NOVEL THERAPEUTICS IN THE PREVENTION OF FLEXOR TENDON ADHESION FORMATION

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Declaration

I, Benjamin Robert Klass, confirm that the research forming the basis of this thesis is original and the ideas were developed in conjunction with my supervisors. I performed the majority of experiments myself with guidance and technical assistance from the scientific and laboratory staff in each of the institutes where the work was undertaken. Histological processing was performed by the RAFT histopathologist, Liz Clayton, and adhesion analysis was performed in a blinded fashion by a mixed group of scientists and surgeons from the RAFT institute. The institutes include The RAFT Institute, Mount Vernon Hospital and the Biological Services Department, The Royal Free Hospital. Where I have drawn on the work, ideas and results of others this has been appropriately acknowledged in the thesis.

This work has been partly funded by Britannia Pharmaceuticals Ltd. (suppliers of Pumactant) however the research undertaken has been performed independently without any personal financial interest.

Ben Klass

Abstract

Tendon injuries of the hand are common with nearly one-third of a million digital flexor tendon injuries per year in the United States. Injuries in zone II of the flexor tendon are notoriously difficult to repair and the main complications are either tendon rupture or adhesion formation. Adhesions remain a problem despite many attempts at prevention using various chemicals and physical barrier techniques. The overall aim of this thesis was to further understand the biology of tendon adhesion formation and to develop novel treatments targeting this process.

Uninjured flexor tendons were obtained from New Zealand White rabbits. Tenocytes derived from different parts of the flexor tendon-sheath complex were grown using standard tissue culture techniques. Each of the three different cell types (endotenon, epitenon and tendon sheath) was subjected to various assays (proliferation/toxicology, cell adhesion, and mRNA expression,) using TGF-β1 and our proposed treatments; epigallocatechin-3-gallate (EGCG), Resveratrol and Pumactant.

A further study then compared the three treatments *in vivo*. New Zealand White rabbits (n=8 per group; 32 in total) were anaesthetised and the flexor digitorum profundus (FDP) of digits 2 and 4 of the forepaw was subjected to a partial tenotomy. The three treatments (compared with control groups) were infiltrated into the flexor sheath of immobilised tendons and the wound was then sutured closed. After two weeks the tendons were harvested and randomised to either mechanical or histological assessment of adhesion formation.

The major findings from the *in vitro* study were as follows: TGF-β1 showed a statistically significant increase in collagen type I gene expression in epitenon cells at 24 and 48 hours and an increase in collagen type III in sheath cells between 6 and 24 hours. There was a statistically significant down-regulation of collagen type III in endotenon and epitenon cells at various time points. Resveratrol showed a statistically significant increase in collagen type I gene expression in epitenon cells with a corresponding down-regulation of fibronectin and PAI-1 in both epitenon and sheath cells. Resveratrol also up-regulated collagen type III at late time points in tendon

sheath cells. Pumactant also showed some therapeutic advantages at the gene expression level with a statistically significant increase in collagen type III in endotenon cells at late time points, corresponding with an overall down-regulation of PAI expression in the same cell type and sheath cells.

The results from the *in vivo* study were that all three treatments showed a statistically significant reduction of tendon adhesion formation when compared to operated controls in both mechanical and histological assessments ($p<0.05$). However, Pumactant was the only treatment to demonstrate a statistically significant reduction in adhesion formation when compared to the H_20-group using both methods ($p<0.05$).

All three treatments displayed potential therapeutic advantages. However, Pumactant showed the most promising *in vivo* results and it would be worthwhile investigating this further with an *in vivo* model of tendon healing and if successful a pilot clinical trial. Hopefully this could act as a suitable adjunct to tendon repair in the future and improve the lives of patients with disabling tendon injuries.

Acknowledgments

There are numerous people that I would like to thank without whom none of this research would have been possible. Firstly, I would like to thank my supervisors; Mr Addie Grobbelaar for his continued support, advice, direction, precious time and enthusiasm for this project and Dr Kerstin Rolfe for the laboratory skills training, technical support, editing and proof reading, understanding and constant friendly encouragement throughout the last few years. I was extremely grateful to have regular structured meetings with both of my supervisors and their constant guidance helped me to develop my scientific thoughts and plan experiments appropriately.

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Finally, a huge thank you to my family who have supported me through everything I have ever done and achieved. In particular my wife, Claire who has shown amazing encouragement and understanding and my children Archie and Jemima who I have watched grow and blossom since the dawn of this thesis. I dedicate this work to them.

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List of Abbreviations

A	adhesion
A1-5	annular pulleys
ACTB	beta actin
ASPA	Animals (Scientific Procedures) ACT 1986
C	claw
C1-3	cruciate pulleys
cDNA	copy deoxyribonucleic nucleic acid
Col	Collagen
CV	coefficient of variation
DEPC	diethylpyrocarbonate
DIP	distal interphalangeal
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic nucleic acid
dNTP	deoxyribonucleotide triphosphate
DP	distal phalanx
ECG	epicatechin gallate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGCG	epigallocatechin-3-gallate
EU	European Union
FCS	fetal calf serum
FDA	Food and Drug Administration
FDP	flexor digitorum profundus
FDS	flexor digitorum superficialis
FITC	fluorescein isothiocyanate
Fn	fibronectin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCG	(-)-gallocatechin gallate
GSH	glutathione
HA	hyaluronic acid

H&E	haematoxylin and eosin
IL-1	interleukin-1
IMS	industrial methylated spirit
IP	interphalangeal
JNK/SAPK	c-jun N-terminal kinase pathway
MC	metacarpal
MCP	metacarpophalangeal
MDA	malondialdehyde
MMP	matrix metalloproteinase
MMP-1	matrix metalloproteinase-1
mRNA	messenger ribonucleic acid
n	number in sample
N	newtons
NF	nuclear factor
NGM	normal growth medium
NO	nitric oxide
NS	no statistical significance
NSAIDs	non-steroidal anti-inflammatory drugs
NZW	New Zealand White
OD	optical density
OUT	operated untreated
p	probability
P1	Pumactant 1mg/ml
P2	Pumactant 2mg/ml
PA	palmar aponeurosis
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PF	peak force
PIP	proximal interphalangeal
PP	proximal phalanx
PT	partial tenotomy
PxT	proximal transection
QRT-PCR	quantitative real time polymerase chain reaction

RAFT	Restoration of Appearance and Function Trust
RDS	respiratory distress syndrome
RES 25	Resveratrol 25μM
RES 50	Resveratrol 50μM
REST-MCS©	relative expression software tool
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
SFM	serum free media
T	injured tendon
T1-5	flexor tendon zones of injury of the thumb
TGF-β	transforming growth factor beta
TGF-β1	transforming growth factor beta-1
TGFβRI-III	transforming growth factor beta receptor I-III
TIMP-1	tissue inhibitor of metalloproteinase-1
t-PA	tissue plasminogen activator
TI	site of tendon injury
TIFF	tagged image file format
TS	flexor tendon sheath
UOUT	unoperated untreated group
UV	ultraviolet
Z1-5	flexor tendon zones of injury of the digits

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

Tendon injuries of the hand are common and can result directly from trauma or indirectly from chronic diseases such as rheumatoid arthritis. These injuries provide a large proportion of the workload for plastic and reconstructive hand surgeons. Repair of tendons is normally achieved using standard surgical techniques. However, although the repair is often satisfactory and strong enough to withstand early mobilisation, the problem remains that of the formation of excess scar tissue, otherwise called 'adhesions'. Adhesions form between the tendon and the surrounding structures and thus inhibit the movement or natural glide of the tendon through the tendon sheath. Subsequently, this leads to a reduction in the range of movement of the affected digit and overall, a poor functional outcome (Gelberman and Manske, 1985).

It is estimated that there are nearly one-third of a million digital flexor tendon injuries per year in the United States (Pennisi, 2002). Adhesion formation within the digital synovial sheath is particularly severe (Potenza, 1962) with repair to zone II flexor tendon injury being optimal in 69% (Peck et al., 1998) to 89% (Kitsis et al., 1998) of cases. Adhesions may be the single most common and costly surgical problem.

Tendon adhesions are not exclusive. Many other surgical conditions are also caused or complicated by adhesions. These restrictive fibrous bands of extracellular matrix form between normally separate gliding tissue layers following surgery, injury or inflammation. This process invariably results in alteration to the tissue or organ structure and ultimately a loss of function. Occurring after more than 90% of abdominal surgery (Liakakos et al., 2001), they are also common following gynaecological and urological surgery. Adhesions may lead to debilitating pain, infertility, visceral obstruction and death (Jeekel, 1997; Parker et al., 2001).

The overall aim of this thesis was to further understand the biology of tendon adhesion formation and to develop novel treatments targeting this process. If successful this may lead to clinical trials and eventually improve the functional outcome for patients undergoing surgical tendon repair.

1.2 Histology and Structure of Tendons

The structural components of the tendon are the collagen fibrils of which consist of proteoglycan matrix and fibroblasts, arranged in parallel rows, although most tendons are relatively acellular. The primary collagen present is type I, which accounts for approximately 86% of the tendon dry weight, and is composed of glycine, proline and hydroxyproline. There are also small quantities of collagen type III and IV. Collagen fibrils are orientated in a longitudinal fashion to from fibres (Figure 1.1), which together with the tenocytes (tendon fibroblasts), are encased by a loose connective tissue. Tendon size and thickness depends on the relative size and strength of the muscle acting upon it. Digital flexor tendons can either be intrasynovial or extrasynovial i.e. with or without a surrounding fibrous synovial sheath.

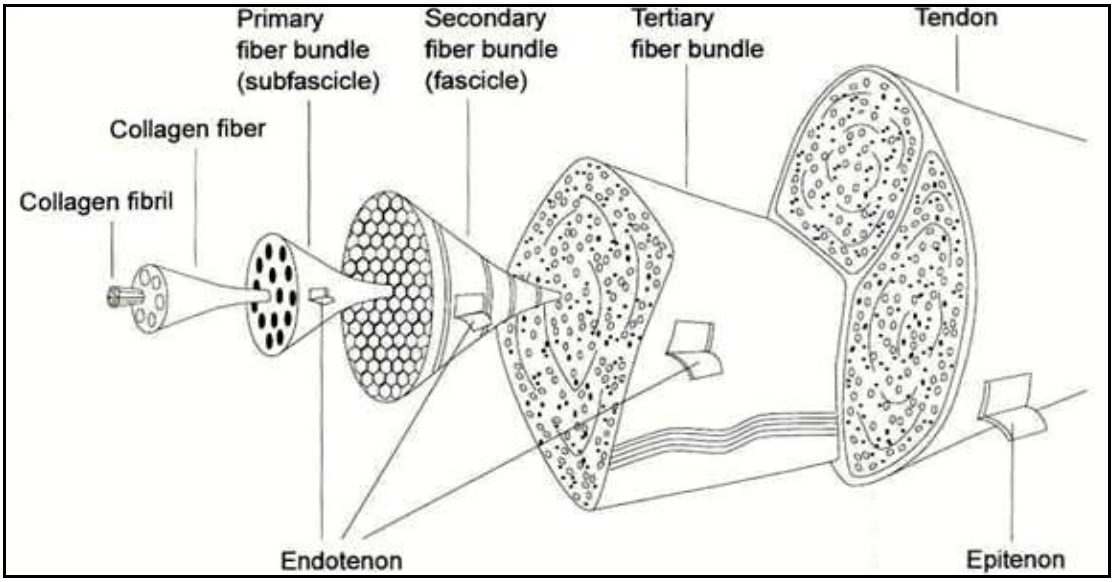


Figure 1.1 The hierarchical structure of a tendon (Kastelic et al., 1978).

Fibroblasts are found in all parts of the tendon and at different levels may represent different sub-populations. Each has a modified role in cell migration and matrix production in response to wounding. Other cell types, in addition to the indolent sub-populations of fibroblast may be found within the tendon substance. These can include endothelial and nerve cells as well as an inflammatory infiltrate in response to injury, made up of macrophages, neutrophils and lymphocytes.

Classically, the tendon inner core has been referred to as the endontenon 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. For the purpose of this thesis, sub populations of fibroblasts are of three types (i) endontenon (core-derived) (ii) epitenon (surface-derived) or (iii) tendon sheath (sheath-derived).

1.3 Flexor Tendon Anatomy

There are two main digital flexor tendons in the human forearm (excluding flexor indicis and flexor pollicis longus, the flexors to the index finger and thumb respectively): The flexor digitorum superficialis (FDS) and the flexor digitorum profundus (FDP).

1.3.1 Flexor Digitorum Superficialis

Function

The FDS is a deep flexor muscle of the forearm and acts to flex the proximal interphalangeal (PIP) joint, the metacarpophalangeal (MCP) joint, only after flexion has been initiated, and the wrist along with other muscles, which flex the wrist.

Anatomy

The FDS lies superficial to the FDP in the anterior compartment of the forearm. This muscle is the largest of the superficial forearm flexors, and arises from two heads. The ulnar head arises from the common flexor origin, (the medial epicondyle), the anterior band of the ulnar collateral ligament, the medial side of the coronoid process, and the intermuscular septa. The radial head arises from an area distal to the radial tuberosity.

Progressing distally, the superficialis muscle forms four distinct bundles in the middle aspect of the forearm. The tendons to the middle and ring fingers lie superficial to the tendons of the index and little fingers and enter the carpal tunnel in the same orientation. Once in the palm, the tendons bifurcate, allowing the transmission of the deeper profundus tendon, which itself becomes more superficial at a site corresponding to the level of the A2 pulley. At the point of bifurcation the central fibres continue into two equal slips. After the two slips have formed, they insert into

the anterior surface of the middle phalanx, blending with the fibro-osseous junction of the A4 pulley.

Blood supply

The arterial blood supply of this muscle is provided by the branches of the artery of the median nerve, muscular branches of the ulnar artery and, occasionally branches of the radial artery. The FDS tendons to the index, middle, ring and little fingers insert into the respective middle phalanx of the digit.

Nerve supply

Innervation to the superficialis muscle is provided by the C7, C8 and T1 distribution of the median nerve.

1.3.2 Flexor Digitorum Profundus

Function

The FDP is a deep flexor muscle of the forearm and acts to flex the distal interphalangeal (DIP) joint, as well as the PIP joint, the MCP joint (only after flexion has been initiated) and the wrist.

Anatomy

The FDP originates from the proximal three quarters of the medial and anterior surfaces of the ulna, the deep forearm fascia and the interosseous membrane. Occasionally, there is a proximal attachment to the radius just distal to the radial tuberosity. As the single muscle belly travels distally deep to FDS in the forearm, it separates into an ulnar bundle and a radial bundle. It then divides into four tendons, which gain entry into the palm via the carpal tunnel deep to the flexor retinaculum. At the musculotendinous junction, the radial bundle forms the profundus tendon of the index finger. The ulnar bundle forms interdigitating slips covered by a single paratenon, ultimately forming the individual profundus tendons of the middle, ring and little fingers. The insertion is the palmar base of each respective distal phalanx.

Blood Supply

The blood supply of the FDP is comprised of muscular branches from the ulnar artery, anterior interosseous artery, and common interosseous artery.

Nerve Supply

Motor branches from the C8 and T1 distribution of the ulnar nerve provide innervation for the ring and small finger muscle bellies. The anterior interosseous branch of the median nerve (C8 and T1) innervates the index and middle finger muscle bellies.

1.3.3 The Flexor Tendon Sheath

The flexor tendon sheath is a fibro-osseous tunnel, which encases the long flexors to the fingers and thumb. It is lined with synovium, which produces synovial type fluid. There are, as with other body cavities, two layers: an outer parietal, and inner visceral layer, which is adherent to anything within the sheath, such as the tendon surface, vinculae, and nerves. The thumbs' sheath continues into the palm, as does that of the little finger. The thumbs' sheath commences approximately 2cm distal to the radial styloid (Doyle and Blythe, 1977). In the little finger, the proximal aspect is in common with the palmar flexor sheath, which envelops all superficialis and profundus tendons as they emerge from the carpal tunnel. It is for this reason that infections, such as a paronychia of the little finger, may spread proximally so rapidly as there is no physical barrier. The anatomy of the sheaths encompassing the middle three digits is different. They commence at the level of the distal palmar crease, which equates to the metacarpal neck. All finger digital sheaths terminate at, or just beyond, the distal interphalangeal joint, whilst the thumbs sheath ends just distal to the interphalangeal joint.

Function

The synovial sheath has been proposed to have three main functions:

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

(ii) As a bursa, containing synovial fluid, to bathe the tendons and aid in its nutrition (Lundborg et al., 1980).

(iii) 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).

Biomechanical Function

Biomechanical laws dictate that the greater the distance from the axis of joint rotation, the greater the moment, resulting in less motion around that joint, and vice versa. Hence, the closer the tendon is held to the bones and joints, the more the resulting movement. Loss of portions of the digital pulleys significantly alters the normal integrated balance between the flexor, intrinsic, and extensor tendons. The A2 and the A4 pulleys (Figure 1.2) are the most biomechanically important; the loss of a substantial portion of either may diminish digital motion and power and lead to flexion contractures of interphalangeal joints or a bowstringing deformity.

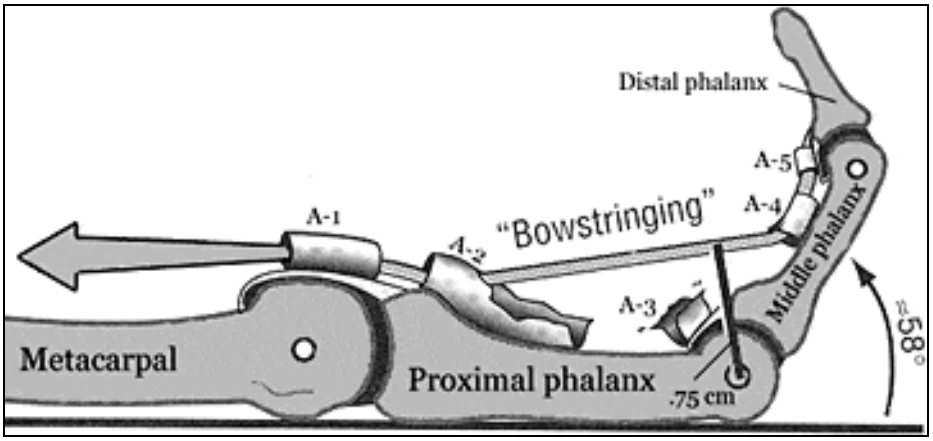


Figure 1.2 Diagram demonstrating the importance of the A2 and A4 pulleys in maintaining biomechanical function and tendon position during flexion (Neumann, 2002).

Recent advances in our understanding of the mechanism of tendon sliding (Guimberteau et al., 2010) have challenged previous theories. Their study suggested that tendon sliding is explained by the existence of a mechanical adaptable multimicrovacuolar network and fibrillar tissue. This tissue enables complete sliding without any dynamic influence on the surrounding tissues. This new theory is based on a polyhedric fibrillar framework, apparently chaotic and complex, subtending the microvacuolar gel, a concept that is to be found everywhere in the human body.

The Pulley System

The fingers have five thickenings of the sheath whose collagenous fibres arch transversely over the tendons. They vary in size, position and functional importance [Figure 1.4]. Initially, it was thought that there were only four pulleys present until the description of the fifth annular pulley by Doyle (1988). The narrow A1, A3 and A5 pulleys all lie over joints: the MCP, PIP and DIP joints respectively. As mentioned above, the functionally more important are the A2 and A4 pulleys, which are broader and lie over the base of the proximal and middle phalanx respectively. There are also three cruciate pulleys (C1-3), which run diagonally across the tendon and perpendicular to each other. C1 lies between A2 and A3, C2 lies between A3 and A4, and C3 lies between A4 and A5. Finally there is the singular palmar aponeurosis believed to be the mouth of the flexor sheath situated at the level of the distal palmar crease.

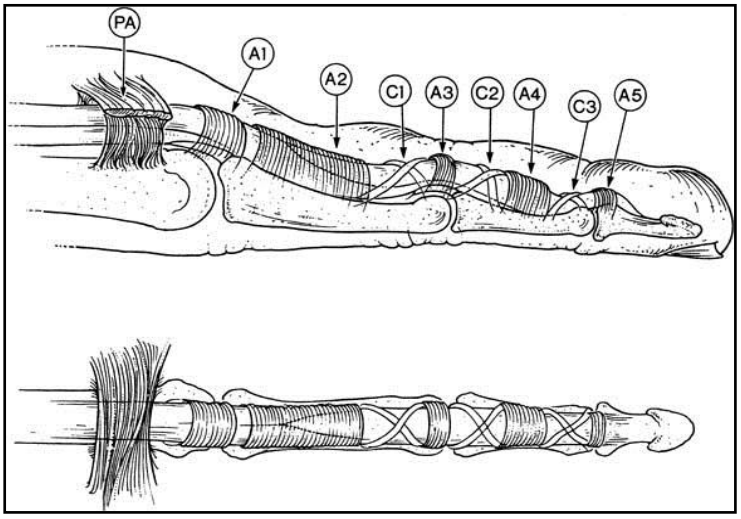


Figure 1.3 The anatomy of the digital sheath within the human finger. Specialised transverse thickenings within the sheath (annular pulleys) are labelled A1 to A5. The three cruciate pulleys are labelled C1 to C3. The palmar aponeurosis is labelled PA (Strickland, 1999).

The thumb has a slightly different pulley system having one oblique and two annular pulleys (A1 and A2) that encase the flexor pollicis longus. A1 lies over the MCP joint and A2 lies over the interphalangeal (IP) joint, with the oblique pulley lying between the two (Figure 1.4). The latter was believed to be more important and it runs from the

ulnar to the radial border proximal to distal (Doyle and Blythe, 1977). A further anatomical and biomechanical study showed a distinct pulley between A1 and oblique pulleys (Bayat et al., 2002). This was named the variable annular pulley or Av pulley (with 3 discrete forms, type I to III). This study challenged previous understanding as they found that the oblique pulley did not prevent bowstringing when A1 and Av pulleys were sectioned. This has implications for clinical procedures such as pulley reconstruction and trigger thumb release.

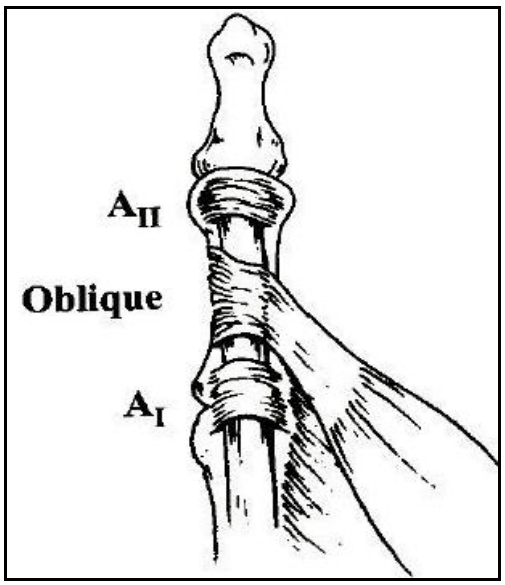


Figure 1.4 The anatomy of the digital sheath within the human thumb. The transverse orientated specialised thickenings of the sheath (annular pulleys) are labelled A1 and A2. In between these is the larger oblique pulley of the thumb (Kaplan, 1965).

Tendon Injury Zones

Tendon injuries of the hand can be divided up into zones. For surgeons, this is a helpful way of describing such injuries as the anatomy differs in each zone (Figure 1.5). The five zones are as follows: 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. The thumb has the same number of zones but different anatomical demarcations: zone 1, distal to IP joint; zone 2, from the A1 pulley to the IP joint; zone 3, the thenar eminence; zone 4, carpal tunnel; zone 5, proximal to the carpal tunnel.

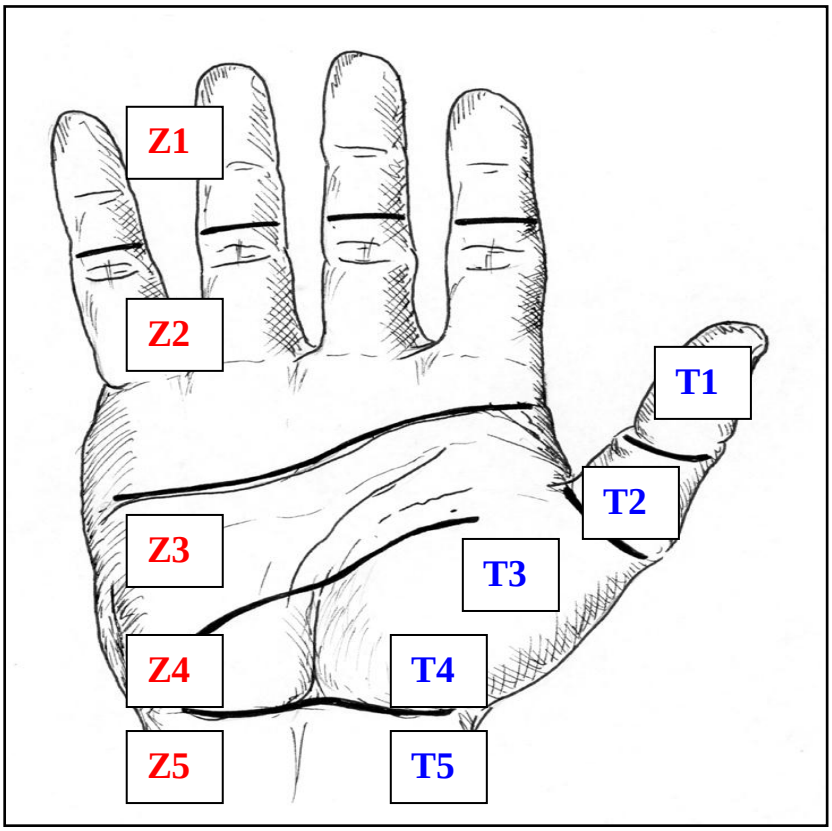


Figure 1.5 The zones of injury of the flexor tendons to the digits of the hand. The four fingers all have the same boundaries to their zones (Z1-5). Z1, distal to the FDS insertion; Z2, from the A1 pulley to the FDS insertion; Z3, from the distal end of the carpal tunnel to the A1 pulley; Z4, carpal tunnel and Z5, proximal to the carpal tunnel. The thumb flexor has slightly different zones of injury (T1-5). T1, distal to the interphalangeal joint; T2, from the A1 pulley to the interphalangeal joint; T3, thenar eminence; T4, carpal tunnel and T5, proximal to the carpal tunnel (Spivak, 1999).

1.4 Tendon Nutrition

It was not until the late nineteenth century that tendons were found to contain blood vessels (Ludwig, 1872). Since then attention has focused on the source and importance of these vessels. Tendons receive their blood supply from three main sources: the musculotendinous junction, the osseotendinous junction, and the vincular vessels as shown by Mayer (1916). He documented palmar avascular zones within the intrasynovial segment of the FDP tendon. Later studies showed that the three sources as described by Mayer in fact contributed in different proportions; the segmental vincular blood supply was the most important and the two other sources relatively minor.

There have been many different anatomical studies examining the blood supply of tendons. These have been performed using cadaveric and post-mortem material, not only from humans, but also from other species. Perfusion studies have included the use of India ink (Lundborg, 1976), methyl methacrylate (Ochiai et al., 1979) and latex-gelatin mixtures (Warren et al., 1988). Due to the variety of injection materials and species used there have been some differences in the description of flexor tendon blood supply. Lundborg and Myrage (1977) reported a sizeable avascular zone on the palmar aspect of the tendon relating to an area between the A2 and A4 pulley 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, a later study, using canine forepaw long flexors, showed that the avascular area in this species was total with an absence of blood vessels in both dorsal and palmar aspects (Gelberman et al., 1991a).

Extrasynovial Tendon Vasculature

From their musculotendinous origin to the level of the A1 pulley, the flexor tendons receive their blood supply from the surrounding paratenon. The paratenon is a thin layer of connective tissue that invests the tendon. Segmental blood vessels arise within the tissue, enter the tendon, and run longitudinally between fascicles to supply nutrients.

Intrasynovial Tendon Vasculature

When the tendons enter the flexor sheath, they are no longer surrounded by paratenon but are incorporated within the visceral lining of the synovium. Here blood can penetrate into the tendons from major branches via vinculae.

1.4.1 The Vinculae

Vinculae are remnants of mesotenon and provide blood supply and nutrition to the flexor tendons. One short and one long vinculum supply each FDS and FDP tendon (Figure 1.6). The FDS receives a short vinculum at the level of the PIP joint. The FDP receives a short vinculum at the level of the DIP joint. The vinculae receive their blood vessels through transverse communicating branches of the proper digital artery located on the dorsum of the flexor tendons. Vinculae provide the blood supply that is

essential to the early healing of flexor tendons and they also serve as a checkrein to limit proximal retraction of a lacerated tendon.

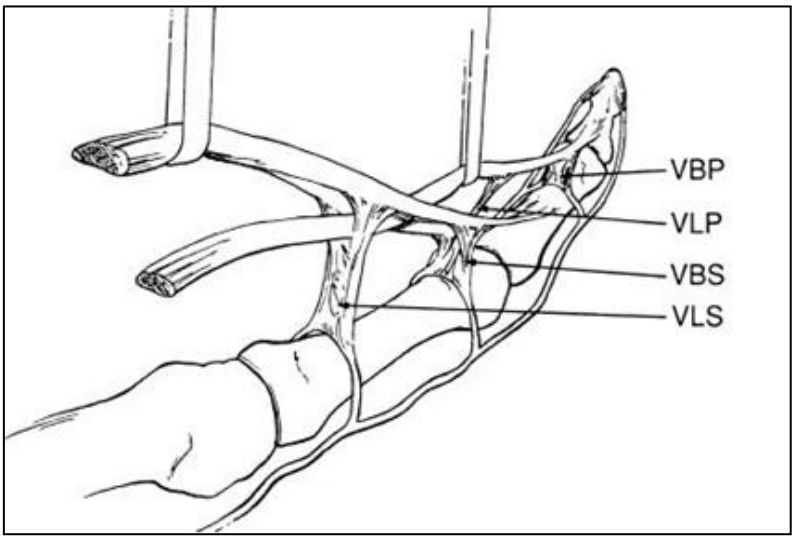


Figure 1.6 Diagram showing the anatomical relationship between the short and long vinculae supplying the FDS and FDP tendons. The vincula are labelled as follows: vinculum brevis profundus (VBP), vinculum longus profundus (VLP), vinculum brevis superficialis (VBS) and the vinculum longus superficialis (VLS) (Strickland, 1999).

1.4.2 Synovial Fluid

Potenza in 1963, proposed that the metabolic requirements of a tendon could be met entirely by the diffusion of synovial fluid into the tendon substance. He deduced this by enclosing a vascularised intrasynovial tendon in a synthetic tube. The tendon was thus isolated from the synovial fluid, and started to degenerate. This seemed to be corroborated by work carried out by Matthews (1976), who devascularised a portion of a rabbit's long flexor tendon in an intact synovial sheath, and found that the tendon survived. It was found that the segments were free from adhesions in all digits and had a smooth surface formed from new collagen. All but the very central cells appeared to remain viable. A year later, McDowell and Snyder (1977) examined the interface between the vinculae and longitudinal vessels within the tendon. They hypothesized that these 'capillary loops' acted as a dialysing membrane with the exuding fluid made available to the tendon substance for metabolism.

With the advent of radioisotope technology Manske et al., (1978) and others performed radiolabelled uptake studies, confirming the importance of synovial derived nutrition over and above the tendons blood supply. These findings began to challenge the widely held surgical belief that volar sutures were detrimental, that grafts required adhesional vascular in-growth for survival, and that the sheath should be resected. However, in the light of studies in the early eighties it is now thought prudent to close the sheath wherever possible, a fact hinted at earlier by Codivilla in 1889. Codivilla is credited as being the first to recognise the importance of sheath preservation.

In practice, trauma to the digits requiring repair is never as exact as that produced in the laboratory setting. Therefore, the general consensus is that both vascular and synovial fluid, do play a role in tendon nutrition, but there is still debate as to what proportion and in what setting.

1.5 Tendon Healing

Tendon healing studies have mostly been performed on transected animal tendons or ruptured human tendons. The mechanisms by which tendons repair have been closely studied and mostly understood, however we are still far from knowing the exact pathways involved.

Tendon healing occurs in three phases: inflammatory, proliferative and remodelling phases. During the inflammatory phase erythrocytes and inflammatory cells, in particular neutrophils enter the site of injury. In the first twenty-four hours, monocytes and macrophages predominate and phagocytosis of necrotic material occurs. Vasoactive and chemotactic factors are released which leads to increased vascular permeability, initiation of angiogenesis, stimulation of tenocyte proliferation, and recruitment of more inflammatory cells (Murphy et al., 1994). Tenocytes slowly migrate into the wound and Type III collagen synthesis commences (Oakes, 2003).

The proliferative phase begins after a few days. Collagen type III synthesis peaks during this stage and lasts for a few weeks. Water content and glycosaminoglycan concentrations remain high during this period (Oakes, 2003).

The remodelling phase begins after approximately six weeks, with a reduction in cellularity and a reduction of collagen and glycosaminoglycan synthesis. This phase can be further sub-divided into a consolidation stage and maturation stage (Tillman, 1996). The consolidation stage begins at about six weeks and continues for up to ten weeks. During this time the tissue changes from cellular to fibrous. The metabolism of tenocytes remains high and tenocytes and collagen fibers become aligned in the direction of stress (Hooley and Cohen, 1979). A higher proportion of collagen type I is produced throughout this stage (Abrahamsson, 1991). The maturation stage then occurs at around ten weeks, with a gradual change of the fibrous tissue to scar-like tendon tissue over the course of one year (Abrahamsson, 1991; Hooley and Cohen, 1979; Farkas et al., 1973). Towards the end of this stage tenocyte metabolism and vascularity decline (Amiel et al., 1983).

Tendon healing can occur intrinsically, by proliferation of epitenon and endontenon tenocytes, or extrinsically, by invasion of cells from the surrounding sheath and synovium (Gelberman et al., 1984; Manske and Lesker, 1984; Potenza, 1962). Epitenon tenoblasts initiate the repair process through proliferation and migration. Cells from the epitenon alone can achieve healing of severed tendons without reliance on adhesions for vascularity or cellular support (Gelberman et al., 1986). Endotenon cells contribute to the intrinsic repair process and secrete larger and more mature collagen fibers than the epitenon cells (Fujita et al., 1992). It has been shown that initially collagen is synthesised by the epitenon cells, with the endontenon cells producing collagen later (Ingraham et al., 2003; Lundborg and Rank, 1980; Russell and Manske, 1990; Becker et al., 1981). The relative contribution of each cell type may be influenced by the type of trauma sustained, the anatomical location, the presence of a synovial sheath, and the amount of stress induced by motion after repair has taken place (Koob, 2002).

In theory intrinsic healing should result in better biomechanics and fewer complications; in particular, a normal gliding mechanism within the tendon sheath is preserved (Koob and Summers, 2002). In extrinsic healing, scar tissue results in adhesion formation, which disrupts tendon gliding (Strickland, 1999).

Wounding and inflammation also stimulates the release of growth factors and cytokines from platelets, polymorphonuclear leukocytes, macrophages and other inflammatory cells (Evans, 1999; Woo et al., 1999; Sciore et al 1998; Chang et al., 1998; Chan et al., 1997). These growth factors induce neovascularisation, chemotaxis, proliferation and collagen synthesis in fibroblasts and tenocytes (Marui et al., 1997; Abrahamsson and Lohmander, 1996).

1.6 Animal models in the study of tendon healing and adhesion formation

For advances to be made in tendon research animal models are essential. They allow for the investigation of the normal tendon healing response, disease pathogenesis and the study of potential treatments. There are important considerations to be made when choosing a model so that any conclusions formed can be directly compared, as accurately as possible, to the human condition under investigation. '*The size of the 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).

Many different animal models have been employed for the investigation of tendons including the rat (Harrison et al., 2003; Matthew et al., 1987; McNeilly et al., 1996), chicken (Becker et al., 1981), mouse (Michna, 1987), rabbit (Abrahamsson et al., 1989; Abrahamsson et al., 1991; Chang et al., 1997; Chang et al., 1998; Kang and Kang, 1999; Kakar et al., 1998; Khan et al., 1996; Manske et al., 1984; Matthews and Richards, 1976; Rank et al., 1980), dog (Gelberman et al., 1991a; Gelberman et al., 1984; Lundborg et al., 1980; Potenza, 1962), cow (Rodeo et al., 1994; Vogel and Meyers, 1999), monkey (Russell and Manske, 1990), and even horse (Smith et al., 2003; Gaughan et al., 1991; Watkins et al., 1985) and comparison between species have been conducted (Gelberman et al., 1984).

In all these different animal models the type of injury inflicted has varied greatly. It may range from complete transection and repair (Frykman et al., 1993), partial transection (Manske et al., 1984), crush injury, avulsion injury or partial window tenotomy (Khan et al., 1996; Iwuagwu and McGrouther, 1998). The conclusions

drawn have often been contradictory or in part confusing. It is perhaps unsurprising due to the range of material studied.

For the purpose of this thesis the rabbit model has been chosen, as it is the most commonly used *in vivo* flexor tendon model and has gained popularity due to its ease of use and accessibility of the deep flexor tendons of the forepaw. The rabbit flexor tendon essentially functions in the extended position in contrast to the human where it functions mainly in flexion; however there are anatomical and physiological similarities, described below, which outweigh this difference and allow its usefulness as an animal model. The partial tenotomy injury model has been chosen for comparing the effect of our different treatments. This is a good flexor tendon model as the injury can be easily made with a scalpel; a measured standard tenotomy window can be produced; the tenotomy area of tendon can be returned to the intrasynovial region after releasing traction; there are few confounding factors such as suture material or tendon sheath injury; the formation of adhesions is reliable; and processing of samples is straightforward. This flexor tendon model has been refined and used on several occasions at RAFT and has been described in studies from this research unit (Jones et. al., 2002; Branford et al., 2008).

Unfortunately, there have been some inconsistencies in the literature regarding the number of annular pulleys observed along the anatomical path of the long flexor tendons of both the hind and forepaw. This number varies between 3 (Hagberg and Gerdin, 1992), 4 (Abrahamsson et al., 1989; Wiig et al., 1996) and 6 in number (Hanff and Hagberg, 1998). Other authors have given a description of the site of injury by relating it to either the proximal (Matthews and Richards, 1976) or distal pulley (Moro-oka, T, 1999). Further anatomical studies by Jones, at RAFT (2001) confirmed there were actually 5 annular pulleys when the Lop-eared Rabbit was studied. Although the fifth pulley was often small it was consistently present. His work showed that in fact there is an even greater similarity between this species of rabbit and humans confirming its usefulness as an appropriate tendon-healing model. He also showed that adhesion formation in injured rabbit tendons was a more reliable finding when compared to the rat tendon model.

1.7 Tendon Adhesion Formation

Tendon adhesions are fibrous bands that form post tendon injury and connect the affected area to its' surrounding anatomical structures. Work by Potenza in the early 1960's found that they were made from fibroblasts and endothelial cells. 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 (Potenza, 1962, 1964). Although acting as conduits for neovascularisation of the injury, adhesions have been shown to be detrimental to mobility and lead to a reduction in hand function (Gelberman and Manske, 1985). 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 and Richards, 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. The general consensus is that adhesions represent an inflammatory response to the traumatic stimulus. A recent study supports this view in a genetically tractable mouse model of flexor tendon adhesion formation (Wong et al., 2009). They confirmed that immobilization of the injured tendon led to increased adhesion formation and that the subcutaneous tissue was always the site of greatest inflammatory, proliferative, synthetic, vascularization and apoptotic activity.

1.7.1 Tendon Adhesion Types

There are few studies in the literature that have focused on the structure, biological composition or mechanics of tendon adhesions tissue. Weeks and Wray (1973) classified adhesions into two groups (i) loose adhesions, which are easily released by physical activity to allow improved tendon glide, and (ii) dense adhesions, which are not easily released. Furthermore, they observed that the adhesive tissues arising from the subcutaneous tissue and the fat became loose adhesions, while those from the palmar fascia, volar plate, tendon sheath and the periosteum became dense adhesions. Masuda et al. (2002), described adhesions histologically depending on the location of

the adhesion in relation to the sheath. The scar tissue in their outside-sheath group was composed of long, coarse fibrous elements (loose adhesions), while in their sheath-excised group; the fibrous tissue was composed of a crossing arrangement of dense fibrous elements (dense adhesion). Following tenorraphy, biochemical analysis of adhesion tissue samples from each of these groups was performed. The ratio of collagen type III to collagen type I in the outside-sheath group was higher than that in the sheath-excised group at 12 weeks and approached an almost normal value at 24 weeks. The collagen type III/I ratio remained low in the sheath-excised group. They concluded that biochemical properties are different between loose and dense adhesions and pathological collagen turnover occurs even at 24 weeks after tendon repair.

Recent research undertaken at RAFT, (Branford personal communication) has attempted to further classify adhesion types based on the micromechanical properties of the cells within adhesion tissue. For the first time confocal microscopy has been used to observe the dynamic properties of tendon adhesion tissue under local strain. Various adhesion types have been observed using this method. In some, the labelled nuclei appear to stretch easily in the direction of the applied force, whereas in others they move in a more random pattern unrelated to the directional forces. It may be that the former are the so-called ‘loose adhesions’ and the latter are the ‘dense adhesions’ as described by previous authors. Non-specific shearing and tearing of adhesion tissue has also been demonstrated in both adhesion types.

When studying adhesions histologically various quantitative and qualitative methods have been employed. Tang et al. (1994) classified adhesions into four grades: no adhesions, slight adhesions, moderate adhesions, and severe adhesions. This grading was based on the presence of adhesions, the extending length of adhesions, and the density of filamentous fibres. Akali et al. (1999) described adhesions histologically as the substance that obliterates the synovial space, thus separating the tendon from the synovial sheath. Consequently, no clear margin can be delineated between the tendon and the synovial sheath. They devised a system for assessing the magnitude of adhesion formation with the aid of a graticule placed in the light microscope’s eyepiece. They measured: (i) synovial thickness at the site of adhesion commencement (inflection); (ii) cell counts within a standardised area at the site of

inflection; and (iii) the proportion (percent) of the volar surface of the tendon covered by adhesions. Qualitative assessment using cell morphology was also used to compare samples. Accurately defining and measuring adhesions is challenging and has led to difficulties in comparing studies. However, for the purpose of this thesis rabbit flexor tendon adhesions will be examined using a combination of the latter two methods.

1.8 Methods used for the reduction of tendon adhesion formation

Tendon adhesions still complicate surgical repair despite recent advances in surgical technique and materials used. Attempts to reduce the occurrence of adhesions, has involved manipulation of the healing response to tendon injury not only at the time of operation, but also both pre and post operatively.

It is well recognised that motion prevents adhesion formation (Strickland, 1999; Gelberman et al., 1986; Gelberman and Manske, 1985; Gelberman et al., 1981; Green et al., 1986). There is not a specific amount of motion that is needed, only a relative amount i.e. enough to overcome the problems of scarring and adhesion formation. If it were possible to decrease the amount of adhesion formation independently of movement, less motion would be required. This approach would eliminate the need for aggressive postoperative motion protocols that place the tendon repair site at risk of dehiscence. If the ideal inhibitor of adhesion formation could be found, the patient could be treated with post-operative immobilisation in a cast or splint until the tendon healed, allowing the repair to heal in a mechanically protected environment without concern about adhesions.

1.8.1 Pre-operative methods of adhesion reduction

Many different methods of pre-operative adhesion reduction have been studied over the last fifty years. For example the systemic administration of different drugs has been investigated with varying degrees of success. Systemic or local injection of steroids has been shown to reduce adhesion formation in vivo. One study examined the role of parenteral Cortisone in reducing adhesion formation of rabbit extensor hallucis longus tendons following surgery (Carstam, 1953). If started several days prior to surgery and continued post-operatively, Cortisone therapy resulted in suppression of adhesion formation, as measured by tensiometer testing. This was in contrast to those rabbits started on parenteral steroids three weeks after repair. Also,

groups that received no treatment or local application of steroid showed no significant reduction in adhesion formation. Even though these findings were promising pre- and post-operative parenteral steroid courses have not led to routine clinical use due to the associated increased rupture and infection rates (Douglas et al., 1967). Other researchers then investigated the role of Beta-aminoproprionitrile and demonstrated that it could reduce peritendinous adhesions. However, their success was later dampened as the compound produced side effects, such as hepatotoxicity, fever and dermatitis after 20 days of administration (Peacock and Madden, 1969). Adhesion formation was thought to be part of an inflammatory process hence the focus turned towards the use of non-steroidal anti-inflammatory drugs (NSAIDs). Ibuprofen, administered parenterally, was shown to reduce intra-abdominal adhesion formation in a rabbit model (Bateman et al., 1982). Kulick et al., (1984) then injected a topical application of ibuprofen at the time of tendon repair and showed over a 50% reduction in adhesion formation. Further work by this group also showed the benefits of oral ibuprofen in reducing tendon adhesions in a primate model. However, there was also a reduction in breaking strength of the completely divided and repaired tendons (Kulick et al., 1986). This latter finding suggests that ibuprofen may also lead to an increase in tendon rupture rates.

1.8.2 Intra-operative methods of adhesion reduction

While studies on pre-operative methods of adhesion prevention were being carried out, research was also focusing on ways to reduce tendon adhesion formation at the time of surgical repair. The strategies employed included different techniques of tenorrhaphy, different suture materials and the use of barrier methods and the application of various chemicals around the repair site.

The observation that mobilisation led to a reduction in adhesion formation, as shown by some researchers (Matthews and Richards, 1976), led to further work to optimise tendon repair. The theory being, that if the strength of tendon repairs could be improved then post-operative mobilisation would rely less on splinting and allow an earlier and more aggressive active mobilisation regime. The ideal repair would be strong enough to mobilise without rupture but allow for healing between the damaged tendon ends. Additionally, it should be simple to perform and prevent gap formation or bunching at the repair site. Ketchum (1985) demonstrated that during the

immediate post-operative period the strength of repair was dependent on two main factors: the suture material and the technique of insertion. Most suturing techniques have two components, a central core suture and an outer peripheral (epitendinous) suture. The role of the epitendinous suture was initially believed to be solely aesthetic but there is evidence that it does in fact improve overall strength and reduce gap formation (Wade et al., 1989; Trail et al., 1992; Kubota et al., 1996). This benefit may have been due to the specific placement of the peripheral suture as Mashadi and Amis (1992) showed that if tendon fibres were included the repair was 83% stronger than the classic pure epitendinous suture.

Although it can often be difficult to compare new techniques of suture repair due to the huge variety in methodology, there is an overall agreement that two strand methods are comparable. Generally, the greater the number of times the suture crosses the repair site the stronger it is. However, Savage and Risitano (1989) showed that a six-strand method was at least three times the strength of the most popular two strand Modified Kessler technique. Other studies have corroborated these findings (Thurman et al. 1998; Wagner et al., 1994). This technique has been shown to withstand the additional forces of early active mobilisation (Sanders et al., 1997). However, this repair has not been widely accepted as it has been argued that the increase in suture material with the multiple strand method would impair normal tendon healing.

The type of suture material has greatly improved during recent years. The ideal suture should be strong, securely knottable, easy to handle, non-reactive and inelastic. The most commonly used materials are either; a synthetic braided (e.g. Ticron), or monofilament suture (e.g. Prolene). The former resulting in less gap formation, however the latter has the advantage of ease of use. When a repaired tendon ruptures it is usually at the repair site. Some authors, with this in mind, have studied several synthetic material analogues with a degree of success. An example of this is a human cadaveric investigation of an internal tendon Dacron splint (Aoki et al., 1994). This had similar strength when compared to the Savage six-strand method and also showed significantly lower gap formation than any other repair method tested. However, there were potential limitations as the material added too much bulk to the repair site and would therefore increase the work of flexion.

Meticulous repair of the tendon sheath has been recognised as an important factor in reducing adhesion formation. Codivilla of Bologna (1889) had first documented this at the end of the nineteenth century (Adamson and Wilson, 1961). This finding has been supported by more recent experiments (Matthews and Richards, 1976; Lister, 1985). In fact such importance has been placed on sheath presence that its reconstruction has been encouraged in situation where primary closure is not possible. Autologous methods have included veins (Strauch et al., 1985), synovial bursae, sheaths from toes and even neighbouring digits (Rank et al., 1977). Synthetic methods have included cellulose tubes (Ashley et al. 1959), silastic (Ashley et al. 1964), Sterispon wrapping (Austin and Walker, 1979) and expanded polytetrafluoroethylene diffusible membrane (Hanff and Hagberg, 1998). 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 reduce or prevent adhesion formation.

One example of a commercial physical barrier to adhesions is ADCON T/N (Wright Medical Group Inc). This is a resorbable carbohydrate gel that was initially approved in the European Union (EU), but following a negative Food and Drug Administration (FDA) review in 2005, further product development was suspended. An initial study showed promising results. 59 patients were randomised into control or ADCON-T/N treated groups and all followed an early mobilisation regime following tendon repair. Tendon rupture rates were comparable between the control and ADCON-T/N treated patients. At six months follow-up, the ADCON-T/N treated group had better proximal interphalangeal motion (Liew et al., 2001). However, a prospective double blind randomised controlled clinical trial including 45 patients with 82 flexor tendon repairs in 50 digits, demonstrated higher rupture rates (33%) when compare to the control group (20%), although this difference was not significant. However, the mean time to rupture in the ADCON-T/N group was 3 weeks, compared to 2 weeks in the control group ($p=0.016$) suggesting that ADCON-T/N interferes with tendon healing in some way. Also, the final ranges of motion at six months were the same (Golash et al., 2003). Essentially, there appears to be no clinical advantage from using ADCON-T/N and although not significant, tendon rupture in one third of patients was deemed unacceptable.

Another liquid barrier is lubricin, also known as superficial zone protein (SZP) and proteoglycan 4 (PRG4), found in synovial fluid, cartilage, meniscus and tendon (Rees et al., 2002). It is best-known for its excellent boundary lubricating properties and appears to be expressed ubiquitously in the canine FDP tendon with variations relating to regional mechanical requirements (Sun et al., 2006). More recently, a lubricin-containing gel compound has been shown to decrease post-operative flexor tendon adhesions in a canine model, although there was some impairment of tendon healing (Zhao et al., 2010). Additionally, lubricin, phospholipids and hyaluronic acid all appear to be involved in maintaining low gliding resistance of tendons (Sun et al., 2008). One of the most promising recent advances in gel barrier technology was a randomized controlled multicentre clinical trial using a hyaluronan-based gel, Hyaloglide, in the prevention of recurrence of adhesions after tenolysis of flexor tendons in Zone II (Riccio et al., 2010). Patients (45 total, 19 controls, 26 treated) in the Hyaloglide treated group had a statistically better recovery of finger motion at all time intervals and returned earlier to work and daily activities. There was no apparent increase in complication rate however there was no discussion regarding its use in primary Zone II flexor tendon repairs and the potential direct effects on tendon healing.

Various chemicals have been applied to the repair site, at the time of surgery, with the aim to modify the inflammatory response and reduce excessive scarring or adhesion formation around the healing tendon. As already mentioned in the pre-operative section, topical ibuprofen has also been studied intra-operatively, again due to its anti-inflammatory actions (Kulick et al., 1984). Other substances include dextran 70 (Green et al., 1986), sodium Hyaluronate (Hagberg, 1992; Salti et al., 1993; Weiss et al., 1986), aprotinin (Komurcu et al., 1997), 5-Fluorouracil (Moran et al., 2000; Khan et al., 2000), neutralising antibodies to transforming growth factor beta-1 (TGF-β1) (Chang et al., 2000) and fibrin sealant (Frykman et al., 1993) or a human-derived form of fibrin sealant called Vivostat® (Jones et al, 2002), all with differing results. Fibrin sealant has been used for almost half a century. It is now regularly used as a surgical adjunct for haemostasis in hepatic and cardiothoracic surgery (Chisholm et al., 1989; Holcomb et al., 1999; Matthew et al., 1990). Most sealants are synthetic or derived from pooled human plasma. Therefore there is a risk of foreign body reactions, or the transmission of blood born pathogens. Unfortunately, this is an

unacceptable risk for the commercial use of a fibrin sealant as a treatment for the prevention of tendon adhesions.

1.8.3 Post-operative methods of adhesion reduction

The importance of mobilisation following tendon surgery has been documented as early as 1912 by Erich Lexer, of Jena. He was the first to describe a series of ten free flexor- tendon grafts in the hand and emphasised the importance of avoiding adhesions by careful suturing and early motion six days after operation. He also recognised that local skin flaps needed to be shifted to prevent skin-to-tendon scar adherence and the need for long and patient post-operative care (Adamson and Wilson, 1961). This theory of movement following tendon repair was challenged by Potenza (Potenza, 1961). It was his belief that a period of immobilisation was required to allow adhesion formation, which he thought was required for adequate tendon healing. This once gold standard treatment is now only accepted for very young or uncooperative children. The key turning point in post-operative management followed the work of Kleinert (Kleinert et al., 1972), who confirmed that post-operative mobility was essential for improved results. Controlled passive motion after surgery led to 87% of cases with either good or excellent results. The Kleinert regime incorporated a rubber band fixed to the nail of the affected digit, which is then attached to the flexor aspect of the forearm allowing active extension but passive flexion.

Some authors have shown improved outcome with passive mobilisation, and displayed evidence that it enhances healing by stimulating maturation of the tendon wound in parallel with the remodelling of the tendon scar (Gelberman and Manske, 1985). Others have added to these studies with their observations that active (in contrast to passive) mobilisation applies stress to the sutured tendon, increases the strength of the repair, enhances the biological response, and obviates the loss of tensile strength during the first three weeks of healing (Hitchcock et al., 1987; Aoki et al., 1994). Small in the late 1980's reported the results of patients that had routine tenorrhaphy followed by early active mobilisation. He achieved 77% of patients with either good or excellent results with a rupture rate of 9.4% (Small et al., 1989). In the same year there was a smaller series that reported 64% of cases with either good or excellent results with a rupture rate of 6% (Cullen et al., 1989). Later, other studies

confirmed slightly improved results with early active mobilisation when compared to the Kleinert post-operative regime (Elliot, 1994; Baktir et al., 1996). When considering all of the research to date, 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.

Adhesions once formed, are likely to cause a restriction of movement to the affected digit. After extensive post-operative physiotherapy, if the passive range of movement is greater than the active range, tenolysis is indicated. It should be remembered that this is a salvage procedure and might not improve the situation but may even make it worse. It involves the surgical release of the restrictive adhesion to free the adherent tendon and therefore allow an improved range of movement.

1.9 Rationale for proposed anti-adhesion treatments

The increased rupture rates, foreign body reactions and other adverse events associated with the chemical and barrier treatments as mentioned above, have forced a change to our anti-adhesion treatment strategy. In this study, we therefore examined the role of more naturally occurring compounds in the search for an optimal treatment. A treatment that ideally can reduce tendon adhesions, allow normal or improved tendon healing but without these potential complications. The three compounds chosen for this particular study are as follows: Epigallocatechin-3-gallate (EGCG), an extract from green tea; Resveratrol, a natural extract from several plants including grapes; and lastly Pumactant, a mixture of two naturally occurring phospholipids (supplied by Britannia Pharmaceuticals).

1.9.1 Epigallocatechin-3-gallate (EGCG)

During the last phase of in vitro experiments at RAFT, Mount Vernon Hospital, a natural product was being tested for its potential clinical application in tendon adhesion reduction. A naturally occurring product if found to have beneficial clinical effects would have the advantage of being relatively easy to manufacture, have low costs and potentially have less side effects when compared with previous treatments.

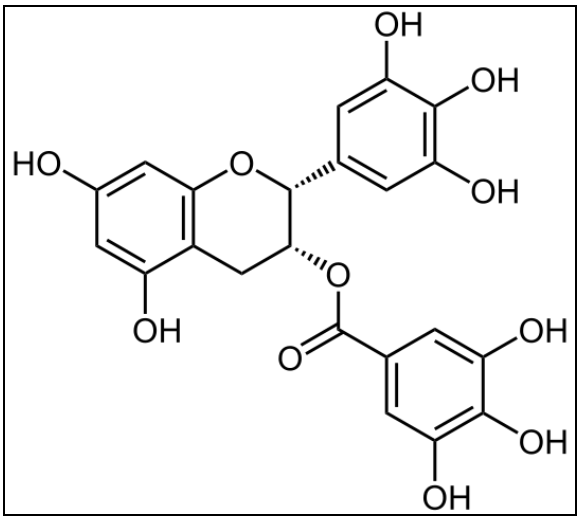


Figure 1.7 Chemical structure of epigallocatechin-3-gallate (EGCG) (Wan, 2005).
Molecular formula: $C_{22}H_{18}O_{11.}$
Molecular weight: 458.372 g/mol.

The product used was Epigallocatechin-3-gallate (EGCG) (Figure 1.8) an extract from green tea (Latin name: *Camellia sinensis*). It is the main active and water-soluble catechin from green tea. Other less active catechins in green tea include epicatechin gallate (ECG), (+)-epicatechin and (-)-gallocatechin gallate (GCG). It accounts for 9-13% of green tea in net weight. EGCG possesses strong anti-oxidant activities and plays an important role in preventing cancer (Ahmad et al., 1997) and cardiovascular disease (Basu et al., 2007). Recent studies have shown that EGCG suppresses collagen production and may have therapeutic potential for keloid scarring and liver fibrosis (Zhang et al., 2006). Further, EGCG appears to be able to prevent transforming growth factor-beta 1 (TGF-β1) stimulated collagen contraction through a number of mechanisms, including reducing myofibroblast differentiation and the reduction of connective tissue growth factor expression (Klass et al., 2010). Other mechanisms of action have been studied; one theory is that EGCG reduces interleukin-1 (IL-1) stimulated expression of collagenase and stromelysin messenger ribonucleic acid (mRNA) via c-jun N-terminal kinase (JNK/SAPK) pathway (Corps et al., 2004). Other theories suggest that EGCG inhibits extracellular matrix (ECM) gene expression, by interrupting TGF-β1 signalling through attenuating oxidative stress (Yumei et al., 2006), or that it enhances transcription of type 1 collagen (although collagen production was actually inhibited in this study) and tissue inhibitor of metalloproteinase-1 (TIMP-1) and reduces the transcription of matrix metalloproteinase-1 (MMP-1) (Nakumata et al., 2005). The specific collagen types affected by EGCG have not been examined. Also, due to its action on collagen synthesis and the ECM its possible role in tendon healing and prevention of adhesions should be explored.

1.9.2 Resveratrol

Another naturally occurring compound, in this study, is Resveratrol (trans-3, 4, 5-trihydroxystilbene) (Figure 1.9). This is a natural polyphenolic phytoalexin found in some plants including grapes and their related products. Phytoalexins are antibacterial and antifungal chemicals produced by plants as a defence against infection. It is also a stilbenoid, a derivative of stilbene, and is produced with the help of the enzyme stilbene synthase. Resveratrol has also been produced by chemical synthesis and is sold as a nutritional supplement.

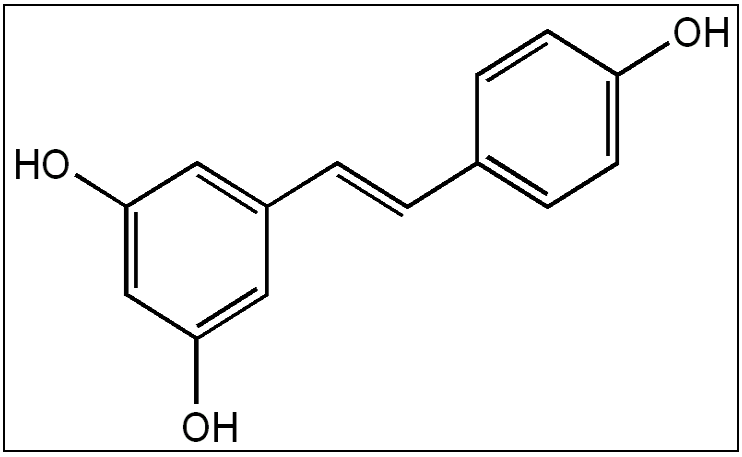


Figure 1.8 Chemical structure of resveratrol (trans-3,5,4'-trihydroxystilbene) (Choi, 2010)
Molecular formula $C_{14}H_{12}O_3$
Molecular weight 228.25g/mol

A recent study using a rat model demonstrated a reduction in peritoneal adhesions following laparotomy and intra-operative application of resveratrol when compared to saline (Sogutlu et al., 2006). Twenty-one Wistar-Albino rats were assigned into three groups (7 in each). After a midline laparotomy a 1cm area of caecum was abraded in two of the groups. They were either given resveratrol (Group 1) or saline (Group 2) intraperitoneally. Group 3 rats (sham operation) received no treatment, without serosal damage. After two weeks the rats were killed and the adhesion score was determined according to Mazuji's adhesion grade scale. The mean adhesion grade in the resveratrol group was 1.0 ± 0.0, in the saline group 2.57 ± 1.51, and zero in the sham operated group ($p<0.05$ between the resveratrol and saline groups). Tissue levels of malondialdehyde (MDA) and nitric oxide (NO) were significantly reduced in the resveratrol group whereas glutathione (GSH) levels were increased. From these findings they concluded that resveratrol reduces surgically induced peritoneal adhesions possibly through reduction of lipid peroxidation products.

Other studies have shown that resveratrol has anti-inflammatory, antioxidant, anti-platelet and anti-tumour effects (Brakenhielm et al., 2001; Sgambato et al., 2001; Surh

et al., 1999). In a dermal wound-healing mouse model, Resveratrol treatment was associated with a more well-defined hyperproliferative epithelial region, higher cell density, enhanced deposition of connective tissue and improved histological architecture (Khanna et al., 2002). Another study showed that Resveratrol can prevent fibrosis in a rat cirrhosis model, and that its action is probably associated with its ability to reduce nuclear factor-kappa (NF-kappa) activation and thus reduce levels of profibrinogenic transforming growth factor-beta (TGF-β) (Chavez et al., 2007). There are close associations between wound healing, fibrosis, excess scarring and adhesion formation. Hence, following these studies it would be worthwhile investigating the effects of resveratrol on tendon healing and whether peri-tendinous adhesion formation can be reduced.

1.9.3 Pumactant

Cells on the tendon surface are known to contain lipid-containing vesicles and it has been estimated that lipids account for up to 43 percent of the epitenon matrix whereas collagen accounts for only 23 percent (Banes et al., 1988).

Pumactant is a mixture of two naturally occurring phospholipids (a 7:3 mixture of dipalmitoylphosphatidycholine and unsaturated phosphatidylglycerol). It was originally developed for neonatal respiratory distress syndrome (RDS) and results of a large multi-centred trial showed a 48 percent reduction in neonatal mortality in babies less than 32 weeks gestation. The product was withdrawn due to commercial reasons in 2000 following unfavourable comparison with the market leader at that time. However, Pumactant has been investigated in the prevention of adhesion formation, focusing initially on post-surgical abdominal adhesions in two randomised, and partially double blinded clinical trials. The results of two Phase III clinical trials were equivocal in respect to efficacy. The confidence and interpretation of the results were confounded by reports of inadequate and unsatisfactory delivery. Improvements have been made since then and in a murine abdominal adhesion model there was a dose dependent reduction in adhesion formation (unpublished studies at Cardiff University).

Pumactant has also been found to accelerate mesothelial and keratinocyte cell proliferation (Topley & Woodcock, 2010). This proliferation appeared to be unique to

this specific compound, as other phospholipids appear to inhibit cell proliferation and cause a reduction in cell viability. A number of publications have either identified phospholipids in tendons and tendon sheath fluid (Mills et al., 2005) or a role for phospholipids in the prevention of adhesion formation (Moro-oka et al., 2000). In the latter study two experiments were conducted using Japanese White rabbit flexor tendon models: the first investigated the effect on the friction coefficient using a mixture of dipalmitoyl phosphatidylcholine and sodium hyaluronate (PHA), saline or sodium hyaluronate alone (HA). In this experiment the hindpaw flexor tendons were fixed using a clamp and strain gauge at either ends. The type of lubricant was varied while the load and preload were kept constant. The friction force was obtained from the difference between the tensions at the ends of the tendon, as measured by the strain gauge. They concluded that the decrease in friction coefficient in the PHA group indicates that dipalmitoyl phosphatidylcholine complements the boundary-lubricating ability of the tendon. In the second experiment, rabbit hindpaw flexor tendons were subjected to injury and then immobilised. Three weeks later the rabbits were terminated, the tendons were harvested and prepared for mechanical testing. In the HA group there was a tendency for less work to be required to tear off the adhesion but this was not significant ($p=0.171$). In the PHA group, the difference between the right (treated paw) and left paw (operated but untreated) showed a statistically significant difference ($p=0.027$). In summary the PHA mixture was shown to significantly reduce tendon friction and adhesion formation. Hyaluronic acid is known to be a naturally occurring joint lubricant and in a similar study it was again combined with a phospholipid (Kawano et al., 2003). Phospholipids appear to have an additional effect (when combined with HA) on reducing tendon friction and joint lubrication when compared to just HA alone. In neither study did they investigate the actions of phospholipid alone. For the purpose of this current study Pumactant was used alone. In addition there is evidence (Topley & Woodcock, 2010) to suggest that Pumactant may achieve some of its effects through the induction of HA synthase, which presents the possibility that phospholipids might aid lubrication and also participate, through HA production, in tendon repair and healing.

The ideal treatment would promote tendon healing and reduce or modify adhesion formation thus allowing the tendon to glide freely within the tendon sheath and allow for optimal function.

1.10 Rationale for in vitro mRNA gene selection

In this thesis various genes known to be involved in healing and fibrosis were selected. In general, collagen type I and collagen type III are important for tendon healing, whereas fibronectin, plasminogen activator inhibitor 1 (PAI-1) and tissue plasminogen activator (t-PA) are associated with adhesion formation. By studying the different gene responses to each of the treatments better insight into the molecular processes involved could be gained. This in turn, should allow a more targeted approach to modifying tendon healing and adhesion formation.

1.10.1 Collagen type I and Collagen type III

Collagen (as already discussed in sections on histology, structure and tendon healing) is known to be important in maintaining the mechanical properties of the tendon and transmitting the forces generated by muscle fibers to the skeletal system (Benjamin et al., 2002; Purslow, 2002). Although there are numerous collagen types, collagen type I and type III appear to play the most significant role in tendon healing. In the uninjured tendon approximately 95% of the collagen is type I. Following injury the tendon ECM undergoes remodelling with breakdown of collagen fibres and the production of a new collagen framework. Collagen type III levels increase in the early inflammatory and proliferative stages of tendon healing whereas collagen type I production is increased in the later remodelling stages of tendon healing replacing collagen type III. Our treatments should aim to allow normal or optimised transcription and deposition of these collagen sub-types. Any reduction in production or alteration of collagen ratios may lead to inadequate tendon healing and probable tendon rupture.

1.10.2 Fibronectin

Fibronectin is a high molecular-weight glycoprotein containing approximately 5% carbohydrate and binds to membrane spanning receptors also known as integrins. It can also bind to ECM components such as collagen and fibrin.

Fibronectin is deposited during the granulation stage of tendon healing, and it has been proposed that this preliminary fibronectin matrix allows tendon cells to move into the wound site (Williams et al., 1984) and remodel the ECM forming an adhesion-bridge between the tendon and tendon sheath.

Wojciak and Crossan (1993) investigated the accumulation of inflammatory cells in synovial sheath and epitenon during adhesion formation in healing rat flexor tendons. They concluded that the presence of inflammatory cells in the synovial sheath and epitenon during tendon healing induces synovial fibroblasts and epitenon cells to increase their production of fibronectin, which provides a scaffold for subsequent adhesion formation. Our treatments, if able to block the expression and excess production of fibronectin may inhibit adhesion formation via this mechanism.

1.10.3 Plasminogen Activator Inhibitor 1 (PAI-1) and Tissue Plasminogen Activator (t-PA)

During the normal fibrinolytic process (Figure 1.10) plasminogen is converted to plasmin via the action of t-PA. Plasmin then converts excess fibrin to fibrin degradation products allowing for clot dissolution around the site of healing. Excess fibrin around the site of tendon healing is believed to form the framework for adhesion formation between the tendon surface and its surrounding sheath. Low expression of t-PA would lead to a reduction of plasmin, a persistence of fibrin and therefore increase the potential for adhesion formation around the tendon. Furthermore, increased levels of PAI-1 would inhibit the effect of t-PA and exacerbate this effect. The cells most associated with these changes are those of the tendon surface and sheath. If this process occurs at the surface-sheath interface during tendon healing then there is a greater likelihood of adhesion formation at the site that will impair normal tendon gliding.

Indeed, studies have shown that PAI-1 may play a role in the formation of peritoneal adhesions (Cheong et al., 2001). In contrast, increased t-PA has been found to decrease adhesion formation (Dorr et al., 1992; Orita et al., 1991; Doody et al., 1989; Menzies & Ellis, 1991). Fibrinous adhesions appear to be the precursors of fibrous, permanent adhesions in the pelvis (Holmdahl, 1997). Other studies (Di Filippo et al., 2006; Ivarsson et al., 1998) have shown that post-surgical peritoneal adhesions show an alteration of local fibrinolytic components, with the formation of adhesions being the result of an imbalance between fibrinogenesis (excess PAI-1) and fibronolysis (increased levels of tissue plasminogen activator; t-PA). These compounds have yet to be examined in tendons and warrant further investigation.

Ideally treatments should aim to increase plasma and tissue levels of t-PA but reduce the levels of PAI-1. This, in theory, would allow more efficient fibrin degradation and reduce the risk of adhesion development.

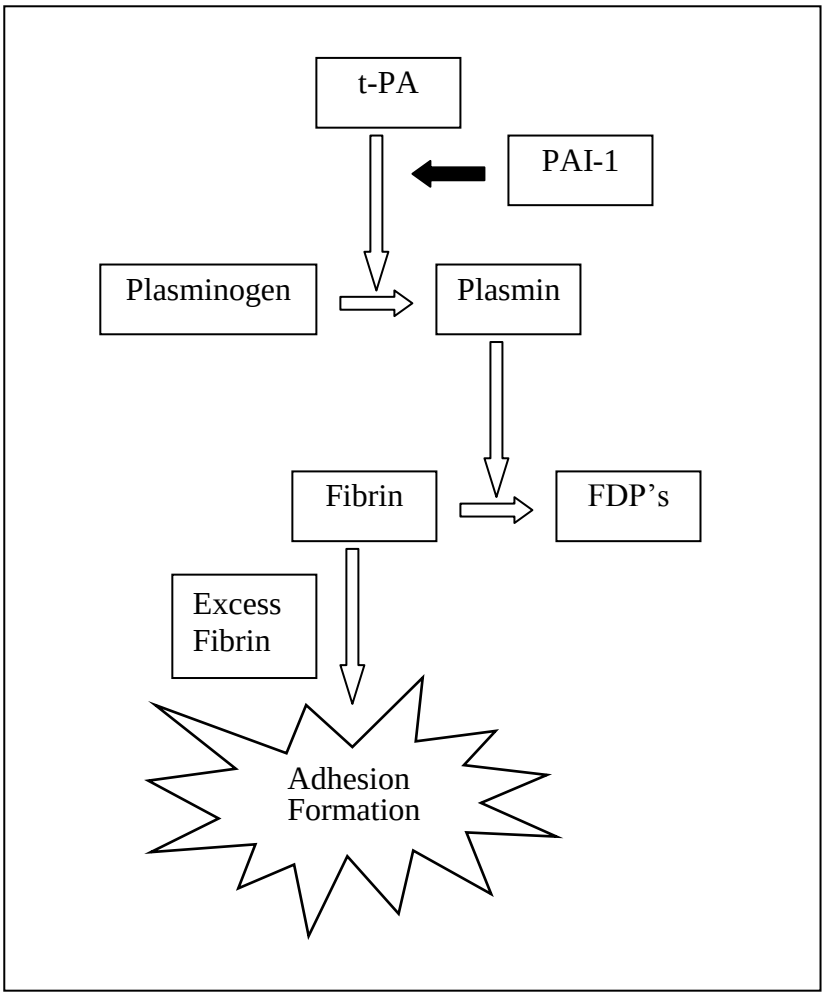


Figure 1.9 A flow diagram showing the influence of the fibrinolytic pathway on the formation of adhesion formation involving plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (t-PA). White arrows represent activation and the black arrow represents inhibition.

1.11 Rationale for Thesis

Flexor tendon injuries are common and can be surgically repaired but are frequently complicated by either rupture or peritendinous adhesion formation. Adhesion formation leads to a reduction in movement of the affected digit and overall hand function for patients. Although early post-operative mobilisation has reduced the occurrence of some adhesions this is not without an increased risk of tendon rupture. To date there is still no accepted or effective treatment to prevent adhesions from occurring altogether. The treatments that have shown promise have also had unwanted side effects. The naturally occurring compounds that have been carefully selected for this study have all shown potential as anti-adhesion treatments and should also allow for normal or optimised tendon healing. Should one or all of these treatments show positive results then clinical trials will follow on from this work. Ultimately, the aim is to improve hand function and the lives of patients following tendon injury and surgical repair.

1.11.1 Thesis Hypothesis

The aim of this thesis was to test the following hypothesis:

'The three novel compounds, EGCG, Resveratrol and Pumactant are able to reduce or modify flexor tendon adhesion formation'.

1.11.2 Thesis Objectives

- Establish rabbit tendon cell cultures, including endotenon, epitenon and tendon sheath cells, to allow *in vitro* testing on the each part of the tendon-sheath complex using our three proposed treatments.

- Determine the effect of transforming growth factor beta-1 TGF-β1 on flexor tendon cells of different origin.

- Perform cell proliferation studies to determine optimal doses of specific treatments.

- Determine the *in vitro* effect on the production of collagen sub-types and other extracellular matrix components important for tendon healing or adhesion formation using the different treatments on each cell type.

- Determine the effect that the treatments have on *in vitro* cell adhesion with each tendon cell type.

- Determine if the treatments can reduce adhesion formation following tendon injury using an *in vivo* flexor tendon model.

- Compare and explain the differences between the three treatments and various cell types.

CHAPTER 2

MATERIALS AND METHODS

2.1 Tissue Sampling and the Isolation of Cells

2.1.1 Tendon Sampling

Uninjured fresh New Zealand White (NZW) rabbit forelimbs were obtained following Schedule 1 Termination (procedures were carried out following the regulations of the Home Office Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (1989) and the Animals (Scientific Procedures) ACT 1986 (ASPA)). The limbs were submerged in industrial methylated spirit (IMS) for 1 minute and the skin was carefully removed and washed three times with warmed phosphate buffered saline (PBS, Gibco) prior to dissection. All dissection was carried out using a standard operating light microscope (Leica), a sterile scalpel and forceps.

Under sterile conditions the forepaw flexor tendons were exposed at the level of the mid-metacarpal region (A1 pulley) to the level of the distal phalanx (A5 pulley). Three separate cell components from this flexor tendon region (Zone II) were dissected out in preparation for tissue explants including: the tendon core (endotenon), the tendon surface (epitenon) and the tendon sheath (Figure 2.1). The flexor digitorum superficialis (FDS) tendon was excised to further expose the larger flexor digitorum profundus (FDP) tendon. The sheath was easily removed from the latter of these tendons thus exposing the whole length of the tendon and pulley system. The sheath tissue was kept in a sterile container until further processing. The pulleys were released to allow removal of the FDP tendon. The tendon itself was then dissected into surface and core. The surface tissue was identified as the outside layer of cells approximately 0-0.5mm maximum depth.

After the surface tissue was removed the core was then identified. Each of the three tissue types were placed in sterile containers and the tissue finely diced. The tissues were placed into ten T25 tissue-culture flasks (plastic ware from Greiner) per tissue type containing pre-warmed normal growth medium (NGM; Dulbecco's Modified Eagle Medium containing 4500mg/L glucose and 4mM L-glutamine, Gibco) supplemented with 10% fetal calf serum (FCS, Gibco) and 1% 5000U/ml penicillin and 5000mg/ml streptomycin, (Gibco) and incubated under standard cell culture conditions (37ºC and 5% CO_2). After one week, growth of tendon fibroblasts was observed under light microscopy, the media was replaced with NGM (as described above) and the original tissue specimen carefully removed and discarded.

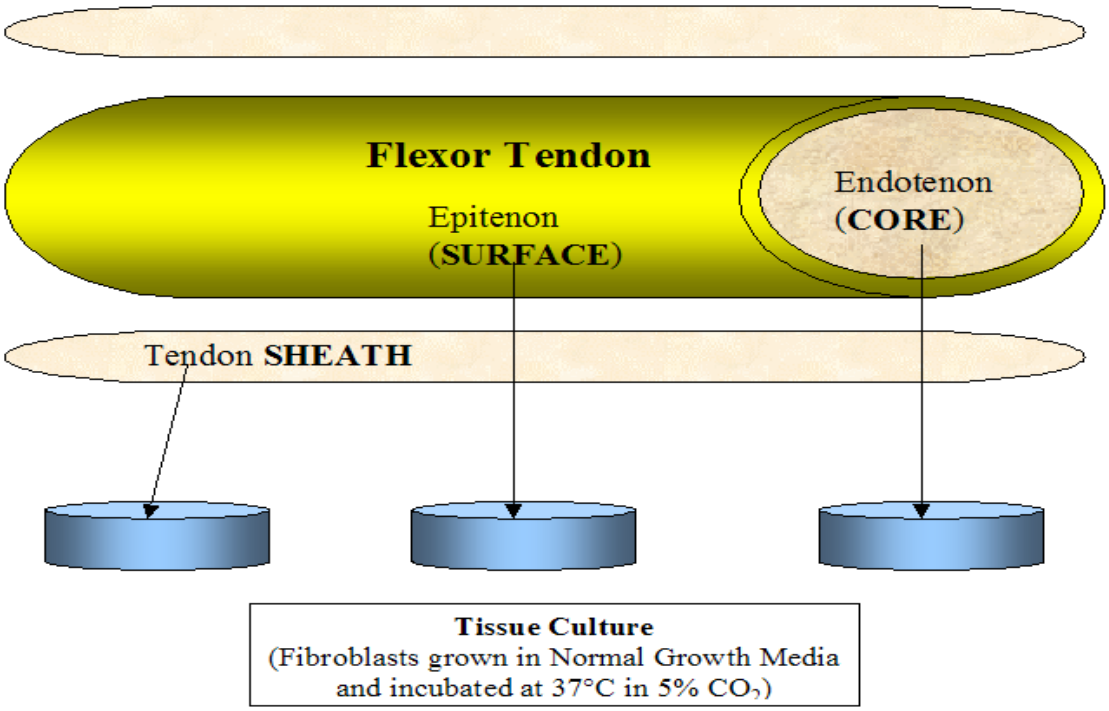


Figure 2.1 Showing the location within the tendon-sheath complex that each cell type was sampled from in preparation for tissue culture.

2.2 Cell Culture Techniques

2.2.1 Cell Passage and Counting

All cell culture techniques were performed using class II laminar flow hoods. When confluence was reached in T25 flasks, cells were then transferred to T75 tissue culture flasks, and eventually maintained in T175 tissue culture flasks. Cells were passaged when 85-90% confluence was reached. At this confluence level media was removed and the cells were washed with Versene (Gibco). This was removed and a pre-warmed Trypsin/Versene solution was added to the cell layer and incubated under standard cell culture conditions for 5 minutes, then knocked gently, until the cells detached from the base of the flask. 10ml of NGM was then added to the cell suspension to inactivate the Trypsin and the solution centrifuged at 1000 rpm for 5 minutes. Only cells of passages 1-6 were used for experimentation.

2.2.2 Cryopreservation

Exponentially growing cells were trypsinised and neutralised as above. After centrifuging, the supernatant was removed and 3mls of cryopresevative solution

(4.5mls FCS and 0.5mls dimethyl sulfoxide (DMSO; Sigma-Aldrich)) added slowly to the cell pellet. Cell suspension aliquots were transferred into labelled cryogenic vials. Insulated cryovials were slowly frozen by placing into a -80°C freezer for 24 hours before being transferred to liquid nitrogen for long-term storage.

To culture cells from frozen the cryovials were rapidly thawed in a water bath at 37ºC. Cells were then plated into T75 tissue culture flasks and cultured as previously described.

2.3 In Vitro Flexor Tendon Cell Assays

All experiments were performed on passage one to six cells and n=3 for each individual cell strain: endotenon (tendon core), epitenon (tendon surface) and tendon sheath-derived cells unless otherwise specified.

2.3.1 Toxicology Assay

Tendon cells were seeded at 3 x 10^3 cells/well, of a 96-microtitre plate (Greiner, Stonehouse, Glos) and left to attach and spread overnight in minimal media (Dulbecco's Modified Eagle Medium (DMEM) with 0.4% FCS). One plate was removed at time zero (no treatment added) and other plates had their media aspirated and replaced with minimal media and serial dilutions of the treatment being studied over the time course of 3, 7 and 10 days. The media and treatment were changed twice a week.

Cell number was assessed using the WST-1 assay (Roche, Lewes). Due to the viscosity or colour of treatment a prewash step had to be performed. Treatment and media was carefully aspirated from the wells and each well was washed twice with 200µl of minimal media. Minimal media was then replaced and 20µl WST-1 was added to each well (10µl WST per 100µl of minimal media) and left for 1 hour (previously optimised) in standard tissue culture conditions. The plate was shaken for one minute before reading of absorbance on a Bio-Rad plate reader at a frequency of 440nm. WST-1 is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. The intensity of the dye is proportional to viable cells.

2.3.2 Cell Adhesion Assay

Matrix Coating

Matrix proteins: collagen type I, collagen type III and fibronectin were diluted in ice cold Dulbecco's PBS (without calcium and magnesium, Gibco) to achieve 10µg/ml, 0.5mg/ml and 3.57µg/ml respectively. 50µl of each solution was pipetted into separate Nunc maxasorb 96 well culture plates. Triplicate wells were set up for each treatment. Plates were incubated for 1hour at 37ºC. Excess matrix was aspirated and replaced with a blocking solution, PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich, tissue grade), for 1hour at room temperature and washed with PBS.

Cell Preparation

Cells were removed from tissue culture flasks using Acutase (Innovative Cell Technologies) and the cells were re-suspended into NGM at $2x10^4$ cells/well in 100µl. Cells were incubated with treatment at 37ºC for 30 minutes in suspension.

Assay Set Up

The PBS in each well was aspirated, cells were re-suspended and 100µl aliquots of treatment/cell suspension were plated into the pre-coated wells. Plates were incubated at 37ºC in humidified 5% CO_2 for 2 hours. Cells were observed, using inverted microscopy, for signs of attachment prior to the next stage. The treatment was carefully aspirated from the wells and the wells were gently washed with 100µl of PBS. Adhered cells were fixed and stained with 0.5% crystal violet (Sigma-Aldrich) (0.5% crystal violet, 5% formal saline, 50% ethanol, 0.85% NaCl). Excess crystal violet was removed and the wells carefully washed with 200µl of PBS and drained by inverting onto tissue. Bound crystal violet was solubilised in 100µl of 33% acetic acid and left for 10mins. Optical density was measured at 595nm with a Bio-Rad plate reader.

Data Analysis

Cell adhesion was compared between treated and untreated cells on the different extracellular matrices in each cell type. Statistical analysis was performed using the Student t-test.

2.3.3 Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

Cell Culture

Experimental groups were placed in serum free media (DMEM, 2mM glutamine, 100U/ml penicillin and 100mg/ml streptomycin; SFM) for 24 hours before addition of treatment. Treatments were as follows: SFM only, recombinant TGF-β1 (R&D Systems), 2ng/mL; Resveratrol 50µM, EGCG 50µM and Pumactant 1mg/mL (following optimisation).

A comprehensive time course (0, 1, 2, 4, 6, 8, 16, 24 and 48 hours) was used to study early changes in gene activation. The time zero (untreated) was used to compare with treatments at various time points.

RNA Isolation

Quiescent rabbit tendon cells (endotenon, epitenon and sheath) were either treated or untreated for 0-48 hours in SFM. Total RNA was extracted using TRIzol® (Gibco, Paisley Invitrogen).

Trizol Method for RNA Extraction

Trizol (500µl) was added to each cell monolayer at the appropriate time points. The flasks were then incubated at room temperature for 5 minutes and cells were removed from the base using a rubber policeman. The TRIzol® /cell suspension was placed in (RNA/DNA free) centrifuge tubes and stored at 4ºC until ready for RNA preparation. 100µl of chloroform was added to each tube, shaken vigorously for 15 seconds to mix well, and incubated at room temperature for 3 minutes. The mixture was then centrifuged at 10,000g at 4ºC for 10-30 minutes to separate into phases. The upper phase was removed and placed in a fresh sterile centrifuge tube. 0.5ml of isopropanol (molecular grade; Sigma-Aldrich) was added mixed and left to incubate at room temperature for 10 minutes. The tubes were centrifuged at 11,000g at 4ºC for 30 minutes to pellet RNA. The supernatant was removed and 1ml of 75%-ethanol (Hayman Ltd.) dissolved in diethylpyrocarbonate (DEPC; Sigma-Aldrich) treated H_2O was added and placed in a vortex on low speed for 5-10 seconds to wash the pellet. A further centrifuge at 6,000g at 4ºC for 5 minutes was required to re-pellet the RNA. The supernatant was then removed and the pellet allowed to air-dry at room

temperature for 5-10 minutes. Finally, the dried pellet was dissolved in DEPC-H_2O (30µl) by gentle pipetting and incubated for 5-10 minutes. Samples were then stored at -80°C until ready for use.

Quantification of RNA (Quant-iT™ Ribogreen® RNA Reagent)

Background

The Quant-iT™ Ribogreen® RNA Reagent (Invitrogen) is a fluorescent nucleic acid stain used for quantitating RNA in solution. The excitation maximum for Quant-iT™ Ribogreen® RNA Reagent bound to RNA is ~500nm and the emission range is ~525nm. Sensitivity is higher than ethidium bromide assays by 200-fold and exceeds the ultraviolet (UV) absorbance by 1,000 fold.

Protocol

Two different dye concentrations are required to achieve the full linear dynamic range of the assay. Before preparing the working solutions it was necessary to establish if a high range (20ng/ml to 1µg/ml RNA) or a low range assay (1ng/ml to 50ng/ml) or both was required. For the purpose of this thesis a high range assay was found to be appropriate for all samples.

Assay Buffer Preparation

Sterile (nuclease and DNA free) TE buffer (10mM Tris-HCL, 1mM ethylenediaminetetraacetic acid (EDTA), pH 7.5) was used for diluting the Quant-iT™ Ribogreen® RNA Reagent and for diluting RNA samples. TE working solution was prepared by diluting in DEPC- H_2O. Quant-iT™ Ribogreen® RNA Reagent was then diluted in TE.

For a high range assay: There was a 200-fold dilution e.g. the working solution was prepared for 20 samples in 200µl volumes, 10µl Quant-iT™ Ribogreen® RNA Reagent was added to 1.99mls TE. The working solution was protected from light by covering in foil, as it is susceptible to photo degradation.

RNA Standard Curves

A 2µg/ml solution of RNA was prepared in TE. Ribosomal RNA (100µg/ml) was diluted 50-fold in TE to make a 2µg/ml working solution (Table 2.1).

Volume (µl) of TE	Volume (µl) of 2µg/ml RNA stock	Volume of 200 fold diluted Quant-iT™ Ribogreen® RNA Reagent	Final RNA concentration in Quant-iT™ Ribogreen® Assay
0	100	100	1µg/ml
50	50	100	500ng/ml
90	10	100	100ng/ml
98	2	100	20ng/ml
100	0	100	0

Table 2.1 Showing protocol for preparing a high-range standard curve: After dilution of the 2µg/ml RNA as shown in the table above, 200µl of the working solution was added to a 96 well polymerase chain reaction (PCR) plate (Stratagene). This was mixed well and incubated for 2-5 minutes at room temperature in the dark. The fluorescence was measured using the fluorescein isothiocyanate (FITC) wavelength and end point plate recorder on the MX3000P. The fluorescence value of the reagent blank was subtracted from that of each of the samples. Corrected data was then used to generate a standard curve of fluorescence versus RNA concentration.

Sample Analysis

The experimental RNA solution was diluted in TE to a final volume of 100µl. 100µl of aqueous working solution of the Quant-iT™ Ribogreen® reagent was added to each sample. This was incubated for 2-5minutes at room temperature and protected from light. A blank 100µl TE and 100µl Ribogreen was run as a control. The fluorescence of the sample was measured using the Stratagene™ MX3000P. The fluorescence value of the reagent blank (100µl TE and aqueous solution) was subtracted and the RNA concentration of each sample was determined from the standard curve.

To check the RNA for quality and minimal DNA contamination the RNA samples were run on a 1% agarose gel to ensure the integrity of the total RNA samples by visualisation of both the 18S and 28S ribosomal RNA as discrete bands.

First-Strand cDNA Synthesis Protocol

This procedure is optimised to convert 1ng to 5ng of total RNA or up to 250ng of poly (A) + RNA into first strand cDNA. This protocol is optimised for the first step of a two-step RT-PCR procedure.

The first-strand cDNA synthesis reaction was prepared in a DNA/RNAase free microcentrifuge tube by adding the following components in order: 1ng-5µg of total RNA (quantity determined previously), RNAase-free water to total volume 15.7µl and 1.0µl of oligo (dt) primer (0.5 µg/µl; all from Stratagene™) and incubated at 65°C for 5 minutes. The reaction was cooled at room temperature for ~10 minutes to allow the primers to anneal to the RNA. The following components were added, in order, for a final reaction volume of 20µl: 2.0µl of First Strand Buffer, 0.8µl of deoxyribonucleotide triphosphate (dNTP) mix (25mM each dNTP), 0.5µl RNAase Block Ribonuclease Inhibitor (40 U/µl) and 1µl of AffinityScript Multiple Temperature RT (all from Stratagene™). The reaction components are mixed gently, and the tubes placed in a temperature-controlled thermal block at 45°C for 1hr. Finally the reaction is terminated by incubating at 70°C for 15 minutes. The completed first-strand cDNA was frozen at -80°C until ready for use.

Primers were all confirmed on BLAST and were purchased from MWG and diluted with DEPC-water to a dilution of 100pmol/µl. Primers were optimised to ensure minimal primer dimmer formation by analysing the PCR reaction using different concentrations of both forward and reverse primers and performing a dissociation curve. Primer efficiency was required for the mathematical logarithm (REST-MCS©) used for analysis (described later), each primer following optimisation was used for amplification using a range of cDNA (ng). The mathematical logarithm determines the primers efficiency.

Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

Two microlitres of the first-strand cDNA product was used for amplification in triplicate in a 25µL reaction solution containing 12.5µL of SYBR Green PCR Master Mix (Stratagene™) and 10pM of each primer as per the manufacturer's instructions. The primer sequences are shown in Table 2.2. The PCR reaction was performed on an MX3000P (Stratagene™). The PCR program consisted of an initial denaturation where the reaction was incubated for 10 minutes at 95°C; in the second step the DNA was amplified for 40 cycles of 30 seconds at 95°C, with an annealing of primers for 1 minute at 60°C and an extension at 72°C for 30 seconds. A dissociation curve was performed to ensure that no primer-dimers were present at the end of each PCR run.

Gene	Sequence
Collagen type I (α2)	F 5’ TTCTTGGTGCTCCTGGCATTC 3’ R 5’ GCAATCCGTTGTGTCCCTTTATG 3’
Collagen type III (pro α1)	F 5’CAAACCTCTTCCTGAAGCC 3’ R 5’ATTATAGCACCATTGAGAC 3’
Fibronectin	F 5’ TCGGGAGGAAGAAGACAGATGAGC 3’ R 5’ ACCACTGCCAAAGCCTAAGCAC 3’
PAI-1	F 5’ GGATTTGGCCGCATTG 3’ R 5’ CAACATCCACTTTGCCAGAGTTAA 3’
t-PA	F 5’ GAGGCTCACGTCCGGCTGTACCCCTCCA 3’ R 5’TCCTTCTGCCCACAGCCCAGCCCCCAG C 3’
GAPDH	F 5’ GGATTTGGCCGCATTGG 3’ R 5’ CAACATCCACTTTGCCAGAGTTAA 3’
ACTB	F 5’ GCTCGTCGTCGACAACGGCTG 3’ R 5’ CAAACATGATCTGGGTCATCTTCTC 3’

Table 2.2 Showing primers used: PAI-1, plasminogen activator inhibitor-1; t-PA tissue plasminogen activator; ACTB, β-actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase**.**

QRT-PCR Data Analysis

The relative expression software tool (REST-MCS©) was used for the calculation of the relative expression levels in real time PCR. This mathematical model is based on the PCR efficiencies and the mean crossing point deviation between the treated and control cells (Figure 2.2). The target gene expression was normalised by the two housekeeping genes: glyceraldehyde-3-phosphate dehyrdogenase (GAPDH) and β-actin (ACTB). Statistical analysis of group differences was performed by the Pair Wise Fixed Reallocation Randomisation Test©, implemented in the REST-XL software (Pfaffl et al., 2002), and the coefficient of variance was given.

$$R = \frac{(E_{target})^{\Delta CP target\ (MEAN\ control - MEAN\ sample)}}{(E_{ref})^{\Delta CP ref\ (MEAN\ control - MEAN\ sample)}}$$

Figure 2.2 Showing equation used by the relative expression software tool (REST-MCS©).

2.4 In Vivo Model of Flexor Tendon Adhesion Formation

All procedures were carried out following the regulations of the Home Office Code of Practice for the Housing and Care of Animals Used in Scientific Procedures (1989) and the Animals (Scientific Procedures) ACT 1986 (ASPA).

New Zealand White (NZW) rabbits were anaesthetised for surgery using general anaesthesia with recovery. A partial tenotomy was conducted on digits 2 and 4 of the right forepaw of 8 NZW rabbits in each treatment group, with injured untreated tendons acting as adhesion controls (n=8). All injured tendons were immobilised via a proximal tendon transection (described below). Only one paw per rabbit was subjected to an operation so as to minimize suffering to the animal after recovery from anaesthesia. The left paw acted as the unoperated untreated control.

General anaesthesia, using induction with Hypnorm® (VetaPharma Ltd.; Fentanyl citrate and fluanisone) 0.5ml/kg and diazepam (Diazemuls®, Alpharma) 0.1ml/kg via intramuscular injection, was followed by maintenance with inhaled Isoflurane (Baxter) 2-3% mixed with O_2 2L/minute, was performed in rabbits.

Antibiotic, enrofloxacin (Baytril®, Bayer HealthCare) was administered on induction via subcutaneous injection 0.3ml/kg. Analgesia, buprenorphine (Vetergesic®, Reckitt Benckiser Healthcare (UK) Ltd.) 0.01-0.05mg/kg, was administered peri-operatively and if necessary continued for up to 24 hours post operatively.

2.4.1 Operative Procedure

The right paw was shaved and cleaned with betadine (Figure 2.3). General anaesthesia was administered as described above. A ‘V’-Shaped incision was made in the palmar aspect of the right forepaw (Figure 2.4), proximal to the metacarpophalangeal (MCP) joints to access both flexor digitorum profundus (FDP) tendons (Figure 2.5) and to avoid direct injury to the synovial sheath. The flexor digitorum superficialis (FDS) tendons were excised limiting adhesions to those formed between FDP and the sheath. The FDP was then retracted to expose the intrasynovial tendon. With a scalpel a partial tenotomy was performed (Figure 2.6) i.e. a section of tendon was removed whilst maintaining the continuity of the tendon (half the width of the tendon and 5mm in length). Synovial sheath injury was avoided

(to reduce added injury variables). In order to immobilise each specific tendon, a proximal transection (a transverse cut across the whole width of the tendon) was performed at the level of the carpus (Figure 2.7).

The tendon sheath was infiltrated with the treatment (0.5mls of Sterile H_2O or 1mM EGCG or 10mM Resveratrol or 10 mg/ml Pumactant per sheath) using a 24-gauge cannula with the needle removed (Figure 2.8). The partial tenotomy controls received no treatment. Both digits 2 and 4 of each paw were given the same treatment to allow for leakage between tendon compartments. Following the administration of treatment the tendon was allowed to return to the intrasynovial position by extending the digits and the wound closed. Two weeks following partial tenotomy and the application of treatment the rabbits were humanely killed by anaesthetic overdose (Schedule 1). Tendons and associated tissues were harvested at post mortem (Figure 2.9).

Harvested digits were then randomised to either mechanical assessment of adhesion strength or histological analysis.

Figure 2.3 Showing a New Zealand White rabbit right forepaw prior to dissection of volar tissues.

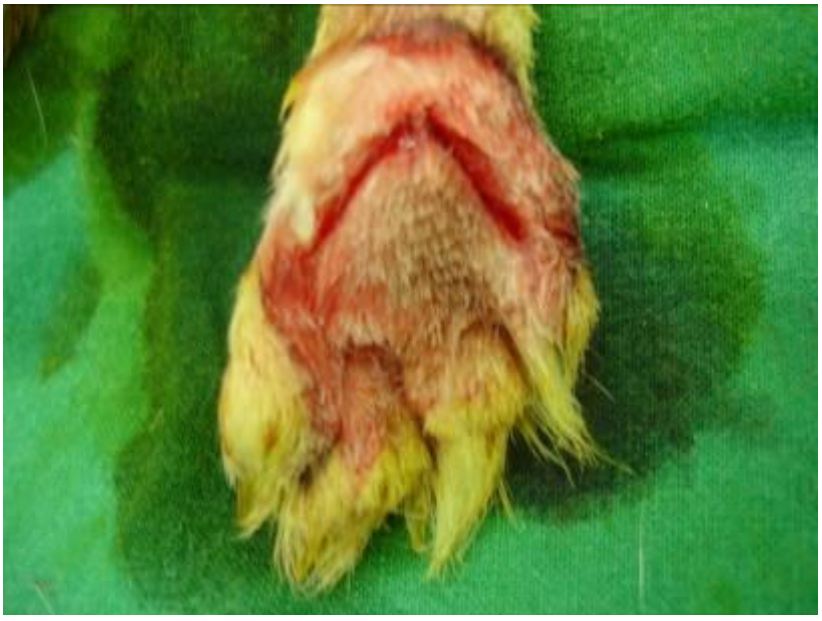


Figure 2.4 Showing the ‘V’-shaped skin incision to expose flexor tendons of a New Zealand White rabbit right forepaw.

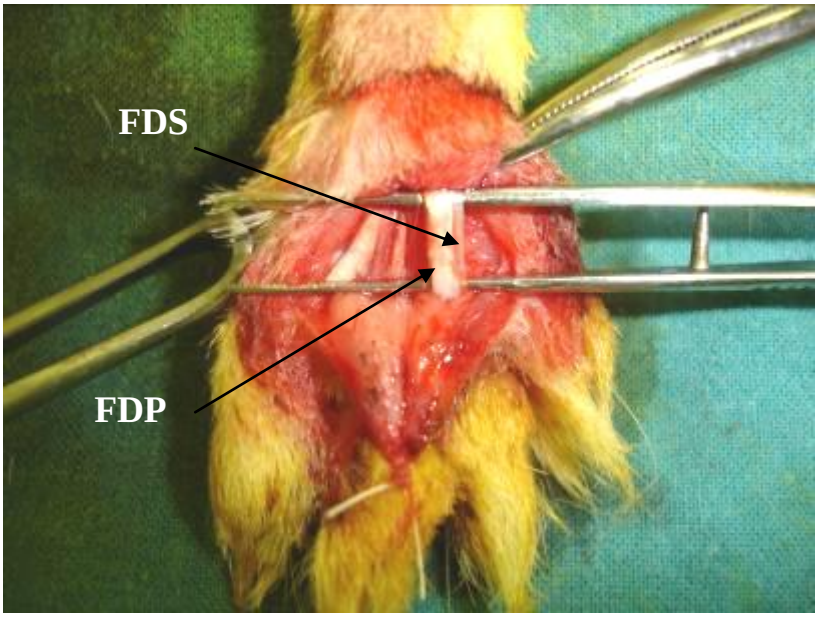


Figure 2.5 Showing the skin flap retracted proximally to expose the FDS and FDP tendons.

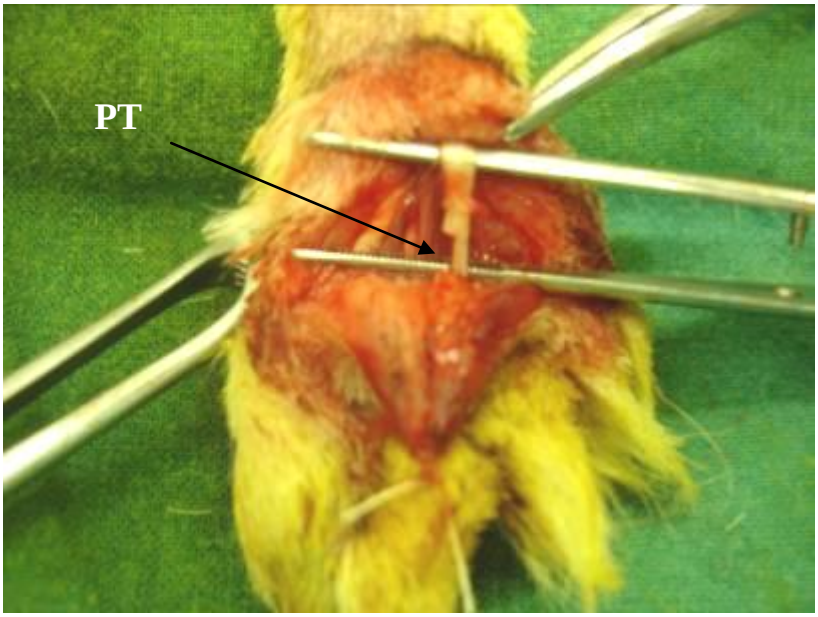


Figure 2.6 Showing a partial tenotomy (PT) created in the FDP of the 4th digit.

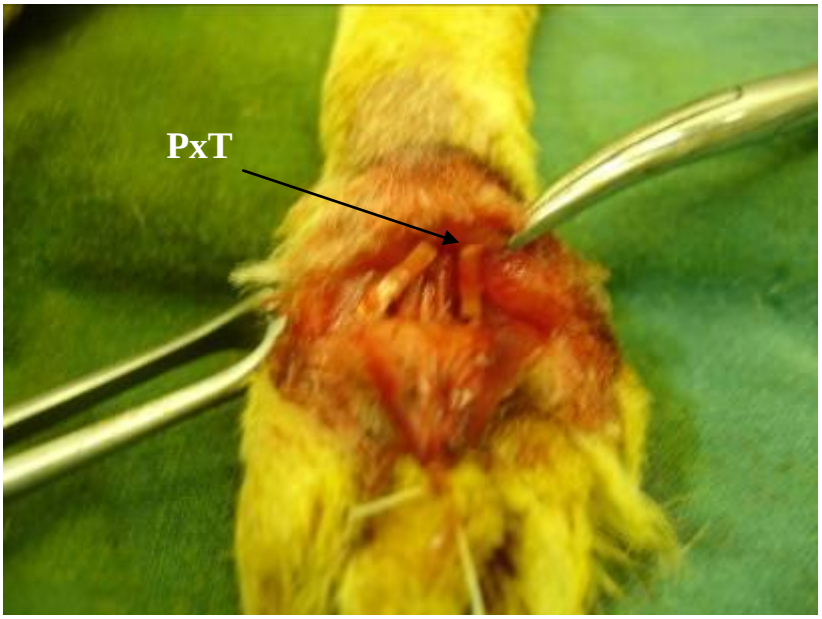


Figure 2.7 Showing the tendon immobilisation technique via proximal transection (PxT) of the FDP's to the 2nd and 4th digits.

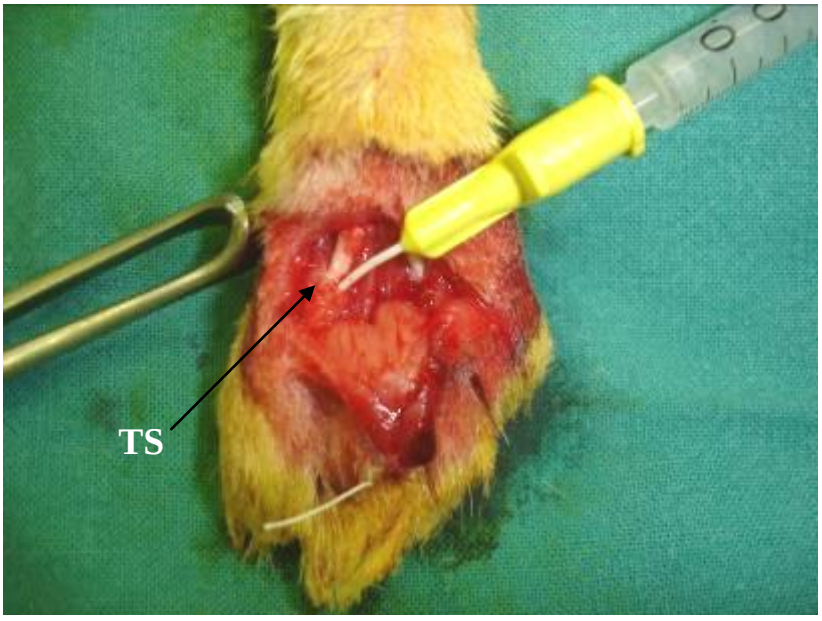


Figure 2.8 Showing infiltration of treatment into the flexor tendon sheath (TS) via a paediatric 24-gauge cannula.

2.4.2 Mechanical Assessment of Adhesion Strength

Forepaws were freshly assessed. Prior to testing the V-shaped wounds were re-opened and the proximal free ends of each treated tendon were identified. A distal transection was made over the insertion of the FDP into the distal phalanx. This allows the tendon to lie freely within the sheath, disconnected at either end such that a force applied proximally would measure solely the strength of any adhesion between the tendon and the sheath. The digital claw was held using a clamp and the proximal FDP tendon transfixed to a tensile testing machine (Mecmesin) (Figure 2.10). The proximal tendon was then pulled from its sheath at a rate of 5mm/min until the adhesion failed (Figure 2.11A & 2.11B). The peak force was measured in newtons via a connecting laptop computer using Mecmesin Emperor Software (Figure 2.12).

2.4.3 Histological Analysis

This is necessary to assess microscopic evidence of tendon adhesion formation. The tendon-adhesion complex of each digit (Figure 2.13) was fixed and paraffin embedded. Haematoxylin and Eosin (H&E) staining was carried out (Appendix I). Presence of a characteristic dense cellular band, connecting tendon to the surrounding tissues, was recorded as an adhesion. Semi-quantitative analysis was carried out using the method described by Tang (Tang et al, 1996) (Table 2.3). Tendon and adhesion specimens were also stained with Masson's trichrome stain to identify collagen fibril formation (Appendix I).

The average cell count for each adhesion was assessed by examination under a light microscope (x40 magnification) following H&E staining. Images were recorded (using a digital camera and saved as tagged image file format (TIFF) files) and with the use of a 100 x 100μm grid {Akoli et al, 1999} and image analysis software (Adobe Systems, Inc., San Jose, Calif.), the average cell count was calculated. This was performed by counting the number of cells in 5 random squares and the mean used to determine cell number.

For both scoring methods described above three independent blinded observers were used and mean scores recorded.

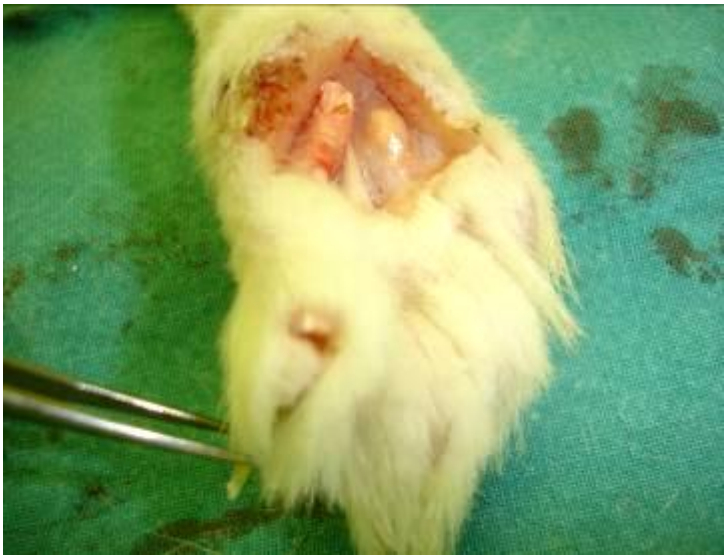
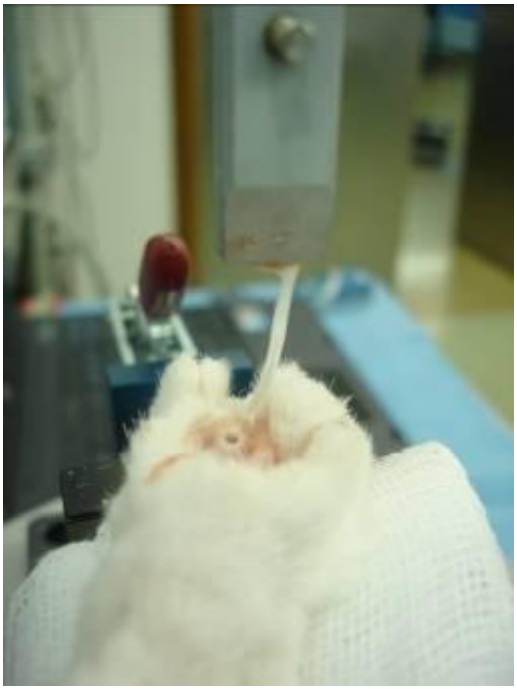


Figure 2.9 Showing preparation and harvest of the flexor tendons (FDP's to 2nd and 4th digits) two weeks following operation. A distal transection of the tendon was made at the level of the DIP joint to allow the tendon to lie freely within the tendon sheath. Hence, the only structures restricting movement of the tendon were adhesions or vinculae.

Figure 2.10 Showing the mechanical tensiometer device (Mecmesin) connected to a laptop. Force readings were measured and recorded using Emperor Software (supplied by Mecmesin).

A



B

Figure 2.11A Showing the flexor tendon (FDP) being pulled out from its respective flexor tendon sheath.

Figure 2.11B Showing the tendon completely pulled out following rupture of an adhesion and the hollow sheath is now visible.

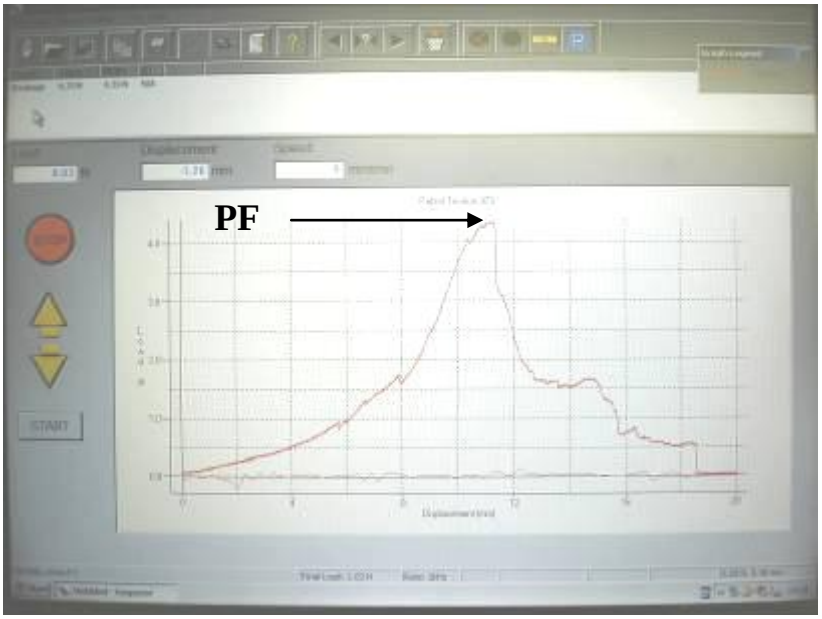


Figure 2.12 Showing a typical reading from an adhesion pullout (Mecmesin Emperor Software). The displacement (mm) lies along the x-axis and the force (N) lies on the y-axis. The peak force (PF) required to break the adhesion is recorded.

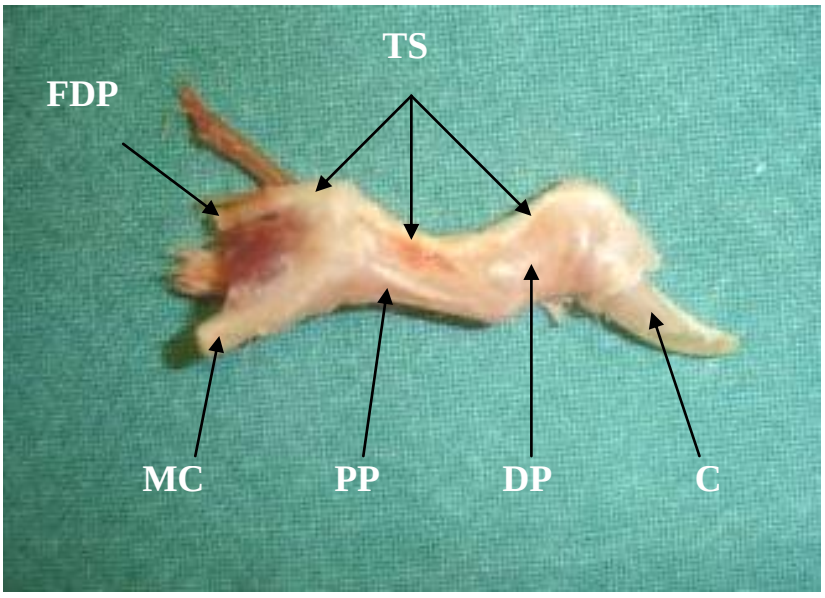


Figure 2.13 Showing a histology specimen before being place in formalin for fixation and further processing. The specimen is a rabbit forepaw digit including the metacarpal (MC), proximal phalanx (PP), distal phalanx (DP), claw (C), FDP and respective flexor sheath (TS).

Points	Features of Adhesions
	Quantity
0	No apparent adhesions
1	A number of scattered filaments
2	A large number of filaments
3	Countless filaments
	Quality
0	No apparent adhesions
1	Regular, elongated, fine, filamentous
2	Irregular, mixed, shortened, filamentous
3	Dense, not filamentous
	Grading of adhesions
0	No adhesions
2	Slight adhesions
3,4	Moderate adhesions
5,6	Severe adhesions

Table 2.3 Showing the grading criteria of adhesions in histological evaluation (Tang, et al., 1996).

2.4.4 Data Analysis

For all *in vitro* and *in vivo* data statistical analysis was performed using the Student t-test or unpaired Rank Sum Mann-Whitney Test where appropriate.

For the QRT-PCR the relative expression software tool (REST-MCS©) was used as described previously (Pfaffl et al., 2002).

CHAPTER 3

THE *IN VITRO* FLEXOR TENDON CELL RESPONSE TO TGF-β1

3.1 Introduction

Flexor tendon injuries of the hand are common and despite accurate surgical repair and rehabilitation, tendon surgery remains a clinical challenge. The main complications involve rupture at the repair site or adhesion formation. Studies attempting to modify adhesion formation using either chemicals or physical barriers have as yet proved unsuccessful (as discussed in Chapter 1). To develop a more targeted treatment, a better understanding of the molecular basis of tendon repair and adhesion formation is required.

Transforming growth factor-β1 (TGF-β1) is part of a family of multipotent growth factors that are consistently associated with fibrosis and scarring in a number of tissues including tendon (Coker and Laurent, 1998; Okuda et al., 1990; Bissell et al., 2001; Chang et al., 1997; Klein et al., 2002). TGF-β1 is also known to affect a number of cellular processes: proliferation, differentiation, extracellular matrix (ECM) deposition, immunosuppression and apoptosis in a number of cell types. There are three mammalian isoforms of TGF-β1, β2 and β3 sharing 60-80% homology. TGFβ-1 is known to play an important role in both wound healing and scarring (reviewed in Klass et al., 2009). One group has studied the impact of neutralizing TGF-β1 via antibodies in a murine surgical model of abdominal adhesions which showed little effect (Gorvy et al., 2005) however, recent studies have shown the importance of TGF-β1 in flexor tendon healing and adhesion formation (Chang et al., 1997; Chang et al., 2000; Klein et al., 2002).

Collagens are known to be essential ECM components of tendons, and their formation and configuration are important factors determining tendon structure, function and repair. Although there are numerous collagen types, collagen type I and type III appear to play the most significant role in tendon healing. In general, collagen type III levels increase in the early inflammatory and proliferative stages of tendon healing whereas collagen type I production is increased in the later remodelling stages of tendon healing replacing collagen type III.

TGF-β1 is known to be able to regulate extracellular matrix turnover and has been shown to increase the deposition of fibronectin in fibroblasts (Balza et al., 1988). Fibronectin has been shown to be deposited during the granulation phase of tendon

healing. While others have proposed that this preliminary fibronectin matrix allows tendon cells to move into the wound site, remodel the ECM, and form an adhesion-bridge between the tendon and tendon sheath (Williams et al., 1984).

Plasminogen activator-1 (PAI-1) is a known early TGF-β response gene (Kurtz et al., 2001) and may play a role in the formation of peritoneal adhesions (Cheong et al., 2001). Other studies (Di Filippo et al., 2006; Ivarsson et al., 1998) have shown that post-surgical peritoneal adhesions show an alteration of local fibrinolytic components, with the formation of adhesions being the results of a balance between fibrinogenesis (excess PAI-1) and fibronolysis (increased levels of tissue plasminogen activator; t-PA) (Figure 1.10).

The role of TGF-β1 in gene expression in a number of genes known to be involved in both tendon repair and adhesion formation was studied. A comprehensive time course studying the stimulatory effect of TGF-β1 on quiescent cells derived from different regions of the tendon-sheath complex (sheath, endotenon, epitenon) using real time PCR was employed. The time course was selected as others have shown that *in vitro* TGF-β1 stimulated gene expression can occur in a short time span (Rolfe et al., 2007) which has also been seen in tendon derived cells in vitro (Fu et al., 2005).

3.1.1 Hypothesis

The aim of this chapter was to test the hypothesis:

TGF-β1, a multipotent growth factor involved in tendon healing and adhesion formation, has different effects on proliferation and gene expression on each cell type within the tendon-sheath complex.

3.1.2 Objectives

- Establishment of tendon cell lines of different origin (endotenon, epitenon, and tendon sheath) using tissue culture techniques.
- Assessment of the effect of TGF-β1 on the proliferation of these different cell types.
- Assessment of the transcription of specific genes known to be involved in either tendon healing or adhesion formation, using the QRT-PCR method, following TGF-β1 treatment on each cell type.
- This study will be used as a baseline *in vitro* investigation to compare our proposed anti-adhesion treatments (Chapter 4).

3.2 Materials and Methods

3.2.1 Cell Culture

All animal care complied with the "UK Home Office Guide for the Care and the Use of laboratory animals" 1996. Uninjured New Zealand White rabbit digital tendon complexes were isolated and dissected into synovial sheath, epitenon, and endotenon. Fibroblasts from these different areas were established using the method described in Chapter 2. All experiments were performed on passage one to six cells and n=3 for each cell type. Experimental groups were placed in serum free media (DMEM, 2mM glutamine, 100U/ml penicillin and 100mg/ml streptomycin; SFM) for 24 hours before stimulation with recombinant TGF-β1 (R&D Systems, Abingdon, Oxon), and viability was verified using trypan blue (Sigma Dorset, UK, data not shown). The concentration of TGF-β1 used throughout the experiments was 2ng/mL following optimisation.

3.2.2 Proliferation (Toxicology) Assay

Cells were trypsinised, normal growth media (NGM) added, then centrifuged, counted and plated (as described in Chapter 2, section 2.3.1). One plate was removed at time zero (no treatment added) and other plates had their media aspirated and replaced with minimal media with various concentrations of treatment (TGF- β1) to be studied over a time course of 24, 48 and 72 hours. TGF-β1 treatment was diluted as per manufactures instructions at a stock concentration of 2ng/µl. Concentrations of 4ng/ml, 2ng/ml, 1ng/ml, 0.5ng/ml and 0.25ng/ml were diluted in minimal media (DMEM with 0.4% FCS). WST-1 (Roche) reagent was added to each well and absorbance read on a plate reader (as described in Chapter 2, section 2.3.1).

3.2.3 RNA Isolation and Real-Time Polymerase Chain Reaction (QRT-PCR)

A comprehensive time course was used to study early changes in gene expression that were more likely to be a direct response to TGF-β1. The time zero (untreated) acted as the control for all samples.

Quiescent rabbit tendon cells (endotenon, epitenon and sheath) were treated with or without TGF-β1 (2ng/ml) for 0-48 hours in SFM. RNA extraction and real-time polymerase chain reaction was performed (as described in Chapter 2, section 2.3.4)

3.2.4 Data Analysis

For the proliferation assay data was compared using the Student T-test where appropriate. SigmaStat 3.5 (Systat Software, Inc.) software was used to confirm statistically significant findings.

The relative expression software tool (REST©) (Pfaffl et al., 2002) was used for the calculation of the relative expression levels in real time PCR. This mathematical model is described in full detail in Chapter 2, section 2.3.4. Sigma Plot was used to illustrate the logarithmic graphs.

3.3 Results

3.3.1 Proliferation Assay

Endotenon cells also showed no significant increase in proliferation at 24, 48 or 72 hours with TGFB-1 for any concentration when compared to cells grown in minimal media (Figure 3.1).

Epitenon cells in TGF- β1 showed no significant change compared to cells in minimal media at 24 or 48 hours (Figure 3.2). However, at 72 hours epitenon cells in 2ng/ml of TGF-β1 showed a statistically significant increase ($p= 0.003$) compared to cells grown in minimal media.

Tendon sheath cells showed no statistical increase in response to any concentration of TGF-β1 at 24 or 48 hours (Figure 3.3). However, at 72 hours there was a statistically significant increase ($p=0.001$) identified at 0.5ng/ml of TGF-β1 compared to cells grown solely in serum free media.

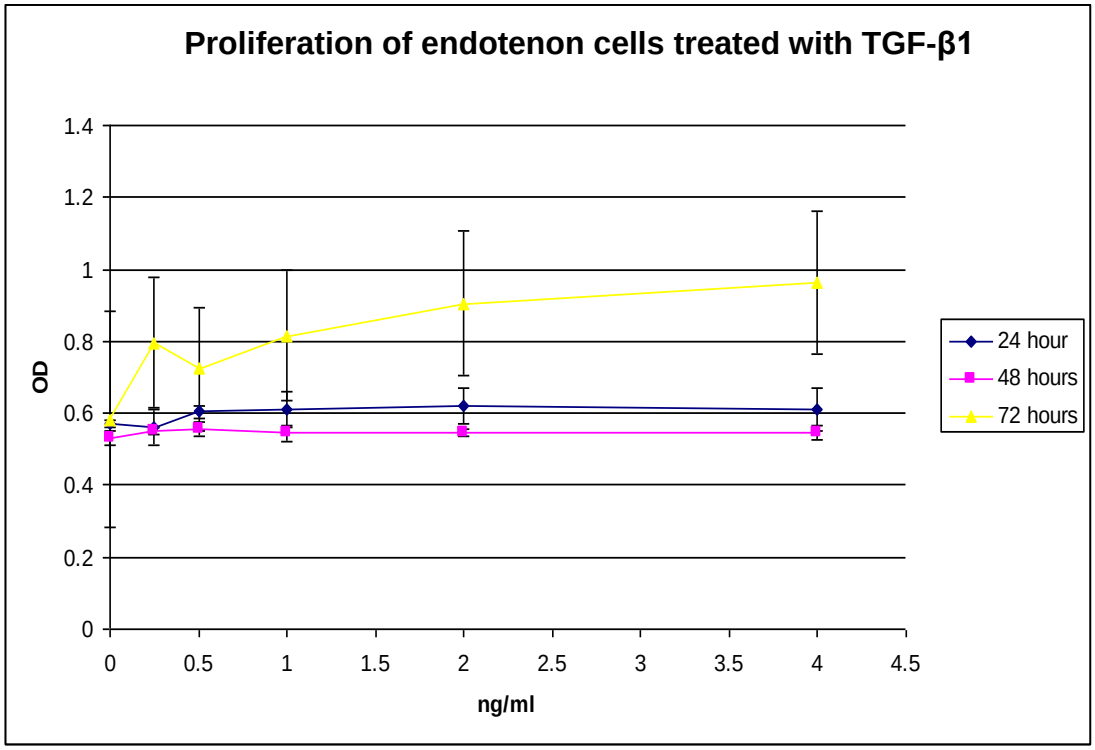


Figure 3.1 Graph represents the average (n=3) optical density (OD as measured using absorbance on a Bio-Rad plate reader) and SD for endotenon cells at 24, 48, and 72 hours after treatment with various concentrations of TGF-β1.

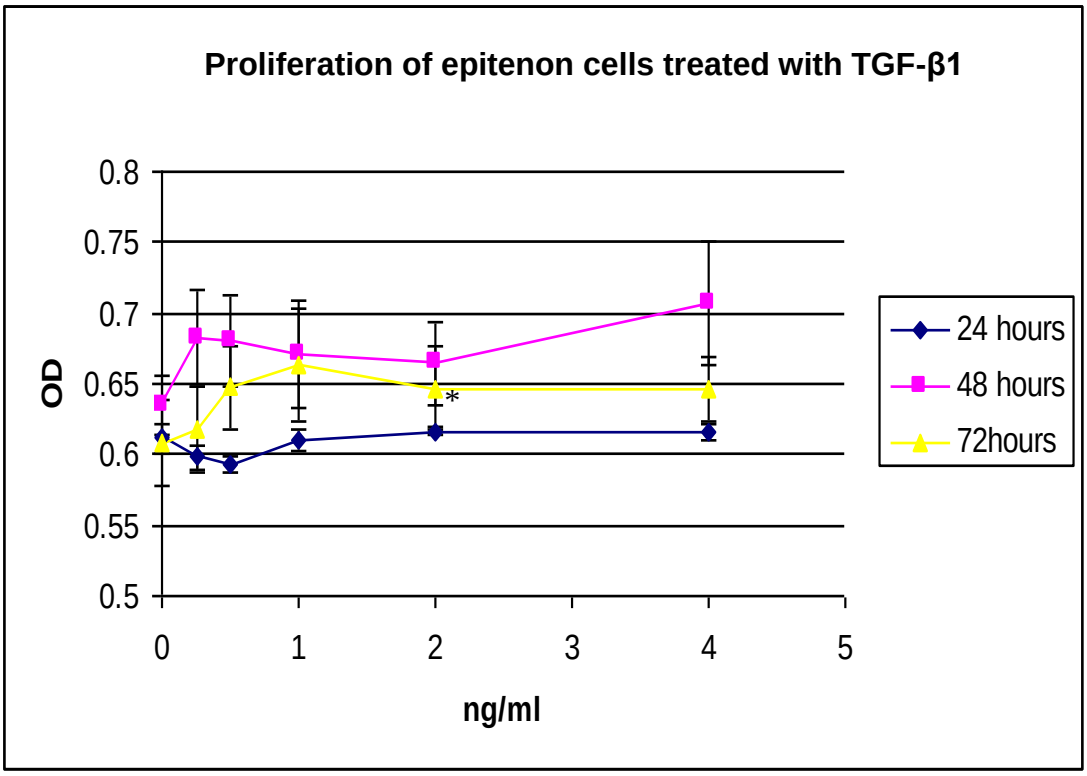


Figure 3.2 Graph represents the average (n=3) optical density (OD as measured using absorbance on a Bio-Rad plate reader) and SD for epitenon cells at 24, 48, and 72 hours after treatment with various concentrations of TGF-β1. Statistically significant difference: *p= 0.003.

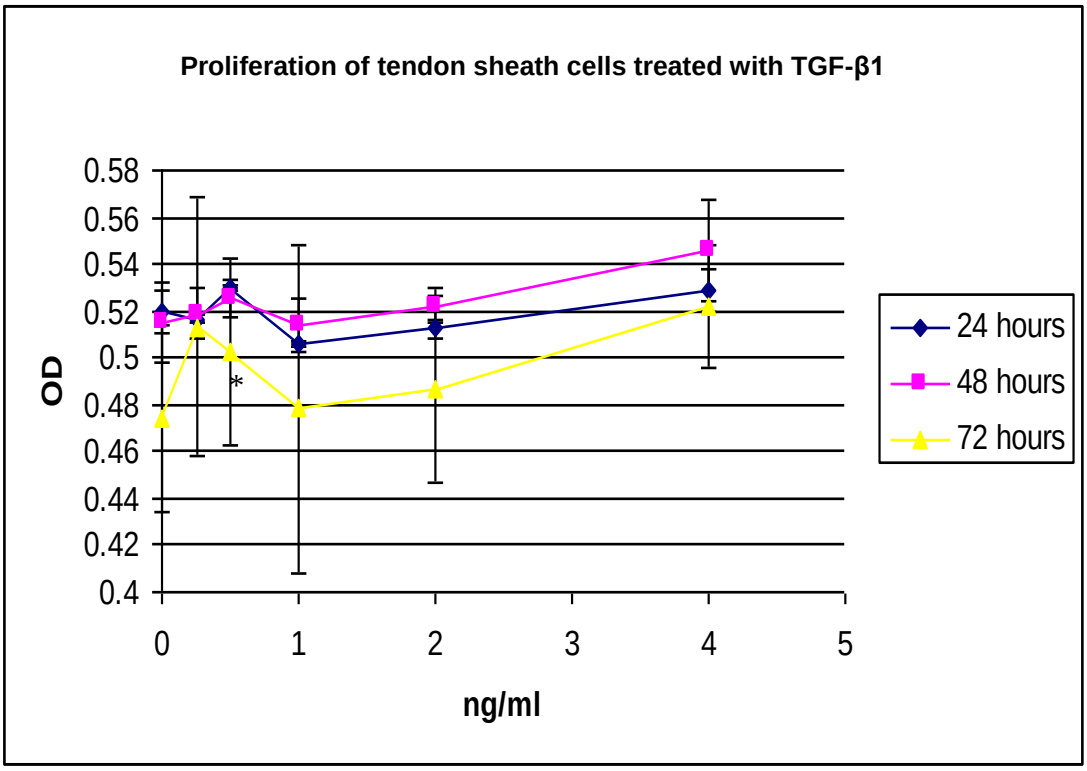


Figure 3.3 Graph represents the average (n=3) optical density (OD as measured using absorbance on a Bio-Rad plate reader) and SD for tendon sheath cells at 24, 48, and 72 hours after treatment with various concentrations of TGF-β. Statistically significant difference: *p= 0.001.

3.3.2 Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

The expression of five different genes: collagen type I, collagen type III, fibronectin, plasminogen activator inhibitor I (PAI-1), and tissue plasminogen activator (t-PA), were examined.

All genes examined were expressed at detectable levels in the three cell types in the absence of stimulation with TGF-β1.

Time Zero

Time zero (or equivalent of cell types before treatment with TGF-β1) shows a statistically significant difference between epitenon and sheath cells with collagen type I gene expression ($p=0.007$). There was also a statistically significant difference between endotenon and epitenon cells with collagen type I ($p=0.032$) and collagen type III ($p=0.009$) (Figure 3.4).

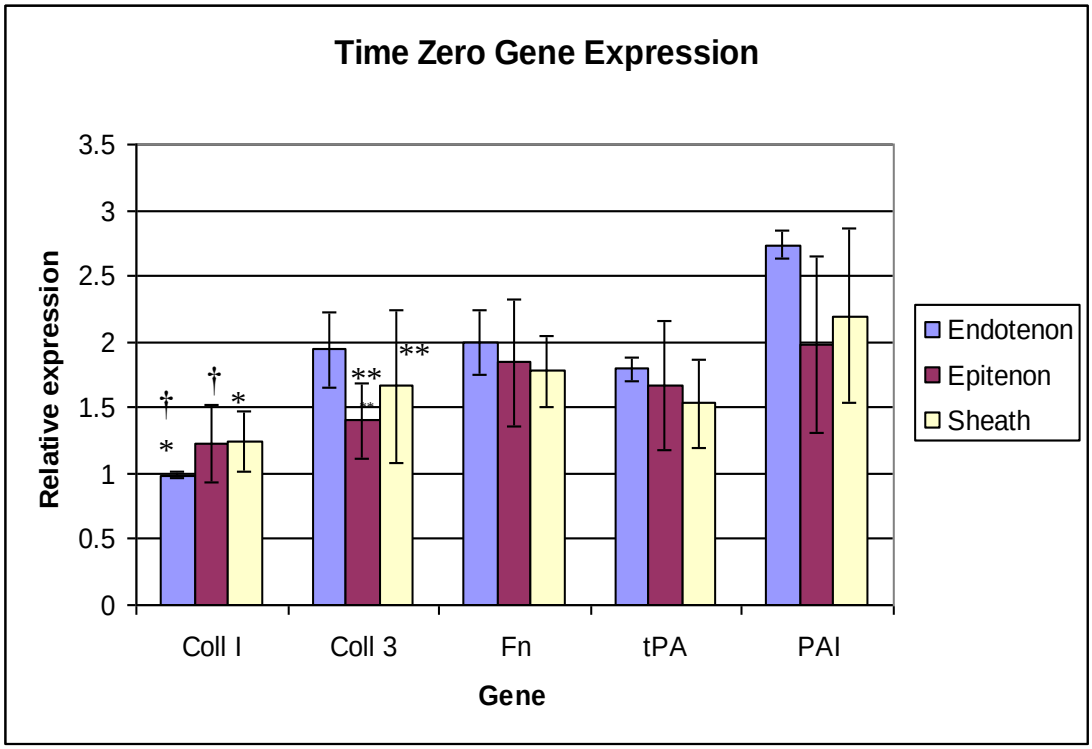


Figure 3.4 Showing time zero gene expression for TGF-β1 treated endotenon, epitenon and sheath derived cells (all were corrected for the housekeeping gene GAPDH). Statistically significant differences are shown as follows: *p= 0.007; †p= 0.032; **p=0.009. Error bars=SD, n=3.

Endotenon Cells

Endotenon cells showed no statistical change in collagen type I gene transcription over the 48-hour time course (Figure 3.5). Whereas collagen type III gene transcription showed a statistically significant down regulation in the early stages of the time course 2, 6 and 8 hours ($p=0.03$), however, at the later time points collagen type III showed an up regulation which did not reach statistical significance (Figure 3.5).

Fibronectin gene transcription (Figure 3.5) showed at 6 hours a statistically significant down regulation ($p=0.024$), which was later, reversed with a statistically significant up regulation at 24 and 48 hours ($p<0.05$). PAI-1 (Figure 3.6) showed an up regulation of gene transcription at 2 and 4 hours but this was only statistically significant at 2 hours ($p=0.001$). However, t-PA (Figure 3.6) showed a statistically significant down-regulation in gene transcription at 1, 2, 6, 8 and 24 hours ($p=0.01$) and 16 hours ($p=0.002$).

Epitenon Cells

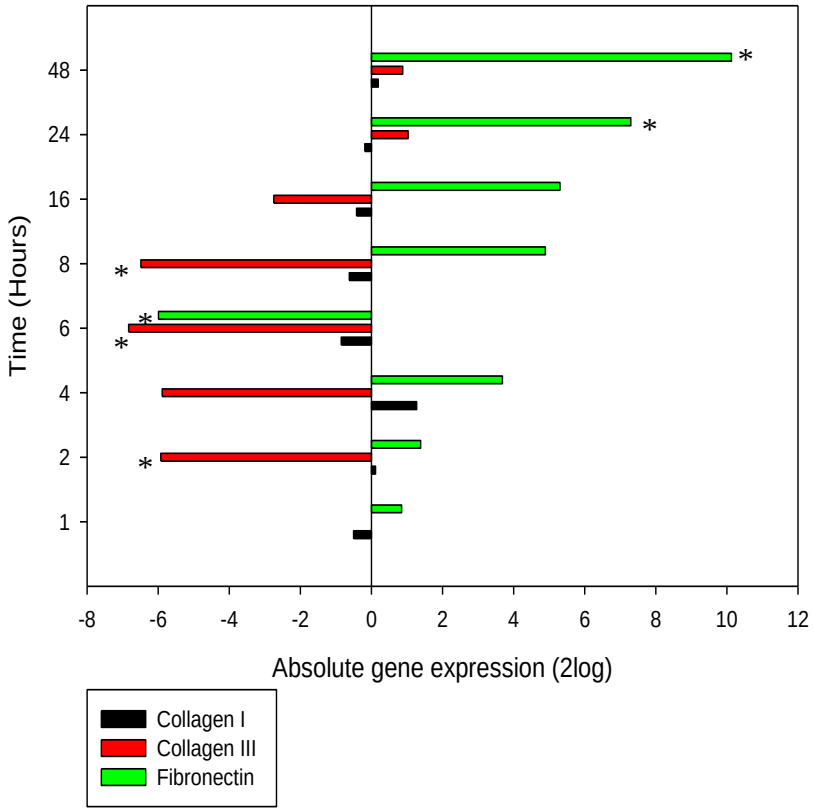
Epitenon cells stimulated with TGF-β1 showed a statistically significant down-regulation of collagen type III at various points throughout the specified time course: 1, 2, 6, 16 and 48 hours ($p<0.05$) (Figure 3.7). In contrast, there was a statistically significant up-regulation of collagen type I at later time points: 24 ($p=0.025$) and 48 hours ($p=0.04$) when compared to untreated controls, despite the relatively high sample variation. Fibronectin showed a statistically significantly up-regulation at 24 hours ($p=0.03$). Also, there was a statistically significant up-regulation of PAI-1 at 4 hours ($p=0.026$) however there was not a significant change in the expression of t-PA at any of the time points examined (Figure 3.8).

Tendon Sheath Cells

Tendon sheath cells treated with TGF-β1 demonstrate a statistically significant up-regulation of collagen type III gene expression at various time points: 6, 8, 16, and 24 hours ($p<0.05$) when compared to untreated controls (Figure 3.9). This is in contrast to the endotenon and epitenon cell types that both showed a significant down-regulation of collagen type III as mentioned above. Unexpectedly, collagen type I and fibronectin gene expression levels were relatively unchanged at all time points.

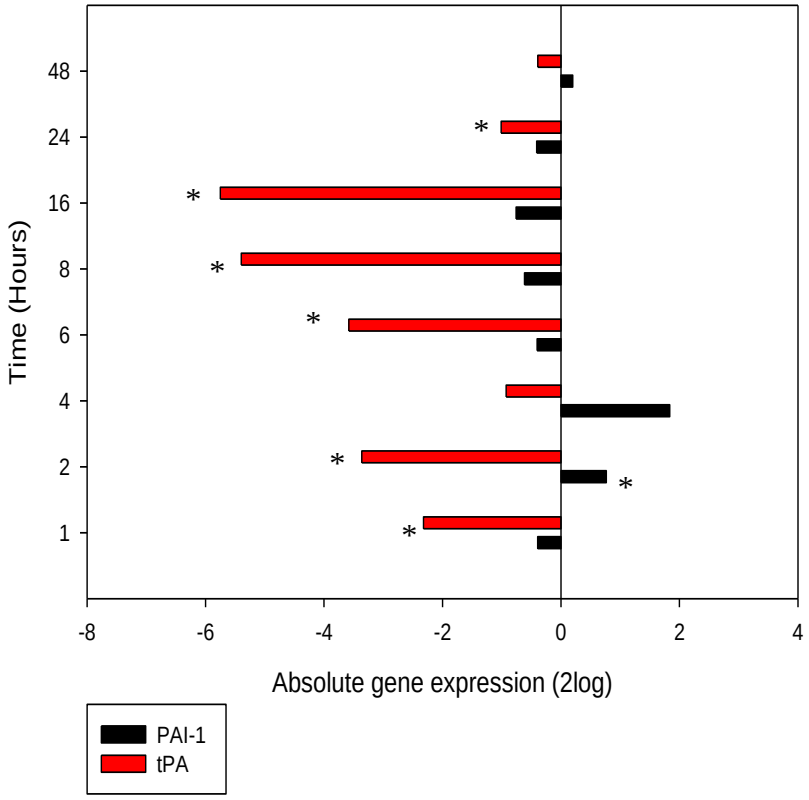
However, collagen type I expression in sheath cells showed high sample variation, especially at early time points and this may account for the lack of significance. There was a significant down-regulation of t-PA at early and late time points: 2 hours ($p=0.013$), 4 hours ($p=0.001$) and 24 hours ($p=0.001$), respectively, when compared to untreated controls (Figure 3.10). This finding was similar to the down-regulation of t-PA seen in the endotenon cells (Figure 3.6). In comparison, there was an apparent increase in PAI-1 levels in sheath cells but these changes were not statistically significant.

A summary of the statistically significant findings is shown in Figure 3.11.



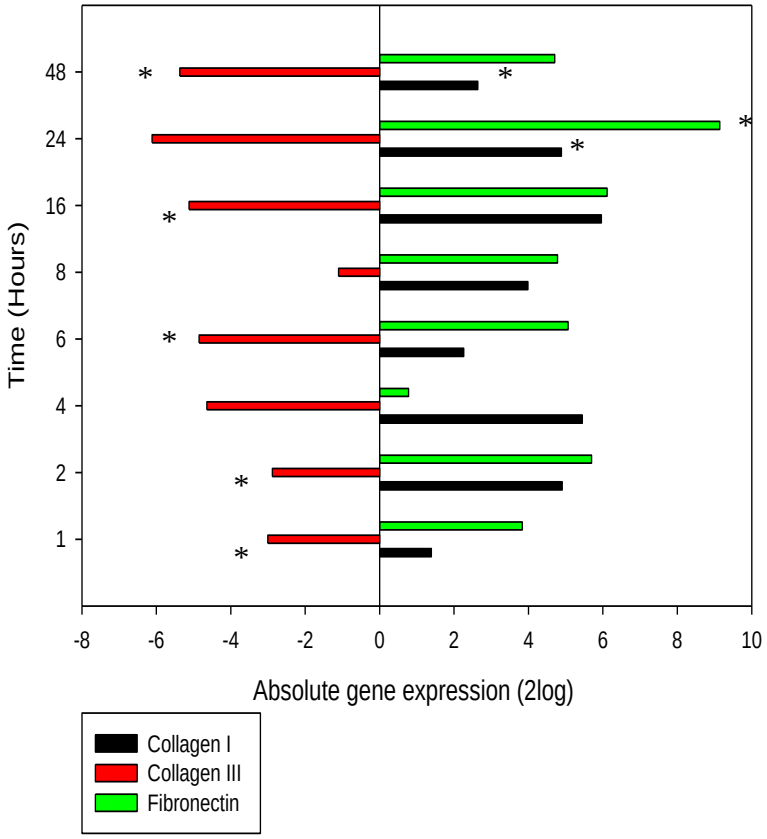
Time	Coll I	Coll III	Fn
48	5.77	3.77	12.85
24	6.81	1.03	4.57
16	3.17	0.89	0.29
8	4.35	1.96	0.27
6	11.07	3.53	0
4	8.87	1.52	1.38
2	6.27	1.07	0.34
1	3.57	0	0.7

Figure 3.5 The absolute gene regulation of endotenon cells (n=3) from basal levels induced by transforming growth factor beta 1 (TGF-β1) is plotted over time (normalised with two house-keeping genes) for collagen type I, collagen type III, and fibronectin. The table demonstrates the coefficient of variation, CV (%), between each gene. * = Statistical Significance.



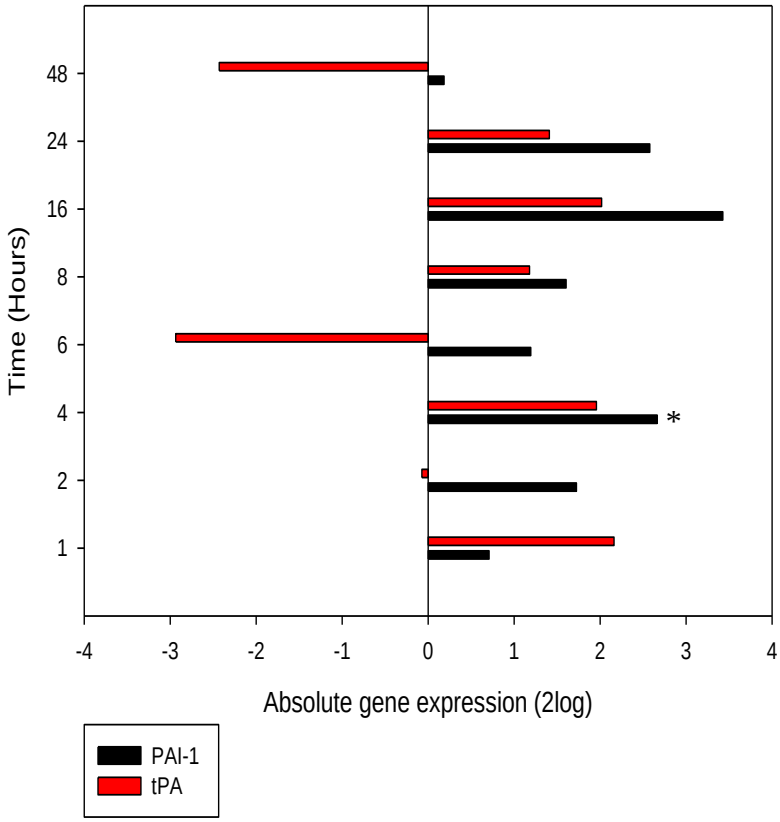
Time	PAI-1	t-PA
48	1.2	1.28
24	0.88	0.67
16	0.34	0.67
8	0.04	0.57
6	2.76	3.73
4	0.61	4.66
2	0.53	3.26
1	0.8	3.6

Figure 3.6 The absolute gene regulation of endotenon cells (n=3) from basal levels induced by transforming growth factor beta 1 (TGF-β1) is plotted over time (normalized with two house-keeping genes) for plasminogen activator inhibitor 1 (PAI-1) and tissue plasminogen activator (t-PA). The table demonstrates the coefficient of variation, CV (%), between each gene. * = Statistical Significance.



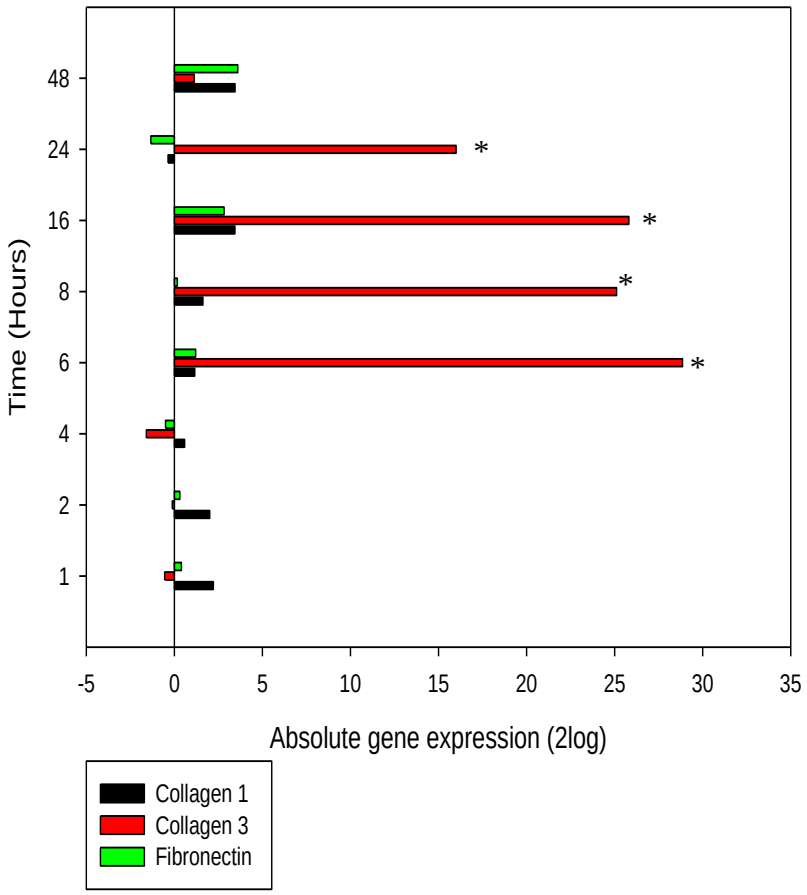
Time	Coll I	Coll III	Fn
48	12.41	2.98	15.17
24	16.9	2.4	1.99
16	2.19	2.8	3.62
8	14.12	0.63	6.18
6	8.46	4.04	11.52
4	22.31	1.88	4.7
2	2.91	0.82	2.29
1	11.61	2.41	8.27

Figure 3.7 The absolute gene regulation of epitenon cells (n=3) from basal levels induced by transforming growth factor beta 1 (TGF-β1) is plotted over time (normalised with two house-keeping genes) for collagen type I, collagen type III, and fibronectin. The table demonstrates the coefficient of variation, CV (%), between each gene. * = Statistical Significance.



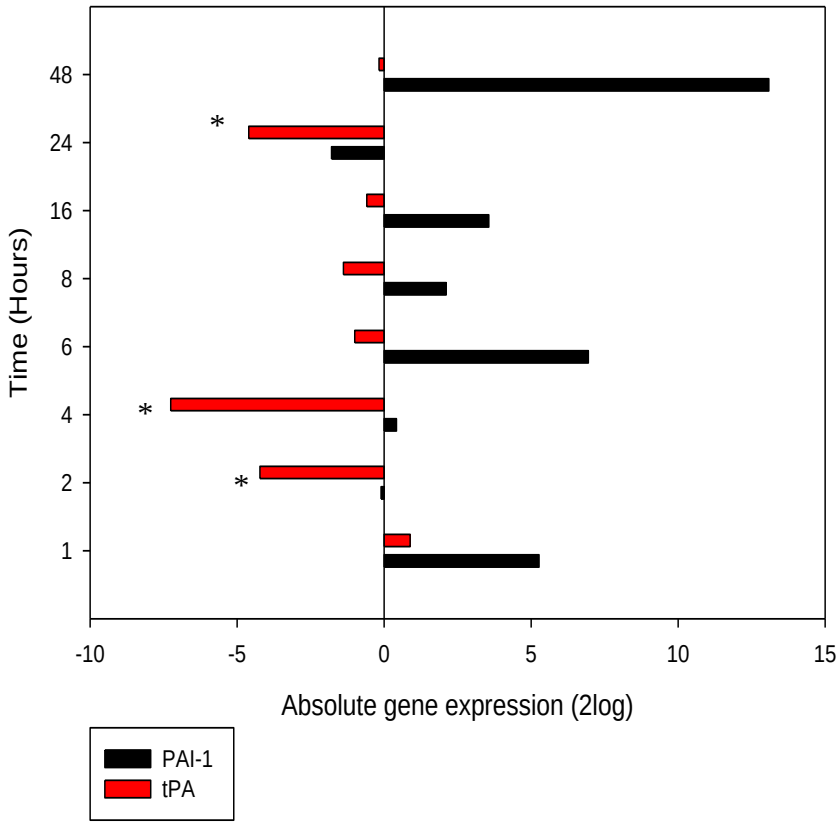
Time	PAI-1	t-PA
48	1.28	1.2
24	3.96	0.17
16	4.32	3.94
8	6.83	4.0
6	9.38	0.45
4	7.82	2.08
2	4.9	1.81
1	5.99	2.22

Figure 3.8 The absolute gene regulation of tendon epitenon cells (n=3) from basal levels induced by transforming growth factor beta 1 (TGF-β1) is plotted over time (normalised with two house-keeping genes) for plasminogen activator inhibitor 1 (PAI-1) and tissue plasminogen activator (t-PA). The table demonstrates the coefficient of variation, CV (%), between each gene. * = Statistical Significance.



Time	Coll I	Coll III	Fn
48	15.56	0.99	0.91
24	13.55	9.15	16.19
16	25.95	0.13	20.94
8	20.92	1.08	10.9
6	24.14	1.02	2.8
4	14.86	0.43	11.38
2	31.66	0.43	19.7
1	32.21	3.15	23.09

Figure 3.9 The absolute gene regulation of tendon sheath cells (n=3) from basal levels induced by transforming growth factor beta 1 (TGF-β1) is plotted over time (normalised with two house-keeping genes) for collagen type I, collagen type III, and fibronectin. The table demonstrates the coefficient of variation, CV (%), between each gene. * = Statistical Significance.



Time	PAI-1	t-PA
48	0.65	2.48
24	12.41	2.91
16	12.89	2.86
8	12.14	0.86
6	2.45	1.39
4	7.93	2.48
2	13.7	1.15
1	13.94	1.98

Figure 3.10 The absolute gene regulation of tendon sheath cells (n=3) from basal levels induced by transforming growth factor beta 1 (TGF-β1) is plotted over time (normalised with two house-keeping genes) for plasminogen activator inhibitor 1 (PAI-1) and tissue plasminogen activator (t-PA). The table demonstrates the coefficient of variation, CV (%), between each gene. * = Statistical Significance.

(i) **Endotenon**

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

(ii) **Epitenon**

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

(iii) **Tendon Sheath**

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Figure 3.11 Summary of results showing tendon cell gene expression induced by transforming growth factor beta 1 (TGF-β1) for collagen type 1, collagen type III, fibronectin, plaminogen activator inhibitor 1 (PAI-1) and tissue plasminogen activator (t-PA) in (i) endotenon cells; (ii) epitenon cells and; (iii) tendon sheath cells. Grey = no significant change; white = statistically significant gene up-regulation ($p<0.05$) and ; black = statistically significant down-regulation ($p<0.05$).

3.4 Discussion

TGF-β1 has been implicated in both tendon healing (Chang et al., 1997; Klein et al., 2002; Tsubone et al., 2006) and tendon adhesion formation (Chang et al, 2000). Chang in his early study (1997) demonstrated that TGF-β1 mRNA was produced by normal unwounded tenocytes and tendon sheath cells and that it was transcribed in the tendon wound environment. This same group also demonstrated that the TGF-β receptors were up-regulated after injury and repair and there was a strong correlation between the expression of the receptors and ligands (Ngo et al., 2001) indicating that tenocytes may be responsive to TGF-β signalling. Further, during tendon healing other groups have shown an increase in TGF-β1 m-RNA (Berglund et al., 2006) suggesting that TGF-β1 may play a role in this process (Hsu et al., 2004). Although neutralising antibodies to TGF-β1 have been shown to prevent tendon adhesions (Chang et al., 2000), others have shown that TGF-β1 neutralisation can decrease tensile strength and enhance the deterioration of collagen fascicles in a tissue-cultured tendon matrix (Azuma et al., 2007). Berglund et al., (2006) noted that after injury there were a number of differences in gene expression both in the spatial and temporal response of the tendon. Therefore it is important to understand the effect that TGF-β1 plays on the different regions of the uninjured tendon which may allow a greater understanding of the biology of both tendon healing and adhesion formation. Understanding the role that TGF-β1 has on the different regions of the tendon-sheath complex should help the development of strategies to reduce adhesion formation in association with optimal tendon healing.

Three distinct membrane receptors have been identified (TGFβRI, TGFβRII and TGFβRIII) which bind the TGF-β isoforms with varying degrees of specificity. Tendon injury and repair have been shown to cause the up-regulation of all three receptors with the highest expression being seen in the sheath and epitenon derived cells (Ngo et al., 2001). TGF-β1 is known to stimulate a wide variety of genes that can be categorized into basic cell functions e.g. metabolism, protein synthesis, gene transcription, genes of unknown function along with genes associated with matrix formation and remodelling (Chambers et al., 2003; Verrechia et al., 2001). In this study a number of genes known to be involved in tendon healing and adhesion formation were examined but focus on specific TGF-β1 receptor levels could not be explored due to time constraints.

In this study the proliferation results demonstrate that when sheath cells were treated with TGF-β1 there was a statistically significant increase at 0.5ng/ml at 72 hours but not at 24 or 48 hours. Epitenon cells showed a similar significant response with FCS compared to SFM. Also, although there was not an early increase (24 and 48 hours) in proliferation with TGF-β1 compared to cells grown in minimal media there was a statistically significant increase at 72 hours when using a higher dose (2ng/ml). Endotenon cells showed no significant increase in proliferation with TGF-β1 when compared to SFM and this is likely to be due to the general slow proliferation and non-reactivity of this cell type demonstrated throughout this thesis. It appears that epitenon and sheath cells are behaving differently to endotenon cells in such a way that reflects modern tendon healing concepts (Berglund et al., 2006).

It has been established in fibroblasts derived from other tissues that collagen gene expression is increased in response to TGF-β1 (Rolfe et al., 2007; Varga et al., 1987; Tiggleman et al., 1995; Chai et al., 2003). Collagen is known to be important in maintaining the mechanical properties of the tendon and transmitting the forces generated by muscle fibres to the skeletal system (Benjamin et al., 2002; Purslow, 2002). However, collagen type III is composed of small fibrils deficient in cross-linking resulting in less tensile strength when compared to collagen type I. Collagen type I is the main constituent in uninjured tendons. While collagen type III is rapidly deposited after injury its presence appears only temporary as during the remodelling phase of tendon healing collagen type III is replaced with collagen type I (Jaibaji, 2000). Similarly to our study, but in an injured *in vivo* model, Gelberman et al found that the epitenon cells showed an increase in collagen type I, whereas the endotenon cells showed no detectable expression during the early healing phases (Gelberman et al., 1992). Klein using cells derived from an uninjured tendon demonstrated that all three isoforms of TGF-β caused an up regulation of protein expression for both collagen type I and III (Klein et al., 2002). It has been suggested that the surface tendon fibroblasts are more active in both proliferation and in the production of collagen type I in tendon repair restoring the gliding surface and tensile strength of the tendon (Gelberman et al., 1992). Others have also suggested that endotenon cells play a minimal role in tendon repair, which certainly appears the case in this current study when studying production of collagen type I gene expression (Russell and Manske, 1990).

Interestingly stimulation of epitenon cells with TGF-β1 showed no increase in collagen type III expression over the whole time course though both sheath and endotenon derived cells showed some statistically significant increase during the time course. Similarly Berglund et al., (2006) also showed an increase in collagen type III in both tendon and tendon derived sheath cells in an *in vivo* model, over a longer period of time and in a post injury model, however their tendon cells were not classified as either epitenon or endotenon derived (Berglund et al., 2006). Further Klein et al., (2002) also demonstrated an increase in collagen type III production, following TGF-β1 treatment, in all three cell types at day 4 post treatment (Klein et al., 2002). In this study the time points were associated with uninjured cells under culture conditions rather than days post injury. There were limitations in this study as after 48 hours in SFM the cell number and viability is reduced, therefore meaningful results are not achievable beyond this point.

TGF-β1 is known to be able to alter the production of fibronectin (Saika et al., 1998), further it is also known that fibronectin can alter tendon gene expression in response to TGF-β1 (Fu et al., 2005). Fibronectin is known to be deposited at an early stage of tendon healing (granulation phase) and is thought to play a role in the formation of an adhesion bridge (Kurtz et al., 2001) Fibronectin is also known to bind and activate TGF-β therefore enhancing the effect of TGF-β (Fava and McClure, 1987). This present study showed that all tendon-derived cells showed at some time point a statistically significant increase in fibronectin gene expression compared to the untreated controls. Wojciak and Crossan concluded that the presence of inflammatory cells during tendon healing induces synovial fibroblasts and epitenon cells to increase their production of fibronectin, which provides a scaffold for subsequent adhesion formation (Wojciak and Crossan, 1993). Inflammatory cells are known to secrete TGF-β1 and cause the deposition of the extra cellular matrix (Kehrl et al., 1986). Fibronectin is believed to play a role in adhesion formation possibly through its role in cross-linking of fibrin, and through its role in fibroblast chemotaxis and adherence of cells to the substratum during tendon healing (Gelberman et al., 1991).

TGF-β1 is known to cause the increase in plasminogen activator inhibitor 1 (PAI-1) gene transcription in a number of cell types (Rolfe et al., 2007; Zhao et al., 2006). An increase in PAI-1, and therefore an increase in the inhibition of fibrinolysis has been

shown to be associated with post-surgical peritoneal adhesion formation (Di Filippo et al., 2006) while increased tissue plasminogen activator (t-PA) has been associated with a decrease in adhesion formation in the peritoneum and pelvis (Orita et al., 1991; Doody et al., 1989). PAI-1 gene expression was found to be statistically significantly increased compared to untreated controls in endotenon cells in the early part of the time course, whereas sheath derived cells showed a statistically significant increase at 48 hours. Others, studying mesothelial cells have also shown an increase in PAI-1 gene transcription in response to TGF-β1 and a reduction in t-PA (Falk et al., 2001). Interestingly, all tendon-derived cells showed a reduction in t-PA gene expression over the time course with endotenon and sheath derived cells showing a statistically significant down regulation. The increase in PAI-1 in sheath-derived cells in conjunction with a decrease in t-PA, following treatment with TGF-β1, could potentially lead to adhesion formation. Excess fibrin and the cross-linking with fibronectin around the wound site could form a framework for adhesion formation between the tendon surface and its surrounding sheath. Low expression of t-PA would lead to a reduction of plasmin, a persistence of fibrin and therefore increase the potential for adhesion formation around the tendon (Figure 1.10). Furthermore, increased levels of PAI-1 would inhibit the effect of t-PA and exacerbate this effect. This mechanism could be one explanation for the excess scarring and adhesion formation associated with TGF-β1.

Traditional models of flexor tendon healing have been described as simply intrinsic or extrinsic, however to study tendon healing more precisely it is important to observe the behaviour of cells from the three main areas of the tendon-sheath complex i.e. the endotenon, the epitenon and the sheath. During tendon healing each cell type plays a different role but it is the epitenon-sheath interface where adhesion formation occurs. We acknowledge there are differences with cells grown in culture to those *in vivo.* However, *in vitro* studies allow a tightly controlled environment whereby various experimental conditions can be measured which is not possible in an *in vivo* model. This study allowed the study of the role that TGF-β1 alone plays in gene expression on the cells derived from different areas of the tendon. Though tendon cells *in vitro* are believed to have undergone differentiation due to culture conditions it is also believed that during tendon healing the cells also undergo some forms of differentiation (Gelberman et al., 1985).

Having studied the effects of this important growth factor on flexor tendon cells a baseline investigation has been established to compare against our anti-adhesion treatments. Additionally, the three novel treatments may act via TGF-β1 pathways which may be further manipulated to achieve an optimal therapy.

CHAPTER 4

THE *IN VITRO* FLEXOR TENDON CELL RESPONSE TO 3 NOVEL ANTI-ADHESION TREATMENTS:

EPIGALLOCATECHIN-3-GALLATE (EGCG)

RESVERATROL

& PUMACTANT

4.1. Introduction

There have been many different chemical (Kulick et al., 1984; Green et al., 1986; Hagberg, 1992; Moran et al., 2000; Chang et al., 2000) and physical barrier (Austin and Walker, 1979; Hanff and Hagberg, 1998; Golash et al., 2003) methods studied to improve tendon repair, but to date none have proven to be successful. Problems with previous treatments have included increased rupture rates, infection, side effects and increased bulkiness preventing tendon glide. The aim of this study was to look at specific treatments, which could potentially inhibit adhesion formation and allow optimal tendon healing, but without unwanted side effects.

4.1.1 Epigallocatechin-3-gallate (EGCG)

EGCG is the main active catechin extract from green tea (*Camellia sinensis*). Other less active catechins in green tea include epicatechin gallate (ECG), (+)-epicatechin and (-)-gallocatechin gallate (GCG). EGCG accounts for 9-13% of green tea in net weight. EGCG possesses strong antioxidant activities and may play a role in preventing cancer (Ahmad et al., 1997) and cardiovascular disease (Basu et al., 2007). EGCG appears to alter a number of fibrotic mechanisms: reduces the expression of collagenase and stromelysin mRNA via various pathways (Corps et al., 2004), inhibits extracellular matrix (ECM) gene expression, by interrupting TGF-β1 signalling through attenuating oxidative stress (Yumei et al., 2006), inhibits collagen production and tissue inhibitor of metalloproteinase-1 (TIMP-1) and reduces the transcription of matrix metalloproteinase-1 (MMP-1) (Nakumata et al., 2005). Recent studies have shown that EGCG suppresses collagen production and may have therapeutic potential for keloid scarring and liver fibrosis (Zhang et al., 2006; Abe et al., 2007). Further, EGCG appears to be able to prevent TGF-β1 stimulated collagen contraction through a number of mechanisms, including reducing myofibroblast differentiation and the reduction of connective tissue growth factor expression (Klass et al., 2010). EGCG appears to play a role in not only preventing fibrosis but also appears to enhance wound healing (Kapoor et al., 2004; Zhang et al., 2006; Nakamuta et al., 2005), and may therefore, have a potential therapeutic role in flexor tendon healing.

4.1.2 Resveratrol

Resveratrol (trans-3, 4, 5- trihydroxystilbene) is a natural polyphenolic phytoalexin found in some plants including grapes and their related products. Phytoalexins are

antibacterial and antifungal chemicals produced by plants as a defence against infection. Resveratrol is also a stilbenoid, a derivative of stilbene, and is produced with the help of the enzyme stilbene synthase.

There is evidence to suggest that Resveratrol has the potential to reduce adhesions following tendon repair. Sogutlu et al., (2006) demonstrated a reduction in peritoneal adhesions following laparotomy (with abrasion of the caecum to create adhesions) with an intra-operative application of Resveratrol, compared to saline in an animal model. A more recent study showed a reduction in postoperative adhesion formation in a rat uterine horn model with repeated subcutaneous injections of Resveratrol (Utsűn et al., 2007). Other studies have shown that Resveratrol has anti-inflammatory, antioxidant, anti-platelet and anti-tumour effects in a number of tissue types (Brakenhielm et al., 2001; Sgambato et al., 2001; Surh et al., 1999). In a dermal wound-healing mouse model, Resveratrol treatment was associated with improved histological architecture and faster wound closure (Khanna et al., 2002). Resveratrol appears also to have a role in preventing fibrosis in a rat cirrhosis model, and its action is probably associated with its ability to reduce NF-kappa activation and thus reduce levels of TGF-β (Chavez et al., 2007). There are close associations between wound healing, fibrosis, excess scarring and adhesion formation. It, therefore, appears that Resveratrol may have a role in reducing peri-tendinous adhesions without affecting wound healing.

4.1.3 Pumactant

Pumactant is a mixture of two naturally occurring phospholipids: a 7:3 mixture of dipalmitoylphosphatidylcholine and unsaturated phosphatidylglycerol. It was originally developed for neonatal respiratory distress syndrome (RDS), but this application of use was discontinued for commercial reasons in 2000. However, there has never been any suggestion of toxicity or side effects of Pumactant requiring regulatory action. The use of Pumactant in asthma and other respiratory conditions has also been investigated (Babu et al., 2003). More recently, in a murine post-surgical peritoneal adhesion model it has been shown that Pumactant powder produced a dose-dependent reduction in adhesion formation so long as the powder was completely dissolved when applied to the caecum and sidewall (unpublished studies at Cardiff University).

Pumactant has also been found to accelerate mesothelial and keratinocyte cell proliferation. This proliferation appeared to be unique to this specific compound, as other phospholipids appear to inhibit cell proliferation and cause a reduction in cell viability (Topley and Woodcock, 2010). A number of publications have either identified phospholipids in tendons and tendon sheath fluid (Mills et al., 2005) or a role for phospholipids in the prevention of adhesion formation in animal models (Moro-oka et al., 2000).

The ideal treatment would promote tendon healing and reduce or modify adhesion formation thus allowing the tendon to glide freely within the tendon sheath and allow for optimal function.

4.1.4 Hypothesis

The aim of this chapter was to test the hypothesis:

The three novel compounds, EGCG, Resveratrol and Pumactant are able to influence cell adhesion and gene expression in different cells from the tendon-sheath complex. These effects may lead to a reduction or modification of flexor tendon adhesion formation.

4.1.5 Objectives

- Establishment of tendon cell lines of different origin (endotenon, epitenon, and tendon sheath) using tissue culture techniques.
- Evaluation of cell proliferation to determine optimal doses of specific treatments.
- Assessment of the transcription of specific genes known to be involved in either tendon healing or adhesion formation, using the QRT-PCR method, following the three treatments on each tendon cell type.
- Evaluation of the effect that the treatments have on *in vitro* cell adhesion with each tendon cell type.

4.2 Materials and Methods

To study specific changes occurring at the cellular level initially flexor tendon cells were isolated and cultured in the laboratory. Uninjured New Zealand White rabbits were terminated via a Schedule 1 technique (as described in Chapter 2, section 2.1.1). The fresh uninjured forepaw tendons were dissected under a microscope and three different tissue types were isolated and cultured separately: endotenon (core), epitenon (surface), and tendon sheath cells (as described in Chapter 2, section 2.2.1). Established cell lines were then incorporated into the following assays:

4.2.1 Toxicology Assay

A toxicology assay was performed to determine the optimal dose for each treatment. WST-1 was used over a time course of 10 days (as described in Chapter 2, section 2.3.1).

4.2.2 Cell Adhesion Assay

A cell adhesion assay was performed to determine the adherence of each different cell type to various ECM components in response to the three treatments under investigation (as described in Chapter 2 section 2.3.3).

4.2.3 Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR)

Quantitative real-time PCR was used to determine the effects of each treatment on gene expression. Genes chosen are thought to be involved in tendon healing and/or adhesion formation. The regulation of specific genes *in vitro* will indicate the possible actions *in vivo* and predict the cell type responsible. QRT-PCR was performed using the Stratagene real-time PCR machine (as described in Chapter 2, section 2.3.4).

4.3 Results

4.3.1 Toxicology Assay

EGCG

Endotenon cells treated with various concentrations of EGCG (12.5μM, 25μM, 50μM, 100μM, 500μM and 1000μM) a reduction in proliferation was shown after 25μM although these changes were not statistically significant (Figure 4.1). Epitenon cells treated with various concentrations of EGCG showed a statistically significant increase in proliferation by day 7 (12.5μM, $p=0.01$ and 25μM, $p=0.01$) and day 10

(12.5μM, p=0.01; 25μM, p=0.03 and 50μm, p=0.018). However, at higher concentrations (500μM, p=0.006 and 1000μM, p=0.044) there was a statistically significant increased toxic effect on this cell type by day 3 (Figure 4.2). Lastly, tendon sheath cells showed a statistically significant reduction in proliferation by day 3 at mainly high concentrations (25μM, p=0.034; 100μM, p=0.04 and 500μM, p=0.005) and at day 7 with 50μM of EGCG (p=0.039) (Figure 4.3).

Resveratrol

Endotenon cells treated with Resveratrol at various concentrations (12.5μM, 25μM, 50μM, 100μM, 500μM and 1000μM) showed a marked reduction in proliferation after treatment with a dose greater than 25μM. These findings were not statistically significant. Also, there appears to be an increase in proliferation at very high concentrations of Resveratrol (1000μM) but again this was not found to be significant (Figure 4.4). Epitenon cells show a statistically significant reduction in proliferation at day 3 (12.5μM, p=0.003 and 50μM, p=0.045), day 7 (12.5μM, p= 0.02 and 50μM, p=0.002) and day 10 (50μM, p=0.001). Unexpectedly, there was a statistically significant increase in proliferation at day 10 with 1000μM of Resveratrol (p=0.002) (Figure 4.5). Tendon sheath cells showed a wide variability in response to treatment with Resveratrol (as shown by the large standard deviation error bars); however, there was a statistically significant reduction in proliferation at day 10 with a dose of 25μM (Figure 4.6).

Pumactant

Endotenon cells treated with various concentrations of Pumactant (1mg/ml, 2mg/ml, 4mg/ml, 8mg/ml and 10mg/ml) showed a statistically significant increase in proliferation on day 3 with 10mg/ml (p=0.03). There does not appear to be a significant toxic effect at any concentrations with this cell type (Figure 4.7). Epitenon cells treated with Pumactant also showed a statistically significant increase in proliferation at day 3 (1mg/ml, p=0.04 and 8mg/ml, p=0.01) and day 10 (1mg/ml, p=0.045) (Figure 4.8). Tendon sheath cells treated with Pumactant showed a statistically significant reduction in proliferation at day 7 with 2mg/ml (p=0.027) but a statistically significant increase in proliferation at day 10 with 10mg/ml (p=0.02) (Figure 4.9).

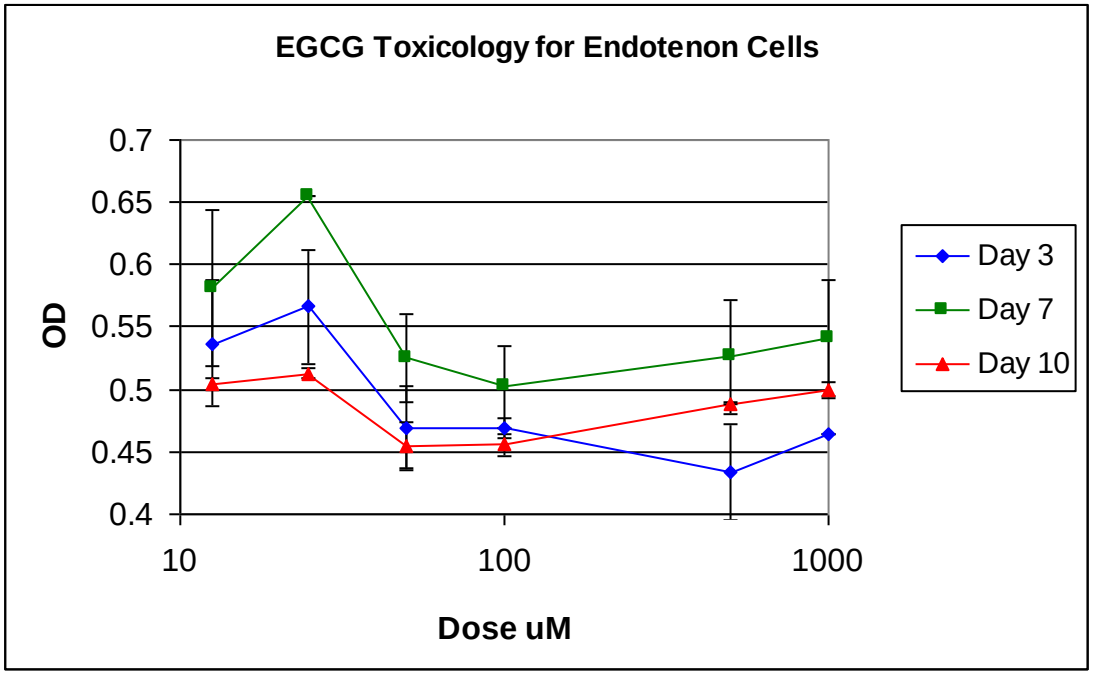


Figure 4.1 Toxicology of EGCG at various doses for endotenon cells at day 3, day 7 and day 10 (n=3; Error bars=SD; N.S. =No statistical significance).

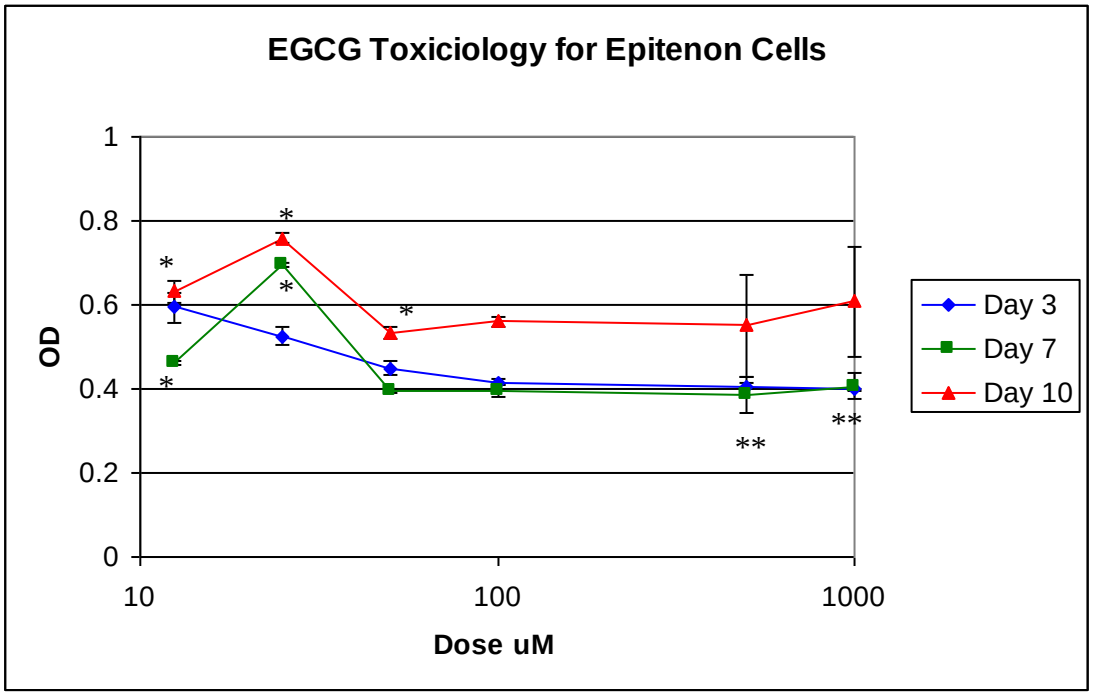


Figure 4.2 Toxicology of EGCG at various doses for epitenon cells at day 3, day 7 and day 10 (n=3; Error bars=SD; *p<0.05; **p<0.05 day 3 only).

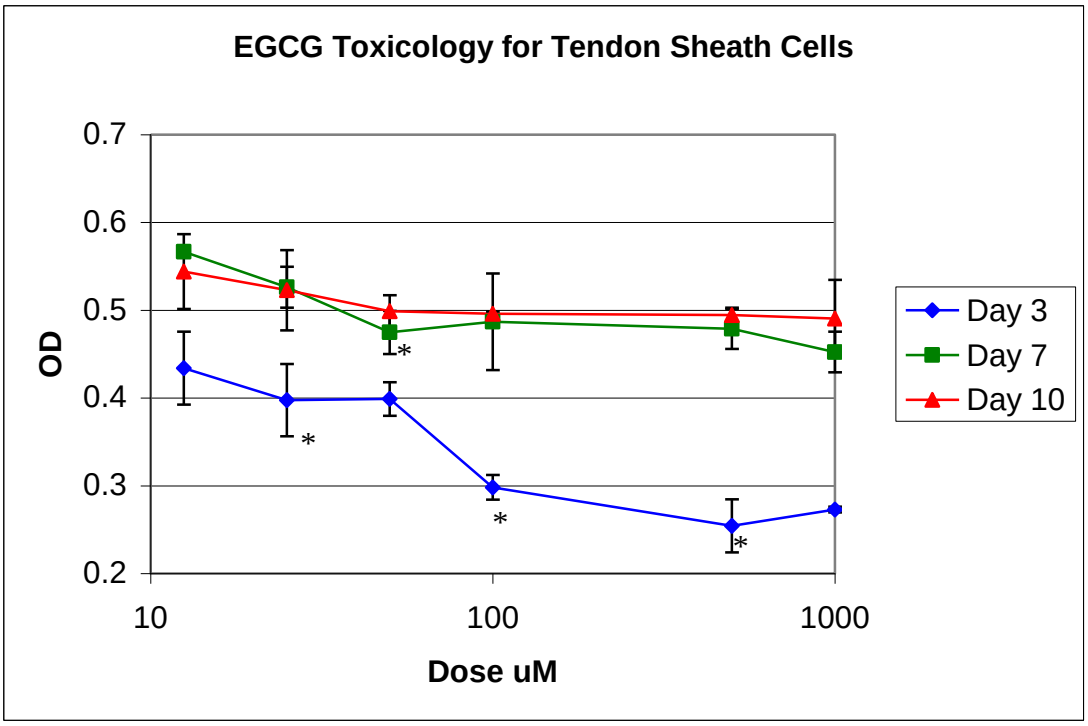


Figure 4.3 Toxicology of EGCG at various doses for tendon sheath cells at day 3, day 7 and day 10 (n=3; Error bars=SD; *p<0.05).

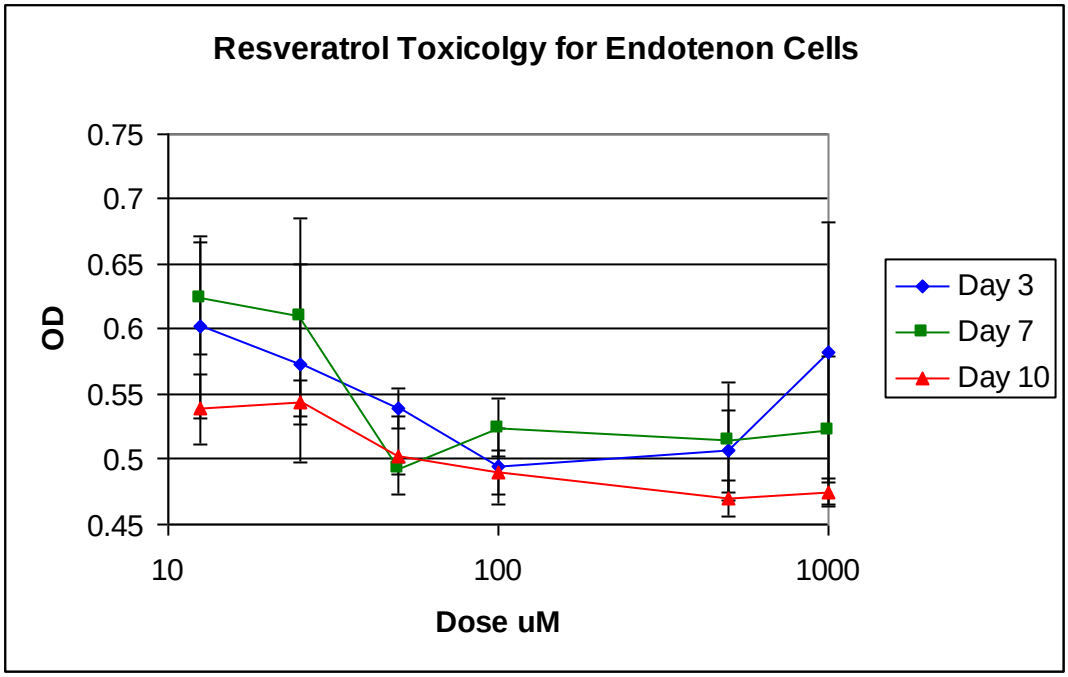


Figure 4.4 Toxicology of Resveratrol at various doses for endotenon cells at day 3, day 7 and day 10 (n=3; Error bars=SD; N.S. =No statistical significance).

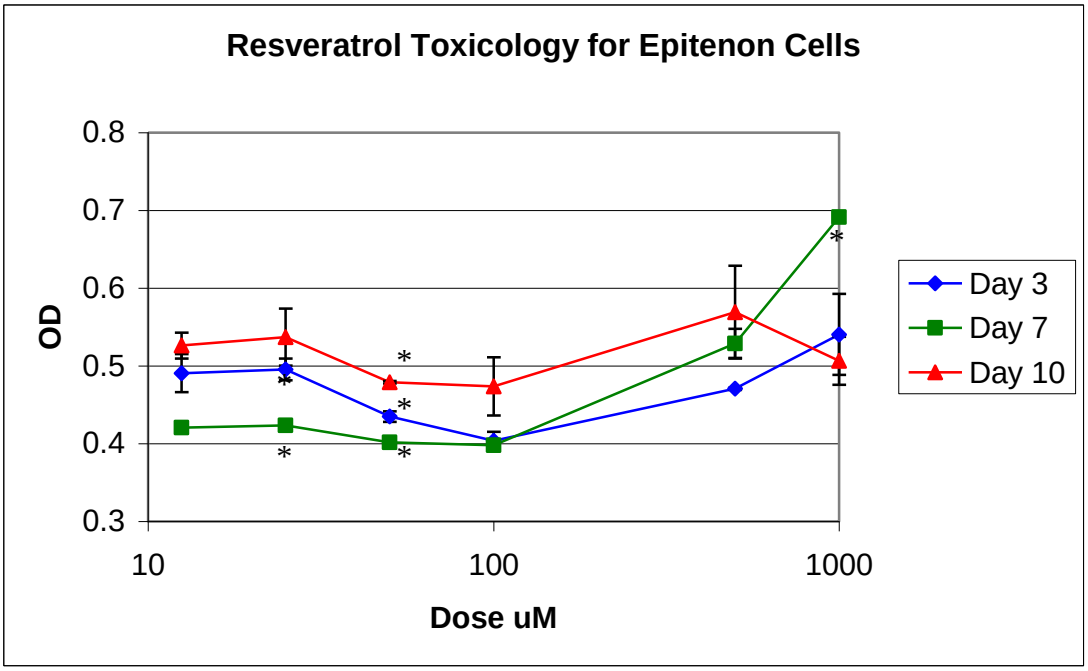


Figure 4.5 Toxicology of Resveratrol at various doses for epitenon cells at day 3, day 7 and day 10 (n=3; Error bars=SD; *p<0.05).

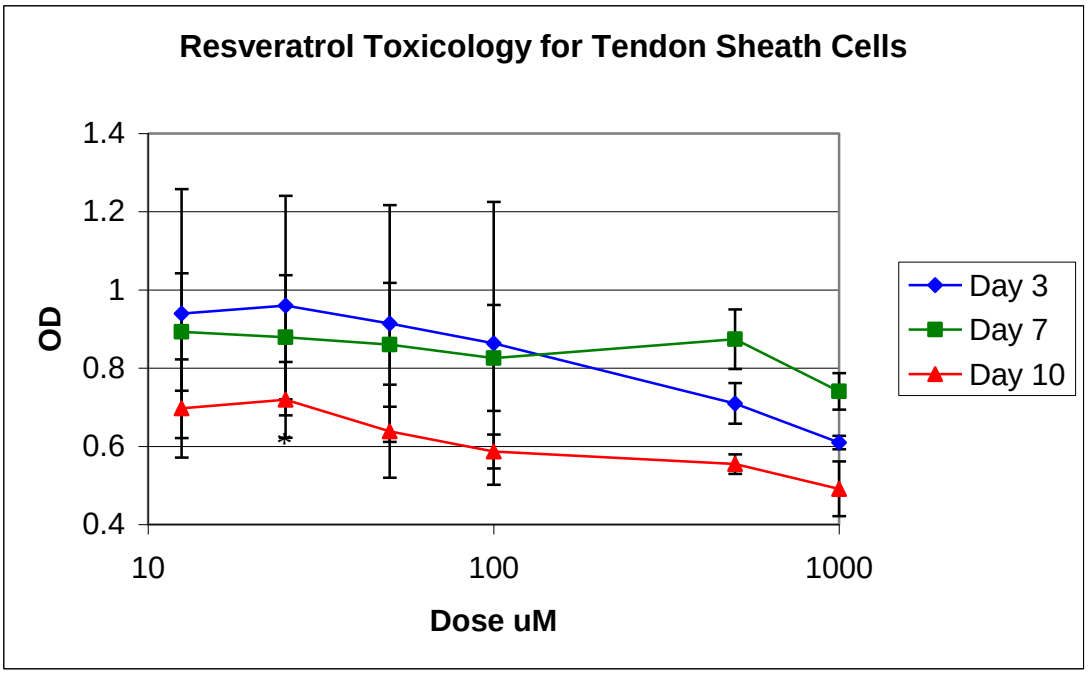


Figure 4.6 Toxicology of Resveratrol at various doses for tendon sheath cells at day 3, day 7 and day 10 (n=3; Error bars=SD; *p<0.05).

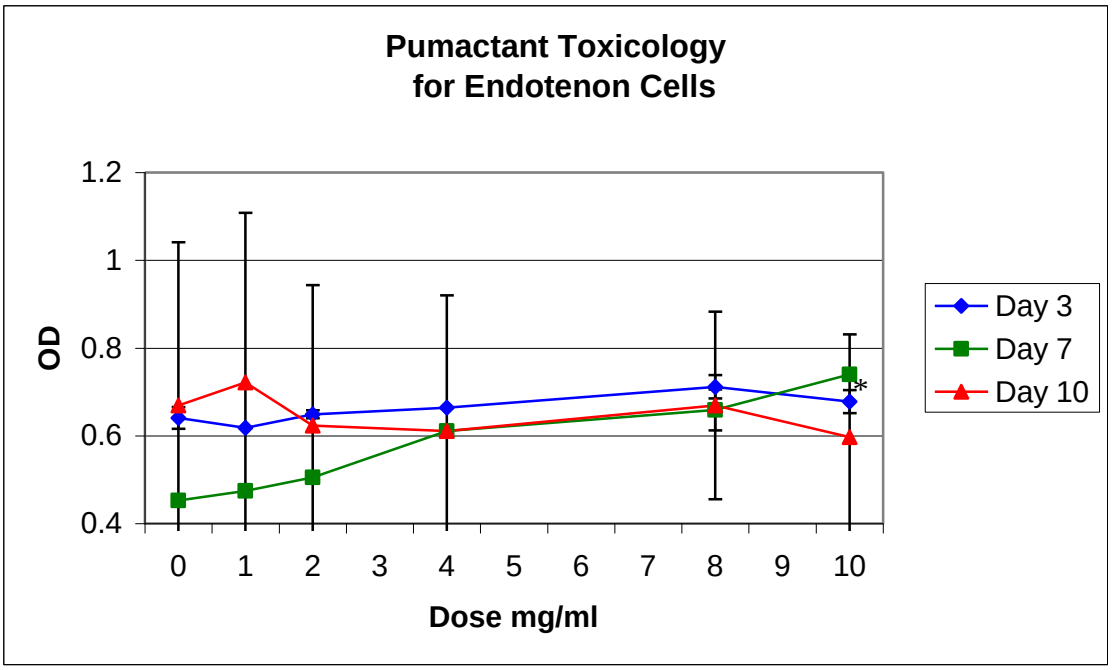


Figure 4.7 Toxicology of Pumactant at various doses for endotenon cells at day 3, day 7 and day 10 (n=3; Error bars=SD; *p<0.05).

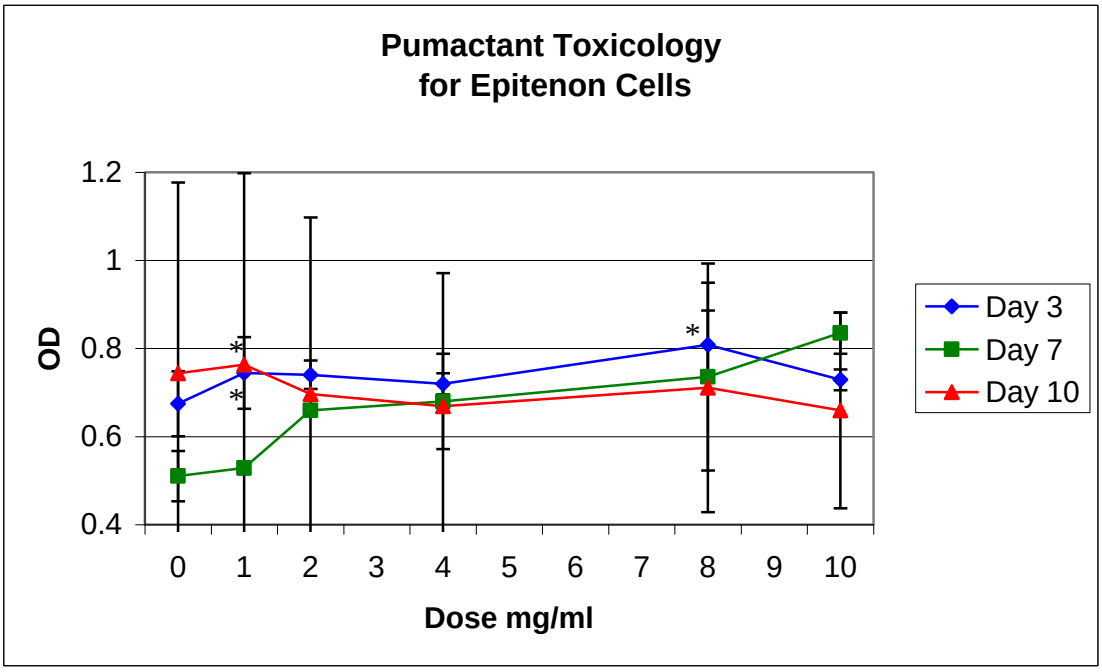


Figure 4.8 Toxicology of Pumactant at various doses for epitenon cells at day 3, day 7 and day 10 (n=3; Error bars=SD; *p<0.05).

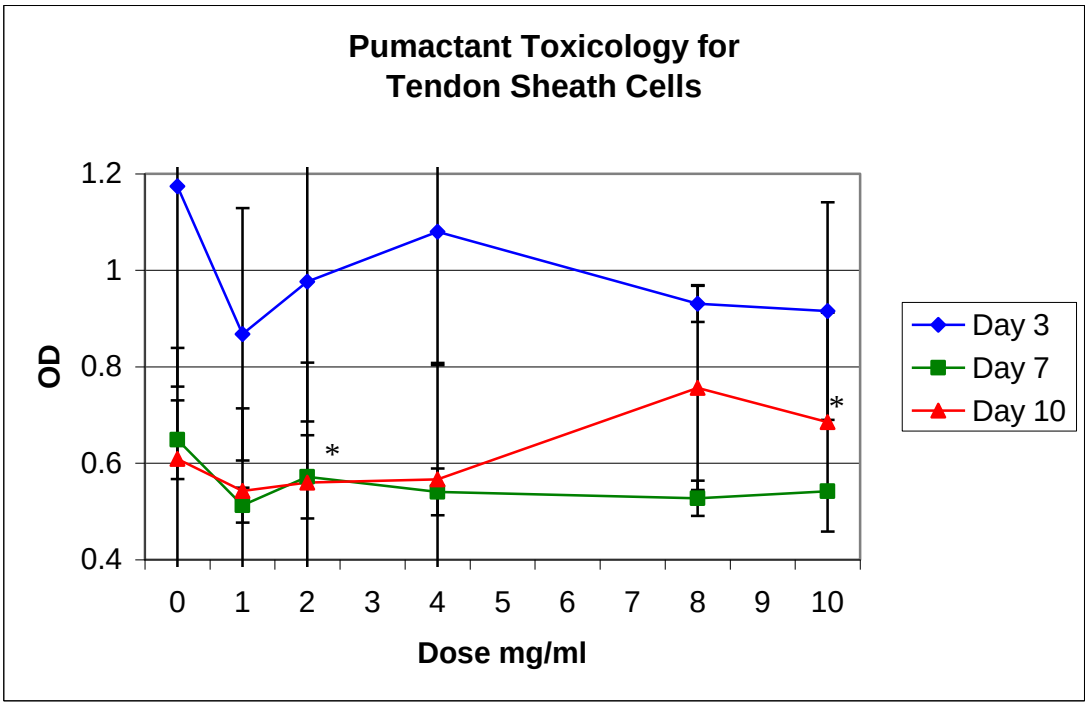


Figure 4.9 Toxicology of Pumactant at various doses for tendon sheath cells at day 3, day 7 and day 10 (n=3; Error bars=SD; *p<0.05).

4.3.2 Cell Adhesion Assay

EGCG

Interestingly treatment with EGCG (Figure 4.10) showed a statistically significant decrease in cells adhering to collagen type I in cells derived from the sheath ($p=0.02$). Conversely, treatment with EGCG made both endotenon and epitenon derived cells more adherent to collagen type III ($p=0.02$ and $p=0.0007$ respectively). Treating cells with EGCG showed no difference in cell adherence to fibronectin.

Resveratrol

Pre-treating the cells with Resveratrol (Figure 4.11) resulted in both epitenon and sheath derived cells showing less adhesion to collagen type I ($p=0.003$ and $p=0.048$ respectively). No other cells or matrices showed any statistical significant differences.

Pumactant

Pre-treatment with Pumactant (Figure 4.12) showed no statistical significance for any group of cells or matrices.

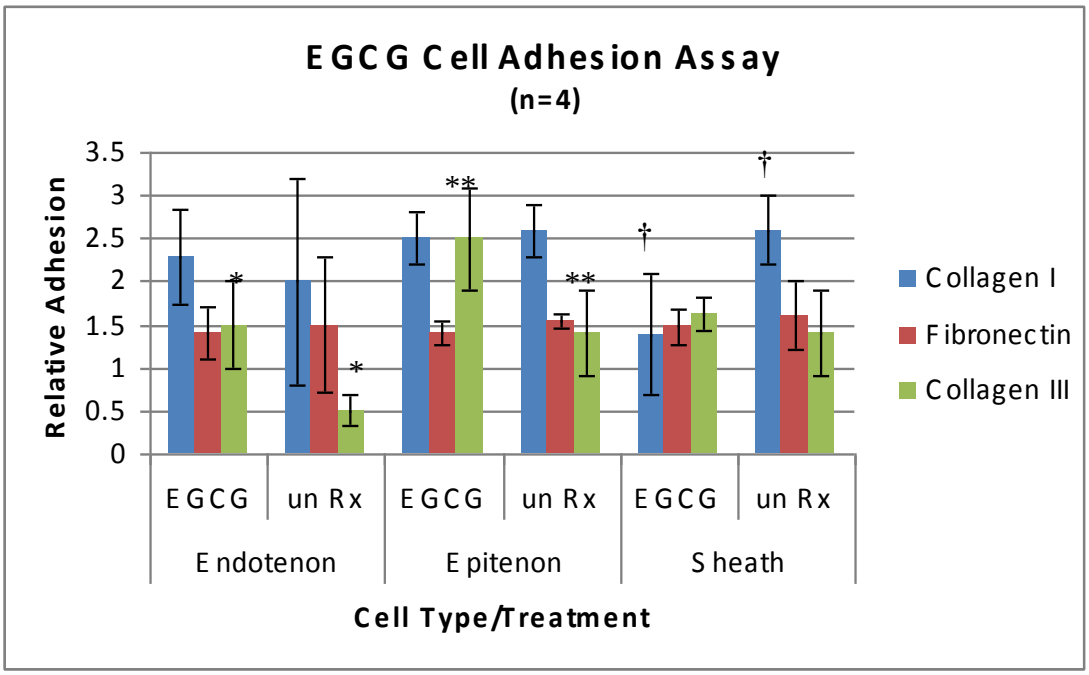


Figure 4.10 Cell adhesion assay for EGCG (50µM), showing relative adhesion for all tendon cell types in the presence of different extracellular matrix components (n=4, Error bars=SEM, *p=0.02, **p=0.0007, †p=0.02).

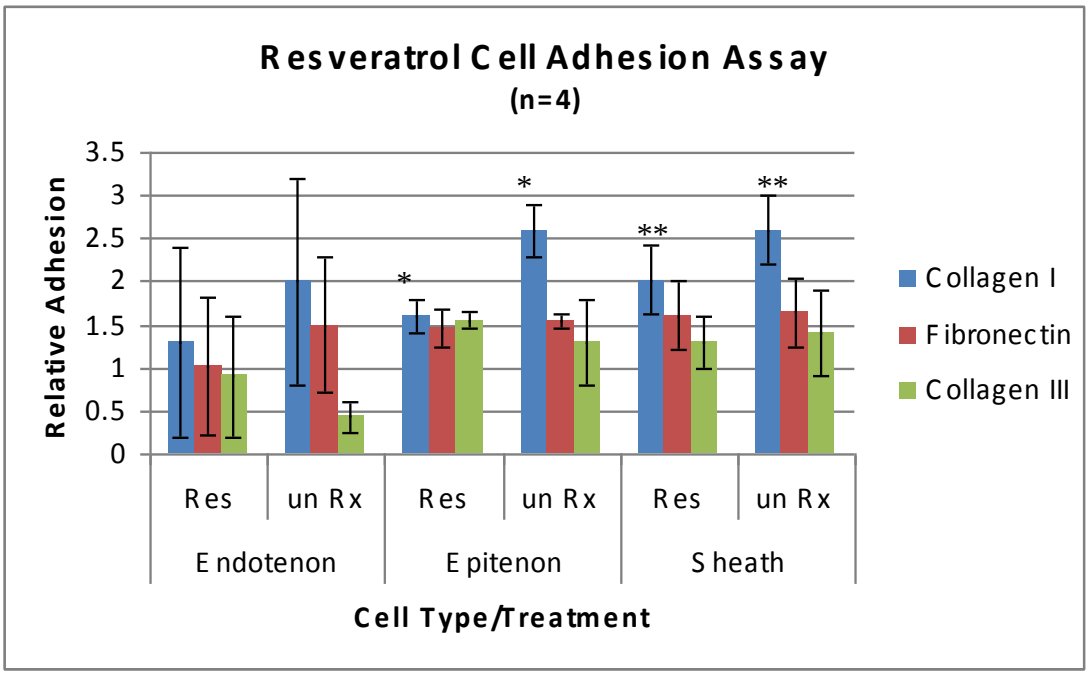


Figure 4.11 Cell adhesion assay for Resveratrol (50µM), showing relative adhesion for all tendon cell types in the presence of different extracellular matrix components (n=4, Error bars=SEM, *p=0.003, **p=0.048).

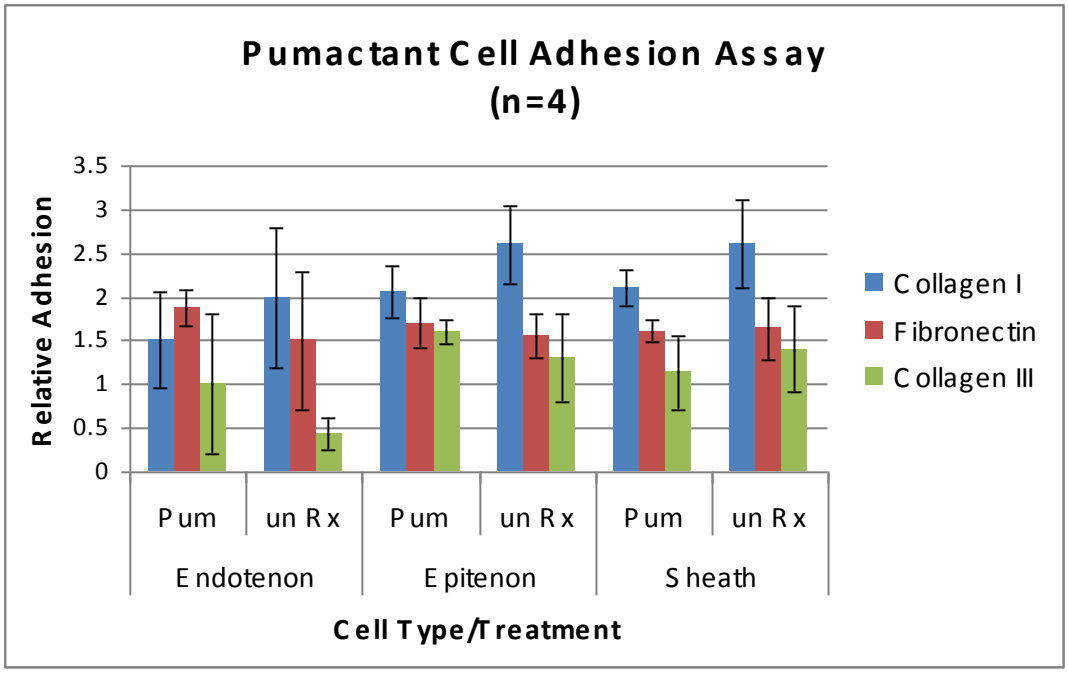


Figure 4.12 Cell adhesion assay for Pumactant (1mg/ml), showing relative adhesion for all tendon cell types in the presence of different extracellular matrix components (n=4, Error bars=SEM).

4.3.3 Quantitative Real-Time Polymerase Chain Reaction (QRT-PCR) (For a summary of results see Tables 4.1-4.12)

EGCG

Quiescent endotenon cells were cultured in an optimal concentration of EGCG (50μM) and absolute gene expression was determined over a time course of 0-48 hours (See Figures 4.13 and 4.14). EGCG caused a down-regulation of gene expression for all three genes studied, but the only statistical significance was seen in collagen type III gene expression at 4 hours (p=0.012, CV% 3.82). Although fibronectin and collagen type I showed a reduction in absolute gene regulation at 8 hours these changes were not significant as the data represents high variance (CV% 12.23 and 5.01 respectively). EGCG showed no effect on gene expression for t-PA. However, the PAI-1 gene was up-regulated up to 6 hours but only statistically up-regulated at 4 hours (p=0.008, CV% 0.91). After 6 hours the gene expression for PAI-1 was down regulated though this was not significant. Interestingly at longer time (16-48 hours) no QRT-PCR results were identified for any of the genes studied. On further examination the EGCG appeared to bind to the RNA, as on extraction the RNA from cells treated over a long time course appeared brown in colour.

Epitenon cells treated with EGCG showed a marked down-regulation of fibronectin at 2, 4, 6, and 8 hours however these changes were not statistically significant (Figure 4.15). However, there was a statistically significant down-regulation of t-PA at 2 hours (p=0.023, CV% 3.91) and at 4 hours (p=0.022, CV% 2.25) (Figure 4.16). Although PAI-1 was also down-regulated these changes were not statistically significant.

Tendon sheath cells treated with EGCG showed a marked down-regulation of collagen type III and an up-regulation of fibronectin and t-PA at most time points however none of these changes were statistically significant (Figure 4.17 and 4.18).

Resveratrol

Endotenon cells treated with Resveratrol showed no statistically significant changes for any specific genes, although there was an increase in collagen type III seen at 6, 8, 16 and 24 hours (Figure 4.19 and 4.20).

Epitenon cells treated with Resveratrol (Figure 4.21) showed a statistically significant up-regulation of collagen type I at 4 (p=0.001, CV% 0.04) and 24 hours (p=0.001, CV% 0.45). Early collagen type III gene expression was down-regulated compared to basal levels and this was statistically significant at 1 hour (p=0.001, CV% 1.61). Epitenon cells stimulated with Resveratrol showed an up-regulation in collagen type III gene expression from 6 hours though this did not reach statistical significance. Resveratrol showed a down-regulation of fibronectin expression which was statistically significant at 6 (p=0.001, CV% 0.73) and 48 hours (p=0.001, CV% 0.06). Resveratrol caused no change in t-PA gene expression apart from a statistically significant down-regulation at 48 hours (p=0.001, CV% 0.19). Conversely, PAI-1 was statistically significantly down-regulated at 6 (p=0.001, CV% 0.16), 8 (p=0.001, CV% 1.34), and 48 hours (p=0.001, CV% 0.09) (Figure 4.22).

Tendon sheath cells treated with Resveratrol (Figure 4.23) showed a down-regulation of fibronectin at all time points and this was statistically significant at 2, 4, 6, and 24 hours (p=0.001, CV% 2.77, 2.94, 1.72 and 4.62 respectively). There was a tendency for down-regulation of Collagen type I at all time points, though this was not statistically significant. Although collagen type III showed a statistically significant down-regulation at 4 hours (p=0.001, CV% 0.16), there was a statistically significant up-regulation at 16 (p=0.001, CV% 1.67) and 48 hours (p=0.001, CV% 0.11). A similar effect was seen with PAI-1 with an early down-regulation at 6 hours (p=0.001, CV% 0.02) followed by a statistically significant up-regulation at 48 hours (p=0.001, CV% 0.21) (Figure 4.24). There were no significant effects on t-PA gene regulation.

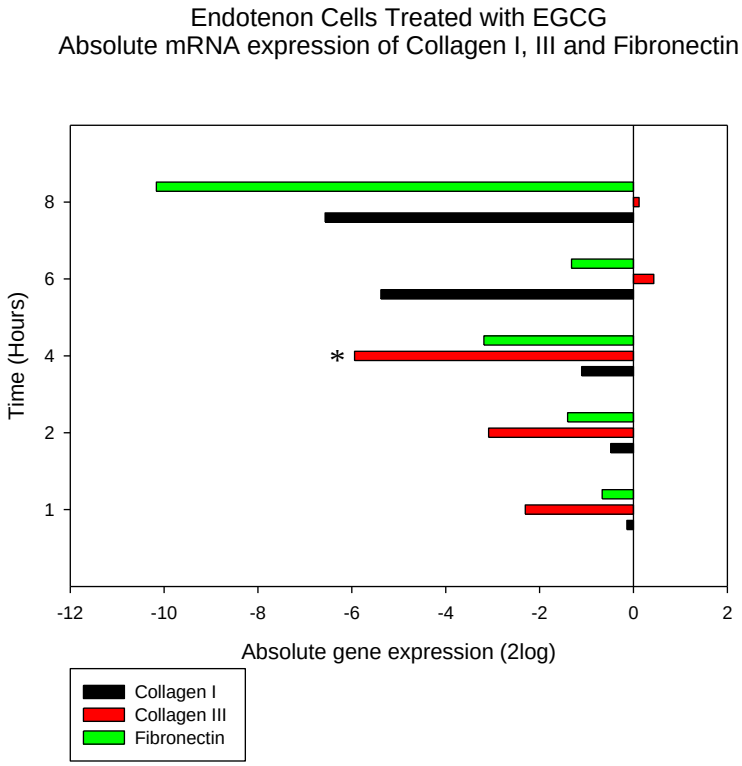
Pumactant

Endotenon cells treated with Pumactant (Figure 4.25) showed a statistically significant up-regulation of collagen type III at 24 (p=0.027, CV% 2.83) and 48 hours (p=0.025, CV% 2.17). Collagen type III was increased at all other time points but these changes were not significant. Conversely, collagen type I demonstrated down-regulated at all time points but this effect was not statistically significant possibly due to high variability between cells (CV% range 14.76-36.99). PAI-1 also showed a statistically significant up-regulation (Figure 4.26) at 1 and 2 hours (p=0.001, CV% 0.47 and 0.22 respectively). Followed by a significant down-regulation at 6, 8, and 16

hours (p=0.001, CV% 0.08, 0.53 and 1.26 respectively). There were no significant effects on fibronectin or t-PA gene regulation in endotenon derived cells.

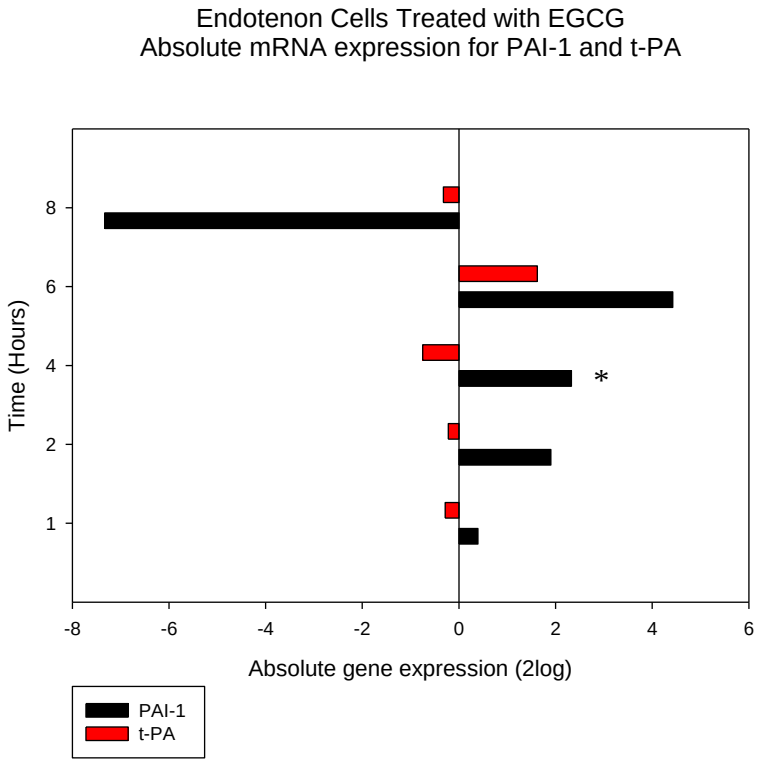
Epitenon cells showed no statistically significant changes in gene expression when treated with Pumactant (Figure 4.27 and 4.28). However, there was an observed down-regulation of collagen type I at all time points though there was also a high variability between cells (CV% range 8.57-15.49). There was also a marked down-regulation of t-PA (CV% range 17.35-21.92) synchronous with an up-regulation of PAI-1 seen at all time points (not statistically significant).

Tendon sheath cells when treated with Pumactant (Figure 4.29) showed a marked increase in fibronectin at all time points but this effect was not statistically significant (high variance; CV% range 24.61-35.27). There was a statistically significant down-regulation of PAI-1 at 1 hour (p=0.001, CV% 0.79). As with epitenon cells, there was a down-regulation of t-PA at all time points but this was not statistically significant (Figure 4.30).



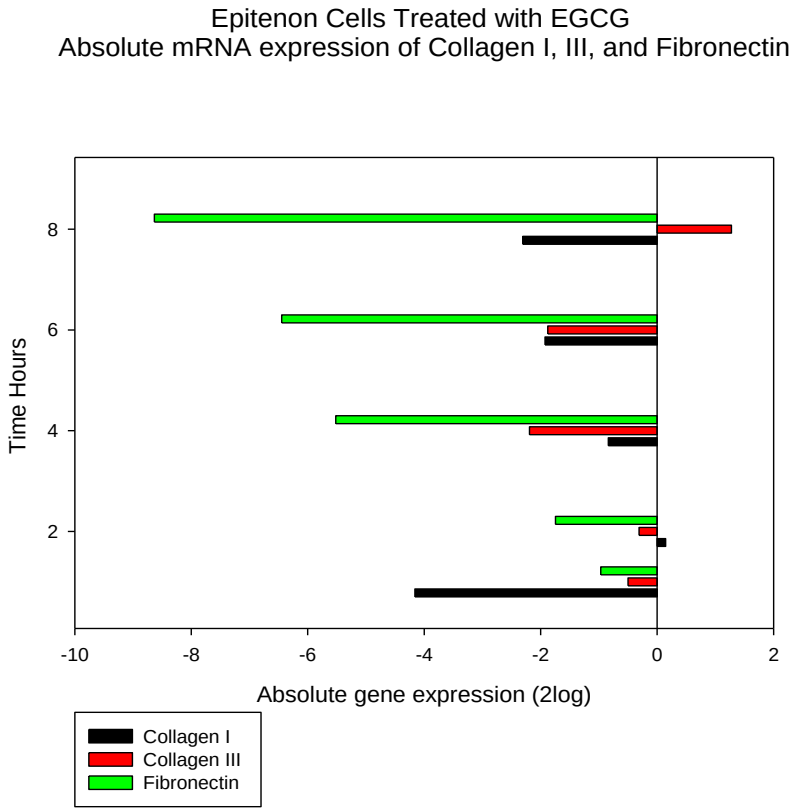
Time	Col I	Col III	Fn
8	12.23	0.361	5.01
6	20.52	0.01	1.76
4	6.94	3.82	5.89
2	0.74	2.4	3.21
1	1.24	2.62	4.67

Figure 4.13 The absolute gene regulation of endotenon cells (n=3) from basal levels induced by EGCG (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for collagen type I, collagen type III and fibronectin. The table demonstrates the coefficient of variation, CV%, between each gene. * = Statistical Significance (p=0.012).



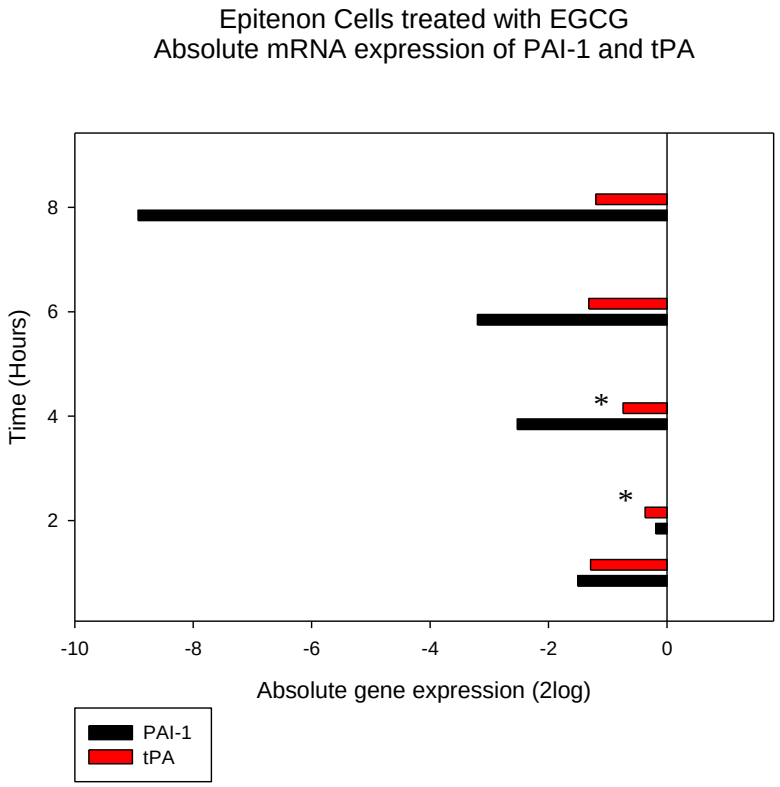
Time	PAI-1	t-PA
8	2.29	0.36
6	0.48	2.84
4	0.91	0.29
2	1.77	2.38
1	2.32	3.16

Figure 4.14 The absolute gene regulation of endotenon cells (n=3) from basal levels induced by EGCG (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for PAI-1 and t-PA. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance (p=0.008).



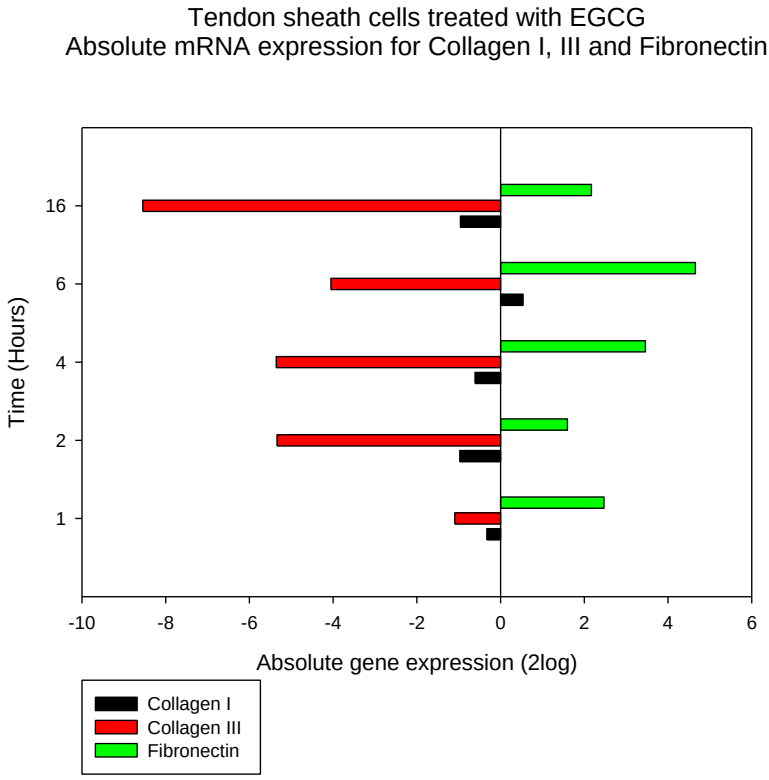
Time	Col I	Col III	Fn
8	2.74	0.74	1.93
6	0.62	0.64	5.11
4	2.74	0.92	1.82
2	2.27	0.03	1.6
1	11.25	0.01	11.06

Figure 4.15 The absolute gene regulation of epitenon cells (n=3) from basal levels induced by EGCG (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for collagen type I, collagen type III and fibronectin. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance.



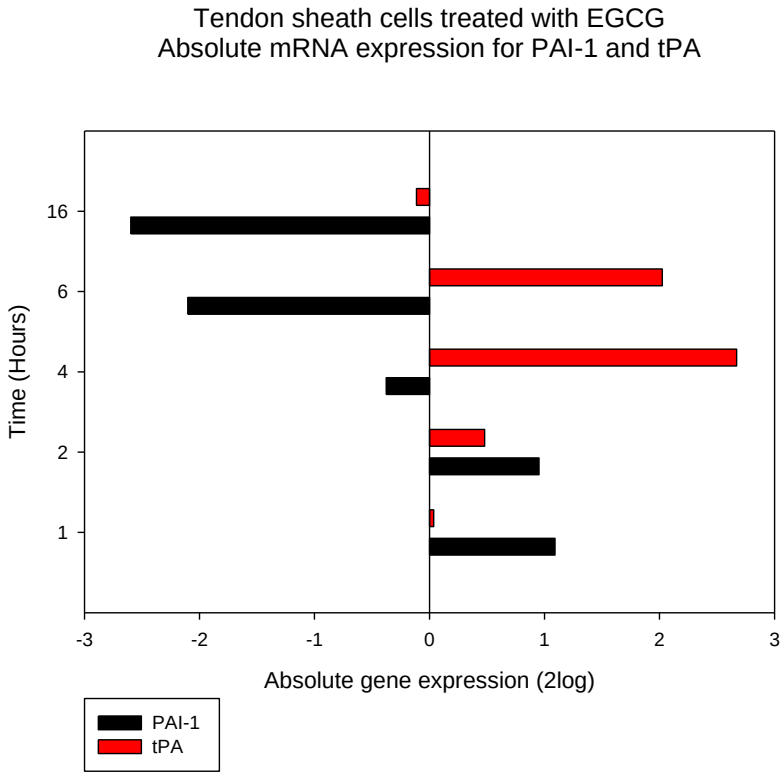
Time	PAI-1	t-PA
8	1.86	3.73
6	1.96	2.06
4	3.26	2.25
2	2.89	3.91
1	7.03	3.11

Figure 4.16 The absolute gene regulation of epitenon cells (n=3) from basal levels induced by EGCG (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for PAI-1 and t-PA. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance (p=0.023 at 2 hours, p=0.022 at 4 hours).



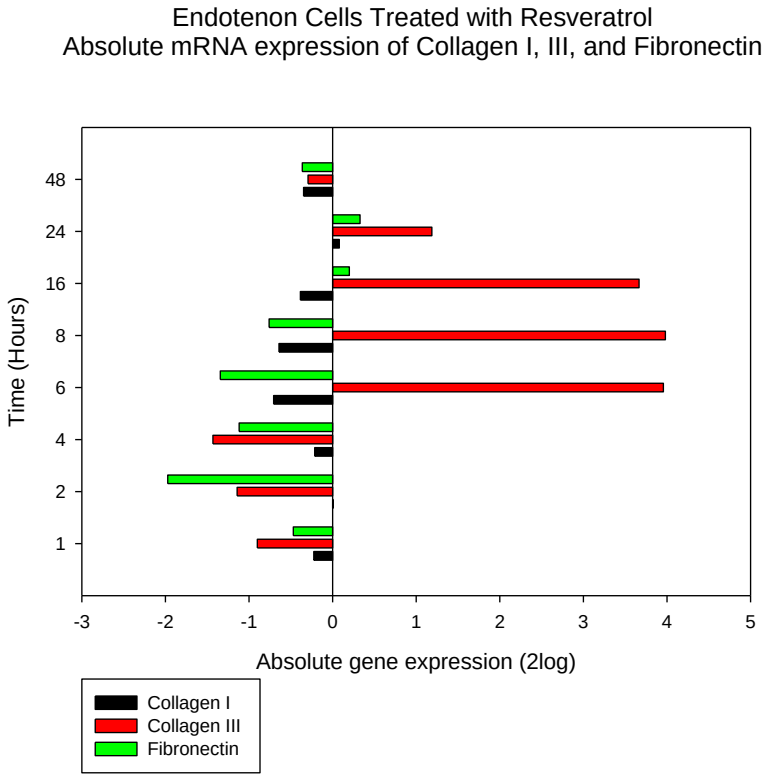
Time	Col I	Col III	Fn
16	0.63	6.47	5.31
6	1.59	12.72	7.79
4	2.92	4.66	6.24
2	4.89	8.6	4.84
1	2.93	9.71	3.65

Figure 4.17 The absolute gene regulation of tendon sheath cells (n=3) from basal levels induced by EGCG (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for collagen type I, collagen type III and fibronectin. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance.



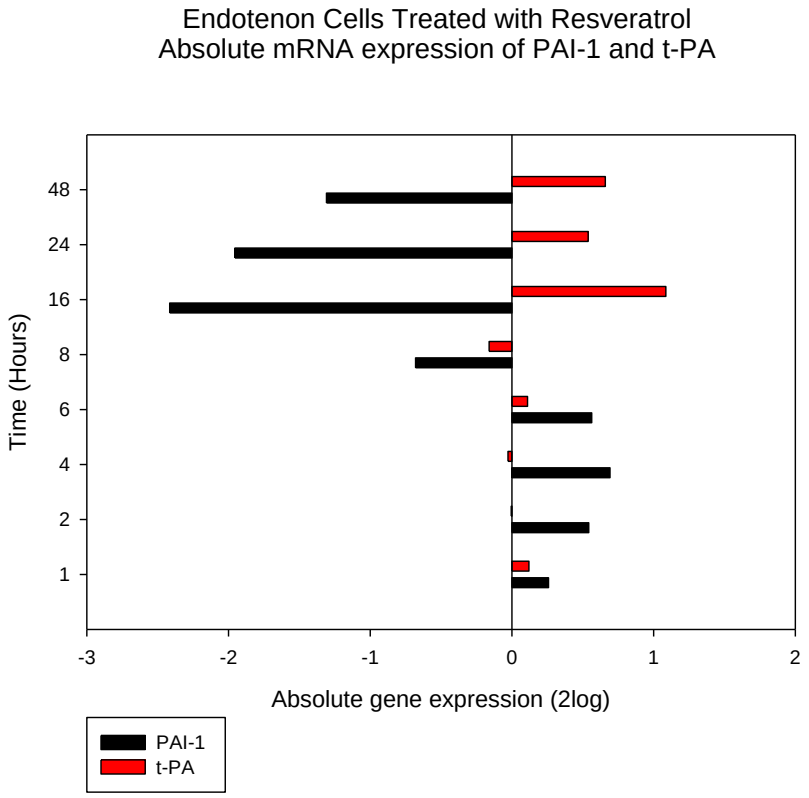
Time	PAI-1	t-PA
16	1.61	1.83
6	1.7	2.22
4	0.34	1.79
2	3.42	1.02
1	3.07	1.09

Figure 4.18 The absolute gene regulation of tendon sheath cells (n=3) from basal levels induced by EGCG (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for PAI-1 and t-PA. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance.



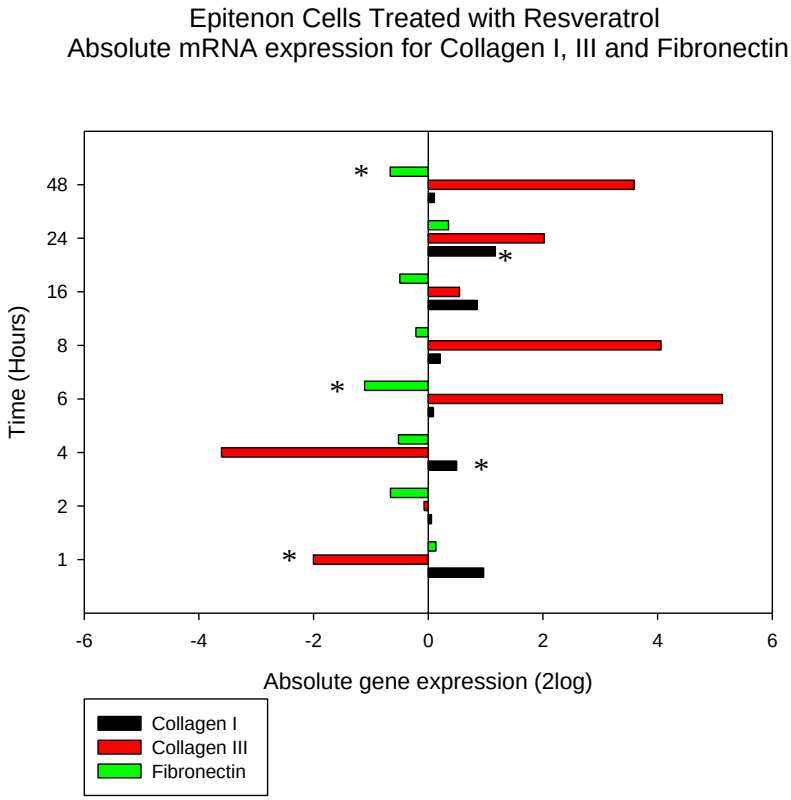
Time	Col I	Col III	Fn
48	0.61	3.01	1.39
24	0.87	3.57	3.49
16	2.13	5.71	7.38
8	0.22	3.99	2.75
6	2.35	6.64	1.16
4	0.22	5.69	3.77
2	1.74	5.31	1.61
1	1.87	6.53	4.23

Figure 4.19 The absolute gene regulation of endotenon cells (n=3) from basal levels induced by Resveratrol (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for collagen type I, collagen type III and fibronectin. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance.



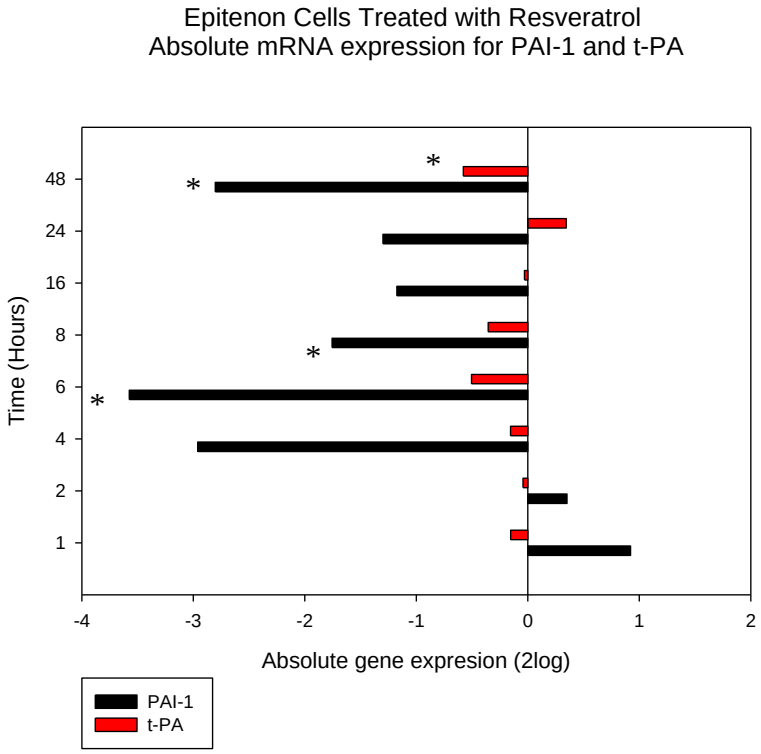
Time	PAI-1	t-PA
48	1.57	2.84
24	1.39	2.09
16	1.21	1.53
8	1.03	3.61
6	1.39	3.78
4	0.67	2.77
2	0.85	4.52
1	3.48	4.61

Figure 4.20 The absolute gene regulation of endotenon cells (n=3) from basal levels induced by Resveratrol (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for PAI-1 and t-PA. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance.



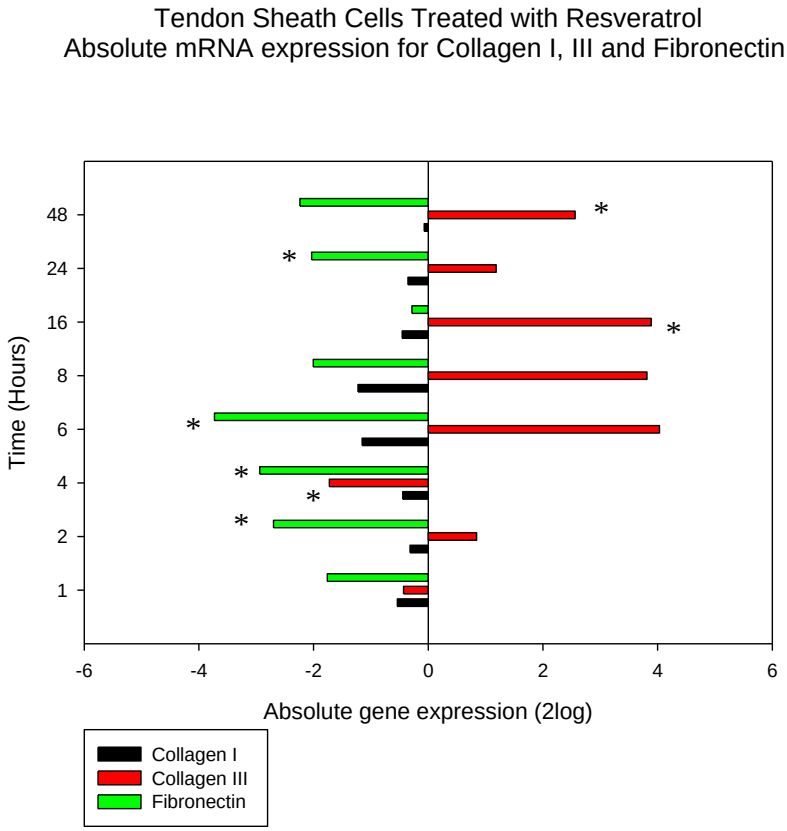
Time	Col I	Col III	Fn
48	0.24	2.55	0.06
24	0.45	3.83	0.44
16	0.82	1.13	0.92
8	2.64	2.66	1.48
6	0.94	5.71	0.73
4	0.04	5.83	0.09
2	2.34	0.81	1.98
1	0.07	1.61	1.47

Figure 4.21 The absolute gene regulation of epitenon cells (n=3) from basal levels induced by Resveratrol (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for collagen type I, collagen type III and fibronectin. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance (p=0.001).



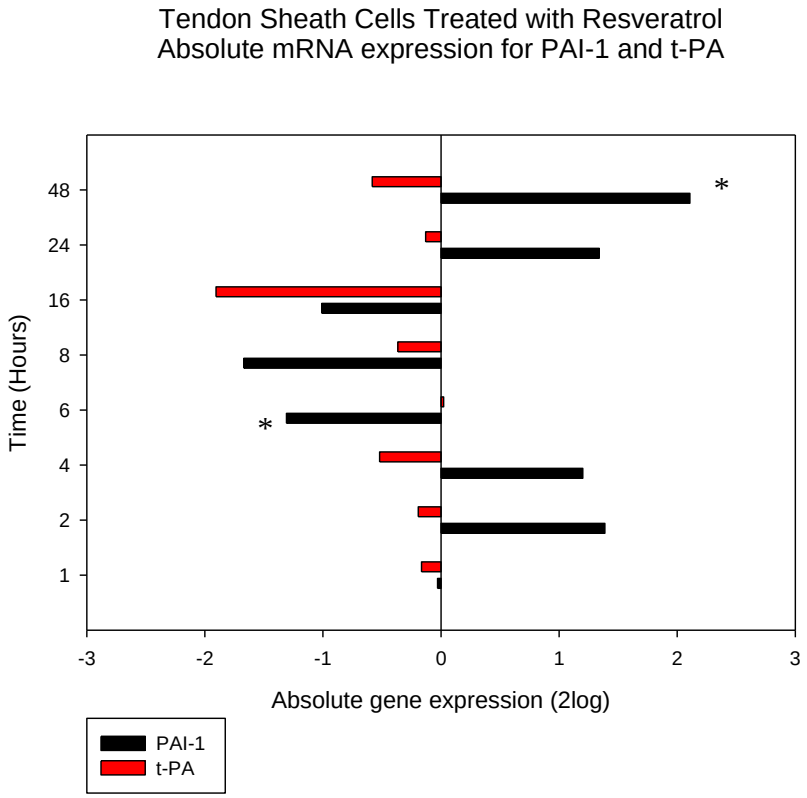
Time	PAI-1	t-PA
48	0.09	0.19
24	0.73	1.42
16	0.25	0.65
8	1.34	0.49
6	0.16	0.01
4	1.91	0.22
2	1.18	0.81
1	3.42	0.25

Figure 4.22 The absolute gene regulation of epitenon cells (n=3) from basal levels induced by Resveratrol (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for PAI-1 and t-PA. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance (p=0.001).



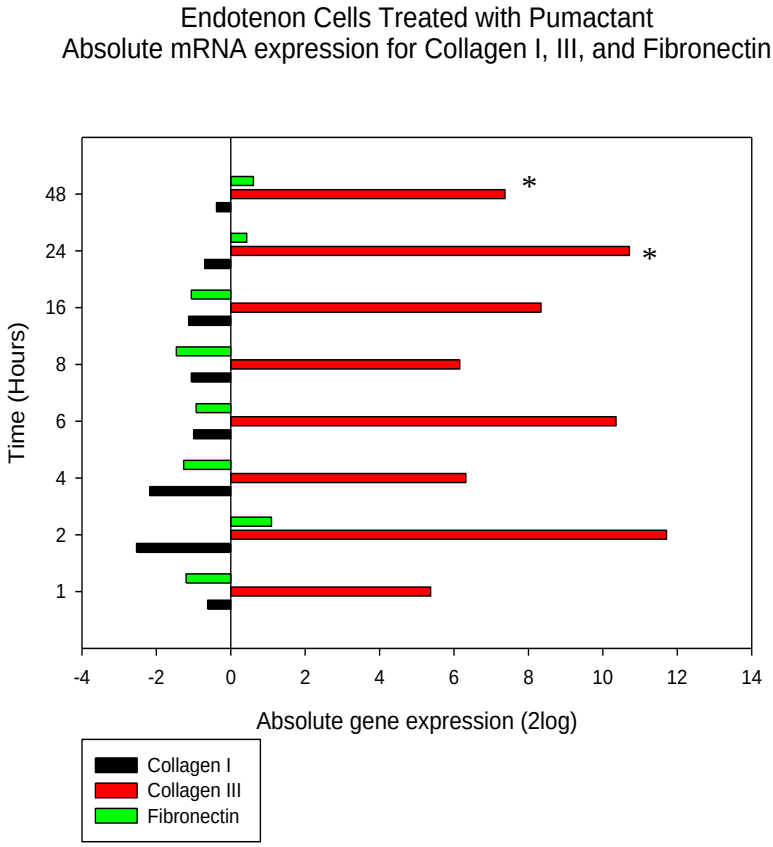
Time	Col I	Col III	Fn
48	0.11	0.21	1.79
24	0.21	0.11	4.62
16	1.67	4.39	0.74
8	0.03	4.09	3.29
6	0.51	0.38	1.72
4	0.16	0.11	2.94
2	0.19	0.12	2.77
1	0.92	1.61	2.76

Figure 4.23 The absolute gene regulation of tendon sheath cells (n=3) from basal levels induced by Resveratrol (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for collagen type I, collagen type III and fibronectin. The table demonstrates the coefficient of variation, CV%, between each gene. * = Statistical Significance (p=0.001).



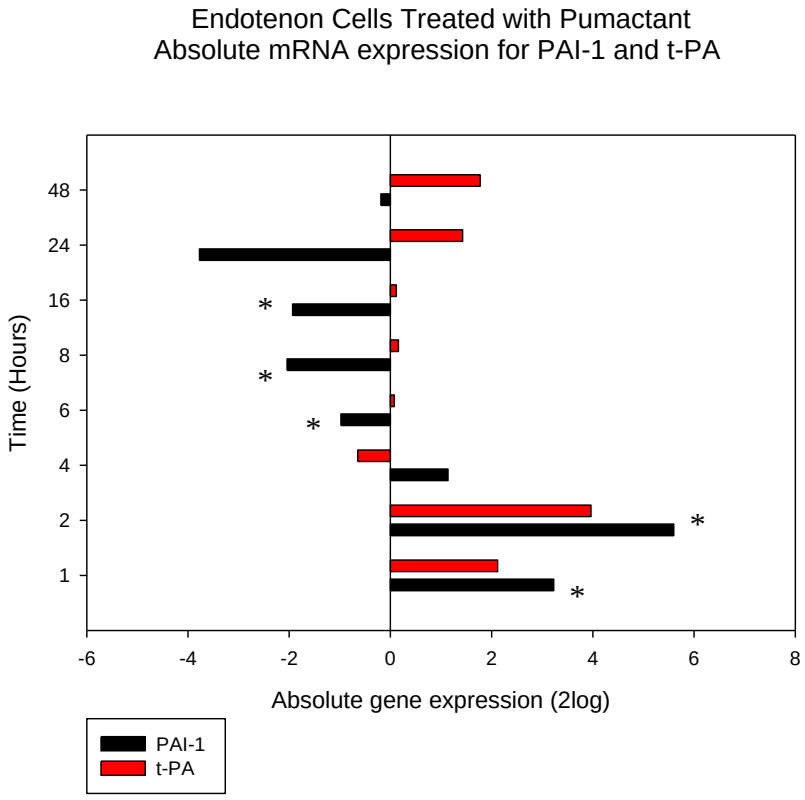
Time	PAI-1	t-PA
48	0.21	1.53
24	0.64	0.59
16	7.79	8.57
8	1.95	1.89
6	0.02	0.67
4	0.83	0.47
2	0.18	1.56
1	1.08	0.51

Figure 4.24 The absolute gene regulation of tendon sheath cells (n=3) from basal levels induced by Resveratrol (50μM) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for PAI-1 and t-PA. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance (p=0.001).



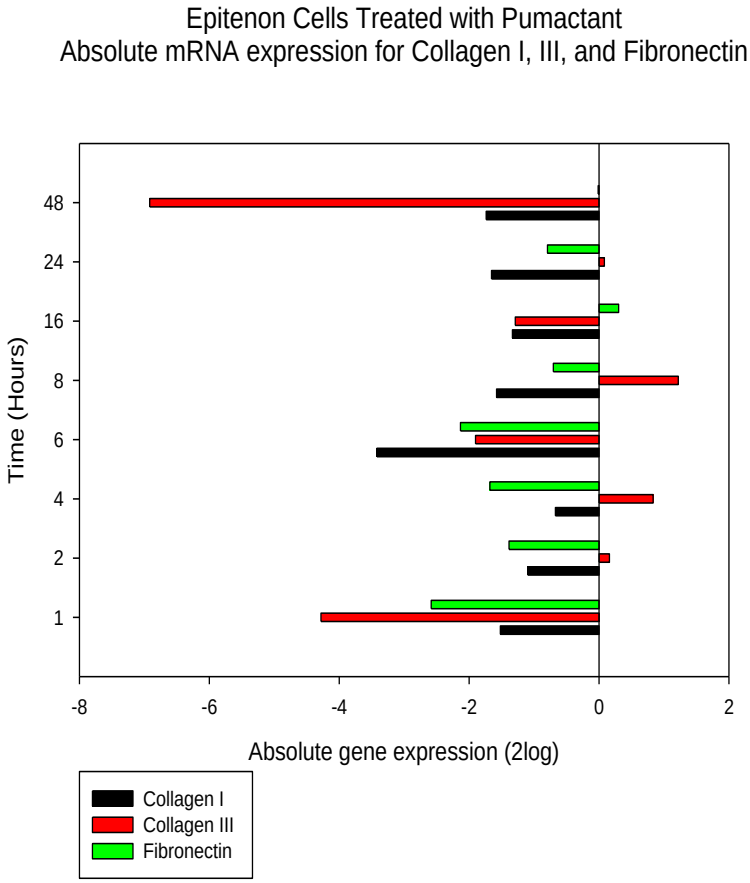
Time	Col I	Col III	Fn
48	21.54	2.17	12.25
24	16.92	2.83	11.97
16	17.84	2.34	11.65
8	15.53	11.71	12.99
6	19.71	9.48	13.31
4	14.76	12.74	13.69
2	36.99	6.79	14.12
1	25.83	7.66	18.99

Figure 4.25 The absolute gene regulation of endotenon cells (n=3) from basal levels induced by Pumactant (1mg/ml) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for collagen type I, collagen type III and fibronectin. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance (p=0.027 at 24 hours, p=0.025 at 48 hours).



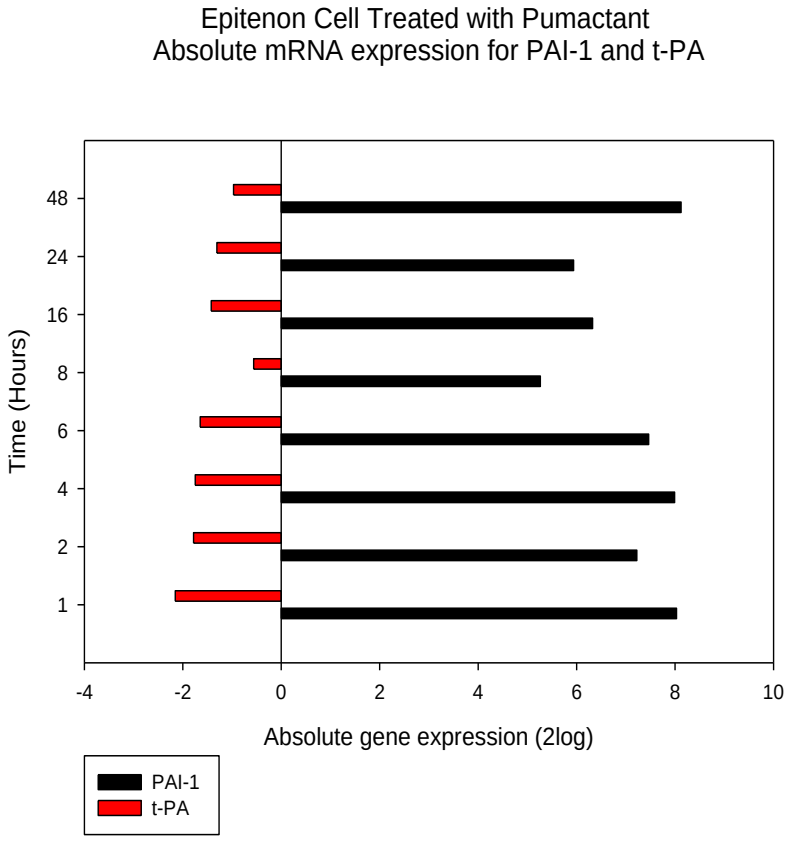
Time	PAI-1	t-PA
48	0.04	0.71
24	7.16	0.61
16	1.26	0.38
8	0.53	0.89
6	0.08	0.17
4	0.64	0.91
2	0.22	1.25
1	0.47	2.05

Figure 4.26 The absolute gene regulation of endotenon cells (n=3) from basal levels induced by Pumactant (1mg/ml) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for PAI-1 and t-PA. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance (p=0.001).



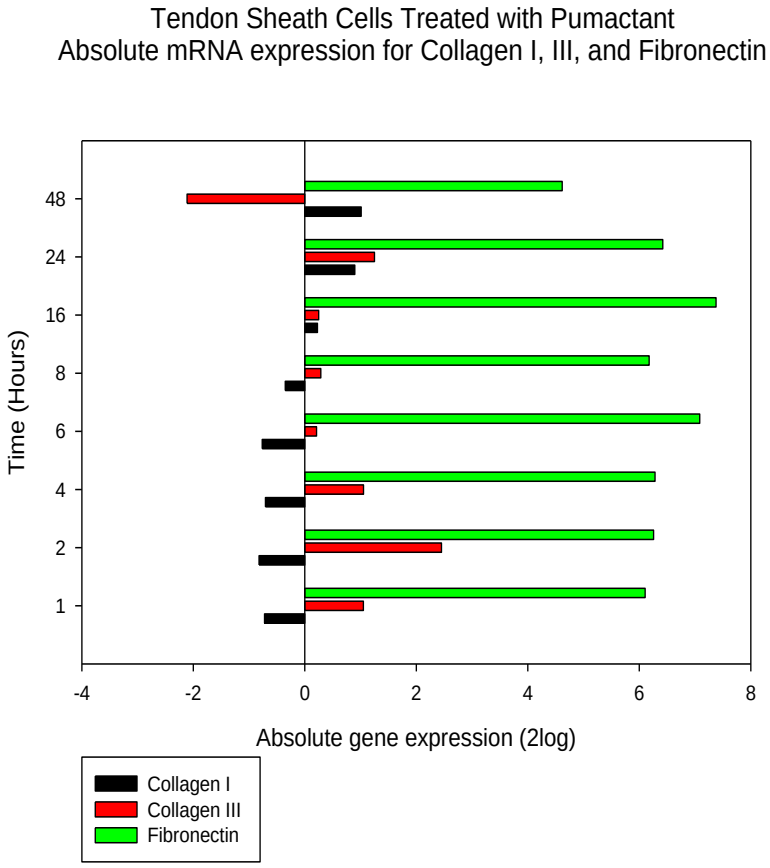
Time	Col I	Col III	Fn
48	11.73	19.29	9.24
24	15.31	11.49	8.89
16	15.49	10.67	8.87
8	13.45	0.75	8.88
6	8.57	6.36	8.86
4	15.17	6.63	15.35
2	14.39	4.41	16.78
1	15.48	17.81	18.03

Figure 4.27 The absolute gene regulation of epitenon cells (n=3) from basal levels induced by Pumactant (1mg/ml) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for collagen type I, collagen type III and fibronectin. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance.



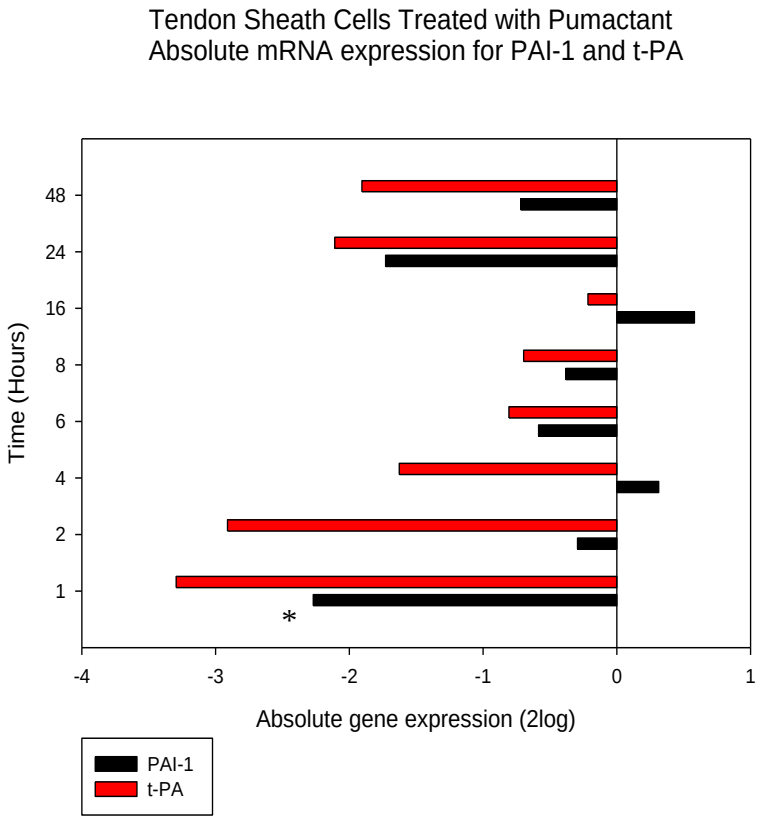
Time	PAI-1	t-PA
48	0.59	19.33
24	0.08	18.85
16	0.75	18.56
8	0.54	21.92
6	0.87	17.89
4	1.03	17.76
2	0.93	18.39
1	1.53	17.35

Figure 4.28 The absolute gene regulation of epitenon cells (n=3) from basal levels induced by Pumactant (1mg/ml) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for PAI-1 and t-PA. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance.



Time	Col I	Col III	Fn
48	13.92	4.23	23.24
24	11.95	6.24	24.61
16	16.55	3.81	35.27
8	13.59	4.67	28.95
6	10.96	2.75	27.34
4	12.77	4.74	28.38
2	11.78	6.19	27.74
1	14.76	2.29	27.23

Figure 4.29 The absolute gene regulation of tendon sheath cells (n=3) from basal levels induced by Pumactant (1mg/ml) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for collagen type I, collagen type III and fibronectin. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance.



Time	PAI-1	t-PA
48	0.02	5.86
24	0.04	5.74
16	0.05	3.75
8	0.12	5.27
6	0.09	5.88
4	0.05	5.44
2	0.09	9.15
1	0.79	5.61

Figure 4.30 The absolute gene regulation of tendon sheath cells (n=3) from basal levels induced by Pumactant (1mg/ml) is plotted over time (normalised with two house keeping genes: GAPDH and β-Actin) for PAI-1 and t-PA. The table demonstrates the coefficient of variation, CV%, between each gene. * =Statistical Significance (p=0.001).

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.1 Endotenon cells in response to TGF-β1. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.2 Epitenon cells in response to TGF-β1. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.3 Tendon Sheath cells in response to TGF-β1. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.4 Endotenon cells in response to EGCG. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.5 Epitenon cells in response to EGCG. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.6 Tendon sheath cells in response to EGCG. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.7 Endotenon cells in response to Resveratrol. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.8 Epitenon cells in response to Resveratrol. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.9 Tendon sheath cells in response to Resveratrol. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.10 Endotenon cells in response to Pumactant. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.11 Epitenon cells in response to Pumactant. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

	1h	2h	4h	6h	8h	16h	24h	48h
Collagen I								
Collagen III								
Fibronectin								
PAI-1								
t-PA								

Table 4.12 Tendon Sheath cells in response to Pumactant. Grey shows no statistical change in gene transcription. White demonstrates a statistically significant up-regulation in gene transcription whereas black demonstrates a statistical significant down-regulation in gene transcription.

4.4. Discussion

The purpose of this investigation was to test our three proposed tendon anti-adhesion treatments *in vitro* to discover their potential to prevent adhesions and optimise the environment for tendon healing. This was assessed by first performing toxicology studies on cultured tendon cells derived of different origin (endotenon, epitenon and tendon sheath) to determine the appropriate doses to be used in later experiments. A cell adhesion assay was used to determine if treatment affected the cell binding properties of each cell type to different extracellular matrix components. Unfortunately, there are no ideal *in vitro* models of tendon healing or adhesion formation. However, the methods used in this study are based on similar studies (Berglund et al., 2006; Klein et al., 2002) investigating mRNA expression of various extracellular matrix components in flexor tendons following injury.

4.4.1 EGCG

EGCG is the major polyphenol and most potent antioxidant found in green tea (Ahmad and Mukhtar, 1999) and has been associated with a widespread biological activity (Cooper et al., 2005; Nagle et al., 2006). EGCG has been shown to play a role in treating a number of diseases (Suzuki and Isemura, 2001; Abe, 2007) including the reduction of fibrosis in a number of organs and improving scarring in wound healing (Kapoor et al., 2004; Nakamuta et al., 2005; Zhang et al., 2006; Klass et al., 2010).

Epitenon cells showed a statistically significant increase in proliferation with EGCG at day 7 and day 10 with doses from 12.5μM-50μM. At higher concentrations there was an increase in toxicity. Although tendon sheath cells showed a slight reduction in proliferation at day 7 with a dose of 50μM, this dose was selected for further work as endotenon and epitenon cells showed no significant toxicity. Also, other studies have used a dose of 50μM to achieve non-toxic effects in stromal fibroblasts and activated hepatic stellate cells (Hung et al., 2005; Chen et al., 2002; Nakamuta et al., 2005).

EGCG has shown that it has an ability to prevent the adhesion of a number of cell types particularly tumour cells to various components of the matrix (Sen et al., 2009; Lo et al., 2007). This has also been demonstrated in stromal fibroblasts which following pre-treatment with EGCG were unable to adhere to collagen type I, fibronectin and fibrinogen (Hung et al., 2005). This current study also showed that

fibroblasts derived from the tendon sheath showed a statistical significant reduction in adhesion to collagen type I following treatment with EGCG, though other tendon derived cells showed no significant difference. Hung et al, demonstrated that this was through EGCG interfering with α2β1 the major collagen type I receptor. EGCG treated endotenon and epitenon cells, however, showed no difference with adhesion to collagen type I, it is possible that they express more α1β1 also a collagen type I receptor. Others have shown in tendons derived from the patellar that fibroblasts from outside the tendon complex express different integrins than fibroblasts from within (Ikema et al., 2005). Unlike Hung et al., (2005) in this current study EGCG showed no effect on fibroblast adhesion to fibronectin, it remains unclear why EGCG appears not to alter the binding of fibroblasts derived from the tendon-sheath complex to fibronectin. Interestingly, both endotenon and epitenon derived cells showed a statistical significant increase in adhesion to collagen type III following treatment with EGCG. Collagen type III binds by both α1β1 and α2β1 and is known to have a novel binding site, which may explain the difference (Kim et al., 2005). Interestingly, EGCG prevented any results being obtained from QRT-PCR over the long time course. Others have suggested (Kuzuhara et al., 2006) that EGCG can bind to both DNA and RNA *in vitro*. It is therefore feasible that in the current QRT-PCR the EGCG had bound to the RNA and therefore prevented the amplification of the genes being studied when the cells were cultured with EGCG over a long time course.

Scar tissue, including adhesion formation, is characterized by the excessive production of collagen. However, collagen production is also a major component of tendon healing; therefore equilibrium must be maintained. EGCG down-regulated all the extracellular matrix genes being studied in endotenon and epitenon cells, though only collagen type III showed a statistically significant reduction when compared to quiescent untreated cells. Sheath derived cells also showed a down-regulation of both collagen types, but an up-regulation of fibronectin though none reached statistical significance. Other experimental studies have shown that EGCG inhibits collagen type I production in a number of cell types (Zhang et al., 2006; Nakamuta et al., 2005; Hashem et al., 2008) even when the cells are exposed to a fibrotic stimuli (Klass et al., 2010). In particular, collagen type I mRNA and protein levels were reduced in a dose-dependent manner with *in vitro* EGCG treated keloid fibroblasts (via inhibition of the STAT3-signalling pathway) and remarkably the same group showed suppression of

nodule development and collagen production in an *in vivo* keloid model (Park et al., 2008). However, others have suggested that the collagen that is synthesised is of a better quality as EGCG appears to improve the maturity and organisation of the collagen fibres (Kapoor et al., 2004; Kim et al., 2008). Also, in human tendon fibroblasts, EGCG has been shown to reduce the IL-1 stimulated expression of collagenase mRNA (mediated by inhibition of the JNK/SAPK pathway), perhaps leading to higher collagen levels (Corps et al., 2004).

Low levels of t-PA and high levels of PAI-1 are associated with peritoneal adhesion formation (Cheong et al., 2001; Ivarsson et al., 1998). PAI-1 is associated with adhesion formation as increased levels leads to excess fibrin deposition which in turn is believed to act as a scaffold for adhesion formation (Wojciak and Crossan, 1993). EGCG appeared to have little effect on PAI-1 gene expression with only endotenon derived cells showing a significant up-regulation at 4 hours. However, others have shown that green tea polyphenols inhibit PAI-1 expression and its release from endothelial cells (via PI3K/Akt pathway) (Liu et al., 2009).

4.4.2 Resveratrol

Resveratrol (trans-3, 4, 5-trihydroxystilbene) is a natural polyphenolic phytoalexin found in some plants including grapes and their related products. Resveratrol has been shown to reduce adhesions in animal models of peritoneal (Sogutlu et al., 2006) and pelvic (Ustűn et al., 2007) adhesion formation. A further study showed that Resveratrol can prevent fibrosis in a rat cirrhosis model, and that its action is probably associated with its ability to reduce NF-kappa activation and thus reduce levels of profibrinogenic TGF-β (Chavez et al., 2007). There are close associations between wound healing, fibrosis, excess scarring and adhesion formation. Hence, following these studies it was deemed worthwhile investigating the effects of Resveratrol on tendon healing and whether peri-tendinous adhesion formation can be reduced.

Resveratrol is known to prevent the proliferation in a number of cell types and this was seen in this current study. However, unexpectedly, there was a statistically significant increase in proliferation at day 10 with 1000μM of Resveratrol. This could be due to experimental error as at high concentrations, Resveratrol was found to form precipitate which may not have been removed during the washing stage. Resveratrol has been found to prevent the adhesion of some cell types to collagen and fibrinogen

(Olas et al., 2002; Cao et al., 2005). In this current study Resveratrol also reduced the adhesion of sheath and epitenon derived tendon cells to collagen type I but there was no effect on adhesion to collagen type III and fibronectin in any cell type. There have been no studies on the effect of Resveratrol on the integrins involved in collagen adhesion or fibronectin, though the integrin αvβ3 has been shown to contain a binding site for Resveratrol (Lin et al., 2006).

In a mouse dermal wound healing model, Resveratrol treatment has been associated with enhanced formation of epidermis, deposition of connective tissue and improved histological architecture (Khanna et al., 2002) which implies an organised production of ECM collagen. However, in cultured human liver fibroblasts, although Resveratrol reduced proliferation of myofibroblasts and decreased expression of alpha smooth muscle actin (associated with reduced scarring and fibrosis) there was inhibition of expression of type I collagen mRNA (Godichaud et al., 2000) and this was also seen in osteoblast-like cells (Rucinski et al., 2006). However, this current study showed a statistically significant up-regulation of collagen type I (at 4 and 24 hours) in epitenon derived cells and up-regulation of collagen type III in sheath derived cells (at 16 and 48 hours).

Epitenon cells treated with Resveratrol showed a statistically significant down-regulation of fibronectin (6 and 48 hours). Similarly, tendon sheath cells treated with Resveratrol showed a statistically significant down-regulation of fibronectin (2, 4, 6, and 24 hours). Recently, Resveratrol has been shown to reduce fibronectin protein synthesis in glomerular epithelial cells (Lee et al., 2010). A low level of fibronectin has a therapeutic advantage as there would be less scaffolding for tendon adhesion formation (Wojciak and Crossan, 1993).

Resveratrol has been shown to reduce PAI-1 gene expression in adipocytes (Ahn et al., 2007). This finding was mirrored in this current study with epitenon and sheath derived cells showing a statistically significant down-regulation, with the exception at the long time course in sheath cells at 48 hours. This equates to more fibrinolysis around the site of tendon injury at early and late time points. In theory, an increase in fibrinolysis leads to reduced fibrin and less adhesion formation. Also, an increase in t-PA at 48 hours corresponds well with a down-regulation of PAI-1 at the same time

point and may be synergistic to a reduction in adhesion formation. This result is expected as physiologically t-PA and PAI-1 are opposing forces in the fibrinolytic pathway. In addition polyphenolics, including Resveratrol, have been shown in cultured human endothelial cells to increase t-PA protein and mRNA levels (RT-PCR), concomitant with an increase in sustained (24 hours) surface localised fibrinolytic activity (Abou-Agag et al., 2001). This increase in local fibrinolytic activity, has labelled Resveratrol together with ethanol, as the responsible compounds for reducing cardiovascular risk associated with moderate wine consumption (Pasten et al., 2006). Moreover, Resveratrol has been shown to have a direct inhibitory action on cardiac fibroblasts (inhibition of cardiac proliferation and myofibroblast differentiation) and might be useful in inhibiting the progression of cardiac fibrosis (Olson et al., 2005).

4.4.3 Pumactant

Pumactant is a mixture of two naturally occurring phospholipids: a 7:3 mixture of dipalmitoylphosphatidylcholine and unsaturated phosphatidylglycerol. In a murine peritoneal adhesion model it has been shown that Pumactant produced a dose-dependent reduction in adhesion formation when applied to the caecum and sidewall following surgery (unpublished studies at Cardiff University). A number of publications have either identified phospholipids in joints, tendons or tendon sheath fluid (Mills et al., 2005; Hills et al., 1996), or more specifically shown a role for phospholipids in the prevention of tendon adhesion formation using animal models (Moro-oka et al., 2000). Pumactant could act as a boundary lubricant in improving tendon glide, as a barrier preventing adhesions, and may show advantages in cell proliferation and tendon healing.

Endotenon cells treated with various concentrations of Pumactant showed a statistically significant increase in proliferation on day 3 (10mg/ml). Epitenon cells treated with Pumactant also showed a statistically significant increase in proliferation at day 3 (1mg/ml and 8mg/ml) and day 10 (1mg/ml). Although, tendon sheath cells treated with Pumactant showed a statistically significant reduction in proliferation at day 7 (2mg/ml) there was a statistically significant increase in proliferation at day 10 (10mg/ml). In accordance with this study, Pumactant has been shown to accelerate mesothelial and keratinocyte cell proliferation. This proliferation appeared to be

unique to this specific compound, as other phospholipids appear to inhibit cell proliferation and cause a reduction in cell viability (Kremlev et al., 1994; Topley and Woodcock, 2010).

No matrix or cell type showed any statistical significant difference when cells were pre-treated with Pumactant. Phospholipids in other studies have been shown to inhibit gastric cancer cell adherence to collagen type IV, laminin and fibronectin (Jansen et al., 2004). The mechanism of action was unknown but may be due in part to the barrier properties of phospholipids. This current study did not show the same effect.

Endotenon cells treated with Pumactant showed a statistically significant up-regulation of collagen type III mRNA at late time points (24 and 48 hours). Collagen type III is produced in the initial stages of tendon healing and later this is replaced with collagen type I. An increase in collagen type III gene expression in endotenon cells at the core of the tendon may allow for optimisation of tendon healing. There was also a statistically significant up-regulation of PAI-1 at early time points (1 and 2 hours) which was followed by a significant down-regulation (6, 8, and 16 hours). Also, tendon sheath cells when treated with Pumactant showed a statistically significant down-regulation of PAI-1 at 1 hour. Overall there appears to be a reduction in PAI-1 between the different cell types which may be beneficial in preventing adhesions, as already described (Cheong et al., 2001; Ivarsson et al., 1998). To date there has been no previous work studying the effect of Pumactant on gene expression in tendons or any other tissues.

4.5 Conclusions

- The optimal *in vitro* doses for each treatment based on this study and the current literature are as follows: EGCG 50μM, Resveratrol 50μM and Pumactant 1mg/ml.

- EGCG caused increased adhesion of pre-treated endotenon and epitenon cells to collagen type III. Conversely, there was decreased adhesion of EGCG pre-treated sheath cells to collagen type I.

- Resveratrol caused reduced adhesion of pre-treated epitenon and tendon sheath cells to collagen type I.

- Treatment with Resveratrol caused the most optimal changes to gene expression as collagen type I, collagen type III and t-PA were up-regulated, whereas fibronectin and PAI-1 were down-regulated. These changes were seen mainly in epitenon and tendon sheath cells.

- Pumactant treated endotenon cells up-regulated collagen type III gene expression at late time points and there was an increase in PAI-1 gene expression early but then a later down-regulation. There was also a down-regulation of PAI-1 in tendon sheath cells. These results indicate a moderate therapeutic advantage.

Tendon healing and the formation of adhesions is a multi-factorial process with many different biological pathways involved. This study and work of others has alluded to some of the mechanisms involved but does not complete our understanding. Following our work with TGB-β1 and our three potential treatments *in vitro* we next developed an *in vivo* model to further assess the efficacy of each treatment.

CHAPTER 5

THE *IN VIVO* FLEXOR TENDON RESPONSE TO THREE NOVEL ANTI-ADHESION TREATMENTS:

EPIGALLOCATECHIN-3-GALLATE (EGCG)

RESVERATROL

& PUMACTANT

5.1 Introduction

Having studied the *in vitro* effects of EGCG, Resveratrol and Pumactant using tenocytes from different areas of the tendon-sheath complex, this study aims to assess the effects on flexor tendon adhesion formation using a standard *in vivo* model. The latter is essential to prove efficacy and safety prior to human trials. From Chapter 4 it is clear that all three treatments show potentially beneficial changes in flexor tendon cells. The Resveratrol-treated group showed the most optimal effects when examining changes in genetic expression (up-regulation of collagen type I, collagen type III and t-PA with a down-regulation of fibronectin and PAI-1). These effects were mainly found in the epitenon and tendon sheath cells i.e. the tendon-sheath interface. However, Pumactant had more of an influence on the endotenon cells (gene up-regulation of collagen type III and a late down-regulation of PAI-1) and some effect on tendon sheath cells (down-regulation of PAI-1). Although, EGCG showed no significant changes in genetic expression there were positive findings in our cell adhesion study (i.e. a reduction of epitenon cell adhesion to fibronectin). There was certainly evidence to justify further investigation of all three treatments using a flexor tendon *in vivo* model.

The rabbit model has been used in many previous studies and is well established (Abrahamsson et al., 1989; Abrahamsson et al., 1991; Chang et al., 1997; Chang et al., 1998; Kang and Kang, 1999; Kakar et al., 1998; Khan et al., 1996; Manske et al., 1984; Matthews and Richards, 1976; Moro-oka et al., 2000; Rank et al., 1980). In particular, this model has been used at RAFT by previous researchers and techniques have been accordingly modified and refined (Branford et al., 2008; Jones et al., 2000; Jones et al., 2002).

5.1.1 Hypothesis

The aim of this chapter was to test the hypothesis:

'The three novel compounds, EGCG, Resveratrol and Pumactant are able to reduce or modify flexor tendon adhesion formation using an in vivo model'.

5.1.2 Objectives

- Selection and design of an appropriate flexor tendon adhesion animal model.

- Evaluation of each treatment using mechanical testing.
- Evaluation of each treatment using histological scoring methods.

5.2 Materials and Methods

All animal care complied with the "UK Home Office Guide for the Care and the Use of Laboratory Animals" 1996. New Zealand White (NZW) Rabbits were anaesthetised for Surgery using general anaesthesia with recovery. A partial tenotomy was conducted on digits 2 and 4 of the right forepaw of 8 NZW rabbits in each treatment group, with injured untreated tendons acting as adhesion controls (n=8). All injured tendons were immobilised via a proximal tendon transection. Only one paw per rabbit was subjected to an operation so as to minimize suffering to the animal after recovery from anaesthesia. The left paw acted as the unoperated untreated control.

5.2.1 Mode of Anaesthesia

General anaesthesia was induced as previously described (Chapter 2, section 2.4.1).

5.2.2 Preparation of Treatments

EGCG

A 0.45mg/ml (1mM) solution of EGCG was dissolved in sterile water and incubated for 1hour at 37ºC in a water bath to ensure adequate dispersal. The solution was then drawn up into a 1ml sterile syringe immediately prior to surgery in preparation for infiltration.

Resveratrol

A 2.28mg/ml (10mM) solution of Resveratrol was dissolved in sterile water and incubated for 1hour at 37ºC in a water bath to ensure adequate dispersal. The solution was then drawn up into a 1ml sterile syringe immediately prior to surgery in preparation for infiltration.

Pumactant

A 10mg/ml (unknown molecular weight) solution was dissolved in sterile water in a sterile 50ml container. Following brief agitation the sample was incubated for 1 hour

at 37ºC in a water bath to ensure adequate dispersal. The solution was then drawn up into a 1ml sterile syringe immediately prior to surgery in preparation for infiltration.

5.2.3 Operative Procedure

The procedure is fully described in Chapter 2, section 2.4. Following induction of general anaesthesia, a V-Shaped incision was made in the palmar aspect of the right forepaw, to access both flexor digitorum profundus (FDP) tendons and to avoid direct injury to the synovial sheath. With a scalpel a partial tenotomy was performed (half the width of the tendon and 5mm in length). To immobilise each specific tendon, a proximal transection was performed at the level of the carpus. The tendon sheaths were then infiltrated with 0.5mls of treatment solution (H_2O, EGCG, Resveratrol or Pumactant solutions as described above) and the wounds were closed. The partial tenotomy controls received no treatment or water only (as diluent for all treatments). Two weeks later the rabbits were humanely killed by anaesthetic overdose (Schedule 1). Tendons and associated tissues were harvested at post mortem. Harvested digits were then randomised to:

1. Mechanical Assessment of Adhesion Strength.

2. Histological Analysis.

5.2.4 Mechanical Assessment of Adhesion Strength

Forepaws were freshly assessed (as described in Chapter 2, section 2.4.2). Prior to testing the V-shaped wounds were re-opened and the proximal free ends of each treated tendon were identified. A distal transection was made over the insertion of the FDP into the distal phalanx. This allows the tendon to lie freely within the sheath, disconnected at either end such that a force applied proximally would measure solely the strength of any adhesion between the tendon and the sheath. The digital claw was held using a clamp and the proximal FDP tendon transfixed to a tensile testing machine (Mecmesin). The proximal tendon was then pulled from its sheath (at a rate of 5mm/min) until the adhesion failed. The peak force was measured in Newtons.

5.2.5 Histological Analysis

The tendon-adhesion complex of each digit was fixed and paraffin embedded (as described in Chapter 2, section 2.4.3). Digits were divided into halves in the sagittal plane, and 5 micron sections were cut from longitudinal equally spaced zones. The area of adhesion or zone of injury was selected and sections were concentrated around this.

Haematoxylin and Eosin staining was performed (Appendix I). Presence of a characteristic dense cellular band, connecting tendon to the surrounding tissues was recorded as an adhesion. A semi-quantitative method using an adhesion score, as described by Tang et al. (1996) was used to further characterise adhesion formation. This scoring system combines the scores for quantity (0-3) and quality (0-3) to give an overall grading score (0-6) for each adhesion (Table 2.3).

The average cell count for each adhesion was assessed by examination under a light microscope (x40 magnification) following H&E staining. Images were recorded (using a digital camera and saved as TIFF files) and with the use of an 100 x 100μm grid and image analysis software (Adobe Systems, Inc., San Jose, Calif.), the average cell count was calculated. This was performed by counting the number of cells in 5 random squares and the mean used to determine cell number.

For both scoring methods described above three independent observers were used and mean scores recorded.

Tendon and adhesion specimens were stained with Masson's trichrome stain to identify collagen fibril formation (Appendix I).

5.2.6 Data Analysis

Statistical assessment of these data was performed using SigmaStat 2.0 (Jandel Scientific Software, San Rafael, Calif.). The Student t-test, Wilcoxon Rank Sum Test and the Pearson correlation coefficient where appropriate.

5.3 Results

5.3.1 Mechanical Assessment of Adhesion Strength

The raw data from the tensile testing machine (Figure 5.1) demonstrates an observable difference between the operated untreated (OUT) and unoperated untreated groups (UOUT). These two groups represent the positive and negative control groups for this study respectively. Tendon injury or surgical tenotomy followed by a period of immobilisation (as in this study) will lead to adhesion formation as identified in the OUT-group. A mean force of 3.78N (Figure 5.1) is a typical finding for this group. As expected, there were no observable adhesions in the UOUT-group. However, in this latter group we do occasionally find small increases in load (0.2-0.6N) as discussed later.

The data in Figure 5.3 represents the mean mechanical force in newtons (N) required for the adhesion to fail in the following groups: unoperated untreated (UOUT); operated untreated (OUT); operated and treated with sterile water (H_2O, diluent for all treatments), EGCG (EGCG), Resveratrol (RES) or Pumactant (PUM). There was an observable difference between the OUT-group (positive control) and the treatment groups. Clearly, the OUT-group produced the most adhesions over the two-week period with a mean force of 2.81N ±1.13. There was a statistically significant difference between the OUT- and UOUT-groups ($p<0.001$). Interestingly, treatment with H_2O shows an observable reduction in the force required to rupture adhesions compared to the OUT group, however this difference was not shown to be statistically significant ($p=0.07$). Each treatment showed a statistically significant reduction in the force required for adhesion failure (EGCG 1.66N ±0.486, $p=0.02$; Resveratrol 1.24N ±0.621, $p=0.006$; Pumactant 0.81N ±0.824, $p=0.001$) when compared to the OUT-group. The diluent (sterile H_2O) appears to show some biological effect in reducing adhesion formation as there was no statistically significant difference between the H_2O-group and the EGCG ($p=0.706$) or Resveratrol groups ($p=0.128$). However, Pumactant showed a statistically significant reduction in force required compared the OUT-group ($p=0.001$) and the H_2O ($p=0.034$). Also, all the groups showed a statistically significant difference compared to the UOUT-group ($p<0.001$) except for the Pumactant treated group ($p=0.07$). This demonstrates that the amount of adhesion formation in the Pumactant treated group is comparable to the negative control group (UOUT).

5.3.2 Histological Assessment

All unoperated and untreated (UOUT) samples showed, as expected, no evidence of adhesion formation on macroscopic or microscopic examination (Figure 5.4). Masson's trichrome staining demonstrated a more organised collagen fibril formation in the unoperated compared to the operated tendon groups. It also showed new extracellular matrix (ECM) deposition as demonstrated by red staining (Figure 5.5). Operated untreated (OUT) tendons frequently showed a dense and irregular adhesion pattern between the tendon and tendon sheath (Figure 5.6).

Adhesions and repairs were scored based on the system developed by Tang. The OUT-group showed a mean adhesion score of 5.13 ±0.3; H_2O-group 4.2 ±0.37; EGCG 3 ±0.53; Resveratrol 3.13 ±0.67 and Pumactant 1.88 ±0.74 (Figure 5.7). There was no statistically significant difference in the adhesion scores between the OUT and H_2O groups ($p=0.08$). The EGCG and Resveratrol groups also showed no statistically significant difference when compared with the H_2O-group (EGCG $p=0.14$; Resveratrol $p=0.26$) but they did show a statistically significant difference when compared with the OUT-group (EGCG $p=0.004$; Resveratrol $p=0.016$). Pumactant was the only treatment that did show a statistically significant difference when compared with both the H_2O-group ($p=0.04$) and the OUT-group ($p=0.001$). In fact in some tendons treated with Pumactant no adhesions could be identified (Figure 5.8).

Tendon adhesion cell numbers were recorded using the counting method as previously (Chapter 2, section 2.4). Although there was a reduction in mean cell number when comparing all treatments to the OUT and H_2O groups, these changes were not statistically significant (Figure 5.9). Tendons treated with Pumactant that developed adhesions showed the lowest cell number though, this was not statistically significant ($p=0.11$). Interestingly, there was a greater correlation between adhesion cell number and the scoring by Tang when tendons were treated with Pumactant (Figure 5.11, $r = 0.81$) compared to other treatments (Figure 5.12, $r = 0.43$).

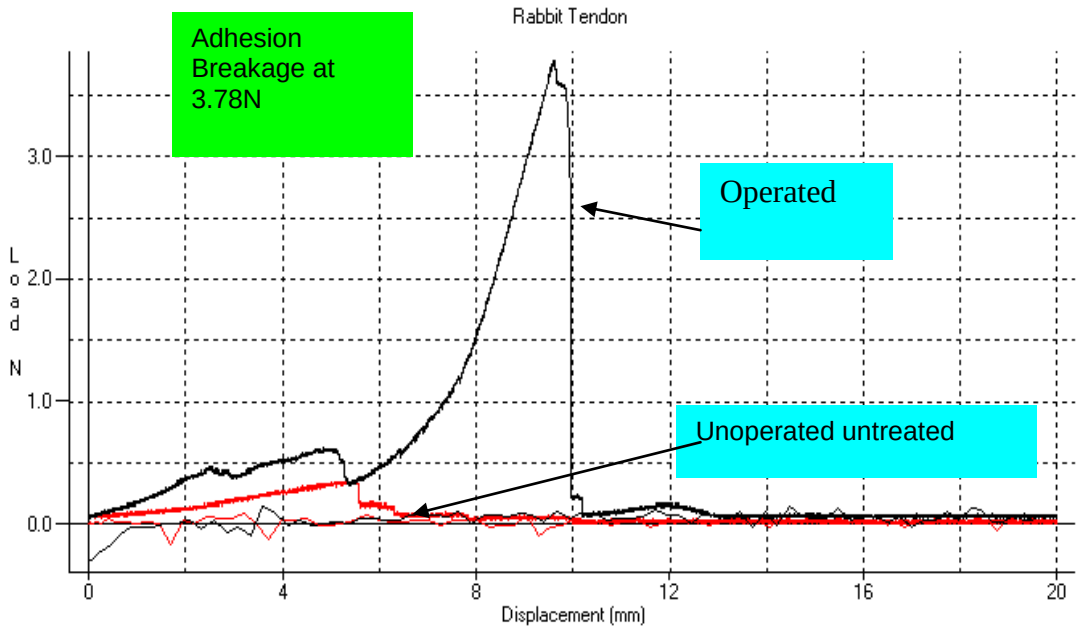


Figure 5.1 This graph represents a typical example of the raw data from the mechanical tensile testing machine (Mecmesin) using the Emperor™ Software. It shows the load (force in Newtons, N) as a rabbit tendon is displaced (mm) from its tendon sheath (at a rate of 10mm/min). The peak force measured is the point when the adhesion fails. This graph compares the two control groups: operated untreated and unoperated untreated.

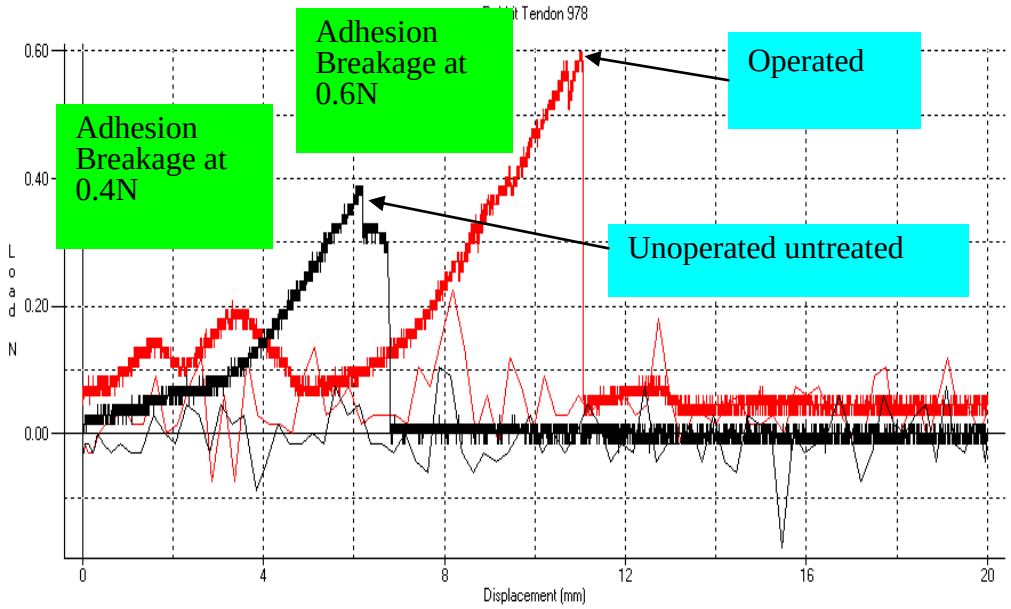


Figure 5.2 This graph represents a typical example of the raw data from the mechanical tensile testing machine (Mecmesin) using the Emperor™ Software. It shows the load (force in Newtons, N) as a rabbit tendon is displaced (mm) from its tendon sheath (at a rate of 10mm/min). The peak force measured is the point when the adhesion fails. This graph compares the operated Pumactant treated group with the unoperated untreated group.

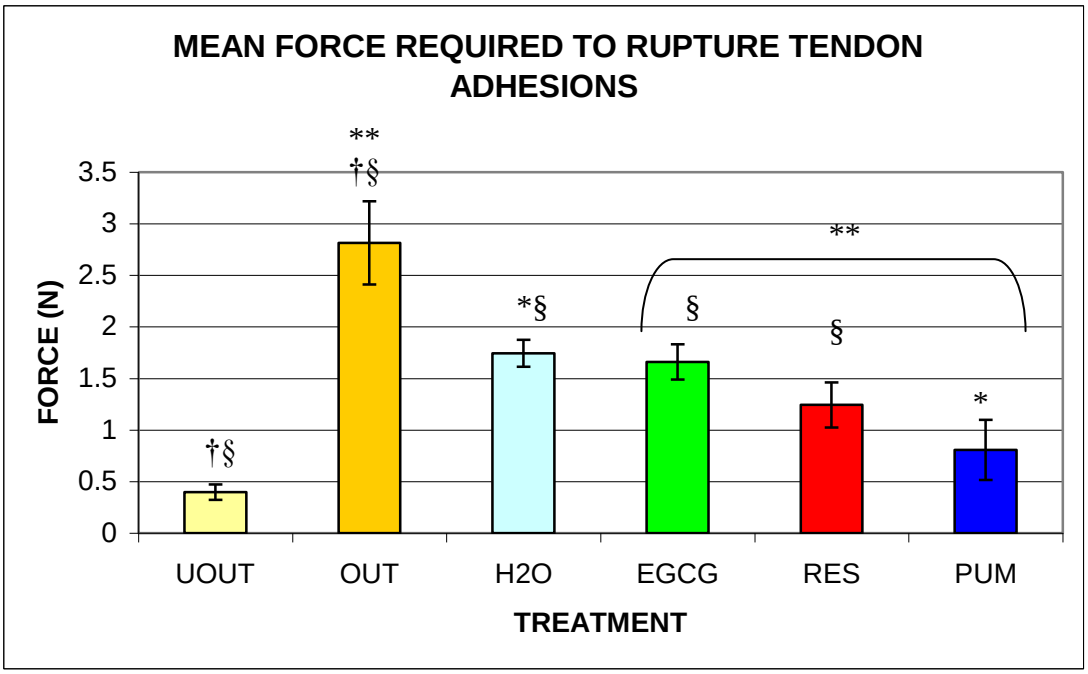


Figure 5.3 Graph representing the mean force in Newtons (N) for the tendon adhesion to fail for each group (n=8): UOUT (unoperated untreated control), OUT (operated untreated control), H_2O (sterile H_2O control), EGCG, RES (Resveratrol) and PUM (Pumactant). Error bars=SEM. A statistically significant difference was demonstrated between: OUT and EGCG (**p=0.02), RES (**p=0.006), PUM (**p=0.0012) and UOUT (†p=0.0005) groups. Pumactant was the only treatment to show a statistically significant difference when compared with its diluent, H_2O (*p=0.03). There was also a statistically significant difference between UOUT and OUT, H_2O, EGCG, RES groups (§p<0.001).

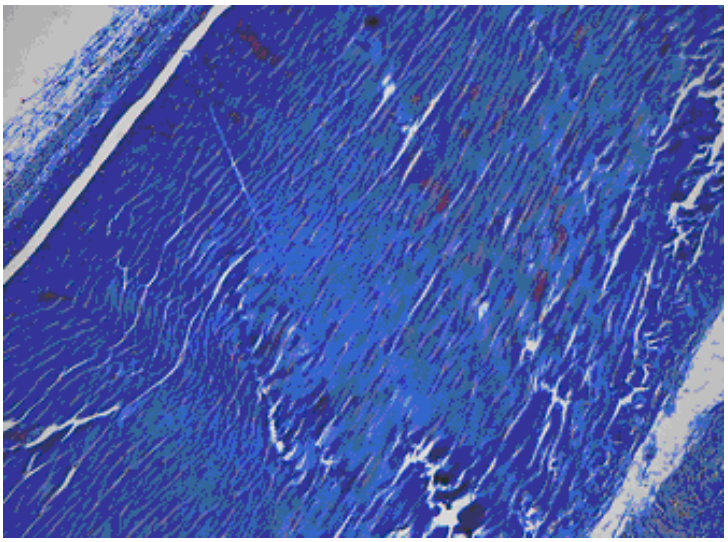


Figure 5.4 Light microscopy photograph (x40 magnification) of unoperated untreated (UOUT) tendon, showing bundles of collagen fibrils stained blue (Masson's Trichrome Stain).

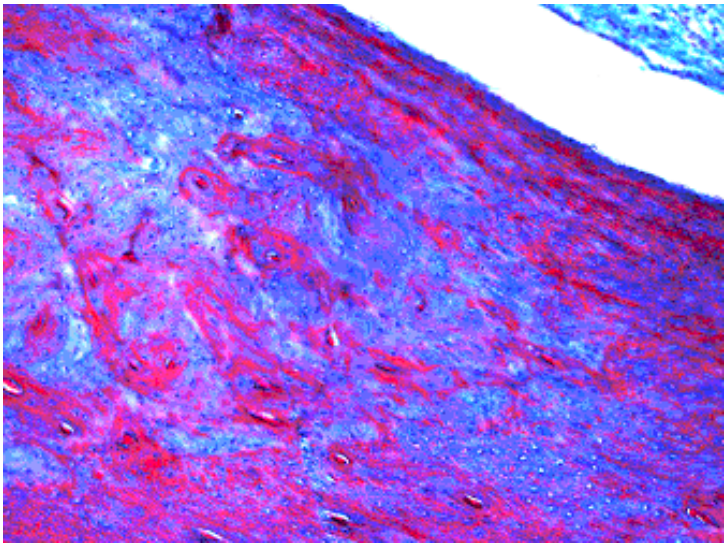


Figure 5.5 Light microscopy photograph (x40 magnification) of injured tendon, showing blue (Masson's Trichrome Stain) collagen fibrils but also new matrix being laid down in the tendon (stained red).

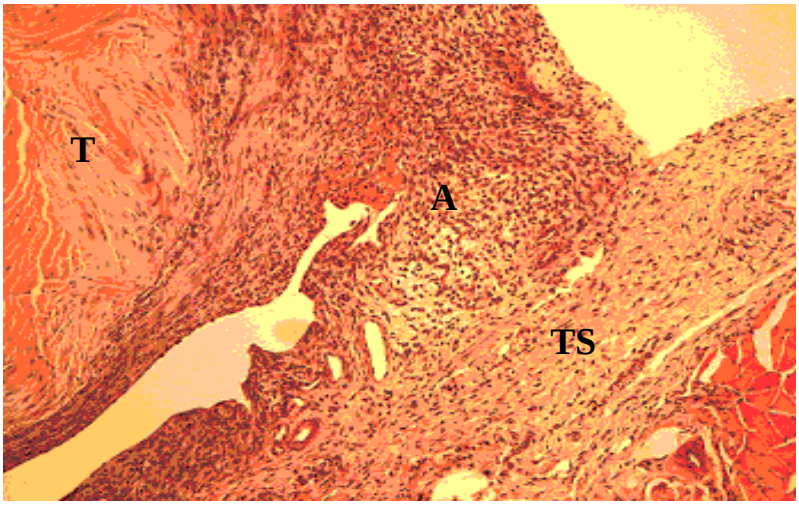


Figure 5.6 Light microscopy photograph (x40 magnification, H&E Stain) of an injured tendon (T) without treatment showing an obvious ‘adhesion’ bridge (A) between the tendon and the tendon sheath (TS).

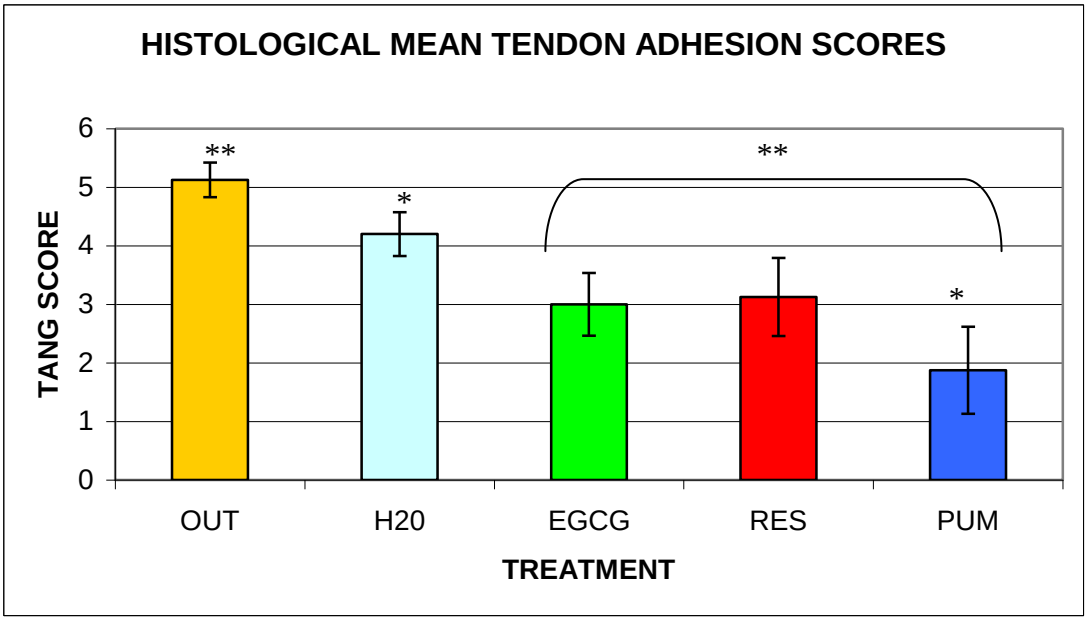


Figure 5.7 Graph representing the histological adhesion scores, according the Tang adhesion scoring system, comparing the mean values between the following groups (n=8): OUT (operated untreated control), H_2O (sterile H_2O control), EGCG, RES (Resveratrol) and PUM (Pumactant). Error bars=SEM. A statistically significant difference was demonstrated between: OUT and EGCG (**p=0.003), RES (**p=0.02), PUM (**p=0.001) groups. Pumactant was the only treatment to show a statistically significant difference when compared with its diluent, H_2O (*p=0.04).

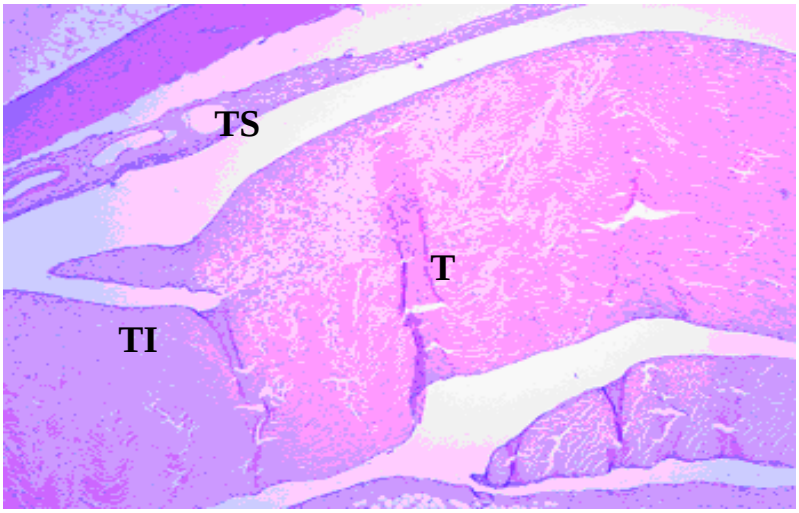


Figure 5.8 Light microscopy photograph (x40 magnification, H&E Stain) of an injured tendon (T) treated with Pumactant showing no evidence of adhesion formation between the tendon sheath (TS) and the site of tendon injury (TI).

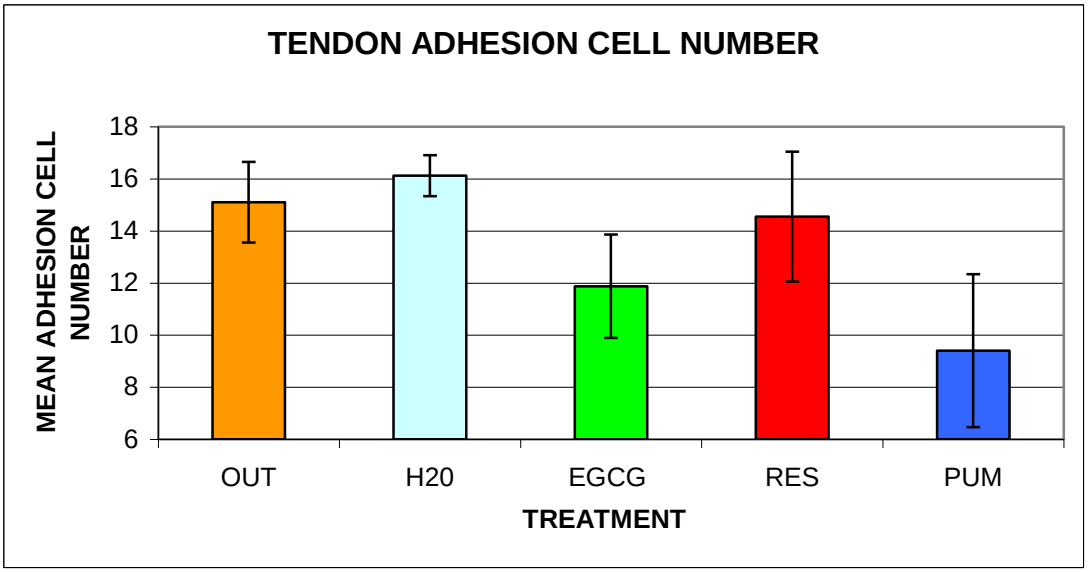


Figure 5.9 Graph representing the mean tendon adhesion cell numbers, comparing the mean values between the following groups (n=8): OUT (operated untreated control), H_2O (sterile H_2O control), EGCG, RES (Resveratrol) and PUM (Pumactant). Error bars=SEM. There was no statistically significant difference between groups.

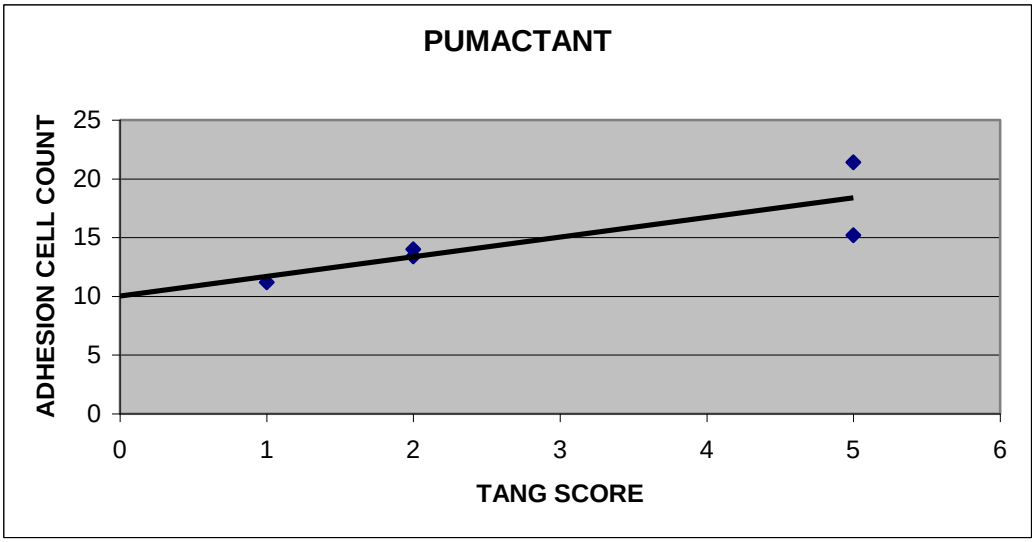


Figure 5.10 Scatter plot showing linear relationship between histological adhesion scores (Tang) and adhesion cell number (n=5) with Pumactant treated flexor tendons (correlation coefficient, r = 0.81).

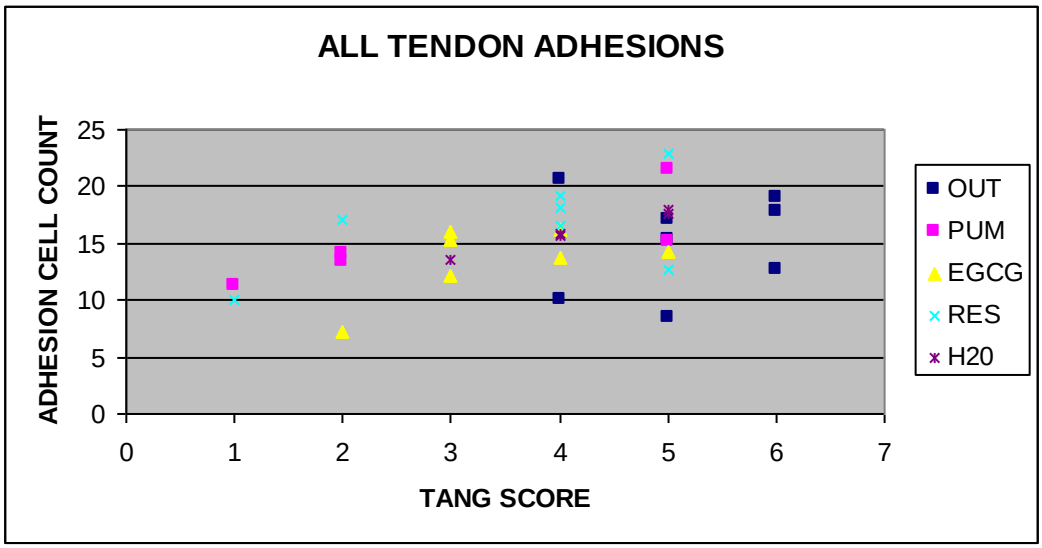


Figure 5.11 Scatter plot showing linear relationship between histological adhesion scores (Tang) and adhesion cell number for all flexor tendon adhesions (correlation coefficient, r = 0.43).

5.4 Discussion

Despite the best surgical technique, optimal suture material and careful post-operative rehabilitation, tendon adhesions still occur. Further, it can be difficult to predict which patients will develop adhesions following surgery. Many different chemical modulators (Chang et al., 2000; Frykman et al., 1993; Green et al., 1986; Hagberg, 1992; Jones et al, 2002; Khan et al., 2000; Komurcu et al., 1997; Kulick et al., 1984; Moran et al., 2000; Salti et al., 1993; Weiss et al., 1986) and physical barriers (Ashley et al. 1959; Ashley et al. 1964; Austin and Walker, 1979; Golash et al., 2003; Hanff and Hagberg, 1998; Rank et al., 1977; Strauch et al., 1985) have been used in the past in an attempt to reduce adhesion formation. Unfortunately, there have been various problems with each of these treatments including: increased rupture rates, poor gliding of the tendon within the sheath and unwanted systemic effects. This study has set out to assess three novel treatments that may prevent tendon adhesion formation but ideally without added complications.

In vitro work at RAFT (see Chapter 4) has demonstrated that EGCG, Resveratrol and Pumactant show potential beneficial effects on gene expression associated with tendon healing and production of adhesions. It was therefore necessary to trial these compounds using an *in vivo* model of flexor tendon adhesion formation before a pilot clinical trial could be undertaken. A rabbit model was selected as it has been widely used in similar studies (Abrahamsson et al., 1989; Abrahamsson et al., 1991; Branford et al., 2008; Chang et al., 1997; Chang et al., 1998; Jones et al., 2000; Jones et al., 2003; Kang and Kang, 1999; Kakar et al., 1998; Khan et al., 1996; Manske et al., 1984; Matthews and Richards, 1976; Moro-oka et al., 2000; Rank et al., 1980), and is a well established model. Furthermore, adhesions can be produced reliably following tendon injury in a rabbit model.

Some tendon adhesion studies have focused on quantifiable or histological assessments (Akali et al., 1999; Tang et al., 1996) but few have combined biomechanical and histological methods (Strick et al., 2004; Branford et al., 2008). The combination of mechanical tensile testing with histological scoring methods allows for a thorough investigation of adhesion formation by observing the physical properties and the histological architecture respectively. This particular study has not

assessed *in vivo* tendon healing as others have done, but suggestions for further work to be carried out in this area are made later.

From the mechanical (tensiometer) data it is clear that Pumactant appears to have the most marked effect on the reduction of force required for adhesions to fail (H_2O v PUM, $p = 0.03$). The other two treatments showed a reduction in adhesion formation compared to the OUT-group ($p<0.05$) but not when compared to the H_2O-group. Interestingly, there appears to be a reduction in adhesions seen in the H_2O-group compared to the OUT-group although this effect was not statistically significant ($p=0.07$). Sterile water appears to be having some form of biological or physical effect reducing the formation of adhesions. This difference may be due to the physical injection into the tendon sheath at the time of surgery. In the OUT-control group although a tenotomy is performed there is no solution infiltrated into the tendon sheath. There is an observable degree of tendon sheath expansion that occurs during infiltration and this may lead to a greater gap between the tendon and the sheath, therefore increasing the distance that adhesions need to form to create a bridge between the two structures. The injection of water may also clear away any fibrin clot which can act as a precursor for adhesion formation. Tendons have a high percentage weight of water and gliding function is optimal in a moist environment. However, water also exerts an osmotic effect on tissues such that they swell and may interfere with cellular function and possibly tendon healing.

There was no statistical difference between the Pumactant group and the UOUT-group, which suggests that the use of Pumactant on operated tendons makes them behave like negative controls (i.e. no tenotomy). Paradoxically, in the UOUT-group there were occasionally small increases in load (0.2-0.6N) as displacement increased. This increase in load may be explained by the vinculae that supply blood and nutrients to the tendon. However, as the forces involved are small their influence should not affect the overall outcome of this study.

The histological findings support the mechanical data extremely well as the same pattern of adhesion reduction emerges. The Tang scoring showed again that Pumactant was the only treatment to reduce tendon adhesions with a statistically significant difference compared to the H_2O group ($p= 0.04$). However, there was a

significant reduction in scores when the EGCG and Resveratrol groups were compared to the OUT-group ($p<0.05$). Although, there appeared to be a reduction of adhesion cell number between the Pumactant (mean = 9.4 ± 2.94) and H_2O (mean = 16.12 ± 0.78) or OUT (mean = 15.1 ± 1.54) groups this change was not significant. The Tang scores combined with the results from adhesion cell number confirm that Pumactant reduces the size and density of adhesions, but does not significantly reduce cellularity in any adhesion formed. However, the strong correlation between Tang scoring and adhesion cell number with Pumactant compared to other treatments (r = 0.81, 0.43 respectively) suggests there is a direct relationship between these factors i.e. a predictable response.

It has been previously established, that cells on the tendon surface are known to contain lipid-containing vesicles. Further, it has been estimated that lipids account for up to 43% of the epitenon matrix (whereas collagen accounts for only 23% of the matrix) (Banes et al., 1988). Pumactant is a mixture of two naturally occurring phospholipids: a 7:3 mixture of dipalmitoylphosphatidycholine and unsaturated phosphatidylglycerol. Phospholipids have a biomembrane with two important roles (Ansell and Hawthorne, 1964; Briscoe et al., 2006; Higaki et al. 1998; Moro-oka et al., 2000): the physical role of forming a hydrophobic boundary-lubricating environment, and the functional role as a second messenger or agonist. It is most likely that Pumactant is acting as a barrier to preventing adhesion formation, but there may be secondary functional properties specific to this phospholipid mixture. Certainly, in our study (Chapter 4) Pumactant has demonstrated a significant biological effect with the gene up-regulation of collagen type III and late down-regulation of PAI-1 in endotenon cells combined with the down-regulation of PAI-1 in tendon sheath cells.

Previously, Pumactant has shown a dose dependent reduction in abdominal adhesion formation in a murine model (unpublished studies at Cardiff University). Pumactant was found to accelerate mesothelial and keratinocyte cell proliferation (Topley and Woodcock, 2010). This proliferation appeared to be unique to this specific compound, as other phospholipids seem to inhibit cell proliferation and cause a reduction in cell viability (Kremlev et al., 1994; unpublished work performed at Cardiff University). Our study corroborates an increase in proliferation and is demonstrated in tendon cells

(all types) with various doses of Pumactant (8-10mg/ml) (Chapter 4). Phospholipids have been identified in joints, tendons or tendon sheath fluid (Mills et al., 2005; Hills et al., 1996) and have been shown to play a major role in joint lubrication (Hills et al., 1989; 2002b). Others have shown a role in the prevention of articular cartilage degeneration (Kawanao et al., 2003) and a deficiency has been associated with osteoarthritis in humans (Hills and Monds, 1998). Moreover, phospholipids have been shown to prevent flexor tendon adhesion formation in a rabbit model (Moro-oka et al., 2000). In the latter study a mixture of phospholipid and hyaluronic acid reduced adhesion formation compared to saline or hyaluronic acid alone but phospholipid alone was not tested (as previously discussed in Chapter 1, section 1.9.3). This group measured the friction coefficient of the gliding tendon followed by the work required to tear the tendon from the sheath (a similar mechanical method as used in our study). There is also evidence (Topley and Woodcock, 2010) to suggest that Pumactant may achieve some of its effects through the induction of hyaluronic acid synthase, which presents the possibility that phospholipids might aid lubrication and also participate, through hyaluronic acid production, in tendon repair and healing.

A final mechanism of action is the possibility that phospholipids interact with collagen type I fibril formation, which may interfere with the extracellular matrix organisation within the adhesion complex (Martinez Del Pozo et al., 1989). This current study did not examine the effect of Pumactant on tendon healing, however, as phospholipids are present in the normal tendon sheath fluid and joints it is unlikely to cause any impairment. Further, this current *in vivo* study did not demonstrate excess inflammation nor was there evidence of Pumactant itself present two weeks following surgery.

EGCG and Resveratrol showed a statistically significant reduction in force required for adhesions to fail and a reduction in the grade of adhesion formation by the Tang method but only compared to the OUT and not the H_2O group. As discussed previously H_2O appears to be having unknown physical or biological effects.

The results in this current study for Resveratrol are comparable to a study using a rat model, which demonstrated a reduction in peritoneal adhesion formation following laparotomy and intra-operative application of Resveratrol when compared to saline

(Sogutlu et al., 2006). In the above study, tissue levels of malondialdehyde (MDA) and nitric oxide (NO), markers of tissue injury and inflammation, were significantly reduced in the Resveratrol group whereas glutathione (GSH), a cellular anti-oxidant, showed increased levels. From these findings, they concluded that Resveratrol reduces surgically induced peritoneal adhesions possibly through reduction of lipid peroxidation products. *In vitro* work discussed in Chapter 4 demonstrates that Resveratrol could be having an anti-adhesion effect via the down-regulation of both fibronectin and PAI-1 (changes seen mainly in epitenon and tendon sheath cells). Other studies with Resveratrol have shown a wide biological effect in various tissues (Brakenhielm et al., 2001; Sgambato et al., 2001; Surh et al., 1999). Furthermore, Resveratrol has been shown to prevent fibrosis in a rat cirrhosis model, and its action is probably associated with its ability to reduce the activation of a transcription factor, NF-kappa, and thus reduce levels of profibrinogenic TGF-β (Chavez et al., 2007). These mechanisms may be occurring in tendons, but further work is required to measure these specific compounds.

EGCG may still offer a therapeutic benefit in the reduction of tendon adhesion formation. So far studies have shown EGCG suppresses collagen production and may have a role in the treatment of keloids and liver fibrosis (Zhang et al., 2006). Various mechanisms of action have been investigated: One study, using human tendon-derived fibroblasts, showed that EGCG reduces chemokine (IL-1) stimulated expression of collagenase and stromelysin (MMP's) mRNA via JNK/SAPK pathway (Corps et al., 2004). Other theories suggest that EGCG inhibits ECM gene expression, by interrupting TGF-β1 signalling through attenuating oxidative stress (Yumei et al., 2006), or enhancing the transcription of collagen type 1 (although collagen production was actually inhibited in this study) and TIMP-1 and reduces the transcription of MMP-1 (Nakumata et al., 2005). Chapter 4 of this study shows that EGCG reduce the adhesion of epitenon cells to fibronectin. Conversely, Hung et al., (2005) in melanoma derived stromal fibroblasts, showed that EGCG binds to fibronectin and fibrinogen but not to collagen. This group also showed that EGCG attenuated the antibody binding to the fibroblast integrin receptor α2β1, indicating an effect on the expression and affinity of integrin α2β1. Others (Suzuki and Isemura, 2001) showed that EGCG inhibited cell adhesion to fibronectin by its binding to the carboxyl-

terminal heparin-binding II domain and not to the cell binding domain. Further research into this area may be a potential strategy for adhesion reduction.

The doses of EGCG, Resveratrol and Pumactant used for this *in vivo* study were higher than used in our *in vitro* work. This is based on several factors. Firstly, as rabbit numbers were limited it was decided to optimise doses so that maximum therapeutic effects could be assessed. A range of doses would have led to higher rabbit numbers and have cost implications. EGCG toxicity has been studied in detail and it has been demonstrated that oral administration of 500, 1000 or 2000mg EGCG/kg in mice or intravenous injection of 10, 25 and 50mg EGCG/kg/day to rats showed absence of genotoxic effects (Isbrucker et al., 2006, Part 1). Also, the dietary administration of EGCG to rats over 13 weeks was not toxic at doses up to 500mg/kg/day. Furthermore, although topical EGCG preparations caused minor irritation in rats and guinea pigs, there was no effect in rabbits (4 hour dermal exposure to 0.47g EGCG under semi-occluded patch) (Isbrucker et al., 2006, Part 2). In this study an EGCG dose of 0.45mg/ml (1mM solution) should not have induced any adverse reaction as this was a local injection into the tendon sheath and well below the topical or oral dose used in the studies as cited above. In a phase 1 pharmacokinetic study (Chow et al., 2001) EGCG has been shown to be present in the plasma (predominantly in conjugated form) and urine after oral administration. The systemic availability of EGCG increased at higher doses possibly due to saturable pre-systemic elimination of the orally administered green tea polyphenols. The Resveratrol dose in this study was also increased as high doses (10mg) have been shown to be effective in the prevention of surgery-induced peritoneal adhesions in a rat model (Sogutlu et al., 2006). Also, high doses of Resveratrol (10mg/kg/day) were administered orally to successfully alleviate bleomycin-induced lung injury in rats (Sener et al., 2007). In male Sprague-Dawley rats, 20mg Resveratrol/kg/day given for 28 days did not induce systemic toxicity (Juan et al., 2002). In the current study a Resveratrol dose of 2.28mg/ml (10mM solution) was infiltrated into the tendon sheath, well below the doses used in the studies as mentioned above. Resveratrol is rapidly absorbed after oral intake with a maximal plasma concentration after 30-60minutes. In one study the level of Resveratrol in the blood was found to be low, likely caused by rapid metabolism to glucuronide and sulphate conjugates (Walle et al., 2004). Lastly, a Pumactant dose of 10mg/ml was found in this study to

significantly increase proliferation in all tendon cell types (Chapter 4.3.1) and therefore deemed the appropriate dose for use in the *in vivo* assessment. Pumactant is non-toxic and produced no apparent toxic affect when administered to rats as a single oral dose of 2000mg/kg bodyweight (communication with the Britannia Pharmaceuticals).

Unfortunately, the tissue distribution and absorbance rate of EGCG, Resveratrol & Pumactant were not studied in this work but it would be worthwhile for future pre-clinical studies.

5.5 Conclusions

All three treatments showed a statistically significant reduction of tendon adhesion formation when compared to the operated untreated controls in both the mechanical and histological assessments ($p<0.05$). However, Pumactant was the only treatment to show a statistically significant reduction in adhesion formation when compared to the H_2O-group in both of these assessments. There was no significant difference in adhesion cell counts between groups; however, Pumactant cell counts showed better correlation with the histological adhesion scores (Tang scores) indicating more of a predictable response compared to other treatments.

Pumactant has been shown to reduce adhesion formation following partial tenotomy in a flexor tendon model. When combined with an active post-operative mobilization regime, functional improvement in outcome appears a distinct possibility for patients. Furthermore, Pumactant may also improve the outcome in patients who were not compliant with their mobilization protocol. Further work is required to confirm the effectiveness of EGCG and Resveratrol however there is still potential for benefit in tendon surgery.

CHAPTER 6

GENERAL DISCUSSION

Although there have been many recent advances in the repair of injured flexor tendons, range of movement, strength of digit and overall hand function are often still far from ideal. Flexor tendon adhesion formation remains a significant problem especially for those with poor compliance to early active mobilisation protocols. This study has set out to examine the potential of three novel treatments which may in the future act as surgical adjuncts in the prevention of tendon adhesions.

6.1 The In Vitro Flexor Tendon Cell Response to TGF-β1

The growth factor TGF-β1 appears to have an important role in both tendon healing (Chang et al., 1997; Klein et al., 2002; Tsubone et al., 2006) and tendon adhesion formation (Chang et al., 2000). TGF-β receptors have also been shown to be up-regulated after tendon injury and there is a strong correlation between the expression of the receptors and ligands (Ngo et al., 2001), indicating that tenocytes may be responsive to TGF-β signalling.

This current study demonstrates that when epitenon and tendon sheath cells were stimulated with TGF-β1 there was a statistically significant increase in proliferation at late time points (72 hours but not at 24 or 48 hours). Endotenon cells showed no significant increase in proliferation with TGF-β1 when compared to those cultured in minimal media and this is likely to be due to the general slow proliferation and non-reactivity of this cell type. It appears that epitenon and sheath cells are behaving differently to endotenon cells in such a way that reflects modern tendon healing concepts (Berglund et al., 2006).

Fibroblasts derived from various tissues have shown that collagen type I gene expression is increased in response to TGF-β1 (Rolfe et al., 2007, Varga et al., 1987, Tiggleman et al., 1995; Chai et al., 2003). Collagen is known to be important in maintaining the mechanical properties of the tendon (Benjamin et al., 2002; Purslow, 2002). Although, collagen type III is rapidly deposited after injury its presence appears only temporary as during the remodelling phase of tendon healing collagen type III is replaced with collagen type I (Jaibaji, 2000). This and other studies (Gelberman et al., 1992), epitenon cells show an increase in collagen type I gene expression, whereas the endotenon cells show no expression during the early healing phases. This increase in gene expression appears to also mirror an increase in protein

expression, though not statistically significant, with an increase in soluble collagen identified in epitenon and sheath derived cells following stimulation with TGF-β1. Collagen types I and III have been shown to be up-regulated in an uninjured tendon model (Klein et al., 2002). In this study, interestingly stimulation of epitenon cells with TGF-β1 showed no increase in collagen type III gene expression though both sheath and endotenon derived cells showed some statistically significant increase during the time course. Similarly, Berglund et al., (2006) also showed an increase in collagen type III in both tendon and tendon derived sheath cells in an *in vivo* model, over a longer period of time and in a post injury model, however, their tendon cells were not classified as either epitenon or endotenon derived (Berglund et al., 2006).

TGF-β1 is known to be able to alter the production of fibronectin (Saika et al., 1998). Fibronectin is known to be deposited at an early stage of tendon healing (granulation phase) and is thought to play a role in the formation of an adhesion bridge (Kurtz et al., 2001; Wojciak and Crossan, 1993). TGF-β1 increased fibronectin gene expression in all cells derived from the tendon-sheath complex. Plasminogen activator inhibitor 1 (PAI-1) gene transcription is increased in a number of cell types (Rolfe et al., 2007; Zhao et al., 2006) in response to TGF-β1. High PAI-1 levels, and therefore the inhibition of fibrinolysis has been associated with peritoneal adhesion formation (Di Filippo et al., 2006), while low levels of tissue plasminogen activator (t-PA) have been associated with a reduction in adhesion formation in the peritoneum and pelvis (Orita et al., 1991; Doody et al., 1989). In this current study, PAI-1 gene expression was found to be increased in endotenon and tendon sheath cells in response to TGF-β1. Although, all tendon-derived cells showed a down-regulation in t-PA gene expression statistical significance was reached in only endotenon and sheath derived cells. Similarly, in mesothelial cells, TGF-β1 increased PAI-1 gene transcription and caused a reduction in t-PA (Falk et al., 2001). This increase in PAI-1 in sheath-derived cells in conjunction with a decrease in t-PA, following stimulation with TGF-β1, could potentially lead to adhesion formation (Figure 1.10). This mechanism could be one explanation for the excess scarring and adhesion formation associated with TGF-β1.

TGF-β1 has been associated with tendon adhesion formation in several studies (Chang et al., 2000; Khan et al., 2000; Ngo et al., 2001). In this current study there is

a demonstrable spatial and temporal variability in response to tendon cells of different origin when treated with a multipotent growth factor, TGF-β1. Additionally, the novel treatments studied in this thesis may affect specific TGF-β1 pathways which could be further manipulated to achieve an optimal therapy.

6.2 The In Vitro and In Vivo Assessment of 3 Novel Flexor Tendon Anti-Adhesion Treatments

Over the years many different chemical (Kulick et al., 1984; Green et al., 1986; Hagberg, 1992; Moran et al., 2000; Chang et al., 2000) and physical barrier (Austin and Walker, 1979; Hanff and Hagberg, 1998; Golash et al., 2003) treatments have been studied, but none have yet proven to be successful in improving the outcome of tendon surgery without significant side effects. The purpose of this investigation was to test three proposed flexor tendon anti-adhesion treatments *in vitro* to discover their potential in the prevention of adhesions and optimise the environment for tendon healing. Epigallocatechin-3-gallate (EGCG) and Resveratrol are naturally occurring plant extracts and Pumactant is a mixture of two naturally occurring phospholipids. The advantages of non-synthetic compounds are their high abundance, relative low cost to manufacture and general low toxicity.

6.2.1 EGCG

EGCG, as previously discussed (Chapter 1, section 1.9.1), is a catechin extract, major polyphenol and the most potent antioxidant found in green tea (Ahmad and Mukhtar, 1999). EGCG has been shown to play a role in treating a number of diseases (Suzuki and Isemura, 2001; Abe, 2007) including the reduction of fibrosis in a number of organs and improving scarring and wound healing in cutaneous wounds (Kapoor et al., 2004; Nakamuta et al., 2005; Zhang et al., 2006; Klass et al., 2010).

Based on this work and other studies (Hung et al., 2005; Chen et al., 2002; Nakamuta et al., 2005) an EGCG dose of 50μM was selected as the optimal treatment dose. Adhesion studies were performed to investigate the effect of each treatment on the adhesion of different tendon cell types to various extracellular matrices. Changes in adhesion properties of cells to specific matrix components may influence formation of adhesion, in particular at the epitenon-tendon sheath interface. Sheath derived cells showed a statistical significant reduction in adhesion to collagen type I following

treatment with EGCG, though other tendon derived cells showed no significant difference. This effect on collagen type I may be due to the effects of EGCG on the α2β1 receptor (Hung et al., 2005). Hung et al showed that EGCG blocks α2β1 either through an interaction with the integrin receptor itself or through the decrease of intracellular H_2O_2. Endotenon and epitenon cells showed no difference with adhesion to collagen type I and it is possible that they express more α1β1, a further collagen type I receptor. Interestingly both epitenon and endotenon derived cells showed an increase in adhesion to collagen type III following treatment with EGCG. Collagen type III binds by both α1β1 and α2β1 and is known to have a novel binding site, which may explain this effect (Kim et al., 2005). In contrast to other studies (Hung et al., 2005), EGCG showed no effect on fibroblast adhesion to fibronectin and this difference remains unclear.

EGCG showed no significant change in collagen type I, type III and fibronectin gene expression in the tendon-sheath derived cells compared to untreated controls, with the exception being endotenon derived cells which showed a statistically significant reduction in collagen type III gene expression. Other studies have shown that EGCG also inhibits collagen type I production in a number of cell types even when the cells exposed to a fibrotic stimuli such as TGF-β1 (Zhang et al., 2006; Nakamuta et al., 2005; Hashem et al., 2008; Park et al., 2008; Klass et al., 2010). However, other studies suggest that though EGCG reduces collagen production it appears to improve the maturity and organisation of the collagen fibres (Kapoor et al., 2004; Kim et al., 2008). Also, in human tendon fibroblasts, EGCG has been shown to reduce the expression of collagenase mRNA, which may lead to higher collagen levels (Corps et al., 2004).

EGCG appeared to have little effect on PAI-1 gene expression with only endotenon derived cells showing a significant up-regulation at an early time point. Others have shown that green tea polyphenols inhibit PAI-1 gene expression and its release from endothelial cells (Liu et al., 2009) i.e. increased fibrinolysis and potential reduction of adhesions. Epitenon derived cells demonstrated a statistically significant down-regulation of t-PA at 2 and 4 hours which could potentially lead to unfavourable conditions for increased adhesion formation. Fortunately, the changes seen in both PAI-1 and t-PA gene expression are seen at early time points and a prolonged effect

may be required for adhesions to develop. In a post-surgical peritoneal model, low levels of t-PA and high levels of PAI-1 have been associated with increased adhesions (Cheong et al., 2001 and Ivarsson et al., 1998), to date there have been no studies looking at the role of PAI-1 and t-PA in tendon adhesion formation.

EGCG *in vivo* showed a statistically significant reduction in both mechanical and histological adhesions when compared to the operated untreated group (OUT-group). However, there was no difference when compared to the diluent or H_2O-group. As discussed in Chapter 5 it appears that water may be exerting an as yet unknown physical or biological effect. Although saline would have been a suitable alternative as a control it was not recommended as a diluent for Pumactant (based on proliferation studies at Cardiff University). An additional saline group in the *in vivo* model would have been useful but it was deemed important to keep animal numbers and cost to a minimum. This study refinement including reduction of animal numbers is a necessary part of animal welfare and the project license application process (Handbook of Laboratory Animal Management and Welfare, Wolfensohn and Lloyd, 2003).

EGCG has previously demonstrated anti-fibrotic potential and in this current study there was a reduction of zone II flexor tendon adhesion formation in an animal model. The effect on tendon healing *in vivo* has not been assessed fully and this would be the next phase of work to be undertaken. Other studies in compromised wounds, such as those in a diabetic mouse model, show that EGCG accelerates re-epithelialisation (Kim et al., 2008) and this suggests that tendon healing may also benefit from such treatment.

6.2.2 Resveratrol

Resveratrol (trans-3, 4, 5-trihydroxystilbene) is a natural polyphenol found in some plants including grapes and has been shown to be effective in preventing fibrosis and peritoneal and pelvic adhesions in animal models (Chavez et al., 2007; Ustűn et al., 2007; Sogutlu et al., 2006). Following previous discussion (Chapter 1, section 1.9.2) it was deemed worthwhile investigating the effects of Resveratrol on tendon healing and whether peri-tendinous adhesion formation can be reduced.

Resveratrol treated epitenon cells showed a statistically significant reduction in proliferation at several different doses. Comparison with other studies suggest using doses between 25-100μM (Olas et al., 2002). For this study a dose of 50μm was selected to maximise any biological effect. Resveratrol reduced the adhesion of sheath and epitenon derived tendon cells to collagen type I but no other extracellular matrix component. Similarly, Resveratrol has been found to prevent the adhesion of some cell types to collagen and fibrinogen (Olas et al., 2002; Cao et al., 2005) and the mechanism for this may be related to its antioxidant properties (Olas et al., 2002). Additionally, the integrin αvβ3 has been shown to contain a binding site for Resveratrol (Lin et al., 2006), and αvβ3 is known to bind to a number of extracellular matrices (Tandon et al., 2005).

Resveratrol treatment has been associated with enhanced formation of epidermis, deposition of connective tissue and improved histological architecture in a cutaneous wound (Khanna et al., 2002) which implies an organised production of collagen. Resveratrol in other studies can inhibit expression of type I collagen gene expression in a number of cell types (Godichaud et al., 2000; Rucinski et al., 2006). This current study showed a statistically significant up-regulation of collagen type I gene expression in epitenon derived cells and up-regulation of collagen type III in sheath derived cells. Epitenon cells and tendon sheath cells treated with Resveratrol showed a statistically significant down-regulation of fibronectin. Similarly, Resveratrol has been shown to reduce fibronectin synthesis in other tissues (Lee et al., 2010). An intact fibronectin framework is critical for maintaining the composition of cell-matrix adhesion sites (Sottile and Hocking, 2002). Hence a reduction of fibronectin production could lead to less tendon adhesion formation.

Epitenon and sheath derived cells treated with Resveratrol showed a statistically significant down-regulation of PAI-1 gene expression, with the exception at 48 hours in sheath derived cells. This reduction in PAI-1 gene expression has been confirmed in a study in adipocytes treated with Resveratrol (Ahn et al., 2007). Essentially, this equates to more predicted adhesion formation (as explained in Chapter 4). Also, there was a synchronous up-regulation in t-PA with a down-regulation of PAI-1 at 48 hours. This result is expected as physiologically t-PA and PAI-1 are opposing forces in the fibrinolytic pathway. Also, polyphenolics, including Resveratrol, have been

shown in cultured human endothelial cells to increase t-PA protein and gene levels (RT-PCR), concomitant with an increase in sustained surface localised fibrinolytic activity (Abou-Agag et al., 2001) thus showing the ability to reduce adhesion formation.

Resveratrol *in vivo* showed a very similar response to EGCG with a statistically significant reduction in both mechanical and histological adhesions when compared to the operated untreated group (OUT-group). However, again there was no difference when compared to the H_2O-group. Resveratrol and EGCG are both potent antioxidants and they possibly share a common anti-adhesion effect via this pathway. Certainly, increased oxidative stress has been associated with adhesion formation in various tissues (Ksiazek et al., 2010; Heydrick et al., 2010) and antioxidants may play a role in prevention by increasing fibrinolytic activity (Heydrick et al., 2010). The effect of Resveratrol on tendon healing *in vivo* has also not been assessed and this would be the next phase of work to be undertaken. Though, others have shown that dermal wounds heal with enhanced re-epithelialisation and therefore indicate that Resveratrol should not affect tendon healing (Khanna et al., 2002).

6.2.3 Pumactant

The properties of Pumactant have been discussed (Chapter 1, section 1.9.3) and the functional role and effects of various phospholipids have been previously described (Chapter 5, section 5.4). Recently, a phospholipid emulsion has been shown to reduce adhesion formation in a rat model of peritoneal adhesions (Aritas et al., 2009). Moreover, phospholipids have been shown to play a role in the prevention of flexor tendon adhesion formation in a rabbit model (Moro-oka et al., 2000).

All tendon cell types showed a statistically significant increase in proliferation when treated with Pumactant. However, tendon sheath cells treated with Pumactant also showed a statistically significant reduction in proliferation at day 7 (2mg/ml). Similarly, Pumactant has been shown to accelerate mesothelial and keratinocyte cell proliferation. This response appeared to be unique to this specific compound, as other phospholipids appear to inhibit cell proliferation and can cause a reduction in cell viability (Kremlev et al., 1994; unpublished work performed at Cardiff University). From this current work and previous studies (unpublished work performed at Cardiff

University) a Pumactant dose of 1 and 2mg/ml was used for further *in vitro* studies and 10mg/ml for our *in vivo* study as described in Chapter 5.

Phospholipids in other studies have been shown to inhibit gastric cancer cell adherence to collagen type IV, laminin and fibronectin (Jansen et al., 2004). The mechanism of action is unknown but may be due in part to the barrier properties of phospholipids. In this current study Pumactant showed no significant effects on cell adhesion to different extracellular matrices and this may be explained by the different cell type and alternative matrices employed.

Pumactant treated endotenon cells showed a statistically significant up-regulation of collagen type III gene expression at late time. An increase in collagen type III gene expression in cells at the core (endotenon) of the tendon may allow for optimisation of tendon healing. Endotenon cells also showed a statistically significant up-regulation of PAI-1 at early time points which was followed by a later down-regulation. Tendon sheath cells showed a statistically significant down-regulation of PAI-1 at 1 hour. Overall there appears to be a reduction in PAI-1 between endotenon and sheath cell types which may be beneficial in preventing adhesions, as already described (Cheong et al., 2001; Ivarsson et al., 1998).

Pumactant *in vivo* appeared to have the most marked effect on the reduction of force required for adhesions to fail. There was also no statistical difference between the Pumactant treated group and the unoperated untreated group (UOUT-group) which suggests that the use of Pumactant on injured tendons makes them behave like uninjured ones. The histological findings (Tang scoring) showed again that Pumactant was the only treatment to reduce tendon adhesions with a statistically significant difference compared to the H_2O group. The Tang scores combined with the adhesion cell number results confirm that Pumactant reduces the size and density of adhesions but does not significantly reduce adhesion cellularity.

Cells on the tendon surface are known to contain lipid-containing vesicles and it has been estimated that lipids account for up to 43 percent of the epitenon matrix, whereas collagen accounts for only 23 percent (Banes et al., 1988). Phospholipids have a biomembrane with two important roles (Ansell and Hawthorne, 1964; Briscoe et al.,

2006; Higaki et al., 1998; Moro-oka et al., 2000): the physical role of forming a hydrophobic boundary-lubricating environment, and the functional role as a second messenger or agonist. Surface-active phospholipids, like Pumactant, commonly form multiple layers when in contact with solid surfaces, providing the basis for lubrication. The lamellar body (enclosed multi-layered structure) is the surface active form of surfactant first described in alveolar type II cells in the lung (Ueda et al., 1984). Lamellar bodies are secreted onto the surface to be lubricated where they unravel and coat the epithelial surface (Ueda et al., 1984; Hills 1989). Boundary lubrication occurs when rubbing surfaces are coated with molecular monolayers. Such monolayers generally consist of amphiphilic (molecule containing both hydrophilic and hydrophobic parts) surfactants anchored by their polar headgroups; sliding occurs at the interface between the layers, greatly reducing friction and especially wear of the underlying substrates. Interestingly, this friction is greatly reduced when the two sliding surfaces bearing surfactant monolayers are immersed in water, to as little as one per cent or less of its value in air (Briscoe et al., 2006).

Pumactant could act as a boundary lubricant in improving tendon glide, as a barrier preventing adhesions, and may show advantages in cell proliferation and tendon healing. Certainly, in this current study (Chapter 4) Pumactant has demonstrated a significant biological effect on gene expression mainly in endotenon cells. A further study showed that a mixture of phospholipid and hyaluronic acid reduced adhesion formation compared to saline or hyaluronic acid alone though the group did not study solely phospholipid (Moro-oka et al., 2000). This same group measured the friction coefficient of the gliding tendon followed by the work required to tear the tendon from the sheath (a similar mechanical method as used in this current study). There is evidence (Topley et al., 2010) to suggest that Pumactant may achieve some of its effects through the induction of hyaluronic acid synthase, which presents the possibility that phospholipids might aid lubrication and also participate, through hyaluronic acid production, in tendon repair and healing. A final further mechanism of action may be that phospholipids interact with collagen type I fibril formation which may interfere with extracellular matrix organisation within the adhesion complex (Martinez Del Pozo et al., 1989). We are unable to comment on the effect of Pumactant directly on tendon healing but as phospholipids are present in normal tendon sheath fluid and joints this is unlikely to cause any impairment. There was

certainly no excess inflammation or evidence of Pumactant present at two weeks following surgery. The effect of Pumactant and the other treatments on tendon healing *in vivo* has not been assessed and further work in this area is required before clinical trials.

6.3 Critique

There are several factors that can affect the interpretation of the current findings. Cells cultured were from previously uninjured rabbit flexor tendons and ideally cells from injured flexor tendons would have offered additional information. Further, there are differences with cells grown in culture to those *in vivo*; as it is impossible to exclude the effects of other cytokines *in vivo* whereas *in vitro* studies allow an environment which can be tightly controlled. Though tendon cells *in vitro* are believed to have undergone differentiation due to culture conditions it is also believed that during tendon healing the cells also undergo some form of differentiation (Gelberman et al., 1985).

Animal models can never fully reproduce the clinical environment. However, they are essential before human trials can be undertaken. New treatments can be fully tested in a controlled manner whilst monitoring efficacy and safety. Refinements, including dosing regimes, routes of administration and pharmacokinetics can be assessed. In this study it was necessary to create an accurate model of tendon adhesion formation but keep confounding factors to a minimum. The New Zealand White rabbit model is widely recognised for use in flexor tendon studies. By incorporating this model the clinical situation could be mimicked very closely as flexor tendons were selected from Zone II of the rabbit forepaw which is equivalent to Zone II in the human hand. A standardised tendon injury was performed (tenotomy) and the treatment infiltrated into the sheath. Classically, injured tendons are repaired with a non-absorbable core suture using a modified Kessler technique and a second non-absorbable epitendinous suture. This was not used in the current model as suture material at the site of adhesion formation could potentially bias the results. However, there is scope for a suture repair model as discussed later.

6.4 Further Work

Testing three treatments required a large number of animals, considerable time and cost however it has been beneficial in that two of the compounds have shown promise and one, Pumactant, has shown great potential in the prevention of tendon adhesions. Further work is required, specifically looking at tendon healing rather than adhesion formation. There will be no clinical benefit if adhesions can be prevented but the tendon ruptures due to poor healing.

To determine the effects of each treatment on tendon healing, a similar rabbit flexor tendon model (as described in Chapter 2) could be employed. However, the surgical tenotomy would be replaced with a complete tendon transection followed by a standard modified Kessler repair using a non-absorbable monofilament suture material. The treatments would be added using the same infiltrating technique but harvesting would not occur until six weeks post-operatively. This would allow adequate time for tendon healing and the formation of adhesions. Tendon healing could be assessed using the same techniques: mechanical tensile testing (ultimate tensile strength or cyclical testing of the healed tendon and the related adhesion) and histological assessment (endotenon and epitenon repair, inflammatory markers and further adhesion scoring). It would also be interesting to use different doses to study if there is a dose dependent effect and use fluorescent typing to follow the absorption rate and metabolism of Pumactant. Lastly, it may be useful to leave a cannula within the sheath so that several treatment doses can be applied over a period of time however this approach may not be clinically applicable.

6.5 Conclusions

6.5.1 Chapter 3

- Proliferation results demonstrate that when sheath cells were treated with TGF-β1 there was a statistically significant increase at 0.5ng/ml at 72 hours but not at 24 or 48 hours.

- Epitenon cells showed a similar significant response with fetal calf serum compared to serum free media. Also, although there was not an early increase (24 and 48 hours) in proliferation with TGF-β1 compared to cells grown in

minimal media there was a statistically significant increase at 72 hours when using a higher dose (2ng/ml).

- Endotenon cells showed no significant increase in proliferation with TGF-β1 when compared to serum free media. It appears that epitenon and sheath cells are behaving differently to endotenon cells in such a way that reflects modern tendon healing concepts {Berglund et al, 2006}.

- Epitenon cells treated with TGF-β1 showed an increase in collagen type I gene expression, whereas the endotenon cells showed no detectable expression during the early time points.

- Stimulation of epitenon cells with TGF-β1 showed no increase in collagen type III expression over the whole time course though both sheath and endotenon derived cells showed some statistically significant increase during the time course

- All tendon-derived cells treated with TGF-β1 showed at some time point a statistically significant increase in fibronectin gene expression compared to the untreated controls.

- PAI-1 gene expression was found to be statistically significantly increased compared to untreated controls in endotenon cells treated with TGF-β1 in the early part of the time course, whereas sheath derived cells showed a statistically significant increase at 48 hours.

- All tendon-derived cells treated with TGF-β1 showed a reduction in t-PA gene expression over the time course with endotenon and sheath derived cells showing a statistically significant down regulation.

6.5.2 Chapter 4

- The optimal *in vitro* doses for each treatment based on this study and the current literature are as follows: EGCG 50μM, Resveratrol 50μM and Pumactant 1mg/ml.

- EGCG caused increased adhesion of pre-treated endotenon and epitenon cells to collagen type III. Conversely, there was decreased adhesion of EGCG pre-treated sheath cells to collagen type I.

- Resveratrol caused reduced adhesion of pre-treated epitenon and tendon sheath cells to collagen type I.

- Treatment with Resveratrol caused the most optimal changes to gene expression as collagen type I, collagen type III and t-PA were up-regulated, whereas fibronectin and PAI-1 were down-regulated. These changes were seen mainly in epitenon and tendon sheath cells.

- Pumactant treated endotenon cells up-regulated collagen type III gene expression at late time points and there was an increase in PAI-1 gene expression early but then a later down-regulation. There was also a down-regulation of PAI-1 in tendon sheath cells. These results indicate a moderate therapeutic advantage.

6.5.3 Chapter 5

- All three treatments showed a statistically significant reduction of tendon adhesion formation when compared to the operated untreated controls in both the mechanical and histological assessments ($p<0.05$).

- Pumactant was the only treatment to show a statistically significant reduction in adhesion formation when compared to the H_2O group in both mechanical and histological assessments ($p<0.05$).

- There was no significant difference in adhesion cell counts between groups; however, Pumactant cell counts showed better correlation with the histological adhesion scores (Tang scores) indicating more of a predictable response compared to other treatments.

This study has examined the effect of a multipotent growth factor, TGF-β1 on cultured uninjured flexor tendon cells derived from different parts of the tendon-sheath complex. Following this three novel treatments were assessed using *in vitro* and *in vivo* methods to determine their potential use in the prevention of flexor tendon adhesion formation. All three treatments have demonstrated an ability to modify tendon adhesion factors and one treatment in particular (Pumactant) has shown a statistically significant mechanical and histological reduction of adhesion formation in a flexor tendon animal model. EGCG and Resveratrol appear to have some biological effects whereas it appears Pumactant may be acting via both biological and physical mechanisms (e.g. steric hindrance) therefore offering a greater therapeutic advantage. A second stage investigation using Pumactant is warranted and ultimately this may prove to be a successful adjunct to surgery in patients who sustain tendon injuries in the future. The overall goal of research into this area is to improve outcome of surgery in patients with hand injuries such that they recover function with minimal physiotherapy, return to work more promptly and develop fewer complications.

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APPENDICES

Appendix I

Histology Protocols

Haematoxylin and eosin stain for paraffin sections

Haemotoxylin and eosin (H&E) staining allows visualisation of cell nuclei (haematoxylin: blue) and cytoplasm (eosin: pink) and is useful for assessing cell morphology.

The sections were de-waxed, hydrated through graded alcohols to water and stained in Harris' haematoxylin for 5 minutes. The sections were washed well with running tap water, differentiated with 1% acid alcohol and then 'blued' in running tap water for 5 minutes or less. Sections were stained with 1% eosin for 10 minutes and washed in running tap water for 1-5 minutes. Finally, sections were dehydrated with alcohols, cleared with xylene and mounted with DePX (BDH).

Masson's trichrome stain

The Masson's trichrome staining method is used for the detection of collagen fibers in various tissues on formalin-fixed, paraffin-embedded sections, and may be used for frozen sections as well. The collagen fibers will be stained blue, the nuclei will be stained black and the background is stained red.

Solution A

Acid fuchsin	0.5g
Glacial acetic acid	0.5ml
Distilled water	100ml

Solution B

Phosphomolybdic acid	1.0g
Distilled water	100ml

Solution C

Methyl blue	2.0g
Glacial acetic acid	2.5ml
Distilled water	100ml

Method

1. Deparaffinise sections and bring to water
2. Wash in tap water
3. Stain nuclei by the Celestin blue-haematoxylin method
4. Differentiate with 1% acid alcohol
5. Wash in tap water
6. Stain in acid fuchsin solution A for 5 minutes
7. Rinse in distilled water
8. Treat with solution B for 5 minutes
9. Drain
10. Stain with solution C for 2-5 minutes
11. Rinse in distilled water
12. Treat with 1% acetic acid for 2 minutes
13. Dehydrate through alcohols
14. Clear in xylene and mount with DePX

Appendix II

Preparation of Solutions

Normal growth medium (NGM) for tendon cell culture

500mls Dulbecco's Modified Eagle Medium containing 4500mg/L glucose and 4mM L-glutamine (Gibco)

Add 50mls (10%) of fetal calf serum (FCS, Gibco)

Add 5mls (1%) of 5000U/ml penicillin

Add 5mls of 5000mg/ml streptomycin (Gibco)

DEPC-ddH_2O

Add 0.1% DEPC to ddH_2O in an RNase free glass bottle

Stir vigorously in a fume hood overnight

Autoclave and store at room temperature

50x TAE buffer

Dissolve 242g Tris-base (BDH) in 800ml sterile ddH_2O

Add 57.1ml glacial acetic acid (BDH) and 100ml 0.5M EDTA (pH 8.0)

Adjust volume to 1L and store at room temperature

Appendix III - Results Data

The Biomechanical Assessment of Adhesion Formation (Chapter 5)

NZW No.	Rabbit Forepaw	Digit	Treatment	Force N	
RT921	Left	D2	UOUT	0.43	
RT921	Right	D2	OUT	3.48	
RT941	Left	D2	UOUT	0.39	
RT941	Right	D2	OUT	2.02	
RT942	Left	D2	UOUT	0.18	
RT942	Right	D2	OT PUM	1.9	
RT925	Left	D2	UOUT	0.19	
RT925	Right	D2	OT PUM	0.19	
RT922	Left	D2	UOUT	0.48	
RT922	Right	D2	OUT	1.88	
RT938	Left	D2	UOUT	0.22	
RT938	Right	D2	OT PUM	2.3	
RT939	Left	D2	UOUT	0.25	
RT939	Right	D2	OUT	1.58	
RT940	Left	D2	UOUT	0.82	
RT940	Right	D2	OT PUM	0.14	
RT979	Left	D2	UOUT	0.36	
RT979	Right	D2	OUT	3.78	
RT980	Left	D2	UOUT	0.31	
RT980	Right	D2	OT PUM	0.43	
RT973	Left	D2	UOUT	0.46	
RT973	Right	D2	OUT	4.35	
RT974	Left	D2	UOUT	0.75	
RT974	Right	D2	OT PUM	0.6	
RT975	Left	D2	UOUT	0.18	
RT975	Right	D2	OUT	3.78	
RT976	Left	D2	UOUT	0.73	
RT976	Right	D2	OT PUM	0.28	
RT977	Left	D2	UOUT	0.22	
RT977	Right	D2	OUT	1.64	
RT978	Left	D2	UOUT	0.4	
RT978	Right	D2	OT PUM	0.61	
RT981	Right	D2	OT RES	0.79	
RT982	Right	D2	OT RES	2.11	
RT983	Right	D2	OT RES	1.45	
RT984	Right	D2	OT RES	1.72	
RT985	Right	D2	OT RES	1.41	
RT986	Right	D2	OT RES	9.82	Result excluded as wound dehiscence
RT987	Right	D2	OT RES	0.25	
RT988	Right	D2	OT RES	0.97	
RT989	Right	D2	OT EGCG	1.08	

RT990	Right	D2	OT EGCG	1.91
RT991	Right	D2	OT EGCG	1.67
RT992	Right	D2	OT EGCG	1.48
RT993	Right	D2	OT EGCG	2.14
RT994	Right	D2	OT EGCG	2.48
RT995	Right	D2	OT EGCG	1.29
RT996	Right	D2	OT EGCG	1.23
RT997	Right	D2	OUT H2O	1.63
RT998	Right	D2	OUT H2O	1.58
RT999	Right	D2	OUT H2O	1.6
RT1000	Right	D2	OUT H2O	1.64
RT1100	Right	D2	OUT H2O	2.27

Results table (raw data) showing force (N) required for tendon adhesion breakage. UOUT, unoperated untreated; OUT, operated untreated; OT PUM, operated and treated with Pumactant; OT EGCG, operated and treated with EGCG; OT RES, operated and treated with Resveratrol; OUT H2O, operated and untreated with H_2O as control.

Appendix III - Results Data

The Histological Assessment of Adhesion Formation using Tang Scoring (Chapter 5)

Slide No.	NZW No.	Rabbit Forepaw	Digit	Treatment	Tang Score
BK2	RT921	Right	D4	OUT	5
BK7	RT922	Right	D4	OUT	4
BK11	RT941	Right	D4	OUT	6
BK14	RT939	Right	D4	OUT	5
BK17	RT977	Right	D4	OUT	4
BK21	RT973	Right	D4	OUT	6
BK26	RT975	Right	D4	OUT	6
BK29	RT979	Right	D4	OUT	5
BK41	RT989	Right	D4	OT EGCG	3
BK42	RT990	Right	D4	OT EGCG	4
BK43	RT991	Right	D4	OT EGCG	3
BK44	RT992	Right	D4	OT EGCG	3
BK45	RT993	Right	D4	OT EGCG	4
BK46	RT994	Right	D4	OT EGCG	0
BK47	RT995	Right	D4	OT EGCG	2
BK48	RT996	Right	D4	OT EGCG	5
BK33	RT981	Right	D4	OT RES	4
BK34	RT982	Right	D4	OT RES	1
BK35	RT983	Right	D4	OT RES	5
BK36	RT984	Right	D4	OT RES	0
BK37	RT985	Right	D4	OT RES	2
BK38	RT986	Right	D4	OT RES	5
BK39	RT987	Right	D4	OT RES	4
BK40	RT988	Right	D4	OT RES	4
BK1	RT938	Right	D4	OT PUM	2
BK4	RT942	Right	D4	OT PUM	0
BK6	RT925	Right	D4	OT PUM	5
BK8	RT940	Right	D4	OT PUM	0
BK19	RT976	Right	D4	OT PUM	5
BK23	RT974	Right	D4	OT PUM	0
BK28	RT978	Right	D4	OT PUM	2
BK32	RT980	Right	D4	OT PUM	1
BK49	RT997	Right	D4	OUT H2O	4
BK50	RT998	Right	D4	OUT H2O	5
BK51	RT999	Right	D4	OUT H2O	4
BK52	RT1000	Right	D4	OUT H2O	3
BK53	RT1100	Right	D4	OUT H2O	5

Results table (raw data) showing histological assessment of adhesion formation using Tang scoring. OUT, operated untreated; OT PUM, operated and treated with Pumactant; OT EGCG, operated and treated with EGCG; OT RES, operated and treated with Resveratrol; OUT H2O, operated and untreated with H_2O as control.

Appendix IV

Publications derived from this thesis

Paper Published:

In Vitro Flexor Tendon Cell Response to TGF-β1: A Gene Expression Study.

B.R. Klass, K.J. Rolfe, A.O. Grobbelaar.

Journal of Hand Surgery, American Volume, 2009, 34A (3), 495-503.