Investigating Tendon Mechanobiology and the potential of High Frequency Low Magnitude loads for Tendon repair and remodelling using an novel *in vitro* loading system

Isaiah Adekanmbi

Mansfield College



Supervisor: Dr Mark S Thompson

Department of Engineering Science

University of Oxford

A thesis submitted for the degree

Doctor of Philosophy

Trinity term 2013

ACKNOWLEDGEMENTS

Undertaking my doctoral thesis has been a character building, emotionally challenging, and mentally demanding journey, but it has not been a solitary endeavour, and I would like to give ‘honour where honour is due’.

It would be difficult to express how grateful I am to my supervisor Dr Mark Thompson, whose constant advice and feedback was always challenging but also thought provoking, edifying, and conducive towards personal and academic progress. Without his committed guidance this thesis would not have been possible. Thank you to Dr Philippa Hulley, for her contributions towards many of the biological aspects of this project. It has been a great privilege to spend the last few years in the Botnar Research Centre at the Nuffield Orthopaedic Hospital, Oxford.

Thank you to my friends, and members of the Oxford Tendon Mechanobiology group, Dr Nasim Zagar-Baboldashti, Russell Tucker, who helped in the proofreading of this thesis, and Tyler Grant, always willing to share discussions on theory and new ideas. Thank you to Dr’s Sarah Franklin, Peter Cain, Osnat Hakimi, Clarence Yapp, Raewyn Poulsen, Cynthia Chang, to Ali Shirazi, and Sharif Hala. Thank you to Prof Liz Tanner who has been a constant voice of encouragement. To my athletics sprints coach, Martey Newman for his practical and undying enthusiasm for Tendon research. Thank you to Dr Chris Martin, my college advisor, whose reassurance helped me to balance competing demands of academic work with personal commitments to OUAC and Achilles AC.

Thank you to members of my Church home groups and the wider congregation at St Ebbes Church, Headington Oxford, for their support and encouragement. Finally, I would like to say a heartfelt thank you to my mother and father for their unconditional love. To my brothers and sisters for their support and in particular John who inspired me to undertake this PhD, along with my father who departed this life before it all ended. I hope this makes you proud.

ABSTRACT

Tendon injuries are ubiquitous in the sporting and occupational environment. Clinically they present a challenge to Orthopaedic surgeons as they account for up to half of all sports injuries and almost half of reported work related ailments. The capacity for tendons to heal subsequent to injury is restricted due to their poor blood supply. Moreover, healed tendon tissue may be inferior to the intact tendon, having diminished biochemical and biomechanical properties and this brings about an ever increasing need for optimized treatment methods for tendon repair.

Mechanobiology is concerned with how mechanical forces influence physiological and pathological aspects of the living tissue. However, the complex and poorly controlled loading environment in living organisms prevent the establishment of direct relationships between mechanical stimuli and tissue response.

By developing a novel *in vitro* loading system (IVLS), the work in this thesis investigates the potential of a new and exciting biophysical loading intervention, High Frequency Low Magnitude (HFLM) mechanical loading, for stimulation of tendon repair and remodelling.

Following a pre-defined stimulation period, healthy rat tail tendon fascicles (RTTFs) were evaluated for tissue viability and metabolism, Glycosaminoglycan (GAG) content, collagen arrangement and tangent modulus, using staining and biochemical assays, together with microscopy techniques, and mechanical testing.

HFLM mechanically loaded tendons showed a trend for a higher tangent modulus than fresh tissue, and significantly higher modulus than unloaded. Further, when varying mechanical loading parameters of frequencies and dosages over clinically relevant ranges, a frequency dependent response was observed with increased tangent modulus and GAG content with

increasing frequency. An association between high tendon crimp pattern and elevated tendon modulus as a result of HFLM mechanical loading was also demonstrated.

Concomitantly, an injury model was developed to evaluate the effects of *in vitro* static, low frequency cyclic and HFLM mechanical loading conditions on the biochemical and biomechanical properties of *in vitro* damaged tendons. HFLM mechanically loaded damaged tendons again demonstrated significantly higher modulus and metabolism than unloaded tissue, although these were reduced below those of fresh damaged tissue.

The findings in this thesis together with the newly developed IVLS reveal the potential for an exciting and unique biophysical therapeutic loading intervention for treatment of tendon injuries, and provide a scientific platform for further investigation of the effects of HFLM mechanical loads, potentially leading to an application within the clinic for enhanced connective tissue repair and remodelling.

Publications and Contributions

Work from this DPhil thesis has contributed towards peer-reviewed manuscripts and conference articles which have been detailed below.

Journal Publications

Adekanmbi I., Franklin S., Zagar-Baboldashti N., Yapp C., Thompson MS., A novel in-vitro loading system for high frequency loading of cultured tendon fascicles; Medical Engineering and Physics: 2012 35(2) 205-210

Conference Papers

Adekanmbi I., Franklin S., Baboldashti NZ., Hulley P., Poulsen R., Thompson MS., Investigating the potential of High Frequency Low Magnitude (HFLM) loading interventions for tendon repair using a novel in-vitro loading system. Histology and Histopathology Cellular and Molecular Biology vol 26, 2011, Tissue Engineering and Regenerative Medicine Conference (TERMIS 2011)

Adekanmbi I., Thompson MS., Low doses of high frequency low magnitude loading increase modulus & maintain Glycosaminoglycan in cultured tendons. Journal of Biomechanics Vol. 45Supplement 1, Page S406 European Society of Biomechanics Conference (ESB 2012)

Adekanmbi I., Thompson MS., A novel in-vitro loading system for investigating the Mechanobiology of tendons; University of Oxford, Engineering Science Symposium 2011

Contents

Table of Figures 6

Table of tables 10

Table of Equations 11

List of Technical Abbreviations 12

CHAPTER 1 General Introduction 14

1.1 Motivation and Scope of Study 14

1.2 Tendon Physiological Background 16

1.3 Tissue Composition 18

1.3.1Tendon Cells 19

1.3.2Tendon Extracellular Matrix (ECM) 21

1.3.3 Tissue level of organisation 22

1.4 Biomechanical Properties 24

1.5 Tendon Mechanobiology 28

1.6 *In vitro* loading systems 29

1.7 Aims of Project 30

1.8 Thesis Outline 31

CHAPTER 2 Tendon injuries, healing and strategies for repair 32

2.1 Introduction 32

2.2 Tendon Injuries 32

2.3 Tendon Healing 36

2.3.1 Biomechanical properties of healing tendons 39

2.4 Strategies for tendon repair 40

2.4.1 Tendon rehabilitation methods 40

2.4.2 Physical Therapies 41

2.4.2.1 Ultrasound therapy 41

2.4.2.2 Extracorporeal Shock Wave Therapy 42
2.4.2.3 Pulsed Electromagnetic field therapy 43
2.4.2.4 Tissue Engineering 44
2.5 Mechanical Loading of tendons 46
2.5.1 *In vivo* forces on tendons 46
2.5.2 Effects of mechanical loading (mobilisation) on healthy tendons 46
2.5.3 Effects of mechanical loading (mobilisation) on healing tendons 47
2.5.4 Effect of load deprivation on tendons 49
2.5.5 High Frequency low magnitude (HFLM) mechanical loading 49
2.6 Summary 51
CHAPTER 3 Materials and Methods Common for all Studies 52
3.1 Introduction 52
3.2 Tissue Culture 52
3.2.1 Tendon harvesting 53
3.2.2 Tendon fascicle isolation and *in vitro* loading model 54
3.2.3 Culture environment 55
3.3 Tissue Viability 55
3.3.1 Live and Dead Staining assay 56
3.3.2 Alamar Blue Assay 57
3.4 Imaging Techniques 58
3.4.1 Confocal Microscopy 58
3.4.2 Multi-Photon Microscopy (MPM) 58
3.5 Biochemical Assay 59
3.5.1 Dimethylmethylene Blue (DMB) 59
3.6 Mechanical Testing 60
3.6.1 Measurements of *in vitro* forces on isolated tendon fascicle 60
3.6.2 Tensile testing 61

3.7 Injury model for tendon fascicles 63

3.8: Statistical techniques 65

CHAPTER 4 A novel *in vitro* loading system for static low frequency and high frequency low magnitude cyclic loading of cultured tendons 67

4.1 INTRODUCTION 67

4.1.1 *In vitro* loading systems for cultured tendon specimens 67

4.1.2 *In vitro* static loading of cultured tendons 68

4.1.3 *In vitro* cyclic loading of cultured tendons 68

4.1.4 Application of High Frequency Low Magnitude (HFLM) loads on tendons 69

4.2 MATERIALS AND METHODS 72

4.2.1 Study Design 1 72

4.3 RESULTS 74

4.3.1 Custom IVLS for low frequency and HFLM loading of tendon fascicles 74

4.3.2 Characterisation of applied load magnitude and frequency using IVLS 76

4.3.3 Tissue Viability 76

4.3.4 Effect of static loading on tendon mechanical properties and GAG content 79

4.3.5 Effect of HFLM loading on tendon mechanical properties and GAG content 81

4.4 DISCUSSION 83

CHAPTER 5 Effects of frequency and dosages of HFLM loading on the biochemical and biomechanical, properties of cultured tendon fascicles 88

5.1 INTRODUCTION 88

5.2 Essential loading parameters for cyclic stimulation 88

5.2.1 *In vitro* cyclic loading frequencies on tendons 89

5.2.2 *In vitro* cyclic loading dosages on tendons 90

5.2.3 Tendon decellularisation and mechano-transduction effects 91

5.3 MATERIALS AND METHODS 93

5.3.1 Study Design 2 93

5.4 RESULTS 96

5.4.1 Characterisation of applied peak cyclic load magnitude 96
5.4.2 Effect of loading dosage periods on tendon modulus and GAG content.....97
5.4.3 Effect of loading frequencies on tendon modulus and GAG content 98
5.4.4 Effect of low temperature culture on the metabolic activity of tendon cells.....100
5.4.5 Effect of low cell activity on the modulus of mechanically loaded tendons 101
5.6 DISCUSSION.....103
CHAPTER 6 Mechanisms for tendon mechanical adaptation to HFLM mechanical loading in cultured tendon fascicles.....109
6.1 INTRODUCTION 109
6.2 Tendon remodelling and the mechanical environment.....109
6.3 Cellular induced remodelling.....110
6.4 Non-cellular induced remodelling 112
6.4.1 Tendon crimp 113
6.5 MATERIALS AND METHODS.....114
6.5.1 Study Design.....114
6.6 RESULTS 118
6.6.1 Tendon modulus during low and normal cell activity 118
6.6.2 Effect of cyclic loading on tendon crimp during normal cell activity 118
6.6.3 Effect of cyclic loading on tendon crimp during low cell activity 121
6.7 DISCUSSION.....123
CHAPTER 7 Effect of static, low frequency cyclic, and HFLM mechanical loading conditions on the *in vitro* repair of damaged tendon fascicles.....128
7.1 INTRODUCTION 128
7.1.1 *In vitro* intrinsic tendon repair 128
7.1.2 *In vitro* injury models for tendon repair.....129
7.1.3 Markers for *in vitro* tendon repair.....130
7.1.4 Mechanical loading conditions for tendon repair 131
7.1.5 Static loading for tendon repair.....131

7.1.6 Low frequency Cyclic loading for tendon repair 131
7.2 MATERIALS AND METHODS 133
7.2.1 Study Design 133
7.3 RESULTS 136
7.3.1 Characterisation of damage model for *in vitro* tendon repair 136
7.3.2 Viability of cultured damaged tendons 136
7.3.3 Effect of scratch damage on tendon modulus 138
7.3.4 Effect of scratch damage on tendon metabolic activity 139
7.3.5 Effect of loading conditions on the modulus of damaged tendons 140
7.3.6 Effect of loading conditions on the metabolism of damaged tendons 141
7.4 DISCUSSION 142
CHAPTER 8 Final Discussion 149
8.1 OVERVIEW 149
8.2 BACKROUND 149
8.3 RESEARCH QUESTIONS 150
8.4 LIMITATIONS OF STUDY 157
8.5 FUTURE WORK 160
8.6 Concluding Remarks 162
REFERENCES 164

Table of Figures

Chapter 1

Figure 1.1 Surgical image of human Achilles tendon

Figure 1.2 Composition for tendons dry mass

Figure 1.3 Confocal microscopy image of live tendon cells from a rat tail tendon fascicle

Figure 1.4 Light microscope image from human Achilles tendon section

Figure 1.5 Proteoglycan interaction with many tendon extracellular matrix components

Figure 1.6 Structural hierarchy of tendon

Figure 1.7 Stress-strain profile illustrating the basic mechanical properties of tendons.

Figure 1.8 Key events of mechanobiology

Chapter 2

Figure 2.1 MRI showing typical rotator cuff tear of the shoulder

Figure 2.2 Stages in tendon healing

Figure 2.3 Wound healing in damaged tendons, illustrating the decline in tensile strength

Figure 2.4 Patient receiving Extracorporeal Shock Wave therapy

Figure 2.5 Tissue engineered tendon construct

Figure 2.6 Illustration of HFLM mechanical load cycles

Chapter 3

Figure 3.1 Image of rat tail and extracted fascicle after sacrifice

Figure 3.2 *In vitro* apparatus for unloaded and static loaded tendon fascicle

Figure 3.3 Apparatus and set-up used for characterisation of the applied cyclic load

Figure 3.4 Bose Machine used for mechanical testing of tendon fascicles

Figure 3.5 Stress strain plot for rat tail tendon fascicle

Figure 3.6 Apparatus and set-up used for application of scratch damage on RTTF

Figure 3.7 Damaged and undamaged tendons following application of 1cm scratch

Chapter 4

Figure 4.1 Experimental set up for study 1

Figure 4.2 The custom *in vitro* loading system for cyclic loading

Figure 4.3 Load-Time plots; Fast Fourier Transform signals for 2 tendon groups

Figure 4.4 Live-dead confocal microscopy images of four tendon groups

Figure 4.4E The proportion of live and dead cells within tendon fascicles.

Figure 4.5A Mean tangent modulus (A) and GAG content (B) after 7 days. Static loaded

Figure 4.6A Mean tangent modulus (A) and GAG content (B) after 7 days. HFLM loaded

Chapter 5

Figure 5.1 Experimental set up for study 2

Figure 5.2 Modification of custom IVLS to allow comparison at constant load magnitude

Figure 5.3 Mean tangent modulus in 5 tendon groups. Varying loading dosages

Figure 5.4 Mean GAG content in 5 tendon groups. Varying loading dosages

Figure 5.5 Mean tangent modulus in 5 tendon groups. Varying loading frequencies

Figure 5.6 Mean GAG content in 5 tendon groups. Varying loading frequencies

Figure 5.7 The metabolic activity of tendon cells

Figure 5.8 Mean tangent modulus in 3 tendon groups at A) 27^{o}C and B) 37^{o}C

Chapter 6

Figure 6.1 Experimental set up for study 3

Figure 6.2 Classification of crimp parameters

Figure 6.3 Crimp angle, amplitude and, wavelength in 3 tendon groups. 37°C.

Figure 6.4 SHG images showing crimp morphology in 3 tendon groups. 37°C.

Figure 6.5 Crimp angle, amplitude, and wavelength in 3 tendon groups. 27°C.

Figure 6.6 SHG images showing crimp morphology in 3 tendon groups. 27°C.

Chapter 7

Figure 7.1 Experimental set up for study 4

Figure 7.2 SHG images of three tendon fascicles after application of a scratch damage

Figure 7.3 Live-dead confocal microscopy images of three damaged tendon fascicles

Figure 7.4 Injury model validation. Tangent modulus in damaged and undamaged RTTFs

Figure 7.5 Injury model validation. Metabolic activity in damaged and undamaged RTTF

Figure 7.6 Mean tangent modulus in 4 damaged tendon groups after mechanical loading

Figure 7.7 Metabolism in 4 damaged tendon groups after mechanical loading

Table of tables

Chapter 2

Table 2.1 Classification of tendon injuries. Adapted with modification from, Leadbetter WB., 1992, Sharma and Maffulli, 2005, Kannus and Jozsa 1991, and Wang, JC., 2006

Chapter 3

Table 3.1 Characteristic properties and function of components used as part of custom *in vitro* system for static and unloaded tendon groups

Chapter 4

Table 4.1 Characteristic properties and function of components of electromagnetic stimulation device used for custom IVLS RTTF

Table 4.2 Magnetic field strength of electromagnetic stimulation device at set frequencies

Table 4.3A The average Modulus, Tensile strength and Strain to failure, in; fresh, unloaded and static loaded tendon groups, after 7 days. For each group n=5. Reported values are mean± standard deviation. Indicated p-values are for SL vs. Unloaded tendon groups (ANOVA)

Table 4.3 B The average Modulus (n=8), Tensile strength (n=5), and Strain to failure (n=5); in fresh, unloaded, and HFLM tendon groups after 7 days. Reported values are mean± standard deviation. Indicated p-values are for HFLM vs. unloaded tendon groups (t-test)

Table of Equations

Chapter 1

$$E = \frac{Stress}{strain} = \frac{\sigma}{\varepsilon} = \frac{F.L}{A.\Delta L}$$

[Where $\sigma = \frac{F}{A}$; $\varepsilon = \frac{\Delta L}{L}$]

Equation 1.1

$$K = \frac{AE}{L}$$

Equation 1.2

Chapter 3

$$Applied\ static\ load\ (N) = mass\ of\ stir\ bar\ x\ 9.81$$

Equation 3.1

List of Technical Abbreviations

CS	Chondroitin Sulphate
CSA	Cross sectional area
DS	Dermatin Sulphate
DMEM	Dulbecco's Modified Eagle Medium
EMG	Electromyography
ECM	Extracellular matrix
ESW	Extracorporeal Shock Wave therapy
FPCL	Fibroblast populated collagen lattice
GAG	Glycosaminoglycan
HFLM	High Frequency Low Magnitude Load
IMS	Industrial methylated spirit
IVLS	*In vitro* loading system
JNK	c-Jun-N-terminal kinases
KS	Keratin Sulphate
LSM	Laser scanning microscopy
LF	Low frequency
LVST	Low Vibration strength trained
MMP	Matrix Metalloproteinase
MIP	Maximum intensity projection
mRNA	messenger Ribonucleic Acid
MVC	Muscle Voluntary Contraction
PBS	Phosphate buffered solution
PPLM	Plane polarised light microscopy
PG	Proteoglycan
PEMF	Pulsed electromagnetic field therapy

RFU	Relative fluorescence unit
RTTF	Rat tail tendon fascicle
SD	Sprague Dawley
SHG	Second Harmonic Generation
SAPK	Stress-activated protein kinase
TIMP	Tissue inhibitor metalloproteinase
US	Ultrasound
VEGF	Vascular endothelial growth factor
WBV	Whole Body Vibration

CHAPTER 1 General Introduction

1.1 Motivation and Scope of Study

Tendons are tough bands of fibrous connective tissue. They function to transmit forces between muscle and bone and by doing so they permit locomotion and ensure joint stability. Tendon injuries may be caused by trauma or degeneration and may last for several months, and even years, often becoming chronic problems. Clinically, the number of tendon injuries presents a challenge to orthopaedic surgeons as they account for up to 50% of sports injuries and 48% of reported occupational ailments [1]. In the US approximately 200,000 patients undergo tendon or ligament repair each year [2] .

The capacity for tendons to heal subsequent to injury is restricted due to their poor blood supply. Moreover, healed tendon tissue may be inferior to the intact tendon, having diminished biochemical and biomechanical properties [3]. This poor healing response and the ubiquitous nature of tendon injuries within sports and occupational environments brings about an ever increasing need for optimized treatment methods for tendon repair.

A number of therapeutic modalities for tendon injuries have been identified, and these are discussed briefly in chapter 2 of this report. Mechanical stimulation has been postulated as critical factor for maintaining tendon health [4] , and is considered as having a high potential as an effective non-drug based treatment method for accelerating tendon repair.

Mechanical stimulation is also recognised as an important factor in enhancing the quality of tissue engineering constructs for tendon repair [5].

However several questions remain to be addressed. These include but are not limited to the following; what is the most effective mechanical loading frequency and magnitude for treatment, is there a critical dosage period of stimulation per day, how is the narrow window between under-stimulation and over-stimulation defined, and what are the risks and safety margins for any proposed stimulation therapy?

Mechanobiology is concerned with how mechanical forces influence physiological and pathological aspects of the living tissue. A major aspect within this interdisciplinary branch of science involves elucidating the mechanisms by which mechanical forces are transduced to biochemical activity within cells which then modify tissue properties [6]. A better understanding of this process, known as mechanotransduction is required in order to answer the questions posed above, and hence provide new, optimised clinical therapies for both sports and occupational tendon diseases.

Physiologically, mechanical loading regimes for tendons depend on the activity of the attached muscle, and studies have measured peak forces in, the human Achilles tendon during running, the patellar tendon forces in goats during standing, walking, and trotting, the rat tail tendons, and the forces in the gastrocnemius muscles of cats [6-9].

Similar loading regimes for tendon specimens can be represented using *in vitro* systems equipped to apply static and dynamic, time varying loading. *In vitro* loading systems (IVLS) enable precise characterisation of cell and tissue response to mechanical loading in a controlled and repeatable way. However, most *in vitro* studies on tendons have been limited to a narrow load frequency range of approximately 0.01-3Hz [10-15] .

High Frequency Low Magnitude (HFLM) loading regimes are mechanical loading interventions which are characterised by cyclic loading frequencies of 10-50Hz and loading magnitudes that are many orders lower than those present during intense physical activity [16, 17]

HFLM loading interventions have shown enormous potential for increasing bone mass density and in counteracting the effects of bone diseases such as osteoporosis [18, 19] however, little is known about the effects of this loading regime on tendon health and tendon repair.

Adaptation to the presence and the absence of mechanical loading is known to be more rapid in damaged tendons than in healthy intact tendons [20], and while studies from a number of laboratories have demonstrated the ability of tendons to display an *in vitro* healing response without the contribution of inflammatory or vascular cells, there is a lack of research into the role of mechanical stimulation on *in vitro* tendon repair. Consequently, this brings about an enormous scope for further investigation into the mechanobiology of tendon repair using *in vitro* loading systems.

The remainder of this chapter provides a background introduction to the anatomy, physiology and physical properties of tendons. This is followed by an overview of the three major aspects involved within this project and include; 1.) The use of *in vitro* loading systems for tendon mechanobiology 2.) Tendon disorders, tendon healing and strategies for tendon repair 3.) Mechanical loading regimes for tendon stimulation. Finally, the aims and outline of the thesis are presented.

1.2 Tendon Physiological Background

In their healthy state, tendons appear brilliant white (figure 1.1)with a fibro-elastic texture and are composed mainly of highly organised collagen fibres.

Anatomically, tendons within the human body may vary considerably ranging from cylindrical cords and strap-like bands, to flattened ribbons or delicate and peel-able sections next to nerve and blood vessels which are known as aponeuroses. Tendons that are connected to powerful muscles such as the quadriceps are short and broad, while those attached to muscles that provide fine movement such as the finger flexors, adopt a longer and thinner structure [21]



Figure 1.1: Surgical image of a Human Achilles tendon; showing A. Discolouration of the tissue in a defected rupture region and, B. the white shiny appearance of the healthy tissue. Taken from Oommen, AT., *et al* 2010 [22].

Intact tendon possess two sites of force transmission which are morphologically and compositionally specialized for their function. These are located at the proximal and distal end of tendons and are referred to as the myotendinous junction (MTJ), and the osteotendinous junction (OTJ), commonly known as the enthesis. The myotendinous junction, is predominantly responsible for transmitting forces generated and is partially responsible for enhancing muscle growth. At the myotendinous junction, collagen fibrils appear to be deep-seated within depressions created between the finger-like processes (endings) of muscle cells. The folding at the basal membrane of the muscle cell endings

markedly increases the contact area between the muscle fibers and the tendon (collagen) fibers by 10 to 20 fold. This architecture minimises stress concentrations at the tendon during a muscle contraction [23, 24]. Nevertheless, the MTJ is still considered the weakest interface of the muscle-tendon complex. The OTJ, ensures that contractile forces are transmitted to bone and is arranged in a way that prevents collagen fiber bending, shearing, and failure [25, 26]. The structure exists in two forms. At a fibrous enthesis, the tendon fastens to the periosteum or bone itself, whereas at a fibrocartilaginous enthesis, the presence of a transitional hyaline fibrocartilage, aids the distribution of mechanical loads [27]. This combination enables the enthesis to withstand, tensile, compressive and shear forces but also means tensile stress at the enthesis maybe up to four times higher than that of the tendon mid-substance [28].

1.3 Tissue Composition

The dry mass of tendons constitutes approximately 30% of its physiological mass and the amount occupied by the ECM in tendons far exceeds that of the cells, figure 1.2

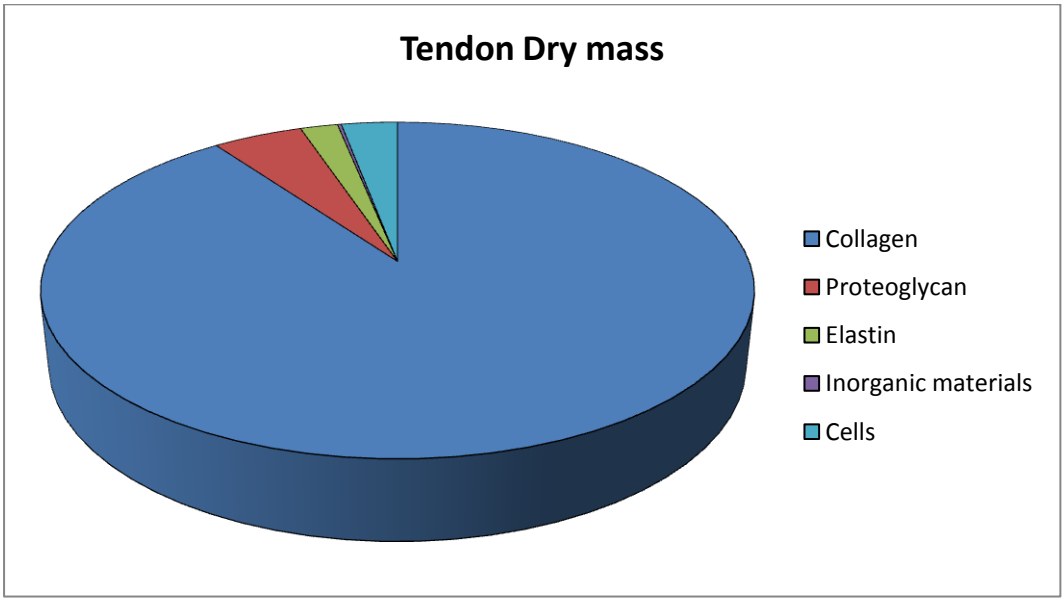


Figure 1.2: Breakdown of tissue composition for tendon dry mass

Under physiological conditions water accounts for approximately 70% of the mass of tendons [29]. Almost 90% of tendons dry mass is made up of collagen, while proteoglycan, and elastin account for approximately 1-5% and 2%, respectively [21, 30, 31]. The remaining inorganic materials account for less than 0.2% of tendon dry mass, and detected minerals include calcium, magnesium, manganese, cadmium, copper, cobalt, zinc, lead, nickel fluoride, phosphor and silicon [32].

1.3.1Tendon Cells

Tendons contain relatively few cells, which account for approximately 1-3% of the total dry mass and are responsible for secretion and deposition of new extra cellular matrix (ECM) components. The predominant cell types, are fibroblasts known in tendon as tenocytes and tenoblasts and these constitute 90-95% of the cellular components of tendon[6, 33]. Fibroblasts within tendons are typically rod or spindle-shaped and are situated in rows between collagen fibres of tendon ECM, as depicted in figure 1.3.

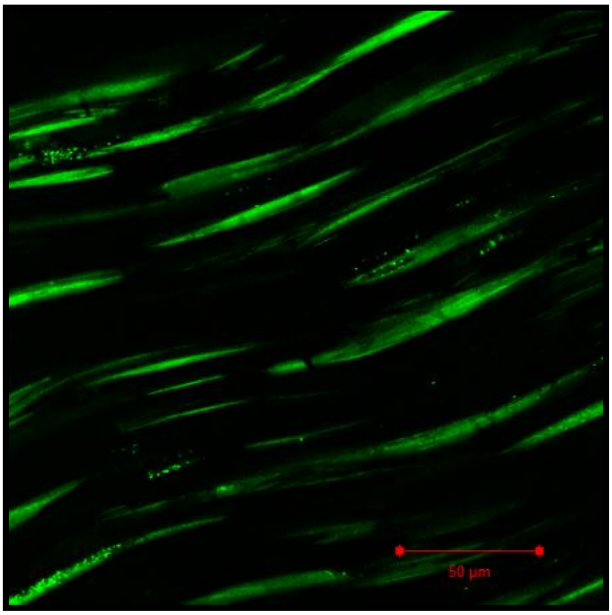


Figure 1.3: Microscopy image of live tendon cells from a rat tail tendon fascicle showing spindle shaped features and characteristic length of healthy tenocyte

The remaining 5-10% consist of chondrocytes, synovial cells, vascular cells, capillary, endothelial cells and smooth muscle cells of arterioles[34] Other cell types such as macrophages, myo-fibroblasts and inflammatory cells may also be present within Tendon tissue during periods of healing.

Immature tendon cells called tenoblasts are approximately 20-70μm in length, and appear spindle shaped. The numerous cytoplasmic organelles contained within these cells are mirrored by their high metabolic activity [21]. The cell-to-matrix ratio of tendons decreases with age. Over time tenoblasts become elongated and transform into mature tendon cells, called tenocytes. The cytoplasm of tenocytes is stretched between collagen fibres and these cells range from 80-300μm in size, figure 1.4. Tenocytes contain fewer cytoplasmic organelles and an increased nucleus to cytoplasmic ratio predisposes them to economical synthesis of new tendon extracellular matrix components [35]. More recently, a unique and rare form of tendons cells demonstrating similar characteristics to stem cells have also been identified as tendon stem/progenitor cells (TSPCs) [36].

In tendon cells, three major pathways of energy are known to be active, aerobic, anaerobic and the pentose phosphate shunt. Shortly after birth, there are equal amounts of energy produced by the aerobic Krebs cycle and the pentose phosphate shunt. Within a few months, physiological transformations take place so that the pentose shunt has a higher energy yield. In later life however, both energy systems decrease in activity, while activity and energy yield of only the anaerobic glycolysis system remains relatively unchanged. Consequently, anaerobic pathways become the main source of energy production with age [21].

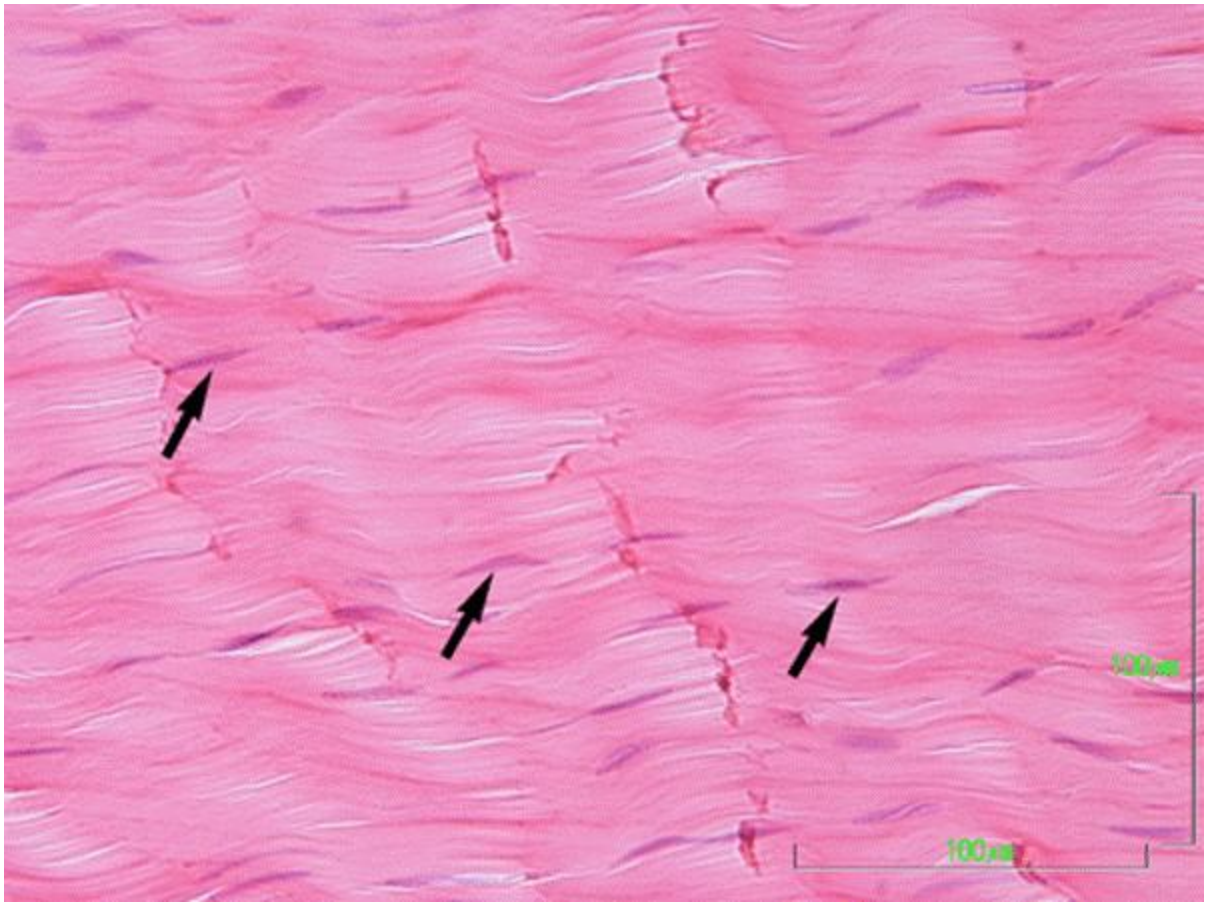


Figure 1.4: Light microscope image from human Achilles tendon section. Bundles of parallel collagen fibers (pink) with interposed tendon fibroblasts (arrows). Taken from Franchi, M., *et al* 2007c [37], with permission

1.3.2Tendon Extracellular Matrix (ECM)

The macromolecular structures found within the ECM of tendons generally fall into three groups, collagen, proteoglycans, and glycoproteins.

Collagen type I, which has a high tensile strength (approximately 50MPa) and high elastic modulus (approximately 1GPa) is the most abundant component in tendons and it accounts for approximately 90% of the total collagen content in tendons [38], as depicted in figure 1.4 Other collagen types found are type II, III, IV and V [21]. Each rod-like collagen molecule (tropocollagen unit), combines end-to-end and side-by-side in a quarter-staggered arrangement; forming overlaps by a quarter of its length with neighbouring rods [39]. This produces the characteristic 64nm-68nm banding pattern of a collagen fibril seen in a light

microscope. The tropocollagen molecules themselves are soluble, but form cross-links thereby adopt an insoluble structure known as the collagen fibril [32]. Surrounding the tropocollagen molecule and between the fibrils and fibers are proteoglycans and other glycosaminoglycans [21].

Proteoglycans (PGs) and their associated side chains are present throughout tendons, and are associated with collagen molecules and collagen fibrils. Proteoglycans consist of a protein core with branching chains of sulphated Glycosaminoglycans (GAGs) such as Chondroitin Sulphate CS, Dermatan Sulphate DS and Keratan sulphate KS. The most abundant PGs found in tendons are Decorin, Biglycan, Fibromodulin, Lumican, Versican and Aggrecan, and the way these molecules interact with other tendon matrix components are highlighted in figure 1.5. Each PG has a specialised role, however collectively they function in regulating collagen assembly[40]. Although proteoglycans constitute a small proportion of tendon dry mass they are believed to contribute to the structural and mechanical properties of tendons by assisting with collagen crosslinking [41, 42].

Elastin is an insoluble protein that is stable in several biochemical procedures such as boiling, and hydrolysis by weak acids and alkalis [43]. It is composed of amino acids of valine and hydroxyproline and believed to have a role in contributing to the recovery of macroscopic crimp subsequent to tendon stretching and straining [44].

1.3.3 Tissue level of organisation

The fibrous features of tendon are formed by collagen sub-units and these are arranged in hierarchical levels of increasing complexity, as shown in figure 1.6. The multi-faceted structure of tendon begins with an α-triple-helix polypeptide chain known as a tropocollagen molecule. These join together end to end and side by side to form a collagen fibril. One fibril is approximately 10-500nm in diameter and approximately 4.26mm long [45].

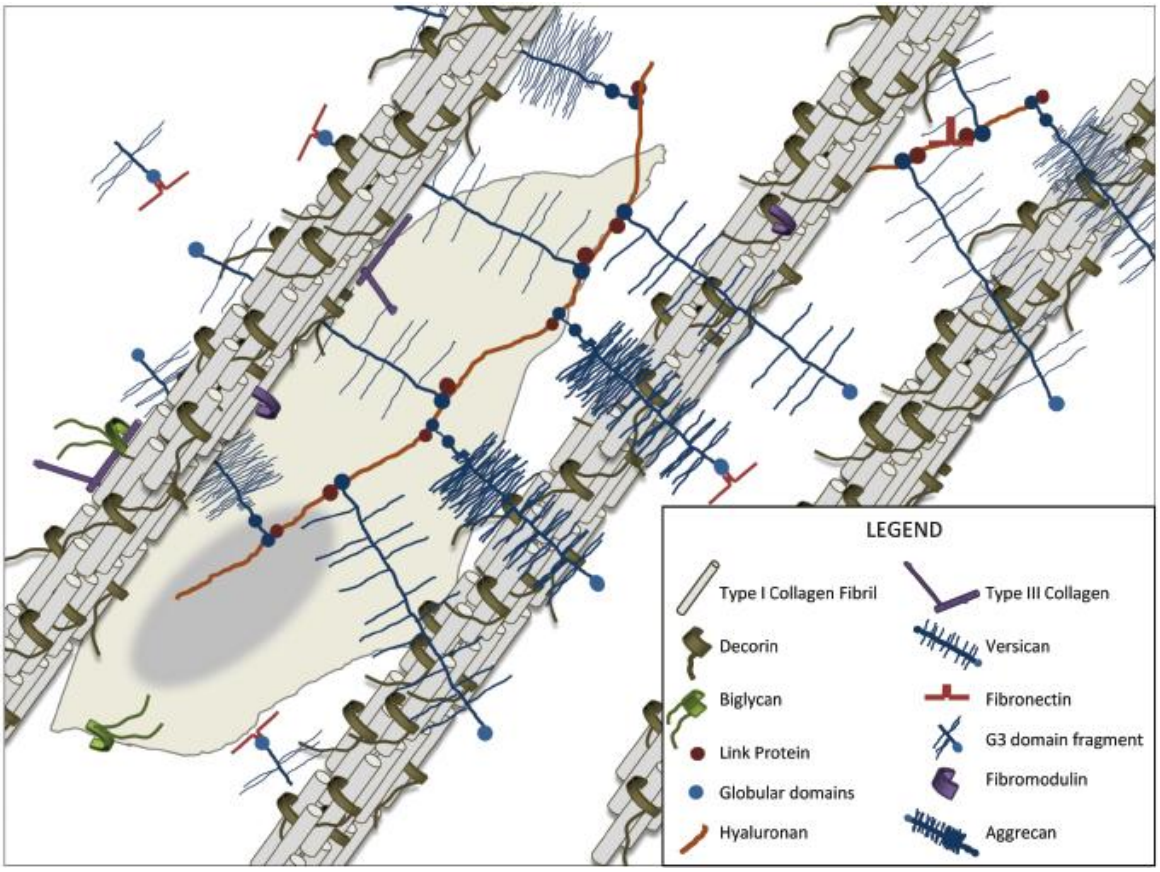


Figure 1.5: Schematic showing Proteoglycan interaction with other tendon extracellular matrix components. Image taken from Parkinson *et al* 2011 [41], with permission

Fibrils pack together to form a collagen fiber, and these fibers come together to form the primary (sub-fascicle), secondary (fascicle) and tertiary fiber bundles [46, 47]. A complete tendon unit consists of a collection of tertiary fiber bundles that are enclosed by epitenon, a fine connective tissue sheath that contains the vascular, lymphatic and nerve supply for the tendon.

Epitenon extends deep within the tendon and between the fiber bundles where it is known as endotenon. Paratenon is the final sheath encompassing a tendon unit and appears as a loose areolar connective tissue which surrounds the entire tendon organ. It consists of type I and type III collagen fibrils, some elastin fibrils and an inner lining of synovial fluid cells.

Epitenon and paratenon make up the so called paratendon which functions to reduce friction between tendons and its surrounding structures [6].

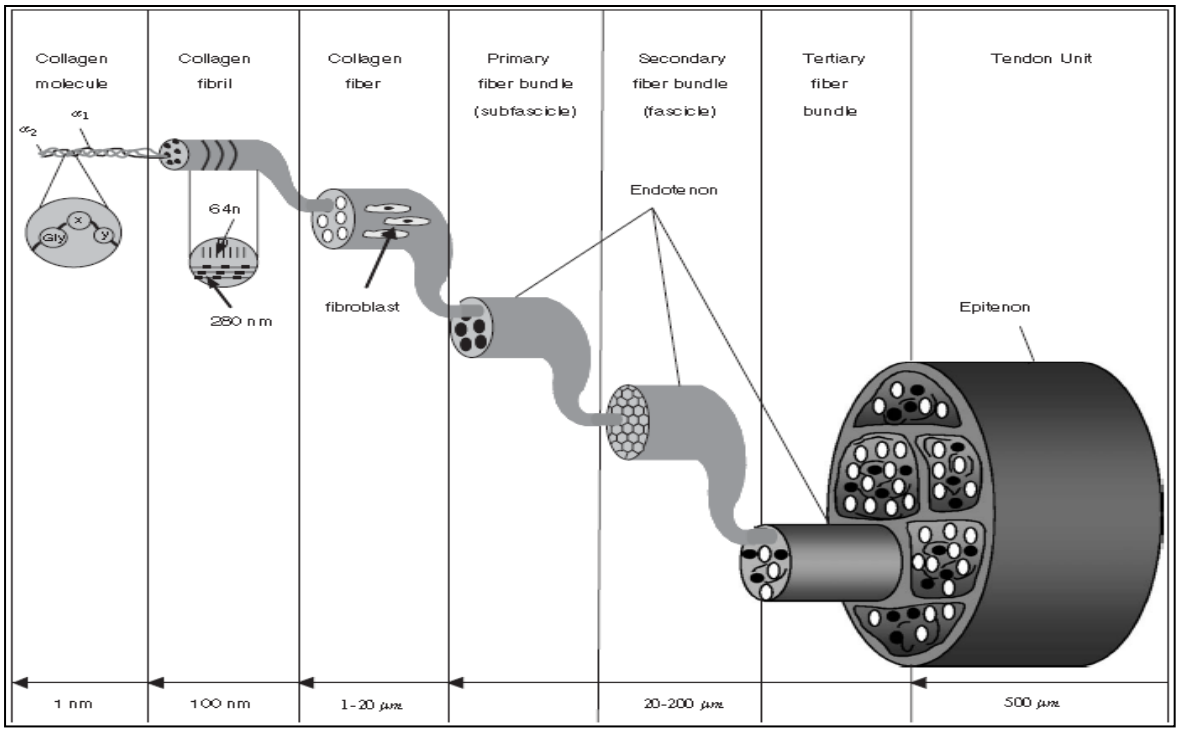


Figure 1.6: Schematic depiction of the structural hierarchy of tendon. Taken from Wang *et al.*, 2006, with permission

1.4 Biomechanical Properties

Tendons posses an exquisite capacity to cope with the physical demands of their external environment by virtue of their multifaceted structure, and this enables them to facilitate efficient body movement. In order to meet these dynamic mechanical demands, the tissues exhibit, high tensile strength, compliance and optimal modulus of elasticity [21]

In addition to their primary role as force transmitters, tendons also act as shock absorbers to muscles by minimising the damage produced from external forces [34]. The major load bearing element of tendons are collagen fibres, and the surrounding proteoglycans contribute to the tissues structural stability and compressive elasticity.

The stress-strain profile of tendons (Figure 1.7), illustrates their mechanical behaviour during exposure to an applied tensile load from slack until rupture (macroscopic failure). Three characteristic regions have been identified, the toe, linear, and failure regions.

In the low strain toe region of the curve, the wave-like undulations or "crimps" in the fibrils, observable on a scale of several microns, are straightened, this transition zone corresponds to the heel zone In the toe region of the curve, tendons remain relatively un-deformed and collagen fibrils show a wave like appearance due to planar undulations or "crimp," and this is observable on a scale of several microns. When tendons are initially stretched, the fibrils are parallel to each other and closely packed, and as deformation continues towards the end of the toe region the fibers straighten, this transition zone corresponds to the heel zone [34]. During the linear region of the curve, further deformation involves both sliding and extension of collagen fibres and fibrils [4].

Tendons typically function within a physiological range of 4% strain, and at these strains they are able to recover their original length when unloaded. Above 4% strain, microscopic damage occurs, and beyond 8-10%, macroscopic failure occurs as a consequence of relative gliding between neighbouring collagen molecules [48].

Different crimp patterns are associated with different properties: tendons with small crimp angles rupture at lower stresses than those with larger crimp angles [49, 50]

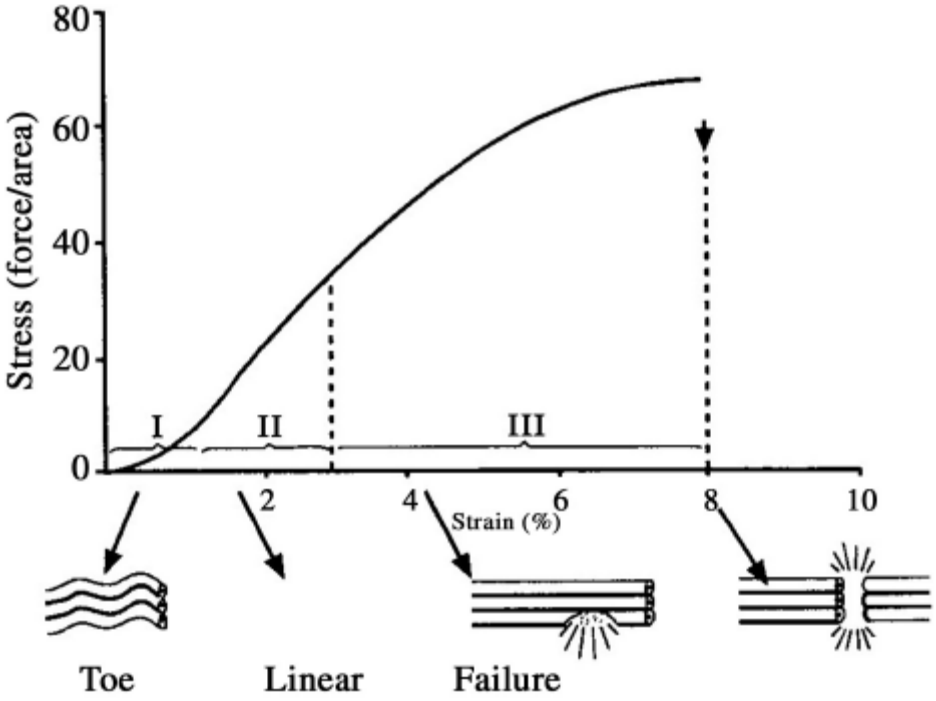


Figure 1.7: Typical stress-strain profile illustrating the basic mechanical properties of tendons. Taken from Sharma, P., and Maffulli, N., 2005, with permission

The slope of the linear portion of the stress strain curve is often quoted as the Young's Modulus. Commonly denoted, E; it can be calculated as the gradient of the tensile stress σ versus the tensile strain ε, as described in Equation 1.1

$$E = \frac{Stress}{strain} = \frac{\sigma}{\varepsilon} = \frac{F.L}{A.\Delta L}$$

[Where $\sigma = \frac{F}{A}$; $\varepsilon = \frac{\Delta L}{L}$]

Equation 3.1

In equation 1.1, F is the applied force, A is the cross sectional area through which the force is applied, ΔL is the change in length of the tissue while L is the original tissue length. The

Young's modulus is a measure of a materials resistance to elastic deformation and studies have shown that the cross linking between the collagen structures increases the Young's Modulus of tendons and reduces its strain at failure [51]. The stiffness of a homogenous tendon sample k can also be calculated as a function of E, as described in equation 1.2.

$$K = \frac{AE}{L}$$

Equation 1.4

Here A is the cross sectional area, L is the sample length and E is the Young's Modulus of the tendon.

Values for the Young's Modulus and stiffness of tendons have been reported to be as high as 0.9-1.8GPa and 161N/mm respectively with mature tendons showing the highest values [52].

The tensile strength of tendons is, the maximum applied stress the tissue can withstand before failure or rupture, and values of up to 100MPa have been reported in mammals [53]. It has been speculated however that published values of tendon tensile strengths should be taken as a lower limit, since stress concentrations formed at specimen grips during measurements by mechanical testing are known to have a major influence on specimen failure [54].

The strain to failure for adult human patellar tendons has been found to be approximately 14±6% and 15±5% for immature and mature patellar tendons respectively[55].

During activities such as running the human Achilles is said to experience forces as high as 12.5 times that of the body weight, or 9kN [37-39]. In animals such as goats, the forces experienced by the patellar tendon during standing and walking have been reported to have reached 270N and 800N respectively [7].

Tendons show a time dependent deformation response under tensile loads and as such exhibit creep, stress relaxation, hysteresis [56].

Mechanical hysteresis is often measured as a fraction of the total work done during tendon loading, and values of approximately 10% have been recorded during tensile tests of *in vitro* tendon specimens [57]. Fatigue damage in tendons can be a result of an extended period of constant or cyclic loading. Tendons with a high fatigue resistance are able to bare greater loads for longer periods of time without rupturing, compared with those which have a low fatigue resistance. Furthermore, it has been proposed that tendon cells are able to modify the surrounding tissue matrix to meet mechanical demands associated with fatigue stress cycles [24]. Overall, these biomechanical properties are frequently used as measures for the functional performance and quality of tendon.

1.5 Tendon Mechanobiology

Tendon mechanobiology is concerned with tendon cell and tissue response to mechanical stimuli, figure 1.8. It looks at how mechanical forces influence physiological and pathological processes. At the microscopic level, it is concerned with the mechanotransduction mechanisms that lead to a cascade of cellular and physiochemical events [58].

Tendons have a unique capacity to alter their structure, composition and mechanical properties in response to the mechanical loading they receive and it is believed that the actors in this process are tendon cells [6]. This mechanical adaptation response is thought to contribute towards the prevention of tissue failure and enables tendon to maintain a constant internal environment [59]. A number of positive and negative effects of mechanical loading

on tendon health and tendon healing have been identified, and these are highlighted in Chapter 2 of the thesis.

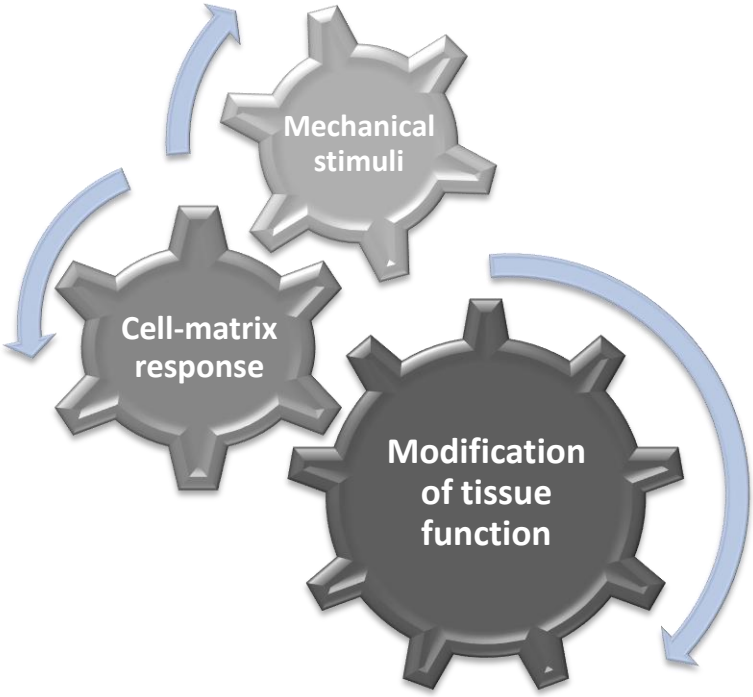


Figure 1.8: Schematic highlighting key events of Mechanobiology

1.6 *In vitro* loading systems

The complex and poorly understood *in vivo* environment in humans, make it exceptionally difficult to determine direct relationships between applied forces and tissue adaptation. For decades, *in vitro* systems have shown continued success in producing scientific discoveries and have contributed to a number of clinical breakthroughs. *In vitro* systems allow for the monitoring of tissue and cell response to environmental changes, in a highly controlled and repeatable way. The use of *in vitro* loading systems (IVLS), is an effective method for studying the influence of mechanical stimuli on healthy and damaged tendons in a laboratory setting. For example using *in vitro* systems, it is possible to make controlled observations by applying a known amount of stress at some frequency onto living tissue and quantify subsequent biochemical and biomechanical changes using assays and mechanical testing.

A number of criteria exists for the design of such a model system intended for studying tendon mechanobiology.

Specifically, such a model system should consist of a dissected tendon that can survive several days in culture, with each tendon portion being well matched in size and mechanical properties to the next and able to facilitate the generation of a precise repeatable damage. The system should be capable of maintaining an oxygen rich and moist environment for supporting metabolic activity in cells, while preventing bacterial contamination[60].

1.7 Aims of Project

The overall aim of this project is to elucidate the effects of various mechanical loading regimes and in particularly HFLM loading on the biochemical and biomechanical properties of healthy and damaged tendons using an *in vitro* loading system. This will be accomplished by employing tissue culture techniques in combination with biochemical assays and mechanical testing. The overall aims will be achieved by undertaking the following objectives;

1) Develop a novel IVLS for application of static, low frequency cyclic and HFLM loading of cultured tendon fascicles. Validate the system by characterisation of the applied cyclic load frequency and magnitude; and with measurements of tissue viability subsequent to culture. Assess the effect of static and HFLM loads on the GAG content and mechanical properties of tendon fascicles.
2) Examine the effect of mechanical loading frequencies and dosage periods on the GAG content and modulus, of healthy tendon fascicles.
3) Investigate the potential mechanisms for tendon mechanical adaptation to mechanical loading by considering cellular and micro-structural effects.

4) Develop and validate an injury model for investigating *in vitro* tendon repair. Establish suitable markers for tendon repair. Examine the potential of RTTFs to demonstrate an *in vitro* repair response. Identify the precise effect of *in vitro* damage on the modulus, viability and metabolism of cultured tendon fascicles.
5) The ultimate aim of this project is to use the above approaches to investigate the effect of various mechanical loading conditions on tendon repair and identify which loading condition provides strongest stimulation for tendon repair as indicated by measures of tendon metabolism and tendon modulus in damaged RTTFs

1.8 Thesis Outline

The following chapters of this report aim to firstly provide a review of the most relevant literature on tendon disorders, tendon repair and tendon response to mechanical loading in Chapter 2. Chapter 3 provides information on the working materials and methods. This includes viability and biochemical assays, together with the mechanical testing protocols and details of techniques used to facilitate tissue culture and the *in vitro* injury model used. In chapter 4 the first of four studies, a novel IVLS for tendon mechanobiology is introduced along with preliminary findings on the effect of static and HFLM loading on healthy RTTFs. Chapter 5 is a study on the effect of HFLM dosages and frequencies on the properties of healthy tendon fascicles. In Chapter 6, the contribution of cellular and non-cellular activities as potential mechanisms for tendon adaptation to mechanical loading is examined. In the concluding study, Chapter 7, the effects of static, low frequency and HFLM cyclic loading on *in vitro* tendon repair are examined. Finally, chapter 8 presents a summary of the thesis findings, the conclusions from the studies that have been performed as part this project and an outline of proposed future work.

CHAPTER 2 Tendon injuries, healing and strategies for repair

2.1 Introduction

In chapter 1 the ubiquitous nature of tendon injuries within the sporting and occupational environment along with the diminished properties of the repaired tissue was presented as part of the motivation for this thesis. This chapter provides a review of tendon injuries, tendon healing, and common strategies used for tendon repair. Also, included is a short review introducing paradigms of mechanical loading as a non-drug based therapeutic method for tendon repair. These key areas are essential in understanding the aims and methods of this project.

2.2 Tendon Injuries

Tendons transmit forces and as a consequence may experience trauma and degeneration. This makes them susceptible to both acute and chronic damage which impairs normal functioning of the tissue. Damage to tissue in this way is referred to as an injury. Tendon injuries are generally considered to be acute, as in the case of ruptures, or chronic as in the case of tendinopathies and resulting from either tensile overload or repetitive micro-trauma [33]. The precise aetiology of tendon injuries is not very well defined and this reflects our somewhat incomplete understanding of the subject [34]. Table 2.1 details some common tendon injuries along with their associated symptoms. These range from inflammatory type disorders to full or partial thickness ruptures, and clinical diagnosis for these disorders often depends on biopsies and histopathological observation.

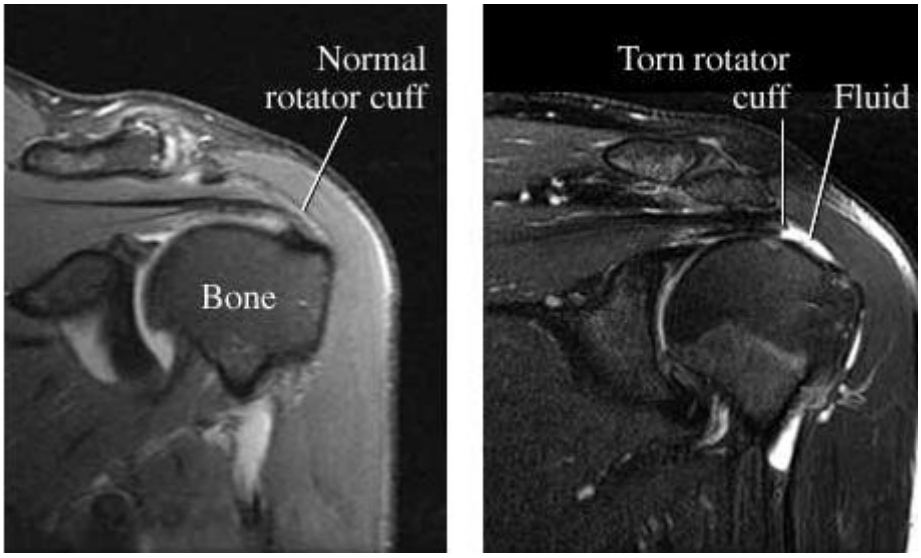


Figure 2.1: MRI showing typical rotator cuff tear of the shoulder with tissue fluid separating torn fibres. Taken from Schultz, JR., 2009.

Tendinopathy is a generic term used to describe tendon overuse injuries which are multi-factorial in origin, since they can be caused by extrinsic factors such as accidents and sports collisions, or intrinsic factors such as tissue degeneration. In sports, tendinopathy is typified by pain, localised tenderness, and swelling at the injury site that often impairs performance [1]. Paratenonitis is brought about by traumatic and excessive loading of the paratenon (the outer layer of connective tissue surrounding tendon) which results in inflammation of the paratenon and can be recognised by oedema and swelling of the joint. The term tendinitis has also been used to describe acute chronic tendon strains, which in the most extreme cases may lead to partial or complete tendon rupture [61]. Tendinosis is characterised by tendon degeneration and is brought about by the incapability of the cell matrix to adapt to varying stresses that are caused by an imbalance between matrix degeneration and synthesis [62].

Enthesopathy disorders arise due to the susceptibility of the osteotendinous junction to tendon overuse injuries [6]. It is estimated that the tensile forces at the enthesis may be four times that of the tendon midsubstance [63], and degenerative histological changes in the fibrocartilage matrix have been correlated with enthesopathy [64].

Tendon ruptures are partial or complete tears within the tendon substance (figure 2.1) [65], and although there precise origin remains enigmatic, the most common histological finding in ruptured tissue is degenerative tendinopathy. Very rarely are tendon ruptures completely "spontaneous" as they are often preceded by warning signs of tenderness, stiffness and pain or discomfort, prior to rupture. In a study by Kannus and Jozsa degenerative changes were found in 865 of 891 tendons that had spontaneously ruptured [66]. In Sports related injuries, an acceleration-deceleration mechanism has been reported to be the cause of up to 90% of Achilles tendon rupture [34].

Tendon degeneration has a debilitating effect, inevitably leading to reduced tensile strength and a predisposition to rupture, and it has been reported that the source of degenerative changes are usually a mixture of hereditary, occupational, and lifestyle factors [21]. Tendons found at different joints in the human body present diverse physiological and pathological characteristics. The Achilles tendon of the heel joint for instance, acts as an energy absorbing spring particularly during activities that require energy conservation such as walking and running [67].The thinnest portion of the Achilles and the region of avascularity which lies 2-6cm proximal to the calcaneus is the most susceptible to tendinitis and rupture. Achilles tendinitis is said to account for up to 15% of all running injuries [68]. In contrast, the Rotator cuff tendons of the shoulder are designed to produce joint stability and control during shoulder rotation. Unlike Achilles injuries, rotor cuff disorders are often age related and it has been suggested that approximately 30% of the population aged older than 65 years will experience a tear or related injury [69]. A tear is usually preceded by impingement syndrome during which tendon is repeatedly 'scraped' against the shoulder blade causing fraying and eventually rupture [70, 71]. The high exposure of tendons to repetitive load cycles makes them susceptible to fatigue damage.

Table 2.1: Classification of tendon injuries. Adapted with modification from, Leadbetter WB., 1992, Sharma and Maffulli, 2005, Kannus and Jozsa 1991, and Wang, JC., 2006

Type of tendon Injury	Description	Histological findings and symptoms
Tendinopathy	Generic term used to describe tendon overuse injuries	Recognised by pain, localised tenderness, swelling and impaired functioning
Paratenonitis	Inflammation of Paratenon	Collagen fibre disorientation, and scattered vascular in growth. Signs of swelling
Tendinitis	Symptomatic degeneration of tendon due to inflammatory repair response	Inflammation, disruption of vascular integrity, hematoma, atrophy related cell necrosis
Tendinosis	Structural alteration in tendon by intratendinous degeneration due to atrophy (aging, micro trauma)	Appearing grey brown and amorphous with loss of healthy glistening white outward appearance. Chief manifestation is rupture
Enthesopathy	Tendon injury at bone-tendon junction	Metabolically active insertion site with ECM is alterations. Loose collagen bundles, accumulation of lipids, and micro-calcification of tendon substance
Tendon Rupture	Tear or mechanical failure of tendon substance	Caused by degenerative Tendinopathy

The presence of ongoing tissue remodelling however prevents tendon fatigue damage from becoming degenerative. In a study by Ker *et al*., 2002 it was revealed that the fatigue quality of each tendon appears to be adjusted to the kind of fatigue loading it experiences in everyday life. It was proposed that tendon remodelling is prompted by fatigue damage, which occurs at a sufficient rate for cells to receive a detectable signal [24].

2.3 Tendon Healing

When tendons are damaged the body initiates a complex wound healing process which is characterised by various biological processes involving tissue inflammation, cellular proliferation and remodelling. In adult humans, this process often manifests as progressive removal of scar formation during a cascade of cellular and biochemical events [33]. Fundamentally, healing may occur by regeneration as is the case with human embryos, and vertebrates such as frogs and salamanders [72], by scar repair, or by combination of both.

Regeneration is a form of repair that produces new tissue that is morphologically and physiologically indistinguishable from the uninjured tendon [61]. This phenomena is known to occur early in gestation with injured fetal tissue being completely reformed without scarring [73-75]. Such regenerative capabilities are thought to be lost prior to birth due to signalling pathways responsible for them being rendered dormant [76].

Repair by scar formation often involves reduction in the size of abnormal tissue and a noticeable diminution of structural and functional properties of the intact tendon. Our current understanding of tendon healing, stems predominantly from studies performed on transected animal tendons or ruptured human tendons, and their relevance to healing of tendinopathic human tendons remains largely obscure [62].

The healing process initiated by the body subsequent to tendon injury can be divided into three overlapping phases and the process is essentially the same for all soft tissue damage. These phases are highlighted in figure 2.2.

Phase 1 is known as the inflammatory phase and occurs almost immediately after damage. During this initial phase, trauma leads to a localised collection of blood (erythrocytes and platelets), such that haemorrhaging is prevented by clotting (hematoma). Following this, phagocytosis of debris and necrotic tissue is brought about by migration of macrophages,

monocytes, and neutrophil. Further, tendon fibroblasts are recruited by the release of vasoactive and chemotactic factors, resulting in an increase in DNA content and other tissue components such as collagen type III [6].

Phase two, known as the Proliferation phase can last for weeks, as depicted in figure 2.3. During this period there is active matrix synthesis, as indicated by the presence of abundant endoplasmic reticulum. Water content and glycosaminoglycan concentrations remain elevated while DNA and collagen III, reach their peak amounts [77]. These changes aid the gradual conversion Type III collagen into type I collagen.

The final phase of healing, Remodelling, begins approximately 6 weeks after initiation of tendon damage, and can last for more than 12 months. During this phase the scar matures and becomes visible as a pinkish-translucent material. Fibroblasts decrease in size and there is a corresponding decline in the synthesis of new matrix material. Type I collagen becomes dominant over type III and the fibres orient themselves longitudinally to the long axis of tendon. A characteristic increase in cross-links between the collagen fibres is accompanied with a rise in the water, GAG and DNA content [33].

Two modes for tendon healing have been proposed, extrinsic and intrinsic healing. During the former, the ability of tendons to heal is largely dependent on the permeation of inflammatory cells and fibroblasts from outside the tendon through the formation of internal scars. These scars form abnormal bands of fibrous tissue at the tendon sheath (adhesions) along with an extra-tendinous blood supply, together with permeation of inflammatory cells and fibroblasts[78]. In the latter, damaged tendon is almost devoid of adhesions, however proliferation of epitenon and endotenon cells have been implicated as primary governors [79]. Evidently, the ability of tendons to undergo intrinsic healing has prompted considerable

number of tissue culture studies on *in vitro* tendon repair and these are discussed further in chapter 7 of this thesis.

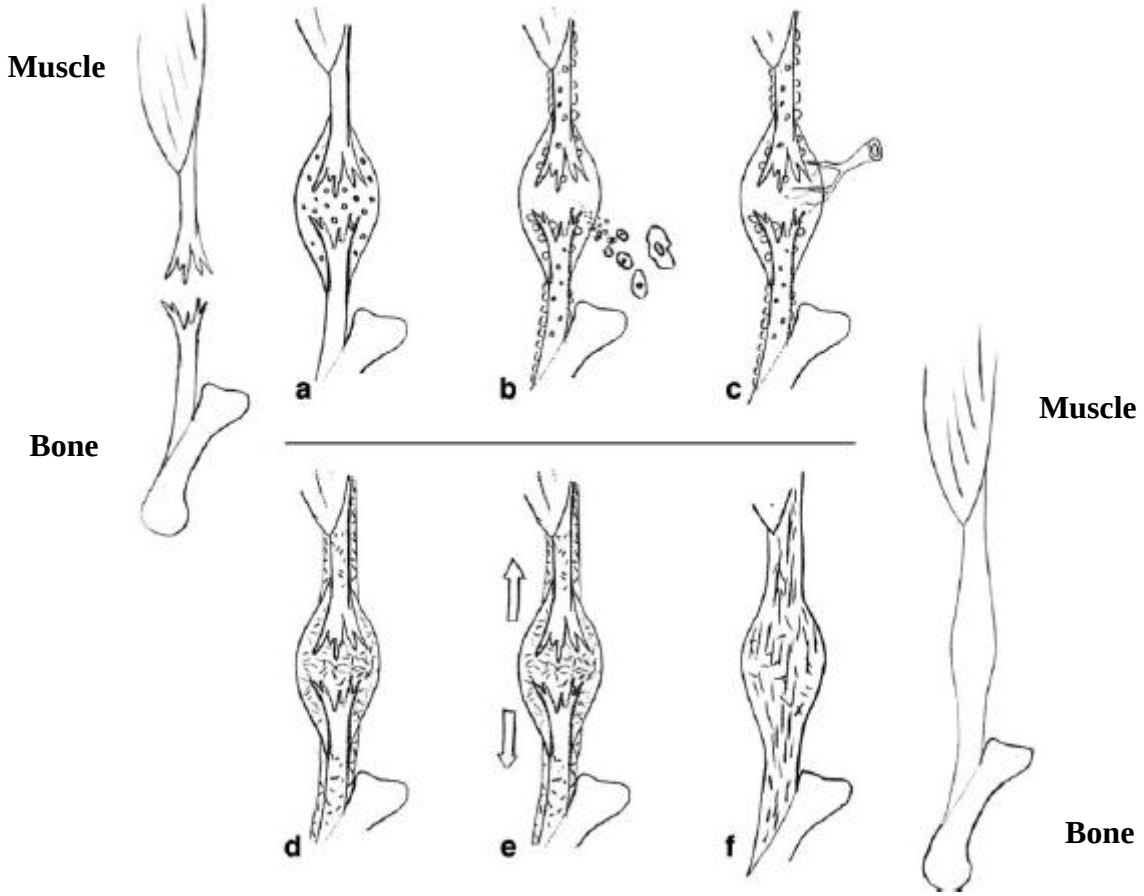


Figure 2.2: Schematic highlighting various stages in tendon healing by repair. a Haematoma; b Invasion of cells and proliferation of paratenon; c Vascular and neuronal ingrowth (angiogenesis); d Loose collagenous callus formation; e Mechanical stimulation; f Maturation and remodelling. Adapted from Aspenberg, P., 2007 [80]. With permission.

As previously mentioned, tendon healing is made possible by a number of cellular and ECM modifications which occur throughout the individual phases. Migration of fibroblasts towards the healing tendon may be encouraged by the epitenon sheath and or the paratenon sheath [81], and tenocytes involved in healing have been reported to initiate collagen synthesis within a only few days subsequent to damage [34] . Further contributions arise from the vast number of glycoproteins present in the ECM of tendon. Amongst these is fibronectin which

is found on collagen surfaces. It is understood that synthesis of fibronectin increases in order to facilitate wound healing and it is believed that the fibronectin is the most essential chemotactic factor for fibroblasts and macrophage activity [82].

2.3.1 Biomechanical properties of healing tendons

Biomechanical properties such as tensile strength of regenerate tendons are generally inferior to that of the intact, undamaged tendon [61] as shown in figure 2.3, and this has previously been attributed to a number of cellular and matrix alterations which occur during healing. More recently however, it has been postulated that the decrease in biomechanical properties of the repaired tendon may be due to the absence or lack of mechanical loading during the healing period[80, 83, 84]which is considered necessary during the initial stages.

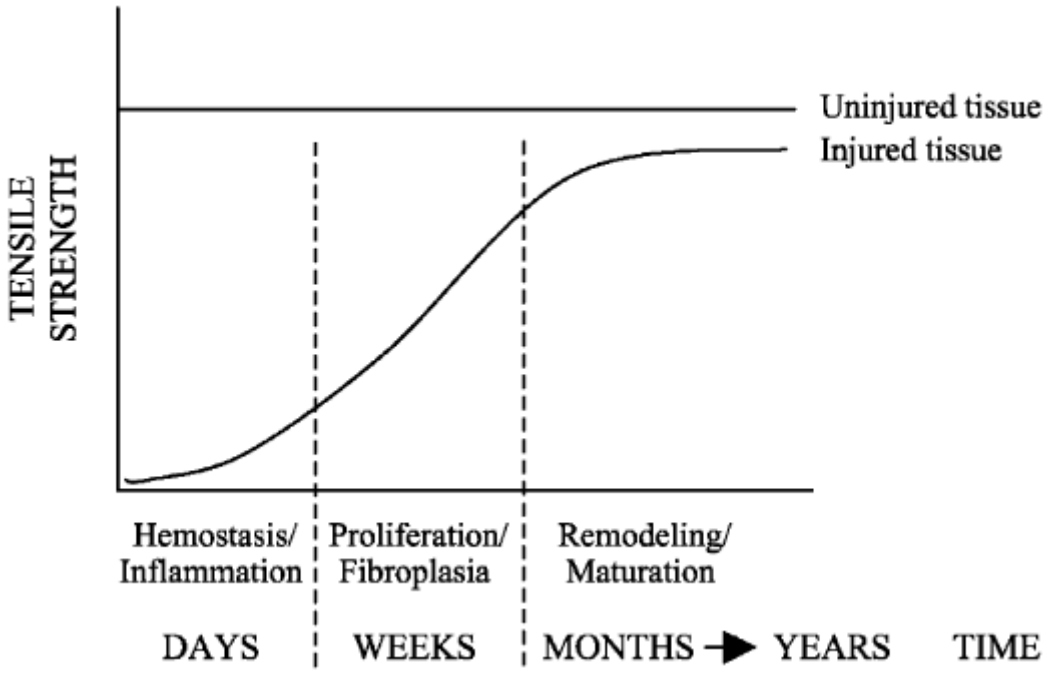


Figure 2.3: Wound healing response in damaged tendons, illustrating the decline in tensile strength and permanent loss of mechanical properties of injured tissue compared with uninjured tissue. Taken from Lin *et al.*,2004 [33], With permission.

A major challenge therefore in the repair of damaged tendons is the use of mechanical stimulation during the early stages of healing. Loading during healing is crucial for avoiding

undesirable adhesions which hinders normal tendon function, but also involves the risk of rupture to the repair site [85, 86]. Correspondingly, it has been suggested that surgical and non-surgically repaired tendons demonstrate great disparity with the repair material of the former allowing earlier motion than non-surgically repaired tendon and showing superior biomechanical properties [3]. Despite the cascade of biological processes which take place subsequent to healing, the biomechanical and indeed biochemical properties of the healed tendon tissue is never fully restored to its uninjured state.

2.4 Strategies for tendon repair

A number of different strategies have been used in the management of tendinopathy and tendon ruptures. These treatment methods have aimed to stimulate tendon healing such that damaged tissue is restored to a healthy functioning state [21]. Nevertheless, current techniques have shown limited and inconsistent success and the quest for a long term successful solution remains the focus of intense research.

2.4.1 Tendon rehabilitation methods

Common rehabilitation methods for tendinopathy and mild tendon wounds are rest, ice, compression, elevation (RICE), or Rest, anti-inflammatory medication, Physiotherapy, steroid injections (RAPS). These are particularly popular amongst sports therapists for treating inflammatory type injuries such as tendinitis and peritendinits [21]. Steroidal anti-inflammatory drugs known as corticosteroids may also be used to provide symptomatic relief but do not provide a long lasting solution [87], and have been implicated in damaging effects on tendon biology [88, 89]

When tendon damage is severe, as in the case of acute and sub-acute ruptures, there is often a need for surgical intervention in order to reconnect the ruptured tissue. The use of braces and casts as an aid for rehabilitation is also widely practised. Furthermore, additional

rehabilitation stages of stretching and eccentric muscle strengthening are often employed to supplement any one of the above mentioned techniques.

2.4.2 Physical Therapies

Physical modalities which have been used to facilitate wound healing typically involve the use of mechanical, thermal, or electrical stimulation, and some of these are highlighted below. Most commonly, these include Ultrasound therapy, Extracorporeal shock wave (ESW) therapy, and Pulsed electro-magnetic field (PEMF) therapy.

2.4.2.1 Ultrasound therapy

Ultrasound, a widely used modality for treating soft tissue injuries uses sound waves with frequencies between 20kHz-200MHz, and its therapeutic effects have been exploited by clinicians over the past 50 years. Common dosages range from 0.125 to 3W/cm^2 at frequencies of 1-3MHz during the inflammatory, proliferative and remodelling phases of tendon healing [90].

When applied onto tendon injuries ultrasound energy waves can be used to convey mechanical and or thermal effects. In its mechanical mode, an ultrasound transducer converts electrical energy into mechanical energy by directing pulses of EM radiation onto biological tissue. It is believed that when used in its mechanical mode the benefits are far greater if administered during the early stages of healing [91, 92].

An increase of 65% in the tensile strength compared with control tendons has been reported and [93] and enhancement of several other biomechanical properties of tendons has been demonstrated using ultrasound [90, 94, 95].

When used for its thermal effects, ultrasound, like most other forms of heat is often applied during the latter stages of tendon healing in combination with stretching exercises and this ultimately improves the flexibility and extensibility of the treated tendon [96].

Ultrasound therapy has been reported to promote a considerable number of biological processes including, stimulation of macrophages [97], conversion of collagen type III to the more dominant type I, modification of collagen fibre alignment[98], and stimulation of mast cell degranulation which increases the rate of histamine delivery to the damage site [99].

In spite of this, the clinical efficacy of ultrasound to treat associated tendon injuries such as chronic tendinitis, peritendinitis, and common ankle sprains is still disputed amongst clinicians since the outcome of several clinical trials have reported no significant difference between treated and untreated patients[100-103].

2.4.2.2 Extracorporeal Shock Wave Therapy

Extracorporeal shock wave (ESW) therapy is a non-invasive treatment method that uses a high voltage spark generated under water to produce an explosive evaporation of water. The resulting high energy acoustic waves that are created are focussed on to specific tissue sites using a semi-ellipsoid reflector. In clinical applications ESW has shown effect in treating tendon-bone junction disorders in addition to Achilles tendinitis, calcifying tendinitis of the shoulder and painful heel syndrome[104-107]. In one animal study, 200 impulses of ESW therapy applied to the Achilles of rats was found to restore the biomechanical and biochemical properties of the healing tendon, 12 weeks after treatment, as revealed by changes in the load to failure, stiffness, DNA, GAG and hydroxyproline content [108]. A typical set-up and apparatus used during ESW therapy are highlighted in figure 2.4 [109].



Figure 2.4: Patient receiving ESW therapy. Taken from Rezk-Allah., *et al* 2013.

2.4.2.3 Pulsed Electromagnetic field therapy

Although Pulsed electro-magnetic field (PEMF) therapy has demonstrated clinical value for the treatment of bone fractures and spinal fusions, there have been a limited number of reported studies on their effects on tendon repair. PEMF therapy is an electrical stimulation method during which magnetic pulses are focussed onto injured tissue. The magnetic pulses induce tiny electrical signals that stimulate tissue repair and healing.

In one *in vivo* study it was shown that the Achilles tendon of Sprague Dawley Rats that had received PEMF therapy for a period of 21 days after laceration and suturing showed a 69% increase in tensile strength at the repair site compared with non-stimulated control animals.

In a double-blind controlled pilot study involving 29 patients with rotator cuff tendinitis which had persisted for at least 3 months, patients receiving PEMF therapy (15) showed a significant benefit over control patients receiving no treatment, as measured by assessments of pain score, pain on resisted movement, painful arc on active abduction, and the total range of active movement [110]. Despite these encouraging signs, the high number of conflicting

and inconsistent results for studies on PEMF therapy[111-114], raise speculation on the reliability of this non-invasive modality for tendon repair.

2.4.2.4 Tissue Engineering

The development of artificial materials for musculoskeletal applications, particularly those in load-bearing conditions has been the focus of much research in recent times. Tissue Engineering is the laboratory based development of living tissue which focuses on using constructs that are implanted into the wound site of tendon in order to facilitate tissue regeneration or in order to act as potential replacement tissue [115-117]. A tissue engineered construct typically consists of three major components either alone or in combination. These include non-immunogenic cells such as mesenchymal stem cells, degradable scaffolds which serve as a 3D template for cell growth and tissue redevelopment, and finally signals, such as growth factors which contribute to the biological processes of healing [87].

Biomimetic tissue replacements such as scaffolds and polymer derived patches particularly in the repair of rotator cuff injuries have been studied extensively. These have shown potential for the mechanical suitability of repair grafts in supporting the human rotator cuff following injury [118-121]. A schematic showing a typical approach to a tissue engineered tendon construct is shown in figure 2.5 [122].

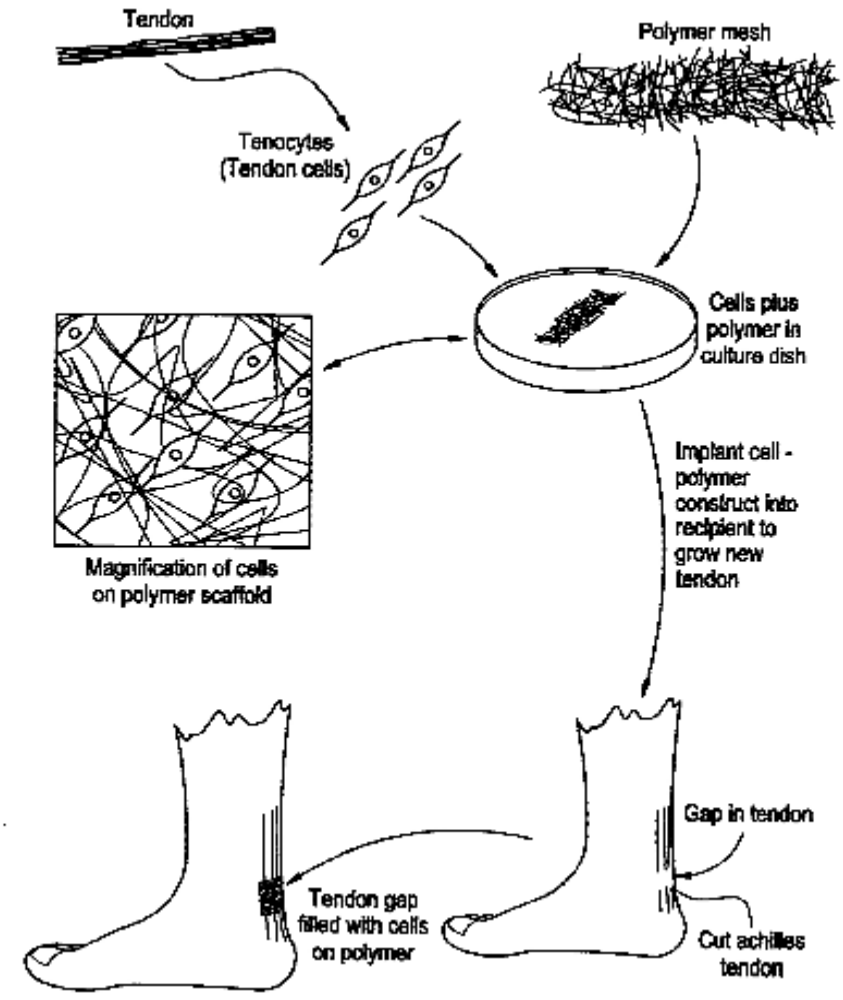


Figure 2.5: Illustration of patented tissue engineered tendon construct using biodegradable synthetic scaffold seeded with tendon cells. Taken from Vacanti CA *et al.,* 2005.

Studies have also been performed using mesenchymal stem cells in gapped-tendon animal models. These studies found minimal improvements for the repair process of tendon and this demonstrates that as with cartilage and ligament repair, stem cell technology for tendon repair is still in its infancy [123]. In Functional tissue engineering the *in vivo* mechanical stresses on uninjured tendons are determined and this information is used to design constructs capable of sustaining *in vivo* loads [115, 124]. Following tendon rupture the use of tissue adhesives, surgical stitches and sutures are often required in order to augment scar healing [125].

2.5 Mechanical Loading of tendons

Tendons are living biological tissues and are therefore responsive to mechanical loading. The appropriate amount of mechanical loading at the physiological level is beneficial to tendons. However, excessive mechanical loading or lack of mechanical loading conveys damaging effects on tendons and a greater understanding of these paradigms are essential for enhancing and optimising tendon repair.

2.5.1 *In vivo* forces on tendons

During muscle contraction forces are transmitted onto bone through tendons and these forces have been measured in animals using implantable force transducers (IFTs). In goats the patellar tendon force was measured to be 207N during standing, while sharply rising to 800N and 1000N during walking and running activities respectively [7]. The physiological loads experienced by human tendons vary with activity and tendon type. Some of the smallest forces experienced by human tendons are those in the wrist and fingers and these range between 1-6N in the wrist and up to 9N in the fingers [6] . In contrast, forces as high as 9kN (over 12 times the body weight), are often transmitted through the human Achilles tendon during running [6, 126]. Moreover, during rest and immobilisation, tendons are believed to experience *in vivo* forces. Passive tension exists since tendons at rest are not always in a slack position between muscle and bone. Subsequent to isometric conditioning, passive tensions as low as 0.2N have been observed in resting tendons [9]. The effects of static loads on tendons are discussed in Chapter 4 of this thesis.

2.5.2 Effects of mechanical loading (mobilisation) on healthy tendons

Mechanical stimuli have been postulated as an important regulatory factor in tendon homeostasis and their influence on tendon cells, and tendon ECM has been the focus of a number of scientific studies. The ability of tendon tissue to modify its structure, composition and mechanical properties in response to mechanical forces is brought about by action of

tendon cells called tenocytes [127]. Tenocytes translate mechanical forces into biochemical signals that trigger physiological and pathological changes and thus permit tissue mechanical adaptation to occur [6].

A number of human studies have shown that mechanical loading by training and exercise in athletes increases the deposition of collagen type I in the Achilles tendon [128, 129]. Several animal models have also been used to study the effect of mechanical loading on tendon tissue. In a study by Curwin *et al*.,1988 it was found that 8 weeks of hard endurance training was enough to bring about a 46% increase in collagen deposition in the Achilles tendons of roosters. This effect was accompanied by a 50% "reduction in pyridinoline" crosslinks. They concluded that prolonged tendon activity resulted in improved collagen turnover, with newly formed fibres however maintaining immature characteristics as shown by the reduced cross-linking [84].

The use of rat Tail tendon specimens is widespread within tendon research and like humans, forces generated within rat tail tendons vary with age, gender, and species. In one investigation, it was found that after 26 weeks of heavy resistance training, male Wilstar rats were capable of lifting up to 800g (8N) or 140% of their individual body mass for four sets of 12-15 repetitions per session [8].

In other animal models, 12 months of running exercises improved the biomechanical properties of the digital flexor tendons of swine[130], and physical exercise was found to increase the cross sectional area but decrease the failure stress of tendons compared to control animals [131].

2.5.3 Effects of mechanical loading (mobilisation) on healing tendons

Adaptation to the presence and the absence of mechanical loading is far more rapid in healing tendons than in healthy tendons [20]. These effects have been demonstrated in a study where

mobilisation after damage in rat tendons resulted in an increased rate of collagen biosynthesis [132].

While mechanical loading of healthy tendons causes thickening of the collagen fibres, in healing tendons, controlled mobilisation activities during the proliferative phase of healing, accelerates the synthesis, and correct alignment of collagen fibres [90]. During the inflammatory phase of repair, it is believed that training and stretching should be avoided so that healing may continue uninterrupted [6]. Controlled mobilisation approximately 1 week after injury however, is believed to improve the quality of healing tendons and has been shown to enhance the tensile properties of the repair site [133].

A major topic of interest within research for tendon healing is concerned with dosages of mechanical loading required for tendon repair. Andersson *et al*., 2009 found that unloaded transected tendons exposed to prolonged treadmill or cage activity for periods 30, 60 and 2x15minutes, each day had no additional effects on improving the healing of tendons with only 15 minutes of treadmill activity each day. They concluded that healing tendons posses a capacity to memorise short loading episodes, which subsequently results in long lasting response of several hours [20]. The required time for immobilization subsequent to tendon injury has been recognised as crucial in facilitating optimal tendon healing [134, 135]. Early voluntary exercise in animals studies has been found to decrease the stiffness and the maximum tensile strength, extend the inflammatory response, and promote long term collagen deposition in injured tendon [135]. Mobilisation of tendons shortly after damage has also been shown to decrease the formation of adhesions during healing of canine flexor tendons [136].

2.5.4 Effect of load deprivation on tendons

Less is known about the effects of inactivity and disuse on tendon tissue, compared with that of other musculo-skeletal tissue [137]. Conventionally, tendon immobilisation is generally used to aid healing during the early phases after tissue damage [33]. Immediate immobilization is generally employed following a partial or complete rupture, although in some extreme instances, the necessity for surgery is inevitable for re-attachment of torn fibers. In order to reduce or alleviate the effects of atrophy during immobilisation, the period of complete inactivity following injury, should be minimised [138]. Studies have shown that tendon immobilisation ultimately results in diminished total weight of the tendon, along with decreased tensile strength and elastic stiffness [139-142].

2.5.5 High Frequency low magnitude (HFLM) mechanical loading

High frequency low magnitude (HFLM) loading regimes are mechanical loading conditions which are characterised by cyclic frequencies of 10Hz and above, and loading magnitudes that are many orders lower than those present during intense physical activity, as depicted in figure 2.6.

HFLM loads have been shown to be effective in increasing bone mass, and in mitigating the effects of osteoporosis [17-19, 143]however little is known about its effects on tendon.

Correspondingly, the positive effects of an eccentric loading regime in the rehabilitation of Achilles tendinopathy have been linked to high frequency loading [144], and high frequency loads of up to 65Hz combined with 1.2mm displacement amplitudes have been suggested to stimulate neuromuscular responses in healthy biceps tendons [145].

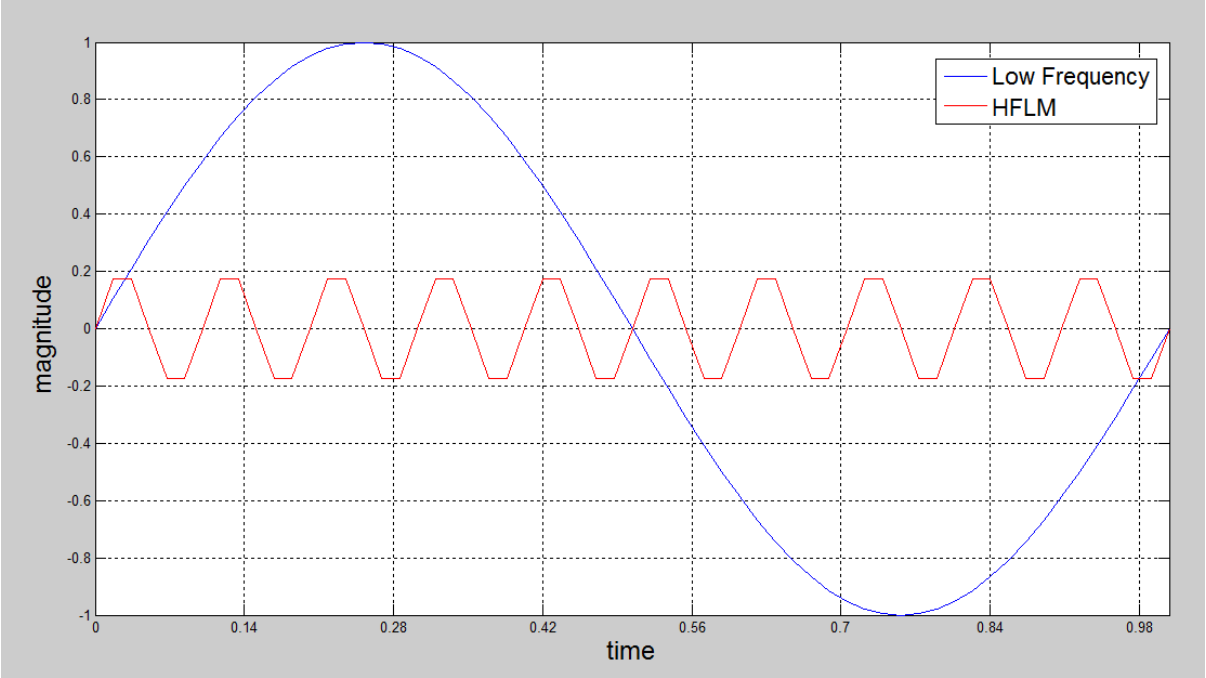


Figure 2.6: Graphical illustration of HFLM mechanical load cycles superimposed on a low frequency mechanical load cycle typically observed during physical activity

Administration of whole body vibrations (WBV) by means of vibrating platforms has been utilised to deliver high frequency mechanical loads and to elicit therapeutic effects in musculoskeletal tissues in both human and animal studies [146, 147]. These studies have reported effects such as increases in trabecular bone mass density (BMD) in rats, [148, 149], increases in BMD of the human spine [150], and increases in muscle strength and cross sectional area of mature and young human subjects [151, 152].

However, drawbacks such as patient discomfort, limited transmissibility of floor vibrations to more proximal structures in living organisms, and potential harm to soft tissue in the upper extremities have been associated with the WBV technique [153] [147]. Further, appropriate standards for use of vibrating platforms have not been established or validated for human patients. Alternative and less risky methods for administration of HFLM loads on tendons are discussed in chapter 4 of this thesis.

Ultimately, the use of HFLM mechanical loading and WBVs for tendon therapy is a field of research that remains in its infancy and appropriate frequencies, durations, and amplitudes of mechanical loads necessary for beneficial responses have not been defined.

2.6 Summary

In this chapter a number of key areas within tendon injuries and tendon healing were discussed. These highlighted the various types of tendon disease and the biological healing stages involved in restoring damaged tendons into a healthy functioning state. This was followed by a brief review of current strategies used for management of tendon injuries in section 2.4, and the effects of mechanical loading on healthy and damaged tendons in section 2.5. The next chapter describes the Materials and Methods used in this thesis.

CHAPTER 3 Materials and Methods Common for all Studies

3.1 Introduction

Chapter 2 presented a review of relevant literature detailing the potential of mechanical loading as a non-drug therapy method for promoting tendon health and tendon healing.

In vitro models enable the complex relationships between mechanical stimulation of tendons and tissue adaptation to be studied in a controlled and repeatable way, allowing greater understanding of the mechanisms driving this therapeutic technique for tendon repair.

This chapter provides a comprehensive description of the developed *in vitro* models used to investigate tendon response to mechanical loading in healthy and damaged fascicles, and the materials and methods used to perform the hypothesis driven experiments have been categorised into the following 1.) Tissue culture techniques and tendon isolation 2.) Tissue viability 3.) Imaging techniques, 4.) Biochemical assays 5.) Mechanical testing, and 6.) Tendon fascicle injury model.

3.2 Tissue Culture

Tissue cultures are useful because they enable connective tissue function to be examined in relative isolation. They provide an ideal model for controlling experimental variables, allowing a detailed analysis of finite changes to experimental conditions [4].

Isolated cultured tendon fascicles derived from rat tails are frequently used to study tissue biochemical, biomechanical and microstructural responses in tendon, with an assumption that cultured fascicles contain essential biochemical features for tissue viability, similar to an un-dissected tendon fascicle in its physiological environment.

3.2.1 Tendon harvesting

Tails were removed from male Sprague Dawley Rats (aged between 3-6 months and weighing 300-360g), following animal sacrifice, at the Department of Physiology, Anatomy and Genetics, Oxford University. These rats were killed as part of an unrelated study, with full ethical approval, and where the animal tails were not needed. An image of typical rat tail is shown in figure 3.1, highlighting the dimensions of an individual rat tail tendon fascicle (RTTF). The superficial skin and fascia of rat tails were sprayed and disinfected using 70% industrial methylated spirit, IMS (Sigma Aldrich, UK). The tail tips, (consisting of approximately 5 vertebrae) and the outer layer of skin were removed using a scalpel. Tendon fascicles were harvested (from the distal end of the tail, using sterile forceps) and immediately cultured in media within 2 hours of animal sacrifice. All tissue culture techniques were performed under aseptic conditions and using a laminar flow hood.

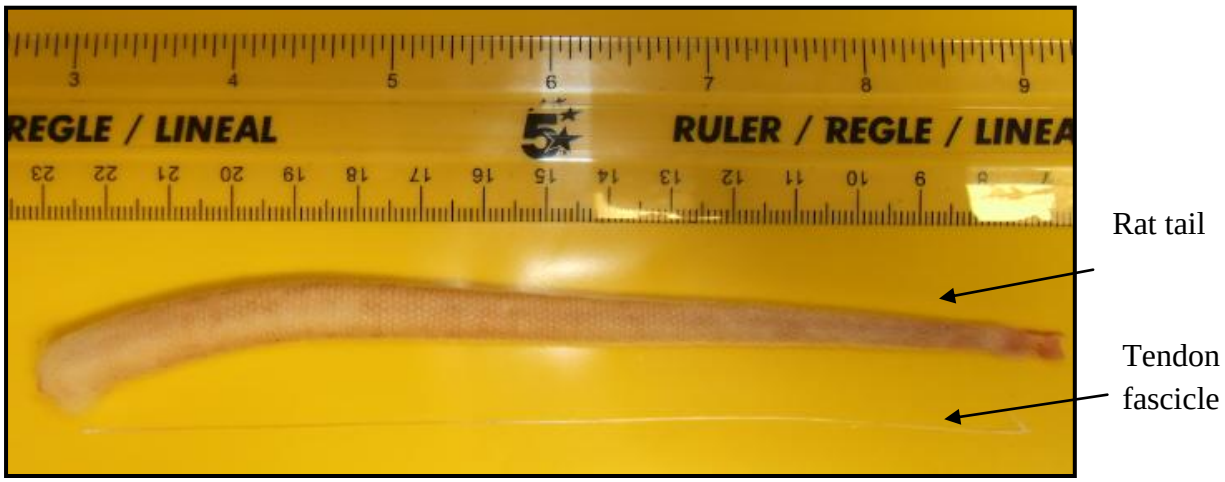


Figure 3.1: Image of rat tail and extracted fascicle after sacrifice from male Sprague Dawley Rat.

3.2.2 Tendon fascicle isolation and *in vitro* loading model

Tendon fascicles were immersed in Dulbecco's Modified Eagle Medium (DMEM) (Lonza-Biowhittaker, Belgium) in petri-dishes immediately after harvesting in preparation for isolation and incubation in one of three loading conditions; no load, static load or cyclic load.

The characteristic lengths of un-dissected rat tail tendon fascicles (14-16cm), which is similar to the rat tail (figure 3.1), makes them ideal for centrifuge tube cultures. An experimental apparatus (figure 3.2) was designed using centrifuge tubes in order to fulfil the *in vitro* loading criteria and table 3.1 gives details of the individual components used to form the static *in vitro* loading system along with their specialised roles.

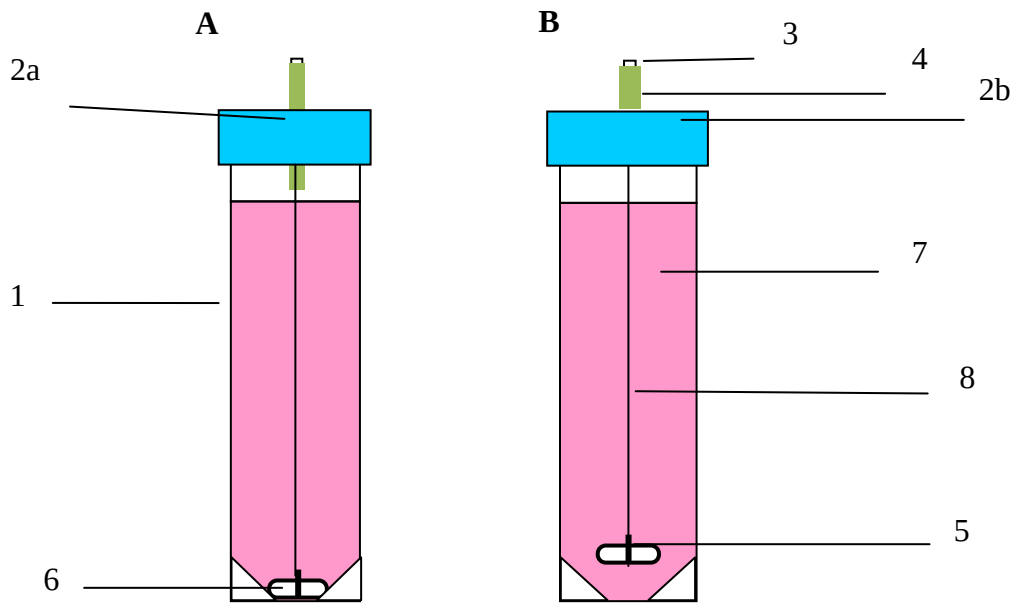


Figure 3.2: Illustration of *in vitro* apparatus for A.) Unloaded tendon fascicle, and B.) Static loaded tendon fascicle. Features labelled 1-8 are described in table 3.1

Rat tail tendon fascicles were cultured and suspended in 50 ml centrifuge tubes (DMEM supplemented with 10% foetal calf serum (Biosera, South America) and 1% penicillin–streptomycin (Sigma Aldrich, UK), for tissue growth survival and antibiotic protection). Four pin sized holes in the tube cap facilitated gas exchange. A custom designed gripping system attached the RTTFs to the cap, wrapping them around an inert rod clamped by a silicone tube. A magnetic stir bar (VWR, UK) (weighing approximately 6.2±0.14g) was glued to the lower end of the RTTF using a biocompatible cyanoacrylate adhesive (RS components, UK). This produced static tension in the tendon fascicle (0.06N±0.010N). Unloaded tendon fascicles were cultured in an identical way to statically loaded fascicles only with the magnetic weight being lowered to the bottom of the centrifuge tube. Fresh tendons were kept in a culture dish with the above described media, before subsequent testing. Cyclic loading of isolated tendon fascicles was achieved using a custom-built electromagnetic stimulation device located below the bottom of the culture tube. A time varying magnetic field agitated the magnet stir bar superposing cyclic loading on the static load on the cultured fascicle. The components and operation of the custom built electromagnetic stimulation device are described in detail in chapter 4 of this thesis.

3.2.3 Culture environment

Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin streptomycin antibiotic was used to culture tendon fascicles and subsequent to fascicle isolation, RTTFs were incubated at 37^{o}C and 5% CO_2, with culture medium being renewed every 3 days.

3.3 Tissue Viability

Staining methods using fluorescent reagents can be used to provide a sensitive measure of tissue viability and cell proliferation. A Live and Dead staining assay and an Alamar Blue assay were used to provide an indication of tissue health within the culture environment.

Table 3.1: Characteristic properties and function of components used as part of custom *in vitro* system for static and unloaded tendon groups

	Component	Material Property	Functional role
1	Centrifuge tube	Bio inert. Ideal length for un-dissected tendon fascicle	Housing for tendon fascicle DMEM, and magnetic stir bar. Enables RTTF isolation
2	Centrifuge tube cap with central hole (a) and surrounding pin-sized holes (b)	Stiff, hard, seal for housing. Inert, autoclavable and re-usable.	Means of entry for gripping system of perspex rod and silicone tube into housing. Provides oxygen pathway for tissue and cell respiration.
3	Perspex rod	Non-toxic and biocompatible with living tissue	Attachment for proximal end of tendon fascicle. Forms inner core of gripping system for RTTF
4	Master-Flex Silicon Tube	Biocompatible, inert, autoclavable, re-usable	Clamp for perspex rod and tendon. Forms outer layer of gripping system for RTTF
5	Cyno-acrylate adhesive	Biocompatible adhesive	Adhesive for attaching tendon fascicle to magnetic stir bar
6	Magnetic stir bar	Magnetic core, with inert polymer coating	Tool for providing static and cyclic tensile load onto RTTF.
7	Dulbecco's Modified Eagle Medium (DMEM)	Cell and tissue culture medium	Provides essential nutrients for tissue survival.
8	RTTF	Biological tissue	Specimen for assessment

3.3.1 Live and Dead Staining assay

Live and dead staining techniques are used to accurately distinguish a population of live cells from the dead cell population within cell and tissue cultures. Live and Dead staining reagents were obtained from Molecular Probes (Invitrogen, UK). Tendon fascicles were removed from

the culture environment and washed in Phosphate Buffered Saline (PBS) (Fishr Scientific, UK) in order to remove serum which could interfere with the staining process. A 2:1 live-dead staining solution (Sigma Aldrich, UK) was formulated by mixing 2µl of calcein -4mM solution in DMSO (live stain); and 1µl of Ethidium Homodimer-2mM solution in 1:4 DMSO/H_2O (dead stain); with 1ml of PBS. Live cells contain an intracellular chemical (esterases) that cleaves the non-fluorescent calcein (live stain) and causes it to become fluorescent green as it permeates the cell membrane. Ethidium (dead stain) will only penetrate the damaged membrane of dead cells and becomes fluorescent red when bound to their nuclei. Tendon fascicles were placed either on a microscope slide, and left to soak in the staining solution for approximately 30minutes before viewing under the microscope. Zen Software (Zeiss) was used in combination with Image J (NIH) to provide image analysis and count the number of live and dead cells. A fascicle was defined as viable if 50% or more of the observed cells were living.

3.3.2 Alamar Blue Assay

Alamar blue is an indigo coloured nontoxic aqueous dye that is used to assess cell viability and cell proliferation in tissue culture experiments. Alamar blue contains a REDOX indicator that both fluoresces and changes colour from blue to pink upon chemical reduction resulting from normal cell metabolism. The innate metabolic activity of cells within tissue can therefore be monitored with Alamar Blue.

Metabolic activity was assessed in healthy tendon fascicles subsequent to culture conditions of low (27^{o}C) and standard (37^{o}C) incubation temperatures using Alamar Blue (AbD Serotec, UK). Additionally, Alamar blue was used to monitor metabolic activity in damaged fascicles cultured under conditions of no load, static load, low frequency cyclic load and HFLM cyclic load.

Immediately after each experiment tendon segments were rinsed in DMEM and supplemented with Alamar blue (Abd Serotec, Kidlington UK), by using a 1:10 (v/v) mixture of the dye reagent and culture media. Following an incubation period of approximately 3 hours the REDOX state of Alamar blue was measured using Fluostar Optima spectrofluorometer (BMG Labtech 413-0120) at an excitation wavelength of 530 nm and emission wavelength of 590 nm, with a 570-nm cut off, and these were used to record the relative fluorescence unit (RFU), a measure of the tissues relative health.

3.4 Imaging Techniques

3.4.1 Confocal Microscopy

Stained RTTFs were inspected using a Zeiss LSM 710 inverted confocal laser scanning microscope coupled to a tunable and pulsed femtosecond Chameleon laser (Coherent, UK) operating at 850nm. For live and dead staining assay, peak emission signals of calcein-AM and ethidium bromide were collected at 510nm and 600nm respectively. A 10× objective lens was used over a central region of each fascicle (2.6 mm × 0.85 mm) with a 6 slice z-stack to a depth of 60µm. Slices were taken from the outside surface of each fascicle with a z-distance of 12µm between each slice. The maximum intensity projection (MIP) was used to provide a 2D image of the sampled section.

3.4.2 Multi-Photon Microscopy (MPM)

MPM was used for examination of tendon fascicle crimp morphology. Second Harmonic Generation (SHG) imaging was performed on unstained tendon fascicles. Samples were rinsed in PBS and viewed under an inverted confocal laser scanning multi-photon microscope (LSM 710; Carl Zeiss), coupled to a tunable and pulsed femtosecond Chameleon laser (Coherent, UK) operating at 850nm. An oil immersion objective (NA=1.0; 10 × magnification) was used for focusing the excitation beam and for collecting the backward SHG signals which were then directed by a dichroic mirror to an external detector through a

narrow band pass filter (384-447 nm). Images were acquired at three regions (2.6 x0.85mm x 3) across the longitudinal section in the mid-substance of each tendon fascicle. The high power, (long-wavelength) laser-light used in multi-photon microscopy, penetrates 200µm in thick tendon sections, avoiding artefacts typically associated with thin sections. This allows for high resolution visualization of the collagen microstructure deep within tendon sections, eliminating the need for staining reagents [154].

3.5 Biochemical Assay

3.5.1 Dimethylmethylene Blue (DMB)

The DMB assay is used to provide a quantitative measure of the Glycosaminoglycan (GAG) (Sigma Aldrich, Germany) present within biological samples. The assay is based on the ability of sulfated GAGs to bind the cationic dye 1,9-dimethylmethylene blue leading to changes in the absorption spectrum of the dye. GAG content was measured for healthy tendon fascicle groups subsequent to a culture period of 7 days and the protocol used was adopted from studies by Farndale *et al* 1986 and 1982 [155, 156]. Briefly, portions of each RTTF were dehydrated, weighed, dissected into small pieces, and digested with aliquots of papain enzyme in eppendorf tubes for 7 h at 65^{o}C. The DMB colour reagent was formulated using 40.55 mM glycine, 40.55 mM NaCl, 9.5 mM 0.1 M HCl, and 1.6 mg of 0.0016% [w/v] DMB. Chondroitin sulphate (CS) solution (Sigma–Aldrich, Gillingham, UK) was used at five concentrations (500µg/ml, 250µg/ml, 125µg/ml, 50µg/ml and 0µg/ml) to generate a standard curve along with blank papain buffer solution alone used for the 0µg/ml concentration. 40µl of the fascicle digest solution and 20µl of each CS concentration was transferred into individual wells in a 96 well plate. Each sample and standard was duplicated and 100µl of the colour reagent was added to each well, containing either sample or standard. The GAG concentration of duplicates of each RTTF digest was determined from absorbance at 520 nm using a Spectra Max Plus spectrophotometer (Molecular Devices, Sunnyvale, CA).

Measured concentrations for each fascicle were normalised by RTTF weight, and the resulting GAG content used to assess the relative amounts of GAG in fresh, unloaded, static loaded and HFLM loaded tendon fascicles.

3.6 Mechanical Testing

3.6.1 Measurements of *in vitro* forces on isolated tendon fascicle

The static load applied to fascicles was measured by weighing 10 individual magnets on a commercially available balance. The average mass was computed and equation 3.1 was used to calculate the static load applied by the magnetic stir bar. In the equation, accl is gravitational acceleration, 9.81m/s^2, and M_b is the mass in grams of the magnetic stir bar.

$$Applied\ static\ load\ (N) = Mb\ x\ g$$

Equation 3.1

The mechanical signal delivered to isolated cultured RTTFs was recorded using a load cell, and a Fast Fourier transform was used to identify the applied frequency. A clamp stand was used to position a load cell directly above an uncapped culture tube. The top end of the tendon fascicle was connected to the load cell, and the attached magnet at the bottom end of the fascicle was left suspended in the culture tube approximately 20mm above the tube base, exactly the same distance from the field coil as in a stimulated culture specimen. During application of a pulsed magnetic field, the resulting force magnitude applied onto the tendon fascicle was recorded and measured using the connected load cell. Force-time data sets were acquired (Wintest software) and used to identify the frequency of loading applied onto tendon fascicle (MATLAB) at set magnetic field frequencies. Data acquisition was performed at 100Hz for 10 seconds, (2 minutes after the initial application of cyclic load) at applied field frequencies of 1, 5, 10, 15, 20Hz. A schematic of the set-up used for characterising the applied load onto isolated tendon fascicles is shown in figure 3.3.

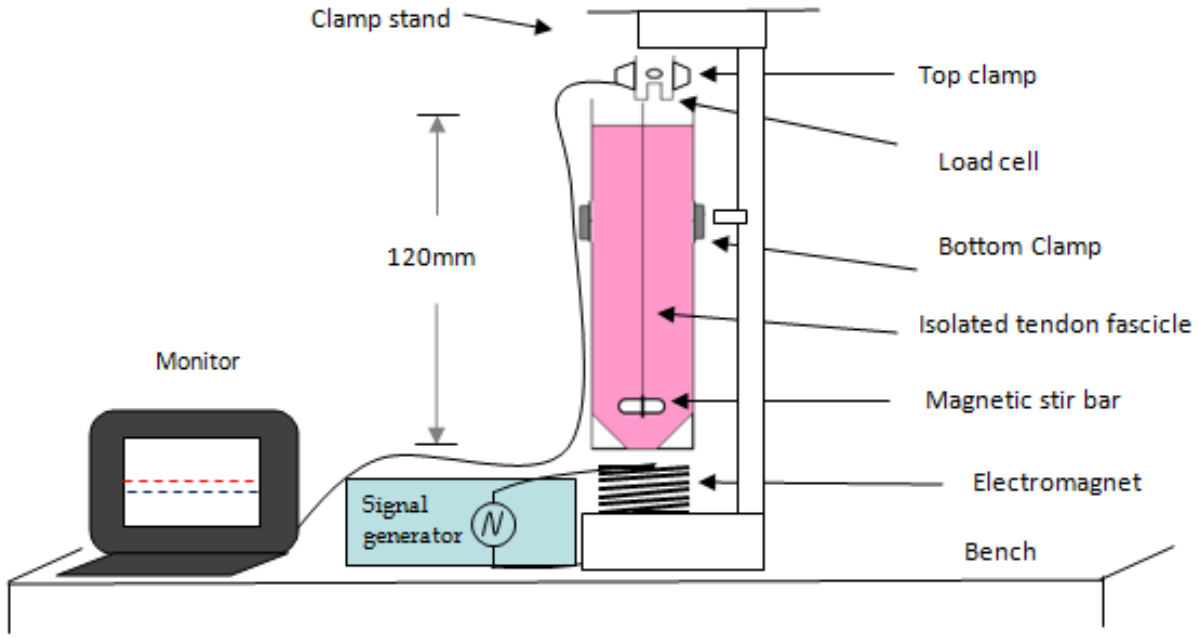


Figure 3.3: Illustration of apparatus and set-up used for characterisation of the cyclic load applied to isolated tendon fascicle

3.6.2 Tensile testing

Biomechanical properties of rat tail tendon fascicles from tendon groups were determined using tensile testing. Tensile testing of RTTFs was performed using a bench top tensile test machine equipped with a 22 N load cell (Bose Electroforce, Eden Prairie, MN). RTTFs were mounted with a grip to grip length of 15 mm using a custom made gripping system formed by wrapping the ends of each fascicle around an acrylic rod clamped by a silicone tube. Pre-conditioning of tendon specimens was performed using a 1 mm displacement sine waveform at 0.5 Hz for 30 cycles. RTTFs were tested to failure at 0.05 mm s. Tendon specimens were kept moist by spraying with PBS. Force and displacement data were captured at 10 Hz. Tendon specimen diameters were measured using a Keyence High Speed High precision

digital micrometer, prior to testing. Specimen diameters were determined by taking the mean of 7 measurements perpendicular to the long axis of the tendon and this was used to calculate the cross sectional area (CSA) of each fascicle. Testing was carried out at room temperature (20^{o}C).



Figure 3.4: Bose Machine used for mechanical testing of tendon fascicles

Subsequent to mechanical testing, load-displacement data from the Bose machine was normalised with tendon fascicle cross sectional area and original length respectively to produce stress-strain data for each fascicle and this used to produce a stress-strain plot (figure 3.5). Tangent modulus was calculated as a function of strain to estimate the maximum modulus. Tensile strength was measured as the maximum stress in the failure region of the curve and strain to failure was recorded as the strain at the point of maximum stress.

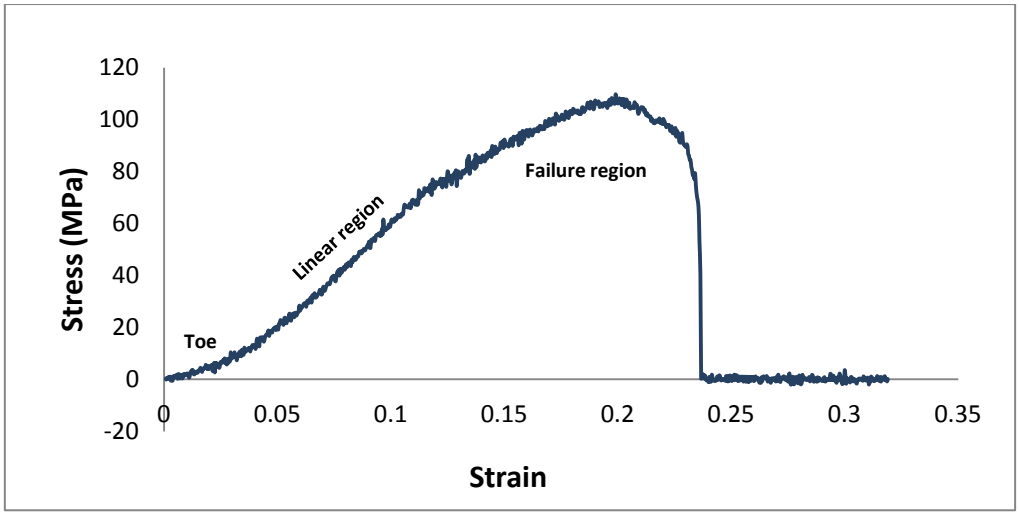


Figure 3.5 Typical stress-strain plot for rat tail tendon fascicle measured using Bose Electroforce, Eden Prairie, MN

3.7 Injury model for tendon fascicles

In order to examine the influence of mechanical loading conditions on tendon repair in a controlled laboratory setting, an injury model was developed for cultured tendon fascicles in collaboration with Dr Nasim Zargar Baboldashti, a former DPhil student in the Oxford Mechanobiology group.

The most repeatable and uniform injury as assessed by both live & dead staining and mechanical testing was caused by application of a scratch damage. Marker dots located 1cm apart were positioned in the centre and on the inside face of a petri-dish lid. Freshly harvested fascicles were placed on the outer face of the petri dish lid, superimposing the 1cm target length outlined by marker dots (figure 3.6), and fascicles were immediately moistened with DMEM, to avoid dehydration during the process. A sterile microlance 23 gauge insulin needle was used to apply 2 x 1cm scratches along the length of tendon fascicles.

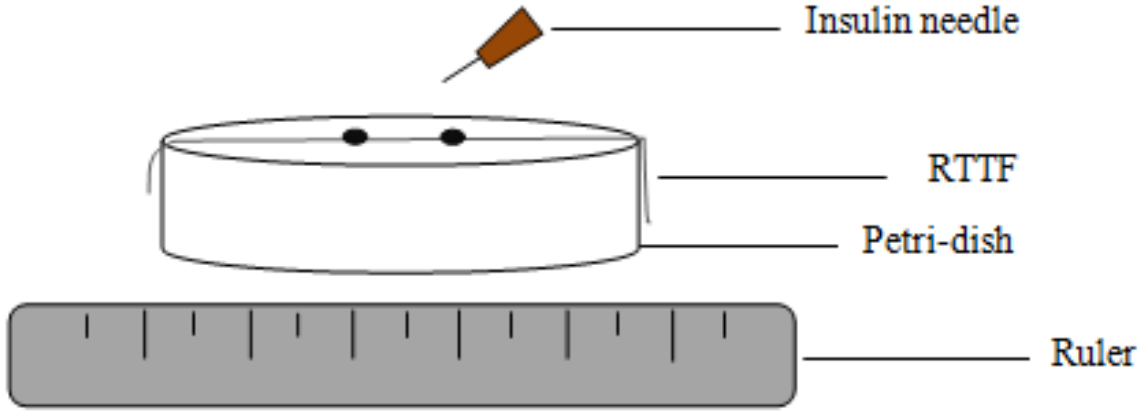


Figure 3.6 Illustration of apparatus and set-up used for application of scratch damage on RTTF

After the scratch was applied, both ends of the fascicle handled by the operator were removed by surgical scalpel and the fascicle was immediately placed in DMEM, ready for *in vitro* culture using the custom made IVLS. Intact and freshly harvested tendon fascicles from the tails of male Sprague Dawley Rats are typically 14-16cm in length, allowing the ends to be handled and leaving an untouched working length of at least 10cm. Each step of the procedure was performed under aseptic conditions and using a laminar flow hood. Macro-structural changes in the tendon fascicle were visible with the naked eye following application of a 1cm scratch damage and this is shown in figure 3.6.

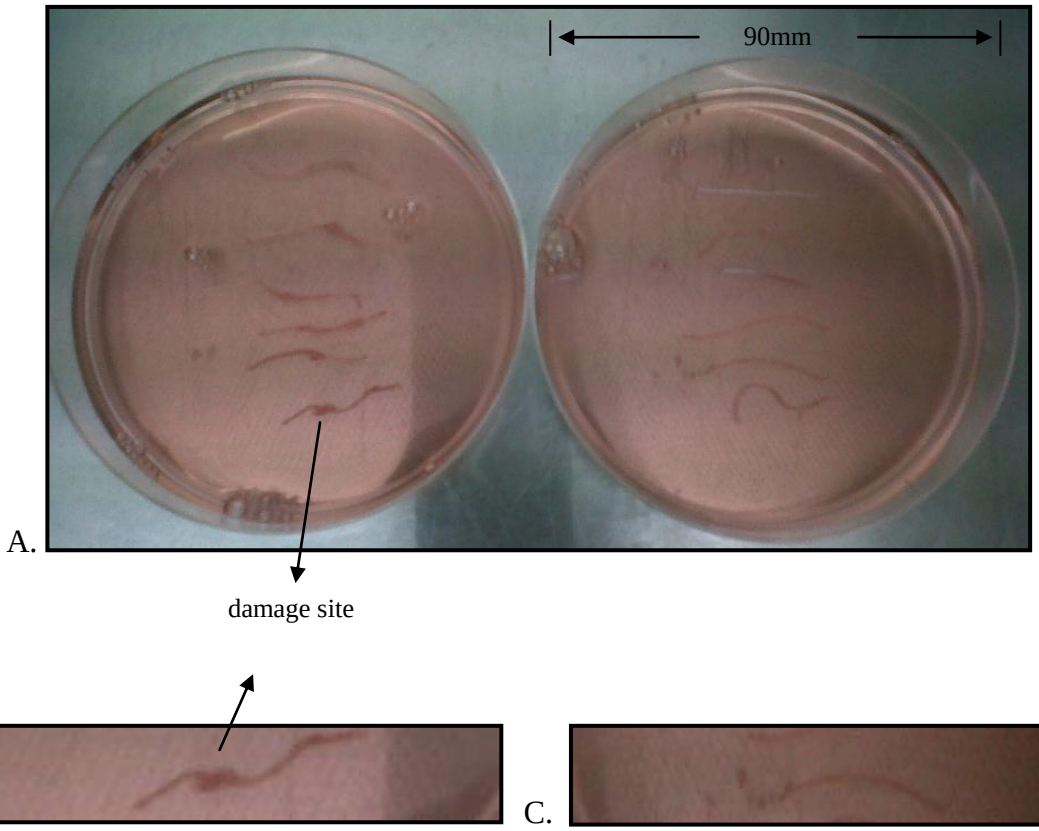


Figure 3.7: Images highlighting difference between A. damaged (left) and undamaged (right) tendons following application of 1cm scratch to cultured RTTFs. Images B. and C. show enlarged views of one damaged and undamaged tendon RTTF respectively.

3.8: Statistical techniques

Statistical tests (Student's t-test and one-way ANOVA analysis) were performed using Prism v.5 (GraphPad Software, La Jolla, CA). Both t-tests and one way ANOVA were used to identify statistically significant differences between control and treated tendon groups. The former method is used to compare between two experimental groups while the latter is used to analyse two or more data groups.

ANOVA was used as the statistical tool for the majority of experiments conducted in this thesis. However, t-tests were performed to assess the effect of dosage periods on tendon modulus and tendon GAG content (figure 5.3 and 5.4), since data fresh fascicles included for both experiments are taken from separate experiments. T-tests were also used for validation

experiments on the effect of scratch damage on tendon modulus and tendon metabolism (figure 7.4 and 7.5) since comparisons made in these experiments were between damaged and undamaged tendons only.

Error bars were used to indicate the variation of data points from a mean value. Graphs were plotted as either mean± standard deviation or mean± standard error. Standard error was used to provide an estimate of the standard deviation and was used when extreme outlier values were present in data sets.

CHAPTER 4 A novel *in vitro* loading system for static low frequency and high frequency low magnitude cyclic loading of cultured tendons

4.1 INTRODUCTION

In chapter 3 the experimental techniques and apparatus used to investigate the effects of *in vitro* mechanical loading on healthy and damaged tendon fascicles were detailed.

This chapter presents the design of a novel *in vitro* loading system (IVLS) useful for performing *in vitro* studies on tendon mechanobiology. In particularly, this unique and custom developed system enables application of High Frequency Low Magnitude (HFLM) loading onto isolated tendon specimens allowing tendon response to unconventional mechanical loads to be monitored in a controlled laboratory setting. Preliminary studies assessing the biochemical and biomechanical response of cultured tendon fascicles to static and HFLM loading were performed. The design was validated through magnetic flux, load cell and viability measurements and has shown the potential of clinically relevant HFLM loading for influencing tendon biology. No system has been developed previously for applying high frequency loads onto isolated cultured tendons and part of this work has been published in the journal Medical Engineering and Physics[157].

4.1.1 *In vitro* loading systems for cultured tendon specimens

IVLSs enable tissue response to be monitored closely while applying mechanical loads in a controlled and repeatable way. For example using IVLSs, it is possible to make controlled observations on the biochemical and biomechanical changes by applying a known amount of stress at some frequency onto living tendons. Various IVLSs have been developed, for static and cyclic stimulation of cultured tendon specimens, some being simple, and others, showing increasing levels of complexity. These have been constructed using various laboratory apparatus and techniques such as stainless steel weights suspended within culture tubes [158]pulley systems within bioreactor chambers [159]and culture tubes hanging from the arm

of a rotating cam [160]. In a more recent design, Legerlotz *et al.*, 2013 used stainless steel chambers containing media to isolate individual tendon fascicles end to end. Each chamber was connected to an actuator arm and secured within a mechanical load frame which holds up to 16 chambers. Newly developed IVLSs such as these highlight the need for more effective methods of performing tendon research, within this exciting branch of bioengineering [161].

4.1.2 *In vitro* static loading of cultured tendons

Although static loads are not a complete reflection of the *in vivo* loading environment, they have been found to play a role in the mechanotransduction response of connective tissue and these have contributed to developing current knowledge on the mechanisms responsible for the beneficial effects of mechanical loading on tendons.

Using IVLSs with static loads tendons have shown increased modulus, altered tensile strength, strain to failure, and decreased stress relaxation alongside increased glycosaminoglycan content, reduced water content, and a dose-dependent inhibition of MMP1-mRNA [159, 160, 162]. In one study, a statistically significant correlation, was observed between applied static stress and tensile strength of collagen fascicles from the rabbit patellar tendon, and the maximum tensile strength (16.7MPa) was obtained at an applied load of 0.1N (applied stress of 1.2MPa), after a culture period of 1 and 2 weeks [163]. Experimental studies with *in vitro* static loading systems have also been used to monitor deleterious effects of immobilization and stress deprivation on the structural and functional properties of tendons. In general these have reported a decrease in tendon biochemical and biomechanical properties with loss of homeostatic tension [158, 164, 165].

4.1.3 *In vitro* cyclic loading of cultured tendons

Extensive research has been performed using cyclic *in vitro* loading systems and such systems are generally considered to provide a more relevant approximation for the *in vivo* loading environment, and thus an enhanced model for understanding tendon mechano-

biological effects. Cyclic *in vitro* loading at various frequencies between 0.017 and 4 Hz has shown increased tendon modulus and tensile strength as well as up-regulated collagen synthesis, elimination of MMP-1 expression, reduced water content and increased cell viability in cultured tendons [4, 165-168].

Moreover, *in vitro* cyclic loading at frequencies of 1Hz has been shown to significantly increase proliferation, and increase expression of VEGF in tendon cells after 24 hours, while stimulation at 2Hz for just 30mins has been demonstrated to increase expression of Stress activated protein Kinases (SAPK), a protein that has been linked with regulation of cell proliferation and cell morphological changes and immune responses [13, 169, 170] . These studies highlight the immediate responsive nature of tendon cells to *in vitro* cyclic loads.

Characterisation of the effects of cyclic loading of tendon tissue is crucial, as excessive or prolonged loading cycles may lead to fatigue, and cause incessant tissue damage, and this in turn may contribute to tendon degeneration and eventually lead to rupture [171, 172].

4.1.4 Application of High Frequency Low Magnitude (HFLM) loads on tendons

High frequency low magnitude (HFLM) loading regimes have been shown to be effective in increasing bone mass [18], and in mitigating the effects of osteoporosis in both animal models and clinical trials [143, 173].

A limited number of *in vivo* studies have employed various techniques for delivering of HFLM loads onto tendons. As previously mentioned, administration of whole body vibrations (WBV) by means of vibrating platforms are commonly used to deliver HFLM mechanical loads onto musculo-skeletal tissues such as tendons, and studies using these techniques have been highlighted in chapter 2. Localised administration of HFLM loads however, present less harm to other soft tissues and proximal structures in living organisms than WBV techniques which use whole body vibration platforms.

In one study surface electrodes were placed over the belly (skin) of the gastrocnemius and tibialis anterior muscle and these were used in connection with constant current stimulator to deliver electrical pulses and evoke a twitch in the muscle, thus loading the Achilles tendon [174]. A foot plate with a force transducer was used to record the resulting high frequency load applied onto the Achilles tendon, assuming no other structures were resisting moment around the ankle. The set up was used to deliver 1 h of daily vibrations at a loading frequency of 50 Hz (amplitude: 0.2 mm) to healthy patients in a seated and resting position. After 14 days of stimulation, there was an increase in the force developed in the plantar flexor during voluntary contractions as indicated by increased electromyograms (EMG), but no sign of hypertrophy

A similar technique was employed by Uiyshyama *et al*., 2005 who used a mechanical stimulator consisting of vibratory shaft and strap for administering localised high frequency loads onto the Achilles tendon of patients. It was discovered that prolonged vibration at 100Hz for 30mins to the Achilles tendon resulted in the suppression of maximal voluntary contractions (MVC) of the plantaris muscle [175], indicating a possibly harmful effect of excessive HFLM loads on neurophysiologic functions in tendons, as with any form of excessive mechanical loading.

Vibration exercises during bed-rest have also been administered by placing healthy male subjects in a supine position with only their feet resting on a suspended vibration platform and applied vibration frequencies of 18-26Hz with amplitudes of 3.5-4mm were used to stimulate tendons in the lower limb [176]. After a total of 89 sessions with two 30mins sessions per day for 5 days a week, vibration in combination with load resistive exercises was found to reduce atrophy in the extensors of the knee and ankle, but have no effect on the hamstrings when compared with inactive controls.

A similar technique was used in an animal study where male Wilstar rats were maintained in an un-weighted position with the animal's body and right knee of the hind limb being firmly fastened, while leaving free range of movement in the ankle. Mechanical vibrations were applied perpendicularly to the Achilles tendon of the right hind limb by means of a Teflon probe (3 mm in diameter) connected to a moving coil of an electromechanical vibrator fixed in a independent holder, and driven by a sinusoidal signal generator. Tendon vibration for 14 days during hindlimb unloading was found to significantly mitigate but not prevent the 'loss of muscle mass (by -17 vs -41%), fiber size', the decrease in fibre cross sectional area of type IIa (-28vs -50%)' fibres and 'the decrease in maximal twitch (-3 vs by -54%)', when compared to non stimulated controls, indicating that tendon vibration can be used as to counteract atrophy related processes caused by unloading of limbs in the lower extremities[177].

Despite these various techniques, until now no studies have been made on HFLM loading for cultured tendons, and although IVLS have been reported for application of static and low frequency (0.1-4Hz) cyclic loading to our knowledge, no system has been developed for applying HFLM loads onto isolated tendons in culture.

The aim of the current study was to develop and validate a novel IVLS which uses an applied magnetic field to produce time varying mechanical loads in cultured tendon fascicles and use the developed system to examine the biochemical and biomechanical response of cultured tendon fascicles to static and HFLM loading. Our hypothesis is that HFLM loading will enhance the tangent modulus and glycosaminoglycan content of cultured tendons in comparison to unloaded tendons.

4.2 MATERIALS AND METHODS

4.2.1 Study Design 1

Study 1 was divided into 3 experiments and included characterisation of the custom built *in vitro* loading system. The breakdown of experiments performed is illustrated in figure 4.1.

Tendon fascicles from the tails of 8 male Sprague Dawley rats (aged 3–5 months), were harvested within 2 hours of sacrifice. All fascicles serving as fresh controls were analysed immediately after harvesting. Cultured fascicles were separated into 3 groups, unloaded (U), static loaded (SL), and high frequency low magnitude (HFLM) loaded groups. HFLM loaded tendons were stimulated at a frequency of 20Hz and magnitude of 0.12N. SL and loaded fascicles were stimulated for 24h/day and 1h/day respectively, with HFLM fascicles receiving 5 days of loading and 2 days of no loading during culture to represent a clinically feasible pattern of mechanical stimulation.

In experiment 1; two fascicles from fresh, unloaded, static loaded and HFLM tendon groups were analysed for tissue viability. Live-dead staining and confocal scanning laser microscopy techniques were used to quantify tissue viability after 7 days of culture. The experiment was designed to assess whether tendon fascicles could survive the IVL culture environment over a 7 day culture period.

In experiment 2; Fifteen RTTFs out of a total of eighteen tendon fascicles (from 4 rats) were divided into 3 groups (n=6) and used to examine GAG content after 4 days and tangent modulus after 7 days of incubation in fresh fascicles, unloaded and static loaded cultured fascicles. The experiment was designed to assess the effects of static loading and load deprivation on the biochemical and biomechanical properties of cultured tendons using the custom IVLS.

In experiment 3; Twenty four RTTFs out of a total of thirty tendon fascicles (from 4 rats) were separated into 3 groups (n=10) and used to examine GAG content and tangent modulus in fresh fascicles, unloaded and HFLM loaded cultured fascicles after 7 days of incubation. The experiment was designed to assess the effects of HFLM loading on the biochemical and biomechanical properties of cultured tendons using the custom IVLS.

Validation and characterisation of the custom IVLS was achieved through magnetic flux, load cell and viability measurements and the techniques used to perform these tests are described in chapter 3 of this thesis. The field strengths used to provide HFLM loading in our experiments are shown in table 4.2. Moreover, preliminary experiments were conducted to ascertain that the magnetic field strength used to stimulate the suspended magnet did not produce any changes in the biomechanical and biochemical properties (data not shown).

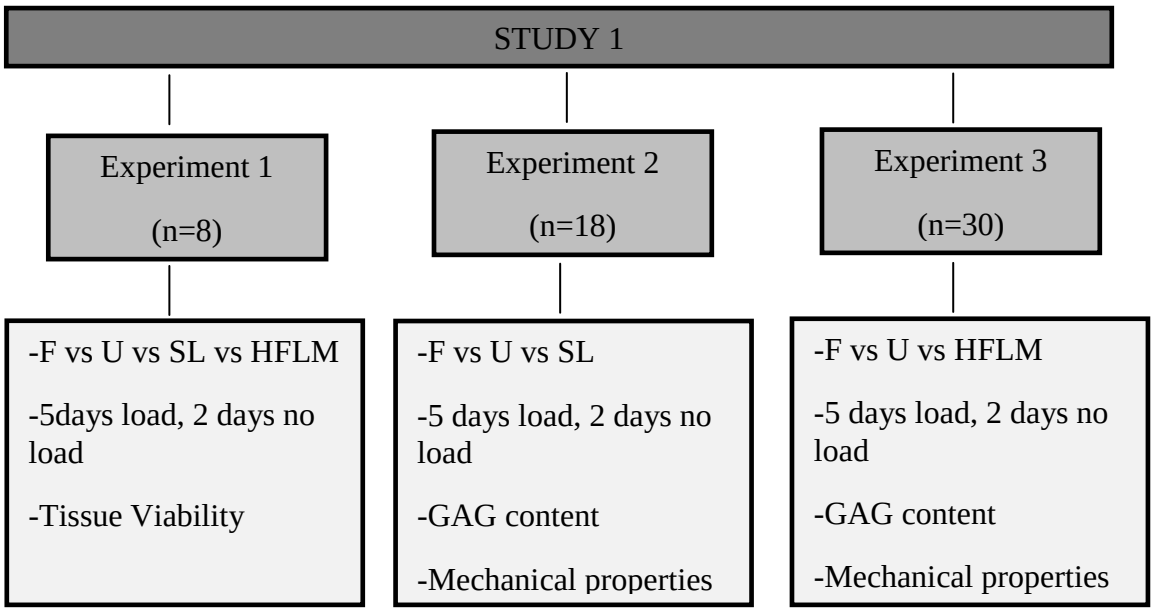


Figure 4.1: Experimental set up for study 1. The study consisted of three experiments with separate research questions; 1. Can tendon fascicles survive the *in vitro* loading culture environment over a 7 day culture period?, 2. What is the effect of *in vitro* static loading on the mechanical properties and GAG content of cultured tendons?, 3. What is the effect of *in vitro* HFLM loading on the mechanical properties and GAG content of cultured tendons?

4.3 RESULTS

4.3.1 Custom IVLS for low frequency and HFLM loading of tendon fascicles

A custom built stimulation device was developed to provide, low frequency (LF) cyclic loading and HFLM loading for the IVLS and for cultured tendon fascicles. Figure 4.2 shows an image of the complete IVLS used for stimulation of RTTFs and a schematic of the apparatus set-up used to apply cyclic force applied onto isolated tendon fascicles. The electromagnetic stimulation device was used in combination with the static IVLS described in chapter 3 (section 3.2.2) to produce cyclic stimulation of isolated tendon fascicles.

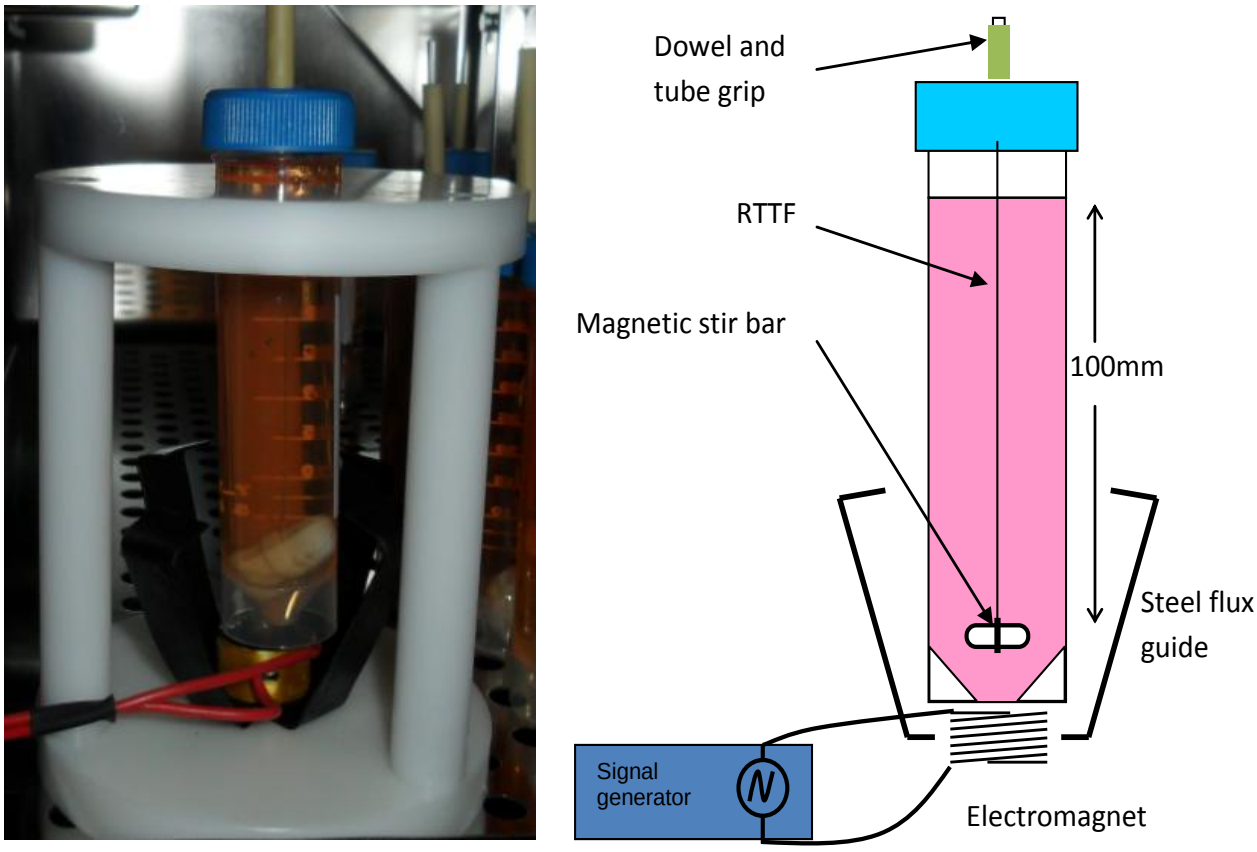


Figure 4.2: The custom *in vitro* loading system for low frequency cyclic and HFLM loading of cultured tendons. Drilled holes in tube cap allow gas exchange. An electromagnetic stimulation device positioned below the tube base was used to provide a PEMF in combination with a signal generator and cyclic perturbations of the magnetic stir bar.

Details of the individual components used to form the stimulation device are outlined in Table 4.1. Briefly, the custom-built electromagnet (steel core, copper coils) driven by a sinusoidal alternating current from a signal generator (Thurlby-Thandar TG210 2MHz, Huntingdon, UK) was used to provide an alternating electromagnetic field, with steel elements positioned vertically around the culture tube to increase local flux density, Figure. 4.2 The time varying magnetic field agitated the magnet superposing cyclic loading on the static load on the cultured fascicle and using the magnetic stir bar cyclic stresses of 0–0.9MPa were produced in the tendon fascicle. During this study, the IVLS was used to investigate the effects of static and HFLM loads on cultured tendons.

Table 4.1: Characteristic properties and function of components of electromagnetic stimulation device used for custom IVLS RTTF

Component	Material Property	Functional role
Steel Elements	Conductor of magnetic field	Positioned vertically around the culture tube to increase local flux density
Electromagnet	Steel core, copper coils induce magnetic field	Generates electromagnetic filed
Signal Generator	Generating electrical signal for electromagnet	Provide sinusoidal alternating current to drive alternating magnetic field from electromagnet. Generates desired waveform. Control unit for applied load frequency and magnitude
Plastic Housing	PP hard, durable robust	Provides stability and enables easy mounting onto laboratory bench tops and incubator environment

4.3.2 Characterisation of applied load magnitude and frequency using IVLS

The magnetic field strengths produced by the electromagnet at 5 set frequencies are shown in table 4.2. Field strength decreased with increasing frequency from 20 G at 1 Hz to 1.67 G at 20 Hz. The highest field strength produced by the electromagnetic stimulation device at HFLM cyclic frequencies was found to be 4.16gauss and occurred at10Hz.

The static load applied to isolated tendon fascicles was 0.06 ± 0.0014 N. For cyclic loading, force–time data, acquired in 4 separate 10 s duration tests, showed the average peak load magnitude of the cyclic loading superimposed on the static load varied with applied field frequency from 0.03 up to 0.09 N at 1 and 10 Hz respectively. At 15Hz and 20 Hz, a cyclic load magnitude of 0.06 ± 0.010 N was superimposed on the static load, giving an over-all force signal varying from 0 to 0.12N. Fast Fourier transform (FFT) confirmed that the load frequency was identical to the applied field frequency at 1, 10, 15, and 20Hz. Force-time plots and FFT signals are shown in figure 4.3 for tendon groups loaded at 1Hz and 20Hz.

Table 4.2: Magnetic field strength of electromagnetic stimulation device at set frequencies

Frequency (Hz)	Field Amplitude (v)	Magnetic Field (gauss)
1	20	20
10	20	4.166667
15	20	3.333333
20	20	1.666667

4.3.3 Tissue Viability

In order to verify that tendon fascicles being assessed were capable of surviving the test environment a live and dead staining assay was performed on all tendon groups. Tendon fascicles in all groups were confirmed to be viable after 7 days culture. The percentage of live cells to that of all cells within the sampled region in fresh, unloaded, static loaded and HFLM

loaded tendon groups was at least 73%, and the proportion of live cells in tendon fascicles exposed to the *in vitro* loading environment did not decrease over a 7-day period when compared to fresh controls.

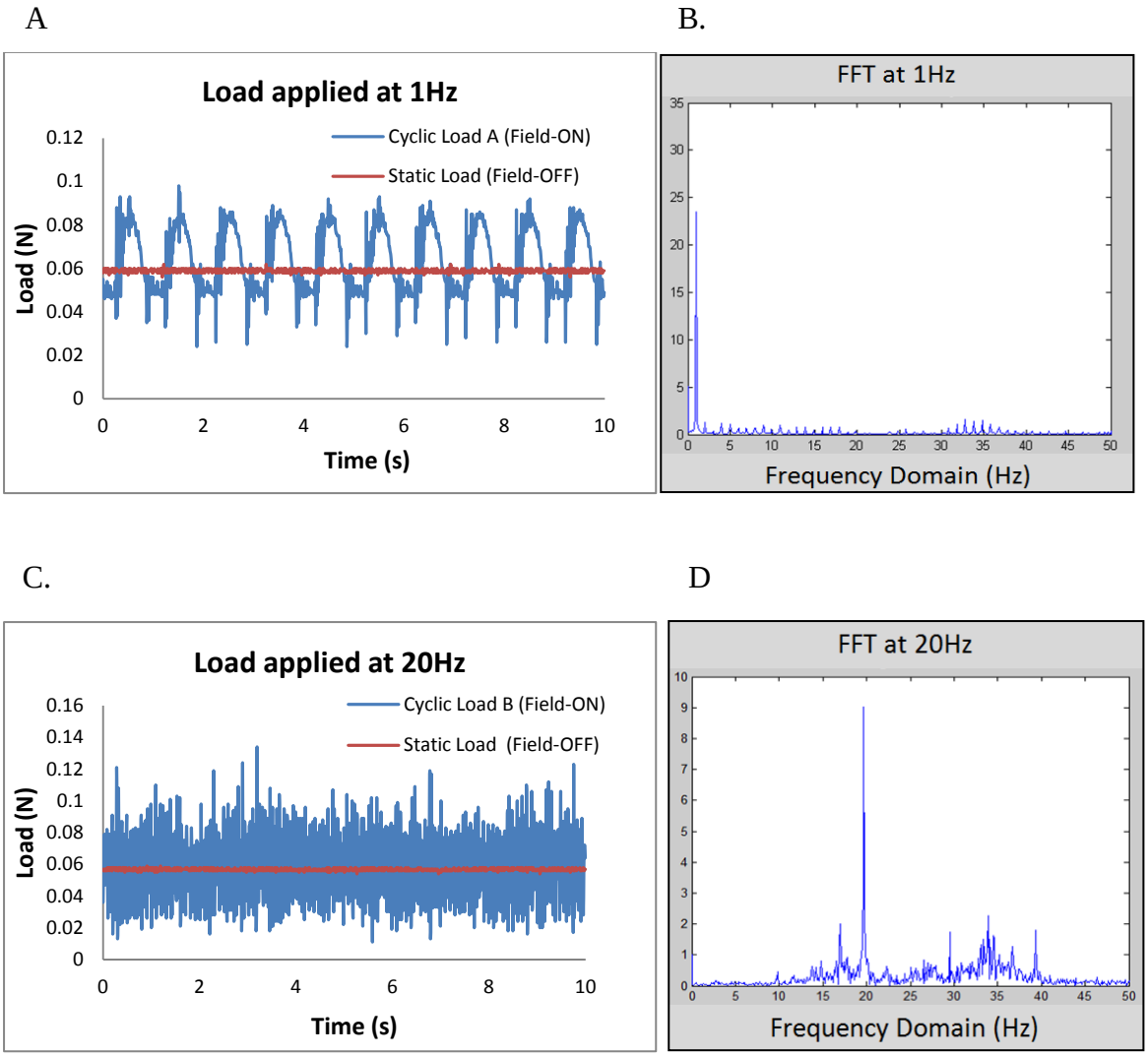


Figure 4.3: Load-Time plots (A and C) and the corresponding Fast Fourier Transform signals (B and D) for 2 tendon groups; Data acquisition was captured 4 times and for 10 seconds, 2 minutes after the initial application of cyclic load at set frequencies. In each data set the mean of the 3 highest load readings was taken as the peak load. For each tendon group, the average peak force from the 4 data sets was recorded as the load applied. The FFT plot shows the cyclic load frequency applied to tendons at the specified field frequency from the signal generator. In plots B and D, Minor peaks between 33-35Hz correspond to the resonant frequency of the tendon fascicle used.

.

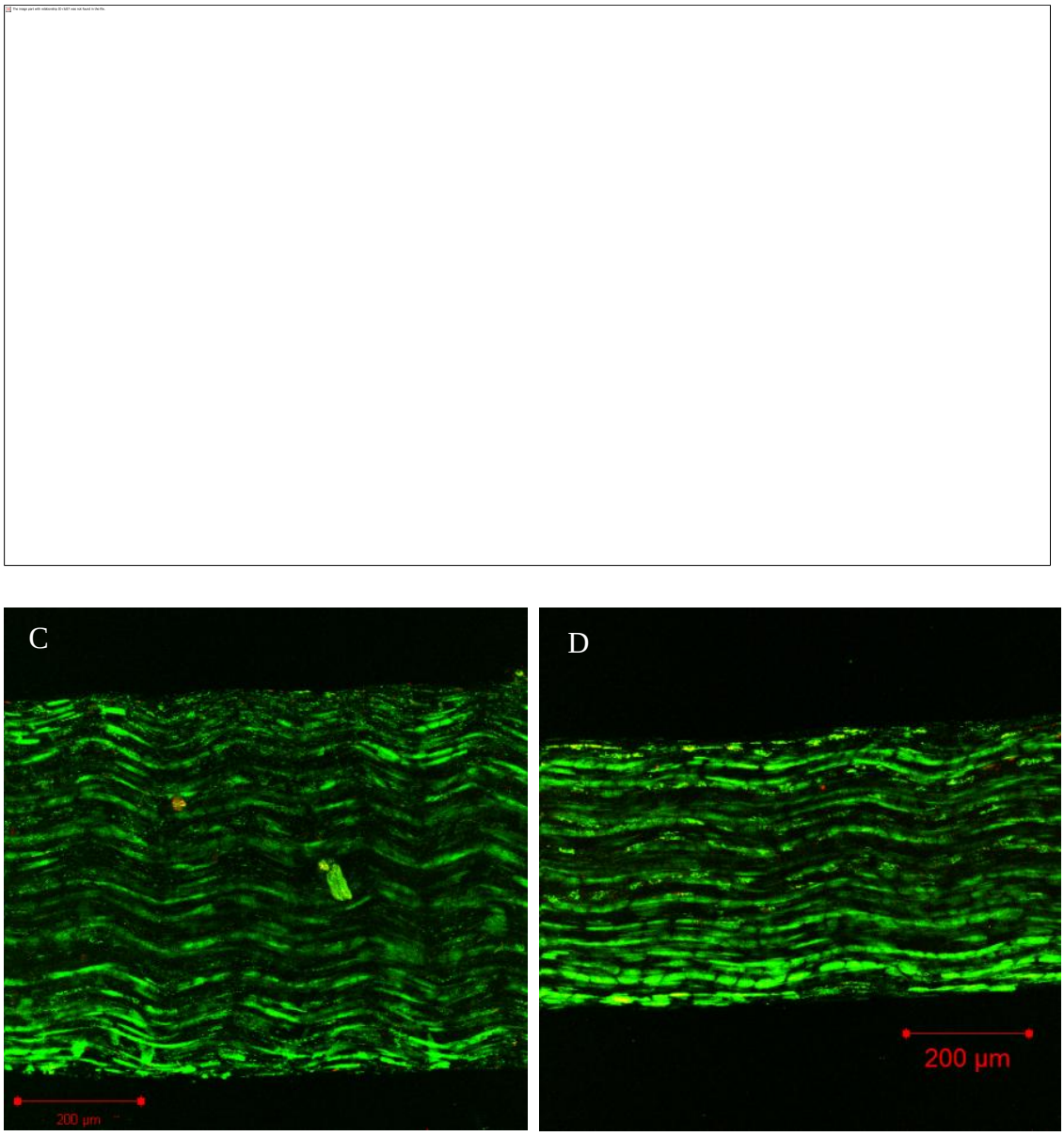


Figure 4.4: Live-dead confocal scanning laser microscopy (maximum intensity projection) of **A** fresh and **B** 7 day unloaded, **C** 7 day static loaded and **D**, 7 day HFLM loaded cultured tendon fascicles, showing live (green elliptical) and dead (red spherical) cells. Images shown are a subset centre region (area; 0.85mmx0.85mm) from the larger 3D tiled images (2.54x0.85mm) used to quantify viability. Scale bars indicate 200µm.

No significant difference was observed between the number of live cells in tendon fascicles in the four experimental groups and mean values. Figure 4.4A-D shows live-dead staining

images for a fresh tendon fascicle and fascicles cultured for 7 days of unloading, static loading and HFLM loading, and figure 4.4E presents the quantitative breakdown for the number of live and cells within each of the tendon groups.

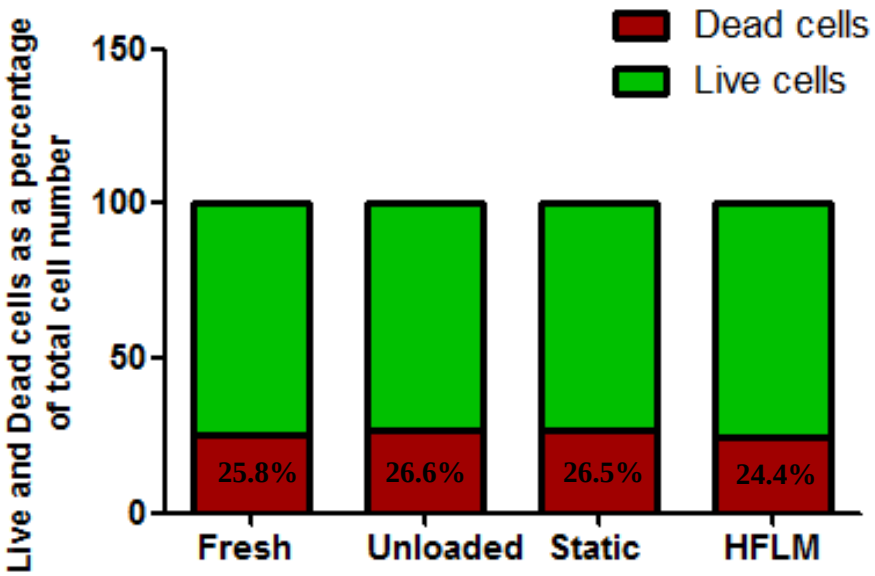


Figure 4.4E: The proportion of live and dead cells within tendon fascicles. Two tendon fascicles from 4 groups were analysed. Live-dead staining, confocal scanning laser microscopy techniques and image J were used to quantify tissue viability

4.3.4 Effect of static loading on tendon mechanical properties and GAG content

Stress–strain data from the tensile tests were smoothed using a 5 point moving average filter (every 2.5second intervals) and the tangent modulus calculated as a function of strain to estimate the maximum modulus for each fascicle. After 7 days, static loaded RTTFs showed a trend to higher maximum modulus values than unloaded, while unloaded RTTFs showed significantly lower ($p<0.05$) modulus than fresh fascicles (Figure 4.5A). Tensile strength was highest in fresh tendon fascicles and strain to failure was highest in unloaded tendon fascicles (Table 4.3A), however differences between tendon groups for tensile strength and strain to failure were not statistically significant (One-way ANOVA).

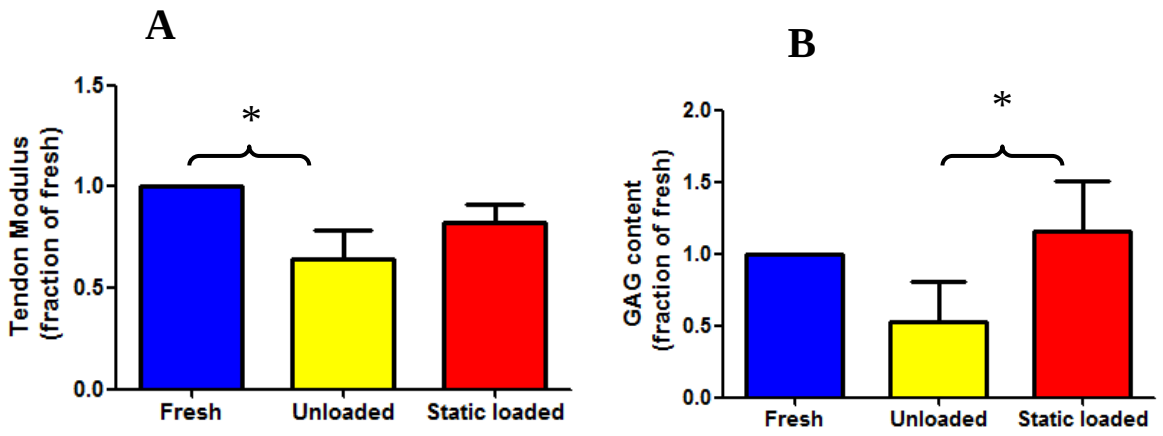


Figure 4.5 A The mean tangent modulus after 7 days of culture (n=5) and, **B** The mean glycosaminoglycan (GAG) content after 4 days of culture (n=5), expressed as a fraction of fresh, unloaded and static loaded tendon fascicles. * Denotes a significant difference between groups, $p < 0.05$. The bars shown represent the standard deviation.

Table 4.3A: The average Modulus, Tensile strength and Strain to failure, in; fresh, unloaded and static loaded tendon groups, after 7 days. For each group n=5. Reported values are mean± standard deviation. Indicated p-values are for SL vs. Unloaded tendon groups (ANOVA)

	Fresh	Unloaded	Static Loaded	Population	p-value
Modulus (MPa)	432.1±66.07	277.52±62.20	357.02±38.87	15	>0.05
Tensile Strength (MPa)	46.34±16.56	36.19±8.80	27.74±6.21	15	> 0.05
Strain to failure (% of total strain)	20.99±6.40	22.746±5.08	22.55+6.3±6.39	15	>0.05

The DMB assay was used to determine the GAG content of fresh, unloaded and static loaded tendon groups. Using a condroitin sulphate (CS) standard curve it was demonstrated that static loaded tendon fascicles had significantly higher GAG content than unloaded tendons, ($p < 0.001$) (Figure 4.5B), and marginally higher GAG content than fresh tendons fascicles.

Failure and infections during culture, GAG testing, and tensile testing resulted in a discrepancy between the number of fascicles harvested for experiments and the number of fascicles used to assess the experimental outcomes. These are described in the discussion section of this chapter. The population of tendon fascicles used for assessment of tendon mechanical properties in the experimental groups are outlined in table 4.3A and 4.3B.

4.3.5 Effect of HFLM loading on tendon mechanical properties and GAG content

Application of the 20 Hz HFLM loading regime resulted in a statistically significant two-fold difference in tangent modulus compared to unloaded groups after 7 days (Figure 4.6A). HFLM loaded tendon fascicles showed higher tensile strength than unloaded and fresh fascicles, although differences between groups were not statistically significant (Table 4.3B).

Further, as HFLM loaded tendons displayed significantly higher modulus and a trend to higher tensile strength than fresh controls, static loaded tendons did not, suggesting that

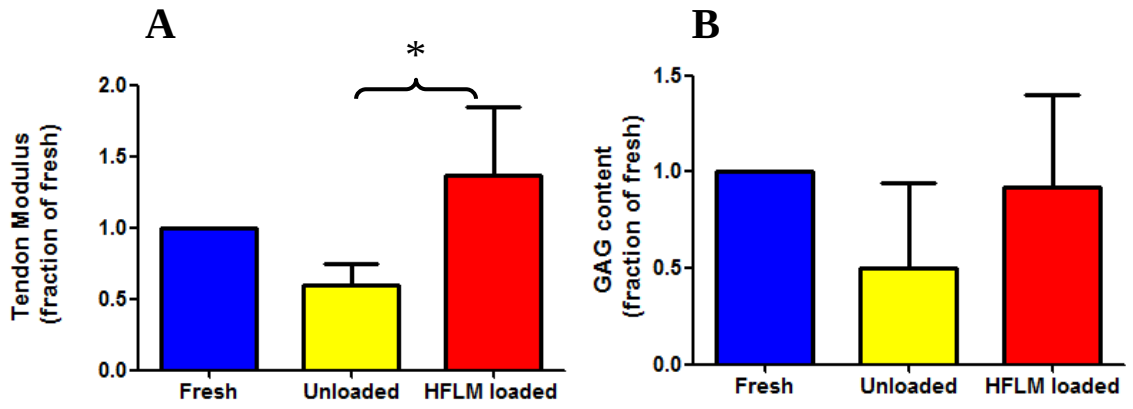


Figure 4.6: A The mean tangent modulus after 7 days of culture (n=8) and, **B** The mean glycosaminoglycan (GAG) content after 7 days of culture (n=8) expressed as a fraction of fresh, unloaded, and HFLM loaded tendons. * Denotes a significant difference between groups, $p < 0.05$. The bars shown represent the standard deviation

HFLM loading provides greater stimulation than static loading for enhancing tendon mechanical properties in healthy tissue.

HFLM loaded tendon fascicles showed a trend to higher GAG content than unloaded tendon fascicles but no significant difference was found (Figure. 4.6B). Fresh tendons displayed highest GAG content compared with unloaded and HFLM loaded tendon groups. While HFLM loaded tendon fascicles produced a stronger effect on tendon modulus than static loading, static loading appeared to provide marginally stronger stimulus for promoting tendon GAG content than HFLM loading.

Table 4.3 B: The average Modulus (n=8), Tensile strength (n=5), and Strain to failure (n=5); in fresh, unloaded, and HFLM tendon groups after 7 days. Reported values are mean ± standard deviation. Indicated p-values are for HFLM vs. unloaded tendon groups (t-test).

	Fresh	**Unloaded**	**HFLM Loaded**	**Population**	**p-value**
Modulus (MPa)	296.8±81.1	175.7±46.1	405.7±143.1	24	<0.001
Tensile Strength (MPa)	22.37±4.3	18.27±5.1	25.47±11.7	15	>0.05
Strain to failure (% of total strain)	21.3±5.6	20.8±6.2	20.9±7.0	15	>0.05

4.4 DISCUSSION

A major outcome in this thesis has been the creation of a novel IVLS for static, low frequency cyclic and HFLM loading of cultured tendons. Although IVLS have been reported for application of static and low frequency cyclic loading [167, 178, 179] to our knowledge, no system has been developed for applying high frequency loads onto isolated tendon fascicles. The developed IVLS allowed culture of viable tendon fascicles for over 7 days (average cell viability 74.2% at 7 days) and was successfully used to study the influence of static and HFLM loading on the biochemical composition and the biomechanical properties of cultured fascicles.

The percentage of live cells to that of all cells within the sampled region of fresh, unloaded, static loaded and HFLM loaded cultured fascicles was 74.2, 73.4, 73.5, 75.6% respectively, indicating maintenance of tissue viability between 0 and 7 days of culture. A comparable study by Yamamota *et al.,* 2002, also revealed that the number of live cells in cultured collagen fascicles does not diminish with time and the proportion of live cells to that of all cells in cultured fascicles was reported to be 0.88 ± 0.05 and 0.87 ± 0.04, after 1 and 2 weeks respectively [163]. Our results on tendon fascicle viability are also in agreement with those published by Screen *et al.,* 2005, who reported no statistically significant differences between mechanically loaded and unloaded tendons fascicle following *in vitro* culture [4]. Although in this current work a culture period of 1 week was used, studies using similar IVLS have reported maintenance of tissue viability in tendon fascicles for culture periods of up to 3 weeks [180], and this highlights the enormous potential for further clinically relevant studies on tendon response to HFLM loads using the developed culture model.

Characterisation of the electromagnetic stimulation device revealed that the field strengths produced by the custom IVLS, varied from 20 - 4.16 G between 1 and 20 Hz. Stimulation at

field strengths below 50G showed no significant effect on rabbit ligaments after 1 week [181].

The field strengths used to provide HFLM loading in our experiments are far smaller and the exposure times shorter, and thus are likely to present no biological stimulus for cultured tendon fascicles

Fast Fourier transform confirmed that loads of 0.06 N at 20 Hz were successfully applied onto isolated tendon fascicles using the custom IVLS. Loading at frequencies of 20Hz is considerably greater than loading applied onto tendons in the lower limbs during walking and running activities (1–4 Hz), and have previously been described in literature as high frequency loads [173].

Contrary to our findings, Legerlotz *et al* 2007 performed *in vivo* studies and found that application of vibration strength training at loading frequencies of 25Hz for 12weeks did not induce any changes in the procollagen I and III mRNA content of the Achilles tendons of Sprague Dawley Rats when compared to non-active age matched controls. Although care must be taken when comparing *in vivo* data with *in vitro* data particularly when there is a mismatch in the experimental study period, it is possible that the dissimilar findings on the stimulatory effects of HFLM loading may be attributed to the discrepancy in load frequency used between the studies [182].

Statically loaded tendon fascicles showed a twofold higher GAG content than unloaded fascicles, mirroring the differences in their mechanical properties, an association that has been seen in previous *in vitro* RTTF experiments [159]. The results obtained are consistent with the hypothesis that tensile loads prevent catabolism of GAGs and proteoglycan molecules within tendons [158], and also with previous work suggesting cyclic loading promotes collagen synthesis [4, 168].

The range of tangent moduli for fresh, unloaded and static loaded fascicles (200–300 MPa) are in agreement with those published in similar studies for cultured rat tail tendon fascicles [159, 163].

Static loaded tendons showed higher moduli than unloaded fascicles, however the differences were not statistically significant. Abreu *et al* 2008., found significantly higher moduli in 1 week statically loaded RTTF than in both "fresh" and unloaded fascicles, however in contrast to our study, their "fresh" control fascicles had been frozen at −20 ◦C [159]. In our study, unloaded tendons showed distinctively lower moduli than fresh tendons, consistent with several studies of stress deprived cultured tendon fascicles [159, 183, 184]

Studies have shown that tendons exhibit increased tensile strength in response to appropriate physical loading [185-187]. In this study tensile strength of HFLM mechanically loaded tendon groups was found to be noticeably higher than unloaded controls, but markedly lower for tendons receiving static loading in comparison to unloaded controls. These results suggest that tensile strength of tendon fascicles may be dependent on the frequency of applied load and not simply the magnitude of loading received by the tendon, and that high frequency loads maybe more effective for enhancing tendon strength than static loads. Strain to failure did not vary significantly between fresh, unloaded and static loaded tendon groups and these results are in agreement with those reported by Yamamota *et al* 1999., who found no significant difference in strain to failure for rabbit patellar tendons fascicles loaded under static stress (1.2-1.3MPa) and fresh control groups [187].

Our study shows significant biomechanical and biochemical effects with lower magnitude cyclic loading than these previous studies, which we attribute to the higher frequencies used here. In a clinical context a continuous static load represents a severe and possibly damaging stimulus, whereas HFLM as it was applied in these experiments for only 1hour every day

with two rest days per week could have the same anabolic potential without posing a threat of tissue damage.

The structural and mechano-transduction mechanisms by which short doses of high frequency low magnitude loads may provide greater stimulus than low frequency stimulation in tendons are not clear. Tendon is a biphasic material, so potentially similar high frequency strain amplification mechanisms to those proposed for bone may be in operation [188].There were no significant differences in the measured cross sectional area between fresh, unloaded, static loaded and HFLM loaded fascicles thus, the reported changes in mechanical property cannot be ascribed to differences in specimen geometry [189], and time in culture did not result in significant changes to fascicle diameter.

The study suffers from a few limitations. Fascicles were lost during the culture due to contamination, or sudden detachment of the RTTF at top and bottom attachment points in the culture tube were caused by operation errors. Further, incomplete closure of the eppendorf tubes during fascicle digestion for GAG measurements resulted in loss of samples, and fascicle rupture during preconditioning of mechanical tests resulted in the additional loss tendon specimens. Table 4.3A, B, highlights tensile strength and strain to failure values for all fascicles tested to rupture.

Another limitation of these studies is the discrepancy in the load magnitude applied for static (0.06N) and HFLM (0.12N) loading of tendons, and the discrepancy between exposure times of tendons to static and HFLM stimulation. Thus, any comparison between static and HFLM stimulation remain speculative and require more detailed experimental studies.

The rat tendon fascicle model has been used effectively for progressing tendon research and studies have shown that when RTTFs are cultured *in vitro*, anabolic and catabolic processes of tissue remodelling similar to those seen *in vivo* can also be observed [190]

Finally, the considerable variation between the tangent modulus of fresh tendons used for static and HFLM loading experiments can be attributed to the variation in animal sizes and age ranges. Immature rats with noticeably lower weights and sizes were used to perform experiments on the effects of HFLM loading on tangent modulus.

However, for each experiment, the population of tendon fascicle used in all tendon groups were drawn from the same rat (s), as described in section 4.3.1 of this chapter. Therefore, our results carry significance for assessing the biochemical and biomechanical potential of HFLM loading of tendons compared with fresh and unloaded tendons.

The developed IVLS is designed to isolate tissue-based mechanotransduction effects in tendon and so any vascular or systemic effects due to mechanical loading in living animals are neglected in this model. Further, the isolated fascicles are not exposed to the same complex loading conditions experienced by bulk tendons *in vivo* [163]. Future experiments will investigate the optimal frequency and loading periods for stimulation and the effects of mechanical stimuli on damaged tendon tissue. In conclusion, the developed system provides a novel platform for investigating tendon mechanobiology under controlled, repeatable settings and allows for characterisation of optimal cyclic loading parameters for tendon remodelling and repair. We have demonstrated significant effects of clinically relevant low doses of HFLM loading on cultured tendon tissue. Data from the use of this system will inform future development of biomechanically based therapies for tendon and other soft musculoskeletal tissue.

CHAPTER 5 Effects of frequency and dosages of HFLM loading on the biochemical and biomechanical, properties of cultured tendon fascicles

5.1 INTRODUCTION

In chapter 4 a novel IVLS was presented for investigating tendon mechanobiological effects and it was highlighted that such a system would allow for characterisation of optimal mechanical loading parameters for tendon remodelling and repair.

In this chapter two important mechanical loading parameters, dosage periods and loading frequencies, essential for maintaining tendon homeostasis were examined. These loading parameters were varied over feasible and clinically relevant ranges in order to provide an indication of the most favourable cyclic loading conditions for tendon remodelling using the developed IVLS. The contribution of cell activity towards biomechanical modification of tendon properties subsequent to mechanical stimulation was also investigated, in order to enable consideration of the underlying mechanisms governing mechanical adaptation of tendons to mechanical loading.

5.2 Essential loading parameters for cyclic stimulation

Characterisation of optimal cyclic loading stimulation parameters for tendon tissue health is crucial, as frequent, or prolonged, loading cycles may lead to sustained tissue damage or tendon degeneration, and mechanical overload may lead to sudden rupture.

Cell and tissue culture *in vitro* loading systems have been used to study a considerable number of loading parameters essential for regulation of tendon health and homeostasis. Amongst these critical parameters, loading frequencies [11, 191], loading magnitudes[11, 192] daily dosage periods [20, 166] and total administration periods [1, 193] have been studied extensively.

More crucially, these studies highlight the possibility that lower magnitudes and increased frequencies of cyclic loading may be beneficial for tendon mechanical properties [166, 193], and that shorter dosages of repetitive cyclic loading, may provide similar stimulation to longer dosages for tendon healing [20].

5.2.1 *In vitro* cyclic loading frequencies on tendons

The effects of frequency of cyclic loading on the biochemical and mechanical properties of tendons have been studied using cell and tissue culture techniques.

Previous work by Lavagnino *et al*., 2003 showed a reduction of MMP1 expression in cultured tendon fascicles with increasing frequency of stimulation and increasing strain amplitude , however complete elimination of MMP1 was possible at the lowest strain, 1%, and highest frequency 1Hz investigated [11].

Cyclic *in vitro* loads applied onto tendons cells at varying frequencies have also been shown to affect expression of biochemical factors such as vascular endothelial growth factor (VEGF). In a study by, Peterson *et al* 2004 it was shown that stretching of rat Achilles fibroblast cells at a frequency of 1 Hz resulted in an increased expression of VEGF while stretching at low frequency (0.5 Hz) reduced VEGF expression to control levels [169]. The study indicates therefore that the applied frequency of cyclic loading is important in the regulation of VEGF expression in tendon tissue.

Tangent modulus and tensile strength of fascicles from rabbit patellar tendons have also been found to be significantly higher in tendons cultured under cyclic loading conditions of 4Hz for 1hr a day than those cultured at 1Hz for 1hr, over a 1 and 2 weeks period [166]. During the study, the time period for application of cyclic load was also varied so that the total number of load cycles applied to tendons per day in tendon groups stimulated at 1Hz and 4Hz

was 14, 400 cycles, indicating the biomechanical effects shown were not due to number of load cycles, but due to the frequency of applied cyclic load.

5.2.2 *In vitro* cyclic loading dosages on tendons

Mechanical stimulation plays a key role in tendon homeostasis and tissue remodelling, however prolonged and excessive mechanical loading may result in tendon damage [172, 194], and have been implicated in the etiology of tendon overuse injuries. It is therefore critical to evaluate the impact of applied dosages during daily mechanical loading regimes.

In one animal study prolonged treadmill running for 17 m/min at a 10^{o} decline, for 1 hour a day for 5 days a week, was shown to reduce tensile strength and tissue modulus in the Achilles tendon of male Sprague Dawley rats, when compared with rats receiving normal and free cage activity [195].

Extended cyclic mechanical stress durations have also been found to inhibit proliferation of tendon fibroblast cells. Barkhausen *et al.,* 2003 showed that cyclic *in vitro* stress of 1Hz applied to human tendon fibroblasts seeded onto flexible silicone dishes for 6, 12, and 24 hours a day resulted in a significant increase in cell proliferation, however a similar stress pattern applied over a two day period produced a reduction in cell proliferation [13]. Varying dosage periods of low frequency (2Hz) *in vitro* cyclic strain applied onto tendons cells between 15, 30, 60, and 120 min has also been reported to influence activation of JNK (c-Jun N-terminal kinase), a transcription factor associated with regulation of cellular processes such as cell proliferation [192]. During the study by Arnoczky *et al.*, 2002, activation of JNK2 was found to reach maximum levels after 30 min of stimulation but returned to near resting levels after 120 min of cyclic loading, indicating that it is necessary to determine the precise cyclic loading dosage periods necessary for optimal functioning of tendon cell processes.

5.2.3 Tendon decellularisation and mechano-transduction effects

Mechanotransduction is concerned with the mechanisms by which the body converts mechanical stimuli into cellular and or tissue responses, and this often results in modification of the tissues biochemical and biomechanical properties.

A proposed mechanism of mechanotransduction hypothesizes that extracellular matrix deformation conveys localised strain to cells through their integrin based cell-matrix connections. This may be transmitted through the cell's cytoskeleton to the cell nucleus and results in specific cellular responses[196-199]. Stimulation of the cell nucleus maybe important for tissue remodelling since synthesis of new matrix material is initiated and regulated by the cell nuclei.

A precise characterisation of the mechanisms of action of HFLM loading interventions has yet to be established, however, one approach to investigating mechano-transduction effects in tendons has been consideration of the participation (or lack of participation) of cellular activity in tendons. Various techniques have been used to minimise and eliminate cell activity within tendon specimens, rendering tissue biologically inert. These include freeze-thaw cycles in liquid nitrogen, a process that has been demonstrated to result in 100% cell death [160, 164, 165], chemical treatment with 0.1% sodium dodecyl sulfate in 0.1% ethylenediaminetetracetic acid[200], and addition of Tri(n-butyl)phosphate (TBP), which has been found to produce a 70%–90% reduction of intrinsic cells and similar biomechanics despite morphological changes in the tissue [201]. Previous *in vitro* studies examining the effects of cyclic loading on the material properties of cultured tendons have suggested cell mediated mechanisms since no significant difference in the stiffness or tensile strength was observed between loaded and unloaded patellar tendon allografts following 3 weeks of transplantation [165, 202]. In contrast, preservation of mechanical properties in acellularised human tendons [200], and a significant decrease in tensile strength observed between

unloaded and loaded acellular patellar tendons [164], show that alterations in the mechanical properties of tendon tissue can take place without the contribution of cell activity.

These conflicting results indicate that much work is still required in order to fully elucidate the mechanisms governing stress induced changes in HFLM and mechanically loaded tendons.

The aim of the work presented in this chapter was to examine the biochemical and biomechanical properties of cultured tendon fascicles subsequent to applied loading frequencies of 1, 10, 15, and 20Hz for 1 hour a day, and dosage periods of 1, 2 and 4 hours per day at 20Hz. I will test the hypothesise that tendon properties are maximised at higher loading frequencies even with short dosage periods, as determined by measurements of tendon Modulus and GAG content.

Further, tendon modulus subsequent to stimulation under low temperature culture conditions will be examined to test the hypothesis that the observed changes in tendon modulus are independent of cell activity.

5.3 MATERIALS AND METHODS

5.3.1 Study Design 2

Study 2 was divided into 4 experiments and included characterisation of the load magnitude applied onto tendon specimens at set frequencies.

The aim of the current study was to monitor the effects of dosages and frequencies of HFLM loading on the biochemical (GAG content) and biomechanical (modulus) properties of cultured tendons. To determine the effect of HFLM loading frequencies on tendons it is essential to constrain all other loading parameters such as loading magnitudes and loading dosages. Similarly, to determine the effects of dosages of HFLM loading on tendons, it is essential to ensure that the applied load magnitude and frequency remain constant when varying dosage periods of loading.

Further, to consider the contribution of cell activity to the observed changes in tissue properties, tendon modulus subsequent to stimulation under low temperature culture conditions was examined. The hypothesis that the observed changes in tendon modulus are independent of cell activity was thereby examined.

Cyclic *in vitro* loading was applied onto tendon fascicles using the previously described custom built IVLS. Rat tail tendon fascicles (RTTFs) were carefully dissected and suspended in 50ml centrifuge tubes, at either 37°C or 27^{O}C. The fascicles were loaded at low frequency (1Hz), and HFLM (10, 15 and 20Hz), with both fresh (F) and unloaded cultured (U) tendon fascicles serving as controls. Cultured fascicles were separated into groups based on the above described environmental conditions, and as outlined in figure 5.1

In Experiment 1, sixty six tendon fascicles from 4 rats (out of 70 fascicles originally harvested and cultured) were used to monitor the effect of HFLM dosages on the modulus and GAG content of cultured tendons fascicles. Cyclic loads at 20Hz were applied onto

tendon groups for 1, 2, or 4hrs per day for 5 days, with 2 days of no load in culture, followed by tensile testing and then GAG assessment.

In Experiment 2, eighty two tendon fascicles from 4 rats (out of 90 fascicles originally harvested and cultured) were used to monitor the effect of HFLM loading frequencies on the modulus and GAG content of cultured tendon fascicles. Cyclic loading at frequencies of 1, 10, 15 or 20Hz were applied onto tendon groups for 1 hour a day for 5 day, with 2 days of no load, followed by tensile testing and GAG assessment.

In Experiment 3, fifteen tendon fascicles from 1 rat were used to monitor the effect of low temperature on the metabolic activity of tendon cells. Tendon groups were cultured under conditions of no load at 4^{o}C, 27^{o}C, or 37^{o}C. Metabolic activity of tendon cells was measured after 1, 3 and 7 days using the Alamar blue assay described in chapter 3. One tendon fascicle from each tendon group was randomly chosen and used to examine tendon metabolic activity.

In Experiment 4, thirty six tendons fascicles from 3 rats were used to examine the effect of low cell activity on the modulus of mechanically loaded tendons. Fascicles subject to no load, 1Hz-low frequency (LF) cyclic loading or 20Hz (HFLM) cyclic loading for 1 hour a day and 5 days, for 1 week were cultured under conditions of 27^{o}C and 37^{o}C. Tensile tests were performed on tendon fascicles subsequent to a culture period of 7 days.

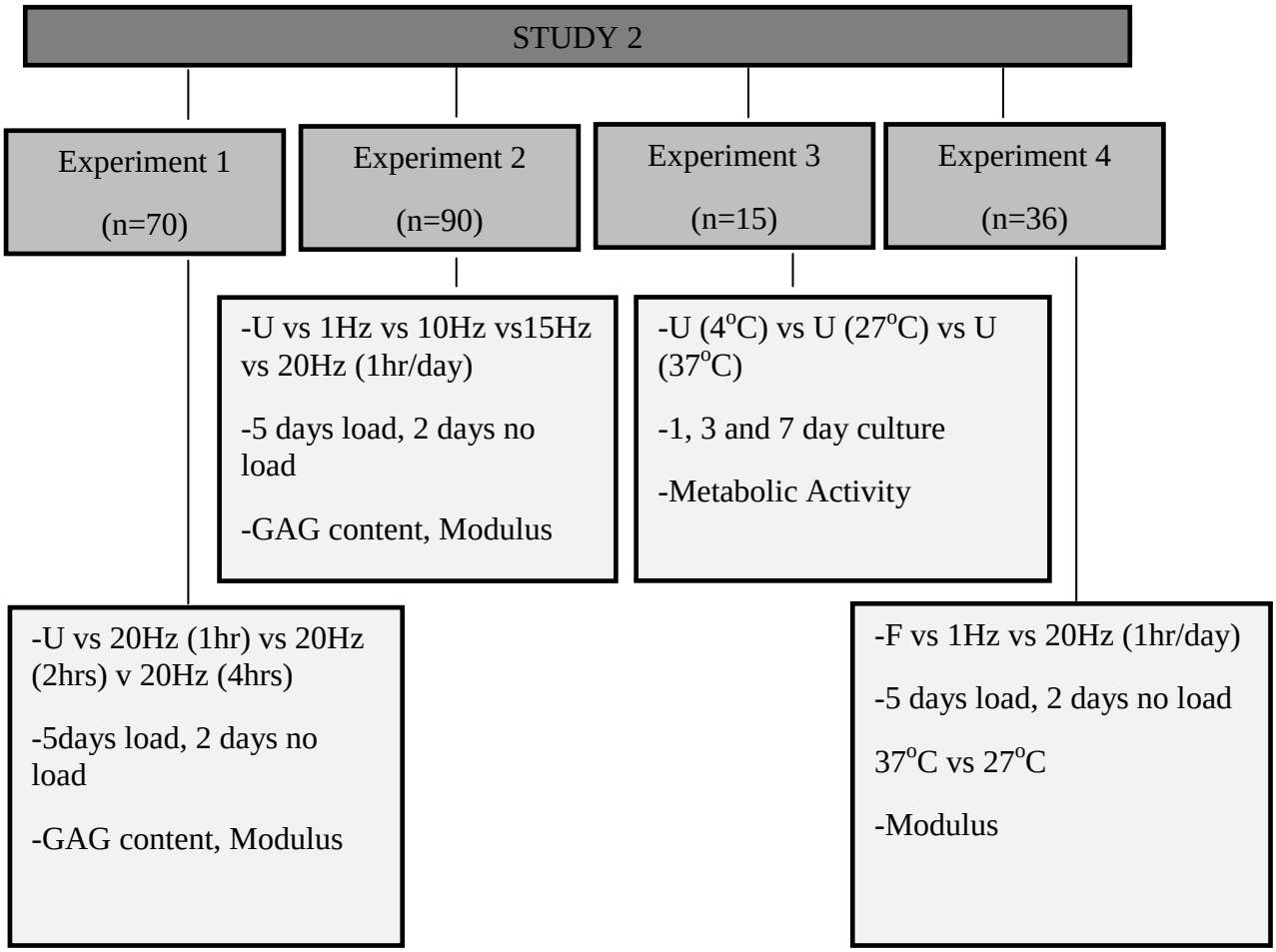


Figure 5.1: Experimental set up for study 2. The study consisted of four experiments.

5.4 RESULTS

5.4.1 Characterisation of applied peak cyclic load magnitude

As described in chapter 4, the average peak load magnitude of the cyclic loading varied with applied max field frequency from 0.09 up to 0.15 N at 1 and 10 Hz respectively, while tendons loaded at frequencies of 15 and 20Hz received peak load magnitudes of 0.12N. In order to enable comparison of tendon properties subsequent to stimulation at different loading frequencies, the IVLS was modified and the field amplitude from the signal generator adjusted to ensure that the average peak load magnitude applied to fascicles from all cyclic loaded groups was constant.

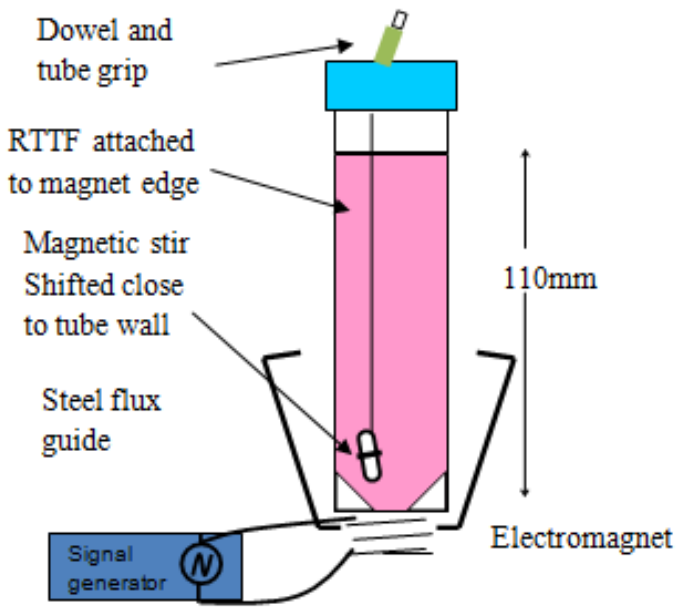


Figure 5.2: The custom IVLS was modified to allow comparison of tendons loaded at various frequencies and dosages with constant load magnitude. For RTTFs loaded at 1Hz, the bottom end of each fascicle was attached to the edge of the magnet stir bar (rather than the centre), and the magnet stir bar was lowered towards the tube base (positioned approximately 10mm above tube base). At loading frequencies of 10Hz, the magnetic field amplitude from the signal generator was adjusted accordingly (reduced) in order to lower the peak cyclic load magnitude applied onto isolated tendon fascicles from 0.15N to 0.12N.

Figure 5.2 illustrates the modification performed to help achieve the desired load magnitude at loading frequencies of 1 and 10Hz. Force-time data was captured at set frequencies of, 1 and 10Hz, using the maximum field amplitude, and a field amplitude of approximately 25% of the maximum respectively, was used to confirm that the peak cyclic load applied onto tendon groups at 1 and 10Hz, subsequent to modification of the custom IVLS was 0.12N. The level of cyclic stress applied to the cultured fascicles therefore ranged between 0-0.9MPa.

5.4.2 Effect of loading dosage periods on tendon modulus and GAG content

After 7 days of culture, tendon groups loaded at frequencies of 20Hz for 1 and 2 hours a day, showed significantly higher average tangent modulus ($p<0.05$) than unloaded tendon groups. There was however, no significant difference in Modulus between tendons loaded for 4 hours a day at 20Hz and unloaded tendons (figure 5.3). Fresh tendon groups showed highest tangent modulus and significantly higher modulus than unloaded cultured tendons.

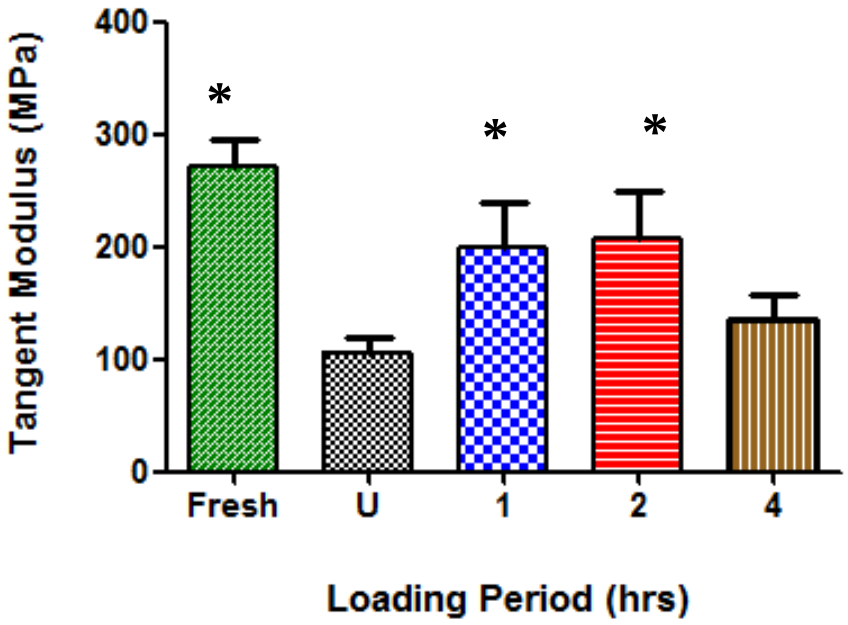


Figure 5.3: Mean tangent modulus of fresh tendon fascicles and fascicles under culture conditions of 20Hz for 1, 2, and 4 hours a day, for 7 days and unloaded tendons. * Denotes a significant difference with unloaded tendon groups, $p < 0.05$ (t-test). Data for fresh fascicle shown are taken from experiment 4.

The DMB assay was used to quantify GAG content within tendon groups. Tendon groups receiving 1, 2, and 4 hours of daily HFLM loading (20Hz) displayed higher GAG contents than unloaded tendon fascicles after 7 days (figure 5.4). Although these differences were not shown to be statistically significant, tendon groups loaded for 1 hour and 4 hours per day showed a two-fold difference with unloaded tendon groups. Fresh tendon groups displayed highest GAG content and a 3-fold significant difference with unloaded tendons.

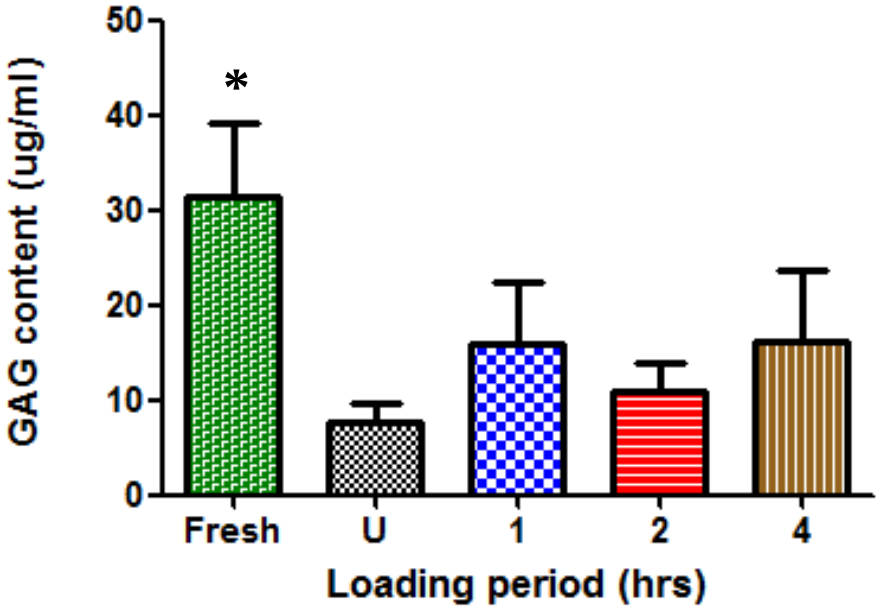


Figure 5.4: Mean GAG content for fresh tendon fascicles and fascicles under culture conditions of 20Hz for 1, 2, and 4 hours a day after 7 days. * Denotes a significant difference with unloaded tendon groups, $p < 0.05$ (t-test). A two-fold difference was observed between unloaded tendon groups and tendon groups loaded for 1 and 4 hours a day. Data for fresh fascicles shown are taken from experiment 2

5.4.3 Effect of loading frequencies on tendon modulus and GAG content

When varying loading frequencies, the average tangent modulus of cultured tendon fascicles increased with frequency over a period of 7 days (Figure 5.5). Only tendon fascicles

stimulated at 20Hz for 1 hour a day showed significantly higher tangent modulus than unloaded tendons. Tendons loaded at frequencies of 15Hz for 1 hour a day displayed similar average modulus to those loaded at 10Hz. Cyclic loading of tendons at frequencies of 1Hz and 10Hz for 1 hour, had no pronounced effect on average modulus when compared with unloaded tendon groups (Figure 5.5). Average tangent modulus was highest in fresh tendon groups, and significantly higher than unloaded tendons

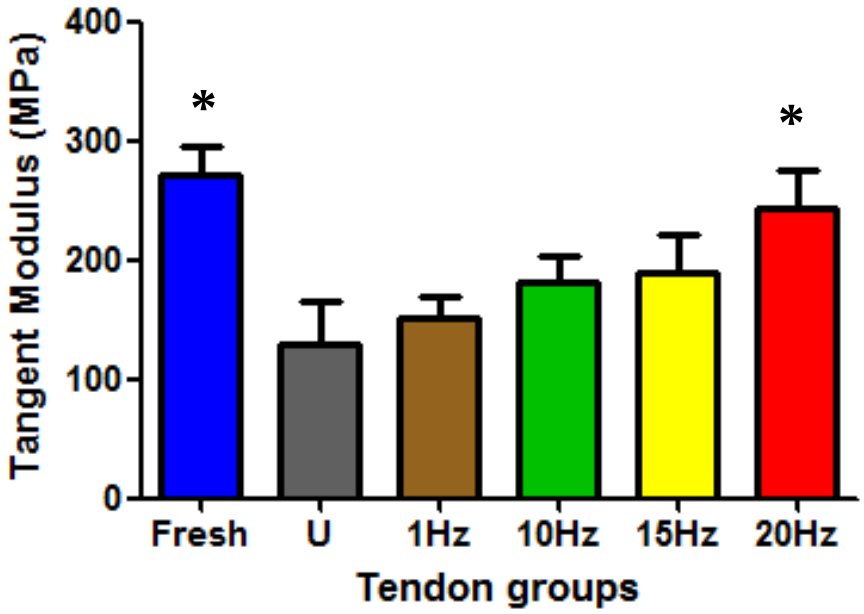


Figure 5.5: Mean tangent modulus of fresh tendon fascicles and fascicles under culture conditions of no load, 1, 10, 15 and 20Hz cyclic loading after 7 days. * denotes a significant difference with unloaded tendons and the bars shown represent standard error. Data for fresh fascicles shown are taken from experiment 4.

Tendon fascicles loaded at 10, 15 and 20Hz showed higher GAG contents than those loaded at 1Hz and unloaded but not higher than fresh controls and there were no statistically significant differences between tendon groups (figure 5.6). GAG content was found to be at a minimum in tendon groups loaded at frequencies of 1Hz and maximum at 15Hz. There was

no significant difference in GAG content between cyclic loaded tendon groups and unloaded tendon groups.

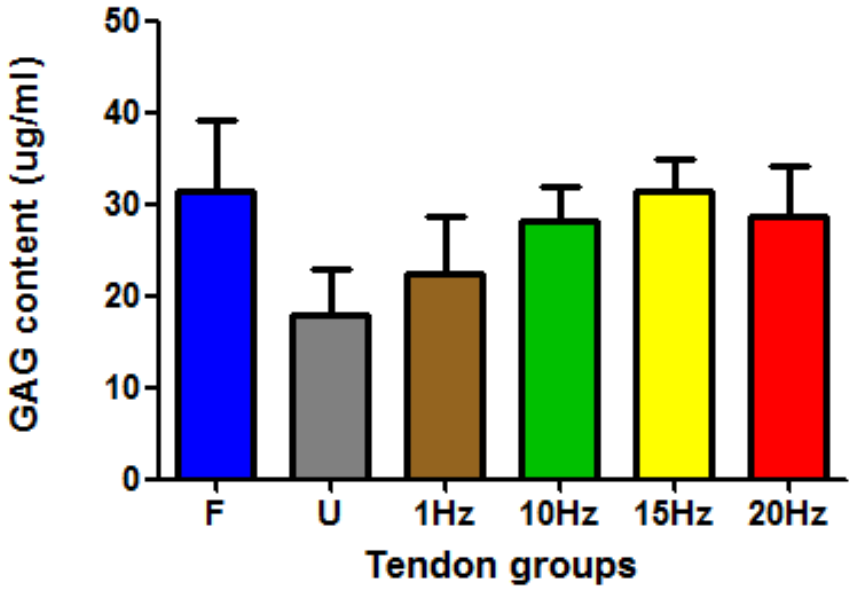


Figure 5.6: Mean GAG content of fresh tendon fascicles and fascicles under culture conditions of no load, 1, 10, 15 and 20Hz of cyclic loading for 1 hour per day, after 7 days. The bars shown represent the standard error.

5.4.4 Effect of low temperature culture on the metabolic activity of tendon cells

Alamar blue assay was used to quantify the metabolic activity of tendon cells within 3 tendon groups. At each time point of measurement, one tendon fascicle was chosen at random (from a starting population of 5 RTTFs) in each tendon group for assessment. Metabolic activity of tendon cells was found to increase with increasing temperature at all days (1, 3, and 7) (figure 6). By day 7, metabolic activity was approximately 82% higher in tendons fascicles cultured at 37^{o}C than in those cultured at 27^{o}C (figure 5.7). Metabolic activity was lowest at 4^{o}C on all days and showed little variation from day 1 to 7. Metabolic activity of tendon cells peaked between day 3 and day 7 for tendon fascicles cultured at 27^{o}C and 37^{o}C.

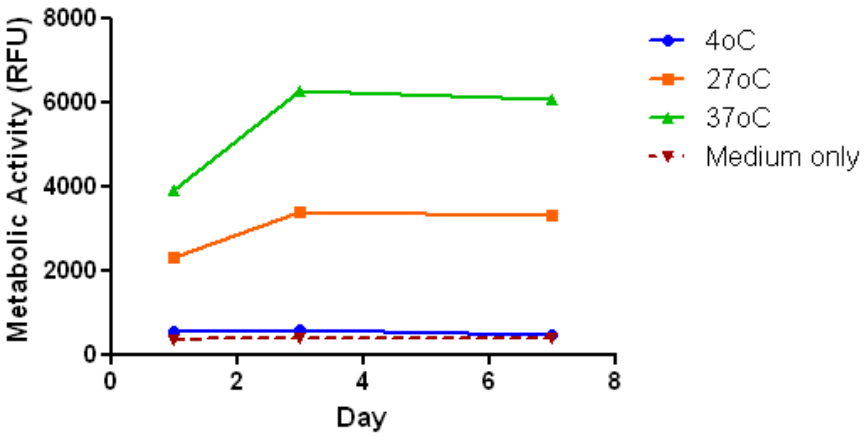


Figure 5.7: The metabolic activity of tendon cells within fascicles cultured at 4^{o}C, 27^{o}C and 37^{o}C as revealed by the relative fluorescence of the REDOX state of Alamar blue after 1, 3 and 7 days of culture. One fascicle from each group was chosen at random and used for assessment of tendon metabolism.

5.4.5 Effect of low cell activity on the modulus of mechanically loaded tendons

Tensile tests were performed on fresh tendon fascicles and fascicles loaded for 1hour per day and 5 days out of 7 at 1Hz and 20Hz, during low temperature (27^{o}C) and standard culture conditions (37^{o}C). At 27^{o}C, tendons loaded at frequencies of 20Hz displayed higher modulus than those loaded at a frequency of 1Hz by 54.4% and fresh fascicles displayed higher modulus than those cultured at 1Hz. Similarly, at 37^{o}C all tendon groups showed higher modulus than the corresponding groups cultured at 27^{o}C (figure 5.8), and tendons loaded at frequencies of 20Hz showed higher modulus than those cultured at loading frequencies of 1Hz by 33.7%. No significant differences were found between any tendon groups cultured at both 27^{o}C, and at 37^{o}C.

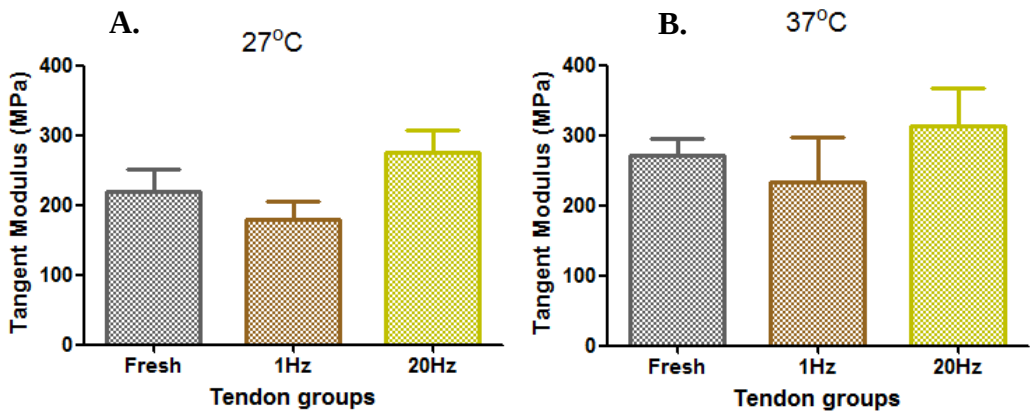


Figure 5.8: Mean tangent modulus for fresh tendon fascicles and fascicles cultured under condition of 20Hz and 1Hz for 1 hour a day after 1 week at A) 27°C (n=8) and B) 37°C (n=4). There were no significant differences between tendon groups at both 37°C and 27°C culture conditions.

5.6 DISCUSSION

Application of appropriate mechanical loads has been implicated as vital for tendon remodelling [165, 192, 203, 204] and excessive repetitive loading has been associated with the aetiology of tendon injuries[87, 205, 206]. These biophysical effects are therefore related not only to the homeostasis of normal tendons but also to the repair of injured tendons.

High frequency low magnitude (HFLM) loading regimes (10-50Hz) have been shown to provide significant anabolic stimulus, reducing the rate of bone mineral loss in osteoporosis[18, 19], and there are indications that similar stimuli may be clinically effective in other musculoskeletal tissues, including tendon [173].

However, to realise the potential of this new and exciting loading intervention for tendon homeostasis and repair, it is critical to identify optimum loading parameters.

In the present contribution, effects of HFLM dosage periods and frequencies were monitored in order to ascertain the most favourable loading conditions for tendon biochemical and biomechanical properties by means of a custom IVLS.

Force time data subsequent to modification of the IVL set-up and applied field frequency was used to confirm that the load magnitude (force signal) applied to tendon fascicles for all loading frequencies varied between 0-0.12N.

The results of our study demonstrate that a loading frequency of 20Hz produced highest tangent modulus in tendon fascicles subject to cyclic loads of either 1, 10, 15, 20Hz for 1 hour a day for 1 week (figure 5.5). There have been no previous reports of HFLM loading

frequencies on cultured tendons and perhaps this can be ascribed to the difficulty of experimental techniques required. However these results reflect those reported by Hazell *et al* 2007, who studied the effects of different frequencies of WBVs (25, 30, 35, 40 and 45Hz) on

optimal muscle electromyography (EMG) activity, a phenomena linked to tissue strength. It was demonstrated that higher WBV frequencies of 35, 40, 45 Hz resulted in the greatest increases in EMG activity in vastus-lateralis muscles, indicating possible improvements in musculoskeletal tissue strength with application of higher frequency mechanical loads [207]. It should be remembered that while high frequency loads of 20Hz may provide a more desirable option than low frequency loads (1Hz) for improved tendon mechanical properties, further studies are needed to elucidate the effects of HFLM loads on tendons at frequencies above 20Hz.

No significant difference was found in GAG content between tendon groups loaded at 1, 5, 10, 15, 20Hz, although tendons loaded at the highest frequencies of 15 and 20Hz showed the maximum GAG content (figure 5.6). These findings indicate that the effect of loading at different HFLM loading frequencies is more pronounced on tendon mechanical properties (modulus) than on tendon biochemical properties (GAG content). To date no *in vitro* studies have examined the effect of HFLM frequencies on the biochemical properties of tendons. However, in an *in vivo* study by Legerlotz *et al*, 2007, no adaptation in mRNA concentration of collagen I, III, and TIMP-1 was found in the Achilles tendon of male Sprague Dawley rats receiving low vibration strength training and high vibration strength training (25Hz) after12 weeks[182], and *in vitro* studies have shown that tendon cells do not display a cyclic frequency dependent response with activation of stress activated protein kinases (SAPKs), a protein that has been linked with regulation of several biological processes [192].

When varying dosages, tendon fascicles receiving 1 hour of HFLM loading at 20Hz, displayed significantly higher modulus than unloaded tendons, and extending the loading period to 2 hours had minimal further effect on tangent modulus (figure 5.3). Moreover, a prolonged HFLM loading period of 4 hours was found to reduce tangent modulus by approximately 33% compared with groups loaded for 1 hour a day. These results suggest that

short dosage periods of HFLM loading at 20Hz provide a sufficient stimuli for increasing tendon mechanical properties, and prolonged exposure times to HFLM mechanical loads may have a harmful effect on tendon properties via fatigue, a relationship that has been observed during *in vivo* studies [205], which have reported mechanical weakening in tendons following prolonged periods of exercise.

When assessing the influence of HFLM (20Hz) dosage periods on tendon GAG content no clear trend was observed (figure 5.4). However, extending the loading period beyond 1 hour to 2 and 4 hours per day did not increase tendon GAG content, and these findings reverberate those found for their mechanical properties, since loading for a period of 1 hour per day had almost the same effect as the optimum loading period in each experiment.

An interesting observation was the discrepancy between tangent modulus and GAG content, where there was a noticeable drop in modulus after 4 hours, but no decline in the GAG content after a 4 hour HFLM loading period. These results reinforce that the tendon GAG content alone should not be used as a primary indicator of the tensile mechanical properties of tendons [159], and other (ECM) factors, such as collagen fibril morphology and integrity may play a more crucial role on the tensile properties of tendons.

The effects of varying *in vitro* HFLM dosages on tendon biochemical properties have not been described in literature. However, our results are in agreement with those published by Lergolotz *et al* 2007, who conducted animal experiments and found that the Achilles tendons of SD rats receiving low strength vibration training (LVST) at 25Hz produced considerably higher levels of mRNA levels of procollagen I, III, and TIMP1 than rat tendons receiving high strength vibration training although differences observed were not statistically significant). More importantly Lergerlotz *et al* 2007 concluded that changes in mRNA expression of procollagen I, III, and TIMP 1 of tendon, essential biochemical components, do

not seem to affect the mechanical properties of tendon, as we have also discovered within our study with expression of GAG, another major tendon biochemical component.

Our findings on GAG and modulus indicate that dosage periods of 1 hour a day provide an optimum balance of safety and effectiveness in promoting tendon biochemical and biomechanical properties.

Furthermore, fresh tendons showed significantly higher tangent modulus and GAG content than unloaded tendon groups (figure 5.3-5.5), highlighting the rapid loss of tendon properties subsequent to tissue culture. It follows then that HFLM mechanical loads present a protective effect for tendon health by preventing deterioration of tissue mechanical and biochemical properties.

Observation of tendon metabolism by Alamar Blue revealed that tendon fascicles cultured at 37^{o}C contained cells with considerably higher metabolic activity than those cultured at 27^{o}C after day 1, day 3 and day 7 of culture by 70.13, 99.50, and 82.50% respectively. This means cell activity is approximately halved by the drop in temperature from 37 to 27 degrees.

Our findings on metabolism which show higher metabolic activity in tendons cultured at 37^{o}C than in tendons cultured at 27^{o}C are coherent with studies which demonstrate that temperature governs metabolism in biological structures through an Arrhenius type relationship, and this has been attributed to the distinct effect of temperature on biochemical reaction rates[208-210]. Furthermore, our measurements of metabolism in rat tendons are similar to those previously reported in literature for control tendons[89, 211].

As expected, tendons loaded at 20Hz showed higher modulus than fresh tendons and those loaded at 1Hz during culture conditions of 27^{o}C and 37^{o}C. Further, a similar pattern in modulus was observed between tendon groups cultured at 27^{o}C as that observed for tendon

groups cultured at 37^{o}C indicating that the changes observed in modulus are able to take place during conditions of both low and high cell activity.

To date, no study has been performed on the mechanical response of cultured tendons to HFLM mechanical loads during conditions of low cell activity. However, studies have subjected tendon specimens to freeze-thaw cycles in liquid nitrogen prior to culturing and mechanical analysis, a process which has demonstrated 100% cell death in tendons [212].

During the investigation by Hannafin *et al* 1995, it was found that tendons subject to repetitive freeze-thaw cycles displayed no change in modulus after 2 and 4 weeks of *in vitro* stress deprivation, indicating that complete cell death may not always be desirable for observation of tendon adaptation effects to mechanical loading.

It should also be remembered that administration of complete cell death during culture by noxious treatment to connective tissues may result in a cascade of catabolic processes leading to tissue degradation [213-215]rendering the tissue biochemically and biomechanically unresponsive. Our approach for studying cell contribution to tissue remodelling and mechanotransduction effects in cultured tendons is an advancement to techniques previously described in literature, using complete cell death for studies on tendon biomechanics [164, 165, 200, 202].

While it may be difficult to monitor the structural and mechanical response of tendons to HFLM mechanical loading independently of cell activity and without comprising cell death, reducing metabolic activity by culturing under conditions of low temperature may provide the correct cues.

While HFLM loading represents a stimulus of remarkably small magnitude and seemingly short duration, a 20Hz stimulus for 1 hour is equal to 20 x 3600 , or 72,000 cycles which may

represent 72,000 strides at stress cycles of 0-0.9MPa. Our present study indicates that based on the parameters investigated, HFLM loading frequencies of 20Hz and dosage periods of 1hr a day provide an optimal balance for promoting the modulus, and GAG content in cultured tendon fascicles.

There is still no complete understanding of the relationship between cyclic stress induced changes on tendon biochemical and biomechanical properties. We have found no evidence that cell activity is responsible for the variation in tangent modulus observed between tendons loaded at low frequency (1Hz) and those loaded at high frequency (20Hz), and these events may indicate a structural mediated mechanism for tendon adaptation to HFLM mechanical loads.

CHAPTER 6 Mechanisms for tendon mechanical adaptation to HFLM mechanical loading in cultured tendon fascicles

6.1 INTRODUCTION

In chapter 5, different HFLM frequencies, magnitudes and durations for promoting the biochemical and biomechanical properties of tendons were considered using the custom made IVLS, and high frequency loads of 20Hz with daily dosages of 1 hour were found to produce the most desirable effects for tendon properties amongst the loading parameters considered (see section 5.3.1). It was also highlighted that differences observed in modulus of tendon groups subsequent to mechanical stimulation occurred regardless of the level of cell activity as controlled by culture temperature.

In this chapter, we investigate the non-cellular factors in tendons which may play a role in the mechanical adaptation mechanisms leading to tissue remodelling. Taking data from experiments in chapter 5 as well as additional studies performed on the matrix organisation of tendon fascicles we examine the association between tendon crimp during low and normal cell activity and changes in tendon mechanical properties.

6.2 Tendon remodelling and the mechanical environment

Tendon matrix remodelling has been defined as "a broad concept that involves changes in the amount and or organization of the matrix constituents" [159], and in healing it is a process that may last for several weeks [33]. The experiments reported in this thesis take place over the course of a maximum of 7 days, so the definition of remodelling considered will be over this relatively short timescale.

The influence of the tendons mechanical loading environment on tissue remodelling has been recognised in literature and these studies have used changes in the structural and mechanical

properties of tendon synonymously to characterise tendon remodelling [159, 216]. Moreover, organisation of the collagen architecture within tendons as indicated by crimp morphology has been associated with the tissues mechanical properties. For instance, tendons possessing fibrils with low crimp angles have been found to rupture before those with larger crimp angle [50], and decreases in strain at failure in rabbit patellar tendons have been positively correlated with decreases in crimp angles in tendons fascicles [193]. However, there is no clear understanding of the mechanisms governing this association since crimp is usually believed to govern only the low strain behaviour of tendon.

Furthermore, it remains unclear whether the mechanisms driving significant changes in tendon mechanical properties subsequent to HFLM loading over the 7 day time course used in this study, are mediated by cells, or mechanical perturbations of the collagen architecture at the fibril level, or a combination of both.

6.3 Cellular induced remodelling

A considerable number of studies have ascribed the mechanisms for tendon remodelling wholly to cellular responses [196, 217-219]possibly since cells can adapt to mechanical loads by increasing protein synthesis and depositing tissue components into the extracellular matrix environment. This was demonstrated in study by Screen *et al.*, 2005 where application of 5% cyclic strain on RTTFs of male Wistar rats at 1Hz for 24 hours resulted in a statistically significant up-regulation of collagen synthesis. Furthermore, a 44.3% retention of newly synthesised collagen was reported in strained fascicles compared with 40.0% retention in unstrained RTTFs [4]. Such changes in the matrix composition of tendon are characteristic of tissue remodelling via cellular driven events. In skeletal tissue application of appropriate mechanical signals has resulted in larger and stronger bone, and stimulation of bone remodelling[17, 19, 220, 221].

Brown *et al* 1998 investigated the mechanical response of fibroblast cells to changes in tensional mechanical loads applied across a fibroblast populated collagen lattice (FPCL) through a computer-controlled force transducer. It was found that subsequent to a culture period of 24 hours resident cells within collagen-GAG sponges reacted to modify tension within the collagen matrix in the opposite direction to an externally applied load such that increased external loading was followed immediately by a reduction in cell mediated contraction whilst decreased external loading elicited increased contraction. They concluded that fibroblasts regulate the mechanical response (and properties) of connective tissue to tensional loads by actively increasing and decreasing cellular contractions [222]. Modification of tendon matrix mechanical properties by cell mediated activity in this way is characteristic of cellular induced tissue matrix remodelling.

It has also been proposed that externally applied mechanical loads can be used to stimulate tissue remodelling in tendons by causing movement of integrin proteins which bridge the intracellular and extracellular matrix and this activates biological pathways such that mechanical stimulus from the cells external environment promote intracellular processes leading to matrix remodelling [223].

Cell deformation subsequent to collagen fibre strain has also been shown to cause tension in the cell cytoskeleton which can provide a signal to the cell nucleus via a tensegrity system, in such a way as to influence matrix remodelling [198, 224-227]. In such instances, it is believed that arrival of a suitable signal at the nucleus initiates a process of ‘transcription’ and transportation of mRNA to the endoplasmic reticulum in the cells cytoplasm where it is converted into protein via a process of ‘translation.’ These events are followed by secretion deposition of protein into the tissues extracellular matrix thereby resulting in matrix remodelling [228].

Ingber demonstrated that control of the cell shape by extracellular matrix and alteration of the intracellular cytoskeleton structure regulates tissue morphogenesis, and stress induced changes in the cytoskeleton cause local rearrangement as well as deformation of tensile elements [229].

6.4 Non-cellular induced remodelling

There has been substantial interest in the potential to manipulate the remodelling process in connective tissue using various techniques and chemical agents[89, 230, 231]. Amongst these techniques is the use of mechanical stimuli.

While tendon cells have been recognised for their role in the secretion of new extracellular matrix materials [4], non-cellular induced mechanisms for tendon remodelling have also been implicated. Majima *et al* 1994., studied the effects of stress deprivation on the mechanical and histological properties of in-situ frozen patella tendon autografts. Complete stress shielding was found to significantly reduce tensile strength and tangent modulus compared with loaded acellular tendons, and significant changes in tensile strength took place after 3 and 6 weeks of stress shielding. Furthermore, histological examination was used to demonstrate that remodelling of the patellar tendon took place in the absence of cells within the tendon fascicles [164], confirming paradigms of non-cellular induced tendon remodelling.

In vitro studies with acellular flexor tendons have also been used to try and elucidate the contribution of cells to the mechanical response of the tissue following a loading stimulus [232]. Further, studies reporting preservation of mechanical properties in human flexor tendons after 24 hours of accellularisation by treatment with 1% sodium dodecyl sulphate suggest further work is needed to confirm whether participation of cell activity is essential for tendon remodelling [200].

A final school of thought indicates both cell and structural mediated mechanisms for tendons adaptation to mechanical loading. For instance, movement and deformation of fibroblasts have been shown to modify the morphology and organisation of collagen extracellular matrix and present collagenous tissue with extremely high mechanical strength [233-235]. These events have also been ascribed to cells which are capable of generating sufficient contractile force necessary to initiate physical changes in the collagen architecture [222, 233-238].

6.4.1 Tendon crimp

Collagen structures within tendon constitute over 85% of the extracellular matrix, and collagen fibrils are organised in closely packed bundles which align parallel to the long axis of tendon. In unloaded tendon these fibril bundles buckle to form an undulating architecture called ‘crimp,’ and these structures are thought to act as shock absorbers during initial stages of tensile loading, and to play an important role in the elastic properties of tendon [239-241]

The ‘toe region’ of a typical tendon stress strain curve where there is an initial extension in tendon length upon application of a tensile load, is brought about by the presence of crimp [242] and although it is unknown how these structures are formed it is widely believed that tendons develop the wavy crimp pattern during embryonic stages of life [243].

Crimp morphology can be characterised by measuring three parameters, wavelength, wave amplitude, and crimp angle [163, 193] and these parameters are defined in figure 6.2.

One study relating changes in tendon mechanical properties to changes within tendon crimp showed that the crimp angle decreased as a result of the increasing applied cyclic stress in an in vitro culture model of tendon loading. Further, strain to failure was lower for the tendons loaded at higher stress, and these tendons also had reduced crimp angles [193].

In a later study, the same authors speculated that, the frequency of cyclic loading may have a pronounced influence on the micro-structural changes within the collagen fibrils [166].

Stress deprived tendons associated with decreased modulus have also been shown to display disrupted collagen architecture and altered crimp pattern after 8 weeks of culture and histological examination [165]. Although it was not discussed whether these events were a consequence of mechanical unloading or enzymatic effects of cell death, the observed changes indicate that differences in tendon modulus maybe associated with re-organisation of tendon extracellular matrix. More recently, Foolen *et al* 2010., evaluated the possibility that fibrous tissue mechanical properties are related to the structural organisation of collagen network. They applied stretches of 0.85,0.90, 0.95, 1.00 or 1.05 times the in vivo length to dense fibrous tissue from the tibiae of chick embryos during a 3 day culture period. It was found that changes in fibre crimp caused by mechanical perturbations of the matrix and cell contractions during culture were responsible for maintaining the mechanical properties of fibrous tissue. More importantly, the observations made indicate that both cell activity and matrix reorganisation play a key role in tissue adaption to mechanical loading [244].

The purpose of the study reported in this chapter was to examine the contribution of cell activity to changes in crimp morphology and tendon modulus following periods of in vitro cyclic loading of tendons. The work tests the hypothesis that changes in collagen crimp morphology in tendon fascicles subject to cyclic loading may be correlated with the change of the tendon's mechanical properties.

6.5 MATERIALS AND METHODS

6.5.1 Study Design

Study 3 was divided into 2 experiments and included consideration of the results and findings from experiment 3 and experiment 4 in chapter 5 of this thesis. These experiments were used to assess the effect of low temperature culture conditions on tendon cell metabolism (metabolic activity), and the effect of low temperature culture conditions on the modulus of mechanically loaded tendons. It was shown that cell activity is approximately halved by the

drop in temperature from 37°C to 27°C, and trends in mechanical properties between tendon groups cultured at 37°C were mirrored at 27°C.

In this study, tendon fascicles from the tails of 4 male Sprague Dawley rats (aged 3–5 months), were harvested and cultured within 2 hours of sacrifice as described in chapter 3 of this thesis. All fascicles serving as fresh controls were analysed immediately after harvesting. Cultured fascicles were exposed to either low frequency loading (LF, 1 Hz), or high frequency loading (HFLM, 20 Hz) for 1h/day, with 5 days of loading and 2 days of no loading during culture. Experiments were performed under conditions of normal cell activity (37°C) or low cell activity (27°C) as highlighted in figure 6.1.

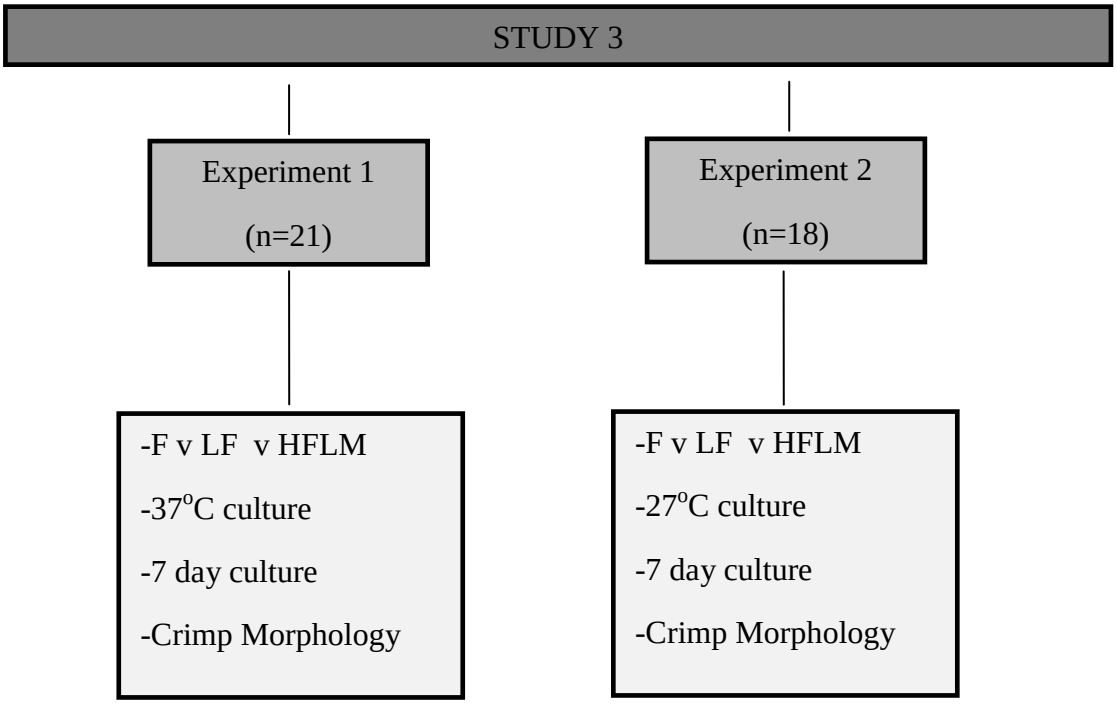


Figure 6.1: Experimental set up for study 3. The study consisted of two experiments used to examine two research questions. 1.) How does cyclic mechanical loading influence tendon crimp morphology during low and normal cell activity, and does low cell activity interfere with/impede/hinder mechanically induced changes in tendon crimp morphology?, 2.) Are differences in tendon crimp induced by cyclic loading linked to differences in tendon modulus?

In experiment 1; twenty one tendon fascicles (2 rats) were divided into 3 groups (n=7) and used to examine crimp morphology in fresh fascicles, and fascicles cultured under conditions of LF and HFLM loading after 7 days of incubation at 37^{o}C. The experiment was designed to assess the effects of HFLM and LF mechanical loading on the crimp morphology of cultured tendon fascicles during normal cell activity.

In experiment 2; eighteen tendon fascicles (2 rats) were divided into 3 groups (n=6) and used to examine crimp morphology in fresh fascicles, and fascicles cultured under conditions of LF and HFLM loading after 7 days of incubation at 27°C. The experiment was designed to assess the effects of HFLM and LF mechanical loading on the crimp morphology of cultured tendon fascicles during low cell activity.

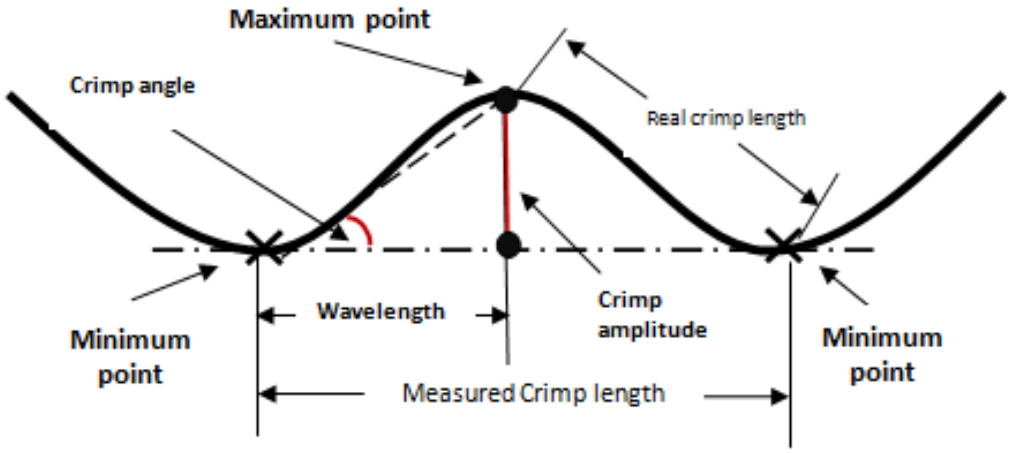


Figure 6.2 Classification of crimp parameters as defined by Yamamoto et al. The measured crimp length was taken as the horizontal distance between two similar points (2 minimum points) in a single waveform. The wave length was calculated from the measured crimp length (half the measured crimp length). The wave-amplitude is defined as the vertical distance between the maximum and minimum point of a single waveform. Wave angle is the angle between the diagonal line connecting the maximum and minimum point of a waveform and the horizontal line connecting two minima points. Adopted from Yamamota *et al* 2002, [163], with permission.

Crimp morphology was characterised by measuring three parameters, wavelength, wave amplitude, and crimp angle using Zen Light edition 2009 software. These parameters are defined in figure 6.2.

Thirteen tendon fascicles from fresh, HFLM (20Hz) and LF (1Hz) cyclic loaded groups were assessed. Tendons fascicles were mounted onto microscope slides and covered with glass cover-slips. Samples were viewed under a Zeiss LSM 710 inverted confocal laser scanning microscope and second harmonic generation (SHG) images were acquired with a 10x objective as described in chapter 3 of this thesis. Collagen fibrils on the outer surface of tendon fascicles were assessed at a depth of approximately 60µm into the z-axis of the tissue and the centre region from a 5cm portion of a fascicle was used to examine crimp morphology.

The measured crimp length was taken as the horizontal distance between two similar points (distance between 2 minimum points) in a single waveform. The wave length was calculated from the measured crimp length and recorded as half the measured crimp length. The wave-amplitude is defined as the vertical distance between the maximum and minimum point of a single waveform. Wave angle is the angle between the diagonal line connecting the maximum and minimum point of a waveform and the horizontal line connecting two minima points.

6.6 RESULTS

6.6.1 Tendon modulus during low and normal cell activity

In chapter 5 we recall that tensile tests were performed on fresh tendons and tendons loaded at 1Hz and 20Hz, during conditions of normal (37°C) and low cell activity (27°C). A similar pattern in modulus was observed between tendon groups cultured at 27°C as that observed for tendon groups cultured at 37°C, indicating that mechanically induced differences observed in modulus are able to take place during conditions of both low and high cell activity (figure 5.8). These were the same tendons as are here reported in Study 3 Experiments 1 and 2 respectively.

6.6.2 Effect of cyclic loading on tendon crimp during normal cell activity

Observation of crimp morphology under conditions of normal cell activity (37°C) revealed that crimp wave angle was largest in fascicles loaded at 20Hz (14.28±0.40°) and lowest in fascicles loaded at 1Hz (11.99±1.79°) (figure 6.3). Wave amplitude was noticeably higher in fascicles loaded at 20Hz (27.9±1.08μm) than in fresh controls (26.41±2.30μm) and those loaded at 1Hz (22.85±1.73μm), however, no significant difference was found in crimp parameters between groups.

Crimp wavelength was almost the same in fresh fascicles (112.50±3.86μm) and those loaded at 1Hz (112.70±8.58μm), but was smallest in fascicles loaded at 20Hz (109.80±1.63μm).

Second Harmonic Generation (SHG) images revealed detailed collagen fibril organisation in tendon fascicles cultured at 27°C and 37°C, (figure 6.4 and 6.6) and RTTFs in all groups exhibited highly aligned and parallel collagen fibrils.

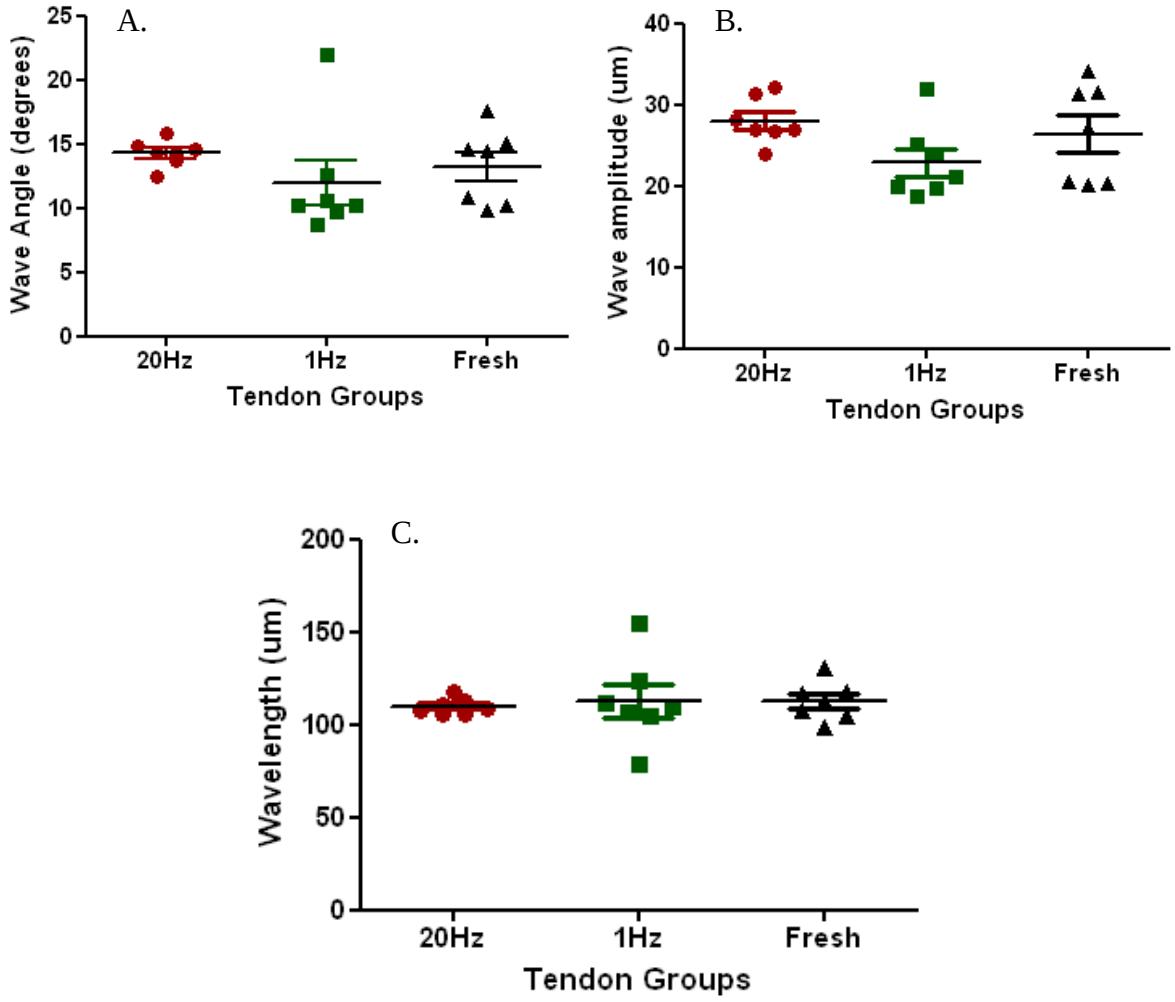


Figure 6.3: Crimp A.) angle B.) amplitude and, C.) wavelength in fresh tendon fascicles, and tendon fascicles loaded at 20Hz and 1Hz at 37˚C. The centre regions of seven tendon fascicles were assessed and mean values for each group are represented as black horizontal lines

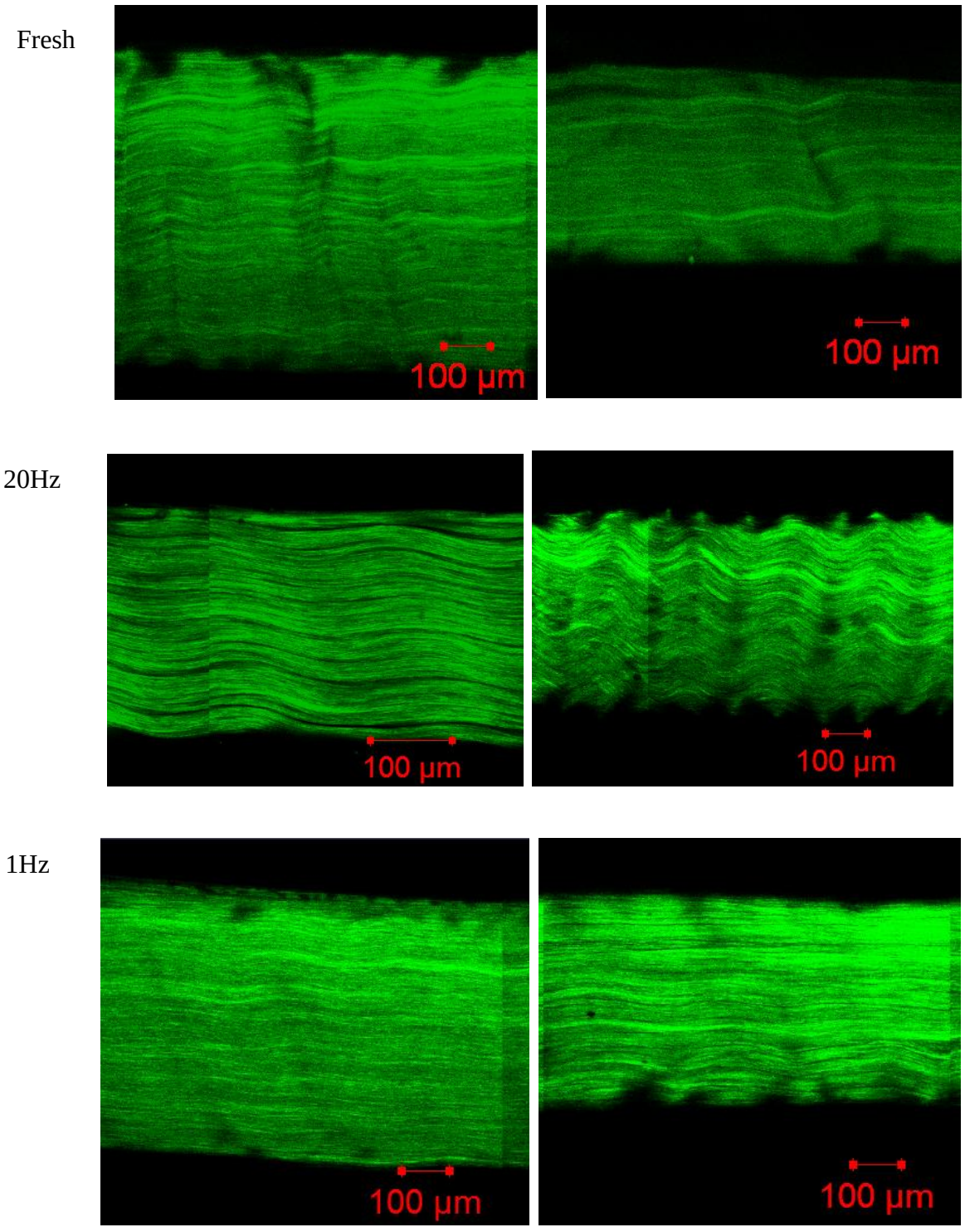


Figure 6.4: SHG images showing crimp morphology in tendon fascicles cultured at 37°C. Two representative fascicles were randomly selected from fresh fascicles, and fascicles loaded at 20Hz and 1Hz

6.6.3 Effect of cyclic loading on tendon crimp during low cell activity

During conditions of low cell activity (27°C) crimp wave angle and amplitude was larger in HFLM loaded fascicles (12.96±1.20°, 26.23±2.68µm) than in fascicles loaded at 1Hz (9.41±1.0°, 19.58±2.63µm) and fresh controls (12.32±0.57°, 25.99±0.73µm). However, wave

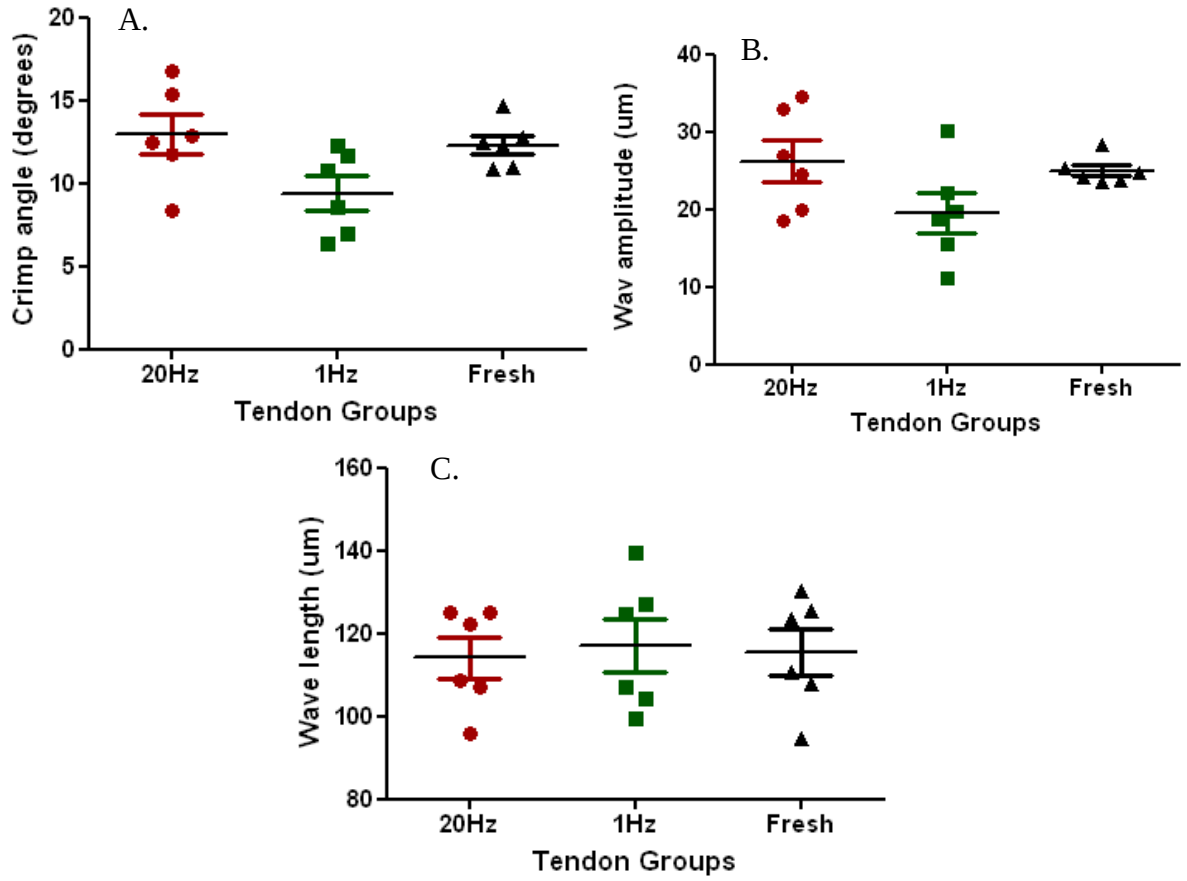


Figure 6.5: A.) crimp angle B.) amplitude and C.) wavelength in fresh tendon fascicles, and tendon fascicles loaded at 20Hz and 1Hz at 27°C. The centre region of six tendon fascicles were assessed, and mean values for each group are represented as black horizontal lines. *Denotes a significant difference between tendon groups loaded at 20Hz

length was larger in tendons loaded at 1Hz (117.1±6.40µm) than in tendons loaded at 20Hz (115.5±5.47µm) and fresh tendon fascicles (114.10±4.93µm), figure 6.5.

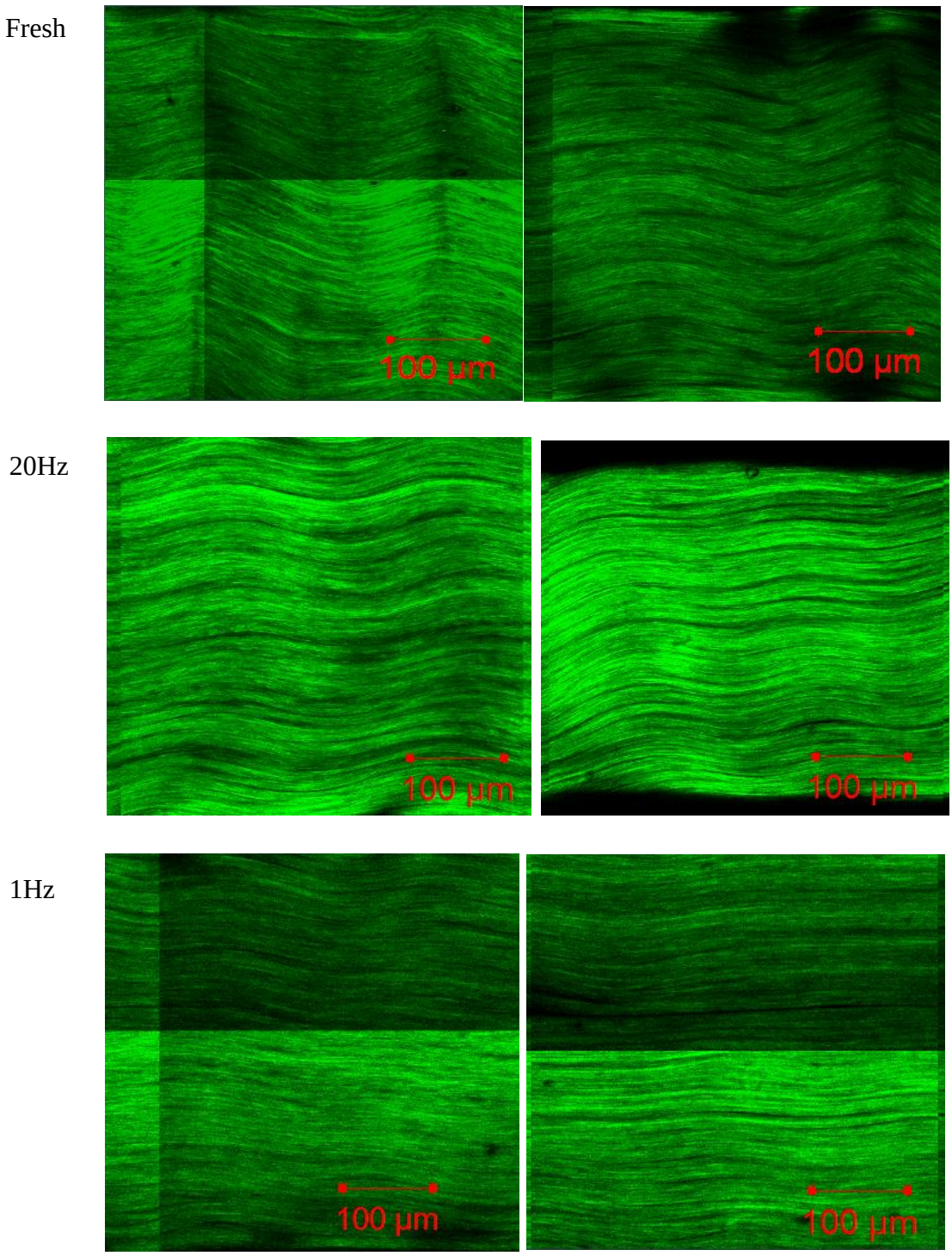


Figure 6.6: SHG images showing crimp morphology in tendon fascicles cultured at 27°C. Two representative fascicles were randomly selected from each of the tendon groups

Crimp morphology was similar in fresh and HFLM loaded fascicles (figure 6.6), while the most pronounced differences in crimp wavelength and wave amplitude was observed

between fascicles loaded at 20Hz and those loaded at 1Hz. A significant difference was observed in wave angle between tendon fascicles loaded at 20Hz and those at 1Hz.

No significant difference was observed between respective crimp parameters and tendon groups at 27°C and 37°C.

6.7 DISCUSSION

Tendons are able to modulate their mechanical and structural properties in response to mechanical loading and whilst the benefit of cyclic mechanical loading for tendon mechanical function is clear, there is as yet no complete understanding of the mechanisms which are responsible for these changes.

Recent interest on the structure-function behaviour of tendons has led to investigations on the relationship between tendon crimp morphology and tendon mechanical properties [163, 193, 244].

In contrast, a number of studies have identified the role of cells as primary mediators [198, 225-227, 229] in converting mechanical signals into biochemical responses within tendons. However, it is unclear whether the biomechanical response of tendons subsequent to mechanical loading is a consequence of cell activity, reorganisation of the tendon architecture, or a combination of both. In this report we consider the contribution of cell activity and changes in tendon crimp as mediators for changes in tendon properties subsequent to LF and HFLM mechanical loading.

In the previous study (chapter 5) we reported that tendon fascicles cultured at 37^{o}C for 7 days contained cells with considerably higher metabolic activity than those cultured at 27^{o}C by

approximately 83%, and this trend towards lower metabolic activity with decreasing temperature has been confirmed in literature.

Our results on tendon metabolism therefore allow us to assess the participation of cell activity in the structural and mechanical response of tendons to HFLM mechanical signals. Furthermore, a similar pattern in modulus was observed between tendon groups (Fresh, LF, and HFLM loaded) cultured at 27^{o}C as that observed for tendon groups cultured at 37^{o}C indicating that tendons are able to demonstrate similar mechanical responses to mechanical signals during low cell activity as they would during conditions of normal cell activity. It follows then that non-cellular mechanisms may play a more significant role in tendons mechanical adaptation to HFLM mechanical loads then cell mediated mechanisms.

Examination of tendon crimp morphology revealed that crimp angle and crimp amplitude were found to show moderate and strong positive correlation with elevated tangent modulus in tendon fascicles during conditions of low and moderate cell activity respectively. R was measured at 0.908 and 0.910 for crimp angle and amplitude (compared with tangent modulus) during low cell activity while R was measured at 0.997 and 0.969 for the same parameters during normal cell activity (data not shown).

Further, during conditions of low cell activity (27^{o}C), crimp angle and tangent modulus were higher in tendon groups loaded at 20Hz than in tendons loaded at 1Hz by 38 %, and 54% respectively, and during conditions of normal (37^{o}C) cell activity these values were 19% and 34% respectively, although not one of these differences was statistically significant.

While it is possible to observe the detailed microstructure and collagen architecture within tendon fascicles using multi-photon microscopy, it would seem that limitations in the technique used to measure crimp pattern exist since sizeable differences between tendon groups shown in the crimp images (figure 6.4 and 6.6) were not reflected in the numerical

and statistical calculations. This observed irregularity may have been caused by the use of a 2-dimensional software measurement technique (Zen 2009) to quantify 3-dimensional structures. Difficulties for accurate quantification of crimp pattern are not unique to this study since several laboratories have adopted a similar approach, using plane polarised light microscopy (PPLM) or polarised light microscopy in combination with Axioplan 2-axiovison software (a 2D planar technique) to quantify crimp morphology and organisation of collagen bundles within tendons [242, 245, 246].

Nevertheless, the association observed in this study between crimp changes and mechanical property changes subsequent to periods of mechanical loading during low and normal cell activity indicate that adaptation of tendon modulus to mechanical loads may involve a mechano-structural induced mechanism and not simply a cell mediated mechanisms as has been suggested in literature [222, 244].

It is beyond the scope of this study to detail why modification of crimp properties and tangent modulus subsequent to HFLM stimulation were found to be higher during conditions of low cell activity; however, it is possible that ongoing catabolic processes contributing to degradation of the matrix and mechanical properties were less dominant during low temperature culture conditions [247]

Our results on tendon crimp parameters are similar to those by Yamamota *et al* 2003, who found that tendon fascicles subject to low frequency cyclic loads of 4Hz and an applied stress of 1.8MPa displayed lower crimp angle (14.2 ± 4.6^{o}) than fresh controls (15.7 ± 3.8^{o}) in addition to higher crimp wavelength (48.7+11.4 vs 47.5+17.3MPa) after a culture period of 1 week. Second Harmonic Generation images of tendon microstructure in our study (figure 6.4 and 6.6) are comparable to those published by Sereysky *et al* .,2011 and Yamamota et al

2002, 2003 for rat patellar and fascicles obtained from rabbit patellar tendons [163, 193, 248].

There are numerous examples in literature where changes in the mechanical properties of the tendon have been suggested to be associated with changes in tendon crimp [249, 250].

Flint *et al* 1980., demonstrated that any alteration in the amount or nature of loading is followed by changes in matrix morphology and functional properties of tendon [251].

Nagel *et al* 2012., developed a phenomenological model that uses a natural configuration of collagen networks with angular reorientation in a continuum mechanical framework to study stress and deformation driven remodelling processes. They reported that the mechanical properties of the collagen network within soft biological tissues were determined by its orientation and degree of crimp and not simply its biochemical composition and degree of cross-linking. Moreover they stipulated that since collagen fibres bear tensile loads, the level of fibre crimp must determine the deformation at which it becomes critically stressed [252].

Collectively these studies are in agreement with our findings which suggest a structurally mediated modification of tendons to HFLM mechanical loading.

Some authors have reported an initially increased stiffness in fatigue damaged connective tissues. This has been attributed to fibre activation that redistributes loads from fatigue damaged to undamaged fibres during cyclic loading [253]. This may provide an explanation of the results in this chapter that tendons subject to higher frequency loading displayed higher tangent modulus. A higher frequency loading for the same period exposes the tendon to a higher number of cycles and hence a greater amount of fatigue damage may have accumulated.

Fung *et al* 2010 proposed that the population of fibres (fibrils) within tendon have variable lengths and material properties, and that kinks or increased crimp results from stretching a small population of fibres into their plastic zone of deformation. As these fibres become damaged and unloaded the elastically elongated fibres in parallel shorten, allowing increased elongation with subsequent stretching, and this reflects a load sharing mechanism where local stresses are redistributed between tendon fibres [154].

In conclusion, in the system reported in this thesis, observed over a 7 day stimulation period, observations of similar collagen architecture changes with both normal and reduced cell activity suggest that the mechanical property changes following HFLM stimulation are not solely due to cell activity. Collagen is by far the most abundant ECM component in tendons and constitutes almost 90% of the material, and since the mechanical properties of materials are dependent on the structural makeup, changes in collagen architecture are likely reflected in altered mechanical properties.

We have seen no evidence that increased cell activity increases tendon crimp pattern, or indeed that reduced (two fold drop in) cell activity significantly reduces tendon modulus. However, we have found that changes in tendon crimp pattern as a result of HFLM mechanical loading are associated with elevated tendon modulus during normal and low cell activity. These events indicate the participation of a structural induced mechanism for tendons mechanical adaptation to HFLM mechanical loading.

CHAPTER 7 Effect of static, low frequency cyclic, and HFLM mechanical loading conditions on the *in vitro* repair of damaged tendon fascicles

7.1 INTRODUCTION

In chapter 1 the potential for HFLM loading interventions in counteracting bone diseases such as osteoporosis was detailed, and the lack of knowledge on effects of HFLM loading for tendon repair was also highlighted as part of the motivation for this thesis. In this chapter the *in vitro* biochemical and biomechanical response of damaged tendons to various mechanical loading conditions was considered. Tensile tests and metabolic assessments were performed on damaged tendons fascicles as markers for tissue repair following *in vitro* mechanical loading. These assessments were used to compare three loading conditions static, low frequency (1Hz) and HFLM (20Hz) cyclic loading and provide an indication of the most favourable mechanical loading environment for *in vitro* tendon repair.

7.1.1 *In vitro* intrinsic tendon repair

In chapter 2 concepts of extrinsic and intrinsic modes of healing were introduced. Extrinsic healing is dependent on the permeation of inflammatory cells and fibroblasts from outside the tendon and through the formation of internal scar tissue. The paradigm that tendons have the capacity to display an intrinsic repair response, that is, repair without the participation of inflammatory (vascular and synovial) cell sources subsequent to damage has been the subject of *in vitro* experimental studies from-numerous laboratories [254-257], and more recently for *in vitro* suture repairs [258, 259] . Early support for this concept is based largely on histological and immunohistologic examination of damaged partially severed flexor tendons from humans [260-262] and several animal studies [263, 264] and relies on the proliferation, migration and collagen synthesis of active fibroblasts within the tissue and remote from the injury site [265, 266]. Experimental studies on intrinsic tendon repair have focused mainly on using tendons from selected animals and anatomical regions including digital flexor

tendons from the hands and feet of primates (monkeys) [256, 267], digital flexor tendons from the forepaws and toes of white rabbits[254, 257, 263], digital flexor tendons from the feet of Leghorn chickens[254, 268] canine (dog) forepaws[254, 255], indicating the capacity of specific animals and tendon types to display an intrinsic repair response *in vitro.*

The ability of rat tendons to display a similar repair response has also been recognised in literature. Oshiro *et al* 2003 used an *in vivo* rat tendon laceration and repair model to investigate the mechanisms for tendon healing and found that newly formed collagen fibres crossed the repair site by day 28 and gene expression of type I collagen decreased initially and then returned gradually to the initial level by day 28, highlighting the rapid repair response of rat tendons and their potential (suitability) for performing studies on intrinsic tendon repair *in vitro* [269].

Inherent to the development of an *in vitro* repair tissue model is the requirement of the optimal culture environment. Vitamin C, also known as ascorbic acid, is required for the synthesis of collagen, and so its supply at levels much higher than in standard culture medium is critical [257].

7.1.2 *In vitro* injury models for tendon repair

A variety of tissue engineering and surgical techniques have been used to simulate tendon injuries which occur *in vivo*, and these have all been established with the common aim of facilitating full tissue reparative evaluation. Classic *in vitro* techniques for creation of tissue damage involve performing transverse laceration across the midsection of tendon segments [255, 267, 270, 271], and this is believed to mimic partial ruptures of varying severity within tendons *in vivo*. Graham *et al* 1984 used an injury model in which defined portions of tendon explants (plugs) were used to study the intrinsic healing capacity of the tissue. The explants contained only inner tendon bundles (fascicles) with endotenon, and fibroblast proliferation

and migration was assessed in the severed region of the explants [268]. Finally, repetitive loading cycles resulting in the accumulation of micro-tears is a technique that has also been used to simulate tendon overuse injuries *in vitro* [272].

7.1.3 Markers for *in vitro* tendon repair

There are differences between the *in vitro* and *in vivo* characteristics of tendon repair. For instance tissue culture *in vitro* repair experiments do not display common inflammatory effects of *in vivo* healing and repair and hence are ideal for studying intrinsic repair.

Therefore, to monitor *in vitro* repair responses in damaged tendons it is essential to measure parameters of the damage area (injury site) to provide a direct indication of the intrinsic healing progression. Investigators have used biological and biochemical assays, in addition to morphological, and biomechanical parameters as markers for tendon healing. These have ranged from measurements of cell proliferation, migration, and metabolism, to quantification of levels of DNA, proteoglycan, fibronectin, and collagen synthesis [254, 267, 273, 274]. Morphologically, cellularity, connective tissue thickening, and laceration site closure have been used as markers for tissue repair [254, 270]. Finally, biomechanical testing for tissue stiffness and tensile strength (breaking force) has provided early indication for the presence or absence of tendons repair response [259, 275].

Investigators have also used a combination of markers. For instance in a study by Morizaki *et al* 2010., tendon viability and biomechanical testing was performed to evaluate *in vitro* repair in flexor tendons following application of surgical sutures with a Bone Marrow Stromal cell-seeded collagen patch at the repair site [276].

7.1.4 Mechanical loading conditions for tendon repair

Both animal and human studies have suggested the importance of mechanical stimulation for encouraging tendon repair [80, 277, 278]. Furthermore, considerable interest around the intrinsic healing capacity of tendons has led to further studies involving *in vitro* stimulation of tendon repair by static and low frequency (<4Hz) cyclic mechanical loading conditions [270, 279]. These studies have provided a theoretical foundation that sufficient dosages of static and low frequency mechanical loading may be beneficial for tendon repair, but have yet to examine the potential of high frequency low magnitude loads for tendon repair.

7.1.5 Static loading for tendon repair

Studies have reported inconsistent findings for the effect of static loads on the intrinsic repair response of tendons [277, 279, 280]. Wray *et al.,* 1981 found that application of *in vivo* static loads produced by continuous loading from 0.1-1N for 48 hours on chicken flexor tendons had no significant effect on the motion or the stiffness of the damage (scar) site formed after repair by surgical graft [281].

However, Mass *et al.,* 1993 demonstrated that constant *in vitro* static loading with 3.1g weights (approximately 0.03N) on partially transected rabbit flexor tendons significantly increased true maximum stress, the energy required for failure, and the tangent modulus when compared to un-incubated unloaded controls after six weeks [279]. These conflicting results highlight the need for further consideration and studies on static *in vitro* loading for tendon repair.

7.1.6 Low frequency Cyclic loading for tendon repair

Low frequency cyclic loading (frequencies less than 4Hz) have also been shown to play a role in the repair response of damaged tendons, with biochemical and biomechanical effects.

Kubota *et al* 1996 used an *in vivo* animal model to study the effect of motion and tension on injured tendons. They demonstrated that 4 weeks of free cage walking activity subsequent to partial laceration of flexor profundus tendon of leghorn chickens resulted in a significant increase in breaking strength and cellular activity compared with controls receiving no motion or tension. This occurred following the use of restrictive casts and application of proximal tenotomies to flexor tendons [277].

In an *in vitro* study by Tanaka *et al*., 1995 segments of partially lacerated chicken flexor tendons in culture media were subjected to cyclic loads of 2cycles/min (0.033Hz). Subsequent to a culture period of 21 days immunohistological analysis revealed that cyclic stimulation of lacerated tendons at 0.03Hz significantly enhanced proliferation and migration of fibroblasts when compared with lacerated unloaded controls. Laceration sites in the cyclic tension group were completely bridged by bundles of thickened spindle-shaped fibroblasts 10-20 cells thick while in the no tension group the laceration site was covered by round fibroblasts in a layer 5-6 cells thick.[270].

The aim of the study presented in this chapter was to investigate the effects of various mechanical loading conditions for *in vitro* tendon repair. To achieve this, an *in vitro* damage model of tendon was evaluated. Biomechanical and biochemical parameters of tendon modulus and tissue metabolism were used as markers for *in vitro* tendon repair. Damaged tendons receiving either, static, low frequency, or high frequency mechanical loading were evaluated.

The hypothesis tested was that *in vitro* tendon repair as indicated by biomechanical and biochemical parameters of tendon modulus and tissue metabolism would be maximised in damaged tendons receiving HFLM cyclic loading, compared with static and low frequency cyclic mechanical loading conditions.

7.2 MATERIALS AND METHODS

7.2.1 Study Design

Study 4 consisted of two experiments and included three validation tests on an *invitro* injury model first proposed by Nasim Zargar Baboldashti (DPhil thesis, 2011). This model uses a microlance 23 gauge insulin needle for application of 1cm scratch across the longitudinal section of a tendon fascicle. The breakdown of experiments performed is shown in figure 7.1

15 tendon fascicles from 4 rats were used to perform validation tests. During the validation tests, each tendon fascicle was dissected into two segments with one segment serving as part of the unloaded damaged group while the other segment serving as part of the unloaded undamaged tendon group. Three validation tests were performed to assess the effect of *in vitro* damage on tissue viability, tendon modulus and tendon metabolism and to answer five separate questions;

1. Can a consistent scratch length be applied onto RTTFs?
2. What is the effect of scratch damage on the collagen architecture of RTTFs?
3. Do damaged RTTFs remain viable subsequent to *in vitro* culture?
4. Does introduction of *in vitro* scratch damage significantly decrease tendon modulus?
5. Does introduction of *in vitro* scratch damage significantly decrease tendon metabolism?

For the main study 96 tendon fascicles (out of 118 fascicles) from the tails of 7 male Sprague Dawley rats (aged 3–5 months), were harvested and cultured within 2 hours of sacrifice, as described in chapter 3 of this thesis. All fascicles serving as fresh (F), and fresh damaged (FD) controls were analysed immediately after harvesting. Cultured fascicles were separated into 5 groups, unloaded (U) v unloaded damaged (UD), static damaged (SD) low frequency cyclic loaded damaged (LF-D), and HFLM damaged (HFLM-D) loaded damaged fascicles.

Mechanically loaded tendon fascicles were stimulated for 1h/day, with 5 days of loading and 2 days of no loading during culture.

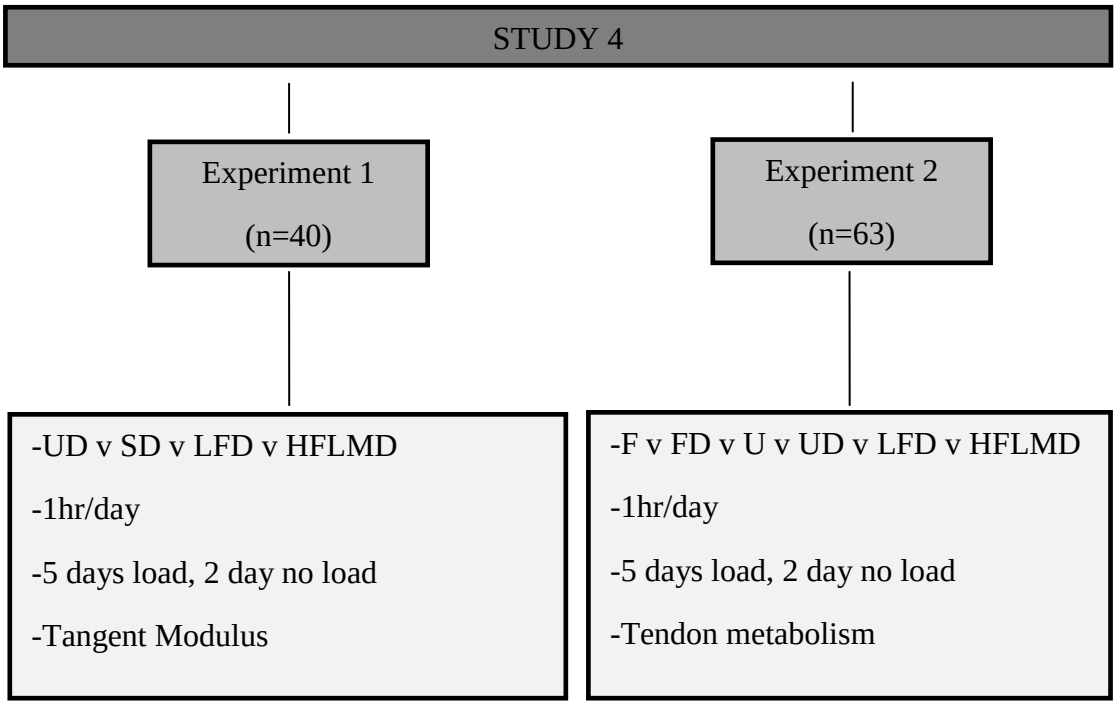


Figure 7.1: Experimental set up for study 4. The study consisted of two experiments used to examine two research questions. Which mechanical loading condition provides strongest stimulation for *in vitro* tendon repair as measured by differences in 1.) tangent modulus; and 2.) tissue metabolism.

In experiment 1; thirty two tendon fascicles (from a starting population of 40 RTTFs) (2 rats), were damaged using the described scratch method (2 x1cm scratches), and cultured in DMEM immediately after application of a scratch to investigate the effects of various mechanical loading conditions on the modulus of damaged tendons. Tangent modulus of damaged RTTFs receiving no load, was compared with those receiving static load, 1Hz cyclic load and 20Hz cyclic load.

In experiment 2; forty-nine tendon fascicles (from a starting population of 63 RTTFs) (2 rats) were used to investigate the effects of various mechanical loading conditions on the metabolism of damaged tendons. Assessments of tissue metabolism in damaged RTTFs receiving no load, static load, 1Hz cyclic load and 20Hz cyclic load was performed by supplementing fascicles with Alamar blue reagent (using a 1:10 [v/v] mixture of the dye reagent and culture media). Following culture and an incubation period of approximately 3 hours with the dye, the REDOX state of Alamar blue was measured using a Fluostar Optima spectrofluorometer and this was used to record the relative fluorescence unit (RFU), an indication of the tissues relative health.

7.3 RESULTS

7.3.1 Characterisation of damage model for *in vitro* tendon repair

SHG images revealed that a scratch length of 0.77±0.6cm was consistently achieved in harvested RTTFs using the described method. Scratch lengths were measured using the Ortho function on Zen 2009 light edition software from a population of 3 RTTFs.

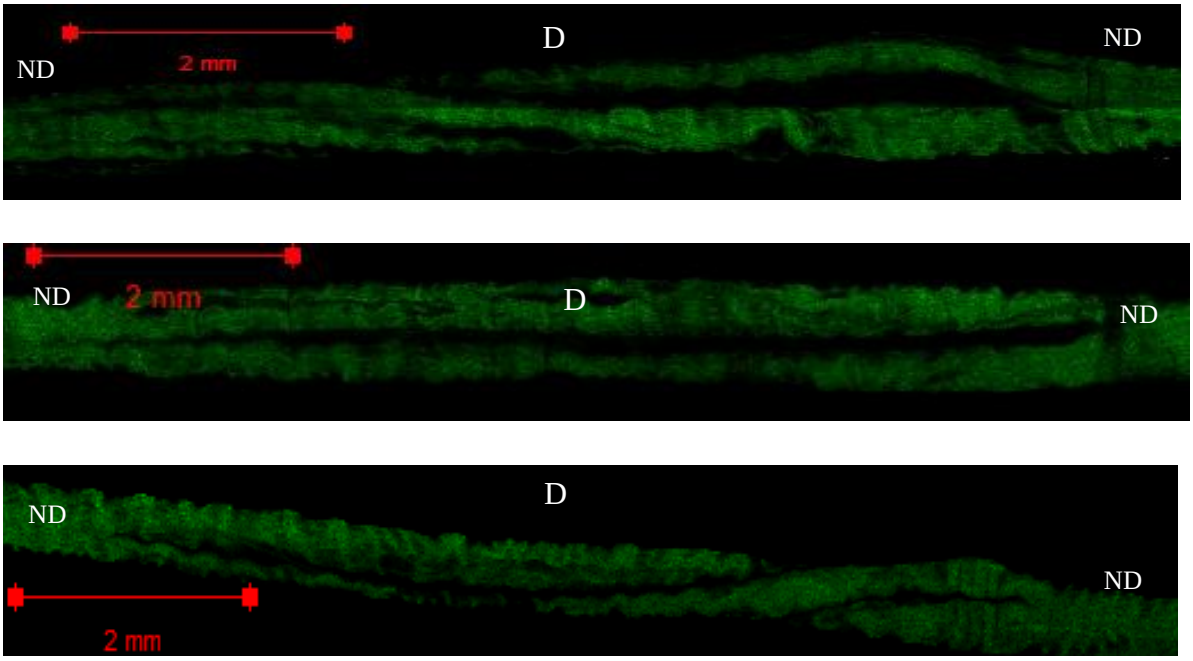


Figure 7.2: Second Harmonic Generation images of three tendon fascicles after three days of application of a scratch damage and *in vitro* culture. Scale bars are shown on the right hand side. In the image 'D' denotes a damaged region, while 'ND' denotes non-damaged regions.

Tightly packed collagen fibrils showing parallel alignment and crimp pattern were observed in the non-damaged regions of cultured tendon fascicles. In contrast damaged regions displayed loose disorganised fibres with disruption of parallel fibril alignment and interruption of crimp organisation as can be seen in figure 7.2.

7.3.2 Viability of cultured damaged tendons

In order to verify that damaged tendon fascicles being assessed were viable and capable of

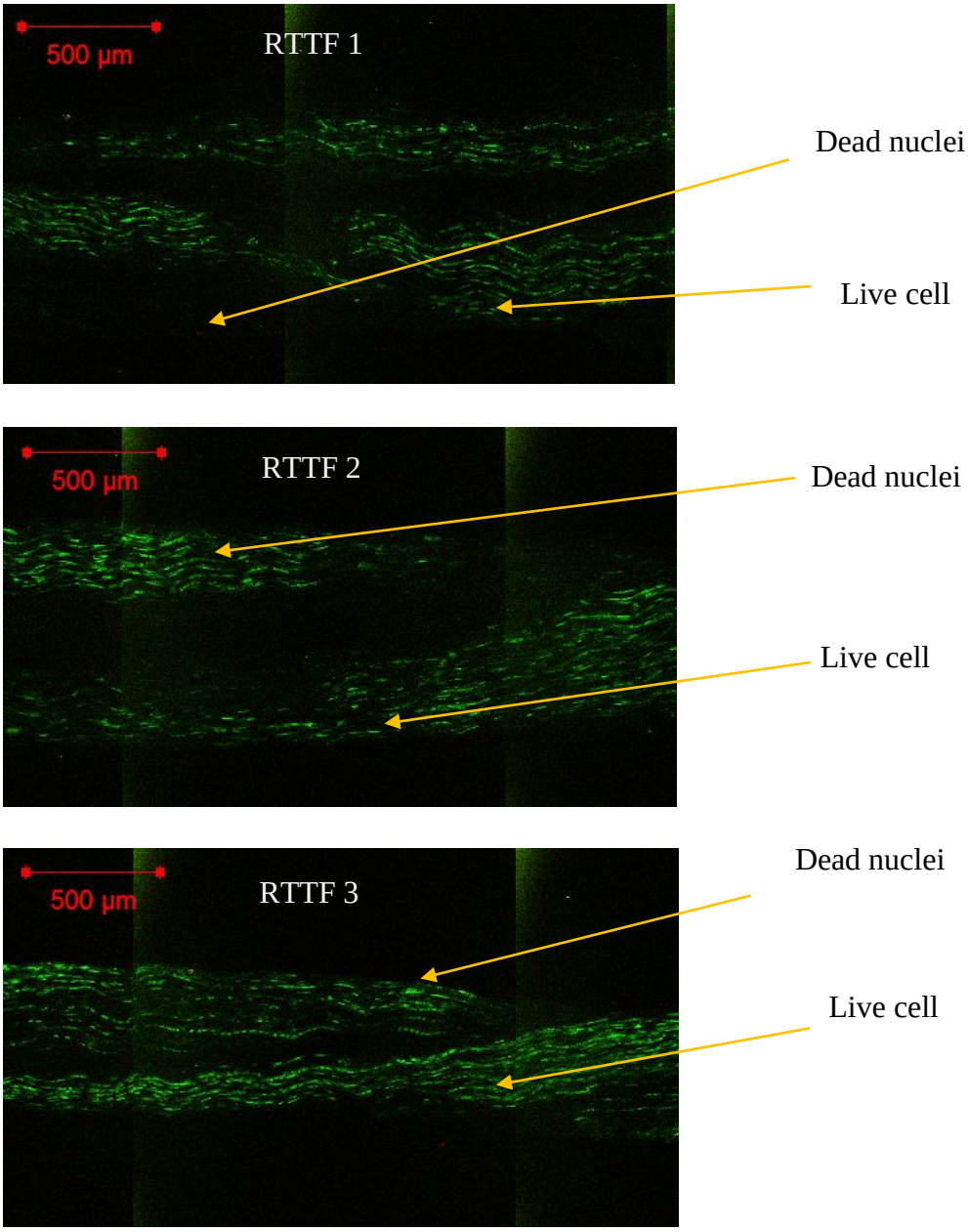


Figure 7.3: Live-dead confocal microscopy images (maximum intensity projection) of three damaged tendon fascicles after 3 days of *in vitro* culture showing live cells (green elliptical features) and dead nuclei (red spherical dots). These images are sub sections extracted from regions in the complete RTTF indicated in figure 7.2

expressing biochemical and biomechanical responses *in vitro*, a live and dead staining assay was performed after application of a scratch damage, on three RTTFs subsequent to 3 days of *in vitro* culture. Tendon fascicles were confirmed to be viable after application of a scratch injury and 3 days of *in vitro* culture (Figure 7.3). Qualitative visual observation by confocal

microscopy and Zen software techniques revealed a similar number of live and dead cells within the damaged region, demonstrating the ability of cultured tendon fascicles to maintain tissue viability 3 days after application of a scratch damage.

7.3.3 Effect of scratch damage on tendon modulus

Four tendon fascicles were used to test the effects of damage on tendon modulus. Each fascicle was dissected into 2 segments, one tendon segment received a scratch injury and the other segments remained undamaged, allowing for paired experimental analysis. Application of a scratch injury along the longitudinal length of unloaded tendon segments resulted in a two-fold significant decrease in the tangent modulus ($p < 0.05$, Student's t-test), when compared with undamaged unloaded controls of the same fascicle after 7 days of culture, figure 7.4. This highlights the severity of the applied damage.

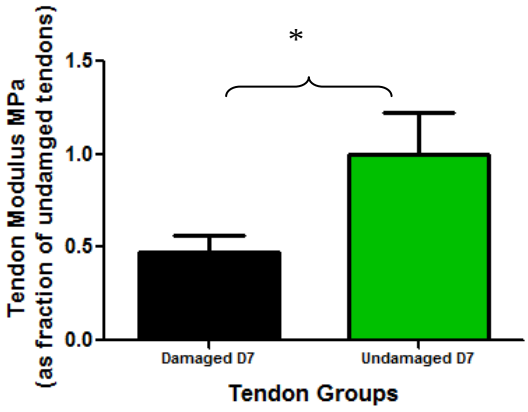


Figure 7.4: Mechanical validation of the scratch injury model showing tangent modulus in damaged and undamaged tendons after 7 days of culture under conditions of no load. Four fascicles were assessed in each tendon group. Values shown are the mean and the standard deviation, expressed a fraction of the undamaged unloaded tendon. * denotes a significant difference with undamaged tendons (Student's t-test)

7.3.4 Effect of scratch damage on tendon metabolic activity

Eight fascicles were used to test the effect of damage on tendon metabolism, (four fascicles for each time point). Each fascicle was dissected into 2 segments, one tendon segment received a scratch injury and the other segments remained undamaged, allowing for paired experimental analysis.

Application of scratch damage resulted in a significant drop in the metabolic activity of tendon segments after 3 and 7 days of *in vitro* culture when compared with unloaded undamaged tendons (figure 7.5) ($p < 0.01$ and 0.05 respectively, Student's t-test).

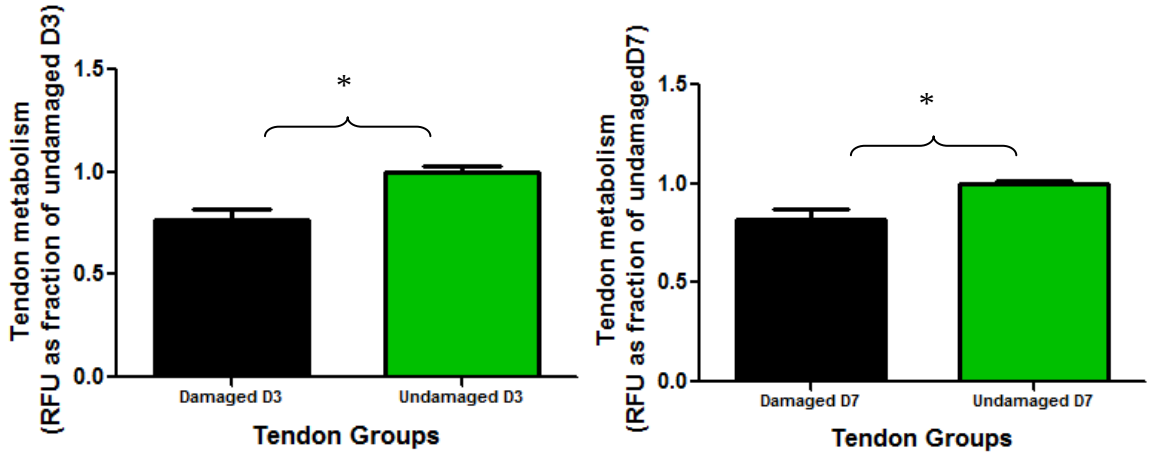


Figure 7.5: Biological validation of scratch injury model showing metabolic activity in damaged and undamaged tendons after A.) day 3 and B.) day 7 of culture under conditions of no load. Four RTTFs were used for assessment at each time point and mean relative fluorescence units (RFUs) for each group are expressed as fractions of undamaged tendon groups at each time point. * denotes a significant difference with undamaged tendons (Student's t-test)

Between day 3 and day 7 of culture, there was no significant change in the metabolism of damaged tendon fascicles. However, at day 7 of culture tendon metabolism (RFU) was 14% higher in damaged tendon segments than after 3 days of culture.

7.3.5 Effect of loading conditions on the modulus of damaged tendons

Tensile tests were performed on damaged tendon fascicles receiving static, 1Hz low frequency cyclic, 20Hz HFLM cyclic and tendons receiving no mechanical loading after 7 days of culture. Stress-strain data was smoothed using a 50 point moving average filter and the tangent modulus was calculated as a function of strain to estimate the maximum modulus for each fascicle. No preconditioning tests were performed prior to testing until failure.

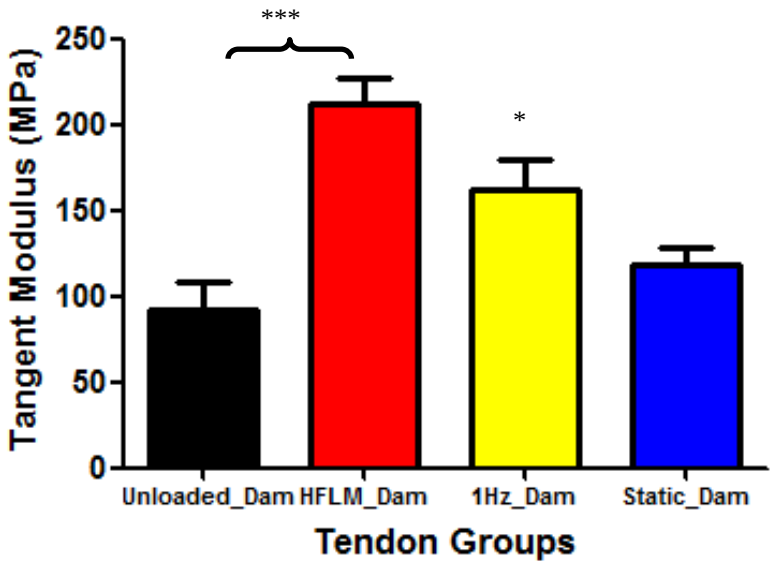


Figure 7.6: Mean tangent modulus in damaged tendon groups under various mechanical loading conditions. * ($p < 0.05$) and *** ($p<0.0001$) denotes a significant difference between unloaded damaged tendon groups. The bars shown represent the standard deviation

When varying mechanical loading conditions, damaged tendons fascicles receiving HFLM mechanical loading displayed significantly higher modulus than unloaded damaged tendons ($p<0.0001$), and damaged tendons receiving static loading ($p<0.0005$). Figure 7.6 Damaged tendons receiving low frequency cyclic loading (1Hz) also displayed significantly higher modulus than unloaded undamaged tendons, but to a lesser extent than HFLM loaded tendons ($p<0.05$). Amongst the mechanically loaded tendon groups, damaged fascicles receiving static loading displayed the lowest modulus compared with unloaded damaged tendons, and no significant difference were found between the two groups.

7.3.6 Effect of loading conditions on the metabolism of damaged tendons

Metabolic activity was assessed in damaged tendon fascicles receiving static, 1Hz low frequency cyclic, 20Hz HFLM cyclic and no mechanical loading after 7 days of culture. Fresh, unloaded (non-damaged) and fresh damaged tendons were also examined and served as respective control groups. Amongst mechanically loaded damaged groups, only tendons stimulated at 20Hz showed significantly higher metabolism than unloaded damaged tendons ($p<0.05$, ANOVA). (Figure 7.7).

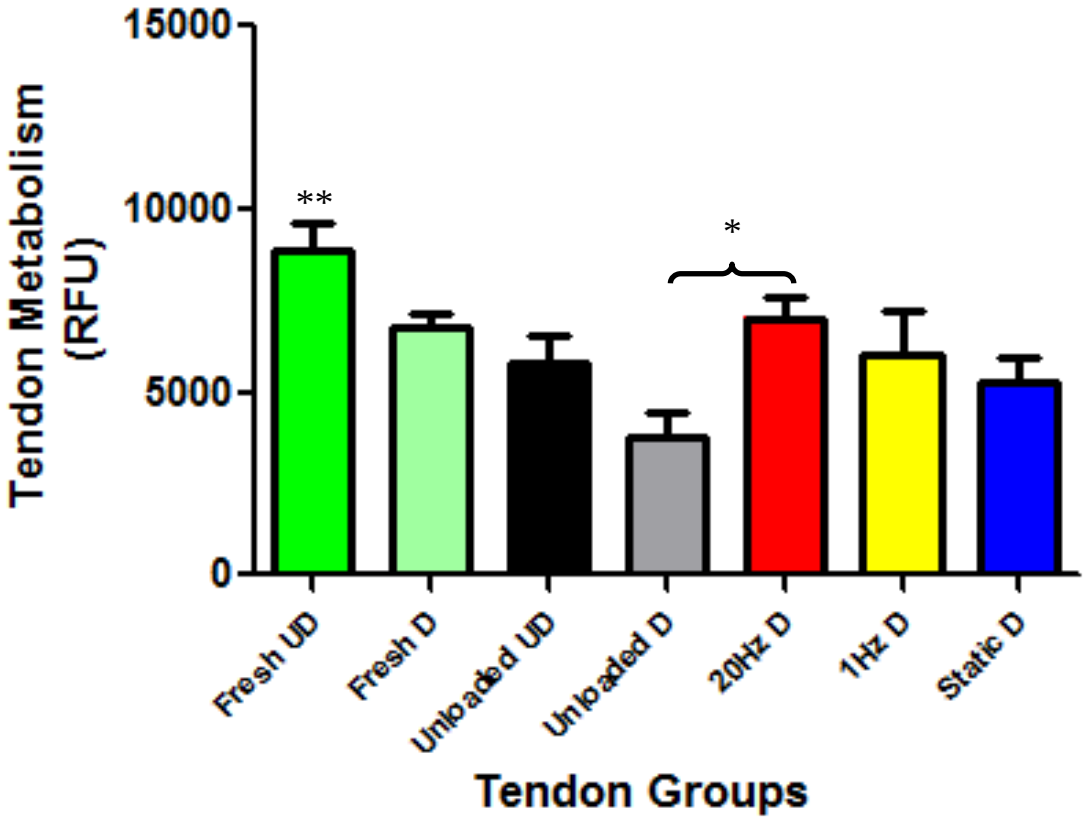


Figure 7.7: Metabolism of damaged RTTF groups under static, low frequency cyclic, and HFLM mechanical loading conditions; with fresh damaged, fresh non-damaged and unloaded non-damaged RTTFs serving as complimentary controls. *Denotes a significant difference with unloaded damaged tendons.

Damaged tendon fascicles loaded at 1Hz displayed higher metabolism than those receiving static loading, and static loaded tendons displayed the lowest metabolism amongst mechanically loaded and damaged groups

As expected fresh non-damaged tendons demonstrated highest metabolism and tendons receiving a scratch injury displayed noticeably lower metabolism than in fresh non-damaged tendons.

Unloaded damaged tendons displayed lowest tissue metabolism, and metabolic activity was marginally higher in Fresh-Damaged tendon groups than in Unloaded-Undamaged tendons.

7.4 DISCUSSION

The role of mechanical forces in regulating tissue healing and repair has been recognised for over a century [76]. Moreover musculoskeletal tissues such as tendons respond in a variety of ways to different types of mechanical loading conditions, and strategies for stimulating tendon repair through the delivery of mechanical loading interventions, has attracted considerable interest [80, 277, 278].

We have previously shown that HFLM loading interventions maintain the properties of healthy tendons [157], however no previous experimental or clinical studies have compared the effect of static, low frequency cyclic and HFLM loading conditions for the repair of damaged tendons. In this study we examined the potential of three mechanical loading conditions for *in vitro* tendon repair and HFLM cyclic loading was found to provide the strongest stimulation as indicated by measurements of tendon metabolism and tendon modulus. Stimulation at 20 Hz maintained both modulus and metabolic activity in 7 day cultured tendons at similar levels to tendons immediately following damage. However, there was no evidence of actual repair as the modulus of cultured stimulated tendons did not

increase above freshly damaged levels. While these results do not show that an *in vitro* repair process is stimulated by HFLM, they repeat the observation in previous work in this thesis that HFLM loading can maintain the properties of cultured tendons.

Tendon modulus and tendon metabolism were used as markers for *in vitro* tendon repair, and these markers have shown effects during *in vivo* animal studies which have used mechanically induced defects to investigate tendon repair [282]. Preliminary tests were performed, in order to validate the *in vitro* damage model used and the suitability of experimental parameters used as markers for *in vitro* tendon repair. This was necessary since there are differences in the *in vitro* and *in vivo* characteristics of tendon repair.

Validation tests revealed that cultured RTTFs remained viable 3 days after application of biomechanical and biochemically relevant scratch damage, and consistent scratch lengths of approximately 0.7cm were achieved in individual fascicles despite an initial target scratch length of 1cm. This may be due to shrinkage as a result of the scratch itself or gap closure due to cross-linking of previously disconnected collagen fibrils to the tendon fascicle by GAGs, which are associated with the bridging of adjacent collagen molecules [283]. Validation tests also revealed that application of 2 x 0.77± 0.6cm scratches along the length of tendon fascicles significantly reduced tangent modulus from186.6MPa to 86.4MPa. These results are in agreement with *in vitro* studies on lacerated flexor tendons which show that the structural properties of tendons are increasingly unfavourably affected as laceration size increases, with significant decreases occurring in stiffness with increasing degree of partial laceration [255].

Although matrix morphology was not identical in damaged tendon fascicles damaged regions in all RTTFs presented disordered collagen arrangement with disruption of parallel fibril alignment and interruption of crimp organisation.

This lack of standardisation reflects to some extent the variability of rupture injuries in the living organism, although of course the mechanism for injury may be quite different. However, despite this variable morphology, the effects of the scratch on mechanical property and tissue metabolism are consistent, with standard deviations of 17.5% and 9.3% respectively after 7 days of damage and tissue culture.

Tendon metabolism (RFU) was 14% higher in damaged tendon segments after 7 days than after 3 days of culture, and although differences were not statistically significant the results indicate that rat tail tendons may possess an intrinsic capacity for healing in the absence of inflammatory or vascular cells. These results also imply that metabolic activity of damaged tendon cells increases with culture time and this may be due to an increase in proliferation of tendon cells with culture time.

Additionally, the percentage difference in tendon metabolism (RFU) between damaged and undamaged tendons was reduced from 30.5% at day 3 to 21.8% by day 7 of culture. This suggests that cellular activity peaks early on in this damage model and declines thereafter, supporting paradigms of intrinsic healing in RTTFs. This seems feasible since in all validation tests performed treated tendons receiving a scratch damage and control tendons, were derived from the same tendon fascicle in order to minimise the effects of confounding variables and increase the statistical strength of the experimental observations.

To date, there have been no studies confirming intrinsic healing responses in rat tail tendon fascicles, however, studies on tendon healing in the rat have highlighted the suitability of using rat tendons from various anatomical regions as a model for expanding our knowledge and understanding of tendon repair [269, 284, 285], and these studies have used tissue stiffness, histological analysis and measurements of gene expression as markers for tendon repair.

Static, low frequency (LF) cyclic loaded and HFLM loaded damaged tendon groups displayed higher tangent modulus than unloaded damaged tendons after 7 days in culture. Only LF and HFLM cyclic loaded damaged tendons displayed significant differences with unloaded damaged tendons. These results indicate that static loads offer the least stimulation for tendon maintenance or perhaps present an overstimulation effect in damaged tissue. Further the higher modulus found in tendons stimulated at higher frequencies, mirrors findings on healthy tendon fascicles detailed in chapter 5 of this thesis.

In an *in vivo* study, Takai *et al* 1991 investigated the effects of low (0.0167Hz) and higher (0.2Hz) frequency mechanical loads on the healing of flexor tendons. Flexor tendons of adult mongrel dogs were initially lacerated and following repair were mechanically loaded *in vivo* using a controlled passive motion device. They showed that tensile properties (stiffness and ultimate load) were significantly improved in lacerated flexor tendons receiving higher frequency mechanical loads. They concluded that the frequency of controlled motion rehabilitation is a significant factor in accelerating the healing response of injured tendons [286].

Numerous *in vitro* and *in vivo* studies have monitored the biomechanics of healing in damaged tendons, revealing that cyclic and static mechanical loading increases the tensile properties of the repair site with noticeable changes in as little as 10 days [258, 275, 277, 287]. An experimental period of 7 days was chosen to monitor the mechanical and biochemical response of damaged tendon to *in vitro* cyclic loading.

Procollagen synthetic activity has been reported to occur after 3 days of *in vitro* cyclic tension in lacerated flexor tendons, with proliferation of new fibroblasts occurring within 7 days of culture at the laceration site [270].

Furthermore, it has been suggested that adaptation to the presence and the absence of mechanical loading is far more rapid in damaged tendons than in healthy tendons with one study showing effects after 14 days of mechanical loading to surgically damaged Achilles tendon of Sprague Dawley rats [20] . Similar effects have been demonstrated in a study where mobilisation after damage in rat tendons resulted in an increased rate of collagen biosynthesis after only 3 days of remobilization from a 7-day cast immobilization [287]. Seven days therefore seemed to be an adequate time period to monitor *in vitro* repair responses in tendon fascicles subsequent to mechanically induced damage.

Examination of tendon metabolism in damaged fascicles subsequent to various mechanical loading conditions revealed a cyclic loading frequency dependency on damage site metabolic activity as was demonstrated in experiments on tendon modulus, and only RTTFs stimulated at 20Hz showed significantly higher metabolism than unloaded damaged tendons.

Static loaded tendons displayed the lowest metabolism amongst mechanically loaded damaged tendon groups (although displaying higher metabolism than unloaded damaged tendons), while damaged tendons loaded at Low frequency (1Hz) displayed a trend to higher metabolism than unloaded damaged and static loaded damaged RTTFs.

Tanaka *et al* 1995 investigated the effects of low frequency (0.03Hz) cyclic tension on the *in vitro* healing of chicken flexor tendons. They found that cyclic tension enhanced collagen synthesis, and proliferation and migration of fibroblasts [270], and these results are in agreement to our findings on metabolism.

The result that fresh undamaged tendons displayed the highest metabolism in all tendon groups is unexpected, since no repair process is taking place in these specimens and all other tendons show lower metabolic activity. However, a remarkable outcome was the comparable

metabolism observed in fresh damaged tendons and damaged tendons stimulated with HFLM mechanical loads.

Further assessments would be needed to definitively confirm tissue recovery and heightened reparative effects of HFLM mechanical stimulation on damaged tendons. This would include observation of reduced metabolism subsequent to damage and culture of an individual tendon fascicle, followed by an incremental rise in tissue metabolism within the same fascicle.

A limitation of this study is that accurate and repeatable measures of tendon cell metabolism within tissue cultures using the Alamar blue assay are difficult to achieve over a several day time course, and this prevented any attempts at performing tests on the recovery of tissue metabolism in damaged tendons. Similar *in vivo* and *in vitro* studies have reported significant improvements in tendon metabolism after stimulation by chemical treatment methods such as platelet rich plasma (PRP) and supplementation of cultures with ascorbic acid [257, 288]. We have shown positive and significant effects of mechanical stimulation with HFLM mechanical loading on tendon metabolism.

It is unknown whether the observed differences in metabolism between damaged tendon groups were caused by the presence of a higher number of cells (cellular proliferation and migration to the damage site), or the presence of higher metabolic activity within the same number of cells. Nonetheless, it seems appropriate to attribute either or both of these events to the heightened stimulatory effects of HFLM mechanical loading, and to a lesser extent LF and static mechanical loading.

The mechanisms by which high frequency low magnitude (HFLM) mechanical loading significantly improved the maintenance of the modulus and the metabolism characteristics in damaged tendons is beyond the scope of this study, however, this seems in part a question of how cells and tissue matrix interact and influence one another following mechanical

perturbations, a question that has been central to cell and developmental biologists for many years [289]. Moreover, although the complete repair process in damaged tendons may last between several weeks to several months, synthesis of new collagen material is believed to begin within days of wound healing in tendons [33]. Further, evidence demonstrating collagen synthesis within 7 days of *in vitro* mechanical stimulation of tendon fascicles [4], suggests that significant differences observed in tissue metabolism between HFLM loaded and unloaded damaged tendons within the relatively short time period of 7 days used in this study may be indicative of early biological processes contributing towards the full reparative events within damage tendons.

In conclusion, we have shown that mechanical cues interact with the biological and biomechanical progression of tendon maintenance, and while static and low frequency (1Hz) cyclic loading of damaged tendons provide moderate stimulatory effects in damaged tissue, HFLM mechanical loading conditions have demonstrated the most superior stimulation effects for firstly, preventing deterioration of mechanical properties and secondly for protection against biological deterioration of damage tendons as indicated by measurements of tendon modulus and tendon metabolism. These results tentatively support clinical application of appropriate dosages of HFLM mechanical loading for accelerating and improving tendon repair.

CHAPTER 8 Final Discussion

8.1 OVERVIEW

Chapter 8 reviews the main findings of this DPhil research project. The scientific and clinical motivation for the current work is initially outlined, and this is followed by a summary of the main outcomes presented in each of the study chapters. Important and practical implications of the major findings are also highlighted. Finally, limitations of the experimental approaches used to examine the research questions are discussed, along with potential avenues for future work.

8.2 BACKROUND

Tendon injuries are ubiquitous and clinically, they present a challenge to orthopaedic surgeons as they account for up to 50% of all sporting injuries and reported occupational ailments [1]. In the US approximately 200,000 patients undergo tendon or ligament repair each year [2]. Moreover, tendons heal slowly due to a poor blood supply and rarely attain the structural integrity and mechanical properties of normal uninjured tendons [33].

A number of different strategies for combating tendon injuries, involving ultrasound, extracorporeal shock wave therapy (ESW), Pulsed electro-magnetic field therapy (PEMF) therapy and stimulation by mechanical loading have been used in the management of tendinopathy and tendon ruptures [94, 100, 105, 110], however these have shown limited and inconsistent success. The quest for a long term successful treatment solution therefore still remains the focus of intense research.

Therapies based on mechanical stimuli have shown promise in accelerating bone healing and in treating osteoporosis [173, 290]. However, before similar approaches can be successful in tendons, the lack of basic scientific understanding of tendon response to mechanical load needs to be addressed.

Given the difficulty in determining direct relationships between applied forces and tissue adaptation in living animals and humans, the controlled environments available in *in vitro* systems offer an attractive option for monitoring tissue and cell response to mechanical stimuli in a highly controlled and repeatable way.

The work in this thesis contributes to the current understanding of tendon behaviour at the tissue level of organisation, the mechanical signals that can influence matrix remodelling and tissue repair in healthy and damaged tendons, and possible mechanisms which may govern tendon mechanical adaptation. The current work has been made possible by the development of a custom *in vitro* loading system (IVLS), designed for studying the influence of mechanical stimuli on tendon mechanical and biochemical properties.

8.3 RESEARCH QUESTIONS

The aim of this thesis was to investigate the mechanobiology of tendons and the potential of HFLM loading for tendon repair and remodelling using a custom built IVLS. Categorically, this was achieved by conducting four studies in healthy and damaged tendons.

1. Studies in healthy tendon were used to assess the effect of static and HFLM loads on the GAG content and mechanical properties of tendon fascicles.
2. Optimum *in vitro* HFLM loading parameters were also examined in order to determine which loading frequencies and dosage periods provide strongest stimulation for tendon remodelling, as indicated by measures of tangent modulus and GAG content in healthy tendon fascicles.
3. To investigate the mechanisms for tendons mechanical adaptation to mechanical loading it was asked whether observed differences in the mechanical properties of

tendons are induced by cell activity or structural mechanisms in tendon matrix, as indicated by observations in crimp behaviour.

4. Studies with damaged tendons were used to consider which mechanical loading conditions are most effective for encouraging tendon repair as indicated by measures of tendon metabolism and tendon modulus in damaged RTTFs

A major outcome reported in this thesis has been the development of a novel IVLS for static, Low Frequency Cyclic and HFLM loading of cultured tendons fascicles, details of which are highlighted in chapter 4.

The system was validated through magnetic flux, load cell and viability measurements. Furthermore it was shown that tissue viability was maintained for 7 days under static, low frequency cyclic and HFLM loading conditions and the proportion of live cells in tendon fascicles exposed to the in vitro loading environment did not decrease over a 7-day period when compared to fresh controls.

The newly developed system presented in the thesis was used to examine the biochemical and biomechanical response of healthy cultured tendon fascicles to HFLM loading for the first time ever in an *in vitro* system.

Application of the 20Hz HFLM cyclic loading resulted in a statistically significant two-fold difference in tangent modulus compared to unloaded groups after 7 days ($p \leq 0.05$). Further HFLM loaded tendon fascicles showed higher tensile strength than unloaded and fresh fascicles, although differences between groups were not statistically significant.

Static loaded rat tail tendon fascicles (RTTFs) showed a trend towards higher maximum modulus values than unloaded, while unloaded RTTFs showed significantly lower modulus

than fresh control tendons. These results are consistent with several studies of cultured tendon fascicles in which increasing magnitude and frequency of applied load were shown to increasingly inhibit the expression of MMP1, a matrix digesting enzyme [11, 159, 167, 184].

The significant trends observed in tendon modulus with static loading mirror the non-significant trends to differences in their biochemical properties (GAG content), an association that has been seen in previous in vitro RTTF experiments [159].

Together, these data provide evidence that the developed system provides a novel platform for investigating the effects of HFLM loading on tendon mechanobiology and that HFLM loading of tendons has potential as an anti-catabolic stimulation therapy.

It was also demonstrated that the biomechanical benefits of HFLM stimulation at 20Hz for 1 hr a day is greater than the biochemical benefits presented using the same loading parameters, since HFLM loaded tendons showed noticeably higher tangent modulus but lower GAG contents than fresh tendons. This result may seem uncharacteristic of tendon behaviour since the biomechanical and biochemical properties are thought to be positively correlated and exclusively interdependent [159].

However, more recently studies have shown that changes in tendon mechanical properties may not always reflect changes in the biochemical (protein) expression, and no correlation was found between tendon modulus and tendon GAG content [42].

Ultimately, elevated biomechanical properties would seem more critical to the role of tendons in transmitting tensile forces than elevated biochemical content and this further highlights our encouraging results which demonstrate increased tangent modulus with HFLM loading. Characterisation tests revealed that the developed IVLS was capable of providing local administration of selective frequencies, dosages, and loading magnitudes onto cultured

tendons by careful modification of the electromagnetic field frequency and amplitude, and this facilitated studies on optimum HFLM *in vitro* loading parameters.

In chapter 5, loading frequencies and dosages were varied over feasible and clinically relevant ranges in order to provide an indication of the most favourable cyclic loading conditions for tendon remodelling using the developed IVLS.

It was demonstrated that HFLM (20Hz) loading dosages of 1 and 2 hours per day produced significantly higher tangent modulus than unloaded cultured tendon fascicles, but loading for 4 hours per day did not. It is well recognised that excessive repetitive loading of tendons induces fatigue damage which may lead to degenerative changes in the tendon-matrix composition and organisation [21, 213, 291], and this may explain the noticeable drop in GAG content observed between 2 and 4 hours of stimulation at 20Hz.

When varying loading frequencies between 1 and 20Hz in culture over a period of 7 days, the average tangent modulus and GAG content of cultured tendon fascicles increased with frequency up to 15Hz. Similar *in vitro* studies have suggested a cyclic loading frequency dependence for the mechanical and biochemical properties of cultured tendon tissue [11, 166, 183]

Ultimately, our findings on modulus indicate that HFLM frequencies of 20Hz and dosage periods of 1 hour a day provide an optimum balance for providing anti-catabolic stimuli in cultured tendons

Observation of tendon metabolism revealed that tendon fascicles cultured at 37^{o}C contained cells with considerably higher metabolic activity than those cultured at 27^{o}C after 1, 3 and 7 days of culture by 70.1, 99.5, and 82.5% respectively. This means cell activity is approximately halved by the drop in temperature from 37°C to 27°C.

A similar pattern in tangent modulus was observed between tendon groups cultured at 27^{o}C as that observed for tendon groups cultured at 37^{o}C indicating that the changes observed in modulus are able to take place during conditions of both low and high cell activity. These observations give rise to the possibility that alternative events other than cell mediated mechanisms are responsible for tendons adaptation to HFLM mechanical loading.

Chapter 6 therefore explored the changes that take place in HFLM stimulated cultured tendons in conditions of low cell activity. It focused on relationships between tendon crimp and tangent modulus in RTTFs for a greater understanding of tendon remodelling process after applications of HFLM mechanical loads.

Tendon remodelling has been described as modification of the extracellular matrix composition and or architecture [159], and it is well understood that the mechanical properties of tendon tissue are largely determined by the constituents and arrangement of its ECM [50, 159, 292]. It follows then, that changes in tendons ECM and mechanical properties are indicative of tissue remodelling [159].

High crimp angle and crimp amplitude were strongly correlated with elevated tangent modulus in tendon fascicles during culture conditions of low and normal cell activity.

The data show that static and lower frequency cyclic loads un-crimp collagen fibres, while HFLM appears to maintain crimp organisation similar to the fresh tendon.

A similar relationship was highlighted by Yamamota et al 2003, who found that tendon fascicles subject to low frequency cyclic loads of 4Hz displayed lower crimp angle (14.2 $\pm$ 4.6^{o}) than fresh controls (15.7$\pm$3.8^{o}) in addition to longer crimp wavelengths (48.7+11.4 vs 47.5+17.3MPa) after a culture period of 1 week.

Evidently, observations of similar collagen architecture changes in our study with both normal and reduced cell activity in healthy tendons suggest that the mechanical property changes following HFLM stimulation of healthy tendons are not due to cell activity, however, they indicate that structural mediated mechanisms caused by mechanically induced perturbations in collagen architecture may be responsible for tendons mechanical adaptation to HFLM mechanical loading.

An interesting result was that HFLM crimp architecture was similar to fresh tendons, yet their mechanical properties were noticeably higher than those of fresh tendons. These observations may also be a reflection of similar effects to that reported in the literature on pre-conditioning where repeated *in vitro* cycles of tendon loading and unloading between 0.1 N and 0.5 N for 10 cycles at a frequency of 0.2260Hz in the supraspinatus of twenty-two Sprague Dawley rats was found to significantly increase the amount of collagen fibre realignment in such as way as to modify the tendons mechanical properties [293].

Increasing the number of *in vitro* load cycles by extending preconditioning times from 30s to 600s during application of repeated stretch cycles at 2% strain in the Achilles tendon of Sprague Dawley rats has also been shown to significantly increase ultimate failure strength. Furthermore, ultimate tensile load was found to be significantly higher in preconditioned tendons than in fresh control (frozen) tendons receiving no cyclic load, and these results mirror our findings which show enhanced mechanical properties in HFLM cyclic loaded tendons compared with fresh tendons, despite the similar crimp pattern observed in both groups [294].

In chapter 7, the potential of HFLM and various mechanical loading conditions for tendon repair was investigated.

The previously described IVLS for HFLM loading (chapter 4) was used in combination with a scratch injury model in rat tail tendon fascicles, and evaluation of metabolic activity and tangent modulus were used to monitor *in vitro* repair responses in damaged tendons.

This work developed a biomechanical and biochemically relevant damage in cultured tendons fascicles, suitable for studies on tendon repair.

Damaged tendon fascicles receiving HFLM mechanical loading showed highest tangent modulus and both LF and HFLM cyclic loaded tendons displayed significantly higher modulus than unloaded damaged tendons.

Static loaded tendons displayed the lowest tangent modulus of all mechanically loaded damaged tendon groups, indicating that continuous static loads may present a severe and possibly damaging stimulus, though not as damaging as unloading, which in a clinical context may represent mechanical immobilisation.

This is the first study to compare effects of static, low frequency cyclic, and HFLM mechanical loading conditions on the modulus of damaged tendons.

Examination of tendon metabolism in damaged fascicles subsequent to various mechanical loading conditions showed that only RTTFs stimulated at 20Hz had significantly higher metabolism than unloaded damaged tendons.

As expected, fresh undamaged tendons displayed the highest metabolism in all tendon groups. Also, damaged tendons stimulated with HFLM mechanical loads displayed higher metabolism than fresh damaged tendons. These observations point towards the heightened ability of HFLM loads in counteracting the effects of tissue degeneration subsequent to mechanically induced damage, and therefore potentially also in stimulating tendon cells towards tissue repair.

It is possible that the observed differences in metabolism between mechanically loaded damaged tendon groups were a result of increased cellular proliferation of tendon cells, however we speculate that mechanical loading of the same number of viable cells in damaged tendon groups stimulated intracellular processes and these events were conductive to higher metabolic activity in the same number of cells.

Although the mechanisms by which HFLM loading prevent the reduction of tangent modulus and increased metabolism in damaged tendons is beyond the scope of this study, it would seem that intrinsic tendon activity of any form is in part a biological process requiring cell activity. We speculate therefore that this result in damaged tendons is not exclusively due to structural mediated changes within the tendon, unlike the processes reported in healthy-undamaged tendons in chapter 6 of this thesis.

Mechanical cues are essential for the biochemical and biomechanical progression of tendon repair and remodelling processes, and while static and low frequency (1Hz) cyclic loading of damaged tendons provide moderate stimulatory effects, HFLM mechanical loading conditions have demonstrated the most superior stimulation for preventing reduction of tangent modulus, and tissue metabolism in damaged tendon fascicles.

8.4 LIMITATIONS OF STUDY

There are several limitations in the approaches used to carry out the experimental studies in this thesis.

Firstly, the developed IVLS was designed to isolate tissue-based mechanobiological effects in tendon and so any vascular or systemic effects due to mechanical loading in living animals were excluded in this model. This means that characteristic healing processes such as angiogenesis cannot be observed.

Furthermore, the developed IVLS was used study the effects of various HFLM loads by controlling electromagnetic field frequencies using an electromagnet and signal generator to apply cyclic perturbations of a magnetic stir bar.

The highest magnetic field frequency and amplitude that could be generated was therefore constrained by the material properties of the components of the electromagnet and this in turn restricted cyclic mechanical loading of the magnet and attached fascicles to a frequency of 20Hz in the developed system. In order to elucidate the effects of higher frequency loads, future work should incorporate a higher performance electromagnet into the developed IVLS, for application of loading frequencies of up to 50Hz.

It must also be remembered that, the isolated fascicles were not exposed to the same complex loading conditions experienced by bulk tendons in vivo, therefore more advanced mechanical loading set ups are required to better reflect forces applied to tendons in the physiological environment.

This thesis investigated the mechanobiology of tendons using a novel *in vitro* loading system. It is therefore critical to take care when extrapolating *in vitro* data to interpret the behaviour of tissues in vivo, and obtain a considerable number of repeatable results before drawing any important conclusions. Moreover, tendon fascicles used in this study were obtained from male Sprague Dawley rats aged between 4-6 months. Therefore parameters such as genetic variation and the disparity in the weight of individual rats must be considered when interpreting the results of this study.

In this thesis, tensile tests were performed at room temperature, (between 17-21^{o}C), in order to allow for comparison of data with the numerous studies on tendon biomechanics and mechanobiology. However, these temperatures are considerably lower than average body temperature (37^{o}C) and temperatures consistently recorded in the core of tendons during

exercise and physical activity (45^{o}C) [295, 296]. The use of accurately controlled environmental chamber during tensile testing would therefore be an improvement to the current approach, and offer a more precise and accurate indication of the true mechanical performance of tendons in a physiological environment

The injury model used in this thesis was introduced by a manual operator using an insulin needle. Although the damage inflicted onto tendon fascicles were shown to have consistent scratch lengths, manifestation of the uncertainties associated with human error is to be expected. Development of an automated method for generation of a scratch damage in RTTF using appropriate equipment would therefore minimise confounding variables of manual work and offer a more precise and reproducible injury in an extensively large population.

The experiments reported in this thesis take place over the course of a maximum of 7 days, so the definition of repair and remodelling considered will be over this relatively short timescale. A major limitation of the *in vitro* damage repair model used is therefore the short term nature of the experimental culture period used to monitor repair and remodelling responses. Although damaged tendons fascicles showed significant responses to HFLM mechanical loading, further investigations are required in order to elucidate the long term effects of this exciting biophysical loading intervention.

Despite the limitations of the experimental approaches used in the thesis, the findings reported are important for understanding the remodelling and repair events in healthy and damaged tendons following stimulation by various mechanical loads.

8.5 FUTURE WORK

Investigate the effects of higher frequency (20-50Hz) *in vitro* cyclic loads on the biochemical and biomechanical properties of healthy tendons

As previously mentioned, the IVLS used for HFLM loading of tendon fascicles was limited to application of cyclic loads of 20Hz because of the restricted field frequencies that could be sustained by the electromagnetic. Upgrading the IVLS with a high performance electromagnet capable of delivering elevated field frequencies (beyond 20Hz) would facilitate HFLM loading studies at even higher cyclic loading frequencies than those used in this thesis. HFLM mechanical signals of up to 90Hz have shown anabolic effects in bone tissue [297], and application of 50Hz loading frequency to the Achilles of human patients by means of a portable vibrator has been found to increase the contractile properties of plantaris flexors, and these advocate the requirement for further HFLM *in vitro* studies beyond 20Hz.

These studies would not only enable the optimisation of stimulation parameters for future HFLM tendon therapies, but will also help to determine whether or not there is a frequency threshold beyond which application of HFLM stimulation becomes ineffective and or induces degenerative changes in tendon matrix.

Investigate the effects of various load magnitudes of *in vitro* HFLM on tendon homeostasis

Although several *in vitro* loading conditions and loading parameters were assessed in this thesis, the precise level of load magnitude required for mechanobiological stimulation of tendon homeostasis was not examined. It is important to understand the effects of different HFLM loading magnitudes on tendon remodelling as this may play an essential role in the quest towards optimising mechanical loading interventions for tendon repair. Such a study can be performed by isolating the effects of frequency and dosages, that is, applying

mechanical loads onto tendon specimens at the same cyclic loading frequency and for the same dosage periods while varying the applied load magnitude.

Implementing HFLM mechanical stimulation in the clinic

In this thesis the potential of HFLM loading for tendon repair and remodelling was investigated and experimental studies were performed using tendon fascicles. To implement tendon stimulation using HFLM mechanical loading in a clinical environment and indeed confirm the results of this study in clinical trials it would be necessary to develop a portable device capable of being securely fastened around whole tendon organs at the desired anatomical region for local administration of HFLM loading. Such a unit should include a battery powered control panel for adjustment of loading frequencies and intensities (magnitudes), in addition to electrical cables connected to a vibrating member, and should function independently without involvement of muscle activity. Although such a device may not firmly guarantee application of tensile loads it would provide a source of high frequency mechanical stimulus with localised administration. Further tests would be needed to confirm the potential of HFLM loads applied perpendicular to the length of a tendon portion.

Similar devices have been developed which use an electrical stimulator to carry electrical pulses to a major nerve such as the posterior tibial nerve, and this induces a twitch in a selected muscle group such as the gastrocnemius over which surface electrodes are positioned, and subsequently results in tensile load acting on the Achilles tendon [174]. Such methods however rely primarily on muscle activity and EMG signals in neighbouring muscle groups and this makes it difficult to accurately control the intensity (magnitude), and frequency of load applied onto the tendon and places a limit on the frequency of load that can be applied onto the tendon.

Scale up of *in vitro* loading system

A progression to our studies on tendon fascicle tissue cultures would be the execution of similar experimental procedures on whole tendon organs. Designing a larger IVLS would enable incorporation of whole organ tendon samples. Furthermore, the mechanisms by which tendons adapt their form and structure in response to mechanical loading is a complex subject, due to the multifaceted interwoven collagen structures (fascicles, fibrils and fibres) presented in tendon. Experimentation with whole organ tendon cultures in combination with highly detailed microscopy techniques (time lapse microscopy imaging) would allow for detailed images of tendon microstructure at various experimental time points and facilitate a deeper understanding of the structure-function relationships governing tendons adaptation to mechanical loading. However, such a system may pose challenges for tissue viability since diffusion gaps for delivery of oxygen and nutrients to tendon cells deep within the tissue may be too far or close to the critical distance.

8.6 Concluding Remarks

An elementary feature of all biological tissues is their ability to adapt to the external environment. Tendons are no different. In fact mechanical loads can encourage modification of tendons in such a way as to enhance tissue biochemical and biomechanical properties both during health and during repair. To benefit from these effects, an optimal balance must be achieved between mechanical loading frequencies and dosages that are too low and those that are too high.

Noteworthy questions concerning current challenges within tendon research were highlighted in chapter 1of this thesis. While all of these questions have not been addressed in the study chapters presented, we have shown that clinically relevant dosages of HFLM loading at

frequencies of 20Hz and loading magnitudes of 0.12N for 1 hour a day and for 5 days a week provides an optimal balance for maintaining tangent modulus and GAG content in healthy tendon specimens. When extended towards damaged tendons, this exciting biophysical loading intervention demonstrated the most superior stimulatory effects for maintaining tendon modulus and tendon metabolism amongst static and low frequency (1Hz) cyclic loading conditions.

As with several therapeutic treatment options it is crucial to consider potential risks and safety margins and although a complete examination of these themes is beyond the scope of this study, we have seen that HFLM loading of tendon specimens at cyclic loading frequencies of 20Hz for periods longer than 1hour a day is not required for optimum stimulation of healthy and damaged tendons and this information may contribute towards prevention of potential risks when considering safety margins of administration.

In conclusion the newly developed IVLS together with the *in vitro* studies performed provide a scientific platform for investigating the potential for localised administration of HFLM mechanical loads as a clinical therapy to enhance tendon repair and tendon remodelling.

REFERENCES

1. Gardner, K., Arnoczky, S.P., Caballero, O., Lavagnino, M., *The effect of stress-deprivation and cyclic loading on the TIMP/MMP ratio in tendon cells: An in vitro experimental study.* Disability and Rehabilitation, 2008. **30**(20-22): p. 1523-1529.

2. Pennisi, E., *Tending, tender, tendons.* Science 2002. **295**(5557): p. 1011.

3. Twaddle, B., and Poon, P., *Early Motion for Achilles Tendon Ruptures: Is Surgery Important?* The American Journal of Sports Medicine, 2009. **35**(12): p. 2033-2038.

4. Screen, H., Shelton, JC., Bader, DL., Lee, DA., *Cyclic tensile strain upregulates collagen synthesis in isolated tendon fascicles.* Biochemical and Biophysical Research Communications, 2005. **336**(2): p. 424-429.

5. Butler, D., Juncosa-Melvin, N., Boivin, GP., Galloway, MT., Shearn, JT., Gooch, C., *Functional tissue engineering for tendon repair: A multidisciplinary strategy using mesenchymal stem cells, bioscaffolds, and mechanical stimulation.* J. Orthop. Res, 2008. **26**: p. 1-9.

6. Wang, J., *Mechanobiology of tendon.* J Biomech, 2006. **39**(9): p. 1563-1582.

7. Korvick, D., Cummings, JF., Grood, ES., Holden, JP., Feeder, SM., Butler DL., *The use of an implantable force transducer to measure patellar tendon forces in goats.* J Biomech, 1996. **29**: p. 557-561.

8. Duncan, N.D., Williams, D A., Lynch, G S, *Adaptations in rat skeletal muscle following long-term resistance exercise training.* Eur J Appl Physiol 1998. **77**: p. 372-378.

9. Whitehead, N.P., Gregory J E., Morgan, D L., Proske, U., *Passive Mechanical Properties of of the Medial Gastrocnemius muscle of the cat.* J Physiol, 2001. **536**(3): p. 893-903.

10. Arnoczky, S., Tiana, T., Lavagnino, M., Gardner, K., Schuler, P., Morse, P., *Activation of stress-activated protein kinases (SAPK) in tendon cells following cyclic strain: the effects of strain frequency, strain magnitude, and cytosolic calcium.* J Orthop Res, 2002. **20**: p. 947–952.

11. Lavagnino, M.H., J.A., Arnoczky, SP., Hoonjan, A., Torzilli, PA.,. *Effect of amplitude and frequency of cyclic tensile strain on the inhibition of MMP-1 mRNA expression in tendon cells: An in vitro study.* Connective Tissue Research, 2003. **44**(3-4): p. 181-187.

12. Griensven, M., Zeichen, J., Skutek, M., Barkhausen, T., Krettek, C., Bosch U, *Cyclic mechanical strain induces NO production in human patellar tendon fibroblasts – a possible role for remodelling and pathological transformation.* Exp Toxic Pathol, 2003. **54**: p. 335–338.

13. Barkhausen, T., Griensven, M V., Zeichen, J., Bosch U., *Modulation of cell functions of human tendon fibroblasts by different repetitive cyclic mechanical stress patterns.* Exp Toxic Pathol 2003. **55**: p. 153–158.

14. Slack, C., Flint, MH., Tompson, BM., *The effect of tensional load on isolated embryonic chick tendons in organ culture.* Connect Tissue Res, 1984. **12**: p. 229-247.

15. Almikinders, L., Baynes, AJ., Bracey, LW., *An in vitro investigation into the effects of repetitive motion and nonsteroidal antiinflammatory medication on human tendon fibroblasts.* Am J Sports Med, 1995. **23**(1): p. 119 – 123.

16. Fritton, S.P., McLeod, K J., & Rubin, C T., *Quantifying the strain history of bone: spatial uniformity and self-similarity of low magnitude strains.* J. Biomech, 2000. **33**: p. 317-325.

17. Ozcivici, E., Luu, YK., Adler, B., Qin, YX., Rubin, J., Judex, S., and Rubin, CT., *Mechanical signals as anabolic agents in bone.* Nat Rev Rheumatol, 2010 **6**: p. 50–59.

18. Rubin, C., Simon S., Bain, S., Mallinckrodt, C., McLeod, K., *Low mechanical signals strengthen long bones.* Nature, 2001. **412** p. 603-604.

19. Rubin, C., Turner, AS., Muller R., Mittra, E., McLeoad, K., Lin W., *Quantity and quality of trabecular bone in the femur are enhanced by a strongly anabolic, noninvasive mechanical intervention.* J. Bone Miner. Res. , 2002. **17,** : p. 349–357.

20. Andersson, T., Eliasson P., Aspenberg P., *Tissue memory in Healing Tendons: Short loading episodes stimulates Healing.* J. Appl Physiol, 2009. **107**: p. 417-421.

21. Jozsa, L., and Kannus, P., *Human Tendons: Anatomy, Physiology and Pathology.* Human Kinetics Champaign, IL., 1997: p. 1-574.

22. Oommen, A., Poonnoose, PM., Padhy, D., Korula, RJ., *Management of Open Chronic Tendo Achilles Injuries: A case report.* The Foot and Ankle Online Journal 2010. **3**(1): p. 2.

23. Kvist, M., Jozsa, L., Kannus, P., Isola, J., Vieno, T., Järvinen, M., Lehto, M., *Morphology and histochemistry of the myotendineal junction of the rat calf muscles. Histochemical, immunohistochemical and electron-microscopic study.* Acta Anat (Basel), 1991. **141**: p. 199-205.

24. Ker, R., *The implications of the adaptable fatigue quality of tendons for their construction, repair and function.* Comp Biochem and Physiol - Part A, 2002. **133**(4): p. 987-1000.

25. Benjamin, M., Qin, S., Ralphs, JR., *Fibrocartilage associated with human tendons and their pulleys.* J Anat., 1995. **187**: p. 625-33.

26. Evans, E., Benjamin, M., Pemberton, DJ., *Fibrocartilage in the attachment zones of the quadriceps tendon and patellar ligament of man.* J Anat. , 1990. **171**: p. 155-162.

27. Benjamin, M., Evans, EJ., Copp, L., *The histology of tendon attachments to bone in man.* J Anat. , 1986. **149**: p. 89–100.

28. McGonagle, D., Marzo-Ortega, H., Benjamin, M., Emery, P., *Report on the Second international Enthesitis Workshop.* Arthritis and Rheumatism 2003. **48**: p. 896–905.

29. Everts, V., Buttle, DJ., *Methods in studying ECM degradation.* Methods, 2008. **45** p. 86–92.

30. Woo, S.L., An, K., Frank, C., Livesay, G., Ma, A., Zeminski, J., Wayne, J., Myers, B., *Anatomy, biology, and biomechanics of tendon and ligament.* In: Buckwalter, JA., Einhorn, TH., Simon, SR., (Eds) Orthopaedic Basic Science: Biology and Biomechanics of the Musculoskeletal System, 2nd Edition. 2000a: p. 582–616.

31. Woo, S., Debski, RE., Zeminski, J., Abramowitch, SD., Saw, SS., and J. Fenwick., *Injury and repair of ligaments and tendons.* Ann Rev Biomed Eng 200b. **2**: p. 83–118.

32. Kannus, P., *Structure of the tendon connective tissue.* Scand J Med Sci Sports 2000. **10**: p. 312-320.

33. Lin, T., Cardenas, L., Soslowsky, LJ., *Biomechanics of tendon injury and repair.* J Biomech, 2004. **37**: p. 865-877.

34. Sharma, P., and Maffulli, N., *Tendon injury and tendinopathy: healing and repair.* JBJS, 2005. **87**(1): p. 187-202.

35. Ippolito, E., Natali, PG., Postacchini, F., Accinni, L., Demartino, C., *Morphological, Immunochemical, and biochemical study of rabbit Achilles tendon at various ages.* J Bone Joint Surg (Am), 1980. **62**: p. 583-592.

36. Bi, Y., Ehirchio D., Kilts, TM., Inkson, CA., Embree, MC., Sonoyama, W., *Identification of tendon stem / progenitor cells and the role of the extracellular matrix in their niche.* . Nat Med. , 2007. **13**: p. 1219-27.

37. Franchi, M., Trirè A., Quaranta, M., Orsini, E., Ottani, V., *Collagen structure of tendon relates to function.* The scientific world journal, 2007c. **7**: p. 404-420.

38. Balasubramanian, P., Prabhakaran, MP., Sireesha, M., and Ramakrishna, S., *Collagen in Human Tissues: Structure, Function, and Biomedical Implications from a Tissue Engineering Perspective.* Adv Polym Sci, 2012. **251**: p. 1-34.

39. Petruska, J., Hodge, AJ., *A subunit model for the tropocollagen macromolecule.* . Proc. Natl. Acad. Sci. U. S. A. , 1964. **51**: p. 871-76.

40. Yoon, J., and Halper, J., *Tendon Proteoglycans: biochemistry and function.* J Musculoskelet Neuronal interact 2005. **5**(1): p. 22-34.

41. Parkinson, J., Samiric T., Ilic MZ., Cook, J., Handley, CJ., *Involvement of Proteoglycans in Tendinopathy.* J Musculoskelet Neuronal Interact 2011. **11**(2): p. 86-93.

42. Marturanoa, J., Arenaa, JD., Schillera, ZA., Georgakoudia, I., and Kuoa, CK., *Characterization of mechanical and biochemical properties of developing embryonic tendon.* PNAS 2013. **110**(16): p. 6370–6375

43. Ippolito E, P.L., Postacchini F, *Biochemistry and metabolism. The Tendons: Biology-pathology-clinical aspects.* 1986: p. 37-46.

44. Butler DL, G.E., Noyes FR, Zernicke RF., *Biomechanics of Ligaments and Tendons.* Exerc Sport Sci Rev.6, 1978: p. 125-182.

45. Provenzano, P., Vanderby, R., *Collagen Fibril morphology and organisation: Implications for force transmissionin ligament and tendon.* Matrix Biology, 2006. **25**: p. 71-84.

46. Astrom, M., *On the nature and etiology of chronic achilles tendinopathy.* 1997.

47. Movin, T., Kristoffersen-Wiberg, M., Shalabi, A., Gad, A., Aspelin, P., Rolf, C., *Intratendinous alterations as imaged by ultrasound and contrast medium-enhanced magnetic resonance in chronic achillodynia.* Foot Ankle Int 1998. **19**: p. 311-7.

48. Kvist, M., Jozsa, L., Kannus, P, Isola, J., Vieno, T., Jarvinen, M., Lehto, M., *Morphology and histochemistry of the myotendineal junction of the rat calf muscles. Histochemical, immunohistochemical and electron-microscopic study.* Acta Anat (Basel), 1991. **141**: p. 199-205.

49. Wren, T., Beaupré, GS., Carter, DR., *Mechanobiology of tendon adaptation to compressive loading through fibrocartilaginous metaplasia.* JRRD, 2000. **37**(2): p. 135-143.

50. Wilmink, J., Wilson, AM., Goodship, AE., *Functional significance of the morphology and micromechanics of collagen fibres in relation to partial rupture of the superficial digital flexor tendon in racehorses.* Research in Veterinary Science 1992. **53**: p. 354-359.

51. Thompson, J.I., Czernuszka, J.T., *The effect of two types of cross-linking on some mechanical properties of collagen.* Biomedical Material Engineering, 1995. **5**: p. 37–48.

52. Pollock, C.M., Shadwick, R.E., *Relationship between body mass and biomechanical properties of limb tendons in adult mammals.* Am. J. Physiol. , 1994. **266**: p. R1016–1021.

53. Bennett, MB., Ker, RF., Dimery, NJ., Alexander, R., *Mechanical properties of various mammalian tendons.* J. Zool. Lond. A 1986. **209**: p. 537–548.

54. Ker, RF., Wang, XT., Pike, AVL., *Fatigue quality of mammalian tendons.* J. Exp. Biol. 2000. **203**: p. 1317–1327.

55. Johnson, G., Tramaglini, DM., Levine, RE., Ohno, K., Choi, NY., Woo, S.L., *Tensile and viscoelastic properties of human patellar tendon.* . J Orthop Res, 1994. **12**: p. 796–803.

56. Butler, D., Grood, ES., Noyes, FR., Zernicke, RF., *Biomechanics of Ligaments and Tendons.* Exerc Sport Sci Rev.6, 1978: p. 125-182.

57. Maganaris CN, N.M., Maffulli N., *Biomechanics of the Achilles tendon.* Disability and Rehabilitation, 2008. **30**((20-22)): p. 1542-1547.

58. Wang, J., and Thampatty, BP., *An introductory review of cell mechanobiology.* Biomechan Model Mechanobiol, 2006. **5**(1): p. 1-16.

59. Ker, R.F., *The implications of the adaptable fatigue quality of tendons for their construction, repair and function.* Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, 2002. **133**(4): p. 987-1000.

60. Cacho, F., Elbischger, P., Rodrguez, J., Doblar, M., Holzapfel, G., *A constitutive model for fibrous tissues considering collagen fiber crimp.* Int J Non Linear Mech, 2007. **42**(2): p. 391–402.

61. Leadbetter, W.B., *Cell Matrix response in Tendon Injury.* Clin Sports Med, 1992. **11**(3): p. 533-578.

62. Sharma P, MN., *Tendon injury and tendinopathy: healing and repair.* Jounal of Bone Joint Surgery 2005. **87**(1): p. 187-202.

63. McGonagle, D., Marzo-Ortega, H., Benjamin, M., Emery, P.,, *Report on the Second international Enthesitis Workshop. .* Arthritis and Rheumatism 48, , 2003. **48**: p. 896–905.

64. Thomopoulos, S., Williams, G.R., Gimbel, J.A., Favata, M., Soslowsky, L.J., *Variation of biomechanical, structural, and compositional properties along the tendon to bone insertion site.* Journal of Orthopaedic Research, 2003. **21**: p. 413–419.

65. Schultz, J., *MRI images showing torn rotator cuff.* Centeno Schultz clinic Online report, 2009.

66. Kannus, P., and Jozsa L., *Histopathological changes preceeding spontaneous rupture of a Tendon. A controlled study of 891 patient.* JBJS (Am), 1991. **73**: p. 1507-1525.

67. Maganaris, C., Narici, MV., Maffulli, N., *Biomechanics of the Achilles tendon.* Disability and Rehabilitation, 2008. **30**(20-22): p. 1542-1547.

68. James, S., Banes, BT., Osternig, LR., *Injuries in Runners.* Am J Sports Med 1978. **6**: p. 40-50.

69. Galatz, L., Charlton, N., Das, RS., Kim, M., Havlioglu, N., Thomopoulos, S., *Complete removal of load is detrimental to rotator cuff healing.* J Shoulder Elbow Surg 2009. **18**: p. 669-675.

70. Halder, A., Zobitz, ME., Schultz, F., An, KN., *Mechanical properties of the posterior rotator cuff.* Clinical Biomechanics 2000. **15**: p. 45-462.

71. Schneeberger, A., Nyffeler, RW., Gerber, C., *Structural changes of the rotator cuff caused by experimental subacromial impingement in the rat.* J Shoulder Elbow Surg 1998. **7**: p. 375-380.

72. Murawala, P., Tanaka, EM., Currie, JD., *Regeneration: The ultimate example of wound healing.* Seminars in Cell & Developmental Biology 2012. **23**: p. 954– 962.

73. Burrington, J., *Wound healing in the fetal lamb.* J Pediatr Surg 1971. **6**(5): p. 523.

74. Colwell, A., Longaker, MT., Lorenz, HP., *Fetal wound healing.* Front Biosci

2003. **8**: p. 1240.

75. Colwell, A., Longaker, MT., Peter Lorenz, H., *Identification of differentially regulated genes in fetal wounds during regenerative repair.* Wound Repair Regenration, 2008. **16**: p. 450.

76. Agha, R., Ogawa, R., Pietramaggiori, G., M.D., and Orgill, DP., *A Review of the Role of Mechanical Forces in Cutaneous Wound Healing.* Journal of Surgical Research 2011. **171**: p. 700–708

77. Oakes, B.W., *Tissue healing and repair: tendons and ligaments.*

In: Frontera WR, editor. Rehabilitation of sports injuries: scientific basis. Boston: Blackwell Science, 2003: p. 56-98.

78. Mass, D., Tuel, RJ., *Intrinsic healing of the laceration site in human superficialis flexor tendons in vitro.* . Journal of Hand Surgery (American), 1991. **16**: p. 24–30.

79. Potenza, A., *Mechanisms of healing of digital flexor tendons.* Hand, 1969. **1,** : p. 40-41.

80. Aspenberg, P., *Stimulation of tendon repair: mechanical loading, GDFs and platelets. A mini-review.* International Orthopaedics, 2007. **31**(6): p. 783–789.

81. Garner, WL., Koo, M., Khun, C., Weeks PM, *Identification of the collagen producing cells in healing flexor tendons.* Plast Reconstr Surg, 1989. **83**: p. 875-879.

82. Jozsa, L., Lehto, M., Kannus, P., Kvist, M., Reffy, A., Vieno, T., Jarvinen, M., Demel, S., Elek, E., *Fibronectin and laminin in Achilles tendon.* Acta Orthopaedica Scandinavica 1989a **60**: p. 469–471.

83. James, R., Kasturu, G., Balian, G., Chhabra, AB., *Tendon: Biology, Biomechanics, Repair, Growth Factors, and Evovling treatment options.* J hand Surg 2008. **33A**: p. 102-112.

84. Postacchini, F., Accini, L., Natali, PG., Ippolito, E., DeMartini, C., *Regeneration of Rabbit Calcaneal Tendon. A morphological and immunohistological study.* Cell Tiss Res, 1980. **195**: p. 81-97.

85. James, R., Kasturu, G., Balian, G., A.Bobby Chhabra., *Tendon: Biology, Biomechanics, Repair, Growth Factors, and Evovling treatment options.* J hand Surg 2008. **33A**: p. 102-112.

86. Gill, S., Turner, MA., Battaglia, TC., Leis, HT., Balian, G., Miller, MD., *Semitendinosus regrowth: biochemical, ultrastructural, and physiological characterization of the regenerate tendon.* Am J Sports Med 2004. **32**: p. 1173-1181.

87. Sharma, P., Maffulli, N., *Tendinopathy and tendon injury: The future.* Disability and Rehabilitation, 2008. **30**(20–22): p. 1733–1745.

88. Haraldsson, B., Langberg, H., Aagaard, P., Zuurmond, AM., van El, B., DeGroot, J., Kjaer, M., Magnusson, SP., *Corticosteroids reduce the tensile strength of isolated collagen fascicles.* J Sport Med, 2006. **34**: p. 1992-1997.

89. Zargar Baboldashti, N., Poulsen, RC., Franklin, SL., Thompson, MS., and Hulley, PA., *Platelet-Rich Plasma Protects Tenocytes From Adverse Side Effects of Dexamethasone and Ciprofloxacin.* Am J Sports Med, 2011. **39**(9): p. 1929-1935.

90. Ng, C., Ng, GY., See, EK., Leung, MC., *Therapeutic ultrasound improves strength of Achilles tendon repair in rats.* Med Biol 2003, 2003. **29**: p. 1501–1506.

91. Houglum, P.A., *Soft Tissue Healing and its impact on Rehabilitation* J Sport Rehabil, 1992. **1**: p. 19-39.

92. Rivenburgh, D.W., *Physical modalities in the treatment of Tendon injuries.* Clin Sports Med 1992. **11**: p. 645-659.

93. Enwemeker, C., Rodriguez, O., Mendosa, S., *The biomechanical effects of low-intensity ultrasound on healing tendons.* Ultrasound Med Biol, 1990. **16**: p. 801-807.

94. Enwemeka, C., *The effects of therapeutic ultrasound on tendon healing. A biomechanical study. .* Am J Phys Med Rehabil 1989. **68**: p. 283–287.

95. Enwemeka, C., Rodriguez, O., Mendosa, S., *The biomechanical effects of low-intensity ultrasound on healing tendons.* Ultrasound Med Biol, 1990. **16**: p. 801-807.

96. Prentice WE, Malone,T., *Thermotherapy. In Leadbetter WB, Buckwalter JA, Gordons SL (eds).* Sports Induced inflammation. Park Ridge :AAOS 1990: p. 455-461.

97. Young SR, D.M., *Macrophages responsiveness to therapeutic ultrasound.* Ultrasound in Medicine and Biology, 1990. **16**: p. 809-816.

98. Nussbaum, E., *The influence of Ultrasouldnd on healing tissue.* J Hand Ther, 1998. **11**: p. 140-147.

99. Hashish, I., *The effects of ultrasound on post-operative inflammation.* 1986.

100. Lundeberg, T., Northstrom, F., Brodda-Jansen, G., Eriksson, SV., Kjartansson, J., Samuelson, UE., *Pulsed ultrasound does not improve healing of venous ulcers* Scand J Rehabil Med 1990. **22**: p. 195–197.

101. Roberts, M., Rutherford, JH., Harris, D., *The effect of ultrasound on flexor tendon repairs in the rabbit.* Hand 1982. **14**: p. 17–20.

102. Williamson, J., George, TK., Simpson, DC., Hannah, B., Bradbury, E., *Ultrasound in the treatment of ankle sprains.* Injury 1986. **17**: p. 176–178.

103. Turner, S., Powell, ES., Nq, CS.,, *The effect of ultrasound on the healing of repaired cockerel tendon is collagen cross-linkage a factor?* J Hand Surg 1989. **14**(4): p. 428–433.

104. Wang, C., Ko, JY., Chen, HS., *Treatment of calcifying tendinitis of the shoulder with shock wave therapy.* Clin Orthop, 2001. **387**: p. 83-69.

105. Perlick, L., Schifmann, R., Kraft, CN., Wallny, T., Diedrich, O., *Extracoporeal shock wave of the Achilles tendinitis.* Z Orthop Ihre Grenzgeb, 2002. **140**(3): p. 275-280.

106. Rompe, J., Rumler, F., Hpof, C., *Extracoporeal shock wave therapy for calcifying tendinitis of the shoulder* Clin Orthorp, 1995. **321**(196-201).

107. Speed, C., Nichold, D., Richard, C., Burnet, S., Wiles, JT., Humphreys, H., *Extracoporeal shcok wave therapy for tendonitis of the rotator cuff. A double-blind randomised controlled trial.* JBJS, 2002. **84**: p. 509-512.

108. Chen, Y., Wang, CJ., Yang, KD., Kuo, YR., Huang, HC., Huang, YT., Sun, YC., Wang, FS., *Extracorporeal shock waves promote healing of collagenase-induced Achilles tendinitis and increase TGF-beta 1 and 1GF-1 expression.* J Orthop Res, 2004. **22**(4): p. 854-861.

109. Rezk-Allah, S., Abo-El, SN., & El Gendy, AM., *Extra corporeal shock wave therapy is superior to ultrasound in the treatment of lateral epicondylitis: An experimental study.* Journal of medical research and practice, 2013. **2**(7).

110. Binder, A., Parr, G., Hazelmann, B., Fitton Jackson, S., *Pulsed electromagnetic field therapy of persistent rotator cuff tendinitis. A Double-blind Controlled Assessment.* The Lancet, 1984. **31**(1): p. 695-698.

111. Greenough, C., *The effect of Pulsed electromagnetic fields on flexor tendon healing in the rabbbit* J Hand Surg, 1996. **21b**: p. 808-812.

112. Robotti, E., Zimbler, AG., Kenna, D., Grossman, JA., *The effect of pulsed electromagnetics fields on flexor tendon healing in chickens.* J Hand Surg (Euro), 1999. **24B**(1): p. 56-58.

113. Lee, E., Maffulli, N., Li, CK., Chan, KN., *Pulsed Magnetic and electromagnetic fields in experimental Achilles tendon in the rat.* Arch Phys Med Rehabil, 1997. **78**: p. 399-404.

114. Lin, J., Wang, MX., Wei, A., Zhu, W., Diwan, AD., Murrell, GA., *Temporal expression of nitric oxide synthase isoforms in healing Achilles tendon.* J Orthop Res, 2001. **19**: p. 136–142.

115. Butler, D., Goldstein, S., Guilack, F., *Functional Tissue Engineering: the role of biomechanics.* Transactions of the ASME, 2000. **122**: p. 570-575.

116. Woo, S.L., Hildebrand, K., Watanabe, N., Fenwick, J.A., Papageorgiou, C.D., Wang, J.H.,, *Tissue engineering of ligament and tendon healing. .* Clinical Orthopaedics, 1999: p. 312–323.

117. Guilak, F., *Functional tissue engineering: the role of biomechanics in reparative medicine.* Annales of the New York Academy of Science 2002. **961**: p. 193–195.

118. Chaudhury, S., Holland, C., Thompson, MS., Vollrath, F., Carr, AJ., *Tensile and shear mechanical properties of rotator cuff repair patches.* Journal of shoulder and elbow surgery, 2012. **21**.

119. Derwin, K., Badylak, SF., Steinmann, SP., Iannotti, JP., *Extracellular matrix scaffold devices for rotator cuff repair.* J Shoulder Elbow Surg 2010. **19**: p. 467-476.

120. Derwin, K., Baker, AR. Spragg, RK., Leigh, DR., Iannotti, JP., *Commercial extracellular matrix scaffolds for rotator cuff tendon repair. Biomechanical, biochemical, and cellular properties.* JBJS, 2006. **88**(12): p. 2665-72.

121. Chaudhury, S., Gwilym, SE., Moser, J., Carr, AJ., *Surgical options for patients with shoulder pain.* Nat Rev Rheumatol, 2010. **6**(4): p. 217-26.

122. Vacanti, C., Cao, YL., Langer, RS., Vacanti, JP., Paige, K., Upton, Joseph. , *Tissue engineered tendons and ligaments* FPO IP Research ad Communities, 2005.

123. Koob, T.J., *Biomimetic approaches to tendon repair.* Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, 2002. **133**(4): p. 1171-1192.

124. Butler, D., Awad HA., *Perspectives on cell and collagen composites for tendon repair* Clinic Orthopeadics and related research, 1999: p. S324-332.

125. Cilindro de Souza, S., Luiz de Oliveira, W., Fernando de Oliveira, S., Soares, D., Henrique-Briglia, C., Athanázio, PR., Dias de Cerqueira, M., Guimarães, P., Carreiro, M., *Comparative study of suture and cyanoacrylates in skin closure of rats.* Acta Cirúrgica Brasileira 2007. **Vol 22** (4): p. 309-316.

126. Lavagnino, M., Arnoczky, SP., Egerbacher, M., Gardner, KL., and Burns, ME., *Isolated fibrillar damage in tendons stimulates local collagenase mRNA expression and protein synthesis.* Journal of Biomechanics, 2006. **39**(13): p. 2355-2362.

127. Wang, J., Guo, Q., Li, B., *Tendon Biomechanics and Mechanobiology: A mini review of basic concepts and Recent Advancements.* Journal of Hand Surgery, 2011.

128. Langberg, H., Skovgaard, H., Karamouzis, M., Bulow, J., Kaejar, M., *Metabolism and inflammatory mediators in the peritendinous space measured by microdialysis during intermittent isometric exercises in humans* J Physiol, 1999. **515**: p. 919-927.

129. Langberg, H., Rosendal, M., Kaejar, M., *Training induced changes in peritendinous type I collagen turnover determined by microdialysis in humans.* J Physiol, 2001. **534**: p. 297-302.

130. Woo, S., Gomez, MA., Amiel, D., Ritter, MA., Gelberman, RH., Akeson, WH., *The effects of exercise on the biomechanical and biochemical properties of swine digital flexor tendons.* J Biomech Eng, 1981. **103**(1).

131. Soslowsky, L., Thomopolous, S., Esmail, A., *Rotator cuff tendinosis in an animal model: role of extrinsic and oversuse factors.* Ann Biomed Eng, 2002. **30**(8): p. 1057-1063.

132. Karpakka J., V.K., Virtanen P, Savolainen J., Orava S, Takala TES., *The effects of remobilization and exercise on collagen biosynthesis in rat Tendon* Acta Physiol Scan, 1990. **139-145**: p. 139-145.

133. Wada, A., Kubota, H., Miyanishi, K., Hatanaka, H., Miura, H., Iwamoto, Y., *Comparison of postoperative early active mobilization and immobilization in vivo utilising a four-strand flexor tendon repair.* Journal of Hand Surgery [British], 2001. **26**: p. 301–306.

134. Bailon-Plaza, A., van der Meulen, MC., *Beneficial effects of moderate, early loading and adverse effects of delayed or excessive loading on bone healing.* J Biomech 2003. **36**: p. 1069–1077.

135. Godbout, C., O. Ang, and J. Frenette, *Early voluntary exercise does not promote healing in a rat model of Achilles tendon injury.* Journal of Applied Physiology, 2006. **101**(6): p. 1720-1726.

136. Gelberman, H., Manske, PR., Akeson, WH., Woo, SL., Lundberg, G., Amiel, D., *Flexor Tendon Repair.* J Orthop Res, 1986. **4**(1): p. 119-128.

137. Kannus, P., Jozsa, L., Renstrom, P., Jarvinen, M., Kvist, LM., Oja, P., Vuori, I., *The effect of training, immobiliztaion, and remobilization on musculoskeletal tissue. Part 1: Training and immobilization.* Scand J Med Sci Sports, 1992a. **2**: p. 100-118.

138. Holch M, B.A., Thermann H, Zwipp H *Non operative treatment of acute Achilles tendon ruptures: A clinical concept and experimental results.* Sports Exerc Injury, 1994. **1**: p. 18-22.

139. Woo, S., Gomez, MA., Woo, YL., Akeson, WH., *Mechanical properties of Tendons and ligaments. II. The relationship between immobilization and exercise on tissue remodelling* Biorheology 1982. **19**: p. 397-408.

140. Tipton, C., Vailas, AC., Mathes, RD., *Experimental studies on the influences of physical actvity on ligaments, tendons, and joints. A brief Review.* Acta Med Scand, 1986. **71**: p. 157-168.

141. Amiel, D., Woo, SL., Hardwood, FL., Akeson, WH., *The effect of immobilisation on collagen turnover in connective tissue. A biochemical-biomechanical correlation* Acta Orthop Scand, 1982. **53**(325-332).

142. Tipton, C., Mathes, RD., Maynard, JA., Carey, RA., *The influence of physical activity on ligaments and tendons.* Med Sci Sports Exerc, 1975. **7**: p. 165-175.

143. Rubin, C., *Transmissibility of 15hertz to 35hertz vibrations to the human hip and lumbar spine: determining the physiologic feasibility of delivering low‐level anabolic mechanical stimuli to skeletal regions at greatest risk of fracture because of osteoporosis* Spine 2003. **28**: p. 2621–2627.

144. Henriksen, M., Aaboe J., Bliddal, B., Langberg, H., *Biomechanical characteristics of the eccentric Achilles tendon exercise.* Journal of Biomechanics 2009. **42**: p. 2702–2707.

145. Luo, J., Clarke, M., McNamara, MB, Moran, K., *Influence of resistance load on neuromuscular response to vibration training.* Journal of Strength & Conditioning Research, , 2009. **23**(2): p. 420-6.

146. Prisby, R., Lafage-Proust, MH., Malaval, L., Belli, A., Vico, L., *Effects of whole body vibration on the skeleton and other organ systems in man and animal models: What we know and what we need to know.* Ageing Research Reviews, 2008. **7**: p. 319–329.

147. Rittweger, J., *Vibration as an exercise modality: how it may work, and what its potential might be.* Eur J Appl Physiol 2010. **108**: p. 877–904.

148. Rubinacci, A., Marenzana, M., Cavani, F., Colasante, F., Villa, I., Willnecker, J., Moro, and S. G.L., L.P., Ferretti, M., Guidobono, F., Marotti, G., *Ovariectomy sensitizes rat cortical bone to whole-body vibration.* Calcif. Tissue Int., 2008. **82** (4): p. 316–326.

149. Flieger, J., Karachalios, T., Khaldi, L., Raptou, P., Lyritis, G., , *Mechanical stimulation in the form of vibration prevents postmenopausal bone loss in ovariectomized rats.* . Calc. Tissue Int. , 1998. **63** (6): p. 510–514.

150. Rubin, C., Recker, R., Cullen, D., Ryaby, J., McCabe, J., McLeod, K., *Prevention of postmenopausal bone loss by a low-magnitude, high-frequency mechanical stimuli: a clinical trial assessing compliance, efficacy, and safety.* . J. Bone Miner. Res. , 2004. **19** (3): p. 343–351.

151. Verschueren, S., Roelants, M., Delecluse, C., Swinnen, S., Vanderschueren, D., Boonen, S., *Effect of 6-month whole body vibration training on hip density, muscle strength, and postural control in postmenopausal women: a randomized controlled pilot study.* . J. Bone Miner. Res. , 2004. **19**(3): p. 352–359.

152. Gilsanz, V., Wren, T.A.L., Sanchez, M., Dorey, F., Judex, S., Rubin, C., *Low-level, high-frequency mechanical signals enhance musculoskeletal development of young women with low BMD.* J. Bone Miner. Res., 2006. **21**(9): p. 1464–1474.

153. Kiiski, J., Heinonen, A., Ja¨rvinen, T., Kannus, P., Sieva¨nen, H., *Transmission of vertical whole body vibration to the human body* J Bone Miner Res, 2008. **23**(8).

154. Fung, D., Wang, VM., Andarawis-Puri, N., Basta-Pljakic, J., Li, Y., Laudier, DM., Sun, HB., Jepsen, KJ., Schaffler, MB., Flatow, EL., *Early response to tendon fatigue damage accumulation in a novel in vivo model.* Journal of Biomechanics, 2010. **43**: p. 274–279.

155. Farndale, R., Sayers, CA., Barrett, AJ., *A direct spectrophotometric microsassay for sulfated glycosaminoglycans in cartilage.* Connect. Tissue Res 1982. **9**: p. 247-248.

156. Farndale, R., Buttle DJ., Barrett AJ., *Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue.* Biochim Biophys Acta 1986. **883**: p. 173-177.

157. Adekanmbi, I., Franklin, S., Zagar-Baboldashti, N., Yapp, C., Thompson, MS., *A novel in vitro loading system for high frequency loading of cultured tendon fascicles.* Med Eng & Phys, 2012.

158. Arnoczky, S., Tian, T., Lavagnino, M., Gardner, K., *Ex vivo static tensile loading inhibits MMP-1 expression in rat tall tendon cells through a cytoskeletally based mechanotransduction mechanism.* J Orthop Res, 2004. **22**(2): p. 328-333.

159. Abreu, E., Leigh, D., Derwin, KA., *Effect of altered mechanical load conditions on the structure and function of cultured tendon fascicles.* J Orthop Res, 2008. **26** (3): p. 364 - 373

160. Hannafin, JA., and Arnoczky, SP., *Effect of Cyclic and Static Tensile Loading on Water-Content and Solute Diffusion in Canine Flexor Tendons - an in vitro Study.* Journal of Orthopaedic Research, 1994. **12**(3): p. 350-356.

161. Legerlotz, K., Jones, GC., Screen, HRC., Riley, GP., *Cyclic loading of tendon fascicles using a novel fatigue loading system increases interleukin-6 expression by tenocytes.* Scand J Med Sci Sports 2013. **23**: p. 31-37.

162. Arnoczky, S.P., Tian, T., Lavagnino, M., Gardner, K., *Ex vivo static tensile loading inhibits MMP-1 expression in rat tall tendon cells through a cytoskeletally based mechanotransduction mechanism.* J Orthop Res, 2004. **22**(2): p. 328-333.

163. Yamamoto, E., Iwanaga, W. Miyazaki, H., Hayashi, K., *Effects of static stress on the mechanical properties of cultured collagen fascicles from the rabbit patellar tendon.* Journal of Biomechanical Engineering-Transactions of the Asme, 2002. **124**(1): p. 85-93.

164. Majima, T., Yasuda, K., Yamamoto, N., *Deterioration of mechanical properties of the autograft in controlled stress-shielded augmentation procedures. An experimental study with rabbit patellar tendon.* . Am J Sports Med 1994. **22**: p. 821-9.

165. Hannafin, J.A., Arnoczky, SP., Hoonjan, A., Torzilli, PA., *Effect of stress deprivation and cyclic tensile loading on the material and morphologic properties of canine flexor digitorum profundus tendon: An in vitro study.* Journal of Orthopaedic Research, 1995. **13**(6): p. 907-914.

166. Yamamoto, E., Kogawa, D., Tokura, S., Hayashi, K., *Effects of the Frequency and Duration of Cyclic Stress on the Mechanical Properties of Cultured Collagen Fascicles From the Rabbit Patellar Tendon.* Journal of Biomechanical Engineering, 2005. **127**: p. 1168-1175.

167. Lavagnino, M., Frank, K., *Collagen fibril diameter distribution does not reflect changes in the mechanical properties of in vitro stress-deprived tendons.* J Biomech . 2005. **38:**: p. 69–75.

168. Maeda, E., Shelton, JC., Bader, DL., Lee, DA., *Time dependence of cyclic tensile strain on collagen production in tendon fascicles.* Biochemical and Biophysical Research Communications, 2007. **362**: p. 399-404.

169. Petersen, W., Varoga, D., Zantop, T., Hassenpflug, J., Mentlein, R., Pufe, T., *Cyclic strain influences the expression of the vascular endothelial growth factor (VEGF) and the hypoxia inducible factor 1 alpha (HIF-la) in tendon fibroblasts.* J Orthop Res, 2004. **22**: p. 847-853.

170. Arnoczky, SP., Tiana, T., Lavagnino, M., Gardner, K., Schuler, P., Morse, P., *Activation of stress-activated protein kinases (SAPK) in tendon cells following cyclic strain: the effects of strain frequency, strain magnitude, and cytosolic calcium.* Journal of Orthopaedic Research 2002. **20**: p. 947–952.

171. Schechtman, H., Bader, D.L., , *In vitro fatigue of human tendons.* Journal of Biomechanics, 1997. **30**: p. 829-835.

172. Magnusson, S., Langberg, H., and Kjaer, M., *The pathogenesis of tendinopathy: balancing the response to loading* Nat. Rev. Rheumatol., 2010. **6**: p. 262-268.

173. Ozcivici, E., Luu, YK., Adler, B., Qin, YX., Rubin, J., Judex, S., and Rubin, CT., *Mechanical signals as anabolic agents in bone.* Nat. Rev. Rheumatol., 2010 **6**: p. 50–59.

174. Lapole, T., Pérot, C., *Effects of repeated Achilles tendon vibration on triceps surae force production.* Journal of Electromyography and Kinesiology 2010. **20**: p. 648–654.

175. Ushiyama, J., Masani, K., Kouzaki, M., Kanehisa,H., and Fukunaga T., *Difference in aftereffects following prolonged Achilles tendon vibration on muscle activity during maximal voluntary contraction among plantar flexor synergists.* J Appl Physiol . 2005. **98**: p. 1427–1433.

176. Belavý, D., Miokovic, T., Armbrecht, G., Rittweger, J., Felsenberg, D., *Resistive vibration exercise reduces lower limb muscle atrophy during 56-day bed-rest.* J Musculoskelet Neuronal Interact 2009. **9**(4): p. 225-235.

177. Falempin, M., and Fodili in-albon, S., *Influence of brief daily tendon vibration on rat soleus muscle in non-weight-bearing situation.* J Appl Physiol 1999. **87**: p. 3-9.

178. Nabeshima, Y., *Uniaxial tension inhibits tendon collagen degradation by collagenase in vitro.* J Orthop Res 1996. **14:1**: p. 23–130.

179. Cousineau-Pelletier, P., Langelier, E., *Relative Contributions of Mechanical Degradation, Enzymatic Degradation, and Repair of the Extracellular Matrix on the Response of Tendons When Subjected to Under- and Over- Mechanical Stimulations In Vitro* MECHANICAL STIMULATION OF TENDON EXPLANTS, 2010. **28**: p. 204-210.

180. Yamamotoa, E., Kogawab, D., Tokurac, S., Hayash, K., *Biomechanical response of collagen fascicles to restressing after stress deprivation during culture.* J Biomech, 2007. **40**: p. 2063-2070.

181. Lin, Y., Ryohei, N., Nozaki, K., Sasaki, N., Kadosawa., T., Goto, N., Date, M., and Takeuchi, A., *Effects of pulsing Electromagnetic Fields on the ligament Healing in Rabbits* J Vet Med Sci 1992. **54**(5): p. 1017-1022.

182. Legerlotz, K., Schjerling, P., Langberg, H., Bru¨ggemann, GP., and Niehoff .,, *The effect of running, strength, and vibration strength training on the mechanical, morphological, and biochemical properties of the Achilles tendon in rats.* J Appl Physiol 2007. **102**: p. 564–572.

183. Lavagnino, M., Arnoczky, SP., Frank, K., *Collagen fibril diameter distribution does not reflect changes in the mechanical properties of in vitro stress-deprived tendons.* J Biomech, 2005. **38**: p. 69–75.

184. Yamamoto, E., Hayashi, K., Yamamoto, N., *Mechanical properties of collagen fascicles from the rabbit patellar tendon.* J Biomech Eng 1999(121): p. 124–131.

185. Viidik, A., *The effect of training on the tensile strength of isolated rabbit tendons.* Scandinavian Journal of Plastic and Reconstructive Surgery, 1967. **1**: p. 141-147.

186. Viidik, A., *Tensile strength properties of Achilles tendon systems in trained and untrained rabbits.* Acta Orthopaedica Scandinavica, 1969. **40**: p. 261-272.

187. Yamamoto E, Hayashi, K.,.K., Yamamoto N., *Mechanical properties of collagen fascicles from the rabbit patellar tendon.* J Biomech Eng 1999(121): p. 124–131.

188. Han, Y., Cowin, SC., Schaffler, MB., Weinbaum, S. , *Mechanotransduction and strain amplification in osteocyte processes.* Proc Natl Acad Sci USA 2004. **101**: p. 16689–94.

189. Legerlotz, K., Riley, G., Screen, HRC., *Specimen dimensions influence the measurement of material properties in tendon fascicles.* Journal of Biomechanics, 2010. **43**: p. 2274–2280.

190. Slack, C., Bradley, G., Beaumont, B., Poole, A., Flint, M., *Changes in the morphology and synthetic activity of cultured rat tail tendon.* Cell and Tissue Research, 1986. **245**(2): p. 359-68.

191. Yamamoto, E., Kogawa, D., Tokura, S., Hayashi, K., *Effects of the Frequency and Duration of Cyclic Stress on the Mechanical Properties of Cultured Collagen Fascicles From the Rabbit Patellar Tendon.* Journal of Biomechanical Engineering, (2005). **127**: p. 1168-1175.

192. Arnoczky, S.P., Tiana, T., Lavagnino, M., Gardner, K., Schuler, P., Morse, P., *Activation of stress-activated protein kinases (SAPK) in tendon cells following cyclic strain: the effects of strain frequency, strain magnitude, and cytosolic calcium.* Journal of Orthopaedic Research (2002). **20**: p. 947–952.

193. Yamamoto, E., Tokura, S., Hayashi, K., *Effects of Cyclic Stress on the Mechanical Properties of Cultured Collagen Fascicles From the Rabbit Patellar Tendon.* Journal of Biomechanical Engineering, 2003. **125** p. 893-901.

194. Arnoczky S P., L.M., Egerbacher M., *The response of tendon cells to changing loads: Implications in the etiopathogenesis of tendinopathy.* Encyclopedia of Sports Medicine, 2007: p. 46-59.

195. Haung, T., Perry, SM., and Soslowsky, LJ., *The Effect of Overuse Activity on Achilles Tendon in an Animal Model: A Biomechanical Study.* Annals of Biomedical Engineering 2004. **32** (3): p. 336–341.

196. Banes, A., Tsuzaki, M., Yamamoto, J., Fischer, T., Brigman, B., Brown, T., *Mechanoreception at the cellular level: The detection, interpretation, and diversity of responses to mechanical signals.* Biochem Cell Biol 1995. **73**: p. 349 – 365.

197. Sachs, F., *Mechanical transduction in biological systems* Crit Rev Biomed Eng 1988. **16**: p. 141-69.

198. Wang, N., Butler, JP., Ingber, DE., *Mechanotransduction across the cell surface and through the cytoskeleton.* Science New Series, 1993. **260**: p. 1124-1127.

199. Watson, P., *Function follows form: generation of intracellular signals by cell deformation* FASEB J 1991. **5**: p. 2013-9.

200. Pridgen, B., Woon, CYL., Kim, M., Thorfinn, J., Lindsey, D., Pham, H., Chang, J., *Flexor Tendon Tissue Engineering: Acellularization of Human Flexor Tendons with Preservation of Biomechanical Properties and Biocompatibility.* Tissue Engineering: Part C, 2011. **17**(8).

201. Cartmell, J., Dunn, MG., *Effect of chemical treatments on tendon cellularity and mechanical properties.* J Biomed Mater Res, , 2000. **49**: p. 134–140.

202. Muneta, T., Lewis, J., Stewart, N., *Effects of controlled load on graft healing: autograft vs allograft.* Trans Orthop Res Soc, 1993. **18**(4).

203. Arnoczky, S.P., Tian, T., Lavagnino, M., and Gardner, K., *In situ tensile load modulates inhibition of MMP-1 expression in rat tail tendon cells in a dose-dependent manner through a cytoskeletally-based mechanotransduction mechanism.* Trans. Ortho. Res. Soc. . 2003. **28:791**.

204. Banes AJ, T.M., Yamamoto J, Fischer T, Brigman B, Brown T, *Mechanoreception at the cellular level: The detection, interpretation, and diversity of responses to mechanical signals.* Biochem Cell Biol 1995. **73**: p. 349 – 365.

205. Archambault, J., Wiley, JP., Bray, RC., *Exercise loading of tendons and the development of overuse injuries.* Sports Med 1995. **20**: p. 77–89.

206. Soslowsky, L., Thomopoulos, S., Tun, S., Flanagan, CL., Keefer, CC., Mastaw, J., Carpenter, JE., *Overuse activity injures the supraspinatus tendon in an animal model: A histologic and biomechanical study* J Shoulder Elbow Surg 2000. **9**(2): p. 79 – 84.

207. Hazell, T., Kenno, KA., *The effects of whole-body vibration on upper- and lower-body EMG during static and dynamic contractions.* Applied Physiology of Nutrition and Metabolism 2007. , 2007. **32**(6): p. 1156–1163.

208. Clarke, A., and Fraser, KPP., *Why does metabolism scale with temperature?* Functional Ecology, 2004a. **18**: p. 243–251.

209. Clarke, A., *Is there a Universal Temperature Dependence of metabolism?* Functional Ecology, 2004b. **18**: p. 252-256.

210. Hemmingsen, A., *The relation of standard (basal) energy metabolism to total fresh weight of living organisms* Reports of the Steno Memorial Hospital and the Nordisk Insulinlaboratorium 1950. **4**: p. 1-48.

211. Alomar, A., Gawri, R., Roughley, PJ., Haglund, L., and Burman, M., *The Effects of Chlorhexidine Graft Decontamination on Tendon Graft Collagen and Cell Viability* American Journal of Sports Medicine 2012. **40**(7).

212. Hannafin, J.A., Arnoczky, SP., Hoonjan, A., Torzilli, PA., *Effect of stress deprivation and cyclic tensile loading on the material and morphologic properties of canine flexor digitorum profundus tendon: An in vitro study.* Journal of Orthopaedic Research, (1995). **13**(6): p. 907-914.

213. Jones, G., Corps, AN., Penninton, CJ., *Expression profiling of metalloproteinases and tissue inhibitors of metalloproteinases in normal and degenerative human Achilles tendon.* Arthritis Rheum, 2006. **54**: p. 832–842.

214. Farber, E., *Programmed cell death: necrosis versus apoptosis.* Mod Pathol 1994. **5**: p. 605-609.

215. Yuan, J., Wang, MX., Murrell, G., *Cell death and tendinopathy.* Clin Sports Med, 2003. **22**: p. 693– 701.

216. Rumian, A., Draper, ERC., Wallace, AL., Goodship, AE., *The influence of the mechanical environment on remodelling of the patellar tendon.* Jounal of Bone Joint Surgery (Br), 2009. **9**(4): p. 557-564.

217. Banes, A., Tsuzaki, M., Hu, P., Brigman, B., Brown, T., Almekinders, L., Lawrence, WT., Fischer, T., *PDGF-BB, IGF-I and mechanical load stimulate DNA synthesis in avian tendon fibroblasts in vitro.* J. Biomech, 1995a. **28**(12): p. 1505–1513.

218. Wall, M., Banes, AJ., *Early responses to mechanical load in tendon: role for calcium signaling, gap junctions and intercellular communication.* J Musculoskelet Neuronal Interact. , 2005. **5**(1): p. 70-84.

219. Arnoczky, S., Lavagnino, M., Whallon, JH., Hoonjan, A., *In situ cell nucleus deformation in tendons under tensile load; a morphological analysis using confocal laser microscopy.* J Orthop Res 2002. **20**: p. 29-35.

220. Frost, H.M., *Bone "mass" and the "mechanostat": a proposal.* Anat Rec 1987. **219**(1): p. 1-9.

221. Frost, H., *Bone's mechanostat: a 2003 update.* Anat Rec A Discov Mol Cell Evol Biol, 2003. **275**(2): p. 1081-101.

222. Brown, R., Prajapati, R., Mcgrouther, DA., Yannas, IV, and Eastwood, M., *Tensional Homeostasis in Dermal Fibroblasts: Mechanical Responses to Mechanical Loading in Three-Dimensional Substrates.* Journal of cellular physiology, 1998. **175**: p. 323–332

223. Chicurel, M., Singer, RH., Meyer, CJ., *Integrin binding and mechanical tension induce movement of mRNA and ribosomes to focal adhesions.* Nature, 1998. **392**: p. 730–3.

224. Arnoczky, SP., Lavagnino, M., Whallon, JH., Hoonjan, A., *In situ cell nucleus deformation in tendons under tensile load; a morphological analysis using confocal laser microscopy.* J Orthop Res 2002. **20**: p. 29-35.

225. Ingber, D., *Tensegrity: the architectural Basis of cellular Mechanotransduction.* Annu. Rev. Physiol. , 1997. **1997**: p. 575–99.

226. Ingber, D., *lntegrins as mechanochemical transducers.* Current Opinion in Cell Biology 1991. **3**: p. 841-848.

227. Banes, A., Tsuzaki, M., Yamamoto, J., Fischer, T., Brigman, B., Brown, T., Miller, L., *Mechanoreception at the cellular level: the detection, interpretation, and diversity of responses to mechanical signals.* Biochem Cell Biol. 1995b. **73**(7-8): p. 349-65.

228. Alberts, B., Johnson, A., Lewis, J., *Molecular biology of the cell.* Garland Science, 2002.

229. Ingber, D., *The riddle of morphogenesis: A questions of solution chemistry or molecular cell engineering* Cell 1993. **75**: p. 1249-1252.

230. Hayashi, R., Kondo, E., Tohyama, H., Saito, T., Yasuda, K., *In vivo local administration of osteogenic protein-1 increases structural properties of the overstretched anteriorcruciate ligament with partial midsubstance laceration: a biomechanical study in rabbits.* J Bone Joint Surg [Br] 2008. **90b**: p. 1392-400.

231. Riley, G., Currya, V., DeGrootb, J. van Elb, B., Verzijlb, N., Hazlemana, BL., Bankb, RA., *Matrix metalloproteinase activities and their relationship with collagen remodelling in tendon pathology.* Matrix Biology 2002. **21**: p. 185–195.

232. Devkota, A., Weinhold, PS., *Mechanical response of tendon subsequent to ramp loading to varying strain limits.* Clinical Biomechanics, 2003. **18** p. 969–974.

233. Harris, A.K., Stopak,D.,Wild,P., *Fibroblast traction as a mechanism for collagen morphogenesis.* Nature 1981. **290**: p. 249–251.

234. Grinnell, F., Lamke,C.R., *Reorganization of hydrated collagen lattices by human skin fibroblasts* J.CellSci., 1984. **66**: p. 51–63.

235. Stopak, D., Harris, AK., *Connective tissue morphogenesis by fibroblast traction. Tissue culture observations.* Dev. Biol., 1982. **90**: p. 383–398.

236. Sawhney, RK., Howard, J.,2002., *Slow local movements of collagen fibers by fibroblasts drive the rapid global self-organization of collagen gels.* J.CellBiol. , 2002. **157**: p. 1083–1091.

237. Guidry, C., and Grinnell,F., *Studies on the mechanism of hydrated collagen gel reorganization by human skin fibroblasts.* J.CellSci, 1985. **79**: p. 67–81.

238. Meshel, A., Wei, Q., Adelstein, RS., Sheetz, MP., *Basic mechanism of three-dimensional collagen fibre transport by fibroblasts.* Nat.CellBiol., 2005. **7**: p. 157–164.

239. Diamant, J., Keller, A., Baer, E., Litt, M., Arridge, RG., *Collagen; ultrastructure and its relation to mechanical properties as a function of ageing* Proc R Soc Lond B Biol Sci 1972. **180**(60): p. 293–315.

240. Hansen, K., Weiss, JA., Barton, JK., *Recruitment of tendon crimp with applied tensile strain.* J Biomech Eng 2002. **124**(1): p. 72–77.

241. Sasaki, N., Odajima, S., *Elongation mechanism of collagen fibrils and force-strain relations of tendon at each level of structural hierarchy* J Biomech 1996. **29**(9): p. 1131–1136.

242. Herchenhan, A., Kalson, NS., Holmes, DE., Hill, P., Kadler, KE., Margetts, L., *Tenocyte contraction induces crimp formation in tendon-like tissue.* Biomech Model Mechanobio, 2011. **11**: p. 449-459.

243. Shah, J., Palacios, E., Palacios, L., *Development of crimp morphology and cellular changes in chick tendons. .* Dev Biol, 1982. **94**(2): p. 499–504.

244. Foolen, J., vanDonkelaar, C., Soekhradj-Soechit, S., Ito, K., *An adaptation mechanism for fibrous tissue to sustained shortening.* Journal of Biomechanics, 2010. **43**: p. 3168–3176.

245. Tomiosso, T., Nakagaki, WR., Gomes, L., Hyslop, S., Pimentel, ER., *Organization of collagen bundles during tendon healing in rats treated with L-NAME.* Cell and Tissue Research, 2009. **337**(2): p. 235-242.

246. Fung, D., Wang, VM, Laudier, DM., *Subrupture tendon fatigue damage.* J Orthop Res, 2009. **27**: p. 264–273.

247. Arnoczky, S., Lavagnino, M., Egerbacher, M., Caballero, O., Gardner, K., *Matrix Metalloproteinase Inhibitors Prevent a Decrease in the Mechanical Properties of Stress-Deprived Tendons An In Vitro Experimental Study.* Am J Sports Med 2007 **35**(5): p. 763-769.

248. Sereysky, J., Andarawis-Puri, N., Ros, S., Flatow, EL., *Automated image analysis method for quantifying damage accumulation in tendon.* Journal of Biomechanics, 2010. **43**: p. 2641–2644.

249. Wood, T., Cooke, PH., Goodship, AE., *The effect of exercise and anabolic steroids on the mechanical properties and crimp morphology of the rat tendon.* Am J Sports Med, 1988. **16**(2): p. 153–158.

250. Magnusson, S., Narici, MV., Maganaris, CN., Kjaer, M., *Human tendon behaviour and adaptation, in vivo.* J Physiol, 2008. **586**(1): p. 71–81.

251. Flint, M., Gillard, GC., and Menilees, MJ.,, *Fibrous Proteins:* Scientific, Industrial and Medical Aspects, , 1980. **2**: p. 107-119.

252. Nagel, T., Kelly, DJ., *Remodelling of collagen fibre transition stretch and angular distribution in soft biological tissues and cell-seeded hydrogels.* Biomech Model Mechanobiol 2012. **11**: p. 325–339.

253. Thornton, G., Shrive, NG., Frank, CB., *Healing ligaments have decreased cyclic modulus compared to normal ligaments and immobilization further compromises healing ligament response to cyclic loading.* J Orthop Res. , 2003. **21**(4): p. 716-722.

254. Manske, P., and Leske, PA., *Biochemical Evidence of Flexor Tendon Participation in the Repair Process An in Vitro Study.* J Hand Surg [Br] 1984. **9**: p. 117.

255. McCarthy, D., Tramaglini, DM., Chan, SS., Schmidt, CC., Sotereanos, DG., Herndon, JH., Pittsburgh, PA., *Effect of Partial Laceration on the Structural Properties of the Canine FDP Tendon: An In Vitro Study.* J Hand Surg, 1995. **20A**: p. 795-800.

256. Russell, J., and Manske, PE., *Collagen Synthesis During Primate Flexor Tendon Repair In Vitro.* Journal of Orthopaedic Research, 1990. **8**: p. 13-20.

257. Russell, J.E., and Manske, PR., *Ascorbic Acid Requirement for Optimal Flexor Tendon Repair in Vitro.* Journai of Orthopaedic Research, 1991: p. 9714-719.

258. Hopfner, M., Windolf, J., Logters, TT., Hakimi, M., Celik, I., *Flexor tendon repair using a new suture technique: A comparative in vitro biomechanical study.* Eur J Trauma Emerg Surg, 2011. **37**: p. 79-94.

259. Everett, E., Barrett, JG., Morelli J., and DeVita R., *Biomechanical Testing of a Novel Suture Pattern for Repair of Equine Tendon Lacerations.* Veterinary Surgery, 2012. **41**: p. 278-285.

260. Mass, D., Martell, J., Tuel, R., *Human flexor tendon healing in vitro.* Orthop Trans 1988. **12**(9).

261. Matthews, P., Richards, H.,, *The repair potential of digital flexor tendons. An experimental study.* . J Bone Joint Surg [Br], 1974. **56**: p. 618-625.

262. Lundborg, G., Rank, F., *Experimental studies on cellular mechanisms involved in healing of animal and human flexor tendon in synovial environment* Hand 12, 1980. **12**: p. 3-11.

263. Lundborg, G., *Experimental flexor tendon healing without adhesion formation- a new concept of tendon nutrition and intrinsic healing mechanisms.* The Hand, 1976. **8**(3).

264. Lundborg, G., Rank, F., *Experimental intrinsic healing of flexor tendon based on synovial fluid nutrition.* J Hand Surg, 1978. **3**: p. 21-31.

265. Abercrombie, M., Heaysman, JEM., and Pegrum, S., *The locomotion of fibroblasts in culture.II. "ruffling".* Exper Cell Res, 1970. **60**: p. 437-444.

266. Peter, D., and Prockop, DJ., *Synthesis and Extrusion ofCollagen by Freshly Isolated Cells from Chick Embryo Tendon* Biochim Biophys Acta, 1971. **240**: p. 358-369.

267. Gelberman, R., Manske, PR., Vande Berg, JS., Lesker, PA., and Akeson, WH., *Flexor Tendon Repair In Vitro: A Comparative Histologic Study of the Rabbit, Chicken, Dog, and Monkey* J Orthop Res, 1984. **2**: p. 39-48.

268. Graham, M., Becker, H., Cohen, K., Merritt, W., and Diegelmann, RF., *Intrinsic Tendon Fibroplasia: Documentation by In Vitro Studies.* Journal of Orthopaedic Research, 1984. **1**: p. 251-256.

269. Oshiro, W., Lou, J., Xing, X.,Tu, Y., Manske, PR., *Flexor tendon healing in the rat: a histologic and gene expression study.* The Journal of Hand Surgery, 2003. **28**(5): p. 814-823.

270. Tanaka, H., Manske, PR., Pruitt, DL., Larson, BJ., Louis, St., *Effect of Cyclic Tension on Lacerated Flexor Tendons in vitro.* J Hand Surg 1995. **20A**: p. 467 473.

271. Rank, F., Eiken, O., Bergenholtz, A., Lundborg, G., Erkel, LJ., *Flexor tendon specimens in organ cultures.* Scand J Plast Reconstr Surg. , 1980. **14**(2): p. 179-183.

272. Asundi, K., Kursa, K., Lotz, J., and Rempel, DM., *In Vitro System for Applying Cyclic Loads to Connective Tissues Under Displacement or Force Control.* Annals of Biomedical Engineering. , 2007. **35**(7): p. 1188–1195.

273. Wiig, M., Abrahamsson, SO., Lundborg, G., *Tendon Repair--Cellular Activities in Rabbit Deep Flexor Tendons and Surrounding Synovial Sheaths and the Effects of Hyaluronan: An Experimental Study in Vivo and in Vitro.* J Hand Surg1997. **22A**: p. 818-825.

274. Iwuagwu, F., McGrouther, DA., *Early Cellular Response in Tendon Injury: The Effect of Loading.* Plastic & Reconstructive Surgery, 1998. **102**(6): p. 2064.

275. Boyer, M., Gelberman, R., Burns, M., Dnopolous, H., Hofem, R., Silva, M., *Intrasynovial flexor tendon repair: An experimental study comparing low and high levels of in vivo force during rehabilitation in canines* JBJS 2001. **83a**(891-899).

276. Morizaki, Y., Zhao, C., An, KN., Amadio, PC., *The Effects of Platelet-Rich Plasma on Bone Marrow Stromal Cell Transplants for Tendon Healing In Vitro.* The Journal of Hand Surgery, 2010. **35**(11): p. 1833-1841.

277. Kubota, H., Manske, PR., Aoki, M., Pruitt, DL., Larson, BJ., *Effect of Motion and Tension on Injured Flexor Tendons in Chickens.* J Hand Surg, 1996. **21a**: p. 456-463.

278. Sharma, P, and Maffulli, N., *Tendon injury and tendinopathy: healing and repair.* JBJS, 2005. **87**(1): p. 187-202.

279. Mass, D., Tuel, R., Labarbera, M., Greenwald, D., *Effects of Constant Mechanical Tension on the Healing of Rabbit Flexor Tendons.* Clinical Orthopaedics & Related Research, 1993. **296**.

280. Becker, H., Diegelmann, RF., *The influence of tension on intrinsic tendon fibroplasia.* . Orthop Rev 1984. **13**: p. 153-159.

281. Wray, R., Ollinger, H., Lowrey R., and Weeks, PM., *Effect of continuous load on the mechanical properties of tendon adhesions.* The Hand, 1981. **13**(1).

282. Bosch, G., van Schie, H., de Groot, MW., Cadby, JA., van de Lest, CHA., Barneveld, Ab., van Weeren, PR., *Effects of Platelet-Rich Plasma on the Quality of Repair of Mechanically Induced Core Lesions in Equine Superficial Digital Flexor Tendons: A Placebo-Controlled Experimental Study.* J Orthop Res, 2010. **28**: p. 211–217, .

283. Danielsen, C., *Mechanical properties of reconstituted collagen fibrils. Influence of a glycosaminoglycan: Dermatan sulfate.* Connect Tissue Res 1982. **9**(4): p. 219–225.

284. Greenlee, T., and Pike, D., *Studies of tendon healing in the rat: Remodeling of the Distal Stump after Severance.* Plastic & Reconstructive Surgery, 1971. **48**(3): p. 260-270.

285. Sandhu, E., Mile, JD., Dauhners, LE., Keller, BV., Weinhold, PS., *Whole body vibration increases area and stiffness of the flexor carpi ulnaris tendon in the rat* J of Biomechanics, 2011. **44**: p. 1189-1191.

286. Takai, S., Woo, SL., Horibe, S., Tung, DK., Gelberman, RH., *The effects of frequency and duration of controlled passive mobilization on tendon healing.* J Orthop Res, 1991. **9**(5): p. 705-13.

287. Ditsios, K., Burns, ME., Boyer, MI., Gelberman, RH., Silva, MJ., *The rigidity of repaired flexor tendons increases following ex vivo cyclic loading.* Journal of Biomechanics 2002. **35**: p. 853–856.

288. Karpakka, J., Vaananen, K., Virtanen, P., Savolainen, J., Orava, S., Takala, TES., *The effects of remobilization and exercise on collagen biosynthesis in rat Tendon* Acta Physiol Scan, 1990. **139-145**: p. 139-145.

289. Woodley, D., O'Keefe, EJ., Prunieras, M., *Cutaneous wound healing: a model for cell-matrix interactions.* J Am Acad Dermatol. , 1985. **12**: p. 420-433.

290. Busse, J., Kaur, J., Mollon, B., Bhandari, M., Tornetta, P., Schünemann, HJ., Guyatt, GH., *Low intensity pulsed ultrasonography for fractures: systematic review of randomised controlled trials.* British Medical Journal, 2009. **338:b351**.

291. Jarvinen, M., Jozsa, L., Kannus, P., Jarvinen, TL., Kvist, M., Leadbetter, W., *Histopathological findings in chronic tendon disorders.* Scand J Med Sci Sports 1997. **7**: p. 86–95.

292. Kajaer, M., *Role of Extracellular Matrix in Adaptation of Tendon and Skeletal Muscle to Mechanical Loading.* Physiol Rev 84: 2004. **84**: p. 649–698.

293. Miller, K., Edelstein, L., Connizzo, BK., Soslowsky, LJ., *Effect of Preconditioning and Stress Relaxation on Local Collagen Fiber Re-Alignment: Inhomogeneous Properties of Rat Supraspinatus Tendon.* Journal of Biomechanical Engineering, 2012. **134**(3): p. 1-6.

294. Teramoto, A., Ping Luo, Z., *Temporary tendon strengthening by preconditioning.* Clinical Biomechanics, 2008. **23**: p. 619-622.

295. Birch, H., Wilson, AM., AND Goodship, AE., *The effect of exercise-induced localised hyperthermia on tendon cell survival.* The Journal of Experimental Biology, 1997. **200**: p. 1703–1708

296. Birch, H., Wilson, AM., Goodship, AE., *The effect of exercise-induced localised hyperthermia on tendon cell survival.* Journal of Experimental Biology, 1997. **200**(11): p. 1703-8.

297. Rubin, C., and Lanyon, LE., *Dynamic strain similarity in vertebrates; an alternative to allometric limb bone scaling.* J Theor Biol.1984. **107**: p. 321–327.