A NEW APPLICATION OF DEMINERALISED BONE AS A TENDON GRAFT

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Declaration

I, Sherif Elnikety, confirm that the work presented in this thesis is my own.

Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Tendon injuries present a challenging situation for orthopaedic surgeons. In severe injuries, a tendon transfer or a tendon graft is usually used. The aim is to find a biocompatible substance with mechanical and structural properties that replicate those of normal tendon. Because of its structural and mechanical properties, we propose that Demineralised Cortical Bone (DCB) can be used in the repair of tendon and ligament, as well as for the regeneration of the enthesis. I hypothesise that DCB grafted in a tendon environment will result in remodelling of the DCB into tendon and produce a fibrocartilaginous enthesis.

DCB was prepared according to a modified Urist technique, the effect of gamma irradiation and freeze-drying on the tensile strength of the DCB was examined. These are two common methods used in medical practice to sterilise biological products and prolong over the shelf life. In the second part of the study, four models of repair of a patellar tendon defect were examined for their strength to failure in order to identify a suitable technique for an in vivo animal model.

In the final part of the study, animal study was performed using DCB as a tendon graft to treat defect in sheep patellar tendon. Animals were allowed to mobilise immediately post-operatively and were sacrificed after 12 weeks. Pre and post operative force plate analyses were done as well as X-ray Radiographs, pQCT scans and histological analyses.

My results show that DCB remodelled into a ligament-like structure with evidence of neo-enthesis. No evidence of ossification; instead, DCB retrieved was cellularised and vascularised with evidence of crimp and integration into the patellar tendon.

My results prove that DCB can be used as a biological tendon graft; this new application of demineralised bone has the potential for solving one of the most challenging injuries. Combined with the correct surgical techniques, early mobilization can be achieved, which results in the remodelling of the DCB into a normal tendon structure.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ACL ..... Anterior Cruciate Ligament

ASAD ..... Arthroscopic Subacromial Decompression

B ..... Bone

FGF ..... Fibroblast Growth Factor

BMP..... Bone Morphogenetic Protein

CFC ..... Calcified Fibrocartilage

CI ..... Confidence Interval

COMP ..... Cartilage Oligomeric Matrix Protein

CTGF ..... Connective Tissue Growth Factor

DBM ..... Demineralised Bone Matrix

DCB ..... Demineralised Cortical Bone

EDTA ..... Ethylenediaminetetraacetic acid

FD ..... Freeze Drying

FDA ..... Food and Drug Administration (American)

F_{max} ..... The Mean GRF_z

FWB ..... Functional Weight Bearing

GHJ..... Glenohumeral Joint

GI ..... Gamma Irradiation

GRFz ..... Ground Reaction Force

HCL ..... Hydrochloric Acid

IMS ..... Industrial Methylated Spirit

ISO ..... The International Standards Organisation

LHB ..... Long Head of Bicep

MSCs ..... Mesenchymal Stem Cells

NaCl ..... Sodium Chloride

PBS ..... Phosphate Buffered Saline

PDGF..... Platelet-Derived Growth Factor

pQCT ..... peripheral Quantitative Computerized Tomography

PROMs ..... Patient Reported Outcome Measures

PRP ..... Platelet Rich Plasma

RC ..... Rotator Cuff

ROM ..... Range of Movement

RSD ..... Radiation Sterilization Dose

T ..... Tendon

TGF ..... Transforming Growth Factor

UFC ..... Uncalcified Fibrocartilage

Chapter 1

Introduction

1.1 Aims and Hypothesis

This thesis aims to investigate the novel application of demineralised cortical bone (DCB) as a biological tendon graft. I hypothesise that DCB, when exposed to a normal tendon environment and subjected to tendon stresses and strains will remodel into tendon tissue.

1.2 Study background

This study is part of an ongoing research project at the John Scales Centre for Biomedical Engineering, Institute of Orthopaedics and Musculoskeletal Research, University College London. The main research project aims to investigate various aspects and applications of demineralised bone in musculoskeletal science. Multiple publications and presentations were produced out of this ongoing research project (Sundar et al., 2009d, Sundar et al., 2009c, Pendegrass et al., 2006, Oddy et al., 2005, Pendegrass et al., 2004, Sundar et al., 2009a, Elnikety et al., 2013b, Elnikety et al., 2013d, Elnikety et al., 2013c, Aderinto and Blunn, 2006, Sundar et al., 2009b, Elnikety et al., 2013a). A number of both undergraduate and postgraduate research students were granted academic degrees based on their contributions to this overall project. My project was specifically associated with utilizing DCB to repair a tendon defect associated with a tendon insertion. It is a functional model that tries to mimic a tendon repair where it has retracted from the bone surface. This is a condition that is often associated with chronic failure and tendon avulsion, but because of the nature of my model it was associated with an acute defect.

Demineralised bone is a well-known material in orthopaedic practice; it is widely used as a bone graft substitute and has multiple applications such as in non-union of fractures and impaction bone grafting for joint replacement surgeries. Prior to my involvement in the research project, the research group proved in a less severe model than my own that the use

of demineralised bone as interface between tendon and bone results in direct enthesis and enhanced healing of the tendon bone interface. These findings led to the development of my research project to answer the question of whether demineralised bone can be used as a graft when there is deficient tendon that has pulled away from the bone surface.

In the tendon-enthesis-bone complex, the demineralised bone proved to be beneficial in the healing and augmentation of the bone and the enthesis. The question that remained unanswered was whether there was a role for the demineralised bone in tendon repair and healing. Answering this question would not only potentially result in the production of new biological tendon grafts, but could lead to a new perspective on demineralised bone and may have significant implications on tumour surgery, major tissue resections, disuse tendon atrophy and osteoporosis.

1.3 Study overview

Tendon injuries are common and they present a wide variety of musculoskeletal disorders. Millions of people are reported to be affected by tendon disorders every year (Almekinders and Temple, 1998). In the USA, nearly 50% of musculoskeletal injuries involve soft tissues, including tendons and ligaments (Urwin et al., 1998). Although tendon injuries are not age related, the prevalence of tendon disorders varies within different age groups. Traumatic tendon injuries are more prevalent in younger age groups, while degenerative disorders are more common in older groups (Nyyssonen et al., 2008, Sivakumar et al., 2008).

Tendons are present in all anatomical regions of the human body; nevertheless, the prevalence of tendon disorders varies from one region to another. This variation is dependent on the anatomy and the biomechanics of the involved tendons (Krivickas, 1997). For

example, tendon injuries are not known to be a common disorder of the back, while shoulder tendon disorders are common among different age groups. Similarly, Achilles tendon disorders are a common presentation in musculoskeletal clinics, while disorders involving tendons of the intrinsic foot muscles are rare. There exists a wide variety of tendon disorders; traumatic injuries, including partial and complete tears, overuse tendon disorders, including tendinitis, tendinosis and paratendinitis (Maffulli et al., 1998b, Maffulli et al., 1998a, Selvanetti et al., 1997), which are additional to chronic tendon disorders (Almekinders, 1998). Several treatment modalities are available for treating tendon injuries; immobilization, non-steroidal anti-inflammatory medications, steroids, physiotherapy and surgical intervention are the most commonly used.

Many factors influence the treatment decision for tendon injuries; the nature and the aetiology of the injury, the chronicity of the injury, the condition of the surrounding structures, the pre-injury condition of the injured tendon, as well as socioeconomic factors, including the profession of the patient and treatment cost.

Complete tendon rupture is a special category of tendon injuries; it develops either due to direct trauma to the tendon or indirect trauma, typically as a result of an acceleration/deceleration injury. Complete tendon rupture can also occur as a result of predisposing tendon conditions such as tendinitis (Birch et al., 1998). Tendons have low potential for regeneration; besides, they usually retract due to resting muscle tone, resulting in gap formation between the bone and the tendon end. Complete tendon ruptures heal by scar tissue filling the gap, which sometimes happen in Achilles tendon rupture; however, this scar tissue is biomechanically inferior to tendon and often results in elongation of the tendon with poor functional outcome. In other cases, as in rotator cuff tears, tendons retract with no scar

formation, resulting in disuse atrophy of the muscles and degenerative changes in the shoulder joint leading to loss of function. This may be associated with local osteopenia of the bone associated with the region of tendon attachment, as this is no longer loaded to the same degree.

Treatment of complete tendon injuries is either surgical or non-surgical. Non-surgical options involve pain relief and rehabilitation, and the use of the compensatory muscles, for example, the deltoid may compensate for the supraspinatus when there is a tear in the supraspinatus tendon. Non-surgical options can result in satisfactory outcomes, especially with low demand patients and those with other co-morbidities making surgical treatment unsafe. Multiple surgical treatment options are available that include direct repair, tendon transfer and tendon grafting.

In this study, I discuss a new biological tendon graft material; in order to be able to produce a biological tendon graft, a detailed understanding of tendon structure and function is needed. In this introductory chapter, I discuss tendon anatomy in detail. I also discuss rotator cuff pathology and current treatment practice as a clinical application for my research. I then move on to describing current tendon graft materials and explain why I consider DCB to be ideal material for tendon grafting.

1.4 Tendons

The tendon is a highly specialized connective tissue and forms an anatomical part of skeletal muscles. It has an important role in the musculoskeletal system, as it concentrates the muscular force and transmits it from the muscles into bone; intact, healthy tendon is paramount for efficient muscular function. Tendons attach to the skeletal system via

transitional zones called enthesis, which have characteristic features that will be discussed later in this chapter.

Tendons are formed mainly of densely packed collagen fibres; these fibres are arranged in longitudinal bundles that are parallel to the long axis of the tendon.
Water makes up approximately 70% of tendon composition, while collagen fibres form nearly 75% of the dry weight of the tendon, of which 95% is Collagen type I. Other collagen types are present to a lesser extent and have a minimal influence on tendon function and structure (Kjaer, 2004, Banos et al., 2008). Tendons contain other non-collagenous proteins; elastin, glycosaminoglycans and glycoproteins which form the extra cellular matrix (Hoffmann and Gross, 2007, Kannus, 2000, O'Brien, 1997, Kjaer, 2004). Collagen fibres are responsible for the elastic tensile response of the tendon, while proteoglycans provide the viscoelastic properties of the tendons (Robinson et al., 2004).

Tendons are fibroelastic in texture and can take different forms; cords, bands or flattened, they can be classified as intrasynovial or extrasynovial. Intrasynovial tendons are surrounded by thin layers of synovial sheath filled with synovial fluids, while extrasynovial tendons are surrounded by loose connective tissue only. Tendons have poor vascularity in general; they obtain nutrition and maintain the body's metabolism via the extracellular and the synovial fluids that are contained within the tendon sheath. Micro-blood vessels were observed in the tendon tissue either crossing the myotendeneous junction, feeding vessels from the nearby periosteum, or as segmental vessels passing across the tendon sheath into the mesotenon (Ahmed et al., 1998, Carr and Norris, 1989, Fenwick et al., 2002). One of the functions of the tendons is to provide neural feedback mediated by the proprioceptive nerve endings; tendon

nerve supply is usually received from neighbouring nerves that typically supply the nearby joint.

1.4.1 Tendon ultrastructure

Tendons have a highly organized microstructure. The base unit is collagen I (Tropocollagen) molecules; these molecules join to form subunits of the hierarchical tendon structure (Figure 1.1), which in turn join to form a further subunit of bigger diameter. Collagen I is made up of three polypeptide chains each consisting of about 1000 amino acids. These chains are stabilized together with hydrogen bonds. The three chains are organized in a helically fashioned "Tri-helix", forming the collagen molecules that are about 300 nm in length and 1.5 nm in diameter (Birch et al., 2013). Each group of Tropocollagen (usually five) form a microfibril which in turn forms fibrils. Fibrils are grouped in bundles forming collagen fibres; collagen fibres group to form bundles that are the basic units of fascicles (Hoffmann and Gross, 2007).

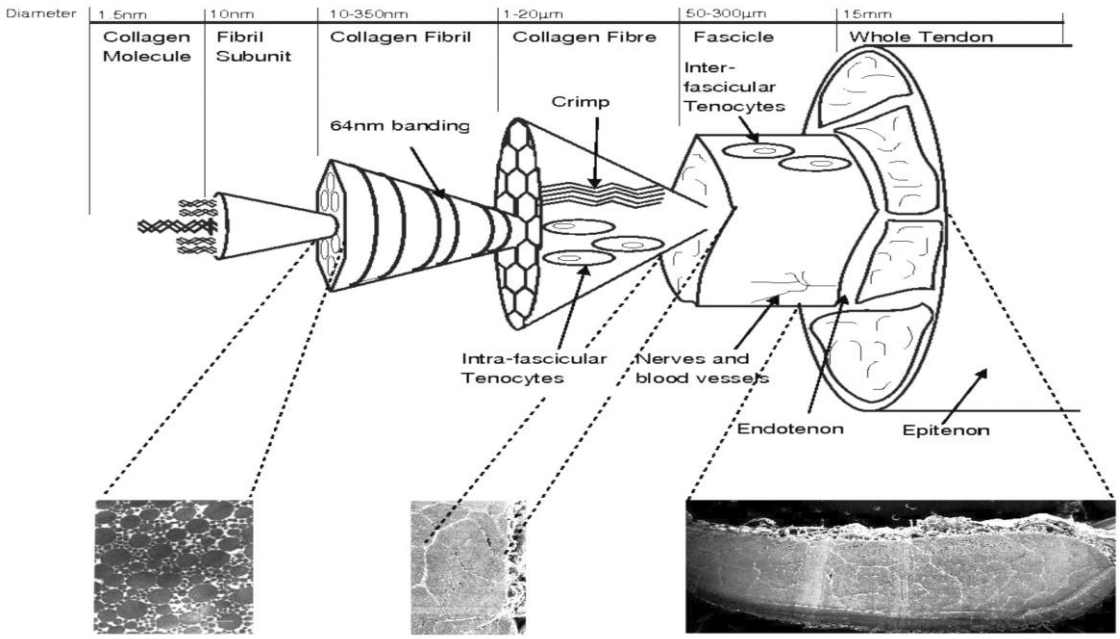


Figure 1.1: Hierarchical structure of tendon and ligament (reproduced from (Thorpe et al., 2010).

Fascicles are connected with loose connective tissue called endotenon, while epitenon is a layer of connective tissue surrounds the outer surface of the tendon structure (Towler and Gelberman, 2006, Sharma and Maffulli, 2006a). The epitenon is a loose connective tissue sheath containing the vascular, lymphatics and nerve supply to the tendon, which in turn is surrounded by the paratenon, another layer of loose connective tissue that is lined with synovial cells and responsible for tendon gliding (Kastelic et al., 1978, Banes et al., 1988).

Besides collagen I, tendons have other collagens, mainly collagen types III, V, XII and others (Birch et al., 1999). Tendons also contain non-collagenous proteins such as elastin, proteoglycans and other glycoproteins. These different types of proteins form the extracellular matrix of the tendon, which plays an important role in tendon development and maturation (Yoon and Halper, 2005). The extracellular matrix also affects the biomechanical properties of the tendons; it is thought that the non-collagenous proteins are responsible for the fatigability, compressive properties and sliding ability of collagen fibres (Esther et al., 2008, Shepherd et al., 2014, Legerlotz et al., 2013). The distribution of these non-collagenous proteins vary between tendons; it is also found to be more concentrated in areas exposed to compressive forces such as tendons crossing over joints (Birch, 2007, Rees et al., 2002, Taguchi et al., 2008).

1.4.2 Cellular content of tendons

Tendons are cellular structures; they are dominated by specialized fibroblast cells, called 'tenocytes'. Tenocytes have a large cytoplasmic area with a heterochromic nucleus; they are responsible for the formation and maintenance of the tendon's structure. In immature and healing tendons, active cells are called tenoblasts, which represent active tenocytes and contain prominent nuclei, the endoplasmic reticulum and the Golgi apparatus. Tenocytes are

arranged three-dimensionally; they are aligned mainly along the longitudinal axis of the tendon and are connected in-between the collagen bundles via the long cytoplasmic processes (McNeilly et al., 1996). Previous studies have shown physiological variation in the cellularity of the tendon, where it is thought to be higher in young patients with immature tendons and also higher in healing tendons; the number of cells gradually reduces with age (Oryan and Shoushtari, 2008, Kannus, 2000). About five to 10% of the cellular contents of the tendons are not tenocytes, but a mix of chondrocytes, synovial cells, immune cells and vascular cells (Kannus, 2000, Jozsa et al., 1979, Sharma and Maffulli, 2005a).

1.4.3 Mechanical properties of the tendons

Tendons and ligaments share unique mechanical characteristics; they have high mechanical strength, high flexibility and a level of elasticity. Besides transmitting forces from muscles to bones, tendons also act as a buffer, absorbing external forces to protect the body from muscular injury (Kirkendall and Garrett, 1997, Oxlund, 1986).

The anatomical and molecular structure of the tendons gives the tendons their unique mechanical characteristics. At resting tension, the collagen fibres are crimped; as load increases, these fibres stretch and become straight. The crimp feature of the tendons is interesting; it is not clearly understood why it happens or how and it was found that the degree of crimp in tendons is different than those in ligaments. Crimping might occur as a result of weak biochemical bonds between the components of the collagen chains. Collagen I molecules are formed out of triple helix chains; under tensile stress, these chains stretch and the collagen molecules slide on each other, giving the tendon its elastic properties. This unique arrangement of tendon structure allows the tendons to absorb energy when exposed to

forces, protecting the muscular tissue and dissipating this energy on returning back to the resting tension (Shadwick, 1990).

Several factors affect the mechanical properties of tendons; at a microstructural level, the amount of collagen fibres, the percentage of collagen I to other extracellular content, the hydration of the collagen and the inter- and intra- molecular bonds of the collagen molecules all contribute to the mechanical behaviour of tendons. At a macroscopic level, the thickness and length of the tendon have a direct relation with the mechanical strength of the tendons (Sharma and Maffulli, 2005b).

When tendons are exposed to tensile forces they initially act with a gradual increase in the length of the tendon and minimal increase in stress. This is due to the straightening of tendon crimp; as the force increases, stress builds up in the tendon tissue. Due to its viscoelastic nature, tendons can withstand much higher forces applied at a slow rate, more so than these forces at a quick rate. It is estimated that tendons can accommodate tensile stresses of 500 to 1000 Kg/cm^2 (Shadwick, 1990). Visco-elastic properties of the tendons allow for time-dependant strain-relaxation for strains limited to < 4%. Within this limit, tendons maintain their elastic behaviour, while higher strain will result in damage to the tendons (Jozsa and Kannus, 1997).

1.4.4 Enthesis

The biomechanics of tendons and bone are vastly different. The enthesis is thought to be a transition zone between the two structures that enables load transition and distribution without disruption (Cook and McDonagh, 1996). Entheses have been categorized into two types: either fibrous (periosteal) or fibrocartilaginous (chondral), depending on the character

of the interface between the tendon and the bone (Benjamin and Ralphs, 1997, Woo and Buckwalter, 1988). In the case of the fibrous type, tendon attaches either to the bone or the periosteum by strong dense connective tissue, while in the fibrocartilaginous type (Figure 1.2) the tendon attaches through a region of fibrocartilage, which anchors to the bony surface via a mineralised zone. Some authors have found the two types of enthesis in the same tendon attachment (Benjamin and Ralphs, 1998, Benjamin and Ralphs, 2000).

Some authors refer to the fibrocartilaginous enthesis as direct enthesis and the fibrous enthesis as indirect enthesis. However; this nomenclature is not universal, sometimes indirect enthesis refers to post traumatic healed enthesis or enthesis that results after surgical repair. I find the terms direct and indirect to be confusing, as they may indicate different scientific terms and the superiority of one type over the other. I have therefore avoided using these two terms; instead, I referred to the two types as either fibrocartilaginous or fibrous in the current study.

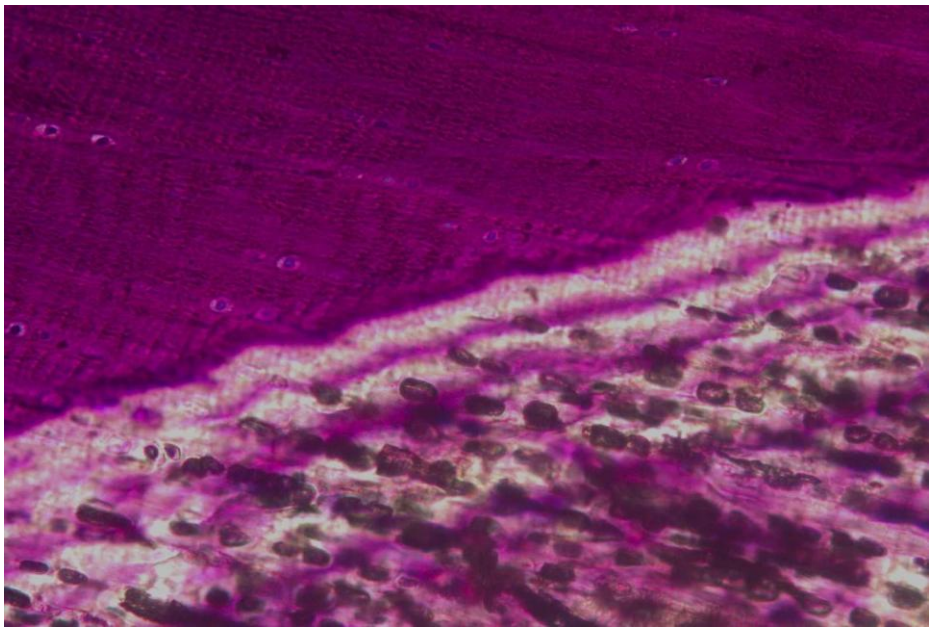


Figure 3.2: Microphotograph of a fibrocartilaginous enthesis of the normal patellar tendon (patellar attachment) X 20.

The fibrocartilaginous enthesis is usually found in the diaphyseal region of the long bone. It has four distinct zones with a clear tidemark between calcified and non-calcified cartilage (Cooper and Misol, 1970, Oegema et al., 1997). These four zones were also found to vary in thickness and distribution along the enthesis, with the thickest region in the middle of the enthesis. This is thought to be an adaptive mechanism concerning the increased compressive stresses in the centre of the enthesis (Matyas et al., 1995, Benjamin et al., 1992). In the enthesis, tenocytes are predominant in the tendon layer; as the enthesis develops into cartilaginous layers, chondrocytes become more predominant with the characteristic cartilage arrangement and lacunae structures (Claudepierre and Voisin, 2005).

In fibrous type enthesis, authors have described characteristically dense collagenous fibres, known as 'Sharpey's fibres' that cross the enthesis, anchoring the tendon to a deeper layer of bone tissue (Jones and Boyde, 1974, Benjamin et al., 2002). Sharpey's fibres are not seen in all entheses; this characteristic feature is reported to be present in the fibrous enthesis only. Although some authors debate whether these fibres are present in both types of enthesis, it is more acceptable that Sharpey's fibres are a feature of the fibrous enthesis (Benjamin et al., 2002, Francois et al., 2001). This debate might be explained by the fact that both types of enthesis were found in the same tendon attachment, thereby explaining the presence of Sharpey's fibres alongside the fibrocartilaginous layers. One can argue that these two types of entheses are interchangeable and that the body adopts one type over the other depending on local stimulants and the loads exerted on the tendons.

1.4.5 Treatment of tendon injuries

Not all tendon injuries require surgical treatment. Surgical management is indicated only when non-surgical treatment fails to restore function or control pain. Indeed, most tendon injuries, except in the case of complete rupture, respond satisfactorily to non-surgical treatment. Except in cases of complete tendon rupture, in most cases, physical therapy and anti-inflammatory medication are all that is required for treating tendon injuries. In these situations physical therapy aims to improve the efficiency of the intact part of the tendon and to enhance the function of the synergistic muscles to compensate for any functional loss incurred. To have an impact, physical treatment is needed for an average of three to six months and can take up to a year. The aim of the anti-inflammatory medications, either steroidal or non-steroidal, is to decrease the inflammation, which in turn will decrease pain and result in better functioning.

Tendons have poor regenerative potential; after complete tendon rupture, tendons either fail to heal as in the case of rotator cuff (RC) tears and hand flexor tendon rupture, or they heal with disorganized fibrous tissue, as in some Achilles tendon ruptures. In both situations, the outcome might be poor, with loss of functional capacity and muscle power. If left untreated, it can lead to disuse muscle atrophy and degenerative changes in the surrounding tissues, which in turn affect quality of life and the individual's ability to work. Similar to other tendon injuries, non-surgical treatment has a role in the treatment of complete tendon rupture. In Achilles tendon rupture, for example, non-surgical treatment is considered a valuable treatment option. Some surgeons prefer this treatment option compared to surgical treatment if the patient accepts the slightly higher risk of re-rupture. Similarly, in RC tears, patients with good deltoid muscle function can compensate extremely well for the loss of supraspinatus muscle function. Unfortunately, it is difficult to predict whether a patient will

improve with non-surgical treatment; therefore, some surgeons advocate early surgical intervention in complete tendon rupture to prevent instances of disuse muscle atrophy and degenerative changes in the nearby joints. Supporters of this view also argue that even if patients are able to compensate for the lost muscle function through non-surgical treatment, degenerative changes will still take place and early surgical intervention is needed to prevent this.

Surgical options include direct tendon repair, tendon grafting and tendon transfer. Surgical options usually result in better outcomes compared to non-surgical treatments. Although surgical treatment delivers better outcomes, none of these options are without risks and potential complications. Direct tendon repair usually requires a period of post-operative immobilization resulting in a prolonged rehabilitation period. Direct repair outcome is unpredictable; it has higher incidence of re-rupture compared with other surgical options and scaring and adhesions may also limit functional outcome (Taras et al., 1994). Direct repair is not possible in all situations; traumatic tendon injuries may result in loss of tendon substance, making direct repair impossible; chronic tendon ruptures result in tendon retraction and stiffness, which makes direct repair difficult to achieve. Tendon transfer is not a common procedure; it requires the satisfaction of certain criteria to be applicable and results in suboptimal function of the donor tendon function (Mastrokalos et al., 2005). Tendon grafting also its limitations and risks attached, which will be discussed in detail later on in this chapter.

Other surgical interventions for specific tendon conditions are also available. In De Quervain's tenosynovitis, for example, tendon tunnel release is a surgical option;

additionally, subacromial decompression for subacromial impingement is also common surgical procedure.

1.4.6 Tendon healing

Although tendons are cellular structures, tendon cells are low density with low mitotic activity, which results in poor regenerative function (Calve et al., 2004). Similarly, although tendons are vascular structures, this vascularity is poor, which limits the regenerative potential of the tendons. Healing of tendon ruptures happens over three overlapping phases; the acute inflammatory phase, repair phase and the remodelling phase (Lin et al., 2004). The completion of this process can take up to two years.

The acute inflammatory phase starts immediately following the tendon injury. As in other acute inflammatory responses, it is characterized by haematoma formation, which later turns into organized haematoma and aggregation of inflammatory cells mediated by different factors as a result of the trauma itself, as well as the release of cytokines and prostaglandins from the inflammatory cells (Wong et al., 2003, Rodeo et al., 1993). This phase usually lasts for two to three weeks and is characterized by pain and other symptoms of acute inflammation (Sharma and Maffulli, 2006b).

The repair phase starts a few days after incurring the injury and can last for months. During this phase, fibroblasts invade the haematoma and a disorganized fibrous scar tissue gradually replaces the haematoma. In an attempt to produce functional tissue, the healing tendon undergoes remodelling and maturation of the scar tissue into a more organized tendon-like fibrous structure takes place. Collagen fibres assume tendon-like organization, bridging the rupture gap and integrating into the two ends of the tissue. This fibrous structure is capable of

withstanding reasonable loads and providing functional capabilities that might be adequate for low demand individuals. In most cases, however, this outcome is suboptimal and patients always notice a reduced level of activities following non-surgical treatment of complete tendon ruptures (Sharma and Maffulli, 2006b).

Most tendon ruptures occur at the enthesis; mid-substance ruptures are not that common and in these cases the fibrous tissue repair bridges the gap between the two ends of the tendon. After the process of maturation and remodelling, the tendon becomes one continuous unit, though it might not be functional or have adequate biomechanical properties.

When tendon rupture happens at the enthesis the healing process is different, as the tendon heals to bone rather than to tendon. This process of tendon healing is similar to that which results after surgical repair, where the gap between the tendon and the bone is filled with inflammatory cells and haematoma (Wong et al., 2003, Liu et al., 1997). This soon develops into granulation tissue with collagen fibres disorganized in the gap. As the process of repair and maturation takes place, organized collagen fibres connect the two surfaces and new bone formation occurs. During this phase, anchoring fibres similar to Sharpey's fibres connects the tendon to the bony surface and attaches to a deeper layer of bone (Rodeo et al., 1993). The bony surface invades the gap with new bone formation and provides more stability to the anchoring fibres (Shaieb et al., 2000, Oguma et al., 2001, Walsh et al., 2004). This process usually results in neo-enthesis similar in structure to a fibrous type enthesis. Some authors argue that this fibrous type progresses over time to form fibrocartilaginous enthesis. The time frame for this process is variable; it is estimated to happen over years, as the enthesis matures over the stages of repair that have already been outlined (Walsh et al., 2004, Koike et al., 2005).

Despite repair and remodelling, and due to the reasons mentioned earlier, the outcome is biomechanically inferior to normal tendon and lacks the elasticity and flexibility of a normal tendon.

If tendon rupture happens with major retraction of the tendon ends due to the resting muscle tone, or where the tendon is not contained in a confined space as in a RC tear, the healing process fails to happen. In the case of a RC tear, the haematoma involves the whole of the glenohumeral joint with the presence of synovial fluid preventing formation of a blood clot, which is the initial step for organized haematoma formation and initiation of the repair process. In this case, the tendon ends degenerate and an atrophic process takes place in the tendon and the muscle.

1.5 Rotator cuff

'Rotator cuff' is an anatomical term that describes four muscles that act on the glenohumeral joint (GHJ) and are responsible for a considerable amount of the joint's movement. Although the term 'shoulder joint' usually refers to the GHJ, some authors use this term to describe joints of the shoulder girdle, including the acromioclavicular joint and thoraco-scapular articulation. To avoid confusion, I will use the terms 'GHJ' and 'shoulder girdle' in this study.

The RC muscles are the supraspinatus, infraspinatus, subscapularis and the teres minor. These four muscles originate from the scapula and insert on the greater tuberosity of the humerus, with the exception of the subscapularis, which attaches to the lesser tuberosity. These tendons belong to three groups of muscles acting about the shoulder girdle: the scapular stabilisers group, the scapulohumeral group and the axiohumeral group. These provide the core strength and stability for upper limb movement.

The three muscles that insert onto the greater tuberosity blend with the joint capsule and form a conjoint insertion over a wide area of the greater tuberosity. Although tendon coalition is not a unique feature of RC, it represents an important anatomical structure (for example, similar coalition is present in the hip muscles and conjoint tendon of the abdominal muscles). RC injury usually results in debilitating disability and loss of function. As it involves the GHJ, RC injury has a high socioeconomic impact as it results in loss of the ability to work and injured individuals may also require increased levels of social and personal care.

The GHJ is located underneath the arch of the acromion process of the scapula; the acromion, along with the coracoacromial ligament, constitutes superior constraint to the shoulder joint. The subacromial bursa occupies a region between the superior surface of the RC tendons and the under surface of the acromion, and this space measures around 10 mm in height (Flatow et al., 1994). Both the subacromial bursa and the under surface of the acromion have important roles in the pathogenesis of the RC impingement syndrome (which will be detailed at a later stage).

The shoulder is a highly mobile joint and can achieve up to 360˚ of circumduction, To allow this high level of mobility and, unlike other ball and socket joints in the body, the bony anatomy has to be non-congruent. In fact, the bony GHJ is much like a golf ball on a golf tee with large ball size compared to smaller socket. This incongruence results in poor bony stability; as a result, GHJ stability is provided mainly by the soft tissue structures; glenoid labrum, muscles and tendons, ligaments and the joint capsule.

The glenoid labrum is a cartilaginous structure that is thought to provide depth to the glenoid surface and increases GHJ congruency without compromising the high range of motion. The ligamental structure of the GHJ is extremely complex; it involves multiple ligaments that

provide different restraints to the GHJ movement in different directions. These ligaments can be categorized into two main groups: an intrinsic group, which includes the glenohumeral capsular ligaments and an extrinsic group, such as the coracoacromial ligament. The labrum, the ligaments and the capsule form the static restraints and stabilisers of the GHJ, while shoulder dynamic stability is provided mainly by the muscles. Out of the different muscle groups acting on the shoulder girdle, the RC muscles are the main dynamic stabilisers for the GHJ.

1.5.1 Pathology

Shoulder pain is a common complaint in musculoskeletal clinics; most of these are related to RC pathology. It is estimated that about 30% of the world's population above 60 years are affected by RC pathology. However, this might not be symptomatic or evident in cases of radiological investigation (Reilly et al., 2006). Other reports show an even higher prevalence, with up to 80% reporting RC tears in individuals above 80 years old (Milgrom et al., 1995).

Several pathological conditions can affect the rotator cuff and these conditions can be categorized into two main groups. The first group are the conditions that affect the collagen tissues and joint diseases such as rheumatoid arthritis (Sivakumar et al., 2008), osteoarthritis and genetic collagen disorders. The second group are those specific to the GHJ; these are usually linked to anatomical and biomechanical features of the joint. The most common of these conditions are RC tearing (Lahteenmaki et al., 2006, Sorensen et al., 2007), either full or partial thickness (Moosmayer et al., 2010b) and RC impingement; other less common conditions include calcific tendinitis and adhesive capsulitis.

RC tears can be either due to acceleration /deceleration injury, as experienced by athletes for example, or can occur as a secondary to predisposing RC tendon condition; in most cases it is a combination of both. RC tearing is thought to be a complication of an impingement disorder of the GHJ. Neer described impingement as a cause of rotator cuff tendinitis, which is usually associated with pain. If impingement persists a RC tear results, this tear can be a partial or full thickness tear (Neer, 1972). RC tearing can also result from degenerative changes affecting the tendons. For example, progressive degeneration affects the tendon's biomechanical properties and can cause tearing to result either spontaneously or following minor trauma (Seitz et al., 2011, Kannus and Jozsa, 1991).

A specific subgroup of degenerative RC changes concerns osteoarthritic changes in the GHJ. In this subgroup, the RC changes are often clinically referred to by RC arthropathy, indicating that these changes are part of the osteoarthritis pathology and treatment of the RC in this situation should also target osteoarthritis, forming part of the treatment of the GHJ as whole (Oh et al., 2012).

If RC tear is left untreated it may propagate, resulting in a non-reparable massive tear; this might further affect other shoulder muscles, leading to increased loss of function, muscle atrophy and degenerative changes (Lee, 2013, Andarawis-Puri et al., 2011).

The blood supply to the RC may explain part of the pathogenesis of the RC injury; it was found that the Supraspinatus tendon has an area of relative avascularity compared to other RC tendons. This area of relative avascularity is the site of impingement lesions (Rathbun and Macnab, 1970). It was also found that the articular surface of the supraspinatus tendon has lower concentrations of arterioles compared to the bursa surface (Lohr and Uhthoff, 1990).

Shoulder impingement is thought to be due to the anatomical structure of the GHJ with the RC tendons confined in a narrow space (Figure 1.3) between the acromion and the coracoacromial ligament above and the humerus below (Neer, 1983).

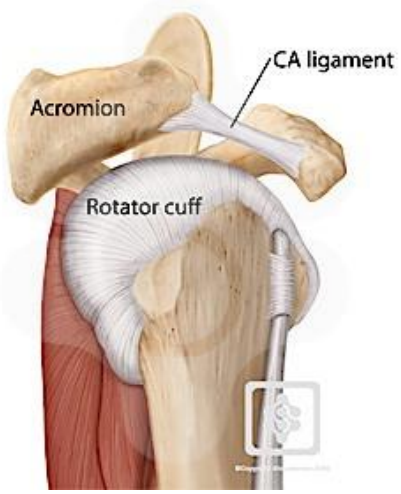


Figure 1.3: Diagrammatic representation (lateral view) of the subacromial space (reprinted from www.shoulderdoc.co.uk, accessed January 2014).

Neer also suggested bony overgrowth of the inferior antrolateral surface of the acromion, resulting in a bony spur (or more likely to be a bony ridge) that causes pressure and compression on the RC tendon, resulting in initial tendinitis which my progress to RC tear (Figure 1.4). This belief is supported by other studies that have reported increased incidence of RC tears with a type III acromion (hook-shaped acromion) (Bigliani and Levine, 1997, Bigliani et al., 1991, Rockwood and Lyons, 1993). Some authors argue that this bony spur (ridge) is a reactive change to the impingement condition and not the primary cause of impingement (Budoff et al., 1998).

Figure 1.4: Diagrammatic representation of different types of acromion (reprinted from www.shoulderdoc.co.uk, accessed January 2014).

More recently, internal impingement has been considered as one of the reasons for increased shoulder pain in athletes. Internal impingement happens when the GHJ is in extreme range of movement, usually in full abduction, internal rotation and extension, which results in impinging of the articular surface of the RC tendon against the postrosuperior aspect of the glenoid (Gartsman and Milne, 1995). This internal impingement results in pain and if it persists, can lead to tendinitis and RC tear (Brossmann et al., 1996, Neviaser and Neviaser, 1990, Hyvonen et al., 1998).

1.5.2 Treatment modalities of RC injury

Treatment of RC conditions follows similar principles to other tendon pathologies. The first line of treatment is non-surgical in the form of anti-inflammatory and analgesic medications, along with physical therapy. If this line fails to cure the injury, surgical intervention is always required to treat the condition. Surgical treatment could be ahead of non-surgical options in certain situations, especially in full thickness RC tear (Moosmayer et al., 2010a). As discussed earlier, the non-surgical options aim to control pain, reduce inflammation and

improve function. In RC conditions, non-surgical treatment is usually recommended for six to 12 months before surgical intervention is required (Morrison et al., 1997).

Surgical intervention for shoulder treatment has evolved significantly over the past three decades. Previously complete or partial full thickness acromionectomy had been performed for impingement syndrome (Armstrong, 1949, Smith-Petersen et al., 1943).These procedures are now obsolete; nowadays, as the primary procedure for treatment of impingement syndrome, open surgery is considered against the best evidence. Over the past 20 years, arthroscopic procedures have evolved greatly; arthroscopic shoulder surgery is now established as the preferred option over open surgery. This is supported by early recovery and fewer complications.

Surgical treatment for RC conditions varies according to the disease involved. Impingement syndrome is usually treated by arthroscopic subacromial decompression. Arthroscopic subacromial decompression (ASAD) was first described by Ellman in the 1980s (Ellman, 1987) and is now considered the gold standard for subacromial impingement (Olsewski and Depew, 1994). Several studies have reported long term success in 70 -90% of cases (Speer et al., 1991, Lindh and Norlin, 1993, Roye et al., 1995). Ten to 30% of patients are usually not satisfied with surgical outcomes and report having persistent pain, either due to progression of the RC tendinitis into the RC tear, or because of inadequate resection of the acromion with the residual bony spur/ridge. Fewer patients have persistent pain after ASAD due to neuropathic pain syndromes (Coderre et al., 1993).

Partial thickness RC tears are usually treated arthroscopically by either debridement alone or with direct repair using suture bone anchors, depending on the degree of the tear and the

condition of the RC (Itoi and Tabata, 1992, Strauss et al., 2011). GHJ osteoarthritis with associated RC arthropathy is treated by shoulder arthroplasty, either hemiarthroplasty, total shoulder arthroplasty or reverse polarity total shoulder arthroplasty, depending on the disease severity and the condition of the RC (Drake et al., 2010, Mulieri et al., 2010).

Full thickness RC tears represent a special category of conditions. In this case, the treatment modalities available are either arthroscopic, open or mini-open procedures; these include direct repair, tendon transfer or tendon grafts. Treatment of full thickness RC tears is discussed separately below and in greater details, as it is more relevant to my thesis.

1.5.3 Surgical treatment of full thickness RC tears

Full thickness RC tears represent a challenging problem to orthopaedic surgeons. In the majority of cases they are always debilitating conditions with poor and unsatisfactory outcomes if managed non-surgically (Itoi et al., 1997, Zingg, 2007). Non-surgical management in these situations aims to control pain and improve the function of synergistic muscles. The outcome might be satisfactory for a low demand individual or those who cannot undergo surgical procedures (Zingg et al., 2007, Baydar et al., 2009). Nevertheless, most full thickness RC tear patients end up having surgical intervention.

In the USA, it is estimated that around 75,000 surgical cuff repairs are performed annually (Vitale et al., 2007). If the full thickness tear is not surgically treated it can progress into a massive tear, resulting in greater loss of function. Untreated full thickness tears may also result in the alteration of shoulder biomechanics (Hansen et al., 2008), as well as degenerative changes that affect both tendon and muscle tissues (Kannus and Jozsa, 1991). Unfortunately, surgical treatment is not always successful, with the failure rate of direct

repair reported to be as high as 94% in one study (Galatz et al., 2004). The more accepted failure rate for direct repair among shoulder surgeons is between 20 to 40% (Bishop et al., 2006, Cho et al., 2010, Boileau et al., 2005).

Surgical treatments for full thickness RC tears are arthroscopic debridement, direct repair, partial repair (Burkhart et al., 1994, Duralde and Bair, 2005), margin convergence (Burkhart et al., 1996, Burkhart et al., 2001), direct repair with augmentation, tendon transfer and tendon grafting (Nho et al., 2010, Moser et al., 2007). Arthroscopic debridement is a limited procedure that aims to control pain and is indicated when non-surgical treatment cannot control the pain, and other surgical options are not applicable. Arthroscopic debridement is usually successful in controlling pain, but has a doubtful long-term outcome (Delaney et al., 2012).

1.5.3.1 Direct repair

Direct repair is the option of choice for most surgeons and yields reliable results and a higher success rate compared to other surgical treatments. Direct repair can be done by either arthroscopic, open or a mini-open approach. The open approach is now rarely practiced due to the degree of trauma to the deltoid muscle inflicted during the process, which is a synergistic muscle to the RC group and leads to delayed recovery with an associated unsatisfactory outcome. A recent retrospective study reviewed patients after open RC surgery and reported a re-rupture rate of 94% (Vastamaki et al., 2013); other studies reported similar outcomes regardless of the approach used (Duquin et al., 2010, Ide et al., 2005).

Current practice is either arthroscopic or mini-open repair. Both techniques have their advocates, as well as reported results for having comparably satisfactory outcomes (Kim et

al., 2003, Pearsall et al., 2007). A recent study comparing quality of life after RC repair using both techniques showed no difference in terms of pain and function (Osti et al., 2010).

Advocates of arthroscopic cuff repair argue that it causes less trauma and that post-operative recovery is faster with less pain and satisfactory outcomes (Cho et al., 2012a, Kasten et al., 2011). Mini-open surgery is relatively quicker; the technique is to longitudinally split the deltoid raphe (longitudinal fibrous junction on the lateral aspect between the anterior and the postrolateral parts of the deltoid muscles), which does not damage the deltoid muscle fibre and causes minor trauma (Cho et al., 2012b). It is a relatively faster procedure and the technical skills required are less than for arthroscopic repair (Nho et al., 2007, Yamaguchi, 2001, Yamaguchi et al., 2001, Baysal et al., 2005).

Bone anchors are widely used to secure the RC tendon onto the bony attachment (foot print); the other option involves using transosseous bony tunnels. These tunnels are currently not widely used, as they cause more trauma and their application is time consuming. The continued use of bony tunnels might be driven mainly by their cost (bony anchors are more expensive), as the strength of the bone anchors is adequate for secure repair. Few studies reported lower re-rupture using bony tunnels compared to bone anchors, although this view is not widely adopted (Zhang et al., 2013).

1.5.3.2 Direct repair with augmentation

Recent trends for repair of massive RC tears suggest utilizing an augmentation device to strengthen the repair and decrease the possibility of re-rupture. Several augmentation devices have been tested that are either biological or synthetic. Several studies reported using human dermal allograft for augmentation of large and massive RC tears with high success rate.

Human dermal allografts are acellular human matrix derived from the skin and have been found to increase the ultimate failure load in the repair of cadaveric human RC tears (Barber et al., 2008, Omae et al., 2012). Barber and his colleagues conducted a prospective study on the use of human dermal allograft for augmentation of large RC tear and reported better outcomes with a 15% re-rupture rate compared to 60% in non-augmented repairs (Barber et al., 2012). Several other studies have reported favourable healing and better outcomes using the human dermal allograft GraftJacket (Wright Medical Technology, Arlington, TN), with a lower re-rupture rate compared to non-augmented repairs (Agrawal, 2012, Gupta et al., 2012, Bond et al., 2008, Wong et al., 2010, Burkhead Jr et al., 2007, Modi et al., 2013). Similar successful results were reported using synthetic augmentation material for RC repair.

A non-resorbable reticulated polycarbonate polyurethane patch (Biomerix, Fremont, CA) was used to augment RC tears in 10 patients; with a 12-month follow up, one patient (10%) had experienced re-tearing, while the other patients showed satisfactory healing and improved function (Encalada-Diaz et al., 2011). A polyester (Dacron) ligament was also clinically used with similar success; Nada and colleagues reported a failure rate of two out of 21 patients at 12 months after Dacron augmentation of a repaired massive cuff tear (Nada et al., 2010, Petrie, 2013). Similar synthetic devices have been preclinically tested. For example, Poly-L-Lactide and poly- (ε-caprolactone) scaffolds have shown promise in animal and cadaveric models. Unfortunately, to date, these devices have not been widely used in clinical practice (Derwin et al., 2009, Beason et al., 2012, McCarron et al., 2010, Santoni et al., 2010).

Although some studies have reported the successful use of xenografts in augmentation of RC repair (Badhe et al., 2008), the success with allograft and synthetic augmentation devices have not been matched with xenografts. Trials using porcine small intestine submucosa for

RC repair augmentation did not show similar successes, with no improved outcome in humans (Iannotti et al., 2006, Walton et al., 2007, Sclamberg et al., 2004, Phipatanakul and Petersen, 2009).These successes with augmentation devices are based on direct repair of the RC tear, where improved mechanical strength of the construct was thought to enhance biological healing. In un-repairable RC tears, whether due to large defect, severe retraction or poor tendon quality, there is a need for other treatment modalities. The augmentation devices are not suitable for filling in a defect in the tendon by acting like a tendon graft; therefore, tendon transfers and tendon grafts are needed.

1.5.3.3 Tendon transfer

In extreme cases of RC tear with severe retraction and muscle atrophy, direct repair with or without augmentation is impossible to achieve (Gladstone et al., 2007). Although non-surgical treatment might be used to control pain and improve function, results are usually unsatisfactory (Zingg, 2007). In patients where there is associated GHJ arthritis, reverse polarity shoulder arthroplasty is an option; however, it is not a valid option for patients with no or minimal arthritis. In these situations, tendon transfer is considered a viable option. Deltoids, the pectoralis major, the long head of triceps and latissimus dorsi muscles have been reported to be used in tendon transfer for un-repairable RC tears.

Of these different muscles, the latissimus dorsi was the most widely used for RC tendon transfer. Its use was first described by Gerber in 1988 as a salvage operation for non-repairable RC tears (Gerber et al., 1988). In 2006, Gerber reported the largest series of latissimus tendon transfers with 69 cases; he concluded that transfer resulted in durable and substantial improvement, provided that the subscapularis tendon is intact (Gerber et al., 2006).

Weening reviewed 16 cases of latissimus dorsi transfer (Weening and Willems, 2010) and found substantial clinical improvement in cases with an intact subscapularis tendon. His results were similar to those reported by Gerber and his colleagues. Several other authors reported similar successful results (Lehmann et al., 2010, Nove-Josserand et al., 2009, Zafra et al., 2009, Aoki et al., 1996, Birmingham and Neviaser, 2008, Namdari et al., 2012, Longo et al., 2011, Miniaci and Macleod, 1999, Warner and Parsons, 2001).

Deltoid flap tendon transfer is also used to treat un-repairable RC tears. Several studies have reported successful results using this technique. One study reported follow-up on 57 patients for a mean of six years; they reported 91% of cases with no or mild pain and 14% re-rupture rate (Schneeberger et al., 2012). Similar successful results were reported by Hadjicostas et al. (2008) on deltoid tendon transfer in 61 patients with an average of 46 months follow up. In this study , three patients had aseptic necrosis of the flap, while 80% of patients reported excellent results. Other published reports reflect similar success rates (Spahn et al., 2006, Gedouin et al., 2002, Lu et al., 2008, Boehm et al., 2004, Vandenbussche et al., 2004, Gille et al., 2009).

Three papers discussed pectoralis major transfer for treatment of un-repairable RC tear. The first paper reported a successful outcome with a re-rupture rate of two cases out of 13 at an average of 37 months follow up (Gavriilidis et al., 2010). In this series, researchers transferred the sternal head in the case of rupture of the subscapularis, combined with supraspinatus with or without infraspinatus tears. Jost and colleagues (Jost et al., 2003) reported improved outcome of pectoralis major tendon transfer for subscapularis, but in cases with additional tears, results were poorer. Results reported in the third study contradict the findings reported in the above two studies. In the third study, researchers used the sternal

head of the pectoralis major for subscapularis tendon transfer in three different groups, one with failed shoulder stabilization, another with shoulder replacement and the last with a massive RC tear. The results were unsatisfactory and the researchers advised against using this technique (Elhassan et al., 2008).

One group of surgeons used the long head of triceps for tendon transfer as early as 2002 with successful results. In 10 cases with 12 months follow up, Sundine and Malkani reported improved functional and subjective outcomes (Sundine and Malkani, 2002); they have since produced other reports with similar successful results (Malkani et al., 2004, Keen et al., 2006, Schulz et al., 2008).

1.5.3.4 Tendon grafting

In massive RC tears associated with major retraction and muscle atrophy, surgical options are limited; if direct repair is not achievable, reverse polarity shoulder arthroplasty yields reasonable outcomes if the tear is associated with GHJ arthritis. If there is no or mild arthritis, reverse shoulder arthroplasty is not the best option, as it does not result in acceptable outcomes. The salvage operations in this situation are either a tendon transfer or a tendon graft. Tendon transfers, as discussed earlier, yield reasonable results, provided that rigorous patient selection is performed. However, the re-rupture rates and patient satisfaction are below average for other shoulder conditions. None of the available studies reviewed the functional loss and long term outcome resulting from transferring the tendon, whether this be the pectoralis major, latissimus dorsi or others. Additionally, there are no long term results for tendon transfer and no randomized control studies have been conducted to provide the evidence needed to support such techniques. To justify tendon transfer, certain criteria need to be fulfilled, rendering this technique not suitable for all patients.

Tendon grafting provides additional solutions for massive RC tears; it offers a salvage solution for patients with a massive defect that is not treatable with other techniques. Tendon graft material can be autograft, allograft, xenograft or synthetic.

The long head of bicep (LHB) tendons is the most widely used tendon graft in RC repair. Although it is sometimes described as augmentation graft material for RC repair, it is usually used as a graft material to fill in a RC defect. Furthermore, the techniques described for using the LHB tendon in RC repair clearly indicate that it is not a tendon transfer, as in tendon transfer, the tendon is detached from its insertion and reattached onto a new insertion point with the muscle still in continuity with the tendon. In the case of LHB, the tendon is detached from the muscle bulk and used as a free graft. In most large RC tears, the LHB tendon is damaged and tenodesis or tenotomy is usually indicated. LHB tendon rupture is also frequently seen in large RC tears (Lo and Burkhart, 2004, Ditsios et al., 2012). With such tears, the LHB tendon sometimes dislocates out of the biceptal groove, causing pain and loss of function, which are indications for tenodesis or tenotomy (Boileau et al., 2007). When tenotomy or tenodesis is indicated, the LHB tendon is considered a good option for use as a tendon graft it is readily available, with no need for extra surgical wounds and no concerns regarding graft rejection, immune reaction and cross infection. Unfortunately, there are limitations for using LHB as a tendon graft; the size of the graft is limited and the LHB tendon is usually damaged in shoulders with massive RC tears and might not be suitable for grafting.

The first available series using LHB as a tendon graft was published in 2001 (Guven et al., 2001). In this study, 14 cases were reported with a minimum of 26 months follow up. LHB tenodesis was done using the open approach and the proximal segment was used as tendon

graft. Satisfactory results were achieved in 85.7% of cases, with improved function and pain relief. Sano et al. (2010) reported a similar outcome; they clinically and radiologically assessed 14 patients with an average 28-weeks follow up. Only one case showed minor re-tear and all patients had improved functional outcome. Similar outcomes were reported in a number of published studies (Rhee et al., 2008, Nassos and Chudik, 2009, Cho et al., 2009, Obma, 2013).

Trials using xenografts as tendon graft materials did not reveal successful outcomes. Solar and his colleagues reported the failure of porcine dermal collagen used as a bridging construct in massive RC repair (Soler et al., 2007). Multiple tendon grafting materials are available for applications in RC repair and other tendon repairs and these materials are based on tissue engineering concepts, which will be discussed in detail in the following section.

1.6 Tissue engineering of tendons and ligaments

Regeneration of tendons and ligament has been the focus of many tissue engineering researchers for a number of years. Tendons, as described earlier, have poor regeneration potential and regardless of the treatment given, damaged tendons may not fully recover functional capacity. Current tissue engineering strategies aim to regenerate tendon tissue and biologically augment the healing process to achieve better functional outcomes. Tendon tissue engineering has three components: the scaffold, cellular enhancement and biological enhancement.

1.6.1 Scaffolds

The aim of scaffold in tissue engineering is to provide a stable platform to the host cells for integrating and regenerating tendon tissue. In these cases, the scaffold is expected to provide

mechanical strength to the construct until the regeneration process is complete and the newly regenerated tendon can take up the mechanical load. In addition, scaffolds are used to provide a vehicle for cellular and biological components. Scaffolds can be either biological or synthetic; biological scaffolds are either allografts or xenografts, while synthetic scaffolds can be either absorbable or non-absorbable (Coons and Alan Barber, 2006) and are usually polymeric.

1.6.1.1 Biological scaffolds

Several biological scaffolds are available in the market for clinical use. GraftJacket, as described earlier, is an allograft that has been used in augmentation of RC tears with successful results. GraftJacket and Allopatch HD are allografts made of a human dermal matrix, while Allopatch Fascia Lata is made of human fascia; all these allografts are acellular freeze dried collagen based scaffolds (Table 1.1). Xenograph scaffolds have been manufactured from different origins, including porcine small intestine such as CuffPatch, Restore Orthobiologic Implant and Permacol, porcine dermal matrix like collagen repair patch, equine pericardial collagen matrix such as OrthAdapt and porcine skin matrix such as TissueMend and BioBlanket (Derwin et al., 2006, Coons and Alan Barber, 2006).

These biological scaffolds are all based on an acellular collagen matrix. Some of these materials have been used for RC repair with variable results; currently biological scaffold use in tendon tissue engineering is limited by host tissue reaction and failure to integrate (Zheng et al., 2005).

Product	Material	Manufacturer
Allografts		
GraftJacket	human dermal matrix	Wright Medical Technology, Arlington, TN, USA
Allopatch HD	human dermal matrix	Musculoskeletal Transplant Foundation, USA
Alloderm	human dermal matrix	Life Cell Corporation, Branchburg, NJ, USA
Allopatch Fascia Lata	human fascia	Musculoskeletal Transplant Foundation, USA
Xenografts		
CuffPatch	porcine small intestine	Organogenesis, Canton, MA, Arthrotek, Warsaw, IN, USA
Restore Orthobiologic Implant	porcine small intestine	DePuy Orthopaedics Inc., Warsaw, Indiana, USA
Permacol (Collagen repair patch)	porcine dermal matrix	Tissue Science Laboratories, Covington, GA, Zimmer, Warsaw, IN, USA
TissueMend	porcine skin matrix	TEI Biosciences, Boston, MA, Stryker Howmedica, Kalamazoo, MI, USA
BioBlanket	porcine skin matrix	Kensey Nash Corporation, Exton, PA, USA
OrthAdapt	Equine pericardial collagen matrix	Pegasus Biologics, Irvine, CA, USA

Table 2.1: Commercially available biological scaffolds

Biological scaffolds go through a rigorous manufacturing process. This process includes sterilization of the graft to prevent transmission of infection, decellularisation to decrease the immune reaction and alteration of the construct, such as crosslinking of the collagen, to improve mechanical properties. This extensive processing of the graft material might alter the properties of the scaffold. To date, only allografts (GraftJacket) have proven to be beneficial in RC tear augmentation (Adams et al., 2006, Ide et al., 2009), whereas other materials have not consistently shown the same positive results as those observed when using GraftJacket. Although xenografts have been successfully used in soft connective tissue repair, their mechanical properties suggest that they have a limited role in the augmentation of tendon repair (Derwin et al., 2006, Sclamberg et al., 2004, Iannotti et al., 2006, Walton et al., 2007).

Several studies have recently investigated the use of periosteum for augmentation of tendon-bone healing; primary, results on rabbits are promising, with improved healing at six weeks but some doubtful improvement when compared to bone marrow biological augmentation at 12 weeks (Karaoglu et al., 2009). These results were supported by other researchers working on small animal models, proving that wrapping of periosteum around the tendon results in improved tendon bone healing at early stages (Kyung et al., 2003, Chen et al., 2003a, Youn et al., 2004). Li et al. reviewed the use of periosteum for tendon-bone healing and concluded that it has strong potential for improving the healing of the enthesis (Li et al., 2012). Unfortunately, this application has not yet been widely investigated.

1.6.1.2 Synthetic scaffolds

Several resorbable or non-resorbable synthetic materials have been investigated for tendon grafting and augmentation. Different materials have been used (Table 1.2), such as polyester, Dacron, silicone, polypropylene and nylon (Post, 1985, Ozaki et al., 1986, Kain et al., 1988).

Different designs have been developed, including mesh that produces porous scaffolds (Santoni et al., 2010) and aligned fibre scaffolds (Beason et al., 2012). Some of these synthetic scaffolds resulted in host tissue reactions, while others did not indicate any better outcomes when used in tendon grafting (Wredmark and Engstrom, 1993, Fukubayashi and Ikeda, 2000, Miller et al., 2006, Debnath et al., 2004).

Product	Material	Manufacturer
Biodegradable		
Artelon	porous polyurethaneurea	Artimplant, AB, Weden
Sportsmesh	polyurethaneurea	Biomet Sports Medicine, Warsaw, IN, USA
Biomerix 3D	reticulated polycarbonate polyurethane patch	Biomerix, Fremont, CA
Non biodegradable		
Gore-Tex	Microporous polytetrafluoroethylene	Gore and Associates, Flagstaff , AZ, USA
LARS ligament	Terephthalic polyethylene polyester	LARS, Arc-sur-Tille, Dijon, Burgundy, France
Leeds-Keio	polyethylene terephthalate (polyester)	Xiros plc, Neoligaments, Leeds, UK

Table 1.2: Commercially available synthetic tendon grafts

Few synthetic scaffolds are currently commercially available and beneficial as tendon and ligament scaffolds; these include Gore-Tex (WL Gore and Associates, AZ, USA), which is an elastic microporous material made of polytetrafluoroethylene. In a clinical study using Gore-

Tex for massive RC repair as a graft material, Hirooka concluded that it is suitable material, despite reporting three revision surgeries in 28 patients (Hirooka et al., 2002). Kollender also reported successful tendon augmentation with Gore-Tex in the repair of the knee extensor mechanism (Kollender et al., 2004).

The LARS Ligament was also developed as a non-absorbable ligament prosthesis. It is made of polyethylene polyester and has shown satisfactory outcomes in a randomized control study against an ACL autograft (Nau et al., 2002). Similarly, Leeds-Keio graft, another polyester, has been successfully used in tendon repair and augmentation, with multiple studies supporting its beneficial use (McLoughlin and Smith, 1992, Jones et al., 2007, Tanaka et al., 2006).

Absorbable (biodegradable) synthetic materials have also shown success as tendon grafts. Artelon (Artimplant AB, Sweden) is made of slow degrading polyurethane urea and has proven to be biocompatible and capable of stimulating cell ingrowth in animal studies (Liljensten et al., 2002, Gretzer et al., 2006).

Despite the availability of multiple synthetic scaffolds for tendon grafting, very few studies have been conducted on humans; most of the available information have been based on laboratory and animal models. Very little is known about the biocompatibility of these products and their long term effect on tendons (Derwin et al., 2010a). The ability of the biological scaffolds to stimulate host cell ingrowth appears to be uncontrolled and non-specific (Guidoin et al., 2000). There is also a wide variability in the tissue used for commercially available tendon grafts, which might be related to processing techniques, the sources of the graft material and the graft components (Valentin et al., 2006).

1.6.2 Biological factors

The role of growth factors and other biological molecules is promising for tissue engineering and adequate control of these factors might lead to the regeneration of tissues. These factors modulate the healing process. As already indicated, tendon bone healing in the RC works through three phases of inflammatory response. These responses and progression are mediated mainly by cytokines. It is thought that controlling these factors might prevent scar tissue formation and lead to the formation of neo-enthesis with similar characteristics to normal enthesis (Gulotta and Rodeo, 2009, Edwards et al., 2011). Scientists have found that postnatal healing is different; scar tissue is not produced and instead, tissue regeneration occurs, producing normal tissue (Rodeo, 2007). This phenomenon is usually limited to the first few post-natal days and is thought to be mediated by cytokines (Galatz et al., 2007).

Several studies have investigated the role of growth factors (including cytokines) on the healing of tendon and enthesis. These studies have shown multiple growth factors to be involved, their concentration found to be fluctuating during the healing process of tendon injury. It is likely that signalling cascade of multiple factors are needed and this cascade is thought to be affected by the cellular and extracellular structure of the damaged tissue (Anderson et al., 2001). Factors such as basic fibroblast growth factor (bFGF), bone morphogenetic protein 12 (BMP-12), BMP-13, BMP-14, cartilage oligomeric matrix protein (COMP), connective tissue growth factor (CTGF), platelet-derived growth factor-B (PDGF-B) and transforming growth factor-beta-1 (TGF-beta 1) were found to play a role in tendon healing and healing of the enthesis (Wurgler-Hauri et al., 2007, Rodeo, 2007, Kobayashi et al., 2006, Angeline and Rodeo, 2012).

One of the aspects that are under current investigation for tendon healing and regeneration is platelet rich plasma (PRP). PRP have increased concentrations of the growth factors and cytokines. It is usually generated from an autologous source during surgery and circumvents the risk of disease transmission and immune reactions. Previous animal studies have shown increased vascularity and enhanced tendon healing with the use of PRP (Aspenberg and Virchenko, 2004, Lyras et al., 2009). The use of PRP in the treatment of tendon injuries in humans has yet to be indicated as beneficial. Multiple studies have investigated autologous PRP on human tendons and although some of these studies have shown some improvement in outcomes, it is not clear if this is due to the use of PRP, as these findings were not found to be universal (Jo et al., 2011, Bergeson et al., 2012, Castricini et al., 2011, Randelli et al., 2011).

1.6.3 Cells for tissue engineering

The utilization of cells in tendon tissue engineering is being extensively investigated. Cells are the source of the extracellular matrix that gives the tendons its biomechanical characteristics. For tendon tissue engineering, two types of cells are being investigated: mesenchymal stem cells (MSCs) and fibroblasts (Liu et al., 2008a, Arthur et al., 2009). Whilst tendon cells (tenocytes) and ligament cells are known to be specialized forms of fibroblasts, their response, growth and surface markers vary according to cell source (Scutt et al., 2008, Brune et al., 2007). There is an ongoing debate whether fibroblasts are a good source for tendon and ligament tissue engineering (Ge et al., 2005, Bellincampi et al., 1998). Some authors have found that ACL fibroblasts present the most suitable source of cells for tendon and ligament tissue engineering (Cooper et al., 2006, Brune et al., 2007), while others found MSCs to give superior results (Van Eijk et al., 2004, Liu et al., 2008a).

MSCs have been the centre of attention of tissue engineering researchers for many years, since their discovery in 1976 showed that cells isolated from bone marrow have the potential

to differentiate along different cell lineages, potentially making them ideal for tissue regeneration. While the optimal cell source for tendon tissue engineering is not clearly known, MSCs provide promising results (Ge et al., 2005, Cheng et al., 2010). Traditionally MSCs have been harvested from bone marrow, adipose tissue, umbilical cord and other sources (Yates et al., 2012). Although little is known about the lineage that MSCs from different sources will differentiate into and how to modulate this differentiation, studies suggest that MSCs can differentiate into fibroblasts and produce ligament- and tendon-like structures (Fan et al., 2009, Moreau et al., 2005, Fan et al., 2008).

Multiple research groups are investigating the autocrine and paracrine characteristics of MSCs. These properties are thought to play an important role in the differentiation of MSCs and the regulation of the extracellular matrix. Autocrine effect is an autonomic cell signalling mechanism that influences cell activity, while paracrine effect is the signalling mechanism to nearby cells. Jaiswal and his colleagues found that cultured MSCs can be stimulated into osteoblastic differentiation with evidence of autocrine and paracrine effects, based on changes in culture media (Jaiswal et al., 1997). Chen and others investigated the paracrine effect of bone marrow MSCs on wound healing and found improved healing with increased recruitment of macrophages and epithelial cells as a result of the paracrine effect of MSCs (Chen et al., 2008b). MSCs were found to have a regenerative effect in bone fracture healing; this effect was a result of autocrine and paracrine properties of the MSCs. It was also found that this effect is dependent on intact cell signalling and receptors (Granero-Molto et al., 2011, Gnecchi et al., 2008). Little is understood about the autocrine and paracrine effects of MSCs; it is believed that controlling these effects have an important role in tissue engineering and regeneration.

One of the new advances in tissue engineering is the use of bioreactors. These bioreactors regulate the in vitro chemical and physical environment and stimulate and activate cells into specific cellular lineage and tissue production. Currently, tissue engineers are studying different bioreactors in a bid to stimulate MSCs into fibroblastic lineage and production of the desired tendon and ligament tissue. Comparing bioreactors to other in vitro tissue culture techniques, it is showing promising results in production of mature tendon and ligament extracellular matrices (Oragui et al., 2011, Plunkett and O'Brien, 2011, Hansmann et al., 2013).

The choice of scaffold was also found to be detrimental in cell differentiation; it was found that seeding cells on a three dimensional construct with oriented ultra-structure facilitates cell migration, proliferation and differentiation into desirable cell lineage (Mahapatra and Khan, 2011, Khaled et al., 2011, Vaquette et al., 2010).

In spite of these marked advances in tissue engineering, its application in the regeneration of human tendons and ligaments is lacking. Further work is needed to fill the gap in current knowledge and to provide a suitable tissue engineered construct for treatment of current tendon disorders. It is clear now that none of the three components for tissue engineering – scaffolds, biological factors and cells – will on their own lead to regeneration of normal human tissue. These components overlap and influence the biological reaction of one another; combined strategies for modulating and controlling these three factors are needed to be able to produce tissue engineered structures and regenerate normal human tissue (Kovacevic and Rodeo, 2008, Lui et al., 2011, Chen et al., 2011).

1.7 Demineralised bone

Demineralised bone is an acellular matrix of collagen I and other proteins; it is bone that has been treated, resulting in the removal of its mineral content and death of the cellular content. It was first described by Urist in 1965 to be osteoinductive (Urist, 1965). Reddi and Anderson found that demineralised bone results in bone induction through endochondral ossification, which is the formation of cartilage that later turns into bone by the process of mineralisation of the extracellular matrix (Reddi and Anderson, 1976). It is thought that the osteoinductive ability of the demineralised bone is mediated by its BMPs and the content of other growth factors (Urist et al., 1979, Sampath and Reddi, 1984). Following on, the isolation of active proteins from demineralised bone eventually led to the discovery of BMPs.

Demineralised bone is made of collagen I, other collagen types such as collagen III at much lower ratios and amidst this scaffold a mix of different growth factors and non-collagenous proteins. These components, the collagen I scaffold and the growth factors are significant for tissue engineering researchers.

The collagen scaffold, as explained earlier, is being investigated for tendon grafting and augmentation. Current commercially available materials are not produced from a bone extracellular matrix but are instead derived from dermal and internal organs matrices (such as small intestine, fascia and heart). Collagen of the demineralised bone is mainly collagen I, mostly oriented in longitudinal fashion following the Haversian system and can be processed from autogenic, allogenic and xenogenic sources. Although the collagen scaffold of the demineralised bone presents a promising material for tendon tissue engineering, very few studies have investigated its potential.

Variable growth factors were found in the demineralised bone at variable concentrations. Several studies reported different concentrations of BMPs content in commercially available demineralised bone (Pietrzak et al., 2006, Blum et al., 2004, Bae et al., 2006). Out of these various growth factors, BMP-2, BMP-4 and BMP-7 were extensively investigated for their role in bone induction and promoting healing of enthesis. Rodeo and colleagues investigated the role of BMP-2 in tendon bone healing and concluded that BMP-2 enhances this healing and results in stronger tendon-bone attachment (Rodeo et al., 1999, Chen et al., 2011, Lovric et al., 2012). Similar findings were also concluded by other research groups (Chen et al., 2008a, Kim et al., 2011a, Kim et al., 2007, Klatte-Schulz et al., 2013). Studies have also shown that the cellular response to different BMPs varies according to cell type, donor age and gender (Klatte-Schulz et al., 2013, Rui et al., 2012, Klatte-Schulz et al., 2012). BMP-2 and BMP-7 were also found to affect collagen I expression and production by human tenocytes derived from RC (Pauly et al., 2012). BMP-2 and BMP-4 were found to have a positive effect on osteocalcin and ALP expression by MSCs as an indication of osteoblastic activities (Rickard et al., 1994, Thies et al., 1992, Mayr-Wohlfart et al., 2001). Moreover, increased ALP and collagen production was found to be a direct effect of BMP-2 on osteoblasts (Takuwa et al., 1991). Asahina found that BMP-7 induced chondrogenic differentiation of murine clonal cells (Asahina et al., 1996).

The effect of the growth factors available in demineralised bone on tendon tissue is not yet clear. Evidence from literature suggests that different BMPs found in demineralised bone result in differentiation of the MSCs into osteogenic, chondrogenic and tenogenic lineage (Kovacevic and Rodeo, 2008, Lui et al., 2011). The cascade of events that lead to differentiation into each pathway is not yet clearly understood; it is thought that different BMPs are present in different concentrations and express variable effect on the MSCs

(Wildemann and Klatte, 2011). Demineralised bone has strong potential for being a tendon graft material; it has the ideal scaffold needed and contains variable growth factors that are thought to enhance tendon healing and regeneration.

1.8 Study outline

Based on the information presented in this chapter; massive rotator cuff tears represent a difficult condition to treat; to date, there is no ideal treatment option. During our trial to find a solution for this clinical problem, our research group investigated the use of demineralised bone as a potential biological tendon graft material. I hypothesise that DCB, when exposed to a normal tendon environment and subjected to tendon stresses and strains, will remodel into tendon tissue.

In order to answer the research question, I have designed my study to consist of five chapters.

1.8.1 Chapter 1: Introduction

In this chapter, I explain the purpose of my study and the previous research conducted by our research group that led to the development of my study. I use the rotator cuff tear as a clinical application for my research. I extensively studied the available literature discussing several aspects of the rotator cuff: anatomy, pathophysiology, aetiology of cuff injury, current treatment practice and outcome of available treatment options. I also investigated tendon graft options, its manufacturing, properties and applications. This is followed by a discussion on demineralised bone, its manufacturing, content, properties and applications.

1.8.2 Chapter 2: Tensile properties of Demineralised Cortical Bone

The aim of this study was to investigate the tensile properties of DCB. Tendons are under constant tensile stress; therefore, the process of developing a tendon graft material should take into consideration that it must be able to withstand these tensile stresses. In this chapter, I examine the tensile properties of DCB and the effect of freeze-drying and gamma irradiation on these properties. In order to produce a tendon graft material for clinical application it must go through multiple processing steps to provide a sterile product. Ideally, the graft should be available off the shelf. I chose freeze-drying, as it is a common procedure for prolonging the shelf life of medical and biological products, while gamma irradiation is one of the most common sterilization techniques in the medical field.

1.8.3 Chapter 3: Cadaveric model of patellar tendon repair using DCB as a tendon graft

The aim of this study was to identify a suitable technique for repairing patellar tendon defects using DCB as tendon graft. In order to comply with research ethics and laws regarding scientific research on animals, this cadaveric study was designed. Using the results from Chapter 2, I developed four cadaveric models for repairing a defect in the patellar tendon using DCB. The four models were tested for maximum tensile strength to failure. Based on the outcome of this study, I conducted live animal study (explained in Chapter 4). This process was important and not only supports the subsequent chapters, but also provides evidence on the suitability of DCB for use as a graft material in tendon repair in humans.

1.8.4 Chapter 4: Augmentation and repair of tendons using DCB: animal study

The aim of this study was to investigate the main research hypothesis: can DCB be used as a biological tendon graft and will it remodel into tendon tissue? Based on the outcome of Chapter 2 and Chapter 3, I designed an in vivo study to repair a patellar tendon defect with

the involvement of the enthesis in an ovine model. Six animals underwent surgical resection of distal 1 cm of the right patellar tendon; this defect was repaired using DCB as tendon graft. Animals were mobilized immediately post-operatively and the construct was harvested after 12 weeks. Force plate analysis, X-ray radiographs, pQCT scans and morphological and histological analyses were done.

1.8.5 Chapter 5: Discussion

In the final chapter, I discuss my results, defend the findings and discuss the available literature that supports the outcome of my study. I also touch on possible future research projects and expected expansion of the ongoing research project.

Chapter 2

Tensile Properties of Demineralised Cortical Bone

2.1 Introduction

Demineralised bone has been used in clinical practice since 1889 (Senn, 1889), when it was first used as a bone graft material in osteomyelitis. However, it was not until 1965, when Urist identified its osteoinductive properties (Urist, 1965) that demineralised bone has been widely used in the medical field. The focus of its use is its osteoinductive properties. Several studies have investigated the biological properties of demineralised bone, while others have focused on the quantification of its growth factor content. Demineralised bone is usually delivered in non-structural forms to facilitate its processing and utilization; therefore, few studies have to date investigated the mechanical properties of demineralised bone.

The mechanical properties of demineralised bone are expected to be directly related to its collagen content (Bowman et al., 1999). These properties were also found to be affected by collagen fibre orientation (Novitskaya et al., 2011, (Gigante et al., 2009). The compressive strength of immature demineralised bone was found to be higher than mature demineralised bone; this was not the case in non-treated (mineralised) bone. This difference in compressive properties is thought to be related to increased collagen content in immature bone (Manilay et al., 2013).

Bowman and his colleagues (Bowman et al., 1996) studied the tensile behaviour of DCB of bovine origin using EDTA for demineralisation. They found that demineralised cortical bone behaved in a similar fashion to other collagen tissues that have organized collagen matrix, such as tendons and ligaments. The authors were able to produce a typical stress-strain curve with initial non-linear toeing followed by a linear segment (Figure 2.1). These findings were supported by another study that also reported heterogeneity in the tensile strength of demineralised cortical bone (Catanese et al., 1999). Similar stress-strain curve was found at a

nanostructure level; collagen fibrils were found to exhibit non-linear segments, followed by a linear stress-strain curve (Hamed and Jasiuk, 2012). A similar curve was also produced when studying the nanocomposite structure of the bone (Ji and Gao, 2006). These studies have identified the importance of the non-mineralised phase in influencing the mechanical properties of the bone.

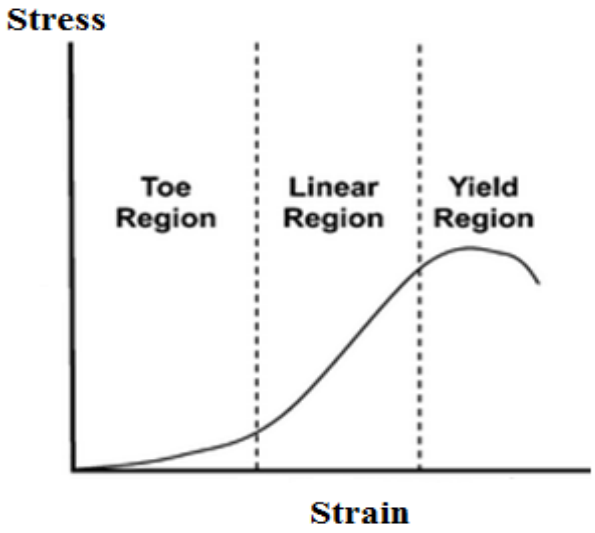


Figure 2.1: Standard collagen tissue stress-strain curve.

The toeing phase of collagen-based structures is a result of uncrimping of the collagen fibres. During this phase, stresses remain low and the collagen fibres stretch to form straight bundles. Following this phase, the collagen fibres begin to elastically deform in response to increased tensile stress. This elastic deformation happens mainly due to sliding of the collagen fibres and the characteristic tri-helix configuration of the collagen molecule. As the stress continues, the collagen fibres fail to absorb any more energy and a short plastic deformation phase begins prior to break and failure of the collagen fibres (Freeman et al., 2007). The mechanical properties of collagen-based structures represented by the stress-strain

curve depend on several factors of the structure, including the stiffness and modulus of elasticity.

In ligament tissue engineering, biological and mechanical properties are usually studied to ensure integration of the tissue-engineered structure into host tissues and to ensure it is mechanically able to withstand the forces acting on the graft after implantation. Tensile forces are the main forces that act on ligaments and tendons in human body. To assess the tensile mechanical characteristics of tissue-engineered graft measurement of the maximum tensile strength, tensile strain and modulus of elasticity is usually performed to prove the mechanical suitability of the graft (Vieira et al., 2009, Cooper et al., 2005).

Processing of demineralised bone for medical application requires sterilization. Sterilization techniques for medical applications often involved irradiation such as gamma irradiation (Puolakkainen et al., 1993), chemical sterilization using for example formaldehyde, glutaraldehyde (Munting et al. 1988), ethanol, hydrogen peroxide (Carpenter et al., 2006), ethylene oxide (Munting et al., 1988, Dahners and Hoyle, 1989, Doherty et al., 1993), aseptic harvesting, heat sterilization, either dry or with steam (Actis et al., 2004), microwave (Dunsmuir and Gallacher, 2003, Singh and Singh, 2012) and gas-plasma (Ferreira et al., 2001). Several studies have been conducted to investigate the effect of these different sterilization techniques on the properties of demineralised bone. The aim of these studies was to identify an adequate sterilization technique without compromising the biological and mechanical properties of demineralised bone. Most of the available methods either have a negative effect on demineralised bone properties or results were found to be controversial, with no clear evidence of superiority of one technique over another.

Gamma irradiation is widely used in the medical field for sterilization of healthcare products; it has deep penetration ability, packages remain sealed and it does not interact with most healthcare products. The International Standards Organisation (ISO) has set standards for using irradiation as a sterilizing technique in healthcare production (ISO11137-2, 2013). The ISO standards recommend doses between 15 KGray and 25 KGray, depending on the biological burden of the products (bioburden), the ability of gamma irradiation to penetrate the products and the resistance of the microorganisms to gamma irradiation. The estimation of the bioburden varies depending on the source of the product and processing technique. In tissue banks, a wide variation in bioburden measurement was found (Nguyen et al., 2008, Baker et al., 2005, Grieb et al., 2005) and 25 KGray was widely adopted by tissue banks as radiation sterilization dose (RSD). In bone banks, however, low dose gamma irradiation was found to be a more appropriate RSD due to relatively small production size (Nguyen et al., 2008, Nguyen et al., 2011).

Multiple studies have investigated the effect of gamma irradiation on demineralised bone. These studies did not show consensus on the effect of gamma irradiation on the biological or mechanical properties of demineralised bone. Ijiri and his colleagues ((Ijiri et al., 1994) found that gamma irradiation with 25 KGray had a negative effect on the osteoinductivity of BMPs. Others have found that low does gamma irradiation (between 15 and 19.4 KGray) has no effect on osteoinductive properties or the TGF-ß content in demineralised bone (Puolakkainen et al., 1993, Pekkarinen et al., 2005). These results contradict the findings of previously published studies that have reported the enhanced osteoinductive potential of demineralised bone irradiated with doses between 30 to 50 KGray. Gamma irradiation with 25 KGray did not compromise the osteoinductive potential of demineralised bone (Wientroub and Reddi, 1988, Hallfeldt et al., 1995). Gamma irradiation was also found to have a negative

effect on the mechanical properties of cortical bone, showing dose-related decreased toughness in samples irradiated with doses higher than 15 KGray (Nguyen et al., 2013). The effect of gamma irradiation on the mechanical properties of tendon allograft was not different from that of cortical bone. De Deyne and Haut found that gamma irradiation with 20 KGray resulted in deceased tensile strength and reduced Young's modulus of human patellar tendon allograft (De Deyne and Haut, 1991). This was contradicted in a later study, which showed no effect of low and moderate doses of gamma irradiation (18.3-28.5 KGray) on the tensile strength and modulus of elasticity on multiple human bone and tendon grafts, including the patellar tendon (Balsly et al., 2008).

It is widely acceptable nowadays that sterilization of biological products like allografts and demineralised bone should be applied at doses lower than 20 KGray, as this will minimise the negative effects of gamma irradiation on the biological and the mechanical properties of the graft (Nguyen et al., 2013, Nguyen et al., 2007).

Some studies have investigated the effect of gamma irradiation on the mechanical properties of demineralised cortical bone. Although it is expected that gamma irradiation will result in denaturing and cross linking of the collagen scaffold of the demineralised bone (Nguyen et al., 2007), it is not clear if this effect is dose dependant or whether it will result in considerable changes in the mechanical properties of DCB. Summitt and Reisinger studied different mechanical aspects of demineralised bone as a potential ligament replacement (Summitt and Reisinger, 2003), namely failure stress, elastic modulus and percentage of failure strain. They also studied the effect of sterilization techniques, including gamma irradiation, on these mechanical aspects and concluded that demineralised bone can be a

suitable material for tendon replacement. Additionally, they reported no effect of gamma irradiation between 25 and 35 KGray on the mechanical properties of demineralised bone. In this study, I also investigated the effect of freeze-drying on the maximum tensile strength of demineralised cortical bone. Freeze drying is a widely used technique in medical and non-medical production for prolonging over the shelf life time of products. Freeze drying can also facilitate handling of products and it minimises the contamination risk of products. In the field of orthopaedic surgery and traumatology, freeze-drying of tissue grafts like tendon grafts and demineralised bone is a common practice (Jackson et al., 1987, Curtis et al., 1985, Glowacki, 2005).

Some studies have investigated the effect of freeze-drying on the osteoinductive properties of demineralised bone. Hosny and others found that freeze-drying did not affect the osteoinductive capability of demineralised bone up to six months post processing (Hosny et al., 1987). Cornu and colleagues studied the effect of both freeze-drying and gamma irradiation on the mechanical properties of cancellous bone and concluded that freeze-drying had a negative effect on mechanical properties, but did not affect work to failure. The addition of gamma irradiation to freeze dried samples had a more obvious negative effect on the mechanical properties of the demineralised cancellous bone and work to failure (Cornu et al., 2000).

In this chapter, I investigate the effect of freeze-drying and gamma irradiation on the tensile strength of demineralised cortical bone. I aim to identify the ideal processing technique for demineralisation, sterilization and storage of DCB to be used as biological tendon graft material. I hypothesise that freeze-drying and gamma irradiation do not compromise the mechanical strength of DCB.

2.2 Materials and methods

2.2.1 Study overview

In this study, I examined the tensile strength of cortical demineralised bone and the effect of gamma irradiation and freeze-drying on this strength. I prepared strips of DCB and allocated it into four groups that were processed differently, either freeze dried or gamma irradiated or a combination of both. The strips were tested for tensile strength and stress-strain curves were also produced.

2.2.2 DCB preparation

2.2.2.1 DCB manufacture

DCB was manufactured according to the Urist technique (Urist, 1965), with some modifications. Tibias of skeletally mature female ewes aged between two and three years were harvested immediately post euthanasia; all soft tissues and periosteum were removed and tibias were cut into longitudinal strips using a band saw (Exact, Hamburg, Germany). Proximal and distal ends of each tibia were excised and the shafts were cut into three longitudinal strips corresponding to the three surfaces of the triangular shape of the tibia. Each strip was of average 3-4 mm in thickness, 17 mm (±2 mm) wide and average length was 18 cm. Strips were allocated into two groups, either for tensile strength testing or for cadaveric models of patellar tendon repair (chapter 3). Strips for tensile testing had both ends dipped in paraffin wax (2-3 cm length) to prevent demineralisation of the end to facilitate their attachment on the testing clamp. Strips were then demineralised in 0.6 N hydrochloric acid (HCL) at room temperature. The solution was changed every eight to 12 hours until complete demineralisation. Each strip was demineralised in 1 L of the HCL solution with an average two to three changes until complete demineralisation. Demineralisation was confirmed with X-ray radiographs (300 seconds, 30 kV, Faxitron Corporation, Illinois, USA)

before washing with phosphate buffered saline (PBS) several times until pH was around (7.4 ±0.1). Each strip was washed in PBS at least four times, with a minimum of 30 minutes for each wash and one wash for 12 hours (Figure 2.2).

Figure 2.2: DCB strip.

DCB strips were then randomly allocated into four groups:

A) Non-freeze-dried, non-gamma irradiated

B) Freeze-dried, non-gamma irradiated

C) Non-freeze-dried, gamma irradiated

D) Freeze-dried, gamma irradiated

2.2.2.2 Freeze drying

Strips were placed in a -20˚ C environment for at least two hours prior to placement in lyophiliser (Edwards Girovac Ltd, Crawley, West Sussex, UK). Samples were kept in the lyophiliser for a total of three days at -70˚ C in a continuous vacuum. After freeze-drying, each strip was heat sealed in double layers of plastic bags prior to gamma irradiation.

2.2.2.3 Gamma irradiation

Specimens allocated in gamma irradiated groups were gamma irradiated at 15 KGray (Isotron Limited, Reading, UK). Specimens allocated in the freeze-drying and gamma irradiation group were freeze dried first then irradiated. Specimens were irradiated on dry ice to minimize the effect of heat produced by irradiation on DCB. Following gamma irradiation, specimens were stored in a -20˚ C environment.

2.2.3 Maximum tensile strength of DCB

DCB strips of each group were rehydrated in PBS for two hours before trimming into a dog bone shape for tensile testing (Figure 2.3). The thickness and width of the narrowest section of each specimen were recorded. The pull out clamp was custom made, designed and manufactured at John Scales Biomedical Engineering Centre. Four 2 mm diameter surgical K-wires were used to secure the specimen to the clamps, two at each end.

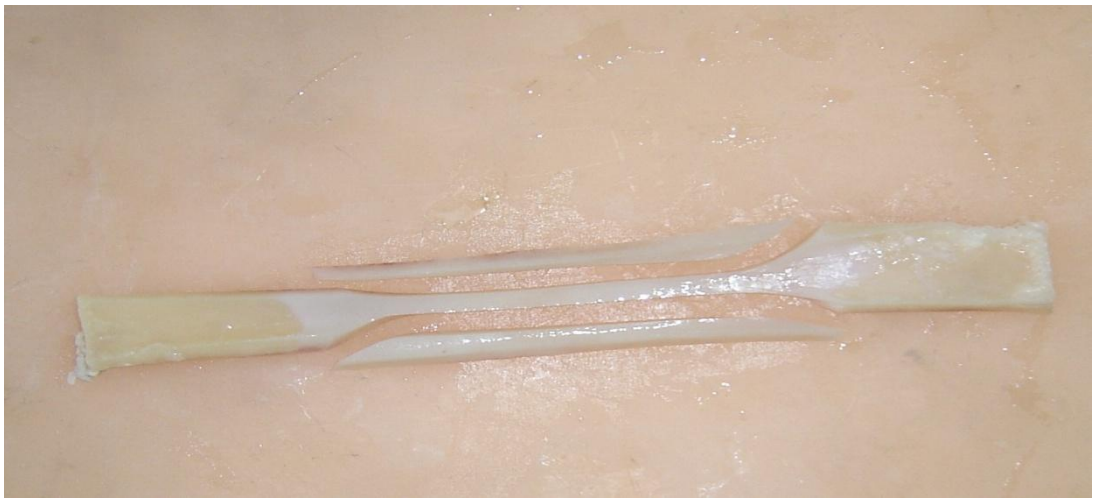


Figure 4.3: DCB strip cut into dog bone shape prior to mechanical testing

The samples were tested until failure at a displacement rate of 200 mm per minute without preconditioning. A stress-strain curve was produced and the ultimate tensile strength (UTS) was recorded for each strip.

2.2.4 Native ACL maximum tensile strength

Specimens were harvested and tested immediately after euthanasia. Dissection from mid-thigh to mid-tibia was done to test for ACL strength; all soft tissue was removed except for the ACL. For the purpose of this study, we assumed that the ACL was in the shape of a cylinder; the radius was measured to calculate the stress. ACL is a strong ligament, exposed to high forces during normal weight bearing activities; measurement of it tensile strength is easily accomplished with available resources and it has been investigated extensively in scientific publications, which makes it a suitable comparison to my results.

2.2.5 Mechanical testing

The specimens were mounted on a testing machine (Zwick/Roell Group, Ulm, Germany) using custom made clamps and secured with surgical grade 2 mm K-wires.
K-wires were introduced through two longitudinally placed holes in the clamps, transecting the specimens in the antroposterior direction (Figure 2.4).

2.2.6 Statistical analysis

All statistical analysis was done using SPSS v.21 software (Statistical Package for Social Sciences, SPSS Inc., Chicago, Illinois, USA). Because of the sample size and the expected variance in each group, normal distribution was not assumed and a non-parametric Mann-Whitney U test was performed in this study.

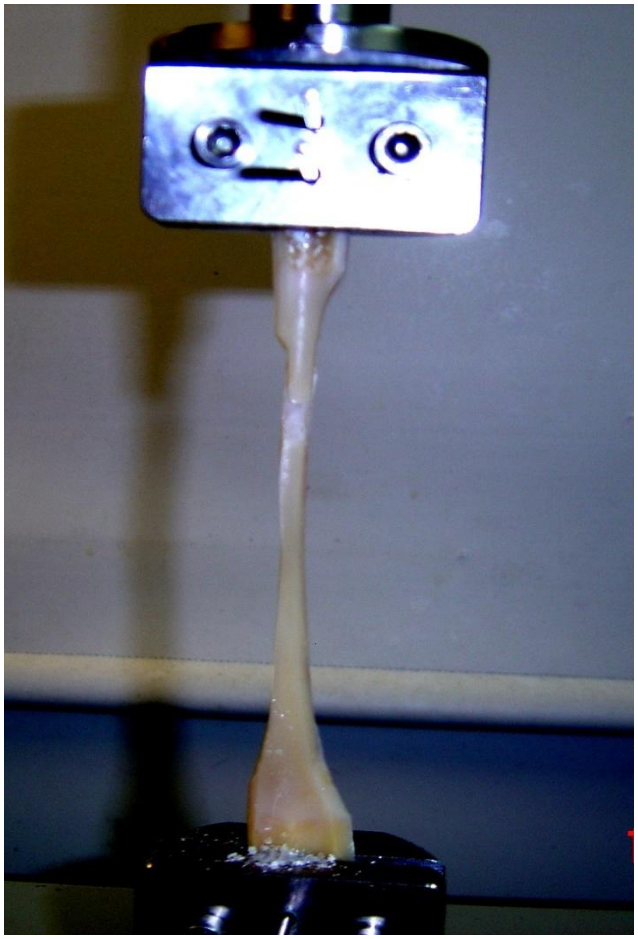


Figure 2.4: Custom made clamp for mechanical testing of the DCB strip.

2.3 Results

2.3.1 Maximum tensile strength of the native ACL

Three specimens were tested for the maximum tensile strength of the native ACL. The median force was 983 N (95% C.I. 728.9-1371.0); the three specimens failed mid substance. Stress-strain curve of the tensile strength of the native ACL was produced showing initial toeing followed by elastic deformation (Figure 2.5).

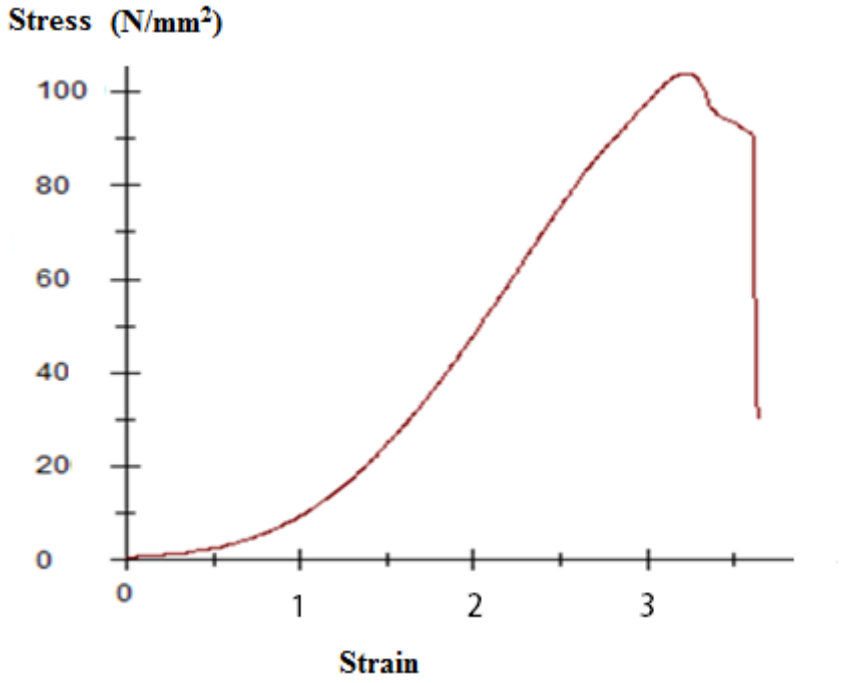


Figure 2.5: Stress-strain curve of native ACL.

2.3.2 Maximum tensile strength of the DCB

Six samples were tested in each group; all samples failed mid-substance. The maximum tensile strength in group A showed the lowest results with a median force of 218 N (95% C.I. 147.9-284.7). Group D showed the highest strength with a median of 676 N (95% C.I. 127.0-1094.9).

The median for group B was 306 N (95% C.I. 154.1-488.6) and for group C was 263 N (95% C.I. 227.8-315.6) (Table 2.1 and Figure 2.6). Stress-strain curve for each sample was also produced and showed similar findings of the native ACL curves; initial toeing segment followed by elastic segment (Figure 2.7).

	Median (N)	**95% CI**
Group A	218	147.9 – 284.7
Group B	306	154.1 – 488.6
Group C	263	227.8 – 315.6
Group D	676	127.0 – 1094.9
Native ACL	983	728.9 – 1371

Table 2.1: The median and C.I. for the maximum tensile strength for DCB groups and native ACL

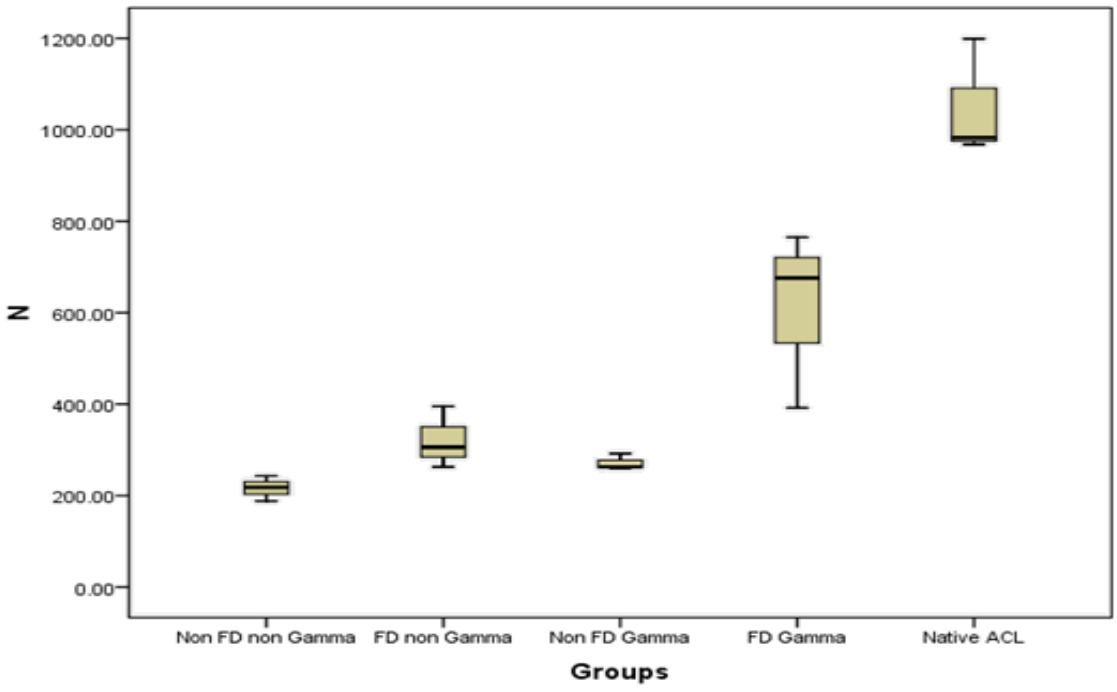


Figure 2.6: Box and whiskers plot showing the maximum tensile strength of different DCB groups and the native ACL (FD = freeze dried)

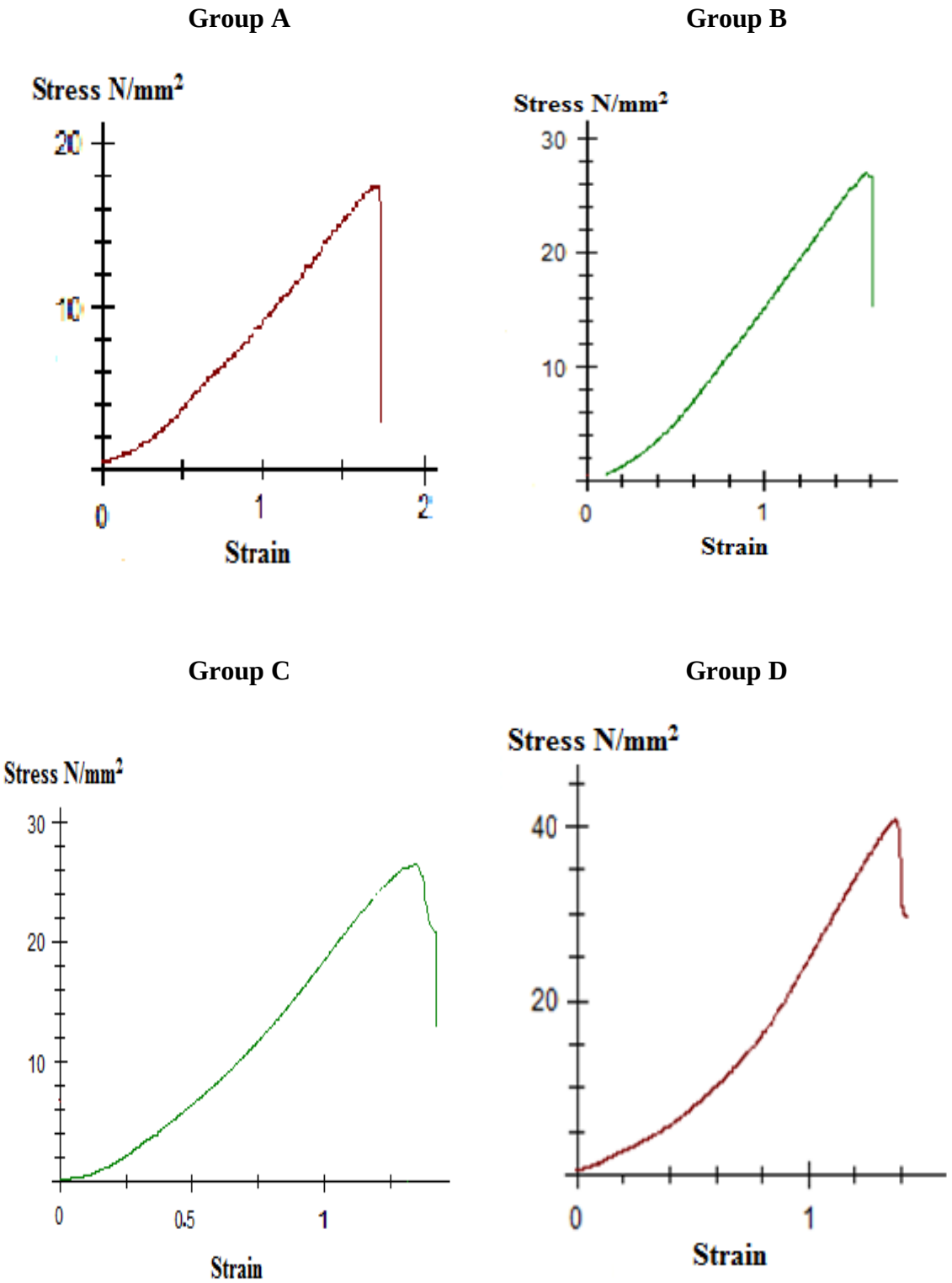


Figure 2.7: Stress-strain curve of representative samples of DCB in each group

There was no statistical difference between groups B and C (p=0.184) or between groups B and D (p=0.127). However, statistical difference was found between other groups (Table 2.2).

	Group A	Group B	Group C	Group D	Native ACL
Group A					
Group B	0.05				
Group C	0.05	0.184			
Group D	0.05	0.127	0.05		
Native ACL	0.05	0.05	0.05	0.05	

Table 2.2: Statistical significance (p-values) between DCB groups

2.3.3. Maximum tensile stress for DCB

The maximum tensile stresses for DCB groups showed corresponding results to the maximum tensile forces. Group A had the lowest stresses with a median of 15 N/mm^2 (95% C.I. 8.8-21.5). Group D showed the highest stresses with a median of 54.6 N/mm^2 (95% C.I. 7.9-90.6) (Table 2.3).

The median maximum tensile stresses for groups B and C were 41 N/mm^2 (95% C.I. 31.4-50.8) and 18.7 N/mm^2 (95% C.I. 12.8-22.4), respectively (Figure 2.8).

	Median (N/mm^2)	95% CI
Group A	15	8.8 – 21.5
Group B	41	31.4 – 50.8
Group C	18.7	12.8 – 22.4
Group D	54.6	7.9 – 90.6
Native ACL	89.4	66.3 – 124.6

Table 2.3: The median and C.I. for the maximum tensile stress for DCB groups and native ACL.

Unlike the maximum tensile strength results, there was no statistical difference between groups A and C (p = 0.127), while statistical difference was found between groups B and C (p= 0.05). Similar to maximum tensile strength results, no statistical difference was found between groups B and D (p = 0.51). Statistical difference was found between other groups (p = 0.05) (Table 2.4).

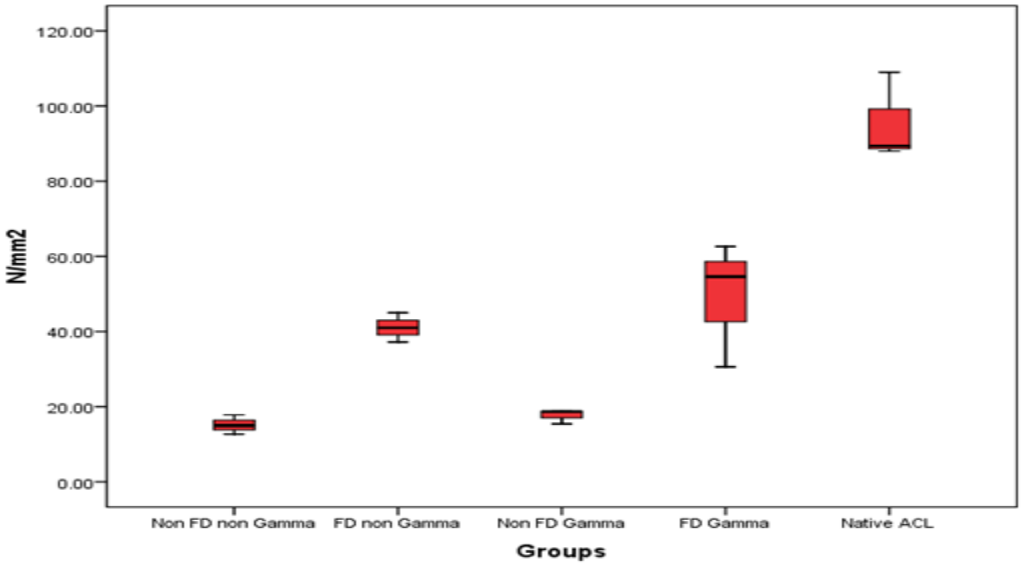


Figure 2.8: Box and whiskers plot showing the maximum tensile stress of different DCB groups and the native ACL (FD = freeze dried).

	Group A	Group B	Group C	Group D	Native ACL
Group A					
Group B	0.05				
Group C	0.127	0.05			
Group D	0.05	0.51	0.05		
Native ACL	0.05	0.05	0.05	0.05	

Table 2.4: Statistical significance (p-values) between groups

2.4 Discussion

2.4.1 Maximum tensile strength of native ACL

Native ovine ACL tensile strength has been reported in the published literature on several occasions. Ovine ACL is frequently used for preclinical animal studies on ACL properties and for trials testing new ligament grafting materials. Measurement of ACL tensile strength was also easily performed with the resources available to me. As such, I found measuring the tensile strength to be a suitable method for comparing my technique and results of tensile strength measurement with the available studies.

Weiler studied the tensile strength of sheep ACL as part of his research on ACL fixation techniques and reported strength of 1513 +/- 180 N (Weiler et al., 2002c). Similar results were reported by Kondo et al. (2012), with maximum load of average 1500 N (this value was estimated from a chart, exact strength was not mentioned in the text) (Kondo et al., 2012). Different groups of researchers have reported lower forces; in a comparative animal study of three ligament replacement prostheses, Dürselen et al. (1996) reported force to failure of the native ACL of 1118 ±244 N. Yoshikawa reported average ultimate failure load of around 800 N (Yoshikawa et al., 2006) and similar values were reported by Hunt with a maximum load to failure of 888 +/_ 139 N (Hunt et al., 2005). Milano reported load to failure of 723 ± 12 (Milano et al., 2005).

Fresh frozen ACLs were also studied with maximum load to failure of 725 +/-77 N (Fabbriciani et al., 2005), while Scheffler reported a much higher load to failure in ovine fresh frozen ACL of 1670 ±375 N (Scheffler et al., 2008). Meller reported ACL maximum load to failure in immature sheep; his results were 759 ±114 N (Meller et al., 2008). Other studies reported results within the range of those mentioned (Viateau et al., 2013, Seitz et al.,

2013, Rogers et al., 1990, Bolton and Bruchman, 1985, Bercovy et al., 1985). This obvious variation in the maximum tensile strength of native ovine ACL might be due to different measurement and processing techniques, as well as differences in the breed and variation of ACL size. My results are within the reported range, which reflects the adequacy of my technique.

2.4.2 Effect of gamma irradiation on the tensile strength of DCB

The obvious contradiction in the reported effects of gamma irradiation and freeze-drying on demineralised bone might be attributed to different techniques in demineralisation, irradiation and freeze-drying. There are no standard techniques for demineralising bone for medical use; instead, each manufacturer has developed their own technique, usually modified from the Urist technique (Urist, 1965). Wildemann studied the concentration of eight different growth factors in three commercially available demineralised bone products and found significant differences in concentrations of these growth factors among samples from each product, as well as between the three products (Wildemann et al., 2007). This work was confirmed in a similar study by Gruskin and co-workers (Gruskin et al., 2012), who comprehensively reviewed 35 different commercially available demineralised bone products and found wide variation in the processing of the products, as well as significant differences in the growth factor content of each product. In her review of osteoinductive testing methods and processing of demineralised bone, Dr Glowacki called for regulating the processing of demineralised bone and validation of osteoinductivity testing techniques (Glowacki, 2005).

In my study, gamma irradiation of DCB strips did not show consistent results. Group C strips (non-freeze-dried + gamma irradiated) did not show statistical significance compared to group B (freeze-dried + non-gamma irradiated). Although Group D strips (freeze-dried +

gamma irradiated) resulted in higher tensile strength, there was no statistical significance compared to Group B. The relative increase in tensile strength in Group D could not be attributed to the effect of gamma irradiation, as it was not consistent with the findings in the other gamma irradiated group (Group C).

2.4.3 Effect of freeze-drying on the tensile strength of DCB

All freeze dried samples were rehydrated in PBS for two hours; samples were of similar texture, characteristics and morphology to the non-freeze-dried samples after rehydration. Cornu (Cornu et al., 2000) rehydrated freeze dried cancellous bone for mechanical testing for 30 minutes in normal saline and stated that this was enough to hydrate the samples to normal levels. Similar to the findings in gamma irradiated groups, freeze-drying did not have a consistent effect on the tensile strength of DCB. Although freeze dried groups (Groups B and D) did have higher tensile strength compared to the non-freeze-dried groups, there was no statistical significance between Groups B and C. The relative increase in the tensile strength of the freeze dried groups might be explained by incomplete rehydration of the DCB strips; however, this is a weak theory, as samples were flexible and evidently fully rehydrated on gross morphology.

Overall, Group D strips (freeze dried + gamma irradiated) showed the highest tensile strength, while Group A strips (non-freeze-dried + non-gamma irradiated) had the lowest tensile strength, the difference between both groups was found to be statistically significant. In the introduction section to this chapter (section 2.1), I previously identified published studies supporting my results; however, I am unable to explain these findings based on my study alone.

2.4.4 Tensile properties of DCB

Bowman examined the tensile properties of the demineralised bovine humerus without gamma irradiation or freeze-drying (Bowman et al., 1996) and reported the demineralised cortical bone to be a flexible, rubber like material. They also reported stress-strain curves similar to my own findings. Bowman suggested that the initial toe region in the stress-strain curve was due to straightening of the coiled collagen fibres, while stretching of the collagen triple helix explains the linear segment of the curve.

The stress-strain curve produced by exposing DCB to constant tension resulted in a curve similar to other collagen- based structures. The toe region in the curves produced by tensile forces acting on the DCB strips is relatively short compared to curves produced by native ACL and the native patellar tendon; this can be explained by the absence of crimp nature of the collagen content in the DCB. As the strips were derived from cortical bone with a Haversian organization, the inherent collagen orientation must affect the tensile properties.

A previously published study of the compressive mechanical properties of demineralised, deproteinised and untreated bone showed that both deproteinised bone and demineralised bone have low Young's modulus of elasticity, and the two phases of the bone (minerals and proteins) have a synergetic effect on the compressive properties of the bone (Chen and McKittrick, 2011). The same group of researchers also published another study showing anisotropy of the different types of bone (demineralised, deproteinised and untreated) in response to compressive forces in different directions; they attributed this anisotropy to the microstructure of the bone (Novitskaya et al., 2011).

The maximum tensile loads were inferior compared to those of the native patellar tendon and native ACL; however, I hypothesise that the use of DCB as biological tendon graft will remodel into ligament tissue. In the next chapter of my study, I develop a cadaveric model of patellar tendon repair with the initial mechanical strength provided by the suture material.

2.5 Conclusion

My results represented in this chapter shows that DCB has tensile mechanical properties similar to those of other collagen-based tissues such as tendons and ligaments. Based on my findings, gamma irradiation and freeze-drying was shown to not compromise the tensile strength of DCB. In the following chapter, I develop the methods utilizing DCB to replace the patellar tendon, with a view to utilizing this in an in vivo model.

Chapter 3

Cadaveric Model of Patellar Tendon Repair Using Demineralised Cortical Bone as a Tendon Graft

3.1 Introduction

Tendon injuries are a common musculoskeletal problem that occurs in all age groups. Traumatic injuries more commonly affect younger patients, while degenerative disorders are more prevalent in older age groups. Tendon rupture and loss of tendon substance can be a complication of predisposing tendon pathology or as a result of direct trauma. Treatment of these conditions is usually either by direct tendon repair or by tendon grafting. Tendons have poor regenerative properties and often spontaneous repair of complete tendon rupture cannot be achieved (Gigante et al., 2009). Due to the resting muscle tone, complete rupture of the tendons results in tendon retraction and gap formation, with spontaneous healing resulting in fibrous tissue filling the gap and leading to elongation of the tendon and suboptimal functional outcome.

Direct tendon repair has its limitations; it is not usually possible to achieve, especially in chronic tendon injuries where a period of immobilization is required to protect the repair until healing; however, this cautious approach often leads to a high rate of suboptimal results and re-rupture (Brodie et al., 2011). Chronic tendon ruptures present an even more challenging situation, with tendons usually retracting and becoming more stiff, making it impossible to approximate the edges or reattach to the bone. Tendon rupture is also associated with disuse muscle atrophy and fatty degeneration (Thomopoulos et al., 2003, Sharma and Maffulli, 2006b).

Over the past 20 years, tissue engineering has been the focus of scientists trying to produce functional tissues for use as a replacement for diseased and malfunctioning organs. Tendon tissue engineering is thought to be a viable treatment option for tendon injuries and multiple

published studies have investigated different aspects of tendon tissue engineering. Tissue engineering of tendons has three components: a scaffold, cellular content and enhancing factors like growth factors and other proteins. Liu (Liu et al., 2008b) identified five requirements for the ideal scaffold for tendon tissue engineering. The scaffold should be 1) biodegradable; 2) biocompatible; 3) mechanically strong; 4) bio-functional, allowing cellular invasion, differentiation and proliferation; 5) processable. The common tissue engineering tendon scaffolds are collagens, polysaccharides and polyesters. Each of these scaffolds has limitations; polyesters have low cellular affinity (Wan et al., 2003) and can result in local tissue reactions as a result of its metabolites (Bostman and Pihlajamaki, 2000a, Bostman and Pihlajamaki, 2000b). Polysaccharides also have variable tissue adhesions and its involvement in cell signalling and immune response is unclear. Collagens have processing limitations, they can produce immune reactions and they do not have suitable mechanical strength (Liu et al., 2008b).

Despite these limitations, tendon tissue engineering is promising, with multiple research centres developing strategies to overcome these limitations through approaches such as surface modification (Chen et al., 2003b), the combination of more than one material (Gentleman et al., 2006) and cellular enhancement. These are some of the strategies that have been used to enhance the properties and the biocompatibility of the available scaffolds (Davies et al., 2013).

Collagen scaffolds are highly biocompatible, resorbable, have high cellular affinity and facilitates vascular invasion and diffusion of the nutrients, leading to high levels of integration within the host tissues (Meimandi-Parizi et al., 2013, Chen et al., 2009). Thus, in spite of their limitations, in terms of strength and rates of remodelling, collagen scaffolds are

considered to be an excellent scaffold for tendon tissue engineering (Meimandi-Parizi et al., 2013, Oryan A, 2012, Calve et al., 2004).

Collagen materials can be either from allogenic or xenogenic in origin. Collagen I is the main component of an extracellular matrix of tendons and ligaments (>95%), making it an ideal engineering scaffold for reproduction and regeneration of tendon tissues (Badylak et al., 1999). In a review of tendon scaffolds available for clinical use in rotator cuff repair in the USA (approved by the FDA), Derwin listed eight biological (one human and seven xenographic in origin) and two synthetic materials (Derwin et al., 2010a). Clinical reports did not show superiority of any of these materials over the others; in fact, reports stated mixed results for some of these materials. Gigante and colleagues demonstrated the effect of collagen fibre orientation on cellular behaviour (Gigante et al., 2009). They proved that collagen fibres could be tissue engineered to match the orientation desired. and demonstrated improved cellular adhesions and proliferation with better mechanical properties with oriented collagen fibre scaffolds.

Multiple products have been examined for their suitability as a tendon scaffold. Most preclinical experimental work has been conducted on small animals. There is an obvious gap of knowledge in tissue engineered tendon studies; there are limited translation studies in which the promising scaffolds used in small animal studies have been applied to large animal models, in which stresses and forces on the scaffolds more closely resemble those encountered in humans. Indeed, the translation of in vivo preclinical studies into human clinical practice is limited (Shearn et al., 2011, Davies et al., 2013).

In an effort to co-ordinate tissue engineering studies into realistic protocols and criteria for tendon and ligament tissue engineering, the Functional Tissue Engineering concept was introduced by a group of researchers (Butler et al., 2010, Bodle et al., 2011, Donaldson et al., 2010). Although the term "Functional Tissue Engineering" is not widely used by other research groups, the concept is widely accepted and aims to translate laboratory work into solutions for clinical challenges through preclinical animal studies and clinical evaluation (Guilak et al., 2001, Butler et al., 2004, Ricchetti et al., 2012, Butler et al., 2008).

Preclinical animal studies represent a critical part of tissue engineering research. In the USA, preclinical animal data is a pre-requisite for approval of a medical device by the FDA. In rotator cuff tears, for example, it was found that rat shoulders have the greatest similarity to human shoulder anatomy (Soslowsky et al., 1996) and multiple studies investigated new techniques of RC repair on rats. However, the data generated from rat studies was found insufficient to reflect the experience seen in human shoulders and there was a need for large animal models to supplement this data (Derwin et al., 2010b). Large animals may have different anatomy to humans; however, they have similar forces acting on tendons and represent more appropriate biomechanical models (Edelstein et al., 2011).

It is now commonly accepted that no single animal model resembles human anatomy closely enough in terms of biology and biomechanics, and although animal studies are required, the data that they generate needs to be strongly supported by phase one and two clinical trials. Multiple studies of scaffolds in different animal models are needed for more accurate predictions of human performance (Derwin et al., 2010b, Edelstein et al., 2011).

The patellar tendon has been used to investigate scaffold incorporation for tendon tissue engineering in several studies in both large and small animal models (Juncosa-Melvin et al., 2007, Juncosa-Melvin et al., 2006, Nirmalanandhan et al., 2009). The patellar tendon of animals have similar mechanical stresses to those of human patellar tendons, with large animal patellar tendons being exposed to forces that react similarly to those on human tendons. In some animal models such as sheep, the patellar tendon is the sole mechanism to transmit the forces, with no other compensatory structures; this is similar to what is encountered in humans, making it the ideal tendon to use for investigating different aspects of tendon pathology and treatment. Assessment of patellar tendon function can be done through monitoring the gait and range of motion, as well as force plate analysis; the patellar tendon is subcutaneous, surgically accessible with average size for surgical interventions.

Tissue engineered tendons is still in the early stages of development and more efforts are needed to direct research centres toward collaborative work and multi-centre studies. There is a need for funding bodies to lead tendon tissue engineering research into translational studies and pre-clinical and clinical studies (Evans, 2011).

In Chapter 2, I examined some of the tensile properties of DCB; my results prove that DCB has sufficient mechanical strength to be considered as a tendon graft. I also examined the effect of gamma irradiation and freeze-drying on DCB. In this chapter, I examined four different models of repair for patellar tendon rupture, using demineralised bone as tendon graft material in cadaveric sheep knees. The techniques developed in this chapter will be used to repair the patellar tendon in an ovine model, using DCB strips as a tendon graft and this will be reported on in Chapter 4.

The use of DCB as a tendon graft is a novel application and no previous studies have investigated an adequate surgical technique that might provide initial mechanical stability for the DCB graft when attached to the host tendon. The aim of this study is to examine different models of repair in order to identify the most suitable models, which I can then use in an in vivo ovine preclinical study.

3.2 Materials and methods

3.2.1 Study overview

In this study, I developed and tested models for patellar tendon repair using DCB as a tendon graft and using bone anchors for the repair. The patellar tendon of mature female ewes was surgically dissected and detached off the tibial tuberosity immediately after euthanasia by sharp surgical dissection using a scalpel. The distal 1 cm of the patellar tendon was excised creating a 1 cm gap between the end of the tendon and the insertion site on the tibial tuberosity. DCB was used as a tendon graft and was stitched to the patellar tendon using suture bone anchors. Four different models of repair were tested for pull-out strength, one model with one bone anchor, another with two bone anchors and the last two using different off-loading techniques. This chapter presents the cadaveric work of patellar tendon repair prior to applying it in vivo on the ovine patellar tendon model that is presented in Chapter 4.

3.2.2 Native patellar tendon and maximum tensile strength

As the effect of the demineralised bone was to be tested in an ovine model where the patellar tendon was resected and replaced, the strength of the ovine patellar tendon was measured and compared with the strength of DCB. Specimens were harvested and tested immediately after euthanasia. The patella-patellar/tendon-tibia complex was dissected and all soft tissue were removed, except for the intact patellar tendon.

3.2.3 Models for cadaveric repair of patellar tendon

2.2.3.1 Using two suture bone anchors

The patella, patellar tendon and tibia of skeletally mature ewes aged between two and three years were immediately harvested after euthanasia. Careful dissection of the specimens with

an intact patellar tendon was carried out. The distal end of the patellar tendon was surgically dissected off the tibial tuberosity and the distal 1 cm of the tendon was excised.

The tibial tuberosity was osteotomised using an oscillating saw and two drill holes were made using size 2.5 mm drill pits. Holes were placed vertically on the tibial tuberosity at the upper and lower edges of the shaved surface 1 to 2 cm apart. Two corkscrew bone anchors (Arthrex Inc., Naples, Florida, USA) were inserted into the drilled holes; each anchor had four stitches of # 2 FiberWire (Arthrex Inc., Naples, Florida, USA).

Freeze dried and gamma irradiated DCB strips were rehydrated for at least two hours in PBS. Rehydrated DCB strips were cut to length measured from the proximal margin of the tendon with the patella to the distal junction of the patella tendon with tibial tuberosity. The width of the strips was adjusted to the width of the patellar tendon. Threads from the bone anchors were used to stitch the DCB strip to the patellar tendon.

The study was designed to mimic loss of a part of the patellar tendon. The DCB strip bridged the gap caused by excising the distal 1 cm of the patellar tendon. The original patellar tendon was not in contact with the tibia. The DCB strip was stitched to the patellar tendon using four FiberWire threads, two on each side, one Krackow stitch and one continuous stitch on each side. The first loop of each thread involved the tendon and the DCB substances; no loops were made in the DCB alone (Figure 3.1). Using the reconstruction techniques outlined below, specimens were mechanically tested for tensile strength to failure and mode of failure.

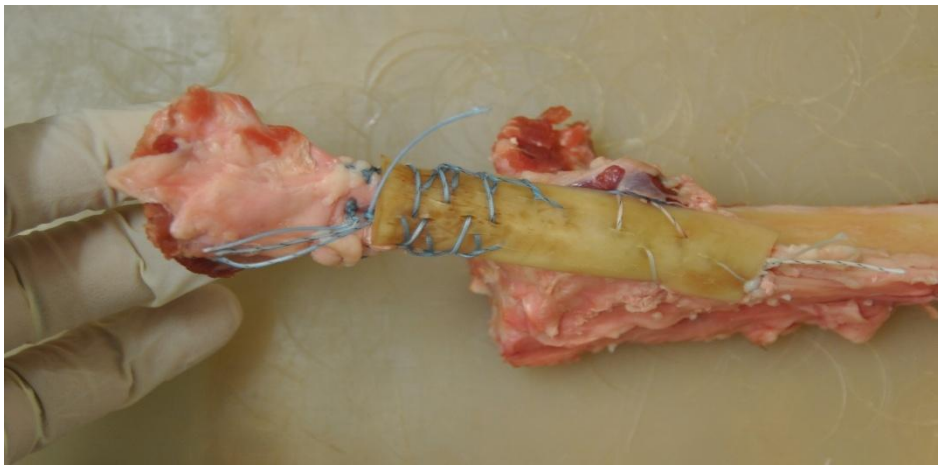
3.2.3.2 Using one suture bone anchor

In this study, we used a similar technique to the two bone anchors study (section 3.2.3.1) but with only one bone anchor inserted at the proximal end of the tibial tuberosity. Four threads from the anchor were used to stitch the DCB strip to the patellar tendon in the same manner as the two anchor study (section 3.2.3.1). A separate 2/0 Vicryl (Ethicon, Cincinnati, Ohio, USA) thread was used to stitch the distal part of the DCB strip to the tissue around the tibial tuberosity.

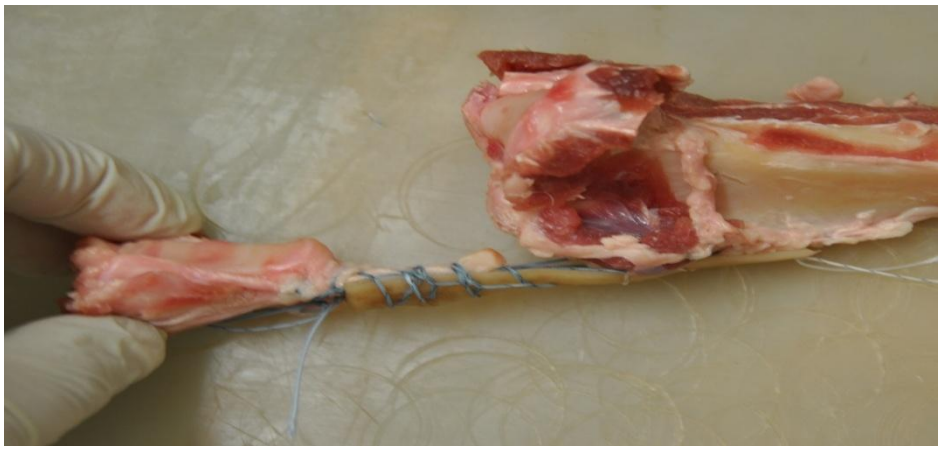
3.2.3.3 Off-loading the patellar tendon augmentation by FiberWire loop

A similar construct used in the two bone anchors study (section 3.2.3.2) was used, with off-loading achieved by augmentation with a loop of FiberWire. Two horizontal medial to lateral bony tunnels were drilled, one in the proximal part of the patella and the other 1 inch distal and posterior to the tibial tuberosity. Continuous FiberWire thread looped twice was used to off-load the augmentation. Specimens were mechanically tested for force to failure using failure of the FiberWire loop as end point (Figure 3.2).

A)



B)



C)

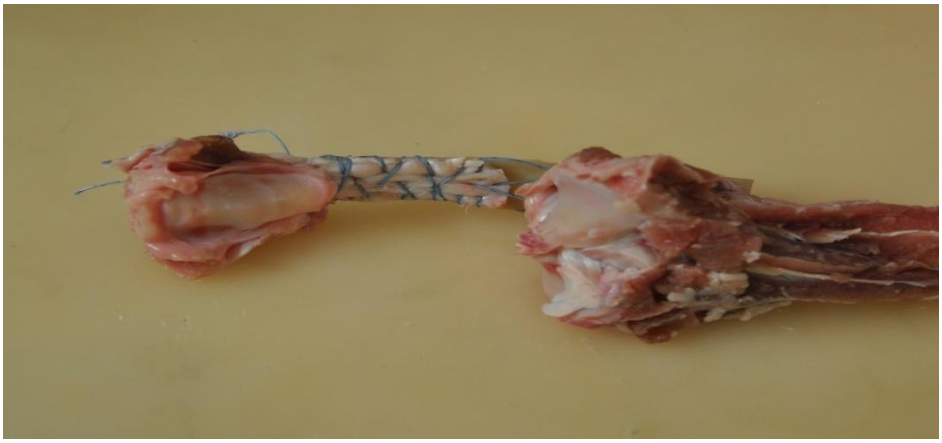
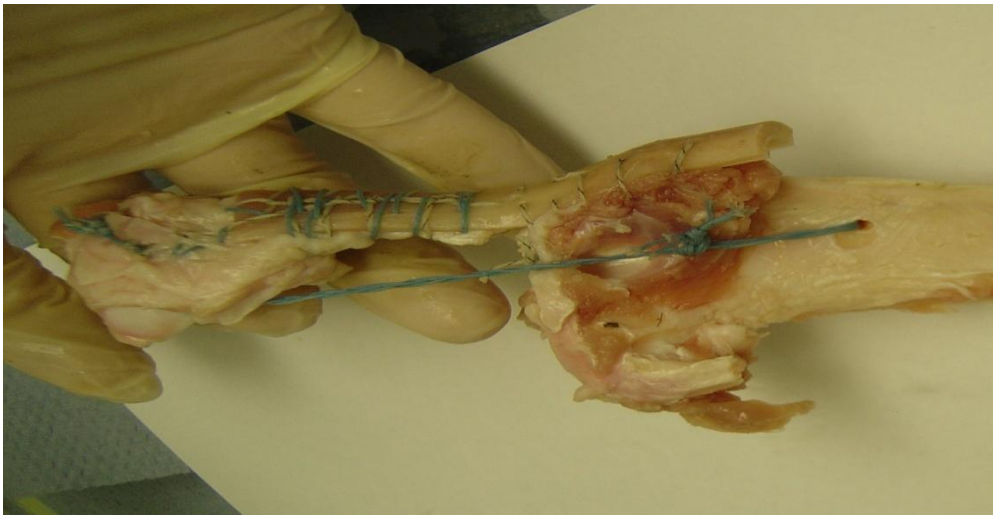


Figure 3.1: Cadaveric model of patellar tendon repair using two suture bone anchors:

(A) anterior view, (B) lateral view, (C) posterior view.

A)



B)

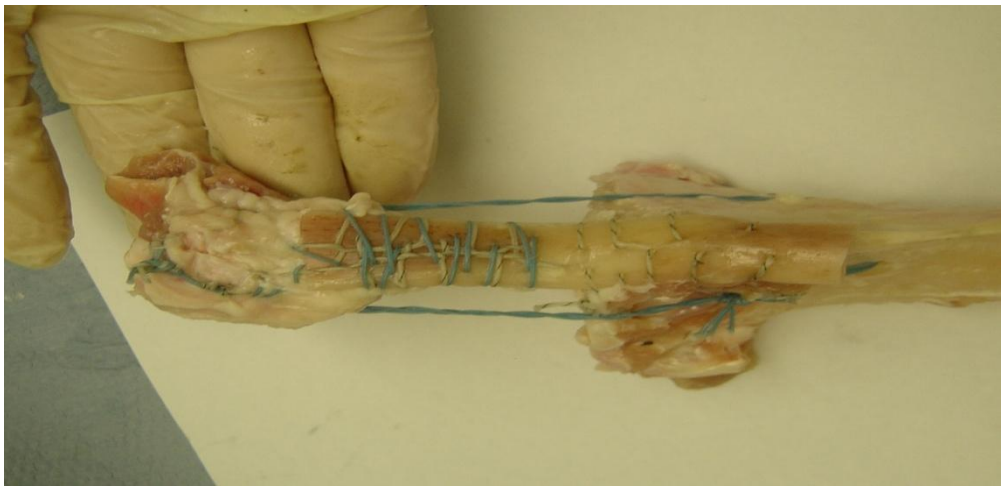


Figure 3.2: Off-loading patellar tendon repair using FiberWire loops:

(A) lateral view, (B) anterior view.

3.2.3.4 Off-loading the patellar tendon augmentation by three threads of FiberWire loop

This study is similar to the previous study (section 3.2.3.3), with three FiberWire threads instead of continuous FiberWire thread looped twice. The three threads were hand braided together to ensure equal distribution of the load (Figure 3.3).

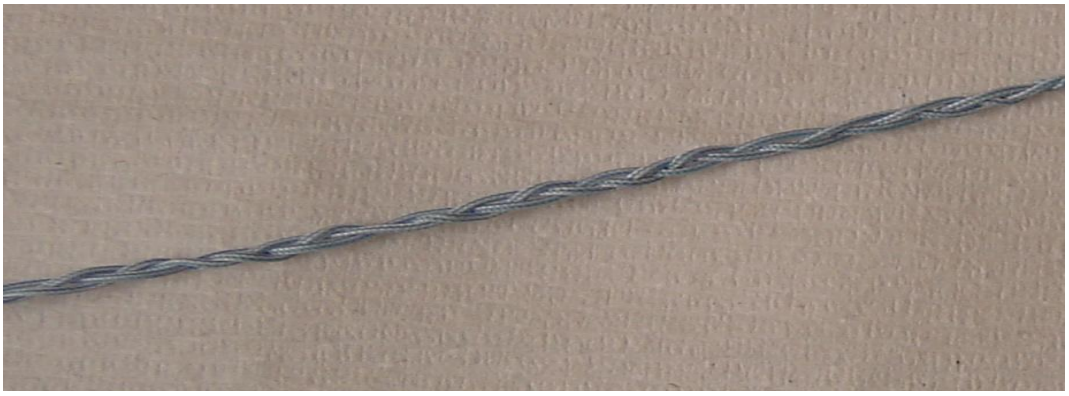


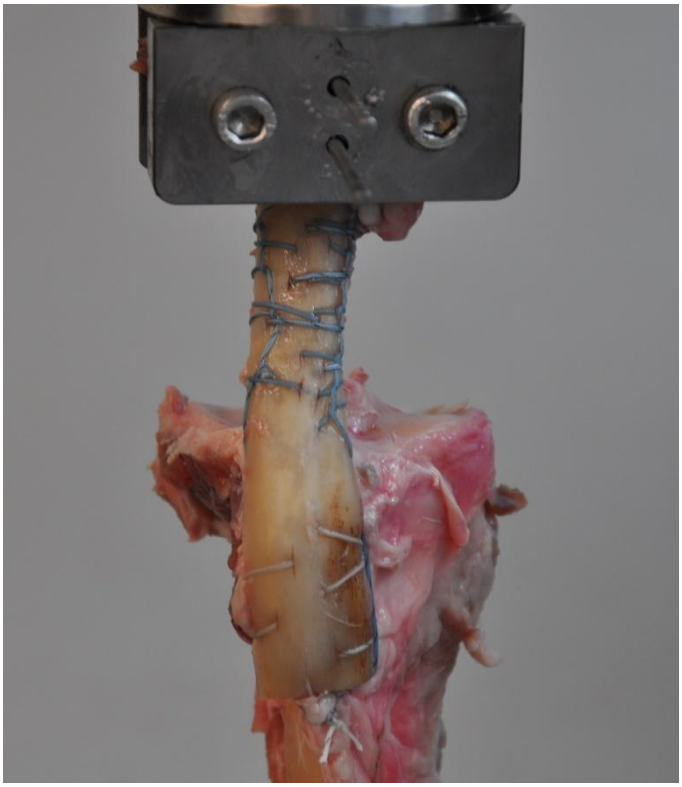
Figure 3.3: Three hand braided FiberWire threads.

3.2.4 Mechanical testing

The specimens were mounted on a testing machine (Zwick/Roell Group, Ulm, Germany) using custom made clamps and secured with surgical grade 2 mm K-wires. K-wires were introduced through two longitudinally placed holes in the clamps, transecting the specimens in the antro-posterior direction. The patellar at the proximal end of the construct was inserted and secured in the clamp by the two K-wires, while distally the shaft of the tibia was clamped and secured by two K-wires.

The patella and proximal end of the tibia were clamped in vertical orientation; continuous vertical distraction was used. The force needed to produce failure was recorded for each specimen (Figure 3.4) and the mode of failure was examined.

A)



B)

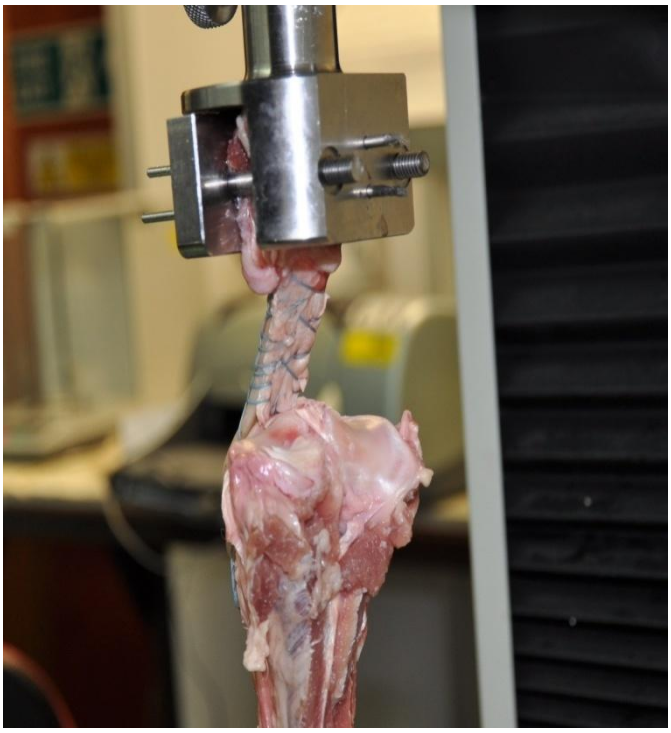


Figure 3.4: Mechanical testing of the cadaveric patellar tendon repair models

(A) anterior view, (B) posterior view

3.2.5 Statistical analysis

All statistical analyses were done using SPSS v.21 software (Statistical Package for Social Sciences, SPSS Inc., Chicago, Illinois, USA). A non-parametric Mann-Whitney U test was performed in this study, as it was thought to be more suitable for the sample size and the non-normal distribution of the samples.

3.3 Results

3.3.1 Maximum tensile strength of the native patellar tendon

Three specimens were tested for the maximum tensile strength of the patellar tendon. The median force was 2743 N (95% C.I. 188.15-4450.52). Two specimens failed mid-substance, while the last failed at the distal K-wire insertion in the patella. A stress-strain curve was also produced with similar characteristics to the native ACL curve, as discussed in Chapter 2 (Figure 3.5).

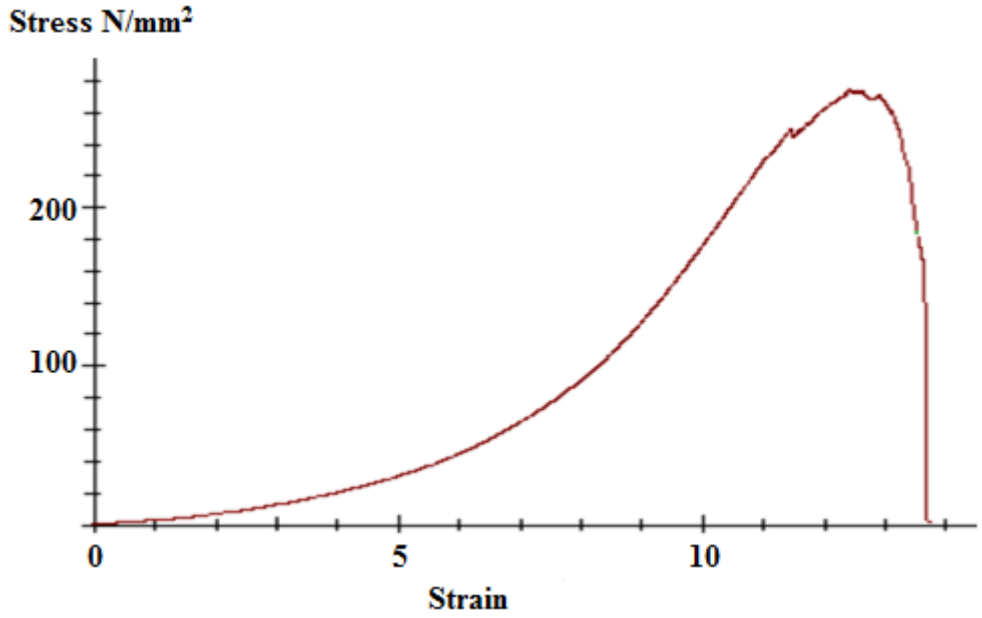


Figure 3.5: Stress-strain curve of the native patellar tendon.

3.3.2 Maximum tensile strength of the repair models

3.3.2.1 One bone anchor model

In the cadaveric repair of the patellar tendon model using one suture bone anchor, the median force to failure was 250 N (95% C.I. 235.39-287.41). Two specimens failed due to sutures pulling out of the anchors, while the other three failed by the tendon pulling out (cheese-wired) of the stitches.

3.3.2.2 Two bone anchor model

In the model using two suture bone anchors, the median force to failure was 290 N (95% C.I. 197.11-396.09). All specimens failed due to the tendon pulling out of the stitches (cheese-wired), except for one specimen that failed by the suture cutting off the anchor.

3.3.2.3 Off -loading models

The two loops continuous thread off-loading model had a median force to failure of 767 N (95% C.I. 730.28-812.06), while the three threads off-loading loop had a median failure force of 934 N (866.62-974.72). Loop failure was considered the end point in all specimens (Table 3.1 and Figure 3.6).

	Median (N)	**95 % C.I.**
Native tendon	2743	188.15 – 4450.52
1 anchor	250	235.39 – 287.41
2 anchors	290	197.11 – 396.09
2-thread loop	767	730.28 – 812.06
3-thread loop	934	866.62 – 974.72

Table 3.1: Results of the patellar tendon cadaveric repair models.

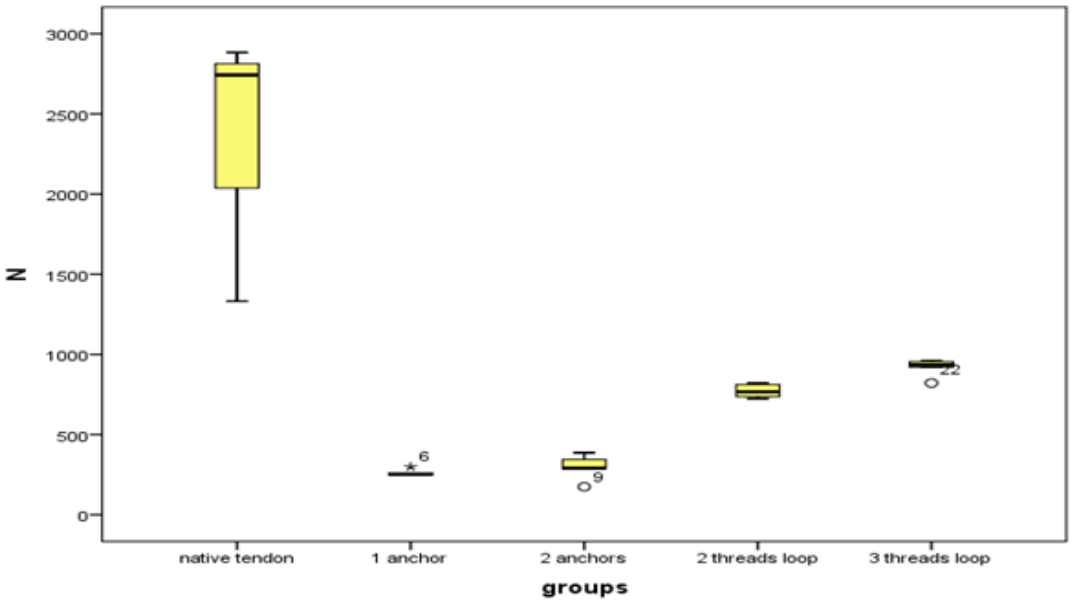


Figure 3.6: Box and whiskers plot showing the maximum force to failure in different models of cadaveric patellar tendon repair and native patellar tendon.

There was no statistical difference between the one suture bone anchor model and the two suture bone anchors model. Statistical significance was found between all other models (Table 3.2).

p value	Native tendon	1 anchor	2 anchors	2-thread loop	3-thread loop
Native tendon					
1 anchor	0.024				
2 anchors	0.025	0.249			
2-thread loop	0.020	0.006	0.006		
3-thread loop	0.020	0.006	0.006	0.005	

Table 3.2: Statistical significance (p-values) between different models.

The stress-strain curves of the repair models did not reproduce similar curves to the native patellar tendon with minimal or no toeing segment (Figure 3.7).

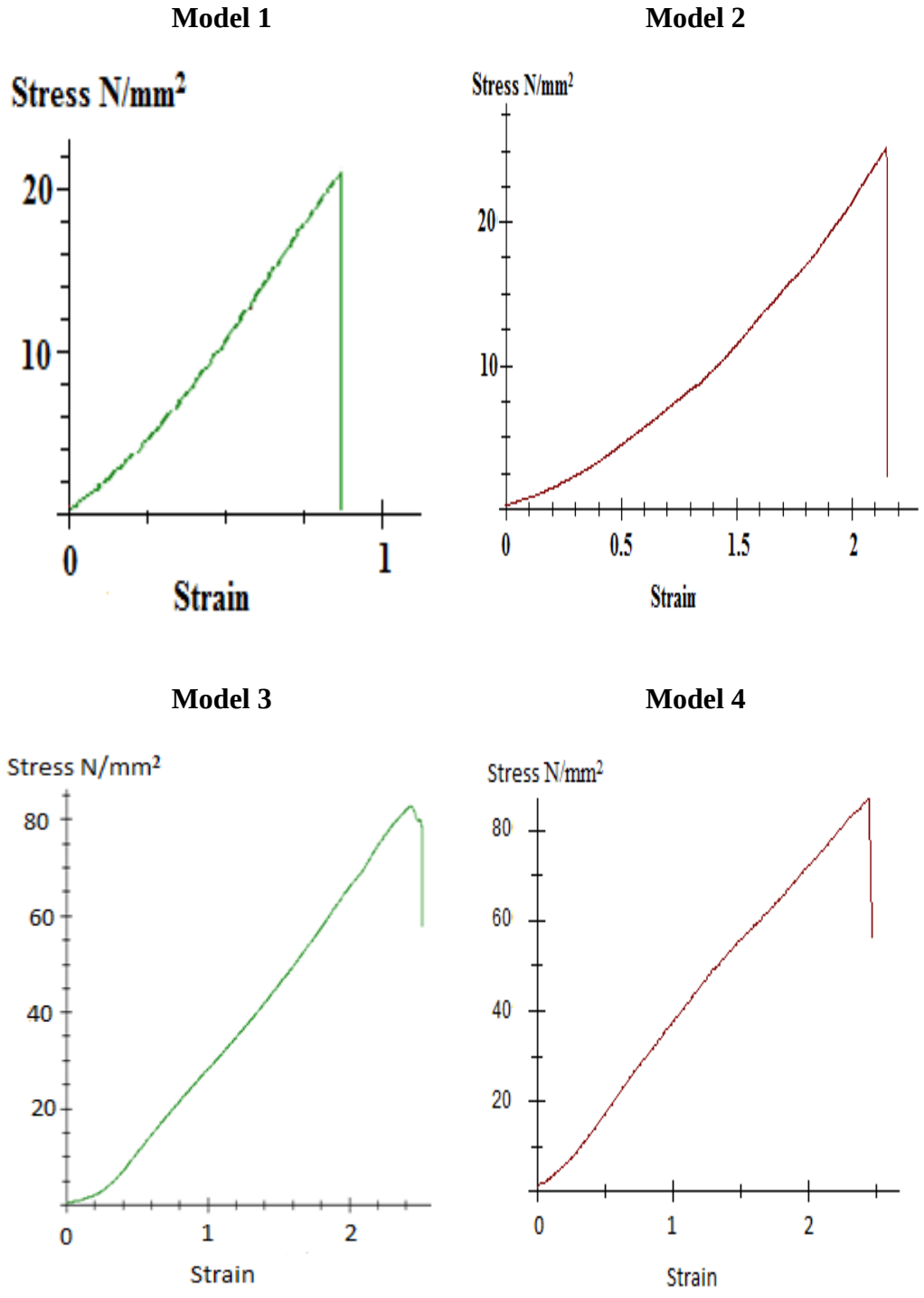


Figure 3.7: Stress-strain curve of a representative sample of each model of patellar tendon repair.

3.4 Discussion

In clinical practice, it is common to use off-loading metal wire for protecting the patellar tendon repair. This wire requires removal after healing of the tendon to allow for a full range of movements and prevents complications as a result of the wire breaking (Marder and Timmerman, 1999). Previously, tendon repair used transosseous tunnels to secure the suturing threads to the bone; however, following the invention of bone anchors, transosseous tunnels have been less favourably used (Capiola and Re, 2007, Gaines et al., 2009).

Two models of repair using off-loading mechanisms were examined to study the mechanical strength of the off-loading mechanism and to compare it to other models with no off-loading mechanisms. In the current study design, using metal wire to off-load the construct was found to be unsuitable, because if the metal wire broke, its sharp edges would cause trauma to the tissues and might damage the DCB graft. Additionally, it would cause an increased amount of pain in a live animal. The decision was made to us FiberWire material as offloading wire; it is strong and had already been used in the study as part of the bone suture anchor. Two off-loading techniques were used; first, using one FiberWire thread looped twice through the bony tunnel and the second was implemented by using three hand braided threads to form the off-loading loop. Both techniques were tested to find out which was more mechanically strong to support the construct.

FiberWire is a strong durable thread made of non-absorbable material; in clinical practice, if it is used as an offloading mechanism, its failure will not result in local tissue trauma as in the case of metal wire. Furthermore, it does not need to be removed; however, if the offloading mechanism is no longer needed, the FiberWire can be cut with a sharp scalpel through a

small percutaneous incision, X-ray guidance could be used to localize the bony tunnels on lateral view for more accurate percutaneous incision.

In the two bone anchors model, no off-loading mechanism was used; instead, the repair was done by bone anchors only, which simulated the repair of tendon rupture in clinical practice. This technique was described for patellar tendon repair (Gaines et al., 2009, Capiola and Re, 2007, Bushnell et al., 2008), quadriceps tendon repair (Kim et al., 2011b, Bushnell et al., 2007) and rotator cuff tendon rupture (Lee et al., 2013, Denard and Burkhart, 2013). It is now widely accepted clinical practice to use bone anchors only, with no off-loading mechanism in the repair of tendon ruptures. There was no statistical significance between the mechanical strength of one bone anchor and two bone anchor models. Therefore, in the animal study detailed in Chapter 4, I used two bone anchors, as this provides more stability and security of the repair in case of pull-out or failure of one of the bone anchors. The use of off-loading wire contradicts the purpose of my study; as I hypothesise remodelling of the DCB into ligament tissue, DCB should be in a tendon-like environment and should be exposed to normal stresses and forces going through the native patellar tendon. The use of off-loading wire eliminates these conditions and interferes with the mechanical forces acting on the DCB.

The stress-strain curves produced by the two models with no off-loading mechanisms did not follow the usual findings of the collagen-based structure curves. The main reason for this was that the construct was supported by the bone anchors and the suture material did not react in a similar way to collagen structures with the initial toe region; instead, a linear relationship between load and displacement was more evident. While the stress-strain curves for the other two models with off-loading mechanisms showed an initial non-linear toe region, this

probably reflected tensioning of the offloading threads and straightening the slackness of the offloading threads prior to building up the tension in the construct.

Sheep animal models have been extensively used in experimental biomechanical and biological studies due to their similarity in terms of body weight to the human body and the similarity of the forces acting on the knee and its surrounding structures (Weiler et al., 2002b, Weiler et al., 2002a, Hunt et al., 2005, Bosch and Kasperczyk, 1992, Bergmann et al., 1999). The patellar tendon is of special importance in this model, as it is routinely used by surgeons as an autograft for ACL reconstruction in humans (Allen et al., 1998, Bosch and Kasperczyk, 1992). Previous studies on sheep knees found that forces acting on the joint are of similar magnitude to those acting on human knees. Furthermore, ligament and tendon structures are of high similarity, rendering the sheep model of great importance as a preclinical model in the development of tendon and ligament substitutes. Several studies have investigated the forces acting on the hind limbs of sheep. Hutzschenr calculated the forces acting on the patellar tendon in the stance phase to 831 N (Hutzschenreuter et al., 1993). In his cadaveric work leading to a published study (Sundar et al., 2009c), Sundar achieved 114 N as the maximum tensile force for cadaveric repair (unpublished data, UCL PhD thesis, Sundar, 2007). In my study, the two bone anchor model had a median of 290 N, which is far greater compared to Sundar's results. Therefore, my repair model can be considered suitable for application on an animal model.

3.5 Conclusion

This study, along with the study presented in Chapter 2 of this thesis, provides evidence that gamma irradiated freeze dried DCB can be used as a tendon graft, given the initial mechanical strength, by using two bone anchors. The construct has sufficient mechanical strength to be used in a live animal model.

Chapter 4

Augmentation and Repair of Tendons Using Demineralised Cortical Bone in an Ovine Patellar Tendon Defect Model

4.1 Introduction

Tendons are connective tissue that serves to transmit the mechanical load from the muscle to the bone. They connect to the muscle via the myotendinous junction and to the bone via the enthesis.

Tendon injuries affect millions of people every year (Almekinders and Temple, 1998) and are common amongst a range of age groups. The pattern of injury and the mechanism varies according to age. Overuse and traumatic tendon injuries are more common in athletes and young adults, whereas degenerative injuries are more common in the elderly. Tempelhof found that 51% of people above 80 years of age had full thickness rotator cuff tears (Tempelhof et al., 1999). Achilles tendon injury, which is the second most common form of tendon rupture, is estimated to affect one in every 10 persons below the age of 45 years (Gaida et al., 2010) and is a traumatic failure associated with tendon degeneration.

Treatment of tendon injury depends on many factors, including the mechanism of injury, type of injury, patient fitness and the severity of the tendon damage. The most widely used treatment options are anti-inflammatory medications, physiotherapy and surgical treatment. In Achilles tendon rupture, a period of immobilization using a cast, followed by gradual dorsiflexion is common treatment. In complete tendon rupture, acute surgical repair is usually the option of choice for restoring function, avoiding muscle atrophy and preventing degenerative changes in the surrounding structures (Rockwood et al., 1991).

Complete tendon rupture is considered to be one of the most difficult types of tendon injuries and is a very challenging condition to treat. It can result from direct trauma, indirect trauma (typically in athletes with acceleration/deceleration injury) or as a progression of degenerative or inflammatory changes in the tendon (Lin et al., 2004, Sharma and Maffulli,

2005a). The mechanism of tendon injury plays an important role in the treatment decision; trauma might be associated with loss of tendon substance, which necessitates the use of a tendon graft to restore length. Inflammatory and degenerative changes in the tendon might indicate poor tendon quality and predisposal to the failure of surgical repair. The duration of the tendon rupture prior to the repair affects the surgical treatment decision; chronic tendon rupture results in tendon stiffness, tendon retraction, muscle atrophy and loss of function of the related joint. In chronic tendon rupture, direct repair might not be possible; in such a case, a tendon graft is usually needed to restore length and ensure adequate repair (Wapner et al., 1993).

Many tissue engineers are investigating different types of synthetic and biological tendon grafts (Bagnaninchi et al., 2007). Biological grafts are usually favoured due to their higher ability for integrating, which can involve resorption, remodelling and replacement of the graft by the host tissues. However, biological graft tissues are limited by several factors, including donor site morbidity in autografts, immunological reaction, cost effectiveness and the risk of transferring pathogens in xenografts and allografts. Advances in synthetic tendon grafts involve enrichment with cellular seeding and cytokines (Wang et al., 2012). The aim is to provide a scaffold that ideally has adequate initial mechanical strength, does not trigger an immune reaction and allows host cells to remodel its structure. Some studies have suggested enriching the tendon graft with MSCs and growth factors, resulting in enhanced tenogenesis and better integration of the graft (Bagnaninchi et al., 2007, Hoffmann and Gross, 2007).

Tendon ruptures usually occur at the tendon bone interface rather than mid-substance, affecting the tendon enthesis. Repair of the tendon usually results in indirect enthesis with a scar-like tissue. Indirect enthesis may result in suboptimal outcome and loss of function.

Research in tissue engineering is trying to produce biologically active grafts that provide adequate scaffold strength with no immunological reaction and remodelling potential. These grafts are expected to allow host tissue invasion and act as a carrier for growth factors that modulate the production of direct enthesis.

The techniques used in surgical repair have been established for a long period of time. Direct end to end repair is usually weak and has a high incidence of re-rupture. The most common techniques used to reattach tendon to bone are either bone anchors or transosseous tunnels (threading the sutures through bony tunnels) to secure the suturing threads to the bony attachment (Capiola and Re, 2007, Ettinger et al., 2013). Both techniques are usually coupled with a whipstitch suturing technique to secure the tendon onto the bone and have proven efficiency and satisfactory outcomes (Lighthart et al., 2008, Capiola and Re, 2007).

The initial phase of the biological healing process of tendon injury takes an average of six weeks (Marder and Timmerman, 1999) and complete healing can take up to one year for maturation (Sharma and Maffulli, 2006b, Wang, 2006). The initial mechanical strength of tendon repair is mainly dependant on the surgical technique and the material used for repair. After six weeks, the strength of the native tissues as a result of biological healing is enough to withstand some of the mechanical forces exerted on them (Olsson et al., 2013). A period of post-operative protection of the tendon repair is usually recommended by most surgeons; this period varies between four to six weeks before the start of the rehabilitation programme.

Rehabilitation of tendon injuries depends on the severity of the injury and the treatment given. In cases of surgical repair of complete tendon rupture, rehabilitation usually starts with passive movement two to four weeks post-operatively and prior to active movement exercises

(Olsson et al., 2013). Recent studies have shown that earlier loading and mobilization of the repaired tendon usually results in better outcomes (Lin et al., 2004).

Previously, research by our work group (Sundar et al., 2009c) proved that the use of a demineralised bone matrix for tendon repair as interface between tendon and bone results in better healing and direct enthesis. In Chapter 2, I examined the strength of DCB and examined the effect of gamma irradiation and freeze-drying on the tensile properties of DCB. In Chapter 2, I concluded that gamma irradiation and freeze-drying did not compromise the tensile strength of DCB. In Chapter 3, I developed a model of repair for tendon defect using demineralised cortical bone in a cadaveric ovine patellar model. My preferred method of repair, as explained in Chapter 3, was using two suture bone anchors that had average tensile force to failure of 290 N. In the cadaveric study leading to the previously published research by our work group (Sundar et al., 2009d), the average tensile force to failure was 114 N. The cadaveric model of repair in my study demonstrated higher tensile strength. Therefore, in the current chapter, I describe performing surgical repair of a patellar tendon defect, created surgically and using demineralised cortical bone.

The aim of my study as presented in this chapter was to determine whether demineralised cortical bone can be used in repair and augmentation of a tendon defect. This study tests the hypothesis that demineralised cortical bone can be incorporated in local tissue and be remodelled into the patellar tendon, producing a direct enthesis and providing functional and competent repair.

4.2 Materials and Methods

4.2.1 Study Overview

Six skeletally mature non-pregnant female Friesland ewes, two to three years old, weighing between 78 and 97 kg were selected for this study. All six animals underwent surgical excision of the distal 1 cm of the right hind limb patellar tendon; the defect was repaired using a strip of demineralised cortical bone. The left hind limbs of the same animals were used as controls; animals were freely mobilized in individual pens and specimens were retrieved after 12 weeks. Force plate analysis, X-ray radiographs, pQCT scans and histological analysis were performed. The surgical procedures and husbandry took place at the Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Hertfordshire, AL9 7TA, UK. This study was done in compliance with the Animal Act 1986 for scientific procedures. An institutional animal licence and personal animal licences were obtained prior to conducting this study.

4.2.2 DCB Manufacture

DCB was manufactured using the same technique described in Chapter 2, all DCB strips used in this study were freeze dried then gamma irradiated as described in Chapter 2. DCB strips were rehydrated separately in 1 L of PBS at least two hours before surgery under aseptic conditions.

4.2.3 Force plate analysis

Kistler force plate analysis was conducted to determine the functional weight bearing (FWB) status of each hind limb in each animal. The assessments were done pre-operatively and at weeks three, six, nine and 12. All measurements were performed in the Gait Analysis

Laboratory, Biological Science Unit at the Royal Veterinary College, by walking the animals over a force plate (Kistler Biomechanics Limited, Alton, UK). For each animal in the study, the 12 readings of the vertical components of the ground reaction force (GRFz) for both the left and right hind limbs were recorded. Excessively high or low readings (10 standard deviations outside the average) were rejected. Readings were also discarded if more than one limb was in contact with the force plate or contact was for more than 900 ms. The mean GRF_z was calculated (F_{max}) and normalized against the animals' weight (F_{max}/weight) at the time of the study. The FWB was calculated as the mean GRF_z of the right hind limb (operated limb) over the mean GRF_z of the left hind limb (non-operated limb) expressed as a percentage.

4.2.4 Surgical Procedure

All surgeries were done in a designated operating theatre at the Biological Science Unit, Royal Veterinary College under strict aseptic conditions; medications and anaesthesia were administered by an experienced veterinary technician. Animals were fasted overnight and pre-operative sedation was given using 0.1 mg/kg of Xylazine 2% (Bayer Health Care, Newbury, Berkshire).

Anaesthesia was induced by intravenous combination of 2.5 mg Midazolam (Hypnovel, Roche Products Limited, Welwyn Garden City, UK) and 2 mg/kg Ketamine (Ketaset, Fort Doge Animal Health Ltd, Southampton, UK). Anaesthesia was maintained with 2% Isoflurane (Abbott Laboratories Ltd, Maidenhead, Berkshire, UK) mixed with pure oxygen via endotracheal tube.

Perioperative antibiotic prophylaxis was also given in the form of 2 g of intramuscular Cefalexin (Ceporex®, GSK, Brentford, Middlesex, UK) preoperatively and 1 g intramuscularly every 12 hours post-operatively for three days.

Preparation of the surgical field was done by shaving the area around the right hind limb stifle joint and decontaminated with Povidone-iodine antiseptic surgical scrub (Videne, Ecolab Inc., St. Paul, MN, USA) and Chlorhexidine surgical scrub (Hydrex, Ecolab Inc., St. Paul, MN, USA).

The animal was positioned on the operating table in a supine position with the right hind limb tied in full extension to the end of the operating table. A non-scrubbed assistant was able to un-tie the limb prior to closure to ensure full range of motion after the repair was done. After the second preparation with Povidone-iodine and appropriate draping, a longitudinal midline skin incision was made extending from above the patella distal to the tibial tuberosity.

Dissection was done in layers; the patellar tendon was identified along with the patella and the tibial tuberosity. Minimal handling and dissection of the patellar tendon was done to ensure that the blood supply was maintained to the tendon. Homeostasis was maintained during the procedure and a frequent normal saline (0.9% NaCl) wash was given to maintain the hydration of the tissues.

The proximal end of the patellar tendon insertion into the tibial tuberosity was identified; 1 cm of the patellar tendon proximal to it was surgically incised and the distal insertion into the tibial tuberosity was surgically avulsed by sharp dissection. Bilateral patellar tendon strips

that attach separately into the tibial tuberosity were also dissected. Complete surgical avulsion of the patellar tendon insertion was confirmed by retraction of the patellar tendon away from the tibial tuberosity. The footprint of the insertion of the patellar tendon on the tibial tuberosity was osteotomised and two holes were drilled 1 cm apart on the osteotomised surface (Figure 4.1). The size of the DCB strip was adjusted to match the length and the width of the patellar tendon prior to tendon repair.

Two bone suture anchors (Corkscrew, Arthrex, Naples, Florida, USA) were used to stitch the DCB to the patellar tendon. Anchors were inserted in the drilled holes and sutures from both anchors were used to stitch the DCB to the patellar tendon using the whipstitch technique. The distal end of the DCB strip overlying the tibial tuberosity was also stitched to the surrounding tissue to ensure direct contact with the bony surface. After the repair, flexion and extension of the operated limb was examined to rule out any mechanical blocking of movement by the repair prior to closure. Closure of the surgical wound was done in layers using absorbable Vicryl sutures (Ethicon Inc., Somerville, NJ, USA). Skin was closed with subcuticular undyed Vicryl sutures and OpSite surgical dressing spray (Smith & Nephew, London, UK) was used.

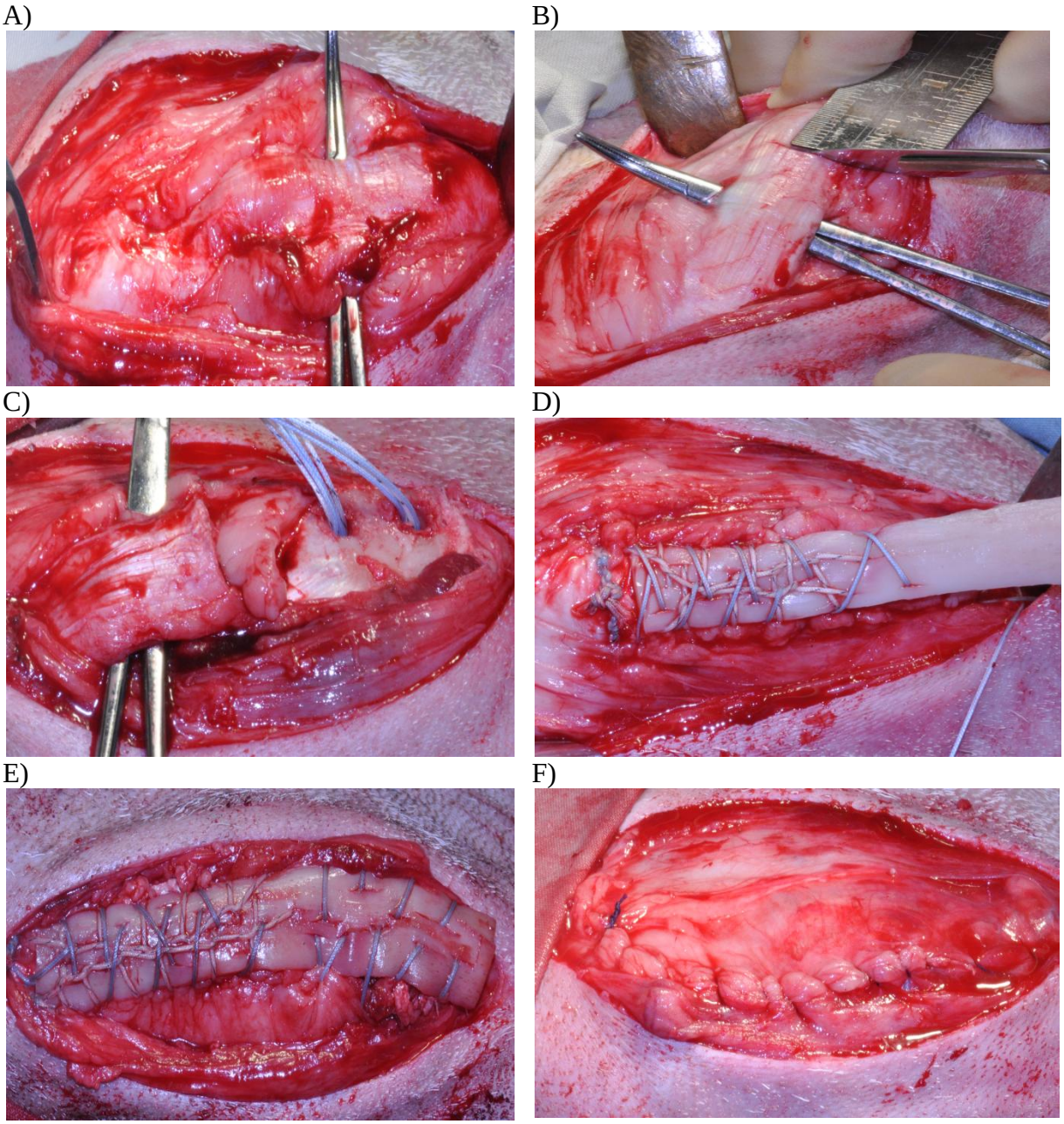


Figure 4.1: Step by step surgical technique: A) identification of patellar tendon, B) distal 1 cm resected, C) tibial tuberosity osteotomised and two suture bone anchors inserted, D) DCB measured to match the size of the patellar tendon ,E) DCB stitched in position, F) closure in layers.

4.2.5 Post-operative care

After recovery, animals were moved to individual pens of average size 2 m X 2 m. There was no weight-bearing protection and animals were allowed free weight-bearing immediately post-operatively. No limitations were applied on range of movement; the operated limb was freely mobile as pain allowed. Post-operative analgesia was maintained by Fentanyl transdermal patches (Duragesic®, Janssen Pharmaceuticals, NJ, USA), the first one 12 hours post-operatively and the second one 60 hours after the first patch.

At weeks three, six, nine and 12, animals were moved to the gait analysis laboratory for the force plate analysis, as previously described.

4.2.6 Euthanasia and retrieval of the specimens

At week 12, after the force plate analysis, animals were given an overdose of 0.7 mg/kg of 20% Pentobarbital (J. M. Loveridge, Southampton, UK). Digital lateral X-ray radiographs of both hind limbs were taken in full flexion and full extension (Raymax, Elstree, Middlesex, UK). Flexion and extension angles of each digital radiograph were measured using ImageJ software (ImagJ 1.48g, National Institutes of Health, USA). Two lines parallel to the longitudinal axes of both femur and tibia in each radiograph were drawn and the angle at the transaction of these two lines was measured and recorded.

Immediate retrieval with dissection of the patella, patellar tendon construct and proximal tibia all in block was done for both hind limbs of each animal; the operated and the non-operated knees. Morphological assessment of the construct and the surrounding tissues was documented during the dissection. Then, a pQCT scan (XCT2000, Norland Stratec, Norland, Wisconsin, USA) was done to look for peripheral ossification prior to fixation in a 10% formaldehyde solution.

4.2.7 Sample processing and sections production

Samples were fixed in a 10% formaldehyde solution for 10 days. Dehydration was carried out in ascending concentrations of 50%, 75%, 85%, 95% and 100% of industrial methylated spirit (IMS). Samples were dehydrated in each concentration for three days with daily change of the solution. Samples were then treated with 100% chloroform for five days with change of the solution twice daily. Following this, samples were further processed into 100% IMS for another three days with a twice daily change of solutions prior to infiltrating in methyl methacrylate resin (LR White Hard Grade Resin, London Resin Company Limited, Reading, UK). Samples were infiltrated in a solution of 50:50 100% alcohol and resin for three days with daily change, then in 100% resin for nine days under a continuous vacuum, with change of solution every three days. Embedding was achieved by polymerizing the resin using a chemical accelerator.

Blocks of hard resin containing the retrieved specimens were cut in half transversely through the middle of the patellar tendon, this construct was created for ease of handling and sections were prepared from different regions of the construct (Figure 4.2).

Zone 1: DCB-tendon interface, examining the area of the construct containing both the patellar tendon with overlying DCB.
Zone 2: DCB alone, examining the area where DCB strip bridges the gap between the end of the patellar tendon and the tibial tuberosity.
Zone 3: DCB neo-enthesis, examining the area of the new tendon enthesis over the tibial tuberosity.

Sections were mounted on acrylic slides using the Technovit 7100 plastic embedding system (Heraeus Kulzer, GmbH, Wehrheim, Germany) and a precision diamond-encrusted band saw (Exakt, Apparatebau GmbH, Norderstedt, Germany) was used to cut a section of 300 μm. Sections were then grinded using the Exakt Micro-grinding system, going through progressive gritting papers until a final thickness of 60-120 μm.

Finally, sections were polished using an alumina suspension (Struers Ltd, Rotherham, UK) and stained with Toluidine blue and Paragon for 20 minutes each.

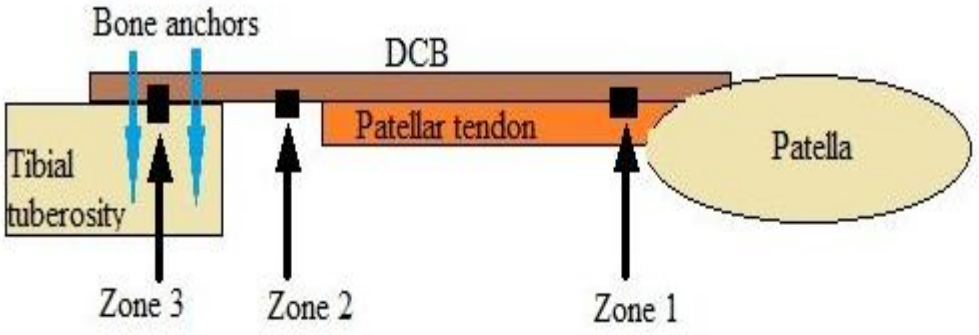


Figure 4.2: Graphic representation of the repaired tendon with the three zones examined.

4.2.8 Histological examination and analysis

A qualitative histological analysis using a light microscope (Zeiss, Hamburg, Germany) with associated Axiovision image processing software was carried out. All sections were examined for evidence of ossification, inflammatory cells, cellularisation, vascularisation and collagen fibre crimp. Zone 1 sections were also examined for interactions between DCB and the patellar tendon, while Zone 3 sections were additionally examined for collagen fibre orientation and formation of the neo-enthesis.

A semi-quantitative analysis was conducted for the neo-enthesis according to the criteria highlighted in Table 4.1. Multiple sections of each neo-enthesis were examined for semi-quantitative analysis by three different researchers and an average of the score for each section was taken as the final score. Results of the semi-quantitative analysis were compared with the contralateral control knees.

score	criteria
1	No fibrocartilage No mineralised fibrocartilage
2	Fibrocartilage present No mineralised fibrocartilage
3	Fibrocartilage present Mineralised fibrocartilage present Disorganized arrangement
4	Fibrocartilage present Mineralised fibrocartilage present Organized graduation between distinct regions but no tidemark
5	Fibrocartilage present Mineralised fibrocartilage present Organized graduation between distinct regions with tidemark

Table 4.1: Criteria for the semi-quantitative analysis of the neo-enthesis.

The number of tenocytes and chondrocytes from different regions of the specimens were counted using an objective of X 40. Six distinct areas were used for cell counting (see Figure 4.3).

Area 1: Neo-enthesis, DCB above tibial tuberosity

Area 2: New tendon bone interface

Area 3: DCB cellularisation

Area 4: Patellar tendon, below the tendon-DCB interface

Area 5: Tendon-DCB interface

Area 6: DCB above the patellar tendon.

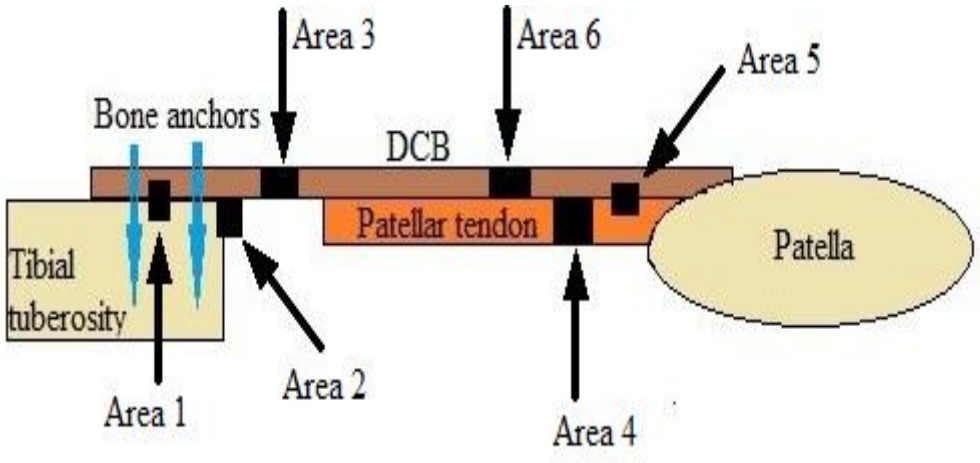


Figure 4.3: Graphic representation of different areas examined for cell counting.

A grid pattern was used to assist cell counting in defined areas and the average number of cells on an area basis was calculated. Cell counting at the neo-enthesis was compared to the cell counting of the normal enthesis of the control knees (semi-quantitative analysis of the neo-enthesis and cell counting were performed by Mr Charlie Holden, undergraduate medical student at UCL, as part of a BSc thesis, London, UK, under my supervision).

4.2.9 Statistical analysis

Statistical software package SPSS v.21 (SPSS Inc., Chicago, Illinois, USA) was used in this study; non-parametric analysis was conducted, as data did not match the requirements for parametric tests. A p-value of <0.05 was considered appropriate for statistical significance.

4.3 Results

4.3.1 Failure rate

All six animals survived the duration of the study and none had post-operative infection. Five animals showed satisfactory progression according to force plate analysis, while one animal failed to show similar progression. A senior veterinary officer's advice was sought and the decision was made to keep the animal included in the study for the period of 12 weeks. Results of this animal were excluded from the force plate analysis and included in all other aspects of the study. On retrieval of specimens, the animal that failed to show satisfactory progression did not have any evidence of infection or inflammatory reaction. All suture anchors were well-positioned in the bone and suture material was intact. The demineralised cortical bone was well integrated into the patellar tendon with evidence of neo-enthesis. X-ray radiographs showed evidence of patella alta (Figure 4.4). Failure of progression was explained by elongation of the patellar tendon, secondary to cheese-wiring of the suture material through the DCB and tendon substance; this might have been a result of poor surgical technique.

A) B)

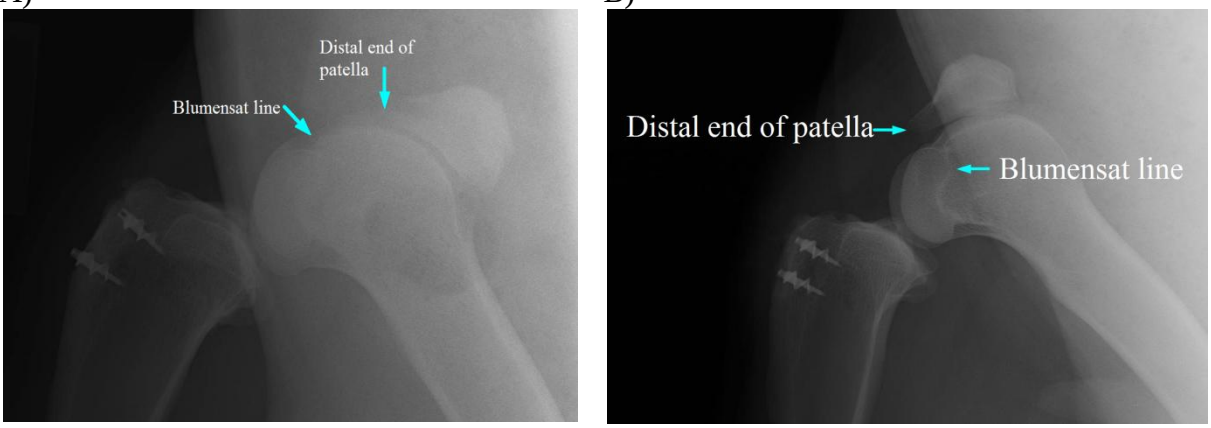


Figure 4.4: A) lateral radiograph of the operated right knee of the failed animal showing patella alta; B) lateral radiograph of a non-failed animal showing normally positioned patella, both at full flexion.

4.3.2 Gait observation

All the remaining animals had normal pre-operative gait with no obvious limp seen. Obvious antalgic gait was observed post-operatively in the operated hind limb. Except for one animal that was diagnosed with patella alta, progressive improvement in the gait was observed over the study period. At six weeks, the antalgic gait was very mild and unrecognisable except by trained observers; at 12 weeks, all animals except for the one with patella alta had normal gait and no limping was observed.

4.3.3 Force plate analysis

Force plate analysis examined pre-operatively, in week three, week six, week nine and week 12 just before euthanasia showed five animals exhibiting satisfactory progression over time (Figure 4.5).

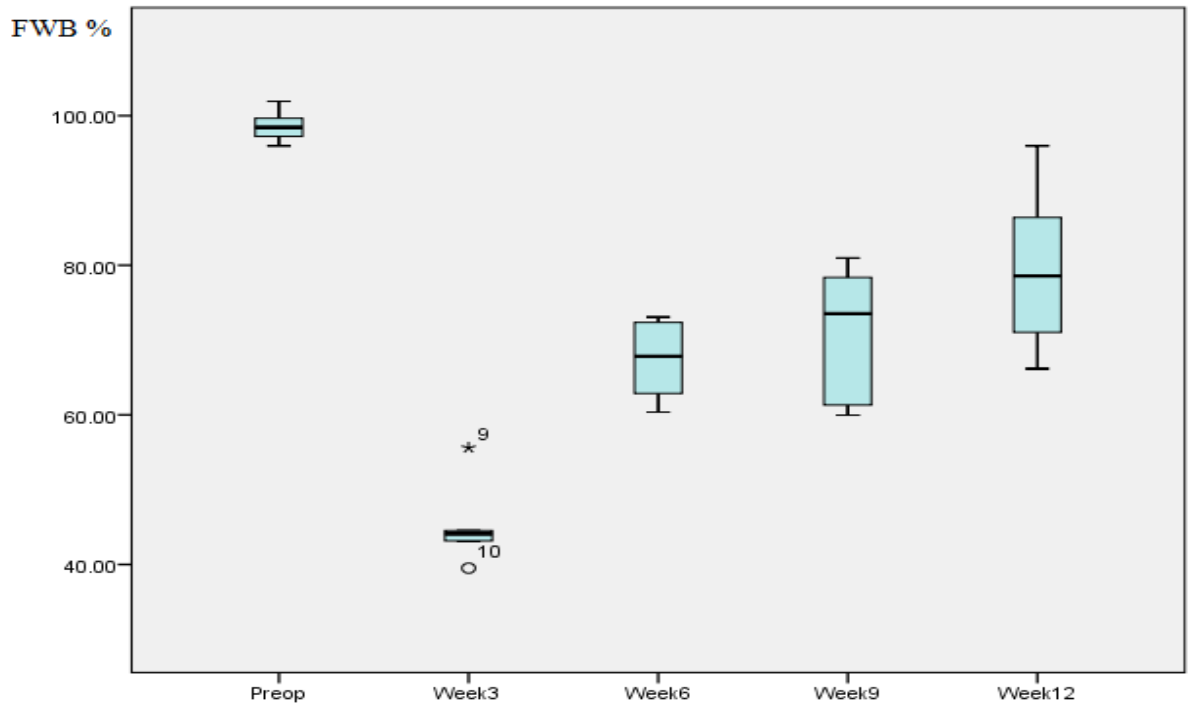


Figure 4.5: Box and whiskers plot showing progression of the FWB over time.

The GRF_z of the right hind limb (operated limb) showed a significant drop at week three, followed by steady recovery between week three and week 12. The opposite was observed for the left non-operated hind limb, that is, a mild increase in the GRF_z at week three with steady decline over the subsequent assessment points (Table 4.2, Figures 4.6 and 4.7).

	Median (N)	**95% CI**
Rt Preop	372.94	312.64 – 426.22
Lt Preop	374.22	312.14 – 438.04
Rt Week 3	201.87	153.7 – 266.38
Lt Week 3	457.84	379.86 – 542.16
Rt Week 6	264.84	214.71 – 344.8
Lt Week 6	438.64	346.3 – 481.79
Rt Week 9	293.53	249.31 – 347.88
Lt Week 9	446.96	335.37 – 518.62
Rt Week 12	324.42	261.65 – 410.62
Lt Week 12	431.36	322.04 – 533.27

Table 4.2: Medians and 95% confidence intervals of right and left hind limbs GRF_z at different time intervals.

Pre-operatively, the median FWB was 98.6% with 95% confidence interval 95.8%-101.5% (Table 4.3). The Mann-Whitney U test showed no statistical significance between the right and left hind limbs at pre-operative assessment (p=0.754).

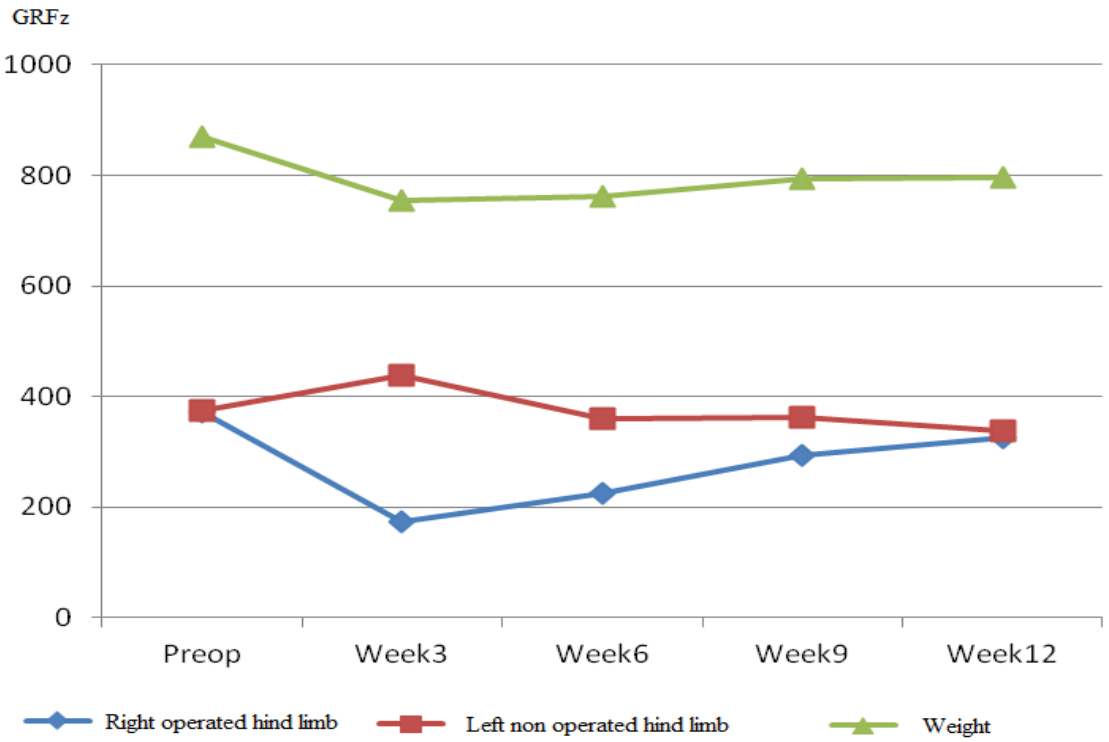


Figure 4.6: Graphic representation of changes in GRFz of both hind limbs over time.

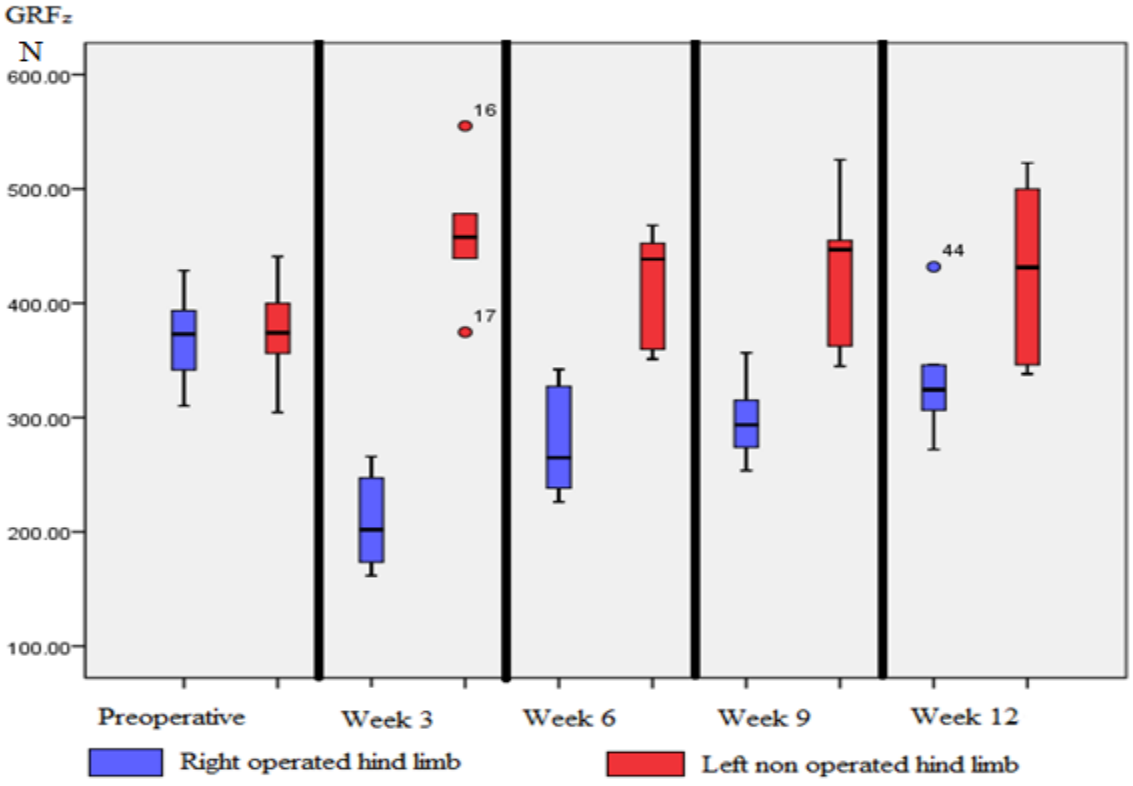


Figure 4.7: Box and whiskers plot of GRFz of operated and non-operated hind limbs.

	Median (%)	95% C.I.
Preop	98.6	95.8 – 101.5
Week 3	44.1	37.9 – 52.9
Week 6	67.8	60.3 – 74.3
Week 9	73.5	58.8 – 82.9
Week 12	78.6	64.8 – 94.4

Table 4.3: Median and 95% confidence interval of the FWB at different time intervals

4.3.3.1 At week three

The median FWB at three weeks post-operatively was 44.1% with 95% confidence interval of 37.9%-52.9%. Mann-Whitney U test showed statistical significance at this time interval compared to preoperative values (p=0.009) (Table 4.4); the same finding with statistical significance was found at week three when comparing right and left GRF_z (p=0.009) (Table 4.5).

	Preop	Week3	Week6	Week9	Week12
Preop					
Week3	0.009				
Week6	0.009	0.009			
Week9	0.009	0.009	0.465		
Week12	0.009	0.009	0.117	0.251	

Table 4.4: P-values showing the statistical significance of the FWB of the operated limb at different time interval.

	Rt Preop	Lt Preop	Rt Wk3	Lt Wk3	Rt Wk6	Lt Wk6	Rt Wk9	Lt Wk9	Rt Wk12	Lt Wk12
Rt Preop										
Lt Preop	0.754									
Rt Week3	0.009	0.009								
Lt Week3	0.028	0.047	0.009							
Rt Week6	0.047	0.028	0.117	0.009						
Lt Week6	0.175	0.347	0.009	0.175	0.009					
Rt Week9	0.047	0.047	0.016	0.009	0.465	0.016				
Lt Week9	0.175	0.251	0.009	0.347	0.009	0.754	0.016			
Rt Week12	0.347	0.251	0.009	0.016	0.175	0.028	0.347	0.047		
Lt Week12	0.251	0.465	0.009	0.465	0.016	0.917	0.028	0.754	0.076	

Table 4.5: P-values comparing GRF_z of right and left hind limbs at different time intervals.

4.3.3.2 At week six

The animals continued to show satisfactory progression with median FWB of 67.8%; the 95% confidence interval was 60.3%-74.3%. The Mann-Whitney U test showed statistical significance in comparison to pre-operative values (p=0.009). Statistical significance was also found when comparing GRF_z of right and left hind limbs at this time interval (p=0.009).

4.3.3.3 At week nine

At nine weeks post-operative assessment, the median FWB was 73.5% with a 95% confidence interval at 58.8%-82.9%. Statistical significance was found with a Mann-Whitney U test (p=0.009) comparing to preoperative values, and statistical significance of the GRF_z was also found comparing right and left hind limb (p=0.016).

4.3.3.4 At week 12

The FWB of the operated on limbs showed progressive improvement (Figure 4.8); the median FWB at week 12 was 78.6% and 95% confidence interval was 64.8%-94.4%. No statistical significance was found by the Mann-Whitney U test at this time interval when comparing right and left GRF_z (p=0.076), while statistical significance was found when comparing FWB to the pre-operative values.

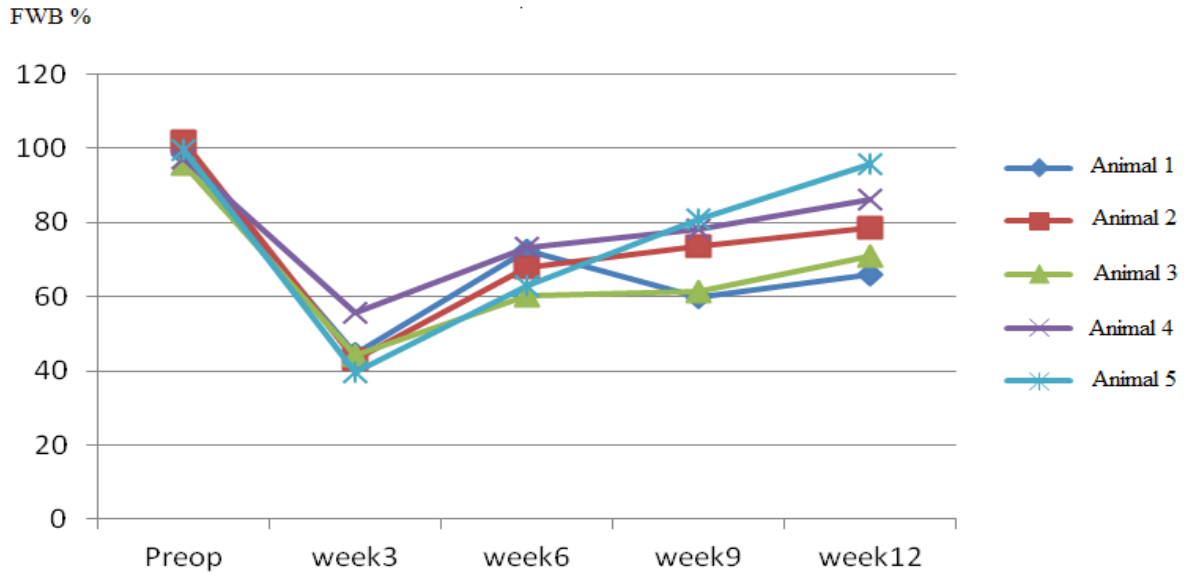


Figure 4.8: Graphic representation of FWB of each animal over time.

4.3.4 Morphological assessment during retrieval of the specimens

All animals showed similar findings and no evidence of superficial or deep infection was found. Normal post-operative scar tissue was present at the expected amount; no evidence of inflammatory reaction or granulation was seen (Figure 4.9).

A) B)

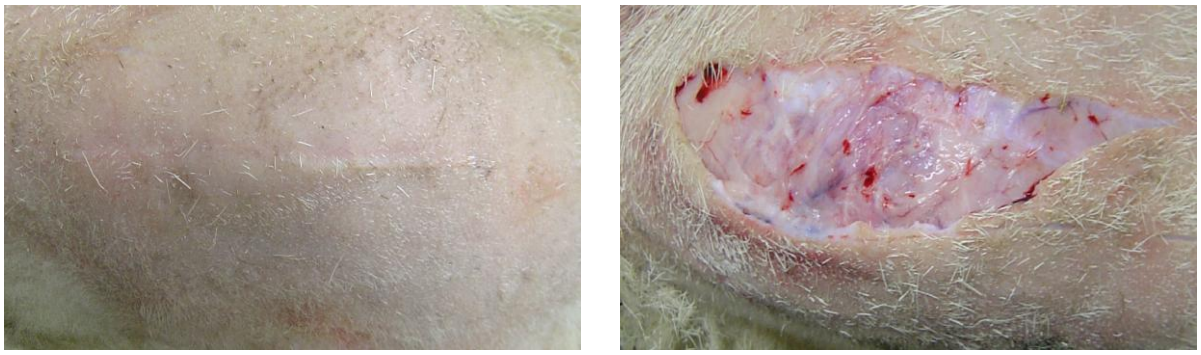
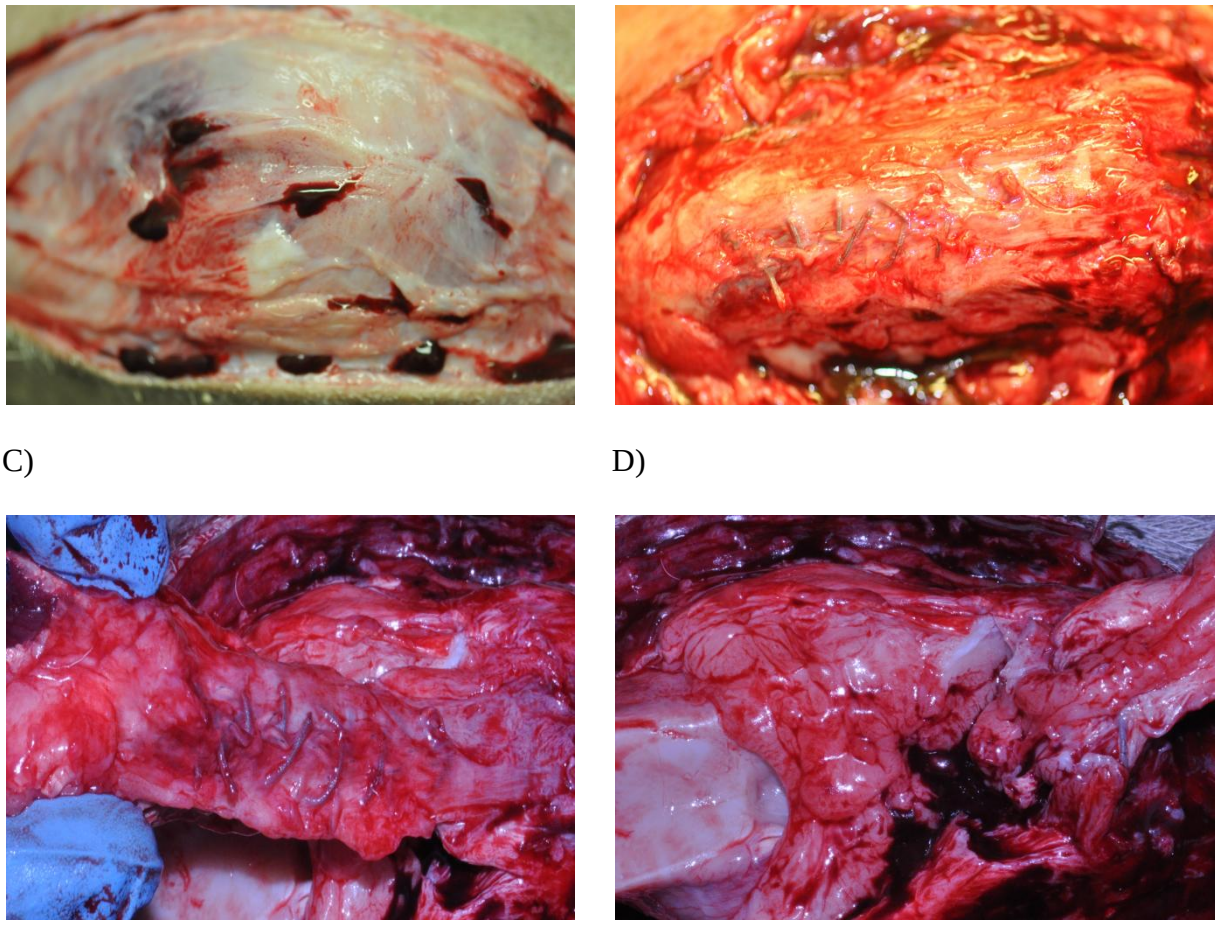


Figure 4.9: A) skin and B) subcutaneous tissues at retrieval.

Normal vasculature was also seen with no excessive hyperaemia or vasculitic lesions. Synovial fluid from the knee joint was in adequate amount with no evidence of effusion or reactive synovitis. The DCB was well integrated into both the tibial tuberosity and the patellar tendon. The distinction between the DCB and the patellar tendon was very difficult to discern visually (Figure 4.10).

A) B)



C) D)

Figure 4.10: Retrieval of the specimens A) superficial tissues showing normal tissues with no inflammation or excessive scaring, B) deep tissue dissection showing well integrated DCB, C) and D) anterior and posterior aspects of the patellar tendon-DCB construct, respectively, showing adequate integration into surrounding tissues and no inflammatory tissues.

The suture bone anchors maintained their position in the bone with no evidence of pull-out or migration. All the stitch materials were found intact with no evidence of rupture or failure. The infra-patellar fat pad was found to be attached to the posterior aspect of the patellar tendon, which is a normal finding as seen in the non-operated knees.

4.3.5 Radiographic assessment

Lateral X-ray radiographs for both hind limbs in full flexion and full extension were taken of each animal immediately after euthanasia. No evidence of calcification of DCB was seen in any of the six animals; some bony irregularity was present at the neo-enthesis over the osteotomised surface of the tibial tuberosity (Figure 4.11).

A) B)

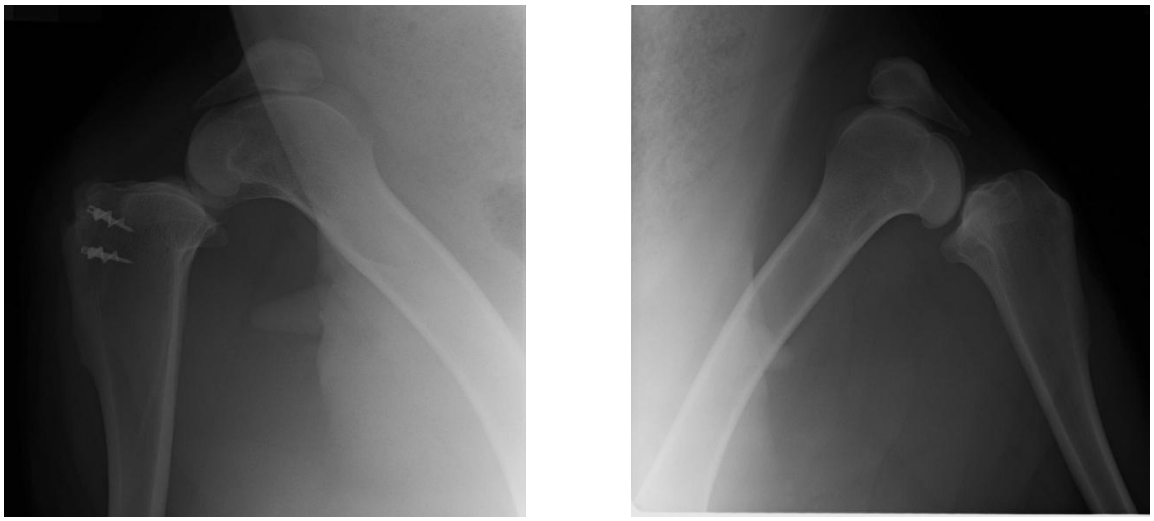


Figure 4.11: A & B: lateral radiographs of both knees of the same animal in full flexion.

The ranges of movement of the knees for both hind limbs were recorded (Table 4.6). The ranges of movement of the operated on limbs were comparable to those of the non-operated limbs (Figure 4.12). The Mann-Whitney U test did not show any statistical significance between the right and left hind limb full flexion angles ($p=0.065$) or between the full extension angles ($p=0.394$).

	Right hind limb knee		Left hind limb knee	
	Flexion	Extension	Flexion	Extension
Animal 1	48	117	57	113
Animal 2	46	108	55	121
Animal 3	43	116	62	129
Animal 4	47	104	42	111
Animal 5	40	109	63	106
Animal 6	54	125	51	129

Table 4.6: Flexion and extension angles of both hind limb knees of each animal (in degrees).

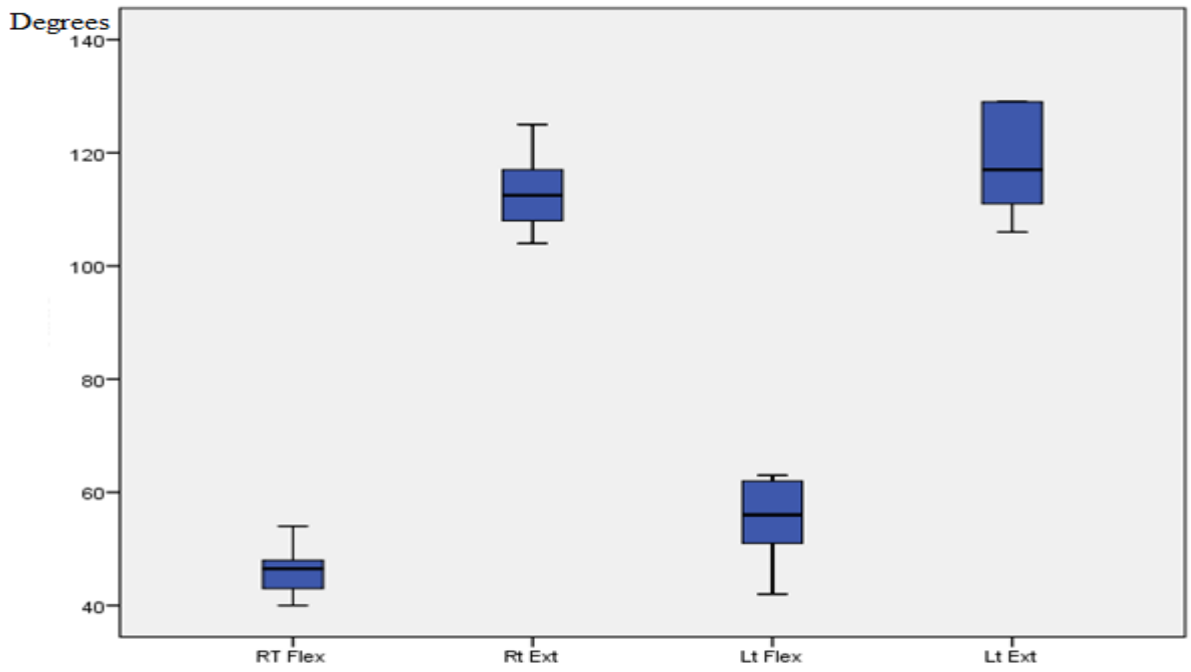


Figure 4.12: Box and whisker plot of the ROM of operated on and non-operated on knees.

4.3.6 pQCT Scan

pQCT Scanning was performed to examine for ossification within the patellar tendon, the DCB and surrounding tissues; 5 mm sections were taken through the tibial tuberosity, patellar tendon/DCB and patella. None of the specimens showed any evidence of ossification within the DCB or the substance of the patellar tendon (Figure 4.13).

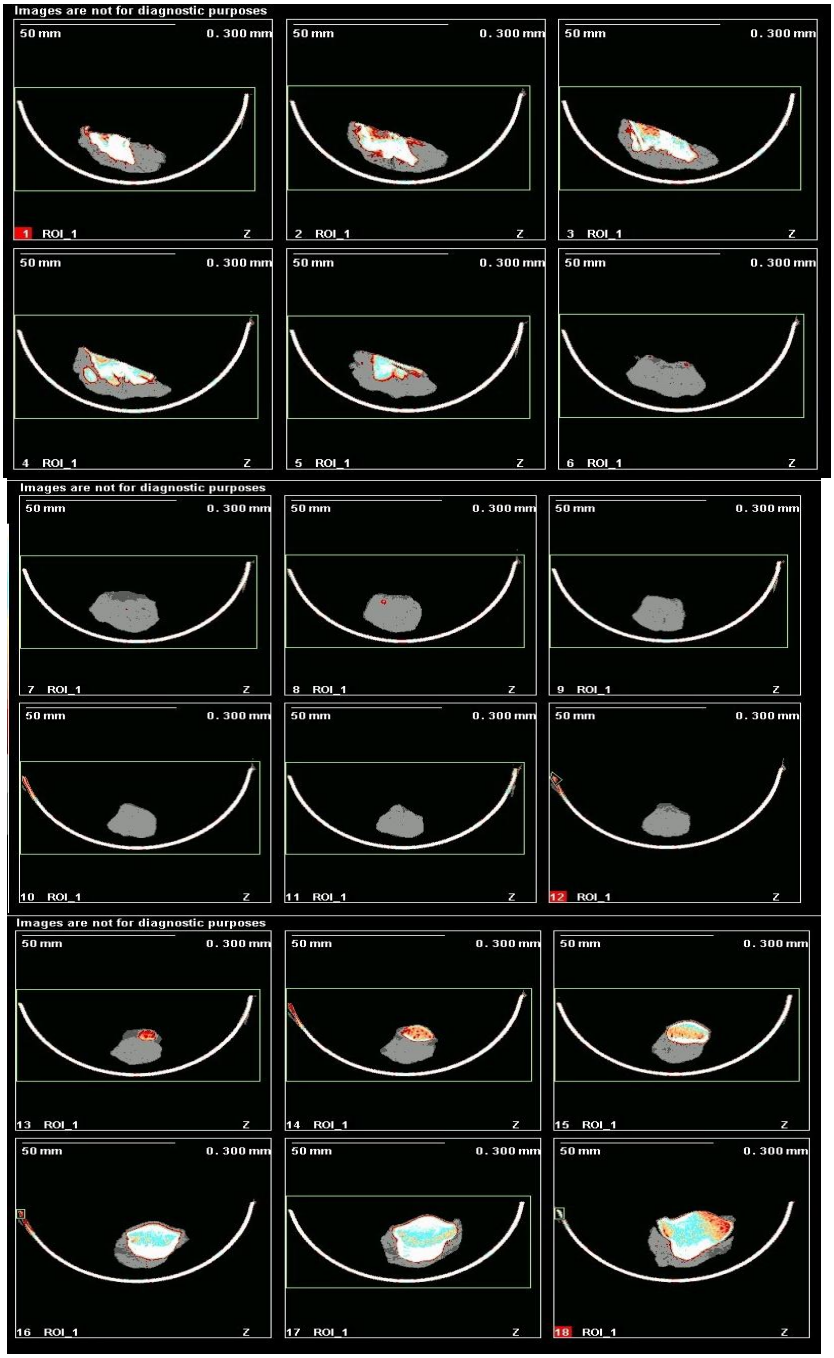


Figure 4.13: pQCT Scan images showing no ossification within the patellar tendon or DCB.

4.3.7 Histological analysis

4.3.7.1 Qualitative assessment

Sections from all animals were examined (including the one that failed to recover its gait). All animals showed strong evidence of remodelling and integration. In all sections there was cellularisation (Figure 4.14); tenocytes and chondrocytes were seen in all parts of the DCB and spindle shaped cells were arranged longitudinally with the oval shaped nuclei arranged in a longitudinal pattern parallel to the longitudinal axis of the patellar tendon (Figure 4.15).

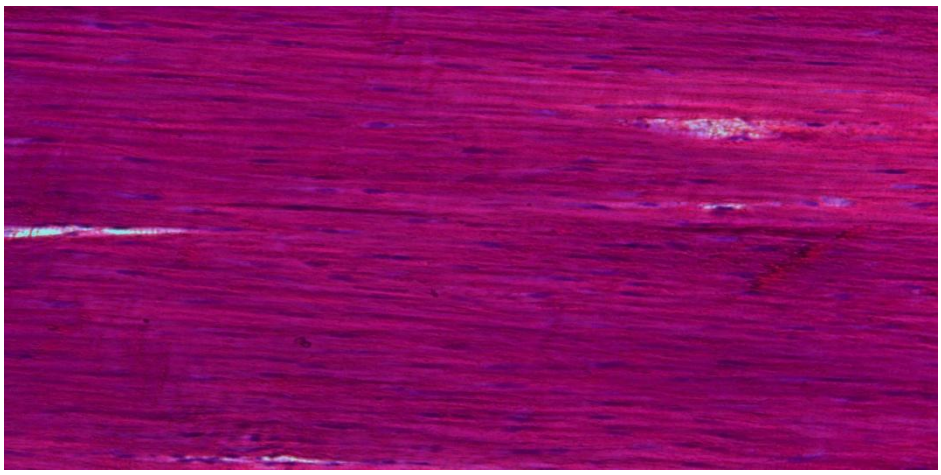


Figure 4.14: Microphotograph (X 20) of DCB showing cellularisation.

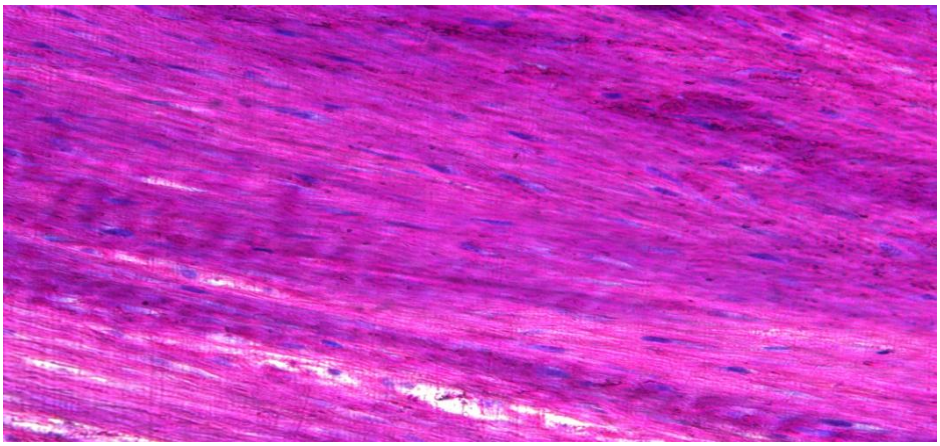


Figure 4.15: Microphotograph (X 20) of DCB showing oval nuclei longitudinally arranged.

Mature blood vessels were also found in the tissue of DCB (Figure 4.16). Collagen fibres were organized in a longitudinal pattern parallel to the longitudinal access of the patellar tendon. Collagen fibres showed evidence of characteristic crimping on polarized microscopy in different areas of DCB (Figures 4.17 and 4.18).

A) B)

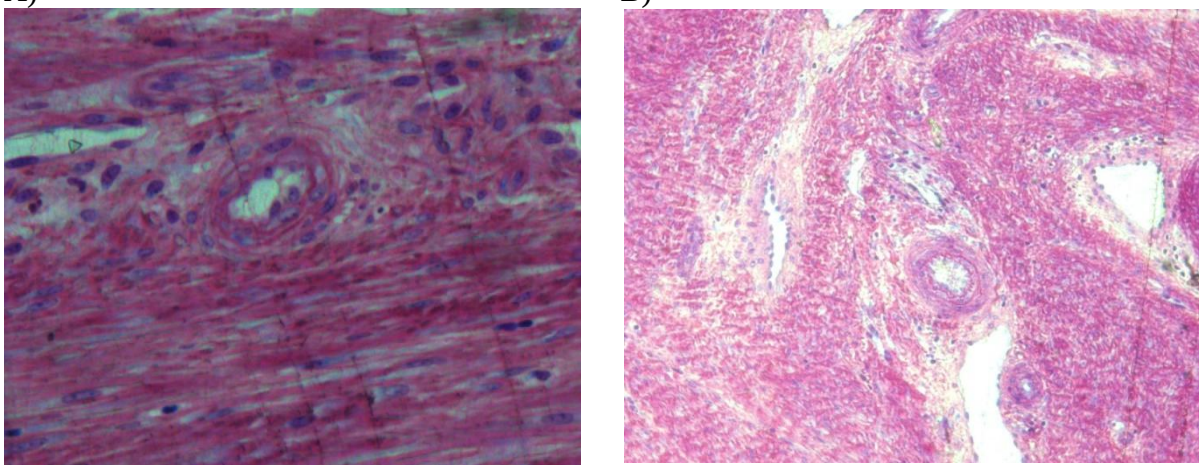


Figure 4.16: Microphotograph (A: X 20, B: X 10) of mature blood vessels within DCB.

A) B)

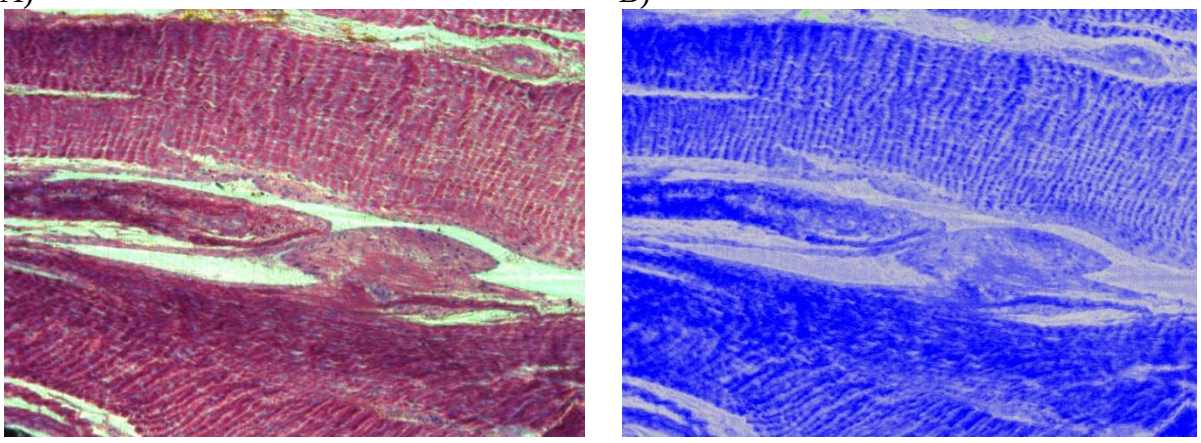
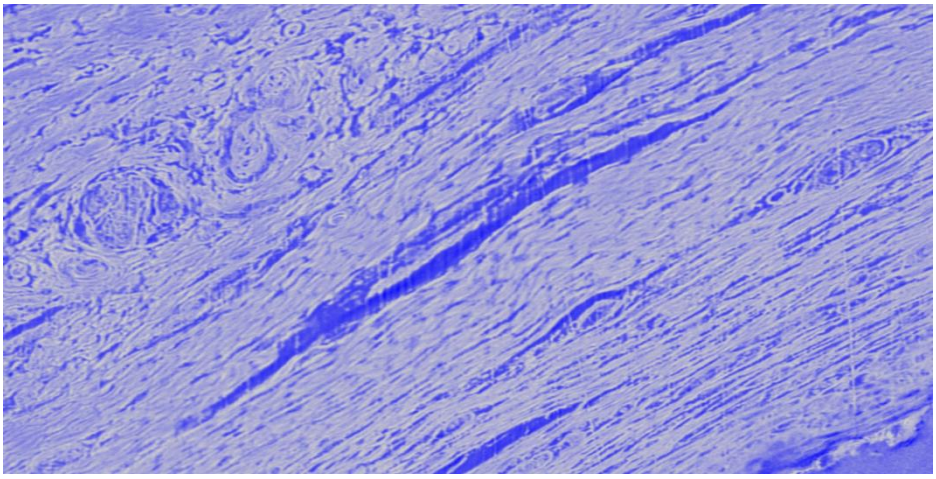
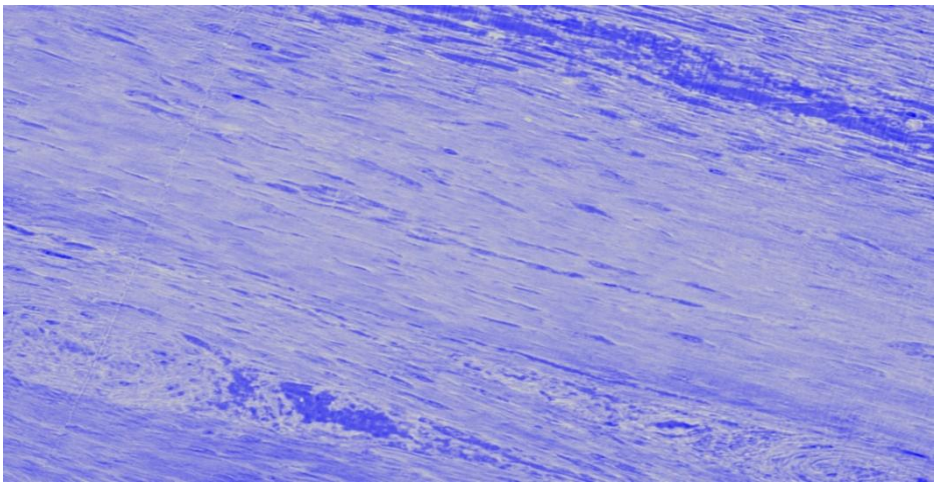


Figure 4.17: Microphotograph (X10) A) pre- & B) post-polarization showing evidence of crimping.

A)



B)



C)

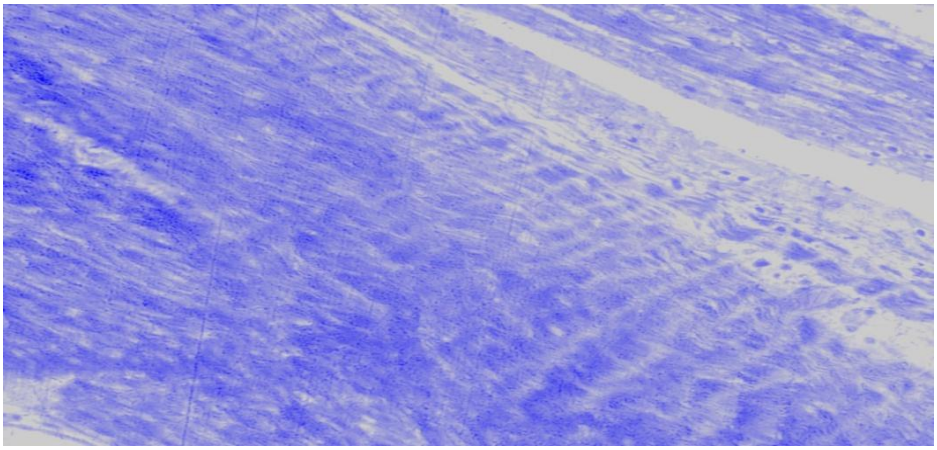


Figure 4.18: Microphotograph (X 20) polarized microscopy showing evidence of DCB crimping.

No changes were seen in the native patellar tendon and there was no evidence of inflammatory cells in either DCB or the patellar tendon; no lymphocytes, macrophages or other immune response cells were found in any of the sections. No evidence of resorption of DCB or heterotrophic ossification was found.

4.3.7.2 Zone 1: interactions between DCB and the patellar tendon

All sections examined from all the animals showed strong interconnections between DCB and the native patellar tendon (Figure 4.19). In some areas, DCB was well integrated into the patellar tendon with no clear demarcation between either (Figure 4.20).

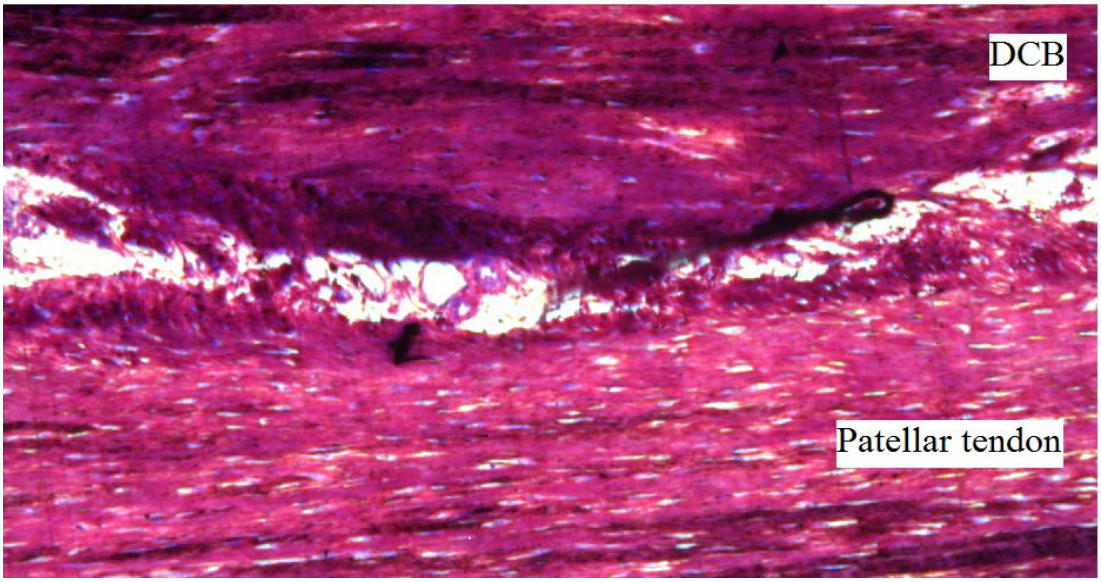


Figure 4.19: Microphotograph (X5) of zone 1 showing interconnections between DCB and the patellar tendon.

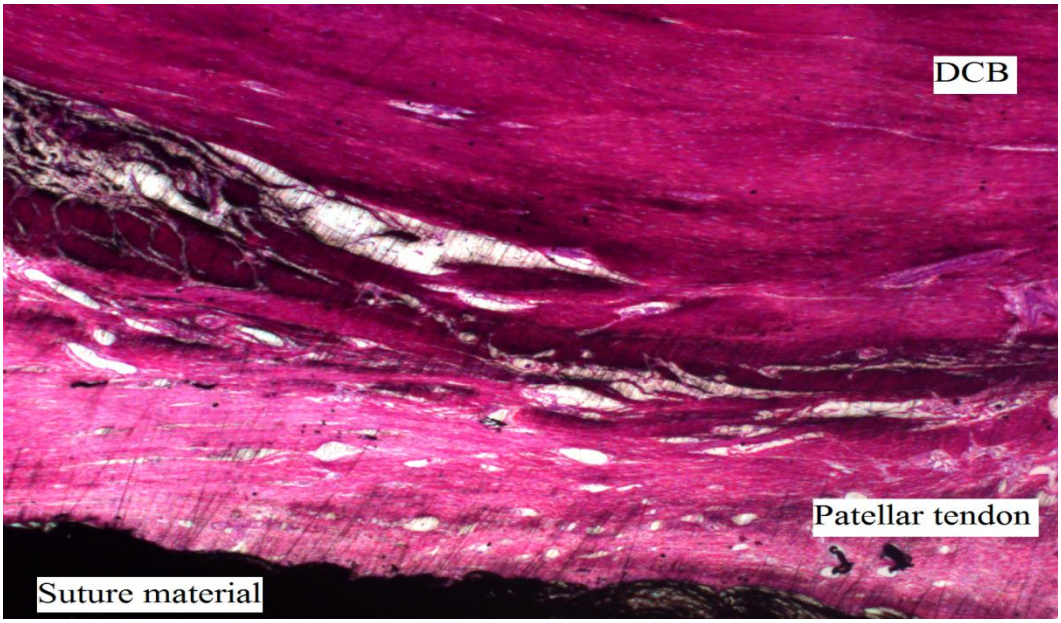
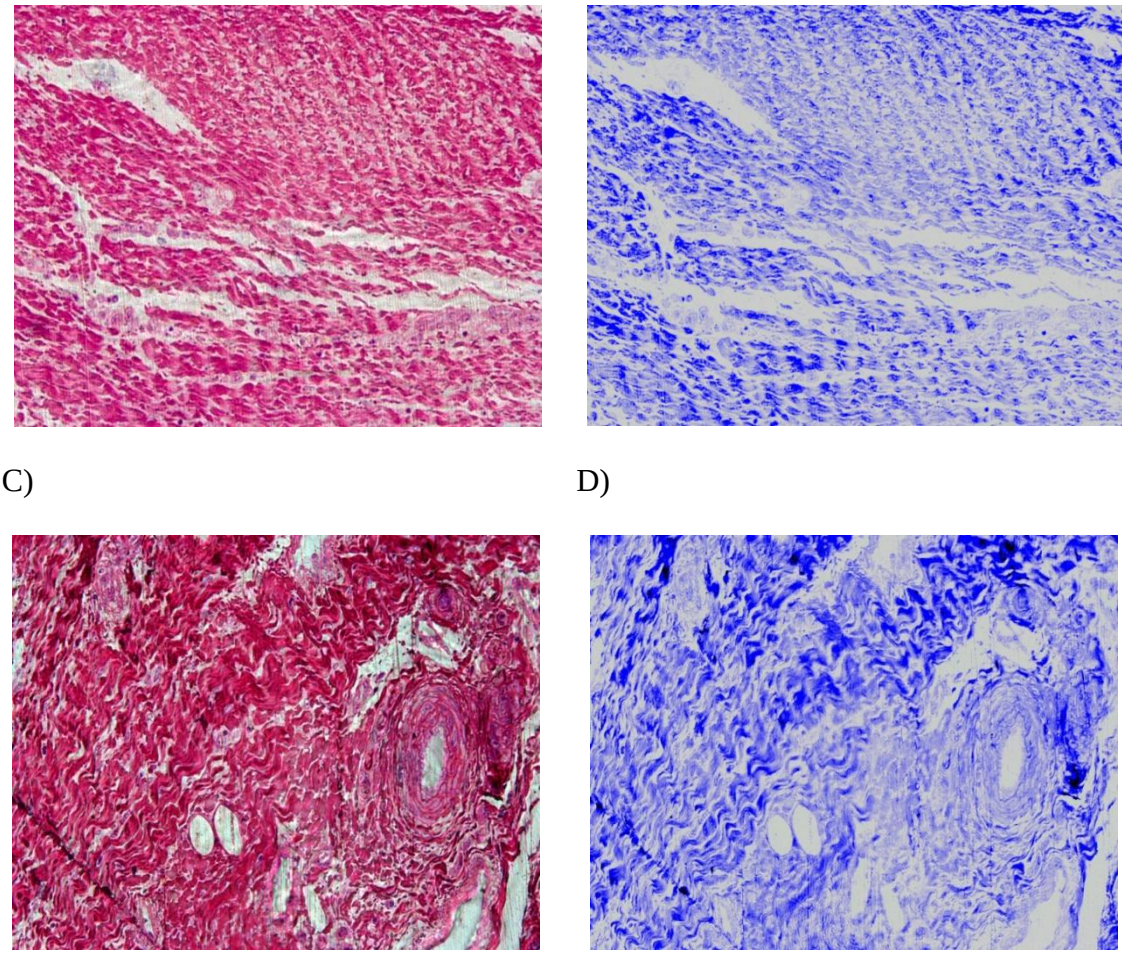


Figure 4.20: Microphotograph (X2.5) of zone 1 showing interconnections between DCB and the patellar tendon.

4.3.7.3 Zone 2: remodelling of the DCB

Zone 2 is the region where the DCB bridges the gap between the end of the patellar tendon and the tibial tuberosity (Figure 4.2). In this zone, all the sections showed strong evidence of remodelling in the form of cellularisation, vascular invasion and crimped collagen fibres (Figure 4.21).

A) B)

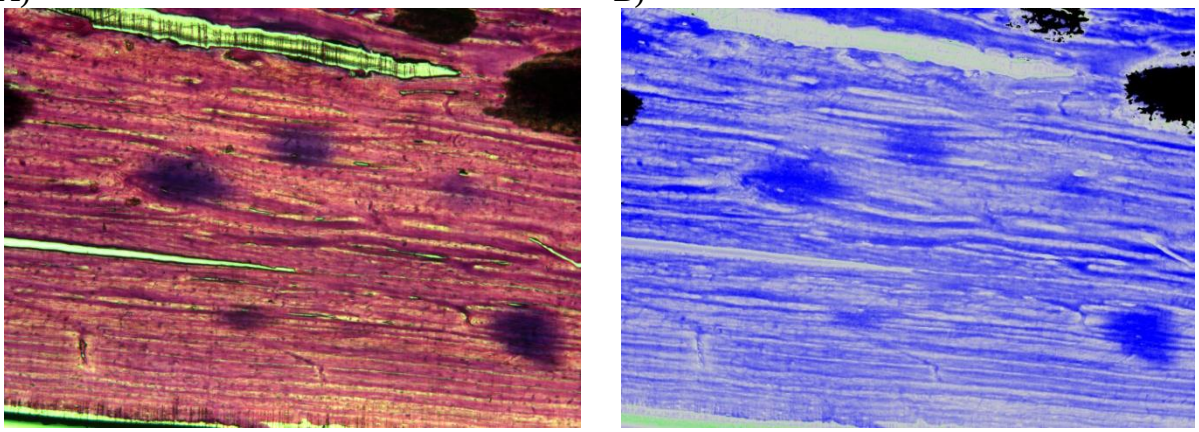


C) D)

Figure 4.21: Microphotographs (X 20) of Zone 2 DCB; B & D: polarized images of A & C, respectively, showing evidence of crimping, cellularisation and vascularisation.

All cells were aligned longitudinally, with oval nuclei also aligned longitudinally in line with the long access of the patellar tendon. Collagen fibres were all in longitudinal fashion; the characteristic crimp of the normal tendon was found in the absence of the Haversian system of DCB (Figure 4.22).

A) B)



C) D)

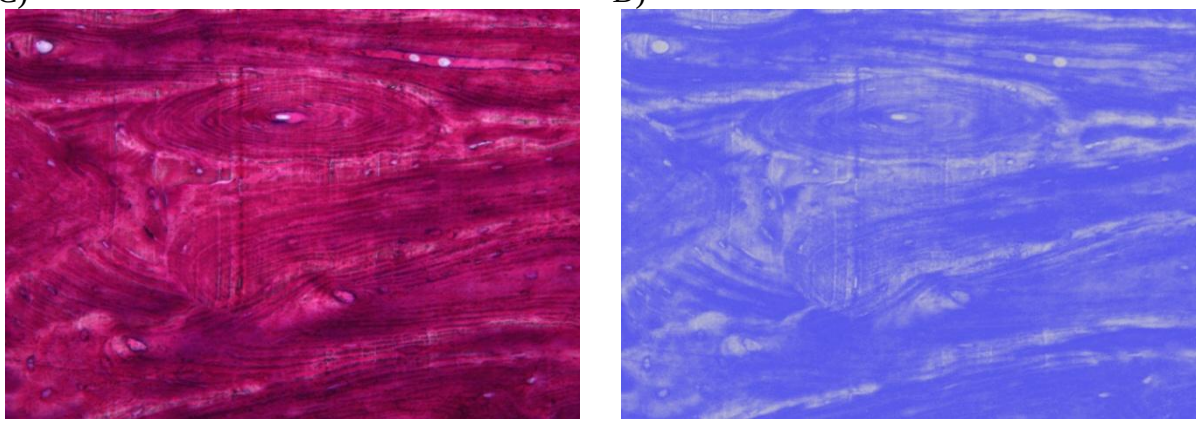
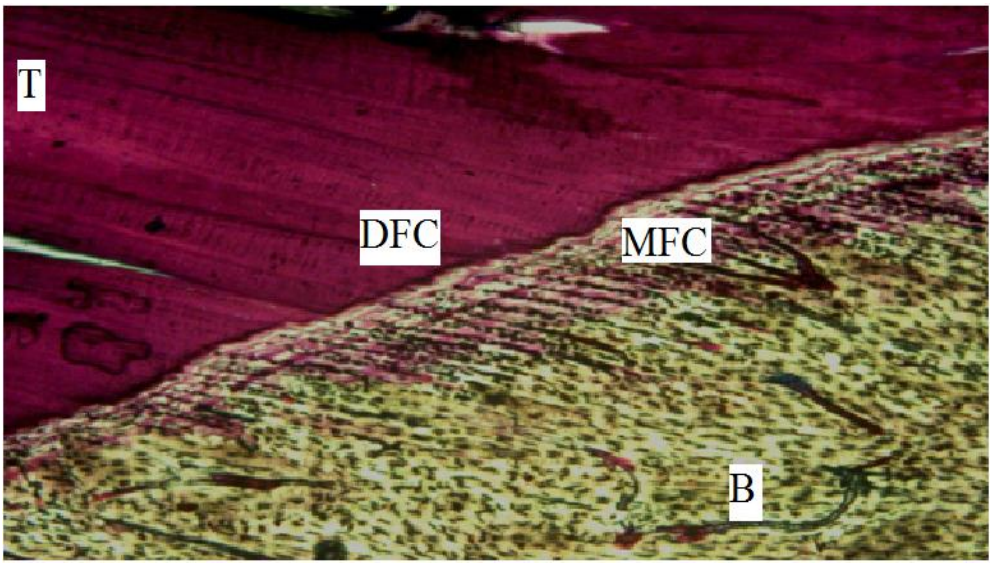


Figure 4.22: Microphotograph (X 5): DCB without implantation in animals; B & D: polarized images for A & C, respectively, showing a characteristic Haversian system and absence of crimping.

4.3.7.4 Zone 3: formation of neo-enthesis

Zone 3 describes the attachment of the DCB onto the osteotomised surface of the tibial tuberosity to form the neo-enthesis. In all the specimens, a direct type enthesis was found with the characteristic transition between bone, mineralised fibrocartilage, demineralised fibrocartilage and tendon (Figures 4.23 and 4.24). Some of the sections showed a clear tidemark, differentiating between these zones with clear demarcation. Although in others no clear tidemark was seen, the four zones were observed with gradual transition between the four zones of the enthesis.

A)



B)

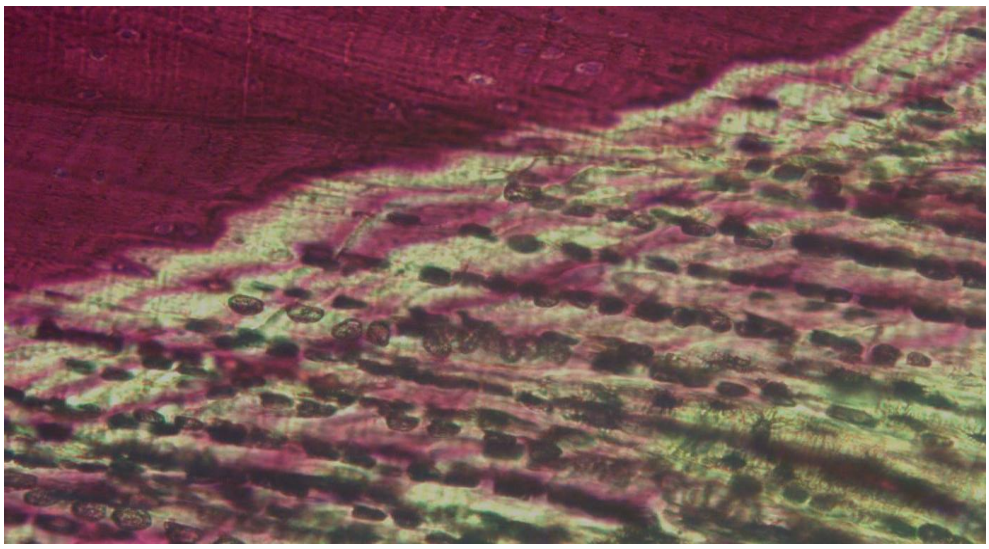
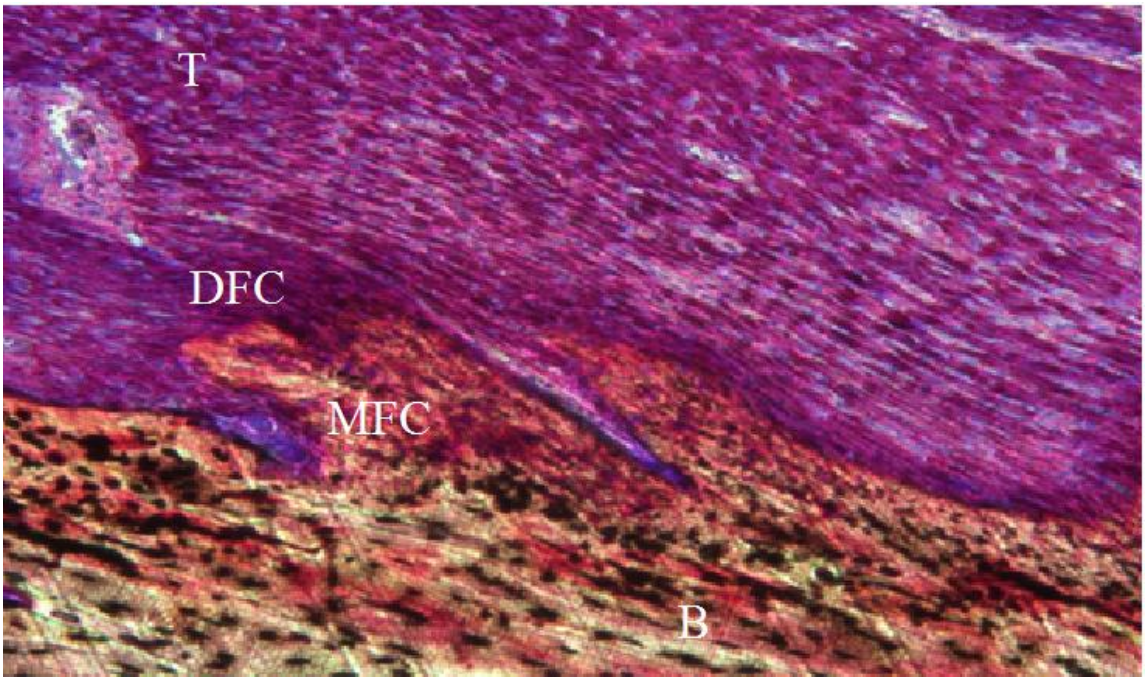


Figure 4.23: Microphotographs of normal tendon enthesis showing the various levels of the zones on these photos (same section at different magnification A: X 10, B: X 20) (T = Tendon, B = Bone, MFC = Mineralised fibrocartilage, DFC = Demineralised fibrocartilage).

A)



B)

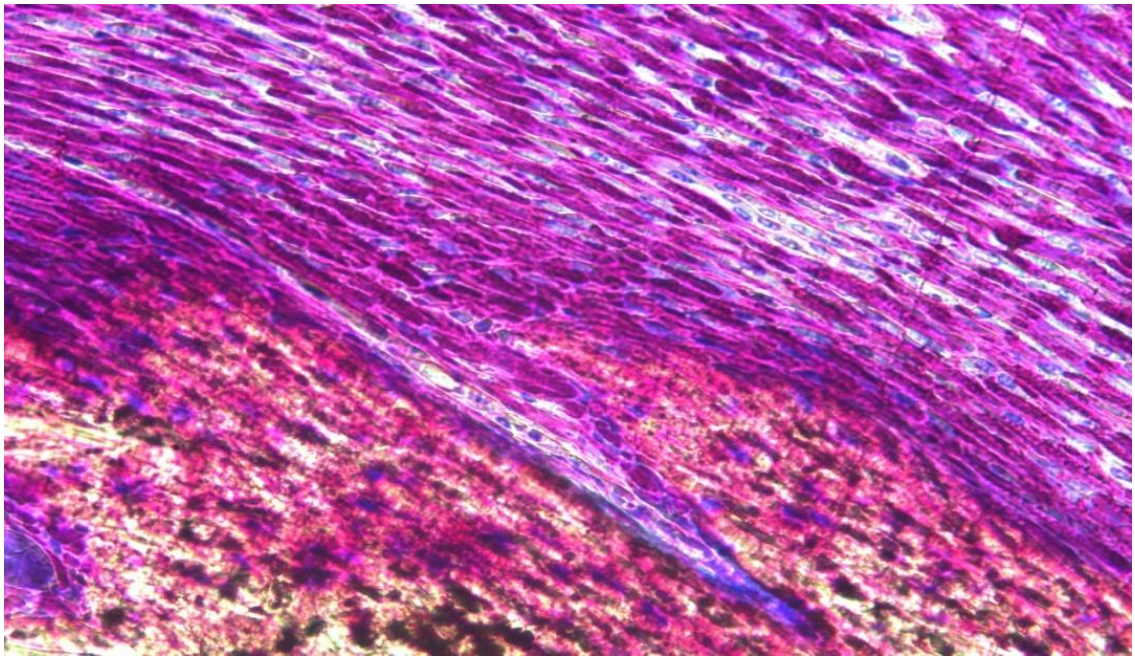
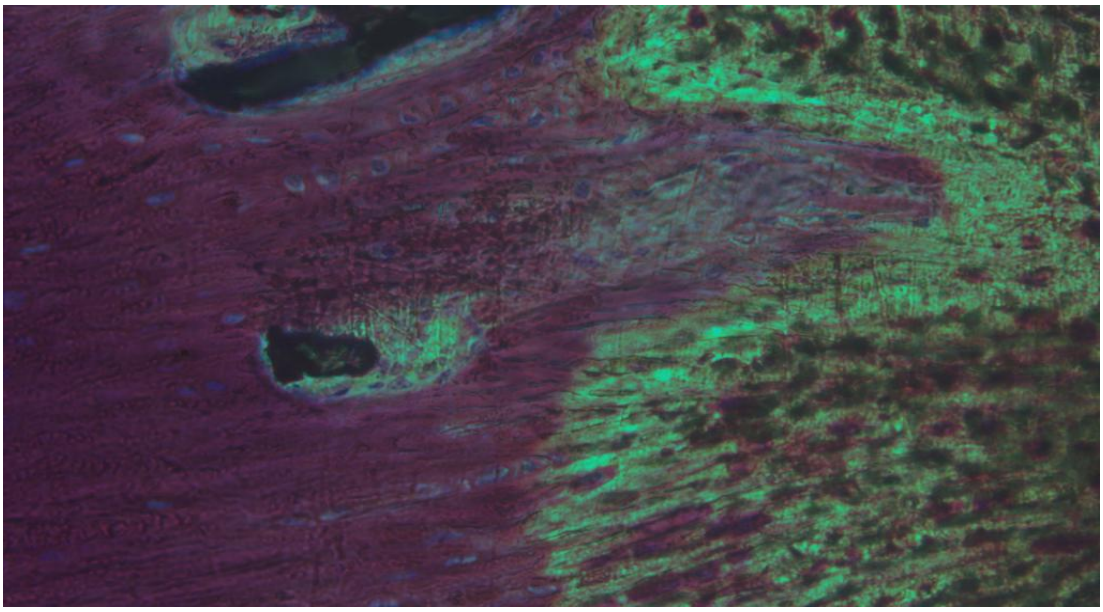


Figure 4.24: Microphotographs of the neo-enthesis, with the four zone transition (same section different magnification A: X 10, B; X20).

Collagen fibres were also seen penetrating the fibrocartilage into the bony tissue in all sections and running parallel to the long access of the collagen fibres of DCB, which were in turn parallel to the long access of the patellar tendon and in line with the direction of tensile loading of the tendon (Figure 4.25). At the neo-enthesis, cells were arranged longitudinally and in line with the collagen fibres (Figure 4.26).

A)



B)

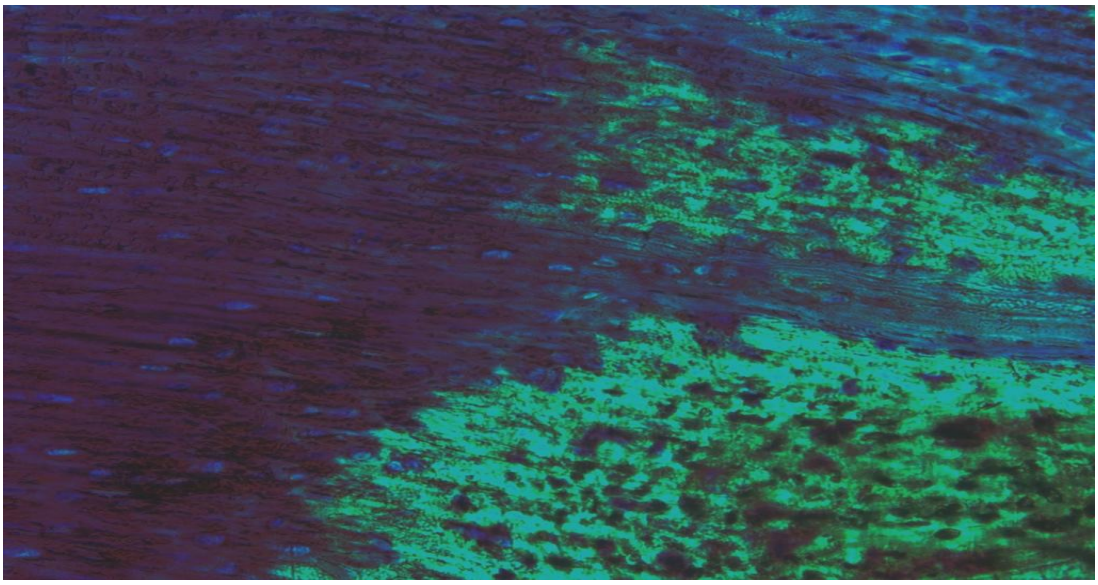
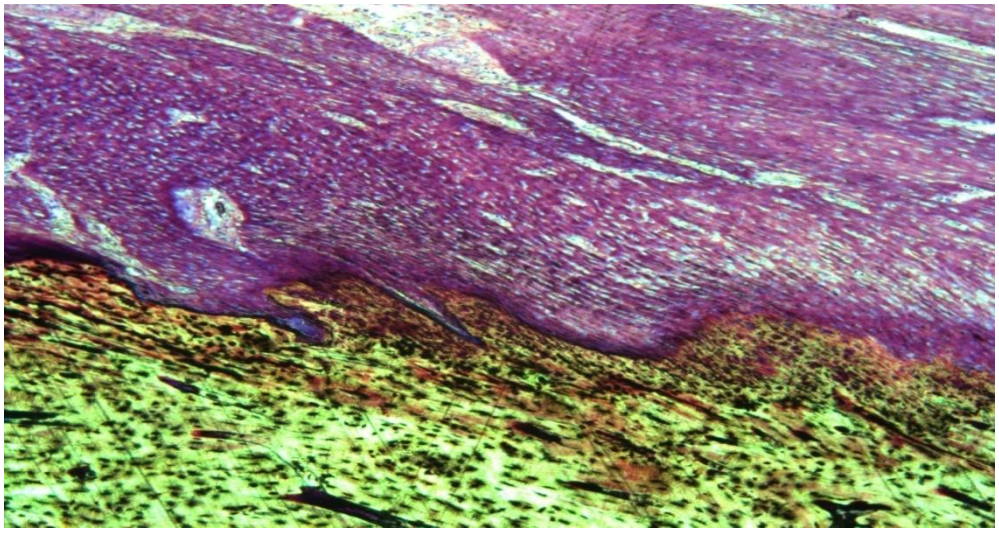
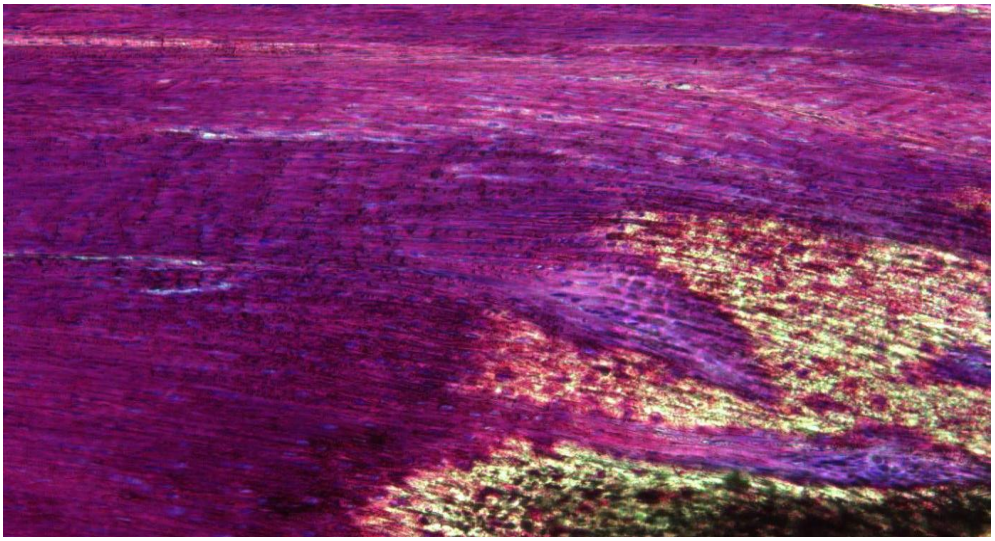


Figure 4.25: Microphotographs (X 20) of the neo-enthesis.

A)



B)



C)

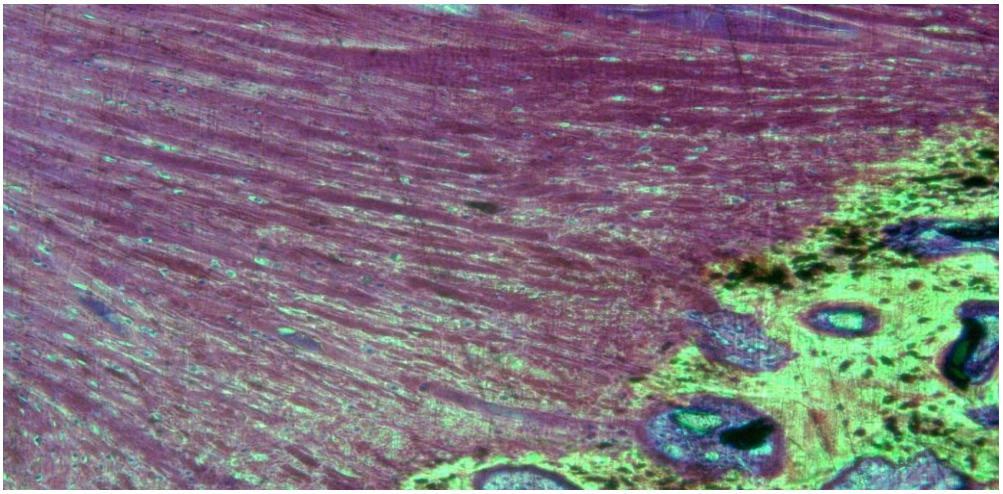


Figure 4.26: Microphotographs of the same section of the neo-enthesis showing cellular and collagen fibre alignment (A: X 10, B: X10, C: X20).

4.3.7.5 Semi-quantitative analysis of the neo-enthesis

A semi-quantitative analysis of the neo-enthesis was conducted as detailed in section 3.2.8. This analysis aimed to examine the maturation of the neo-enthesis, the presence of the four zones and clear distinct transition between these zones.

In all sections examined, the four zones of the direct enthesis were seen; there were fibrocartilage and mineralised fibrocartilage zones in all sections. The transitions between the zones were not distinct in some of the sections; all sections scored between three and five by all the independent observers (Figure 4.27). There was statistical significance between the scores of the neo-enthesis and the contralateral control enthesis on the Mann-Whitney U test (p= 0.03).

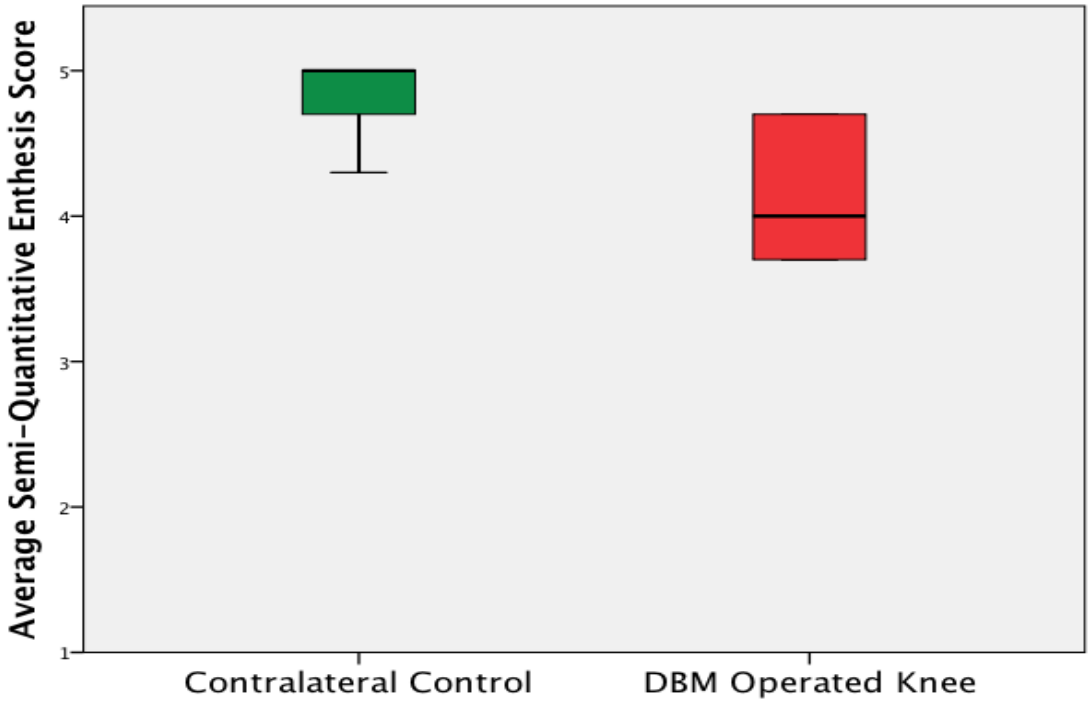


Figure 4.27: Box and whisker plot of the semi-quantitative analysis of the neo-enthesis.

4.3.7.6 Cell counting

Both chondrocytes and tenocytes were counted in different areas of the specimens as described in section 3.2.8. The median and confidence interval for each cell type were calculated for different areas of the specimens (Table 4.7 and Figure 4.28).

	Chondrocytes Median (95% Confidence interval)	Tenocytes Median (95% Confidence interval)	p-value Chondrocytes: tenocytes
Area 1	1727 (985 – 2312)	377 (167 – 681)	0.004
Area 2	1076 (547 – 1489)	88 (0 – 319)	0.010
Area 3	608 (88 – 1504)	380 (66 – 1355)	0.520
Area 4	89 (20 – 209)	1014 (453 – 2076)	0.004
Area 5	258 (97 – 403)	700 (374 – 1138)	0.016
Area 6	1930 (419 – 2721)	687 (224 – 1205)	0.262
Control enthesis	469 (332 – 542)	57 (0 – 112)	0.004

Table 4.7: Chondrocyte and tenocyte cell counts in different areas of the specimens (cell/mm^2).

In areas one and two, where the DCB was in close proximity to the bone, the number of chondrocytes was significantly higher compared to tenocytes, whereas in the patellar tendon substance and the DCB-tendon interface (areas four and five), the number of tenocytes was significantly higher. In areas three and six, where the DCB was not in contact with either tendon or bone, there was no statistical significance between the number of chondrocytes and tenocytes (Figures 4.29 and 4.30).

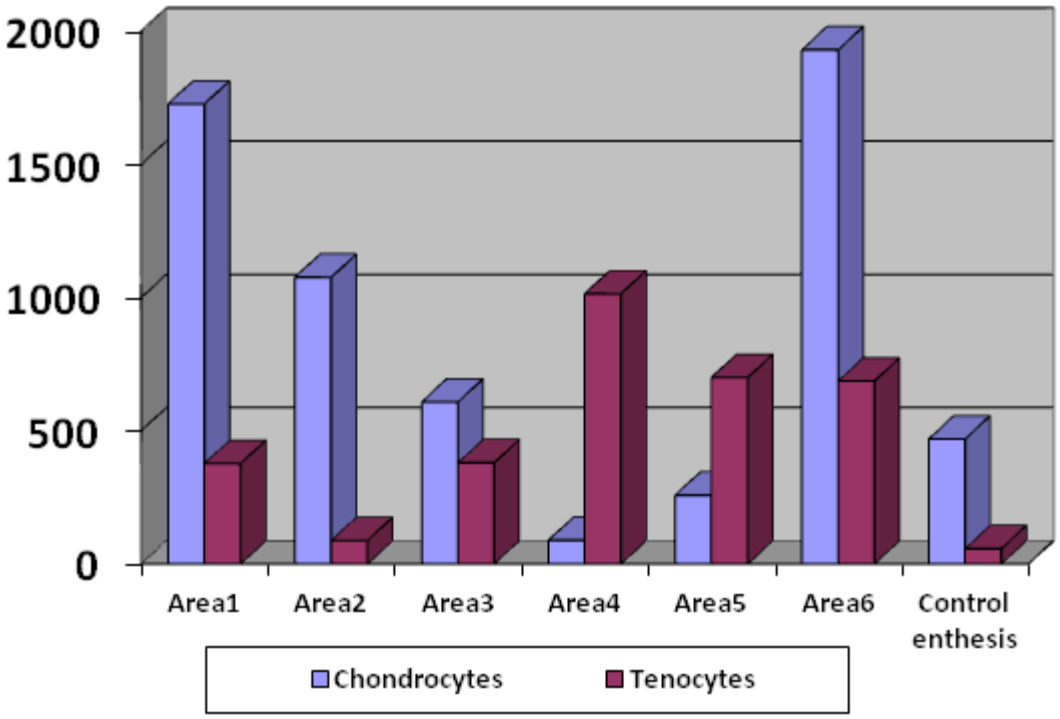


Figure 4.28: Graphic representation of cell counts in different areas.

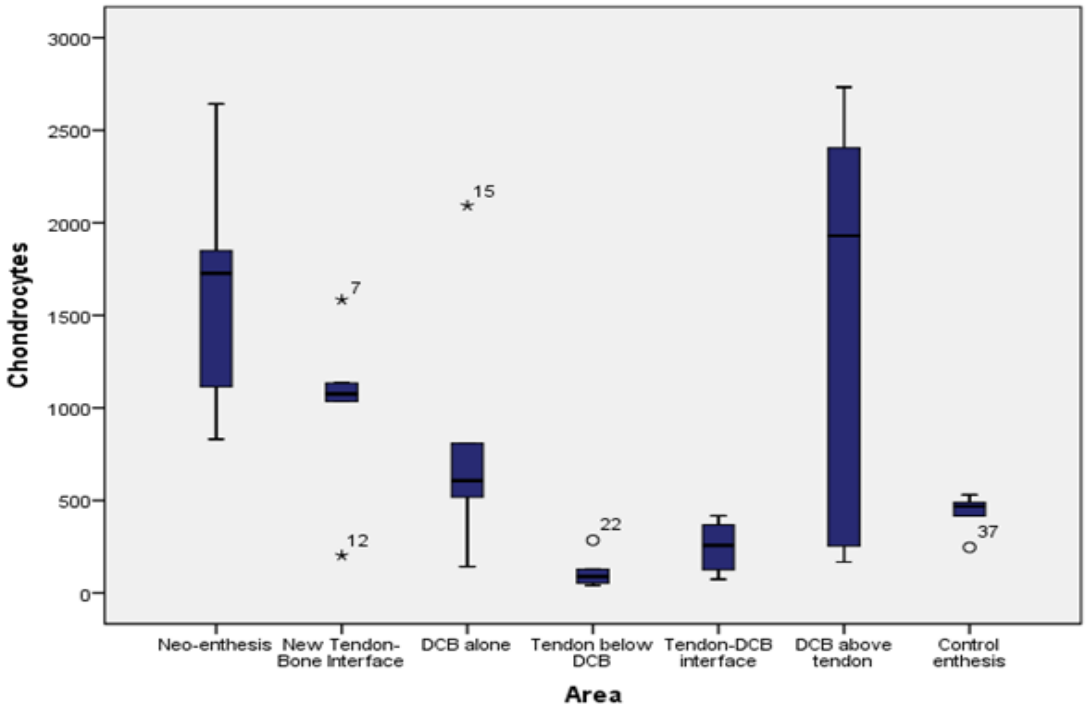


Figure 4.29: Box and whisker plots for A) chondrocyte cell counts in different areas of the specimens.

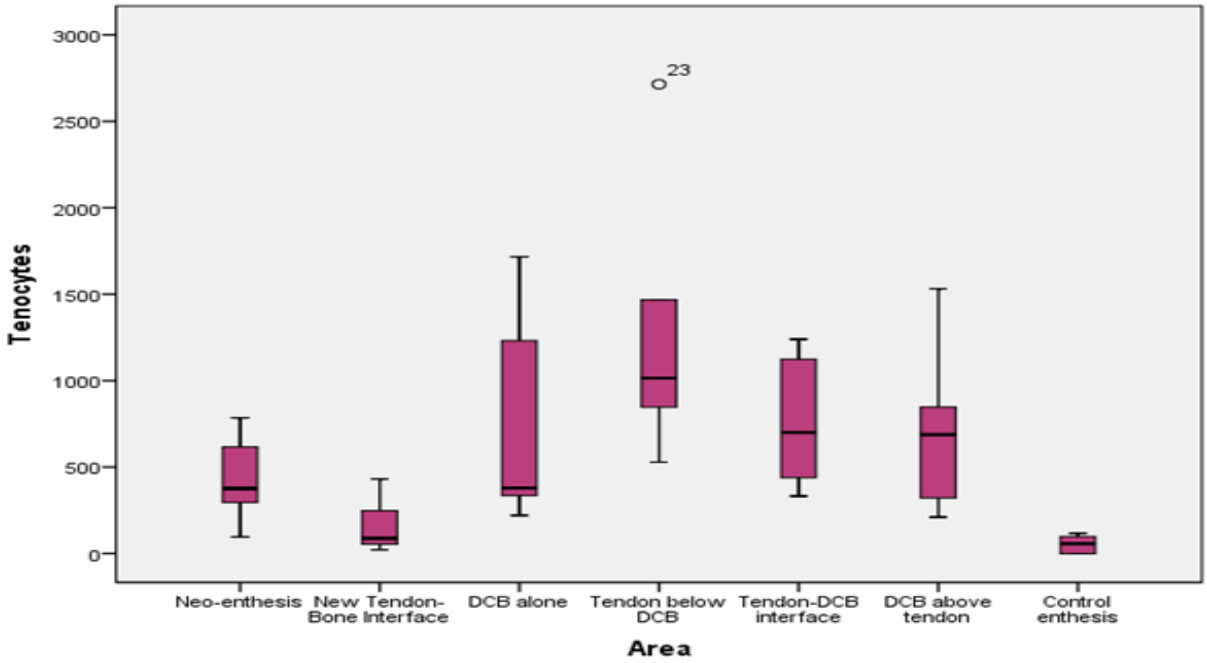


Figure 4.30: Box and whisker plots for tenocyte cell counts in different areas of the specimens.

4.4 Discussion

4.4.1 Failure rate

One animal out of six (16.7%) failed to demonstrate satisfactory progression on FWB status as examined on force plate analysis. This animal was excluded from the force plate study; however, it was included in all other aspects of this research. Radiological and morphological assessments of the repaired patellar tendon construct of this animal have proven the presence of patella alta (high riding patella) as demonstrated in Figure 4.3.1. There were no other findings to explain the failure of this animal. On morphological assessment at retrieval, no difference was found between this animal and the other five animals.

In humans, the length of the patellar tendon is on average 44 mm (range 35-55 mm), according to results of cadaveric study on human knees (Reider et al., 1981). Clinicians usually refer to the Insall-Salvati ratio (Aglietti et al., 1983, Insall and Salvati, 1971) as one of the radiological features of abnormal patellar position. This is the ratio of the patellar height to the length of the patellar tendon and is normally between 0.8 and 1.2.

Unfortunately, there was no available study to demonstrate similar anatomical features on ovine animals. My diagnosis of the patella alta in the failed animal was based on radiological findings of the position of the distal pole of the patella in relation to the Blumensaat line on the lateral radiograph of all the animals at similar degrees of flexion (the Blumensaat line is a radiological feature on the lateral X-ray radiograph representing the roof of the intercondylar notch). In all five animals, the distal pole of the patella was distal to the horizontal line passing through the Blumensaat line at > 100 ˚ of flexion. In the failed animal, the distal pole was significantly proximal to the similar line.

Whipstitch techniques in tendon repair have been widely practised in clinical settings (da Assuncao et al., 2013, Florian Gebhard, 2008) and is considered superior to direct end to end stitching. In this study, a combination of both Krackow (McKeon et al., 2006, Krackow et al., 1988) and running whipstitch techniques were used in each animal.

One of the known drawbacks of the whipstitch techniques is tendon elongation and gap formation at the repair site (Krushinski et al., 2010). In my opinion, the failure in the one animal was due to either stretching and elongation of the patellar tendon or cheese-wiring of the suture material through the DCB.

4.4.2 Gait observation and force plate analysis

The use of force plate analysis in the assessment of FWB and the effect of surgical intervention on weight bearing status has been established in medical and biomechanical fields (Jevens et al., 1996, Ballagas et al., 2004). Previous studies have established a direct relationship between the vertical component of the ground reaction force and the forces going through the limb, which reflect on the weight-bearing status of the limb (Korvick et al., 1996).

During the first three weeks of this study, the animals showed the expected post-surgical reaction of antalgic limp. During this period, the forces going through the patellar tendon were probably solely transferred through the suture material, which provided the initial mechanical strength for the repair.

After three weeks, the animals started to recover abnormal gait with gradual progression into normal non-antalgic gait. The initial phase of healing usually last for four to six weeks;

during this phase, the haematoma and the inflammatory reaction takes place. In the second phase of tendon healing, formation of collagen fibres starts and bridging fibres connect both ends of the tendon. In our study, fibres connected DCB to the osteotomised surface of the tibial tuberosity. These fibres have enough mechanical strength to transmit some of the forces, which was evident during gait observation and confirmed by the force plate analysis at six weeks.

In my study, the median FWB at six weeks was 67.8%, improved from 44.1% at three weeks. This 23.7% improvement can only be explained by the biological process of healing of the repair, as in ovine models, forces are only transmitted through the patellar tendon with no other compensatory mechanism to explain the improvement in FWB (Allen et al., 1998).

As explained in the introduction, complete tendon healing can take up to a year. Over the duration of this study, animals continued to show satisfactory progression, which can only be explained by successful repair of the patellar tendon.

While forces going through the operated on limbs were gradually increasing over time, the forces going through the non-operated limbs increased post-operatively at week three, with gradual reduction to baseline preoperative values. This in itself represents a clear indication of progressive healing and recovery of the operated on patellar tendon.

4.4.3 Radiographic assessment and pQCT scan

The traditional expectation of demineralised bone is that it ossifies after implantation through endochondral ossification. This probability is based on Urist's work, which shows that demineralised bone contains osteoinductive cytokines and it provides a suitable scaffold for

osteogenesis (Reddi, 1998, Van de Putte and Urist, 1965, Urist, 1965). In his studies, Urist found that ossification of the demineralised bone implanted in soft tissue pockets can be evident as early as four weeks. The radiographic assessment and pQCT scan results in my study contradict this common belief; no evidence of ossification of DCB was seen at 12 weeks. In my opinion, the DCB was implanted in a tendon environment, which would have locally regulating proteins and cytokines from the patellar tendon. In addition, the mechanobiology for the construct means that colonization of MSCs from the adjacent Hoffa fat pad may have led to regulation of the MSCs' differentiation into tenogenic and chondrogenic pathways.

4.4.4 Histological analysis

As acute tendon rupture usually occur at the enthesis, treatment of this condition aims to restore normal enthesis. Several studies have found that anatomical healing of tendon ruptures results in poorly differentiated enthesis. The healing usually starts with disorganized collagen fibres filling in the enthesis gap, which later progresses into fibrous scar-like tissue, with anchoring to the bone via increased ossification (Oguma et al., 2001, Uhthoff et al., 2002). In a two-year follow-up study on the repair of enthesis in sheep, it was found that the enthesis remodels into a functional type attachment with increased organized fibrous tissue, rather than the normal four zones attachment (Newsham-West et al., 2007).

Previous studies by our group found that using a demineralised bone matrix during the repair of tendon rupture as an interface between the tendon and bone results in improved healing, with four zone enthesis rather than fibrous enthesis (Sundar et al., 2009c). In my research presented in this chapter, I investigated the possibility of using DCB to replace part of the

tendon and to reproduce neo-enthesis that has the same characteristics as the normal fibrocartilaginous type.

On histological analysis of the DCB, there was strong evidence of remodelling of DCB into a ligament/tendon like structure. The Haversian system arrangement of the bony tissues was not seen, nor was the characteristic bony lacunae; instead, it was replaced with collagen fibres arranged longitudinally in line with the long axis of the tendon, with the axis of stress loading of the tendon. The DCB is an acellular, avascular structure that is expected to ossify when implanted in live tissue, as previous studies have shown (Urist, 1965, Van de Putte and Urist, 1965). In our specimens, DCB was invaded by host cells that differentiated into chondrocytes and tenocytes. Additionally, the DCB was found to be vascularised with mature blood vessels and no ossification or calcification was found.

The DCB overlaying the patellar tendon was found to have variable degrees of integration with the native tendon. I expect this to be part of the remodelling process, which occurs over a long period of time, potentially up to two years, as previous studies have shown (Newsham-West et al., 2007, Wang, 2006). Furthermore, a variable degree of crimping of the collagen fibres of DCB was seen in all the sections as a part of the remodelling process taking place in DCB tissue. The lack of ossification or any evidence of resorption of DCB with the presence of cellularisation, vascularisation and crimping support the remodelling process of DCB into ligament structure.

Multiple studies have proven the osteoinductive and osteoconductive capabilities of demineralised bone (Spampata et al., 1992, Han et al., 2003). In my study, the DCB failed to ossify; instead, it turned into a ligament like structure. It is not clear how the remodelling

process was regulated. I believe the presence of DCB in a mechanical environment with close proximity to the patellar tendon and immediate post-operative mobilization influenced the remodelling process, leading to ligamentisation over ossification.

During the surgical procedure, no or very minimal dissection of the Hoffa fat pad was done, which is known to be a rich source of MSCs (Wickham et al., 2003). These MSCs are known precursors for the tenocytes and chondrocytes needed for DCB remodelling into ligament, which was found in DCB specimens in my study (Uysal and Mizuno, 2010). Tenocytes and chondrocytes were present in all different zones of DCB at different concentrations and different ratios; it is not clear how this was modulated. In my opinion, chondrocytes play a major part in the remodelling of DCB into a tendon-like structure, as it regulates the extracellular matrix. My argument is that the remodelling process starts with chondrocytic activities, with adsorption of the DCB bony-like extracellular matrix and layout of the tendeneous extracellular matrix for the tenocytes to form the tendon structure.

At the neo-enthesis, a fibrocartilaginous enthesis was seen with its characteristic four zone transition; these findings support the previous study conducted by our work group (Sundar et al., 2009c). The four zones were present in all sections examined, while tidemarks between transitions were absent in some of the sections. These tidemarks are known not to be present in all areas of the normal enthesis; in fact, its presence varies according to the differentiation of the enthesis and the thickness of the different zones of the enthesis (Benjamin et al., 1986).

4.5 Conclusion

This study proves that DCB can be used to repair and augment tendons. The results of my research show that it remodelled into a ligament-like structure regulated by host cells. DCB produced fibrocartilaginous enthesis with its characteristic four zones. Demineralised cortical bone therefore has a strong potential for use as a biological tendon graft, which presents solutions to the current clinical limitations and restrictions concerning available grafts.

Chapter 5

Discussion

5.1 General discussion

The current treatments for most of the musculoskeletal disorders are dependent on the human body's ability to heal and regenerate, this healing is either spontaneous or guided by medical or surgical intervention. However, this healing process might not result in optimal outcome, as in some tendon injuries. If this process fails, or if the outcome is not favourable, the treatment options are limited, surgeons usually consider replacement of the damaged tissue as a last resort aiming to improve function and control pain. Replacement of human tissue in musculoskeletal disorders has proven to be successful treatment strategy in some conditions, mainly in severe joint arthritis. Hip and knee joint replacement using artificial joints is now standard practice giving a successful outcome in most of the cases. Unfortunately this is an end stage procedure which is a result of current medical technology being unable to repair and replace articular cartilage. The situation is even worse when it comes to tendons and ligaments, there is no commercially available product for tendon replacement that gives a successful outcome which is of the same order as that seen for joint replacement. The most successful tendon and ligament replacement so far are either allografts or autografts but these two options are not without risks and limitations of donor site morbidity, infection and inflammation. The promise of tissue engineering is to enhance and improve healing, to provide a replacement tissue with better functional outcome and fewer associated risks. However to date and despite considerable investment, tissue engineering has not really delivered this promise.

The current commercially available tendon grafts have shown some success, though limitations and complications means that they are of limited value. The ideal tendon graft has yet to be found and extensive research is being carried out to identify a suitable graft material and processing technique. One of the most important areas is identify or

manufacture a graft material to repair and reattach the tendon back onto bone with the development of a functional enthesis. This has been the focus of my study and I have presented strong evidence that demineralised bone could be used as a graft which enhances reattachment of the tendon back onto the bone. My study is based on the assumption that implantation of the demineralised bone in a tendon environment with suitable mechanical stresses will result in ligamentisation of the demineralised bone rather than ossification.

The available knowledge supports the use of demineralised bone as a tendon graft, as it contains well aligned collagen I fibres. The majority of its collagen fibres are longitudinally organised following the Haversian system, longitudinal orientation was also found to be beneficial and improves cellular adhesions and remodelling (Vaquette et al., 2010, Mahapatra and Khan, 2011). Demineralised bone contains biological factors; growth factors such as BMPs which are proven to enhance healing of enthesis and regeneration of tendon substances (Gulotta and Rodeo, 2009, Edwards et al., 2011, Rodeo et al., 2012, Angeline and Rodeo, 2012, Wurgler-Hauri et al., 2007, Klatte-Schulz et al., 2013). The production of demineralised bone is not a complicated process, several techniques are available and each manufacturing institution has its own technique. Commercially, demineralised bone is available at a reasonable price.

Out of the available tissue engineered tendon graft materials; GraftJacket is showing promising results. It is used mainly as augmentation device for massive RC repair. Snyder and his colleagues (Snyder et al., 2009) retrieved a GraftJacket specimen after 3 months implantation in human shoulder for treatment of massive rotator cuff tear. They found cellular invasion, vascularisation, tenocytes and collagen fibre orientation to be a strong evidence of incorporation and remodelling of the graft material. Sano and his colleagues

(Sano et al., 2002) studied the changes in fascial autograft as augmentation graft for RC repair, he found neo-enthesis at 8 weeks with evidence of remodelling of the graft tissue into tendon. Similar to the findings in these studies, I found evidence in my study supporting the suggestion that DCB has similar potential to be used as tendon graft.

In my study demineralised bone implanted in live tissue did not ossify, it turned into ligament tissue, this contradicts findings by Urist who suggested that DBM formed bone by endochondral ossification in a soft tissue site in rats (Urist, 1965, Sampath et al., 1984). Interestingly studies on osseointegration of bone graft substitutes indicate that whilst mineralisation in ectopic sites readily happens in lower order animals it is less frequently seen in higher order animals such as in primates (Ripamonti et al., 1991).

The available knowledge supports remineralisation of the demineralised bone once implanted in live tissue; this remineralisation takes place as early as 4 weeks post implantation and becomes clearly evident by 12 weeks. While in my study the demineralised bone turned into ligament with evidence of ligamentisation by 12 weeks with complete absence of ossification. In my opinion this was as a result of difference in mechanobiology; in my study the DCB was implanted in tendon environment where DCB was exposed to immediate mobilisation after implantation with the associated mechanical forces found in tendon and ligament tissue. The DCB was also in direct contact with patellar tendon; a source of tenocytes and tendon growth factors. Furthermore, Reddi and Anderson identified the ossification process of the demineralised bone to be enchondral (Reddi and Anderson, 1976). In my study the DCB overlaying the enthesis formed fibrocartilage and calcified fibrocartilage while that overlaying the tendon turned into ligament. In the DCB tissue bridging the gap in the tendon, there was no evidence of bone or cartilage formation, the

characteristic chondrocytes and cartilaginous lacunae of the cartilage were not seen, nor the characteristic Haversian system of the bone. Within 12 weeks the DCB remodelled into fibrocartilage at the enthesis and ligament at the tendon, that denotes that 12 weeks are enough to remodel or show evidence of remodelling DCB into different tissues. If the demineralised bone was to ossify by enchondral process as traditionally expected or other ossification process, there would have been evidence of ossification or cartilage formation within the DCB within those 12 weeks; in my study no ossification process took place.

Failure of ossification in the DCB in my study might be due to biological and mechanical factors. Although the DCB was in direct contact with osteotomised bone (tibial tuberosity), which acts as a source of bone growth factors and osteoblasts, this direct contact resulted in neo-enthesis formation rather than ossification. This indicates that the mechanical factors may play an important role in non-ossification of the DCB.

In fracture hypertrophic non union it is widely accepted that excessive movement is the main reason for failure of union, in this situation the bony ends are usually covered with a sclerotic fibrous layer. Similarly in fibrous union of the fracture, excessive movement is thought to be the main cause of failure of ossification. These two examples support my assumption that the mechanical environment of the DCB in my study is the main reason for failure of ossification of the demineralised bone and resulted in remodelling into ligament. In a review of tendon and ligament engineering mechanical loading has been identified as an important factor in tissue engineering of tendons and ligaments (Hoffmann and Gross, 2006), and it has been postulated that the mechanical environment and loading is crucial for collagen orientation in early phase which will subsequently lead to maturation of tendon and ligament tissue. Similarly other studies identified the mechanical factors to play important role in remodelling

and regeneration of tendon tissue (Butler et al., 2008, Liu et al., 2008b, Mahapatra and Khan, 2011).

Interestingly, Jackson and his colleagues studied the use of demineralised bone in ACL reconstruction in goat model (Jackson et al., 1996), they came to similar findings of remodelling of the demineralised bone into ligament like construct with enhanced enthesis formation. Unlike my study, the demineralised bone was manufactured differently; also the demineralised bone graft was used intra-articularly surrounded by the synovial fluid which is thought to contain elements to inhibit ossification, while in my model the demineralised bone graft was used in extra-articular extra-synovial and arguably subcutaneous. In this study they also reported ossification of the demineralised bone graft in the bony tunnels indicating site-specific ossification which also confirms my findings and supports the mechanical theory behind the lack of ossification and the process of remodelling in the DCB graft.

Cellular invasion and vascularisation were clearly evident in my study, tenocytes and chondrocytes are seen in all areas of the DCB graft. Tenocytes are needed for graft remodelling and is expected to be found in all graft areas while chondrocytes are not known to be a part of tendon or ligament formation, its role in remodelling of the DCB into tendon structure is unclear. Chondrocytes are usually present in normal enthesis to maintain the fibrocartilaginous structure; they are expected to be found in the neo-enthesis for formation and remodelling of the DCB graft attachment to bone. Further research is needed to identify the role of chondrocytes in the remodelling of DCB. The enthesis acts as interface between tendon and bone, these two structures are biomechanically different, the importance of the enthesis is to transmit the forces between these two structures without disruption. It is subject to both tensile and compressive loads, the presence of chondrocytes is vital to maintain the

structure and regeneration of the enthesis and prevent permanent damage (Benjamin et al., 1992, Matyas et al., 1995, Cook and McDonagh, 1996, Claudepierre and Voisin, 2005). In my opinion chondrocytes might have been in the DCB graft for metabolism of the extracellular matrix and its protein content in preparation for the layout of the tendon extracellular matrix (Sampath et al., 1984, Fibbe, 2002). The other possibility is that the initial response for the implantation of the DCB in the host tissue is to ossify the DCB, the ossification of the demineralised bone is endochondral process as proved previously by Urist and by Reddi therefore chondrocytes invaded the graft tissue which later on failed to ossify the DCB as the mechanobiology of the graft tissue was in favour of remodelling over ossification, but this assumption does not explain the persistent presence of the chondrocytes in the DCB graft tissue 12 weeks after implantation.

My study has some limitation, there was no control group due to lack of funding, to overcome this I used the contralateral limb for each animal as a control. Although this is not ideal control group, studying the changes in force plate results in the contralateral limb revealed valuable information in support of my study. The sample size for my study was statistically defined; ideally the study should contain larger sample size to investigate the remodelling process at different time points and perhaps over a longer period. This would provide more understanding of the remodelling process and it would give stronger evidence of remodelling over longer time period. Also increased sample size would have allowed for mechanical testing of the construct alongside the histological analysis, mechanical testing in my study was not possible as it will result in destruction of the tissues with loss of histological evidence of remodelling.

Although histological remodelling of the DCB into ligament tissue was evident my study, immunohistological analysis would have added more value to the study. Investigating the presence of chondrogenic, osteogenic and fibrogenic markers would give better understanding of the remodelling process and the cascade of events. Immunofluorescence study of the neo-enthesis would confirm the presence of fibrocartilage and mineralised fibrocartilage layers, it would give more insight on the distribution of the collagen fibres and it might help understanding the role of chondrocytes in the DCB tissue.

5.2 future work

The data presented in my study gives a valuable insight into tendon grafting, demineralised bone is usually thought of as bone graft material, it is usually expected to ossify when implanted in live tissue. My study opens a new prospective for the demineralised bone; demineralised bone can be a suitable graft material for both soft and hard connective tissues. However more work is needed to develop this application; little is understood about regulation of MSCs and differentiation into different cellular lineage. It is known that MSCs can differentiate into osteoblasts, chondrocytes , fibroblasts and adipogenic cells (Fibbe, 2002, Ge et al., 2005) the control of this differentiation and the effect of the surrounding environment are not yet clearly understood. More work is also needed to understand the cascade behind the differentiation of MSCs and their autocrine and paracrine effects (Hoffmann and Gross, 2006). Moreover the biomechanical environment of the healing tendon needs more investigations; the current research activities are lacking strong evidence in favour of early or delayed, active or passive mobilisation. The mechanobiology of the healing tendon might need different post operative rehabilitation strategies if demineralised bone is used as soft tissue graft, with further work needed to identify the biomechanical factors that lead to ligamentisation of the demineralised bone and how these factors differ from those

leading to ossification of the demineralised bone. It might be valuable to conduct a similar study looking specifically at chondrogenic, osteogenic and fibrogenic markers of the DCB remodelling at different time points and over a longer time period. This might provide more understanding of the remodelling process and might explain the reason behind the presence of different cell types in the DCB tissue.

Demineralised bone is known to have variable content of growth factors including BMPs, cytokines and others, to date there is no study that investigated the exact number of factors expected to be found in the demineralised bone. Also, the quantification of these factors and the ratios of these factors to each other are not known. This might be a difficult task as the presence of these growth factors and their quantities might differ from person to another and variations related to age, gender, ethnicity and other parameters are expected. Previous studies have proven that the recruitment and concentration BMPs and other growth factors vary according to the phase of the remodelling process following a cascade of events (Anderson et al., 2001), this variation and the exact cascade is not understood, further research is needed to quantify these factors at different phases of remodelling.

In my study I have induced a model of tendon loss with acute repair; this is usually associated with local haematoma and recruitment of acute inflammatory response. In chronic tendon injuries there is a lack of this response with no local haematoma formation. In clinical practice, part of the procedure for repair of chronic tendon injury is freshening of the edges of the repaired tendon to stimulate acute reaction which is thought to promote healing. This acute reaction and the local haematoma might have an effect on remodelling of the DCB and recruitment of local cells, research is needed to investigate this effect and to identify factors in the haematoma that can influence this remodelling process. In chronic tendon injuries,

disuse tendon atrophy is seen and the outcome of repair in chronic injuries is usually less favourable when compared with acutely repaired tendon injury. DCB might prove to be beneficial in chronic tendon injuries as it enhances remodelling and healing, it might stimulate the atrophic tendon tissue and induce regenerative hypertrophic effect. The effect of DCB grafting on healing of chronic tendon injuries needs further research to explore a wider scope for DCB applications in tendon healing and regeneration.

Xenograft tissue has been used in human body with marked success; in cardiac surgery for example the use of porcine heart valve is a well known procedure with widely reported success. In Jackson et al study as well as my study, the demineralised bone grafts were obtained from animals of the same species, therefore no host tissue immune reaction was found, it is not clear if a xenograft was used instead of an allograft, an immune reaction would have occurred or not. Studying the immunological response to the demineralised bone from different species and the methods to control and suppress this response might prove to be of great value as it can lead to development of the DCB of animal source to be used as tendon graft in humans. This will lead to increased availability of the graft materials, avoid the donor site morbidity and decrease the cost of the graft material.

Although demineralisation techniques usually follow Urist protocol or a modification of it, manufacturing and processing of demineralised bone vary widely. Several studies investigated the commercially available demineralised bone for its processing techniques and the growth factors content, the general consensus among these studies that there are obvious variations between different products as well as marked differences between different patches of the same product (Wildemann et al., 2007, Gruskin et al., 2012). Standardisation of the

demineralised bone manufacturing is needed to limit the effect of different processing and sterilisation methods on the quality and quantity of growth factors (Glowacki, 2005).

In my study I have investigated the effect of gamma irradiation on the tensile strength, more research is needed to investigate the effect of irradiation on the biological activity and the growth factors available in the demineralised bone. Also more investigations are needed to compare the effect of other sterilisation techniques on the demineralised bone. Similarly, demineralisation can be achieved by treating the bone with various substances; comparative research is needed to identify the effect of each substance on the properties of the demineralised bone.

Optimisation of the surgical technique is also needed, in my study one animal failed to show satisfactory progression on force plate analysis, I found patella alta on plain radiographs, this is thought to be due to either elongation of the graft or cheese wiring of the stitches through the tendon-DCB construct. The DCB has similar characteristics to tendon when it comes to stitching; the stitch can cut through easily (cheese-wire) if force used in longitudinal parallel direction to the tendon fibres, while resistance is seen when the force on the stitch was applied in perpendicular direction to tendon fibres. Therefore, as in clinical practice, whipstitch gives better control and hold of the DCB graft and hence used in my study. Several whipstitches and tendon stitches are used in clinical practice; comparative study is needed to identify the most suitable stitching technique to be used on DCB.

In medical practice, Patient Reported Outcome Measures (PROMs) tools are becoming more important in assessment of treatment outcomes. Its value comes from involving the patients in the assessment of the treatment as well as taking in consideration the demographic,

financial and quality of life impact (Black et al., 2014, Coronini-Cronberg et al., 2013). Currently in UK four procedures are investigated with PROMs, namely; total hip replacement, total knee replacement, varicose vein excision and inguinal hernia repair. It is expected that PROMs tools will expand to involve all surgical procedures including tendon repair, early development of these tools for assessment of tendon repair procedures is expected to help in treatment decisions and guide the improvement in tendon research and graft development.

In our laboratory, the investigation of different applications of demineralised bone continues, research proposal for preclinical human trials using demineralised bone for tendon – bone healing is underway. Also animal study with demineralised bone used as tendon graft enhanced with MSCs and other growth factors is under consideration. Although my study provides strong evidence of ligamentisation of the DCB; the DCB was retrieved after 12 weeks, perhaps longer period is needed to investigate the long term outcome and whether full ligamentisation will happen or not, also to exclude delayed ossification.

Demineralised bone has proven to be versatile material; it provides the scaffold and biological factors, further research is needed to investigate the possibility of seeding different types of cells and controlling the medium to initiate ossification or ligamentisation process. Achieving this might open the door for in vitro production of tendons and bones for repair and replacement of damaged tissue, this production can be patient specific by recruiting different types of cells from the patient. This will eliminate the risk of tissue rejection, immune reaction, donor site morbidity and suboptimal outcome which usually result by using the conventional graft materials currently available.

Chapter 6

Conclusion

I have shown that Allogenic DCB in strip form can be used to replace the distal 1 cm of the ovine patellar tendon adjacent to the tibial tuberosity. This results in the formation of an enthesis, which is similar in morphology to a normal enthesis and over time functional weight bearing significantly increased from 44% at 3 weeks post surgery to 79% at week 12. On retrieval none of the specimens showed any evidence of ossification of the DCB.

Histological analysis proved formation of neo-enthesis with presence of fibrocartilage and mineralised fibrocartilage in all the specimens. DCB grafts contained host cells and showed evidence of vascularisation. Remodelling of the collagen leading to ligamentisation of the DCB was proved by the presence of crimp in the DCB graft on polarized microscopy.

My research proves that DCB can be used to repair and augment tendons. The results of this research show that it remodelled into a ligament-like structure regulated by host cells. DCB produced fibrocartilaginous enthesis with its characteristic four zones. Demineralised cortical bone therefore has a strong potential for use as a biological tendon graft, which presents solutions to the current clinical limitations and restrictions concerning available grafts.

Published abstracts related to this study

A new application of demineralised bone as tendon substitute; animal study

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Repair of tendon injuries aims to restore length, mechanical strength and function. We hypothesise that Demineralised Cortical Bone (DCB) present in biological tendon environment will result in remodelling of the DCB into ligament tissue. A cadaveric study was carried out to optimize the technique. The distal 1cm of the patellar tendon was excised and DCB was used to bridge the defect. 4 models were examined, Model-1: one anchor, Model-2: 2 anchors, Model-3: 2 anchors with double looped off-loading thread, Model-4: 2 anchors with 3 threads off-loading loop. 6 mature sheep undergone surgical resection of the distal 1cm of the right patellar tendon. Repair was done using DCB with 2 anchors. Immediate mobilisation was allowed, animals were sacrificed at 12 weeks. Force plate assessments were done at weeks 3, 6, 9 and 12. Radiographs were taken and pQCT scan was done prior to histological analysis. In the cadaveric study, the median failure force for the 4 models; 250 N, 290 N, 767 N and 934 N respectively. In the animal study, none of the specimens showed evidence of ossification of the DCB. One animal failed to show satisfactory progress, X-rays showed patella alta, on specimen retrieval there was no damage to the DCB and sutures and no evidence of anchor pullout. Functional weight bearing was 79% at week 12. Histological analysis proved remodelling of the collagen leading to ligamentisation of the DCB. Results prove that DCB can be used as biological tendon substitute, combined with the use of suture bone anchor early mobilisation can be achieved. (Elnikety et al., 2013a)**.**

The use of demineralised cortical bone for tendon and ligament repair

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Treatment of tendon and ligament injuries remains challenging; the aim is to find a biocompatible substance with mechanical and structural properties that replicate those of normal tendon and ligament. We examined the mechanical properties of Demineralised Cortical Bone (DCB) after gamma irradiation (GI) and freeze drying (FD). We also used different techniques for repairing bone-tendon-bone with DCB in order to measure the mechanical performance of the construct. DCB specimens were allocated into 4 groups; FD, GI, combination of both or none. The maximum tensile forces and stresses were measured. 4 cadaveric models of repair of 1cm patellar tendon defect using DCB were designed; model-1 using one bone anchor, Model-2 using 2 bone anchors, Model-3 off-loading by continuous thread looped twice through bony tunnels, Model-4 off-loading with 3 hand braided threads. Force to failure and mode were recorded for each sample. FD groups results were statistically higher ($p=<0.05$) compared to non-FD groups, while there was no statistical difference between GI and non-GI groups. The median failure force for model-1: 250 N, model-2: 290 N, model-3: 767 N and model-4: 934 N. There was no statistical significance between model-1 and model-2 ($p=0.249$), however statistical significance was found between other models ($p=<0.006$). GI has no significant effect on mechanical strength of the DCB while FD may have positive effect on its mechanical strength. Our study shows that a tendon rupture can be successfully augmented with DCB giving initial appropriate mechanical strength suitable for in vivo use providing the biological reactions to the graft are favourable.

(Elnikety et al., 2013b).

Effect of gamma irradiation and freeze drying on the maximum tensile strength of the cortical demineralised bone matrix

S Elnikety, C Pendegrass and G Blunn

Introduction: Demineralised Bone Matrix (DBM) is widely used in Orthopaedics and dentistry as a bone graft substitute and may be used to augment bone formation in load bearing applications.In this study we examine the effect of gamma irradiation and freeze drying on the tensile strength of Demineralised Cortical Bone (DCB).

Methods: Tibias were harvested from mature ewes and cut into bony strips. Demineralisation was done using 0.6M HCL and confirmed by X-ray. Specimens were washed until a pH of 7.0 +/_ 0.2 was achieved in the washing solutions. Specimens were allocated into 4 groups; group (A) non freeze dried non gamma irradiated, group (B) freeze dried non gamma irradiated, group (C) non freeze dried gamma irradiated mention the level of gamma irradiation and group (D) freeze dried and gamma irradiated. The maximum tensile force and stress were measured. Statistical analysis using the Mann-Whitney U test was carried out.

Results: The Median of maximum tensile force for group (A) was 218 N, group (B) was 306 N, group (C) was 263 N and for group (D) was 676 N.

Group (D) results were statistically higher ($p =< 0.05$) compared to group (A) and (C), while there was no statistical significance compared to group (B).

Conclusion: Previously published studies suggested the possibility of using DCB as ACL graft substitute. We examined the effect of gamma radiation as the most common sterilisation technique in medical field and the freeze drying as a possible technique for long term storage on the tensile strength of the DCB. Freeze drying significantly increases the tensile strength of the DCB while gamma irradiation has no significant effect. Our results indicate that freeze dried gamma irradiated DCB can be used as a ligament substitute.

(Elnikety et al., 2013c).

The use of cortical demineralised bone matrix (DBM) for repair and augmentation of patellar tendon; cadaveric study

S Elnikety, C Pendegrass and G Blunn

Introduction: Tendon injuries remain challenging, secondary healing and prolonged immobilisation result in suboptimal outcome. Previous study by our group showed that demineralised bone matrix can result in faster healing of a tendon enthesis. The aim of this study is to test different ways augmenting tendon with DBM to enhance tendon regeneration.

Methods: DBM strips were prepared from tibias of mature ewes. Patella, patellar tendon and tibias were dissected and the distal 1 cm of the patellar tendon was excised.

4 models were designed; Model-1, DBM strip was used to bridge the gap between the tendon and the tibial tuberosity. The DBM strip was stitched to the tendon using one bone anchor. Model-2, similar to model 1 with the use of 2 anchors. Model-3, similar to model 2, construct was off loaded by continuous thread looped twice through bony tunnels sited in the patella and in the tibial tuberosity. Model-4, similar to model 3 with 3 threads as off loading loop. All models were tested for pullout force and mode of failure.

Results: The median failure force for model-1 (N=5) was 250 N while for model-2 (N=5) was 290 N. In model-3 and model-4 failure of the off loading loop was used as end point, 6 samples were tested in each model. Median failure force of model-3 was 767 N and for model-4 was 934 N. There was no statistical significance between model-1 and model-2 ($p=0.249$), however statistical significance was found between other models ($p=< 0.006$).

Discussion A study published in 1996 proved that cortical DBM can be used as ACL graft with evidence of ligamentisation. DBM provides a biologic scaffold with potential for use as ligament and tendon replacement. Our study shows that a tendon rupture can be augmented with DBM giving intial appropriate mechanical strength suitable for *in-vivo* use.

(Elnikety et al., 2013d).

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