# Platelet Rich Plasma and Mechanical Loading in Regenerative Tendon Repair

Nasim Zargar Baboldashti

Lady Margaret Hall


Supervisors:

Dr Mark S. Thompson

Dr Philippa A. Hulley

Department of Engineering Science

University of Oxford

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## Abstract

Tendon injuries and tendinopathy are a growing problem in the aging but physically active population as well as athletes. Tendons have highly ordered matrix and undergo complex changes during the remodelling phase of tendon healing. Moreover, anaerobic metabolism and poor vascular network contribute to slow adaptation of tissue to the remodelled matrix which consequently results in slow and compromised healing. Such a destitute and slow healing process necessitates development of new and effective therapies and to combine therapies to obtain possibly synergistic effects.

Addressing this clinical requirement, the work presented in this thesis investigates the role of two emerging treatment options, platelet rich plasma (PRP) and mechanical loading, on tendon healing.

The effects of PRP, a rich autologous source of growth factors, on tendon cells was studied by modelling important stages of tendon healing *in vitro*. Key parameters such as cellular migration, chemotaxis, viability and senescence were investigated by means of different culturing and staining techniques together with microscopic analyses.

PRP significantly increased migration and chemotaxis in human primary tenocyte culture. Moreover, PRP protected human tenocytes against challenging environments created by known tendon damaging drugs, dexamethasone and ciprofloxacin, as well as the injury relevant condition of hypoxia.

Concurrently, an *in vitro* rat tail tendon injury model and static loading device was developed to assess the effect of static mechanical loading and PRP on the biochemical and biomechanical properties of tendon at the tissue level. This *in vitro* system was also used to investigate the synergistic effects of PRP and mechanical loading on tendon healing. Both PRP and mechanical loading helped to improve the biomechanical and biochemical properties of damaged tendon *in vitro*.

In conclusion, the positive effects of PRP on key cellular parameters such as cell survival, migration and chemotaxis and also mechanical and biochemical properties of tendon tissue make it an important option for faster and less invasive tendon treatment. Additionally, an *in vitro* tendon injury model together with the mechanical loading device provide a new tool to investigate the mechanical boundary conditions suitable for treating different types of tendon disorders.

The findings from the current study points towards the significant contribution of PRP and mechanical loading to the healing process in tendons and could serve as a promising starting point for developing integrated therapeutic modalities to improve the quality and speed of recovery from tendon injury.

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## Publications and Contributions

The following peer-reviewed papers were written and presented during and based upon the contents of this D.Phil thesis:

**Journal Papers:**

**Zargar Baboldashti N,** Poulsen RC, Franklin SL, Thompson MS, Hulley PA, Platelet Rich Plasma Protects Tenocytes from Adverse Side Effects of Dexamethasone and Ciprofloxacin, American Journal of Sports Medicine, 2011;39(9):1929-1935

**Zargar Baboldashti N**., Thompson MS., Hulley PA., PRP induces migration and chemotaxis in human tenocytes , *Manuscript in preparation*

**Zargar Baboldashti N**., Hulley PA., Thompson MS., PRP improves the mechanical properties of injured tendon *in vitro. Manuscript in preparation*

Liang M, Cornell H, **Zargar Baboldashti N.,**Thompson MS., Carr AJ.,HulleyPA., Regulation of hypoxia induced cell death in human tenocytes, *In revision Journal of cell biology*.

Franklin SL., **Zargar Baboldashti N.,** Thompson MS., Hulley PA., Intracellular Signalling of Tendon Cells Subjected to Factors Released by Activated Platelets as Found in Platelet-Rich Concentrate, *Manuscript in preparation*

**Conference Papers**

**Zargar Baboldashti N,** Poulsen RC, Franklin SL, Thompson MS, Hulley PA, Platelet Rich Plasma Protects Tenocytes from Drug-induced Senescence and Death, Tissue Engineering and Regenerative medicine (TERMIS) 2011, Poster Presentation

**Zargar Baboldashti N,** Poulsen RC, Franklin SL., Thompson MS, Hulley PA., The effect of PRP in Protection and Migration of Tenocytes (TERMIS) 2010, Oral Presentation and Best Abstract Award

Franklin SL., **Zargar Baboldashti N,** Philippa Hulley, Mark Thompson, Human Tenocyte Cellular Response to Platelet-Rich Concentrate, (TERMIS)2010 Poster

Franklin SL., **Zargar Baboldashti N**., Willett K., Hulley P., Thompson M. (2010) Human tenocyte cellular signalling, Bors 2010, Oral presentation

## Contents

# Chapter 1 General introduction

## 1.1 Motivation and scope of the study

Tendons are uniaxial connective tissues interposed between muscles and bones. They transmit contractile forces created in the muscle to bone, resulting in joint movement.

Tendon injuries are frequent and are responsible for considerable morbidity in athletic and occupational settings. Although most tendons have the ability to heal after injury, biochemical and biomechanical properties of healed tendon appear never to match those of intact tendon and the repaired tissue has increased risk of further injury[256, 293]. This limited healing capacity appears to be caused in part by poor vascularisation of this connective tissue [89, 223]. Therefore even with the best available treatment strategies tendon injuries give rise to substantial morbidity that may last for several months and cause a considerable challenge for clinicians [254].

The prevalence of orthopaedic disorders, including tendon diseases, is increasing considerably as the population ages and remains active [228]. Fast recovery from tendon injuries with less invasive treatment methods is of great importance particularly in the elite athletes. With this prospect, novel therapies such as platelet rich plasma (PRP) are being used as a potential standalone or adjunctive treatment for tendon injuries.

Platelets play a crucial role in the early steps of healing by releasing different growth factors and proteins. It has been suggested that the secreted growth factors are responsible for enhancing cell proliferation, inducing chemotaxis and cell migration in

various tissues. Moreover, they are involved in synthesizing essential proteins such as collagen as well as revascularising the damaged area [125, 169, 185, 191].

The use of autologous platelets in the form of PRP has emerged as a promising treatment option for tendon disorders. However, biological studies and clinical trials conducted to understand the role of PRP in tendon healing are all at an early stage and far from conclusive. Therefore, it is essential to understand the complete mechanism behind PRP function and its influence on human tendon cells and tissue during different stages of healing. This can be addressed by investigating the key parameters involved in tissue healing such as cellular proliferation, viability and migration with the help of tissue culture techniques, biochemical assays and microscopic analyses.

As well as restoring mechanical function, the healing process is known to be influenced by mechanical stimuli. It has been shown that mechanical stimulation is an effective contributing factor in tendon healing and the absence of mechanical stimulation is damaging [23]. Research into understanding the influence of the mechanical environment on tendon healing has been carried out for several years [293, 310]. However, our knowledge on the mechanisms responsible for this effect is limited and there is no conclusive study on the appropriate dose and duration of mechanical stimulation for tendon healing. Therefore, the scope for further investigation on the role of mechanical stimulation in improving tendon healing is immense.

Obtaining *in vitro* biomechanical and biochemical information on cultured tendon tissue would enormously improve our understanding of tissue response to mechanical stimulation and PRP therapy at different stages of healing. However, most *in vitro* studies on tendon biomechanics have been performed on dead animal tissue or human cadavers [252, 255]. Therefore, it is clearly important to design a mechanical set-up

which enables us to culture healthy and injured tendon fascicles and also to optimise the mechanical testing protocols which determine the effect of mechanical loading together with PRP therapy on the structural and functional properties of cultured tendon.

The remainder of this chapter aims to give an introduction to the three key areas related to the work that has been undertaken in this thesis: 1) The relationship between tendon physiological background and its function, 2) Tissue hierarchy and tendon mechanical properties and 3) PRP therapy. The model systems used in this study and the aims and outline of the thesis have also been discussed.

## 1.2 Tendon physiological background

It is necessary to investigate the relationship between biochemical structure and physiological function of connective tissues in order to understand their biomechanical properties. Physically, healthy tendons appear as glistening white bands with a fibrous texture. Biochemically, collagen is the dominant component of tendon tissue, but also other structural proteins such as proteoglycans and glycoproteins exist in the surrounding extracellular matrix.

Tendons are considerably varied in shape and in the way that are attached to bone and muscles. Tendon shape ranges from cylindrical to flattened ribbons. The tendons for large, powerful muscles like the quadriceps, are short and broad whereas those attached to muscles which control delicate movements such as finger flexors are long and thin [33, 128].

Tendons are connected by functionally specialized regions to bone and muscle at their ends. The myotendinous junction (MTJ) transfers forces created in muscles to

the tendon [35] via the deep recesses formed by myofibroblasts in the muscle [155, 270]. The structure of MTJ decreases the tensile stress applied on tendon tissue during the muscle contraction.[122].

The tendon-bone junction or enthesis can be divided into two types; the fibrous enthesis and the fibrocartilaginous enthesis. In a fibrous enthesis, the tendon is directly attached to the bone whilst in the fibrocartilaginous enthesis, a transitional zone of fibrocartilage which is important in distributing mechanical load exists. Each of these zones is specialized to prevent bending, shearing and failure of collagen molecule or fibres [34].

The complex structure of tendons at both micro and macro levels mean that the tissue is able to withstand high and varying mechanical demands to which it is subjected during activities of everyday living. Tendons are exposed to longitudinal, transverse and torsion forces *in vivo*. However, in the majority of tendons tensile stress along the longitudinal axis is the greatest; therefore most of the collagen fibres are aligned longitudinally in the tensile regions.

Collagen fibres can take up a variety of fibre-crossing arrangements in order to adapt to their mechanical environment. Images from scanning electron microscopy (SEM) and transmission electron microscopy (TEM) show that beside the longitudinal orientation, collagen fibres can run transversely and horizontally creating plaits and spirals [221]. Figure 1.1 shows electron microscopy images of mature rat tail tendon, the tissue which has been used as a model system in this study.

### 1.2.1 Tissue hierarchy

The tendon has a hierarchical structure composed of collagen molecules, fibrils, fibre bundles (primary bundles), fascicles (secondary bundles) and tendon units that

align with the long axis of tendon. This hierarchical structure, shown in figure 1.2, begins at the molecular level with tropocollagen, a triple- helix polypeptide chain. The soluble tropocollagen molecules structure cross-links to form insoluble collagen molecules. The insoluble collagen molecules aggregate to create collagen fibrils. Fibril diameters range from 10 to 500 nm and largely depend on species, age and the anatomical location of the tendon [182, 195]. A collagen fibre is formed from a collection of fibrils and is the smallest tendon unit which can be used for mechanical testing and is detectable under light microscopy [253, 295]. However, a larger fibre bundle makes testing more reliable [204].

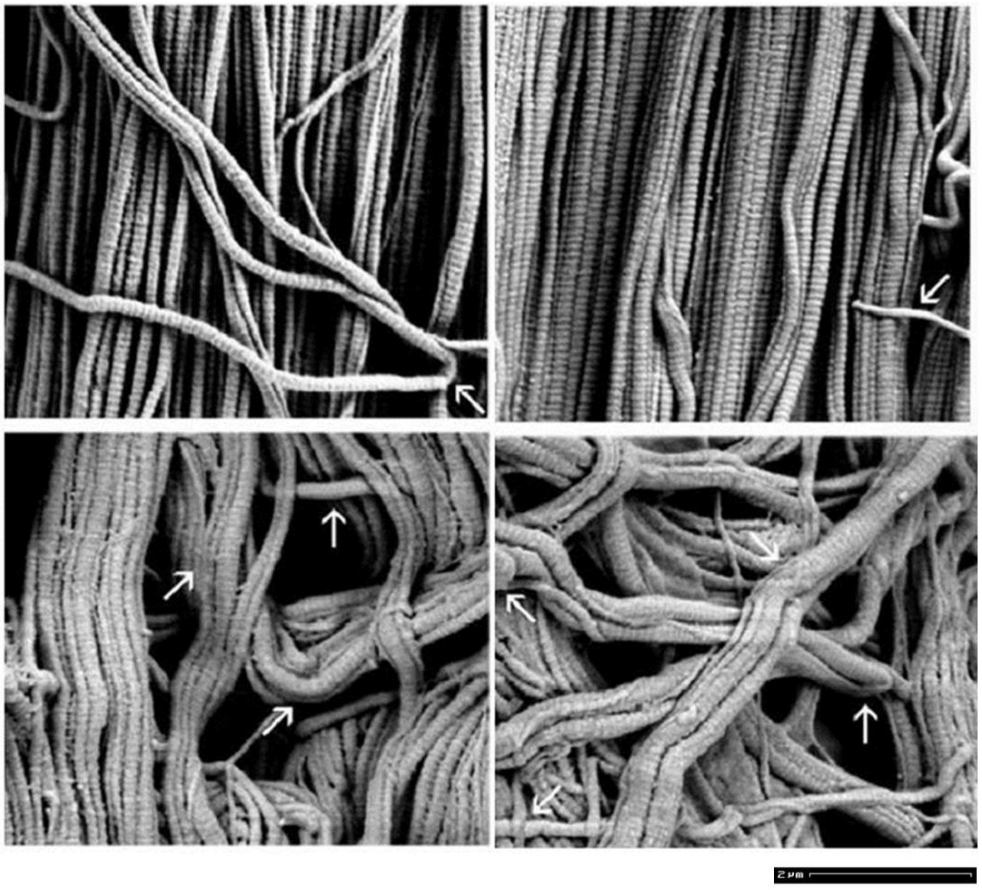

**Figure 1.1** Electron microscopy images of collagen fibril in mature tendon. Variety of fibre arrangements are present (scale bar = 2µm). Figure adapted from Provenzano and Vanderby, 2006 [221].

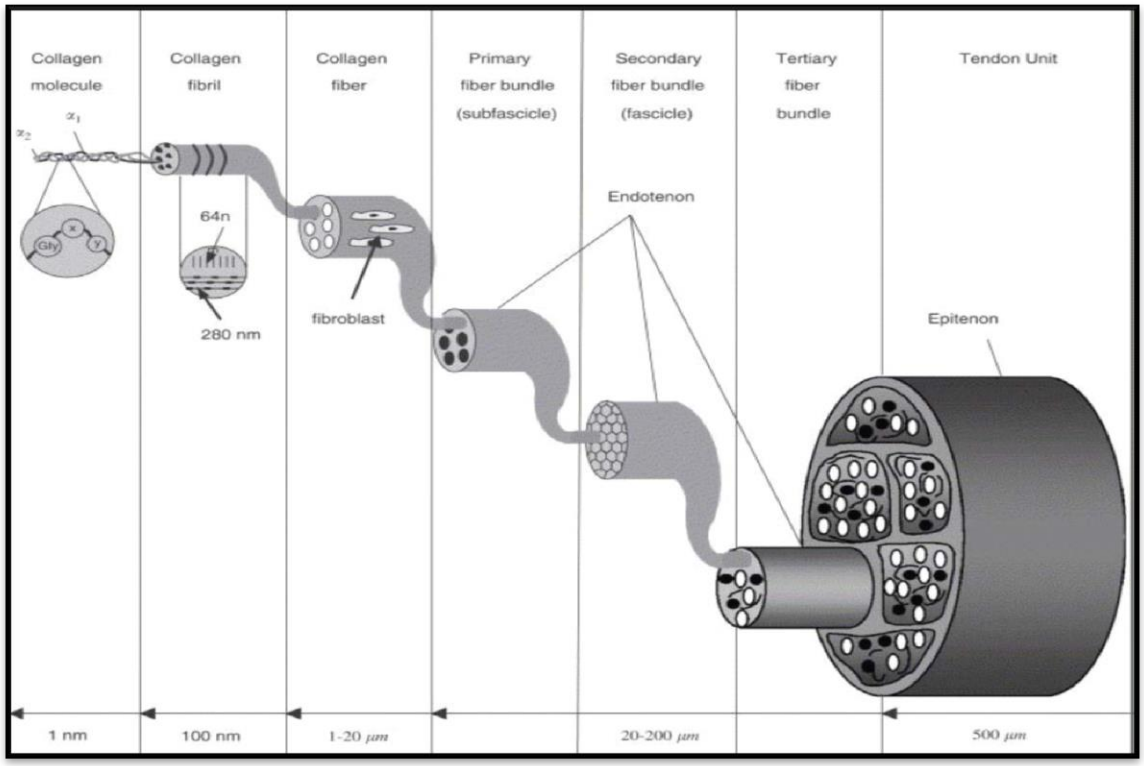


Figure 1.2: Schematic description of the hierarchical arrangement of tendon. Adapted from Kastelic *et al.*, 1978 [133] and Wang *et al.*, 2006 [293] .

A fine sheath of connective tissue which is referred to as epitenon and contains blood vessels, lymphatics and nerves encloses the tendon body. A thin sheath of connective tissue, endotenon, encloses collagen fibres and binds the fibres together [128, 133, 252, 253]. Tendons are also enclosed by paratenon which is a loose areolar connective tissue and superficially surrounds epitenon. The paratenon tissue is largely made of type I and type III collagen fibrils and synovial cells can be found in its inner lining. The interactions at different levels of tendon hierarchy determine the biomechanical and biochemical properties of the tissue.

The surrounding structures of tendons also have a significant role in mechanical function of the tissue. Synovial sheaths comprise an outer fibrotic sheath and inner

synovial sheath that produces synovial fluid [75]. Synovial sheaths are present in the sites such as hands and feet to provide essential lubrication for the tissue.

### 1.2.2 Tendon blood supply

Immature tendons have high metabolic activity and are therefore supplied with rich vascular networks [211]. In contrast mature tendons have poor vascular networks and are mainly dependent on synovial fluid diffusion for their nutrition.

Tendon blood supply is usually provided from three different sources: the intrinsic sources, myotendinous junction and osteotendinous junction (enthesis), and the extrinsic system through the surrounding connective tissues such as paratenon, mesotenon or synovial sheath [267]. Vessels originating from myotendinous junction run longitudinally and are unlikely to extend through the whole length of the tendon. Tendons also receive limited blood supply at the tendon insertion zone of osteotendinous junctions. Branches from major arteries which pass through meseotenon and reach to the synovial sheath are responsible for contributing to the blood supply of the superficial part of tendon. In the absence of the synovial sheath the vascular supply of tendon is provided by the paratenon vascular network.

Regions of reduced vascularity exist at junctional zones and sites of torsion and compression in tendons. In addition, tendons such as supraspinatus, biceps tendon and Achilles tendon appear to have regions with compromised vascularity [89]. These regions of reduced vascularity have been commonly linked to tendon degeneration and rupture. However, there is no solid evidence on the role of hypovascularity in predisposing tendons to degeneration, as not all the hypovascular zones in tendons have the same predisposition to pathology.

### 1.2.3 Tendon innervation

Tendon innervation originates from three main sources: cutaneous, muscular, and peritendinous nerve trunks. Nerve fibres cross paratenon and some of them branch through epitenon. At the myotendinous junction nerve fibres cross tendon sheaths and penetrate endotenon septa. However, the majority of tendon nerves do not penetrate the main body of tendon and function as mechanoreceptors, the Golgi tendon organs, on the surface of the tissue. A golgi tendon organ is a thin fibrous capsule which contains a group of myelinated nerve fibres and is mostly found at tendon-muscle insertion sites.

### 1.2.4 The extracellular matrix and tendon cells

The tendon extracellular matrix (ECM) provides support for the resident cells, and variations in its complex structure and in the proportion of its constituents determine the local physical properties of the tissue.

Approximately 70wt% of tendon is water. Collagen accounts for 65% to 80% of the dry mass of tendon tissue. Collagen type I is the most abundant type of collagen in tendon and compromises about 95% of the total collagen content. The remaining 5% of collagen content is mainly collagen types III and V with other collagens including types II, IX, VI, X and XI present in small but traceable quantities [76]. Another major protein of tendon ECM is elastin which accounts for about 2% of tendon dry mass [128, 204].

Proteoglycans (PGs), together with glycoproteins and other small molecules comprise the ground substance which surrounds and supports collagen fibres in connective tissues [204, 271]. Proteoglycans are heavily glycosylated and hydrophilic proteins which are involved in the diffusion of water-soluble molecules and facilitate cell migration. Different types of proteoglycans such as aggrecan and decorin are

present in different quantities at load bearing sites of tendon. Also, glycoproteins such as fibronectin, thrombospondin and tenascin-C are present in tendon ECM and participate in the processes responsible for tissue regeneration [60, 128]. It has also been shown that tenascin-C functions as an elastic protein and may contribute to the mechanical stability of ECM by configuring collagen fibre orientation and alignment [171].

All these elements are produced by tenocytes and tenoblasts that lie between the collagen fibres. Tenoblasts and tenocytes are the main types of cells in the tendon extracellular matrix and make up to 90-95% of the cellular elements within the tendon.

Other cellular elements such as chondrocytes, synovial cells and vascular cells are also present in tendons and constitute about 5 to 10% of the tissue cellular content. Inflammatory cells and macrophages can be found in healing tendon or in various pathological conditions [126].

The size and shape of tendon cells undergo different changes during tissue development. Immature tendon cells, tenoblasts, have high cell-to-matrix ratio and metabolic activity and are spindle-shaped. However, as tendon ages the mature cells, tenocytes, become elongated and their metabolic activity decreases. [128].

Tenocytes are located along and between collagen fibres and are responsible for synthesis of collagen and secretion of other matrix components. Tendon cells are active in energy production through the aerobic Krebs cycle, anaerobic glycolysis, and the pentose phosphate shunt [153, 254].

Tendons use approximately 7.5 times lower oxygen compared to skeletal muscles [282]. The low metabolic rate of the tendon tissue together with the well-developed anaerobic energy production pathways is thought to facilitate tendon

function while exposing to mechanical loading and tension for long periods of time with no risk of ischemia and necrosis. However, a likely drawback of a low metabolic rate is slow healing and recovery after tendon injury.

## 1.3 Biomechanical properties

The complex hierarchical structure of tendon provides the mechanical strength and flexibility necessary for the tissue to withstand high mechanical demands. It is also known that tendons change their structure, composition and mechanical properties in response to changes in their mechanical environment [175] and further that the mechanical conditions prevailing during the healing process have a major influence on the clinical outcome [293].

As a result of this adaptive response, mechanical loading is considered as a significant contributing factor to the healing process and also a major causative factor in disorders associated with the pathological changes in tendons.

Tendons are responsible for transmitting forces from muscle to bone and hence are subject to constant dynamic mechanical loading *in vivo*. The unique mechanical behaviour of tendon depends on the alignment and orientation of fibres at the tissue level and the number and types of collagen cross-links at the molecular level. Figure 1.3 shows a typical stress-strain curve that helps to characterize the mechanical behaviour of the tendons. At rest, collagen fibres and fibrils display a wavy or crimped configuration. The initial concave portion of the stress-strain curve, which is also referred to as toe region, represents the stretching-out of the crimp configuration of fibres, where the tendon is strained up to 2%.

Mechanical properties of tendons are believed to be influenced by differences in the crimp pattern angle and length, for example fibres which have a small crimp angle fail before those with a larger crimp angle [302, 310, 311]. Beyond the toe region, the straightened collagen fibres lose their crimp pattern as a result of intramolecular sliding of collagen triple helices and gradually transit into the linear region. The slope of this linear region is referred as the Young's modulus, *(E),* a measure of stiffness of the tendon which is dominated by the material behaviour of the straightened collagen.

$$E = \frac{Tensile\ stress}{Tensile\ strain} = \frac{\sigma}{\epsilon} = \frac{\frac{F}{Ao}}{\frac{\Delta L}{Lo}} = \frac{FL.}{A.\Delta L}$$

Where F is the force applied, $A_o$ is the original cross-sectional area through which the force is applied, ΔL is the change in length of the tissue, and $L_o$ is the original length of the tissue. The stiffness of the sample with a uniform Young's modulus is given by:

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

Where A is the cross-sectional area and E is the Young's modulus.

In the linear region of the stress-strain curve, if the strain remains less than 4% tendon behaves elastically and returns to its original length when the force is removed. However, if the tissue stretches over 4% microscopic failure of the tendon fibres occurs. Beyond 8% to 10% widespread microscopic failure of fibre bundles occurs in a stochastic manner as a result of the intrafibril molecular slippage with the weaker collagen links giving way first. Above this range tendon rupture happens.

Tendons like other soft tissues such as ligaments have time and history-dependent viscoelastic properties which result from both viscoelastic properties of the

solid phase and interaction between water and extracellular matrix elements. As a result of its viscoelastic properties, tendon deformation mostly occurs at low strain rates. In this case tendons absorb more energy, but are less efficient in transferring load. On the other hand tendons deform less at high strain rates, having a high degree of stiffness, which helps in transferring loads in a more effective way [52, 119].

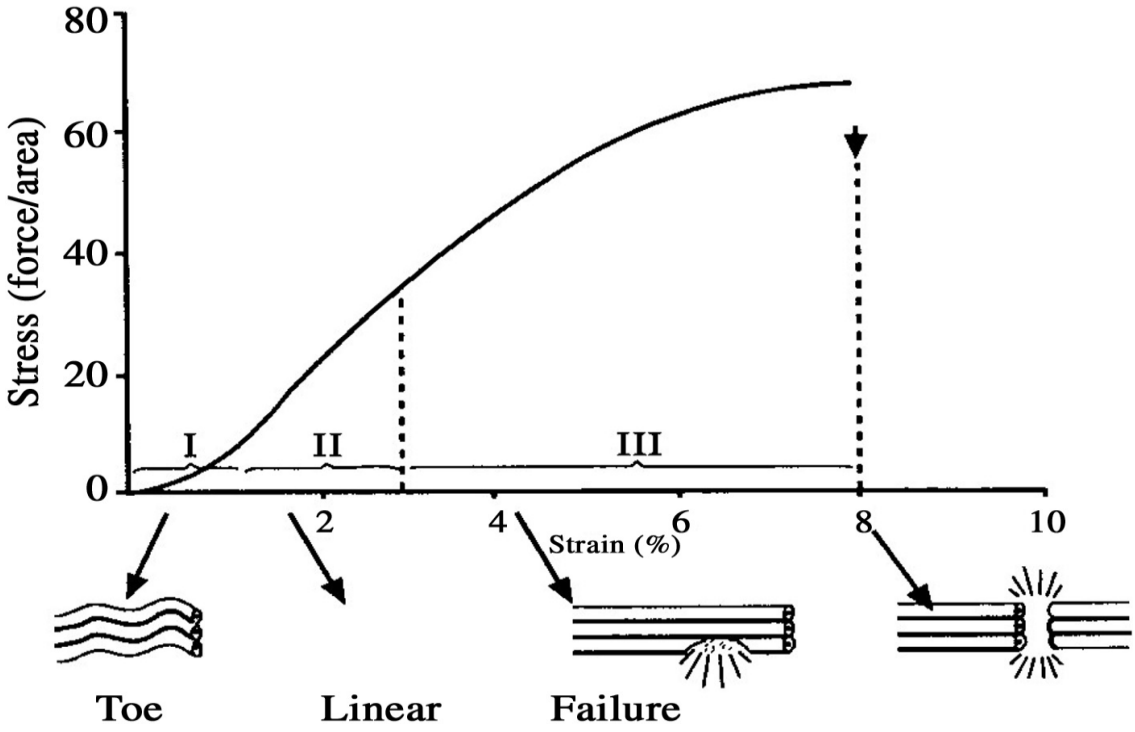


Figure 1.3: Typical tendon stress-strain curve. Crimping occurs at the micrometre scale and can be seen using light microscopy. Tissue failure happens at the millimetre scale. Adapted from Sharma *et al.*, 2005 [253] .

*In vivo,* tendon forces have been reported in different animals. In a study performed on goat using an implantable force transducer (IFT) the patellar tendon (PT) force while standing measured on average 270 N whereas, the PT force reached up to 800 N during walking and to maximum 1000 N during trotting [148]. In humans, using implantable

devices the peak force transmitted through the Achilles tendon during running has been recorded of up to 9 kN which is equivalent to 12.5 times body weight [145].

Although, the *in vivo* measurement of tendon forces gives us a valuable insight into the pathophysiologic features of tendons at specific time points throughout the pathogenetic process, there is a possibility of the interference of the measurement itself with the overall outcome when the method is invasive (e.g., implanted mechanotransducers). Moreover, the complexity of the *in vivo* environment makes it extremely difficult to measure the relationship between mechanical force and tissue remodelling. Therefore, the development of non-invasive approaches such as *in vitro* mechanical studies and imaging techniques is important to monitor the long-term effect of the mechanical environment on tendon damage and healing.

*In vitro* mechanical studies can provide repeatable measurements of load magnitudes under which microscopic and macroscopic failures occur. In addition, it is easier to investigate the influence of different amplitudes, frequencies and time points under controllable and repeatable conditions *in vitro*. The major limitation of the *in vitro* mechanical studies is change in mechanical properties due to the lack of viability of tissue *in vitro*. Developing mechanical testing set-ups which work in the tissue culture setting could maintain the viability of the tissue during mechanical testing and ultimately provide an environment which is more similar to *in vivo*.

To address the following issues this project aims to design a novel mechanical set-up to culture healthy and injured tendon fascicles and optimize current procedures used to determine the effect of different mechanical loading conditions on the structural and functional properties of cultured tendon fascicles. In addition, this study aims to incorporate both tissue culture and mechanical loading with microscopy analysis which

provide valuable data at both cellular and tissue levels under different mechanical loading conditions.

## 1.4 Platelet rich plasma (PRP)

Platelet rich plasma (PRP) is, by definition, a volume fraction of the plasma, which has platelet concentration above baseline (whole blood) [92, 185]. Platelets are active participants in different healing processes in the body [16]. Platelet α-granules are rich in growth factors and other bioactive molecules that play a major role in different stages of tendon healing. Several growth factors such as platelet derived growth factor (PDGF), endothelial growth factor (EGF), insulin-like growth factor (IGF-I), transforming growth factor-β-I (TGF-β-I) and vascular endothelial growth factor (VEGF) are released by platelets after activation [16, 70]. Growth factors and proteins secreted by platelets play an active role in the healing cascade by promoting key cellular parameters such as proliferation and migration, regulating the synthesis of extracellular matrix proteins and supporting angiogenesis and the vascularisation process in the tissue. The role of each growth factor in healing processes is discussed in more detail in chapter 2.

The current treatment options available for tendon injuries are associated with long healing times and poor outcomes (discussed in section 2.3.2). Therefore, novel treatment strategies are required to be developed in order to improve and accelerate tendon healing. Several studies have been initiated to investigate biological options include stem cells, gene therapy and autologous growth factors [58, 200, 261]. However, all of these approaches are at experimental stages and not clinically applicable at present.

Growth factors and cytokines within PRP meet many criteria of the ideal tendon treatment [192, 193]. PRP is an autologous portion of blood which makes disease transmission or immune rejection unlikely. PRP is usually prepared using a simple procedure at the time of the surgery, making it a fast and less costly treatment option in comparison with stem cell therapies which require long periods of cell culture and preparation.

Although PRP has been recently emerged as a promising treatment option for tendon disorders the complete mechanisms by which PRP initiates and stimulates various cellular processes are not fully understood and results of PRP studies are far from conclusive [191, 193, 240]. Therefore, further biological studies and extensive clinical trials are required to fully elucidate the potential effects of PRP on tendon tissue and cells.

The purpose of the present PRP study is to investigate the role of releasates from platelet rich plasma on parameters such as proliferation, migration, protection and chemotaxis in human tendon cell culture. In addition, this study investigates the effect of platelet rich plasma at the tissue level using rat tendon fascicles *in vitro*.

Moreover, the project takes a new approach to study the potential synergistic effect between PRP and mechanical loading by applying static mechanical load in combination with PRP in whole organ culture.

## 1.5 Model systems

In order to make significant progress in addressing issues related to mechanical loading and PRP therapy it is necessary to move beyond simple, static descriptions of effects of PRP on monolayer tenocytes and develop an *in vitro* tendon injury system.

Critically, this system must consist of intact, undissected tendon fascicles which are small enough to survive in culture for several weeks. They must be uniform in size and shape to permit repeatable, quantitative mechanical testing paradigms and to facilitate generation of a precisely repeatable damage. The small size will allow whole organ imaging of the defect over time as it heals. The only system available which matches these criteria is the rat tail fascicle which can be extracted entirely and undamaged, and cultured long term in whole organ culture. Fascicles from rats of the same age and strain are well matched in size and mechanical properties. No human tendon source has all of these attributes and perhaps the only way to obtain the necessary precision with human cells would be to repopulate a ghost (devitalised) rat tail fascicle or seed cells onto a synthetic construct, with all the attendant caveats introduced by associating tenocytes with a foreign matrix.

The strength of this project lies in the robust repeatability and measurability of the system and the capacity for 3D imaging of signalling responses in the healing tissue. The major drawback is that the vascular compartment is not effectively represented and therefore the response of blood vessels to PRP in the injury model will not be evaluated. Responses to PRP will be evaluated initially in human primary tenocytes in monolayer, followed by the rat tail fascicle injury model. These steps will provide the critically lacking background for future 3D studies using human tendon constructs.

## 1.6 Aims of the project

The overall aim of this thesis is to understand the role of platelet rich plasma and mechanical loading in repairing tendon damage. This is achieved by employing a range

of cell and tissue culture techniques together with mechanical testing to address the following objectives:

1) To study the effect of Platelet Rich Plasma on tendon cells and tissue by modelling the important stages of tendon healing *in vitro*. Key parameters such as cellular proliferation, viability, protection and migration and chemotaxis have been investigated by means of different culturing techniques, biochemical assays and microscopic analyses.
2) To design the mechanical set-up to culture healthy and injured tendon fascicles and optimise the mechanical testing protocol to determine the effect of static mechanical load conditions on the structure and function of cultured tendon fascicles.
3) To validate a 3D *in vitro* injury model for tendon repair which can exhibit healing responses following static loading and PRP therapy at a tissue level
4) Finally, the project aims to combine the above approaches to support the ultimate goal of the project which is investigating the influence of mechanical loading together with platelet rich plasma on tendon tissue.

## 1.7 Thesis outline

Addressing the overall aim of this thesis requires fundamental scientific understanding of different stages of tendon healing and the role of growth factors and mechanical loading in the healing process. With this theme in mind chapter 2 presents a detailed review on the established work on tendon healing, studies on tendon biomechanics and mechanobiology and published work regarding the role of growth factors in the healing process.

The biochemical assays and in-vitro injury model developed to facilitate different cell and tissue culture work is described in chapter 3. Using the principles and techniques presented in the chapter 1-3 the role of platelets in protecting tenocytes in challenging environment was also investigated (chapter 4). Moreover, the effects of PRP on chemotaxis and migration were studied in tenocyte culture (chapter 5). Chapter 6 describes the role of static loading and platelet rich plasma both separately and in combination with each other in improving healing properties of tendon tissue. Chapter 7 presents a summary of the thesis contributions and conclusions derived from the work that has been carried in this study. Finally, avenues for future work are proposed in chapter 8.

# Chapter 2 Tendon injury: repair and healing strategies

## 2.1 Introduction

Chapter 1 presented the prolonged and insufficient current treatment of tendon disorders and compromised properties of healing tissue as the clinical motivation of this thesis. This chapter provides a brief introduction to different types of tendon disorders, followed by biological background required to understand the healing process in tendon. The notion that mechanical loading and growth factor therapy may be the hallmarks of tendon healing is also presented in this section. Finally, tendon clinical perspectives are discussed in relation with PRP treatment.

## 2.2 Tendon disorders

Numerous terms have been used to describe tendon injuries and disorders (table 2.1), this reflects our limited understanding of the etiology of tendon diseases. The term tendinopathy has been used as a generic descriptor of various clinical conditions affecting tendons. The spectrum of tendon disorders ranges from paratenonitis to tendonitis and tendinosis to full-thickness rupture. In paratenonitis, paratenon is thickened and inflamed and usually adherent to tendon tissue [239]. Tendinosis is asymptotic and chronic degeneration of tendon and occasionally misdiagnosed as tendonitis which is the inflammation of tendon associated with acute increase in activity [192, 222]. The clinical diagnosis of the above mentioned disorders mainly relies on biopsy and histopathological confirmation. Therefore, conditions characterized by a combination of pain, swelling and impaired performance in tendons are mainly referred to as tendinopathy prior to histopathologic tests.

Tendon injuries are multi-factorial in origin. It has been claimed that interaction between intrinsic and extrinsic factors can contribute to tendinopathy. For example, intrinsic factors such as age-related degeneration together with extrinsic factors, such as hooked acromion in the shoulder, could be responsible for tendinopathy.

Intrinsic factors like biomechanical malalignments have been linked to two-thirds of Achilles tendon disorders in athletes [122, 154]. Both hyperpronation and cavus foot may lead to increased prevalence of Achilles tendon problems [239]. Repetitive impact loading during vigorous physical training is known to be one of the major causative factors in tendon degeneration [253]. Moreover, the risk of excessive loading in causing tendon degeneration may increase in the company of intrinsic factors.

Tendon rupture is one of the main conditions affecting tendons with high prevalence in the athletic settings. 90% of sport-related Achilles tendon rupture has been linked to acceleration-deceleration mechanism during sport activities [262]. Although the etiology of tendon rupture is not completely understood, the most common condition reported in the histological studies is degenerative tendinopathy [300]. A study on the histopathological features of ruptured Achilles tendon in seventy-four patients showed degenerative changes in all the cases and the authors hypothesized that all of these changes existed due to intrinsic abnormalities prior to the incident of the rupture [19]. In another study by Kannus and Jozsa degenerative changes were observed in 97% of tendons that had spontaneously ruptured and only in 33% of tendons from the control group [124, 127].

Tendon degeneration may also be responsible for a decrease in tensile strength of tendon which ultimately increases the risk of rupture. During physical exercise,

athletes tendons are exposed to excessive mechanical loading which could be a major pathological stimulus for tendon degeneration [310].

**Table 2.1** Classification of tendon Inflammation and degeneration. Adapted with modification from Wang *et al.*, 2006 [295], adapted from Saltzman *et al.*, 1998 [239] and Mishra *et al.*, 2009 [193].

| Type of tendon problem | Definition and findings |
|---|---|
| Tendinitis or Tendonitis | Tendon inflammation associated with acute increase in activity *e.g.* patellar tendonitis with hill running |
| Tendinosis | Chronic intratendinous degeneration due to atrophy. Can be caused by aging, micro-trauma and vascular compromise *e.g.* tennis elbow |
| Tendinopathy | Generic term for tendon problems |
| Paratenonitis | Inflammation of the paratenon |
| Peritendinitis<br>(tenosynovitis, tenovaginitis) | Inflammation of the peritendon ( peritenon and tendon sheath) |
| Spontaneous tendon rupture | Rupture without any pre-injury symptoms |
| Partial rupture | Partial tear of the tendon |
| Enthesopathy or enthesitis | Tendon-bone junction disorders |

Repetitive overloads which are above physiological threshold in tendon can result in inflammation of the tendon sheath or appearance of degenerative features in the tendon body [295]. However, overloading is not the only pathological stimulus for tendon degeneration and tendons respond to mechanical stresses in different ways. For example, repetitive submaximal loading which causes fibril damage on microscopic level could increase the risk of failure in tendons [140]. Moreover, if fatigue damage remains unrepaired, the tendon starts weakening and consequently the tendons become more susceptible to rupture [135].

Other factors such as different types of oxidative stress, high temperature and drugs can all contribute to tendon damage in different ways [41, 42, 64, 135] .

## 2.3 Tendon healing

Tendon injuries produce substantial morbidity, and disabilities that they cause may last up to several months due to poor healing mechanisms in tendons. Therefore, management of tendon disorders including tendon injuries creates a substantial challenge for clinicians. Our knowledge of tendon healing is mainly based on studies performed on tendons transected from animals or ruptured human tendons, consequently the relevance of data obtained from these studies to the healing of tendinopathic human tendons *in vivo* is not clear [254]. The following section reviews different phases of healing and possible strategies for optimizing tendon healing.

Tendon healing largely occurs in three overlapping phases [24, 194, 252]. In the initial stage which is also referred to as inflammatory phase, erythrocytes, platelets, and inflammatory cells (*e.g.* neutrophils, monocytes, and macrophages) enter the site of injury. The phagocytosis of necrotic materials occurs within the first 24 hours by

monocytes and macrophages present at the wound site. In the mean time, vaso-active and chemotactic factors are secreted and the vascular permeability significantly increases which result in the stimulation of angiogenesis. Moreover, growth factors stimulate the tenocyte proliferation during the same period [177, 227, 254]. Gradually, more inflammatory cells and tenocytes migrate to the site of injury and tenocytes start synthesizing collagen type III [99, 100].

A few days after the injury, the proliferative stage of tendon healing begins. In this phase which lasts a few weeks, synthesis of collagen type III reaches its maximum level. During the proliferative phase, the glycosaminoglycan and water content remain high.

After about six weeks the remodelling phase commences. The main feature of the remodelling phase is decreased cellularity together with a decline in collagen and glycosaminoglycan synthesis.

The remodelling phase of tendon healing can be divided into two main stages, consolidation and maturation [254]. The consolidation stage, which starts about six weeks after the injury and carries on for up to ten weeks is characterized by high tenocyte metabolic activity and change of repaired tissue from cellular to fibrous. Moreover, during the consolidation stage the collagen fibres and the tenocytes resided on them align towards the direction of stress [63, 117]. The synthesis of type I collagen is also increased in this phase [1]. The maturation period begins after ten weeks and continues for up to one year. During this period the fibrous tissue gradually transforms to scar-like tissue. In the latter stage of the remodelling phase the number of covalent bonds within collagen fibres increases which ultimately results in higher stiffness and tensile

strength in the repaired tissue [293]. Also, both metabolic activity and tendon vascularity decrease during the later stage of the remodelling phase [13].

Tendon healing occurs in two forms: intrinsic and extrinsic. Proliferation of the epitenon and endotenon tenocytes is responsible for intrinsic tendon healing. Extrinsically, cells from surrounding sheaths and synovium invade the injury site to initiate tendon healing [178, 217, 218]. Epitenon tenoblasts begin proliferation and migration to the site of injury to initiate the healing process [99].

Internal tenocytes are responsible for initiating the intrinsic healing cascade. Collagen fibres produced by internal tenocytes are larger and more mature compared to the collagen synthesized by epitenon cells [96]. This suggests that although both epitenon fibroblasts and tenocytes secrete collagen, these different cells may synthesize different types of collagen at different time points during the healing process. The contribution of each cell type to that collagen synthesis may be determined by factors such as the anatomical location of the injury, the presence/absence of synovial sheath, the type of injury and the amount and frequency of the stress induced by post-injury activities [147, 265].

Moreover, the region of tenocyte origin plays an important role in the cellular function. It has been demonstrated that tenocytes that reside in tendon sheaths release less collagen and glycosaminoglycans than epitenon and endotenon cells. However, in tendons such as flexor tendon sheath tenocytes proliferate with a faster rate [121, 252]. Our knowledge on the phenotypic variation of tenocytes and their function during different stages of healing is limited to few studies. Full understanding of this phenotypic variation can be helpful in optimizing treatment strategies for tendon healing.

Healing patterns in tendons are influenced by the anatomical location of trauma. For example, extrinsic healing predominates in rotator cuff injuries. Adhesion formation as a result of scar tissue is one of the drawbacks of extrinsic healing which compromise tendon gliding. It seems that complications associated with tendon gliding are less common in intrinsic healing. Moreover, it has been shown that where intrinsic healing is prevailing tendons tend to have better biomechanical properties [146, 147].

Matrix metalloproteinases (MMPs) synthesised by tenocytes have an essential role in regulating different stages of tendon healing. MMPs adjust and control the remodelling process in the extracellular matrix network and their levels change during the repair [121, 288]. Changes in concentration of different types of MMPs have been reported in a rat flexor tendon laceration model at different time points. This study suggests that MMP-9 and MMP-13, peak during the second week of healing, are only responsible for collagen degradation in this model whereas MMP-2, MMP-3 and MMP-14 are constantly active for 28 days after surgery and contribute to both collagen degradation and remodelling [208].

Upon tissue damage platelets in blood at the site of the damage undergo a highly regulated set of functional responses including releasing growth factors and cytokines and recruiting inflammatory cells. Each of these proteins and cells have specific role in different stages of healing. The detailed information on the role of platelet growth factors has been presented in section 2.5.

Nitric oxide is one of the other important factors involved in tendon healing. Nitric oxide is a short-lived free radical that can induce angiogenesis and vasolidation. Moreover, it has been shown that the presence of nitric oxide could stimulate apoptosis in inflammatory cells [83, 233]. A study on rat Achilles tenotomy showed an increase in the

concentration of nitric oxide after 7 days of the surgery. Moreover, the inhibition of nitric oxide syhthase in the same study resulted in the compromised mechanical properties of the healing tissue. [198, 199].

### 2.3.1 Limitations of tendon healing

Despite all of the complex biological reactions after injury the biochemical and biomechanical properties of healed tendon never attain that of intact tendon tissue. It has been reported that the failure force in healed sheep Achilles tendons was only 56.7% of controls after one year [51]. Researchers believe that one of the main reasons for incomplete tendon healing is absence of mechanical loading during the healing period as a result of immobilization.

Moreover, disruption of the synovial sheath which results in adhesion formation poses a major challenge for the clinicians [177]. In intrasynovial tendon injury, damage to synovial sheath either during surgery or injury causes granulation tissue and tenocytes from the neighbouring tissues to attack the damaged site. Following the invasion of the neighbouring cells, the number of exogenous cells increases over endogenous tenocytes and results in adhesion formation by attaching the surrounding tissue to the repair site.

### 2.3.2 Strategies for tendon healing

Many different strategies have been used in optimizing tendon healing and management of tendon disorders. Physical modalities such as extracorporeal shock wave therapy and pulsed magnetic fields have been used to improve tendon healing. 14 kV extracorporeal shock waves applied to a rabbit model at a rate of 500 impulses over 20 minutes induced neovascularisation and increased secretion of angiogenesis-related markers such as vascular endothelial growth factor (VEGF) [291]. In a study of 144

patients with calcific tendinopathy of the rotator cuff, patients that received extracorporeal shock wave therapy had a higher Constant and Murley score compared to untreated controls [102]. However, in a randomized trial including 74 patients with chronic noncalcific rotator cuff tendinopahthy no significant difference in pain scores was observed between the patients treated with the extracorporeal shock wave for three months and controls.

Pulsed magnetic fields have also been used in treating Achilles tendinopathy in animals. A study on Achilles tendinopathy in a rat model pulsed magnetic current with a frequency of 17 Hz resulted in increased collagen fibre alignment [164]. *In vitro* study on rabbit tendons showed that pulsed electromagnetic led to increase in syntheses of type I collagen and a decrease in adhesion formation, however, in another study on lacerated flexor tendons, no difference in adhesion was noticed after electromagnetic field stimulation [108].

Another physical modality that has been used to optimize tendon healing is laser therapy. Increases in collagen production have been reported in rabbits receiving laser phototherapy after tenotomy [226].

Gene therapy and tissue engineering with mesenchymal stem cells can also alter the healing environment of tendons. Genetic materials (DNA) can be delivered to the cells using gene therapy techniques. Delivery of gene materials allow adaptation of cellular function via viral or non-viral vectors or direct gene transfer [202]. The expression of individual proteins can also be facilitated in different cells by means of gene therapy methods [201]. The feasibility of gene transfer as a potential tendon therapy technique has been investigated using animal models. For example, a hydrolase enzyme such as β-galactosidase was delivered to injured rat patellar tendon by means of hemagglutinating

virus of Japan (HVJ)-liposome constructs [200]. Several studies have investigated the potential of gene transfer in tendon tissue [95, 209]. However, additional research is needed to optimize the more efficient techniques for gene therapy in human tendons

Mesenchymal stem cells have the potential to differentiate into specialized tissues such as tendon and ligament [202, 261]. Different delivery techniques such as carrier matrix have been developed to deliver mesenchymal stem cells to the site of injury, but issues such as ideal scaffold materials and optimal cell seeding density needed to be addressed in order to make tissue engineering techniques applicable for tendon treatment.

Although some of the discussed strategies are currently being used in clinic, there is an emerging need to conduct more controlled and well-designed clinical trials together with *in vitro* studies to confirm the conclusive results.

There is also clearly a need for development of new and effective therapies and to combine therapies to obtain possibly synergistic effects. Two promising strategies which may combine well are growth factor therapy and mechanical loading.

## 2.4 Biologic response of tendon to mechanical loading

Tendons have the ability to change their dimensions and properties in response to the functional demands made on them; this phenomenon is referred to as tissue remodelling which defines the stress-dependent homeostatic response of load bearing tissues such as tendons and ligaments. Many studies have shown that tendon tissue responds differently to various types of mechanical loading. The following sections discuss the effect of different loading conditions on tendon cells and tissue.

### 2.4.1 Tendon response to mobilization and immobilization

Several animal and human models have been used to study the response of tendon tissue to different forms of mechanical loading. For example, it has been reported that 40 weeks of training in rabbits increased the ultimate strength and absorbed energy at failure point in peroneus brevis tendon [283, 284]. In swine 12 months of running exercise improved the biomechanical properties of digital flexor tendon [306, 307]. In other animal models training or exercise increased the cross sectional area (164% after 16 weeks), but maximum stress of failure decreased compared to controls [263]. Moreover, number and size of the fibrils of digital flexor tendons increased in mice after one week of treadmill exercise [188].

In human studies, vigorous exercise in elite athletes increased the secretion of collagen type I in the Achilles tendon [157-159].

In general, suitable training or exercise has positive effects on tendons. However, repetitive submaximal loading with high frequency and for long periods may induce degenerative changes in tendon [116].

The effects of stress deprivation and immobilization on tendons have also been investigated in different studies. In humans the mechanical properties of tendon such as stiffness and tensile strength were decreased by a certain period of immobilization [13, 271, 272]. In animal models, changes in the collagen fibre alignment and orientation and size of veins and capillaries have been linked to immobilization [318]. Moreover, it has been demonstrated that stress deprivation due to immobilization could be a major contributing factor to tendon degeneration [317, 318]. Mechanical properties such as Young's modulus and tensile strength were significantly decreased in rabbit patellar tendon after 3 weeks of stress deprivation [314].

In tissue culture studies, 8 weeks of stress deprivation caused remarkable alteration in cell morphology, cell number and alignment of collagen fibres in canine flexor digitorum profundus tendon. Decrease in Young's modulus has also been reported in the same study [109]. However, Young's modulus was significantly increased compared to stress deprived tendons after 4 weeks of cyclic tensile loading in the same study. In addition, cyclic mechanical loading resulted in retention of the normal histological patterns such as the orientation of collagen fibres in tendon.

It has also been verified that immobilization reduces the tensile strength, stiffness and the total weight of the tendon. However, as a result of compromised vascularity and low metabolic activity in tendons the effect of immobilization is less dramatic and happens at a slower rate compared to skeletal muscles [173].

In general, studies demonstrated that exercise and training lead to an increase in cross sectional area and Young's modulus, whereas immobilization is most likely to reduce the discussed biomechanical properties.

### 2.4.2 Effects of mechanical loading on biochemical properties of tendon

Mechanical loading can also induce biochemical changes in tendons. For example, collagen deposition increased by 46% in the Achilles tendon in roosters after 8 weeks of training [67]. In the same study, the number of pyridinoline collagen cross-links significantly decreased which suggested that although training enhances collagen turnover, it may negatively affect the collagen maturation in tendon. The influence of training and exercise on biochemical components of extra cellular matrix has also been determined in humans using microdialysis techniques. It was demonstrated that training

affects the turnover of collagen I by inducing both the synthesis and degradation of collagen in the peritendinous Achilles region [158].

In another study on human Achilles tendon, secretion of inflammatory mediators increased significantly in response to exercise. The concentration of inflammatory mediators, prostaglandin $E_2$ ($PGE_2$) and thromboxane $B_2$, was more than doubled during exercise. The concentration of these two inflammatory mediators remained high after a recovery period of 1 hour [159]. Increase in production of inflammatory mediators could be as a result of tendon response to mechanical loading but excessive concentration of $PGE_2$ has also been linked to tendinopathy [9, 166]. However, in other studies, *in vivo* measurements showed no major changes in PGE2 concentration in response to exercise on different types of tendons in both healthy subjects and those suffered from tendinopathy symptoms [7, 8, 10]. Therefore, further investigation is required to understand the effects of mechanical loading on the levels of inflammatory mediators.

Repetitive mechanical loading has also been linked to the changes in the expression of substance P, a neuromodulator, which could be responsible for mediating the remodelling and adaptive response of tendon to mechanical loading including regulating tendon matrix genes and enzymes, innervations and vascularity [4].

The interaction between MMP-1 gene expression and mechanical loading has also been investigated *in vitro*. Applying static tensile stress (2.6 MPa) on rat tail tendons resulted in gradual inhibition of MMP-1 mRNA expression, while stress deprivation for 24 hours caused remarkable up-regulation of MMP-1 expression [22]. The same study reported a decline in MMP-1 mRNA expression at 1% strain and entire

inhibition at 3% and 6%. The authors suggested that the stretching frequency was responsible for the alteration of MMP-1 mRNA expression [161].

In addition to the fact that mechanical loading induces collagen synthesis, inflammatory mediator production and gene expression, there are also interactions between mechanical loading and different growth factors. It has been shown that loading could induce synthesis of growth factors such as insulin-like growth factor-I (IGF-I) which is actively involved in collagen production and cell proliferation [257, 266]. In a study on bovine articular cartilage, IGF-I increased the production of protein and proteoglycans. However, combination of both IGF- I and static mechanical loading caused a decline in production of proteoglycan after 4 hours, followed by a raise to the initial levels at 24 hour [45]. The mechanism by which IGF-I interacts with loading is not fully understood. It has been suggested that transportation of IGF-I through the extra cellularmatrix is affected by compression caused by mechanical loading. Moreover, growth factors and cyclic strain additively upregulate synthesis of matrix components, sometimes via different pathways, with strain dependent on Rho kinase signalling and growth factor requiring ERK [60, 197, 266].

### 2.4.3 Effects of mechanical loading on tendon cells

As discussed in the preceding sections, tendons change their structure, composition and mechanical properties in response to different mechanical conditions.

Tenocytes are the main cell type in tendons. Therefore, it is believed that tenocytes are regulating various physiologic and pathologic changes in tendons in response to different mechanical loading conditions. Although the processes involved in the mechanobiological response of tendon cells are not fully defined, tenocytes are

considered to be responsible for these changes by regulating the expression of ECM proteins such as collagen and metalloproteases at different conditions [27, 36, 142]. These adaptive cellular responses facilitate the repair and remodelling of damaged tendon. However, the response of tendon cells to their mechanical environment may result in developing pathological changes which could cause disorders such as tendinopathy (figure 2.1) [162].

It is important to be able to control experimental conditions in order to study the effects of mechanical loading on tendon cells. Therefore, different methods have been developed to simulate stretching in *in vitro* systems.

Using a biaxial stretching system, it has been demonstrated that tendon cells at the surface edges change their orientation to be perpendicular to the direction of radial stretching. In this system minimal surface deformation is expected to happen in the direction of radial stretching [49].

In addition, it has been found that cyclic stretching induces the synthesis of $PGE_2$ and $LTB_4$ in human finger flexor tendon cells [11]. $PGE_2$ and $LTB_4$ are usually present in inflamed or injured connective tissues including tendons, therefore it is possible that the increase in expression of these molecules as a result of repetitive mechanical loading plays a part in the development of tendinopathy.

In an *in vitro* model system designed to mimic alignment, shape and repetitive uniaxial stretching conditions of human Patellar tenocytes, tendon cells in serum-free conditions showed insignificant increase of proliferative activity at 4% and 8% compared to non-stretched controls [293].

It has been suggested that cyclic stretching could induce gene expression and synthesis of collagen type Ⅰ and TGF-β1. The changes in the level of gene expression

and protein synthesis under cyclic stretching condition were dependent on the stretching magnitude [315].

Moreover, cyclic stretching increased the synthesis of cyclooxygenase (COX) in human patellar tendon cells [166]. It was found that there is a direct relationship between the COX expression levels and magnitude and frequency of repetitive loading. In a similar study using human patellar tendon cells, the synthesis of procollagen peptides for collagen type I and III and fibronectin was significantly increased by cyclic stretching where the increase was dependent on the stretching magnitude and period of stretch [48].

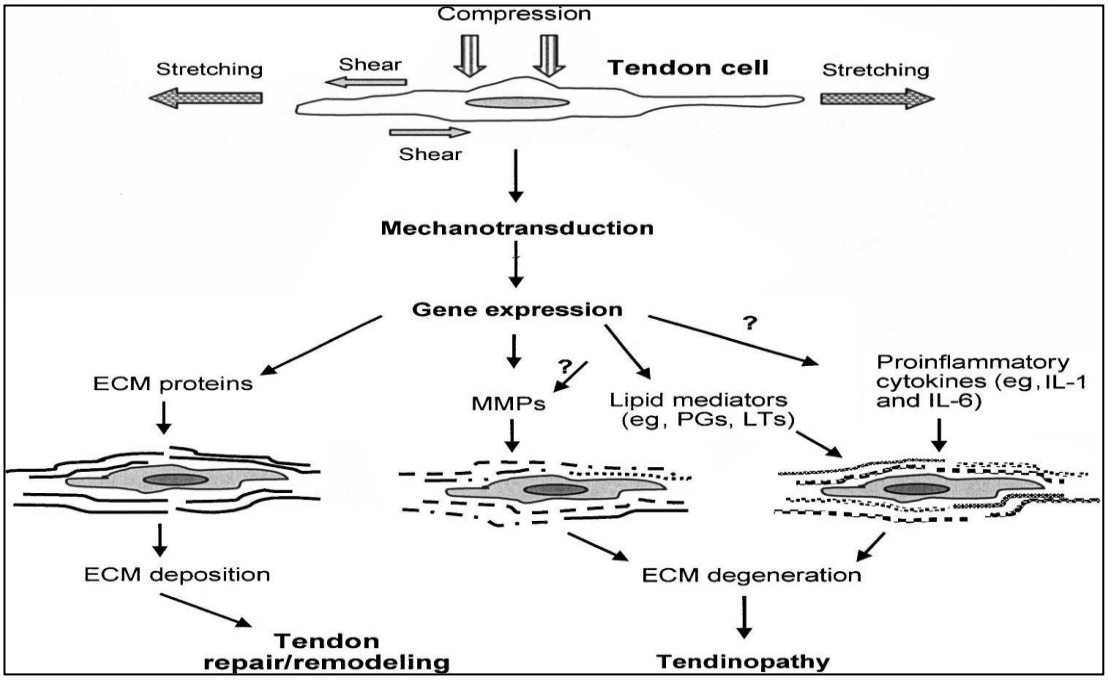


**Figure 2.1** A conceptual illustration of the biological response of tendon cells to different conditions of mechanical loading. Depending on the nature of mechanical loading mechanobiological reactions may lead to tendon physiologic remodelling or pathologic changes such as tendinopathy. Adapted from Wang *et al.,* 2006 [295].

Mechanical loading may also be responsible for the changes in metalloprotease expression. The expression of MMP-1 increased in tendon cells embedded in relaxed

collagen gels, whereas 1% cyclic stretching decreased the level of MMP-1 mRNA expression [156, 183]. Moreover, the production of collagen XII and tenascin-C increased in tendon cells in the stressed gels [156, 183]These results suggest the importance of tension induced by tenocytes on extracellular matrix for maintaining the structural integrity of tendon [160].

### 2.4.4 Effects of mechanical loading on tendon healing

Depending on the phase of healing, mechanical loading could be both beneficial and deleterious to the tendon healing. Generally, training and stretching is not recommended during the inflammatory phase to avoid disruption of the healing cascade. However, it has been reported that controlled mobilization about 1 week after injury could improve the biomechanical properties of healing tendon [292, 294]. In an injury model developed by surgical laceration in canine flexor digitorum profundus, applying active mobilization significantly increased the ultimate strength of the healing tissue [289]. In another study on canine flexor tendons early mobilization of injured tendon resulted in improved mechanical properties and enhanced excursion properties [99]. In a collagenase-induced injury model of rat Achilles tendon, mechanical loading improved healing properties of tendon by affecting collagen realignment and inducing tenocyte proliferation [68].

Generally, tendon treated post operatively with early mobilization showed better biomechanical and biochemical properties compared to those of immobilized tendons. Moreover, early mobilization also resulted in a decrease of adhesion formation during the healing [13, 99].

## 2.5 PRP as an autologous growth factor therapy

### 2.5.1 Biological properties of platelets

Platelet rich plasma (PRP) which is also known as platelet rich concentrate (PRC), autogenous platelet gel or platelet releasate is, by clinical definition, a portion of an autologous blood having a platelet concentration above baseline [181]. Different studies suggest various concentrations of platelets for PRP. Marx states a concentration of 1,000,000 platelets/μL to be the working definition of PRP, with lesser or greater concentration unable to provide enhancement in healing [180]. By contrast, another study used the platelet count higher than 300,000 platelets/μL as PRP [16]. The number of platelets in blood is normally between 150,000 platelets/μL to 350,000 platelets/μL with an average of approximately 200,000 platelets/μL. The uncertainty in concentration ratio determination is likely to be affected by different variables including type of injury, PRP volume delivered and method used to measure platelet concentration [17, 215].

Five important areas of research which could contribute to our understanding of the role of PRP in tendon healing are discussed in this chapter in the following order: Platelet origin and biology, growth factors within PRP, preparing, handling and application of PRP, and the role of PRP in clinical fields including tendon repair.

### 2.5.2 Platelet origin and biology

Platelets are derived from cytoplasmic fragmentation of megakaryocytes. The megakaryocytes are a type of white blood cell which are formed in the marrow [112]. Platelets are small in size, approximately 2 μm in diameter, and are oval or disc-shaped. Platelets have no nucleus but contain principal structures such as microtubules,

mitochondria and granules (alpha, delta and lambda). The α-granule is a unique secretory organelle surrounded by a membrane and formed throughout maturation of the megakaryocytes. The α-granules are approximately 200 to 500 nm in diameter, and 50 to 80 are found per formed platelet [110, 111]. The α-granules contain a wide variety of bioactive proteins, which are significantly involved in both haemostasis and tissue healing cascades. The properties of PRP mainly rely on the synthesis and release of different growth factors from α-granules upon platelet activation. The roles and categories of the growth factors secreted by α-granules are discussed in the following section.

Platelets start secretion of growth factors and bioactive proteins within 10 minutes of clotting with more than 95% of pre-synthesized growth factors released within the first hour [94]. After an initial increase in growth factor secretion, the platelets synthesize and release more growth factors for the remaining period of their life span which is between 5 to 10 days [172]. It has been suggested that the concentration of the growth factors is linearly proportional to the platelet concentration in PRP. This linear relationship has been reported in growth factors such as PDGF, TGF-β, VEGF and EGF [81]. However, further studies using the same principal are needed to confirm the status of other growth factors in PRP.

Understanding the combined action of growth factors within the PRP requires a wide range of research. Each of the growth factors released by platelets may have a different influence on healing of particular tissues. In addition, the interaction of growth factors with each other may activate different sets of signalling pathways. The healing cascade is also influenced by the type of the growth factors released by PRP as well as the dynamics of the injury environment and the location of injury [80, 180].

The complex and combined processes involved in the functions of the growth factors released by platelets are not fully understood and our knowledge on the role of PRP in soft tissue healing is limited to a few studies. Therefore, extensive biological and clinical studies are required to elucidate the potential effects of PRP in improving the healing properties of different tissues including tendon.

### 2.5.3 Growth factors in PRP

The level of growth factors secreted from platelets is usually quantified by enzyme-linked immunosorbent assay (ELISA) [55]. Table 2.2 is a summary of important growth factors released from the platelets including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF), platelet-derived epidermal growth factor (PDEGF), platelet-derived angiogenesis factor (PDAF), insulin-like growth factor 1 (IGF-1), and platelet factor 4 (PF-4)

#### 2.5.3.1 Platelet-derived Growth Factor (PDGF)

PDGF is a family of polypeptide growth factors and is secreted during the clotting cascade from platelet α-granules. It has been reported that the exposure to PDGF can induce synthesis of proteins and DNA in bone. Moreover, PDGF can be active in angiogenesis, macrophage activity, fibroblast proliferation, collagen synthesis and matrix formation. PDGF is also present in other kind of cells such as endothelial cells, monocytes and fibroblasts. Furthermore, PDGF acts as a significant chemoattractant for smooth muscle cells, fibroblasts, macrophages and leukocytes [185, 238].

### 2.5.3.2 Transforming Growth Factor-β (TGF-β)

TGF-β is a 2 chain polypeptide and exists in three different isoforms: β1, β2, β3. The β1 isoform has a high concentration in platelets. TGF-β is involved in almost all the stages of healing. It has been demonstrated that TGF- β enhances fibroblast proliferation, induces collagen Ⅰ synthesis and supports extracellular matrix production [185, 238]. TGF- β has an ability to regulate pathways for synthesising other growth factors such as PDEGF and PDGF. TGF-β action is not only limited to the surrounding environment of the cells that synthesise it, but also has endocrine circulation which is extensively involved in the pathogenesis of chronic fibrotic and autoimmune diseases [92].

### 2.5.3.3 Platelet- derived Epidermal Growth Factor (PDEGF)

PDEGF was first discovered in 1962 [241]. PDEGF induces the proliferative activities in keratinocytes and dermal fibroblasts which enhances wound healing. It also regulates the production of other growth factors.

### 2.5.3.4 Platelet-derived Angiogenesis Factor (PDAF)

PDAF stimulates vascular endothelial cells and in this way induces vascularisation in its environment. This factor is also active in a process by which new blood vessels penetrate devascularized tissue [144]. PDAF is up-regulated by many cytokines and growth factors such as IGF-1, PDGF and PDEGF. The expression of PDAF is significantly increased by hypoxic conditions [240].

### 2.5.3.5 Insulin-like Growth Factor-1 (IGF-1)

IGF-1 has 47% homology with insulin and is chemoatactic for fibroblasts and stimulates cartilage growth. IGF-1 also enhances bone matrix formation by replication

and differentiation of osteoblasts. The concentration of IGF-1 in PRP is measured to be 84.2 ± 23.6 ng/ml. It is known that the combination of IGF-1 and PDGF can promote the rate and quality of wound healing [81, 180].

### 2.5.3.6 Platelet Factor 4

PF-4 is secreted from α-granules and acts as a chemoattractant for fibroblasts. It has been claimed that PF-4 may be partially involved in initial attraction of neutrophils into a site of injury [144]. This factor also enhances blood coagulation by regulating the influence of heparin-like molecules [240] .

## 2.5.4 Preparation of PRP

PRP can be prepared in a laboratory or clinic room from blood drawn from a patient ideally before surgery, because surgery itself can result in platelet activation that may interfere with PRP preparation [176].

There are three main techniques to prepare PRP: 1) gravitational platelet sequestration (GPS) technique, 2) standard cell separators and salvage devices and 3) autologous selective filtration technology or plateletpheresis.

The GPS is a table top centrifuge system. Following the centrifugation, three layers become evident: the bottom layer which consists of red blood cells, the middle layer comprised of platelets and white blood cells and the top plasma layer. It is not possible to collect the red blood cells separately in this method and they are therefore discarded [84]. Different studies have used various rate of spin for obtaining PRP. For example, 5 ml PRP could be obtained following 12 minutes spin at 3200 rpm.

Standard cell separators use a continuous flow centrifuge bowl or continuous flow disk separation technology. Platelet concentration from 2-4 times above baseline

**Table 2.2** Summary of growth factors released by platelets [12].

| Growth Factor | Target | Effect |
|---|---|---|
| PDGF | Fibroblasts, Smooth muscle cells, Macrophages, Neutrophils, | - Stimulates chemotaxis and proliferative activities in fibroblast and smooth muscle cell<br>- Induces synthesis of collagen<br>- Regulates release of collagenase<br>-Stimulates the proliferation of bone cells |
| TGF-β | Fibroblast, Marrow stem cells, Endothelial cells, Epithelial cells, Preosteoblasts, | -Stimulates the proliferative activity of fibroblasts and osteoblasts<br>- Regulate synthesis of type I collagen<br>- Collagenase secretion<br>- Regulates mitogenic effects of other growth factors<br>- Stimulates deposition of bone matrix<br>- Inhibits osteoblastic mitogenesis |
| PDEGF | Endothelial cells, Fibroblasts, Epithelial cells, | -Stimulates endothelial chemotaxis/angiogenesis,<br>- Regulates collagenase secretion<br>- stimulates epithelial and mesenchymal mitogenesis<br>- Enhances wound healing |
| PDAF | Endothelial cells, | - Increases angiogenesis and vessel permeability<br>- Stimulates mitogenesis in endothelial cells<br>- Up-regulates cytokines and growth factors including IGF-1, TGF alpha and beta, PDGF and PDEGF |
| IGF-Ⅰ | Fibroblasts, Osteoblasts, Chondrocytes, | -Stimulates cartilage growth<br>-Stimulates bone matrix formation<br>- Chemotactic for fibroblasts<br>-Induces protein synthesis in combination with PDGF can enhance the rate and quality of wound healing |
| PF-4 | Fibroblasts, Neutrophils, | - Chemoattractant for neutrophils/fibroblasts<br>- Induces initial influx of neutrophils towards wound region<br>- Potent antiheparin agent |

has been reported for this method [136]. The red blood cells can be collected separately and returned to the patient in this method [84, 86].

Devices that use autologous selective filtration technology are usually small, compact office systems. These devices differ widely in their ability to make PRP, from less than a two-fold to about an eight-fold increase in platelet concentration above baseline [85, 86]. The filtration device is a single-use disposable unit which eliminates the need for a blood centrifuge which may result in platelet fragmentation.

It is important to avoid fragmentation during production of PRP because fragmentation could cause secretion of high levels of growth factors but with compromised bioactivity. The use of anti-coagulant citrate dextrose A (ACD-A) has also been suggested to avoid coagulation during the process. In ACD-A citrate binds calcium and prevents coagulation, while the dextrose and other ingredients support integrity of the platelet membrane and its viability.

### 2.5.5 Handling and application of PRP

The prepared PRP is stable for 8 hours or longer in the anti-coagulated state. This allows blood to be drawn before the procedures and be kept for lengthy surgeries and experiments [16, 240]. In order to activate the PRP a solution of 1000 units of bovine thrombin per millilitre of 10% calcium chloride ($CaCl_2$) is added to PRP. This forms a clot which enables platelets to release growth factors and maintain the presence at the application site [136].

Man *et al*., used a technique in which the PRP and calcium chloride/thrombin solution are mixed in a 10:1 (v/v) ratio using a dual syringe system [176]. The PRP was drawn into a 10-ml syringe and activating solution is drawn into a 1–ml syringe. Both syringes outputs were connected to each other to facilitate mixing of two solutions as

they are applied to surgical bed. Most commercially available PRP preparation devices have a PRP delivery technique similar to Man *et al,*.

In another study Marx *et al.,* developed a technique in which 6 ml of PRP, 1 ml of calcium chloride/thrombin and 1 ml of air were mixed inside a 10-ml syringe [180]. The syringe was agitated for 6 to 10 seconds to start making clots and then the clot is delivered to the site of injury. It is important that the delivery of clot should happen within 10 minutes of clot activation otherwise released growth factors may have compromised bioactivity.

There were a few reports of complications associated with cross reactions between human clotting factors and anti-bovine antibodies used in surgeries [61]. However, in current methods of processing bovine products more bovine factor V contamination is separated to avoid development of anti bovine antibodies in human body.

### 2.5.6 Clinical use of PRP

Studies suggest that the benefits of applying PRP include enhanced bone and wound healing and decrease in postoperative infection, pain, and blood loss [16, 54, 240]. There have been several publications regarding the application of PRP in various clinical field, including periodontal and oral surgery [15, 94, 243], maxillofacial surgery [298], cosmetic surgery [176], spinal fusion [168], heart bypass surgery [72], and treatment of chronic skin and soft tissue ulcers [143, 179]. In clinical studies, the details of the quantity of PRP and technique employed are procedure-specific. Application of PRP appears promising in the vast majority of these studies. However, most available literature on PRP is limited to case studies or series.

The following section reviews the role of PRP in tendon injuries in both human/animal culture and also clinical studies.

### 2.5.6.1 Actions of PRP in tendon disorders

It has been reported that the application of PRP increases the proliferation of human stromal and mesenchymal stem cells *in vitro* [169] . On the other hand, PRP inhibits the proliferation of macrophage and production of pro-inflammatory cytokines such as IL-1 during the first 72 hours of exposure [308, 309]. This gap in the induction of different types of cells as a result of using PRP may play an important role in tendon healing. This process could be important in preventing the formation of the fibrous scar tissue that is caused by macrophage-mediated tendon-to-bone healing [134].

*In vitro* equine and human studies have supported the application of PRP in improvement of healing process in tendon injuries and disorders. An increase in collagen type I gene expression has been reported in cultured tenocytes treated with PRP [243]. This study showed no remarkable increase in the concentration of the catabolic molecules such as MMP-3. However, another study suggested a minor increase in the synthesis of MMP-3, together with the increase in tenocyte proliferation and collagen expression in PRP-treated tendon [70].

In an animal study, greater maturation in tendon callus has been reported after using PRP for augmentation of rat Achilles tendon tears. The same study has also reported higher ultimate stress and point of failure in animals which received PRP treatment [25].

A recent study showed that circulation-derived cells were mobilized to the area of PRP injection. They also reported an increase in the synthesis of type I collagen and macrophage proliferation after 3 and 7 days [125]. However, due to the lack of

information related to macrophage activity in the initial 48 hours the comparison of this study to other works on macrophage PRP-induced suppression during the first 48 hours of injection, is not possible.

The injection of PRP in surgery of chronic severe elbow tendinosis was first reported by Mishra and Pavelko [192]. All of the patients involved in this study had not responded to standardized non-operative treatment protocols. The authors reported improvement in pain scores in the patients that received PRP therapy 8 weeks after the treatment.

Faster recovery has been reported in athletes undergoing PRP treatment for Achilles tendon injury. In this study, patients treated with surgery and PRP recovered range of motion earlier. Moreover, patients that have undergone PRP therapy had no wound complication [16, 193]. The ultrasound images from the same study showed a smaller cross sectional area in PRP treated tendons. Figure 2.2 is MRI scan of Achilles tendon rupture before and 4 months after PRP treatment. The partial tear in the PRP treated tendon has been fully recovered after 4 months [193].

A recent case study on patients receiving PRP for rotator cuff repair reported enhanced healing properties and no wound complication after the surgery [225].

### 2.5.6.2 Discussion and future consideration of PRP studies

It is obvious that PRP is capable of inducing key cellular parameters such as proliferation and migration in different cell types [169, 273, 285, 325]. It has also been demonstrated that PRP enhances the recruitment of reparative cells to the site of the injury [125]. This may explain why a single application of PRP can have a long term effect on the healing cascade. It has also been suggested that through interaction with macrophages PRP may direct the inflammatory response and as a result, enhance tissue

healing and regeneration. Moreover, PRP stimulates collagen deposition and enhances the gene expression of matrix-degrading enzymes.

All these studies suggest that PRP application could be beneficial in improving different phases of tendon healing. However, the precise mechanism by which PRP stimulates these activities is not fully understood and is currently being investigated. Studying the exact gene expression pathways *in vitro* and *in vivo* can contribute to more detailed comprehension of the mechanism of PRP activity.

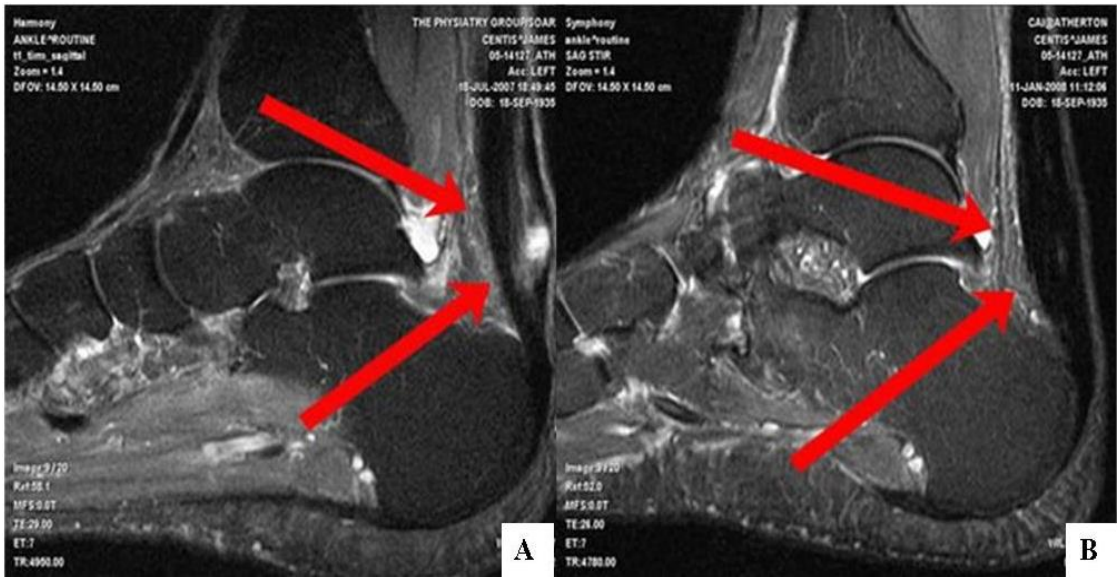


**Figure2.2** Achilles MRI before and after PRP treatment. A) MRI before PRP injection, partial Achilles tear. B) MRI 4 months after PRC, healing of partial tear. Adapted from Mishra *et al.*, 2009 [193].

The clinical investigation of PRP in orthopaedic surgery and tendon injuries has presented encouraging outcomes. However, many of these studies include a limited number of non-randomized subjects with no controls. Therefore, more well-designed randomized human and animal trials need to be carried out to confirm the effectiveness of PRP treatments. Moreover, it is important to evaluate the types of tendon disorders that could benefit from PRP treatment.

Finally, as the clinical trials for PRP progress it is important to develop rehabilitation techniques to obtain the best clinical outcome. The post-procedure protocols including loading tendon may help to enhance the healing process after PRP therapy.

## 2.6 Summary

This chapter has discussed some key issues in tendon disorders and tendon healing. Firstly, the main characteristics of tendon disorders were introduced (section 2.1). Next, the different phases of tendon healing, limitations in tendon healing and current strategies for improving the healing process were discussed (section 2.2). Finally, different aspects of mechanical loading and PRP treatment, two main focused strategies of this study, and advantages and limitation of each was reviewed (section2.3& Section 2.4). The next chapter describes the material and methods used in this thesis.

# Chapter 3 Materials and methods

## 3.1 Introduction

Chapter 2 reviewed important literature on the role of mechanical loading and PRP in tendon healing. Detailed knowledge of the mechanisms for action of new treatments are required to enable their optimal and safe application, and especially to investigate any possible interactions. The controlled setting *in vitro* models and validation methods based on biological and mechanical investigation are therefore required. This chapter provides a detailed description of the *in vitro* models developed to investigate the responses to PRP and mechanical loading at both cellular and tissue levels and the materials and methods used throughout this study are described in six main categories: 1) Cell culture techniques 2) PRP preparation 3) Staining and imaging techniques 4) Biochemical assays 5) Fascicle culture and injury model 6) Mechanical set up and testing.

## 3.2 Tenocyte culture

### 3.2.1 Tenocyte isolation

Samples of hamstring tendon were obtained from the Oxford Musculoskeletal Biobank with informed donor consent (donors aged 24-55 yr) and in full compliance with United Kingdom Human Tissue Act, and the Declaration of Helsinki. Tendon samples were cut into 1 $mm^2$ pieces and were cultured in 50% fetal calf serum (FCS, Biosera,UK) with DMEM: F12 (1:1) (Lonza,UK) and 1% penicillin/streptomycin (Sigma, Poole, UK) in 6 well plate culture dishes (Corning, UK) for 10 days under 5% CO2 and atmospheric oxygen. After this period the explants were removed and cells

were cultured in DMEM: F12/10% FCS with 1% penicillin/streptomycin. Once the tenocytes reached 70-80% confluence the cells in each well were replated in a 10cm petri-dish and were fed with DMEM: F12/10% FCS with 1% penicillin/sreptomycin until 70-80% confluent. Cells were then frozen in 10% dimethylsulfoxide (Sigma, Poole, UK)/FCS and placed in liquid nitrogen for future use.

All experiments were performed on cells below passage 3. These cells are well characterized, showing no phenotypic drift of major tenocyte markers, collagen I, aggrecan and scleraxis until after passage 5 in our hands (unpublished results). It has also been demonstrated by other groups that major tenocyte markers such as cell shape, total collagen, the ratio of collagen type I to type III, and decorin expression changes significantly after passage 5 [316].

### 3.2.2 Hypoxic culture

For hypoxia experiments, tenocytes were cultured in 10% PRP-conditioned medium with 0.1% oxygen for 48hours with 5% $CO_2$, balance $N_2$, using a miniGalaxy incubator (RS Biotech, Irvine, US). Tenocytes cultured in 10% FCS with atmospheric oxygen served as control. Induction of hypoxia was confirmed in a parallel project by Western blot of HIF 1alpha levels (Hannah Cornell thesis).

## 3.3 Platelet rich plasma preparation

Platelet Rich Plasma was generated from fresh whole human blood using a table-top centrifuge. 55ml blood from a healthy volunteer was collected directly into a syringe containing 5ml acid citrate dextran anti-coagulant (Sigma, Poole, UK) to prevent platelet activation. The blood was centrifuged for ten minutes at 200G. After this 3 layers of blood were obtained and the upper two thirds of the plasma layer were

used as PRP. 1ml of PRP was activated with bovine thrombin and $CaCl_2$ (Sigma, Poole, UK) 10: 1 (V/V) and the resulting clots left to condition 9ml of standard culture medium containing 0% FCS overnight in petri-dish. PRP conditioned media (10% PRP) was collected after 24 hours and kept frozen in -80 freezer prior to the experiments. The age and sex of the donors were

Also, 1ml of fresh PRP was immediately sent to Oxford Haemophilia Centre for platelet count. The number of platelets in the fresh PRP obtained from 6 donors was 353,000/µl, 420,000/µl, 586,000/ µl, 397,000/µl, 483,000/µl, 837,000/ µl.

## 3.4 Drug treatments

Certain classes of drugs are thought to predispose tendons to rupture, presenting clinically relevant tools with which to investigate damage mechanisms and potential treatment techniques. This study investigated the effects of three different classes of drugs, fluoroquinolone, glucocorticoids and etoposide, on tenocyte culture. Effective doses and times of treatment were established in pilot experiments (data not shown). Moreover, the pro-survival efficacy of PRP was tested in the presence of these drugs.

Five different doses (10, 20, 30, 40 and 50 µg/ml) of ciprofloxacin (BioMYC C, GeneFlow, UK) were used, although it should be noted that ciprofloxacin is poorly soluble so concentrations are approximate. Dexamethasone (Sigma, Poole, UK) $10^{-2}$M stock solution was prepared in ethanol. Also, cells were treated with etoposide (Sigma) (500 µM) as a positive control for cellular death. Cells were exposed to these three drugs with/without PRP in 96 well plates for 4 days, followed by removal of PRP and replacement with basal medium containing 0% FCS for 30 minutes to wash cells. After

the above treatments parameters such as viability, death and senescence were measured using the techniques described in the following sections.

## 3.5 Viability assays

### 3.5.1 Live&Dead staining

Live& Dead kit was obtained from Molecular Probes (Invitrogen, Paisley, UK). 2 µl of calcein AM and 1 µl ethidium homodimer were added to 1000 µl of PBS to make the staining mixture. Culture medium was removed and cells were washed twice in PBS to remove serum which could interfere with staining. This was followed by adding 50 µl of staining mixture to each sample. Samples were kept at room temperature for 30 minutes. After this, samples were washed with PBS and imaged using an Olympus BX40 microscope with DP70 camera and Cell^F 2.2 software (Olympus BioSystems, Tokyo, Japan). Ethidium homodimer (Dead stain) is excluded by live cells only. Calcein-AM (Live stain) is colourless until cleaved by intracellular esterases in living cells when it fluoresces green.

### 3.5.2 Alamar Blue

Viable cell number relative to the control was determined using the Alamar Blue® assay. Alamar blue contains a REDOX indicator that both fluoresces and changes in colour (from blue to pink) upon chemical reduction resulting from normal cellular metabolism. Alamar Blue® (Abd serotec, Kidlington, UK) was added 1:10 (v/v) to the cultures, followed by 3 hours of incubation. The REDOX state of Alamar blue was measured using a Spectramax Gemini XS microplate reader Molecular

Devices, Sunnyvale, CA, USA) at an excitation wavelength of 530 nm, and emission wavelength of 590 nm, with a 570 nm cut-off.

## 3.6 Senescence- associated β-Galactosidase staining

Tendon cells were plated in 24-well dishes, treated with dexamethasone with/without PRP, and grown for 72 hours (based on a previously established treatment and staining protocol from Dr Raewyn Poulsen, Postdoc in the group). Medium was removed and cells were washed in PBS, and fixed in 1% formaldehyde/0.25% glutaraldehyde for 5 minutes at room temperature. After two washes in PBS, cells were incubated overnight with β-galactosidase stain solution containing 1mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside, 5mM ferrocyanide, 2mM magnesium chloride, 150mM sodium chloride in citric acid/sodium phosphate buffer at pH 6. Neutral red solution was used as a counterstain. Cells were then imaged using a Nikon, Eclipse TE300( Japan) and Olympus BX40 upright fluorescence microscope (Southend-on-Sea, UK). Image J software was used to quantify the number of senescent cells.

## 3.7 Biochemical assays

### 3.7.1 Dimethylmethylene Blue (DMB) assay

The GAG content was measured using the method adapted from studies by Farndale et al., [88]. 45 tendon fascicles from male Sprague Dawley rats (aged between 4-6 months) were used to measure the relative amounts of GAG in 15 test groups. Briefly, 2 cm of fascicle was air dried and weighed before digestion in 250 µl of papain digestion buffer solution, (papain 300 µg/ml, 20 Mm sodium phosphate buffer, 1mM EDTA, 2mM dithiothreitol DDT pHed with NaOH)(all chemicals from sigma, Poole,

UK) for 7 hours in shaking incubator (Stuart Scientific) at 65º C. The DMB colour reagent was made using 40.55 mM glycerine, 40.55 mM Nacl, 9.5 mM 0.1M HCL, and 1.6 mg of 0.0016% [w/v] DMB. Chondroitin sulphate (CS) (5mg/ml) from (Sigma, Poole, UK) was purchased to prepare 5 different CS concentrations of 500 µg/ml, 250 µg/ml, 125 µg/ml, 50 µg/ml and 0 µg/ml for the chondroitin sulphate standard curve. Papain digestion buffer was used as blank. 100 µl of the colour reagent was added to sample or CS concentration and corresponding values were obtained at 520 nm using a Spectra Max plus, spectrophotometer from Molecular Devices. Each sample and standard was triplicated. GraphPad Prism 5.03 software was used to produce the standard curve and determine GAG concentration in each sample.

### 3.7.2 Picogreen assay

The same samples that were digested in the papain digestion buffer as described in the previous section were sonicated for 15 seconds. Double-stranded DNA (dsDNA) in the digestion buffer was measured using Quant-iT™ PicoGreen® dsDNA quantitative kit (contains PicoGreen reagent, λ dsDNA, 20 X TE buffer). Briefly, 6 different concentration of λdsDNA stock were prepared for the standard curve. 10 µl of digested samples were loaded into the 96 well plate in triplicate. 100 µl of 1 X TE buffer and 100 µl of Picogreen dsDNA quantitation reagent were added to the wells containing standards and samples and left at room temperature in the dark on a plate shaker for 30 minutes. The fluorescence was measured at excitation 490 nm, emission 540 nm and cut off 530 nm with the Spectramax Gemini microplate spectrofluorimeter.

## 3.8 Migration and chemotaxis assays

### 3.8.1 Wound healing scratch assay

A wound healing assay was used to study directional cell migration *in vitro*. The wound was stimulated by introducing a scratch in a confluent cell monolayer (96-well) using a P200 pipette tip (Figure 3.1). The cell debris was removed by washing once with medium. The cells on the edge of the scratched area moved towards the denuded area closing the gap and establishing new cell-cell contacts.

The monolayer recovery was observed over a time course of 6 days using Nikon eclipse TE300 inverted microscope with phase contrast to establish healing times. Using different wells for each time point the cells were stained with calcein AM (Live stain from Live&Dead kit) to facilitate image analysis. It was found that Live stain was toxic if used repeatedly on the same well, hence use of different wells for later time points. The entire scratch was imaged in 2 low power shots per well and 5 different wells per treatment group and time point were imaged at the beginning and regular intervals during the progress. Image J 1.41 software (NIH) was used to quantify the area covered by cells after different intervals using a saved average ROI from the fresh scratch.

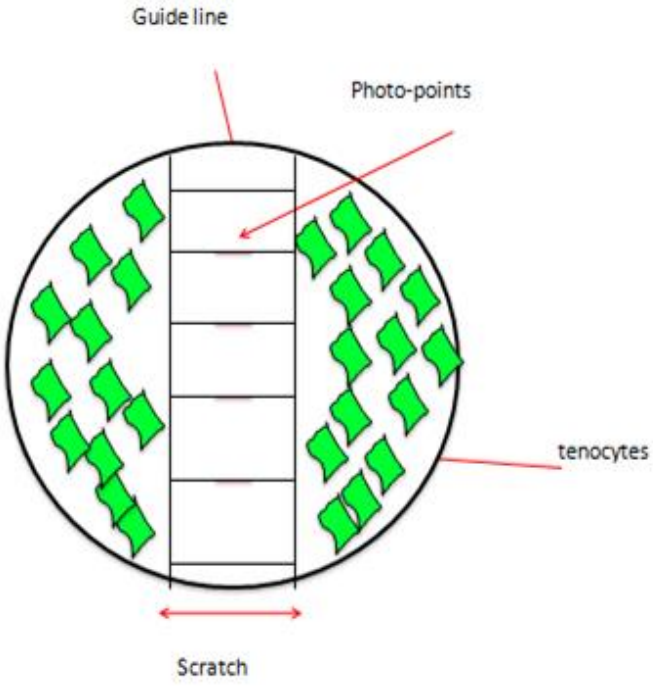


**Figure 3.1** Cartoon of scratched area, wound healing scratch assay.

### 3.8.2 Transwell migration assay

Transwell chambers (VWR,UK) with 8.0 μm pores were used to assess the migration and chemotaxis boosting effects of PRP on human tendon cells over the course of 6 days. A Transwell chamber consists of two compartments which are separated by a micro-porous membrane and are supported in a well full of medium. Tenocytes were placed on the upper layer of the membrane and were allowed to migrate through the pores of the membrane into the lower compartment, towards the base of the well on which a clot of chemotactic agent, 100%PRP, was placed. The cells on both sides of the membrane were stained with Live stain from Live & Dead kit (Invitrogen, Paisley, UK). The transparent filter was imaged before and after the complete removal of cells from the top compartment, using a cell scraper. Focal plane

was different for top and bottom of filter. Image J software was used to count the cells remaining on the lower membrane surface.

## 3.9 Tendon fascicle culture

Rat tails were harvested immediately after Sprague Dawley rats (Male, aged between 3-6 months, weighted between 300-360 grams) were sacrificed in the Department of Physiology Anatomy and Genetics, University of Oxford. These rats were in use for another study with full ethical approval and the tails were not needed. Rat tails were rinsed in 70% IMS to sterilize and cleaned of their superficial skin and fascia prior to fascicle harvesting. The tail tips (5 vertebrae) were removed by scalpel and the fascicles were extracted using forceps and rinsed in DMEM: F12 Medium (Lonza, UK) supplemented with 10% fetal calf serum (FCS) and 1%penicillin/streptomycin. Individual fascicles are typically 10-15cm long, allowing the ends to be handled and leaving an untouched working length of at least 5cm. Figure 3.2 shows the fascicles harvested from rat tail tendon.

## 3.10 Damage model

Fresh fascicles were harvested from the rat tail and placed on a glass slide. The glass slide was marked at 1 cm intervals and the fascicle was glued to the both end of the slide to avoid slipping while making the injury.

A variety of crushing and piercing strategies were tested using capillary clamps, forceps or fine pins but the most repeatable and uniform injury as assessed by both Live& Dead stain and mechanical properties was caused by scratching.

23 gauge insulin needles were used to create 1cm scratches along the length of a region of fascicle. The fascicles were moistened with DMEM : F12 medium to avoid dehydration during the process. After the injury was made both glued ends of the fascicle were removed by surgical scalpel and the fascicle was immediately placed in DMEM : F12 media. The fascicles were then cut into 2 cm and 5 cm pieces with the damaged area in the middle and were used for biochemical and biomechanical tests, respectively.

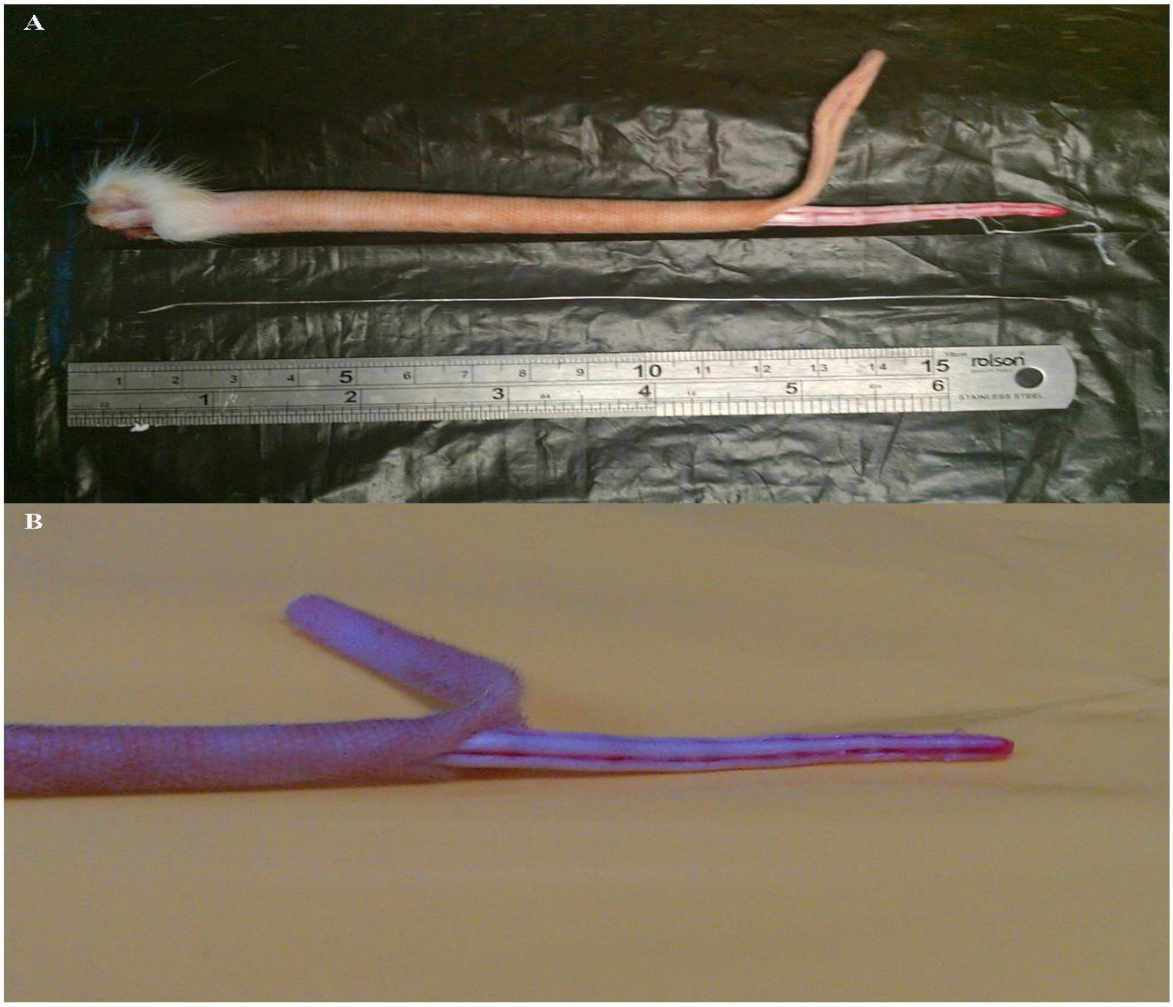


**Figure 3.2** A) Image of rat tail after excision from Male Sprague Dawley Rat. B) Image of fascicle bundles prior to harvesting. Tendon fascicles were obtained from several rat tails throughout the course of this study.

## 3.11 Mechanical testing

Injured tendon fascicles were cultured in DMEM : F12/5% FCS or platelet rich conditioned medium for up to 7 days. In order to investigate the synergistic interaction between mechanical loading and PRP a set-up was designed in collaboration with a fellow PhD-student to culture fascicles under static load in the presence of PRP (Figure 3.3). The proximal end of the fascicle was connected to a test tube cap via a holder consisting of a rod and a silicon tube. The distal end of the fascicle was attached to a magnet (load) using cynoacrylated adhesive. The magnetic weight (2.25 g) was then suspended in a DMEM-F12 medium filled test tube in order to apply static load of approximately 0.02N. The force applied onto the tendon was measured in-situ using a load cell. Load cell was positioned directly above an open centrifuge tube using a clamp stand. At all times the magnet and fascicle were fully immersed in the centrifuge tube filled with medium. Data acquisition of the force applied was then captured using the Wintest software. The function and role of individual components shown in figure 3.3 is described in table 3.1.

Injured tendon fascicles were cultured in DMEM: F12 5% FCS with 1% penicillin/sreptomycin or platelet-rich conditioned medium for up to 7 days. Viability of tenocytes, GAG and DNA content were measured by Live & Dead, DMB and Picogreen assays respectively (as detailed above) to gain biochemical properties of healthy, damaged and treated tendon tissues.

It should be noted that a method to perform accurate Sircol assays on fascicle tissue could not be developed in the time available, since specimens were either

incompletely digested or overdigested. A variety of acid and pepsin extraction protocols were attempted at different temperatures and over several days.

It is important to note that the Sircol assay is optimised for use on soluble collagen released into tissue culture and for cell monolayers. Future attempts would involve lyophilisation of the sample and crushing into a powder before digestion and assay or use of the hydroxyproline content assay.

Prior to the mechanical testing fascicles were rinsed in PBS and a digital area micrometre (Keyence, UK) was used to measure the exact cross sectional area of fascicles. Tensile testing machine (Bose, UK) (Figure 3.4) and Wintest software version 4 (Bose, UK) was used to measure the biomechanical properties of different groups of fascicles.

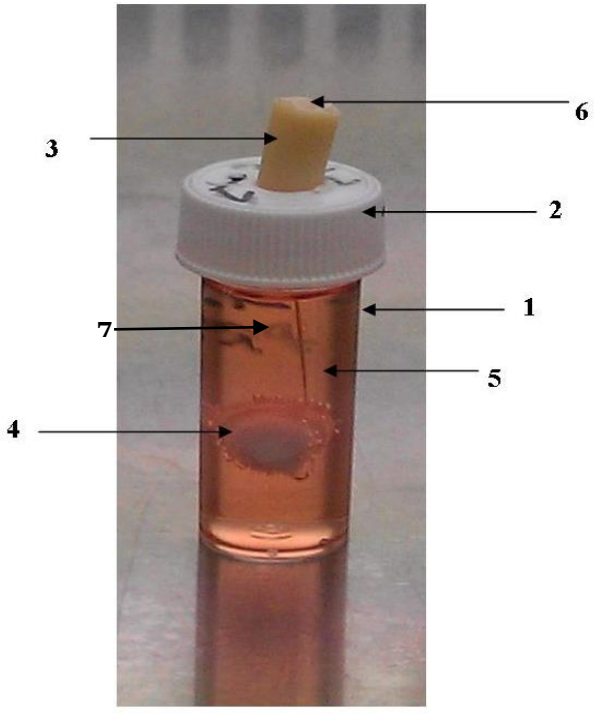


**Figure 3.3** *In vitro* set-up for statically loaded tendon fascicle. Fascicles were cut into 5 cm pieces for static loading.

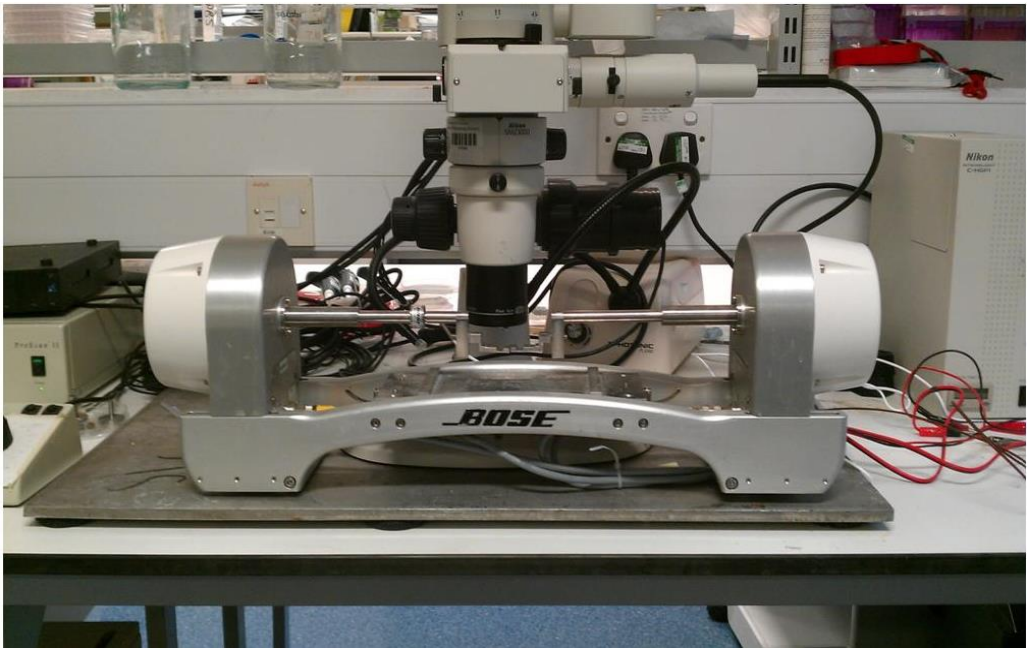


**Figure 3.4** Bose machine for mechanical testing of tendon fascicles

**Table 3.1** Function and role of individual components of static loading system (Key to Figure 3.3)

| | **Component** | **System function** |
|---|---|---|
| 1 | Test tube | Storage for the fascicle, Medium and weight. |
| 2 | Test tube cap | Entry for silicon tube, surrounding pin-sized holes provide gass necessary for cells to survive. |
| 3 | Master-Flex silicon tube | Holds tendon fascicle and perspex rod in place. |
| 4 | Weight(magnet) | Attached to the distal end of fascicle and provides static load onto fascicle (2.25 g). |
| 5 | DMEM : F12 | Provides nutrients for tissue survival. |
| 6 | Perpex rod | Used to hold proximal end of tendon fascicle in place. |
| 7 | Fascicle | Fascicle was attached from proximal and distal ends to the cap and magnet, respectively. |

### 3.12 Statistical analysis

Results are expressed as mean± SEM. Experiments were repeated on separate occasions using tenocytes derived from 3 different donors and data is presented as an average of all donors except where otherwise indicated. All conditions are expressed as n-fold difference from the baseline control at the corresponding time point. Statistical analysis was performed using GraphPad Prism 5.03 software. Repeated-measures analysis of variance (ANOVA) and Tukey post-hoc test were used to compare different sets of data. P values of less than 0.05 were considered to indicate statistically significant difference

# Chapter 4 PRP protects tenocytes against adverse effects of dexamethasone, ciprofloxacin and hypoxia

## 4.1 Introduction

Chapter 3 presented different techniques used in this thesis to investigate the effects of platelet rich plasma and mechanical loading on tendon tissue and tendon cells.

This chapter provides evidence of an entirely new mode of action of PRP in tendons, in which PRP has the ability to protect tenocytes from damaging environments. These studies have focused on two important drugs, glucocorticoids and fluoroquinolones, which both have clinically observed negative effects on tendon. The ability of PRP to mitigate the adverse effects of these drugs together with its protective effect against hypoxia is presented in this chapter, pointing towards new clinical uses of PRP. Part of this work has already been published in the American Journal of Sports Medicine [323].

## 4.2 Effects of dexamethasone and ciprofloxacin on tenocyte culture

Certain classes of drugs interfere with the innate healing processes and are thought to predispose tendons to rupture, presenting clinically relevant tools with which to investigate damage mechanisms in tendon cells. Moreover, understanding the mechanisms responsible for the deteriorating effects of these drugs provides an interesting paradigm to test the efficacy of potential treatments such as PRP.

### 4.2.1 Glucocorticoids

Glucocorticoids are a class of steroid hormones which exert various effects on different type of cells. They mediate several physiological and cellular processes which could be divided into two major categories: anti-inflammatory and metabolic.

Glucocorticoids are involved in regulating different types of anti/pro inflammatory proteins, glucose homeostasis and carbohydrate metabolism [69, 167]. They also play essential roles in fetal development and homeostasis of T lymphocytes [210, 231].

As a class of drugs, glucocorticoids are extensively used for treatment of immune and inflammatory conditions such as asthma, rheumatological, allergic rhinitis, ocular and dermal pathologies [97, 98] . In addition, glucocorticoids are widely used to treat painful and inflammatory musculoskeletal soft-tissue disorders including tendon diseases. In the United Kingdom glucocorticoid therapy is among the most prescribed anti-inflammatory agents used to treat musculoskeletal conditions with around 500,000 glucocorticoid injections administered for tendon and joints problems annually [219]. However, severe degenerative effects such as bone loss and ocular hypertension have been reported in association with glucocorticoid treatment [53, 206].

Several reports have also indicated the risk of spontaneous tendon rupture and tendinopathy as a consequence of glucocorticoid treatment in human and animal models [106, 184] and there is concern that systemic glucocorticoid injection leads to the same deleterious effects in tendon tissue as in bone and skin.

Experimental studies performed to understand the mechanism behind the negative effects of glucocorticoids on tendons are limited. There are some indications that the glucocorticoids have degenerative side effects on tenocytes. It has been suggested that glucocorticoids reduce the cell proliferation and viability as well as

collagen synthesis in cultured human tenocytes [249, 274, 305]. In animal models glucocorticoid administration resulted in compromised biomechanical properties in tendon [131].

As a consequence, the use of glucocorticoids (*e.g*, dexamethasone) in treatment of painful tendon conditions remains controversial.

### 4.2.2 Fluoroquinolones

The second class of drugs discussed in the current study is fluoroquinolones. Fluoroquinolones are broad spectrum antibiotics used for treatment of infectious diseases [137]. The fluoroquinolone antibiotics are known to have good tissue diffusion, gastrointestinal absorption, and a long half life [40, 322]. This class of antibiotics (*e.g.* ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin) are widely used to treat respiratory conditions, urinary tract and intestinal infections. In addition, fluoroquinolone drugs are used for treatment of musculoskeletal infections such as septic arthritis and osteomyelitis [301].

Certain side effects have been reported in association with the use of fluoroquinolone in both humans and animals [118, 137, 214, 249]. Conditions such as myalgia, arthralgia and arthritis have been described as adverse effects of fluoroquinolone antibiotics. Moreover, several cases of tendinopathy and tendon rupture have been observed in patients undergoing fluoroquinolone treatment [137].

In clinical practice fluoroquinolones are known to have a negative impact on tendon health and are therefore relatively contra-indicated in patients with a history of tendinopathy and in children [138, 214]. Experimental evidence has shown a decrease in proliferation, collagen and proteoglycan synthesis and dysregulated matrix

metalloproteinase activity following fluoroquinolone exposure in human tenocyte culture [65].

It has also been suggested that fluoroquinolone drugs increase inflammation of paratendon in animal models which may contribute to tendon disorders associated with ciprofloxacin [132]. However, studies performed to understand the effects of fluoroquinolones on tendons are not conclusive and the role of the fluoroquinolone in tendon disorders remains controversial.

Experimental evidence on the influence of glucocorticoid or fluoroquinolone induced cellular death in tendons is even more limited. In addition, the role of these drugs in inducing cellular processes such as senescence, which could cause a general decline in the structural and functional properties of tendon, has not been addressed in the literature.

## 4.3 Platelet rich plasma as a protective strategy in drug-induced tendon problems

It is necessary to continue using glucocorticoids since they are highly effective in the treatment of a broad spectrum of musculoskeletal conditions. Therefore, research attention must be focused on developing new strategies to minimize their adverse effects on tendons.

Because of its pro-healing properties, PRP may have the potential to be used as a protective strategy to eliminate the side effects of glucocorticoid treatment while maintaining their anti-inflammatory activity.

The hypothesis for this study is that the pro-healing response induced by PRP would protect human tenocytes against the cytotoxicity of both the synthetic

glucocorticoid, dexamethasone, and the fluoroquinolone, ciprofloxacin. The following section discusses the role of PRP in protecting tenocytes treated with ciprofloxacin, dexamethasone and etoposide.

## 4.4 Effects of ciprofloxacin, dexamethasone and etoposide on tenocyte culture in the presence of platelet rich plasma

### 4.4.1 Tenocyte viability

The experiments were performed over a wide range of ciprofloxacin doses (10, 20, 30, 40 and 50 µg/ml). Alamar blue fluorescence was measured as a reflection of metabolic activity and viability of tenocytes. The viability of the cells, treated with ciprofloxacin, decreased dose-dependently over the course of 4 days. The viability of cells exposed to 40 µg/ml and 50 µg/ml of ciprofloxacin was significantly decreased to 48% and 45% of the control respectively ($P<0.01$and $P<0.005$).

10% PRP alone induced a strong increase in the number of viable cells. Concentrations of PRP ranging from 1% to 10% demonstrated dose-dependent effects, with 10% consistently the highest. Figure 4.1 shows the dose-dependent response to different concentration of PRP after 48 hours and 4 days of treatment.

In the presence of 10% PRP the viability of tenocytes co-treated with 40 µg/ml ciprofloxacin was significantly higher than with ciprofloxacin alone. PRP was not able to significantly protect against the highest dose, 50µg/ml ciprofloxacin (Figure 4.2).

The viable number of PRP treated cells with lower doses of ciprofloxacin exceeded the level of controls suggesting that PRP not only protected tenocytes and preserved their viability but also induced proliferative activity in the presence of

ciprofloxacin.

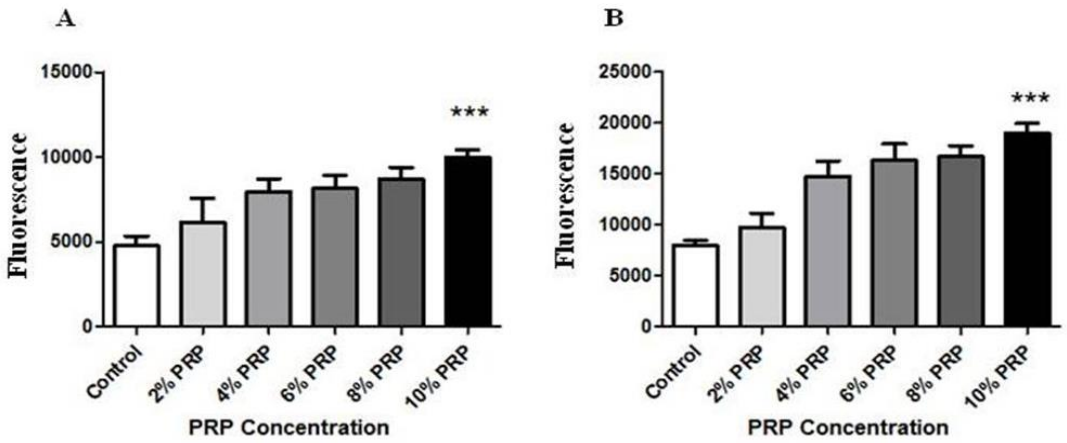


**Figure 4.1** The effect of different doses of PRP on the *in vitro* viability of human hamstring tenocytes after A) 48 hours B) 4 days. Tenocytes showed dose dependent response to PRP, with 10% consistently the highest. The values are shown as the mean and standard error of the mean of the values obtained from three different patients, with 6 replications per subject. Asterisks denote comparison with untreated control, with significance values (***P<0.005).

Alamar blue was also used to investigate the effects of dexamethasone on the viability of tenocytes. Cell viability significantly decreased by 30% after a 4-day treatment with a pharmacologically relevant dose of 1 μM dexamethasone (Figure 4.3.A). However, exposure to PRP completely restored the viability of tenocytes treated with dexamethasone (P<0.01).

The chemotherapy agent, etoposide (500 μM) was used as a strong inducer of cellular death in tenocytes to investigate the role of PRP in protecting tenocytes in pro-apoptotic conditions. Etoposide is known to inhibit re-ligation of DNA strands which induces apoptosis in cancer cells. The viability of tenocytes was increased from 45% of the control in etoposide treated (P<0.05 vs control) to about 94% in PRP-etoposide co-treatment (P<0.05) (Figure 4.3.B)

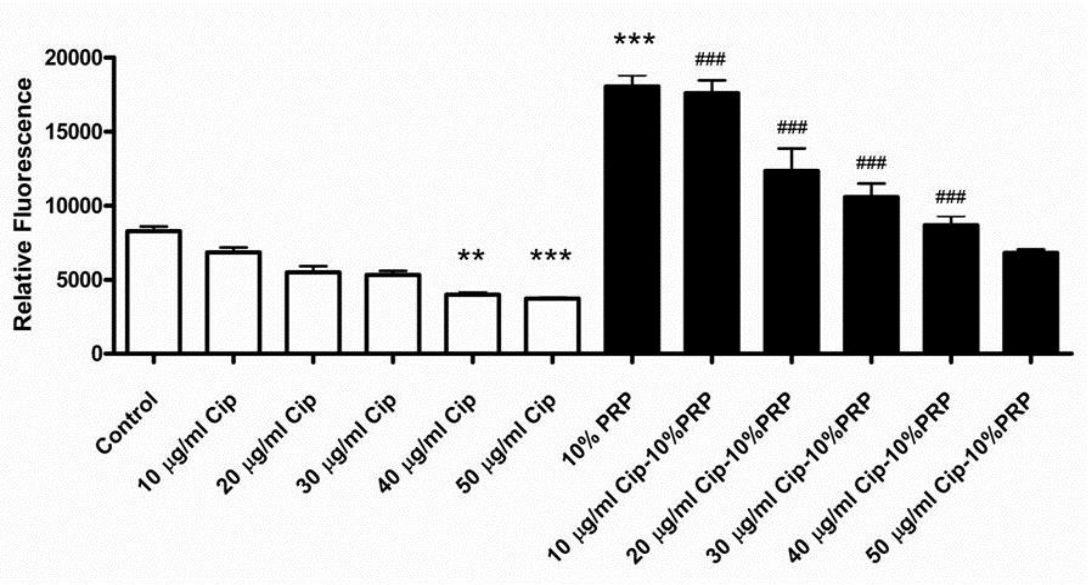


**Figure 4.2** Protective effect of Platelet rich plasma against ciprofloxacin: The effect of different doses of ciprofloxacin (10-50 µg/ml) on the *in vitro* viability of hamstring tenocytes with/without PRP after 4 days. The addition of ciprofloxacin decreased viability of tenocytes, assessed by Alamar blue assay, in a dose dependent manner, whereas the addition of 10% PRP together with ciprofloxacin significantly increased the viability of cells. The values are shown as the mean and standard error of the mean of the values obtained from three different patients, with four replications per subject. Asterisks denote comparison with untreated control, with significance values **P<0.01, ***P<0.005. Hash marks denote comparison of ciprofloxacin doses with the corresponding ciprofloxacin/10% PRP condition, with significance values ###P<0.005. DMEM:F12 was used as control.

Fluorescence-based LIVE&DEAD$^{TM}$ assay was used to assess the actual induction of cell death after exposure to ciprofloxacin. Dexamethasone, while reducing viable cell number, did not induce cell death with the pharmacological dose of 1 µM used for up to 7 days. Cellular death was detected in tenocyte culture after 4 days of treatment with the highest dose of ciprofloxacin (Figure 4.4 B, F). However, the percentage of dead cells was clearly decreased in the presence of PRP (Figure 4.4 D, H).

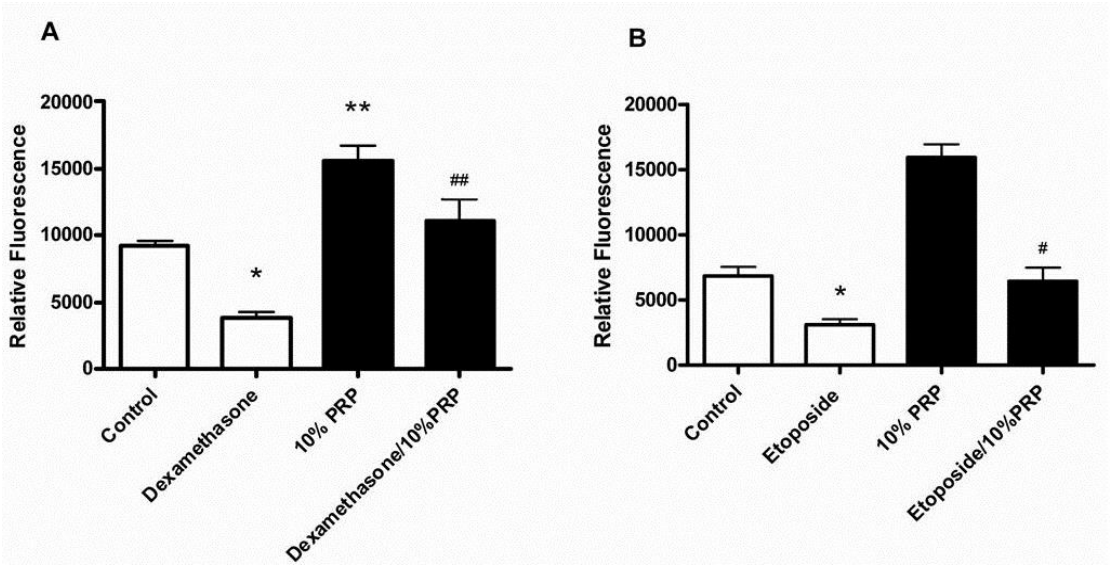


**Figure 4.3** The effect of dexamethasone and etoposide on tenocyte viability after 4 days treatment: A) Alamar blue assay of untreated controls and cells treated with $10^{-6}$ M dexamethasone, 10% PRP or co-incubation of $10^{-6}$ M dexamethasone together with 10% PRP. The values are shown as the mean and standard error of the mean of the values obtained from three different patients, with four replications per subject. *P<0.05, **P<0.01 compared to the values of control, with significant values ## P<0.01 comparing Dexamethasone with Dexamethasone/10%PRP B) Viability of untreated controls and treatment with 500µM etoposide, 10% PRP or 500µM etoposide together with 10% PRP. The values are shown as the mean and standard error of the mean of the values obtained from three different patients, with four replications per subject. *P<0.05 compared to the values of control, #P<0.05 comparing etoposide with etoposide/10% PRP. DMEM: F12 was used as control.

## 4.5 Replicative Senescence (RS)

Senescence or biological aging is defined as a progressive, deteriorating decline in physiological function of whole organism as it ages after maturation [299]. Senescence is a complex phenomenon and may be associated with variety of cellular and sub-cellular factors.

Two major theories have been advanced to explain cellular senescence: programmed and stochastic theories of aging [141].

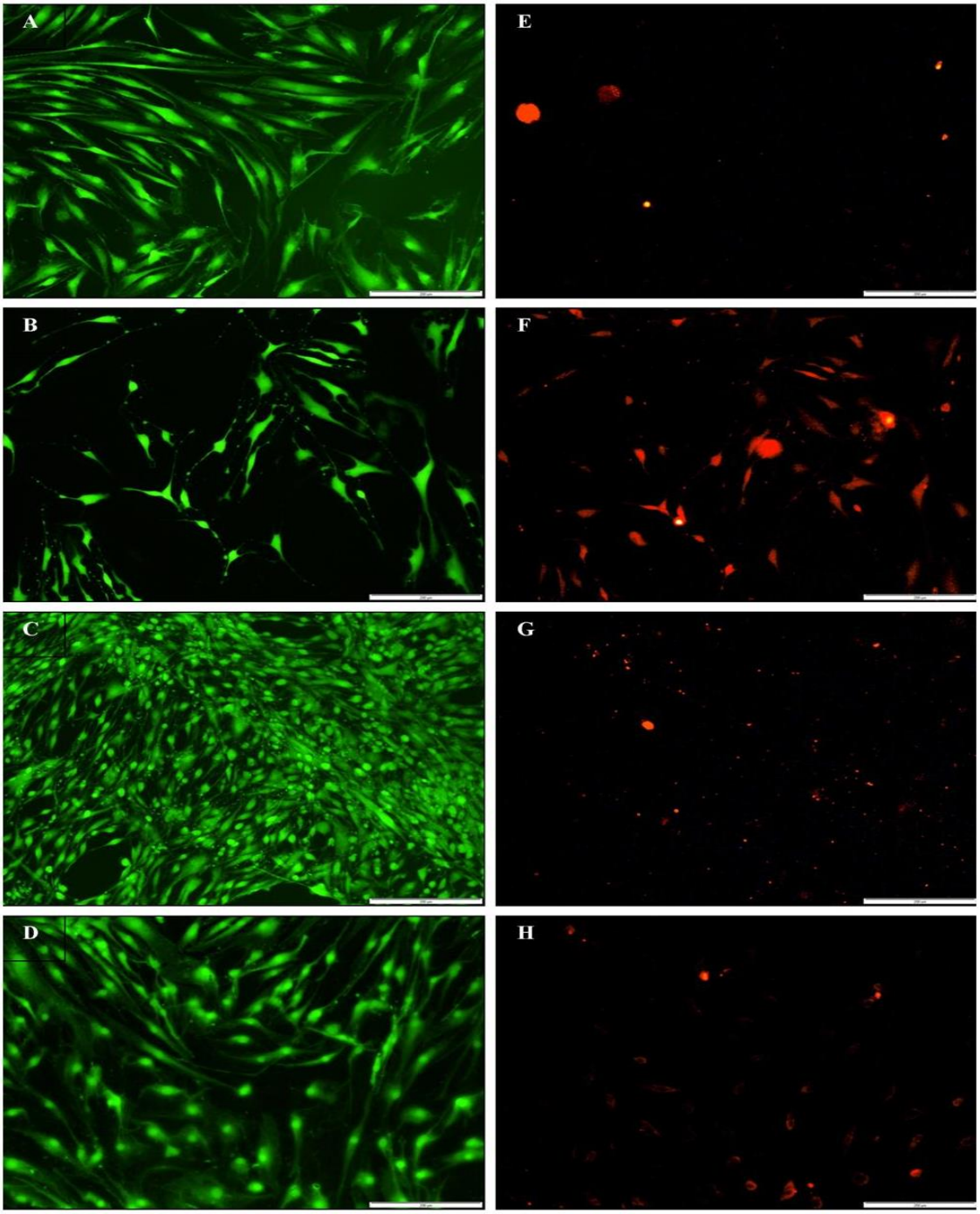


**Figure 4.4** 10% PRP protects tenocytes against drug-induced cell death. Live & dead staining of tenocytes following treatment of cells for 4 days with ciprofloxacin in presence/absence of 10% PRP. Control (DMEM: F12)(A, E), with Ciprofloxacin (50µg/ml) (B,F), with 10%PRP alone (C,G) and Ciprofloxacin plus 10%PRP (D,H). Photos (A-D) show Live cells - green fluorescence (ex/em 495/515nm) was captured with a FITC filter and photos (E-H) show Dead cells - red fluorescence ex/em 495/635nm) was captured with rhodamine filter, (scale bar = 200 µm).

In general, the programmed theory implies that senescence is likely to be a genetically-determined process and is regulated by the changes in gene expression which controls maintenance, repair and defence functions in different systems. In contrast, stochastic theory is based on the environmental stresses which result in cumulative damage on molecular to tissue levels.

The process of senescence was first described by Hayflick and Moorhead in the 1960s. They showed that the cells from embryonic tissues can only divide for a limited number of times, the Hayflick limit, in culture and lose their capability for further proliferation in response to mitogens [114].

According to the Hayflick theory there are three different phases of cell culture: Phase 1 is described as primary culture and is characterised by rapid proliferation. Phase 2 is represented by vigorous cell division followed by gradual decline in the rate of cell division. Eventually, cells stop replicating and reach the senescent state. The Hayflick limit varies between different species and cell types and could be up to 60-80 cumulative population doublings (CPDs) in fetal or neonatal human cells.

Replicative senescence has been reported in a variety of cell types including fibroblasts, glial cells, keratinocytes, endothelial cells, lymphocytes, vascular smooth muscle cells, chondrocytes and tenocytes [104].

Although growth arrest has been described as the hallmark of replicative senescence, the phenotype of replcative senescence is characterised by a series of biomarkers such as changes in cellular morphology and altered growth properties. The following section reviews the major biomarkers of senescence.

### 4.5.1 Biomarkers of replicative senescence

Growth arrest is the most important feature of senescence in cell culture. Senescent cells are arrested in the G1 phase of cell cycle and are not able to progress to phase S of the cycle in response to mitogens [66, 260]. Although many of the genes remain responsive to the physiological mitogens, those that are essential for the transition of cell cycles become suppressed. It is believed that multiple, dominant acting genes are responsible for regulating the senescence process. Although the presence of growth factors and mitogens cannot reverse the growth arrest phase in the senescent culture, the senescent cells remain metabolically active and do not turn into apoptopic cells for an extended period of time.

It has also been shown that senescent cells have altered morphology. Senescent cells are bigger in size and senescent culture has different phenotypes in comparison with the cells in earlier passages [32].

Senescence-associated β-galactosidase is another important biomarker of senescence. β-galactosidase is a lysosomal hydrolase and is cytochemically detectable in most mammalian cells at pH 4.

In 1995 Dimri *et al.,* discovered that β-galactosidase has an abnormal activity in senescent human fibroblast culture at pH 6 [74]. β-galactosidase activity at pH 6 has been identified in different senescent cell types and consequently became known as senescence-associated β-galactosidase (SA- β-galactosidase) [163]. The mechanism behind this phenomenon is not fully understood but some studies suggest that SA- β-galactosidase abnormal activity at pH 6.0 reflects the increase in lysosomal content in the senescent cells [152, 235].

It has also been reported that senescent cells have a compromised ability for synthesizing heath shock proteins both *in vivo* and *in vitro* [44, 46, 87]. In addition, studies suggest that the expression levels of some proteins such as osteonectin, fibronectin and apolipoprotein change in senescent fibroblast culture [77, 105, 150].

### 4.5.2 Senescence phenomenon in tendons

In general tendons are subjected to early degeneration. Structural and functional properties of tendon decline with aging. Mechanically, tensile strength decreases and tendons become stiffer with age. It is believed that the decline in mechanical properties of tendon is caused by changes in collagen content and fibril morphology with age [203]. Senescence has been associated with a decrease in collagen turnover and reduction of collagenolytic activity, essential for remodelling in tendons [128, 129]. Moreover, the mechanical property of collagen molecule decline with aging as a result of changes in collagen cross-linking [251].

The number of tenocytes, cell morphology, protein production and enzyme activity also change with age [279].

It has been suggested that different types of stressors including chemical agents such as ethanol and mitomycin C and oxidative stress can induce senescence in fibroblast culture [73, 276]. However, our knowledge of the features associated with replicative senescence in tendons is limited. The following section investigates the effect of drug-induced senescence in tenocyte culture and the potential role of PRP in protecting tenocytes against the side effect of the drug.

### 4.5.3 Role of PRP in tenocyte senescent culture

β-galactosidase activity at pH 6 (senescence-associated β-galactosidase activity; SA β-gal) was detected by visualisation of the hydrolysis product of 5-bromo-4-chloro-3-indolyl-β galactoside which forms a blue precipitate under aqueous conditions. Among all three drugs (ciprofloxacin, etoposide and dexamethasone), the number of SA-β-gal positive cells was significantly higher in dexamethasone-treated cultures. To understand the efficacy of PRP in protecting tenocytes from entering the senescence stage in a stress-inducing environment the cells were treated with dexamethasone with/without PRP. To reduce the role of serum deprivation in inducing senescence in tenocytes the DMEM-F12 medium was supplemented with 5% FCS, in contrast to all other experiments with PRP where serum was removed prior to treatment to remove confounding serum-deprived growth factor effects.

β-gal expression was measured in untreated control (Figure 4.5A), dexamethasone (Figure 4.5B), PRP-dexamethasone (Figure 4.5C) and PRP alone conditions (Figure 4.5D).

The percentage of SA β-gal positive cells was 20% of total cells in control and about 7% in PRP alone (Figure 4.6).

The percentage of SA β-gal positive cells was increased to 50% in dexamethasone treated cultures, however, in the presence of PRP this was decreased significantly to 8% ($P<0.005$).

## 4.6 Effects of hypoxia on tenocytes

Until recently tendons have been considered to be metabolically inactive mainly because of their avascular appearance. However, recent studies suggest that despite

their poor vascular network tendons are more dependent on oxygen compared to other joint tissues such as cartilage [253].

It has been shown that three main pathways of energy generation, aerobic Krebs cycle, anaerobic glycolysis and the pentose phosphate shunt exist in tendon cells [174, 282].

With tendon aging, metabolic pathways shift from aerobic to anaerobic glycolysis. During the healing process, tendon relies on oxidative posphorylation suggesting a higher metabolic activity in the tissue [300].

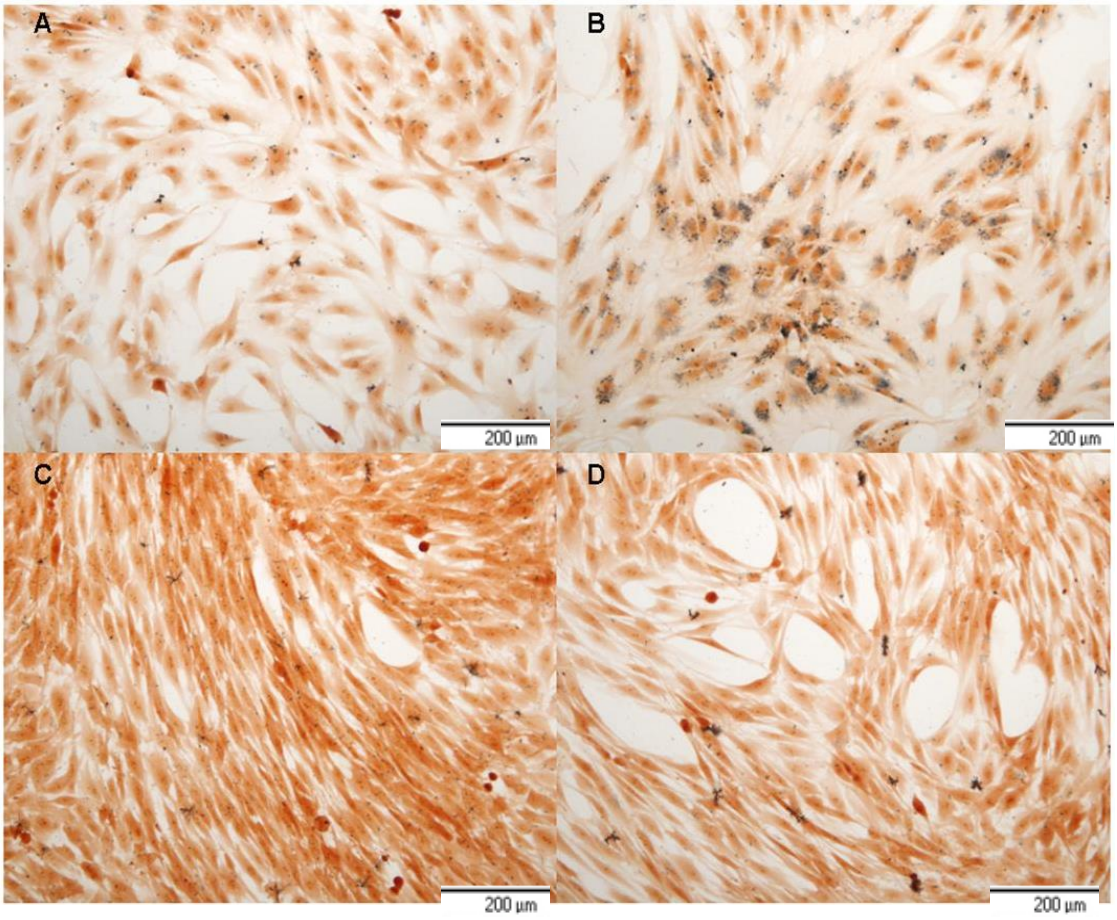


**Figure 4.5** 10% PRP protect tenocytes against senescence induced by 1µM dexamethasone (72 hours). Senescence-associated β-galactosidase activity was detected by visualisation of the hydrolysis product of 5-bromo-4-chloro-3-indolyl-β galactoside which forms a blue precipitate under aqueous conditions A) Untreated controls (DMEM:F12/ 5% FCS) B) 1µM Dexamethasone C) 1µM Dexamethasone together with 10 %PRP D) only 10%PRP. Neutral Red stain was used as a counter stain, (scale bar = 200 µm).

It is believed that the disruption of the blood networks following tendon rupture leads to localized hypoxia in the damaged area. Moreover, hypoxia may occur as a result of excessive loading during physical activity. The lack of oxygen reduces the metabolic activity and cellular ATP level could not be maintained which may result in slow and compromised healing in tendons [41, 42].

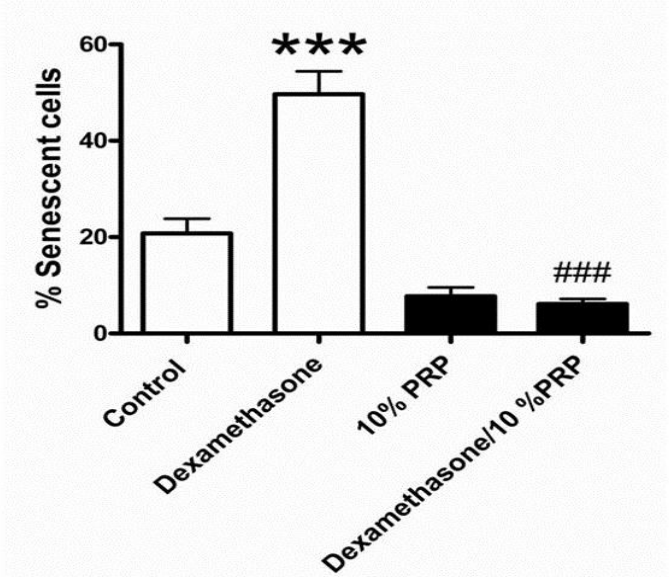


**Figure 4.6** Quantification of senescent cells after 72 hours culture in medium supplemented with 5% FCS. Cells were untreated (control) or treated with 1µM Dexamethasone, 10% PRP or $10^{-6}$ M dexamethasone together with 10% PRP. The values are shown as the mean and standard error of the mean of the values obtained from three different patients, with four replications per subject. ***P<0.005 compared to the values of control. ###P<0.005 Dexamethasone vs Dexamethasone/10%PRP. DMEM: F12/5% FCS was used as control.

Classical hypoxia pathways have been recently described in tendons. High level of hypoxia-induced factor α, HIF-α, vascular endothelial growth factor (VEGF) and hypoxia-induced death gene, BNIP3, are demonstrated in torn rotator cuff tendon [39].

All this data suggests that hypoxia could be a potential contributor to tendon degeneration. Therefore, it is of great importance to develop strategies in order to protect tenocytes negatively affected by hypoxic conditions.

The following section investigates the role of platelet rich plasma as a protective strategy in hypoxic tenocyte culture.

### 4.6.1 Platelet rich plasma protects tenocytes from hypoxia-induced cell death

Tenocyte were cultured in 10% FCS until 70-80% confluence. The medium was replaced with 1% FCS or 10% PRP conditioned media 48 hours prior to hypoxic exposure. Live & Dead staining showed no detectable cell death in normoxic cultures (Fig 4.7 A, B). Following 48hours of hypoxia the ratio of dead to live cells was clearly increased (Fig 4.7B). However, treatment with PRP provided protection for tenocytes in hypoxic conditions, at least over this short time period (Fig 4.7D). PRP reduced the percentage of dead cells from 20% to 8% of total cells (Fig 4.8).

## 4.7 Discussion

Ciprofloxacin and dexamethasone are commonly used drugs for a broad spectrum of disorders. However, application of both drugs has been associated with tendon problems. Little is known about the pathologic mechanisms and cellular alterations which lead to these adverse effects. This thesis provides evidence that both drugs adversely affect primary tenocyte viability, with ciprofloxacin inducing overt cell death and dexamethasone inducing senescence-associated changes in healthy human tenocytes.

Platelet rich plasma is currently being widely tested as a soft tissue healing agent and it is demonstrated here that PRP is strongly protective against tendon damaging drugs at least *in vitro*.

Healthy and viable tenocytes have balanced metabolic activity which is necessary for tendon matrix turn over. It is important to understand the influence of commonly used drugs with known soft tissue side effects on tenocyte viability since this has consequences for tendon function.

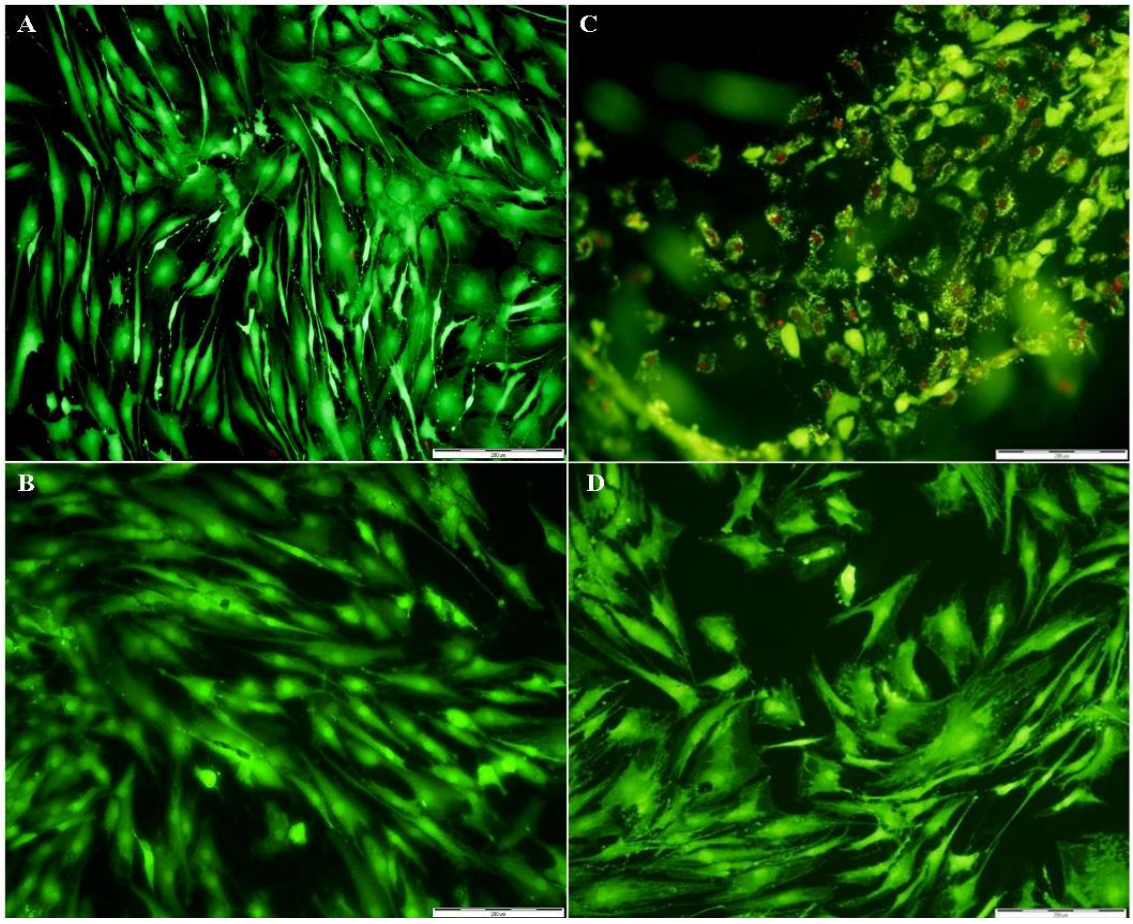


**Figure 4.7** Platelet rich plasma protects tenocytes against hypoxia-induced cell death Live & Dead staining of tenocytes following treatment of cells for 48 hours under normoxia alone (DMEM: F12/1% FSC) (A), 10% PRP conditioned medium (B) and following 48 hours with 0.1% oxygen alone (C) , with addition of 10% PRP conditioned medium (D), (scale bar = 200 µm).

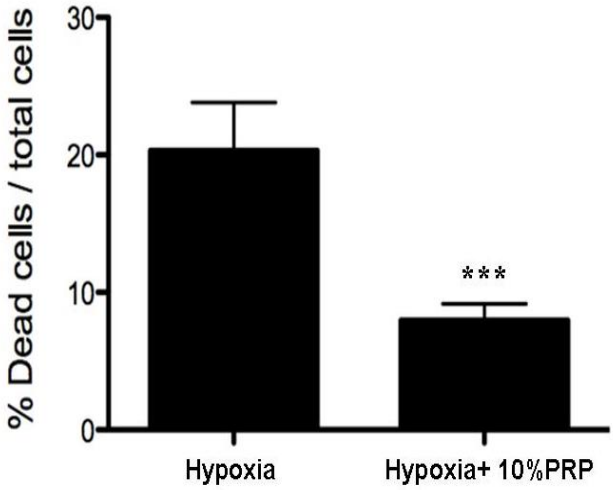


**Figure 4.8** Percentage of dead cells vs. total cells with hypoxia treatment alone or in combination with 10% PRP (mean ± SD, n=5). *** P <0.005 vs Hypoxia

It has been demonstrated in this chapter that ciprofloxacin causes a dose-related decline in tenocyte viability. A decrease in tenocyte viability with increasing concentration of ciprofloxacin (10 to 50 µg/ml) was observed. This could suggest that the development of ciprofloxacin-induced tendon disorders is a dose-dependent phenomenon. It should be noted, however, that the fluoroquinolones are difficult to dissolve and are generally supplied as a suspension. Therefore the absolute concentrations used are approximate and it is therefore difficult to advise on harmful *in vivo* doses.

Studies have shown that glucocorticoids inhibit cell proliferation and reduce cell number in tenocytes [248, 274, 305]. Because of the widespread use of this class of drugs to treat painful joint conditions, it is critical to consider the adverse effects of dexamethasone on tenocyte proliferation particularly in patients undergoing tendon repair where active proliferation is essential.

This study suggested that a therapeutically relevant dose of dexamethasone reduced viable cell number in tenocytes. The effect of dexamethasone on tenocyte viability was less distinct than ciprofloxacin.

This study did not detect an increase in cellular death after dexamethasone treatment suggesting that dexamethasone inhibits cell proliferation rather than causing tenocyte death. Similarly, Wong *et al.,* reported no effect of $10^{-9}$-$10^{-4}$ M dexamethasone on tenocyte apoptosis [305] and an increase in apoptosis was only observed by Hossain *et al.,* in tenocytes when much higher doses of dexamethasone were administered [118].

In the present study dexamethasone treatment also led to a significant increase in the number of senescent cells. To our knowledge, this study is the first work to demonstrate that dexamethasone can induce senescence in tenocyte cultures.

One other study has reported senescence-associated morphological changes in muscle cells following dexamethasone treatment [207] suggesting that glucocorticoid-induced cellular senescence may also occur in other cell types. There are two forms of cellular senescence: replicative senescence and stress-induced premature senescence (SIPS). Increased β-galactosidase activity at pH 6 is a characteristic feature of both forms of senescence [74]. A common feature of senescent cells is increased lysosomal number and/or activity. Lysosomal β–galactosidase is active at a lower pH than other cellular β-galactosidases and hence the increase in β-galactosidase activity at pH 6 in senescent cells is a reflection of the increased lysosomal compartment in these cells [163]. Senescent cells do not proliferate therefore glucocorticoid-induced induction of senescence in tenocytes may contribute to the reduced viable cell number we observed in dexamethasone-treated tenocytes.

In human fibroblasts, senescent cells have been found to synthesise abnormal extracellular matrix proteins [30]. Tenocytes are also of the fibroblastic lineage. Therefore glucocorticoid treatment of tenocytes may not only reduce tenocyte cell number but may also reduce the ability of tenocytes to synthesise normal tendon tissue. These changes are likely to impact on the integrity of tendon tissue and the ability of tendon to heal following injury.

The mechanism behind dexamethasone function in tendons is not fully understood. Recently, it has been shown that dexamethasone induces generation of reactive oxygen species in tendon cells and strongly up-regulates stress-response transcription factors FOXO1 and FOXO3A. FOXO1 in particular represses collagen synthesis. Moreover, phosphorylation of ERK and protein kinase B/Akt, which regulate cell proliferation and also inhibit forkhead activity, was reduced by dexamethasone treatment. It has also been demonstrated that growth factors targeting ERK and PKB are partly protective because they inactivate FOXOs [219]. This may explain part of the protective effects of PRP as it provides a rich source of growth factors.

The results presented in this chapter suggest that PRP effectively protects against the loss of viability of tenocytes induced by both ciprofloxacin and dexamethasone. However, the mechanism by which PRP protects tenocytes from the adverse effects of dexamethasone and ciprofloxacin has yet to be investigated. Moreover, the percentage of senescent cells was significantly decreased by PRP treatment. It was not possible to discern whether PRP prevented or reversed senescence as it was added concurrently with dexamethasone. Therefore, the type of senescence in this study remains unknown.

The PRP was applied to cultures in a concentration that is commonly used in studies related to PRP[5, 6, 16, 70]. A 100% concentration of PRP was not applied in this study because this dose of PRP is unlikely to be achieved during clinical application of PRP to tendons by injection during surgery [16]. It is believed that cells are exposed to 100% PRP for only a few minutes and PRP will be diluted in extracellular fluid soon after injection [70]. However, a major limitation of this work and other studies on PRP applications is the fact that the growth factor concentrations in the individual releasates depend on the activation of platelets which is difficult to standardize and consequently, the comparison of experimental results becomes relatively complicated.

The PRP used in this study, obtained from 6 volunteers, was applied heterologously to tendon cell cultures from patients, unlike in clinical settings where the PRP is prepared from the patient's own blood. This could produce the risk of a graft versus host reaction in culture [16, 70]. Cells responded to PRP conditioned medium with active growth and PRP-treated cultures displayed both fewer dead cells and less senescence than untreated controls, suggesting that graft-host response did not exist or negatively affect the cells in our experiments.

It is necessary to continue using glucocorticoids and fluoroquinolones therapeutically as both drugs are highly effective in treatment of inflammatory and infectious conditions. Therefore, it is important to come up with the new strategies to minimize their adverse effects on tendon. Local steroid injections present the highest risk of damage to tendon but could easily be combined with co-injection of a protective substance such as PRP to block side-effects. Fluoroquinolones are systemically administered but where the patient has a tendon injury, existing or fluoroquinolone-

induced, co-treatment by injection with PRP holds promise as an effective agent to promote healing and repair.

The role of hypoxia in inducing tenocyte death and potential of PRP in protecting tenocytes in hypoxic conditions has also been discussed in this chapter.

Mechanical overload of tendon tissue could result in localized hypoxia and consequently cell death. It has been shown that high mechanical strain could cause apoptosis in rat tendon. Moreover, apoptotic genes have been up-regulated after excessive training in the same study [189].

Here, it was demonstrated that the percentage of dead cells increases after 48 hours exposure to hypoxia. However, in the presence of PRP percentage of dead cells was significantly lower compared to untreated controls. This may suggest that PRP may be effective in preventing cellular death caused by hypoxia in tendon tissue and could be used in the clinical setting to enhance tissue repair.

The mechanisms behind the positive effects of PRP are not fully understood and further investigation, beyond the scope of this bioengineering thesis, is required to understand the pathways responsible for this effect.

# Chapter 5 Platelet rich plasma induces migration and chemotaxis in tenocytes

## 5.1 Introduction

Chapter 2 covered important studies on the role of mechanical loading and platelet rich plasma in tendon healing. Chapter 3 outlined the material and methods used to investigate the overall aim of this thesis. In chapter 4 the role of PRP as a protective strategy in challenging environments created by drugs and hypoxia in tenocyte culture was discussed. This chapter extends the framework presented for the use of PRP in chapter 4 by investigating the role of PRP in inducing migration and chemotaxis in tenocyte culture using a wound healing scratch assay and transwell chamber, respectively.

The hypothesis is that the pro-healing response induced by PRP would stimulate chemotaxis in tenocytes and induce migration of the tenocytes adjacent to the wound. Additionally, this study investigates the effects of dexamethasone on tenocyte migration and the potential merit of PRP co-treatment/co-injection to reduce the undesirable effects of dexamethasone is investigated.

## 5.2 Role of migration and chemotaxis in tendon healing

Tendon healing is a highly regulated process which is mediated by a variety of molecules and cellular processes. Although cell proliferation has a vital role in tendon healing and subsequent tissue regeneration, the cells must first migrate to the wound area for the healing process to begin.

As described in chapter 2 tendon healing can occur intrinsically through proliferation and migration of epitenon and endotenon tenocytes or via extrinsic mechanisms, by migration of cells from the surrounding sheath and synovium [252]. The migration process is an essential stage in both types of healing and requires the coordination of numerous molecules and pathways.

One of the important phenomena involved in regulating cell migration is chemotaxis. This process is characterized by movement of cells along a chemical concentration gradient and is regulated by different molecules, chemicals and cellular pathways [79]. It has been demonstrated that different chemical factors such as growth factors could induce chemotaxis [216, 264, 290, 303].

Chemotaxis plays a critical role in regulating directional cell migration during embryonic development and postnatal homeostasis and healing [213, 244, 250] and is well described in different types of cells including inflammatory cells and fibroblasts [244, 290]. However, our knowledge on the role of chemotaxis in different stages of tendon healing including migration is restricted to a few studies [101]. Moreover, our knowledge on the potential effects of chemotaxis-inducers, i.e. growth factors, on tenocyte migration is even more limited. Therefore, it is important to investigate the role of chemotaxis in cellular migration and other key processes involved in tendon healing.

In addition, identifying potential chemotaxis-inducers in tenocytes will be useful in the development of clinical therapies for tendon disorders.

## 5.3 Role of growth factors in tendon healing

As discussed in the previous chapters growth factors play key roles in initiating and sustaining different cellular processes involved in tissue healing[194]. In vascularised

tissues, the repair process is initiated by the release of different growth factors and cytokines from degranulating platelets. However, although ruptured tendons do make a blood clot, the relatively avascular nature of tendon tissue may compromise this process and contribute to slow and unsatisfactory healing[293].

Understanding the role of growth factors in stimulation of chemotaxis and migration in tenocytes provides a rationale for use of growth factor-based therapies such as PRP in tendon healing.

Chapter 2 provided a summary of important growth factors released from the platelets and their general role in tissue healing. This section reviews some of the recent investigations into the role of the four best characterized growth factors in tendon healing with particular interest in their effect on migration and chemotaxis; platelet-derived growth factor (PDGF), insulin growth factor I (IGF-I), transforming growth factor β (TGF-β) and basic fibroblast growth factor (bFGF)

### 5.3.1 Role of PDGF in tendon healing

PDGF is secreted shortly after tendon injury and regulates synthesis of other growth factors, including IGF-I. *In vitro* studies suggest that PDGF also plays a critical role in the late remodelling stage of tendon healing by stimulating the synthesis of ECM proteins such as collagen [320]. PDGF is a strong chemoattractant for human inflammatory cells, fibroblast and smooth muscle cells [280].

Different isomers of PDGF have been studied to improve healing in tendon. It has been shown that PDGF-BB increases proliferation of tenocytes and promotes collagen remodelling during the flexor tendon healing in dogs [268].

In a different study transfer of exogenous PDGF genes into rat intrasynovial tenocytes increased collagen synthesis which improves the healing properties of tendon[296]. Moreover, PDGF-BB in combination with IGF-I and mechanical loading stimulates DNA synthesis in avian flexor tendon cells [28].

### 5.3.2 Role of IGF-I in tendon healing

It has been demonstrated that the concentration of IGF-I increases significantly at the site of injury during the first few days of healing, suggesting IGF-I involvement in the inflammatory and proliferative phases of tendon healing [245, 278, 286, 287]. In addition, IGF-I plays an active role in stimulating proliferation and migration of fibroblasts to the site of injury [2, 123].

In vivo, treatment with IGF-I improved the biomechanical properties of healing tendon in a rat model. The same study also showed reduced inflammation in tendons treated with IGF-I compared to untreated controls[151]. However, the exact mechanism behind the effect of IGF-I on tendon inflammation is not understood.

### 5.3.3 Role of TGF-β in tendon healing

TGF-β is active in almost every phase of tendon healing. It has been shown that TGF-β stimulates extrinsic cell migration and regulates cell proliferation during wound healing [37, 38, 327]. However, *in vivo* studies suggest that the presence of TGF-β could be associated with adhesion formation in tendons [56]. Different studies have investigated the effects of other members of TGF-β family on tendons. Forslund et al., reported that a single injection of cartilage-derived morphogenetic protein-2 (CDMP-2 or GDF-6) improves the mechanical properties of Achilles tendon of rat [91].

### 5.3.4 Role of bFGF in tendon healing

It has been shown that basic fibroblast growth factor (bFGF) stimulates migration and proliferation in tenocyte culture [56, 194].

A study by Chang et al., showed an increase in expression of bFGF in both tendon parenchyma and tendon sheath during flexor tendon healing in rabbits, suggesting the involvement of both intrinsic and extrinsic cells in synthesis of growth factors [58].

*In vivo* studies demonstrated an increase in proliferation and type III collagen expression in the early phases of healing after treatment with bFGF in rat patellar tendon [57] .

## 5.4 Advantages of PRP therapy versus isolated growth factor therapy

Each of the growth factors discussed in the previous section has crucial and varied roles in tendon healing and could be used to accelerate and improve the healing process. However, delivery techniques such as direct injection and surgical implants are still unreliable and need to be modified in order to facilitate the delivery of sufficient doses of growth factors to the injured site. In addition, many of these growth factors have synergistic influence on each other and are fully functional only in combination with other growth factors.

PRP provides an autologous and relatively cost effective combination of growth factors which could be easily and safely delivered to the site of injury and eliminates the need for growth factor delivery tools such as scaffolds and implants. Moreover, some of the growth factors released by PRP regulate the synthesis of other important growth factors of the healing cascade.

It has been shown that the growth factors within PRP exert mitogenic effects on tenocytes and have been used to accelerate and enhance tendon healing in the clinical setting[12, 16, 193]. However, studies on the role of PRP in stimulating tenocytes migration are limited and there are no reports on chemotactic responses induced in tenocytes by PRP.

The following section reports the results of investigation into the role of PRP in stimulating migration and chemotaxis in human primary tenocytes by using wound healing assays and transwell chambers, respectively.

Moreover, the work presented in this chapter extends the observation concerning the effects of dexamethasone on tenocytes by investigating the role of this drug on tenocyte migration. The effects of PRP on the migratory ability of dexamethasone-treated tenocytes have also been investigated in this chapter. The novel findings and data provided in the current chapter support the hypothesis that PRP could improve the healing process in tendons by inducing migration and chemotaxis in tenocytes.

## 5.5 Results

### 5.5.1 Role of PRP in stimulating human tenocyte migration

The wound healing scratch assay was used to measure directional tenocyte migration in the presence of PRP. The migration of tenocytes towards the denuded area, mimics the *in vivo* migration of neighbouring tenocytes to the wound area during tendon healing.

Migration of tenocytes into scratch area (Figure 5.1 A&D) was measured over the course of 6 days. In the presence of 10% PRP the coverage of the scratched area

was significantly higher compared to the controls after 72 hours. Figure 5.1 B&E and 5.1 C&F show the cell coverage of the scratched region in control and PRP treated conditions, respectively.

After 72 hours, cell coverage of the scratched region was 72% and 25% of unscratched controls in PRP treated and control conditions, respectively (Figure 5.2). After 6 days, tenocytes in PRP treated conditions completely covered the scratched area and were over-confluent. Although this coverage is produced both by migration and proliferation, the gap did not fill in from the edge but throughout the scratch track, indicating not only proliferation but also migration.

### 5.5.2 Role of PRP in stimulating chemotaxis in tenocyte culture

The transwell chamber assay was used to examine the guided migration or chemotactic response of tenocytes to PRP. 100% fresh PRP clot was placed on the bottom compartment of the chamber and was immediately activated by thrombin. Cells were seeded on the upper membrane and were left to migrate from the top chamber to the bottom chamber of the dish.

The fresh PRP clot was used for this experiment to provide a concentrated source of released growth factors from the base of the well over a period of hours to days. Simply filling the chamber with PRP-conditioned medium as used in previous experiments would not have achieved this concentration gradient.

The populations of cells on both sides of the chamber membrane were measured over the course of 6 days using Calcein AM Live stain for highly sensitive imaging of cells. No cells were detected on the bottom side of the membrane in both control and

PRP conditions after 48 hours. However, tenocytes showed an increased migration towards the PRP clot after 4 days. Figure 5.3 shows tenocytes on mainly the upper

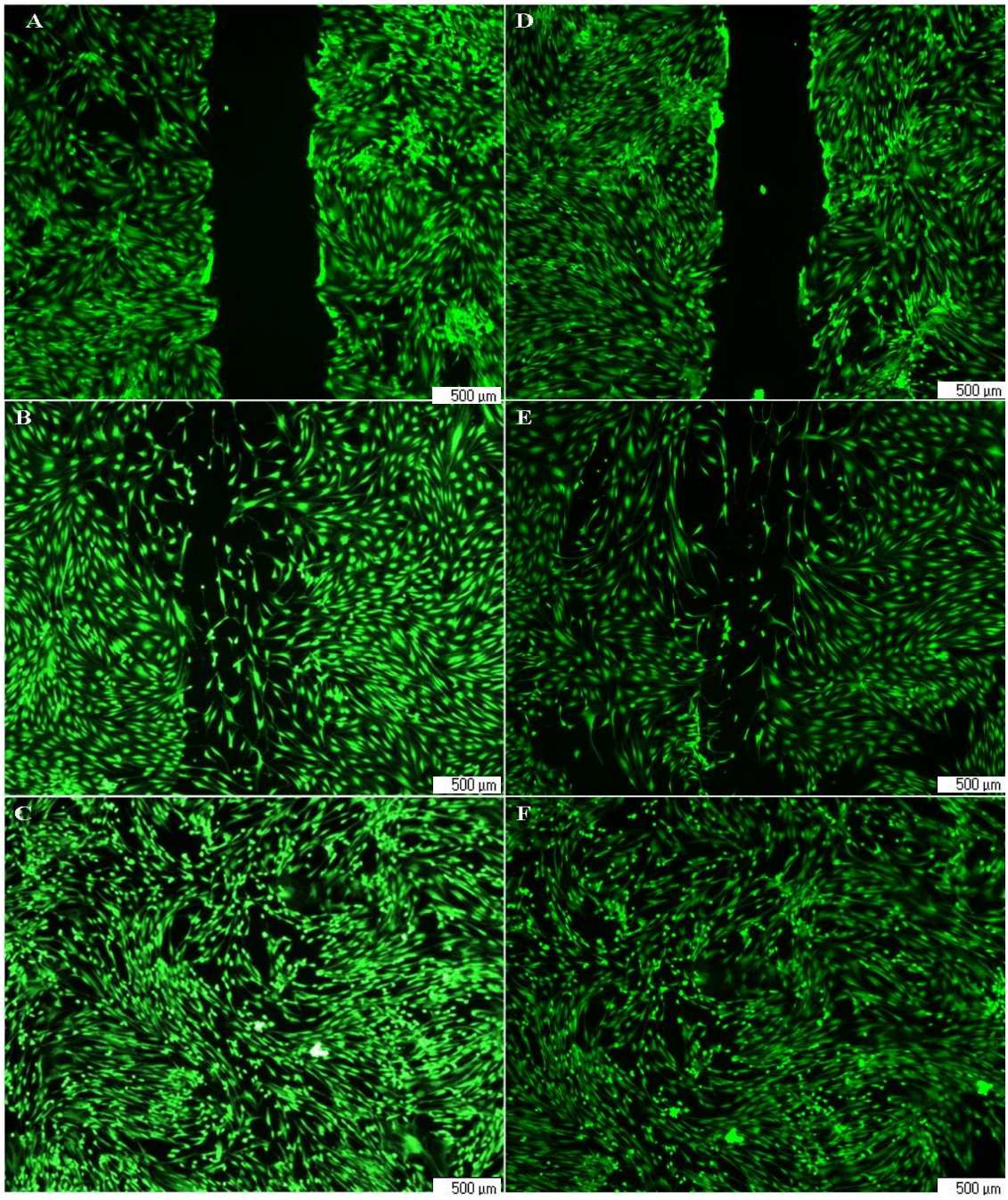


**Figure 5.1** The effect of PRP on migration of human tendon cells in wound healing assay after 72 hours. A&D) Scratch area B&E) Controls (DMEM: F12) C&F) 10% PRP, Live calcein AM stain was used as a fluorescent stain, green fluorescence (ex/em 495/515 nm) was captured with a FITC filter, scale bar= 500 µm

membrane surface in control wells but both sides of membrane in PRP wells after 4 and 6 days.

After 4 days, 3% and 24% of tenocytes had migrated to the bottom layer in control and PRP conditions, respectively. After 6 days, the percentage of migrated cells increased to 14% in controls and 31% in PRP chambers (Figure 5.4).

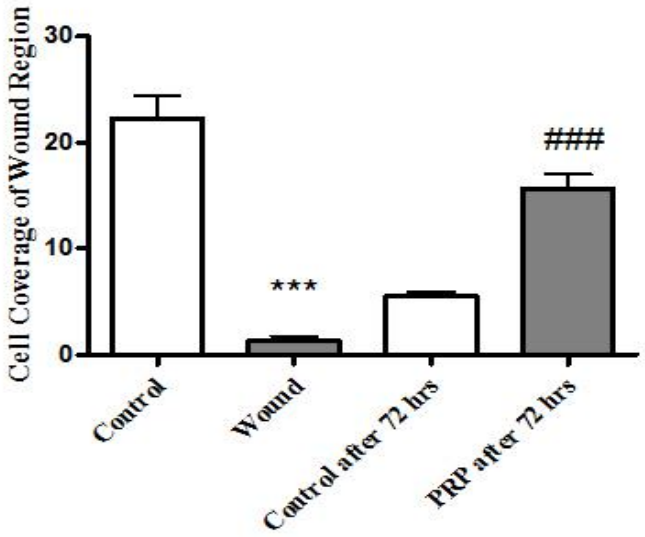


**Figure 5.2** Quantification of cell coverage of scratch region after 72 hours. The values shown are the mean and standard error of the mean of the values obtained from 3 different patients, with 4 replications per subject. Asterisks denote comparison of the unscratched control with wound (***P< 0.005), Hash marks denote statistically significant comparison of 10% PRP with control after 72 hours (### P< 0.005). DMEM: F12 was used as control.

### 5.5.3 PRP induces migration of dexamethasone-treated tenocytes

Dexamethasone is reported to inhibit migration of vascular smooth muscle cells and neutrophils in both animal and human [212, 220]. However, experimental evidence on the influence of dexamethasone in tenocyte migration has not been fully discussed in the literature. This study hypothesised that tenocyte migration is one of the cellular processes that may be influenced by dexamethasone treatment. As a consequence, it is

important to come up with the new strategies which retain the anti-inflammatory activity of dexamethasone while minimising its detrimental effects on tendons.

The wound healing scratch assay was used to investigate the effects of dexamethasone on tenocyte migration. 1μm of dexamethasone significantly decreased the migration and/or proliferation of tenocytes into the wound region in comparison to controls after 72 hours (Figure 5.5 C&G). PRP significantly increased the cell coverage of scratched area in dexamethasone-treated conditions after 72 hours.

Cell coverage of scratched area was approximately 31% of the controls after 72 hours ($P < 0.005$). The cell coverage in PRP/Dexamethasone treated conditions was about 3 times higher than controls during the same period ($P< 0.005$) (Figure 5.6).

## 5.6 Discussion

Tendon healing is a complex process that is initiated by the influx of inflammatory cells and tenocytes to the site of injury and terminated by the remodelling and regeneration of tendon tissue.

Tenocyte migration plays an essential role in the inflammatory and regenerative phases of tendon healing. Migrated tenocytes proliferate and regulate the production of collagen and other fundamental extracellular matrix components.

Platelet rich plasma is a natural source of different growth factors which are involved in tendon healing by regulating different processes shown in this chapter to include tenocyte chemotaxis and migration.

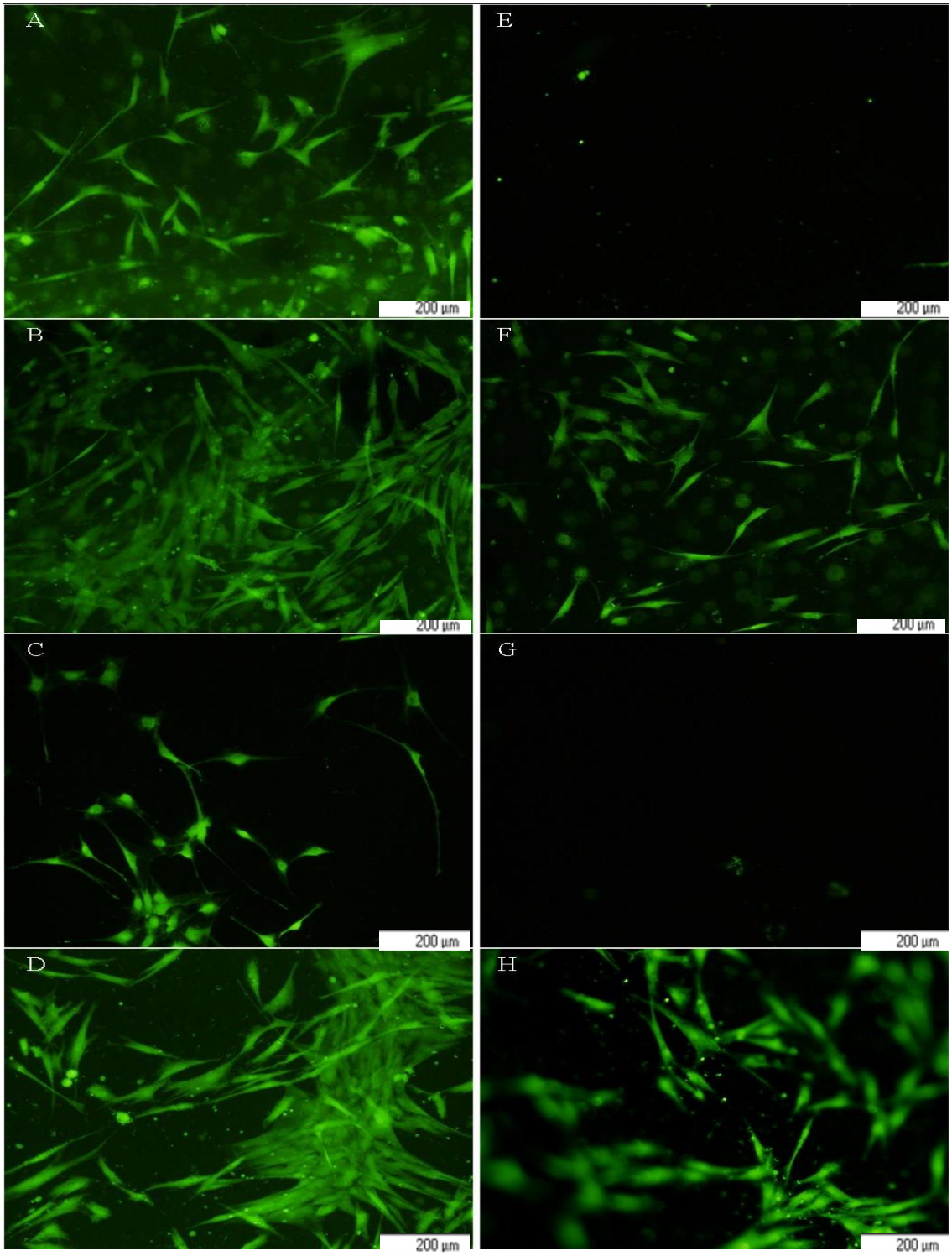


**Figure 5.3** Chemotactic response of tenocytes to PRP clot using transwell chamber assay after 4 and 6 days of exposure. Photographs A through D show the tenocytes on the top layer of membrane. Photographs E through H show the tenocytes migrated to the bottom layer of membrane in response to chemoattractant (PRP). A, E and C, G are controls after 4 and 6 days ,respectively. B, F and D, H are PRP treated wells after 4 and 6 days, respectively, 200µm.

In this section, it was demonstrated that PRP significantly induces migration of tenocytes in a wound healing model. A significant increase in tenocytes number at the wound region was observed after 72 hours treatment with 10%PRP.

Migration of tenocytes to the site of injury could set the stage for tenocyte proliferation and deposition of the abundant extracellular matrix which consequently results in enhanced and accelerated tendon healing.

Chemotaxis, a necessary component of wound healing, is a complex process regulated by a large number of molecules including growth factors. It has been shown that PDGF is chemotactic to macrophages[16] and combination of PDGF, IGF, TGF-β induces chemotaxis in stem cells and osteoblasts[16, 94]. However, our understanding of the role of growth factors in inducing chemotaxis in tenocytes is limited to few *in vitro* studies[194]. Moreover, the application of individual growth factors in tendon healing has been mainly limited to animal models and there has been no report of growth factor therapy in the clinical treatment of tendon disorders[254].

It has been demonstrated that the chemotactic effects of growth factors in PRP could stimulate migration of circulation-derived cells including macrophages towards the site of injury which is essential in the early stages of tendon healing[125]. However, circulation-derived cells are not able to synthesize collagen directly and the presence of tenocytes is essential to initiate synthesis of collagen and other extracellular matrix components[252].

Therefore, it is important to induce migration of tenocytes to the site of injury in order to accelerate and enhance tendon healing in the early stages.

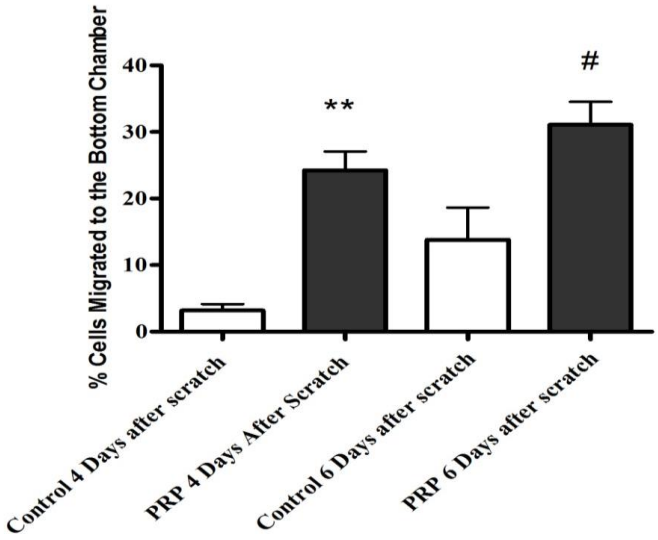


**Figure 5.4** Quantification of the migrated tenocytes after 4 and 6 days with/without PRP. Cells were untreated (control) or exposed to 100% PRP. The values are shown as the mean and standard error of the mean of the values obtained from 3 different patients, with 4 replications per subject. **P<.01 control versus 100%PRP after 4 days; #P<.05 Control versus 100% PR after 6 days

This study took a new approach to study the potential role of PRP as a chemoattractive agent for tenocytes by using transwell chamber. The hypothesis was that PRP stimulates chemotactic responses and induces directional migration in tenocyte culture. Tenocytes showed strong chemotactic response to PRP after 4 days of exposure by migrating towards the PRP clot. However, there was no observable chemotactic response towards the chemoattractant, PRP, within the first 48 hours of exposure. This is comparable to the scratch assay which also showed no strong response to PRP under 48hour. Further studies are required to understand the mechanism and timing behind the chemotactic response of tenocytes to PRP.

Glucocorticoids have been widely used as primary nonsurgical treatment for variety of musculoskeletal and dermal conditions. However, administration of glucocorticoids has been linked to degenerative side effects in skin and bone tissues[53, 205]

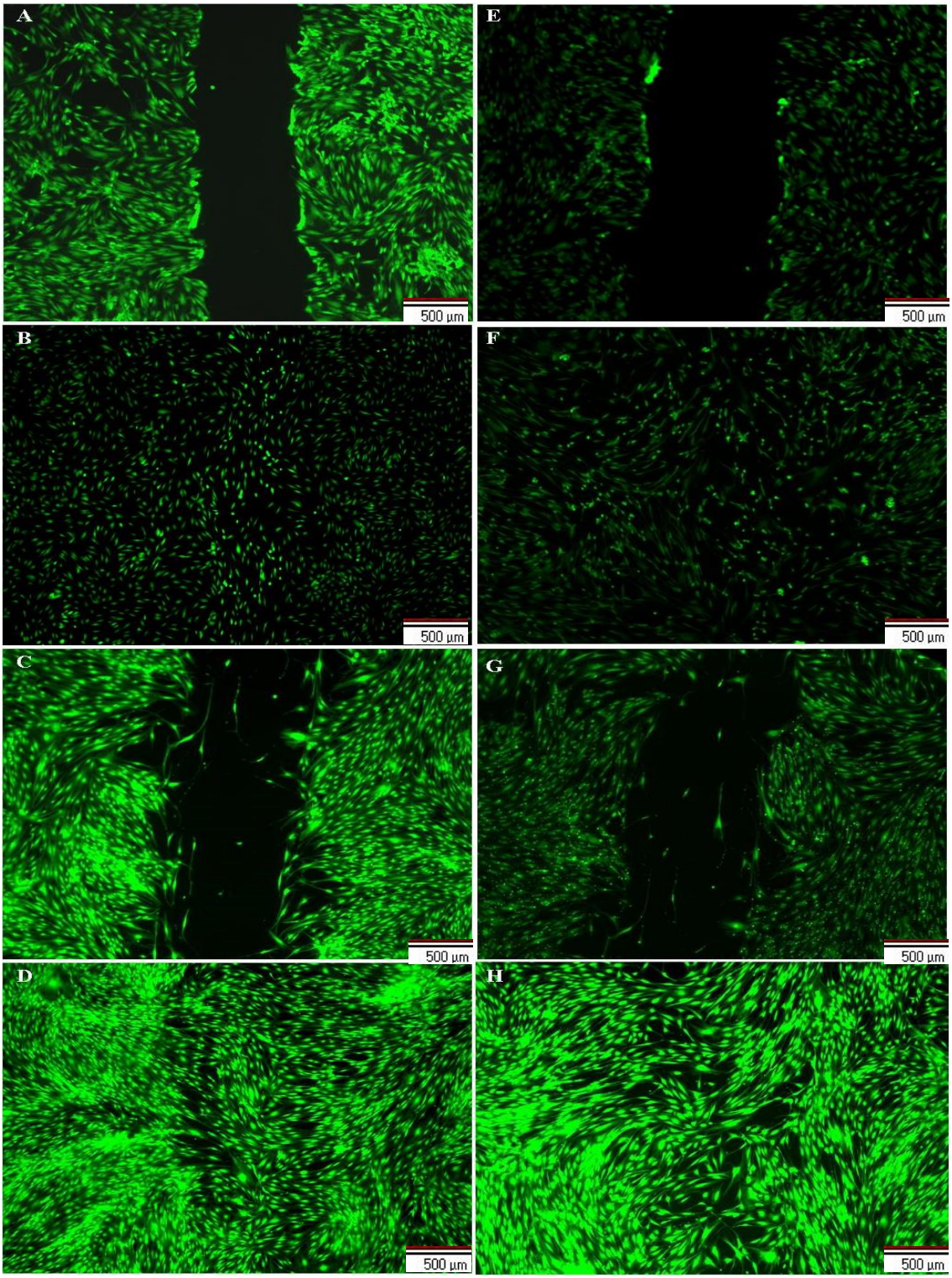


**Figure 5.5** Dexamethasone reduces tenocyte migration but PRP overrides this increase migration in dexamethasone-treated tenocytes after 72 hours. A&E) scratch area, B&F) controls, C&G) dexamethasone-treated D&H) PRP/Dexamethasone treated, scale bar= 500µm

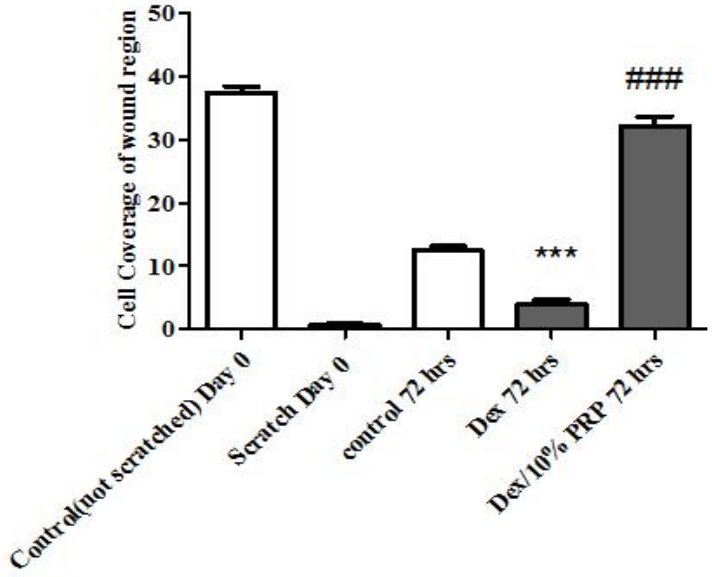


**Figure 5.6** The effect of PRP on migration of tenocytes after 3 days treatment with dexamethasone. Cell coverage of wound region was measured using Image J software for untreated controls, cells treated with $10^{-6}$ M dexamethasone, or co-incubation of $10^{-6}$ M together with 10% PRP. The values are shown as the mean and standard error of the mean of the values obtained from 3 different patients, with 4 replications per subject. ***P<0.005 dexamethasone versus control; ### P<0.005 dexamethasone versus dexamethasone/10% PRP

Also, spontaneous tendon ruptures have been reported in association with glucocorticoid injection[59, 62, 196]. It has also been shown that glucocorticoids cause a decrease in cell number and viability, collagen production and matrix protein output in tenocyte culture[219, 248, 274, 304].

This study used human primary tenocytes to investigate the effect of dexamethasone on migratory ability of this cell type. Migration of tenocytes towards the scratched area was significantly reduced in the presence of dexamethasone. This is likely to contribute to the adverse effects of dexamethasone on tendon tissue. The study by Tsai *et al.*, suggested that the inhibition of tenocytes migration after dexamethasone treatment could be caused by the decrease in the levels of gene expression of alpha-smooth muscle actin [277]. However, the exact mechanism behind this effect is not

understood.

Additionally, as shown in chapter 4 Dexamethasone reduces tenocyte proliferation or viable cell number which will contribute to the slow filling in of the scratch track. To test the relative contributions of proliferation and migration additional experiments should be performed using time lapse microscopy.

It is necessary to continue using dexamethasone as it is highly effective anti-inflammatory agent, therefore new strategies such as PRP have potential merit to be used as co-treatment/co-injection to reduce the undesirable effects of dexamethasone. This study has shown that co-treatment with PRP could negate the negative effect of dexamethasone on the migratory ability of tenocytes. Whether PRP directly neutralizes or simply overrides the inhibitory effects of dexamethasone on tenocyte migration is still unknown.

Two different preparations of PRP, 10% supernatant and 100% fresh PRP were applied to tenocytes in this study. 10% conditioned PRP media used in the scratch assay could be compared to the concentrations reported in the literature. However, fresh PRP was used for the chemotaxis experiments in order to provide a growth factor gradient over several hours/days and to assess the role of growth factors in PRP in inducing chemotaxis immediately after platelet activation similar to the clinical setting.

Unlike the clinical setting where PRP is produced from patients' own blood the PRP used in this study was applied heterologously to tendon cell cultures. Cells responded to PRP conditioned medium with active migration and chemotaxis, suggesting that graft-host response did not negatively affect the cells in this study.

Poorly vascularised tendon tissue may not provide sufficient growth factors for healing after tendon injury, leading to major delays in different stages of tendon

healing. Here, it was demonstrated that PRP significantly increases migration of tenocytes and induces chemotaxis in tendon culture. This suggests that PRP injection could be used as complementary or standalone treatment to accelerate and increase migration of tenocytes to the site of injury. The molecular mechanisms responsible for the positive effects of PRP on chemotaxis and migration of tenocytes require further investigation.

# Chapter 6 Effects of mechanical loading and platelet rich plasma on mechanical and biochemical properties of injured tendon

## 6.1 Introduction

Chapter 4 presented evidence of an entirely new mode of action of PRP in protecting tenocytes from damaging environments created by drugs and hypoxia. Chapter 5 demonstrated the effects of PRP on migration and chemotaxis of tenocytes using a wound model and transwell chamber, respectively. All these studies showed that the application of PRP has a positive effect on key parameters involved in tissue healing at a cellular level.

However, in order to understand the complete role of PRP in improving the healing of tendon tissue and providing a faster recovery of mechanical function it is important to investigate the role of PRP in tendon healing at tissue level. While animal models may demonstrate the efficacy of the treatment, deeper mechanistic understanding can only be obtained using the controlled environment provided by *in vitro* techniques.

Therefore, an *in vitro* tendon injury model which facilitates studying the effects of PRP on mechanical and biochemical properties of tissue during healing is an essential step in assessing the effectiveness of new therapeutic strategies such as PRP.

This chapter presents a novel *in vitro* tendon injury model which allows repeatable mechanical testing, quantitative biochemical assays and whole organ imaging of tendon tissue as it heals.

Mechanical loading has also been postulated as an important contributing factor to tendon healing and regeneration (Chapter 2). This chapter aims to describe the role of static loading in tendon repair using the novel tendon injury system developed for this project. The effects of static loading on biomechanical and biochemical properties of injured tissue have also been investigated by tensile test and different biochemical assays.

Finally, this chapter investigates the ultimate goal of the thesis which is the potential of combined mechanical loading and platelet rich plasma as non-drug based and non-invasive treatment method for tendon healing.

## 6.2 Tendon injury models

*In vivo* animal models have been frequently used to simulate human tendon injury and test the effectiveness of various treatment techniques. Different methods such as walking tracks, electrical stimulation and kicking machines have been used to create tendon injury or induce tendon degeneration in different animal models such as rabbits and rats [18, 26, 29, 187]. Moreover, the tendon laceration injury model has been widely used to mimic tendon rupture and study different stages of tendon repair in various animal models [149, 326].

Although the *in vivo* tendon injury models provide valuable insight to the pathophysiologic features of tendons at specific time points throughout the healing process, it is extremely difficult to control the experimental conditions and provide repeatable measurements using *in vivo* animal models. In particular, longitudinal time courses in the same animal are difficult except where using non-invasive imaging techniques, which are unlikely to deliver results at the cellular level

Therefore, it is essential to develop *in vitro* model systems which facilitate studying the potential of different tendon treatment regimes under controllable and repeatable conditions and which are small enough to permit live cellular imaging. The following section describes the in vitro injury model developed for this thesis.

## 6.3 3D Rat tail tendon (RTT) scratch injury model

In order to make significant progress in addressing the issues related to mechanical loading and PRP therapy, an *in vitro* tendon injury system was developed using rat tail tendon fascicles. A scratch injury was generated using 23 gauge insulin needles along the length of a defined region of the fascicle (Section 3.10).

Rat tail tendon (RTT) fascicles were chosen for the injury model because they are uniform in size and shape and are small enough to allow nutrition by diffusion and thus remain viable in culture for up to 2 weeks. These properties allow repeatable, quantitative mechanical testing paradigms and facilitate generation of a precisely repeatable scratch injury. Moreover, the small size of the rat tail fascicles permits whole organ imaging of the defect over time as it heals.

Different assessment techniques were used to characterize the mechanical and biochemical properties of the injured fascicles in order to validate the 3D RTT *in vitro* injury model. Live& Dead staining, DMB and Picogreen assays and mechanical testing were used to measure the viability, sulphated GAG content, double-stranded DNA (ds-DNA) content and Young's modulus of the injured fascicles, respectively. Collagen content was also assayed but the Sircol cell culture based assay proved to be unsatisfactory for analysis of whole tissue, despite extensive optimisation efforts. Moreover, Environmental scanning electron microscopy (ESEM) images of the injured

and intact fascicles have been presented to show the effects of the scratch injury on the structure of tendon tissue.

The validated injury model was then used to investigate the effects of static loading, PRP and static loading together with PRP (loading/PRP) on injured tendon fascicles after 4 and 7 days in culture using the previously described assessment techniques. The properties of intact fascicles were also assessed and reported as control for each group.

### 6.3.1 The viability of rat tail fascicles in culture

Although tendon fascicles can remain viable in culture for long period of time mechanical and biological properties of tissue may alter during this period. Therefore, it is important to consider the effects of culturing on the fascicle's while investigating different treatment options.

This section discusses the potential effects of culturing on the viability of tenocytes located within and between collagen fibrils and fibres using Live & Dead staining.

The ratio of live to dead cells was estimated every 3 days for two weeks. In the first day of culture, 95% of cells were viable and healthy in culture with only a few dead cells in the areas handled by forceps. After the first week of culture the number of live cells reduced to about 70% of cells with a higher number of live cells in the middle of the fascicle. At the end of the second week the number of live cells decreased to about 50% of total cells with the increase in the number of dead cells particularly on the surface areas of the fascicle. Figure 6.1 shows live & dead staining of fascicle after 1, 7 and 14 days of culture. This data suggests that although the number of dead cells

was increased after two weeks, the culturing technique used in the project could maintain sufficient cell viability within the tissue to enable its use as an *in vitro* model for tendon injury.

Live & Dead staining was also used to study the effect of scratch injury in inducing tenocyte death on both fresh and cultured fascicles. Figure 6.2 shows live & staining of intact and injured fascicles 4 days after the generation of the injury. Although the number of dead cells was increased immediately after injury this number was significantly higher after culturing the fascicles for 4 and 7 days.

### 6.3.2 Environmental scanning electron microscopy (ESEM) of tendon fascicle

The Environmental scanning electron microscope was used to study the structure of intact and injured fascicles. Unlike conventional scanning electron microscopy, ESEM facilitates imaging under auxiliary gases, e.g. nitrogen or nitrous oxide, which keeps the fascicles hydrated and minimizes the need for sample preparation that may affect the structural and mechanical properties of the biological samples.

Freshly harvested fascicles with no chemical preparation were used for ESEM imaging. Injury was generated immediately after harvesting the fascicles from rat tail and before imaging. Figure 6.3 shows ESEM images of intact and injured fascicles. Images from scratch site show significant disarrangement of the fibres and increase in the cross sectional area of the fascicles.

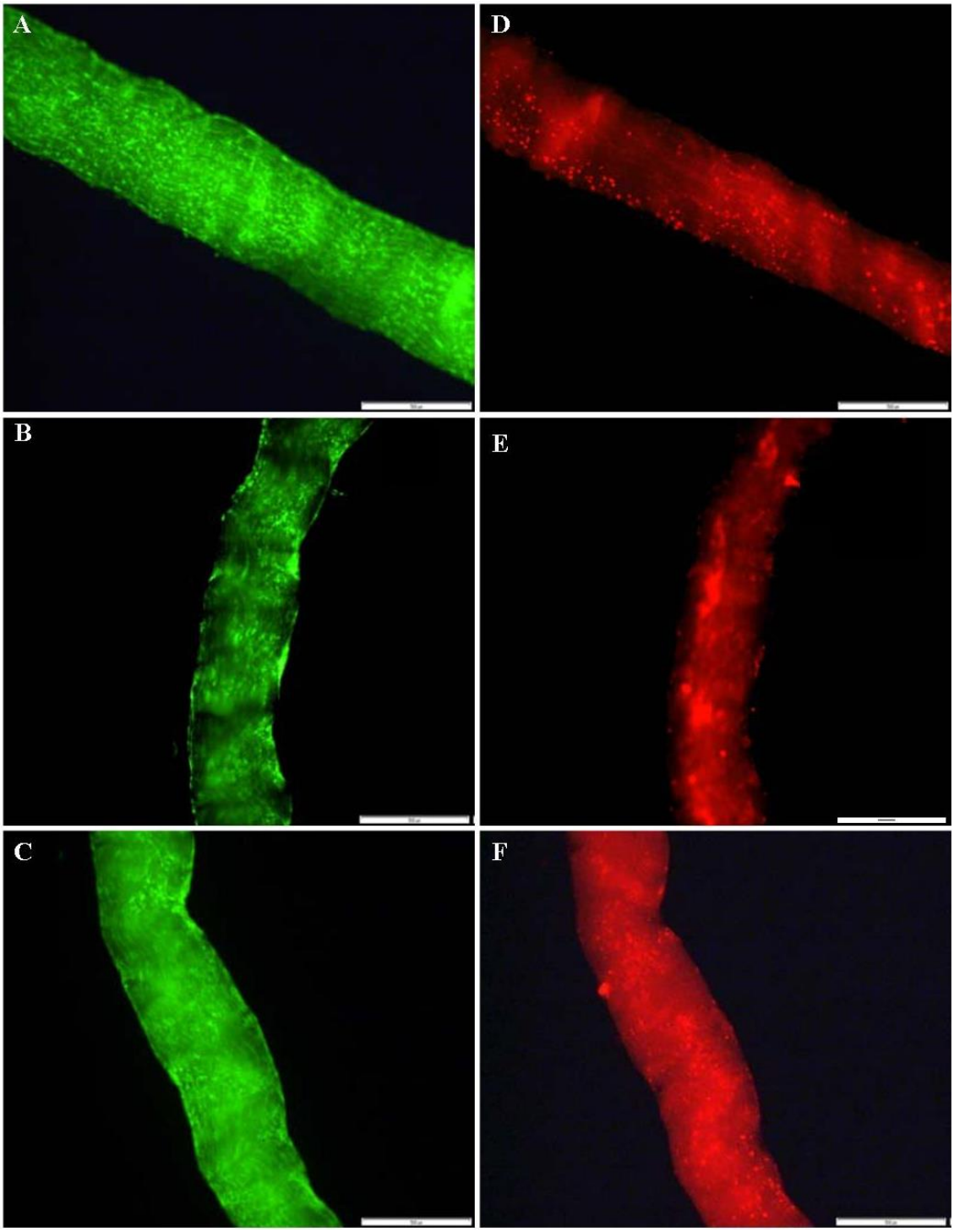


**Figure 6.1** The effect of culturing on the viability of rat tail tendon fascicle after 2 weeks: Live & Dead staining was performed after A&D) 1 day in culture B&E) 7 days in culture C&F) 2 weeks in culture, Photographs A through C show Live cells; green fluorescence (ex/em 495/515 nm) was captured with an FITC filter. Photographs D through F show Dead cells; red fluorescence (ex/em 495/635 nm) was captured with rhodamine filter, scale bar=500µm.

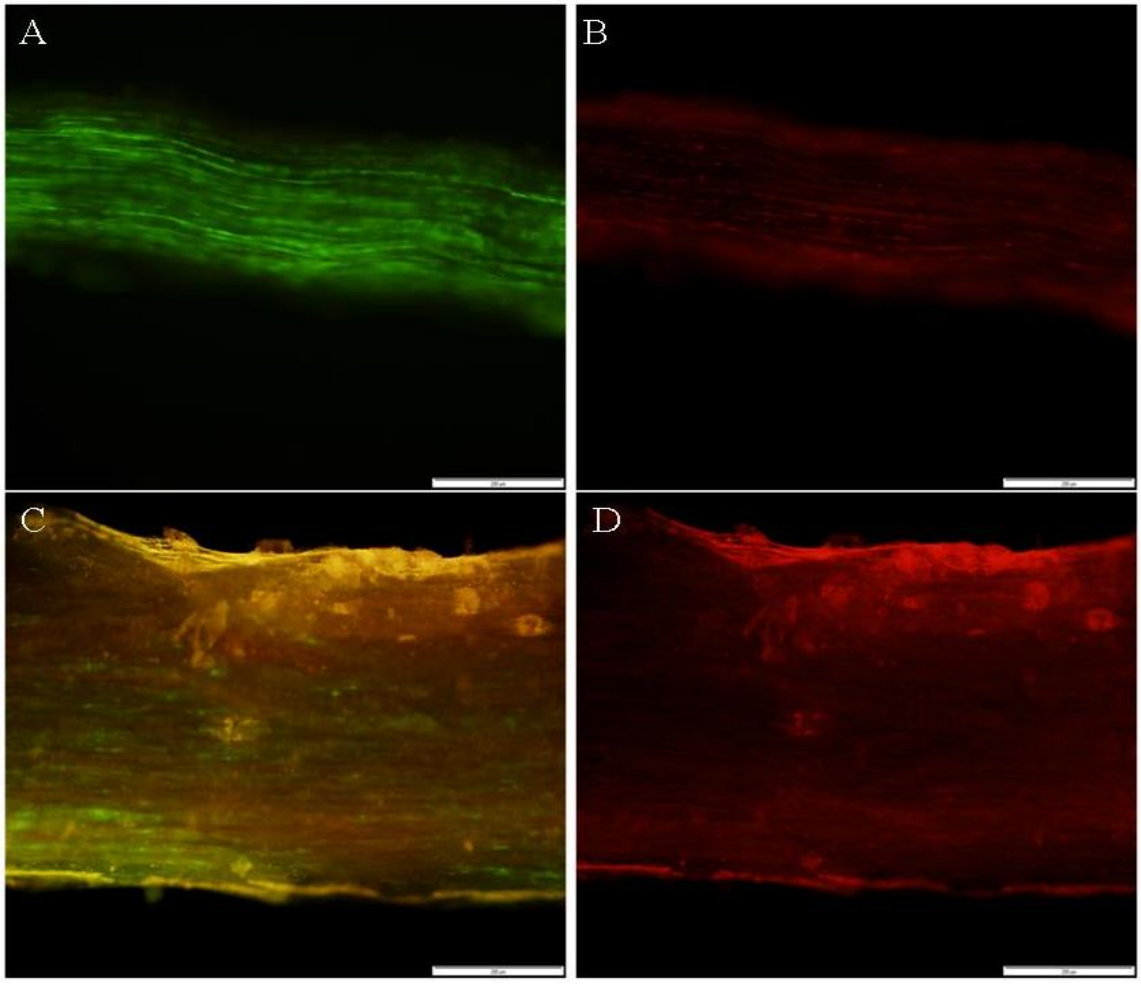


**Figure 6.2** The effect of scratch injury on the viability of fascicles after 4 days in culture. Live & Dead staining of A&B) Intact fascicle C&D) Injured fascicle after 4 days in culture, Photographs A and C show Live cells; green fluorescence (ex/em 495/515 nm) was captured with an FITC filter. Photographs C and D show Dead cells; red fluorescence (ex/em 495/635 nm) was captured with rhodamine filter, scale bar= 200µm.

### 6.3.3 Mechanical validation of scratch injury model

Displacement controlled tensile testing was performed in order to measure the mechanical properties of intact and injured fascicles immediately after harvesting (fresh) and after 4 and 7 days in culture. Figure 6.4 shows the load-displacement curve of a fresh rat tail tendon fascicle. The representative stress-strain curves showed similar regions, toe, linear and failure, as typical tendon stress-strain curves. The cross

sectional area of fascicles was measured using a digital area micrometre and the stress-strain curve was plotted in order to calculate Young's modulus of fascicles in different groups. The values of Young's modulus (*E*) have been reported as a measure of tendon material behaviour during elastic deformation.

This section reports Young's modulus values of injured fascicles immediately after generation of the damage (fresh/injured)[1]and after 4 and 7 days in culture[2]. Moreover, Young's modulus values of fresh/intact fascicles[3] and intact fascicles after 4 and 7 days in culture[4] are reported (Figure 6.5).

The Young's modulus values of injured fascicles decreased to 60% of the fresh intact group immediately after injury ($P<0.005$). Moreover, Young's modulus values of the injured fascicles were decreased to 55% and 46% of intact fascicles in culture after 4 and 7 days, respectively ($P<0.05$).

In order to understand the effects of culturing on mechanical properties of fascicles Young's modulus values of intact/fresh fascicles were compared to intact/cultured fascicles after 4 and 7 days. Young's modulus values of cultured fascicles were significantly lower than fresh fascicles ($P<0.01$). However, there was no significant difference between Young's modulus of fascicles after 4 and 7 days in culture in both intact and injured groups suggesting that major changes in mechanical properties of tissue occurs within the first 4 days of culturing.[127]

[1] **Fresh/injured**: refers to the fascicles that were injured and used for mechanical testing and biochemical assays immediately after harvesting from the rat tail

[2] **Cultured/injured**: refers to the injured fascicles that were kept in culture for 4 or 7 days after generation of the injury

[3] **Fresh/intact**: refers to the fascicle that were used for mechanical testing and biochemical assays immediately after harvesting frorm the rat tail

[4] **Cultured/intact**: refers to intact fascicles that were kept in culture for 4 or 7 days

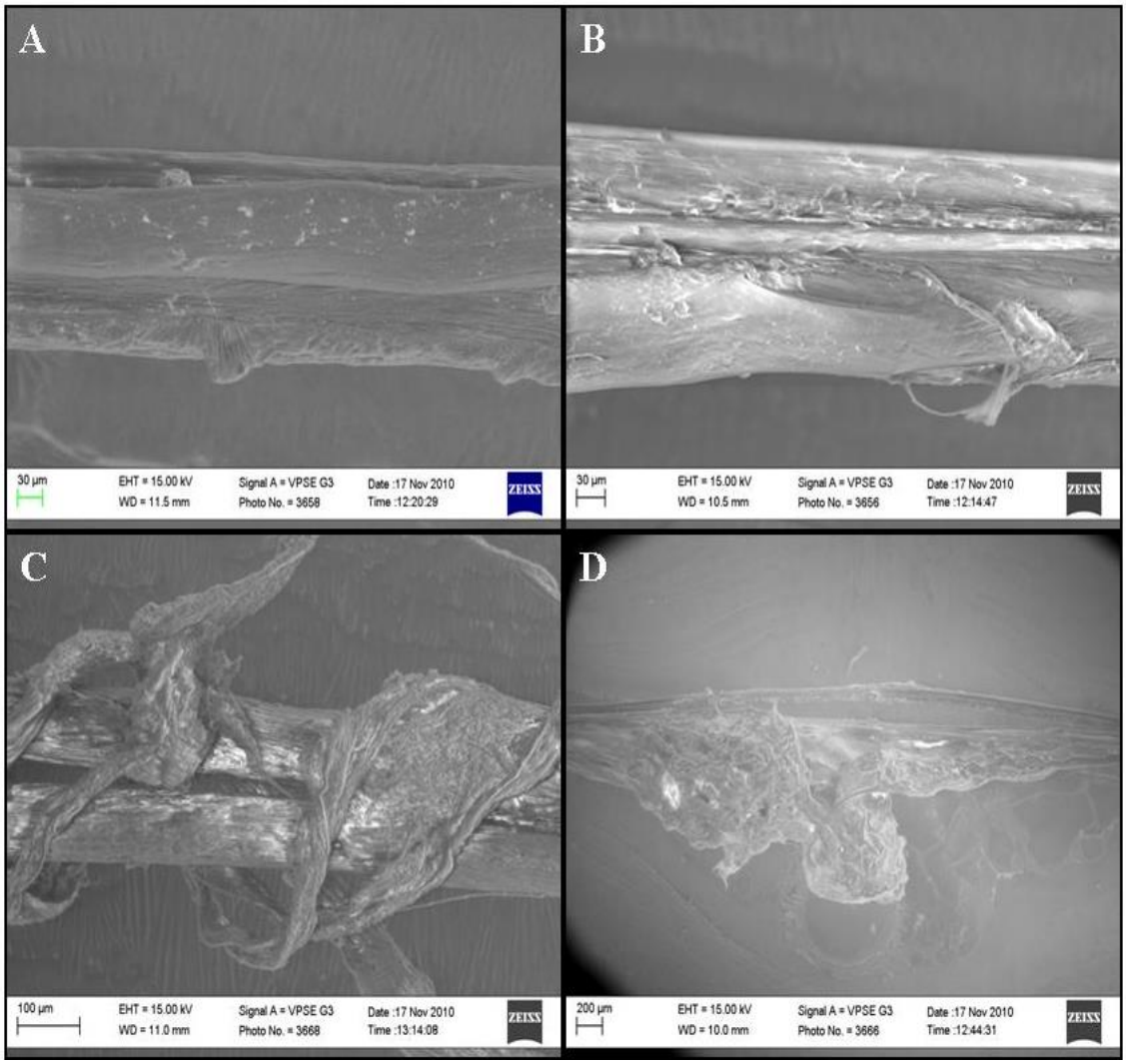


**Figure 6.3** ESEM images of two freshly harvested rat tail tendon fascicles A&B) before and C&D) after scratch injury.

### 6.3.4 Role of glycosaminoglycans (GAGs) in tendon tissue

Glycosaminoglycans are one of the most abundant polysaccharides of tendon extracellular matrix. GAGs attach to a protein core to create proteoglycans which are embedded between collagen fibrils and fibres [128]. Chondroitin sulphate, dermatan sulphate, heparin, heparin sulphate and hyaluronan are six major identified classes of glycosaminoglycans.

The concentration of GAGs varies in different types and zones of tendon tissue. Pressure zones may include up to 5% GAG with chondroitin accounts about 65% of the GAG concentration [186]. However, in tensional zone GAG constitutes 0.2% of tendon dry mass with the dermatan sulphate being the most abundant class of GAGs [14, 128]. Heparin has also been shown in small quantities in myotendinous junction [122, 155]

Although GAGs constitute a small portion of tendon extracellular matrix they are involved in vital physiological processes such as ion transport and water and nutrient diffusion. Moreover, hydrophilic properties of GAGs contribute to the elastic properties of tendon tissue.

It has been reported that the GAG concentration of tendons changes by different factors such as aging [120, 234], mechanical loading[103] , immobilization[281] injury and healing[67].

Increase in the concentration of the major GAG composites, Hyaluronic acid and dermatan sulphate, has been reported during Achilles tendon healing in rabbits [229]. In culture, it has been shown that GAG concentration significantly increases after 48 to 72 hours of mechanical load in isolated embryonic chick tendons [259].

DMB assay was used in this study to measure the relative amounts of sulphated GAG after the scratch injury. Moreover, DMB was used to measure the GAG content in three different groups of static loading, PRP and static loading/ PRP[5] after 4 and 7 days in culture. The following section describes the effect of scratch injury and culturing on the GAG content of fascicles using DMB assay.

[5] Static loading/PRP: Fascicles that were statically loaded in PRP conditioned medium

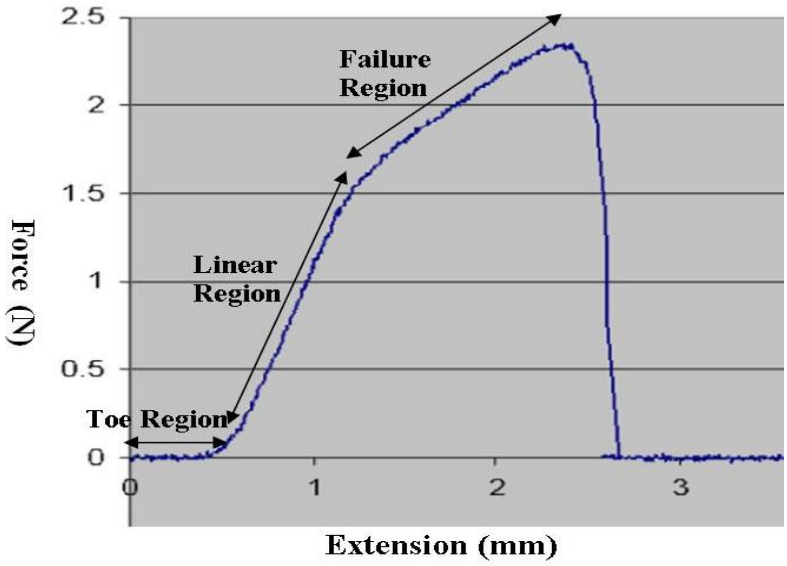


**Figure 6.4** Load-extension curve for rat tendon tail fascicle. Toe, linear and failure regions are labelled. Microscopic failure happens at about 2.4 mm of extension and approximately 2.3 N load.

## 6.3.4.1GAG content of scratch injury model

The DMB assay was performed to measure the GAG content of fresh/injured fascicles and injured fascicles after 4 and 7 days in culture. The GAG content was also measured for the intact fascicles in each group. GAG concentration in each sample was calculated from the absorbance readings of a spectrophotometer at 520 nm, relative to the standard curve (Figure 6.6). The GAG content was then normalized to tissue wet weight (air dried).

There was no statistically significant difference in the GAG content between fresh/intact and fresh/injured fascicles. The GAG content in the injured fascicles decreased significantly after 4 and 7 days in culture ($P<0.05$ and $P<0.005$) (Figure 6.7).

The GAG content of intact/cultured fascicles was about 53% and 51% of intact fresh fascicles after 4 and 7 days in culture, respectively($P<0.005$). There was no

significant difference in the GAG content between the fascicles cultured for 4 and 7 days in both intact and injured groups.

### 6.3.5 DNA content of scratch injury model

The Picogreen assay was performed to measure the double-stranded DNA content as an index of cell number of fresh/injured fascicles and injured fascicles after 4 and 7 days in culture. The dsDNA content was also measured for the intact fascicles in each group.

dsDNA concentration in each group was calculated from the fluorescence readings of a spectrophotometer at excitation of 490 nm, relative to the standard curve (Figure 6.8). The dsDNA content was then normalized to tissue wet weight (air dried).

Scratch injury significantly decreased dsDNA content or cell number immediately after generation of the injury ($P<0.005$). dsDNA content of injured fascicles decreased by about 36% and 46% in comparison with intact/cultured fascicles after 4 and 7 days in culture, respectively ($P<0.05$) (Figure 6.9).

The dsDNA content of intact/cultured fascicles was about 39% and 31% of intact fresh fascicles after 4 and 7 days in culture, respectively($P<0.005$). There was no statistically significant difference in the dsDNA content between the fascicles cultured for 4 and 7 days in both intact and injured groups.

## 6.4 Role of static loading on mechanical and biochemical properties of healing tendon

Chapter 2 reviewed the important literature on the role of mechanical loading in improving the biomechanical and biochemical properties of tendon tissue in both *in*

*vivo* and *in vitro* studies. *In vitro* loading systems have been commonly used in tendon studies in order to assess the effects of different types and magnitudes of mechanical loading on tendons[3, 21, 313].

*In vitro,* the effects of static stress between 0 and 3 MPa was investigated in rabbit patellar tendon. During this study the Young's modulus significantly decreased in the load deprived fascicles. The maximum tensile strength of 16.7 MPa was observed in fascicles subjected to 0.8N static load[313].

In a different study an *in vitro* model system was developed in order to study the relationship between the amount of tendon extracellular matrix major components, glycosaminoglycans and collagen, and mechanical loading in rat tail tendon fascicles. The fascicles were tensioned with static load of 0.116 N (~1MPa) or left free floating (unloaded) in a tray-like bioreactor. 1 week of applied stress resulted in 30% increase in elastic modulus of fascicles whereas 1 week of unloading decreased the elastic modulus by 23% in comparison with controls. It was suggested that the changes in the elastic modulus (Young's modulus) of the fascicles were associated with the changes in the concentration of glycosaminoglycans in the fascicles[3].

*In vitro* loading set-up was used to investigate the effects of static loading and load deprivation on the expression of collagenase (MMP-1) in tendon cells [21]. Rat tail tendon fascicles were loaded at 0.012 N, 0.062N, 0.11N and 0.21N for 24 hours. It was shown that the static load of 0.012 N (~0.16 MPa) and 0.2N (~2.60 MPa) induces the highest and lowest expression of MMP1-mRNA in tendon cells.

This study used the *in vitro* static loading set-up and scratch injury model described in chapter 3 to investigate the effects of static loading on the mechanical and biomechanical properties of tendons.

Briefly, a small magnetic weight (2.25 g) was suspended in a DMEM-F12 medium filled test tube in order to apply static load of approximately 0.02N. An average specimen diameter of 300μm was found for 45 fascicles using a digital micrometre (data not shown). Using the equation $Stress = \frac{Force}{Cross\ sectional\ Area}$ each fascicle was therefore subject to static stress of approximately 0.28 MPa. The amount of stress applied on tendon fascicles is within the range commonly used in *in vitro* studies.

In order to understand the effect of static loading on mechanical and biochemical properties of tendon fascicles controlled tensile tests, DMB and Picogreen assays were used to measure Young's modulus, GAG content and double-stranded DNA (ds-DNA) content of statically loaded and load deprived(unloaded) fascicles.

### 6.4.1 Effect of mechanical loading on elastic modulus of tendon fascicles

Similar to section 6.3.3 the Young's modulus of fascicles was measured using the data obtained from tensile testing. The fascicles were divided in to four main groups of intact/loaded, intact/unloaded, injured/loaded and injured/unloaded and were kept in culture for 4 and 7 days.

After 4 days of culture the Young's modulus of intact/unloaded fascicles decreased by 34% in comparison with intact/loaded fascicles ($P<0.01$). Young's modulus of injured/loaded fascicles was also compared to injured/unloaded fascicles in order to investigate the effect of mechanical loading in improving the mechanical properties of injured tendon. There was a statistically significant difference between the

Young's modulus of injured/loaded fascicle and injured/unloaded fascicles after 4 days of culture ($P<0.05$) (Figure 6.10 A).

Intact/loaded fascicles showed significantly higher values of Young's modulus than intact/unloaded group after 7 days in culture ($P<0.05$). However, there was no significant difference between values of Young's modulus in injured/loaded and injured/unloaded groups after 7 days in culture (Figure 6.10 B).

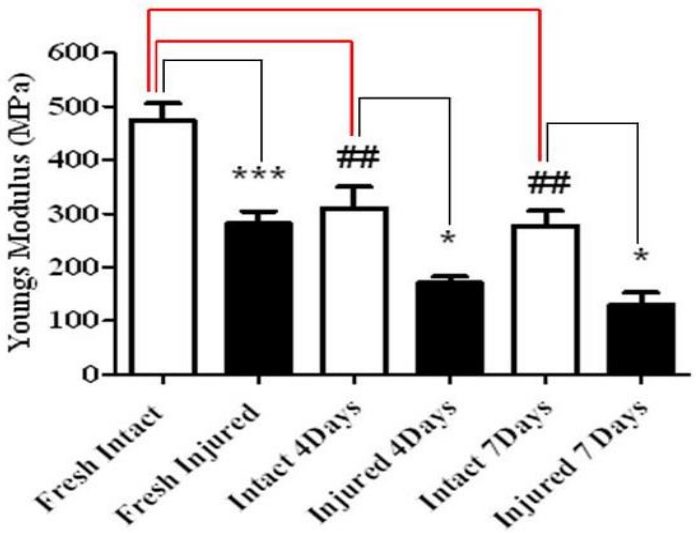


**Figure 6.5** Mechanical validation of the injury model**.** The values are shown as the mean and standard error of the mean of the Young's modulus values calculated from 9 different fascicles in each group. Asterisks denote statistically significant comparison of the intact fascicles with injured fascicles in 3 different time points, fresh, 4days, 7days *$P<0.05$, ***$P<0.005$. Hash marks denote statistically significant comparison of the values of intact groups after 4 and 7 days in culture with fresh intact group with significance values ##$P<0.01$.

## 6.4.2 Effect of static loading on GAG content of tendon fascicles

The DMB assay was performed on statically loaded and unloaded fascicles in both intact and injured groups. Using a CS standard curve it was demonstrated that load deprived intact fascicles possessed lower GAG content in comparison with

intact/loaded fascicles after 4 days in culture (P<0.01). In the injured group, the GAG content was decreased to about 43% of loaded fascicles after 4 days (Figure 6.11 A) (P<0.05).

There was a statistically significant difference between intact/loaded and intact/unloaded fascicles after 7 days in culture (P<0.01). Moreover, the injured/loaded fascicles possessed about three times more GAG in comparison with injured/unloaded fascicles within the same period (P<0.005)(Figure 6.11B)

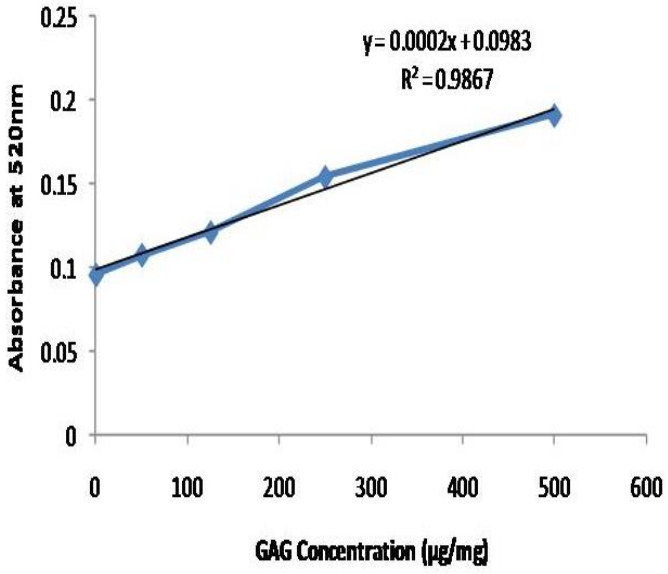


**Figure 6.6** Standard curve for DMB analysis. The figure depicts change in absorbance at 520 nm plotted against increase in chondroitin sulfate concentration.

### 6.4.3 Effect of static loading on DNA content of tendon fascicles

The Picogreen assay was performed on 4 different groups of intact/loaded, intact/unloaded, injured/loaded and injured/unloaded in order to study the effects of static loading on DNA content of fascicles.

Using Picogreen standard curve it was found that the DNA content of intact/unloaded fascicles decreased by 39% in comparison with intact/loaded fascicles after 4 days in culture ($P<0.005$). Moreover, injured/loaded fascicles possessed higher DNA content than unloaded fascicles within the same period ($P<0.01$) (Figure 6.12 A).

There was a significant difference between intact/loaded and intact/unloaded fascicles after 7 days in culture ($P<0.05$). The average DNA content of injured/unloaded fascicles was decreased to about 44% of injured/loaded fascicles after 7 days in culture ($P<0.05$) (Figure 6.12 B).

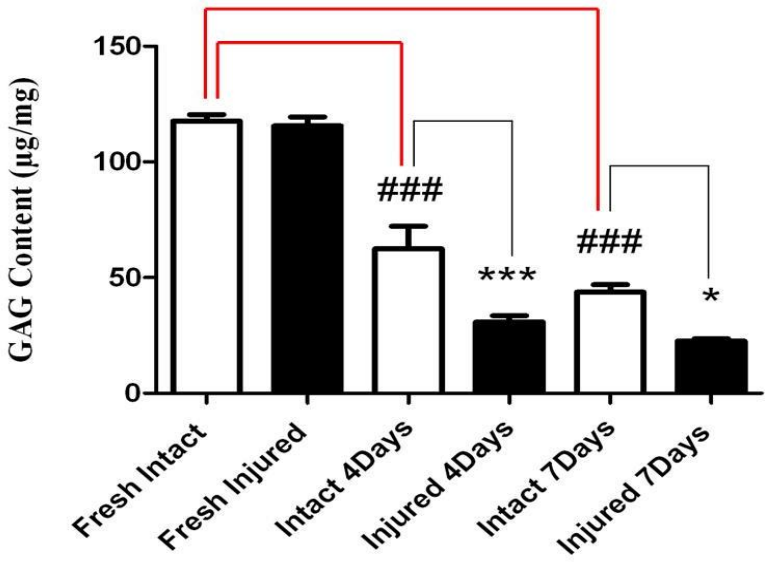


**Figure 6.7** GAG content of intact and injured fascicles after harvesting and 4 and 7 days in culture**.** The values are shown as the mean and standard error of the mean of the Young's modulus values calculated from 9 different fascicles in each group. Asterisks denote statistically significant comparison of the fresh intact fascicles vs fresh injured, Intact 4days in culture vs injured 4 days in culture and intact 7 days in culture vs injured 7 days in culture *$P<0.05$, ***$P<0.005$. Hash marks denote statistically significant comparison of the values of intact groups after 4 and 7 days in culture with fresh intact group with significance values ###$P<0.005$.

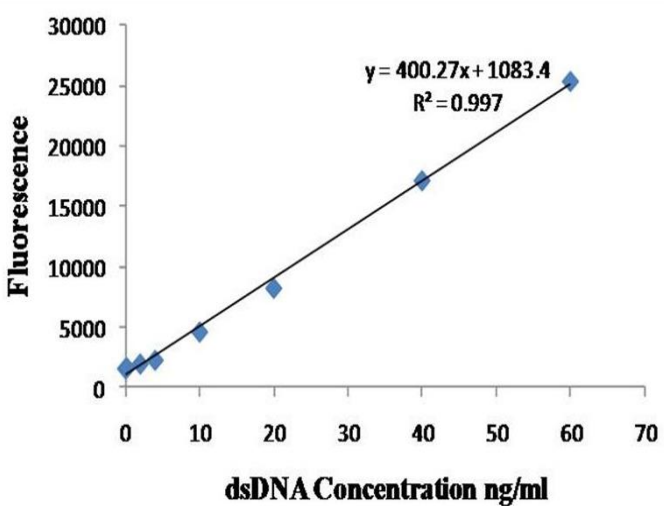


**Figure 6.8** Standard curve for PicoGreen dsDNA quantification. The amount of DNA indicated (ng/ml) at an excitation wavelength of 490 nm, emission wavelength of 540 nm, with a 530 nm cut off.

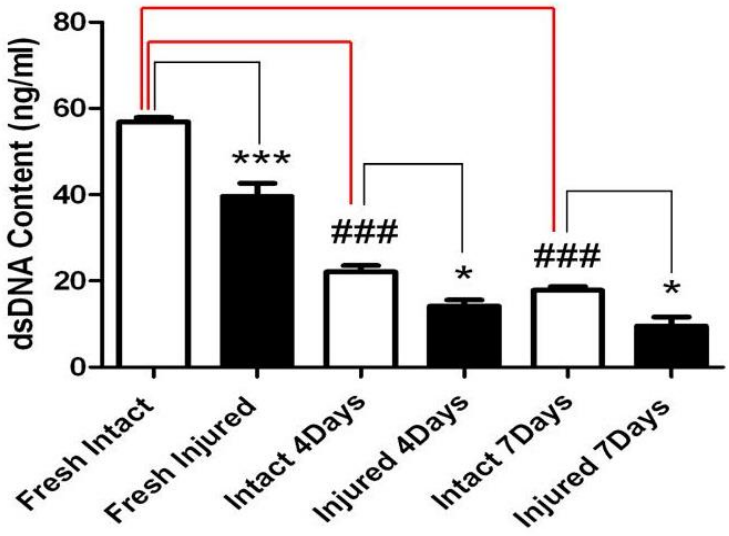


**Figure 6.9** Measurement of dsDNA in the injury model**.** The values are shown as the mean and standard error of the mean of the Young's modulus values calculated from 9 different fascicles in each group. Asterisks denote statistically significant comparison of the intact fascicles with injured fascicles in 3 different time points, fresh, 4days, 7 days *P<0.05, ***P<0.005. Hash marks denote statistically significant comparison of the values of intact groups after 4 and 7 days in culture with fresh intact group with significance values ###P<0.005.

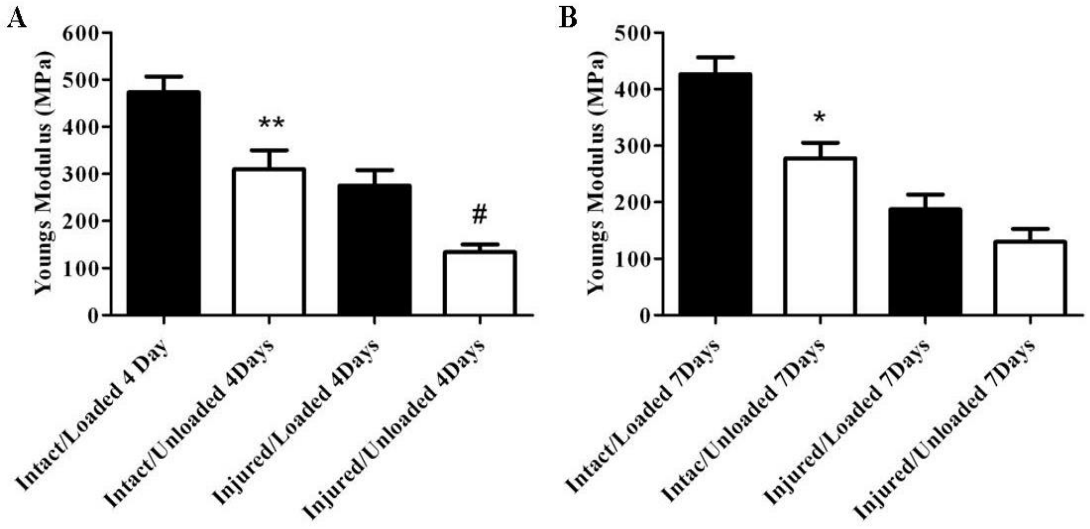


**Figure 6.10** The effects of static loading on the elastic modulus of intact and injured fascicles after A) 4 days B) 7 days in culture. Asterisks denote statistically significant comparison of the intact/loaded fascicles with intact/unloaded fascicles. # denotes statistically significant comparison of injured/loaded fascicles with injured/unloaded fascicles.

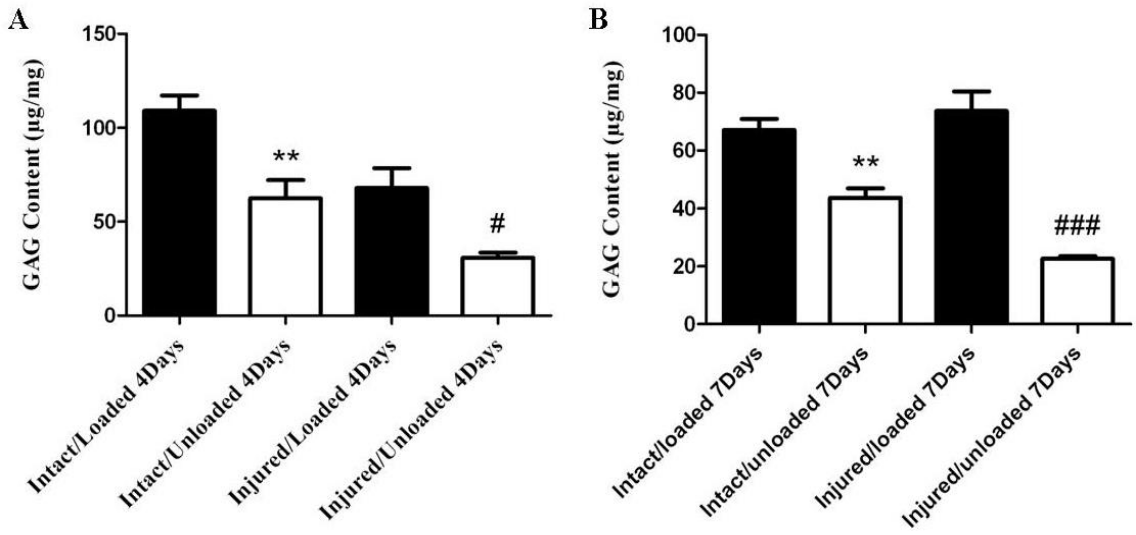


**Figure 6.11** The effects of mechanical loading on the GAG content of intact and injured fascicles after A) 4 days B) 7 days in culture. Intact unloaded vs intact loaded (**P<0.01). # denotes statistically significant comparison of injured/loaded with injured/unloaded (###P<0.005) (#P<0.05).

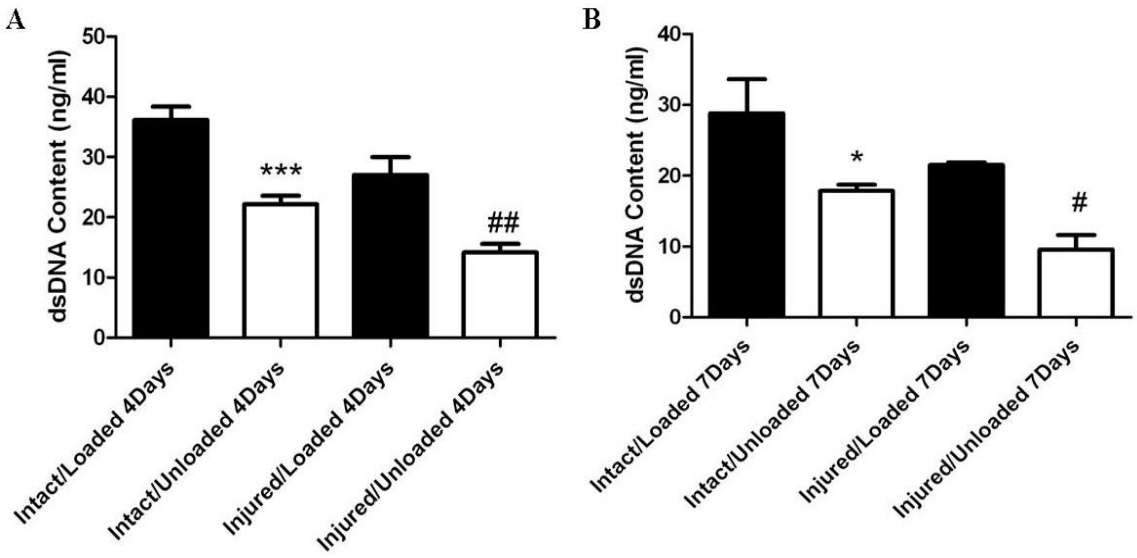


**Figure 6.12** The effects of static loading on double-stranded DNA content of intact and injured fascicles A) after 4 days B) after 7 days. Asterisks denote statistically significant comparison of the intact/loaded fascicles with intact/unloaded fascicles *** P<0.005. # denotes statistically significant comparison of injured/loaded fascicles with injured/unloaded fascicles (#P<0.05) (##P<0.01).

## 6.5 Effect of PRP on mechanical and biochemical properties of tendon

Platelet rich plasma is currently being widely tested as a soft tissue healing agent. It was demonstrated in chapter 4 and 5 that PRP is strongly protective against tendon damaging drugs and hypoxia and induces migration and chemotaxis in tenocyte culture. The following section describes the effects of PRP on mechanical and biochemical properties of tendon tissue.

### 6.5.1 Effect of PRP on elastic modulus of injured tendon fascicles

The injury model was used in order to understand the potential effects of PRP on mechanical properties of healing tendon. Injured fascicles were cultured in PRP-conditioned medium for 4 and 7 days. Tensile testing was performed after measuring

the CSA area of the fascicles and Young's modulus values were calculated using stress-strain curve.

PRP treatment significantly increased the values of Young's modulus in injured fascicles after 4 and 7 days in culture. The values of Young's Modulus were 50% and 91% of intact fascicles after 4 days in culture. Statistical analysis revealed a significant difference between injured fascicles and injured/PRP treated fascicles after 4 days in culture ($P<0.05$) (Figure 6.13 A).

PRP-treated fascicles showed higher Young's modulus in comparison with untreated injured fascicles after 7 days with the Young's modulus values of PRP-treated group reaching to about 91% of cultured/intact fascicles ($P<0.01$) (Figure 6.13 B).

### 6.5.2 Effect of PRP on GAG content of injured tendon fascicles

DMB assay was performed to measure the GAG content of injured fascicles before and after treatment with PRP for 4 and 7 days. PRP significantly increased the GAG content of injured fascicles after 4 days in culture. The average GAG content in injured PRP/treated was about 6 and 2.5 times higher than untreated/injured and intact fascicles after 4 days in culture, respectively ($P<0.005$)(Figure 6.14A).

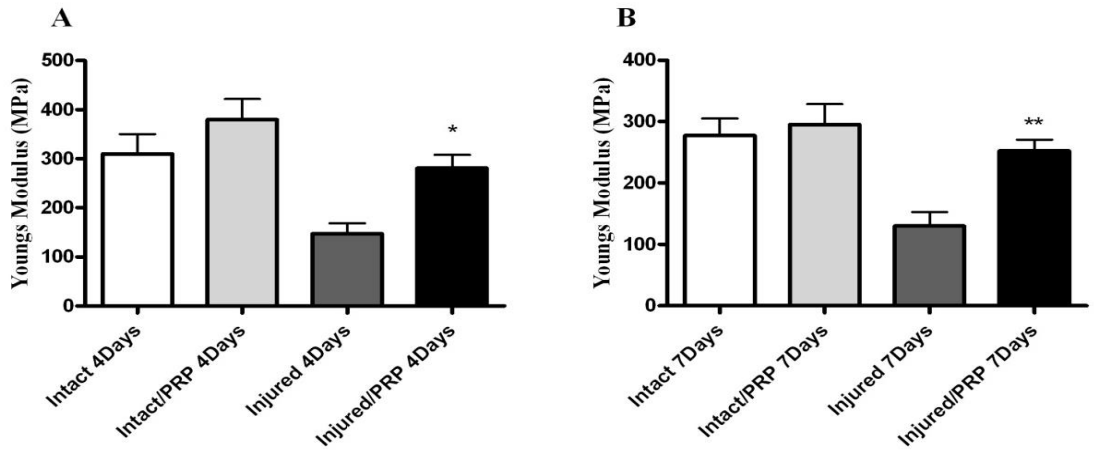


**Figure 6.13** The effect of Platelet Rich Plasma on Young's Modulus of injured fascicles. Asterisks denote statistically significant comparison between injured fascicles in 5% DMEMF-12 and PRP-conditioned medium after A) 4 days B) 7 days in culture(**P<0.01)(*P<0.05).

Moreover, it was demonstrated that injured fascicles treated with PRP possessed higher amount of GAG in comparison to both untreated injured and intact fascicles after 7 days in culture. Furthermore, statistical analysis revealed the differences to be significant between injured/PRP treated fascicles and injured untreated fascicles within the same period (P<0.005) (Figure 6.14B)

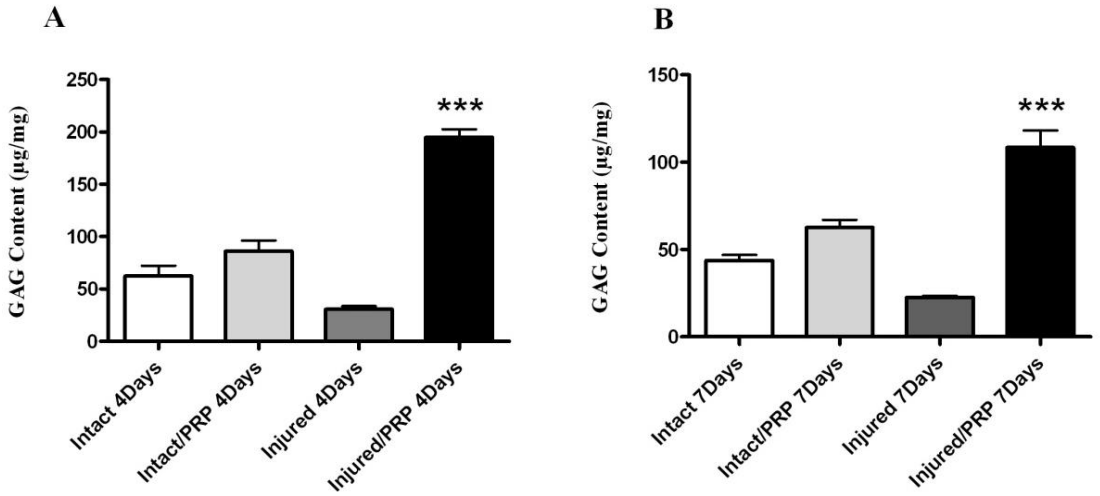


**Figure 6.14** The effect of platelet rich plasma on the GAG content of injured fascicles after A) 4 days B) 7 days in culture. Asterisks denote statistically significant comparison between injured fascicles and injured/PRP fascicles (***P<0.005).

### 6.5.3 Effect of Platelet Rich Plasma on DNA content of injured fascicles

Picogreen assay was also used to measure the DNA (or cell) content in injured fascicles after 4 and 7 days treatment with PRP. PRP significantly increased DNA content of injured fascicles in comparison with both injured and intact fascicles after 4 days of treatment ($P<0.005$).

The average DNA content in injured/PRP treated fascicles was 6 and 3 times higher than untreated injured and intact fascicles after 4 days, respectively($P<0.005$) (Figure 6.15 A). DNA content was also significantly higher in PRP treated injured fascicles in comparison with untreated/injured fascicles after 7 days ($P<0.005$) (Figure 6.15 B). Moreover, the intact/PRP treated fascicles showed higher DNA content in comparison with intact untreated fascicles after both 4 and 7 days.

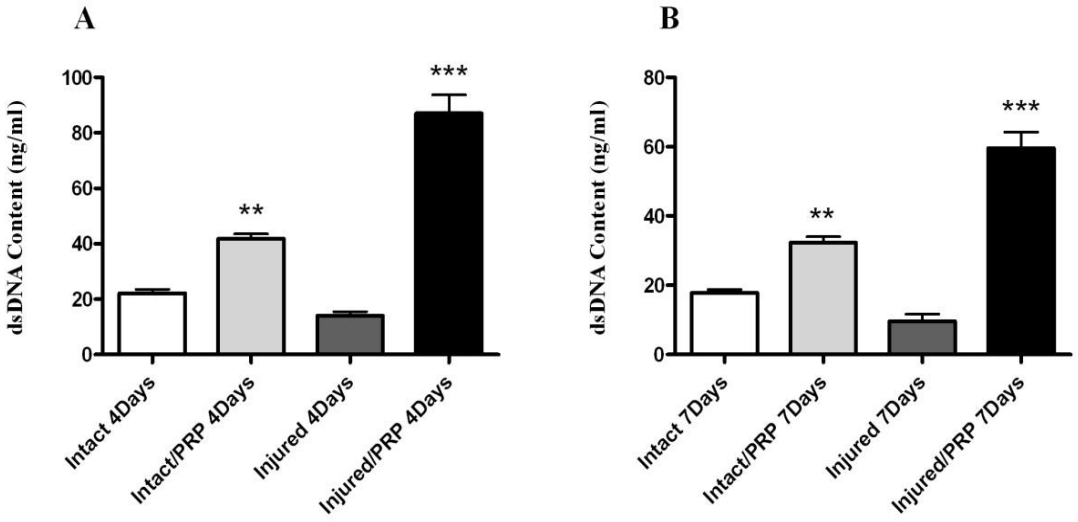


**Figure 6.15** The effect of PRP on DNA content of intact and injured fascicles after A) 4Days B) 7Days in culture. Intact vs intact/PRP and injured vs injured/PRP (**P<0.01) and (*** P<0.005).

## 6.6 Effect of Static Loading together with PRP on mechanical and biochemical Properties of injured fascicles

This section aims to combine the above approaches to support the ultimate goal of the thesis which is investigating the influence of static mechanical loading together with platelet rich plasma on tendon tissue. The injured fascicles were treated with PRP and mechanical loading for 4 and 7 days. Similar to previous sections, tensile testing, DMB and Picogreen assays were performed in order to understand the effects of static loading and PRP on the elastic modulus, GAG content and DNA content of injured fascicles, respectively. The injured fascicles were treated with PRP and mechanical loading for 4 and 7 days. The statistical analysis was performed to compare the effects of different treatments on the mechanical and biochemical properties of injured fascicles.

### 6.6.1 Effect of static loading together with PRP on elastic modulus of injured fascicles

The Young's modulus values were calculated in four different groups of injured, injured/loaded, injured/PRP treated and injured/loaded/PRP after 4 and 7 days in culture. The average Young's modulus values were 134, 275.4, 280.5, 278.4 for injured, injured/loaded, injured/PRP and injured/loaded/PRP, respectively, after 4 days in culture. There were significant differences between untreated injured fascicles and PRP treated, static loaded and loaded/PRP treated groups. However, there was no significant difference in the values of Young's modulus between the treatment groups of static loading, PRP and loaded/PRP after 4 days in culture ($P<0.005$)( $P<0.01$)

(Figure 6.16 A). Statistical analysis revealed significantly higher Young's modulus values of injured/PRP treated and injured/loaded/PRP in comparison with injured fascicles after 7 days of treatment (P<0.005). However, there was no significant difference in Young's modulus values of injured and injured/loaded fascicles after 7days. Moreover, the values of Young's modulus were significantly higher in loaded/PRP treated fascicles in comparison to those which were only loaded (P<0.01) (Figure 6.16B).

## 6.6.2 Effect of static loading together with PRP on GAG content of injured fascicles

The DMB assay showed an increase in the GAG content in all the treatment groups after 4 and 7 days of treatment. The statistical analysis revealed significant differences between PRP treated, loaded and loaded/PRP treated groups in comparison with untreated fascicles. Injured/PRP treated group showed significantly higher GAG content compared to other treatments (P<0.005 and P<0.05) (Figure 6.17 A).

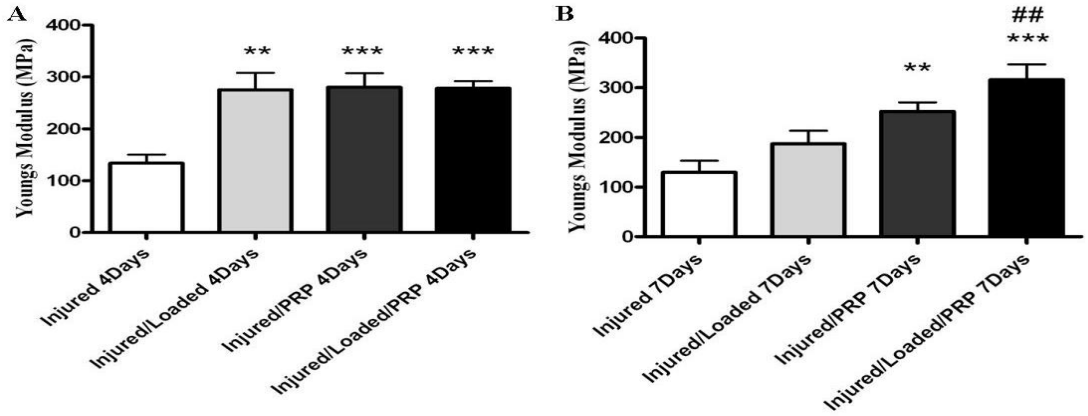


**Figure 6.16** The effect of static loading, PRP and static loading/PRP on the Young's modulus of injured fascicles after A) 4days B) 7days. * denotes statistically significant comparison between each treatment and injured fascicles (***P<0.005) (**P<0.01). (##P<0.01) injured/loaded vs injured/loaded/PRP, n=9 fascicles per group.

Moreover, the GAG content was significantly higher in PRP treated group in comparison with loaded and loaded/PRP groups after 7 days of treatment ($P<0.01$). All three treatment groups possessed significantly higher GAG content than injured untreated fascicles after 7 days in culture ($P<0.005$) (Figure 6.17B).

### 6.6.3 Effect of static loading together with platelet rich plasma on DNA content of injured fascicles

Picogreen assay was performed in order to compare the DNA content in each treatment group after 4 and 7 days in culture. There was no significant difference between injured/loaded and injured/loaded/PRP treated fascicles with untreated/injured group after 4 days. However, injured/PRP showed highest DNA content and there was a significant difference between the two other treatment groups and PRP after 4 days ($P<0.005$) (Figure 6.18 A).

Also, Picogreen assays showed higher DNA content in injured loaded fascicles and PRP treated fascicles in comparison with injured untreated fascicles after 7 days ($P<0.05$ and $P<0.005$) (Figure 6.18B). The highest DNA content was observed in PRP treated fascicles within the same period. Moreover, statistical analysis revealed significant differences between both injured loaded and injured/loaded/PRP with the group treated with PRP only ($P<0.005$).

Furthermore, the relationship between GAG concentration and cell content was determined by measuring the GAG concentration per DNA for all treatment groups (Figure 6.19). The concentration of GAG per DNA was significantly higher in loaded/PRP group in comparison with both PRP alone and with static loading alone after 4 and 7 days in culture (* $P<0.05$ and *** $P<0.005$).

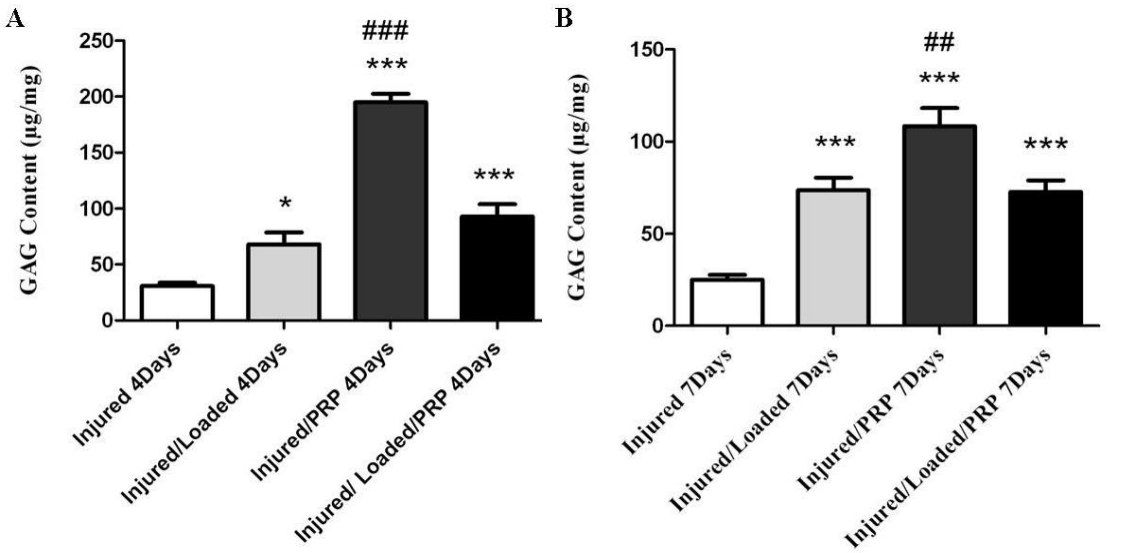


**Figure 6.17** The effect of static loading, PRP and static loading/PRP on the GAG content of injured fascicles after A) 4days B) 7days. * denote the comparison between treatment goups and injured fascicles (***P<0.005) (* P<0.05). # denotes statistically significant comparison between the three treatment groups of loaded, PRP treated and loaded/PRP treated, n=9 fascicles per group.

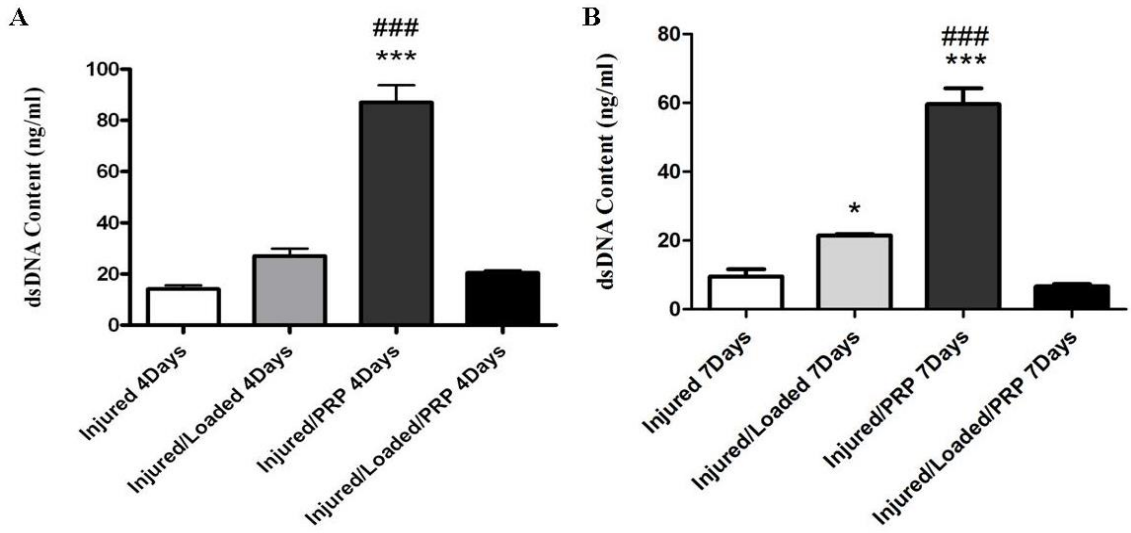


**Figure 6.18** The effect of static loading, PRP and static loading/PRP on the DNA content of injured fascicles after A) 4days B) 7days. * denotes the comparison between treatment goups and untreated injured fascicles (***P<0.005)(* P<0.05. # denotes statistically significant comparison between the three treatment groups of loaded, PRP treated and loaded/PRP treated. n=9 fascicles per group.

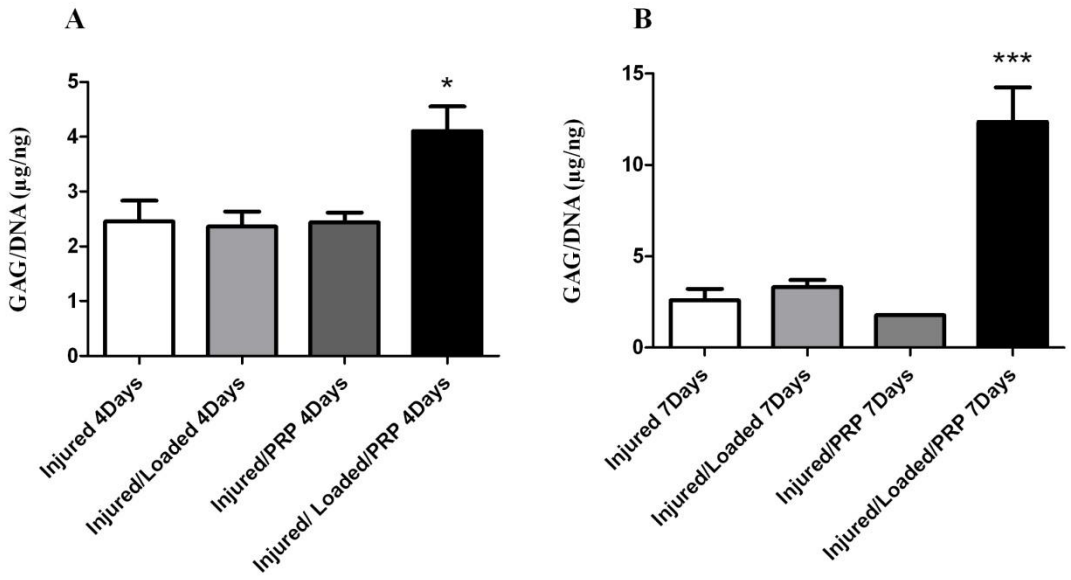


**Figure 6.19** concentration of GAG per DNA in injured, injured/loaded, injured/PRP treated and injured/loaded/PRP treated fascicles A) after 4 days B) after 7 days. * denotes statistically significant comparison between injured/loaded and injured/PRP with injured/loaded/PRP group (***P<0.005)(* P<0.05).

## 6.7 Discussion

This chapter presented a novel *in vitro* model of tendon injury which facilitates studying the effects of different treatment techniques such as mechanical loading and PRP, alone and in combination, on mechanical and biochemical properties of tendon during the healing process. Different validation techniques were used in order to assess the effects of scratch injury on three defining parameters of tendon structure and function; Young's modulus, GAG and DNA content.

The results from tensile testing revealed that the scratch injury significantly decreases the values of Young's modulus in both fresh and cultured fascicles. Young's modulus is an excellent indicator of tendon material property and is known to be altered by tendon degeneration and injury. It has been reported that the Young's modulus of human tendons significantly decreases to about half of intact tendon after

mechanically-induced damage [242, 312]. The calculation of Young's modulus after scratch injury revealed a similar pattern in this study. The values of Young's modulus significantly decreased after generation of injury and remained lower than intact fascicles within 7 days of experiment. The mean average modulus of freshly injured fascicles were about half of the original fascicles and decreased even more with culturing. Therefore, the scratch injury model could provide an interesting paradigm for investigating the effects of mechanical loading and PRP in improving the mechanical properties of tendon after injury.

Tendon remodelling is one of the most important processes involved in tissue development and healing. Remodelling is a complex process which is defined by changes in biochemical and mechanical structure of tendon in response to environmental conditions. Although the complete mechanism behind the remodelling process in tendons is not understood, major components of the extracellular matrix such as proteoglycans (PGs) are known to be involved in this process.

Proteoglycans play a key role in tissue hydration, and hence influence the mechanical properties of tendon tissue. Moreover, it has been shown that proteoglycans control the fibrillogenesis of type I collagen and therefore they play a crucial role in defining the structural properties of tendon tissue [107].

Many of the unique properties of proteoglycans have been attributed to the GAG chains attached to these macro-molecules. It has been revealed that GAGs are active in cell signalling and adjust the interaction of different growth factors and cytokines responsible for tissue growth and repair[82]. Moreover, GAG chains contribute to mechanical stability of tendon tissue. Therefore, measuring the changes in GAG

content in response to injury and different types of treatment helps in assessing the effectiveness of different treatment techniques.

Using DMB assay it was demonstrated that GAG content decreases significantly after 4 and 7 days in culture in both intact and injured fascicles, with the intact fascicles possessing higher amount of GAG per fascicle. Contrary to the effects of scratch injury on the elastic modulus of fresh tendon fascicles, there was no significant difference between the GAG content of fresh/intact and fresh/injured fascicles suggesting the gradual decline in the amount of GAG after injury (differences appearing after 4 days). Moreover, there was no significant difference between the injured fascicles cultured for 4 and 7 days. This may suggest that the major decline in amount of GAG occurs within the first 4 days after the injury.

Nucleic acid concentration is commonly determined using spectrophotometer at the absorbance of 260 nm ($A_{260}$). However, absorbance methods are unable to detect the contribution of nucleotides and single-stranded nucleic acids to the fluorescence signals which may attribute to incorrect estimate of the DNA concentration of samples. To avoid this problem, the project used ultra sensitive fluorescent nucleic acid stain, Picogreen, to measure the double stranded DNA content in injured fascicles before and after static loading and PRP treatment. Here, it was shown that the scratch injury model significantly decreases the DNA content of the fascicles immediately after injury and 4 and 7 days in culture. Decrease in DNA content reflects the loss of tendon cells after injury also observed with increased Dead staining (Figure 6.2).

There is clearly a need for development of new and effective tendon therapies and to combine therapies to obtain possibly synergistic effects. Two promising strategies which may combine well are mechanical loading and PRP therapy.

Using the validated injury model and previously described assessment techniques the effects of mechanical loading and PRP treatment were studied on tendon tissue.

Tensile tests showed that injured fascicles experiencing static load of ~ 0.02N had higher Young's modulus values compared to load deprived injured fascicles throughout the test period. There was a significant difference between injured/loaded and injured/unloaded fascicles after 4 days but not after 7 days. The result from this study highlights the important role of mechanical loading in improving the mechanical properties of injured/cultured fascicles. However, further studies are needed in order to translate the *in vitro* results to *in vivo* application.

Different studies have investigated the effects of loading on cells behaviour *in vitro* [43, 50, 71, 113, 78, 246, 247]. Depending on the type and duration of mechanical loading the nature of activity varies in different cell types. In tendons it has been demonstrated that mechanical loading in an animal model influences the total GAG content in flexor digitorum profundus tendon [103]. Increases in the GAG content affect the elastic properties of tendons due to the hydrophilic capacity of this molecule.

DMB assay was performed in order to understand the role of static loading on biochemical modification of injured fascicles. The hypothesis was that the changes in GAG concentration are directly linked to tenocytes response to injury and mechanical loading. The GAG content was significantly higher in statically loaded fascicles in both intact and injured fascicles after 4 and 7 days in culture than in unloaded fascicles. Increase in the production of GAG has been reported during wound healing in tendons [234]. Static loading has also been shown to inhibit the expression of catabolic enzymes in cultured rat tail tendons which may prevent the loss of mechanical properties [20, 21].

In vitro, it has been shown that mechanical load stimulates proliferative responses in human tenocytes[324] . Using Picogreen assay it was demonstrated that statically loaded fascicles have higher DNA content in comparison with load deprived in both intact and injured group after 4 and 7 days in culture. This may suggest that static loading could influence the healing process by inducing proliferation or preventing death of tenocytes.

Chapter 5 and 6 discussed the role of PRP at cellular level. This chapter investigated the role of PRP in improving the healing properties of tendon tissue using the scratch injury model.

PRP has been shown to improve the mechanical and biochemical properties of tendon tissue in both animal models and human [23, 125, 190, 193]. However, the exact mechanism behind this effect is not fully understood.

Using the previously described assessment techniques, tensile testing and biochemical assays, this study investigated the effects of PRP on Young's modulus, GAG and DNA content of injured fascicles. Data from tensile testing suggested that PRP treated fascicles display superior average Young's modulus than unloaded fascicles after 4 and 7 days in culture. Moreover, untreated fascicles failed more often during tensile testing in comparison with PRP treated fascicles.

Growth factors within PRP regulate the synthesis of different proteins of extracellular matrix such as proteoglycans and glycoproteins. It has been shown that transforming growth factor β regulates the synthesis of proteoglycans with chondroitin and dermatan sulphate GAG chains in mesenchymal (fibroblasts, myoblasts, preadipocytes) and epithelial cells. [31]. In chondrocytes transforming growth factor β stimulates synthesis of glycosaminoglycans and collagen. Moreover, transforming

growth factor β regulates the synthesis of proteoglycans in cartilage [170]. The application of platelet rich plasma has been linked to increase in GAG content of superficial digital flexor tendon in horses [47].

Here, it was demonstrated that PRP significantly increases the GAG content of injured fascicles after 4 and 7 days. The injured/PRP treated fascicles possessed higher GAG per fascicle than intact control. The increase in GAG content as a result of PRP treatment could be indicator of improved healing in the fascicles.

It has been shown that PRP increase the proliferation of different types of cells. In tendons, it has been demonstrated that platelet rich plasma increases tenocyte proliferation, survival and matrix synthesis. This study was designed in order to understand the effects of PRP on DNA content of injured fascicles. The hypothesis was that the pro-healing properties of PRP increase tenocyte proliferation and viability and therefore the PRP-treated samples have higher concentration of DNA.

Using Picogreen assay it was shown that injured fascicles treated with PRP possessed significantly higher concentration of DNA after 4 and 7 days in comparison with untreated controls.

Finally, this chapter investigated the ultimate goal of this thesis which was combining the two important treatment modalities, PRP and loading, in order to study the synergy between these two treatment techniques.

Using the rat model it has been shown that the injection of platelets to a loaded Achilles tendon results in improved material properties of the tissue [23]. Moreover, it has been suggested that the combination of growth factors and mechanical loading stimulate DNA synthesis in tendons [28].

Section 6.6 discussed the effect of mechanical loading together with PRP on the mechanical and biochemical properties of injured fascicles. Moreover, the three treatment methods used in this study, static loading, PRP, static loading/PRP were compared to each other in order to suggest the best treatment approach for tendon injuries.

Results obtained from tensile testing revealed that the fascicles treated with the combination of static loading and PRP showed significantly higher Young's modulus in comparison with untreated injured fascicles. There was no significant difference between loaded/PRP treated fascicles, PRP treated and statically loaded fascicles after 4 days. However, there was a significant difference between the two treatments of loaded/PRP and only loaded after 7 days in culture.

Although the combination of static loading and PRP showed higher GAG content than untreated fascicles the fascicles treated with only PRP possessed the highest GAG content among the three treatment groups after 4 and 7 days in culture.

There was no significant difference in the DNA content between fascicles receiving both PRP and static loading treatments and injured untreated group. The fascicles treated with only PRP showed highest DNA content among all three treatment groups. However, the concentration of GAG per DNA was significantly higher in loaded/PRP group in comparison with both PRP alone and with static loading alone after both 4 and 7 days in culture

Overall, the results from this study suggest that mechanical loading and PRP has positive effects on mechanical and biochemical properties of healing tendon when applied separately.

The combination of two treatments had significant positive effect on mechanical properties of healing fascicles, suggesting potential additive or synergistic signalling mechanisms. It will be interesting to explore in more detail the contribution of repressed matrix degradation enzymes, a known outcome of loading, along with enhanced matrix output. However, in the experiments run for biochemical readouts, fascicles receiving both treatments showed lower GAG and DNA content than either treatment alone. It is possible given that the assays are end-point assays that an initial gain in cell number and GAG with co-therapy could be followed by the start of a loss due to heavy loading which displays itself first in cell number and GAG content and would be followed later by a decline in material properties. The growth factors within PRP may switch off some of the pathways involved in synthesis of GAG and DNA in the presence of certain levels of mechanical loading but the exact mechanism behind this effect is not fully understood. It will be important to repeat this work with viability staining and cell death readouts since the combination of PRP and mechanical loading had a clear negative effect on cell number after 7 days despite both stimuli enhancing cell number alone. Additionally, it was not possible to measure the collagen content and this may correlate more closely with improved material properties.

# Chapter 7 Final discussion

## 7.1 Overview

This chapter summarises the important findings of this work. General introduction of the literature and motivations leading to this DPhil thesis are presented followed by an overview of the research questions and approaches taken to answer them. The significant implications of the results and drawbacks of the project are also highlighted. Finally, the potential avenues for further work are discussed.

## 7.2 Background

Tendon injuries and tendinopathy are a growing problem in the aging but physically active population as well as in young athletes. Rates of Achilles tendon rupture in Finland are reported at up to 18:100,000 [165] while cadaveric studies suggest an incidence of rotator cuff tears of 30% [230].

Tendons have highly ordered matrix and undergo complex changes during the remodelling phase of tendon healing. Moreover, anaerobic metabolism and poor vascular network contribute to slow adaption of tissue to the remodelled matrix which consequently results in slow and compromised healing.

As discussed in chapter 2 different treatment modalities such as pulsed magnetic field[164], cytokines and growth factor therapy[56, 123, 280], tissue engineering with mesenchymal stem cells[232, 261] and mechanical rehabilitation[130, 173] have been used in order to improve the healing properties of tendon tissue in both animal models and humans.

Although some of the discussed strategies are currently being used in clinic, none of them are supported by convincing clinical or scientific evidence so there is a need to conduct more controlled and well-designed biological studies and clinical trials to draw firm conclusion on their efficacy and modes of action.

There is clearly a need for development of new therapies and combining different therapies in order to obtain synergistic effects which may promote healing over a shorter timescale. Two promising strategies which may combine well are growth factor therapy and mechanical loading.

This study used a bottom-up approach to study the effects of platelet rich plasma and mechanical loading, alone and in combination, on both tenocytes and tendon tissue.

## 7.3 Research questions

At cellular level, this thesis investigated the ability of platelet rich plasma as a protective strategy to prevent some of the adverse side-effects of two commonly used and tendon damaging drugs, steroids and fluoroquinolones. In addition, the capacity of total hypoxia (0.1% oxygen) in inducing cellular death in human hamstring tenocytes and the ability of PRP in protecting tenocytes in hypoxic conditions were investigated.

Moreover, the potential of PRP in improving migration and chemotaxis, two important elements of tendon healing, was considered in this thesis.

At the tissue level, using a rat tail tendon injury model, the role of PRP and mechanical loading in improving mechanical and biochemical properties of tendon tissue was questioned. Finally, it was asked whether the combined therapies of PRP and mechanical loading could boost tendon healing through synergistic effects.

To answer these questions, complementary model systems, tenocytes and tendon tissue were used together with different biochemical assays, mechanical testing and imaging techniques.

Chapter 4 investigated the effects of two important drugs, glucocorticoids and fluoroquinolones, on parameters of tenocyte viability, senescence and death. It has been shown that these drugs interfere with innate healing processes and are thought to predispose tendon to rupture [137].

It was demonstrated in this thesis that ciprofloxacin and dexamethasone adversely affect primary tenocyte viability. Although both drugs reduced the viability of tenocytes the effects of dexamethasone on tenocyte viability were less distinct than ciprofloxacin and did not include induction of cell death in the short term. However, other effects were shown, and this is the first study to demonstrate dexamethasone-induced senescence in tenocyte culture [323].

It has been shown that senescent human fibroblasts synthesise abnormal extracellular matrix proteins [30]. Tenocytes are also of the fibroblastic lineage. Therefore glucocorticoid treatment of tenocytes may not only reduce tenocyte cell number but may also reduce the ability of tenocytes to synthesise normal tendon tissue. These changes are likely to impact on the integrity of tendon tissue and the ability of tendon to heal following injury.

It is critical to consider the adverse effects of dexamethasone and ciprofloxacin on tenocyte viability and proliferation particularly in patients undergoing tendon repair where active proliferation is essential. On the other hand, both drugs are highly effective in treatment of inflammatory and infectious conditions. Therefore, it is

important to come up with the new strategies to minimize their adverse effects on tendon.

This thesis provided evidence of an entirely new mode of action of PRP in tendons, in which PRP has the ability to protect tenocytes from damaging environments. This is the first study to show that PRP protects tenocytes against adverse effects of dexamethasone and ciprofloxacin. This suggests that the co-injection of a protective substance such as PRP may block side effects of steroid and reduce the risk of spontaneous tendon rupture as a result of steroid injection.

Fluoroquinolones are systemically administered, but where the patient has a tendon injury, existing or fluoroquinolone-induced, co-treatment by injection with PRP holds promise as an effective agent to promote tendon repair.

In addition, because tendon rupture compromises an already poor blood supply, Live & Dead staining was performed to seek evidence of the role of total hypoxia (0.1% oxygen) on the viability of human tenocytes and thereafter to explore the potential of PRP in protecting tenocytes in hypoxic conditions.

Following 48 hours of hypoxia the ratio of dead to live cells was significantly increased. However, co-treatment with PRP provided protection for tenocytes in hypoxic conditions. PRP reduced the percentage of dead cells from 20% to 8% of total cells. This protective effect suggests that PRP is effective in preventing cellular death caused by hypoxia in tendon tissue and could be used in clinical setting to enhance repair in hypoxia exposed tendon *e.g.* in the acute post-rupture timeframe or immediately following repair surgery. The growth factors within PRP may interfere with the function of the hypoxia-inducible factor 1α (HIF-1 α) which induces hypoxic cell death.

The second question of this thesis was addressed by investigating the role of PRP on migration and chemotaxis in tenocyte culture (chapter 5). Both migration and chemotaxis are necessary components of tendon healing. Therefore it is extremely important to understand the potential of treatment strategies such as PRP in improving migration and chemotaxis in tenocytes.

Here, it was demonstrated that PRP significantly induces migration of tenocytes in a wound healing model after 72 hours of treatment. Increased tenocyte migration to the site of injury should initiate proliferation and deposition of the extracellular matrix which consequently results in enhanced and accelerated tendon healing.

This is the first study to investigate the potential role of PRP as a chemoattractive agent for tenocytes by using transwell chamber. Fresh PRP stimulates chemotactic responses and induces directional migration in tenocyte culture. Tenocytes showed a strong chemotactic response to PRP after 4 days of exposure by migrating towards the PRP clot.

Additional to the effects of dexamethasone on the viability of tenocytes, chapter 5 extended the frame-work of dexamethasone studies by investigating the effects of dexamethasone on tenocyte migration and the potential of PRP in increasing the migration of PRP-treated tenocytes.

In the presence of dexamethasone the migration of tenocytes towards the scratched area was significantly reduced which is likely to contribute to the negative effects of dexamethasone on tendon tissue. PRP significantly increased migration in Dex-treated tenocytes.

These data suggest that PRP injection could be used as complementary or standalone treatment to accelerate and increase migration of tenocytes to the site of injury even in the presence of steroid drugs.

Mechanotransduction is an important physiological process by which cells respond to mechanical loading [93, 224, 269]. It has been demonstrated that the extracellular matrix and cells in tendon are able to respond to mechanical loading by initiating complex cascades of cellular events ranging from gene expression to protein synthesis [139, 319].

In addition to the fact that mechanical stimuli influence various extracellular activities in tenocytes, there are interplays between mechanical loading and growth factors. Increase in production of type I collagen and TGF-β has been reported in the presence of mechanical stimuli. However, expression of both collagen and TGF-β was determined by magnitude and duration of mechanical stimuli [315]. Furthermore increase in secretion of basic FGF (bFGF) and PDGF have been linked to the presence of mechanical loading [258]. It is obvious that the pleiotropic function of growth factors plays an essential role in the process of mechanotransduction by influencing the proliferation, migration of cells and the synthesis of extracellular matrix. However, the type and magnitude of mechanical stimuli and dose of growth factors necessary to improve tendon healing is not understood.

In order to develop effective treatment modalities it is necessary to ensure the optimal transformation of the mechanical loading and growth factor activities to functional adaptation of extracellular matrix. To address this requirement it is important to move beyond cellular level and investigate the role of mechanical loading and growth factors at the more complex tissue level.

An *in vitro* tendon injury model which allows repeatable mechanical testing, quantitative biochemical assays and whole organ imaging of tendon tissue as it heals provides an important tool to investigate the effects of mechanical loading and PRP on mechanical and biochemical properties of tissue during healing.

Using the novel rat tail fascicle injury model together with different biochemical assays and mechanical testing this thesis took a new approach to study the effects of PRP and static loading on defining parameters of tendon structure, Young's modulus, GAG and DNA content.

Results from tensile tests showed that injured fascicles exposed to static load of ~ 0.02N have higher Young's modulus values compared to load deprived injured fascicles throughout the 7 days of experiment. Statistical analyses revealed a significantly higher modulus in injured/loaded versus injured/unloaded fascicles after 4 days. The Young's modulus values of injured/loaded fascicles were higher than injured/unloaded fascicles after 7 days but the statistical difference between two groups was not significant.

This study also investigated the effects of static loading on glycosaminoglycans, an important component of tendon extracellular matrix and one which is reported to change rapidly in injured and healing connective tissues such as cartilage [237]. Moreover, the double-stranded DNA content which represents the quantity of viable tenocytes in tissue was assessed.

Both GAG and DNA content were significantly increased in the presence of static loading in comparison to load deprived fascicles. There is substantial literature reporting the down-regulation of matrix degrading enzymes such as matrix metalloproteinases as well as aggrecanases in loaded skeletal tissues [275], which may

explain the improved GAG levels. Alternatively, this may suggest that mechanical stimuli could result in improved healing properties by inducing proliferation, preventing tenocytes death or promoting GAG secretion.

Similar studies have also suggested alteration in mechanical and biochemical properties of tendon tissue in response to load. It was shown that 1 week of applied static stress increased Young's modulus of rat tail tendon fascicles by 30% while load deprivation decreased the modulus to about 23% of the controls. It was further revealed that the changes in mechanical properties of the fascicles were associated with the changes in the GAG content [3]. It has also been shown that tensile strength and tangent modulus significantly decreased in load deprived rabbit patellar tendon fascicles cultured for 1 week, while tensile strength and modulus were increased in the loaded fascicles within the same period [314].

Although tendons are exposed to the combination of cyclic and static loading *in vivo*, the results obtained from this study show the importance of defining the mechanical boundary conditions suitable for treating different types of tendon disorders.

Data from tensile testing suggested that PRP treated fascicles display superior Young's modulus than unloaded fascicles during 7 days of experiment. Moreover, untreated fascicles failed more often during tensile testing in comparison with PRP treated fascicles. Viable tenocytes have the ability to proliferate and synthesise important components of extracellular matrix such as collagen and GAG altering the mechanical properties of tendon tissue, or to rearrange the local extracellular matrix through cytoskeletally mediated contraction [90, 115]. Therefore, the increase in the

Young's modulus values of PRP treated fascicles may be linked to increase in the proliferation of tenocytes as a result of PRP treatment.

Moreover, it was shown that PRP-treated fascicles possessed the highest GAG and DNA content in comparison with other treatments.

Although values of Young's modulus, GAG and DNA content were higher in both injured and intact tissues statistical analyses did not reveal significant changes in mechanical and biochemical properties of intact tendon fascicles. This may suggest that injury created instability in the tissue resulting in the observed biological response to growth factors present in the environment. However, healthy undamaged tissue is in homeostasis and so cells are not sensitive to added growth factors as there is no need to change the balance of the cellular activities.

Finally, the ultimate objective of this thesis was addressed by investigating the effects of mechanical loading and PRP in combination with each other. The hypothesis of this thesis was that the combination of static loading and PRP improve the mechanical properties, GAG and DNA content of fascicles more than each treatment alone.

The combination of two treatments had significant positive effects on mechanical properties of healing fascicles, suggesting potential additive or synergistic signalling mechanisms. Contrary to the hypothesis of this thesis, the results obtained from biochemical assays revealed that fascicles receiving both treatments have lower GAG and DNA content than either treatment alone. However, the concentration of GAG per DNA was significantly higher in loaded/PRP group in comparison with both PRP alone and with static loading alone after both 4 and 7 days in culture. Firstly it should be noted that different fascicles were used for mechanical loading and

biochemical assays because the fascicles get destroyed by mechanical testing. It is therefore possible that subtle differences in fascicle size, quality of PRP were present between tests. However the fact that both PRP and loading alone were able to increase both mechanical and biochemical properties suggests these differences, if present, were slight. Given that DMB and Picogreen are end-point assays it is possible that an initial increase in cell number and GAG with co-therapy could be followed by the start of a loss of cell viability due to heavy loading which displays itself first in cell number and GAG content and would be followed later by a decline in material properties. Moreover, it has been suggested that only specific concentrations of growth factors and duration of mechanical stimuli could induce synthesis of glycoproteins and other proteins in tenocyte culture [236]. Therefore, it is possible that both loading and PRP strongly stimulate the same pathway which may in combination exceed the beneficial threshold of the individual treatments. One drawback of this study was that collagen I, the major structural protein of tendon, could not be measured at the tissue level and collagen is probably the largest contributor to improved material properties (see further discussion below). A decline in GAG and/or cell number will not necessarily be immediately reflected in collagen content. However, further studies using different doses of mechanical loading and PRP must be carried out to understand the complete mechanism behind this action.

Although PRP therapy and mechanical loading are likely to improve the mechanical and biological properties of tendons, it is well known that the balance between anabolic and catabolic mechanical stimuli in tendon is very fine [20] and high concentration of PRP have been reported as detrimental to cell viability ( Dr Alan Homer, Smith and Nephew, personal communication 2009) [297]. Furthermore, it is also

important to be selective in choosing the right type of tendon disorder which could potentially benefit from these treatments.

## 7.4 Limitations of the study

### 7.4.1 Limitations of the studies performed on tenocytes

One of the major limitations of the drug testing and hypoxia work presented in this thesis is the short term nature of the experiments, 72-96 hours in most cases. Although, cells showed significant response to both drugs and hypoxia, further investigations are required in order to understand the long term effect of these conditions on tenocyte culture.

A further limitation of this work and other studies on PRP applications is the fact that the growth factor concentrations in the individual releasates depends on the activation of platelets, which cannot be standardized; consequently, the comparison of experimental results becomes relatively complicated. Also, the time varying course of different growth factors release from activated platelets is not followed, but releasate from one particular time point after activation is used. Furthermore, this study did not identify the role of specific growth factors in inducing proliferation, protection and migration in tenocyte culture. Moreover, only two concentrations of 10% and 100% were used in this study, although our group have tested a wide range of doses to find the optimal proliferative dose and to describe the activated signalling pathways (manuscript in prep Dr Sarah Franklin). It is important to test other combinations of PRP in order to suggest the optimal dose for clinical treatment.

The PRP used in this study, obtained from 6 volunteers, was applied heterologously to tendon cell cultures from patients, unlike in clinical settings where

the PRP is prepared from the patient's own blood. This could produce the risk of a graft-versus-host reaction in culture. Although cells responded to PRP-conditioned medium with active growth and PRP-treated cultures displayed both fewer dead cells and less senescence than untreated controls, the absence of immune reaction could not be confirmed in this work.

Moreover, the interlinked nature of proliferation and migration processes makes it difficult to differentiate the contribution of each process to the wound coverage in the wound healing scratch assay. This could be further investigated using time laps microscopy and labelled cells.

### 7.4.2 Limitations of the studies performed on tendon tissue

The injury model introduced in this thesis was generated by hand using needle scratch. Although the data obtained from this model was consistent, the uncertainties introduced by manual work may be present in this injury model. Designing an automatic device which would generate precise injury using the same needle size and pressure would reduce the possibilities of manual errors and produce precisely the same injury on a large number of fascicles. Furthermore, the load was applied using a small magnet attached to the fascicle with superglue, which was toxic especially when wet. Although toxicity was minimised by letting the glue dry before submersing the construct in culture medium, a clip-on magnet would be an improvement.

Another major drawback of the rat tail tendon injury model, is that the vascular compartment is not effectively represented and therefore systemic effects and essential healing processes such as angiogenesis cannot be observed in this model.

Although the fascicles used in this study were obtained from Sprague Dawley rats, aged between 4-6 months, the genetic variation and differences in the weight of the rats must be taken in to account when interpreting the results of this study

Tendons are exposed to complex and time dependent loading conditions *in vivo*. Static tensile loading was chosen as a convenient experimental modality to investigate the role of mechanical loading in healing, but of course far more complex mechanical set-ups would be required to mimic the forces applied on tendons *in vivo.*

Only measurements of Young's modulus are reported here since this is the key parameter in describing the material properties of tendon tissue. However, other parameters such as stiffness, tensile strain, critical stress, critical strain and cross sectional area of the fascicles have been measured but time ran out to complete the analysis for presentation in this thesis. However, all the data will be published as part of the completed study.

This thesis investigated the effects of PRP and static loading on the GAG content of tendon tissue. Although proteoglycans are important components of tendon extracellular matrix, collagen is the major protein of interest in tendon tissue. This study used Sircol cell culture based assay to measure the collagen content of tendon tissue after different treatments. However, the assay proved to be unsatisfactory for analysis of whole tissue, despite extensive optimisation efforts, because total solubility of sample was only achieved by measures which also destroyed the alpha helical arrangement of collagen necessary for Sirius red dye binding. Further investigations are necessary to modify the biochemical assays used to measure collagen content in the rat tail tendon model.

Moreover, it is important to note the that mechanical testing was performed on a different set of fascicles than the biochemical assays because the tissue gets destroyed in load to failure and cannot be restored for biochemical tests.

Additionally, our limited understanding of the exact composition of tendon extracellular matrix makes it difficult to investigate the influence of different treatment on structural and functional properties of tendons. The arrival of novel techniques such as proteomics and improvements in mass spectrometry may help to characterize components of tendon matrix and ultimately improve our ability to evaluate different treatment techniques .

## 7.5 Further Work

1) **Use mechanically stimulated/PRP treated human tenocytes to begin studying the detailed molecular mechanisms behind tendon responses to mechanical stimulation and PRP**

Complementary studies using mechanically stimulated/PRP treated human tenocytes would help to understand the detailed molecular mechanisms behind tendon responses to mechanical stimulation and PRP. Using this model the hypothesis that cells respond to the mechanical stimulation and PRP by altering gene expression, matrix synthesis and cell survival could be tested. Biochemical assays such as Sircol and DMB could be used to measure Collagen I and GAG, respectively.

Also, QPCR should be used to quantify the effects of PRP and mechanical stimulation on the mRNA expression of major tendon matrix core proteins such as aggrecan, decorin and versican as well as Collagen I and III and matrix regulators such as TIMPS and matrix metalloproteinases including MMP1, 3 and 13.

In addition to the fact that mechanical stimuli influence various extracellular activities in tenocytes, there is interplay between mechanical loading and growth factors which is of significant importance in studies associated with using PRP as a means of growth factor delivery. Using complementary model systems, tendon tissue and tenocytes, the effect of simultaneous versus sequential treatment with PRP and mechanical loading on the repair process could be assessed. Moreover, the signal transduction pathways activated by the co-therapies should be studies, initially focussing on ERK, PKB and cyclic AMP anabolic pathways which our group has found to be strongly activated by PRP. Activation in cells could be assessed by standard Western blot with active-state antibodies against phosphorylated proteins. Inhibitors of the kinases and other growth factor receptors could be tested. Moreover, it is important to understand the effects of different doses and duration of mechanical stimuli and growth factor therapy on tendons. This could be investigated by using a constant dose of PRP with different loading conditions and constant load with different PRP concentrations at various time points.

**2) Investigate the potential of mechanical stimulation in protecting tendons from adverse effects of steroid drugs**

It was shown in this thesis that dexamethasone decreases viability and induces senescence in human tenocytes and PRP is strongly protective against these adverse effects. Moreover, it has been shown by our group that dexamethasone strongly up-regulates stress-response transcription factor FOXO1 which in particular represses collagen I synthesis[219] . This may affect the mechanical properties of tendons as collagen forms tendon hierarchical structure. The effects of dexamethasone on mechanical properties of tendon could be investigated by measuring Young's modulus, ultimate

strength and ultimate strain in dexamethasone treated tendon tissue. Moreover, the mechanical cyclic loading model developed in our Oxford Mechanobiology group could be used to explore the potential of both PRP and high frequency low magnitude (HFLM) loading regimes in protecting tendon against the adverse effects of dexamethasone.

**3) Imaging studies of the healing tendon**

The injury model developed for this project allows whole organ imaging of the defect over time as it heals. Further studies using time lapse microscopy together with staining techniques could be performed to investigate the activities of labelled cells in injured tissue in order to understand the contribution of migration and proliferation processes to tendon healing. In addition imaging techniques such as confocal Z-stack could be used to improve the quantification of Live and Dead stained cells in the wound region.

**4) Platelet rich plasma, mesenchymal stem cells and scaffolds**

Optimal management of tendon disorders should restore normal extracellular activity and decrease the rate of re-rupture. However, none of the available therapies are particularly convincing. Mesenchymal stem cells are multipotential cells which have the ability to differentiate into different cell types including tendons in three dimensional culture and in the presence of growth factors. It has been shown that delivering mesenchymal stem cell-seeded implants could improve biomechanical properties of injured Achilles tendon [321]. However, it is important to supply stem cells with growth factors in order to obtain optimal repair. PRP could be used as an autologous growth factor delivery method which could facilitate differentiation of stem

cells to healthy tendon cells which could synthesis different proteins necessary for improving tissue repair.

## 7.6 Conclusion

PRP has shown better clinical results in animal models than human studies to date, although many of the human studies have been very small. It is therefore encouraging to observe that PRP acts very clearly to stimulate human primary tenocyte proliferation, survival, migration and chemotaxis. In addition the net effect in organ culture of whole tendon was an improvement in mechanical properties, cell number and GAG content. The possible synergy with mechanical loading is encouraging and taken together the outcome of this thesis is that both PRP and mechanical loading play significant role in defining the biochemical and mechanical properties of tendon and could contribute to development of novel tendon treatment strategies.

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