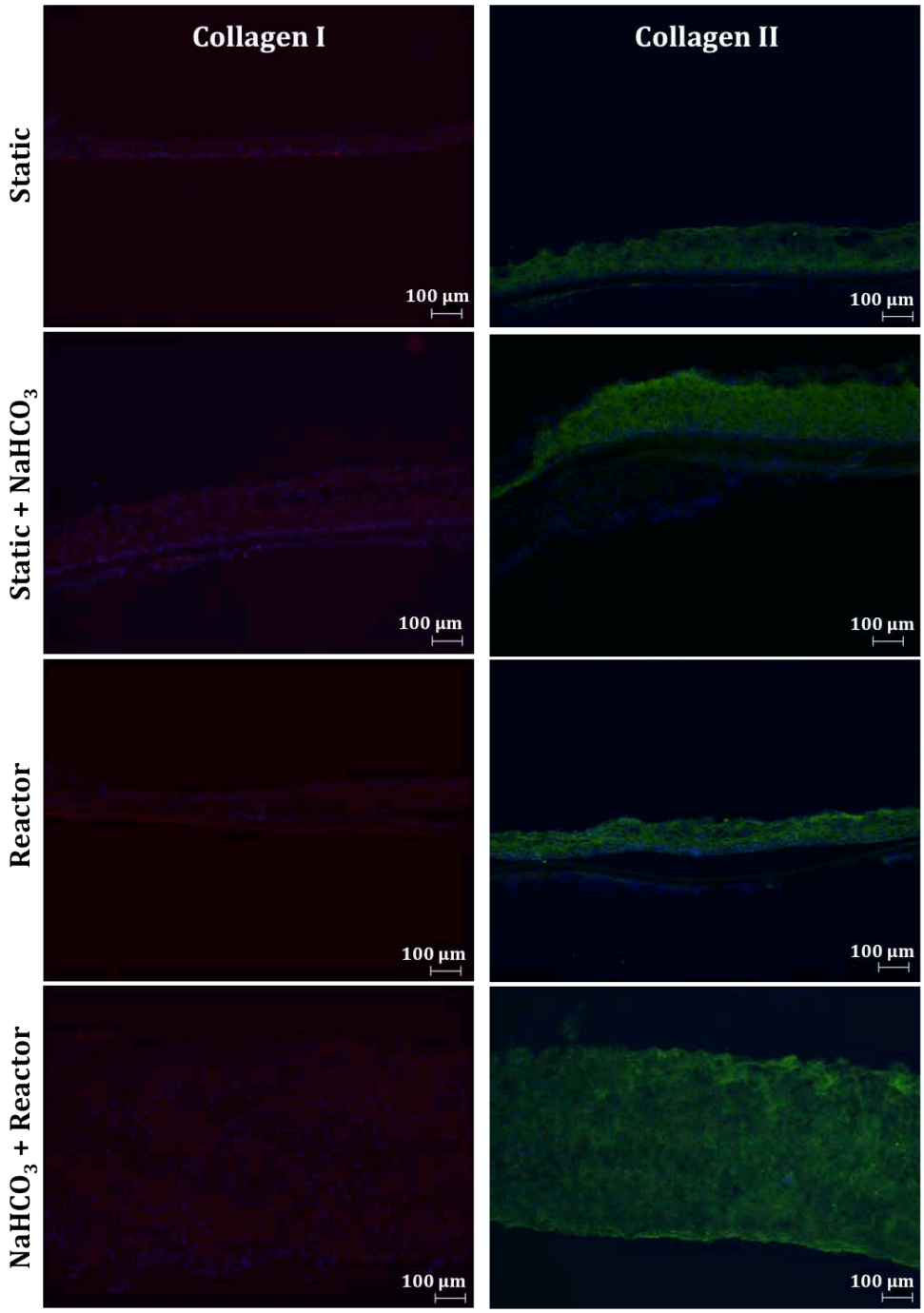

Figure 4.7: Immunohistochemical assessment of cartilaginous tissue constructs after five weeks of culturing under different culturing conditions (static culture, static with $NaHCO_3$-supplemented medium, continuous flow culture without $NaHCO_3$ medium supplementation, and continuous flow culture with $NaHCO_3$ medium supplementation). Tissue sections were stained for collagen types I and II. Scale bar 100 µm.

4.5 Discussion

The purpose of this study was to determine if the incorporation of an additional buffer, sodium bicarbonate, to the culture media in a continuous flow bioreactor [223] would increase extracellular matrix accumulation and cell proliferation.

We found that both continuous culture and $NaHCO_3$ buffering stimulated matrix accumulation and cell proliferation. After five weeks of continuous culture, constructs cultured with $NaHCO_3$ supplementation produced markedly more GAG and DNA, both in the bioreactor and in static cultures, than in constructs with no $NaHCO_3$ supplementation (Figure 4.3 and Figure 4.4; and Table 4.1). Looking at results from the bioreactor cultures, even though the GAG/DNA ratio increased by 65% (Figure 4.3 and Figure 4.4) in the $NaHCO_3$-supplemented system relative to the unsupplemented system, the marked increase in the amount of GAG and collagen produced (Figure 4.3) appears to arise mainly from a 6 fold increase in the number of cells (Figure 4.4). In addition, as found previously [223], chondrocytes cultured under continuous flow produced more matrix than chondrocytes cultured under static conditions (Figure 4.3 and Figure 4.4). Remarkably, in a bioreactor with $NaHCO_3$ supplemented media, the harvested cartilaginous tissue constructs were considerably thicker [a 28-fold increase *($p<0.05$)*] and contained significantly more *($p<0.05$)* extracellular matrix macromolecules [a 98-fold increase in glycosaminoglycans and a 25-fold increase in collagen content] compared to the static control (no-flow) cultures (Figure 4.3). Histological assessment of the developed tissues revealed that all of the tissue sections from the bioreactor stained positive for sulfated proteoglycans. Tissues were immunostained to detect the presence of collagen types II (cartilage-specific) and I (de-differentiation marker). Constructs cultivated in the continuous-flow bioreactor with and without $NaHCO_3$ medium supplementation stained more intensely for type II collagen than for type I collagen (Figure 4.7). Measurements showed that the constructs cultured in the continuous-flow

bioreactor with $NaHCO_3$-supplemented medium had a greater elastic modulus than bioreactor constructs grown without $NaHCO_3$ supplementation; however, in no case was the elastic modulus comparable to that of native articular cartilage.

From the results presented here, it is evident that chondrocytes cultured in a continuous-flow bioreactor where the medium is buffered by both 14 mM $NaHCO_3$ and 20 mM HEPES proliferate more extensively and produce more cartilage matrix than chondrocytes cultured in a similar continuous flow bioreactor with medium buffered only by 20 mM HEPES (Figure 4.3, Figure 4.4 and Figure 4.5). The question then arises, are the effects mediated by improved buffering or by the presence of $NaHCO_3$ itself? Table 4.3 shows that there was a significant difference *($p<0.05$)* between the pH levels of the media with and without $NaHCO_3$ supplementation, with no exposure to cells or tissue when allowed to equilibrate with 5% CO_2 at 37^oC. The medium containing $NaHCO_3$ was at pH 7.4; whereas, without $NaHCO_3$, it was at pH 6.9. However, there was little difference between the two media after exposure to cells. After five weeks of culture in the bioreactor, the pH of the effluent medium with $NaHCO_3$ present was pH 6.89; whereas, in its absence, the effluent pH was pH 6.81 (Table 4.3 and Table 4.4). Thus, in the bioreactor with bicarbonate present, because of increased cell number and activity, the pH fell 0.54 pH units more during the 7 hour residence time than it fell in the bioreactor with no bicarbonate supplementation. Could the differences in inlet (fresh medium) pH (Table 4.4) alone be responsible for the dramatic increase in cell number and matrix accumulation (Figure 4.3, Figure 4.4 and Figure 4.5)? Acidification of the extracellular medium from pH 7.4 to pH 6.8 is reported to reduce the rate of cell proliferation of other cell types [280, 281] and the rate of matrix synthesis by chondrocytes [95, 98, 106, 107, 225] markedly. With no $NaHCO_3$ supplementation, the pHe of the medium fed to the cells was never above pH 7.0 (Table 4.3 and Table 4.4); the acid pH of the medium in contact with the cells over the entire culture period could account, in

part at least, for the lower cell numbers and amount of matrix produced in cultures which are not buffered by $NaHCO_3$.

Does $NaHCO_3$ play a role apart from buffering medium pH? The effect of pHe on cell function is in general mediated through its influence on pHi both in regard to cell proliferation [282, 283] and matrix synthesis [117]. Cells thus have a complex system of membrane transporters, channels and pumps to maintain intracellular ionic homeostasis in the face of extracellular insults [79]. Freshly isolated chondrocytes appear to regulate their pHi mainly through the sodium hydrogen exchanger [58, 79, 87], and unlike other cell types [284, 285], do not appear to utilize Na^+/HCO_3^- dependent membrane transport pathways [117]. However, there are some indications that HCO_3^- transporters may be involved in pH regulation in chondrocytes under some circumstances; bicarbonate transporters have been reported in a chondrocyte cell line [115], in avian chondrocytes [87] and in surface zone articular chondrocytes [118]. The osmolarity of the medium buffered by both $NaHCO_3$ and HEPES (325 mOsm) was approximately 25 mOsm higher than the medium buffered by only HEPES (300 mOsm). Previous studies have shown a 25 mOsm difference in osmolarity to have an influence on cell proliferation and matrix synthesis [92, 283, 286]. From the results obtained from the osmolarity experiment, it is evident that the dramatic cell proliferation and tissue accumulation in the $NaHCO_3$-supplemented media is not solely due to increased osmolarity arising from sodium bicarbonate addition (Table 4.2). It is now important to investigate whether the mechanism of increased tissue growth arises from operating the bioreactor with optimum media pH, or if the presence of bicarbonate ions *per se* is required.

The results here demonstrate the importance of maintaining pH and providing adequate buffering against lactic acid induced acidification in cartilage tissue engineering. Briefly, we compared cartilage constructs engineered in our system supplemented with $NaHCO_3$ to other dynamic bioreactor systems, namely: (i) a mixed-flask bioreactor [221, 287], (ii) a rotating-vessel

bioreactor [74] and (iii) a perfusion bioreactor [217]. From the data reported, it is evident that our constructs accumulated significant amounts of ECM. We postulate this could be due to the continuous supply of medium (15 mL/day per construct), which might have resulted in a near-infinite supply of nutrients. Additionally, $NaHCO_3$ supplementation provided a slightly basic environment (Table 4.3 and Table 4.4), thus, maintaining the pH of the medium close to pH 7.0 for the most part [89, 115], which in turn might have helped regulate the pHi of the chondrocytes to a value close to pH 7.1 [79, 96], thus accounting for high GAG and collagen accumulation.

4.6 Conclusions

In conclusion, this study highlights the advantage of employing a continuous flow bioreactor in conjunction with appropriate buffers in adequate quantities for articular cartilage tissue engineering, thus promoting cell proliferation and increasing matrix accumulation.

Chapter 5

Study to Determine a Feasible Primary Construct for ECM Production

5.1 Relation to Overall Project

The results and discussions in Chapter 5 are an extension of Chapter 4. This study was designed to determine the best seeding technique to produce clinically relevant-sized constructs. The data in this section was presented at TERMIS Europe in 2011 and also published as a conference proceeding in Histology and Histopathology 2011 vol. 26 Supplement 1 [288]. I am the first author of this abstract and was involved in the design and execution of experiments, data analysis and in drafting the abstract. Denver Surrao and Dr. Stephan Waldman are co-authors of this abstract.

5.2 Introduction

Cartilage tissue engineering requires large cell numbers for construct formation, which is a major limitation. Our previous work demonstrated a continuous flow bioreactor, with $NaHCO_3$-supplemented media to improve cell proliferation and ECM deposition, by creating a near-infinite supply of nutrients and by buffering media. Therefore, the aim of this study was to use the above technique to produce clinical-sized constructs (> 1 cm^2) without causing donor site morbidity (~300mg with 2600 cells/mg).

5.3 Material and Methods

The different seeding techniques used to engineer constructs included: monolayer (20,000 cells or 666 cells/mm^2), pellet (200,000 cells), biopsy (5 mm diameter constructs) and minced (5 mm diameter biopsies cut into smaller pieces). The constructs were cultivated in Ham's F-12 media (without $NaHCO_3$ and HEPES and with 10 mM glucose and glutamine) (HyClone, Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS), 100 mg/mL ascorbate and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid). The cultures were grown in an incubator maintained at 37°C and 95% relative humidity supplied with 5% CO_2 : 95% atmospheric air. The culture medium (1 mL per filter) was changed every 48-72 hours, and fresh ascorbic acid was added with each media change. In the bioreactor, cultures were supplied with continuous flow of media further supplemented with 14 mM $NaHCO_3$ at a flow rate of 10 µL/min for 6 weeks. The flow rate corresponded to an average residence time of approximately 7 hours. After 6 weeks of culture, the tissue weight, thickness and ECM deposition were determined.

5.4 Results and Discussions

Monolayer constructs proved superior to all the other constructs investigated in this study, while minced and biopsy constructs recorded inconsistent data (Figure 5.1). Monolayer and pellet constructs recorded the following values: ***thickness:*** 2069 ± 90 and 1600 ± 47 µm, ***DNA:*** 199 ± 33 and 51.6 ± 17 µg/construct, ***GAG:*** 8908 ± 1089 and 3428 ± 458 µg/construct, and ***collagen:*** 2843 ± 150 and 1495 ± 272 µg/construct, respectively. This significant increase in monolayer ECM accumulation could be due to the combined effect of the bioreactor and $NaHCO_3$ supplemented media. Additionally, the large surface to volume ratio per cell in monolayer compared to the pellet construct (chondrocytes, though in larger numbers, were tightly packed

together) could have provided the cells greater accessibility to nutrients while allowing the chondrocytes to divide and synthesize/accumulate ECM in the monolayer without constraint.

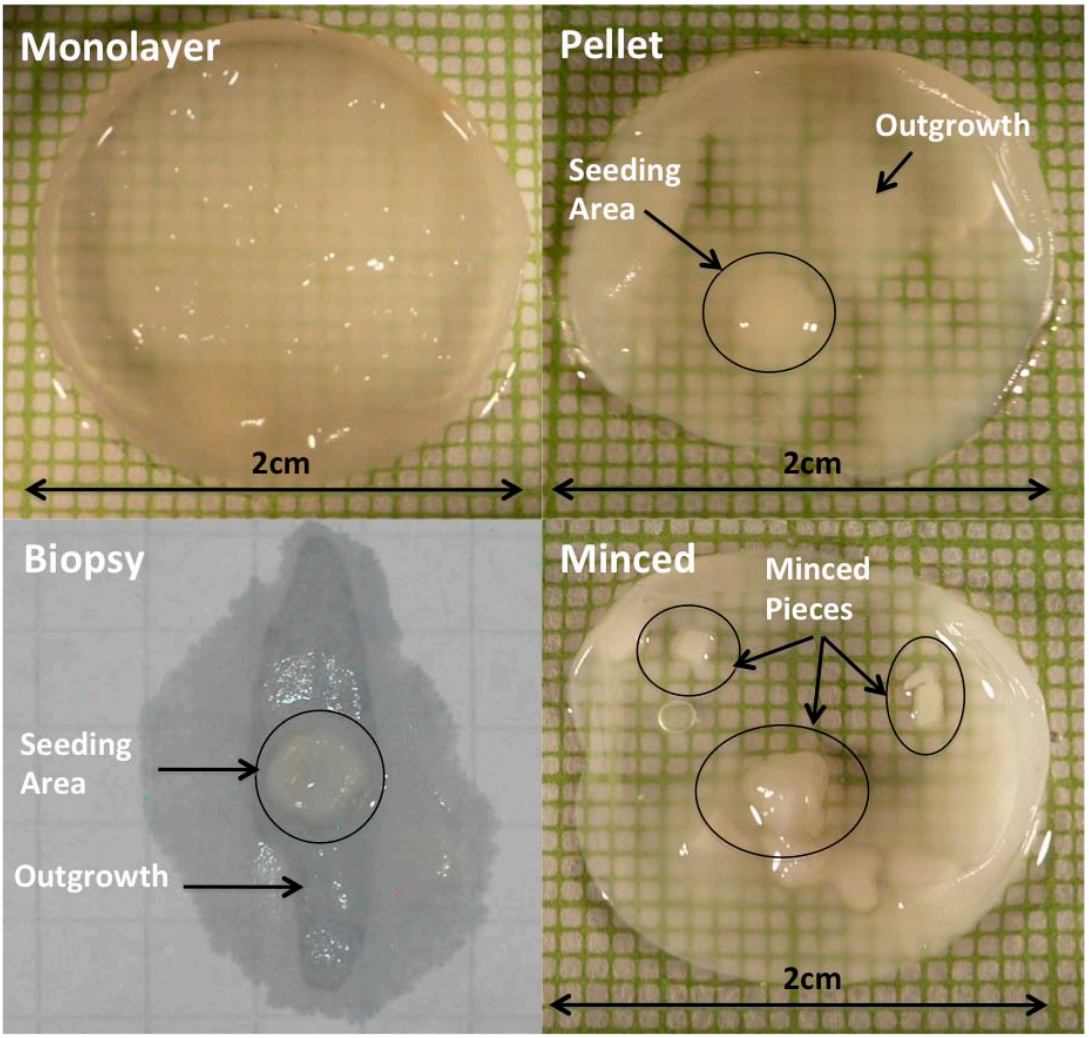

Figure 5.1: Pictorial representation of constructs after six weeks of culture in the bioreactor with $NaHCO_3$ supplemented media.

5.5 Conclusions

Of all the constructs studied, the monolayer constructs outperformed all the other constructs with respect to cell proliferation, biochemical properties and tissue thickness.

Chapter 6

Effects of Extra- and Intracellular Factors on Proliferation of Chondrocytes from Bovine Articular Cartilage

6.1 Relation to Overall Project

The results and discussions in Chapter 6 encompass Objective 3 (Section 2.3), which was to investigate the underlying reasons for the increased proliferation and tissue accumulation as a result of bioreactor cultivation and $NaHCO_3$ supplementation. This chapter will be submitted to the journal, *Osteoarthritis and Cartilage*. I will be the first author on this manuscript and was involved in the design and execution of experiments, data analysis and drafting the manuscript. Navneet Vasistha, Dr. Jill Urban and Dr. Robert Wilkins from the University of Oxford will be co-authors on this manuscript.

6.2 Introduction

Articular cartilage is a highly specialized tissue with a low ability to self-repair, making the treatment of chondral injuries difficult for scientists and surgeons [122]. Autologous Chondrocyte Implantation (ACI) is a cell based replacement technique generally used to treat chondral defects [125, 135, 160, 289]. In this technique, a biopsy sample is taken from a non-load bearing region of the patient's joint. The chondrocytes are expanded *in vitro* and then implanted back into the same patient. Currently, the only source of cells for this treatment are autologous chondrocytes [125, 135, 160, 289]. These cells are present in very low numbers in cartilage, making up only

2% of the total cartilage volume [1-5]. Hence, only a limited number of cells can be taken from the patient, as this leads to donor site morbidity. Due to limited availability of chondrocytes, it is important to establish reliable protocols, aimed at regenerative medicine and tissue-engineering applications. The establishment of reliable protocols requires a better understanding of chondrocyte physiology and behavior under changing physiological and biochemical conditions.

We have previously reported on a continuous flow bioreactor, designed to provide a continuous supply of nutrients, at a constant concentration, while removing the metabolic waste from around the cells [223]. We also have reported how the addition of sodium bicarbonate ($NaHCO_3$) increases the buffering capacity of the medium, influencing chondrocyte behavior [224]. We not only obtained a significant increase in tissue formation in the reactor with bicarbonate addition (a 10-fold increase in glycosaminoglycans and 6-fold increase in collagen compared to controls without bicarbonate addition), but surprisingly, we also obtained a significant increase in cell numbers, (a 6-fold increase compared to control at the end of 5 weeks of culture) [224]. The tissue obtained was tested immunohistochemically for collagen type II, a cartilage marker [119, 290], to determine if the chondrocytes maintained their chondrogenic phenotype in culture. The engineered tissue stained positive for collagen type II. Additionally, the chondrocytes in the flow system (with additional bicarbonate), from the primary construct (collagen-coated filter 8 mm in diameter) expanded and synthesized ECM, forming a secondary construct, 20 mm in diameter together with 8 mm diameter of the primary construct, without a separate expansion phase. We also tested other 3D-seeding techniques along with collagen-coated filters, such as pellet, biopsy, minced and 2D-seeding techniques, such as monolayer cultures, to see if we would obtain similar results [224, 288]. A simultaneous increase in cell proliferation and tissue formation was observed in all cultures when additional bicarbonate was present [224, 288]. The formation of native-tissue-like cartilage in 3D cultures reflected a simultaneous increase in cell proliferation

and matrix formation. In monolayer cultures, it seems that the initial 2D environment was quickly modified into a 3D spatial arrangement which prevented the cells from losing their chondrogenic phenotype.

It is generally believed that chondrocytes obtain most of their energy from the pathway of glycolysis, and hence, produce lactic acid at high rates [56], causing the environment to acidify. The rate of acidification not only depends on the rate of lactate production but also on the buffering power of the cells' surroundings. It has long been known that chondrocyte matrix synthesis rates, and rates of cell proliferation are very sensitive to extracellular pH (pHe) and fall steeply in a pH-dependent manner under acidic conditions [95-99, 106, 107, 225]. Acidic extracellular environments are known to influence the intracellular pH (pHi) of chondrocytes [96, 97]. The pH of cartilage extracellular matrix is between pH 6.9 and pH 7.1 [96]. Chondrocytes have acid-sensing ion channels present that can sense changes in pHe, causing them to respond to their environment [291, 292]. Low pHe causes an influx of H^+ ions into the cytoplasm of the chondrocytes, resulting in chronic acid-loading of the cytoplasm [96]. Intracellular pH in chondrocytes is primarily regulated by the Na^+/H^+ exchanger [79, 87, 88, 96, 97, 115, 118] or at times by HCO_3^- dependent transporter [118]. It is known that bovine articular chondrocytes have a resting pHi of 7.1 and any changes to this pHi have been shown to affect cell metabolism [97]. It has been shown that the reduction in extracellular matrix pH to pH 6.6 and below reduces energy metabolism in the cells [102, 103].

Cultured chondrocytes have been found to behave differently from freshly isolated chondrocytes. In comparison to freshly isolated chondrocytes, cultured chondrocytes may have a higher HCO_3^- transporter activity due to long exposure to compounds or environments that the cells are not normally used to in their native environment [88].

The level of carbonic anhydrase activity, which catalyzes the production of HCO_3^- from hydration of CO_2, has been reported to be low in human chondrocyte cell lines [115, 116]; however, the importance of carbonic anhydrase in chondrocytes remains unclear [118]. In the presence of HCO_3^-/ CO_2 the recovery rate of a human chondrocyte cell line from acidification has been shown to increase [115]. Various studies conducted on freshly isolated chondrocytes have however confirmed that pHi in chondrocytes is primarily regulated by the Na^+/H^+ exchanger, instead of the anion dependent system [79, 87, 88, 96, 97, 115, 118]. Chondrocytes from the superficial zone of (bovine) articular cartilage seem to be an exception in that they regulate pHi by the HCO_3^- dependent transporter [118].

Hence, the aim of this study was to determine the underlying reason for the increase in cell proliferation and extracellular matrix production, when supplementing the cell culture medium with 14 mM $NaHCO_3$ in a 5% CO_2 environment. We hypothesized, firstly, that the increase in matrix production arose mainly from the increase in cell proliferation. Secondly, that this increase in cell proliferation and matrix production was due to differences in buffering capacity between cultures supplemented with HEPES and $NaHCO_3$ under CO_2 in comparison to cultures supplemented with only HEPES under CO_2, and the consequent influences on pHe and pHi. We thus investigated the role of buffering capacity on cell proliferation by studying it in medium supplemented with HEPES and $NaHCO_3$ under 5% CO_2 and in medium supplemented with HEPES in air. Additionally, here we report on measuring pHi, using a confocal microscope, in chondrocytes in 2D culture (monolayer) and in 3D culture (alginate beads), without removal from their attached or encapsulated form to avoid artifacts, as far as possible.

6.3 Materials and Methods

6.3.1 Material

All chemicals and consumables were obtained from Sigma Aldrich Ltd., Poole, UK, unless noted otherwise (e.g. calcium chloride).

6.3.2 Cell Isolation and Culture

Chondrocytes were harvested from bovine (18-24 months old) metacarpal-phalangeal articular cartilage by sequential enzymatic digestion, as described previously [236]. The media used for the first two days of culture was Ham's F-12 (without $NaHCO_3$ and HEPES and with 10 mM glucose and glutamine) (HyClone, Logan, UT, USA) which was supplemented with 5% fetal bovine serum (FBS), 20 mM HEPES (*N*-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid], at a pH of 7.4 in air and a pH of 7.0 under 5% CO_2.

6.3.3 Growth Kinetics

The harvested chondrocytes were seeded in 25 cm^2 T-flasks at a seeding density of 6400 cells/cm^2. Starting at day 2, the media was increased to 10 mL, using Ham's F-12 media (without $NaHCO_3$ and HEPES and with 10 mM glucose and glutamine) supplemented with 20% fetal bovine serum (FBS) and 100 mg/mL ascorbate. Additionally, starting at day 2, the culture medium was replaced every 24 hours, for six consecutive days. At the end of each day, the cells in the flasks were washed with Ham's F-12 media and were incubated with 2 mL of 0.15% collagenase A (w/v) for 30 minutes at 37°C. The collagenase solution was replaced with 1 mL of

0.25% trypsin, and the cells were incubated for another 5 minutes at 37°C. After the trypsin was removed, the flasks were washed with 2 mL of Ham's F-12 media to make sure all the cells were collected and checked under the microscope to make sure all the cells had been removed. The media with the cells were centrifuged at 2000 rpm for 5 minutes. The media was aspirated, and the cells were re-suspended in 0.2 mL to 5 mL media. 1 part cell suspension was mixed with 1 part trypan blue, and the cells were counted using a hemocytometer.

6.3.4 Measurement of Extracellular pH and Lactate Production

The pHe of the media in the T-flask was measured with a pH micro-probe immediately after removal from the incubator. The pH of the media supplemented with $NaHCO_3$ was measured under a constant supply of 5% CO_2, as gaseous exchange can cause a pH drift. The media was also collected from each condition in the T-flask to measure the total lactate production at the end of a 24-hour day. Lactate in the spent media was determined by measuring its absorbance against a standard curve generated from different concentrations (0-4 mM) of lactic acid (Trinity Biotech, Ireland). The absorbance was measured using a plate reader, at a wavelength of 540 nm.

6.3.5 Reagents used to Measure Intracellular pH

The solutions used to measure pHi were made as follows: HEPES buffered solution (HBS), Bicarbonate and HEPES buffered solution (BHBS), and calibration solution [115]. All these solutions contained the following chemicals: 1 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM D-glucose. The pH was adjusted with 5 N NaOH and 5 N HCl. Additionally, the HEPES buffered solution (HBS) with a pH of 7.3 also contained 145 mM NaCl and 5 mM KCl. The

bicarbonate and HEPES buffered solution (BHBS) with a pH of 7.3 also contained: 131 mM NaCl, 5 mM KCl, and 14 mM $NaHCO_3$ with the pH adjusted to 7.3 with 5% CO_2 and 95% air. The calibration solution (high in K^+ ions) also contained 15 mM NaCl and 135 mM KCl. The ammonium pre-pulse solution was the same as above, with the exception that 20 mM NH_4Cl was added, and NaCl concentration was adjusted to keep the final osmolarity of all the solutions the same. A 5 mM stock solution of the membrane dye (permeant acetoxymethyl ester of carboxy SNARF-1-AM) (Invitrogen Ltd., Paisley, UK) was prepared in dimethyl sulfoxide (DMSO), a 3 mM stock solution of nigericin was prepared in ethanol, and aliquots of both were stored at -20°C until required.

6.3.6 Measurement of Intracellular pH in 2D Cultures (Monolayer)

The harvested chondrocytes were seeded on 1.8 cm^2 Lab-Tek coverglass chambers at a seeding density of 6400 cells/cm^2. Starting at day 2, the media was increased to 1 mL using Ham's F-12 medium (without $NaHCO_3$ and HEPES and with 10 mM glucose and glutamine) supplemented with 20% fetal bovine serum (FBS) and 100 mg/mL ascorbate. Additionally, starting at day 2, the culture medium was replaced every 24 hours, for six consecutive days. The pHi of chondrocytes growing in monolayer (2D cultures) was measured on days 3, 5, 6 and 7. The monolayer cultures were washed twice with a HEPES buffered solution (HBS) and then incubated with 10 μM SNARF-1-AM for 20 minutes at 37°C [293-295]. After 20 minutes, the SNARF-1-AM was aspirated, and the monolayer cultures were washed twice with either a HEPES buffered solution (HBS) or a bicarbonate and HEPES buffered solution (BHBS). The BHBS cells were measured under 5% CO_2. The confocal chamber was continuously gassed via a small hole in the lid, large enough to allow a 21G needle to enter, with 5% CO_2 and 95% air via a male connector and a gas

impermeable tube. It is very important that these measurements are taken under 5% CO_2 and 95% air in order to get stable and reliable pHi readings, while maintaining a pHe at 7.3. The confocal chamber was at 37°C. The HBS cells were measured in air with a pHe of 7.3.

Carboxy-SNARF-1 dye was excited by an argon laser at 514 nm and its fluorescence emission was collected ratiometrically at 580 ± 20 and 640 ± 20 nm [293, 294]. The cells were imaged using a Zeiss 710 confocal system (Carl Zeiss Ltd, Cambridge, UK). The ratio of the fluorescence intensity at the two wavelengths was converted to a pH value using a high K^+ nigericin calibration curve method [296]. The ratiometric measurement is very reliable, as it gives a value independent of the dye concentration and intensity losses caused by photobleaching [107, 297]. The $NaHCO_3$-CO_2 measurements were done under a 5% CO_2 stream.

While measuring pHi values, care was taken to minimize background auto fluorescence from the cell-free media or ECM. Additionally, any auto fluorescence exhibited was subtracted from the fluorescence exhibited by the cells. Note that dead/dying cells do not retain the fluorescent dye and would therefore, not be included in the fluorescence measurements.

6.3.7 Measurement of Intracellular pH in 3D Cultures (Alginate Beads)

The harvest chondrocytes were encapsulated in low viscosity alginate beads at a cell density of 4 x 10^6 cell/mL [62, 298]. The 1.2 w/v alginate solution was prepared by dissolving it in a sodium chloride (0.9% w/v) solution and sterile filtering it through a 0.22 μm syringe filter. The harvested chondrocytes were re-suspended in the alginate solution and were carefully added in a drop-wise fashion, using a 21G needle to a 102 mM $CaCl_2$ solution. The $CaCl_2$ helped crosslink the alginate beads, thereby encapsulating the chondrocytes within the beads. The excess $CaCl_2$ was removed, and the alginate beads were washed thrice with Ham's F-12 media containing 5%

FBS. The 3D cultures were grown in an incubator maintained at 37°C and 95% relative humidity supplied with 5% CO_2 : 95% atmospheric air for 2 days. The alginate beads were transferred from the 12 well-plate to a confocal chamber. The alginate beads were incubated with 10 μM SNARF-1-AM for 30 minutes at 37°C to ensure that the centre of the beads was equilibrated with the dye [293-295]. The pHi of the chondrocytes encapsulated in the alginate beads, was measured, as described above.

6.3.8 Calibration

The calibration curve was generated using a well-established nigericin-high K^+ method [296]. The curve was used to calibrate the intracellular fluorescence ratio of SNARF-1 to pHi of *in vitro* chondrocytes. Nigericin is a K^+/H^+ ionophore, which can be used to calibrate a standard curve to convert fluorescence to pH as nigericin equalizes pHi with pHe when K^+ concentration inside and outside the cell is the same. In the absence of a K^+ gradient across the cell membrane, nigericin equilibrates internal and external pH by exchanging internal K^+ for external H^+. Freshly isolated chondrocytes, rather than chondrocytes *in situ*, were used for calibration, primarily because the cartilage ECM hindered accessibility of chondrocytes to the nigericin molecule (results not presented). This observation was also noted by Simpkin *et al*., [118] in chondrocytes, *in situ*. To carry out this procedure, cells that had been seeded in flasks (< 8 days) were enzymatically digested for 30 minutes with collagenase, as mentioned above in section: Growth Kinetics. 150 μL of the isolated cell suspension in HBS solution supplemented with 10 μM SNARF-1-AM was allowed to settle in a confocal chamber for 20 minutes at 37°C. Excess SNARF-1 in the media was washed away, and the cells in the chamber were washed twice with HBS, and finally, with the calibration solution (at the appropriate pH). 3 mM stock solution of nigericin was added to the

calibration media to give a final concentration 6 μM. The pHe range of the calibration solution was from pH 6.5 to pH 8.0. A good correlation was achieved between the fluorescence ratio and the pHe in this range ($r^2 \geq 0.97$) (Figure 1). The confocal measurements were made as mentioned previously.

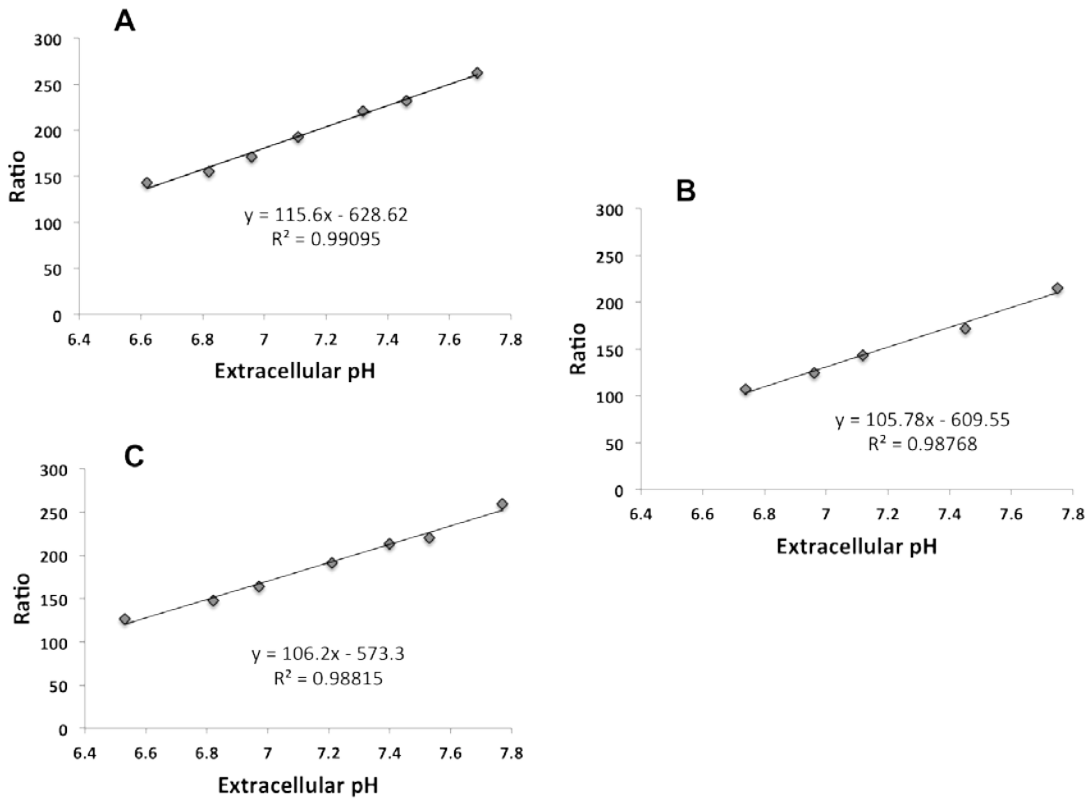

Figure 6.1: Calibration curves **A**, **B** and **C** over the range pH 6.5 and pH 7.8 has a linear relationship between the ratio of the fluorescence intensities of SNARF-1 at the two wavelengths and extracellular pH of the solution. A nigericin molecule in the absence of a K^+ gradient across the cell membrane equilibrates internal pH of the cell with the external pH of the solution.

6.3.9 Measurement of Buffering Capacity

The buffering capacity of the cells in HEPES-air, and the $NaHCO_3$-CO_2 condition at pHe 7.3 were determined by using the ammonium pre-pulse titration technique [299].

The confocal chamber was perfused by making two holes in the lid, one for injecting fresh liquid, and the other to remove spent media. The holes were large enough to insert a 21G needle (Figure 6.2). A perfusion system was used for this experiment, primarily to maintain a steady state environment in the chamber while imaging, and to record changes occurring within the cells, using different solutions over a period of time. The lid on the BHBS media was closed under 5% CO_2 to maintain CO_2 equilibrium while the solution dripped from a needle into the chamber. The needles were adjusted in the chamber to allow continuous flow of media with 0.5 mL media in the chamber at all times. The media bottles were placed in a 37°C H_2O bath and fitted with a cap, which was penetrated by a non-gas permeable tube, placed in liquid. This tube was connected to a peristaltic pump (Gilson Minipuls 3, Anachem, Luton, UK) that pumped media at a flow rate of 1 mL/minute. The outlet tube was connected to another peristaltic pump (Gilson Minipuls 3, Anachem, Luton, UK) that was set at a higher flow rate (1.5 mL/minute) to remove media from the well. The outlet needle was placed slightly higher than the inlet needle to prevent the well from drying out. The media with BHBS, with and without NH_4Cl, was continuously gassed with 5% CO_2 and 95% air, via a tube placed in the media bottle. Once the needles were set, the pumps were started, making sure the volume in the well was maintained. An area of interest was identified, with at least 15 cells outlined, and the change in ratio with time was monitored. The confocal measurements of the cells were performed as explained in the *Measurement of pHi in 2D Cultures* section. Emission was collected at 580 ± 20 and 640 ± 20 nm simultaneously in an XY scanning mode, once every 15.5 seconds. Ratio of the fluorescence intensity at the two wavelengths was measured and converted to a pH value using the calibration curve. The

background fluorescence was offset for all measurements. HEPES-air or $NaHCO_3$-CO_2, chondrocytes were super-fused with either HBS or BHBS (continuously gassed with 5% CO_2) for 3 minutes, to stabilize the chondrocyte pHi, and then the perfusion was switched to NH_4Cl solution for another 6 minutes to alkalise the cells and then switched back to an HBS or BHBS solution to acidify the cells.

The intrinsic buffering capacity (B_I) at pHe 7.3 was measured in HEPES-air cells, and the total buffering capacity (B_T) at pHe 7.3 was measured using the $NaHCO_3$-CO_2 cells. Buffering capacity was measured by change in pHi (ΔpHi) in SNARF-1-AM loaded chondrocytes after the addition of NH_4Cl [299]. The following equation was used to determine buffering capacity.

$$B = \frac{\Delta [NH_4^+]_i}{[\Delta\, pH_i]}$$

$$\Delta [NH_4^+] = \frac{total\,base\,concentration * 10^{(pHo - pHi)}}{1 + 10^{(pHo - pKa)}}$$

where: 'B' is intrinsic (B_I) or total buffering capacity (B_T); delta pHi is the increase in intracellular pH after the addition of NH_4Cl (it is corrected to the point of NH_4Cl addition) [299]; pH_o is the pH of the external solution (pH 7.3; in this instance); the total base concentration was 20 mM, and the pka of NH_4^+ at 37°C is 9.21 [299].

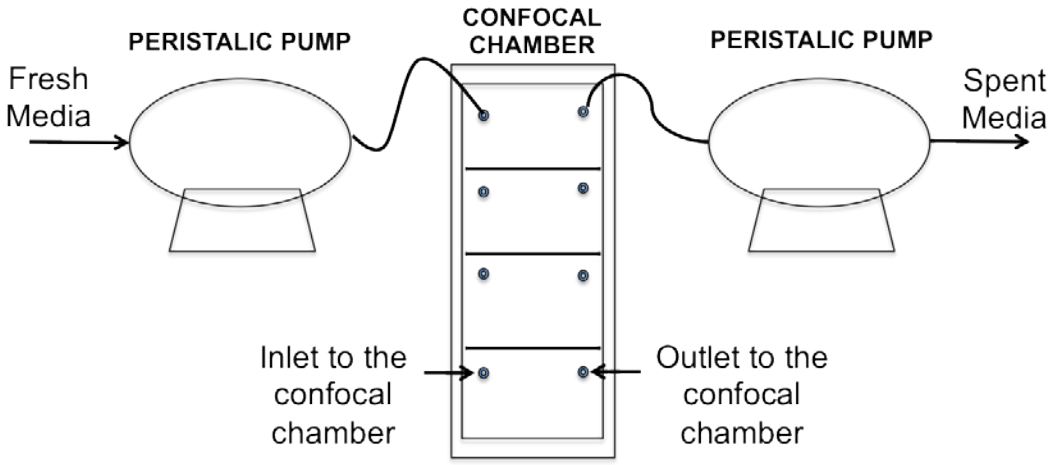

Figure 6.2: Schematic representation of a confocal perfusion system. Cells were seeded in the confocal chamber. During imaging, the solution was perfused in the chamber via a peristaltic pump at a flow rate of 1 ml/min, and the solution was removed from the chamber via a peristaltic pump at a flow rate of 1.5 ml/min. The tube to remove the media from the chamber was adjusted at a height to allow approximately 0.5 ml media in the chamber at all times.

6.3.10 Data Analysis

At least three independent cultures were carried out for each of the experiments outlined in this study. All numerical results expressed were either as ratio or mean ± standard error of the mean (SEM). Independent experiments were conducted with independent cell extractions. Data was compared between each of the experimental groups using a t-test or a one-way ANOVA and the Fisher's LSD post-hoc test (SPSS 18.0, SPSS Inc., Chicago, IL, USA). Significance was associated with *p*-values less than 0.01 or 0.05. It is important to note that the cells used in this study were primary bovine chondrocytes. As mentioned previously, these cells have been documented to possess animal-animal variability [71] in metabolic rates and in proliferation rates. Hence, although within one experiment standard deviations were small, averaging the absolute data from different experiments conducted under the same conditions gave very high standard

errors (Figure 6.3A and Table 6.1). Although the trends seen in each of the experiments were the same, when results were presented as fractional changes, they exhibited significant differences between different experimental conditions (Figure 6.4). Additionally, while measuring pHi values, care was taken to minimize background auto-fluorescence from the cell-free media or extracellular matrix.

Table 6.1: Change in chondrocyte doubling time (hours) in media, with 20 mM HEPES and 14 mM $NaHCO_3$ in CO_2 in comparison with 35 mM HEPES in air, over a period of six days.

	Experiment 1		**Experiment 2**		**Experiment 3**		**Mean of Experiment**	
Day	HEPES/ bicarb	HEPES	HEPES/ bicarb	HEPES	HEPES/ bicarb	HEPES	HEPES/ bicarb	HEPES
2 to 3	11		15		18		15 ± 2	
3 to 4	12	38	16	24	21	50	16 ± 3	37 ± 7
4 to 5	27	19	27	30	25	99	26 ± 1	49 ± 25
5 to 6	24	22	36	26	26	144	29 ± 4	64 ± 40
Data are presented as mean ± SEM								

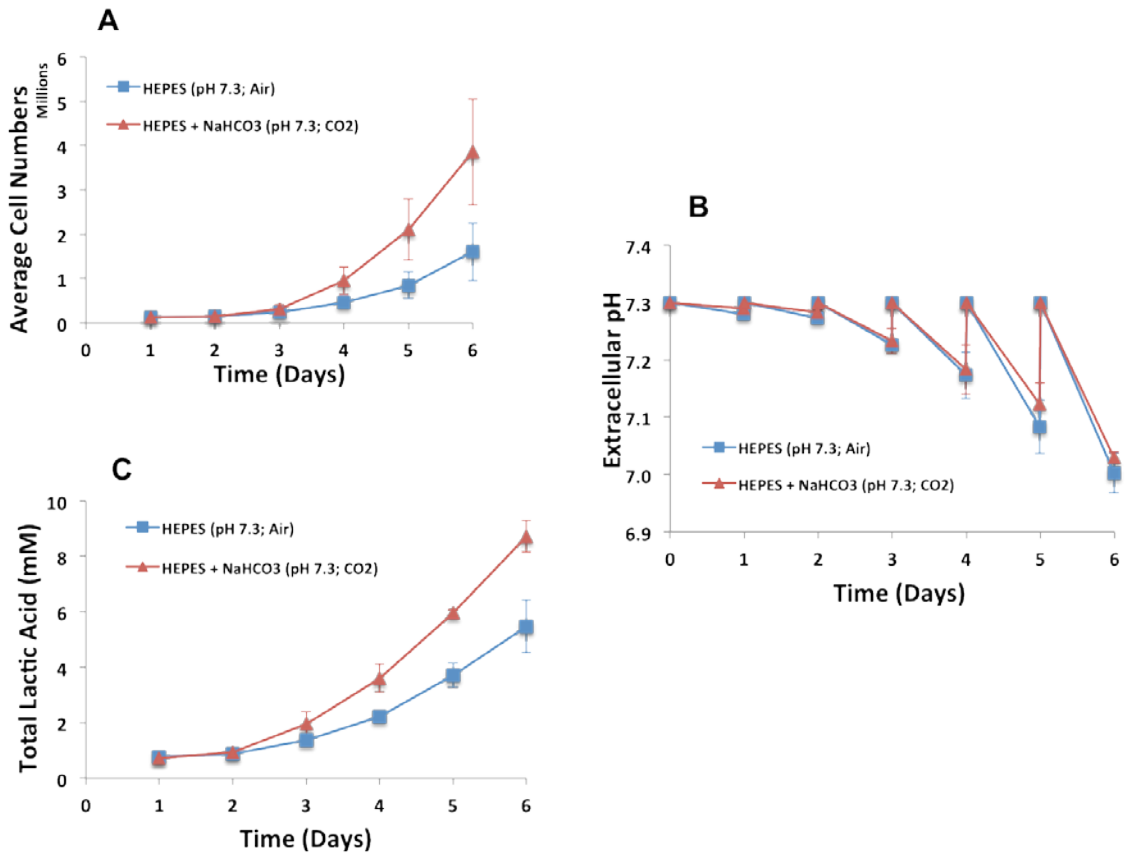

Figure 6.3: Effect of 20 mM HEPES and 14 mM $NaHCO_3$ in 5% CO_2, and 35 mM HEPES in air on cell proliferation, lactate production, and extracellular pH of the media. **(A)**: Average cell numbers every 24 hours post media change. **(B)**: Change in extracellular pH every 24 hours post media change **(C)** Total lactate produced and every 24 hours.

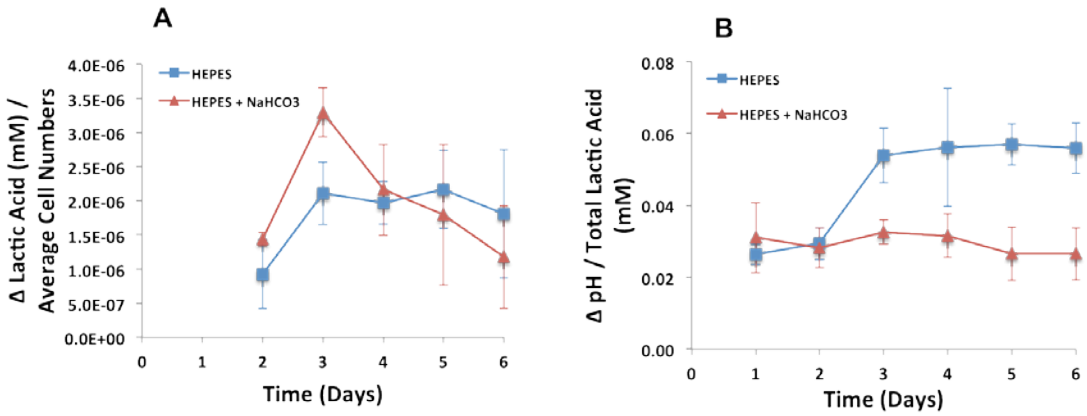

Figure 6.4: Effect of 20 mM HEPES and 14 mM $NaHCO_3$ in 5% CO_2, and 35 mM HEPES in air on cell proliferation, lactate production, and extracellular pH of the media. **(A)** Change in lactate production per million cells every 24 hours **(B)** Change in pH per total lactate production every 24 hours.

6.4 Results

6.4.1 Buffering Capacity of the Media

The media buffering capacity was measured for the two solutions, 35 mM HEPES in air (HEPES-air), and 20 mM HEPES and 14 mM $NaHCO_3$ under 5% CO_2 ($NaHCO_3$-CO_2) at 37°C, by adding known volumes of 1 N lactic acid to the solution and allowing it to equilibrate for a few minutes before recording the pH of the solution (Figure 6.5), which was continuously stirred to allow mixing. The buffering capacity of the HEPES-air media was experimentally calculated to be 20 mmol/L/pH unit while that of $NaHCO_3$-CO_2 media was experimentally calculated to be 50 mmol/L/pH unit (Figure 6.5). The buffering capacity of the $NaHCO_3$-CO_2 media was more than double that of HEPES-air media.

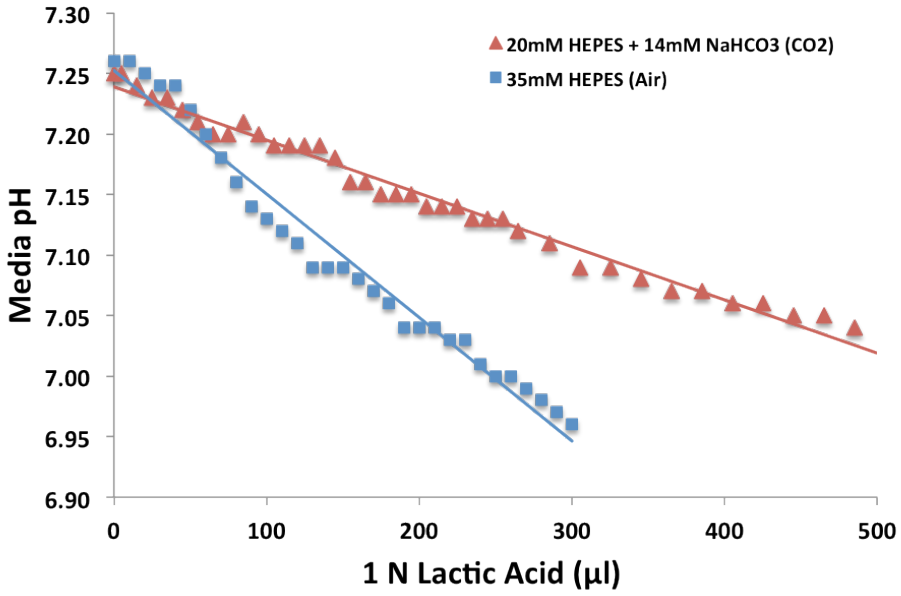

Figure 6.5: Determination of buffering power of the media; 35 mM HEPES in air and 20 mM HEPES and 14 mM $NaHCO_3$ in 5% CO_2 media. Known volume of 1 N lactic acid was added to the media drop-wise, and the pH was recorded after allowing a few minutes of equilibration. The buffering power of the media was determined from the slope of the line.

CELL PROLIFERATION STUDIES IN T-FLASKS

6.4.2 Effect of $NaHCO_3$, CO_2 and Air on Cell Proliferation

This experiment was designed to determine if cells seeded in monolayer at the same initial pHe proliferated at the same rate in media exposed to $NaHCO_3$ and CO_2 as compared to HEPES and air.

First, cells seeded in T-flasks were placed in a 5% CO_2 incubator for 2 days and then were counted; this count was taken as the day 0 count. After the initial two days in the CO_2 incubator, the remaining T-flasks were divided into two batches. The first batch of T-flasks (monolayer

cultures) were fed with $NaHCO_3$-CO_2 and placed in a 5% CO_2 incubator. The second batch of T-flasks were fed with 35 mM HEPES and placed in an air incubator, void of CO_2. The media, for both these conditions had a starting pH of 7.3 and an osmolarity of 325 mOSm (iso-osmolarity obtained by mannitol).

In all the experiments conducted, cultures grown in $NaHCO_3$-CO_2 environment proliferated much more than the cultures grown in HEPES-air environment (Figure 6.3A and Figure 6.6). Furthermore, cell numbers from the $NaHCO_3$-CO_2 environment were significantly *($p < 0.05$)* higher from day 4 onwards, compared to the cell numbers obtained from culture grown in a HEPES-air environment (Figure 6.3A and Figure 6.6).

It is important to note that the cells used in this study were primary bovine chondrocytes. As mentioned previously, these cells have been documented to possess batch-to-batch variability, and therefore, exhibit different proliferation rates. If results from all six experiments were averaged (Figure 6.6), the resulting standard deviation showed no significant difference *($p < 0.05$)* between the two conditions tested ($NaHCO_3$-CO_2 and HEPES-air), even though the cells grown in a $NaHCO_3$-CO_2 environment proliferated substantially better than cells grown in a HEPES-air environment in each of the six different experiments (Figure 6.6).

Additionally, the pH of the media was measured after every 24 hours for both the conditions (Figure 6.3B). The pH drop of the media 24 hours post media change on day 5 and day 6 was less for $NaHCO_3$-CO_2 than pH for the HEPES-air (Figure 6.3B) possibly because of the higher buffering capacity tested for the $NaHCO_3$-CO_2 media (Figure 6.5). Additionally, lactate production was also measured during the culture period for both conditions (Figure 6.3C). Chondrocytes growing in $NaHCO_3$-CO_2 produced more lactate (total production of lactate), compared to chondrocytes grown in HEPES-air (Figure 6.3C) except on day 3 where there was

no significant difference *(p< 0.05)* between the two conditions for lactate production per million cells (Figure 6.4A), while there was a significant difference *(p< 0.05)* in change in pH per total lactate produced (Figure 6.4B). This increase in lactate production per million cells on day 3 coincided with the fastest doubling times in $NaHCO_3$-CO_2 (Table 6.1). The rates/cell then fell again as doubling times increased on days 4 and 6 for HEPES-air cells, while the rates/cell from days 3-6 remained more or less constant (Figure 6.4A Table 6.1). Once again, as mentioned above, the difference in change in pH per total lactate produced between the two conditions could be due to the buffering power of the media (Figure 6.5).

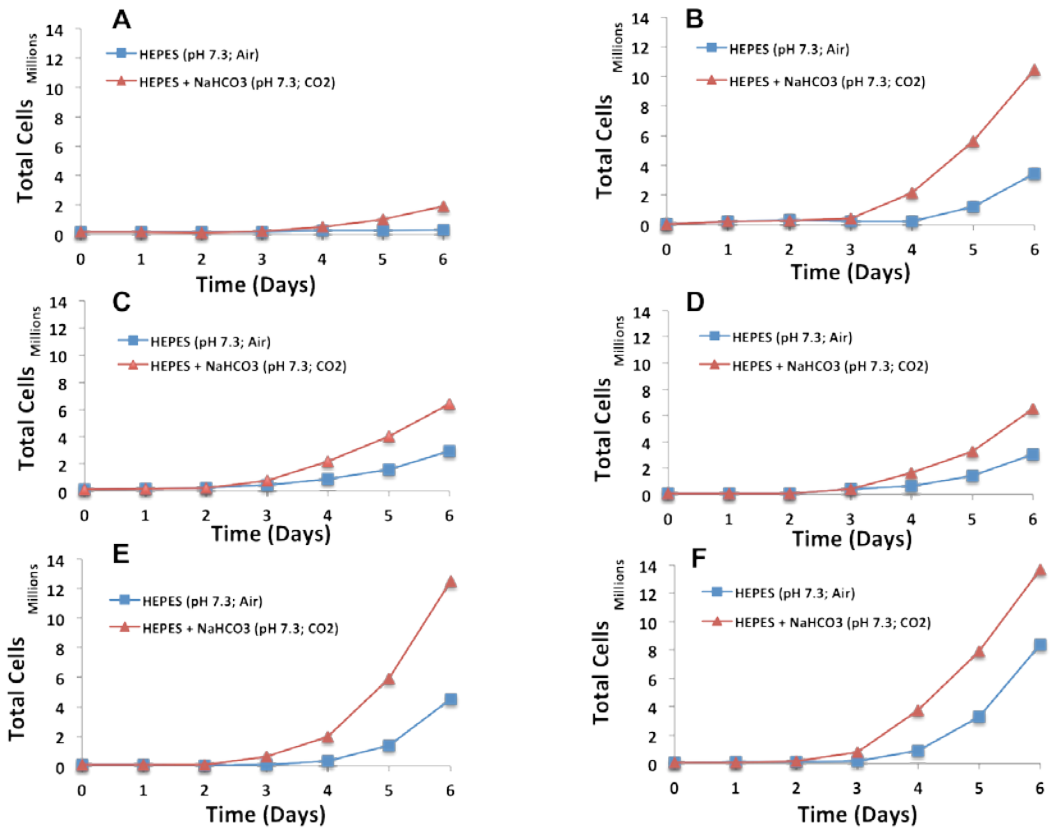

Figure 6.6: Effects on cell proliferation of batch variation in 20 mM HEPES and 14 mM $NaHCO_3$ in 5% CO_2 and 35 mM HEPES in air. Due to the large batch-to-batch variation in the rate of proliferation (**A**, **B**, **C**, **D**, **E** and **F**), common in biological systems, the cell numbers were not pooled together, but the graph accurately depicts the general trend observed in the multiple experiments conducted.

6.4.3 Effect of Extracellular pH on Cellular Proliferation in Solutions Buffered by HEPES, $NaHCO_3$ and CO_2 Compared to Those Buffered by HEPES Alone

After observing that chondrocytes grown in a $NaHCO_3$-CO_2 environment proliferated significantly better than chondrocytes grown in a HEPES-air environment, it was important to determine if this increase in proliferation arose because of differences in pHe arising as a result of buffering power (Figure 6.5).

To determine the role of pHe on cell proliferation in $NaHCO_3$ and CO_2 cultures, we adjusted the pHe of the media to be pH 7.0 and pH 7.3 under 5% CO_2, respectively. Media supplemented with HEPES-air with a starting pH of 7.3 was used as a control. Similarly, increased cellular proliferation trends, as observed in Figure 6.6 and Figure 6.3, were exhibited by both the $NaHCO_3$ and CO_2 cultures at pH 7.0 and pH 7.3, while cultures without $NaHCO_3$ and CO_2 (control) exhibited lower proliferation rates (Figure 6.7A). Furthermore, cell numbers were much higher starting on day 4 in the $NaHCO_3$ cultures (Figure 6.7A). Interestingly, the pHe of the $NaHCO_3$ and CO_2 culture with a starting pH of 7.0 did not drastically affect cell proliferation, even though the pH dropped to pH 6.7 on day 6 (Figure 6.7B). On the other hand, the pHe of the $NaHCO_3$-CO_2 culture with a starting pH 7.3, dropped to pH 7.0 at the end of day 6 (Figure 6.7B).

Additionally, we examined cultures grown in HEPES-air environment, which had a starting pH of 7.0, pH 7.2 and pH 7.4, respectively. Cultures with a starting pH of 7.2 and pH 7.4, proliferated at a similar rate and exhibited a significant *($p < 0.05$)* increase in cell numbers starting on day 4 compared to cultures with a starting pH of 7.0 (Figure 6.8A). Additionally, cultures with a starting pH of 7.2 and pH 7.4 had a significantly *($p < 0.05$)* higher change in pHe starting on day 4 than in cultures with a starting pH of 7.0 (Figure 6.8B). However, under all conditions, proliferation was slower than in media buffered with $NaHCO_3$ and CO_2. We also tested 35 mM MOPS at a starting pH of 7.2 in air as a buffer and results were similar to those of HEPES-air at a

starting pH of 7.2 in air (data not presented). This showed that HEPES itself was not responsible for the relatively low rate of proliferation relative to the rate of proliferation in $NaHCO_3$-CO_2 buffered media.

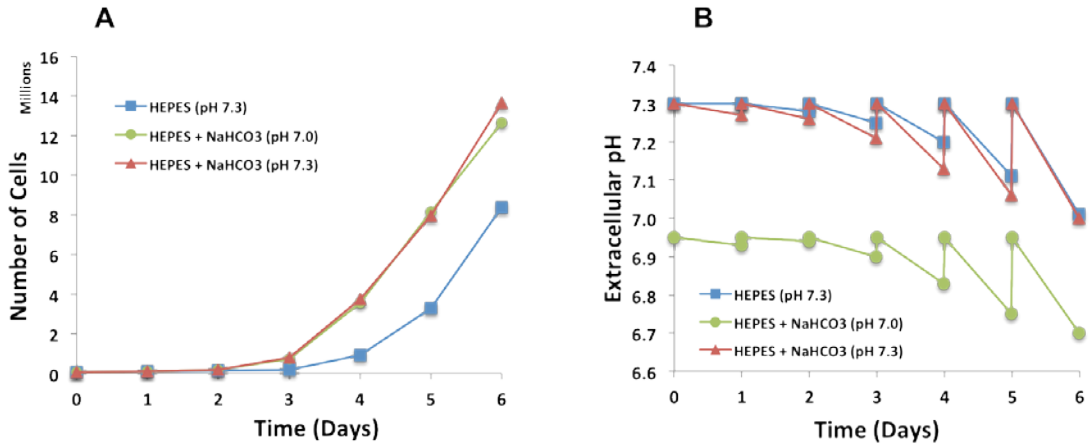

Figure 6.7: Effect on cell proliferation of varying extracellular pH in cultures with HEPES and $NaHCO_3$ in CO_2 and subsequent changes in pHe 24 hours post media change. **(A)**: Cell proliferation in the culture medium with HEPES and $NaHCO_3$ in CO_2 at a starting pH of 7.0 and pH 7.3, and in media supplemented with HEPES in air at a starting pH of 7.3, 24 hours post media change. **(B)**: Change in extracellular pH of the culture medium with HEPES and $NaHCO_3$ in CO_2 at a starting pH of 7.0 and pH 7.3 and in media supplemented with HEPES in air at a starting pH of 7.3, 24 hours post media change.

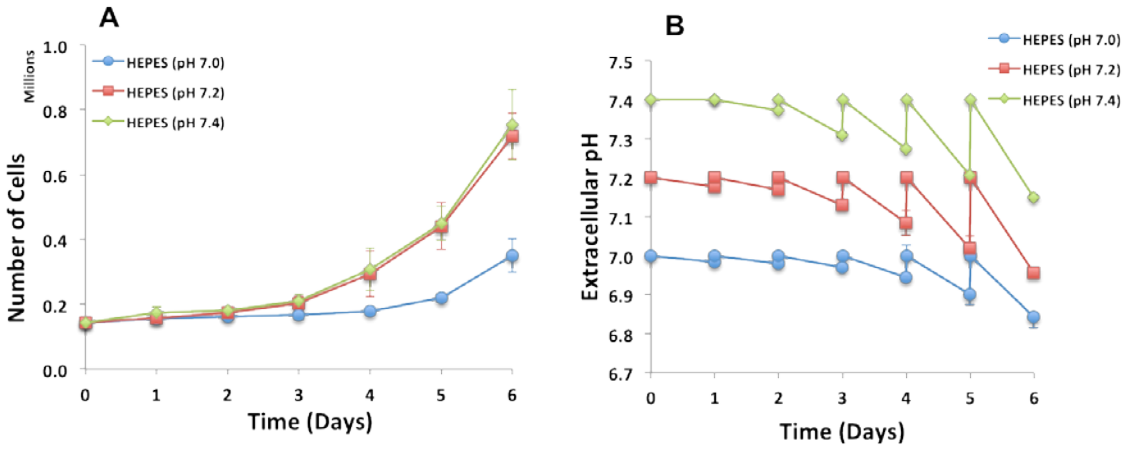

Figure 6.8: Effect on cell proliferation of varying extracellular pH in cultures with HEPES in air, and subsequent changes in pHe 24 hours post media change. **(A)**: Cell proliferation in culture medium 24 hours post media change in media with HEPES in air at a starting pH of 7.0, pH 7.2, and pH 7.4. **(B)**: Change in extracellular pH of the culture medium 24 hours post media change in media with HEPES in air at a starting pH of 7.0, pH 7.2, and pH 7.4.

6.4.4 Lag Phase in Cultures

This study was designed to determine, first, if the chondrocytes growing in a 35 mM HEPES-air condition experienced a longer lag phase due to the absence of $NaHCO_3$ and CO_2 during the initial phase of their culture. Bovine chondrocytes seeded in T-flasks were placed in 5% CO_2 and air incubators, respectively, for 2 days. After this, the cells in the T-flasks from both the incubators were counted and was considered as the day 0 count.

T-flasks in the CO_2 incubator were then divided into two batches. The first batch of T-flasks remained in the CO_2 incubator, under the $NaHCO_3$-CO_2 condition. The second batch of T-flasks were moved to the air incubator, and the media in these T-flasks were replaced with media supplemented with HEPES and were placed in an air environment. Similarly, the T-flasks in the

air incubator were moved to the CO_2 incubator, and the media in these T-flasks were replaced with media supplemented with $NaHCO_3$ and placed in a CO_2 environment.

The results obtained show no lag phase introduced in the culture on account of moving the cultures between the two environments (CO_2 and air) (Figure 6.9A). The cultures moved from air (HEPES-air media) to the CO_2 incubator ($NaHCO_3$-CO_2 media) exhibited an increase in cell numbers (Figure 9A) similar to the increase exhibited by cultures that were grown in the $NaHCO_3$-CO_2 media housed in the CO_2 incubator right from the start. Similarly, cultures that were moved from the CO_2 ($NaHCO_3$-CO_2 media) to the air incubator (HEPES-air media) exhibited a proliferation rate that was similar to that exhibited by cultures that were grown in the HEPES-air media, housed in an air incubator right from the start (Figure 6.9A). Additionally, the change in pHe in the cultures moved from the air (HEPES-air media) to the CO_2 incubator ($NaHCO_3$-CO_2 media) and the ones that remained in the CO_2 incubator ($NaHCO_3$-CO_2 media) was substantially higher starting on day 4 than the cultures moved from CO_2 ($NaHCO_3$-CO_2 media) to the air incubator (HEPES-air) and the ones that remained in the air incubator (Figure 6.9B).

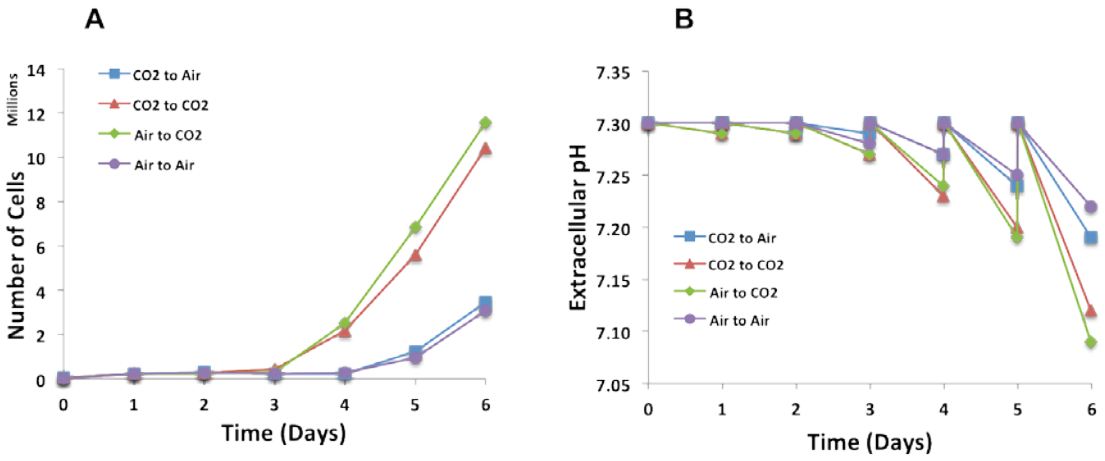

Figure 6.9: Media (20 mM HEPES and 14 mM $NaHCO_3$, and 35 mM HEPES) and environmental conditions (5% CO_2 and air) were exchanged between the cultures to determine if a lag phase existed. **(A)**: Cell proliferation in culture medium 24 hours post media change. **(B)**: Change in extracellular pH of the culture medium 24 hours post media change.

6.4.5 pH Gradient in Cultures

In this study, determining, whether the media in the cultures (T-flasks) exhibited a pH gradient was our primary goal; if that was the case, the pH measured at the end of each culture period would not reflect the same extracellular pH. Cultures grown in a $NaHCO_3$-CO_2 and a HEPES-air media were thus placed in 5% CO_2 and in air, respectively, without mixing, where these T-flasks served as controls. Additionally, cultures grown in a $NaHCO_3$-CO_2 and a HEPES-air media, were placed on shakers, housed in a 5% CO_2 and in an air incubator, respectively. Starting day 0 until day 6, these T-flasks were gently shaken to encourage homogeneous mixing, thereby disrupting any pH gradient that might exist. At the end of each day, the cells were trypsinized and counted, while the media in the other remaining flasks were replaced. Chondrocytes exhibited similar rates

of proliferation under both static and constant mixing conditions (Figure 6.10A) with the rate governed by the buffering conditions, rather than by the degree of mixing (Figure 6.10B).

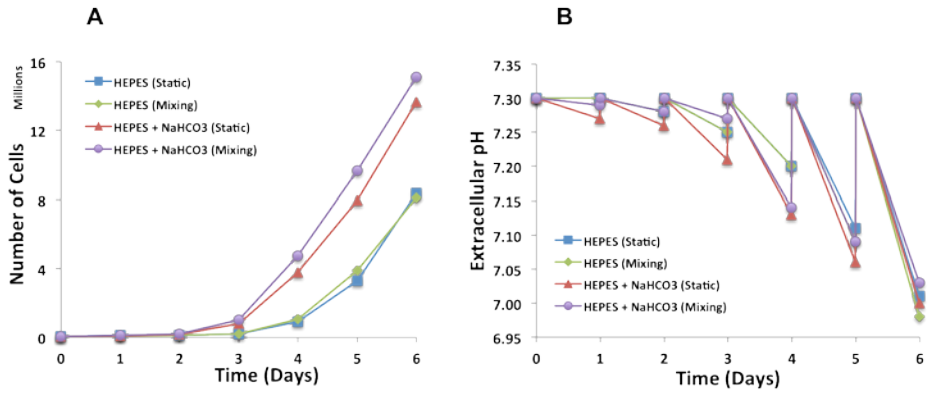

Figure 6.10: Cultures grown in 20 mM HEPES and 14 mM $NaHCO_3$ in CO_2 or HEPES in air, were either mixed or remained static to determine if a pH gradient was present in the cultures effecting cell proliferation. **(A):** Cell proliferation in culture medium 24 hours post media change. **(B):** Change in extracellular pH of the culture medium 24 hours post media change.

INFLUENCE OF CULTURE CONDITIONS ON INTRACELLULAR pH OF PROLIFERATING CHONDROCYTES

We measured pHi of chondrocytes using the dye SNARF-1-AM (acetoxymethyl) that diffuses inside the cell as the acetoxymethyl ester and is trapped there [293]. Intracellular pH was measured while isolated in solution, attached in a monolayer culture, and encapsulated in an alginate bead in culture. Figure 6.11 shows SNARF-1 trapped inside the cells in all three-culture conditions mentioned above (isolated, monolayer and alginate bead) and also shows the overlay of the images at the two wavelengths, eliminating the possibility of a ratiometric errors caused by movement (Figure 6.11C, Figure 6.11F and Figure 6.11I).

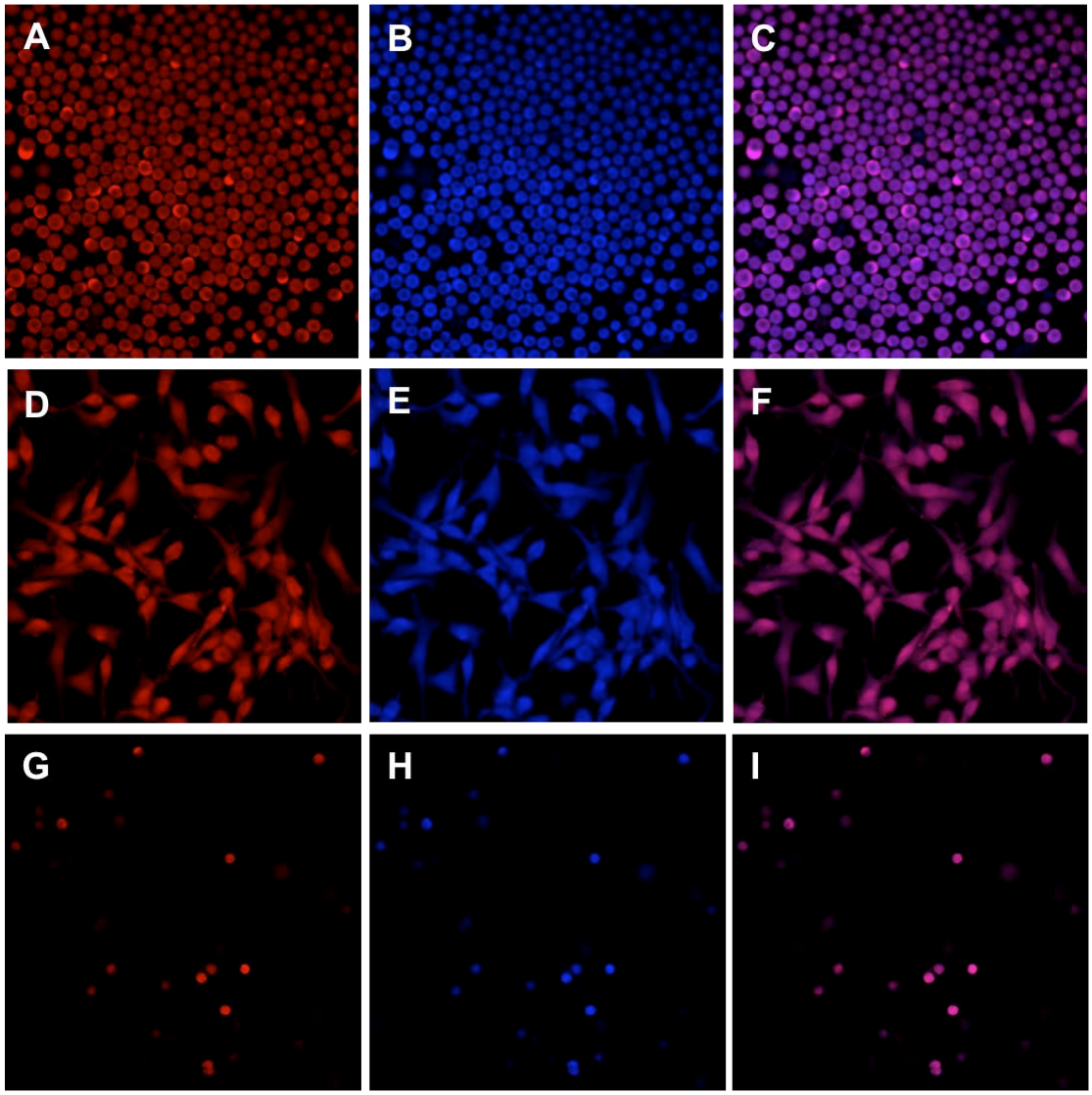

Figure 6.11: Presence of Carboxy-SNARF-1 dye inside the cells excited at 514 nm. All the images have been brightened using Image J, for the purpose of enhancing visibility, thereby making it easier for the reader. For the measurements of pHi, the original images were used. **(A)** Fluorescence emission collected at 580 ± 20 nm. **(B)** Fluorescence emission collected at 640 ± 20 nm. **(C)** Overlay of the emission collected at the two wavelengths 580 ± 20 and 640 ± 20 nm. Chondrocytes in monolayer culture: **(D)** Fluorescence emission collected at 580 ± 20 nm. **(E)** Fluorescence emission collected at 640 ± 20 nm. **(F)** Overlay of the emission collected at the two wavelengths 580 ± 20 and 640 ± 20 nm. Chondrocytes in alginate beads: **(G)** Fluorescence emission collected at 580 ± 20 nm. **(H)** Fluorescence emission collected at 640 ± 20 nm. **(I)** Overlay of the emission collected at the two wavelengths 580 ± 20 and 640 ± 20 nm.

6.4.6 Intracellular pH of Chondrocytes in 2D Culture (Monolayer)

Chondrocytes were seeded in confocal chambers at the same seeding density used in T-flasks, thereby providing them with a 2D environment similar to the one they would be exposed to in a T-flask.

First, chondrocytes were seeded in the confocal chamber and were allowed to settle in a 5% CO_2 incubator for 2 days. After the initial two days in the CO_2 incubator, the cultures were divided into two batches. The first batch was fed with a $NaHCO_3$-supplemented media and placed in a 5% CO_2 incubator. Chondrocytes grown in a $NaHCO_3$-CO_2 environment were gassed for a considerable amount of time before measuring pHi, making sure the CO_2 in the media was at 5% and that the pHe of the media was pH 7.3. The second batch was fed with media supplemented with HEPES and placed in an air incubator, void of CO_2. The pHi for the chondrocytes growing in both these conditions was measured using SNARF-1, a ratio metric dye.

The pHi was measured over a course of 7 days on days 3, 5, 6 and 7, respectively, for both the conditions to determine how different proliferation stages influenced pHi. The results obtained show the pHi of the cells grown in a $NaHCO_3$-CO_2 environment to be significantly lower *($p < 0.01$)* on all the days compared to the pHi of cells grown in a HEPES-air environment (Figure 6.12A). The pHi recorded in $NaHCO_3$-CO_2 cultures ranged between pH 7.1 and pH 7.2 for all 7 days (Figure 6.12A). The HEPES-air cultures recorded a pHi of 7.3 on day 3, which further increased to a range of pH 7.4 and pH 7.5 from day 3 onwards (Figure 6.12A).

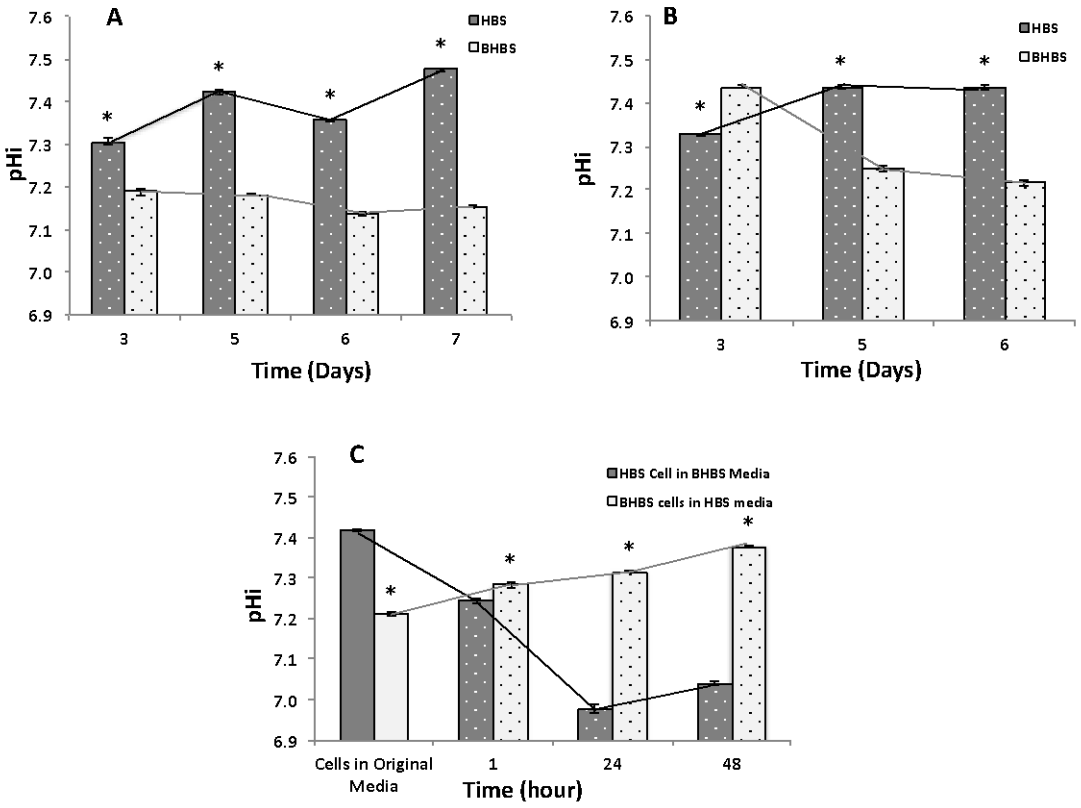

Figure 6.12: Intracellular pH (pH_i) of chondrocytes. **(A)**: Effects of media supplemented with HEPES and $NaHCO_3$ in 5% CO_2 (BHBS), and HEPES in air (HBS) on pHi of chondrocytes in monolayer. **(B)**: Effect of media supplemented with HEPES and $NaHCO_3$ in 5% CO_2 (BHBS), and HEPES in air (HBS) on pHi of chondrocytes encapsulated in alginate beads. **(C)**: Effect of switching media from HEPES and $NaHCO_3$ in 5% CO_2 to HEPES in air, and vice-versa, on pHi of chondrocytes incubated for 1, 24 and 48 hours. * Represents a significant different from the other group *($p<0.01$).*

6.4.7 Intracellular pH of Chondrocytes in 3D Culture (Alginate Beads)

Cells were encapsulated in alginate beads, thereby providing them with a 3D environment. After two days of seeding in the CO_2 incubator, the cultures were divided into two batches. The first batch of alginate beads were fed with a $NaHCO_3$ media and placed in a 5% CO_2 incubator. The second batch of beads was fed with media supplemented with HEPES and placed in an air

incubator void of CO_2. The pHi for the chondrocytes encapsulated with the beads, in both the conditions, was measured using SNARF-1, a ratio metric dye. The pHi of the chondrocytes was measured over a course of 6 days, on days 3, 5 and 6, respectively, for both the conditions. The results obtained show the pHi of the cells grown in a $NaHCO_3$-CO_2 environment to be significantly *($p< 0.01$)* different compared to the pHi of cells grown in a HEPES-air environment (Figure 6.12B). On day 3, the pHi (pH 7.4) of the cells growing in the $NaHCO_3$-CO_2 environment was significantly *($p< 0.01$)* higher than the pHi (pH 7.3) of cells growing in the HEPES-air environment (Figure 6.12B). On days 5 and 6, the pHi (pH 7.4) of cells growing in the HEPES-air environment was significantly *($p< 0.01$)* higher than the pHi (pH 7.2) of cells growing in the $NaHCO_3$-CO_2 environment (Figure 6.12B). The pHi of chondrocytes in the HEPES-air culture increased with culture time, while that of the $NaHCO_3$-CO_2 cultures decreased with time in culture. The pHi of the $NaHCO_3$-CO_2 chondrocytes was, however, greater in cells cultured in alginate beads (Figure 6.12B) than in chondrocytes cultured in monolayer (Figure 6.12A).

6.4.8 Effects of changes in Environment (CO_2 and air) and Composition of the Medium on Intracellular pH

In order to determine if the differences in pHi between the two media arose because of differences in the media rather than because of cellular adaptation, the acute and chronic effects of switching media and environment of the pHi of chondrocytes was noted. The cells in monolayer were grown in their appropriate media until day 5. After the first reading in the original medium, the media was switched (cells in HEPES and $NaHCO_3$ media in a CO_2 environment were switched to a HEPES medium in an air environment, and vice versa).

Intracellular measurements were taken at 1 hour, 24 hours and 48 hours after switching the media. There was a significant *($p< 0.01$)* difference between both the groups at 1 hour, 24 hours and 48 hours (Figure 6.12C). Throughout the experiment, the pHi of the cells that were originally in a HEPES-air environment and were transferred to the $NaHCO_3$-CO_2 environment fell significantly *($p< 0.01$)* (between pH 7.0 - 7.2) below its initial staring value (pH 7.4) (Figure 6.12C). The pHi of these cells was lower after the switch than the starting pHi of cells in the HEPES and $NaHCO_3$ media in a CO_2 environment. The pHi of chondrocytes originally in the HEPES and $NaHCO_3$ media in a CO_2 environment moved to a HEPES media in an air environment gradually increased from an approximate pH of 7.2 to a pH value that was between 7.3 and 7.4 (Figure 6.12C). After 48 hours in the culture, the pHi of these cells was similar to the starting pHi of cells originally cultured in a HEPES-air media (Figure 6.12C). Since adaptation was slow, particularly to the $NaHCO_3$-CO_2 media, it appears that cultured chondrocytes adapt their pH regulatory systems in culture environments and changing to a new environment requires re-adaptation.

6.4.9 Intracellular Buffering Capacity

The ammonium pre-pulse technique was used to determine the buffering capacity of cells grown in HEPES-air media and $NaHCO_3$-CO_2 media, with an external pH of 7.3 [299]. The buffering capacity (mmol/L/pH unit) of cells in a HBS was significantly *($p<0.01$)* lower (27.5 ± 0.4) than the buffering capacity (mmol/L/pH unit) of cells in the BHBS media (40.4 ± 0.9), at the same pHe (Figure 6.13A and Figure 6.13B) as calculated using equation 1.

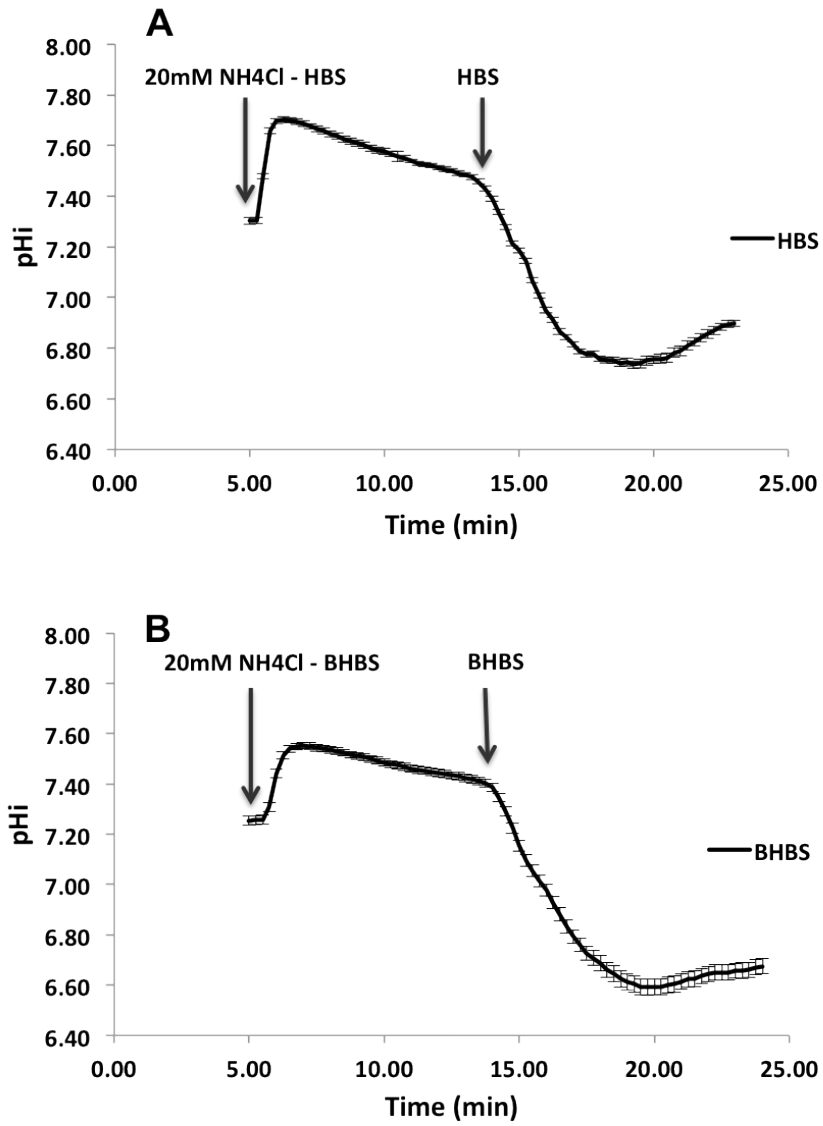

Figure 6.13: Measurement of intracellular buffering capacity of chondrocytes, when the extracellular pH was maintained at 7.3 using 20 mM ammonium prepulse technique: **(A)** 20 mM NH_4Cl was added to a HBS solution at a specific time point indicated by an arrow, which was pumped for 6 minutes and then removed. **(B)** 20 mM NH_4Cl was added to BHBS solution at a specific time point indicated by an arrow, which was pumped for 6 minutes and then removed.

6.5 Discussion

Previously we have reported on a multiple fold increase in cell proliferation and extracellular matrix accumulation in cultures supplemented with HEPES and $NaHCO_3$ in comparison to cultures only supplemented with HEPES in a continuous flow bioreactor [224]. The purpose of this study was to examine: (i) differences in effects on cell proliferation in a $NaHCO_3$-CO_2 medium and a HEPES-air medium, (ii) the effects of pHe on proliferation in both $NaHCO_3$-CO_2 and HEPES-air cultures, (iii) differences in chondrocytes pHi in both the conditions that could potentially influence cell behavior, and (iv) the difference in intracellular buffering capacity in both these conditions.

The medium with 20 mM HEPES and 14 mM $NaHCO_3$ with CO_2 ($NaHCO_3$-CO_2) had a buffering capacity more than double that of 35 mM HEPES in air (HEPES-air) (Figure 6.5). We found that chondrocytes in the $NaHCO_3$-CO_2 medium had significantly more cells than in the HEPES-air medium (Figure 6.6 and Figure 6.3A). As a result of increased proliferation (Figure 6.3A), significantly *($p<0.05$)* higher amounts of lactate (total production) was accumulated in the $NaHCO_3$-CO_2 cultures over the 6 days in relation to HEPES-air cultures (Figure 6.3C), although no significant *($p<0.05$)* difference was seen in lactate production per million cells, between the $NaHCO_3$-CO_2 condition and the HEPES-air condition, except on day 3 (Figure 6.4A). A possible explanation for this might be that the chondrocyte metabolisms in both these conditions were the same (except on day 3). Indeed, other studies have found that although the rate of lactate production by glycolysis is pH dependent, the rate of glycolysis is not affected unless the pH falls below pH 6.9 [300]. Although differences were not significant (except on day 3), the patterns of lactate production per cell were very different between the $NaHCO_3$-CO_2 and HEPES-air media. Both rates increased markedly from day 2 to day 3 (Figure 6.4A), possibly because of the effect

of the serum on rates of glycolysis, which has been reported elsewhere [300]. The effect of mitogens, such as serum, is discussed in further detail below in relation to changes in pHi.

The external pH of the media before each media change under both the conditions ($NaHCO_3$-CO_2, and HEPES-air) was also not significantly *($p<0.05$)* different (Figure 6.3B) even though lactate concentrations were higher in the $NaHCO_3$-CO_2 medium (Figure 6.3C). It is important to note that the $NaHCO_3$-CO_2 environment has a higher extracellular buffering power than the HEPES-air environment (Figure 6.5), and the differences in buffering power account for the differences in the relationship between lactate levels and pHe.

Interestingly, while cell proliferation is not affected by pHe, when it is below pH 7.0 in medium supplemented with HEPES and $NaHCO_3$ in a CO_2 environment (Figure 6.7A and Figure 6.7B) cell proliferation is drastically affected by pHe in the HEPES-air condition, when pHe falls below pH 7.0 (Figure 6.8A and Figure 6.8B). The results obtained in our study for the $NaHCO_3$-CO_2 condition is consistent with Wu *et al.*, [106] which states that no significant increase in cell proliferation was noticed between pH 6.6 and pH 7.3 [106] while our HEPES-air condition is consistent with the results reported by Schwartz *et al.*, [95], which states that chondrocytes proliferate when the extracellular pH is between pH 7.0 and pH 8.0. A possible explanation for cell proliferation halting at pH 7.0 and below in HEPES cultures in air (Figure 6.8A and Figure 6.8B) could be due to: (i) a low intracellular buffering capacity and buffering limited to intrinsic buffering capacity, (ii) an equal concentration of H^+ ions, present between the interior and exterior of the cell, thereby minimizing the function of the Na^+/H^+ exchanger, or (iii) mitogens, such as serum and growth factors, effecting Na^+/H^+ exchanger activity (discussed in detail below).

From our experiments, we can rule out the following as having no effect on cell proliferation: (i) presence of a pHe gradient around the cells (Figure 6.10A and Figure 6.10B), (ii) change in the initial environment from $NaHCO_3$ and CO_2 to air (Figure 6.9A), and (iii) change in medium composition in the air cultures (Figure 6.9A). We also tested a buffer system different from HEPES and one that can be used in air, such as MOPS. Notably, the growth curve for HEPES and MOPS was similar in air (results are not presented here), suggesting that decreased proliferation is not due to HEPES in the media as an extracellular buffer, but could be owing to other advantages that the $NaHCO_3$-CO_2 media might have, such as having the capacity of buffering and maintenance of pHi [87, 88, 118].

As the pHe is known to influence the pHi of chondrocytes [96, 97] we examined how the pHi of chondrocytes is affected by the buffer system used. In monolayer cultures (on all the days measured) and in alginate beads (on all the days measured, except day 3), the pHi of the chondrocytes in the $NaHCO_3$-CO_2 culture was significantly *($p<0.01$)* lower than the pHi of chondrocytes in the HEPES-air culture (Figure 6.12A and Figure 6.12B).

It is important to note that there is not much data available on the pHi of chondrocytes in culture. Interestingly, the steady state pHi values we obtained in our study with the $NaHCO_3$-CO_2 chondrocytes is similar to the pHi value (pHi 7.1) reported for chondrocytes freshly extracted from native cartilage [97]. The pHi in freshly isolated bovine chondrocytes is regulated by a Na^+/H^+ exchanger system [79, 87, 88, 96, 97, 115, 118].

The lower pHi values in the $NaHCO_3$-CO_2 culture could be due to CO_2 permeating through the cell membrane into the cytosol of the chondrocytes [114] and resulting in the acidification of the cytosol. The carbonic anhydrase (if present) in the cytosol can convert the CO_2 into H^+ and HCO_3^- ions [114], which could trigger the activation of the HCO_3^- transporter and the Na^+/H^+

(NHE) exchanger to stabilize the pHi to physiological pH levels [87]. Even though bovine chondrocytes do not show much dependency on HCO_3^- transporters in regulating pHi, other types of chondrocytes, such as avian growth plate chondrocytes [87], human chondrocytes cell lines [115], superficial zone chondrocytes, [118] and isolated intervertebral disc cells (chondrocyte like cells) [89], have shown a Na^+ and HCO_3^- dependent component involved in pHi regulation [79]. Mobasheri *et al.,* [81] have reported chondrocytes, under certain conditions, regulate pHi not only via one system but through multiple systems working together. The chondrocytes in a HEPES-air medium might maintain a more alkaline steady state pHi due to increased activity of the NHE exchanger, hence increasing the removal of H^+ ions from the cell.

However, the pHi values measured in alginate beads on day 3 in the $NaHCO_3$-CO_2 medium were significantly higher *($p<0.01$)* compared to the pHi of the HEPES-air medium (Figure 6.12B). This could be because of the effect of serum on rates of glycolysis [300]. Change in pHi can be a result of how proteins on the cell surface respond to mitogens, such as serum and growth factors [301-304]. It has been shown that the NHE exchanger can be activated by mitogens in the absence of HCO_3^- ions, and as a result, the NHE exchanger affinity increases for the H^+ ions, therefore setting the pHi of the cell to a higher value [301, 305-307]. Mitogens can increase protooncogene expression by various signal transduction pathways at the cell membrane, and one or all of them can influence proliferation [304, 308, 309]. It has been shown by Gillies *et.al.,* [301] that the NHE exchanger and pHi are not effected by mitogens in the HCO_3^- cultures.

The pHi of chondrocytes in the HEPES-air culture in our system is substantially higher than that reported by others from freshly isolated chondrocytes, but are in line with those reported by Browning *et al.,* [115] in human chondrocyte cell lines. Simpkin *et al.,* [118] has also reported that the pHi value of chondrocytes measured *in situ* in the superficial zone of intact cartilage in a $NaHCO_3$-CO_2 medium are approximately 0.3 pH units lower than in a HEPES-air medium.

Xu *et al.*, [107] measured pHi in chondrocytes encapsulated in alginate beads but their results are not in line with our study. They found pHi of chondrocytes in a $NaHCO_3$-CO_2 solution had a slightly more basic pHi than chondrocytes in a HEPES-air solution. The technique used to determine pHi could be the primary reason why our study is different from that of Xu *et al.*, [107]. While we measured the pHi of our chondrocytes *in situ* (encapsulated within the alginate beads), using a confocal microscope under a constant 5% CO_2 environment in the $NaHCO_3$-CO_2 cultures, Xu *et al.*, [107] harvested the encapsulated chondrocytes from the alginate beads and measured the pHi of the chondrocytes in suspension, using a fluoro-spectrometer. It is possible that an environment of 5% CO_2 in the gas phase was not maintained during the latter measurements.

We also wanted to determine if the change in pHi observed in both the cultures occurred over a short period of time (1 hour) or over long periods of time (24 hours to 48 hours). This would give us an idea of the involvement of the transporters used in this process. There was a significant *($p< 0.01$)* difference between both the groups at 1 hour, 24 hours and 48 hours (Figure 6.12C) after the exchange. The pHi of the cells, originally grown in a HEPES-air medium fell significantly *($p< 0.01$)* after the switch; the value after 48 hours was even lower than the pHi of chondrocytes originally in the $NaHCO_3$-CO_2 medium (Figure 6.12C). While 48 hours after switching the media, the pHi of the cells originally in the $NaHCO_3$-CO_2 medium became similar to the starting pHi of cells originally cultured in the HEPES-air medium (Figure 6.12C). Since adaptation was slow, particularly to the $NaHCO_3$-CO_2 medium, it appears that cultured chondrocytes adapt their pH regulatory systems to their culture environment and moving to a new environment requires readapting to that environment. Cells adapting their pH regulatory systems to their culture environment and setting a new steady state pHi has also been mentioned by Roos *et.al*, [310] in other cell types. The transporters in cells originally in a $NaHCO_3$-CO_2 medium that were switched

to a HEPES-air medium might have been up-regulated and expressed at 48 hours. The initial increase in pHi to 7.3 might have been due to the removal of CO_2 and bicarbonate from the cytosol, while the 0.1 pH unit increase between the 24 hour and 48 hour could be a result of up-regulation of the NHE exchanger. The final steady state pHi value after 48 hours was the same as reported in normal monolayer cultures with HEPES-air cells in a HEPES-air medium (Figure 6.12A and Figure 6.12C). When the HEPES-air cells were transferred to the $NaHCO_3$-CO_2 solution, initially the pHi was decreased to pH 7.2. This decrease could be due to the immediate diffusion of CO_2 into the cell, causing an acid loading in the cell, and along side this process, the CO_2 gas might react with water and could be forming HCO_3^- ions. The acidification process could possibly activate the NHE exchanger to stabilize the pHi to physiological pH levels, and within 24 hours, the already present HCO_3^- transporter might have been activated. This has been shown in isolated avian chondrocytes when moved from a HBS solution to a medium rich in HCO_3^- [87].

As expected, the buffering capacity (Figure 6.13) (mmol/L/pH unit) of chondrocytes in a $NaHCO_3$-CO_2 condition was significantly *($p<0.01$)* higher than in the HEPES-air condition at pHe 7.3. These results are in line with the values reported by other researchers [97, 115, 118]. Higher intracellular buffering capacity in $NaHCO_3$-CO_2 chondrocytes will keep the pHi more stable and will minimize changes to pHi as a result of increased cell metabolism and pHe acidification. If the cell metabolism is dependent on the pHi, then higher buffering capacity will work in its favor.

6.6 Conclusions

In conclusion, chondrocyte proliferation is influenced by culture medium. Chondrocytes proliferated more rapidly in media buffered with a HEPES and $NaHCO_3$ in CO_2 than in media

buffered by HEPES in air. Chondrocytes in a $NaHCO_3$-CO_2 medium can proliferate at a lower pHe (pH > 7) while chondrocytes in a HEPES-air medium do not proliferate at pH $\leq$ 7. The different media also influence pHi; the pH in the $NaHCO_3$-CO_2 media being more acidic than in HEPES-air. We found that the pHi of chondrocytes also varied with culture conditions, being more alkaline in alginate beads than in monolayer in the same media. Although elucidating the mechanism, relating pHi to cell proliferation is beyond the scope of this study. Results show the importance of maintaining buffer type and pHe on rates of cell proliferation and the importance of these factors for work on cartilage repair.

Chapter 7

Conclusions and Recommendations for Future Work

7.1 Conclusions

7.1.1 Effect of Continuous Culture on Cartilaginous Tissue Growth *In Vitro*

Engineered tissue construct cultivated in a continuous flow bioreactor at a low flow rate (5-10 μL/min) accumulated significantly more ECM (glycosaminoglycan and collagen) than static cultures. Additionally, the tissue in the continuous culture displayed some features characteristic of stratified morphology of native cartilage, as well as containing stores of intracellular glycogen. The resemblance of the tissue grown in the bioreactor to native tissue could be due to the infinite supply of nutrients and the constant removal of metabolic waste (lactic acid). Furthermore, the shear force at the surface of the tissue, due to media flow, might have also accounted for the formation of native-like tissue.

7.1.2 Beneficial Effects of Media Supplementation with Sodium Bicarbonate

The combination of the continuous flow bioreactor and media supplemented with sodium bicarbonate (Chapter 4), together, had a significant effect on the tissue-engineered constructs. These effects included increased: (i) tissue thickness, (ii) cell proliferation, and (ii) ECM accumulation (glycosaminoglycan and collagen). Moreover, these increases in physical and biochemical properties where significantly higher compared to all other conditions (static cultures with and without $NaHCO_3$, and cultures grown in the bioreactor without $NaHCO_3$

supplementation). In addition, the tissue constructs grown in the bioreactor in the presence of 14 mM $NaHCO_3$ formed a secondary construct that grew out of the primary construct, without a separate expansion phase. This tissue outgrowth was negligible in all the other culturing conditions. Besides that, the chondrocytes seemed to maintain their cartilaginous phenotype as collagen type II was detected throughout the tissue. Thus, this study demonstrates the advantage of employing a continuous flow bioreactor coupled with $NaHCO_3$-supplemented media for articular cartilage tissue engineering.

7.1.3 Determination of a Feasible Primary Construct for ECM Production

A number of different seeding techniques were investigated with the aim to engineer a tissue construct that utilizes a few cells (chapter 5). From the results obtained in Chapter 5, it is evident that the monolayer constructs outperformed all the other constructs investigated in this study, with respect to: (i) dry weight, (ii) thickness, (iii) cell proliferation, and (iv) ECM accumulation (collagen and proteoglycan). The minced and biopsy constructs recorded inconsistent data, which could be due to the presence of ECM, which might have created an additional diffusion barrier for nutrients to get to the cells. The monolayer constructs, on the other hand, were created from single cells, which possessed no ECM-barrier. The absence of an ECM-barrier in the monolayer constructs might have made essential nutrients, required for cell proliferation, readily available. Interestingly, the monolayer constructs accumulated significantly lower amounts of collagen compared to the constructs grown on collagen-coated filters (50 μg of collagen per filter). This increase in collagen accumulation in the filter constructs could be attributed to the initial collagen used to coat the filters. The presence of collagen on the filters might have acted as a signaling

molecule, thereby kick-starting the collagen production in the cells directly seeded on the collagen-coated region of the filters.

7.1.4 Potential Factors Contributing to Increased Growth and Cell Proliferation

In this study we investigate the underlying reason for the increased proliferation and ECM production observed in Chapter 4. Isolated bovine chondrocytes were grown in 2D (monolayer), and 3D (alginate beads) cultures in media with 20 mM HEPES and 14 mM $NaHCO_3$ ($NaHCO_3$-CO_2) in a CO_2 environment, and in media with only 35 mM HEPES (HEPES-air) in an air environment (Chapter 6). The pHi of chondrocytes cultured in media with only HEPES in air was significantly *($p<0.01$)* higher than the pHi of the chondrocytes cultured in a HEPES and $NaHCO_3$ medium in a CO_2 environment. Additionally, the total buffering capacity of chondrocytes cultured in the HEPES and $NaHCO_3$ medium, at pH 7.3 in a CO_2 environment was significantly *($p<0.01$)* higher than the intrinsic buffering capacity of chondrocytes cultured in the HEPES medium, at pH 7.3 in an air environment. Cell numbers in the $NaHCO_3$-CO_2 medium were significantly *($p<0.05$)* higher starting day 4 in comparison to cultures grown in a HEPES-air medium. While cell proliferation was not affected by pHe, in cultures with HEPES and $NaHCO_3$, below pH 7.0, cell proliferation was significantly affected by pHe in cultures with HEPES only once the pHe fell below pH 7.0. Thus, this study demonstrates the importance of the extracellular environment, such as pHe, extracellular buffering capacity, and the presence of carbon dioxide and bicarbonate ions, for chondrocyte proliferation and how extracellular environments can influence intracellular processes.

7.2 Recommendations for Future Work

7.2.1 Tissue Maturation

The tissue engineered in the continuous flow reactor supplemented with $NaHCO_3$ appears to have biochemical and mechanical properties similar to that of immature cartilage (low collagen content and inferior mechanical properties). In order to ensure viability of the tissue post-implantation, this tissue will need to be matured. One of the most effective methods to improve the biochemical composition and mechanical properties of the developed tissue is through mechanical stimulation [238, 279, 311]. While applying mechanical stimuli within the reactor may be problematic, literature has shown that stimulation with ATP [223, 241, 312] or growth factors [313-315] can significantly improve collagen accumulation and, in turn, the mechanical properties of the resultant tissue construct.

7.2.2 Determine how pHe with and without $NaHCO_3$ and CO_2 Influences pHi in Culture

In Chapter 6, the pHi of chondrocytes at pHe 7.3 was measured. It is important to determine the relationship between the pHi and pHe of chondrocytes in the BHBS and HBS media while in culture. The pHi should be measured with varying pHe to determine how pHe influences pHi in the two conditions. This will give a better understanding of chondrocyte behavior in culture.

7.2.3 *In Vitro* Human Chondrocyte Proliferation and Tissue Synthesis

In order to be able to treat a cartilage defect, similar results need to be achieved with human chondrocytes. Media conditions will have to be altered to accommodate for human chondrocytes.

In addition to bicarbonate, media supplemented with growth factors (transforming growth factor β1 (TGF-β1), and fibroblast growth factor 2 (FGF-2)) have shown to encourage re-differentiation of chondrocytes and also increase the production of cartilage-specific ECM molecules [314, 316-321].

7.2.4 *In Vivo* Studies

If the results from the *in vitro* study are promising, future studies could be designed to study tissue integration in large animal models. Prior to implantation, the engineered constructs have to be subjected to a maturation process, as described in Section 7.2.1. A sheep or a goat model is a recommended animal model for the following reasons: (i) these are large animal models that possess close similarities to human articular cartilage, and (ii) the loads experienced in the knee joints of these animal models are similar to the loads experienced in the human knee joint [322, 323]. For these reasons, a sheep or goat model is recommended to effectively study tissue integration and durability of the transplanted tissue-engineered constructs [322, 323]. For clinical trials in humans, I recommend using a construct engineered from the patients own cells to fill the chondral defect. Additionally, using the system outlined in this thesis chondrocytes can also be expanded *in vitro* in a short period of time and used for ACI or MACI, thereby supporting tissue formation at the injury site.

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(54) Title: TISSUE-ENGINEERED CARTILAGE FROM CONTINUOUS FLOW CULTURE

FIGURE 1F

(57) Abstract: Bioreactors are useful for cartilage tissue engineering because traditional batch culture is not optimal for cartilage tissue growth. The present invention attempts to maximize diffusive transport to engineered cartilage constructs by replenishing the culture medium in a continuous flow bioreactor. The medium is supplemented to maintain a pH of 7.0 to 7.6 until the construct reaches a size of at least 1.5 square centimetres. Chondrocytes can be directly cultured from a biopsy without a separate expansion phase. Subject-specific cartilage constructs can be produced from autologous cells. Phenotypically stable, large-sized engineered cartilage constructs that are not hyper-cellularized can be produced. These constructs are desired for articular cartilage repair of damaged joints

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