The Elucidation and Strategic Modification of Flexor Tendon Healing Mechanisms

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A thesis submitted to the University of London for the Degree of Doctor of Medicine

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0.1 THESIS ABSTRACT

0.1.1 Introduction and aims

16,000 flexor tendon repair operations are performed annually in the UK; these are frequently complicated by adhesions, causing significant morbidity. The uncertainty over which cells are responsible for the tendon healing mechanisms persists. We aimed to develop a novel experimental model to elucidate the possible role and migratory response of synovial sheath fibroblasts during tendon healing and to determine the effects of differing environment and stimuli on the cellular processes in the healing tendon.

0.1.2 Materials and methods

Rat synovial sheath cells were labelled with a lipophilic tracer dye, and an injury made in the adjacent flexor tendon. Tendons were then harvested at 1,3,5 and 7 days and frozen sectioned. The location of the labelled fibroblasts was determined using ultra-violet microscopy.

In a separate experimental series, rat flexor tendons received different injury type / mobilisation or immobilisation / and TGF-β1 or saline control application. Tendons were harvested at 7 and 14 days, fixed, sectioned and HandE stained. Cell densities in the injury region were determined.

0.1.3 Results

By 24 hours, labelled synovial fibroblasts were observed to have migrated from the sheath to the zone of injury, with numbers increasing at 3 and 5 days, but diminishing by day 7.

In the second experimental model, tendons injured with a superficial scrape, or immobilised post-injury showed a significant increase in relative cellularity in the region of the injury.

0.1.4 Discussion

These results suggest that synovial cells are involved in the early stages of tendon healing, and migrate from the synovial sheath into the healing tendon. Fibroblast density, in the rat model, is modulated by type of injury, immobilisation, but not TGF-β1 application. This may correlate clinically with collagen deposition and adhesion formation.

0.2 DEDICATION

This thesis is dedicated to my parents.

0.3 ACKNOWLEDGEMENTS

I would like to thank my supervisor, Mr. A O Grobbelaar for his continuous support throughout this period of study and his constant encouragement. I also wish to thank my London advisor, Professor D A McGrouther for his enthusiasm and dedication of the project and Dr V Mudera for invaluable scientific supervision and support. I am indebted to Professor R Sanders, whose vision as the director of The RAFT Institute and faith in appointing me has made this study possible.

I would also like to thank those who have provided additional advice, technical assistance and support in the course of this investigation. Dr C Linge for guidance on cell biology and Mrs. E Clayton for her immuno-histochemical technical expertise and assistance.

I am also grateful to my colleagues at RAFT who made this an invaluable experience, from which I have learned a great deal and enjoyed thoroughly, not least the laboratory manager Mr. J Shelton who maintained an impeccable environment and those who helped look after my cells.

I could not have completed this work without the financial support of the Trustees of The Restoration of Appearance and function Trust, The BUPA Foundation and Smith's Charity.

0.4 DECLARATION OF ORIGINALITY

I declare that the laboratory research for this thesis is original and that the ideas were developed in conjunction with my supervisor and advisors.

I performed the experiments myself with the guidance and technical assistance of the scientific staff at The RAFT Institute.

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0.7 COMMON ABBREVIATIONS USED

5FU	5-Fluorouracil
bFGF	Basic Fibroblast Growth Factor 1
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine percolate
DIP(J)	Distal Interphalangeal (Joint)
ECM	Extra-cellular Matrix
FDP	Flexor Digitorum Profundus
FDS	Flexor Digitorum Superficialis
MCP(J)	Metacarpal Phalangeal (Joint)
MMPs	Matrix Metalloproteinases
NSAIDS	Non-steroidal anti-inflammatory agents
PIP(J)	Proximal Interphalangeal (Joint)
TGF-β1	Transforming Growth Factor Beta 1

CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

This thesis represents 2 years work based at the RAFT Institute of Plastic Surgery, and is a continuation of work commenced by Mr Martin Jones, who primarily investigated digital tendon-surface fibroblast migration in response to injury.

A review of the available literature revealed no direct evidence to support or refute the *extrinsic mechanism* of tendon healing. Chapter 3 describes the development of an animal model to test the hypothesis that extrinsic cells contribute to tendon healing. Chapter 4 describes the results obtained.

It was also apparent that, whilst many different clinical regimes and variables exist in the treatment of tendon injuries, their effects on the tendon fibroblasts were not well documented. Chapter 5 describes the results of experiments designed to investigate this.

Literature is reviewed in the tendon structure and function, tendon healing mechanisms, adhesion formation and information concerning our choices of experimental protocol.

1.2 TENDON STRUCTURE AND FUNCTION

Digital Flexor Tendons are categorised into intrasynovial or extrasynovial, referring to whether the tendon is surrounded by a fibrous synovial sheath. Fibroblasts are present in all parts of the tendon; those at different levels may represent different sub-populations (Hunter JM, 2003); each with a modified role in cell migration and matrix production in term of their response to wounding. Other cell types, in addition to the aforementioned sub-populations of fibroblast are also to be found within the tendon substance; these can include endothelial and nerve cells as well as cells capable of mounting an inflammatory response to injury, made up of macrophages, neutrophils and lymphocytes.

1.2.1 Tendon cell populations and nomenclature

Traditionally, the tendon inner core has been referred to as the endotenon and, in intrasynovial tendons, the outer surface layer is called the epitenon; this surface layer is analogous to the visceral surface of the flexor sheath. In extrasynovial tendons there is a loose covering of connective tissue and elastic fibres, with a vascular network that also contains elastic fibres, referred to as paratenon (Hunter JM, 2003).

In this thesis, consistent with recent trends in the literature, and for the sake of clarity, sub-populations of fibroblasts are referred to "surface-derived fibroblasts", when from the most superficial layers of the tendon and "core-derived' fibroblasts", when from the deeper layers.

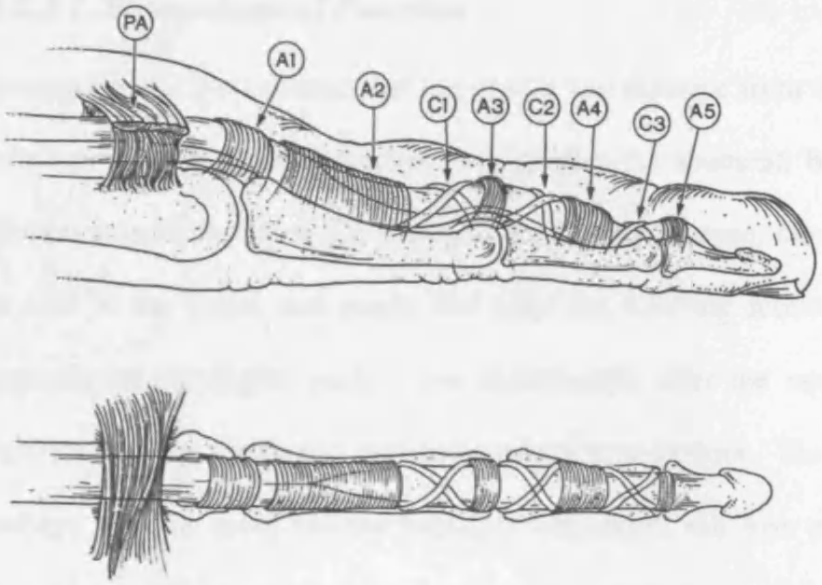


Figure 1 - Lateral (top) and palmar (bottom) views of a finger depict the components of the digital flexor sheath. The sturdy annular pulleys (A1, A2, A3, A4, and A5) are important biomechanically in keeping the tendons closely applied to the phalanges. The thin, pliable cruciate pulleys (C1, C2, and C3) collapse to allow full digital flexion. A recently described addition (Doyle, 1988) is the palmar aponeurosis pulley (PA) which adds to the biomechanical efficiency of the sheath system. Source – Strickland *et al.* (2000).

1.2.2 The flexor tendon sheath - function

The synovial sheath has been proposed to have a 3-fold function (Lundborg, 1980):

(1) To facilitate smooth gliding of the tendons by virtue of their smooth synovial lining.

(2) As a bursa, containing synovial fluid, to bathe the tendon and aid its nutrition.

(3) The retinacular component acts as a fulcrum, adding a mechanical advantage to flexion; acting as an efficient mechanism to hold the tendons close to the digital bone and joint (Idler, 1985).

1.2.2.1 Biomechanical Function

Biomechanical laws dictate that the greater the distance from the axis of joint rotation when a force is applied, the greater the moment, but the less the motion around that joint; the converse is also true. Hence, the closer a tendon is held to the bones and joints, the more the resulting movement. Loss of portions of the digital pulleys can significantly alter the normal integrated balance between the flexor, intrinsic, and extensor tendons. The A2 and the A4 pulleys are the most bio-mechanically important; the loss of a substantial portion of either may diminish digital motion and power and lead to flexion contractures of interphalangeal joints (Idler, 1985).

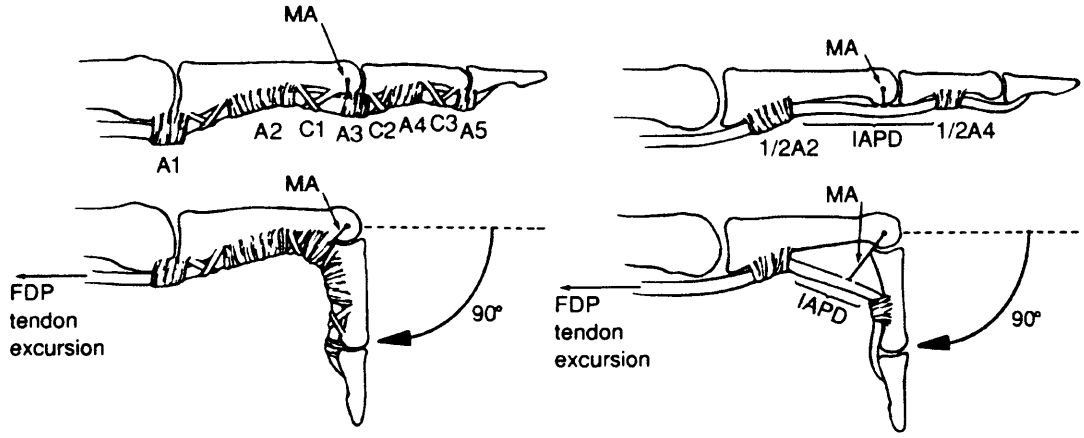


Figure 2 - The function of the digital flexor tendon pulley system. Biomechanical alteration that results from excision of the distal half of the A2 pulley. The distance between the A2 and A4 pulley is the intra-annular pulley distance (IAPD). The moment arm is increased and a greater FDP tendon excursion is required to produce 90° flexion due to bowstringing. From Strickland *et al.* (2000).

1.2.2.2 The Flexor Tendon Sheath - Smooth Gliding Function:

The sheath produces an almost frictionless surface for tendon gliding, improving the efficiency and dexterity of digital movement (Cohen, 1987; Idler, 1985).

1.2.2.3 The Flexor Tendon Sheath - Nutrition function:

The synovial lining produces synovial type fluid which has been shown to be important in tendon nutrition, and thus healing (Lundborg, 1980). Between the fibrous thickenings, the sheath has thin outpouchings that act as reservoirs for fluid.

1.2.2.4 The Flexor Tendon Sheath – Anatomy - Sheath and Pulley System:

The digital flexor sheath is a closed synovial system consisting of both membranous and retinacular portions (Cohen, 1987; Idler, 1985). The membranous portion is comprised of visceral and parietal layers that invest the FDP and FDS tendons in the distal aspect of the hand. The retinacular component consists of tissue condensations arranged in cruciform, annular, and transverse patterns overlying the membranous, or synovial, lining. The membranous portion of the sheath appears macroscopically as a number of cul-de-sacs, or plicae, that interdigitate between both the tendons and the retinacular tissue condensations. The first cul-de-sac is located approximately 10-14 mm proximal to the distal metacarpal head and represents the point of transition between the parietal and visceral layers of synovium. This out-pouching occurs for each separate tendon, in effect forming 2 separate plicae (Doyle, 1988).

The sheath commences at the palmar plate of the metacarpophalangeal (MP) joint, with the A1 pulley. A condensation of the palmar aponeurosis results in the palmar aponeurosis (PA) pulley. Where the tendon overlies a joint, the sheath is thin, resulting in the cruciate pulleys. Where the flexor sheath

overlies the phalanges, it forms the tough annular pulleys A2 and A4. Additional annular pulleys overlie the palmar plates of the MP, PIP, and DIP joints, respectively (A1, A3, A5 pulleys). Distally, the parietal layer of synovium forms plicae between each of the retinacular elements of the pulley system. The synovium ends distally, forming a final single cul-de-sac prior to the insertion of the FDP tendon on the distal phalanx. Ultrastructural analysis by Cohen and Kaplan has confirmed that the flexor tendon sheath is a continuous uninterrupted membrane (Cohen, 1987). A visceral layer of synovium invests all structures entering the sheath. The parietal and visceral layers are qualitatively similar, and synoviocytes are morphologically identical throughout the length of the flexor sheath (Strauch, 1985).

Beneath the annular pulleys and on tendon surfaces distant from the vincula and cul-de-sacs, the synovial layer is attenuated. In these areas, the synovial cells are sparse and widely spaced, and the supporting collagen is thin and laminar; furthermore, capillaries are rarely seen. However, the cul-de-sacs and areas of vincular origin demonstrate a synovial layer several cells thick, complete with capillaries and loosely organised collagen bundles (Strauch, 1985).

1.3 TENDON NUTRITION

1.3.1 Introduction

Although an early account of tendon repair was written by Galen 131-201 AD, quoted in Adamson (1961), it was not until the late nineteenth century that tendons were found to contain blood vessels (Ludwig C, 1872). Since that time interest has centred on the source and the clinical importance of these vessels. Three distinct sources, the musculotendinous junction, the osseotendinous junction, and the vincular vessels have been shown by Mayer to contribute to the vascular network of the tendons (Mayer, 1916).

1.3.2 Extrasynovial tendon vasculature

From their musculotendinous origin to the level of the A1 pulley, (i.e. the extrasynovial area) the flexor tendons have been shown to receive their blood supply from the surrounding paratenon (Doyle, 1988). The paratenon is a thin layer of connective tissue that invests the tendon. Segmental blood vessels arise within this tissue, enter the tendon, and run longitudinally between tendon fascicles to supply nutrients (Doyle, 1988).

1.3.3 Intrasynovial tendon vasculature

At the point that the tendons enter the flexor sheath, they are no longer surrounded by paratenon and are incorporated within the visceral lining of synovium.

1.3.3.1 The Vincula

The vinculae are remnants of mesotenon and provide the blood supply and nutrition to the flexor tendons. The vincular system is supplied by the transverse communicating branches of the common digital artery (Figure 3). One short and one long vinculum supply each FDS and FDP tendon. The vinculae receive their blood vessels through transverse communicating branches of the common digital artery located on the dorsum of the flexor tendons (Strauch, 1985) and may provide the blood supply that participates in early healing of flexor tendons, also serving as a check-rein to limit proximal retraction of a lacerated tendon.

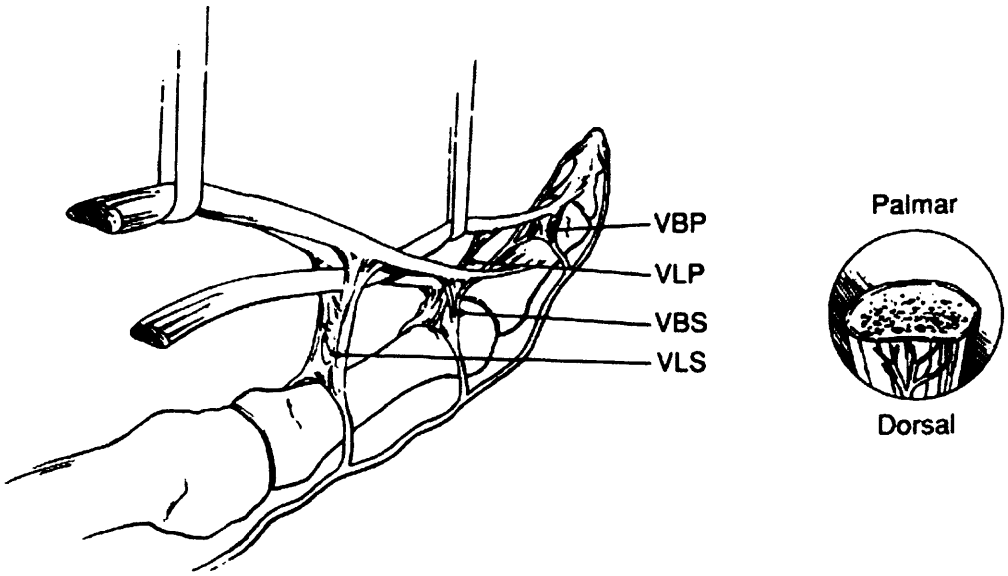


Figure 3 - Vincular system of the digital flexor tendons, supplied by the transverse communicating branches of the common digital artery (Strickland, 2000).

1.3.3.2 Synovial fluid

Synovial fluid is produced by specialised synoviocytes in the synovial lining of the digital flexors, its function has been previously described.

1.3.4 Flexor tendon avascular zones

Mayer (Mayer, 1916) gave an early description of palmar avascular zones within the intrasynovial portion of the FDS tendon. Later that century, these tendons were found to possess deep longitudinal vessels, as well as fine capillary-like anastomoses on the surface (Edwards, 1946). Peacock (1959) later showed that the three sources of blood supply described by Mayer (1916) (Mayer, 1916) in fact contributed in different proportions; that the segmental vincular blood supply was the most important and the two other sources were relatively minor.

Tendon vascularity has been investigated primarily by perfusion studies, and in a variety of animal models. These differences have led to differing reports on intrasynovial vascularity patterns. Lundborg and Myrhage, in 1977 (Lundborg, 1977) reported longitudinal extrasynovial core vessels entering the sheath, but giving way to a sizeable avascular zone on the palmar aspect of the tendon, corresponding to an area between the A2 and A4 pulleys in human cadaveric material. However the dorsum of the tendon in this region was well vascularised due to a continuation of the longitudinal vessels. In contrast, Gelberman *et al.* (1991), using the canine forepaw long flexors, stated that the avascular area in this species was total, with both dorsal and palmar aspects devoid of blood vessels.

Work done by Jones *et al.* in 2000 used a novel technique to assess the rabbit long flexor tendon immunohistochemically, using the endothelial cell surface marker CD31 targeted with a specific monoclonal mouse-anti-human antibody. They demonstrated a consistent deep tendon avascular zone between the A2

and A4 pulley in the rabbit forepaw, although, interestingly, this was not the case in the hindpaw, with dorsally orientated longitudinal vessels coursing the length of the intrasynovial tendon.

This work was furthered by a study by Harrison *et al.* using anti-CD31 monoclonal antibody immunohistochemical visualisation in the human cadaveric FDP tendon. They found that also vessel density ratios varied with anatomic location and that although areas of low vascularity exist on the palmar aspect of the tendon, there seem to be no truly avascular zones (Harrison, 2003), this work also demonstrated that the vascular supply seems to be primary positioned within the dorsal surface of the tendon (Harrison, 2003).

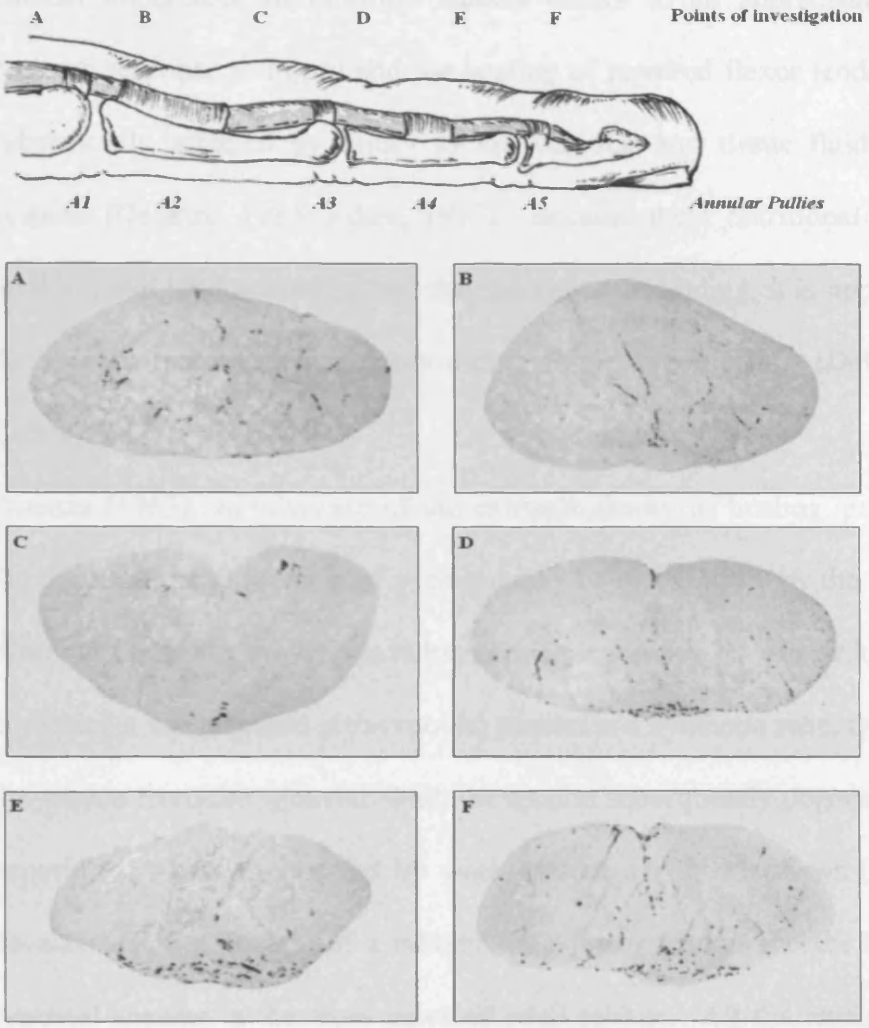


Figure 4 - Immunohistochemical staining patterns at different anatomical positions of the human digital flexor tendon. The locations are illustrated by Figure 4. In each image, top = ventral, left = ulnar.

1.3.5 Importance of vascularity - reflecting healing mechanism

It has long been established that tendons are not inert, but have the capacity to heal, assuming that there is a source from which they can derive nutrition (Skoog, 1954). However, the actual source of this nutrition has been debated; since the turn of the 20th century, controversy has existed as to which method, diffusion or perfusion, is more important for tendon nutrition and healing. The

clinical importance of nutrition studies relates to an appreciation that the biologic response to injury and the healing of repaired flexor tendons may be substantially affected by injury to the vascular and tissue fluid nutritional systems (Delattre, 1983; Eiken, 1977). Because these nutritional sources are vital to rapid tendon healing and the restoration of gliding, it is imperative that the surgeon respect their integrity during all reparative efforts (Delattre, 1983; Eiken, 1977).

Potenza (1963), an advocate of the extrinsic theory of healing, proposed that the metabolic requirements of a tendon may be met entirely by the diffusion of synovial fluid into the tendon substance (Potenza, 1962). He deduced this by enclosing a vascularised intrasynovial tendon in a synthetic tube, thus isolating the tendon from the synovial fluid; the tendon subsequently degenerated. This experiment was corroborated by work carried out by Matthews (1976), who devascularised a portion of a rabbits' long flexor tendon sheaths in the intact synovial sheaths in the front paws of adult rabbits. All the tendon segments were found to have survived as viable "loose bodies", and no adhesions developed. Active remodelling processes occurred over the cut ends of the segments and degenerative changes were confined to the most deeply lying tissue. These experiments confirm the existence of a synovial fluid pathway of nutrition, concerned, it is suggested, with nourishing the more superficial layers of the tendon.

1.4 TENDON INJURY

1.4.1 Aetiology

Tendon pathology and disruption of tendon function has two main aetiologies. Firstly trauma; in a variety of settings e.g. work, home and leisure. Secondarily; resulting from degenerative diseases such as rheumatoid arthritis, a chronic inflammatory disease which weakens tendons, rendering them prone to rupture, sometimes with minimal activity or loading. Typically, a hospital dealing with hand injuries will see between 10 and 20 tendon injuries per week, with over 16,000 recorded "primary tendon repair" operations (Source: NHS OPCS, 2002 figures) carried out every year in the UK, with significant fiscal and temporal burden to the NHS.

1.4.2 Anatomy of Injury

For the purpose of description, tendon injuries of the hand are divided up into zones (Mayer, 1916) (Table 1). Roughly half of all flexor tendon injuries occur in zone II. With respect to the deep and superficial flexors of the fingers, the anatomical location of the tendon insult can be ascribed to one of five zones. They are: zone 1 distal to the FDS insertion; zone 2 from the A1 pulley to the FDS insertion; zone 3 from the distal end of the carpal tunnel to the A1 pulley; zone 4 carpal tunnel and zone 5 proximal to the carpal tunnel. In the thumb, zone nomenclature is similar, but with different anatomical demarcations; zone 1 distal to the interphalangeal joint; zone 2 from the A1 pulley to the interphalangeal joint; zone 3 thenar eminence; zone 4 carpal

tunnel and zone 5 proximal to the carpal tunnel. The most commonly affected zone with the poorest outcome of repair is zone 2.

Zone	Anatomical Area
Zone 1	FDS insertion to FDP insertion
Zone 2	Zone 1 to proximal part of A1 pulley
Zone 3	Zone 2 to distal edge of flexor retinaculum
Zone 4	Within carpal tunnel
Zone 5	Proximal to carpal tunnel

Table 1 - Clinical responses to injury, according to location, based on original work by Verdan, 1972 (Verdan, 1972)

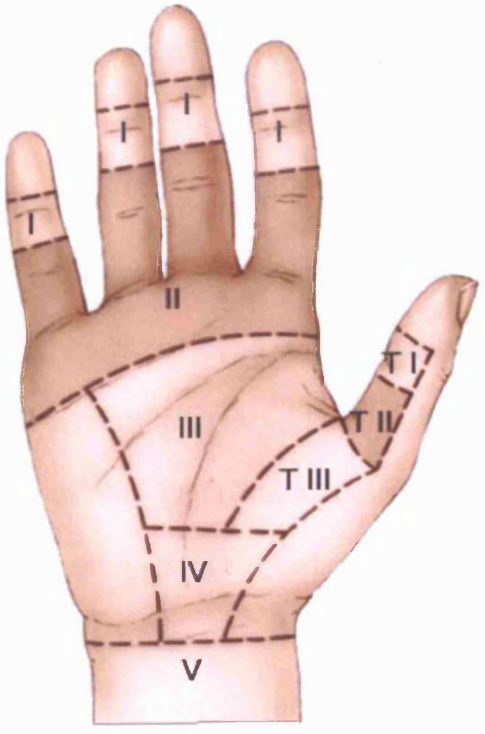


Figure 5 – Anatomical Location of Flexor Tendon Repair Zones (Verdan, 1972)

1.4.3 Adhesion formation

Subsequent to tendon injury, adhesions may form around a tendon, which consist of dense layers of fibrous tissue. Adhesion formation can lead to impaired mobility of the flexor tendon, potentially leading to prolonged post-operative recovery, stiffness and reduced function of the hand. The result is reduced manual dexterity, potentially making everyday tasks such as dressing and eating difficult, affecting recreational activities and frequently impeding the patient's ability to work. Subjective clinical experience confirms that tendon laceration or rupture frequently can have devastating consequences through this disruption of hand function. Any alteration in tendon function can reduce the finger dexterity once taken for granted, making even the simplest of tasks a chore (Vamhidy, 1987). The frequency of such injuries and the high complication rate presents enormous potential implications in terms of the high demand on NHS resources, from surgeons to hand therapists.

1.4.3.1 Adhesion pathophysiology and aetiology

In 1962, Potenza found that adhesions were formed by fibroblasts and endothelial cells (Potenza, 1962). From his observations he deduced that invasion of this granulation tissue was a necessary part of the postulated extrinsic healing process and formed in proportion to the amount of tissue injury with resultant new collagen formation. Although acting as conduits for neovascularisation of the injury, adhesions have been shown to be detrimental to mobility and to lead to a reduction in hand function (Gelberman, 1985). It was shown that in order for adhesions to develop, a combination of tendon suturing, immobilisation and concurrent digital sheath injury were required

(Matthews, 1976). They showed that the counter-experiment of an incompletely divided tendon, without suture material and lying freely within an otherwise uninjured digital sheath, healed normally without the appearance of adhesions. In the same year Furlow (1976) put forward a counter hypothesis to that of Potenza's, concluding that intrasynovial tendon injury healed through an intrinsic capacity and adhesions were not necessary for the process. In addition he showed that early motion prevented or disrupted new adhesion formation.

1.4.4 Significance of Injury

Without the finger dexterity once taken for granted, even the simplest of tasks becomes a chore; and, at worst, loss of livelihood may result. The only proven means of restoring function is surgical repair. If the tendon is cleanly severed, direct suture is often possible. With more extensive trauma, tendon integrity may only be achieved with the use of a tendon graft; this involves harvesting a suitable donor tendon and using it to bridge the zone of injury. Improvements in surgical technique and suture materials have led to better results, but sub-optimal results are still common (Jaibaji, 2000). Post-operative recovery may be prolonged or even sub-optimal due to fibrous tissue in-growth from surrounding structures. These adhesions restrict mobility and lead to a reduction in hand function (Gelberman, 1985).

1.5 TENDON HEALING

1.5.1 History of tendon healing and repair

Flexor tendon injuries were already treated in antiquity by Hippocrates, Galen and Avicenne (quoted in Chamay, 1997). Indeed, Galen, a surgeon to the gladiators, described his observations of forearm flexor tendon and nerve repair around A.D. 160. Interestingly, Galen came to the conclusion that surgery was undesirable due to resultant convulsions! (Kleinert, 1995). Over seven centuries later, Avicenna 980-1087 A.D. reported on direct tendon repair but his observations were largely dismissed as a consequence of Galen's earlier teachings (Kleinert, 1995). Several investigators again tried to change this thinking during the fifteenth and sixteenth century without great success (Guy de Chauliac (14^{th} Century) quoted by Kleinert *et al.* (1995)). In 1677 Job Baster of Zeland performed successful silk-suture repairs in three separate cases; however it was not until the late eighteenth century that tendon healing concepts finally rid themselves of Galen's ideas. Marc Anthony Petit (1720) recorded successful immediate and delayed tenorrhaphy with silk suture and Nuck (1789) performed a successful division and repair of the external flexor in the canine model. Nevertheless, even as late as 1837, tenorrhaphy had its sceptics, Rognetta and Mondiere (1837) denounced the procedure, but as different surgical procedures were introduced, tendon repair was progressively refined. The first recorded importance occurrence of tendon repair with digital sheath preservation at the time of surgery was by Codivilla in 1889. Since Avicenna, other surgeons have attempted to repair flexor tendon injuries, but

outcomes were often poor, probably due to problems related to the use of unsuitable suture materials, ignorance of the basic rules of asepsis and the absence of antiseptics until the second half of the 19th century. The first recorded successful flexor tendon grafts in man were performed by K. Biesalski in 1910, E.Lexer in 1912 and L. Mayer in 1916.

St. Bunnell, in 1918, developed various pull-out direct suture procedures, but faced with the problems of adhesions; he abandoned this technique and proposed not to repair flexors in the digital tunnels but to graft them. He defined the famous zone which he called "No man's land", which subsequently became Claude Verdan's zone II, in 1959. In 1960, Verdan published his first series of tendon repairs, maintained by 2 pins in zone II with comparable results to those obtained after grafting. In 1967, H. Kleinert, with a novel mobile suture technique, pioneered direct tendon repair in zone II. Two-stage grafts were introduced in 1965 under the impetus of J. Hunter, who had revised and popularised the studies conducted by A. Bassett and R.E. Caroll in 1950. (All references quoted in (Adamson, 1961)).

1.5.2 Tendon healing principles

Tendon healing involves an inflammatory phase from 48 to 72 hours after repair, a fibroblastic- or collagen-producing phase from 5 days to 4 weeks, and a remodelling phase that continues until approximately 112 days. During the inflammatory phase of tendon healing, the strength of the repair is almost entirely that which is imparted by the suture itself, with a modest contribution from the fibrin clot between the tendon ends. Strength increases rapidly during the fibroblastic collagen-producing phase when granulation tissue is formed in

the defect. When extrinsic healing predominates, adhesions between the tendon and its surrounding tissues are inevitable, while healing that is largely based on intrinsic cellular activity will result in fewer, less-dense adhesions (All quoted from (Strickland, 2000)).

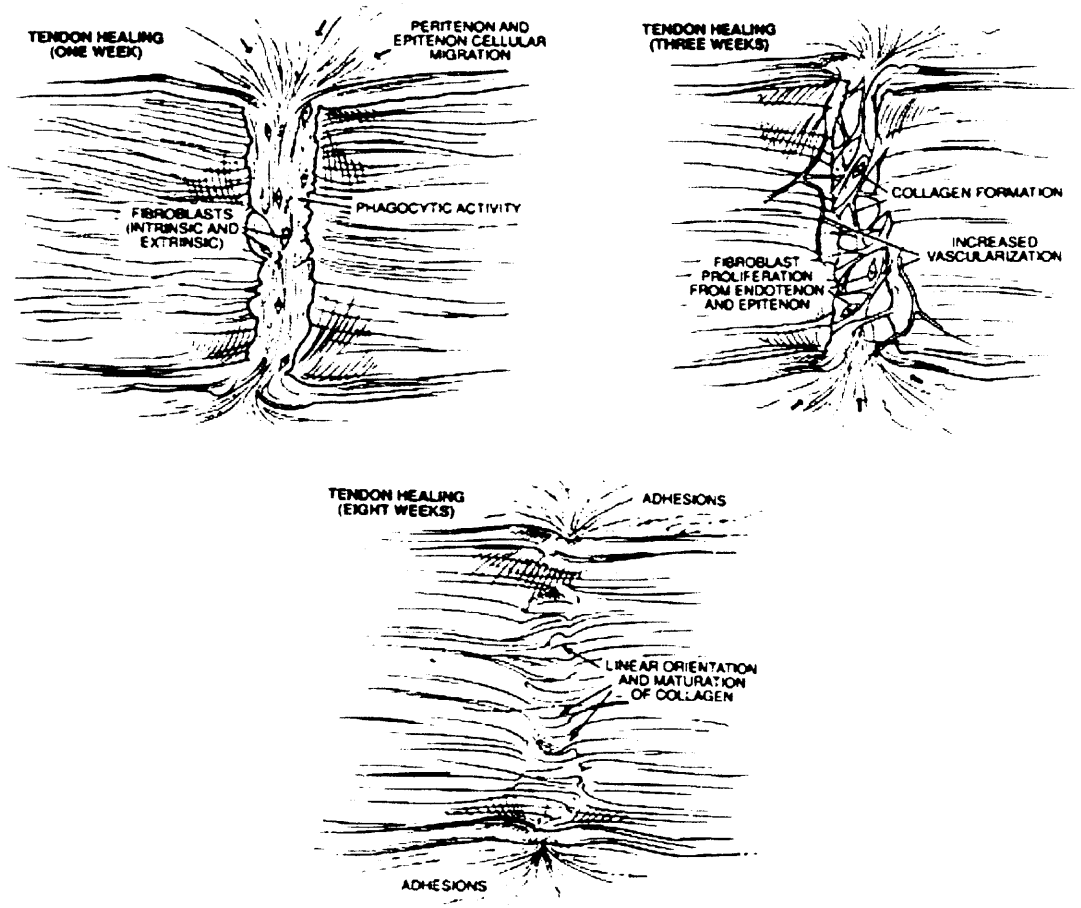


Figure 6 The biologic sequence of tendon healing. At 1 week an inflammatory response predominates and the laceration site is filled with cells. At 3 weeks there is marked fibroblastic proliferation with both synthesis and resorption of collagen. The fibroblasts and collagen can be seen to be irregularly orientated at this stage. At 8 weeks the collagen is more mature and realigned in linear a fashion. From Strickland (2000).

The mechanisms of the initiation and perpetuation of scar tissue formation must be appreciated as it is thought to be in the early stages of established techniques of tendon repair that the predisposition to rupture and adhesion formation occur. Most investigators (Cohen, 1992) agree that the cell at the

centre of the cascade of events leading to scar formation is the fibroblast. These cells, found in many tissues, have the common characteristic of being able to form and re-organise structural proteins. With regards to scar tissue formation, the 2 important features of fibroblasts are their capacity to proliferate to a given stimulus and their capacity to degrade and disorganise the extracellular matrix (ECM). It has not been formally established *in vivo* whether the extent and degree to which this occurs, giving a healing and scarring response, is universally equal for fibroblasts from different sites. Under normal circumstances, fibroblasts are metabolically quiescent and do not appear to divide. Once a stimulus has been introduced, however, there is a burst of activity by local and recruited fibroblasts, resulting in cell migration, division, and ECM synthesis and remodelling (Cohen, 1992).

The extent of ECM remodelling is determined by the degree of formation and breakdown of the matrix. To some extent, remodelling of the matrix is regulated by the cellular secretion of enzymes that break down the ECM; these enzymes are the matrix metalloproteinases (MMPs) (Oshiro, 2003; Riley, 2002 Ragoowansi, 2001). Importantly, synovial fibroblasts produce a greater quantity of the matrix MMPs than the endotenon fibroblasts (particularly on day 7 after seeding) and are better at reorganizing the ECM than endotenon fibroblasts (shown to be statistically significant at days 3 and 7 after seeding in both rabbit and rat cells). The synovial fibroblasts have the capacity to be involved in more ECM breakdown and migration (Khan, 1998).

1.5.3 Intrinsic versus Extrinsic Healing

1.5.3.1 Historical evidence

The debate concerning the origin of the cells responsible for repair after tendon injury has existed for over four decades. Skoog and Persson (Skoog T, 1954) suggested that tendons lack the capacity for intrinsic healing, and that in-growth of cells from surrounding tissues is necessary to enable healing of tendon injuries. Potenza's work agreed with this theory (Potenza AD, 1964), and advocated the extrinsic theory of tendon healing in 1962, hypothesising that tendon healing must rely on the post-injury fibroplastic response of the damaged surrounding tissues. This conclusion was based upon a series of experiments on the flexor tendons of dog, which showed that no intrinsic healing took place in the tendon ends. This theory was challenged a decade later by Eiken *et al.* (1975) and Matthews and Richards (1974). They introduced segments of rabbit flexor tendons into closed synovial compartments, finding cellular fibroplasia and collagen synthesis, i.e. evidence of intrinsic healing. Furlow (1976) subsequently stated that tendons must heal "by a mechanism intrinsic to the tendon, without the aid of extratendinous cells," a concrete hypothecation of the intrinsic healing mechanism, which has subsequently been reiterated by a number of different groups including Lundborg *et al.* (1978).

Many further observations have been made of the biological characteristics of the tendon-derived and synovium-derived tendon cells. It is well established that both cell types can divide, (Becker, 1981; Eiken, 1981; Manske, 1984) migrate (Becker, 1981; Eiken, 1981; Manske, 1984) and modify their matrices

in response to external stress (Eiken, 1981 ; Gelberman, 1982). Becker (1981) observed chicken sublimis tendon explants in tissue culture; tendon cells were shown to migrate concentrically around a 2mm window in the explant and tendon cell migration and proliferation were observed until the window was confluent with cells. These cells were confirmed to be tenocytes, and biochemical analyses confirmed tenocyte collagen synthesis (Becker, 1981). Gelberman, in 1983, examined healing canine flexor tendons, treated with either total immobilisation or mobilisation. Immobilised tendons healed by ingrowth of connective tissue from the digital sheath and cellular proliferation of the endotenon; in contrast, the mobilised tendons healed by proliferation and migration of cells from the epitenon. Epitenon cells exhibited greater cellular activity and collagen production at each interval compared with cells of the immobilised repairs (Gelberman, 1983).

Manske in 1984 looked at rabbit flexor tendons with transverse lacerations, demonstrating that tenocytes have an intrinsic capacity to participate in the repair process in the absence of extrinsic cell sources and without the benefit of nutrition from a circulating blood supply or the influence of synovial fluid. The cellular processes involved differentiation and migration, and collagen synthesis of tenocyte fibroblasts (Manske, 1984). Chang *et al.*, in 1998, investigated bFGF messenger RNA (mRNA) expression in a rabbit flexor tendon wound healing model. Whilst few tenocytes and tendon sheath cells expressed bFGF mRNA in unwounded tendons, tendons subjected to transection and repair exhibited an increased signal for bFGF mRNA, in both resident tenocytes concentrated along the epitenon and infiltrating fibroblasts

and inflammatory cells from the tendon sheath. These data demonstrated normal tenocytes and tendon sheath cells to be capable of bFGF production, and that the upregulation of this angiogenic cytokine occurs in tenocytes as well as in tendon sheath fibroblasts and inflammatory cells (Chang, 1998). Khan *et al.*, in 1996, using immunohistochemical studies, found that both the cells lining the synovial sheath and covering the surface of tendons were relatively more reactive in the early period post insult, as shown by expression of a range of inflammatory markers (Khan, 1996). Whilst these similarities have been determined, some cellular differences have also been observed; for example, the different cell types differ in terms of proliferation rates (Khan, 1996) and extra-cellular matrix synthesis (Abrahamsson, 1996). The tendon surface layer also shows greater vascularity, cellular and biochemical activity than the inner core cells in response to injury; Gelberman *et al.*, in 1992, using mRNA probe techniques, found higher levels of pro-collagen expression in the tendon surface layer, (Gelberman, 1992). Kakar *et al.* have also shown that fibroblasts from the tendon core have a decreased ability to contract a collagen gel in culture, compared to cells of synovial origin (Kakar, 1998).

1.5.3.2 The lack of direct evidence

Whilst differences have been determined between the two cells types, there is no evidence to suggest that the healing response should be localised to one cell type, leading many to suggest that it is a combination of the intrinsic and extrinsic healing mechanisms which together lead to the tendon healing process. Jones *et al.* have demonstrated that epitenon-derived cells migrate into the core of the tendon in an *in vivo* model (Jones ME, 2002).

1.5.4 Adhesions

1.5.4.1 Adhesion formation and control

Methods of controlling adhesion can be divided into pre-operative, intra-operative and post-operative.

1.5.4.2 Pre-operative methods of adhesion reduction

The administration of systemic drug therapies as a method of tendon adhesion reduction both pre and postoperatively, has been attempted for over 50 years, with varying degrees of reported success. Steroids administered both systemically and by local injection have been shown to diminish adhesion formation in animal studies. Carstam, in 1953, examined the role of parenteral Cortisone in the reduction of post-surgical adhesions, finding that if started several days prior to surgery and continued postoperatively; adhesion formation could be suppressed, as measured by tensiometer pulls. In spite of these findings, parenteral steroids have not become standard procedure due to the associated increased rupture and infection rates (Douglas, 1967). With the realisation that adhesions represented an inflammatory process, attention was turned to the investigation of non-steroidal anti-inflammatory agents (NSAIDS). Bateman *et al.*, (1982) showed that a parenteral administration of ibuprofen reduced intra-abdominal adhesion formation in a rabbit animal model. With this in mind, topical application of ibuprofen at the time of tendon repair has been shown to lead to a 50% reduction in adhesion formation, in the animal model (Kulick, 1986).

1.5.4.3 Intra-operative methods of adhesion reduction.

In parallel with the preoperative methods of adhesion reduction, research was being performed into strategies aimed at reducing adhesions at the time of surgical repair. These included different techniques of tenorrhaphy, different suture materials, barrier methods around the repair site and the application of chemical substances around the repair site, as described below.

The intra-operative application of soluble polypeptides, including growth factors, hormones, and chemotactic factors such as fibronectin has been extensively investigated (Abrahamsson, 1991; Amiel, 1989; Chang, 1998; Duffy FJ, 1995; Khan, 1996). These factors play a role in both normal and pathologic processes, and their clinical manipulation could potentially improve the outcome of tendon repair and rehabilitation.

During the immediate postoperative period the entire strength of the repair has been shown to be dependent on both the suture material and its method of insertion (Ketchum, 1985). The aim has therefore been to provide a repair that was strong enough to mobilise without rupture, but that produces the least amount of disruption to the healing ends so as to minimise the interference with tendon neovascularisation. The majority of suture methods have two components, a core suture and an outer epi-tendinous suture. The site of placement of the peripheral suture has also been shown to affect the strength of repair and prevent gap formation. Mashadi, in 1992, found that if the peripheral suture included tendon fibres, it was 83% stronger than the traditional purely epitendinous suture.

There seems to be a general consensus that most two strand methods are comparable in terms of clinical outcome. It has also been shown that the greater number of times the suture crosses over the repair site, the stronger it is (Aoki, 1995; Bhatia, 1992; Mashadi, 1992), confirmed by studies that show that the Savage six-strand technique has three times the strength of the most popular two strand Modified Kessler method (Savage, 1989).

Another important development in adhesion reduction has been the recognition of the need for meticulous repair of the tendon sheath, in fact such importance has been placed on sheath presence, that its reconstruction has been encouraged in situations where primary closure is not possible (Mashadi, 1992). Autologous options have included veins (Strauch, 1985), synovial bursae, sheaths from toes and even neighbouring digits (Eiken, 1977). These inter-positions, whether used to recreate a defective sheath, or placed between an intact sheath and the repair site have all been reported as physical barriers that help to prevent adhesion formation.

Chemical application to the repair site at the time of surgery has also received much attention. The aim is to reduce the 'scarring' of the healing tendon wound. A wide range of experimental substances have been investigated. They include topical ibuprofen (Kulick, 1986), Dextran 70 (Eiken, 1975; Green, 1986), sodium hyaluronate (Hagberg, 1992) and 5-Fluorouracil again with differing results.

1.5.4.4 Postoperative methods of adhesion reduction

As early as 1912, the importance of mobilisation post surgery has been debated. Lexor (quoted from Adamson (1961)), documented the benefits of

mobilising the repair from the sixth day following repair (Adamson, 1961). This concept of movement post-repair was challenged by Potenza (1962), who believed that a period of postoperative immobilisation was required to allow adhesion build-up, which he felt was necessary to effect tendon healing. This once gold standard treatment is now only accepted as postoperative treatment for very young or uncooperative children. The turning point that led to the realisation that post operative mobility held the key to better results came from the observations of Kleinert (1973). 87% of their tendon repair procedures were either good or excellent (researcher's own subjective criteria) when mobilised with controlled passive motion. Their regime employed a rubber band fixed to the nail of the affected digit, and then attached to the flexor aspect of the forearm, allowing active extension but passive flexion.

Gelberman *et al.* (1985 & 1986) improved operative outcome with passive mobilisation, with evidence that it enhanced healing by stimulating maturation of the tendon wound, simultaneously with the remodelling of tendon scar. Hitchcock *et al.* (1987) and Aoki *et al.* (1994) added to these studies with their findings that active (in contrast to passive) mobilisation applies stress to the sutured tendon, enhancing the strength of the repair and the biologic response, obviating the loss of tensile strength during the first 3 weeks of healing. From these reports, (Aoki, 1994 & 1995, Hitchcock, 1987) it has been clinically accepted that the most effective method of returning strength and excursion to repaired tendons involves the use of a strong, gap-resistant suture technique followed by the application of early post-repair controlled motion stress.

1.6 ANIMAL MODELS IN THE STUDY OF TENDON HEALING

Research has, and still does, rely on animal work, in order for improvements in the outcome of tendon repair to be made. These models allow the hypotheses of disease pathogenesis and its treatment to be tested. In the experimental studies performed within this thesis, we have complied with recognised criteria that should be adhered to when choosing an animal model, facilitating comparisons with the human condition under investigation. The size of animal should be large enough to allow adequate and reproducible tissue manipulation; the animal must be available and affordable, giving sufficient numbers to allow statistical comparison; finally the tissue must be amenable to measurement in a controlled reproducible fashion (Carpenter *et al.*, 1999).

Tendon models can broadly be divided into intrasynovial and extrasynovial types. A number of different animal models have been employed in the study of tendons, including mouse, rat, chicken, rabbit, dog, cow, monkey and horse. (Gaughan *et al.*, 1991; Norrie, 1975; Watkins *et al.*, 1985; Williams *et al.*, 1980). Comparisons between species have also been conducted (Gelberman *et al.*, 1984).

Not only have different species been used, but the type of injury inflicted has varied, from complete transection and repair (Frykman, 1993), partial transection (Frykman, 1993 ; Manske, 1984), crush injury, avulsion injury to a partial window tenotomy (Iwuagwu, 1998). The conclusions drawn have often

been contradictory or in part confusing; this is perhaps unsurprising due to the range of material studied.

The migration work outlined in Chapter 3 is, in part, an adaptation of a previously documented partial tenotomy window for use in the flexor tendons of the rat hindpaw, which was originally put forward by Matthew in 1987 (Matthew, 1987), to study collagen fibril formation in the healing wound. McGrouther and Iwuagwu (1998), have also employed this model for investigation into the early cellular response to injury in the rat extensor tendon.

The tendon surface response to injury also uses a rat model, as this model was the smallest in size to allow the procedure to be carried out.

1.7 VITAL DYES AND THEIR ROLE IN CELLULAR STUDIES

Vital dyes are stains that are selectively taken up by living cells, hence labelling them, and therefore can be used to study a number of cellular processes. In this thesis, we have mainly employed DiI, which is a long-chain dialkylcarbocyanine, widely used as a *neuronal tracer* in living and fixed tissues and cells. DiI labelling does not seem to appreciably affect cell viability, development or basic physiological properties. DiI-labelled motor-neurons reportedly have remained viable for up to four weeks in culture and up to one year *in vivo* (McNeilly, 1996). Importantly, the dyes do not transfer from labelled to unlabelled cells. These dialkylcarbocyanines are available in a variety of spectroscopic and cellular labelling properties. Another vital dye employed in this thesis is the lipophilic membrane stain 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine percholate (DiI). Its absorption and emission maxima are 549nm and 565nm respectively. In general it has been shown that there is no transfer from labelled to unlabelled cells, although several reports have documented small amounts of transfer with cell membrane disruption associated with sectioning at temperatures of 40°C (McNeilly, 1996).

Crawford *et al.*, in 1989, utilised vital fluorescent staining of human endothelial cells, fibroblasts, and monocytes in the assessment of surface morphology for studying cell growth and interactions on viable cells colonising artificial heart valves and vascular grafts (Crawford, 1989). McNeilly, Banes

et al. in 1996 demonstrated that vital dyes can be used to give a very high level of detail, using them to delineate gap junctions. They also demonstrated that vital dyes can be used successfully in labelling tendon cells *in vivo.* Their objective was to determine how the tendons respond to mechanical load. Vital dyes were used in unfixed cryosections of adult rat digital flexor tendons, and successfully stained them with DiI to demonstrate cell shape (McNeilly, 1996). For this study, a variety of cellular labels were assessed *in vivo*: DiI had the most appropriate characteristics: lack of leakage from cells, cellular specificity and sufficient longevity. Jones *et al.* (2001) have quantified the migration of tendon surface cells into the core following injury. We have used the same concentration of DiI here in order to selectively stain the synovial fibroblasts. The fading of DiI at seven days could again be due to division of the cells once they reach the core, metabolic irradiation of the dye by the cell, or even apoptosis of the surface-derived cells once they have fulfilled their function.

CHAPTER 2: MATERIALS AND METHODS

2.1 SYNOVIAL SHEATH CELL MIGRATORY RESPONSE TO FLEXOR TENDON INJURY

2.1.1 Introduction

This study was undertaken to test the hypothesis that synovial cells undergo a migratory response to intrasynovial flexor tendon injury. A variety of different methods were assessed to test this hypothesis; these are described in Chapter 3. The experimental method described below is the model successfully developed and utilised in the study. Prior to this study, no model was available which would permit the testing of the study hypothesis, that is to facilitate the selective labelling of the sheath lining (synovial cells) alone, hence the reason for the development of a novel model.

2.1.2 Experimental subjects

A rat model was used to assess cell motility *in vivo.* The primary rationale for this was the previous successful experimental use of a rat model for both flexor and extensor tendon window injuries, originally described by Matthew *et al.*, 1987 and Jones *et al.* 2003. The study by Jones *et al.* (2003) investigated the migratory response of tendon surface cells to injury, this had also been carried out in a rat model. By utilising a similar model and by using a similar methodology, comparison between the experiments would be possible.

Male Sprague Dawley rats, with weights between 250 and 300g, obtained from the Biological Services Department at The Northwick Park Institute for Medical Research, were used for this *in vivo* study. They were housed in

single racked units with sawdust bedding; a period of 72 hours was allowed for acclimatisation after being purchased, during which time they were fed and watered on demand. Regular assessment of the animals' general condition and subsequent surgical wounds were carried out in accordance with the U.K. Home Office Guide for the Care and Use of Laboratory Animals 1996.

2.1.3 Use of vital dye - Pilot studies

Pilot studies had been previously carried out to assess the efficacy and suitability of vital dyes in the assessment of surface fibroblast migration in response to a surgical window injury. Cell Tracker (Molecular Probes, Eugene, USA) was assessed initially, due to its successful use in similar studies, and its apparent specificity and sensitivity. However, our *in vitro* studies, demonstrated that this dye was only retained in cells for a maximum of 48 hours and therefore an alternative vital dye DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine percolate) (Molecular Probes, Eugene, USA) was chosen for the *in vivo* work. This lipophilic long-chain dialkylcarbocyanine tracer has been found to be highly fluorescent and photo-stable when incorporated into and retained by membranes. A degree of lateral plasma membrane diffusion enables entire cell staining. Its absorption and emission wavelengths were 549nm and 565nm respectively. It gave a strong red fluorescence when visualised under the TRITC filter (Excitation wavelength: 525-555 nanometers, Barrier wavelength: 590-650 nanometers). DiI was used at a concentration of 10μM (diluted with 0.9% Sodium chloride) for use *in vivo*.

2.1.4 Mode of anaesthesia

General anaesthesia was induced using intramuscular injection of Hypnorm (Janssen, London, UK) and intra-peritoneal injection of Diazepam (Janssen, London, UK). Animals were positioned on a heated pad throughout surgery to maintain body temperature, and regular assessments of the depth of anaesthesia performed at regular intervals, with top up doses of Hypnorm (Janssen, London, UK) being administered as required.

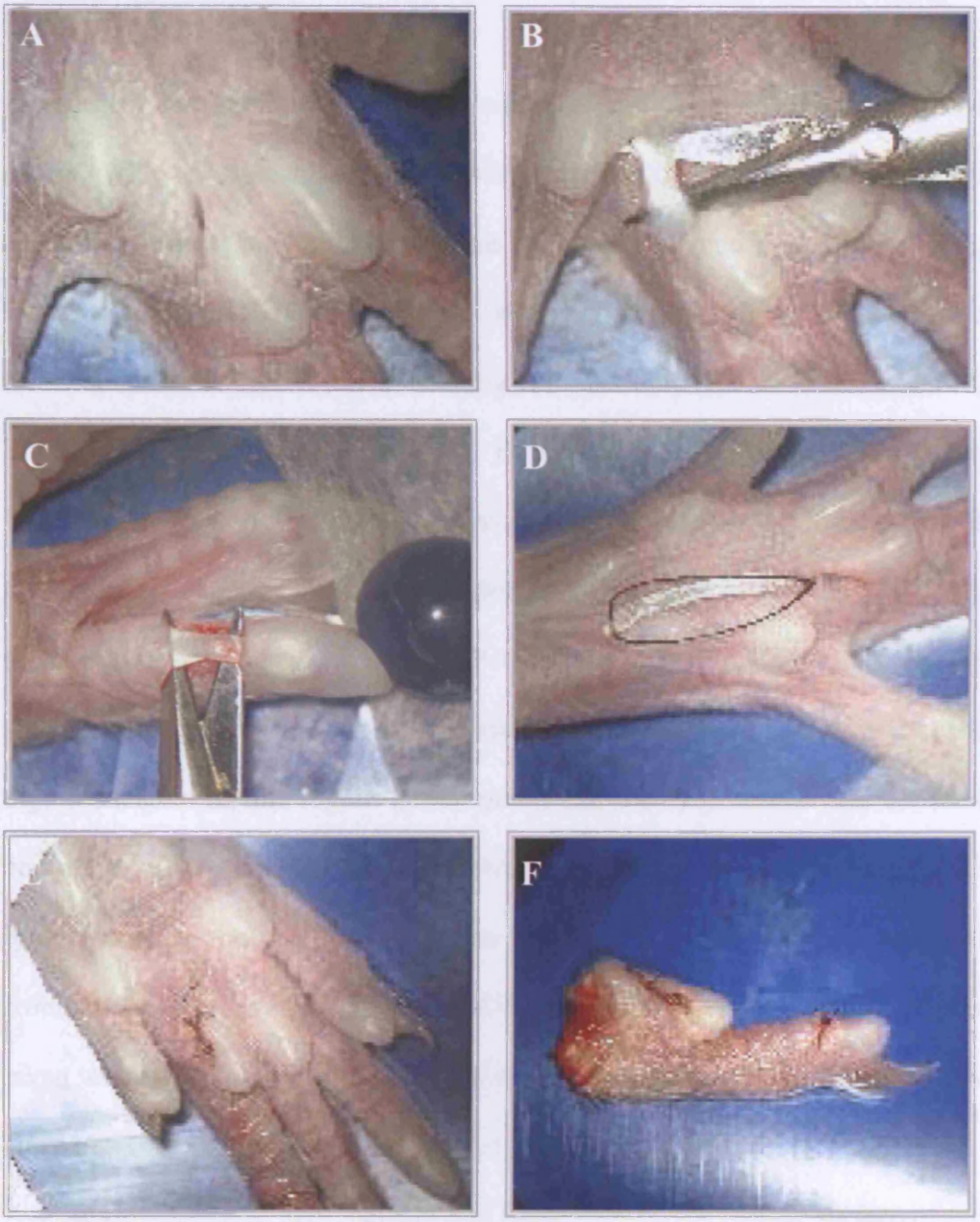


Figure 7. Operative procedure to study the Synovial Sheath Cell migratory response to Flexor Tendon injury. A: The rat hindpaw was used. B: Exposure of the flexor tendon complex C: Distal exposure of the FDP tendon, prior to suture placement. D: FDP tendon exposed through the proximal incision, suture placed to enable tendon to be re-inserted into the synovial sheath. E – Wound closed after tendon re-inserted. F – Digit removed complete for processing.

2.1.5 Operative procedure

All surgery was conducted with the aid of a surgical dissecting microscope (Zeiss, Welwyn Garden City, UK) with variable X2-10 zoom magnification and microsurgical instruments, on digit 3 of the right hind paw (Figure 7A). Under anaesthesia a rubber tourniquet was applied to the right hindpaw of the rat. The foot was then disinfected using chlorhexidine (Baxter Healthcare Ltd, Thetford, UK) 0.5% in spirit (95% Ethanol / 5% Methanol (R.A. Lamb, Eastbourne, UK)). A longitudinal incision was then made in the plantar skin of the hindpaw, exposing the flexor tendon complex in its extrasynovial portion (Figure 7B). The larger rounder FDP tendon was gently separated from the superficial structures and the FDS tendon in the palm, prior to its entry to the digital sheath. In this region it was noted that the deep tendon had bilateral connective tissue connections; these were severed to allow extraction of the intrasynovial portion of the tendon into the wound, and the deep tendon freed from surrounding tissue. Once the FDP tendon was exposed, great care was taken to prevent drying of the tissue with frequent irrigation using sterile 0.9% Normal Saline (R.A. Lamb, Eastbourne, UK).

A separate transverse skin incision was then made on the plantar surface of the operated digit at the distal interphalangeal joint (Figure 7C) and the incision was deepened to expose the synovial flexor tendon sheath. The synovial flexor sheath was carefully opened through a transverse incision, and the FDP tendon exposed. 2 8/0 silk sutures (Ethicon, London, UK) were then placed, perpendicular to each other, within the tendon end; these sutures placed to allow the tendon to be subsequently pulled back into the tendon sheath. The

flexor tendon was then divided transversely, distal to the aforementioned sutures.

Returning to the proximal wound, gentle traction was applied, with the aid of a retractor. This traction, applied to the deep tendon, removed the flexor tendon from its synovial sheath, hence leaving the synovial sheath empty, and into the palmar incision (Figure 7D).

With the FDP (and FDS) tendons removed from the synovial sheath, the synovial sheath was left vacant. A small piece of cotton wool was soaked in a 10μM solution of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine percholate) (Molecular Probes Inc. OR, USA). The cotton wool was rolled into a longitudinal bolster, passed into the synovial sheath, in contact with the synovium, and left *in situ* for 5 minutes. During this period, the microscope light was turned off to prevent drying, and the wound covered to exclude potentially drying surgical light and prevent dye decay. The cotton wool bolster was moistened with the DiI solution at 1.5 and 3 minutes into the application. The cotton wool bolster was removed after a period of 5 minutes and the synovial sheath then irrigated for 1 minute with a slow flow of 0.9% normal saline, again from proximal to distal, to prevent the exposed FDP tendon from becoming coated with vital dye. During this time, the exposed flexor tendon was kept wrapped in saline soaked gauze, and great care taken to prevent any vital dye from coming into contact with the flexor tendon.

A surgical window, as described by Matthew *et al.* and Jones *et al.*, was then created in the intrasynovial portion of the exposed deep tendon to mimic tendon injury, using a size 11 surgical blade. Two parallel through and through

cuts were made in the tendon one third of the way in from the side, measuring approximately 5 mm in length. The resulting "window" was completed by cutting the middle third strip, proximal and distal with a pair of micro scissors (Figure 7C). The proximal extent of the window injury was marked with a single 8/0 prolene suture to guide later sectioning and analysis.

The FDP tendon was replaced within its synovial sheath by means of traction on the distal sutures placed prior to its removal, and returned to its anatomical position, with the window injury now within the intrasynovial environment. At no time was the integrity of the synovial sheath breached (around the wound site). Using the silk sutures placed initially (and used to replace the tendon back into the synovial sheath), the distal tendon continuity was re-established by 2 mattress sutures, joining the tendon at the original line of division.

Skin closure of both incisions was effected with interrupted 8/0 Prolene sutures (Ethicon Ltd). The skin of the palm was dressed with Opsite spray (Smith & Nephew Healthcare, Hull, UK) and the tourniquet released. The rat was nursed in a heated propagator unit until fully recovered and then transferred back to its single cage. Food and water were administered *ad-libitum*. Mobilisation was not hindered in anyway, and all rats were observed to be fully mobile after the procedure.

A total of 28 rats were used in the study, with 1 digit operated per rat.

2.1.6 Tendon harvest

Rats were culled using a 1.5ml intra-peritoneal injection of Lethobarb (pentobarbitone sodium, Virbac Pty Ltd, NSW, Australia). 4 rats were culled at 0 and 2 hours to verify that the vital dye labelling was confined to the

synovial cells. 6 rats were culled at each of the subsequent time periods: 1, 3, 5 and 7 days.

In each case, the operated digit was removed at the MCPJ and snap frozen within a cryo-vial in liquid nitrogen and stored at -80°C (Figure 7F). The reason for taking the entire digit was to keep the deep flexor in extension, the surrounding architecture was also preserved.

2.1.7 Tendon processing

The digit proximal and distal to the zone of injury was excised, leaving the part of the digit containing the experimental injury area (Figures 8A&B). The tendons were then placed in the Cryostat chamber (Model: OTF/AS, Bright Instrument Company Ltd, London, UK) for 20 minutes prior to sectioning to equilibrate the sample to the correct temperature of between -36 and -40°C. The individual tendon digits were embedded 'end-on' onto the metal block (Figures 8C&D) with Cryo-M-Bed (Bright Instrument Company Ltd, London, UK). A further 10-minute period of acclimatisation was allowed.

The digits were then sectioned at 8μm thickness using a cryostat (Figure 9A), with a tungsten blade (Figure 9B), with 4 representative sections cut from each digit. The area of interest, i.e. the surgical window, was delineated by the marker suture placed at the time of operation. The resulting sections were placed onto Polylysine (Sigma-Aldrich, Gillingham, UK) coated slides, to facilitate adhesion.

After 30 minutes air-drying time in a darkened container, the slides were coated in Vectashield (Vector, London, UK) and a cover-slip applied. They

were then placed in slide trays, wrapped in silver foil (to protect them from light) and then stored at -80°C until fluorescent microscopic assessment.

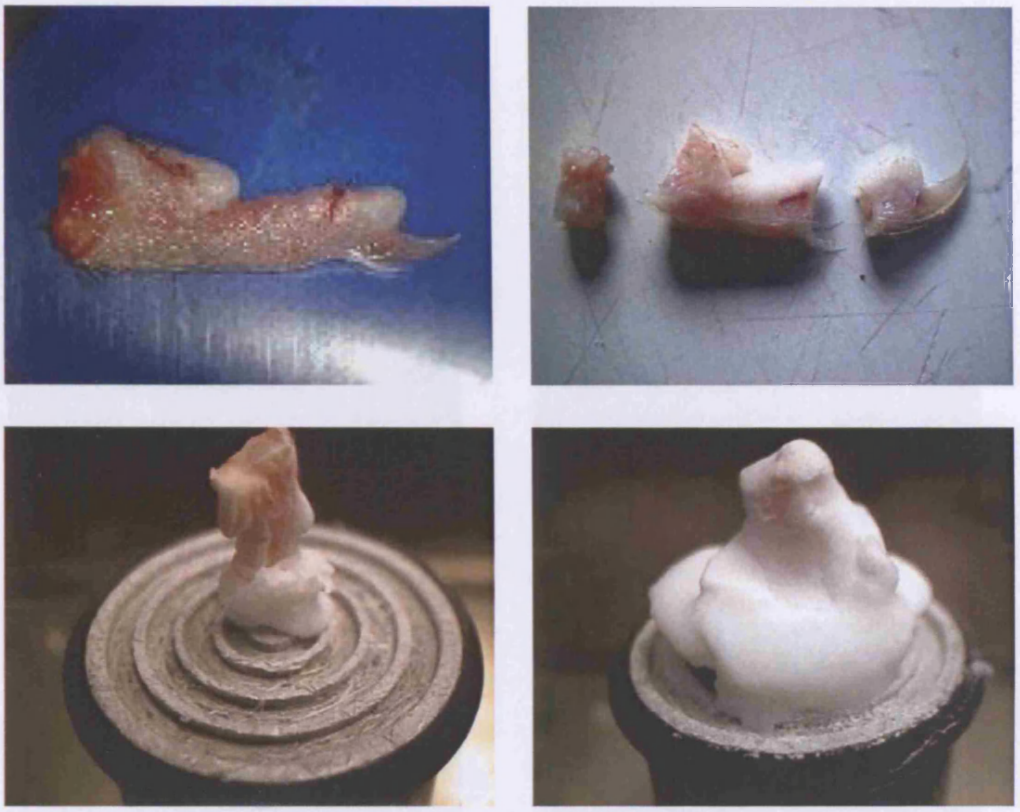


Figure 8. Tendon harvesting and embedding for cryostat sectioning: A: Digit harvested in entirety. B: Digit trimmed to leave zone of interest. C: Digit attached to cryostat chuck using cryoembed. D: Digit prepared for frozen sectioning.

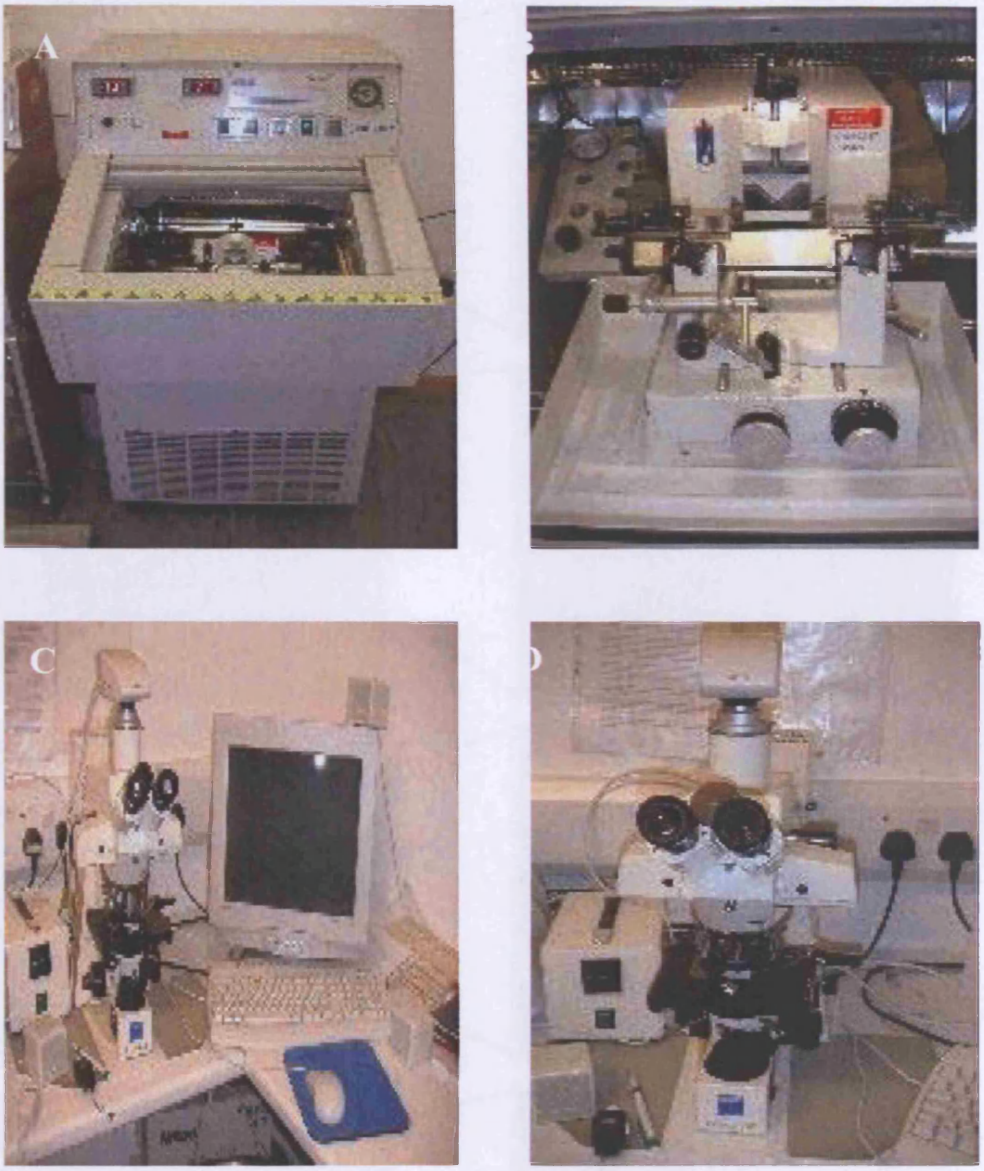


Figure 9. Cryostat sectioning and image capture and analysis equipment A: Bright Instruments Cryostat. B: Tungsten blade used for cutting digits containing bone C: Image capture microscope and computer and D: microscope in close up.

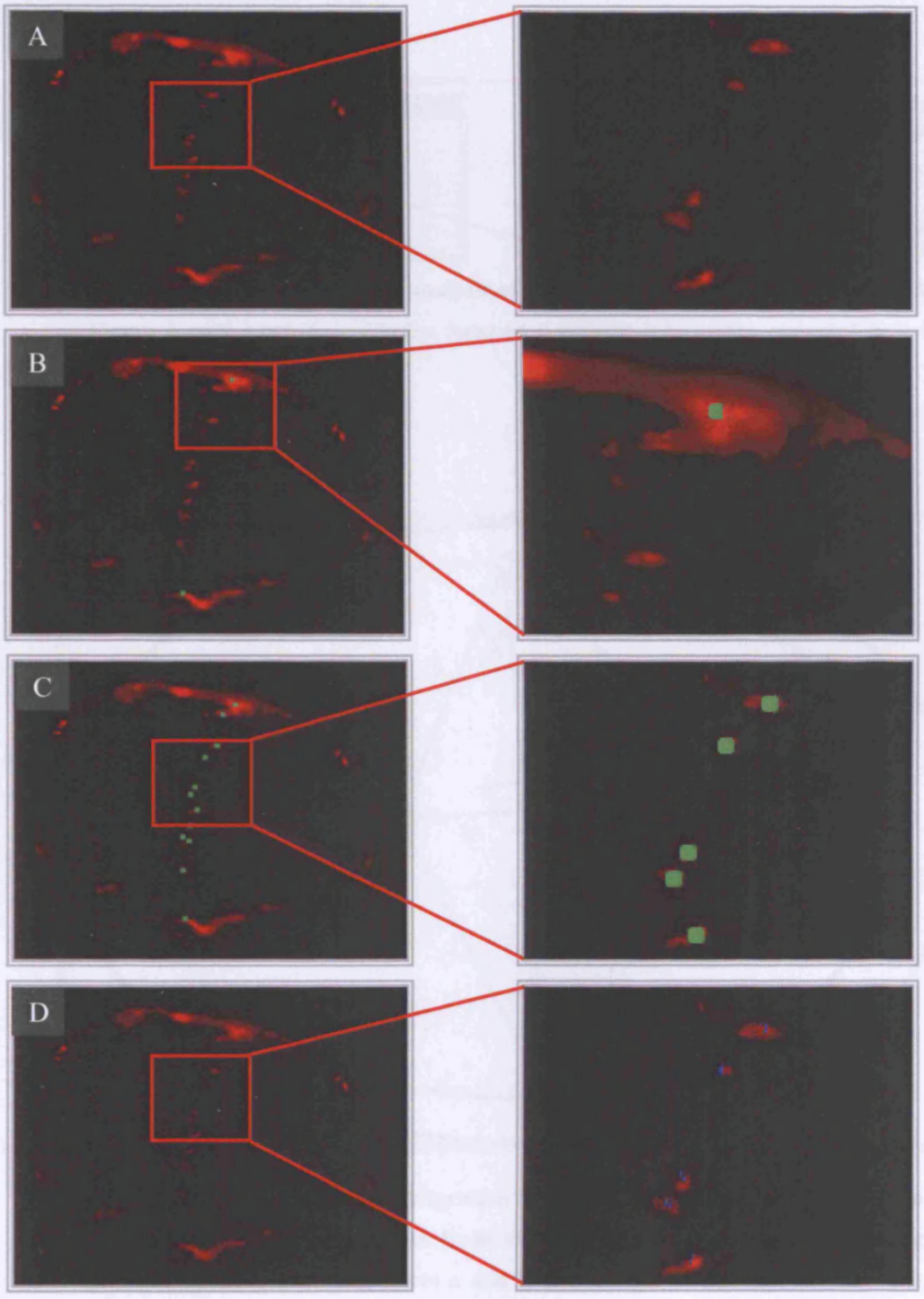
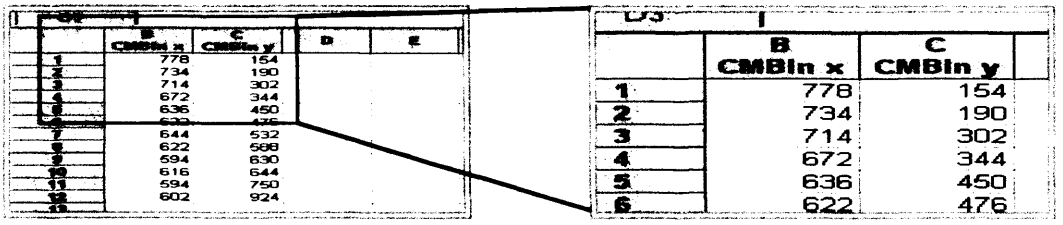


Figure 10 A-D: Method of analysing location of stained cells. A: Transverse (frozen) section of rat digit. The outline of the digit is visible. Stained cells are visualised. B: The edge of the injury is highlighted, to allow the relative location of stained cells to be determined. C: All stained cells were then demarcated using the image analysis software, which D: then numbers and records the position of each cell.



	B CMBIn x	C CMBIn y
1	778	154
2	734	190
3	714	302
4	672	344
5	636	450
6	622	476

Figure 11 – Computer-assisted analysis of stained cell locations. The cells labelled in Figure 4 each have their relative location determined in 2 axis, recorded in a PC spreadsheet.

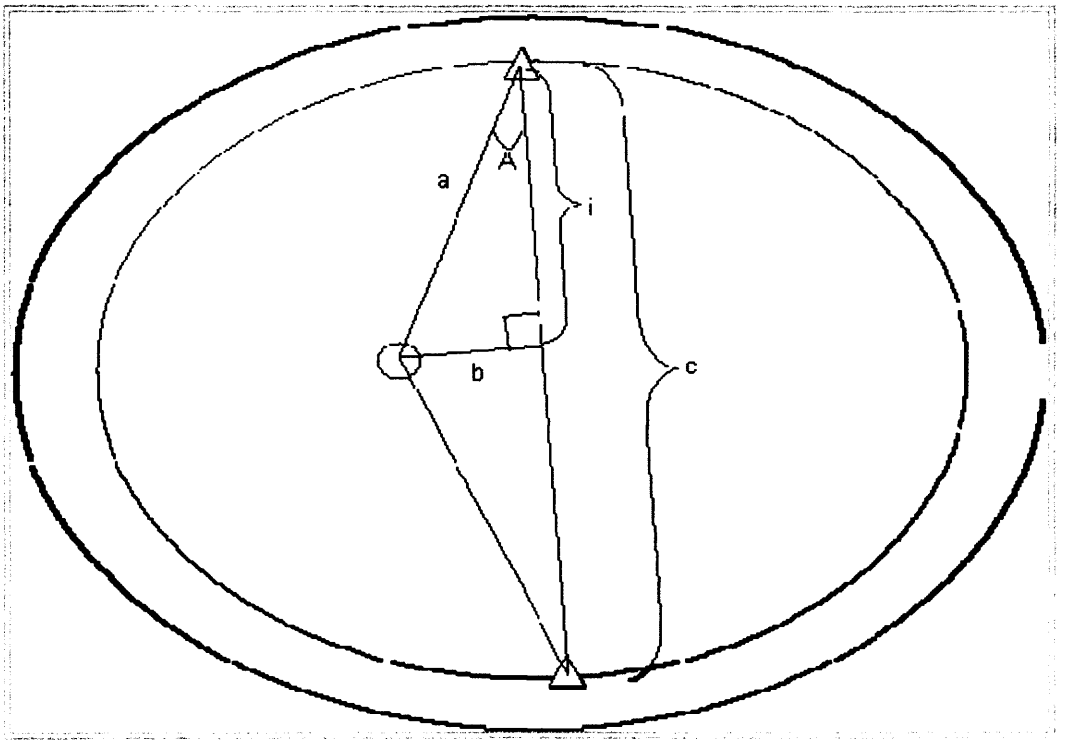


Figure 12. Determination of cell migration distances. The distance of the cell from the line of injury and the distance from the synovial sheath are calculated using trigonometric techniques. Distances a and b are measured directly, distance i is determined.

2.1.8 Photomicrographic assessment

The resulting sections were visualised under light and ultra-violet microscopy. The zone of injury was first determined using light microscopy, then a combination of light and UV micrographs were taken for each specimen examined. Light photomicrographs were taken to enable determination of the zone of injury during subsequent analysis. The DiI labelled cells fluoresced under UV, and could then be visualised with a TRITC (tetramethylrhodamine isothiocyanate) filter (Nikon Bioscience, New York, USA).

To achieve a high level of consistency, the specimens were always placed in the same position on the screen: the tendon was orientated transversely with the operative window placed vertically. All photomicrographs were taken using a Leica DC-200 digital camera (Leica Photographic Systems, Milton Keynes, UK) connected to a MESH computer (MESH, London, UK) running Windows 2000 (Microsoft, OR, USA) (Figures 9C&D). The images were captured using Leica DC image software (Leica Photographic Systems, Milton Keynes, UK), and saved as TIFF (Tagged Image Format File) files.

2.1.9 Slide analysis

Image analysis software (Sigma Scan Pro v5, SPSS UK Ltd, Surrey, UK) was then utilised; the location of each labelled cell was plotted (Figures 10A-D), and the following criteria determined:

- Total numbers of labelled cells in the area of the tendon injury per slide

Each cell was labelled by positioning the cursor over it on the screen, and selecting it using the computer mouse. The image processing software would

then highlight and label the cell, enabling a total cell count per slide to be calculated.

- Minimum perpendicular distance of each cell from the line of injury.

The software would return an X,Y co-ordinates for each cell, as well as co-ordinates for the top and bottom of the incision on each tendon (Figure 11). These co-ordinates could then be converted, trigonometrically into relative distances from the line of incision and the distance from the tendon surface at the point of injury (Figure 12).

- Distance of each labelled cell from the tendon surface, at the point of the injury.

The same co-ordinates could also be converted, using the same trigonometric techniques into a distance from the point of injury.

This procedure was performed for all slides for all tendons at 0, 1, 3, 5 and 7 days.

2.2 *EFFECT OF INJURY TYPE, IMMOBILISATION AND TGFB APPLICATION ON TENDON RESPONSE TO INJURY.*

An animal model was used to observe the effect of different types of injury, immobilisation and application of pro-fibrotic growth factor (TGF-β1) on epitenon cells.

2.2.1 The animal model

As outlined in section 2.1.2 above, there were several indications for the use of a rat model to test the experimental hypotheses of this thesis. The animals used were similar to those described above: Male Sprague Dawley Rats, with weights between 250-300g, were obtained from the same source, kept in the same conditions and treated pre and post-operatively in identical environments.

2.2.2 Anaesthesia

General anaesthesia was performed as described above, using intramuscular injection of Hypnorm (Fentanyl + Fluanisone, Janssen, Beerse, Belgium) and intra-peritoneal injection of Diazepam (Benzodiazepine, CP Pharmaceuticals, London, UK). Heated pads were again used during surgery and top up doses of Hypnorm were administered as required.

2.2.3 Operative procedure

2.2.3.1 Exposure of the tendon:

Under general anaesthesia a rubber tourniquet was applied to the left hindpaw of the rat. The foot was disinfected using chlorhexidine 0.5% in spirit. The

surgery was conducted with the aid of a surgical dissecting microscope (Zeiss with variable x2-10 zoom magnification) and microsurgical operating instruments.

A longitudinal incision was made in the plantar skin of the hindpaw (Figure 13A), exposing the flexor tendon complex in its extrasynovial portion. The larger rounder FDP tendon was gently separated from the superficial structures (Figure 13B) and the FDS tendon in the palm, prior to its entry into the digital sheath. In this region it was noted that the deep tendon had bilateral loose connective tissue connections. These were gently teased apart to allow extraction of the intrasynovial portion of the tendon into the incision described above.

Once the FDP tendon was exposed, great care was taken to prevent desiccation of the tissue using frequent irrigation with 0.9% Normal Saline. Once the deep tendon was free from surrounding tissue, gentle traction was applied with the aid of a human skin hook; to flex the digit, delivering part of the intrasynovial region of the tendon into the plantar wound.

2.2.3.2 Different treatments:

Experimental FDP Tendons received a random permutation of:

- Injury type: Superficial epitenon scrape injury or longitudinal incision injury.
- Mobilisation type: Post-operative immobilisation or mobilisation.
- Pro-fibrotic application: Application of TGF-β1 or saline control.

These treatment permutations are discussed in greater detail below.

Day 0	Day 7	Day 14
Control - No Treatment	Incised – No Immob – Without TGF	Incised – No Immob – Without TGF
Control - Incised	Scraped – No Immob – Without TGF	Scraped – No Immob – Without TGF
Control - Scraped	Incised – Immob – Without TGF	Incised – Immob – Without TGF
	Scraped – Immob – Without TGF	Scraped – Immob – Without TGF
	Incised – No Immob – With TGF	Incised – No Immob – With TGF
	Scraped – No Immob – With TGF	Scraped – No Immob – With TGF
	Incised – Immob – With TGF	Incised – Immob – With TGF
	Scraped – Immob – With TGF	Scraped – Immob – With TGF
	Incised – No Immob – Without TGF	Incised – No Immob – Without TGF
	Scraped – No Immob – Without TGF	Scraped – No Immob – Without TGF
	Incised – Immob – Without TGF	Incised – Immob – Without TGF
	Scraped – Immob – Without TGF	Scraped – Immob – Without TGF
	Incised – No Immob – With TGF	Incised – No Immob – With TGF
	Scraped – No Immob – With TGF	Scraped – No Immob – With TGF
	Incised – Immob – With TGF	Incised – Immob – With TGF
	Scraped – Immob – With TGF	Scraped – Immob – With TGF

Table 2 – List of treatment permutations for rat FDP tendons, at Day 0, Day 7 and Day 14.

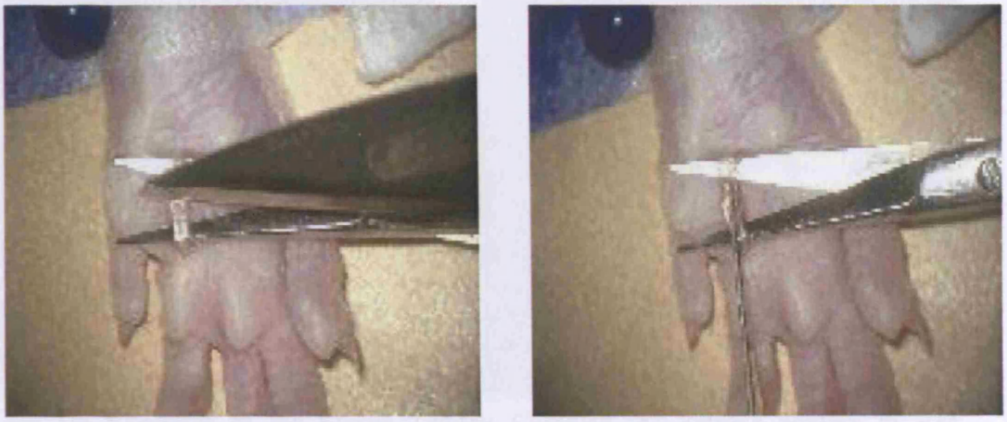


Figure 13: Different Injury Types of the exposed Flexor Tendon Complex. A: Superficial Scrape Injury to the tendon surface B: Longitudinal incision type injury.

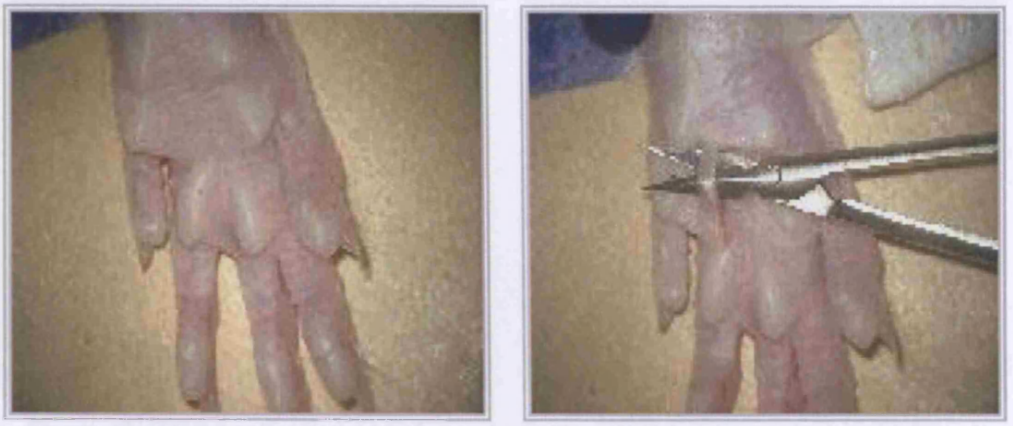


Figure 14: Exposure of the Flexor Tendon Complex. A: Plantar Surface of Rat Hindpaw, prior to procedure. B: Exposed Flexor Tendon complex, indicated by arrowhead.

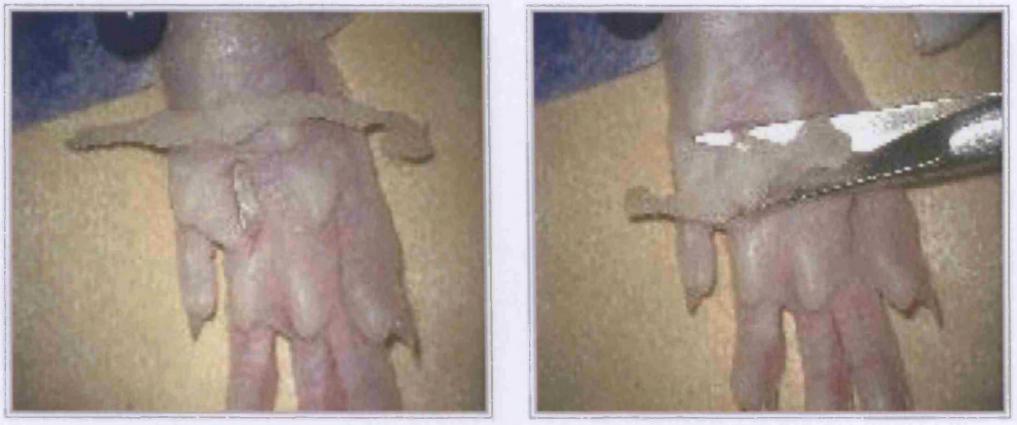


Figure 15: TGF application technique. A: Cotton wool bolster soaked in TGFB or normal saline control. B: Bolster applied and wrapped around exposed tendon.

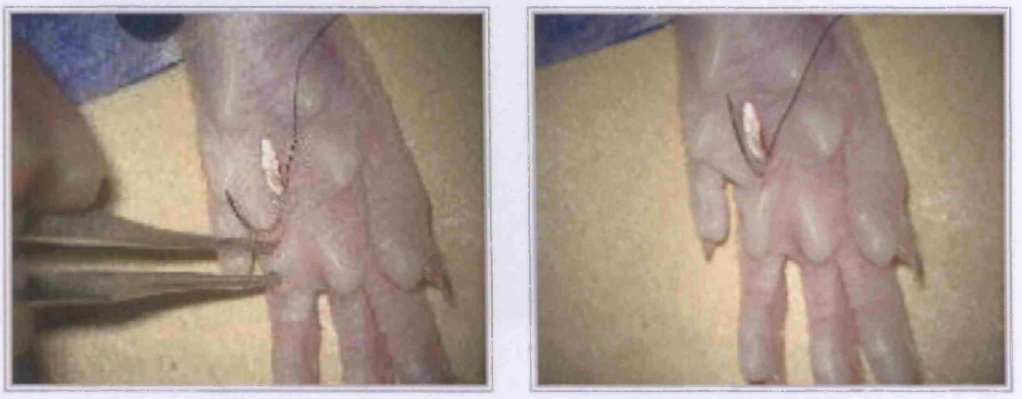


Figure 16: Immobilisation technique. A&B: Suture placed through the tendon body and through the A1 Pulley.

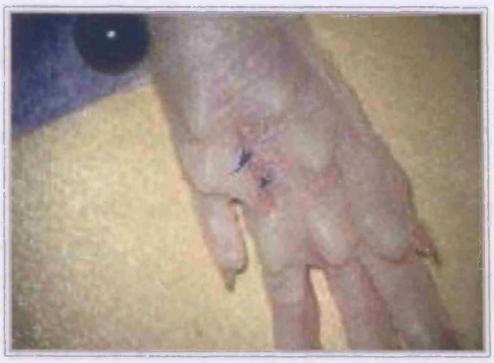


Figure 17 - Wound closure

2.2.3.3 Injury Type:

The experimental injury was performed in one of 2 different ways, according to its allocated treatment permutation (Figure 14). The first was to scrape the palmar surface (i.e. that exposed through the palmar incision) of the tendon with a no. 11 blade scalpel. The scrape injury was approximately 5mm in length; the aim was to remove the very top layer of the tendon surface cells, but not to cause deeper injury. The second was to make a through and through 5mm longitudinal incision in the midline of the tendon, using a no. 11 scalpel blade, using a method similar to that described in the previous section. In both cases, the proximal extent of the window was marked with a single 8/0 Prolene suture (Ethicon, Somerville, NJ, USA) to aid later sectioning.

2.2.3.4 Application of TGFB:

Those tendons subjected to TGFB application (Figure 15) were treated in the following way: A thin piece of cotton wool was rolled to form a longitudinal bolster and soaked in a 7.5ng/ml solution of TGFB (R&D Systems, NY, USA, from RA Lamb, East Sussex, UK) (Brown, RA, *et al.* 2002). This bolster was then wrapped around the exposed tendon for 5 minutes, in direct contact with the surface of the tendon. Care was taken to ensure the rest of the tendon was protected from the TGFB. After 5 minutes, the tendon was rinsed 4 times in sterile 0.9% saline solution. During this period, the microscope light was turned off to prevent drying, and the wound covered to prevent dye decay. The cotton wool was subsequently moistened with the TGFB solution at 1.5 and 3 minutes into the application.

2.2.3.5 Tendon replaced in synovial sheath

The tendon was then returned to its anatomical position by extending the digit, with the window now within the intrasynovial environment. At no time was the integrity of the synovial sheath breached.

2.2.3.6 Mobilisation or immobilisation.

Tendons to be immobilised (Figure 16) were first returned to their synovial sheath. A 7/0 silk suture was placed through the A1 pulley, through the tendon, and out through the pulley on the opposing side. This was sufficient to immobilise the digit at the neutral position of approximately 10 degrees of flexion (this was achieved by lining the digit up with the neighboring digit prior to placement of the suture).

2.2.4 Skin closure

Traction on the tendon was released, and the FDP tendon returned to its anatomical position with the window injury, now within the intrasynovial environment (Figure 17). At no time was the integrity of the synovial sheath breached. Skin closure of both incisions was effected with interrupted 8/0 Prolene sutures (Ethicon, Somerville, NJ, USA). The lack of surgical dressings prevented them from picking at their wounds. The rat was nursed in a heated propagator unit until fully recovered and then transferred back to its single cage. Food and water were administered *ad-libitum*. Mobilisation was not hindered in any way, and all rats were observed to be fully mobile after the procedure.

2.2.5 Tendon harvest, processing and sectioning

Rats were culled at 7 and 14 days post procedure. Each operated digit was removed at the MCPJ, to preserve the anatomy of the digit (Figure 18A). They were then placed into 10% buffered formal saline solution for tissue fixation. After 24 hours in fixative, the specimens were dissected to expose the operative region (Figure 18B&C). The specimens, appropriately orientated, were then paraffin blocked (Figure 18D&E), using a standard 12-hour process (Tissue-Tek Vacuum Infiltration Processor supplied by Miles Scientific, Naperville, USA.). The blocks were embedded in paraffin wax using a Tissue-Tek III Blockmaster (Miles Scientific, Naperville, USA.) Transverse microtome sectioning of the blocks was undertaken at 4-micron thickness (Figure 18F). Due to the inherent difficulty of sectioning specimens containing bone, achieving satisfactory sections required pre-cooling and softening with Mollifex. Sections were placed onto poly-lysine slides (Merk, Upminster, UK) coated in house with 4% Amino Propyl Triethoxysilene (Sigma, Poole, UK). The area of interest, i.e. the surgical window, area being delineated by the marked suture placed at the time of operation.

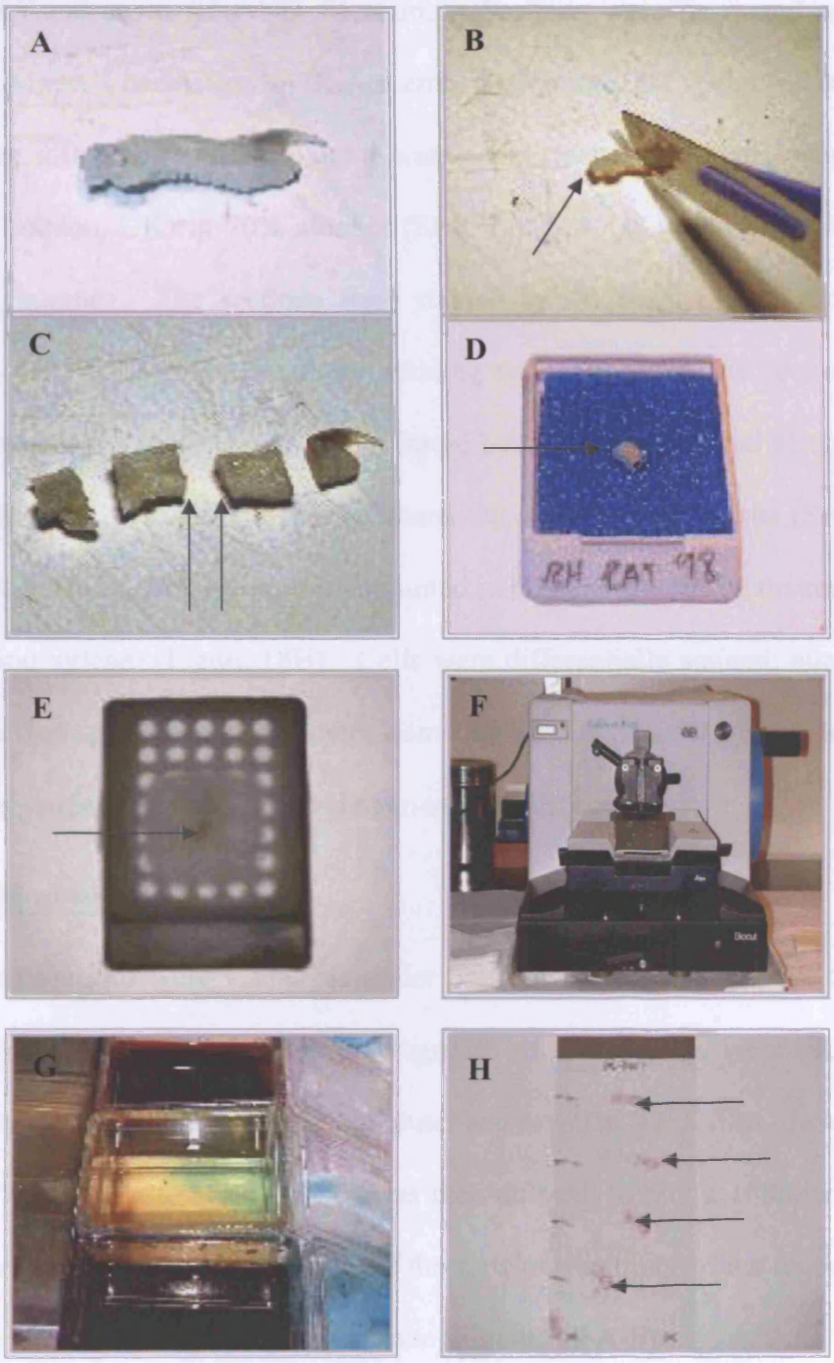


Figure 18A-H: – Tendon harvesting, processing and staining. A: The digits were harvested at the MCPJ to preserve anatomical architecture. B: Scalpel used to dissect the digit (arrowhead). C: Arrowheads show regions of interest. D&E: Wax block embedding (arrowhead = digit). F: Microtome used for sectioning. G: Sections H&E stained. H: Resulting slides and sections (arrowheads).

Haematoxylin and eosin (H AND E) staining Sections were de-waxed and treated with Mayer's haematoxylin (R.A. Lamb, Eastbourne, UK) (Figure 18G) for 5 minutes, followed by washing in tap water, acid alcohol (1% hydrochloric acid (SBP, London, UK) in 70% alcohol (SBP, London, UK)) and tap water again for 5 minutes. The sections were stained in 1% eosin (R.A. Lamb, Eastbourne, UK) for 3 minutes. After staining was completed, the sections were again washed in tap water and dehydrated by treating with serial alcohol (SBP, London, UK) washes and finally cleared by treating with Xylene (SBP, London, UK). The sections were then mounted in DPX (a mixture of distrene, plasticizer and xylene) (Figure 18H). Cells were differentially stained; nuclei appearing a dark purple/blue, the cytoplasm-stained pink, muscle fibres and elastic fibres stained deep pink and collagen-stained light pink.

2.2.6 Section analysis

The resulting sections were visualised under light microscopy, to observe the areas of tendon injury. Light photomicrographs of the sections were taken using a digital camera connected to a computer and saved as TIFF files. Image analysis software (Sigma Scan Pro v5) was then utilised; to plot a 100micron field, centred on the surface and middle of the zone of the injury of the tendon, to count the numbers of cells within that zone (Figures 19 A-F).

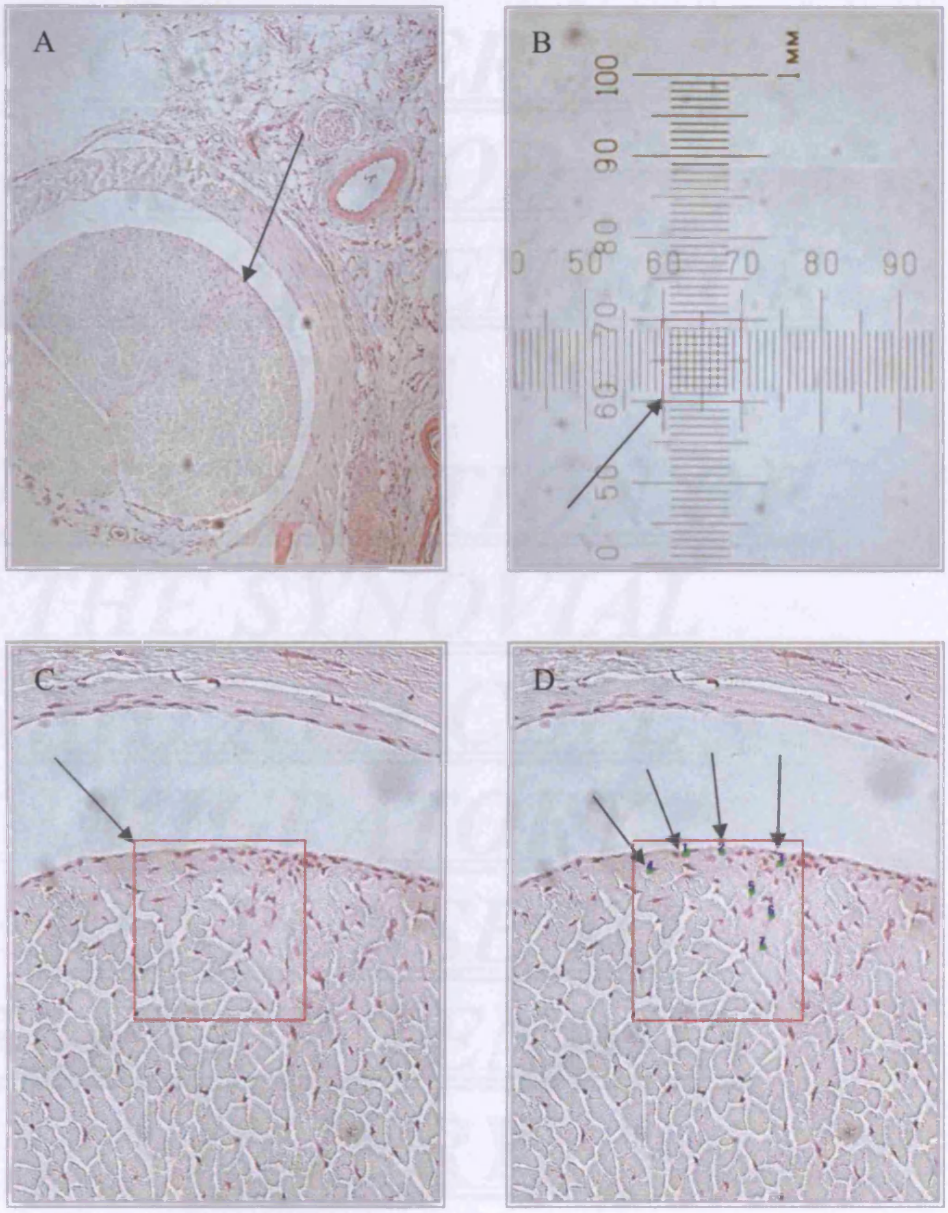


Figure 19 – Image processing of wax-embedded tendon specimens A: 100X light image of transverse section of digit (arrowhead = tendon surface) B: 2 graticule images were superimposed perpendicular to each other forming a 100micron square box to allow accurate cell counting (arrowhead). C: Graticule grid overlaid over area of interest using computer image analysis software, D: Then cells were labelled manually, but counted automatically using computer software-assistance.

CHAPTER 3: METHOD DEVELOPMENT FOR THE INVESTIGATION OF THE SYNOVIAL SHEATH CELL MIGRATORY RESPONSE TO FLEXOR TENDON INJURY

3.1 INTRODUCTION

The debate between the existence and relative roles of *intrinsic* and *extrinsic* healing mechanisms persists. Previously, evidence supporting the role of both synovial- and tendon-derived cells in the healing process has been based on *indirect* evidence (Chapter 1). Relatively recently, Jones *et al.* (2003) have directly observed the migration of tendon surface cells into the tendon healing area, using vital dye staining of the fibroblasts, demonstrating the complexity of an intrinsic healing mechanism with the involvement of different cell populations. No such direct observation to confirm or refute the participation of synovial sheath cells in the repair process has appeared in the literature.

3.2 AIMS

To develop an animal model to allow direct observation of possible synovial sheath cell migratory response to injury. The criteria for this model were:

- To permit the selective and specific labelling of synovial sheath lining cells
- Concurrent creation of a window injury in the adjacent flexor tendon
- To allow post-operative mobilisation of the tendon.

3.3 ANIMAL MODEL SELECTION

Previous work investigating tendon healing has been carried out in a variety of models, including chicken, rabbit, dog, cow, monkey and horse (see references immediately below). Criteria for determining the suitability of an experimental animal model for this study were carefully observed. The rat model was considered to be the most appropriate initial choice, allowing comparison with previous work conducted in our laboratory. It also gave advantages in terms of availability and the size was adequate to conduct the surgical procedure.

Although the rabbit model seems to have been popular in the past (Abrahamsson *et al.*, 1991; Abrahamsson *et al.*, 1992; Chang *et al.*, 1998; Flint, 1972; Gillard *et al.*, 1977; Gillard *et al.*, 1979; Kakar *et al.*, 1998; Khan *et al.*, 1996; Kumagai *et al.*, 1994; Manske *et al.*, 1984; Matthews and Richards, 1976; Rank *et al.*, 1980; Reddy *et al.*, 1999), a number of studies have used the rat model (Iwuagwu and McGrouther, 1998; McNeilly *et al.*, 1996). Matthews *et al.*, in 1987, suggested that the rat model had significant advantages over the rabbit model related to its size and anatomy (Matthew *et al.*, 1987).

3.4 THE CHOICE OF VITAL DYE

3.4.1 Dye selection

Vital dyes were used in the assessment of surface fibroblast migration in response to a surgical window injury. Cell Tracker was utilised for the initial *in vivo* work, however, it was demonstrated to only remain in cells for a maximum of 48 hours. Another vital dye, DiI, had been used previously by McNeilly (1996) to observe that tendons respond to mechanical load by modifying their extracellular matrix, delineating fibroblasts within the tendon body. DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine percolate), is a lipophilic long-chain dialkylcarbocyanine tracer. DiI has been shown to have more appropriate characteristics for the proposed work; cellular specificity and sufficient longevity, high fluorescence and photo-stability when incorporated into cellular membranes. The absorption and emission wavelengths were 549nm and 565nm respectively, resulting in a strong red fluorescence when visualised under a TRITC filter, making it ideal for this study.

3.4.2 Appropriate concentration and timings

The chosen concentration of DiI was 10μM, this concentration having been used successfully by Jones *et al.* (2003). Our aim was to selectively stain tendon fibroblasts, and hence we needed to determine the optimum time of exposure to the DiI solution. This was performed by immersing rat FDP tendons in a 10μM solution of DiI, for varying periods of time: 30 seconds, 1 minute (Figure 10), 3 minutes, 5 minutes (Figure 10) and 10 minutes (Figure 10). The images in Figure 10 below demonstrate the effect of these different

exposure times. 1 minute exposure produced uneven, inconsistent staining. 5 minutes produced even, consistent staining, with good delineation of the tendon surface fibroblasts. 10 minute exposure produced indiscreet staining of the tendon surface, likely to be due to staining of the tendon matrix. There was also some staining of with the core area of the tendon, indicating that the dye is able to leach through the surface matrix; as the core cells were the ones which needed to be stained, this time was deemed to long.

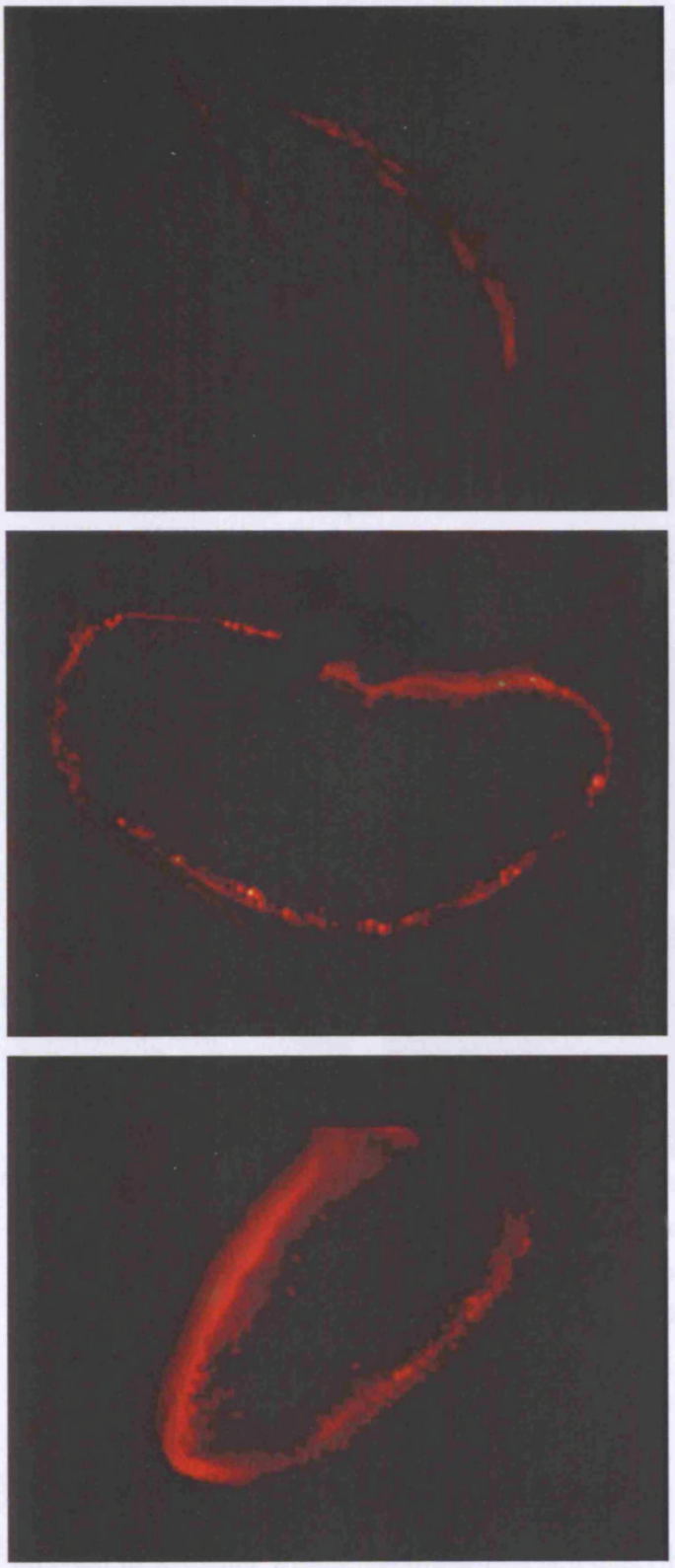


Figure 20 - 1 minute, 5 minutes and 10 minute exposure with 10micromolar solution of DiI.

3.5 *REVIEW OF TECHNIQUE USED BY JONES ET AL. (2003)*

3.5.1 Introduction

Previous work, been performed by Jones *et al.* (2003), investigated the migration of tendon surface cells in response to injury; we aimed to use a similar technique to facilitate comparison with results obtained.

3.5.2 Operative procedure

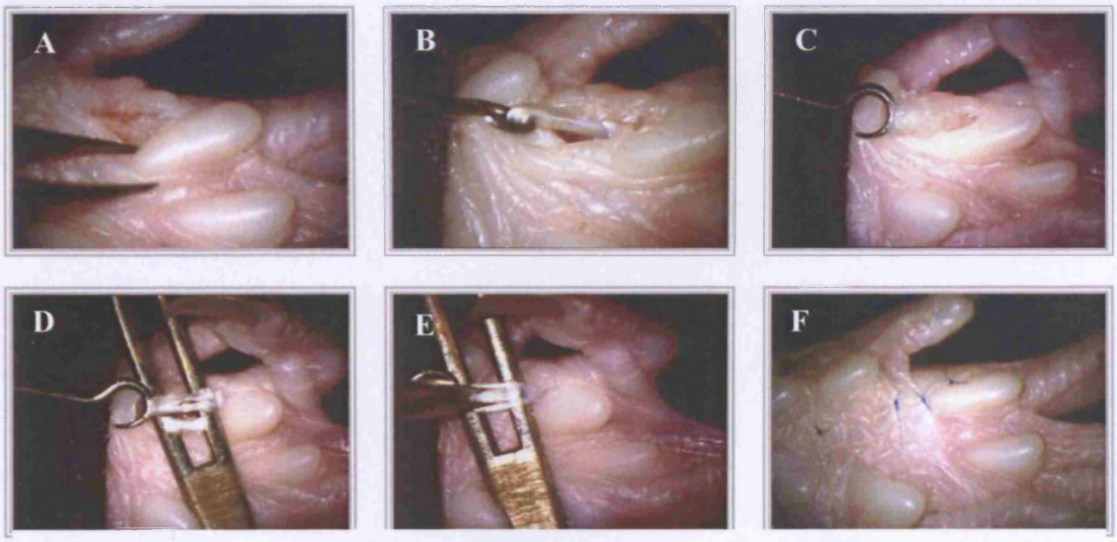


Figure 21: A: The creation of a partial tenotomy after surface cell labelling in a hind flexor tendon of a rat. B: Through a longitudinal incision the intrasynovial portion of FDP was exposed. C: Surface cell staining with was performed using a test substance-soaked cotton wool bolster. D: Thorough irrigation followed. E: Creation of the injury, followed by its return to the sheath. F: The skin wound sutured.

In a rat model, the left hind paw FDP was exposed by retracting it through an incision in a plantar wound, as illustrated and described in Figure 21. Vital dye was applied directly to the exposed tendon, using a cotton wool bolster, applied to the exposed intrasynovial portion of the FDP tendon for 5 minutes, followed

by thorough irrigation. The creation of a surgical window, skin closure and the post-operative mobilisation and care was undertaken as described in Chapter 2.

3.5.3 Resultant staining patterns

Figure 22 illustrates the initial staining patterns obtained by Jones *et al.* (2003). It confirms selective labelling of the tendon surface cells. The resultant migration experiments performed subsequent to this are discussed in more detail in Chapter 4, particular their comparison to the results obtained in this thesis.

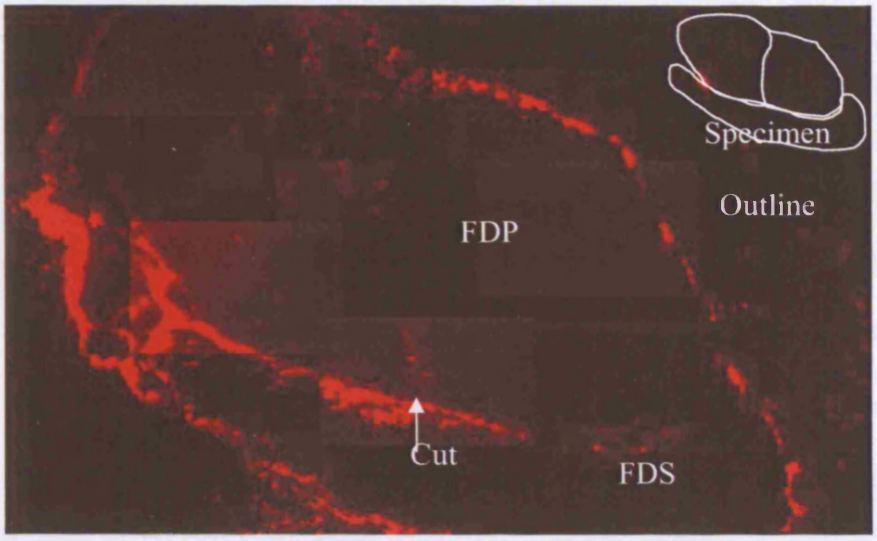


Figure 22: Selective labelling of tendon surface cells by Jones et al. The montage is schematised top right, FDS = flexor digitorum superficialis, FDP = flexor digitorum profundus.

3.6 FACILITATING SELECTIVE DYE APPLICATION

3.6.1 Introduction

Having determined the optimal means of staining tenocytes with vital dye, the next experimental objective was to selectively label the synovial cells, in a model which would allow comparison to the results from the method described in 3.5.2 above, with the criteria outlined in Section 3.2 above. To reiterate for clarity; previous work had selectively stained the tendon-surface fibroblasts to observe their subsequent migratory response to injury, the experimental aim of this work was to selectively label synovial sheath cells alone, and observe their migratory response to injury.

A variety of techniques were attempted, many of which were unsuccessful; however, the methods attempted are noteworthy in determining the final technique utilised and they are described below.

3.6.2 Lysis of tendon-surface tenocytes.

3.6.2.1 Hypothesis

Application of vital dye to the synovial and tendon surface layers could be achieved by the injection of vital dye into the synovial sheath. This technique aimed to allow vital dye to come into contact with both tendon surface and synovial sheath cells; ideally, both would subsequently become labelled. Subsequent lysis of the tendon surface cells could then be performed, such that their participation in the healing process could be prevented.

3.6.2.2 Experimental procedure

To determine the optimal technique, three types of cell lysis were assessed, including application of ethanol (Quest Biomedical, Solihull, UK), DMSO (Dimethyl Sulfoxide, Quest Biomedical, Solihull, UK) and DMF (Dimethylformamide) (Quest Biomedical, Solihull, UK). All 3 compounds are known to be toxic to cells *in vitro* and *in vivo,* by a presumed mechanism of membrane hydrolysis. Ethanol was chosen as a readily available agent, with well recognised cell toxicity effects, by disrupting membrane fluidity, with documented dosage dependent effects. DMSO and DMF are both dipolar aprotic solvent sulfuric compounds which interrupt cellular membrane integrity (See Chapter 1 for references for above).

Rat FDP tendons were harvested from rats euthanased at the termination of other (un-related) experiments, and stored in Phosphate Buffered Saline (PBS) solution, for a maximum of 30 minutes, until use. Tendons were immersed in the test solutions for 30 seconds, 1 minute, 3 minutes and 5 minutes and then washed thoroughly in PBS solution, to remove any residual lytic agent.

The degree of cell lysis was determined by immersion in Propidium Iodide (PI) solution for 60 seconds. PI is a fluorescent marker which binds to nucleotides, and brightly labels the nuclei of most cells, but only after the cells have been fixed or undergone fixation or membrane lysis. PI's peak excitation wavelength is 536 nm, and its emission peak is 620 nm. A 50 microgram/mL solution was used in this instance. PI is also known to label RNA; the fixation in methanol prevents this.

Each tendon was then snap frozen and 8micron frozen sections were taken using a cryostat. Light and UV photomicrographs were taken, using equipment outlined in and the degree of cells stained with PI determined.

3.6.2.3 Results

Corresponding light and UV images for each of the agents used, all shown after 3 minutes of treatment.

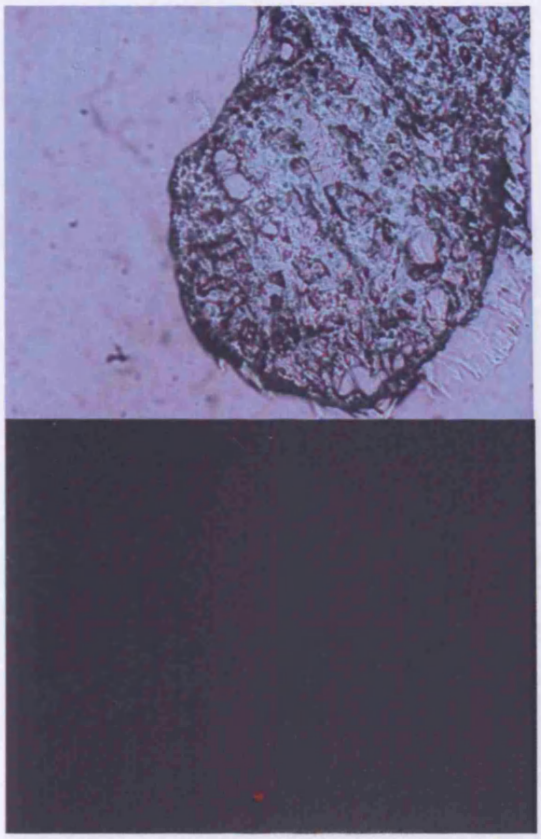


Figure 23: Corresponding light (left) and UV (right) photomicrographs (x40), of tendon treated with DMF for 3 Minutes.

Figure 24: Corresponding light (left) and UV (right) photomicrographs (x40), of tendon treated with DMSO for 3 Minutes.

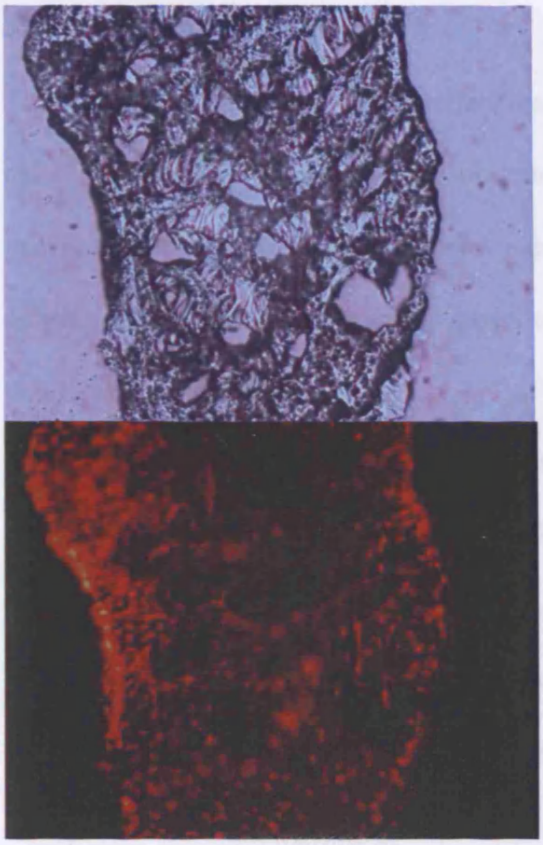


Figure 25: Corresponding light (left) and UV (right) photomicrographs (x40), of tendon treated with Ethanol for 3 Minutes.

3.6.2.4 Outcome

The distinction between tendon surface and tendon core cells is that the top 4-5 cell layer of the tendon to be the epitenon cells (detailed in Chapter 1). To prevent tendon surface cells from participating in the healing response, subsequent to vital dye staining, reproducible, selective and specific lysis of these cells was necessary.

DMF and DMSO did not produce significant cell lysis, with virtually no PI staining, even after 5 minutes of immersion. Ethanol exposure produced inconsistent degrees of lysis, with evidence of cell death to much deeper levels than expected, with evidence of tendon core cell lysis, suggesting that the ethanol was able to penetrate into the tendon fibrillar structure very rapidly.

None of these techniques were able to produce a specific and reproducible region of cell death, and hence this methodology was not deemed successful. It was also postulated that the lysis of the tendon surface cells might interfere with the migration patterns of synovial cells, through the release of cytokines.

3.6.3 Protecting surface tendon cells

3.6.3.1 Introduction

Injection of vital dye into the synovial sheath and subsequent tendon injury had been demonstrated to be possible during early experimental work. By covering and protecting the tendon surface we aimed to prevent labelling of the tendon surface cells, facilitating the selective labelling of synovial cells. After the vital dye was applied, the temporary protective cover could be removed and the surgical window created. Saran wrap (Sainsbury's, UK) was deemed to be appropriate protection, made from the polymerisation of vinylide chloride with

monomers such as acrylic esters and unsaturated carboxyl groups, forming long chains of vinylide chloride, which are impenetrable to liquids and gases.

3.6.3.2 Experimental assessment

In vivo experiments were performed, using an incision in the rat hind paw plantar surface and subsequent exposure of the FDP tendon, using a similar technique to that outlined in Section 5, above. The exposed FDP tendon was wrapped in saran wrap, and then replaced into its original intra-synovial position. The wrap measured around 1.5 cm in the longitudinal axis. Initially, the application of the saran wrap was technically very difficult, primarily due to the size of the tendon, however, it was possible to inject vital dye solution into the synovial space (i.e. not in contact with the tendon) and leave it *in situ* for 5 minutes, followed by thorough irrigation.

Tendons were harvested taking the entire rat digit and subsequently snap freezing the digits. 8 micron frozen sections were taken using the cryostat, using a similar technique to that outlined in Chapter 2. Light and UV photomicrographs were taken, using equipment also outlined in Chapter 2. The areas of vital dye staining were then determined.

3.6.3.3 Results

Figure 26: Light (left) and corresponding UV photomicrographs (right) of tendon protected with Saran wrap to prevent cellular labelling. DiI labelled cells show as red.

3.6.3.4 Outcome

Despite the (apparently complete) envelopment of the tendon in cling film, the above results demonstrate vital dye staining of both the synovium and tendon surface cells. Although the saran wrap was impervious to the vital dye, it was hypothesised that the vital dye penetrated from the proximal and distal margins of the wrap. As the vital dye was able to stain the surface layer of the tendon, this methodology was also deemed inappropriate.

3.6.4 Dual vital dye staining dyes

3.6.4.1 Introduction

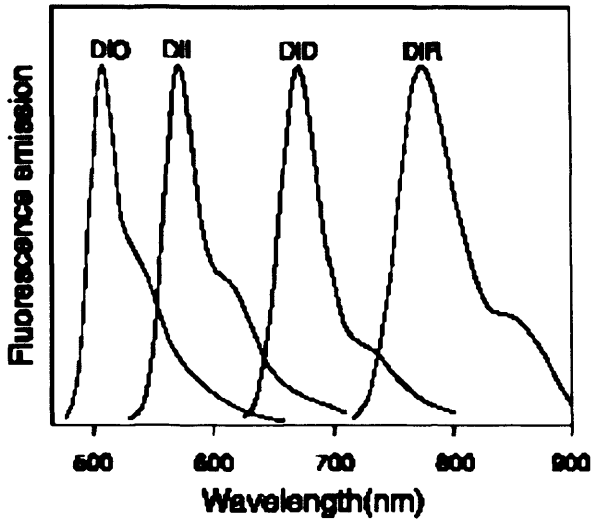


Figure 27: Emission spectra of other vital dyes. DiO and DiI have emission spectra differing by 80nm

From experience with the techniques aforementioned, it was demonstrable that vital dye could be applied selectively to the tendon surface, and non-selectively to the tendon surface and synovium. DiO (3,3-dioctadecyloxacarbocyanine perchlorate), which is also a long-chain dialkylcarbocyanines, has similar characteristics to DiI, but has different emission spectra. It was proposed to use DiI to stain the tendon surface cells, and DiO to stain both the synovial and tendon surface cells. Hence tendon surface cells would be "dual stained" (and be visible under both TRITC and FITC filters on the same sections), and synovial cells would be single stained (only visible under TRITC filter). The participation and migration of synovial cells in the healing process could be determined by the subtraction of tendon cells (dual stained), leaving only single stained cells.

3.6.4.2 Experimental assessment

An *in vivo* experiment to determine whether tendon surface cells would take up both vital dyes with consecutive applications was carried out on the rat model. Using the technique outlined in section 3.5.2 above, DiO was applied selectively to the tendon surface cells, by exposing the FDP tendon through a plantar incision, followed by thorough irrigation. DiI was then applied using the same technique, again followed by thorough irrigation.

3.6.4.3 Results

The results suggest that cells are unable to take up 2 vital dyes with the same efficacy.

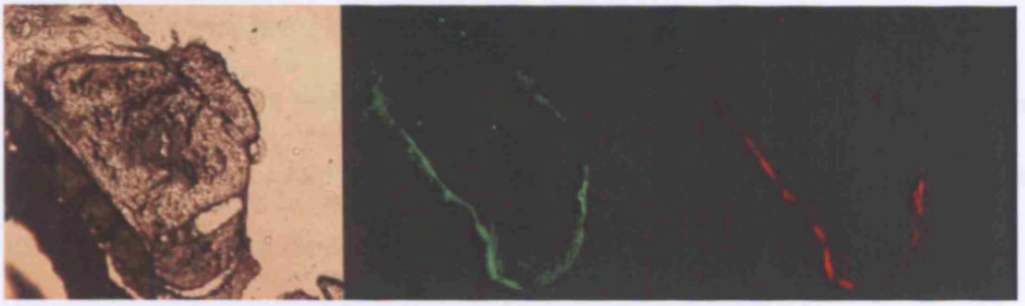


Figure 28: Light (left) and corresponding UV images of DiO (middle) and DiI (right) staining of the tendon surface.

3.6.4.4 Outcome

Although previous work has demonstrated that dual labelling can be effective (Lomo, J *et al.*, 1998) our results suggest that treatment with one vital dye affect the ability of the same cells' uptake of another similar vital dye. Hence, dual labelling and subtraction was deemed to be viable to test the experimental hypothesis.

3.6.5 Removal of the experimental tendon from the synovial sheath

3.6.5.1 Introduction

After indirect methods of vital dye application to the synovial cells had been attempted, direct application of vital dye was attempted as the next step in the method development. Removal of the tendon from the sheath using sutures placed prior to its removal was based on clinical practice - a clinical technique paper by Sourmelis and McGrouther (1987) demonstrated that a paediatric feeding tube could be used to retrieve flexor tendons which had retracted proximally. Whilst the flexor tendon was retracted, the flexor sheath was vacant, and direct access to the synovium was afforded.

3.6.5.2 Experimental assessment

A variety of different techniques were used to replace the rat FDP tendon after removing it from the synovial sheath, all of which included the division of the FDP tendon distal to the A4 pulley, through a separate skin incision.

3.6.5.3 Proximal and distal cut - direct replacement.

After an incision had been made proximal to the A1 pulley on the rat hind paw plantar surface, the FDP tendon could be removed (distally to proximally) from the synovial sheath. However, direct replacement of the tendon back into the synovial space, either manually or instrumented, was not possible.

3.6.5.4 Proximal and distal cut, post staining suture placement

Using the clinical technique described in section 3.6.5.1 above, after the tendon had been removed, a 7/0 silk suture was passed through the distal incision, retrogradely through the flexor sheath into the palm incision. The suture was then attached to the distal end of the FDP tendon and distal traction of the suture used to pull the tendon back into the synovial sheath. This method was difficult to effect as the passage of the sutures through the synovium was difficult as was their placement into the free FDP.

3.6.5.5 Proximal and distal cut, pre staining suture placement

The placement of two mutually perpendicular sutures before the FDP tendon was divided distally avoided the problems of the technique in 3.6.5.4 above, i.e. the inability to replace the tendon back within the synovial sheath at the end of the experiment.

3.6.5.6 Outcome

The placement of sutures prior to removal of the tendon proved to be reproducible and technically possible. The sutures within the synovial sheath did not impede the placement of a cotton wool bolster, soaked in DiI, to allow selective labelling of the synovial cells.

3.7 RESULT ASSESSMENT

3.7.1 Introduction

After tendon surgery, adhesions form between the synovium and the tendon surface. Removing the tendons from the sheath is likely to disrupt the tendon architecture, making assessment of migration of labelled cells difficult.

3.7.2 Fixation and microtome sectioning

The most accurate means of obtaining reproducible sections was by formalin fixation and microtome sectioning. However, formalin fixation interferes with the vital dye staining as it can encourage extracellular leaching of the vital dye.

3.7.3 Confocal imaging

Previous methodologies have relied upon frozen sectioning. However, this can present problems with the use of vital dye due to crystal formation. Hence, confocal imaging was utilised for experimental *in vivo* imaging, with procedural rat hind paws placed directly into the confocal microscope. Although reasonable images were obtained, the collagen autoflouresence at the excitation wavelengths used resulted in extremely long image acquisition times, making this technique unsuitable.

3.7.4 Snap freezing and frozen sectioning

As used by Jones *et al.* (2003), this technique requires the whole rat digit to be removed and snap frozen. The difficulty in obtaining reliable frozen sections, due to the presence of bone within the digit makes this process very difficult. However, with perseverance, reproducible sections were obtained.

CHAPTER 4: RESULTS: SYNOVIAL SHEATH CELL MIGRATORY RESPONSE TO FLEXOR TENDON INJURY

4.1 INTRODUCTION

Until recently, intrinsic and extrinsic tendon healing mechanisms were believed to be mutually exclusive phenomena. Skoog & Persson {Skoog and Persson, 1954} and Potenza {Potenza, 1962} and Eiken *et al.* {Eiken, Lundborg *et al.*, 1975b} were the initial advocates of the extrinsic theory, whilst Matthews and Richards {Matthews and Richards, 1974} proposed the intrinsic healing mechanism, but all this work was all based on indirect evidence. New techniques, such as immunohistochemical localisation and collagen assays, have now allowed these classical concepts to be re-examined.

Novel research techniques have provided evidence that distinct differences exist between the surface and core tendon fibroblasts. After injury the epitenon layer has been shown to demonstrate greater vascularity, cellular and biochemical activity than the inner core cells. Gelberman *et al.*, in 1992, observed greater surface layer procollagen expression during the healing process than in the other layers. Khan *et al.*, in 1996, used monoclonal antibodies to show that both the synovial sheath and the tendon surface tenocytes were relatively more reactive in the early period post insult, compared to the core tenocytes. Khan, in 1998, also showed that tendon core tenocytes were both less reactive and less aggressive than those derived from the synovial sheath in terms of their ability to contract collagen lattice; this led them to conclude that the synovial sheath cells may be involved in the initiation and perpetuation of scarring at the injury site {Khan, Occleston *et al.*, 1998}.

Until 2003, no direct evidence for the migration of either tendon-surface or synovial fibroblasts had been shown. However, in 2003, Jones *et al.*, developed a novel technique to allow specific tendon-surface cell labelling and subsequent observation *in vivo* {Jones, Mudera *et al.*, 2003}. This technique utilised DiI, a fluoroscein-based vital dye, previously used to study the morphology of tenocytes in the rat flexor tendon {McNeilly, Banes *et al.*, 1996a}. They observed that tendon surface cell fibroblasts migrate into the zone of tendon healing, within 24 hours after injury, however, clear evidence of the involvement of synovial fibroblasts in the healing process has not previously been demonstrated.

4.2 AIMS.

- To use the method developed in Chapter 3 to specifically label synovial fibroblasts in a tendon healing model.
- To observe the response of the synovial cells to tendon injury over the course of 7 days.

4.3 *MATERIALS AND METHODS.*

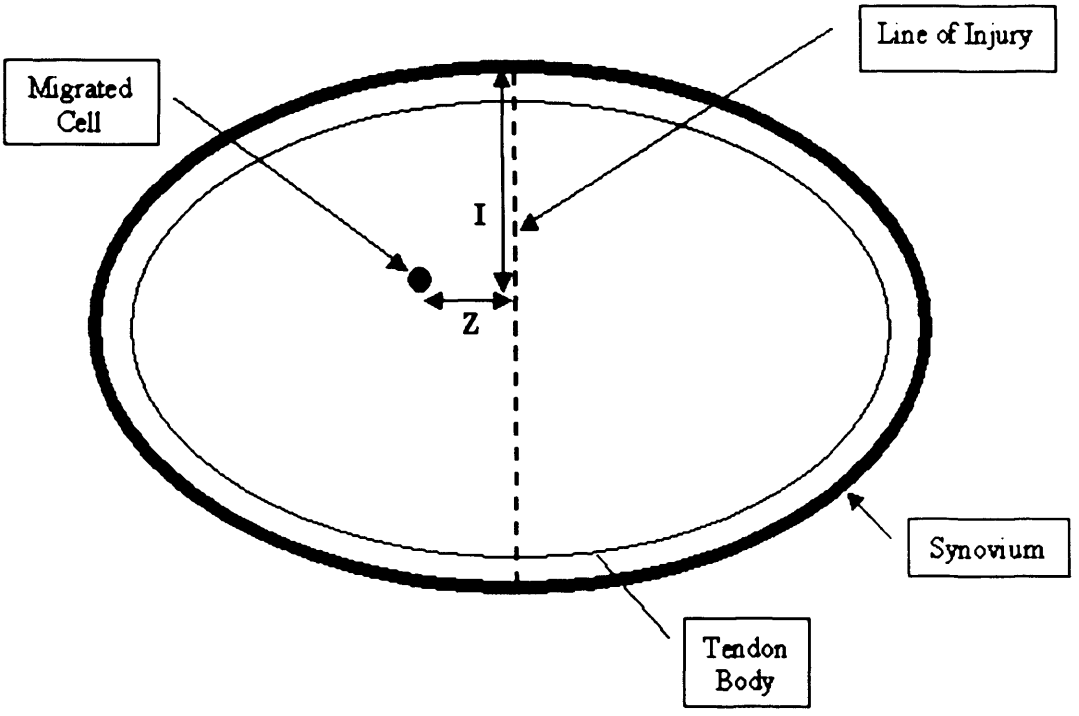


Figure 29: Diagrammatic cross section of a Flexor Tendon depicting the distances migrated. Distance I represents the "vertical" distance migrated from the synovium into the zone of injury (tendon window). Distance Z represents the "horizontal" distance migrated from the injury window into the tendon body. The window cut in the tendon tended to close down under tension to a slit.

4.3.1 Experimental technique.

Visualisation of fibroblast migration from the surface of tendons was investigated in the response to injury *in vivo*. Synovial cells were stained with DiI as described in Chapter 3. Their response to partial tenotomy in the adjacent flexor tendon was observed over the course of a week, with tendons harvested at Days 1, 3, 5 and 7. Transverse frozen sections through the injury area were taken, and ultraviolet photomicrography performed to determine the location of the DiI labelled cells. Counts of labelled cells in the vicinity of the injury were performed, quantifying the total number of cells present, their

distance from the injury, and their distance from the tendon surface, using a computer image analysis software package.

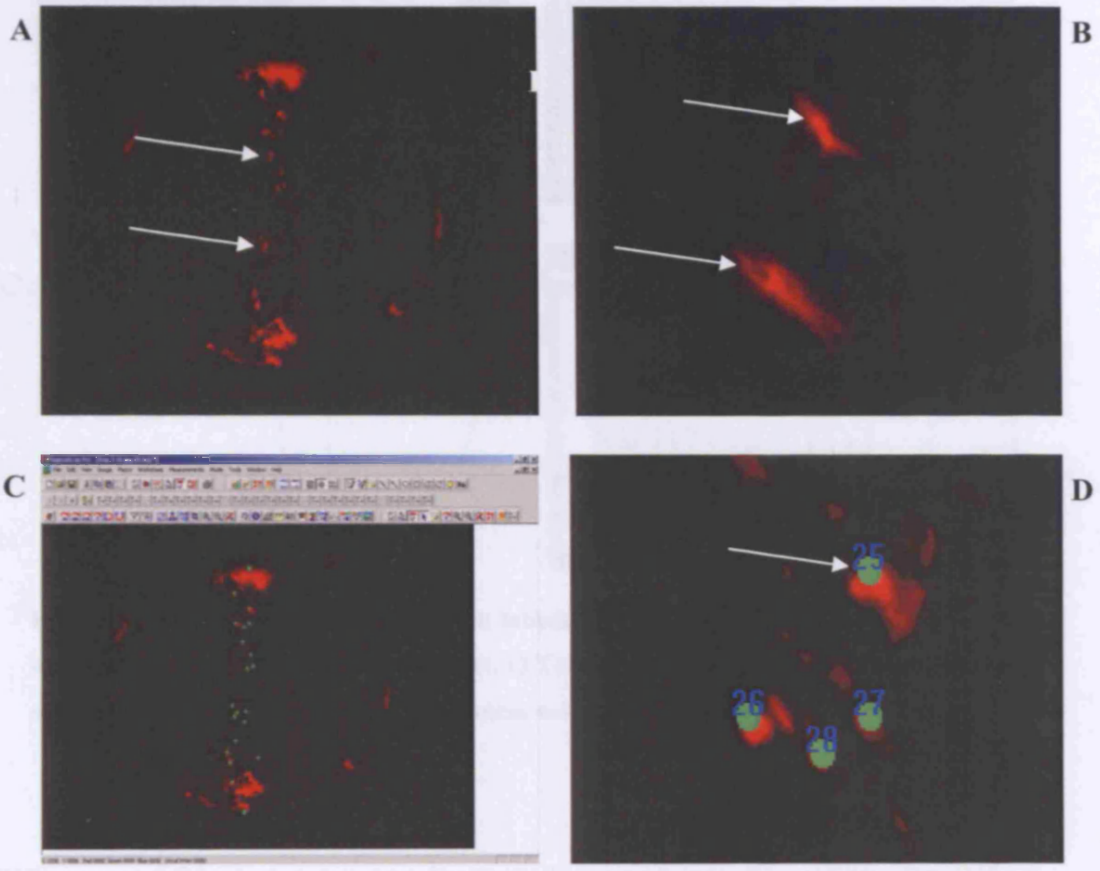


Figure 30 a-d: a) Typical UV photomicrograph obtained, 100X. b) Stained Fibroblasts 800X. c) Image imported into SigmaScan software, and images labelled manually. d) Close up of labelled and counted cells. Arrowheads show stained fibroblasts in each instance.

4.3.1.1 Cell counting technique.

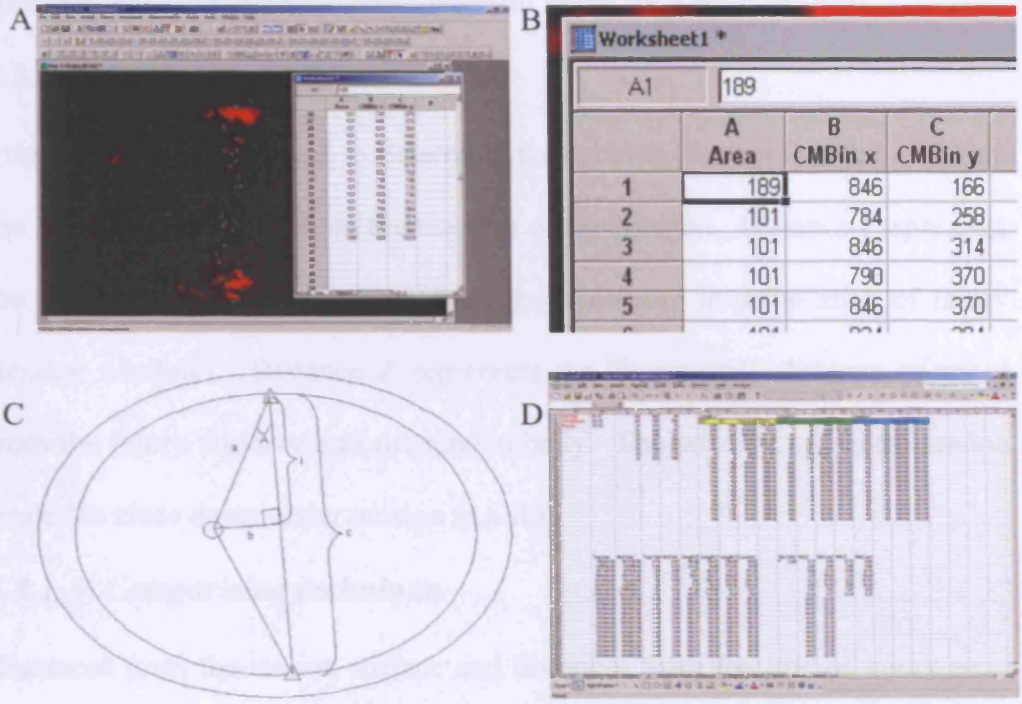


Figure 31: a) X,Y co-ordinates of each labelled cell was generated and converted to spreadsheet. b) Close up of spreadsheet. c) Trigonometry used to determine distances migrated from tendon surface. d) Distances calculated using spreadsheet software.

TIFF image UV photomicrographs were imported into SigmaScan Pro V5. This program facilitated each cell to be identified visually and tagged (labelled by hand) manually, and then the location of each labelled cell (to each other and the the edges of the incision) to be determined. Comparison of each UV image with the corresponding light image allowed the location of the superior and inferior margins of the window injury to be determined, and also marked on the program. The X,Y co-ordinates of each labelled cell, and the injury margins were then determined by the software and a spreadsheet of these

values created. Total cell number counts were also determined. These spreadsheets were imported into Microsoft Excel for further analysis.

4.3.1.2 Counting technique.

Trigonometry was utilised to determine the relative distances of the cell from the line of incision and from the margins of the incision. Distance I represents the "vertical" distance migrated from the synovium into the zone of injury (tendon window). Distance Z represents the "horizontal" distance migrated from the injury window into the tendon body. The window cut in the tendon tended to close down under tension to a slit.

4.3.1.3 Categorising technique.

Distances from the tendon surface and distances from the line of injury were categorised, using Microsoft Excel.

4.4 RESULTS

4.4.1 Number of sections

Frozen sectioning was difficult due to freeze-thaw artefacts or excessive specimen fracture on sectioning in some sections. 12 sections were cut from each of the 24 specimens, and the quality of each individual section was assessed under light microscopy (to ensure no bias in terms of cellular distributions, these only being visible under UV microscopy). The 4 best quality sections were chosen from each, i.e. those with the lowest number of artefacts and highest quality of sectioning, and the rest discarded. Satisfactory specimens were obtained from all procedural digits. Data was therefore collated from 4 sections from each digit, with 6 digits being assessed for each time period, hence, a total of 96 sections were analysed in this study.

4.4.2 Synovial fibroblast migration - the use of dii

DiI is a lipophilic membrane dye which, once taken up, undergoes a certain amount of lateral diffusion allowing the whole of the cell to be visualised. Confirmation of DiI localization in cells is shown in Figure 32. DiI has been shown to be taken up by viable fibroblasts, {Jones, Mudera *et al.*, 2003h}. Counter-staining the same section with H&E confirmed that the vital dye was confined to specific tenocytes.

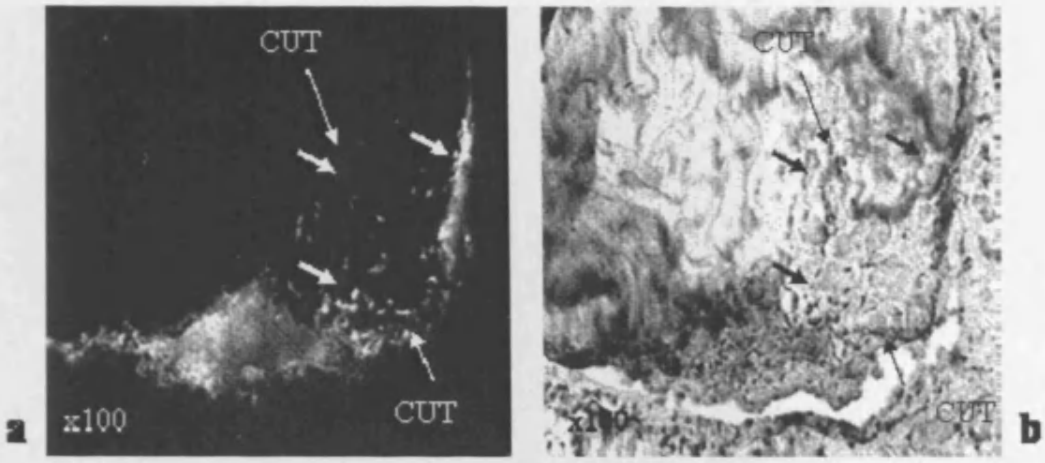


Figure 32: Co-localisation of DiI stained cells with H and E. (a) Photomicrographic image (X400) capture of lower right quadrant of tendon cross-section stained with DiI (10μM) highlighting the surface cells moving into a cut at Day 5. (b) The same section counter-stained with H and E. The nuclei of all cells are delineated confirming that DiI is localised to cells of the tendon. The arrowheads highlight the same sub-populations of cells in both a and b.

4.4.3 Confirmation of selective labelling

Frozen sections taken at 0 and 2 hours confirmed that the vital dye labelling was confined to the synovium at these time points. Tendon-surface cells were not directly stained by free dye or by cell to cell transfer. Similar images were obtained for the other time periods.

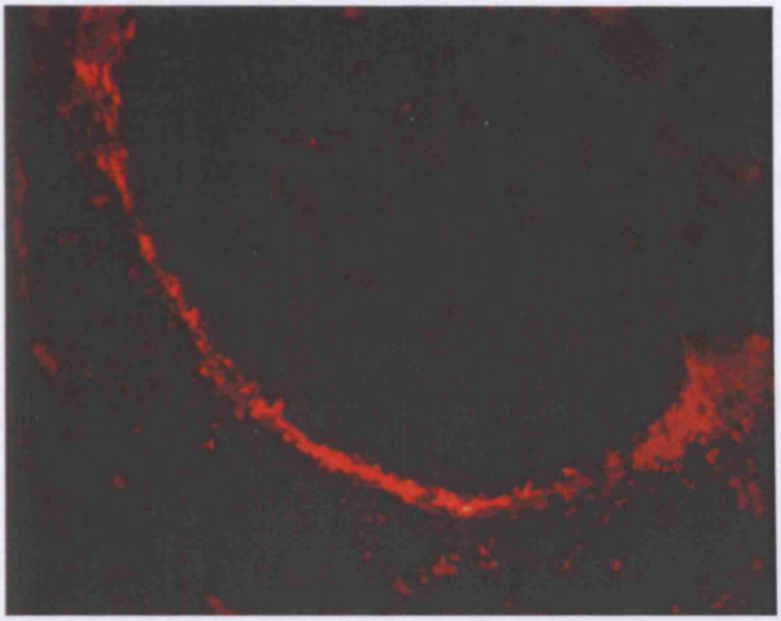


Figure 33: Micrograph of 8 micrometer microtome frozen section of tendon, through sheath & tendon, under UV light at time point t=0. The sheath cells are visible under UV light, confirming that they are stained with DiI. The tendon surface cells are not visible, confirming that they have not been stained with DiI, confirming hat the selective labelling technique outlined in Chapter 3 in effective.

4.4.4 Results description

4.4.4.1 Results description

Frozen sections of sufficient quality to facilitate cell counting were obtained through the injury region of all 24 tendons. Delineation of labelled cells within the injury region was possible. These photomontages are derived from one tendon per time point but were chosen as representative of each group. Figure 33 shows that there are no cells present within the cut at time point 0. By Days 1 and 3 (Figures 34 and 35), cells are present within the injury area and have started displacing laterally into the core substance. Deeper penetration of labelled cells was observed at Day 5 (Figure 36). The cell counts did not fall to below Day 0 levels at any point over the time course of the experiment.

4.4.4.2 Day 1

By Day 1, labelled cells were visible within and around the zone of injury; a distinct contrast to the Day 0 slides is immediately visible (Figure 34). These cells were observed to be diffusely present along the line of the injury. The majority of cells were observed individually, without clumping, with no particular distribution foci around either the tendon surface or centre of the incision. Labelled, synovial cells were frequently observed at points all around the synovial sheath. Some cells were observed to be present on the tendon surface, but the numbers were very small in comparison to those present within the zone of injury. Individual cells were clearly visible and identifiable.

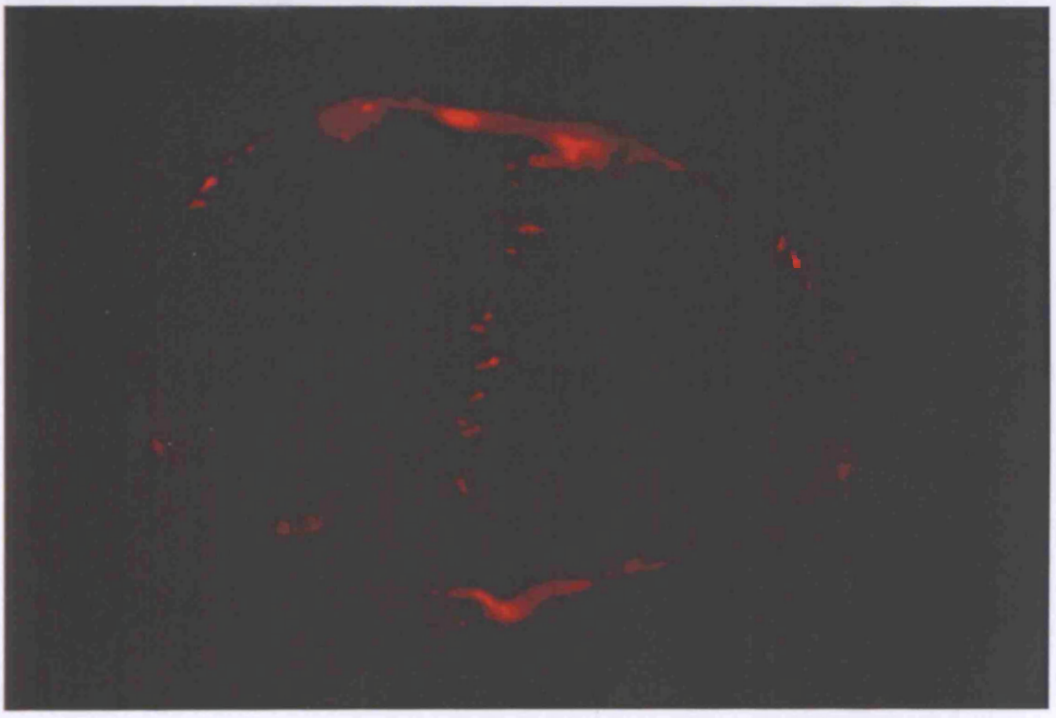


Figure 34 – UV Photomicrograph X200 of Operated Rat Tendon, Day 1 post-procedure. Labelled synovial cells are seen in the centre of image i.e. in the tendon injury zone

4.4.4.3 Day 3

By Day 3, labelled cells were more frequently observed within the zone of injury (Figure 35). Cells were still observed to be present all along the line of injury, but were more frequently observed at a greater distance from the actual line of injury. Labelled, synovial cells were less frequently observed still in situ in the synovial sheath, but to a much lesser degree than the preceding time points.

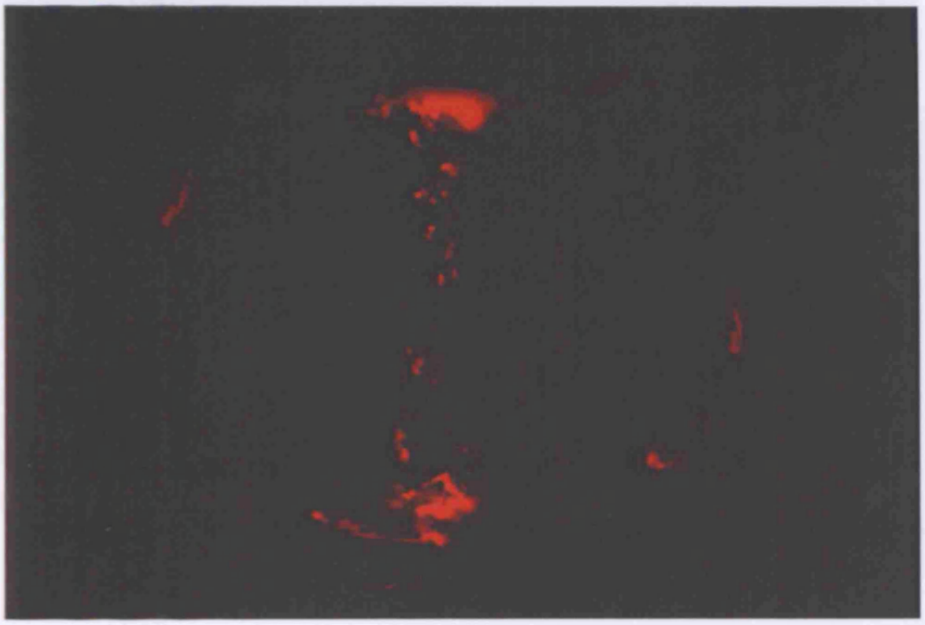


Figure 35 - UV Photomicrograph X200 of Operated Rat Tendon, Day 3 post-procedure.

4.4.4.4 Day 5

Figure 36 is a representative slide of the situation at Day 5, with labelled cells more frequently seen within the zone of injury. Cells were observed to still be present all along the line of injury, with more labelled cells present than either Day 1 or Day 3.

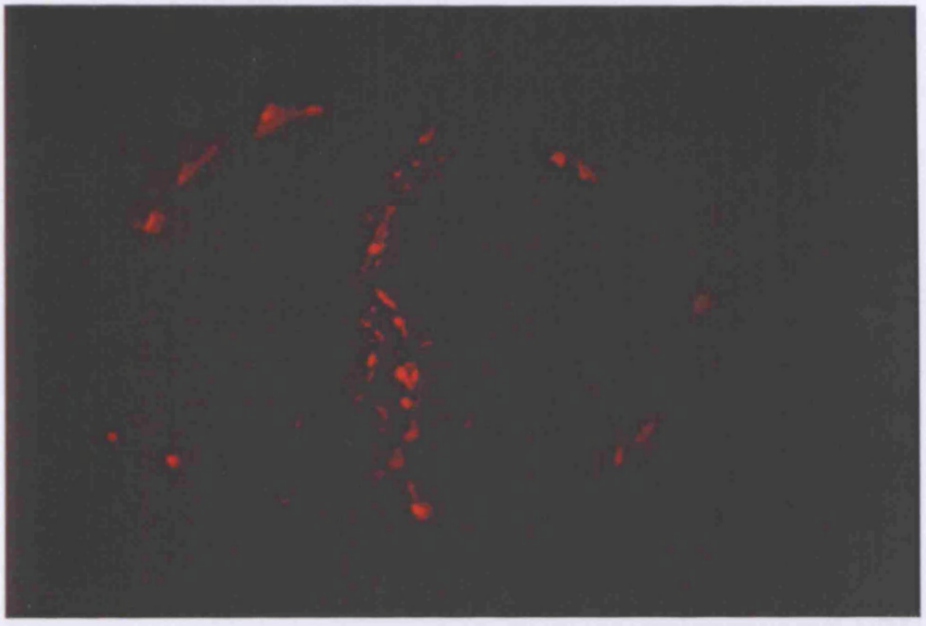


Figure 36 - UV Photomicrograph X200 of Operated Rat Tendon, Day 5 post-procedure.

4.4.4.5 Day 7

By Day 7, although labelled cells were seen less frequently within the zone of injury, some cells were still present all along the line of injury. There appeared to be a reduction in the number of stained cells within the cut, although lateral displacement into the substance of the tendon is still evident. Figure 37 illustrates a typical image from this time point.

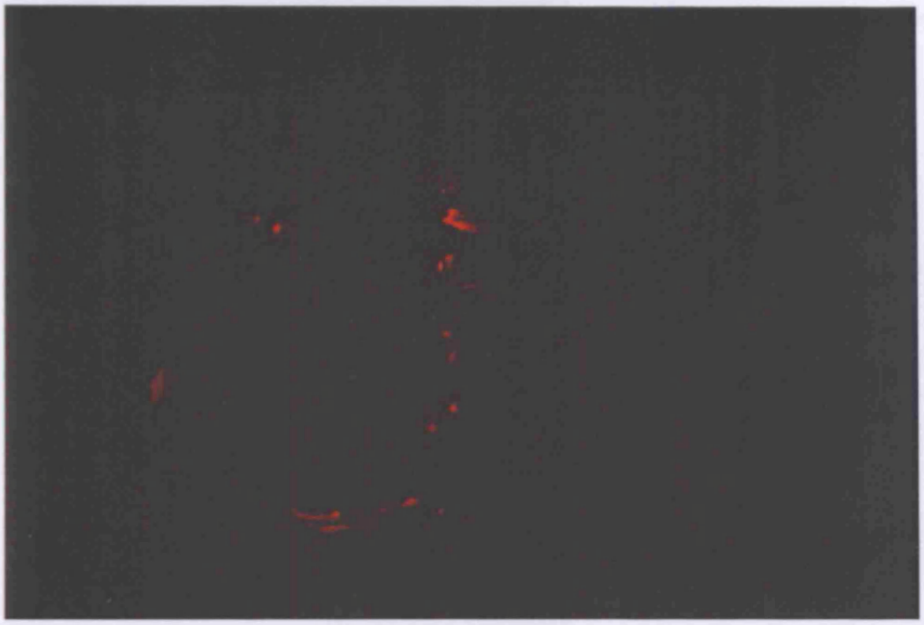


Figure 37 - UV Photomicrograph X200 of Operated Rat Tendon, Day 7 post-procedure. Labelled synovial cells are seen in the centre of image, having been originally confined to the synovium.

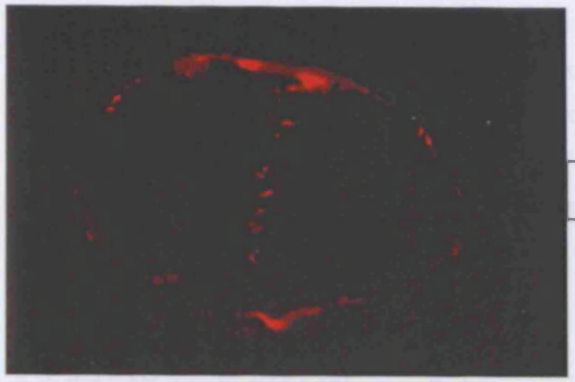
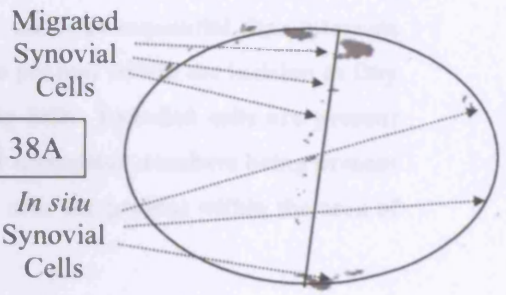


Fig 38A



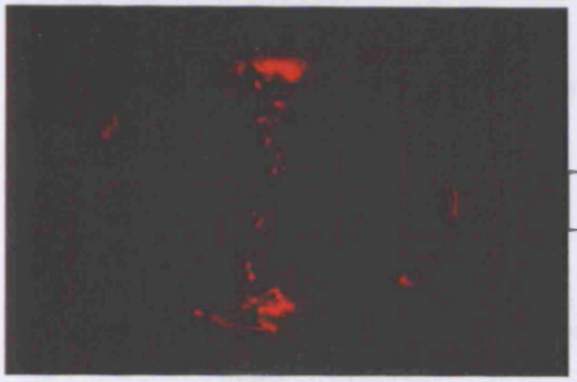
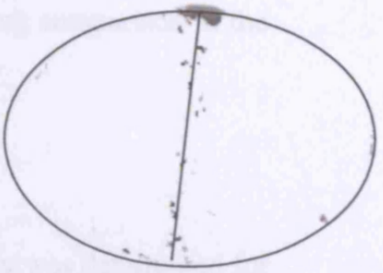


Fig 38B



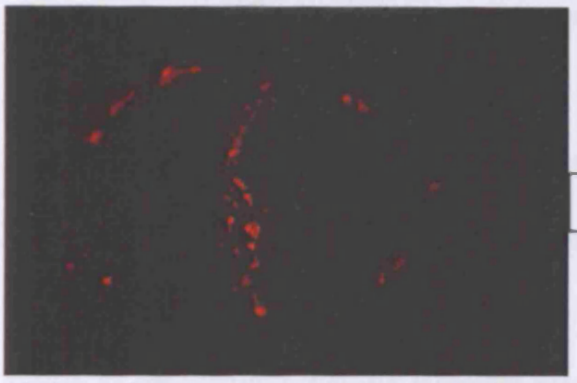
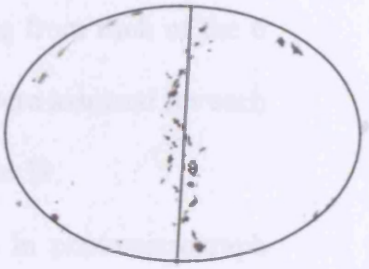


Fig 38C



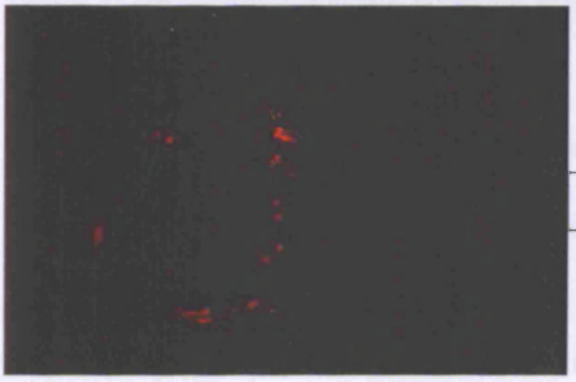


Fig 38D

Figure 38A-D: Caption on following page

Caption for figure 38: Ultraviolet photomicrographs (and corresponding black and white negative images) of transverse tendon frozen sections taken at sequential time intervals after procedure. The sequence illustrates labelled cells present within the incision at Day 1 Fig 38A, Day 3 Fig 38B, Day 5 Fig 38C, Day 7 Fig 38D. Labelled cells are present within the zone of injury by Day 1, with proegressively increasing numbers being present at Day 3 and more at Day 5. By Day 7, fewer labelled cells are present within the area of injury.

4.4.4.6 Photomontage

Figure 10 shows a photomontage of figures 33-36, allowing comparison of the dynamic situation which occurs from Day 1 to Day 7.

4.4.5 Cell numbers

The mean number of cells in and around the zone of injury was determined for each slide and a mean number of migrated cells per slide calculated for each time period. These figures were obtained from 4 sections from each of the 6 tendons at each time period; hence a total of 24 sections were assessed for each time period. The results are illustrated graphically in figure 39.

No migrated cells were present at Day 0, as illustrated in photomicrograph figure 33, confirming that the labelling was specific to the synovial cells. By Day 1 post injury, an average of 6.71 cells was observed in the zone of injury. The mean number of cells number increased progressively at Day 3 and Day 5, with the maximum number of cells being present at Day 5. The increase between Day 1 and Day 3 was 6.63 cells, a 98% increase, the increase between Day 3 and Day 5 was 0.58 cells, a 4.3% increase. By Day 7, the number of cells present fell to 7.08, a fall of 49% from Day 5.

Figure 39 shows that statistically significant differences in cell numbers occurred were between Day 0 and Day 1, Day 1 and Day 3, and Day 5 and Day

7. There was no significant difference in numbers between Day 3 and Day 5. The increase from Day 0 to Day 1 suggests that cells are able to migrate from their synovial environment to the area of injury with 24 hours. The significant increase between Day 1 and Day 3 suggest that cells are still being "recruited" during this period. After the Day 3 time point, there are no further significant increases in cell numbers, suggesting the majority of migration has already occurred. The statistically significant decrease in number from Day 5 to Day 7 could be due to an experimental limitation, such as a serial dilution of vital dye, or due to cell death, migration or division of the labelled synovial cells.

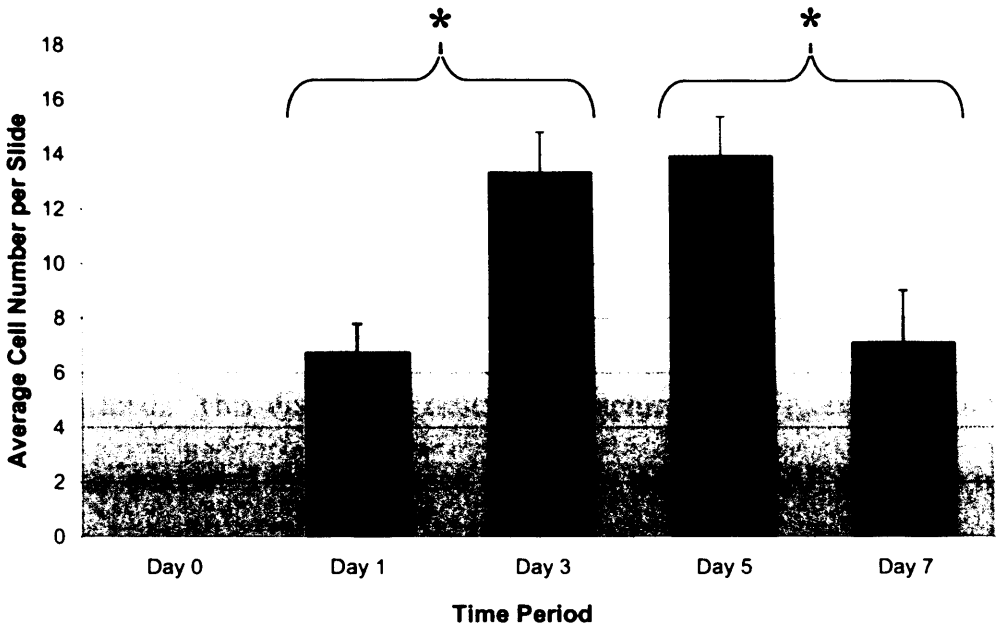


Figure 39 – Average Migrated Cell Number per Slide vs Time
(* = statistically significant P<0.05)

	Day 0	Day 1	Day 3	Day 5	Day 7
Distance	0.00	6.70	13.3	13.9	7.08
Change from preceding time period (cell numbers)	NA	6.71	6.63	0.58	-6.83
Change from preceding time period (percentage)	NA	0.00	98.76	4.37	-49.10

Table 3 - Migrated Cell Number per Slide with Time

4.4.6 Distance I – Distance Migrated from Tendon Surface

4.4.6.1 Qualitative assessment:

One of the important migration variables was the distance migrated from the synovial surface into the line of injury. The method in section 4.3.1.1 above describes how, for each cell, the distance from the tendon surface (distance I) was determined. This distance is described graphically in Figure 40. Each distance was converted into a distance relative to the height (AP diameter) of the tendon and a mean relative migration distance determined for each time period; this is plotted graphically in Figure 12. As each distance is a percentage relative to the AP diameter of the tendon, the maximum distance is 50, and a number greater than this illustrates that more cells are present within the core of the tendon. The mean distance I is 0 at Day 0, as no labelled cells are present within the zone of injury. In contrast to the number of cells, and distance Z, there is little difference between the values for each of the subsequent time periods.

From this point, all Distance I are described as relative percentages. By Day 1, the mean distance I was 27.89, demonstrating that cells have, within 24 hours, redistributed into the zone of injury, a statistically significant increase from Day 0. At Day 3, the mean distance I was 27.01, a fall of 3.16% on the Day 1 value, a non-statistically significant fall. At Day 5, the mean I was 27.01, a 0% change over the subsequent 48 hour period. By Day 7, the mean I is 26.8, a decrease of 0.16 (0.60%). None of the changes are statistically significant, other than the initial change from Day 0 to Day 1. All the figures above display relative cell numbers; they show grouping around the 25% figure,

suggesting that little window injury location-specific migration seems to be occurring.

4.4.6.2 Distances – Graphs & Tables

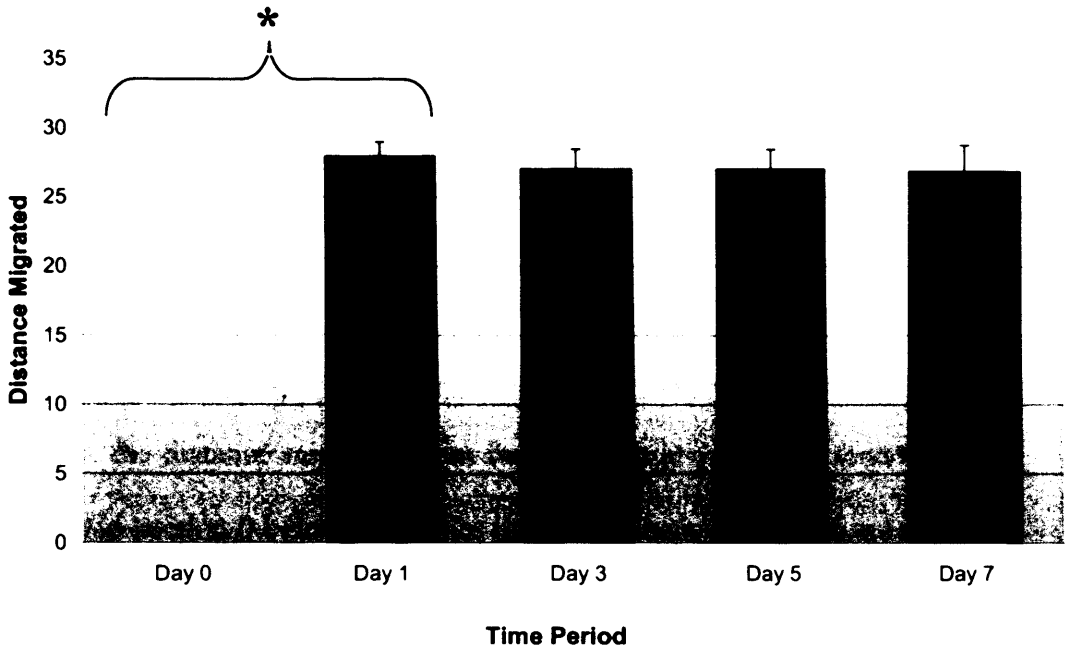


Figure 40 - Graph illustrating the mean distance migrated by labelled synovial cells from the tendon surface with time.

	Day 0	Day 1	Day 3	Day 5	Day 7
Distance	0.00	27.8	27.0	27.0	26.8
Change from preceding time period (cell numbers)	NA	27.89	-0.88	0.00	-0.16
Change from preceding time period (percentage)	NA	0.00	-3.16	0.01	-0.60

Table 4 – Table illustrating the mean distance migrated by labelled synovial cells from the tendon surface with time.

4.4.6.3 Categorised data

The preceding results sub-section describes the *mean* distance migrated from the tendon surface into the window injury. A more detailed and possibly more meaningful method of results description is the categorisation of the total number of cells at different depths from the tendon surfaces. This was carried out in Microsoft Excel, by categorising all cell relative distances from the tendon surface. This distance only represent the "vertical" component of the migration, the distance migrated from the line of injury into the tendon core is described in section 0 below. Changes in cell numbers with depth are shown in figure 41 below. At Day 0, all stained cells confined to the synovium, as described in section 4.4.3 above. Day 0 data is not included in figure 41, but is included in Table 5. No labelled cells were seen at deeper layers at this control point.

The rationale for making all the distances relative is the shapes of tendons are known to vary in man and rabbit, and similar changes were observed in the rat. Although the injury was standardised, some (subjective) variation was still seen in the sections analysed. At 24 hours more cells were observed in the surface areas around the cut, with a few dipping deeper down into the cut. 14 cells were seen within the most superficial 10% of the zone of injury, 30 in the 10-20% zone, 46 in the 20-30% zone, 39 in the 30-40% zone and 32 in the 40-50% zone. The profile of stained cell position altered further by 72 hours post injury. The graph shows that, although larger cell numbers are present, the distribution is still similar to Day 1; cells are evenly distributed throughout the zone of injury, a phenomenon which is still apparent at Day 5. By Day 7,

although overall cell counts have decreased, the distribution is still even throughout the zone of injury.

4.4.6.4 Zoned – Graphs & Tables

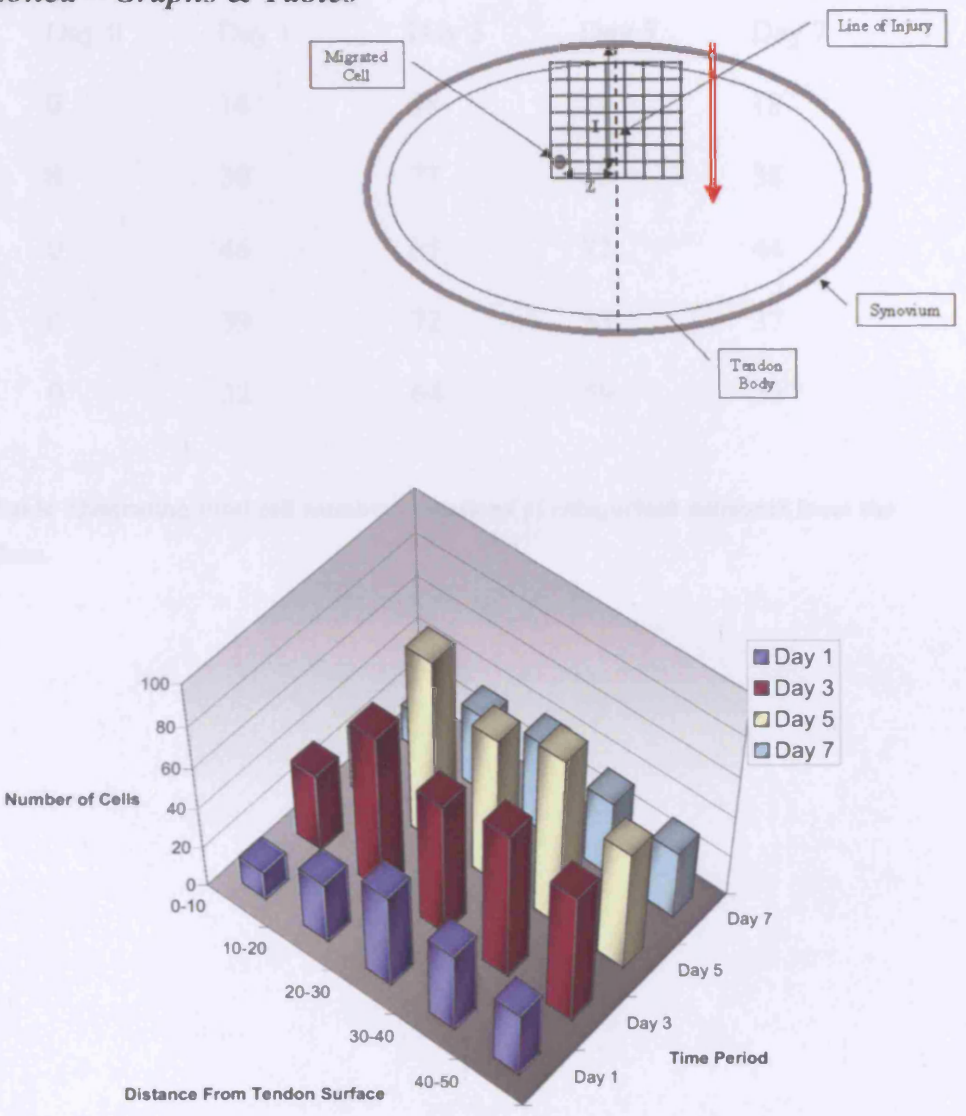


Figure 41 – 3D column plot illustrating total cell numbers locations at categorised distances from the tendon surface

Distance	Day 0	Day 1	Day 3	Day 5	Day 7
0-10	0	14	38	31	18
10-20	0	30	77	89	38
20-30	0	46	65	72	44
30-40	0	39	72	83	37
40-50	0	32	64	59	33

Table 5 – Table illustrating total cell numbers locations at categorised distances from the tendon surface.

Distance Z – Distance from Line of Injury

4.4.6.5 Distances – Writing

The other variable of significant interest is the average distance migrated by cells from the zone of injury into the tendon core, represented by the distance Z. The method in section 3.2 above describes how, for each cell, the distance from the tendon surface (distance Z) was determined; this distance is illustrated and described graphically in Figure 29. For each cell, each distance was converted into a distance relative to the width of the tendon and a mean relative migration distance determined for each time period, and this plotted graphically in figure 42. As no labelled cells are present within the zone of injury, the mean distance Z is 0 at Day 0, as had already been proven during the development of the model. Each distance is relative to the overall *height* of the tendon, to allow comparison of the results to the distance I. The rationale for this being that the tendons varied in height and width; by standardising distance Z to distance I, direct comparisons could be made.

Day 0 is not included in Figure 42, but is included in table 6. No labelled cells were seen at deeper layers at this control point. At Day 1, the distance Z is 5.93, implying that cells have migrated from the zone of injury into the tendon core. By Day 3, the mean Z is 7.98, an increase of 35.65%, although not a statistically significant increase. This indicates that cells are migrating further into the tendon core. By Day 5, the mean Z is 8.13, a non-significant increase of 1.86% compared to the Day 3 value. By Day 7, the mean Z is 9.27, a 14% percent increase on the Day 5 value.

This represents a progressive increasing trend in the mean Z in terms of the distance of the labelled synovial fibroblasts from the line of injury. Although initially, a window injury is made, when the tendon is replaced into the synovial sheath, this potential space is obliterated, as confirmed on visual inspection and on microscopic examination of the slides at Day 0. Usually by Day 1, although it is apparent on light microscopy where the injury has been made, the two sides of the window injury are still in apposition.

4.4.6.6 Distances – Graphs and Table

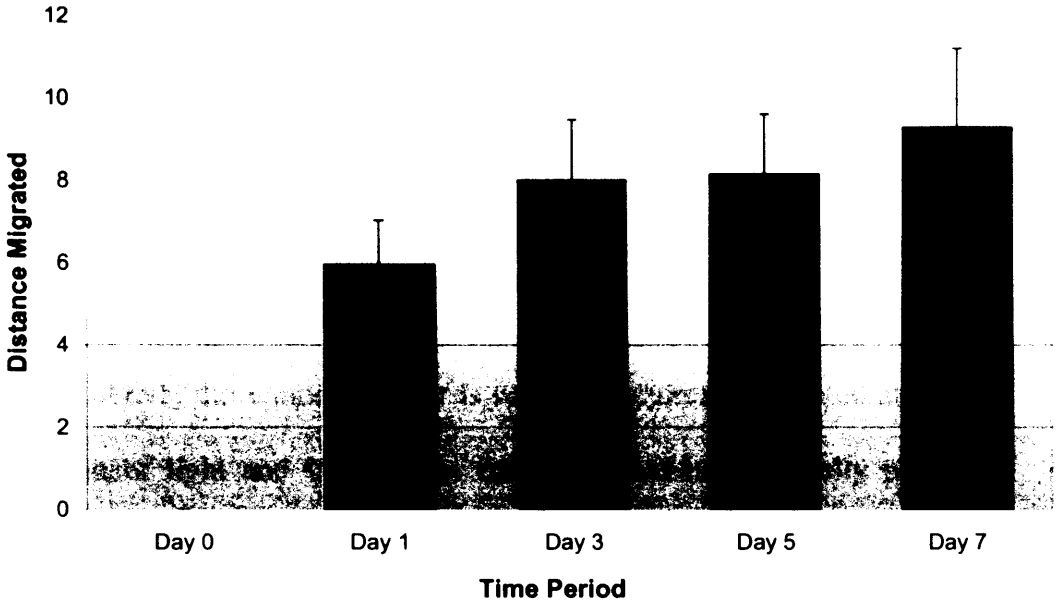


Figure 42 - Graph illustrating the mean distance migrated by labelled synovial cells from the cut into the tendon with time.

	Day 0	Day 1	Day 3	Day 5	Day 7
Distance	0.00	5.93	7.98	8.13	9.27
Change from preceding time period (cell numbers)	NA	5.93	2.06	0.15	1.14
Change from preceding time period (percentage)	NA	0.00	34.65	1.86	13.99

Table 6 – Table illustrating the mean distance migrated by labelled synovial cells from the cut into the tendon with time.

4.4.6.7 Zoned – Writing

Section 4.4.6.5 above (using the Distance I) describes the mean distance migrated from the line of injury into the tendon core and how this distance varies with time. A more detailed and possibly more meaningful mode of results description is the categorisation of the total number of cells at different depths from the tendon surfaces. This was also performed in Microsoft Excel, by categorising all cell movement relative distances from the line of injury. As described above, the window injury is normally obliterated immediately, and comparison of light and UV images confirms what is graphically portrayed above; there is a definite trend to observe; more labelled cells further from the zone of injury with increasing time. It is important to reiterate that distance Z only represents the "horizontal" component of the migration.

A three-dimensional bar chart is plotted in figure 43, demonstrating the cellular profile across the tendon with the cut centred, thus indicating the degree of lateral displacement of stained cells with time. The corresponding data showing standard distribution data, numerical and percentage increases is shown in Table 7; day 0 is not included in Figure 43, but is included in Table 7; no labelled cells were seen in the incision at day 0, and hence are not included in the figure. As described in section 4.4.6.5 above, the distances described are relative to the width of the tendon, from width markings placed at the time of initial tendon assessment in SigmaScan.

At 24 hours post injury, the distribution patterns show that the majority of cells are confined to the 0-5 zone, i.e. in very close proximity to the line of injury. However, some labelled cells were observed up to 20% of the tendon height

from the zone of injury. By Day 3, we have seen already, many more cells are still present within the healing tendon, and although the distribution shows that the majority of cells were still present around the line of injury, many more cells were present much further from the zone of injury. This may be due to "migration", or to random motion of the cells. Whatever migrational influence determined the migration from the synovium is clearly present away from the zone of injury as well. By Day 5, after another increase in actual cell numbers present within the zone of injury, we see a similar pattern of distribution to Day 5. Day 7 sees a diminution of cell numbers, but with cells still present in the more zones a greater distance from the line of injury.

4.4.6.8 Zoned – Graphs & Tables

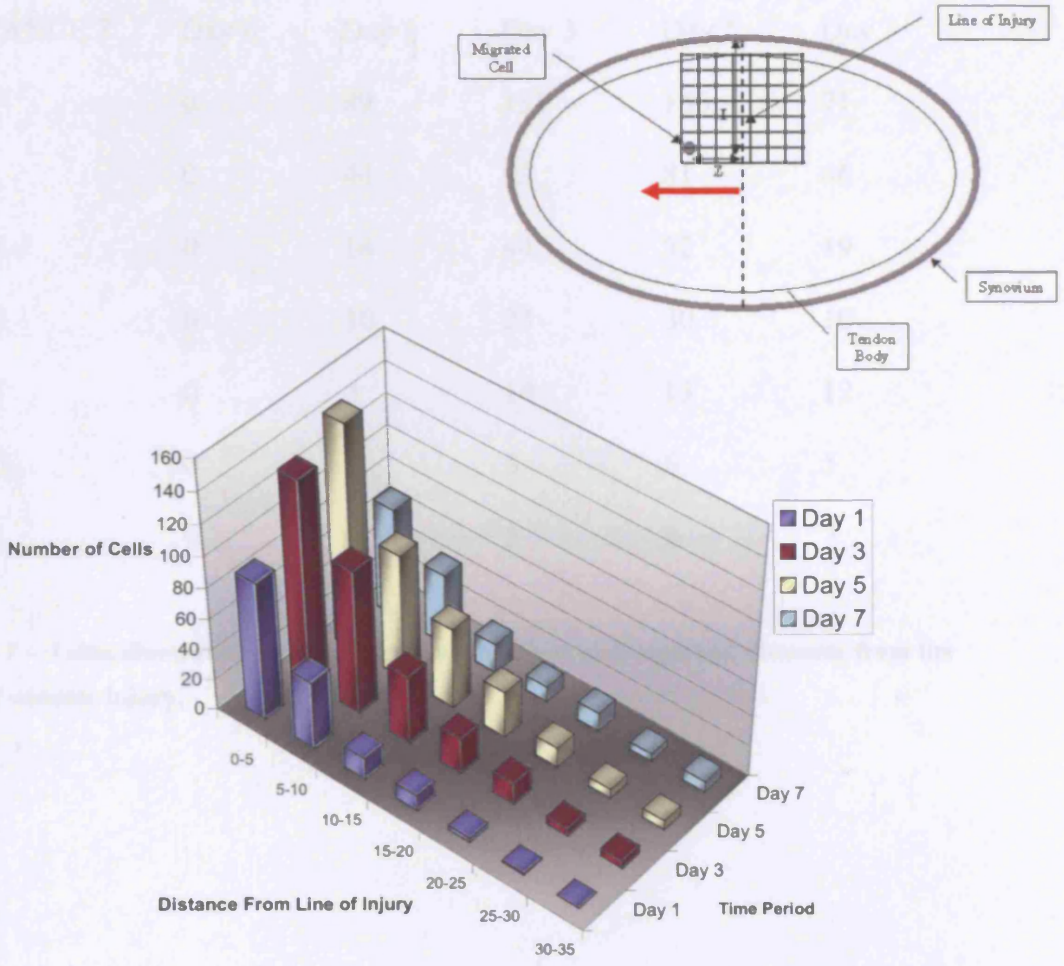


Figure 43 - 3D column plot illustrating total cell numbers locations at categorised distances from line of windows injury.

DISTANCE Z	Day 0	Day 1	Day 3	Day 5	Day 7
0-5	0	89	132	146	71
5-10	0	44	93	81	46
10-15	0	14	44	52	19
15-20	0	10	23	30	10
20-25	0	3	14	13	12
25-30	0	1	5	6	5
30-35	0	0	5	6	7

Table 7 – Table illustrating total cell numbers locations at categorised distances from the line of window injury.

4.5 DISCUSSION

4.5.1 Introduction

The conflicting theories of *extrinsic* and *intrinsic* tendon healing have significant evidence to support themselves; indeed the majority of recent work now suggests their simultaneous and non-mutually exclusive occurrence. However, studies to date have mainly relied upon histological interpretation, and there is no direct evidence to support the existence of the extrinsic mechanism. We aimed to produce a novel model capable of selective labelling of synovial fibroblasts with concurrent tendon window injury and then use this model to attempt to determine whether the extrinsic tendon healing could be observed directly.

4.5.2 Methodology

There are several important aspects of the methodology utilised in this study. The first concerns the use of the animal model, the second the type of injury utilised; the removal of the tendon from the synovial sheath and its subsequent replacement and finally the use of vital dye to facilitate selective tendon labelling.

4.5.2.1 Animal model.

In 1984 Gelberman and Manske investigated the histologic differences in terms of in vitro repair between rabbit, chicken, dog, and monkey {Gelberman, Manske *et al.*, 1984}. Using a tissue culture system, 90% transverse lacerations were made in tendon segments obtained from the different animals and assessed by light and electron microscopy at intervals. The sequence of

repair including epitenon thickening, cellular differentiation, cell migration, and phagocytosis was seen in each of the repaired tendons, and the endotenon cells of several animal tendons appeared to be synthesising collagen. Although the speed of response varied between the animal models, the type and nature of the response was consistent. The rat model was not utilised in Gelberman's study, but the consistency between the other animal models suggest that intra-species variability is not deemed to be significant {Gelberman, Manske *et al.*, 1984}.

The canine and rabbit models have historically been the most popular for investigating tendon injury, however, the rat model has been shown to be suitable for some *in vivo* studies of tendon healing {Bora FW, Lane *et al.*, 1972; Iwuagwu and McGrouther, 1998a}. The model used by Jones *et al.* {Jones, Mudera *et al.*, 2003g} has been shown to be effective for a similar study investigating tendon surface cell migration, and our animal methodology is analogous to this technique, facilitating comparison of results.

The rat model was more difficult to use in terms of requiring operative microscopy equipment and more delicate tissue handling, but with some experience, this did not pose too much of a problem. There were advantages of using the rat model, such as cost, and the ease of conducting anaesthesia.

4.5.2.2 Window Injury.

The *in vivo* simulation of tendon injury has previously been performed in a variety of different ways. The window injury technique was originally utilised by Matthew, Moore and Campbell {Matthew, Moore *et al.*, 1987}, investigating the ultrastructure of collagen fibril formation in the healing

extensor digitorum longus tendon of the rat. Their aim was to produce two healing environments: one (the lesion area) where stress levels were reduced; the other (the non-lesion area) where stress levels were increased. Their results demonstrated that in the lesion area fibrils were synthesised in response to tissue damage and low levels of stress, possibly of type III collagen. In the non-lesion area fibrils of type I collagen were synthesised in response to raised levels of stress. They also suggested that their model would be effective as utilised in this study, i.e. to produce a "through-and-through" tendon window lesion to observe responses to injury. A similar model was also used by Iwuagwu and McGrouther in 1998, to investigate the early cellular response in tendon injury {Iwuagwu and McGrouther, 1998b}. Loaded and unloaded tendon matrix changes in the tendon, window, and tendon-window junction were observed by transmission electron microscopy, observing rapid and extensive change in the tendon structure with rapid loss of definition of the window edge and an increase in cellularity of the tendon substance. This study demonstrated that the cellular response after injury in this extensor tendon model is affected by tensile loading, there being increased cell numbers in both the window and tendon substance in the unloaded tendon, a phenomenon which is discussed in more detail in Chapter 5, in which a similar experiment is performed to observe the effect of tendon mobilisation and immobilisation on tendon healing, in terms of relative cellularity. These successful experiments suggest that the rat model is suitable for testing out experimental hypothesis.

Validation of the use of the window injury model and the vital dye staining technique was performed by Jones *et al.* {Jones, Mudera *et al.*, 2003f}, upon

which this model is based. A comparison of our methodology with that used by Jones *et al.* present in Chapter 3.

4.5.2.3 Vital dyes

To allow the testing of our experimental hypothesis, vital dyes have been utilised extensively for *in vitro* and *in vivo* biological monitoring and observation. Honig and Hume, in 1986, were two of the original proponents for their use, observing that fluorescent carbocyanine dyes allowed living neurons of identified origin to be studied in long-term cultures {Honig and Hume, 1986b}. They had tried to identify a solution to the problem of determining the original cell identity, a prerequisite for many proposed studies of neurons in culture. In this particular instance, Honig & Hume were studying the *in vitro* interactions between spinal cord neurons and sympathetic chain neurons. They described how two highly fluorescent lipid-soluble Carbocyanine dyes, differing in colour but otherwise similar, would incorporate into the plasma membrane, enabling those neurones to be identified in culture. They also observed that some of the labelled membrane became internalised and retained its fluorescence, allowing identification of cells for several weeks in culture. Subsequent tests determined that the uptake of vital dyes did not affect the survival, development, or basic physiological properties of neurons and did not spread detectably from labelled to unlabelled neurons. {Honig and Hume, 1986a}. Honig and Hume went on to confirm that the major mechanism of translocation for these molecules was lateral diffusion in the membrane, rather than fast axonal transport, implying that they could be

used for the study of other tissues, such as fibroblasts {Honig and Hume, 1989}

In the same year, Crawford *et al.* used vital fluorescent staining of human endothelial cells, fibroblasts, and monocytes in the assessment of surface morphology for studying cell growth and interactions on viable cells colonising artificial heart valves and vascular grafts {Crawford, Milford *et al.*, 1989}.

McNeilly, Banes *et al.* have shown that vital dyes can be used to give a very high level of detail, using them to delineate gap junctions. They also demonstrated that vital dyes can be used successfully in labelling tendon cells *in vivo*. Their objective was to determine how the tendons responded to mechanical load. Vital dyes were used in unfixed cryosections of adult rat digital flexor tendons, and successfully stained with DiI to demonstrate cell shape {McNeilly, Banes *et al.*, 1996b}.

For this study, a variety of cellular labels were assessed *in vitro*: DiI had the most appropriate characteristics: lack of leakage from cells, cellular specificity and sufficient longevity. Once attached to cells, the dye is not passed to other cells unless by cell division. In this case, DiI was used to delineate fibroblasts within the tendon body {McNeilly, Banes *et al.*, 1996c}. Jones *et al.* have quantified the migration of tendon surface cells into the core following injury. We have used the same concentration of DiI here in order to selectively stain the synovial fibroblasts.

The fading of DiI at seven days could have been due to division of the cells once they reach the core, metabolic irradiation of the dye by the cell, or apoptosis of the surface-derived cells once they have fulfilled their function.

This suggests that Kakar *et al.* actually visualised apoptosis of surface derived fibroblasts that had migrated deep into the cut by the end of the first week rather than indolent endotenon cells (Kakar *et al.* ., 1998).

4.5.2.4 Tendon removal.

A variety of different surgical techniques were assessed to allow selective labelling of the synovial fibroblasts, as described in Chapter 3. Whilst the tendon surface was accessible proximally by pulling the flexor tendon out of the synovial sheath through flexing the digit, it is more difficult to selectively apply any substance to the synovium lining the sheath alone. Dual labelling with 2 vital dyes, keeping the tendon within the sheath, but physically protecting the tendon with subsequent direct application of the vital dye to the synovium via a per-cutaneous approach were all tried, but the technique described here gave more reliable results. Care was taken to ensure selective staining of the synovium always occurred; whilst removed from the synovium, the FDP tendon was kept moist by wrapping in saline soaked swabs, and therefore any labelled cells found to be present within the cut would be synovial in origin. In order to ensure that this method did not generate an excessive number of adhesions, procedural and control specimens were assessed for evidence of adhesions. There was no evidence of adhesion formation in any treatment or placebo group.

4.5.3 Comparison of results with Jones *et al.*

4.5.3.1 Introduction

Our methodology is based on that of Jones *et al.* {Jones, Mudera *et al.*, 2003e}, as outlined in Chapter 3. The results of this study are presented below with excepts from this work for the purposes of comparison.

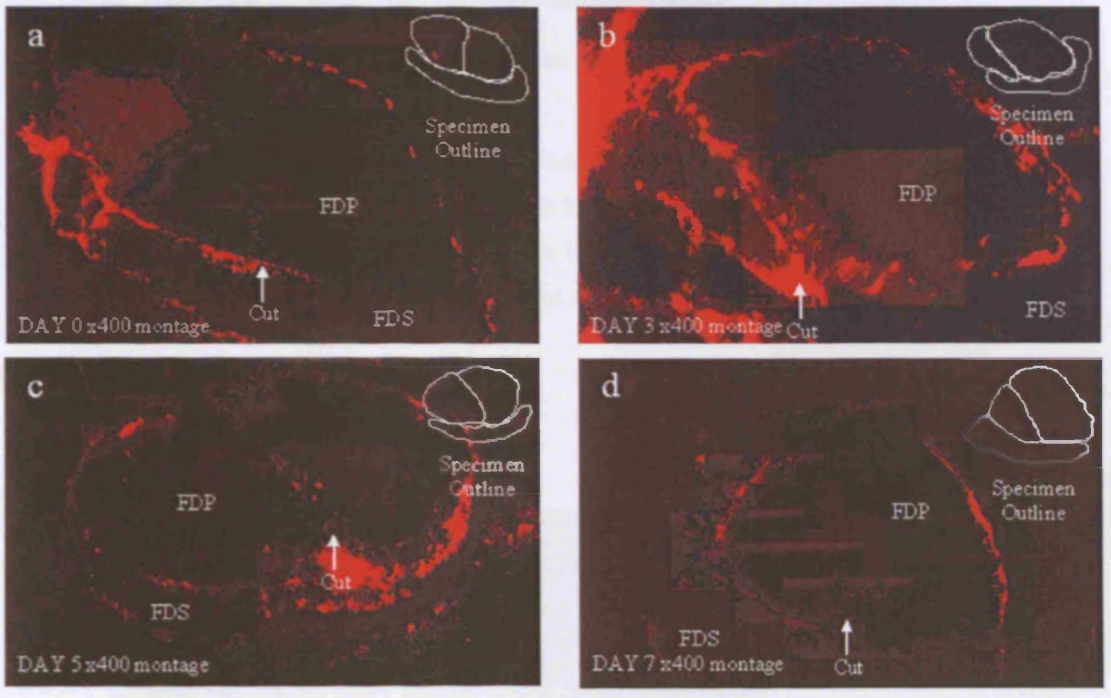


Figure 44 –Jones *et al.* (2003) - montages of DiI-stained tendon cross section in vivo. a to d represent cross sections with the partial tenotomy cut marked at days 0 (a), 3 (b), 5 (c) and 7 (d) respectively. Each montage is labelled for orientation, FDS = flexor digitorum superficialis, FDP = flexor digitorum profundus.

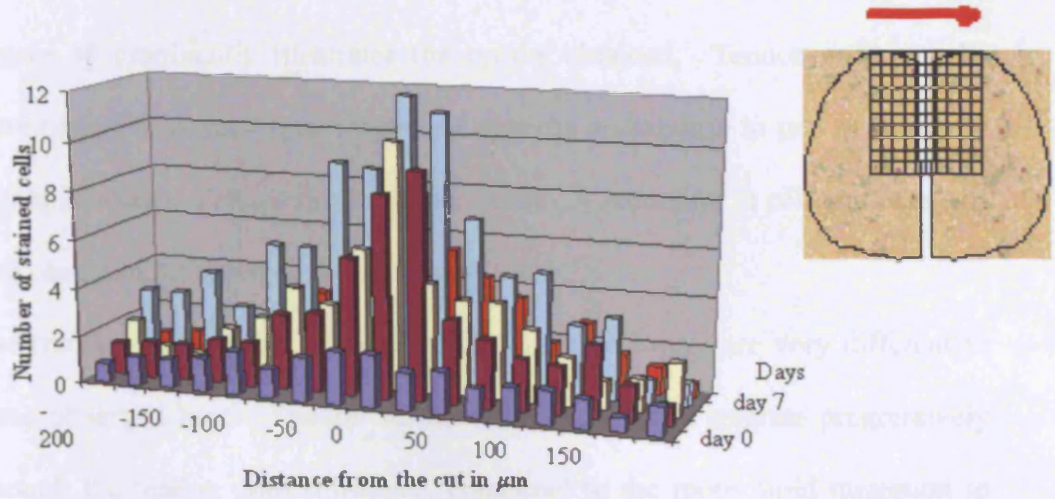


Figure 45 - Cellular profile across the partial tenotomy. (a) The 400-micron grid centered on the vertical tendon cut. Cell counts in columns were performed. The change in cell count per column over time is shown in (b). The middle of the graphs X axis is centred on the vertical cut. {Jones, Mudera *et al.*, 2003d}

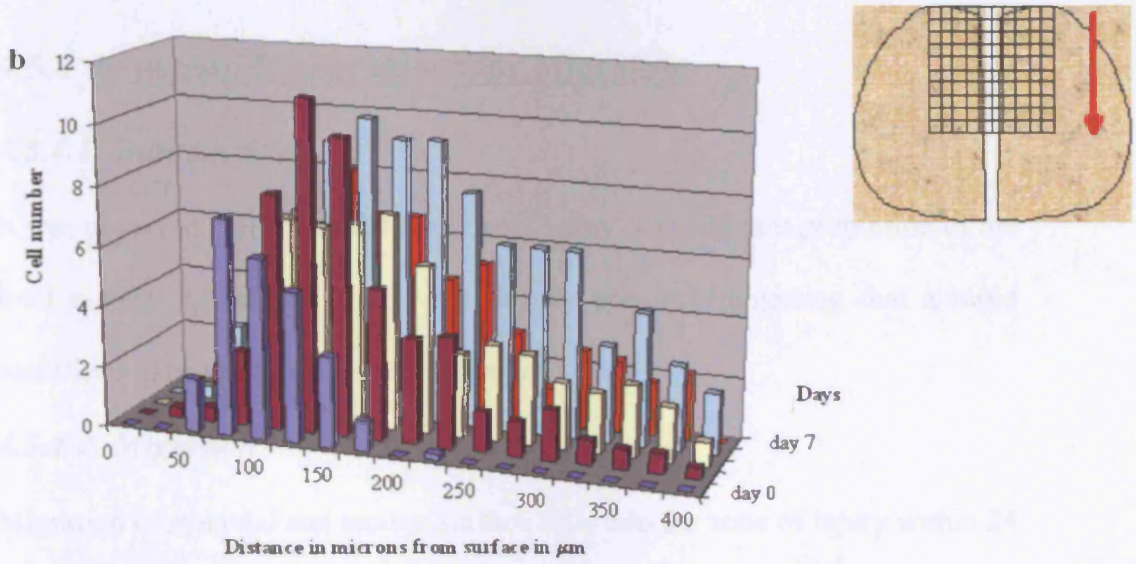


Figure 46 - Cellular profile across the partial tenotomy. (a) Shows the 400-micron grid centred on the vertical tendon cut. Counts in columns were performed. The change in cell count per column over time is shown in (b). The middle of the graphs X axis is centred on the vertical cut {Jones, Mudera *et al.*, 2003c}.

Figure 46 graphically illustrates the results obtained. Tendon surface cells were observed to start their migratory patterns in response to partial tenotomy within 24 hours, a result similar to our study. A reduction in cell numbers was also observed, at seven days post injury.

The trends of migration of cells into the zone of injury are very different to those observed here: Tendon surface cells are seen to migrate progressively through the tendon core substance, compared to the more rapid migration to become evenly distributed throughout the length of the incision seen here.

Surface derived fibroblasts migrated into a surgically created window within 24 hours. At subsequent time points, cells not only continue to migrate into the wound from the surface, but also spread laterally from the window into the core substance. This process is maximal at 5 days.

4.5.4 Reasons / Explanations for Migration

4.5.4.1 Introduction

It was observed that within 24 hours post injury, a significant proportion of the total number of migrated cells are already present, suggesting that a rapid mobilisation and migration process occurs.

4.5.4.2 Migration

Migration of synovial and tendon surface cells into the zone of injury within 24 hours indicates that the processes involved are rapidly activated. Explanation of this phenomenon has been undertaken in a variety of different ways; work by Gelberman *et al.* in a canine model has shown that fibronectin levels increase dramatically in the epitenon within seven days after repair, coinciding with peak epitenon cellular activity. He concluded that fibroblast chemotaxis

and adherence to the substratum is related directly to fibronectin secretion {Gelberman, Steinberg *et al.*, 1991b}. It seems likely that fibronectin does play a part in the migration process, indeed future work, to follow on from this thesis, proposes the use of fibronectin mats placed around the healing tendon to prevent or encourage cellular migration. Meanwhile, Duffy *et al.*, observed significant levels of BFGF, EGF and PDGF, in healing canine intrasynovial flexor tendons, providing compelling evidence that a variety of growth factors are present in healing digital flexor tendons {Duffy FJ, Seiler *et al.*, 1995}. Chang *et al.*, in 1998, also found high levels of BFGF, a cytokine that plays a fundamental role in angiogenesis, in both the tendon and synovium during tendon healing, perhaps indicating that the tendon healing response occurs to an equal extent in both parts of the tendon {Chang, Most *et al.*, 1998b}.

Comparing the data from this study with that of Jones *et al.* {Jones, Mudera *et al.*, 2003b}, it is apparent that synovial sheath derived cells and tendon surface derived cells respond at slightly different times to the healing response. This difference in response corresponds to work by Abrahamsson & Lohmander, in 1996, who determined that IGF1 had differential effects on matrix and DNA synthesis in various regions and types of rabbit tendons. They examined the effects on proteoglycan, collagen, non-collagen protein, and DNA synthesis in short-term explant cultures of intrasynovial intermediate and proximal segments of deep flexor tendons extrasynovial segments of deep flexor tendons. They suggested that the findings demonstrated differences in mitotic capacity between anatomical regions of tendons during culture without

recombinant human insulin-like growth factor-I and in matrix synthesis after stimulation with it {Abrahamsson and Lohmander, 1996b}.

For tendon-surface derived tenocytes to respond differently to synovial fibroblasts, then either there needs to be a different expression of growth factors, or a different response by the different cell types. As we have observed a temporal difference in response, it is interesting to explore the expression of growth factors within the healing tendon. Kang & Kang, in 1999, explored the ideal concentration of growth factors in rabbit's flexor tendon culture, investigating the short-term dose response to IGF, FGF, PDGF and EGF on *in vitro* rabbit's tendon culture. They observed that FCS was the most potent stimulator of cell proliferation and protein synthesis *in vitro* rabbit's tendon culture. The rabbit's tendons underwent a dose-dependent response to PDGF, whereas there was no significant response to FGF {Kang and Kang, 1999}.

Synovium is characterised by an intimal layer of cells, recognised to be a mixture of bone marrow-derived macrophages and specialised fibroblast-like cells. Although there are many similarities between synovium and tendon-surface cells, there are also differences. Edwards explored synoviocyte differentiation in 1994, observing that fibroblast-like cells, or synoviocytes, differ from other fibroblasts in a number of respects, including high activity of uridine diphosphoglucose dehydrogenase (UDPGD) and constitutive expression of VCAM-1. He speculated that mechanical factors may be implicated in the induction of UDPGD activity and VCAM-1 expression, an

interesting observation, which could explain different clinical results with different types of post-operative rehabilitation programmes {Edwards, 1994}. Kakar & Khan, in 1998, observed the cellular changes in the epitenon, endotenon and synovial sheath in a partial transverse laceration rabbit model. Their results showed that the epitenon and uninjured synovial sheath became engorged with fibroblasts and macrophages following injury. The synovial fibroblasts showed the greatest increase in number during the first week after injury; in comparison, the endotenon exhibited a delay in cellular response with initial apoptosis, as judged by delayed positivity in p53 staining. Hypercellular activity was seen within the endotenon at 12 weeks postoperatively {Kakar, Khan *et al.*, 1998a}. Khan *et al.* investigated the relative activity of fibroblasts from the synovial sheath and tendon core. They found that the fibroblasts derived from the fibro-osseous sheath were more active in terms of proliferation and the ability to contract a collagen lattice *in vitro*. In addition, the amount of matrix metalloproteinase activity was found to be greater for the fibro-osseous sheath fibroblasts, implying a greater capacity to degrade and disorganize connective tissue and thus migrate {Khan, Occleston *et al.*, 1998b} Many of these results suggest that synovial and tendon derived fibroblasts do respond differently in response to simulated injury conditions, which support the findings here that synovial cells may be more aggressive migrators. Churei *et al.* in 1999, demonstrated that extensor retinaculum could be successfully used to repair flexor tendons, as assessed using histological and biomechanical outcomes. Proliferation and migration of fibroblasts from both tendon surfaces and the core surface toward the deep layer of the suture site was seen 2 weeks

after operation. New collagen fibres, aligned parallel to the long axis of the tendon, could also be seen 4 weeks after operation, and healing was more advanced than in the coreless model. The maximum force to produce a gap in the core tendon was 82% greater than in the coreless tendon 4 weeks after operation {Churei, Yoshizu *et al.*, 1999}

4.5.4.3 Chemotaxis

Determining those factors that will trigger chemotaxis and migration in the *in vivo* situation would be very difficult, the likely reason that so much *in vitro* investigation has been undertaken. This work, although abstracted from the in vivo situation, allows us to elucidate the triggers and mechanism of chemotaxis. Original work performed by Albini *et al.* in 1983 looked at chemotactic activity of fibroblasts in mucopolysaccharidoses, observing that fibronectin is a potent stimulus for fibroblasts to migrate chemotactically {Albini, Pontz *et al.*, 1983}, whilst Adelmann-Grill *et al.* in 1990, observed that chemotactic migration of normal dermal fibroblasts is stimulated by epidermal growth factor *in vitro* and that platelet-derived growth factor and transforming growth factor-beta can down-regulate this activity. This suggests that *in vivo* these growth factors are part of an intricate network which connects and coordinates proliferation, protein synthesis and chemotactic migration of fibroblasts {Adelmann-Grill, Wach *et al.*, 1990}. In another paper, by the same authors in the same year, using Boyden-type migration chambers, they dispelled a popularly held idea that a constant concentration gradients of attractants in tissues is required for migration to occur. Indeed, they found that only a brief pulse of attractant was required and sufficient to induce prolonged

chemotaxis {Adelmann-Grill and Cully, 1990}. In the tendon healing case, we know that TGFβ1 is expressed in large quantities from as early as 24 hours after injury {Chang, Most *et al.*, 1997a}, and is known to have a significant fibroblast chemotactic ability. Hence, it seems likely that both synovial and tendon surface derived fibroblasts would both respond to even short-lived brief TGFB expression.

Hannafin *et al.* in 1999 tried to characterise this chemotactic migration together with the effect of fibroblast growth kinetics, in canine knee ligament derived fibroblasts. They compared migration of intra-articular and extra-articular ligament fibroblasts in response to cytokines. PDGF, HGF and BMP2 stimulated the migration of all ligament cells in a dose-dependent manner, with optimal migration at 10 ng/ml; EGF preferentially stimulated the migration of intra-articular ligament fibroblasts, whereas IL-1 was more effective with extra-articular ligament fibroblasts. Their data suggested that specific cytokines stimulate the migration of knee ligament fibroblasts providing possible therapeutic approaches to optimize ligament healing. This provides insight into phenotypic differentiation; cells that are anatomical and bio-chemically similar can have significantly different responses to similar cytokine stimulation {Hannafin, Attia *et al.*, 1999}. Suzuki *et al.* undertook similar investigation into the action of cytokines on the migration of fibroblasts derived from the three anatomically and functionally distinct regions of the canine shoulder capsule. They also found differences in terms of responsiveness to specific growth factors {Suzuki, Attia *et al.*, 2001}. This phenomenon has not been investigated in the different tendon subpopulations.

Even subpopulations of the same cytokine, TGF-β can have differing effects on the same fibroblast population; Cordeiro *et al.*, 2000 observed that TGF-β1, β2 and β3 had different effects of Tenon's fibroblast contraction, proliferation, and migration. The different TGF-βs induced different responses, with different functions being maximally stimulated at different concentrations {Cordeiro, Bhattacharya *et al.*, 2000}. Sasaki *et al.* in 2000 observed the effect of heparin and related glycosaminoglycans on lung fibroblast proliferation, chemotactic response and matrix metalloproteinases activity, concluding that heparin and related glycosaminoglycans differentially regulate PDGF-induced lung fibroblast proliferation, chemotaxis and MMPs activity and further that these different effects may have a key role in extracellular matrix remodelling in inflammatory lung disease {Sasaki, Kashima *et al.*, 2000}. The effect of TGF-β on cell migration and proliferation in relation to tendon healing is further investigated in the next Chapter of this thesis.

4.5.4.4 Summary

These results suggest that synovial fibroblasts do migrate migration into the healing tendon area, as early as 24 hours after injury with the number of migrating cells increasing until day 5 and with the response diminishing by day 7.

4.5.5 Review of intrinsic & extrinsic healing mechanisms

4.5.5.1 Intrinsic.

Most hand surgeons and researchers now accept that a combination of both intrinsic and extrinsic healing mechanisms does occur at the time of injury, but the direct evidence has, until now, been lacking on this subject.

Some of the strongest supporting evidence for the ability of the tendon to intrinsically heal itself was presented by Lundborg in 1976 and Lundborg and Rank later the same year. They presented an experimental model which enabled an analysis of the healing process of completely cut and re-sutured free segments of rabbit flexor tendons, kept avascular in a synovial environment and completely isolated from adhesion formation. Under these conditions the cut tendons healed within a few weeks, suggesting that the healing process is a result of intrinsic tendon cell activity only. Lundborg also supplemented this data with further evidence, in which repaired rabbit intra-synovial flexor tendons were enclosed in silicone and placed in a subcutaneous chamber for six weeks. Fibroplasia, cell proliferation and collagen synthesis were interpreted as evidence to support the concept of intrinsic healing {Lundborg, Rank *et al.*, 1985}.

Subsequent studies by Becker *et al.* in 1981 demonstrated in an *in vitro* study that tendons contain active fibroblasts capable of proliferation, migration, and collagen synthesis; utilising chicken sublimis tendon explants in tissue culture. In the absence of tendon sheath, tendon cells were observed to migrate around the margin of a window in the explant, with electron micrography used to confirm the identity of the cells, and collagen synthesis confirmed by biochemical assay {Becker, Graham *et al.*, 1981a}.

Manske *et al.*, in 1984 observed that two cellular processes were involved in the *in vitro* repair process: phagocytosis by differentiation of epitenon-derived fibroblasts, and collagen synthesis primarily within the endotenon cells, implying that not only do tendons have the intrinsic capacity to synthesise new

collagen fibrils, but they can also self-phagocytose. One of their main conclusions was that adhesions do not appear to be an essential component of the repair process {Manske, Gelberman *et al.*, 1984b}.

Abrahamsson & Lundborg, in 1992, investigated the role for endotenon cells in the restoration of the injured flexor tendon surface in a morphological study of the rabbit tendon *in vivo*, using light and scanning electron microscopy. The epitenon layer was carefully excised and the remaining central tendon tissue divided, sutured and placed in diffusion chambers subcutaneously in rabbits. After two weeks of culture, most of the sutured gaps were bridged and the tendons were encapsulated by flattened and spindle-shaped cells which covered a random network of thin collagen fibres. After five and eleven weeks, fibroblast-like cells in multiple layers formed a cobblestone-like surface. They concluded that tendons deprived of their epitenon layer still contained cells which can produce collagen, bridge the gap and restore the injured tendon surface {Abrahamsson, Lundborg *et al.*, 1992}

Jones *et al.*, {Jones, Mudera *et al.*, 2003a} have demonstrated that tendon-surface cells migrate into the healing tendon areas, using a technique outlined in Chapter 3, and similar in principle to that used in this study. Section 4.5.5.3 below, discusses the likelihood that the synovial and tendon-surface derived cell types, although potentially slightly differing in phenotypic behaviour, and are likely to respond to the same mitogenic and migration chemotactic stimuli.

4.5.5.2 Extrinsic

Whilst there have been many proponents of the intrinsic healing mechanism, a large body of evidence to support the existence of an extrinsic healing

mechanism has been developed over the last 5 decades. Eiken *et al.*, in 1975, undertook a study to attempt to elucidate the role of synovial sheath during the healing process. Experimental models in both rabbits and dogs were developed where a free tendon graft would be nourished solely by synovial fluid. Macroscopically, the grafts remained smooth, white and glistening without vascular in-growth or adhesions during an observation period of 12 weeks. By histological and histochemical techniques the grafts as a whole were found viable up to 3 weeks later. They concluded that the synovial sheath contributes to survival of the graft and helps maintain the integrity of gliding surfaces with maximal functional restoration and minimal formation of adhesions {Eiken, Lundborg *et al.*, 1975a}. Matthews, in 1976, observed the "fate" of isolated, devascularised segments of profundus tendon replaced within the synovial flexor sheaths in the front paws of adult rabbits. They discovered that the segments survived as viable "loose bodies", confirming to Matthews the existence of a synovial fluid pathway of nutrition, concerned, it is suggested, with nourishing the more superficial layers of the tendon {Matthews, 1976}. Manske, in 1978 investigated the nutrient pathways to flexor tendons using a hydrogen washout technique. They found hydrogen uptake and decay of a free segment of flexor tendon detached from its blood supply were not significantly different from an intact tendon lying in the synovial bed and no hydrogen uptake by flexor tendons which were mechanically separated from the synovial bed, even though muscular, periosteal and vincular attachments were intact. The authors concluded that the synovium of the flexor tendon was a significant nutrient pathway for the flexor tendon and that the blood vessels did not appear

to play a significant role in the nourishment of the flexor tendon {Manske, Whiteside *et al.*, 1978}.

Many similar works were produced in the two decades subsequent to Manske's paper, the next significant one being by Khan *et al.*, examining the effects of treating the synovial sheath with the anti-metabolite 5-fluorouracil (5-FU). They observed that proliferative and inflammatory responses and the level of activity of TGF-β1 were all reduced in the treated group, suggested that the synovium does play an active role in the formation of adhesions in the healing tendon {Khan, Kakar *et al.*, 2000}.

4.5.5.3 Combination

Recent texts suggest that a combination of the intrinsic and extrinsic healing mechanisms is responsible for the tendon healing process. It is also suggested that the extent and location of the injury will also determine the degree to which the mechanism is responsible. It has been established that cells extrinsic and intrinsic to the tendon have the cellular potential to become involved in the healing process; both have the potential to divide {Becker, Graham *et al.*, 1981c;Eiken, Hagberg *et al.*, 1981a;Manske, Gelberman *et al.*, 1984a}, migrate {Becker, Graham *et al.*, 1981b;Eiken, Hagberg *et al.*, 1981b;Manske, Gelberman *et al.*, 1984c}, modify their surrounding matrices{Eiken, Hagberg *et al.*, 1981c;Gelberman, Woo *et al.*, 1982b}, produce cytokines {Chang, Most *et al.*, 1998a} and express other inflammatory markers{Khan, Edwards *et al.*, 1996}. This work would support some of these findings; for the synovial cells to migrate into the healing area, they must dissociate from their surrounding and migrate into the healing area. Work by Jones *et al.* {Jones ME, Mudera V

et al., 2002} has shown that DiI labelled surface derived cells migrate into the healing area. There is currently no *in vitro* data on the relative migratory, matrix production, apoptosis and proliferation rates of the different cells types involved. However, Khan *et al.* suggest that surface and sheath derived fibroblasts are both more reactive than the core derived cells{Khan, Edwards *et al.*, 1996;Khan, Occleston *et al.*, 1998a}. They also illustrated that surface derived fibroblasts are important in the early response to injury by migrating, supported by *in vivo* work by Jones *et al.* 2002, confirming earlier work which demonstrated epitenon cellular proliferation and subsequent appearance of migration into the cut in *vivo* models (Gelberman *et al.* ., 1991; Gelberman *et al.* ., 1983; Lundborg & Rank, 1978; Manske *et al.* ., 1985b) and in the *in vitro* setting (Gelberman *et al.* ., 1984; Mass and Tuel, 1989). Interestingly, most accounts of surface cell migration are at time points beyond a week from injury. Whilst we can hypothesise that both cell types are involved, we are still uncertain as to the relative roles of each cell type. If differences do exist between the cell types *in vivo*, it may be advantageous for one cell type to be present within the healing tendon rather than another. Differences have been observed *in vitro* including proliferation rates {Khan, Edwards *et al.*, 1996}, matrix production {Abrahamsson and Lohmander, 1996a}, vascularity and biochemical activity{Gelberman, Amiel *et al.*, 1992a} and mechanico-biological capabilities{Kakar, Khan *et al.*, 1998b}, suggesting that the cell types may react differently to injury.

To date, the biology of hand flexor tendon wound healing remains controversial--both intrinsic (resident tenocyte) and extrinsic (tendon sheath

fibroblast and inflammatory cell) processes may contribute to repair. Recent evidence to support dual mechanisms of repair has been provided by researchers such as Khan {Khan, Edwards *et al.*, 1996}and Chang {Chang, Most *et al.*, 1998b}. Khan and Edwards in 1996 examined patterns of cellular activation after tendon injury, assessing the relative response of the cells of the synovial sheath, epitenon and the endotenon to injury. They used a 50% transverse laceration, just outside the synovial sheath on the flexor aspect of the FDP tendon, preserving the synovial sheath. Using monoclonal antibody localisation of specific inflammatory markers, they observed that *both* the synovial sheath and the epitenon are relatively more reactive in the early period after injury. {Khan, Edwards *et al.*, 1996}. Chang *et al.*, in 1998 also found similar dual expression, particularly of BFGF, a cytokine known to play a fundamental role in angiogenesis. They specifically examined BFGF mRNA, using a similar model to Khan *et al.* Few tenocytes and tendon sheath cells expressed BFGF mRNA in unwounded tendons, but tendons subjected to transection and repair exhibited an increased signal for BFGF mRNA in both resident tenocytes concentrated along the epitenon and infiltrating fibroblasts and inflammatory cells from the tendon sheath. Their main conclusion was angiogenic cytokine upregulation occurs in tendon sheath fibroblasts as well as tendon fibroblasts, supporting dual mechanisms for tendon repair. Because transforming growth factor beta-1 is thought to contribute to the pathogenesis of excessive scar formation, they also suggested that perioperative biochemical modulation of transforming growth factor beta-1 levels might help limit flexor tendon adhesion formation {Chang, Most *et al.*, 1997b}.

If upregulation of cytokines occurs in both the tendon and synovial sheath cells, then this lends support to our findings in this study. For cells to become involved in the wound healing process, they must become stimulated, mobilised from their existing matrix, migrate to the site of injury, and then, in the case of tendon healing, undertake new collagen formation and deposition.

4.5.6 Clinical.

Many questions remain unanswered in the clinical domain, for example, the correct timing of tendon repair, which suture type is optimum and whether should great effort be made to close the synovial sheath. Although tendon research has focussed on both basic science and clinical aspects over the last 50 years, trying to integrate the two dichotomous aspects remains difficult.

4.5.6.1 Type of repair and rehab

The need to achieve good clinical results has always been the driving force for research to occur, and frequent audits presenting good results have been published. Richards in 1980 was one of the first of these, presenting the relatively controversial results of his series of 275 tendon repairs. On the basis of his good clinical results, he advocated either primary or delayed primary repair of a divided digital flexor tendon, with preoperative splinting and careful operative technique to avoid damage to the blood supply of the divided tendon {Richards, 1980}. He also advocated closure of the fibrosynovial sheath and postoperative splintage. The alteration in his results when the fibrosynovial sheath was closed lends weight to our results; if synovial cells participate in the healing process, then closure of the sheath around the healing tendon is likely to alter outcome.

Since Richards, even better clinical outcomes from tendon repair series have been reported. Gault, in 1987 {Gault, 1987} undertook a review of 67 patients with 176 repaired flexor tendons, with a mean follow-up of 26.4 months. When evaluated by Buck-Gramcko criteria, the functional results in 92% of the fingers and 69% of the thumbs were graded as excellent or good. Interestingly, patients with partially divided tendons fared no better than those whose tendons were completely divided, but the important phenomenon is that good results are being reported. Singer and Maloon {Singer and Maloon, 1988} in 1988 described their results obtained following primary repair and post-operative controlled mobilisation of flexor tendon injuries. Their experience of 70 patients with 140 injured digits who underwent FDP or FPL tendon injury showed that 67% were rated by Lister's standards as an excellent or good result. However, in zone 2 (intrasynovial area) only 49% where 39 (28%) occurred in no man's land (Zone 2) and only 19 (49%) in this area were rated excellent or good. Gelberman *et al.* in 1982 investigated the effects of early intermittent passive mobilization on healing canine flexor tendons. Early motion, delayed motion, and immobilization groups were compared over a 12-week period for their strength and excursion characteristics. They found that early protected passive mobilisation augments the physiologic processes that determine the strength and excursion of repaired flexor tendons {Gelberman, Woo *et al.*, 1982a}.

Our results do not allow determination of the nature of the role of synovial cells in the tendon healing process, but they do suggest that they migrate in the healing area within 24 hours after the initial insult. If these synovial cells are

more aggressive than those from within the tendon body, then more aggressive adhesion formation might result, if the extrinsic mechanism is predominant. Controlled mobilisation might modulate the degree of synovial fibroblast migration, and hence reduce the degree of adhesion formation. Studies to assess the differential collagen production would assist in answering this question, similar to those undertaken by Gelberman *et al.* in 1992 {Gelberman, Amiel *et al.*, 1992b}, assessing genetic expression for type I procollagen in the early stages of flexor tendon healing. In situ hybridisation was used to assess tendons at 3, 7, 10, and 17 days after repair. Rising levels of procollagen mRNA, indicating increasing levels of synthetic collagen activity, were detected in the healing tendons through 10 days, but started to decrease by 17 days. Interestingly, expression for procollagen mRNA was localised specifically to the epitenon cells on the tendon surface overlying the repair site and to cells in the gap between the tendon stumps. No detectable expression was noted in endotenon fibroblasts. The finding of high levels of expression for procollagen type I mRNA in the surface layer of healing tendons demonstrates that cells intrinsic to tendon epitenon contribute the greatest quantity of native tendon collagen to the repair site during these important early intervals after tendon suture. Unfortunately, this study did not include the synovial sheath, as it would be experimentally difficult, but very revealing to undertake.

Whilst our data suggests that synovial fibroblasts are capable of migrating into the tendon healing area, their subsequent effect on the healing process in uncertain.

4.5.6.2 Timing of repair

We have observed that synovial cells start to migrate into the tendon window and surrounding tendon core by 24 hours after injury, and that more synovial cells migrate into the healing area up to 5 days. There was no increase in the mean distance from the synovium with time. It is impossible from this study to determine how the cells arrive within the injured area, whether it is by slow migration or by "dropping" from the synovium into the injured area. The synovial cells may be shed from the surface and adhere to the wound environment, indeed, the increase in cell numbers may not be purely due to cell migration. It is possible that only some of the visible cells are of synovial sheath origin, and the cells have undergone proliferation, or a combination of these processes; although proliferation would attenuate the dye fluorescence. There were less labelled cells within the incisional area at 7 days, this could be due to fading of the DiI, proliferation or apoptosis of the cells, or migration of the cells in the longitudinal axis. Our *in vitro* work would suggest that the DiI is still visible, even within several successive generations of daughter cells well after seven days post injury.

The rate at which cells are recruited into the tendon healing process has also revealed some interesting data. We observed that maximum cell numbers were present by day 5, many 50% of the maximum present by day 1. Gelberman *et al.* in 1991 described flexor tendon surface cell response within one week of injury {Gelberman, Steinberg *et al.*, 1991a}, noting at day 3 that the epitenon fibroblasts proliferate and appear to migrate towards and into the wound. In this study, (Gelberman, 1991) inflammatory cell presence in the cut at this time

was also noted. He found maximal surface cell migration at day 7, correlating it to the high levels of fibronectin at that time point.

Our results have demonstrated that synovial fibroblasts migrate into the zone of injury in a novel tendon healing model. They appear in the injury within 24 hours and continue to be present in increasing numbers until 5 days after injury. One important clinical question which this data helps to address regards the timing of surgical procedures. Gelberman *et al.* {Gelberman, Siegel *et al.*, 1991}, investigated the importance of the interval from injury to repair, using biomechanical, biochemical, and morphological outcomes in a canine model. There was a significant effect of the delay until repair on biomechanical outcomes in all three groups, but no significant differences in terms of tensile strength or total collagen content (measured by reducible Schiff-base cross-links). The data within this thesis suggests that as early as 24 hours post injury, synovial fibroblasts have migrated into the healing tendon area. The inflammatory component of the healing process will still be ongoing, and collagen deposition will be stimulated. Variance in fibroblastic origin could determine the nature of collagen deposition and hence be responsible for the change in biomechanical outcomes observed in Gelberman's work.

Significant advances in imaging and biochemical analysis techniques have been made over the last decade, and work by Ros *et al.* utilised these technique to provide a detailed description of the morphogenesis and initial differentiation of the long tendons of the (chick) foot, from Day 6 to Day 11 of development {Ros, Rivero *et al.*, 1995}. This insight is significant because the mode of development could be reflected in the mode of subsequent healing

after injury. They determined the existence of an ectoderm-mesoderm interaction as the first stage of the tendon formation together with spatially and temporally differing patterns of distribution of extracellular matrix molecules in developing tendons, with significant variation in the elastic matrix content.

The sources of nutrition within the healing tendon still represent a point of controversy, yet have significant implications in terms of tendon healing; also offering potential in terms of therapeutic intervention. Peacock *et al.* investigated postoperative recovery of flexor tendon function, in 1971. Their study illustrated that the three sources of blood supply described by Mayer (1916) in fact contributed in different proportions; that the segmental vincular blood supply was the most important and the two other sources were relatively minor {Peacock, Jr., Madden *et al.*, 1971}. The debate concerning vascularity is also reflected in the debate over the intrinsic and extrinsic healing capacities of the intrasynovial flexor tendon. Gelberman & Khabie (1991) have produced one of the most seminal works on this subject, investigating the revascularisation of healing flexor tendons in the digital sheath, using a vascular injection study in dogs. Recognising that the role of revascularisation in the nutritional support of repair of the flexor tendons is not completely understood, they explored the extent to which intrasynovial flexor tendons revascularised after transection and suture, using a vascular injection study was carried out in a canine model. Normal tendons demonstrated a well developed mesotenon that provided vascularisation of the proximal portion of the flexor digitorum profundus tendon, with a consistent avascular intrasynovial portion distally, vessels arose from the vinculum breve, supplying the terminal twenty

millimetres of tendon substance. In the experimental tendon group, longitudinal and transverse sections showed consistent revascularisation of the site of repair by proximal vessels in the absence of in-growth of peripheral adhesions. Vessels in the epitenon progressively extended for a distance of ten millimetres, through normally avascular regions, to reach the site of repair by the seventeenth postoperative day. Intra-tendinous vessels around the site of repair consistently originated from surface vessels, rather than from extensions of pre-existing intra-tendinous vessels. New vessels penetrated all areas, including the normally avascular volar segments of tendon, irrespective of previous topical zones of avascularity. Proximal vascular plexi were characterised by large tortuous vessels with frequent circuitous branches. More distal vessels had a longitudinally oriented, feathery appearance {Gelberman, Khabie *et al.*, 1991}.

4.5.6.3 Sheath closure

Richards *et al.* also advocated closure of the fibrosynovial sheath and postoperative splintage. The alteration in his results when the fibrosynovial sheath was closed agrees with our results {Richards, 1980}.

CHAPTER 5: RESULTS: EFFECT OF INJURY TYPE, IMMOBILISATION AND TGF-B1 APPLICATION ON THE FLEXOR TENDON PROLIFERATIVE RESPONSE TO INJURY

5.1 INTRODUCTION

5.1.1 Background

Hand flexor tendon repair is frequently complicated by adhesion formation, caused by tendon fibroblast collagen deposition. Whilst many different tendon repair and mobilisation regimes are employed clinically, their actual effect on the fibroblastic response is uncertain. We aimed to utilise an animal model to observe the effect of different types of injury, immobilisation and application of pro-fibrotic growth factor known to be expressed at the time of injury (TGF-β1) on epitenon fibroblast density.

The type and location of a tendon injury is known to be important. Although Zone 2 is no longer considered "no-man's land", the highest complication rate still occurs in this region and surgical repair is hardest to effect in this region. Whilst the location of tendon injury has been shown to influence clinical outcome, it is also likely that the type of injury is important, but this has not been explored or verified experimentally. The frequency of flexor tendon injury continues to increase with little to categorise them together. Some flexor tendon injuries are sharp incision injuries, some are blunt contusions, and some are superficial scrape injuries, whilst many are a combination of the above. Whilst the severity of injury is likely to correspond to outcome, relatively little research has been performed to investigate the effect of different injury types and outcome, especially in the animal model where responses to injury can be observed scientifically.

As early as 1912, it was postulated that mobilisation post tendon surgery might influence clinical outcome; Lexor documented the benefits of mobilising the

repair from the sixth day following repair (quoted in {Adamson and Wilson, 1961}). This concept of movement post-repair was challenged by Potenza {Potenza, 1962}, who believed that a period of postoperative immobilisation was required to allow adhesion build-up, which he felt was necessary to effect tendon healing. This was once the "gold standard" treatment, but is now only accepted as postoperative treatment for very young or uncooperative children, in whom voluntary immobilisation might be difficult. The turning point that led to the realisation that post operative mobility held the key to better results came from the observations of {Kleinert, Kutz *et al.*, 1973}; 87% of their cases that were either good or excellent when mobilised with controlled passive motion. Their regime (the Kleinert regime) consisted of a rubber band fixed to the nail of the affected digit and also attached to the flexor aspect of the forearm allowing active extension but passive flexion. It was later shown that in order for adhesions to develop, a combination of tendon suturing, immobilisation and, or concurrent digital sheath injury are required {Matthews, 1976}. A large number of different mobilisation programmes are used after flexor tendon injury and an even larger number have been used in the past. Many clinicians and scientists have investigated mobilisation and immobilisation on both patient outcome and in vitro models, but the effect on the peri-injury fibroblastic response has not been observed.

Transforming Growth Factor Beta (TGF-β1) is a stable, multifunctional polypeptide growth factor. Generally, it is stimulatory for cells of mesenchymal origin and inhibitory for cells of epithelial or neuroectodermal origin. It is found in the highest concentration in human platelets and

mammalian bone, but is produced by many cell types {Bennett and Schultz, 1993}. It has been observed to play a significant role in wound healing and has also been implicated in the pathogenesis of excessive scar formation, having been observed to enhance fibroblast contraction of 3D collagen lattices {Brown, Sethi *et al.*, 2002}. Prior to 1995, although a lot of work had been done to investigate the effects of growth factors in bone and soft tissue healing processes, relatively little work had been done to investigate the role of growth factors in flexor tendon healing. Since then *in situ* hybridisation techniques have implicated TGF-β1 in both intrinsic and extrinsic mechanisms of repair and have shown TGF-β1 to stimulate proliferation of tendon fibroblasts in culture {Stein, 1985}. *In vivo* work has shown TGF-β1 to be present in healing digital flexor tendons {Duffy, Jr., Seiler *et al.*, 1995b}, and that infiltration of neutralising antibody to TGF-β1 improves flexor tendon excursion {Chang, Thunder *et al.*, 2000a}.

The study described in Chapter 3, in conjunction with the work of Jones *et al.*, had led us to observe that both synovial and tendon surface cells participate in the tendon healing process. However, the brief introduction above illustrates that relatively little is understood about the differing effect of different post-injury healing environments, such as the degree of immobilisation, the presence of pro-fibrotic growth factors and differing injury types, on the healing capacity of the injured tendon.

5.2 AIMS

To determine the effect of injury and repair conditions:

- Differing injury types.
- Immobilisation and mobilisation.
- Application of a TGF-β1.

On the cellular healing response in an *in vivo* model.

5.3 *MATERIALS AND METHODS*

5.3.1 Experimental procedure

Male Sprague Dawley Rats were anaesthetised and surgery performed on the right hind paw, under local tourniquet, as described in Chapter 2 – Materials and Methods. The FDP tendon was exposed through an incision in the plantar skin, proximal to the A1 pulley. Each tendon was randomised to be treated with an experimental permutation of:

- Injury type: Superficial epitenon scrape injury or longitudinal incision injury
- Mobilisation type: Post-operative immobilisation or mobilisation.
- Pro-fibrotic application: Application of TGF-β1 or saline control.

The zone of injury was marked, the wound closed and all animals mobilised.

5.3.2 Tendon Harvest and Processing

Rats were culled at 7 and 14 days post procedure, and 4 micron thickness paraffin sections taken through the zone of injury using a microtome. Sections were then stained with Haematoxylin and Eosin. Microtome sectioning was problematic at times, due to bone fracturing, disrupting the tendon architecture, however, a 14 day immersion in EDTA de-calcifying solution prior to sectioning, reduced the incidence and significance of this problem.

5.3.3 Section analysis

Light photomicrographs were captured using a digital camera Leica DC-200 digital camera (Leica Photographic Systems) connected to a personal computer (Mesh, Winchester, UK) running Windows 2000 (Microsoft, USA) and image

analysis software (Sigma Scan Pro v5) were used to analyse the resulting images, counting the number of cells on the tendon surface around the zone of injury (as illustrated below).

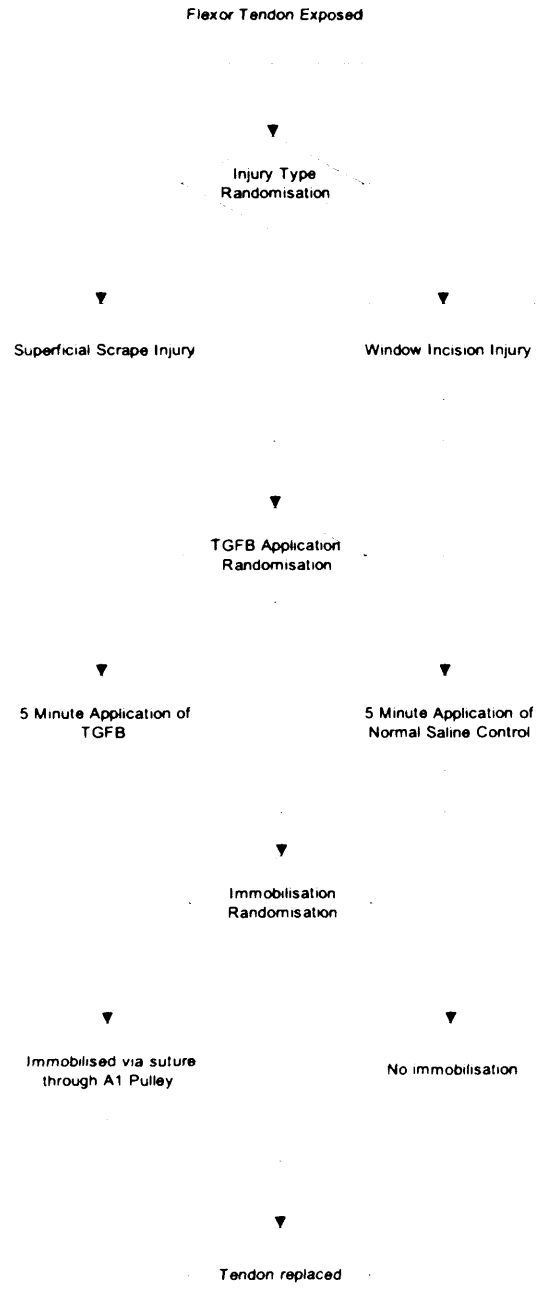


Figure 47. Flow chart of treatment permutation for each tendon.

5.4 RESULTS

5.4.1 Number of sections

Good quality sections and staining were obtained from each experimental digit. Cell counts were obtained from each treated digit, and analysis was facilitated using the computer-aided manual cell counting. 3 sections were cut from each of the 114 procedural tendons, with 6 tendons representative of each of the treatment permutations.

5.4.2 Effect of differing injury types on control specimens

	Raw Cell Numbers	Relative Cell Numbers
Normal Control	11.2 (2.5)	100 (22.2)
Incised Control	11.2 (2.5)	100 (22.2)
Scraped Control	7.5 (2.2)	100 (29.2)

Table 8 – Actual and relative cell numbers (standard deviation in brackets) of normal control, incised control and scraped control specimens. N=6.

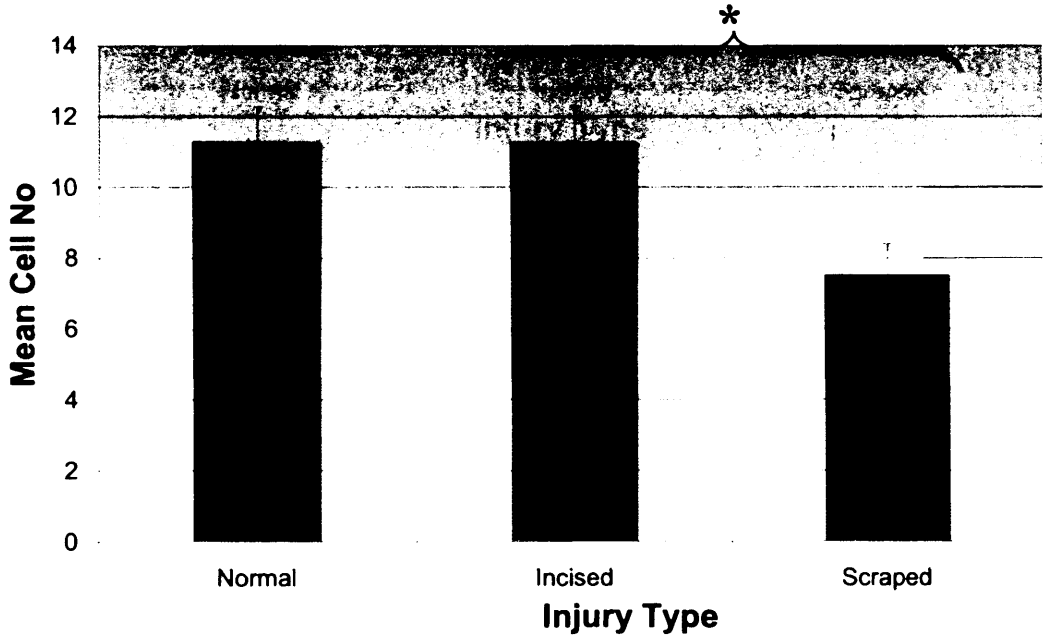


Figure 48 – Histogram representing mean peri-injury cell numbers in different injury tendon control specimens. Note the lower mean cell number in the scraped tendon due to injury at time zero. These mean cell numbers were taken to represent 100% cell numbers for all future data analysis. N=6.

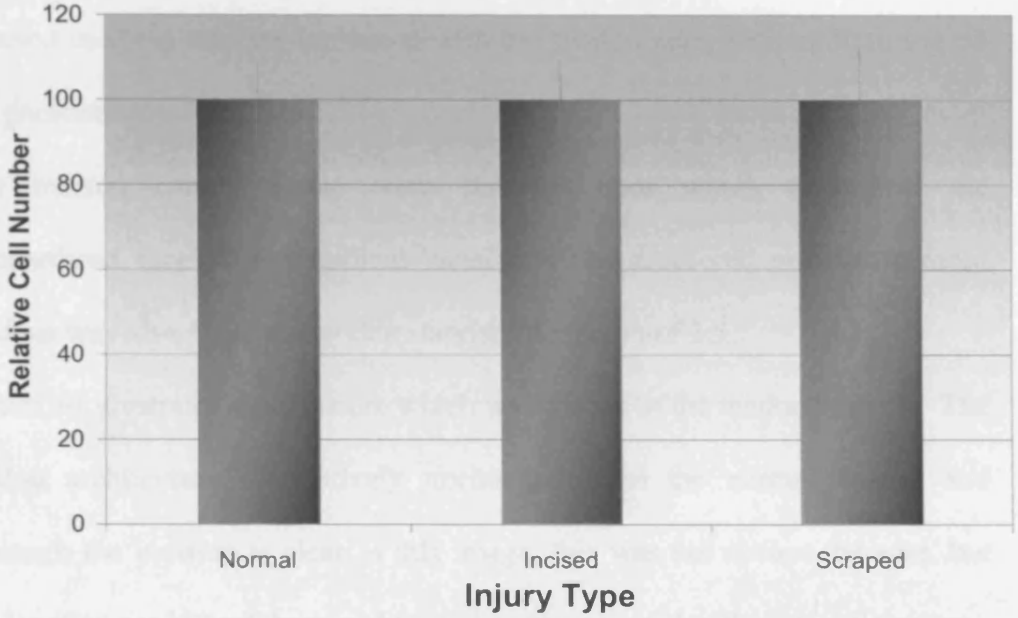


Figure 49 - Histogram representing relative mean peri-injury cell number in different injury tendon control specimens. N=6.

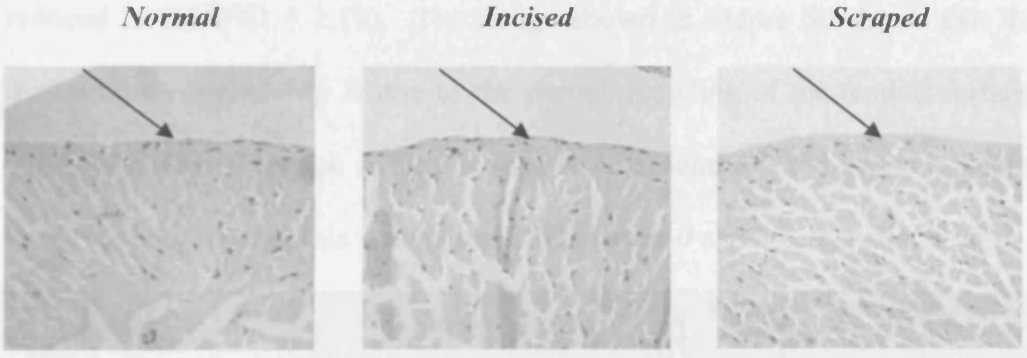


Figure 50 – Photomicrographs of control tendon specimens. H&E Stained. X200. Arrowhead delineates centre of area of injury.

Figure 50 illustrates the mean numbers of cells in the 100 micron grid for normal, incised and scraped control tendons. The mean number of cells observed in the normal tendon was 11.2, with a standard deviation of 2.5.

Figure 50 depicts a representative normal control tendon. A typical cellular distribution is presented, with a relatively cellular tendon surface area and

relatively less densely populated tendon core beneath. Fixing and processing resulted in some artefact formation with the tendon core, seen as fissuring on the photomicrographs above.

The incised control group were those tendons which underwent the standardised surgical longitudinal incision. The mean cell number in these tendons was also 11.2, also with a standard deviation of 2.5.

Figure 50 illustrates the incision, which was placed in the tendon midline. The tendon architecture is relatively unchanged from the normal tendon and although the incision is clear in this image, this was not always the case, but the location could be determined from the suture placed at the time of surgery.

The other control tendons were those which had undergone a superficial scrape injury. The mean number of cells seen within the grid in these tendons was reduced to 7.5 (SD = 2.19). The image shown in Figure 50 shows that the diminution in cellularity is due to the partial denuding of the tendon surface. Whilst the normal tendon surface is seen to be around 3-5 cell layers in depth, in the scraped tendon, this was reduced to between 0 and 2 cell layers.

Day	0	7	14
Normal	100 (22.2)	100 (22.2)	100 (22.2)
Incised	100 (22.2)	128 (40.9)	280 (57.7)
Scraped	100 (29.2**)	220 (48.0)	306 (48.0)

Table 9 - Relative cell numbers (standard deviation in brackets) of control, incised and scraped tendon specimens at 0, 7 and 14 days post injury. N=6. (=Significant difference, P<0.05)**

Figure 48 depicts these results graphically, with the error bars showing the standard error of the mean in each case. Table 9 also depicts the relative cellularity of the control specimens, which in each case is considered, by definition, to be 100; all subsequent tendon analyses are described relative to their corresponding control. The standard deviations have also been calculated relative to the original number. These results are depicted graphically in Figure 49.

5.4.3 Effect of time on cell numbers.

The effect of time on each of the different injury types is presented in Table 9, and Figure 51. Tendons were injured with either an incision or superficial scrape injury and the animal allowed to mobilise for either 7 or 14 days. Tendons were harvested and the degree of cellularity around the injury determined. The results were compared to an uninjured control for each time period and to the injured day 0 control cellularity.

By day 7, the incision injury shows an increase in relative cellularity to 128, not statistically significant compared to the normal control. The scrape injury at day 7 shows a higher increase to a relative cellularity of 220 compared to the day 0 scrape injury. This relative cellularity represents a statistically significant difference compared to the normal control and to the incision, suggesting that the scrape injury has induced a more marked cellular reaction to the injury.

At day 14, the incised tendon has a relative cellularity of 280, compared to the incision control. The scrape injury had a relative cellularity of 306 (NS). The

images in the figure above depict representative images from each of the injury types and time intervals.

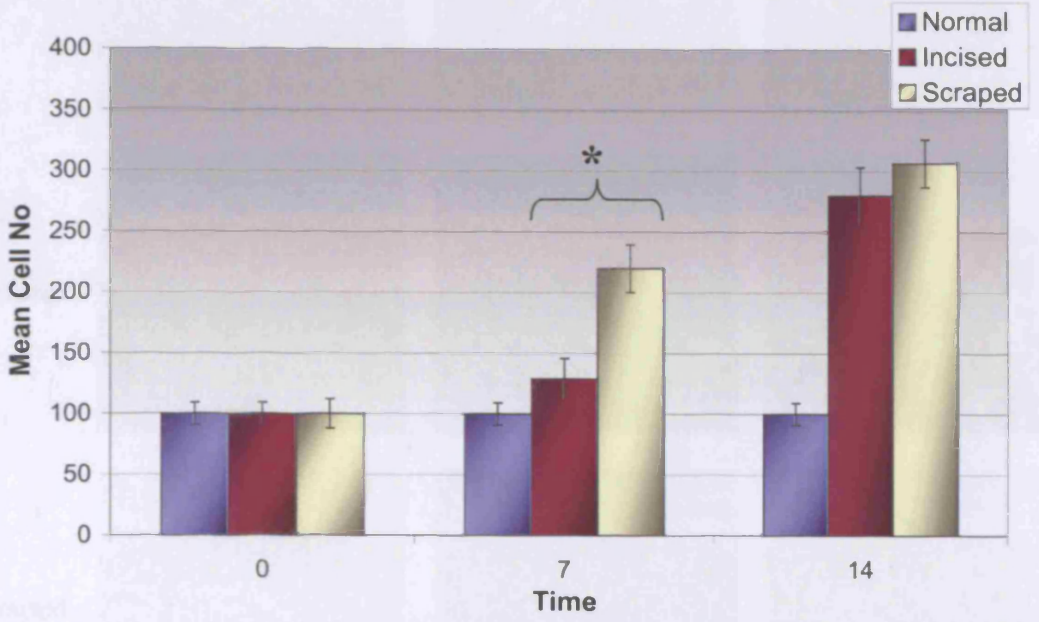


Figure 51 - Relative cell numbers of control, incised and scraped tendon specimens at 0, 7 and 14 days post injury. All tendons are mobilised post treatment. N=6.

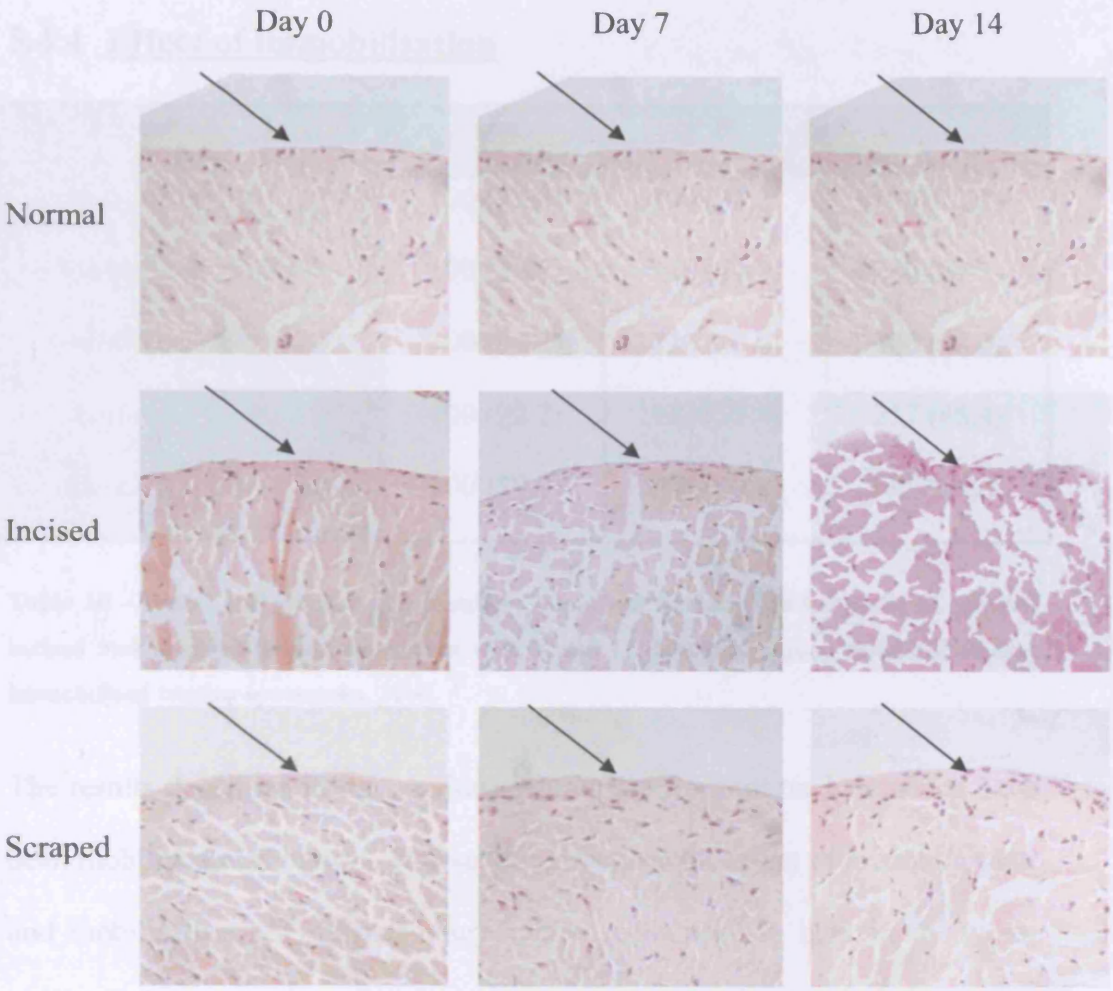


Figure 52 - Photomicrographs of control, incised and scraped tendon specimens at 0, 7 and 14 days post injury. H&E Stained. X200. Arrowheads delineate centre of area of injury.

5.4.4 Effect of immobilisation

Day	0	7	14
Normal	100 (22.2)	100 (22.2)	100 (22.2)
Mobilised - Incised	100 (22.2)	128 (40.9)	280 (57.7)
Mobilised - Scraped	100 (29.2)	220 (48.0)	306 (48.0)
Immob - Incised	100 (22.2)	142 (53.4)	257 (85.4)
Immob - Scraped	100 (29.2)	300 (69.8)	340 (112.0)

Table 10 –Actual and relative cell numbers (standard deviation in brackets) of control, incised and scraped tendon specimens at 0, 7 and 14 days post injury in mobilised and immobilised tendon specimens. N=6.

The results described in the previous section are from rat tendons which have been mobilised post injury. This section describes the effect of immobilisation and mobilisation on the peri-injury tendon cellularity in both injury types. None of the tendons in the section have been treated with TGF-β1. The figures above and below show the results from the different treatment groups and how they change with time. At day 0, the mobilised incised and mobilised scraped data are the same specimens as the immobilised incised and immobilised scraped specimens.

By day 7, the immobilised incised specimens show a relative cellularity of 142, compared to 300 in the immobilised scrape specimens, a statistically significant difference. By day 14, the same specimens show a relative cellularity of 257, compared to 340 in the immobilised scrape specimens, which is also a statistically significant difference.

At 7 days post procedure, the incision injury tendons, which have been mobilised show a mean relative cellularity of 128, compared to 142 in the same injury which had been immobilised. 14 days post procedure, the mobilised tendons have a relative cellularity of 280, compared to the immobilised tendon mean of 257.

Considering the scrape injury tendons, which have been mobilised and immobilised: At day 7, the mobilised scrape injury tendons have a relative cellularity of 220, compared to 300 in the immobilised group, a statistically significant difference ($P<0.05$). By day 14, the margin has narrowed; the mobilised group has a relative cellularity of 306, compared to 340 in the immobilised group.

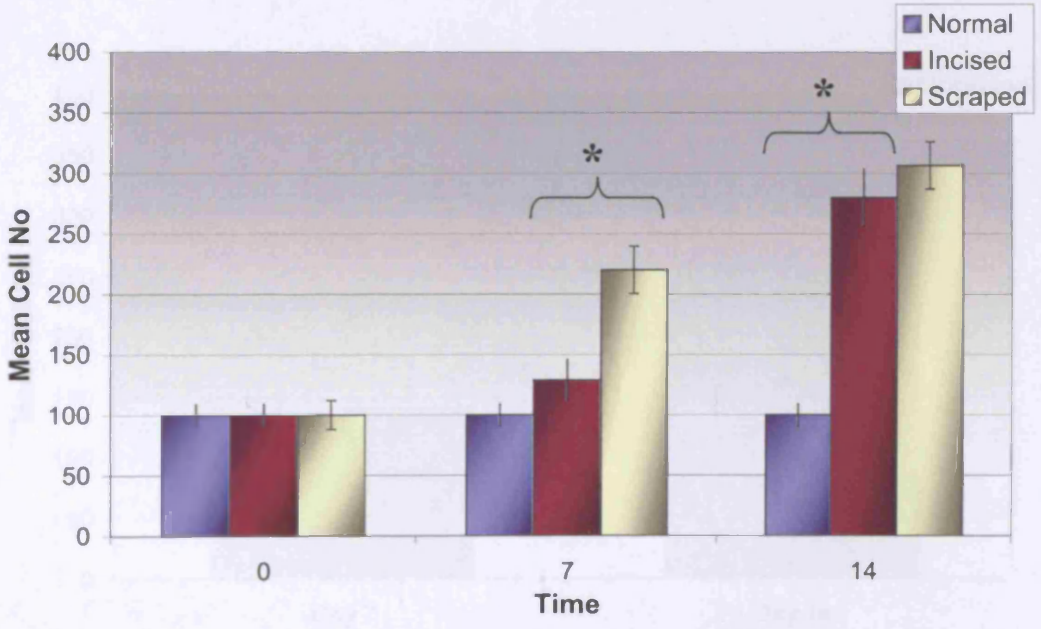


Figure 53 - Histogram representing relative cell numbers of control, incised and scraped tendon specimens at 0, 7 and 14 days post injury. All tendons are mobilised. N=6.

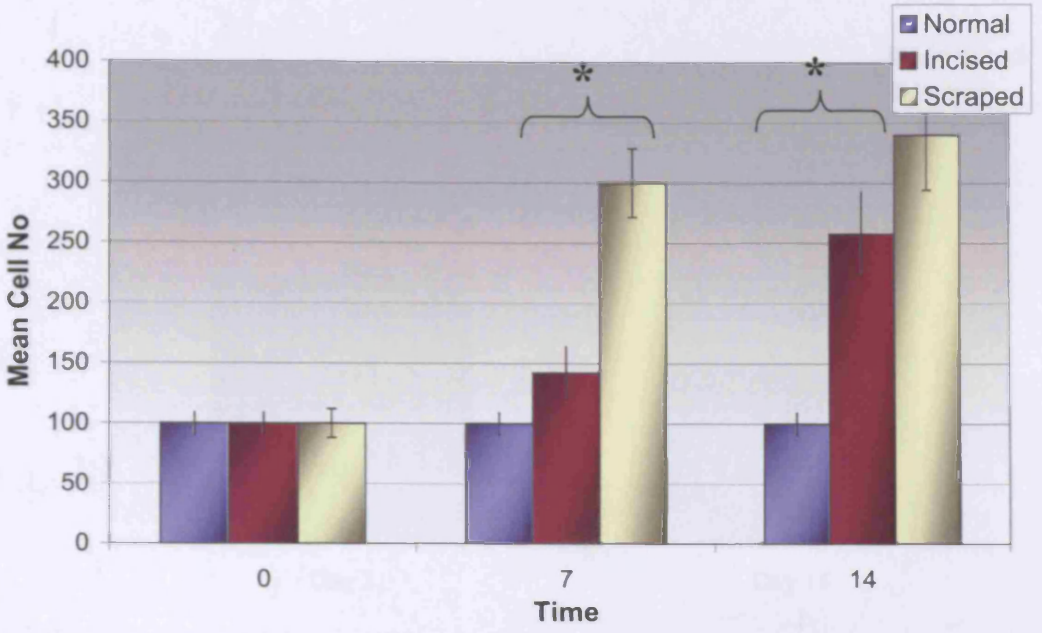


Figure 54 - Histogram representing relative cell numbers of control, incised and scraped tendon specimens at 0, 7 and 14 days post injury. All tendons have been immobilised. N=6.

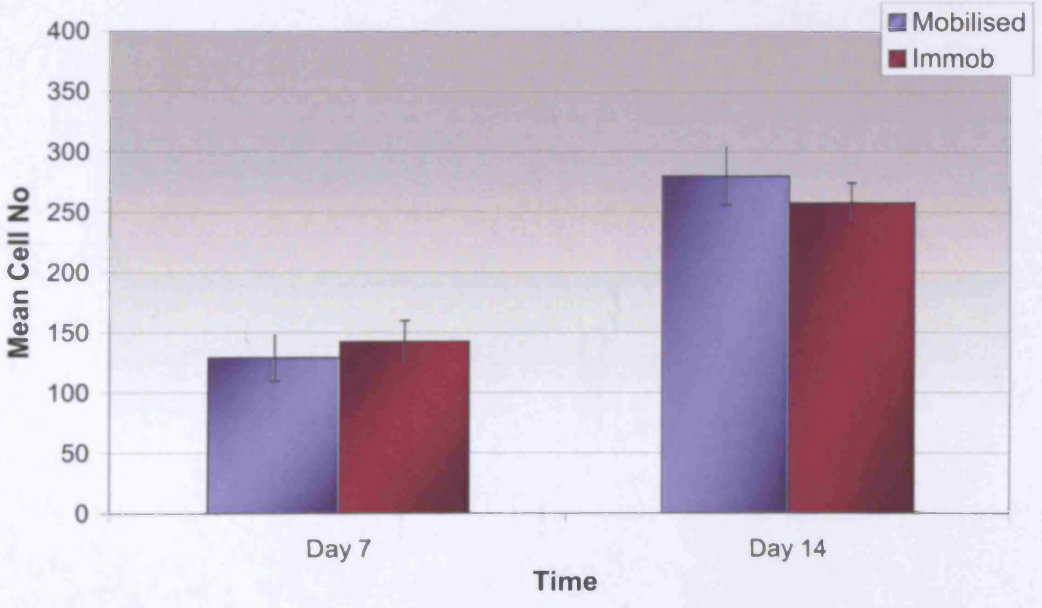


Figure 55 - Histogram representing relative cell numbers of incised tendon specimens at 7 and 14 days post injury. N=6.

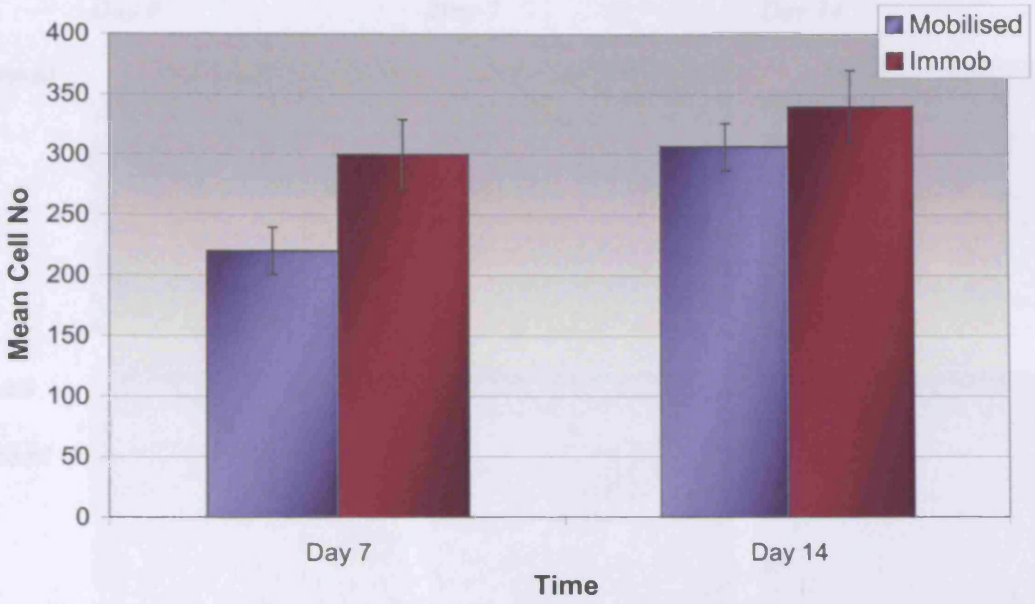


Figure 56 - Histogram representing relative cell numbers of scraped tendon specimens at 7 and 14 days post injury. N=6.

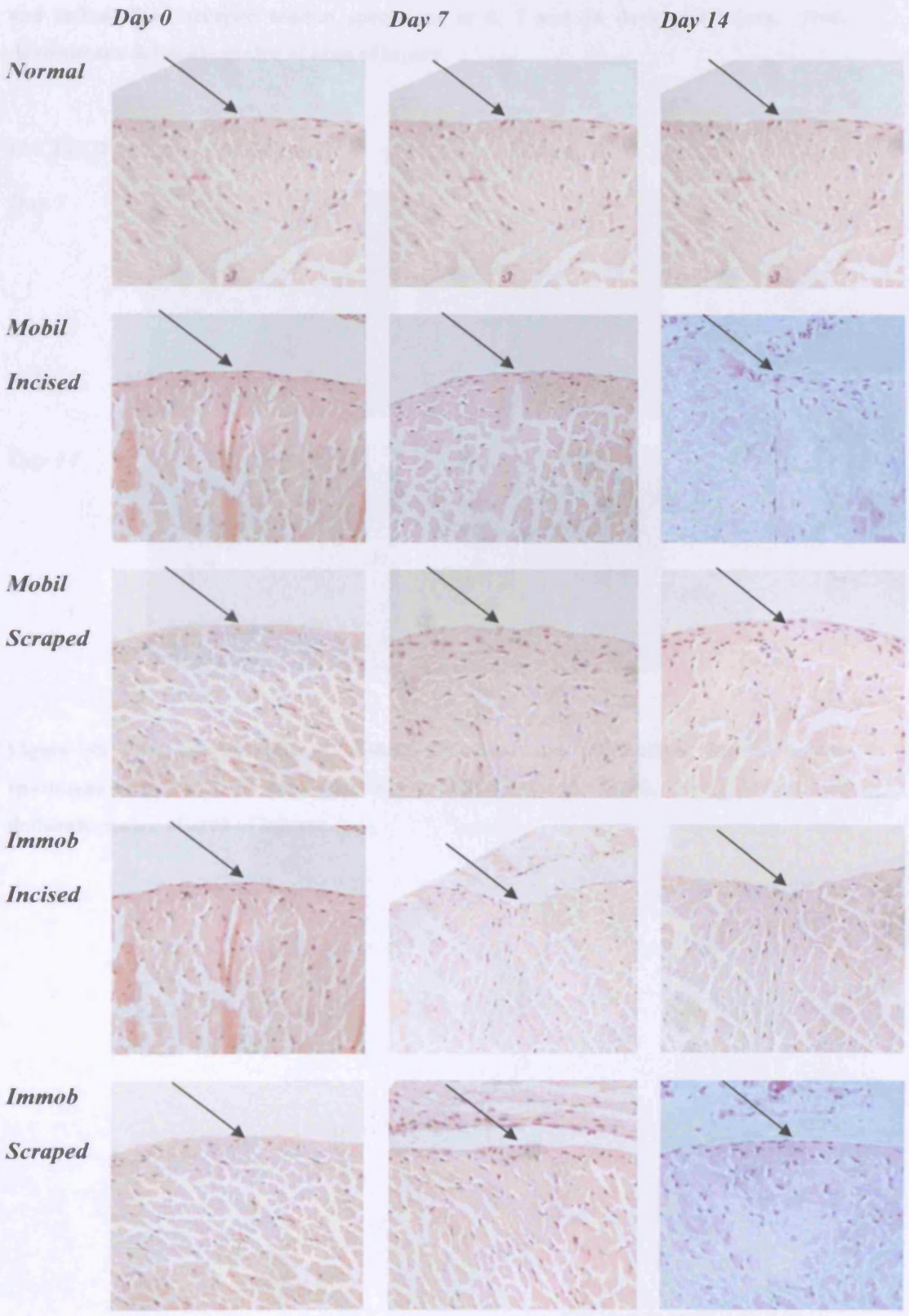


Figure 57 – Caption on next page

Photomicrographs of normal, mobilised incised, mobilised scraped, immobilised incised and immobilised scraped tendon specimens at 0, 7 and 14 days post injury. N=6. Arrowheads delineate centre of area of injury.

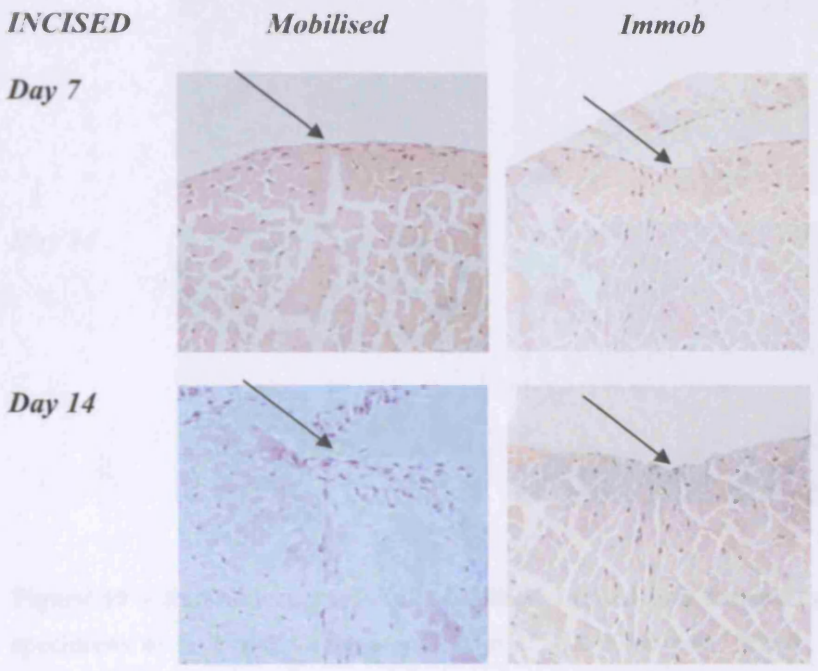


Figure 58 Photomicrographs of Mobilised Incised and Immobilised Incised tendon specimens at 0, 7 and 14 days post injury. H&E Stained. X200. N=6. Arrowheads delineate centre of area of injury.

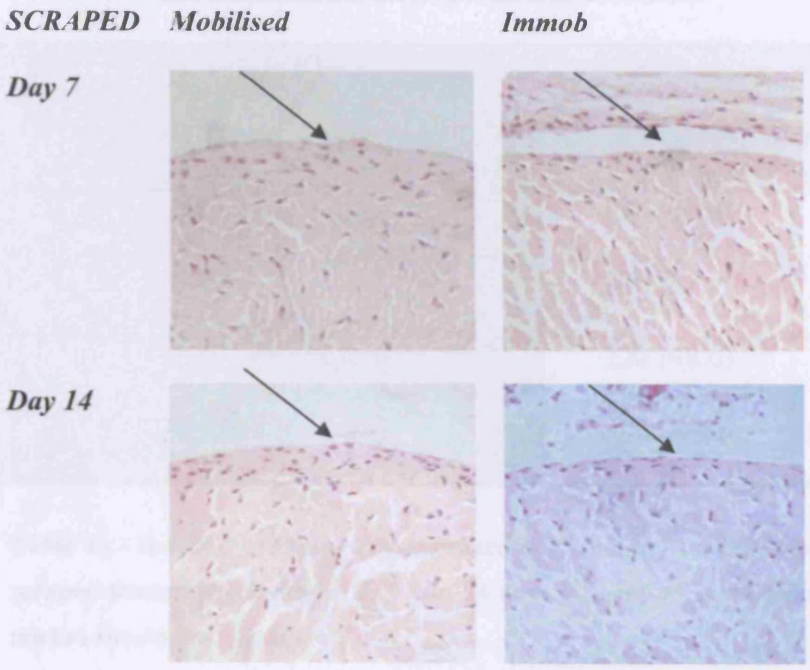


Figure 59 - Photomicrographs of Mobilised Incised and Immobilised Scraped tendon specimens at 0, 7 and 14 days post injury. H&E Stained. X200. N=6. Arrowheads delineate centre of area of injury.

In the initial 7 days after injury, if the specimen tendon is mobilised, the cell numbers around the injured tendon area increases by 97%, compared to 226% in the immobilised tendon, and this difference is statistically significant ($P<0.05$). After 14 days, the increase from baseline in the mobilised tendon is 198%, compared to 208% in the immobilised tendon; this difference is not statistically significant. We can therefore state that, in this experimental model, immobilisation leads to an increase in cellular recruitment.

5.4.5 The effect of the addition of TGF- β 1

INCISED Type (Actual / Relative)	Control Relative	TGF Relative
Day 7 - Incised	128 (40.9)	182 (53.4)
Day 14 – Incised	280 (57.7)	306 (85.4)
Day 7 - Scraped	220 (48.0)	253 (69.8)
Day 14 - Scraped	306 (48.0)	320 (112.0)

Table 11 - Relative cell numbers (standard deviation in brackets) of control, incised and scraped tendon specimens at 0, 7 and 14 days post injury in TGFB and control treated tendon specimens. N=6.

This results section describes the effect of TGF-B1 treatment on the peri-injury tendon cellularity in both injury types. None of the tendons in the section have been immobilised. The mobilised data has already been described, but is presented here to allow comparison to the immobilised tendon data. At day 0, the mobilised incised and mobilised scraped data are the same specimens as the immobilised incised and immobilised scraped specimens.

By day 7, the TGF-B1 treated incised specimens show a relative cellularity of 182, compared to a relative cellularity of 253 in TGF-B1 treated scrape specimens, a statistically significant difference. By day 14, this margin has diminished; the incised and scraped tendons had relative cellularities of 306 and 320 respectively.

At 7 days post procedure, the incision injury which has not been TGF-B1 treated shows a relative cellularity of 128, compared to a relative cellularity of 182 in the same injury which has been TGF-B1 treated. 14 days post

procedure, the non-treated tendon has a relative cellularity of 280, compared to the TGF-B1 treated tendon of 306, which is not statistically different. The effect of TGF-B1 seems to occur very early in the healing process to increase cellularity. This effect is after a single application.

Day	0	7	14
Normal	100 (22.2)	100 (22.2)	100 (22.2)
NO TGF-B1 - Scraped	100 (29.2)	220 (48.0)	306 (48.0)
TGF-B1 - Scraped	100 (29.2)	253 (72.0)	320 (73.3)

Table 12 - Relative cell numbers of scraped tendons which have been TGFB treated or Non-TGFB treated at 0, 7 and 14 days post injury. N=6.

Considering the scrape injury tendons which have been non-TGF-B1 treated and TGF-B1 treated: At day 7, the non-TGF-B1 treated scraped injuries have a relative cellularity of 220, compared to 253 in the TGF-B1 treated group, a statistically non-significant difference. By day 14, the margin has narrowed; the mobilised group has a relative cellularity of 306, compared to 320 in the immobilised group.

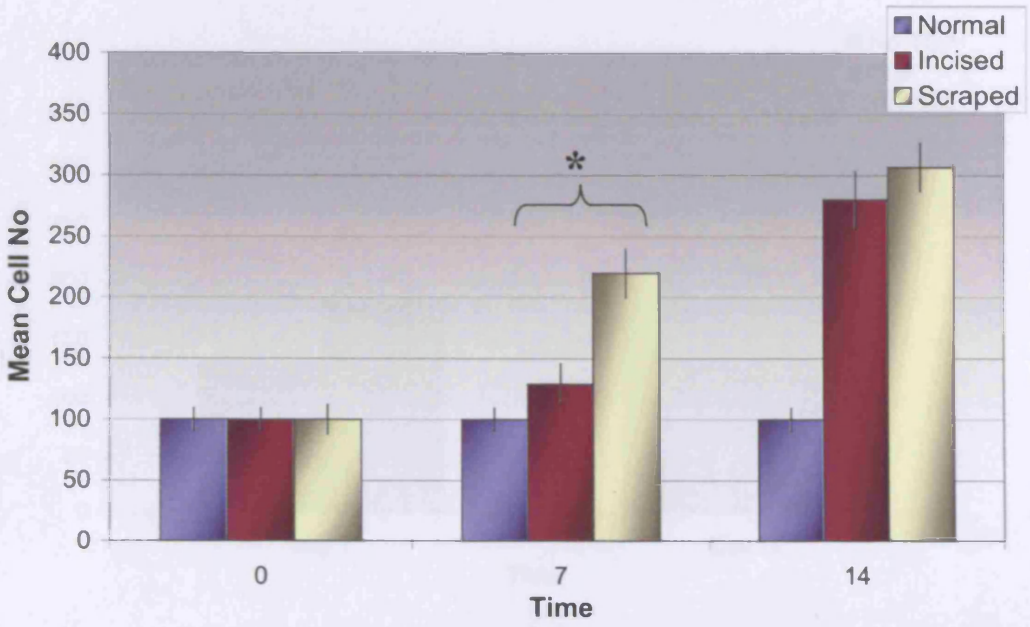


Figure 60 - Histogram representing relative cell numbers of control, incised and scraped tendon specimens at 0, 7 and 14 days post injury. All tendons are non-TGFB treated. N=6.

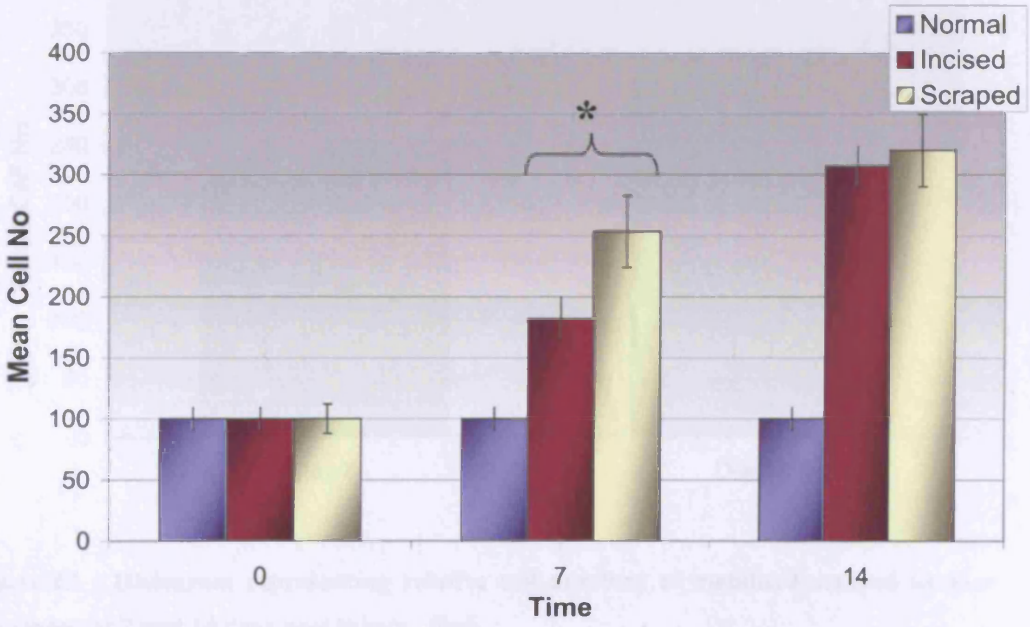


Figure 61 - Histogram representing relative cell numbers of control, incised and scraped tendon specimens at 0, 7 and 14 days post injury. All tendons are TGFB treated. N=6.

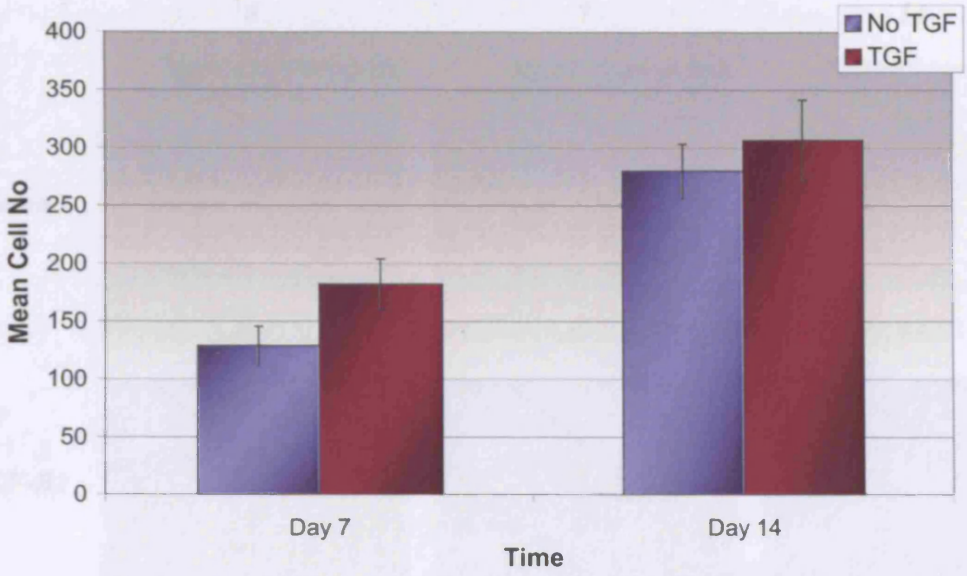


Figure 62 - Histogram representing relative cell numbers of incised tendon specimens at 7 and 14 days post injury. N=6.

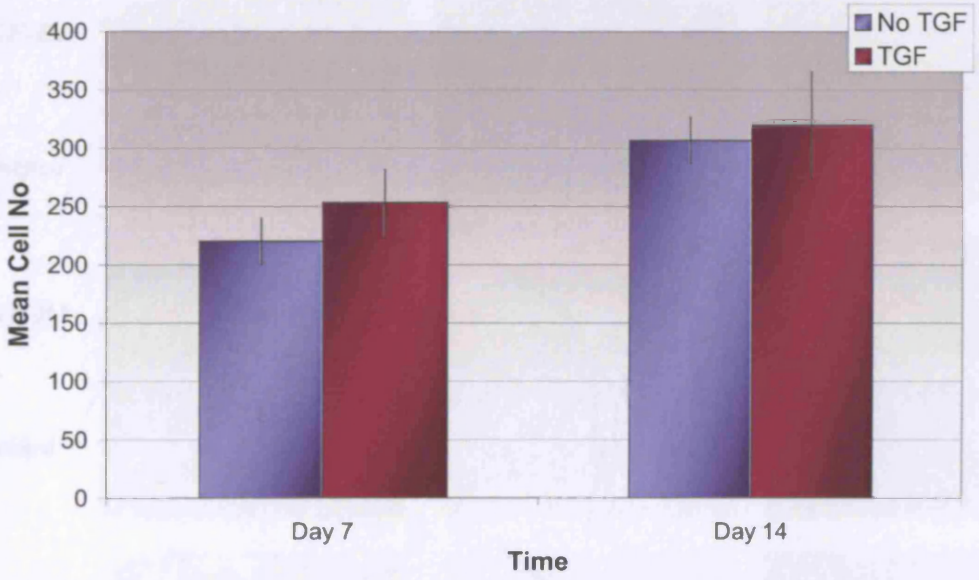


Figure 63 - Histogram representing relative cell numbers of mobilised scraped tendon specimens at 7 and 14 days post injury. N=6.

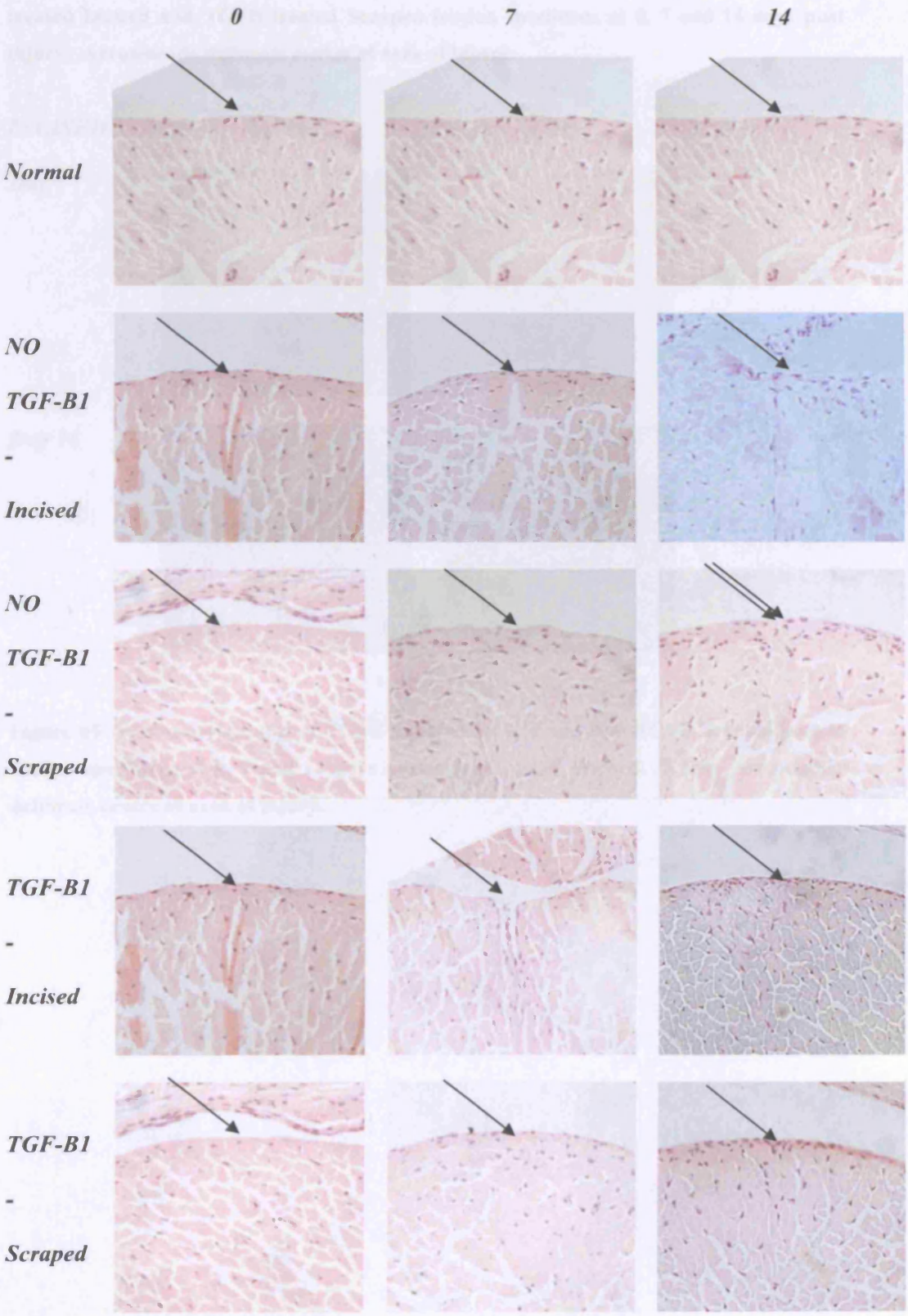


Figure 64 – Caption on next page

Photomicrographs of Normal, Non-TGFB treated Incised, Non-TGFB Scraped, TGFB treated Incised and TGFB treated Scraped tendon specimens at 0, 7 and 14 days post injury. Arrowheads delineate centre of area of injury.

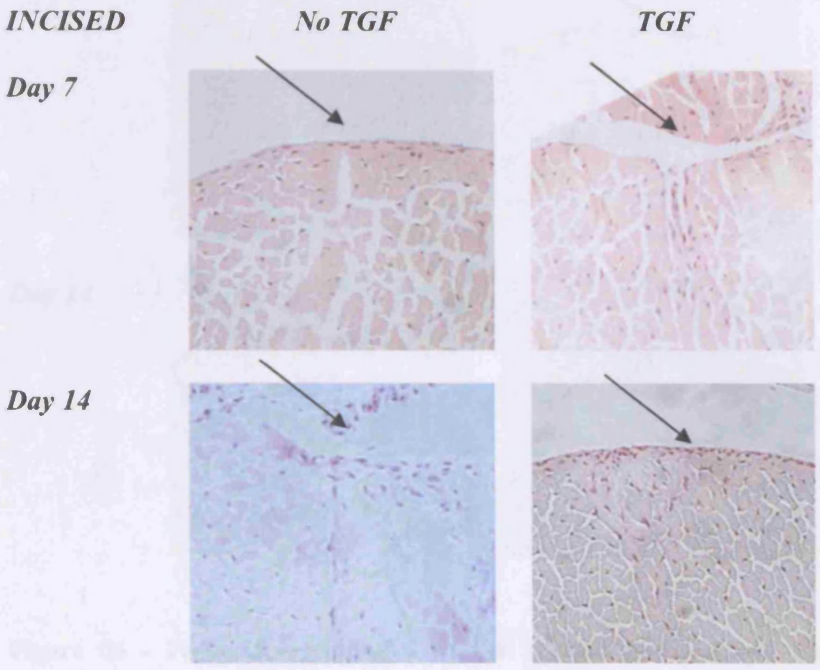


Figure 65 -Photomicrographs of TGFB Treated Incised and Non-TGFB Treated Incised tendon specimens at 0, 7 and 14 days post injury. H&E Stained. X200. Arrowheads delineate centre of area of injury.

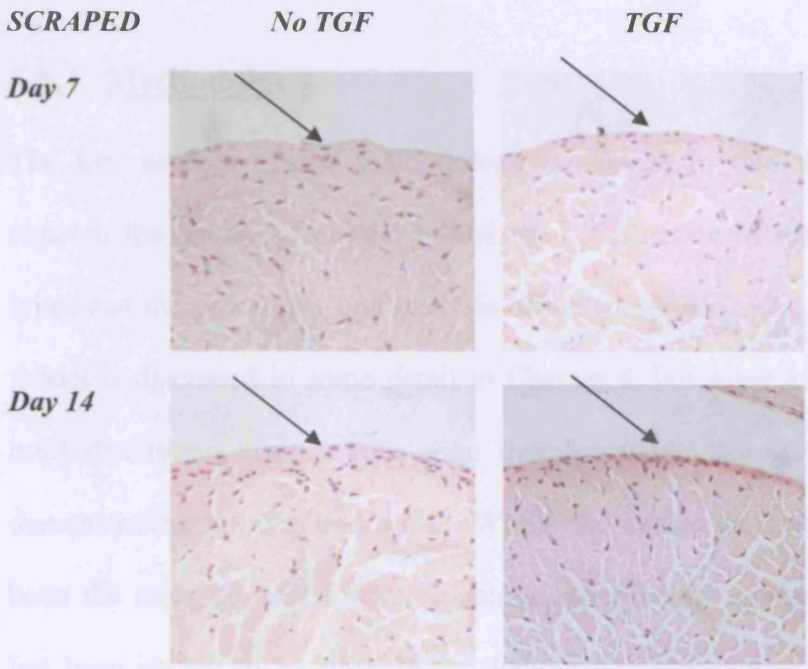


Figure 66 - Photomicrographs of TGFB Treated Scraped and Non-TGFB Treated Scraped tendon specimens at 0, 7 and 14 days post injury. H&E Stained. X200. Arrowheads delineate centre of area of injury.

5.5 *DISCUSSION*

5.5.1 Methodology

The key aspects of the methodology employed in this chapter primarily concern the choice of animal model used, the nature of the different injury types and the processing and analysis of the specimens. The choice of animal model is discussed in some detail in Chapter 4, but some key issues warrant brief discussion again. It is vital that the model chosen was capable of demonstrating the required aims. Whilst the canine and rabbit models have been the most popular for investigating experimental tendon healing, the rat has been shown to be suitable for demonstrating responses to tendon injury {Bora FW, Lane *et al.*, 1972; Iwuagwu and McGrouther, 1998a}. There are also certain advantages to this, in terms of ease and speed of operation, animal handling and the conduct of anaesthesia. The continued use of the rat model in the experimental work within this thesis also facilitated comparison between the different experiments.

There are theoretically an infinite number of permutations of human tendon damage in terms of severity, location, nature of injury and amount of tendon lacerated. To allow investigation of our aims, it was deemed appropriate to choose two injury types which represent the extremes of the injury-type spectrum. The superficial scrape was chosen to represent maximum tendon surface injury, whilst the incision injury was chosen to represent a tendon core type injury. There was no complete means of standardising these injuries, but micrographic investigation revealed a (subjective) consistency. The incision

injury was based on the windows injury, originally utilised by Matthew, Moore and Campbell {Matthew, Moore *et al.*, 1987} and since used by several investigators to study tendon healing {Iwuagwu and McGrouther, 1998b}, {Jones, Mudera *et al.*, 2003}.

Harvesting the entire rat digit was employed to ensure that the tendon anatomy was preserved. Despite the need to microtome through bone, good quality sections were obtained. Routine H&E staining to delineate tendon cellularity produced good quality staining. Delineation of the area of injury was facilitated by appropriate visual inspection and orientation due to the placement of a suture at the time of injury.

5.5.2 Injury type

The data from our experiments suggest that the creation of different injury types has a significant effect of the peri-injury cellular response in the rat model (Figure 49 above). 7 days post procedure, those tendons which had been subjected to a superficial scrape injury showed a more marked cellular response, although this difference was no longer seen by day 14. Whilst it has been well described that both tendon surface and synovial cells are capable of proliferation and collagen production. Becker *et al.* observed that tendon surface cells were able to proliferate, migrate and produce collagen in response to injury {Becker, Graham *et al.*, 1981}. The ability of the tendon surface cells to produce collagen was confirmed using procollagen specific antibody binding in a similar experimental set-up by Garner *et al.* in 1988 {Garner, McDonald *et al.*, 1988}. Whilst tendon surface cells can be shown to be capable of proliferation and collagen production, Abrahamsson *et al.* (1992) have shown

that endotenon cells are capable of similar abilities. They looked specifically at FDP tendons, which had had their epitenon layer removed and then placed in subcutaneously diffusion chambers, finding them capable of restoring the tendon gliding layer. Within 2 weeks in culture, sutured gaps had been bridged and the tendons encapsulated by flattened and spindle-shaped cells which covered a random network of thin collagen fibres, leading to the conclusion that tendons deprived of their epitenon layer still contain cells which can produce collagen, bridge the gap and restore the injured tendon surface {Abrahamsson, Lundborg *et al.*, 1992}.

The evidence described above suggests that both the tendon core tenocytes and tendon surface cells are both capable of mounting a tendon-healing response. The data presented here suggests that the superficial type injury leads, initially, to a more aggressive response. This could be due to this injury involving more of the more reactive tendon surface cells in the healing process. Making analogy to a skin wound, a clean incision injury heals in a very different way to a superficial scrape injury; a large area of skin affected by a partial thickness skin loss quickly mounts a large inflammatory response. If the tendon surface cells are more reactive than tendon core cells, this would explain the more rapid increase in cellularity in the superficial injury type.

Another explanation of these results is that the different injury types predispose the tendon fibroblasts to differing exposure to cytokines, or indeed, that the different planes of injury expose different sub-populations of fibroblast to cytokine exposure. It has been established {Chang, Most *et al.*, 1997} that cytokine expression occurs early within the healing tendon, and also that

expression varies with depth from the tendon surface. Maximal expression seems to occur within the epitenon and synovial tendon layers, with less expression within the central tendon body {Chang, Most *et al.*, 1997}. As discussed in Chapter 4, Hannafin *et al.* {Hannafin, Attia *et al.*, 1999} and Suzuki *et al.* {Suzuki, Attia *et al.*, 2001} have shown that in knee and shoulder tendon fibroblast subpopulations, different cytokines can result in significantly different cellular responses, in terms of proliferation, migration, apoptosis and collagen production. With BFGF also expressed in high levels during the tendon healing process, and with tendon fibroblasts known to undergo proliferation and migration in response to this {Chang, Most *et al.*, 1998}, it is likely that the different proliferative responses are due to either differing exposure amongst the population sub-types, due to the nature of the injury, or that the different cytokines are having differential proliferative responses to the same stimulus, indeed the different response of tendon fibroblasts to the differing growth factors IGF, FGF, PDGF and EGF was observed by Kang & Kang on *in vitro* rabbit's tendon culture {Kang and Kang, 1999}.

The initial observed difference in terms of response to injury is negated by 14 days. This could be due to increased recruitment and proliferation or indeed, apoptosis. One postulate is that the increasing number of cells with time might have a bearing on the increasing strength of the tendon at earlier time point as these cells might be laying down matrix earlier.

5.5.3 Mobilisation and Immobilisation

The effects of immobilisation and mobilisation are shown in the results above. The only significant difference found in these results is that at Day 7, the

superficial scrape injury tendon has a greater cellular response when immobilised. None of the incision injury tendons showed differing responses when mobilised or immobilised at 14 days, the difference between the scraped immobilised and scraped immobilised tendons had diminished. It has been established {Cannon and Strickland, 1985;Tonkin, Hagberg *et al.*, 1988}, that mobilisation or immobilisation after a tendon injury has a significant effect on the clinical outcome, and there are a large number of basic science papers which attempt to explain and investigate the mechanisms behind this, these are discussed below.

The potential benefits of controlled mobilisation have been well described in the clinical literature: In 1980, Strickland and Glogovac assessed 50 consecutive digits in 37 patients and determined that early passive motion appeared to be an effective technique to improve the results of flexor tendon repairs in this area {Strickland and Glogovac, 1980}. Others have commented on the need for close collaboration between the surgeons and hand therapists, noting that that the emergence of therapists specifically trained in the techniques of hand rehabilitation added immeasurably to the ability to return function following upper extremity injury or disease {Cannon and Strickland, 1985}. Tonkin *et al.* assessed 145 tendon grafts and found that the final motion obtained was independent of the post-operative mobilisation regimes utilised, but the rates of graft rupture (nine vs four) and of tenolysis (sixteen vs eight) were higher if immobilised {Tonkin, Hagberg *et al.*, 1988}. The rupture rate found by Hagberg *et al.* has also been investigated in primary acute flexor tendon repairs mobilised by the controlled active motion regimen and found

not to be affected by mobilisation {Elliot, Moiemen *et al.*, 1994}. In summary, these clinical papers tend to show a fall in adhesion strength and/or improved clinical outcome with controlled mobilisation, although some papers find no differences between the groups. It is this clinical literature which stimulated the inclusion of immobilisation as a post-injury variable in this investigation. We found that the only significant difference between the mobilised and immobilised groups to be at 7 days, with the immobilised group showed a higher relative cellularity in the scrape injury type.

These aforementioned clinical results have stimulated basic science investigation into the effects of mobilisation and tension on tendon healing. McGaw looked at the effect of tension on collagen remodelling by fibroblasts; they found a significant decrease in collagen phagocytosis and an increased relative volume of fibroblast cytoplasm in the transected tendon {McGaw, 1986}; this change in volume may alter the tendency of the fibroblasts to proliferate. The effects of tension on tendon healing has also been evaluated *in vitro*; Zeichen *et al.* found that application of mechanical stress to tendon fibroblasts resulted in an alteration of cellular proliferation depending on the stress time. This study may implicate future modifications in the treatment of ligament and tendon injuries {Zeichen, van Griensven *et al.*, 2000}. These experiments were limited to observing cellularities after injury, but other effects may well be occurring which are responsible for the clinical effects seen. For example, mechanical loading in tissues has been observed to influence matrix remodelling, particularly under conflicting guidance cues {Mudera, Pleass *et al.*, 2000} and cyclical stretch to regulate differential

formation of fibronectin and collagen type I and III. {Bosch, Zeichen *et al.*, 2002}. These results were over a very short time interval, compared to our first results which are at 7 days post injury. However, our data suggests that tendons which are immobilised have a higher tendency to proliferate, or recruit fibroblasts from other areas of the tendon, such as the synovium

One possible mode of inducing different cellular responses is that tension alters the expression of cytokines. Skutek *et al.* found that mechanical stress modulates the secretion pattern of growth factors in human tendon fibroblasts, including TGF-beta, bFGF and PDGF after cyclical mechanical stretching {Skutek, van Griensven *et al.*, 2001} may have a positive influence on tendon and ligament healing through stimulation of cell proliferation, differentiation and matrix formation.

We have arrived at two main possibilities to explain the different responses seen in this model. The first is that the nature of scrape incision, when immobilised, stimulates a more vigorous reaction due to the extent of injury in contact with the synovium. The immobilisation might allow the formation of collagen bridges, stimulating the proliferation of tendon surface cells around the injury site. We have observed an increase in cellularity in those tendons which are immobilised, perhaps suggesting that the change in matrix tension modulates the proliferation rate of the fibroblasts.

5.5.4 Effect of TGFB

Tendon healing is a complex and highly-regulated process that is initiated, sustained and eventually terminated by a large number and variety of stimuli. Growth factors represent one of the most important of the molecular families

involved in healing, and a considerable number of studies have been undertaken in an effort to elucidate their many functions. Our data, described in section 5.4.5 above suggests that in the rat model, addition of TGF-B1 does not stimulate an increase in cellularity around the injury region. A comparison of our data to previous *in vitro* work provides interesting results. It is noteworthy that a lot of work had been done to investigate the effects of growth factors in bone and soft tissue healing processes but, prior to 1985, little work has been performed concerning the role of growth factors in flexor tendon healing. Stein, in 1985 observed that TGF-B1 stimulates tendon fibroblasts proliferation *in vitro* {Stein, 1985}. However, our results show that there is no significant change when TGF-B1 is applied to the tendon surface, in either injury type and at both time points. It is perhaps surprising that the addition of TGF *in vitro* stimulates tendon fibroblast proliferation, yet addition of TGF *in vivo* does stimulate a relative increase in cellularity around the zone of injury, although the work by Stein *et al.* did utilise repeated application of TGFB, and used an *in vitro* model.

In addition to the *in vitro* investigation, more recent *in vivo* work has been carried out into the effect of TGF-B1. A comparison of growth factor expression in the uninjured and repair models showed that a variety of growth factors, including TGF-B1 are present in uninjured and healing digital flexor tendons {Duffy, Jr., Seiler *et al.*, 1995a}. The effect of neutralising antibody to TGF-β1 on postoperative range of motion has shown that intra-operative infiltration of neutralising antibody to TGF-β1 improves flexor tendon excursion; and simultaneous infiltration of neutralising antibody to TGF-β2

nullifies this effect {Chang, Thunder *et al.*, 2000b}. This work suggests that TGF-β1 is implicated in adhesion formation, if neutralising its effects improves post-operative range of motion. The recent implication of TGF-β1 isoforms in chronic tendinosis {Fenwick, Curry *et al.*, 2001} adds weight to their involvement in tendon healing TGF-β1 may have differential effects on tendon fibroblasts according to their tendon location – Klein *et al.* in 2002 showed that sheath fibroblasts, epitenon and endotenon tenocytes showed differing responses to TGF-B1 {Klein, Yalamanchi *et al.*, 2002}.

Our methods only investigated the effect of TGF-β1 on the cellularity of the peri-injury region; the cytokine could be having other effects to cause the changes in tendon healing observed. For example TGF-β1 has been shown to increase water content and decrease tendon cross sectional area {Sakai, Yasuda *et al.*, 2002}.

Whilst much clinical and laboratory data exists to support TGF-B1's role in the tendon healing process, our data suggest that TGF-B1 does not lead to an increase in tendon cellularity, regardless of injury type. It is possible that TGF-β1 acts via other mechanisms to alter the tendon healing process, or that it is already maximally expressed in the area of our investigation.

Whilst adhesion strength or number *per se* was not assessed in this model, it is of interest to note that TGFB application did not lead to an increase in the apparent number or strength of adhesion formation.

CHAPTER 6: GENERAL DISCUSSION

6.1 INTRODUCTION

Recent advances in the repair of injured flexor tendons have resulted in improved postoperative results when assessed by objective outcome scores, yet despite these changes, the range of movement, digital strength as a whole are often still far from ideal. With this in mind, this study aimed to examine several aspects of an area that are deemed to be of crucial importance in the healing of digital flexor tendons.

6.2 *STUDY AIMS*

6.2.1 Chapter 3

- To develop a novel experimental model to specifically label synovial sheath fibroblasts during tendon healing in an animal model.

6.2.2 Chapter 4

- To use this aforementioned model to specifically label synovial fibroblasts in a tendon healing model.
- To observe the response of these labelled synovial cells to tendon injury over the course of 7 days.

6.2.3 Chapter 5

To determine the effect of injury and repair conditions:

Differing injury types

Immobilisation and mobilisation

Application of a TGF-β1

On the cellular healing response in an *in vivo* model

To achieve these aims, two animal models were used, both in the Sprague Dawley rat model. We tested the following hypotheses:

6.2.4 Migration.

That synovial cells are not involved in intrasynovial flexor tendon injury over the course of 7 days.

6.2.5 Injury type.

That injury type and repair conditions, such as differing injury types, immobilisation and mobilisation and application of a TGF-β1 do not affect the cellular healing response in an *in vivo* model.

6.3 *HYPOTHESIS TESTING*

6.3.1 Proof or refutation of Migration hypothesis

The theories of *extrinsic* and *intrinsic* tendon healing both have supporting experimental evidence; indeed recent work now suggests their simultaneous and non-mutually exclusive occurrence. We aimed to produce a novel model capable of selective labelling of synovial sheath cells with concurrent tendon injury. A variety of cellular labels were assessed *in vitro*; DiI had the most appropriate characteristics: lack of leakage from cells, cellular specificity and sufficient longevity. Once attached to cells, the dye is not passed to other cells unless by cell division; this type of fluorescent membrane label has been used previously by McNeilly (1996) in a different type of experiment, investigating how tendons respond to mechanical load by modifying their extracellular matrix. In this case, DiI was used to delineate fibroblasts within the tendon body. Jones *et al.* (2002) have quantified the migration of tendon surface cells into the core following injury, using a similar technique. We used the same concentration of DiI here in order to stain selectively the synovial sheath cells. A variety of different surgical techniques (see Chapter 3) were assessed in an attempt to allow selective labelling of the synovial sheath cells. The tendon surface is accessible proximally by pulling the flexor tendon out of the synovial sheath through flexing the digit, but it is more difficult to apply selectively any substance to the synovium lining the sheath alone. Dual labelling with 2 vital dyes, keeping the tendon within the sheath but physically protecting the tendon and direct application of the vital dye to the synovium in a percutaneous

approach were all tried, but the technique described was the only one to give reliable results. Care was taken to ensure selective staining of the synovium always occurred; whilst removed from the synovium, the FDP tendon was kept moist by wrapping in saline soaked swabs, and therefore any labelled cells found to be present within the cut would be of synovial sheath origin.

It was observed that synovial sheath cells were present in the tendon window and surrounding tendon core by 24 hours after injury, and that more synovial sheath cells migrate into the healing area up to 5 days. There was no increase in the mean distance migrated by the cells from the synovium with time. It was not possible to state by which method from this study to determine how the cells arrive within the injured area, whether it is by slow migration or seeding from the synovial sheath into the injured area. The synovial sheath cells may be shed from the surface and adhere to the wound environment, indeed, the increase in cell numbers may not be purely due to cell migration. It is possible that only some of the visible cells are of synovial sheath origin, and the cells have undergone proliferation, or a combination of these processes, however, proliferation would have attenuated the dye fluorescence, which did not occur. There were less labelled cells within the incision area at 7 days, this could have been due to fading of the vital dye, proliferation (and hence serial dilution), apoptosis or migration of the cells in the longitudinal axis. Our *in vitro* work would suggest that the DiI is still visible, even within several successive generations of daughter cells well after seven days post injury.

Work by Jones *et al.* (2003) has shown that DiI labelled tendon surface cells migrate into the healing area. Khan *et al.* (1996) suggest that surface *and*

sheath-derived fibroblasts are both more reactive than the core-derived cells. They also illustrated that surface-derived fibroblasts are important in the early response to injury by migrating, supported by *in vivo* work by, confirming earlier work which demonstrated epitenon cellular proliferation and subsequent appearance of migrated cells into the cut in *in vivo* models and in the *in vitro* setting. Interestingly, most accounts of surface cell migration are at time points beyond a week from injury. Whilst we can hypothesise that both cell types are involved, we are still uncertain as to the relative roles of each cell type. If differences do exist between the cell types *in vivo*, it may be advantageous for one cell type to be present within the healing tendon rather than another. In terms of the relative participation of extrinsic versus intrinsic cells in the repair process, Jones *et al.* (2003) have shown that epitenon cells migrate into the healing area by 24 hours post injury, with a peak at 5 days; similar to the migration profiles of synovial sheath cells in this study. Due to slightly differing methodologies, it is difficult to directly compare cells numbers; we also suspect that the relative contribution of the extrinsic and intrinsic cells will depend on the anatomical location and nature of the injury.

We have observed that maximum cell numbers were present by day 5, with 50% of the subsequent maximum present by day 1. Gelberman *et al.*, in 1991, described flexor tendon surface cell response within one week of injury, noting at three days that the epitenon fibroblasts proliferate and appear to migrate towards and into the wound. In this study, inflammatory cell presence in the cut at this time was also noted. He found maximal surface cell migration at day 7, correlating it to the high levels of fibronectin at that time point.

We have demonstrated, in a novel tendon healing model, that synovial sheath cells migrate into the zone of injury. They appear in the injury within 24 hours and continue to be present in increasing numbers until 5 days after injury. We therefore suggest that synovial sheath cells may play a role within the tendon healing process, and confirm a mechanism, although not exclusively, of extrinsic tendon healing. This is a functional study and DNA array or proteomic studies would be necessary to define whether the cells are participating in functions useful to repair.

Hypothesis Result: Rejected: Synovial cells are involved in intrasynovial flexor tendon injury over the course of 7 days.

6.3.2 Proof or refutation of Injury Type hypothesis

The variety of operative and peri-operative regimes currently used in clinical practice suggests that adhesions are a difficult problem to tackle. Relatively little is understood about the differing effects of different post-injury healing environments, such as the degree of immobilisation, the presence of pro-fibrotic growth factors and differing injury types, on the healing capacity of the injured tendon.

We aimed to utilise an animal model to observe the effect of different types of injury, immobilisation and application of pro-fibrotic growth factor known to be expressed at the time of injury (TGF-β1) on epitenon fibroblast density.

The experimental procedure involved exposure of the FDP tendon and a treatment permutation of differing injury type, mobilisation regime and application of pro-fibrotic application or control solutions. Tendons were

harvested, processed and the relative cellularity of the zone of injury determined.

The results presented in Chapter 5 show that the differing injury type resulted in differing degree of cellular response, with a scrape injury resulting in a more marked cellular response, a difference which is negated by day 14. Both injuries produced relative a hypercellularity, confirming several in-vivo studies {Becker, Graham *et al.*, 1981}, {Garner, McDonald *et al.*, 1988} and {Abrahamsson, Lundborg *et al.*, 1992} and also suggesting that a superficial type injury leads, initially, to a more aggressive response, possibly explained by an increase in localised cellular recruitment, that tendon surface cells are more reactive than tendon core cells, an altered local cytokine response or differential cell population response to the cytokine production; all of these phenomena have previously been observed *in vitro.*

The clinical differences between immobilisation and mobilisation post tendon injury and repair have been well investigated {Cannon and Strickland, 1985;Tonkin, Hagberg *et al.*, 1988}, in general, suggesting a reduction in adhesion formation or improved clinical outcome with controlled mobilisation. Our *in vitro* results suggest significant differences at Day 7, the superficial scrape injury having a greater cellular response whether immobilised or not. None of the incision injury tendons showed differing responses whether mobilised or immobilised at 14 days, the difference between the scraped immobilised and scraped mobilised having diminished. Many explanations have been put forward to explain potential differences in "generic" situations; tension and stretch {Zeichen, van Griensven *et al.*, 2000}, {Mudera, Pleass *et*

al., 2000} {Bosch, Zeichen *et al.*, 2002} and altered response to cytokine production {Skutek, van Griensven *et al.*, 2001}. Our results are possibly due to the scrape incision, when immobilised, stimulating a more vigorous reaction, due to the extent of injury in contact with the synovium and collagen bridge formation inducing proliferation of tendon surface cells around the injury site.

Previous *in vitro* experimental data suggests that cytokines are vital in the wound healing process. Interestingly, our data suggests additional TGF-B1 does not stimulate an increase in cellularity around the injury region. This would seem to contradict previous *in vitro* work {Stein, 1985}, perhaps because TGF-B1 expression is already maximal in such an *in vivo* model. Our study only investigated the effect of TGF-B1 on the cellularity of the peri-injury region; the cytokine could be having other effects to cause the changes in tendon healing observed. Whilst adhesion strength or number per se was not assessed in this model, it is of interest to note that TGFB application did not lead to an increase in the apparent number or strength of adhesion formation. Most *in vitro* data suggests that the effects of TGF-B1 are maximal in the early stages of the healing process.

Hypothesis Result: Rejected in 2 of 3 elements. That injury and injury and repair conditions, such as differing injury types, immobilisation and mobilisation do affect the cellular healing response in an *in vivo* model; the application of a TGF-β1 does not affect the cellular healing response in the same *in vivo* model

6.4 CLINICAL IMPLICATIONS

6.4.1 Nature of original injury

We have shown that the type of injury is a determinant of the cellular response in the tendon healing model. A scrape injury results in a more marked cellular response, although this effect is negated at two weeks post injury and indeed, both injury types produced a relative hyper-cellularity. Although it would be extremely difficult to alter the types of incident tendon injuries, it might be prudent to consider that a superficial denuding injury may require more rigorous follow up and perhaps a more aggressive initial hand-therapy regime. We have also observed that synovial sheath cells participate in the tendon healing process and whilst potentially involved in process of adhesion formation, are likely to be involved in restoration of the tendon gliding surface.

6.4.2 Timing of surgery

Whilst all hand surgeons would advocate early intervention in hand surgery, we have observed that both synovial sheath and tendon core and surface cells have migrated into the healing tendon area as early as 24 hours post injury. A clinical interpretation of these findings is that early intervention and repair is likely to improve the clinical outcome; adhesions may be formed as early as 24 hours post injury.

6.4.3 Nature of surgery

As both tendon and sheath derived tenocytes are involved in the healing process, both of these tissue types are handled as gently as possible to avoid

inadvertent adjuvant damage at the time of surgery. Our data suggests that both tissue types should be anatomically restored, and intra-operative superficial scrape injuries to the tendon surface should be avoided if possible as our results show that this can lead to an aggressive cellular response.

6.4.4 Post operative rehabilitation

That post-operative mobilisation can significantly affect the relative cellularity of the tendon surface after a tendon injury.

6.4.5 Adhesion-reducing interventions

A wide variety of adhesion reducing strategies are being tested in the clinical and laboratory settings. Our results suggest that these strategies should consider:

That both the tendon-derived and synovial sheath-derived tenocytes should be considered to be active and hence involved in the formation of adhesions.

That injury type and the nature of the post-operative mobilisation regime may impact upon the strength of adhesions formed, but that blocking the action of TGF-B1 may not be useful clinically in the reduction of adhesion strength.

6.5 *CRITIQUE*

6.5.1 Methodology

The key aspects of the methods employed in this chapter primarily concern the choice of animal model used, the nature of the different injury types and the processing and analysis of the specimens. The choice of animal model is discussed at Chapter 4, but some key issues warrant require further discussion here. It was vital that the model chosen be appropriate to test the experimental hypotheses. Whilst the canine and rabbit models have been the most popular for investigating experimental tendon healing, the rat has been shown to be suitable for demonstrating responses to tendon injury {Bora FW, Lane *et al.*, 1972;Iwuagwu and McGrouther, 1998a}. There are also certain advantages in terms of ease and speed of operation, animal handling and the conduct of anaesthesia. The continued use of the rat model in the experimental work within this thesis also facilitated comparison between the different experiments.

There are theoretically an infinite number of permutations of human tendon damage in terms of severity, location, nature of injury and amount of tendon lacerated. To allow investigation of our aims, it was deemed appropriate to choose two injury types which represent the extremes of the injury-type spectrum. The superficial scrape was chosen to represent maximum tendon surface injury, whilst the incision injury was chosen to represent a tendon core type injury. There was no complete means of standardising these injuries, but micrographic investigation revealed a (subjective) consistency. The incision

injury was based on the windows injury, originally utilised by Matthew, Moore and Campbell {Matthew, Moore *et al.*, 1987} and since used by several investigators to study tendon healing {Iwuagwu and McGrouther, 1998b}, {Jones, Mudera *et al.*, 2003}.

Harvesting the entire rat digit ensured that the tendon anatomy was preserved. Despite the need to microtome through bone, good quality sections were obtained. Routine H&E staining to delineate tendon cellularity was routine and good quality staining was produced. Delineation of the area of injury was well facilitated by appropriate visual inspection and orientation due to the placement of a suture at the time of injury.

6.5.2 Other

The classical theories of tendon healing represent an over-simplification of what we propose occurs in the initial stages following tendon injury. Modern methods of repair and attempts at adhesion reduction are nonetheless based on these descriptions. Post-operative outcome of tendon repair surgery is far from ideal. If improved clinical outcomes are to be realised, then research has to progress on a number of different fronts. The ultimate aim is for the operative tendon repair to be free from adhesions, able to glide and be as strong as an uninjured tendon; for this to be achieved, it is likely to require a multifactorial approach at the time surgery, as well as during the postoperative period. It is highly unlikely that one single breakthrough will produce a vast difference to the current results. In an attempt to move a step closer to the ultimate repair, this study has looked at several aspects of tendon healing.

6.6 CONCLUSIONS

Chapter 3:

We were able to develop a novel experimental model to elucidate the possible role and migratory response of synovial sheath fibroblasts during tendon healing.

Chapter 4:

This developed model was used to specifically label synovial fibroblasts in a tendon healing model.

The response of the synovial cells to tendon injury was observed over a 7 days course.

Hypothesis Result: Rejected: Synovial cells are involved in intrasynovial flexor tendon injury over the course of 7 days.

Chapter 5:

We aimed to determine the effect of injury and repair conditions:

Differing injury types

Immobilisation and mobilisation

Application of a TGF-β1

On the cellular healing response in an *in vivo* model

Results showed that injury type and injury and repair conditions, such as differing injury types, immobilisation and mobilisation did affect the cellular healing response in an *in vivo* model; the application of a TGF-β1 did not affect the cellular healing response in the same *in vivo* model

6.7 PROPOSALS FOR FUTURE STUDY

We have shown that synovial sheath cells migrate into the zone of injury within 24 hours after injury. We believe this to be important in the repair of the tendon; improvement in surgical outcome is likely to be from a multi-faceted approach that enhances the movement and incorporation of both tendon surface cells and synovial sheath cells, whilst preventing the formation of adhesions. To take a step closer to obtaining tendon repair free from complication, the following projects in tendon research are planned.

6.8 METHODOLOGIES

6.8.1 Introduction

The initial aim would be to build on the research described in this thesis, which has focussed on elucidating the role of synovial and surface-derived fibroblasts in the tendon healing process. The strategies would be:

To progress the innovative research concerning cell migration patterns to selectively target the two different cell types involved in tendon healing, proposing to exclude them from participating in the healing process, and observing the relative contribution of each in the formation of adhesions.

To build on *in vitro* research which suggests that novel fibronectin based biomaterials can inhibit the production of adhesions, without impairing the healing process.

6.8.2 Experiment 1 - selective cellular modification

6.8.2.1 Introduction:

Elucidation of cellular migration patterns using lipophilic tracer dyes has been performed; we have directly observed that both synovial and tendon-surface derived fibroblasts migrate to the tendon healing area within 24 hours after injury. However, the relative contribution of these fibroblasts remains unproven. We propose to selectively lyse each of these cell types in different experiments, and observe the effect of this cell lysis on the cellular mechanisms of healing, on tendon strength and on adhesion formation.

6.8.2.2 Experimental procedure:

New Zealand White Rabbits will be used for this study. Surgery will be conducted under General Anaesthesia, on the right hind paw, under local tourniquet. All animal care will comply with UK Home Office Guidelines concerning the use of laboratory animals. Animals will be randomly allocated to operated treated and operated non-treated groups and to outcome assessment groups - histology and tensiometry. The un-operated left hind paw will act as the control in each case.

6.8.2.3 Tendon Surface Cell Exposure

An incision will be made on the plantar aspect of the paw, proximal to the Metacarpophalangeal Joint (MCPJ). The flexor tendon complex will be exposed and the Flexor Digitorum Profundus (FDP) tendon isolated and removed from the synovial sheath.

6.8.2.4 Synovial Cell Exposure

Two incisions will be made: the first on the plantar aspect of the paw, proximal to the MCPJ; the second distal to the DIPJ. This will allow the FDP tendon to be removed from the synovial sheath.

6.8.2.5 Selective Cell Lysis

Preliminary investigations have demonstrated that a one minute application of 50% ethanol causes cell death in a controlled manner and to approximately 3-4 cell layer depth. Via a cotton wool bolster, 50% ethanol will be applied to either the Tendon Surface or Synovial Cells.

6.8.2.6 Duration of Experiment

Tendons will be harvested at 1,3,5 and 7 days after treatment.

6.8.2.7 Assessment of Adhesion Strength

Tensiometry pilot experiments have already been carried out, allowing reproducible assessment of adhesion strength. A tensiometer manufactured by NE Holm of Denmark will be used to determine the force in grams required to pull the FDP tendon from its sheath. The FDP tendon will be transfixed with a silk 2/0-stay suture, the paw held rigid in a clamp, and the silk tie connected to the tensiometer.

6.8.2.8 Histological and Immunohistochemical Assessments

The remaining digits will be subjected to histological examination and scoring. Whole digits will be removed at the MCPJ and placed in formalin and decalcifying solution to decalcify the bone. After paraffin embedding, 8 micron sections will be taken through the treated area. The following assessment outcomes will be used:

6.8.2.9 Total cell counts:

Sections will be stained with Haematoxylin and Eosin and photomicrographs taken to facilitate cell counts.

6.8.2.10 Apoptosis assessments

Apoptosis is programmed cell death, detectable by the occurrence of endonucleolytic cleavage of chromatin, by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the free 3'-OH of cleaved DNA (the TUNEL test). The biotin-labeled cleavage sites are then visualised by reaction with fluorescein conjugated avidin (avidin-FITC). This will be performed on histological specimens and relative rates of apoptosis compared between different treatment groups and controls

6.8.2.11 Proliferation assessment

Cellular proliferation will be detected by immunohistochemical detection of Ki-67, a cell marker of proliferation (detecting all active stages of the cell cycle (G1,S,M,G2) but not the G0 Stage.) Relative proliferative rates of the treated and control groups will be determined.

6.8.2.12 Statistical power calculations:

Statistical power calculations have determined that to detect a 50% increase in tensiometer-measured force for tendons in operated untreated compared with treated groups, 12 Animals per group will be required, with 80% power.

6.8.2.13 Statistical analysis

Analysis with be performed using the unpaired students' t-test.

6.8.3 Experiment 2 - Application of Novel Biomaterials

6.8.3.1 Introduction:

Two novel forms of biomaterial have been developed in collaboration with the Tissue Repair and Engineering Centre, based at University College London, through the investigation of novel modes of preventing cell attachment during the tendon healing process. All materials are now readily producible, and have worked well in the *in vitro* situation. The materials can be placed around a tendon injury and/or repair and the synovial sheath closed around them. We propose to observe the effect of these materials in the tendon healing model and determine their efficacy in preventing adhesion formation (by down-regulating cell adhesion and cellular realignment).

6.8.3.2 Biomaterial 1 - Fibronectin Mats

Fibronectin (FN) is involved in many cellular processes, including tissue repair, serving as a cell adhesion molecule, anchoring cells to collagen or proteoglycan substrates. It also has a role in organisation of cellular interactions with the extracellular matrix, and cell migration events. An innovative bioengineering technique has allowed the production of FN mats, which are sheets of aligned FN. Our *in vitro* experiments have demonstrated that these fibronectin mats can be used successfully as biocompatible and biodegradable scaffolds to provide orientated cues using contact guidance for cell migration/adhesion and deposition of extracellular matrix.

6.8.3.3 Non-adhesive materials.

We have produced composite fibronectin scaffolds, consisting of a layer of non-adherent material, covered with a layer of adherent material. We hypothesise that the adherent layer will provide a scaffold for collagen deposition and tendon repair, which the non-adherent surface will reduce adhesion formation between the tendon and synovial sheath. The material has tested extensively both *in vivo* and *in vitro* with a number of cell types with promising results.

6.8.3.4 Operative procedure

New Zealand White Rabbits will be used, and surgical conditions will be as outlined above; surgery conducted under general anaesthesia and conducted under Home Office Guidelines.

6.8.3.5 Tendon Surface Cell Exposure

Surgery will be conducted on Digits 2 and 4 of the rabbit hindpaw. Incisions will be made longitudinally to expose the flexor tendon between the A2 and A4 pulleys. The synovial sheath will be exposed and divided to expose the FDP Tendon and a 50% transverse division made in the tendon body. 50% of the repairs will be randomised to receive application of the biomaterial, with the others acting as control. The synovial sheath will be closed over the repair, and the animals mobilised post operatively. The two biomaterials will be tested separately.

6.8.3.6 Outcome measures

Tendons will be harvested at 1,3,5 and 7 weeks post-operatively. Each will be assessed tensiometrically and histologically, as described above, and intra-group statistical comparisons made. Each specimen will also be assessed for degree of degradation of the biomaterial implanted.

6.8.4 Statistical power

Statistical power calculations for the 2 studies has been determined with the assistance of a biomedical statistician, to ensure that statistical differences can be detected, yet minimising the numbers of animals required.

Anticipated Difference in Means (grams)	60.000
Standard Deviation	45.000
Group 1 Size	12
Group 2 Size	12
Alpha	0.05
Power	0.877

6.8.5 Progression to Clinical Trials

Laboratory work carried out so far suggests that above strategies will be extremely useful, but will only be carried out if animal trials demonstrate that the strategies utilised successfully reduce adhesion formation.

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