EFFECT OF CHANGING THE FREQUENCY OF BIAXIAL LOADING ON CHONDROCYTES SEEDED IN AGAROSE GEL

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Abstract

In vivo, articular cartilage is under complex mechanical loadings. It is an accepted idea that mechanical stimulation of engineered articular cartilage may lead to better mechanical properties. *In vitro* biaxial stimulation has been found to be superior to uniaxial loading. Uniaxial loading has shown that change of frequency of stimulation give different effects on chondrocytes. However, the effect of change in frequency of biaxial stimulation is not documented yet. This study aims to test the effect of changes in the frequency of the biaxial loading of chondrocytes seeded in agarose gel. Direct compression amplitude of 10% and shear loading amplitude of 1% will be applied on agarose gel constructs seeded with chondrocytes. Three frequencies of 0.5Hz, 1Hz, and 1.5Hz are tested. Mechanical stimulation is applied for a period of 48hrs with 12hrs ON and 12hrs OFF. Before the start of mechanical stimulation a 24hr pre-culture rest will be allowed. DNA and GAG assays will be used to assess the response of chondrocytes and their production of extracellular matrix. Results obtained are in conformity with previous studies showing the advantage of mechanical stimulation over static culture with the frequency of 0.5 Hz having the highest GAG/DNA production in constructs, which shows that cells are still producing cartilage type extracellular matrix. DNA production in stimulated constructs was less than in static constructs. However the GAG production in all stimulated constructs was higher than in static constructs, which indicates that although cell number is lower in the stimulated constructs, yet their metabolic activity to produce cartilage extracellular matrix is much higher which further confirms the importance of mechanical stimulation in the regulation of cartilage extracellular matrix synthesis by chondrocytes. It is suggested that with a longer duration experiment there is a better chance for the frequencies to show any possible statistically significant differential effects they have on chondrocytes seeded in agarose gel under biaxial stimulation.

Abstrak

Rawan artikular sentiasa berada bawah muatan mekanikal kompleks in vivo. Terdapat satu idea bahawa perangsangan mekanikal atas rawan artikular terjurutera boleh mengembangkan sifat-sifat mekanikal yang lebih baik. Dalam in vitro, perangsangan dwipaksi didapati lebih baik daripada muatan ekapaksi. Perubahan frekuensi perangsangan akan memberi kesan yang berbeza ke atas kondrosit seperti yang ditunjukkan dalam muatan ekapaksi. Walau bagaimanapun, kesan perubahan frekuensi muatan dwipaksi masih tidak didokumenkan. Kajian ini bertujuan mengaji kesan perubahan-perubahan frekuensi muatan dwipaksi atas kondrosit-kondrosit yang dibenih dalam gel agarosa. 10% amplitud mampatan langsung dan 1% amplitud muatan ricih akan digunakan pada binaan gel agarosa dibenih dengan kondrosit-kondrosit. Tiga frekuensi iaitu 0.5Hz, 1Hz, dan 1.5Hz akan dikajikan. Perangsangan mekanikal digunakan selama 48 jam di mana 12 jam dengan perangsangan dan 12 jam tanpa perangsangan. 24 jam rehat pra-kultur dibenarkan sebelum perangsangan mekanikal bermula. Ujian-ujian DNA dan GAG digunakan untuk menilai tindakbalas kondrosit-kondrosit and pengeluaran matriks ekstrasellular mereka. Keputusan yang diperolehi adalah selaras dengan kajian-kajian sebelum ini yang menunjukkan kelebihan perangsangan mekanikal atas kultur statik di mana pengeluaran GAG/DNA di dalam binaan di bawah frekuensi 0.5 Hz menunjukkan sel-sel masih mengeluarkan matriks ekstrasellular jenis rawan. Pengeluaran DNA dalam binaan terangsang adalah rendah daripada binaan statik. Walau bagaimanapun, pengeluaran GAG dalam semua binaan terangsang adalah lebih tinggi daripada binaan statik, menunjukkan bahawa aktiviti metabolik untuk pengeluaran matriks ekstrasellular rawan adalah lebih tinggi walaupun bilangan sel lebih rendah dalam binaan-binaan terangsang. Ini mengesahkan lagi kepentingan perangsangan mekanikal dalam penyelengaraan sintesis matriks ekstrasellular rawan oleh kondrosit-knodrosit. Adalah dicadangkan bahawa frekuensi-

frekuensi lebih berpeluang bagi menunjukkan sebarang kesan perbezaan yang ketara dari segi statistik ke atas kondrosit-kondrosit yang dibenihkan dalam gel agarosa di bawah perangsangan dwipaksi jika tempoh ujikaji yang lebih panjang digunakan.

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: *Yasir Mohammed Abdulqader Al-Saffar* (I.C/Passport No: *G1802546*)

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Name of Degree: *Master of Engineering (Biomedical)*

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

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Acknowledgements

All praise is due to Allah, He has all the knowledge and He gives some of it to whom He wants. I would like to express my great gratitude to my helpful supervisor, Dr Belinda Murphy. The supervision, guidance and support she provided doubtlessly helped the progression and smoothness of this study.

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Table of Contents

List of Figures

List of Tables

List of Symbols and Abbreviations

Analysis of Variance	ANOVA
Dimethylmethylene blue	DMB
Dulbecco's Modified Eagles Medium	DMEM
Earl's Balanced Salt Solution	EBSS
Extracellular Matrix	ECM
Feotal Bovine Serum	FBS
Glycosaminoglycan	GAG
Mesenchymal Stem Cell	MSC
Programmable Logic Controller	PLC

Chapter 1: Introduction

1.1 Tissue Engineering

Many diseases and conditions till date have no definitive cure, and the wish for the availability of body spare parts that can be made ready to be used off the shelf has always been there. This is one ultimate goal that tissue engineering tries to achieve, by rather providing functioning living tissues and organs that can be used for transplant instead of the use of medicines, surgeries, prosthesis, and implants, that don't provide definitive cure for some medical conditions. One of the most exciting results that tissue engineering could achieve is the ability to grow a human ear on the back of a mouse that was achieved by Dr. Charles Vacanti at University of Massachusetts Medical Center. Some other promising results include the growth of pulmonary arteries, jaw bones and teeth (Meyer, 2009; Parry, 2005).

Tissue engineering is relatively a new field and it only get its presence by gathering the knowledge of different disciplines such as medicine, engineering, biology, etc., and apply state of the art technologies in a focused objective. To simplify things however, tissue engineering stands on three main pillars that are: cells, matrix and signaling molecules (Bronzino, 2006). Cells are considered the living part that is in charge of ECM formation. In turn, the ECM once formed it provide mechanical support to the cells and is the route through which the cells get all their needs including nutrition, respiration, waste disposal, and interaction with the signaling molecules that are present among their reach. The precise architecture and harmony of the tissue component is a key for its normal functioning, this is what is called: cellular microenvironment (Wong, Siegrist, & Cao, 1999).

Matrix is any structure that carries the cells and supports them until they integrate with the recipient tissue or body. It primarily provides the basic needed functions of the ECM in an attempt to imitate the cellular microenvironment. It can be made of natural or synthetic materials.

Cells are well known to interact with the surrounding chemical and physical components (Geiger, Spatz, & Bershadsky, 2009) and from here comes the importance of signaling molecules. Signaling molecules such as growth factors are molecules that don't form a bulk of the ECM or tissue fluids, but rather they are molecules that even present in minute amounts, they have big role affecting cellular metabolism, growth, multiplication, differentiation and many vital cellular biological events(Wu, Fannin, Rice, Wang, & Blough, 2011).

Cells need to be obtained from a donor source primarily and if sufficient enough, can be directly transferred to the recipient, either as pure cells or loaded on a scaffold if needed. If cells are insufficient in number, they can be expanded in-vitro or ex-vivo until reach the desired amount and then either be used directly or preserved until it is needed. This simple looking concept is actually not easy to achieve and many challenges throughout the different stages of the process are to be dealt with. The main drive to go through all the challenges is the belief that tissue engineering is the answer for future treatments, and carries a potential to change the whole concept of treatment and disease management (Wirth & Rudert, 1996).

1.2 Articular cartilage

Osteoarthritis is a disease that causes progressive destruction of articular cartilage mainly of the knee and hip joints and commonly affects elderly population. In the US alone, around 27 million suffer of osteoarthritis (Foundation, 2011). It is a common disease that happens either secondary to joint trauma, or more commonly as a primary

disease that affects the older population where 70% of people with osteoarthritis are above the age of 65. The disease is so common that an estimated 5% of total population is affected by it which leads to huge burdening health care costs annually of around $8 billion (Mauck et al., 2000). Osteoarthritis drives researchers around the world to search for a curative treatment, but we need to realize that in addition to osteoarthritis, there are also other diseases that strike human joints and there is hope that researching in tissue engineering may provide a treatment that can be applied to many of the joint disease conditions. With all that said, our focus will be more towards joint diseases problems and the affected articular cartilage. Because in human body there are many types of joints and cartilages where their tissues differ considerably, it is important to know briefly about what type of joint and cartilage we are to study.

1.2.1 Joint

A joint in human body is a place where two or more bones meet each other's, movement may or may not happen. According to the type of tissue that is present between the meeting bony ends, joints can be classified into:

- Fibrous joints: fibrous tissue fills the joint space and minor movement can only happen, e.g: sutures between skull vault bones.
- Cartilaginous joints:
    - Bones are fixed in place by a piece of hyaline cartilage, e.g.: attachment of 1st rib to manubrium sterni.
    - Bones are attached by a piece of fibrocartilage where little movement is allowed. Thin layer of hyaline cartilage covers the bony ends, e.g.: symphysis pubis.
- Synovial joints: In this joint type, a great range of movement is allowed, as there is a joint cavity separating the meeting bones. Bones are covered by a thin layer of hyaline articular cartilage, and the inner joint cavity is

lined by a synovial membrane that secretes a lubricating viscous fluid known as the synovial fluid. The whole joint is protected from the outside by a thick fibrous membrane known as the joint capsule (Plumb & Aspden, 2005) (figure1.1).

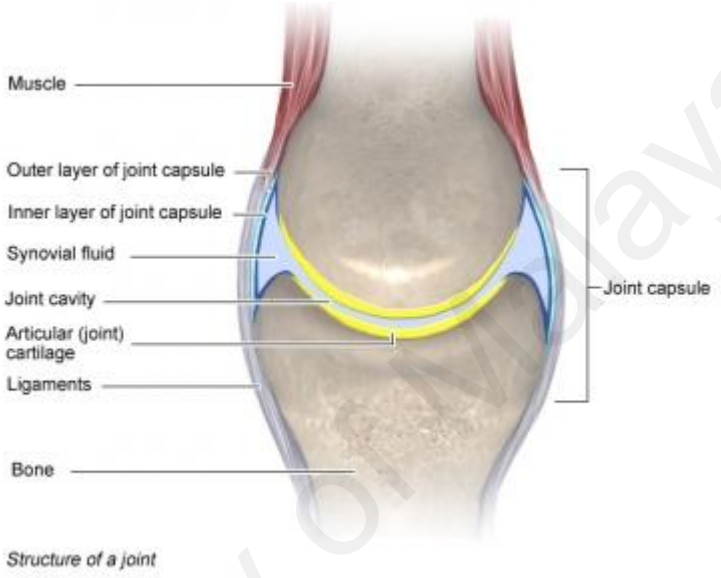

Figure 1. 1: Drawing showing anatomical structure of synovial joint. Reproduced from ((IQWiG), 2009)

1.2.2 Cartilage

Cartilage is one of the connective tissues where cells and fibers are immersed in a gel like matrix. Perichondrium covers the cartilage generally but in articular surfaces, the perichondrium is absent. Human body contain 3 types of cartilage namely, hyaline cartilage, fibrocartilage and elastic cartilage. Hyaline *articular* cartilage is not covered by perichondrium (Plumb & Aspden, 2005).

1.3 Hyaline cartilage:

Hyaline means to have appearance like glass, is predominant type of cartilage in human body. Its colour is bluish opalescent. It's present in synovial joints covering articular surfaces (hyaline articular cartilage), also can be found in costal region and

respiratory tract cartilages or as an epiphyseal growth plate. Hyaline articular cartilage is different because the hyaluronic acid protein complex in its extracellular matrix (ECM) is unique and gives it its viscous and slippery feature that provide a very low coefficient of friction which make it very appropriate for its function in synovial joints (Kerr, 1999).

1.3.1 Hyaline articular cartilage:

In a synovial joint, the hyaline articular cartilage plays the main role in allowing smooth movement of opposing bony ends. It behaves in such a way that it distributes load evenly while allowing a smooth movement therefore protecting the bony ends from abrasion. However, this complex tissue has a very limited ability for repair and damaged areas are usually replaced by fibrocartilage that is inadequate mechanically and biochemically to provide the special function of hyaline articular cartilage, which eventually leads to uneven function of the cartilage in general and spread of damaged area that can progress in time to osteoarthritis (Temenoff & Mikos, 2000). The hyaline articular cartilage as any other type of cartilage is a tissue that is avascular, has no lymphatic drainage and has no nerve supply. However, because articular cartilage has no perichondrium covering, it gets its nutrition via diffusion through the surrounding synovial fluid (Elder & Athanasiou, 2009).

1.3.2 Function of hyaline articular cartilage

Articular cartilage is a fibrillar, hydrated connective tissue. It is widely agreed that cartilage functions as a highly optimised articulation tissue which is able to redistribute and slowly transmit applied stresses to the subchondral bone(A.J. Grodzinsky, 1983; A. Maroudas, 1979, 1985; Mow, 1997; Weightman, 1979), whilst also providing joint lubrication by virtue of the characteristics of its superficial layer. This result in protection of the underlying subchondral bone from being damaged by concentrated

peak stresses and high surface friction.(Eyre, Wu, Fernandes, Pietka, & Weis, 2002; Wolf, Ackermann, & Steinmeyer, 2007).

1.4 Components of hyaline articular cartilage

1.4.1 Chondrocytes

Of the full volume of the articular cartilage, chondrocytes represents only around 1% of it. Even though this low amount, but being the only cell types available in articular cartilage, they carry the whole duty of cartilage matrix synthesis and repair. Being highly metabolically active, their golgi apparatus and endoplasmic reticulum are apparent microscopically. The chondrocyte are able to remodel the cartilage matrix in response to the type of physical load applied. It is suggested that chondrocytes sense the changes in loads through cilia that are projects from some of the chondrocyte into the surrounding matrix (Buckwalter & Mankin, 1998; Temenoff & Mikos, 2000).

The origin of chondrocytes is from the mesenchymal stem cells (MSCs). Through consecutive replications, MSCs start to differentiate into chondrocytes and cartilaginous matrix starts to be produced. Those chondrocytes continue to proliferate and divide. The group of chondrocytes that are located towards the central zone will end up forming the bone, while the ones towards the periphery remains as chondrocytes and deposit the hyaline cartilage matrix. Once reaching maturity, chondrocytes stops proliferating and become entrapped in the matrix inside the lacunae assuming a round shape (Buckwalter & Mankin, 1998; Caplan & Boyan, 1994; Temenoff & Mikos, 2000) (figure 1.2).

1.4.2 Extracellular matrix

As the cellular volume of the hyaline articular cartilage is very small, the understanding of the ECM components and structure is at the heart to the understanding of hyaline articular cartilage unique mechanical properties. It is the fine adjustment of

the amount of each of the ECM components and the architecture in which they are arranged what provide those mechanical properties.

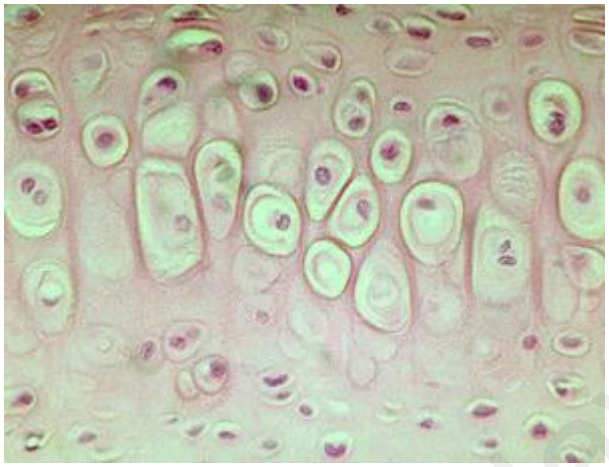

Figure 1. 2: Hyaline cartilage showing chondrocytes morphology and the lacunae surrounding them. Reproduced from (Christoffersen, 2011)

a) Collagen

In articular cartilage we can find different collagens types (II, VI, IX, X and XI). Type II collagen is the most abundant representing 90-95% of all the collagen mass. The high carbohydrate amount bounded to collagen type II gives it its ability to interact more with water. The tensile strength of the cartilage is enhanced by the presence of a mesh formed of interwoven fibrils of types II, IX and XI collagen (Cohen, Foster, & Mow, 1998; Temenoff & Mikos, 2000).

b) Proteoglycans

Those are molecules made up of 95% polysaccharide and 5% protein core. Some un-branched polysaccharides (glycosaminoglycan chains) are associated with the core protein (figure1.3). Those polysaccharides are built of many disaccharides that one of them will at least be negatively charged due to a presence of sulphate or carboxylate group in it. The negativity causes the GAGs to repel other GAGs as well as anions and

to pull cations towards them facilitating interaction with water (Buckwalter & Mankin, 1998; Temenoff & Mikos, 2000; Wirth & Rudert, 1996).

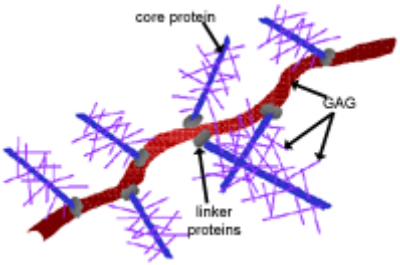

Figure 1. 3: This diagram shows the structure of an aggregate of aggrecan. Reproduced from (Steve, 2011).

Proteoglycans arrange either as large aggrecans or small proteoglycans. The larger ones are usually entrapped due to their bigger size within the fibrillar network of the collagen. With their ability to attract water they are suggested to function as stress distributor providing the resilience of articular cartilage. The smaller proteoglycans are thought to have a role in controlling cell function and help in supporting the collagen fibres, rather than to be a significant player in determining the physical properties of the articular cartilage (Buckwalter & Mankin, 1998; Cohen, et al., 1998).

c) Non-collagenous proteins

Glycoproteins, fibronectin and tenascin are one example of this group. They help stabilising the ECM and play some role in cell-ECM interaction. Some other non-collagenous proteins help anchoring chondrocytes to the ECM (Buckwalter & Mankin, 1998; Temenoff & Mikos, 2000).

d) Tissue fluid

This comprises around 80% of the cartilage wet weight; fluid component is what makes the physical properties of the articular cartilage come to life. Being mainly water that is entrapped inside the ECM, it provides a very effective cushioning effect and allows the cartilage to regain normal shape after being compressed. The presence of

negatively charged GAG in ECM attracts the cations, which in turn get its accompanying water to be entrapped in the ECM.

The tissue fluid is also responsible to provide nutrition and dispose wastes by exchanging them with the surrounding synovial fluid (Buckwalter & Mankin, 1998; Temenoff & Mikos, 2000).

1.5 Architecture of hyaline articular cartilage

Hyaline articular cartilage has a structure that changes according to the depth of the tissue. Three different zones can be identified in hyaline articular cartilage are: superficial zone, middle zone, and deep or radial zone. Differences among these zones include cellular morphology, amount and arrangement of different ECM components (Vanderploeg, Wilson, & Levenston, 2008).

The superficial or tangential layer consist of dense bundles of collagen fibrils which are aligned parallel to the articular surface, and occupies about 5-10% of the matrix volume with a low proteoglycan content (Glenister, 1976; A. Maroudas, 1985). The transitional or midzone layer, which is 40-45% of the matrix volume, contains a significant increase in the proteoglycan content, together with a complex, 3D network of collagen fibrils which exhibit an overall radial arrangement. The deep or radial layer also occupies 40-45 % of the matrix volume with a high proteoglycan concentration (A. Maroudas, 1979; A. Maroudas, Mizrahi, J., BenHaim, E. and Ziv, I., 1987; A. Maroudas, Muir, H. and Wingham, J., 1969; Preston, 1972). The fibrillar meshwork in this region is similar to that in the transitional layer. The calcified cartilage layer, which occupies the region from the tidemark to the cement line (bony end), is heavily impregnated with crystals of calcium salt and is devoid of proteoglycans but contains more smaller chondrocytes in comparison to those in the deep layer (figure 1.4).

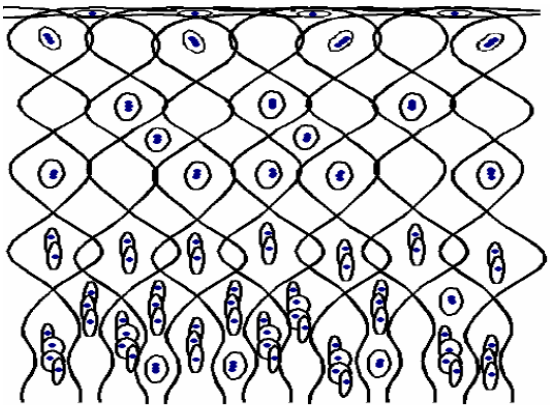

Figure 1. 4: Schematic illustration of type II collagen meshwork and the cells distribution and orientations in the articular cartilage matrix. Reproduced from (Nguyen, 2005)

In consequence, articular cartilage is a highly heterogeneous, anisotropic and multi-phase biomaterial consisting mainly of collagen fibrils, proteoglycans and water. The collagen fibrils in the deep zone (near the bone) are perpendicular to the cartilage-bone interface; they are somewhat randomly oriented in the middle zone, and there is a very thin layer of a maximum of 200 micrometre depth at the surface where the fibrils run parallel to the cartilage surface. Proteoglycans concentration also varies with depth within the cartilage; it's maximum in the middle zone and is significantly reduced near the surface (Urban, 1979).

The differences in the ECM components between the different zones is in part due to the inherent differences in the metabolic activity of the chondrocytes in these different zones as they show different gene expression profiles, another possible factor is the attempt of the cells to adapt to their zone's specific mechanical environment conditions (Darling, Hu, & Athanasiou, 2004; Vanderploeg, et al., 2008).

1.6 Mechanical environment of hyaline articular cartilage

Stress applied on human articular cartilage during normal loading ranges from 0.1-4MPa, with presence of local peak stresses up to 18Mpa (Szafranski et al., 2004; Wolf,

et al., 2007). On the superficial layer, the main type of mechanical load applied by the moving opposing cartilage surfaces is tension and shear forces. An appropriately organised mesh of the collagen fibres running parallel to the surface and being dense, improves the ability of the superficial zone to deal with this type of loads. Stress is absorbed in the middle and deep zones by the effect of negatively charged GAG that are covalently bound to highly concentrated proteoglycans causing attraction of Na+ ions leading to water influx to the matrix, filling up the cartilage and providing a cushioning effect limiting the strain deformity that cartilage sustains (Soltz & Ateshian, 2000; Wolf, et al., 2007). As in daily activities cartilage is usually under cyclical loading, sinusoidal pattern of in vitro stimulation has been explored. Researchers are trying to mimic the mechanical environment in in-vitro models to either attempt to produce engineered cartilage or to study effect of independent mechanical phenomenon on chondrocytes metabolism and ECM properties.(Soltz & Ateshian, 2000)

The importance of cells local mechanical environment comes from the suggestion that mechanical forces play a crucial role in regulating different biological functions. It has been documented that mechanical forces affect a wide range of cellular events including cellular growth, differentiation, migration, gene expression, protein synthesis and even apoptosis (Alenghat & Ingber, 2002; Ingber, 2003). The process by which external mechanical stimuli results in a cell biological response is termed mechanotransduction (Jaalouk & Lammerding, 2009; Wang, Butler, & Ingber, 1993). Mechanotransduction process can be detailed as follow:

1. Signal transduction:
    a. Force transmission
    b. Mechanosensing
    c. Transduction
    d. Signal transmission
2. Signal propagation
3. Cellular response

Signal transduction starts by force transmission which is to conduct the external load down to or even through the cell membrane. The load transmission is conducted by the interaction of ECM molecules situated between load source and cell membrane. Due to tensegrity (positioning of a cell in a specific orientation inside tissue architecture through its external membrane complexes as integrins, desmosomes, etc.), cell rests at a baseline tension, and changes in this tension caused by the external load can then be sensed by the cell through mechanosensing which is the ability of the cell to realize this load and proceed through transduction and signal transmission which are poorly understood processes but likely to include some proteins of the cells cytoskeleton (Geiger, et al., 2009; Jaalouk & Lammerding, 2009; Wu, et al., 2011).

1.7 Hyaline articular cartilage self-repair

Hyaline articular cartilage in normal conditions is in continuous turnover cycle. Different ECM components have different turnover rates, with the collagen fibrils having the slowest turnover time with a half-life of more than 100 days for immature cartilage and up to many years in adult cartilage, resulting in very low or undetectable amount of collagen type II mRNA (Mouritzen, Christgau, Lehmann, Tanko, & Christiansen, 2003; Poole et al., 1995). Once injured, hyaline articular cartilage has low ability to heal properly and repair itself completely. Damaged areas tend to aggravate

with time and cartilage attempts at repairing usually fails ending up by replacing the damaged hyaline articular cartilage with fibrocartilage which is mechanically inferior in properties (Elder & Athanasiou, 2009). Till day, available treatment options as total joint replacement fail to provide long term steady function. Some other trials as extracting cartilage graft, expanding it and replanting it in the damaged area, rely on taking from the healthy part of the patient cartilage that basically introduce more damage to the joint.

As mentioned previously, articular cartilage ability to repair itself is rather limited and usually cellular attempts for repair stops before full regeneration happens. This may be explained by the innate relatively low metabolic activity of chondrocytes and the fact that they maintain the ECM over time without proliferation (Buckwalter & Mankin, 1998). Chondrocytes also have limited ability to migrate to the injured site probably because of their entrapment inside the ECM. Unless the articular cartilage damage is a full-thickness one, access to subchondral progenitor cells is not feasible and cannot thereby fill the damaged area (Ernst, 1999). Also the presence of proteoglycans can alter the ability of cells to attach impairing the repair process further (Temenoff & Mikos, 2000).

1.8 Treatment options available today for articular cartilage diseases

So far, treatments available for treating cartilage diseases does not provide definitive cure and can be summarized as follow:

I. Symptomatic treatment: This is used for large defects and severe cases and is usually the last method used when other treatment modalities and options failed. It is attempted to reduce the pain. Generally done by removing the affected area of the joint and replace it either with nearby healthy parts or by a prosthesis (Shawn, 1998; Temenoff & Mikos, 2000).

II. Cartilage restoration: For small lesions , defect is attempted to be filled by cartilage grafts or by enhancing cartilage self-repair through the use of different techniques ranging from medical to surgical approaches (Shawn, 1998; Temenoff & Mikos, 2000):

a. Tissue grafts: Grafts used can be autografts from a healthy area on the same joint or other healthy joints of the same patient. Cons include donor site morbidity, limited source, and grafts from less weight bearing joints may not be able to compensate for the higher load in the implanting site. The pros include no implant rejection, and decrease in pain for 70% of patients for 2-5 years (Wirth & Rudert, 1996).

b. Cartilage regeneration:

1. Regeneration enhancement: Many techniques are used, the most common being to make a full thickness defect and reach to subchondral bone allowing clot formation to facilitate MSCs to migrate to cite. The outcome is variable from formation of no cartilage, fibrocartilage, or hyaline cartilage. The outcome is highly patient dependent but usually won't harm the patient if it does not benefit him. Other techniques include the use of laser, electrical stimulation and decreased load on the joint in conjunction with continuous passive motion or administration of medication as injecting with growth factors. A lot of research is still needed to identify adequate pharmacological stimulating agents (Shawn, 1998).

2. Tissue engineering: Transplantation of chondrocytes or stem cells has been tried since 1968. The main problem facing at that time is to keep the cells in the location of the defect. Some techniques

such as the use of periosteal have been tried with unequal results. The most recent approach is to load cells onto a scaffold that can maintain the cells in place and become replaced overtime with ECM. This is a promising Technique which is relatively new and one of the problems facing this approach is the availability of cell sources and how to maintain those cells well differentiated while expanded outside the human body before re-implantation (Temenoff & Mikos, 2000).

The ability to expand chondrocytes in-vitro without losing their differentiation is the gate to this new promising treatment approach. As mentioned earlier, chondrocytes as any other cells respond to their surrounding chemical and physical microenvironment stimuli to regulate their biological processes. Due to time constraint, our study will investigate one type of physical stimulation that affects chondrocytes during expansion while trying to maintain differentiation.

1.9 Objectives

1.9.1 Main Objective

To study the effect of mechanical stimulation on chondrocytes

1.9.2 Specific objective

To study the effect of changing the frequency of bi-axial loading on chondrocytes.

Chapter 2: Literature Review

2.1 Introduction

Throughout this chapter a review of previous studies conducted on the same field will be provided. The chapter will explore the mechanotransduction beginning from tissue deformation, followed by cellular deformation and ending in intracellular deformation and response. After that, the process of chondrocytes culture and the mediums and scaffolds used, with an overview of mechanical stimulation protocols conducted in previous studies will be outlined, and the related outcome of each protocol.

2.2 Mechanotransduction

Mechanotransduction as defined earlier, is the cellular ability to generate a signal or biologic activity in response to a mechanical stimuli (Alenghat & Ingber, 2002). When a mechanical load is externally applied on a tissue, it generates a chain of effects that ends on the intracellular level by generating a response. Grossly the load causes tissue deformation accompanied by changes in its ECM and fluid components. The deformation will also be partially conducted to the cells causing their physical deformation too. Once deformation happens at the cellular level, the intracellular deformation will take place (Buschmann, Gluzband, Grodzinsky, & Hunziker, 1995). Below, a description of the process suggested to occur at each level will be outlined.

In endothelial cells integrins act as mechanoreceptors and they transfer mechanical load to the cytoskeleton that then, this tensed cytoskeleton may initiate the biologic response through the cells at multiple locations simultaneously. (Wang, et al., 1993)

2.2.1 Tissue deformation

Gross tissue deformation, resulting in a tissue strain by the stress applied on it, generates some effects other than the deformation itself, which are physiologically significant. When a tissue is at rest, the ECM, ions and charge distribution, and fluid flow through it, with all types of generated pressures by the different components are in a state of dynamic equilibrium (Y.-J. Kim, Bonassar, & Grodzinsky, 1995; Villanueva, Gladem, Kessler, & Bryant, 2010). Once an external load that is strong enough to cause tissue deformation is applied, the arrangements of ECM molecules will change, squeezing fluid, and ions moving them to a lower pressure locations and redistributing ions and freely moving charged molecules till reaching a again a state of dynamic equilibrium. This is true for a case of static compression (figure 2.1).

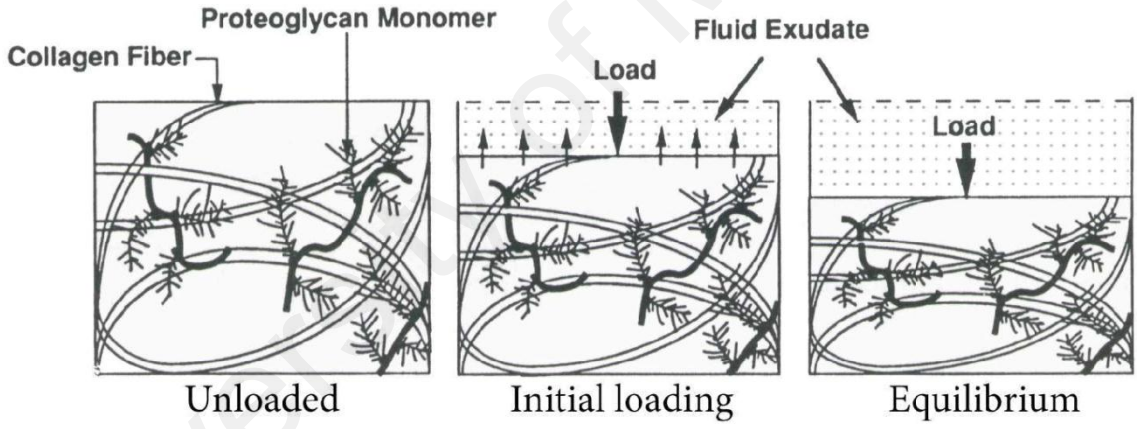

Figure 2. 1: Schematic illustrating the movement of fluid out of the cartilage tissue during compression. Reproduced from. Reproduced from (Geffre, 2010)

Studies has revealed that during static compression of chondrocytes, even the tissue is under increased load, and transport of nutrient, gases, and other molecules are slower, it does not appear that this transport related factor really plays a significant role in chondrocytes response indicating that probably the response is being mediated at lower level (i.e. cellular or intracellular deformation) (Buschmann, et al., 1995).

However, because joint activity in real life includes the cyclic pattern of loading, most investigating studies have rather focused on the dynamic stimulation patterns. During dynamic loading of chondrocytes in unconfined space, the generated fluid flow, streaming potentials, cell matrix interaction appeared to be more important than small increase in fluid pressure, altered molecular transport and matrix independent cellular deformation. To study the effect of fluid flow (transport), a comparison of dynamic loading under confined and unconfined space was done. Fluid flow have been shown to be proportionally related to increase in prostaglandins synthesis, in addition the generated streaming potentials have also been linked to the process but with no clear understanding of the mechanism behind it (Buschmann, et al., 1995; Y.-J. Kim, et al., 1995; Y. J. Kim, Sah, Grodzinsky, Plaas, & Sandy, 1994).

2.2.2 Cellular deformation

This is probably a more curious level of the mechanotransduction pathways. Chondrocytes metabolism towards production of collagen and/or proteoglycans have been shown to increase when compressed dynamically within physiologic ranges (Buschmann, et al., 1995; Chowdhury, Bader, Shelton, & Lee, 2003; Davisson, Kunig, Chen, Sah, & Ratcliffe, 2002; Démarteau et al., 2003; Jin, Frank, Quinn, Hunziker, & Grodzinsky, 2001; D. A. Lee, Noguchib, Freanc, Leesc, & Badera, 2000; Mauck, et al., 2000; Villanueva, et al., 2010; Stephen D. Waldman, Spiteri, Grynpas, Pilliar, & Kandel, 2003). It is important to note that this cellular stimulation is highly dependent on the amount of ECM present around the chondrocytes indicating the need for some interaction between cell surface receptors and some of the ECM components (Buschmann, et al., 1995) (figure 2.2).

When chondrocytes suspended in agarose gel are compressed statically, they show inhibition of synthesis of collagen and/or prostaglandins (Buschmann, et al., 1995; Davisson, et al., 2002; Plumb & Aspden, 2005; Wong, et al., 1999). In addition, the

mechanotransduction pathway seems to be dependent on the presence of ECM surrounding the chondrocytes, suggesting that the mechanical and/or receptor mediated pathway activation is dependent on the physicochemical components of ECM that will probably be increase in NA^+ and decrease in pH in the case of static compression (Buschmann, et al., 1995).

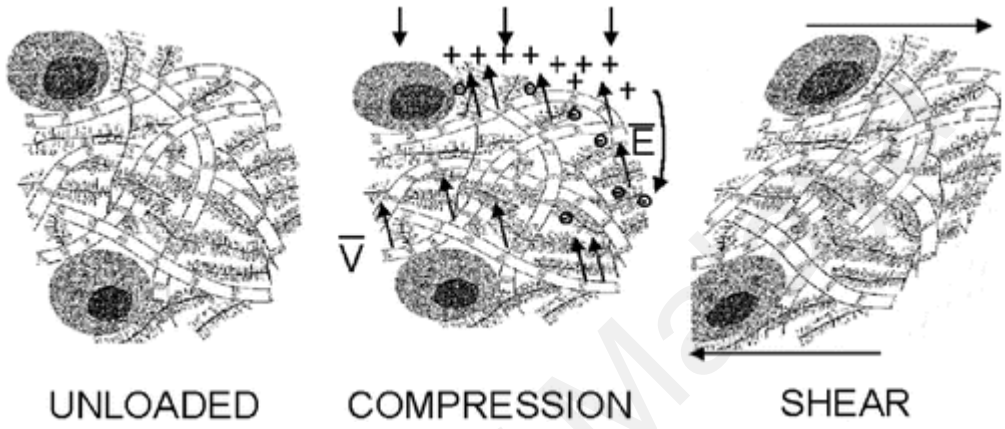

Figure 2. 2: Chondrocyte deformation under different loading patterns, showing changes in ECM molecules orientation and change in cell shape. Reproduced from (Alan J. Grodzinsky, Levenston, Jin, & Frank, 2000)

Interestingly, when chondrocytes are seeded into the agarose gel constructs collagen and/or proteoglycan synthesis whether it is stimulated by dynamic compression or inhibited by static compression, there is a strong association with the presence of ECM directly surrounding the chondrocytes. While the presence of this ECM limits significantly the amount of cellular deformation by forming a partial protective shield around it, yet it apparently plays the major role in the presence of chondrocyte response to whatever the type of mechanical stimulation is (Buschmann, et al., 1995). It is therefore suggested for an experiment to be conducted for at least 3 days to allow enough ECM deposition and chondrocyte better response.

2.2.3 Intracellular deformation

In endothelial cells integrins act as mechanoreceptors and they transfer mechanical load to the cytoskeleton that then, this tensed cytoskeleton may initiate the biologic response through the cells at multiple locations simultaneously (Wang, et al., 1993). For

chondrocytes, as mentioned earlier, intracellular physical deformation caused by cell compression does not seem to have a strong mechanotransduction pathway that is fully independent from the presence of ECM.

When chondrocytes are compressed, its intracellular organelles get deformations in shape accordingly, and this is suggested to be conveyed through the cell cytoskeleton. However, changes in volume of the organelles seem to be more dependent on the osmotic pressure. The organelles appear to have an important role in linking the translational stage to post-translational stage of mechanotransduction that are represented by elongation by elongation and sulfation of GAG in the Golgi apparatus for example (Szafranski, et al., 2004).

Nitric oxide has been shown to have a strong effect as inhibitor for chondrocytes proliferation (Blanco & Lotz, 1995) and synthesis of proteoglycans (Taskiran, Stefanovicracic, Georgescu, & Evans, 1994). It has also been suggested that it plays an intermediate role in the mechanotransduction of chondrocytes especially the cells sub-population isolated from the superficial zone (D. A. Lee, et al., 2000)

2.3 Culture

2.3.1 Medium

Among different studies, minimal variations in the composition of mediums were found. Dulbecco's modified Eagle Medium (DMEM) is obviously the main component with a 10 or 20% Fetal Bovine Serum (FBS) (Buschmann, et al., 1995; Chowdhury, et al., 2003; Démarteau, et al., 2003; D. A. Lee, et al., 2000; Pingguan-Murphy, Lee, Bader, & Knight, 2005; Plumb & Aspden, 2005; Szafranski, et al., 2004; Villanueva, et al., 2010). The use of FBS is to provide growth factors and other stimulating factors present in serum; this is why when attempting to study the effect of growth factor alone,

FBS is not added and instead only the growth factor to be investigated is used (Elder & Athanasiou, 2009).

Some other components that are added to the medium include antibiotics or fungicides to prevent infections, and those are usually adopted according to the need of each specific lab. With the progress of culture, cells metabolism results in disposal of CO_2 leading to increased acidity of medium. As it is more convenient in studies to prepare one standard medium and use for multipurpose (i.e. culture and enzymes preparation), it is better to add a buffer agent that maintains medium pH so that even when used for enzymatic digestion, the pH will be maintained within the active range for the enzymes (Baicu & Taylor, 2002). L-glutamine is of benefit when added to supplement nitrogen in addition to possibly be an assisting energy source as an amino acid for the rapidly multiplying (Nawi, 2011).

2.3.2 Agarose gel

To study effects of mechanical stimulation on chondrocytes, this can be done either by extracting cartilage explants and performing the study on them, or by isolating the cells and then putting them in any type of structure or scaffold that is appropriate. When focusing more on studying the role of mechanical stimulation without the interfering effects of ECM bulk, the second method where chondrocytes are isolated and then cultured on scaffold is used.

Agarose compression have been shown to cause deformation of chondrocytes seeded in it (Buschmann, et al., 1995; Freeman, Natarajan, Kimura, & Andriacchi, 1994). Agarose gel has shown great similarities to hyaline articular cartilage in terms of conduction of mechanical load to the chondrocytes. It seems to preserve some physiologic properties of the chondrocytes and thus can be used for testing physical (David A. Lee et al., 2000; D. A. Lee, et al., 2000) and chemical simulative factors in a

more controlled manner and also fairly similar in responses to that produced with same mechanical stimulation given to cartilage explants. The flexibility that agarose allow, is very suited for studies of mechanotransduction pathways studied at the level of tissue deformation (Buschmann, et al., 1995).

2.3.3 Protocols of stimulation

The type of mechanical loads applied on articular cartilage during normal joints movement are rather complex and if attempted to be studied in a general way, it becomes very complicated. Also it is difficult to replicate physiologic movement in vitro, and it is easier to break down the complex mechanical loading of joint into more simple basic components. This is why many researchers started to apply only one type of mechanical stimulation being either direct compression (Buschmann, et al., 1995; Chowdhury, et al., 2003; M. Knight, Lee, & Bader, 1998; David A. Lee, et al., 2000; D. A. Lee, et al., 2000; Mauck, et al., 2000) or shear (Jin, et al., 2001).

Many researchers apply loads that fall within physiologic range, the amplitudes of mechanical compression ranges from 10-15% and for shear from 1-5%, and the frequencies range from 0.1 – 1Hz in general. From previous studies, the main finding is that dynamic compression causes stimulation of protein and/or GAG synthesis (Buschmann, et al., 1995; Chowdhury, et al., 2003; Davisson, et al., 2002; D. A. Lee, et al., 2000; Mauck, et al., 2000; Villanueva, et al., 2010), this was also true for dynamic shear (Jin, et al., 2001).

Their maybe an age related dependency on how effectively can chondrocytes produce ECM when stimulated by dynamic loading as *in-vitro* dynamic loading was shown not to be able to induce an increase in ECM synthesis when applied on chondrocytes from elderly human cartilage (Plumb & Aspden, 2005).

As most studies have investigated the uniaxial type of stimulation and a documentation of its effects was made, biaxial stimulation is now more appealing as a step to upgrade the in-vitro testing environment to be closer towards physiologic mechanical environment. Only few studies have investigated the biaxial model, with results being also showing that dynamic biaxial loading stimulates collagen and prostaglandin synthesis and that this stimulation is dependent on magnitude of applied compression and shear (S. D. Waldman, Couto, Grynpas, Pilliar, & Kandel, 2007). In a study comparing biaxial to uniaxial dynamic loading, it was found that biaxial stimulation provides a 1.5 times higher GAG synthesis in comparison to the uniaxial stimulation method and that a 24hr pre-culture period improved chondrocytes response (Nawi, 2011).

As biaxial stimulation is showing beneficial results, it would be of good benefit to explore the effect of changing the frequency on the response of chondrocytes as studies has shown that there is a correlation between frequency of uniaxial stimulation and the response of chondrocytes (Davisson, et al., 2002; David A. Lee, et al., 2000; D. A. Lee, et al., 2000; Shelton, Bader, & Lee, 2000; Soltz & Ateshian, 2000).

Chapter 3: Methodology

3.1 Introduction

This chapter describes the method by which our experiment was conducted, starting by description of medium and enzymes used, method of chondrocytes isolation, the preparation of chondrocyte seeded agarose gel constructs, the mechanical system used for the stimulation, the protocol used for conducting the mechanical stimulation and description of the biochemical assays used. The chapter will finish by a description of the statistical analysis preformed to assess the results.

3.2 Culture medium and enzymes

The culture medium was prepared by adding the ingredients shown in (Table 3.1):

Table 3. 1: Ingredients used in medium preparation showing the amount of each ingredient.

Component	Quantity	Concentration used	Brand
Dulbecco's Modified Eagle Medium (DMEM)	500ml	Stock	Sigma-Aldrich, Malaysia
Fetal Bovine Serum (FBS)	100ml	Stock	
Penicillin/Streptomycin	5ml	100units.ml^{-1}/ 10mg.ml^{-1}	
L-Glutamine	5ml	2mM	
Hepes buffer	10ml	2mM	
L-Ascorbic Acid	0.075g	Stock	Duchefa Biochemie

Enzymatic digestion is essential for us as we need to take chondrocytes free of ECM and suspend them in the agarose gel constructs. Different methods have been used before for enzymatic digestion, with usually having a two steps digestion first as a pre-digestion and second step that is considered overnight digestion. In pre-digestion protease is usually used to allow disintegration of ECM matrix and there by easier access of the collagenase to collagen fibrils (Buschmann, et al., 1995; Chowdhury, et al., 2003; David A. Lee, et al., 2000; Nawi, 2011; Nawi & Pingguan-Murphy, 2009 ; Pingguan-Murphy, et al., 2005; Plumb & Aspden, 2005). Two consecutive enzymatic digestions scheme is used in our study starting by 1hr protease followed by 16hr collagenase. Both enzymes are prepared by dissolving the enzyme in the culture medium. For digesting harvested cartilage of each joint a 10ml protease and 30ml collagenase preparations were used (Table 3.2). Culture medium and enzymes used were all passed through a 0.22μm pore cellulose-acetate filter before divided into aliquots.

Table 3. 2: Enzymes aliquots amounts, concentrations, and the duration of their usage.

Enzyme	Aliquot	Concentration used	Duration used	Brand
Protease	10ml	20unit/ml	1hr	Sigma-Aldrich, Malaysia
Collagenase	30ml	200unit/l	16hr	Worthington Biochemical

3.3 Chondrocytes isolation

Many techniques of cell isolation have been tried. Tissue type from which the cells are to be isolated is a big determinant on the method to be used as different tissues have different compositions of ECMs and to allow the release of cells from their matrices the isolation technique used should be appropriate.

Theoretically the source of chondrocytes can be either from human or animal source. Human source is extremely limited, and does not provide a real practical option, especially as the present work is purely for preliminary research purposes and does not involve running any clinical trials. Animal sources on the other hand, provide better availability and most researchers currently use bovine cartilage from different weight-bearing joints of the cow (Chowdhury, et al., 2003; Elder & Athanasiou, 2009; Fan & Waldman, 2010; Szafranski, et al., 2004; S. D. Waldman, et al., 2007; Stephen D. Waldman, et al., 2003).

For chondrocyte isolation, cartilage extraction from a load bearing joint is done first, and then followed by enzymatic digestion where in this process cells are detached from their ECM and released free in suspension. Different research centers tend to use different techniques for chondrocytes isolation, this is usually due to the need of optimization of the isolation method to the best suited due to possible variations in tissue compositions depending on differences in genes and environmental living conditions including diet. And as it is important to use fresh cartilage explants, there is a need to depend on locally available cows, therefore the laboratory where the present work was carried out has developed an optimized method for isolation of chondrocytes from *bos indicus* which is locally available at the abattoir (Nawi, 2011).

The method employed is as published in (Nawi & Pingguan-Murphy, 2009).

From the locally available abattoir at the Department of Veterinary Services, Shah Alam, Selangor, metacarpal-phalangeal joints were collected freshly after slaughter, brought to the lab, washed thoroughly with water and soap, then soaked in 70% Industrial Methylated Spirits (IMS) for about 15 minutes. Under sterile conditions inside class II laminar hood, joints were dissected and joint surface exposed. Full thickness cartilage was removed in slices from the proximal surface, and immersed in DMEM + 20% inside 60mm petri dishes until finishing the removing of whole cartilage from the surface.

Culture medium is then aspirated leaving the cartilage slices which are then transferred into the prepared protease for 1hr for pre-digestion at 37°C inside the hybridization oven where they are continuously rolled to ensure better exposure of cartilage slices to protease. After that, protease preparation is aspirated and replaced with collagenase preparation and put again in hybridization oven at 37°C for 16hr overnight digestion.

After the overnight digestion was done, the supernatant was filtered using a 70μm cell strainer (BD Bioscience, Malaysia). After that the remaining supernatant is centrifuged at 2000rpm for 5 min, supernatant is then removed carefully without disturbing the formed palate and replaced with culture medium for washing. This process of centrifugation and wash is done a total of 3 times for a better removal of the enzymes remnants. After third centrifugation, the palate is re-suspended in 10ml of culture medium using needle and syringe to allow better separation of cells and to prevent formation of clumps.

The cell count measure, using a haemocytometer and trypan blue dye exclusion is done by mixing 20µl of cell suspension with 20µl of trypan blue and suspending them together, then put on haemocytometer under light microscope where cells present on the field are counted visually at 10x power lens. Dead cells appear dark blue as their nucleus stains with the dye. This process is repeated three times and the three total cell counts are averaged to one. The total cell count is done using this equation:

$$\text{Number of cells per ml} = \text{Number of cells per mm}^3 \times \text{dilution factor} \times 10\,000$$

Equation 3.1: Neubauer Ruling

3.4 Agarose gel constructs

After cell counting, if the viability is 90% or above, a cell suspension of $8x10^6$cells.ml^{-1} is prepared. Agarose (Type VII, Sigma-Aldrich, Malaysia) in a concentration of 8% (w/v) was used with EBSS according and prepared according to a previously published method (M. M. Knight, Toyoda, Lee, & Bader, 2006), followed by autoclaving to give a better mixing and sterilization. Then the mixture was put on the roller mixer until the temperature of the gel is reduced to about 37°C. Cell suspension was then added to an equal volume of 8% agarose to get a final desired concentration of cell suspension in agarose of $4x10^6$cells.ml^{-1}. To allow proper mixing, the cell-agarose mixture was allowed to roll on the roller mixer further.

The mold and bioreactors used in this experiments are mentioned in previous publications (Yusoff, Abu Osman, & Pingguan-Murphy, 2011) and all tools used were sterilized prior to use. Agarose was then pipetted and casted carefully to avoid formation of air bubbles between two pores glass bars into the sterilized Perspex mold (Nawi, 2011). Six constructs are formed all of same size of 5mm × 5mm × 5mm (H×W×L). The constructs are allowed to gel at 7°C for few minutes. After gelling, compression and shear bracket were mounted onto their respective porous glass bars

and secured to it by polyethylene screws. By using a specially designed handle, the whole "cell-agarose constructs/ porous glass bars/ compression and shear brackets" were transferred into the bioreactor chamber fitted onto their respective rails, and handle was then removed. The bioreactors chamber was then assembled and then 150ml medium was added through a specific opening in the bioreactor lid totally immersing all constructs. The bioreactor was then transferred to incubator and the 3 motors (2 for compression + 1 for shear) and their water cooling system were assembled and attached to the controller and water pumps outside the incubator (Figure 3.1).

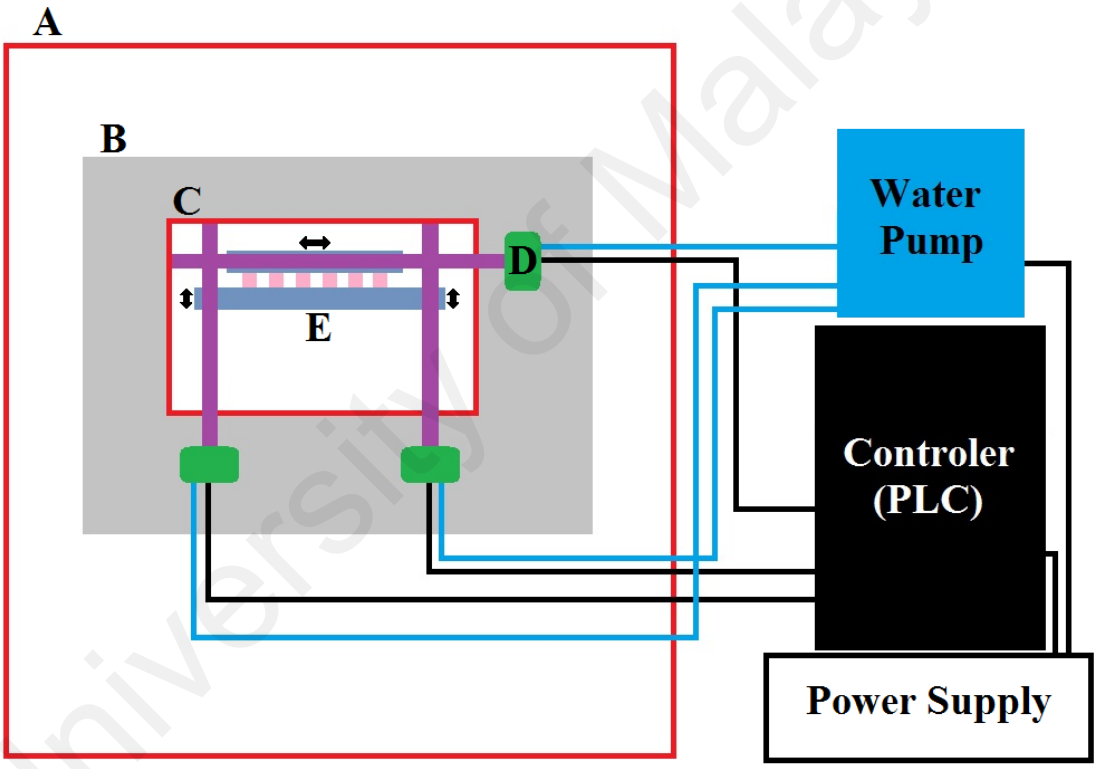

Figure 3. 1: Schematic diagram showing the setup of bioreactor inside the incubator, direction of bracket movements and external connections; A: Incubator; B: Culture tray; C: Bioreactor; D: Stepper motors; E: Cell-agarose constructs attached to brackets.

The unstrained samples were prepared with similar technique but using a different Perspex mould where each construct was attached to two independent porous glass endplates. After gelling constructs were put into a 6 well culture plate, immersed with culture medium, and transferred into incubator.

3.5 Protocol of mechanical stimulation

The mechanical stimulations conducted in previous studies were variable within physiological range. As our work is an extension for our lab team previous studies, the protocol adapted was closely related to it (Nawi, 2011). Three different frequencies were tested (0.5Hz, 1Hz, 1.5Hz) with fixed 10% compression and 1% shear at the three different frequencies. The strain rate at the different frequencies is show in table 3.3.

Table 3. 3: Compression and shear strain rates in relation to frequencies tested.

Frequency	Strain rate	
	Compression	Shear
0.5Hz	1mm/sec	0.1mm/sec
1Hz	0.5mm/sec	0.05 mm/sec
1.5Hz	0.333 mm/sec	0.033 mm/sec

After fixing the whole setup shown in figure 3.1 inside the 37.2 °C and 5% CO_2 incubator, the chondrocytes were left for 24hr of rest as pre-culture and to allow free swelling of agarose constructs without any compression or shear. After the end of 24hr, stimulation was conducted in a 12hr ON – 12hr OFF pattern for a total of 48hr. The stepper motors used were instructed through the programmable logic controller (PLC) to produce the Sin wave compression and shear movements. The medium was changed at day 2 from the time of seeding the chondrocytes into the agarose.

3.6 Biochemical assays

3.6.1 Digestion of chondrocytes agarose constructs

After the end of the experiment, constructs were separated inside the laminar hood from their corresponding porous glass carefully using sterile spatula. Prior to be able to measure the GAG and DNA components, constructs were digested as following.

The constructs were broken into 2 pieces inside bijou tubes and 1ml digest buffer was added and then transferred into oven at 70°C and kept there for 1hr. After that, constructs were allowed to cool to around 37°C and 10 $U.ml^{-1}$ of agarase and 2.8 $U.ml^{-1}$ of papain were added to each construct and then left for overnight digestion at 37°C. After that a further digestion at 60°C for 1hr was allowed (Chowdhury, et al., 2003).

Out of each digested construct, 1ml was aspirated and centrifuged at 10000g at room temperature. Then DNA and GAG assays were conducted.

3.6.2 Deoxyribonucleic acid (DNA) assay

The amount of DNA content was measured using the Hoescht33258 assay which is a commonly used method (Steve, 2011; S. D. Waldman, et al., 2007). The Hoescht33258 dye is DNA specific and it fluoresces upon binding to DNA. A stock solution of 2mg Hoescht33258 per milliliter of distilled $H_2O$was prepared and stored at 4°C in a light tight bottle. Just before doing the assay a working solution is prepared by 10000× diluting the stock solution into TEN buffer (10mM Tris-HCL, 1mM EDTA and 100mM NaCl, pH 7.5) which was also kept in a light tight bottle. This protocol was adapted from (Hoemann, 2004).

To be able to correlate the values of the Hoescht33258 assay with real DNA amount present in samples, a standard DNA is needed. Calf thymus DNA standard is usually used as the reference value when assessing plant and animal DNA contents (Hoemann, 2004). Calf thymus DNA (D1501, Sigma-Aldrich, Malaysia) was used and serial double dilutions from 25 to 0.781µg/ml were prepared in PBE buffer.

A standard curve was made from the serial dilutions of the calf thymus DNA standard to assure its validity, and PBE with Hoescht33258 was used as the blank. FLUOstar Optima microplate fluorometer was used to read the fluorescence level. Settings were adjusted for excitation at 355 nm and for emission at 460 nm (Hoemann, 2004). In a 96-well plate (TPP, Malaysia), 10µl of each sample, standard, and PBE were put in triplicates and toped up with 200µl working solution using a multichannel pipette.

3.6.3 Glycosaminoglycan (GAG) assay

The amount of GAG content was measured using the dimethylmethylene blue (DMB) assay that uses the DMB dye. This dye causes metachromasia when cationic binding of it happens with the sulphate and carboxyl groups present in GAG chains. This Metachromasia from blue to purple is produced by change in pH and salt concentration of the dye produced by the cationic binding (Bronzino, 2006; Goldberg, 1990).

To prepare the assay, 0.016 g DMB (Sigma-Aldrich, Malaysia) was dissolved in 5.0ml 99.7% Ethanol (Sigma-Aldrich, Malaysia) inside a capped glass scintillation vial that is foil-wrapped. A magnetic stirrer (WiseStir MSH10, Daihan Sci., Malaysia) was used for proper mixing. Using a separate clean bottle, 2.00 g of sodium formate was dissolved in 950mL of autoclaved double-distilled H2O (Farndale, 1982). The DMB solution was mixed with the NaCl-glycine solution. The residue of the DMB solution was rinsed with 200µL ethanol. Concentrated formic acid (99%) was used until achieving pH 3.00

(Bronzino, 2006). The solution was topped up with ddH2O until 1L and then stored in a foil-covered bottle at room temperature.

Same as for the DNA assay, GAG assay also needs a standard to get valid readings. The standard used here is chondroitin-4-sulphate standard (C6737, Sigma-Aldrich, Malaysia). Prior to use, chondroitin-4-sulphate was suspended in dH_20 at a concentration of 0.5mg/ml, put in 500µL aliquots and stored at -20 °C. When needed, an aliquot was thawed, vortex mixed and serial double dilutions from 25 to 0.781 µg/ml were prepared.

A standard curve was made from the serial dilutions of the chondroitin-4-sulphate standard to assure its validity and dH_2O and DMB was used as the blank. The FLUOstar Optima microplate fluorometer was used to read absorbance level. Values of OD520-OD590 should be in the range of 0.65 to 0.8 to validate the standard (Nawi, 2011). The fluorometer was set for detecting wavelengths of absorbance between 520 to 590 nm (Yun, 2008). In a 96-well plate (TPP, Malaysia), 10µl of each sample, standard, and PBE were put in triplicates and topped up with 250µl DMB using a multichannel pipette.

3.7 Statistical analysis

Using SPSS ver.18, normal distribution of amount of DNA and GAG was verified by Skewness and Kurtosis methods (sample is normal if value of Skewness and Kurtosis is in the range of -1.96 to +1.96). When results from different runs for the static or a certain frequency of stimulation shows normal distribution, the amount of DNA and GAG can be averaged to give a representative value for each of the frequencies tested and for the static controls alike.

Test of significance for the difference in the results between each group of samples can be done if each sample group is normally distributed. One-way analysis of variance (ANOVA) was used to compare the GAG, DNA, and GAG/DNA production by each group of constructs stimulated by a certain frequency and between them and the static control also. A value of $p<0.05$ was used to indicate statistical significance (Johnson, 2010).

Chapter 4: Results

4.1 Introduction

In this chapter collected data will be presented in a comprehensive way and because in the present study a biaxial mechanical stimulation bioreactor was used under different frequencies of stimulation, the results will be organized by measuring the DNA and GAG products in regard to the frequency of stimulation.

4.2 Measuring cell multiplication and ECM production.

Using the biaxial bioreactor to apply mechanical stimulation according to the protocol mentioned earlier in methodology section, two separate experiments were conducted to measure each frequency. In each experiment six constructs were fitted into the biaxial bioreactor resulting in a total of twelve constructs for each tested frequency and eighteen constructs for the control frequency (Table A.1).

By using the measurement of DNA quantity as a reflection of cell proliferation and the measurement of GAG quantity as representative of ECM production the following readings were found.

4.2.1 Effect of biaxial stimulation frequency changing on the amount of DNA

Cell proliferation under static culture was found to be higher than under any of the stimulation frequencies as shown in Figure 4.1. However, the difference in DNA content was not found to be of statistical significance except comparing static group to 1Hz group (Table A.5).

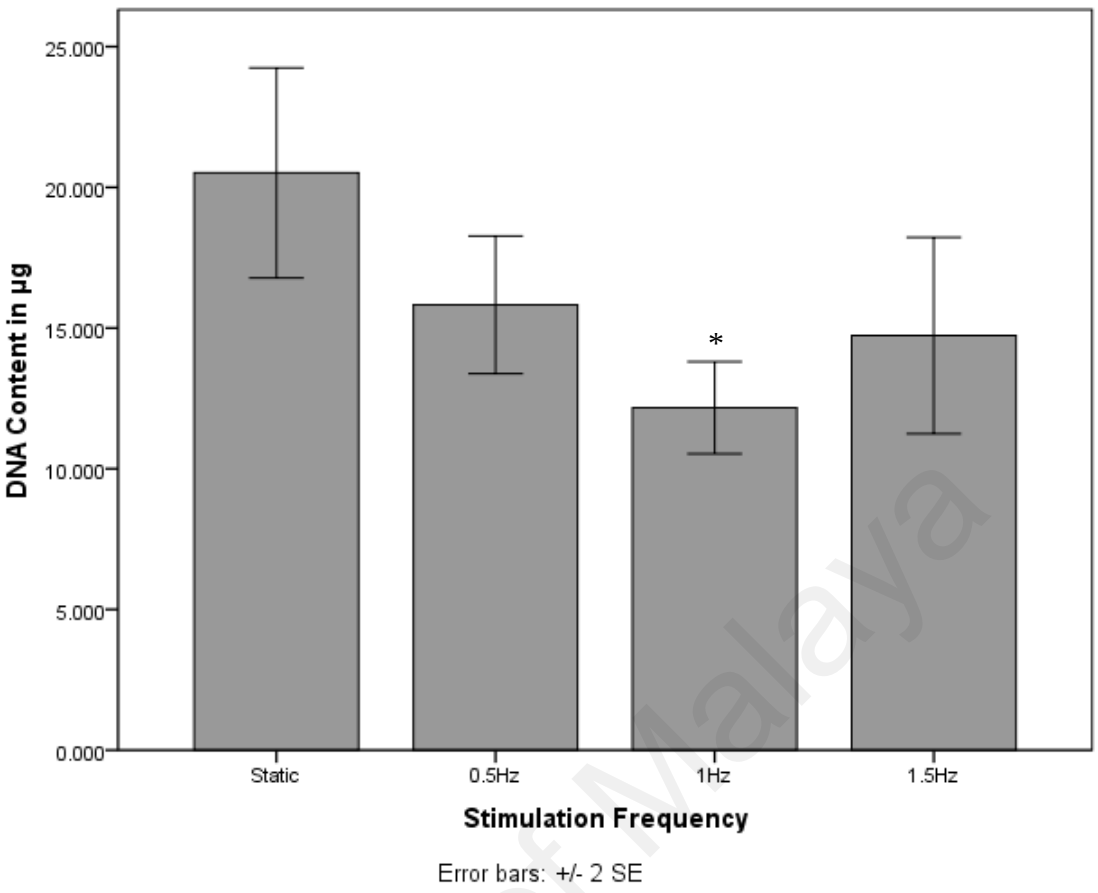

Figure 4. 1: The effect of biaxial compression at different frequencies on the DNA content, shown as means compared to static control group. Error bars represent SEM of replicates (n= 12-18).
***. Difference statistically significant compared to static group (p<0.05).**

4.2.2 Effect of biaxial stimulation frequency changing on the amount of GAG

The GAG content on the other hand was found to be much higher under mechanically stimulated cultured constructs in comparison to static culture with a statistical significant difference (Table A.6). The highest GAG content in the constructs was found at 0.5Hz stimulation frequency with being more than 4 times higher than in static culture (Figure 4.2).

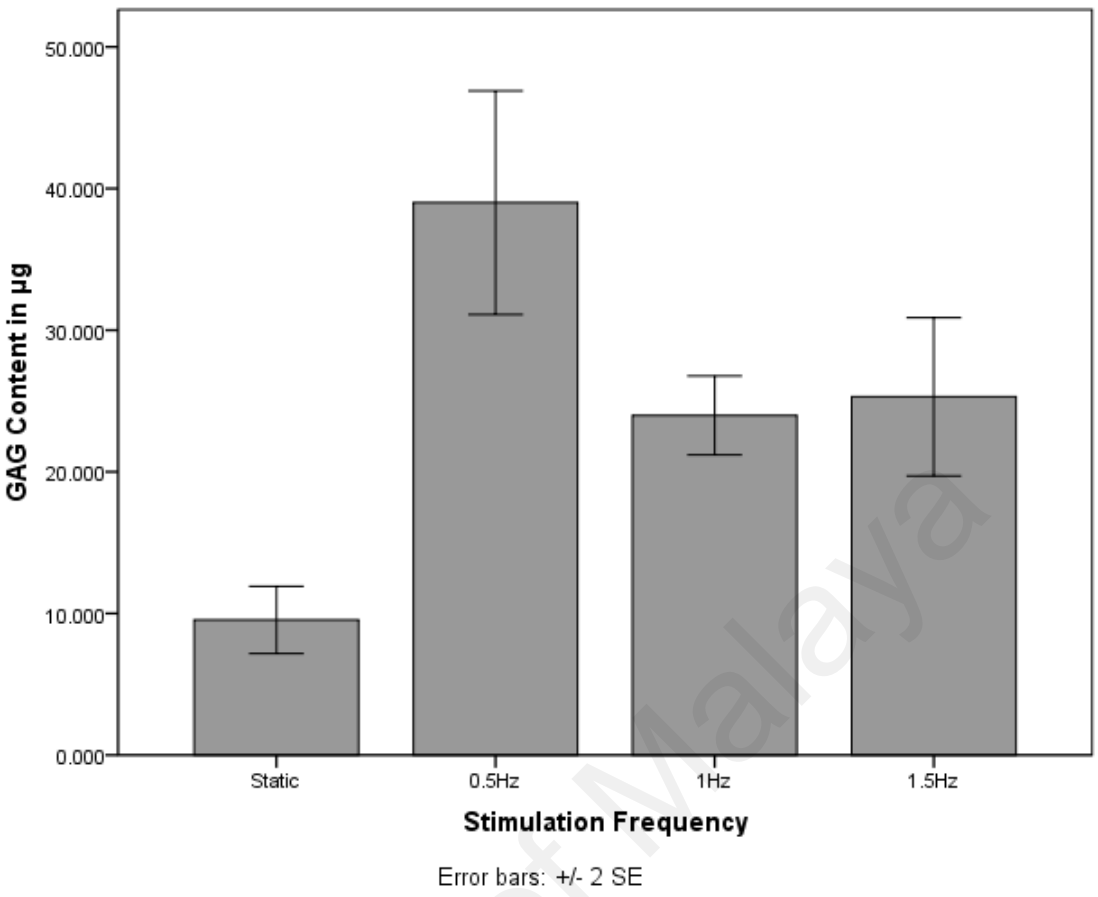

Figure 4. 2: The effect of biaxial compression at different frequencies on the GAG content, shown as means compared to static control group. Error bars represent SEM of replicates (n= 12-18). Difference statistically significant between all groups except 1Hz compared to 1.5Hz ($p<0.05$).

4.2.3 Effect of biaxial stimulation frequency changing on the GAG/DNA ratio

The GAG/DNA ratio is a measurement used to evaluate the cell metabolic activity in producing ECM and reflects the cell's ability to maintain differentiation, where higher values of GAG/DNA reflects better cell differentiation. Figure 4.3 shows again that all constructs under mechanical stimulation had a better cell differentiation maintenance throughout the culture period (Figure 4.3), with a value of GAG/DNA that is statistically significantly higher than constructs under static culture (Table A.7).

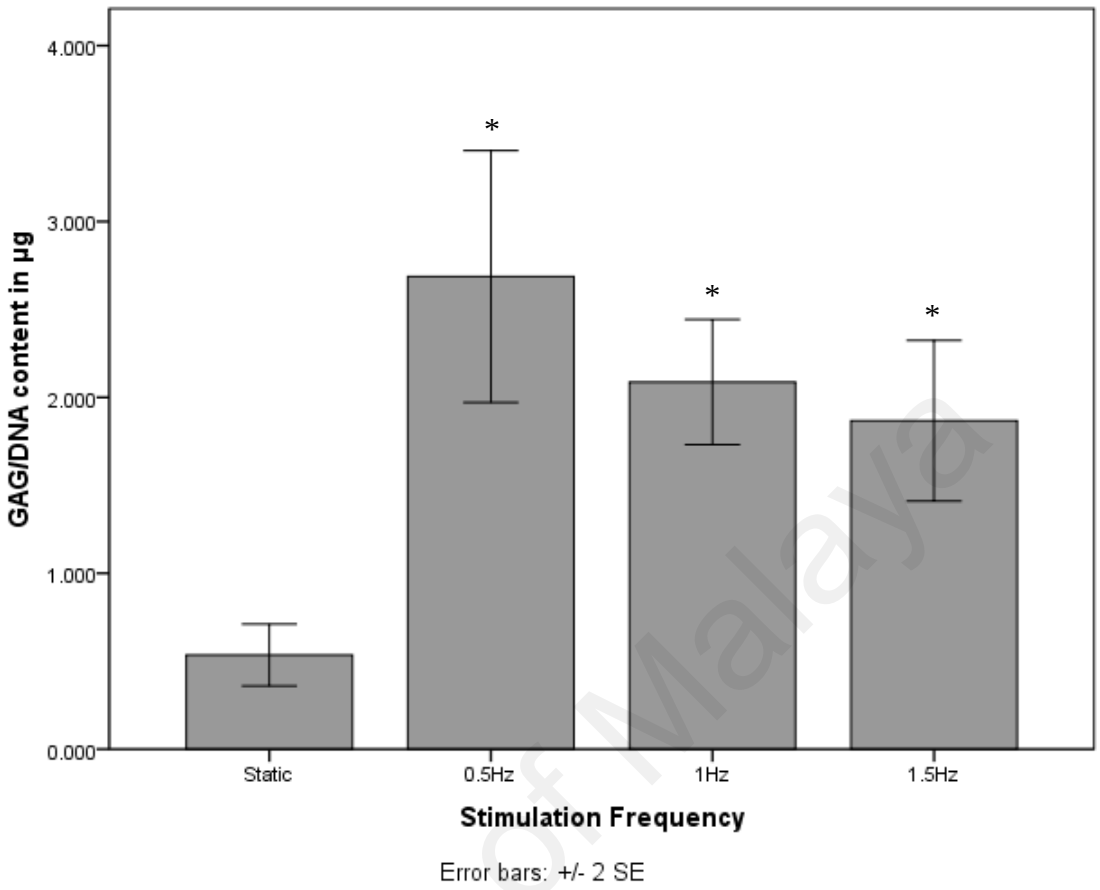

Figure 4. 3: The effect of biaxial compression at different frequencies on the GAG/DNA content, shown as means compared to static control group. Error bars represent SEM of replicates (n= 12-18).
***. Difference statistically significant compared to static group (p<0.05).**

Chapter 5: Discussion

5.1 Introduction

Through this chapter a detailed analysis of the data will be carried out by comparing our results with previous studies to try to get a better understanding of the effect of mechanical stimulation on the chondrocytes and how the chondrocytes respond to changes in the frequency of the stimulation. The chapter will be approached by discussing results in regard to the variable being measured.

5.2 Analysis of DNA results

In a previous study, where the experiment was run for a long period, mechanical stimulation was found to be stimulatory for DNA synthesis by chondrocytes (Buschmann, et al., 1995). In the current study the DNA synthesis was found to be suppressed in constructs under mechanical stimulation, this was also found in a previous study done by our group (Nawi, 2011). This is believed to be due to the short (3 days) run time of the experiment which may be caused by the effect of mechanical stimulation on delaying the entrance of chondrocytes into the s-phase of the cell cycle, decreasing the possibility of cells multiplication and hence is more in favour of ECM production rather than DNA synthesis (Chowdhury, et al., 2003).

5.3 Analysis of GAG results

Mechanical stimulation of chondrocytes has a direct effect on their metabolic activity, as it has been shown in previous studies that cartilage explants exposed to dynamic mechanical loading stimulate GAG synthesis (Guilak, Meyer, Ratcliffe, & Mow, 1994; Jin, et al., 2001; Plumb & Aspden, 2005; Szafranski, et al., 2004; Wolf, et al., 2007; Wong, et al., 1999). Chondrocytes seeded in agarose gel constructs also have been proven to have a better GAG synthesis when exposed to dynamic mechanical stimulation (Buschmann, et al., 1995; Chowdhury, et al., 2003; D. A. Lee, et al., 2000).

Our findings are in conformity with those of the previous studies (Chowdhury, et al., 2003; Nawi, 2011; S. D. Waldman, et al., 2007), where in our study GAG synthesis was more than 4 times higher at 0.5Hz and around 2.5 times higher under 1Hz and 1.5Hz.

It has been suggested in a previous study that GAG synthesis under uniaxial dynamic stimulation is frequency dependant and that there is a window for effective frequencies where lower cut-off frequency lies somewhere between 0.3Hz and 1Hz and upper cut-off frequency lies somewhere between 1Hz and 3Hz (D. A. Lee, et al., 2000). In our experiment, we found that under biaxial mechanical stimulation, 0.5Hz gave a significant ($p<0.05$) increase over either static, 1Hz and 1.5Hz; however, all three frequencies used were still significantly effective in stimulating a higher GAG synthesis in comparison to static, which indicates that under biaxial stimulation the 0.5Hz-1.5Hz range represents a zone of effective stimulation frequencies and that cut-off frequencies if present, will be outside this range. The possible reason for GAG at 1Hz and 1.5Hz to be lower than 0.5Hz is inferior stimulating potential, another possibility being that there is probably a higher amount of GAG leakage from the construct into the medium resulting in a false low reading (Chowdhury, et al., 2003; D. A. Lee, et al., 2000).

5.4 Analysis of GAG/DNA results

GAG/DNA is used to correlate between the available number of cells and their ability to produce ECM. A higher GAG/DNA reflects a metabolically active chondrocyte that is able to produce its own type of ECM indicating further the likeliness of it to be maintained as differentiated chondrocyte. As shown in the results of this study, biaxial loading represents a good stimulator for chondrocytes to be metabolically active in producing their ECM. It also shows that there was no statistical significant ($p<0.05$) difference in the amount that all frequencies stimulated the chondrocytes; however, stimulation with 0.5Hz provided the highest stimulation compared to the other two

frequencies used. Probably a longer duration of the culture under the three different frequencies of mechanical stimulation would have better allowed evaluation of any difference in the amount of GAG and GAG/DNA production, as it is suggested that the more ECM is laid down around chondrocytes, the better their response to mechanical stimulation becomes (Buschmann, et al., 1995). It is noticed from the results collectively that GAG production is not related to the cellularity of the constructs, but rather mainly to the presence of mechanical stimuli, indicating that mechanotransduction is a process that is possibly perceived by the cells individually, mainly depending on presence of ECM matrix around each cell rather than possible well developed cell to cell interaction. The better GAG/DNA results found at 0.5Hz also indicates that mechanotransduction even though is suggested to be more dependent on the effect of the streaming potentials generated by the flow of fluid rather than being dependent on the flow of fluid itself (Villanueva, et al., 2010), it is possible that mechanotransduction is dependent on how long the cycle of streaming potentials last, where at lower frequencies the cycle will take longer and probably allow more time for the cell to sense its presence and react to it.

Chapter 6: Conclusion and future work

6.1 Conclusion

Tissue engineering is rapidly developing and the amount of knowledge about cellular responses is being expanded. Chondrocytes are very specific and sensitive cells that not only respond to chemical stimuli, but also to mechanical stimuli through a process known as mechanotransduction. The importance of mechanotransduction to chondrocytes is not only to regulate their metabolism but is also essential for them to maintain their differentiation. Biaxial stimulation is superior to uniaxial stimulation as it is thought that it better mimics physiological microenvironment phenomena. The change in frequency of biaxial mechanical stimulation showed some difference in the GAG and DNA production. A frequency of 0.5Hz represented the best stimulation frequency. According to the findings of this study, we may speculate that a worth to explore way of joint physiotherapy is to perform joint movement at slow frequencies that can probably provide a natural way to allow joint remodelling.

6.2 Suggestions for future work:

- To expand the time of culture runs to see how the DNA production response will be and to allow a better chance for the frequencies to show if any possible statistically significant different effects they have on chondrocytes seeded in agarose gel under biaxial stimulation.
- To measure the GAG in medium as well to evaluate if there is any significant leak of GAG from the chondrocyte agarose constructs caused by the mechanical stimulation.

Chapter 7: References

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Appendix: SPSS Raw Data and Analysis

Table A. 1: Raw data of chondrocyte seeded agarose gel constructs content of DNA, GAG according to stimulation frequency.

Chondrocyte-agarose gel construct	Stimulation Frequency	DNA	GAG
1	1Hz	18.028	16.190
2	1Hz	12.378	26.370
3	1Hz	10.880	22.600
4	1Hz	10.829	29.420
5	1Hz	10.106	19.920
6	1Hz	10.776	27.040
7	1Hz	12.378	26.370
8	1Hz	18.028	16.190
9	1Hz	10.880	27.260
10	1Hz	10.829	29.420
11	1Hz	10.106	19.920
12	1Hz	10.776	27.040
13	1.5Hz	11.795	12.580
14	1.5Hz	12.684	25.600
15	1.5Hz	14.218	17.320
16	1.5Hz	10.822	16.590
17	1.5Hz	7.220	23.550
18	1.5Hz	10.142	17.660
19	1.5Hz	7.549	26.090
20	1.5Hz	15.825	18.760
21	1.5Hz	23.875	29.090
22	1.5Hz	18.498	43.120
23	1.5Hz	26.407	40.760
24	1.5Hz	17.770	32.470
25	0.5Hz	16.811	53.070
26	0.5Hz	14.291	37.860
27	0.5Hz	23.754	46.860
28	0.5Hz	14.915	55.040
29	0.5Hz	12.987	38.280
30	0.5Hz	14.291	37.860
31	0.5Hz	12.987	38.280
32	0.5Hz	17.129	10.700
33	0.5Hz	24.150	19.950
34	0.5Hz	15.658	28.930

35		0.5Hz	10.179	49.430
36		0.5Hz	12.754	51.793
37		Static	15.097	3.306
38		Static	19.419	3.946
39		Static	34.401	8.921
40		Static	7.702	1.768
41		Static	25.005	5.360
42		Static	34.173	6.113
43		Static	17.244	12.320
44		Static	22.546	19.590
45		Static	15.889	12.050
46		Static	17.750	13.420
47		Static	20.499	15.720
48		Static	7.071	10.880
49		Static	19.419	3.946
50		Static	17.244	12.320
51		Static	34.401	8.921
52		Static	15.889	12.050
53		Static	25.005	5.360
54		Static	20.499	15.720
Total	N	54	54	54

Table A. 2: SPSS study of normal distribution using Skewness and Kurtosis tests for DNA content among chondrocyte-agarose gel constructs for each tested frequency.

	Stimulation Frequency		Statistic	Std. Error
DNA	Static	Skewness	.415	.536
		Kurtosis	.053	1.038
	0.5Hz	Skewness	1.167	.637
		Kurtosis	.841	1.232
	1Hz	Skewness	1.787	.637
		Kurtosis	1.910	1.232
	1.5Hz	Skewness	.717	.637
		Kurtosis	-.203	1.232

Table A. 3: Skewness and Kurtosis values reflecting the distribution of GAG content among chondrocyte-agarose gel constructs for each tested frequency.

	Stimulation Frequency		Statistic	Std. Error
GAG	Static	Skewness	.194	.536
		Kurtosis	-.828	1.038
	0.5Hz	Skewness	-.853	.637
		Kurtosis	.159	1.232
	1Hz	Skewness	-.619	.637
		Kurtosis	-1.127	1.232
	1.5Hz	Skewness	.702	.637
		Kurtosis	-.392	1.232

Table A. 4: Skewness and Kurtosis values reflecting the distribution of GAG over DNA content among chondrocyte-agarose gel constructs for each tested frequency.

	Stimulation Frequency		Statistic	Std. Error
GAG over DNA	Static	Skewness	1.072	.536
		Kurtosis	1.380	1.038
	0.5Hz	Skewness	-.124	.637
		Kurtosis	-.180	1.232
	1Hz	Skewness	-1.219	.637
		Kurtosis	.748	1.232
	1.5Hz	Skewness	1.190	.637
		Kurtosis	.481	1.232

Table A. 5: One-Way ANOVA with Tukey Post Hoc test of significance, comparing DNA mean difference across different tested frequencies.

Dependent Variable	(I) Stimulation Frequency	(J) Stimulation Frequency	Mean Difference (I-J)	Std. Error	Significance
DNA	Static	0.5Hz	4.688556	2.206678	.159
		1Hz	8.347889*	2.206678	.002
		1.5Hz	5.780306	2.206678	.055
	0.5Hz	Static	-4.68856	2.206678	.159
		1Hz	3.659333	2.417294	.437
		1.5Hz	1.09175	2.417294	.969
	1Hz	Static	-8.347889*	2.206678	.002
		0.5Hz	-3.65933	2.417294	.437
		1.5Hz	-2.56758	2.417294	.714
	1.5Hz	Static	-5.78031	2.206678	.055
		0.5Hz	-1.09175	2.417294	.969
		1Hz	2.567583	2.417294	.714

*. The mean difference is significant at the 0.05 level.

Table A. 6: One-Way ANOVA with Tukey Post Hoc test of significance, comparing GAG mean difference across different tested frequencies.

Dependent Variable	(I) Stimulation Frequency	(J) Stimulation Frequency	Mean Difference (I-J)	Std. Error	Significance
GAG	Static	0.5Hz	-29.464944*	3.238162	.000
		1Hz	-14.438833*	3.238162	.000
		1.5Hz	-15.759667*	3.238162	.000
	0.5Hz	Static	29.464944*	3.238162	.000
		1Hz	15.026111*	3.547229	.001
		1.5Hz	13.705278*	3.547229	.002
	1Hz	Static	14.438833*	3.238162	.000
		0.5Hz	-15.026111*	3.547229	.001
		1.5Hz	-1.32083	3.547229	.982
	1.5Hz	Static	15.759667*	3.238162	.000
		0.5Hz	-13.705278*	3.547229	.002
		1Hz	1.320833	3.547229	.982

*. The mean difference is significant at the 0.05 level.

Table A. 7: One-Way ANOVA with Tukey Post Hoc test of significance, comparing DNA mean difference across different tested frequencies.

Dependent Variable	(I) Stimulation Frequency	(J) Stimulation Frequency	Mean Difference (I-J)	Std. Error	Significance
GAG over DNA	Static	0.5Hz	-2.151825*	.290379	.000
		1Hz	-1.552236*	.290379	.000
		1.5Hz	-1.332497*	.290379	.000
	0.5Hz	Static	2.151825*	.290379	.000
		1Hz	0.599589	.318094	.247
		1.5Hz	0.819327	.318094	.061
	1Hz	Static	1.552236*	.290379	.000
		0.5Hz	-0.59959	.318094	.247
		1.5Hz	0.219738	.318094	.900
	1.5Hz	Static	1.332497*	.290379	.000
		0.5Hz	-0.81933	.318094	.061
		1Hz	-0.21974	.318094	.900

*. The mean difference is significant at the 0.05 level.