Extraction and Biomedical Application of Peripheral Blood Stem Cells in Sheep and Horses
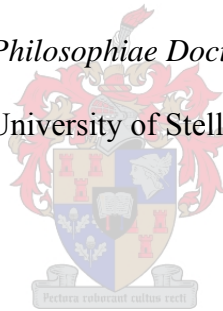
by

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Dissertation presented for the Degree of

Philosophiae Doctor

at the University of Stellenbosch

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December 2007

Declaration: I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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– ABSTRACT –

SUPERFICIAL digital flexor tendon injury has a serious negative impact on the competitive horse industry. Injured horses require up to a year of rest for recovery and likelihood of re-injury upon return to normal activity is as high as 80 %. Tendon healing requires (a) production of collagen by fibroblasts, to provide tensile strength and elasticity to the tendon, (b) minimisation of restrictive fibrosis, which compromises tendon gliding function and (c) minimisation of peritendinous adhesions. We review conventional treatments for tendon healing before exploring stem cell application as a therapeutic alternative. We promote the use of hematopoietic and mesenchymal stem cells derived from adult peripheral blood - as opposed to bone marrow-derived stem cells or embryonic stem cell sources - and review published research output in this regard. In conclusion, we outline our research objectives and present and discuss our results in the chapters that follow.

Mononuclear cells - consisting of hematopoietic stem cells, mesenchymal stem cells and leucocytes – were isolated from the peripheral blood of sheep and horses through red blood cell lysis and blood plasma extraction. Cell counts and propidium iodide dye exclusion viability tests were conducted on the cell pellets. Sheep sub samples were tested for CD45 expression and horse sub samples for CD4 and CD11a/18 cell surface markers by flow cytometry for characterisation purposes. In both cases, separate sub samples were incubated with matched immunoglobulin (IgG) isotypes, conjugated to fluorescein isothiocyanate (FITC), to serve as controls. For the culture of mononuclear cells, 4.5 x 10^6 cells were selected for autologous sheep injections, 3 x 10^6 CD45$^-$ cells for allogeneic sheep injections (the latter excluding leucocytes that may induce an immune response) and 72 x 10^6 cells for horse injections. These cells were incubated with bromo-deoxyuridine (BrdU), cultured and subsets were extracted for a second round of cell counts and viability tests before being resuspended in blood plasma. For the horse samples an additional 1 x 10^6 mononuclear cells were incubated until reaching 60 % confluence and tested for myogenic differentiation. Low cell mortality and lack of fluorescence from IgG-FITC controls reflected effective protocols and a lack of false positive results. The fact that the equine cell population differentiated into myotubes verified the presence of mesenchymal stem cells in injections.

We tested whether surgical incisions or collagenase injections best mimicked naturally occurring tendon injuries and compiled macroscopic and microscopic descriptions of tendon injury sites at seven weeks post-injury. The superficial digital flexor tendons of 27 sheep received an incision, a collagenase injection or a saline control injection. After one week a

number of sheep were sacrificed while the remainder received further saline treatment and were sacrificed after another seven weeks. Tendons were examined through clinical observations, image analysis of maximum tendon diameter, mechanical testing and histological sectioning of affected tissues. Collagenase-induced injury resembled tendonitis more closely than surgically-induced injury. Collagenase-injured tendons (a) induced lengthier lameness in affected limbs, (b) were more swollen and difficult to palpate, (c) assumed the bow appearance characteristic of natural injury, (d) experienced extensive haemorrhage due to collagen lysis, (e) had decreased elasticity and capacity to carry loads and stress, (f) displayed decreased stiffness due to collagen fibre disruption and (g) developed severe inflammation. After seven weeks injured tendons displayed increased vascularisation in the areas of haemorrhage and in the adjacent collagen matrix. High inflammation rates and low collagen levels however still persisted.

Collagenase injections were used to induce tendonitis in the superficial digital flexor tendons of 27 sheep. After one week these tendons received treatment with a control saline solution, autologous peripheral blood mononuclear cells (MNCs) or allogeneic peripheral blood CD45$^-$ MNCs. Healing rates were compared after a further seven week period by conducting ultrasonographic evaluations, clinical observations, image analyses of maximum tendon diameter, mechanical tests and histological investigations. Tendons treated with MNCs displayed an improvement in echogenicity and fibre linearity, higher and more organised collagen levels, stronger mechanical properties and less swelling. Although these improvements were not always significant, they provided strong evidence to suggest marked healing benefits over a longer time period.

Collagenase injections were used to induce tendonitis in the superficial digital flexor tendons of four horses. After one week these tendons received treatment with either a control saline solution or autologous peripheral blood mononuclear cells (MNCs). Healing rates were compared after a further seven week period by conducting ultrasonographic evaluations, clinical observations, image analysis of maximum tendon diameter and histological investigations. Tendons treated with MNCs displayed significant improvements in fibre linearity in the direct vicinity of the lesion, as well as recovery rate thereof, and experienced less swelling when compared with their untreated counterparts. Healing trends suggested that, given a longer period of observation post-injury, more significant improvements may become apparent.

Human adipose tissue is known be an easily accessible and high yielding source of multipotent mesenchymal stem cells. These stem cells could potentially be used for

therapeutic advancement of tendon regeneration. Our first goal was to examine the *in vitro* myogenic differentiation potential of adipose-derived, adherent mononuclear cells (MNCs) from six adult sheep. The second goal was to characterise the population of cells isolated through various available ovine specific, non-mesenchymal stem cell surface markers, namely, CD1, CD31, CD34 and CD45. After incubation, only four of the six MNC cultures started to proliferate. These four cultures all exhibited high myogenic differentiation ability. The isolated cell populations did not express any of the non-mesenchymal stem cell specific cell surface markers.

In conclusion, our data suggests that peripheral blood stem cells and adipose-derived stem cells are important candidate cell types for therapeutic application to improve tendon repair in horses and sheep. Sufficient time must be allowed following injury and prior to stem cell treatment (at least one month) and a controlled exercise program should be followed post-treatment. A larger sample size is required and at least six months of recovery before macroscopic and histological repair can be analysed more accurately and conclusively. Ultrasonography should be carried out on a continuous basis, as it is a non-invasive method of monitoring change over time.

- UITTREKSEL -

BESERINGS van die oppervlakkige digitale buigpees het 'n ernstige negatiewe impak op die meedingende perdebedryf. Beseerde perde benodig 'n rustydperk van tot een jaar, terwyl die waarskynlikheid van herbesering wanneer hulle tot normale aktiwiteit terugkeer so hoog soos 80 % kan wees. Die genesing van pese vereis (a) die produksie van kollageen deur fibroblaste, ten einde treksterkte en elastisiteit aan die sening te verleen, (b) 'n minimalisering in beperkende fibrose, wat glyfunksie van die sening teenwerk, en (c) 'n vermindering van peritendineuse aanhegting. Ons lewer 'n oorsig van konvensionele behandelings van peesbeserings voordat ons die toediening van stamselle as 'n terapeutiese alternatief ondersoek. Ons bevorder die gebruik van hematopoïëtiese en mesenchimale stamselle afkomstig van volwasse periferele bloed - in teenstelling met stamselle afkomstig van van beenmurg of embrioniese stamselbronne - en verskaf 'n oorsig van gepubliseerde navorsing in hierdie opsig. Ter afsluiting verskaf ons 'n opsomming van ons navorsingsdoelwitte en bespreek ons resultate in die daaropvolgende hoofstukke.

Eenkernige selle – bestaande uit hematopoïëtiese stamselle, mesenchimale stamselle en leukosiete – is deur middel van rooibloedsellise en bloedplasma ekstraksie vanuit die periferele bloed van skape en perde geïsoleer. Seltellings en propidiumjodied kleuruitskakeling lewensvatbaarheidtoetse is op die sel-kapsule uitgevoer. Skaap-submonsters is vir CD45-uitdrukking getoets en perd-submonsters is vir CD4 en CD11a/8 seloppervlakmerkers deur middel van vloei sitometerie gekarakteriseer. In beide gevalle is aparte submonsters met gepaste immunoglobulien (IgG) isotipes geïnkubeer wat aan fluoresseïen-isotiosinaat (FITC) gekonjugeer is om as kontroles te dien. Om eenkernige selle te kweek, is 4.5 x 10^6 selle geselekteer vir outoloë skaapinspuitings, 3 x 10^6 CD45⁻ selle vir allogeneïese skaapinspuitings (laasgenoemde uitsluitend leukosiete, wat 'n imuunreaksie kan induseer) en 72 x 10^6 selle vir perdinspuitings. Hierdie selle is met broomdeoksiuridien (BrdU) geïnkubeer, gekweek en submonsters is onttrek vir 'n tweede rondte seltellings en lewensvatbaarheidtoetse voordat hulle weer in bloedplasma gesuspendeer is. Vir die perdmonsters is 'n bykomende 1 x 10^6 eenkernige selle geïnkubeer totdat hulle 60 % samevloeiing getoon het en is daarna vir miogeniese differensiasie getoets. Lae selmortaliteit en 'n gebrek aan fluoressensie in die IgG-FITC kontroles het effektiewe protokolle aangedui en vals positiewe resultate uitgesluit. Die feit dat die perdselbevolking in spierbuisies gedifferensieer het, bevestig die teenwoordigheid van mesenchimale stamselle in ons inspuitings.

Ons het getoets watter een van 'n chirurgiese snit en 'n kollagenase inspuiting 'n natuurlike peesbesering die beste naboots en het makroskopiese en mikroskopiese beskrywings van die ligging van peesbeserings sewe weke ná besering saamgestel. Daar is aan die oppervlakkige digitale buigpees van 27 skape 'n insnyding, kollagenase-inspuiting of 'n soutbevattende kontrole-inspuiting toegedien. 'n Aantal van hierdie skape is ná een week uitgesit, terwyl die res nog soutbehandeling ontvang het en eers ná 'n bykomende sewe weke uitgesit is. Die pese is deur middel van kliniese waarnemings, beeldanalise van hul dwarssnitomtrek, meganiese toetse en histologiese snitte van die aangetaste weefsels ondersoek. Kollagenase-geïnduseerde beserings het natuurlike beserings beter as chirurgies-geïnduseerde beserings nageboots. Kollagenase-beseerde pese (a) het 'n verlengde periode van mankheid in aangetasde ledemate veroorsaak, (b) was meer geswel en moeiliker palpeerbaar, (c) het die boogvorm wat kenmerkend van natuurlike beserings is, aangeneem, (d) het as gevolg van kollageenlise uitgebreide bloeding ondergaan, (e) het verminderde elastisiteit en 'n gepaardgaande verlies van vermoë om laste en stres te dra, ondervind, (f) het verminderde styfheid getoon as gevolg van kollageenveselskeuring en (g) het ernstige ontsteking ontwikkel. Ná sewe weke was daar verhoogde vaskularisasie in die areas waar bloeding voorgekom het, asook in die aanliggende kollageenmatriks. 'n Hoë ontstekings-tempo en lae kollageenvlakke het egter voortgeduur.

Kollagenase inspuitings is gebruik om peesontsteking in die oppervlakkige digitale buigpese van 27 skape te induseer. Ná een week is hierdie senings met 'n kontrole soutoplossing, outoloë periferele bloed eenkernige selle of allogeneïese periferele bloed CD45⁻ eenkernige selle behandel. Die tempo van genesing is ná 'n verdere tydperk van sewe weke deur middel van ultrasonografiese evaluasies, kliniese waarnemings, beeldanalise van dwarssnitomtrek, meganiese toetsing en histologiese ondersoeke vergelyk. Senings wat met eenkernige selle behandel is, het 'n verbetering getoon in eggogenese en vesellineariteit, hoër en meer georganiseerde kollageenvlakke besit, sterker meganiese eienskappe gehad en minder swelling ondervind. Hoewel hierdie verbeterings nie altyd beduidend was nie, het hulle voldoende getuienis verskaf dat aansienlike genesingsvoordele oor die langer termyn moontlik is.

Kollagenase inspuitings is ook gebruik om peesontsteking in die oppervlakkige digitale buigpese van vier perde te induseer. Ná een week is hierdie pese met óf 'n kontrole soutoplossing óf outoloë periferele bloed eenkernige selle behandel. Die tempo van genesing is ná 'n verdere periode van sewe weke deur middel van ultrasonografiese evaluasies, kliniese waarnemings, beeldanalise van dwarssnitomtrek en histologie ondersoek. Pese wat met

eenkernige selle behandel is, het beduidende verbeterings getoon in vesellineariteit in die omgewing van die besering, asook in die tempo van genesing daarvan, en het minder swelling ondervind as onbehandelde pese. Die genesingstendense dui aan dat meer beduidende verbeterings waarskynlik duidelik sou word indien waarnemings vir 'n langer tydperk ná die besering sou voortduur.

Dit is bekend dat menslike vetweefsel 'n toeganklike bron van groot getalle veelvermoënde mesenchimale is. Hierdie stamselle het die potensiaal om in die terapeutiese bevordering van peesregenerasie gebruik te word. Ons eerste doel was om die miogeniese differensiasiepotensiaal van vetafkomstige, adherente eenkernige selle van ses volwasse skape *in vitro* te ondersoek. Die tweede doel was om die bevolking van selle te karakteriseer, soos geïsoleer deur middel van verskillende beskikbare skaapspesifieke, nie-mesenchimale stamseloppervlakmerkers, naamlik, CD1, CD31, CD34 en CD45. Slegs vier van die ses kulture het na inkubasie begin prolifereer. Hierdie vier kulture het almal 'n hoë vermoë tot miogeniese differensiasie getoon. Die selbevolkings wat geïsoleer is, het nie enige van die nie-mesenchimale stamselspesifieke seloppervlakmerkers uitgedruk nie.

Ter afsluiting: ons data dui daarop dat periferele bloedstamselle en vetafkomstige stamselle belangrike kandidaatselsoorte is vir terapeutiese toepassing wat pees herstel in perde en skape kan bevorder. Voldoende tyd moet toegelaat word na die besering en voor stamselbehandeling (ten minste een maand) en 'n beheerde oefenprogram moet na die behandeling gevolg word. 'n Groter monster is nodig en daar moet 'n hersteltydperk van ten minste ses maande gevolg word voordat die makroskopiese en histologiese herstel meer akkuraat en beduidend geanaliseer kan word. Ultrasonografie moet voortdurend uitgevoer word omdat dit 'n ingreepsvrye metode is wat genesing oor tyd kan monitor.

Dedication: To my husband, René Michael Hensen, for his love, support, patience and perseverance – he is my strength, back-up and mentor in many areas of my life.

"God is the only comfort, He is also the supreme terror: the thing we most need, and the thing we most want to hide from." - C. S. Lewis

– ACKNOWLEDGEMENTS –

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AA	Antibiotic-Antimycotic
ADSC	Adipose-derived stem cell
ANOVA	Analysis of variance
BAPN-F	βl-aminopropionitrile fumarate
BrdU	Bromo-deoxyuridine
BSA	Bovine Serum Albumin
MTD	Maximum tendon diameter
DDF	Deep digital flexor (tendon)
DMEM	Dulbecco's Modified Eagle's Medium
D-PBS	Dulbecco's Phosphate-Buffered Saline
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (β-Aminoethylether)-N,N,N'N'-tetraacetic acid
ESW	Extracorporeal shock waves
FACS	Fluorescent activated cell sorting
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
GLM	General linear model
GM-SCF	Granulocyte-monocyte colony stimulating factor
HA	Sodium hyaluronate
HBSS	Hank's Balanced Salt Solution
HCl	Hydrogen chloride
HSC	Hematopoietic stem cell
IGF	Insulin-like growth factor
IgG	Immunoglobulin
IVF	*In vitro* fertilised
MNC	Mononuclear cell
MSC	Mesenchymal stem cell
NSAID	Non-steroidal anti-inflammatory drug
NaF	Sodium fluoride
NaPPi	Sodium pyrophosphate
NP40	Nonidet P40-Substitute (nonylphenylpolyethylene glycol)

PI	Propidium iodide
PMSF	Phenylmethyl sulfonyl fluoride
PSGAG	Polysulphated glycosaminoglycans
RBC	Red blood cell
SBTI	Soybean trypsin inhibitor
SDF	Superficial digital flexor (tendon)
SDS	Sodium dodecyl sulfate
UV	Ultraviolet

- CHAPTER 1 -

ADULT STEM CELLS IN EQUINE TENDON REPAIR: STATUS QUO?

THIS CHAPTER serves as a general introduction to the main body of the thesis. It firstly provides a synopsis of the impact that tendon injuries have amongst performance and competitive horses before evaluating conservative treatments to such injuries. Thereafter it introduces the clinical application of stem cells as an alternative healing method for equine tendon injuries, reviews current research on stem cell therapy and lists commercial ventures promoting such therapy. This information provides a collective argument in support of our research and is concluded by a formulation of our main study objectives, as examined sequentially in the chapters that follow.

1.1 TENDON INJURY AS A COMMON OCCURRENCE IN THE EQUINE INDUSTRY

Tendons and/or ligaments in horses have a limited ability, if any, to adapt positively to exercise after maturity in terms of physiological function and structure (Dowling and Dart 2005). Insufficient training in young horses therefore often results in tendon injuries occurring regularly amongst performance and competitive horses. Of 18 000 performance horses born in Europe and 34 000 born in the United States annually, up to 43 % will at some point be removed from competition due to suspensory ligament (*musculus interosseus medius)* or flexor tendon injuries (Dowling *et al.* 2000). Around 3 500 Thoroughbreds are foaled per annum in South Africa, of which 2 500 – 3 000 are registered for the racing industry or breeding purposes. A similar number are retired from racing each year, mostly due to tendon and ligament injuries (www.jockeyclubsa.co.za, pers. comm.).

Of all tendon and ligament injuries, the superficial digital flexor tendon (*musculus flexor digitalis superficialis;* SDF tendon[1]) is the most common site of injury whereas the deep digital flexor tendon (DDF tendon) is less prone to structural damage (Watkins *et al.* 1985; refer to Figure 1.1 for anatomical localities of these tendons). The small maximum tendon diameter of the SDF tendon, combined with its location in the mid-metacarpal region, predisposes it to mechanical injury (Watkins *et al.* 1985). The SDF tendon and DDF tendon perform different mechanical roles during locomotion, even though both flex the digit of the equine forelimb (Webbon 1977). The SDF tendon is loaded early in the stance phase, acting as an elastic energy store which experiences considerable stresses and strains during high-

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[1] A list of abbreviations is provided on Page xxxix for ease of reference.

Accessory carpal bone

Inferior check ligament

Superficial digital flexor tendon

Deep digital flexor tendon

Suspensory ligament

Proximal sesamoid bones

Superficial digital flexor tendon

Deep digital flexor tendon

Distal sesamoid (navicular) bone

4th Metacarpal (splint bone)

Common digital extensor tendon

3rd Metacarpal (cannon bone)

1st Phalanx (long pastern)

2nd Phalanx (short pastern)

3rd Phalanx (pedal bone)
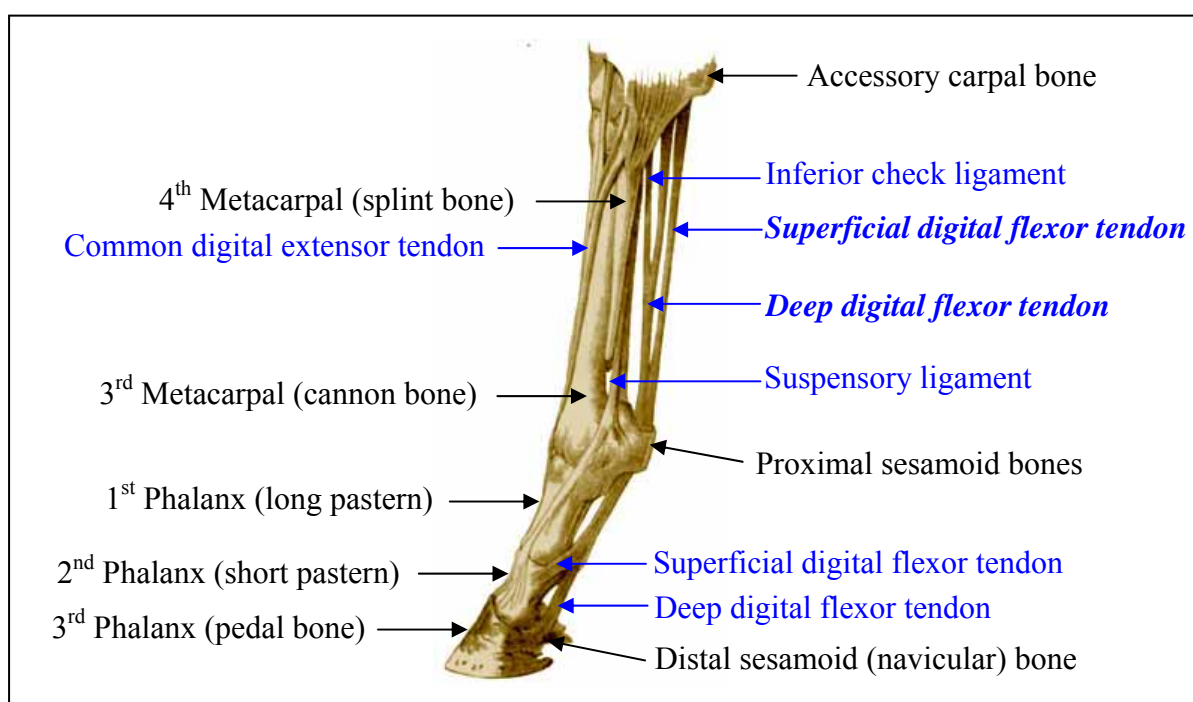
FIGURE 1.1 - Schematic drawing of the lateral aspect of the front left leg of a horse, displaying relative positions of the superficial digital flexor tendon and deep digital flexor tendon.

speed locomotion (Platt *et al.* 1994, Birch *et al.* 1999). During high intensity exercise the SDF tendon in fact operates close to its functional limit (Stephens *et al.* 1989). The DDF tendon, on the other hand, is loaded later in the stance phase and is thus subjected to lower stresses and strains, rendering it less important as an energy store (Platt *et al.* 1994, Birch *et al.* 1999). Furthermore, injuries to the SDF tendon most commonly occur in the mid-metacarpal region; this is where the cross sectional area is smallest and the tendon is not enclosed within a synovial sheath (Webbon 1977, 1978).

Most tendon and ligament injuries require 9 - 18 months of rest for optimal healing (Strömberg and Tufvesson 1969, Strömberg *et al.* 1974, Buckwalter and Hunziker 1996, Woo *et al.* 1999). Conventional treatment of tendon injuries prevents premature formation of collagen cross-links, thereby allowing a controlled exercise regime to improve functionality of scar tissue. However, conventional treatment does not promote regeneration of actual tendon tissue itself and the ensuing scar tissue is less functional than uninjured tissue (Smith *et al.* 2003). Tendon injuries also heal slowly and although up to 60 % of injured horses can return successfully to racing, as many as 80 % of these can sustain re-injury (Sawdon *et al.* 1996, Dowling *et al.* 2000). Such performance horses are often relegated to other equestrian sports, for instance show-jumping and cross-country racing, where the same injuries are likely to recur. If sufficient time is not permitted to maximise the healing capacity of damaged tendons, recurring injuries can lead to devastating effects and eventual elimination of the horse.

1.2 TENDON INJURY

Tendonitis is an injury to a ligament or tendon, caused by excessive pull, twists or repeated application of an injurious force, with the result that the connective fibres are broken, over-stretched or torn away from the bone or muscle (Hays 1926). Injuries to SDF tendons are broadly classified into either (1) diffuse injuries, where large amounts of fibres and fibrils are injured across the tendon, and (2) core lesions, where a focal area of injury exists, displaying an accumulation of fluid and inflammatory products. Diffuse injuries generally have a worse prognosis than core lesions. A combination of intrinsic and extrinsic factors is however required for optimal healing in either scenario. Some intrinsic healing factors include rate of collagen synthesis and cross-link formation, whereas extrinsic factors include nutrition, tendon location and the environment (Lin *et al.* 2004). All these factors are, in turn, influenced by the magnitude of tendon trauma, whether the injury has occurred within or outside the tendon sheath and the degree of blood supply following injury (Lin *et al.* 2004).

The body responds by initiating a process of healing and fibrotic scar formation, which can be divided into four main stages: (1) haemorrhage and oedema, (2) inflammation, (3) proliferation / fibroplasia and (4) remodelling / maturation (Woo *et al.* 1999, Lin *et al.* 2004). Recent studies of single stretch muscle injuries have highlighted that peak damage (micro-injury) occurs at the same time as maximum neutrophil invasion of the injured tissue, which is at stage two of recovery (Brickson *et al.* 2001, Schneider *et al.* 2002). This suggests that, in addition to their classic role, neutrophils may actually cause further micro-injuries (Toumi and Best 2003), possibly via the release of oxygen free radicals, proteases and cytokines (Strömberg 1971, Best *et al.* 1999, Jasti *et al.* 2001, Provenzano *et al.* 2005). Continued microtrauma, which stimulates type III collagen synthesis, may lead to persistent chronic inflammation in an apparently healed tendon (Williams *et al.* 1984a). This is detrimental to normal tendon functioning and can lead to ongoing lameness resulting in imbalanced locomotion. Despite attempts to regain normal collagen concentrations 12 - 14 weeks post-injury (which is 64 % collagen, as opposed to the 37 % in tendon scar tissue), the biomechanical properties of healing tendon remain inferior due to persistently decreased collagen fibril diameter and cross-link formation, as well as altered proteoglycan profiles (Williams *et al.* 1980). This places greater strain on adjacent, uninjured tendon tissue, predisposing them to injury (Smith 2002). As a result, the eventual return of functional strength to tendon lesions can take months to years, with complete restitution being rare (Strömberg and Tufvesson 1969, Strömberg *et al.* 1974, Buckwalter and Hunziker 1996, Woo *et al.* 1999). The frequency of relapse is another indication of inferior functional tensile strength (Strömberg *et al.* 1974).

1.3 SOME CONVENTIONAL TREATMENTS PROMOTING TENDON REPAIR

As discussed above, tendon injuries are slow to repair. Attempts to improve tendon repair aim to (1) fill the defective area with collagen-producing fibroblasts, which mature to provide tensile strength and elasticity, (2) minimise restrictive fibrosis, which compromises the gliding function of the tendon and (3) minimise peritendinous adhesions (Genovese *et al.* 1987).

Generations of orthopaedic surgeons have treated injuries by trying to minimise the progression of tissue damage and, when possible, creating conditions that allowed natural healing to proceed (Buckwalter and Hunziker 1996). They commonly accomplished these goals by immobilisation and thereby stabilisation of the tendon. Veterinarians believed that loading or motion of the tendon would increase the inflammatory response and disrupt tissue

repair. Physical therapies, which include ice application, cold hydro therapy, bandaging and box rest, have been the cornerstones of treatment in acute stages of tendonitis, used in conjunction with anti-inflammatory drugs to curb tissue damage (Bramlage 1992, Rapp *et al.* 1992, Hawkins and Lescun 1997).

Subsequent studies have however shown that, while a brief period of rest may help to avoid further tissue damage and excessive inflammation, early controlled exercise stimulates collagen synthesis, increases tendon strength and aids in the alignment of collagen fibrils (Takai *et al.* 1991, Buckwalter 1996, Genovese 1997, Genovese *et al.* 2000). Controlled exercise programs were consequently developed for the rehabilitation of SDF tendon injuries in horses by Genovese (1997) and Gills (1997). The duration of rehabilitation is critical, with horses rested for less than six months displaying a poorer prognosis for return to racing without sustaining re-injury (Genovese *et al.* 1987, Marr *et al.* 1993a).

Since the development of diagnostic ultrasonography and, more recently, magnetic resonance imaging (both of which greatly enhanced the ability to diagnose tendon and ligament injuries more accurately), a number of additional treatment modalities have been developed. A number of these treatments are described in the following subsections.

1.3.1 *Anti-inflammatory drugs*

Anti-inflammatory drugs have been used extensively by veterinarians treating tendonitis in an attempt to improve healing by minimising neutrophil migration into sites of inflammation (Kallings 1993, Jones and Blikslager 2001). Both corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) can be applied, although the effectiveness of both has been a controversial issue (May and Lees 1996). Administration of short-acting corticosteroids within 24 hours post-injury is known to be beneficial in reducing acute inflammation (Dowling *et al.* 2000). Delayed use of these steroidal drugs has however not proved helpful, since they inhibit the fibroblastic response necessary for tendon healing (Dowling *et al.* 2000). Intralesional use of anti-inflammatory drugs containing corticosteroids has also produced detrimental effects on the healing collagen, inflicting collagen necrosis, hyalinisation and dystrophic mineralization (Pool *et al.* 1980). NSAIDs, consisting of weak organic acids, act by inhibiting prostanoid synthesis and thereby produce anti-inflammatory, analgesic and antipyretic effects (Lees and Higgins 1985, Kallings 1993). This reduces pain and lameness in horses, allowing them to compete without discomfort (Kallings 1993, Moses and Bertone 2002). These drugs do not, however, appear to enhance performance and can have severe toxic effects with adverse reactions including gastrointestinal disturbances and

hypoproteinemia. Permitted dosage levels are therefore a controversial issue in regulations for competing horses, yet NSAIDs are considered to have sufficient therapeutic benefits to justify their application in equine practice (Kallings 1993).

1.3.2 *Intralesional pharmacological therapy*

Some investigations have focused on repair of injured adult tendon through intralesional injections of sodium hyaluronate (HA), polysulphated glycosaminoglycans (PSGAG), βl-aminopropionitrile fumarate (BAPN-F) and insulin-like growth factor (IGF-1), often with disappointing or equivocal results (Genovese 1992, Reef *et al.* 1997, Dowling *et al.* 2000, 2002).

Initial studies on the use of HA intralesional injections reported improved tendon healing based on subjective ultrasonographic evaluation (Spurlock *et al.* 1988, Gaughan *et al.* 1991, Gift *et al.* 1992). However, further research reported limited effects, with none being significant (Oxlund and Andreassen 1980, Salti *et al.* 1993). In an equine study with collagenase-induced injury, no significant benefits were reported (Foland *et al.* 1992, Dyson 1997), indicating that HA was no more beneficial than conservative treatment.

Evaluation of the beneficial effects of PSGAG in clinical tendonitis experiments were based on subjective clinical and ultrasonographic evaluations. Once more the results were not very positive, with no conclusive proof of significantly improved prognosis in comparison with other treatments. Recurrence rates of tendonitis post PSGAG therapy were reported to be as high as 42.5 - 44.4 % (Dow *et al.* 1996, Dyson 1997).

β1-aminoproprionitrile fumarate was proposed to prevent excessive cross-linking in the early stages of tendon repair while promoting linearisation of collagen fibres under the influence of controlled exercise (Genovese 1992). However, long-term data following treated horses' return to racing have been disappointing, with only 45-50 % of these horses regaining maximal athletic activity (Reef *et al.* 1997).

The manufacturer of Tendotrophin™ (active ingredient IGF-1) recommends treatment within 21 days of injury, and two injections are usually needed (www.tendotrophin.com). Studies have shown that IGF-1 levels subside during early stages of tendon healing (Dahlgren *et al.* 2002). Therefore the manufacturer of Tendotrophin™ predicts that supplementation with exogenous IGF-1 could enhance the metabolic response of tenocytes and promote tissue growth and repair (www.tendotrophin.com). A study done by Dahlgren *et al.* (2002) showed a significant increase in collagen-I and collagen-III fibres two months after the first injection. Although these injections can improve the quality of repair, treated tendons displayed

increased rigidity compared to saline treated controls (Dahlgren *et al.* 2002). Whether the elastic qualities and original strength of the tendon can be regained, is not certain. PrimeGRO Ltd., an Australian based biotechnological company that produces Tendotrophin™, is so far the only company that has Australian regulatory approval for a commercial product with IGF-1 as the active ingredient (www.tendotrophin.com).

1.3.3 *Low level cold laser therapy and electromagnetic field therapy*

Low level cold laser therapy is a holistic healing method where injured tendon tissue is irradiated by red and infrared laser light. The damaged tendon absorbs the light, resulting in increased cell metabolism, collagen production and capillary density, ultimately leading to improved blood flow to the damaged area (McKibbin and Paraschak 1983, Marr *et al.* 1993b). In electromagnetic field therapy, a pulsing current is applied to the tendon. This influences cell behaviour by affecting transmembrane ion transport, which is often under enzymatic control (Auer *et al.* 1983). Electromagnetic field therapy has shown to delay collagen maturation in healing tendons. In most cases, the results obtained using the above two methods are varied and do not exceed those results achieved by conventional methods (Dowling *et al.* 2000).

1.3.4 *Extracorporeal shock wave therapy*

Extracorporeal shock waves (ESW) are pressure waves that are generated outside the body and then focused on a specific site within the body. They are differentiated from ultrasound waves by their lower frequency, minimal tissue absorption and an absence of thermal effects. The pressure waves travel through fluid and soft tissue and their effects occur at sites where there is a change in impedance, such as the bone-soft tissue interface (Kersh *et al.* 2004. Wang *et al.* (2002) have shown that ESW therapy promotes growth and differentiation of bone marrow stromal cells into osteogenic progenitors associated with the induction of TGFβ-1, suggesting that this form of therapy stimulates cellular division and cytokine production (Kersh *et al.* 2004). A study conducted by Cheng *et al.* (2004) demonstrated that ESW therapy promotes the healing of Achilles tendonitis, with associated significant increases of TFGβ-1 and IGF-1. In humans, ESW therapy is used successfully to treat conditions such as tennis elbow, non-union fractures and heel spurs (Ogden *et al.* 2001, Kersh *et al.* 2004). In animals, ESW therapy has been shown to induce neovascularisation and tenocyte proliferation at the tendon-bone junction, improving tissue regeneration and repair (Wang *et al.* 2002, Kersh *et al.* 2004). This type of therapy has been used to treat

musculoskeletal diseases in horses (Kersh *et al.* 2004); it however only resulted in a small significant decrease in lesion size at the zone of maximum injury. Moreover, the tendons and ligaments actually displayed increased inflammation.

1.3.5   *Surgical tendon splitting / desmoplasty*

Tendon splitting involves applying multiple stabbing incisions to the section of tendon where swelling has occurred in core lesions, whilst avoiding the infliction of further fibre damage as far as possible (Knudsen 1976, Dabareiner *et al.* 2000). This technique was initially thought to improve vascularisation (Asheim 1964). Subsequent research have shown that splitting actually leads to excessive formation of granulation tissue and progressive degeneration, with no change in collagen production and continued lameness of the affected limb (Strömberg *et al*. 1974, Silver *et al*. 1983). Nevertheless, tendon splitting was again utilised in the early 1990's for experimental purposes in acute lesions, where it facilitated decompression by evacuation of accumulated fluids arising from intratendinous haematoma and oedema. In this scenario it was postulated to reduce lesion size and improve collagen fibril orientation (Henninger *et al.* 1990, Henninger 1992). Although studies demonstrated a 68 % return to racing (Allen 1992), the long-term effectiveness of tendon splitting remains controversial (Henninger 1994). Currently tendon splitting is limited to the treatment of anechoic core lesions (Dowling *et al.* 2000).

1.3.6   *Tissue engineering, autologous and allogeneic transplants and / or grafts*

Tendon tissue engineering involves the creation and use of composites manufactured from various biomaterials and cells to facilitate healing, or to serve as potential replacement tissue. Traditionally it comes in the form of a scaffold seeded with or without cells (Butler and Awad 1999). These resorbable devices were designed to provide an initial load-bearing structure, which would enhance extracellular matrices and growth factor production (via genetic engineering of the cells) and recruit endogenous cells to the site of repair (Young *et al*. 1998, Pittenger *et al.* 2002). It would also serve as a scaffold for both the exogenous cells introduced into the defect site and those recruited to the area. As the materials degrade, they allow transfer of mechanical stresses to the newly formed repair tissue, a step that is critical to increase the diameter of collagen fibrils and thus mechanical functioning (Young *et al*. 1998). Terminally differentiated fibroblasts or chondrocytes have been used in most of these tissue engineering designs (Pittenger *et al.* 2002). Live cell therapy, with fibroblast-seeded

resorbable materials, has been used with some success (Brody *et al.* 1988, Dunn *et al.* 1992, Cao *et al.* 1994).

Grafting produces more rapid reorganisation with well-oriented fibroblasts and collagen fibres and more normal vascularisation than in spontaneously healing tendon lesions (Bergljung 1968, Strömberg and Tufvesson 1977). In the study conducted by Strömberg and Tufvesson (1977), a graft into the DDF tendon rapidly deteriorated yet seemed to act as an "organiser" of invading vessels and fibroblasts, resulting in the production of well oriented collagen. There were however degenerative reactions in the DDF tendon following biopsy. In chronic lesions or lesions with necrotic tissue and scar tissue, tendon grafting is a viable option because the incision into the tendon should be made in such a manner that the necrotic tissue is removed; the transplant is then introduced into the gap (Strömberg and Tufvesson 1977). This has been a successful method in the reconstruction of partial ruptures of Achilles tendons in human athletes (Ljungqvist 1967). Reconstruction of acute lesions with extensive fibre rupture should be performed early (within seven days of injury) in order to prevent excessive invasion of granulation tissue, which would impair complete restitution of the tendon (Strömberg and Tufvesson 1977).

Using the lateral digital extensor tendon as an autologous transplant has shown promising results in horses (Fackelman 1973). Aside from occasional pressure sores and a degree of lameness, which decreased gradually across four months post-operation, no rejection of autologous grafted tissue was reported (Strömberg and Tufvesson 1977). Pedicle grafting however has not produced favourable results. Strömberg and Tufvesson (1977) report the case of a racehorse with a fully ruptured SDF tendon receiving a bipedicle transplant; ten months later it went back into training with a swollen and sore SDF tendon. Post-mortem it was found that the proximal pedicle did not graft properly and was partially ruptured.

Autografts and allografts (Goodship *et al.* 1985, Lieberman *et al.* 1988, Goldstein *et al.* 1989, Wasserman *et al.* 1989, Kato *et al.* 1991, Dunn *et al.* 1992, Salehpour *et al.* 1995), xenografts (Badylak *et al.* 1995), synthetic polymers (Aragona *et al.* 1981, Roberts *et al.* 1983), carbon fibres (Brown and Pool 1983, Vaughan *et al.* 1985) and resorbable biomaterials (Rodkey *et al.* 1985, Ozaki *et al.* 1989, Louie *et al.* 1997) have all been used to repair tendon and ligament ruptures. However, the results have had varying degrees of success and re-injury was a common occurrence.

1.3.7 *Tenocyte cell therapy*

Another potential treatment modality is live tenocyte cell therapy. Flexor tenocytes have been cultured *in vitro* where these cells actively divide, migrate and synthesize new collagen in a cell-free environment (Mass and Tuel 1989). Wigg *et al.* (1997) cultured rabbit flexor tenocytes *in vitro* and demonstrated that the cells exhibit a differential proliferative and healing capacity when compared to those in tendon sheaths. Although this is not technically a conventional treatment for tendon repair, the aforementioned studies clearly provide a foundation for developing hypotheses and designing *in vivo* experiments and potential clinical trials (Lin *et al.* 2004).

1.4 STEM CELL APPLICATION AS POTENTIAL NEW THERAPEUTIC METHOD

The isolation of human stem cells from surplus *in vitro* fertilised (IVF) embryos in 1998 has aroused considerable scientific and economic interest due to their potential therapeutic value (Thomson *et al.* 1998). A summary of the main stem cells types hitherto identified is provided in Table 1.1. The ethical and legal implications of utilising embryonic stem cells remain a controversial topic, hotly debated by media, governments and society. One way to circumvent these problems is to exploit pluripotent adult stem cell sources, such as bone marrow, peripheral blood or umbilical cord blood.

Using adult stem cells for clinical therapy instead would thus provide an obvious advantage. Adult stem cells were initially considered to be of limited use; they were deemed to be developmentally committed to produce specific cell lineages, namely, those of the tissues in which the stem cells reside (Minguell *et al.* 2001). This concept has been challenged by numerous studies, including that of Bjornson *et al.* (1999), where neural stem cells in the adult brain were found to produce early and lineage-committed hematopoietic progenitors. Mesenchymal stem cells (MSCs), which give rise to a variety of mesenchymal phenotypes, can also generate non-mesenchymal cells like mature neural cells (Kopen *et al.* 1999, Reyes and Verfaillie 2001). Moreover, it has been established that adult stem cells can recruit uncommitted progenitors from other tissue sources during elevated tissue growth and repair. This proved to be the case during muscle repair, where MSCs in the bone marrow travelled to skeletal muscle tissue following injury (Ferrari *et al.* 1998).

Despite the various techniques available to improve tendon repair, as illustrated in section 1.3, long-term results of conventional treatments have been discouraging. The therapeutic capacity of adult stem cells in tendon and/or ligament repair is currently under

TABLE 1.1 - Summary of stem cell types and sources.

STEM CELL TYPE	SOURCE OF STEM CELLS	DIFFERENTIATION CAPACITY	POTENCY
Embryonic stem cells	Inner cell mass of embryonic blastocyst	Mature cells of any cell lineage	• Pluripotent • Unspecialised • Long-term self renewal.
Adult stem cells	Bone marrow, peripheral blood, umbilical cord blood, brain, skeletal muscle, pancreas, tooth pulp, fat, skin, etc., of postnatal animal	Mature cells of specific cell lineages	• Pluripotent and Multipotent • Unspecialised • Long-term self-renewal.
Mesodermal progenitor cell	Mesodermal tissue	Mesodermal tissue (Muscle, tendon, cartilage, ligaments, bone, muscle, fat, connective tissue, etc.)	• Unipotent • No self-renewal.
Hematopoietic stem cells	Bone marrow, peripheral blood, umbilical cord blood	Mature blood cells (Red blood cells, white blood cells, platelets, etc.)	• Multipotent
Mesenchymal stem cells	Bone marrow, peripheral blood, umbilical cord blood, adipose tissue skeletal muscle, pancreas, fat, skin etc., of postnatal animal	Mature mesenchymal tissues (Tendons, cartilage, ligaments, bone, muscle, fat, connective tissue, etc.)	• Multipotent

intensive investigation. The most important goals when attempting to achieve full tendon recovery are to promote the regenerative ability of tendons, to reduce scar tissue formation, and to reduce risk of re-injury. MSC-treated injuries showed only slight improvements in material properties of rabbit patellar tendons (Awad *et al.* 1999). More promising results have been advocated by Young *et al.* (1998) and Awad *et al.* (2000). They seeded autologous, bone marrow-derived MSCs in a collagen matrix that was subsequently contracted onto a pretensioned suture. This cell-gel prosthesis was implanted into gap defects in the Achilles tendons of New Zealand rabbits. The prosthesis significantly improved tendon repair biomechanics: structural properties showed a more rapid return to normal function and tendons displayed a significantly larger cross-sectional area. Herthel (2001) reported considerable success in the use of bone marrow aspirates to heal tendon and ligament injuries following direct injection of the marrow into the damaged tendinous area. In 100 horses with suspensory ligament injuries that were treated with bone marrow, the overall prognosis for a return to performance was 84 %, compared to only 15 % in a group of 66 horses receiving conservative treatments (Herthel 2001). Smith *et al.* (2003) similarly used bone marrow-derived cells to treat a five week old, strain-induced injury of the SDF tendon in an eleven year old polo pony. Due to the low yield of MSCs from bone marrow (1 per 10^5 mononuclear cells), the MSCs were first cultured to obtain an amount of 0.64×10^6 MSCs and then injected at the site of injury. The pony showed no signs of lameness or thickening of the tendon, either at ten days or six weeks after live cell therapy application.

The defining characteristics of true MSCs include their ability for long-term self-renewal, an ability to proliferate and finally (when subjected to intrinsic cues) to differentiate into more specialised, mature mesenchymal tissue. Morphologically, MSCs are similar to fibroblasts and possess a consistent set of cell surface markers that has been identified in humans (Pittenger *et al.* 1999, Martin *et al.* 2002). Human MSCs are generally positive for the following surface antigens: STRO-I, SH2, SH3 / CD105, CD29, CD44, CD71, CD90, CD120a and CD124, amongst others. They are negative for the following cell surface markers: CD14, CD34 and CD45, which are markers for the hematopoietic cell lineage (Gronthos *et al.* 1994, Pittenger *et al.* 1999, Reyes *et al.* 2001). It has been proposed that stem cells (which include a long-term subset capable of indefinite self-renewal and a short-term subset with more limited self-renewal capacity) give rise to progenitor cells that have restricted differentiating ability, but eventually differentiate into functionally mature cells (Weissman 2000). When exposed to physical constraints, MSCs appear to respond by organising collagen *in vitro*, indicating their fibroblastic lineage potential (Young *et al.* 1998).

Combined with the osteogenic, myogenic, adipogenic and chondrogenic potentials reported by others (Caplan 1991, Lennon *et al.* 1995, Johnstone *et al.* 1998, Kerr 2000), it suggests a multipotent cell line capable of affecting tendon repair beneficially (Young *et al.* 1998). Considering the high incidence of SDF tendon injuries in performance and competitive horses worldwide, combined with the fact that these tendons are easily accessible, we decided to investigate the efficacy of using stem cell therapy. This was achieved by inducing injuries to the mid-metacarpal regions of SDF tendons, injecting them with progenitor stem cells and analysing recovery parameters. Due to financial constraints, the high price of horses and concomitant unwillingness of horse owners to permit their horses to be part of an experimental study, we used sheep as model animal to establish our methodology and generate initial results. A pilot study with four horses was used to validate these results.

1.5 ESTABLISHING A VIABLE SOURCE OF STEM CELLS

Bone marrow stroma, which fills the intramedullary space in bone, is composed of many cell types such as osteoblasts, adipocytes, reticular cells, endothelial cells, macrophages and monocytes. Progenitors for these cell types are found in the stroma, thus it represents an environment in which stem cell differentiation is inhibited (Pittenger *et al.* 2002). Although both cultured and uncultured bone marrow have been used successfully in treatment of injured horses, there is no published scientific evidence supporting the hypothesis that it is the MSCs specifically in bone marrow which are responsible for healing and regenerating tendon injuries. Bone marrow procurement can be painful and usually requires some sort of analgesia (Zuk *et al.* 2001). As mentioned earlier, bone marrow aspirates also yield very low numbers of MSCs (Zuk *et al.* 2001, Smith 2005). More easily obtainable sources of stem cells that yield higher numbers of MSCs are therefore desirable.

Considerable work has been done on the umbilical cord blood of humans (Belvedere *et al.* 1999, Flores-Guzmán *et al.* 2002, Romanov *et al.* 2003, Yoo *et al.* 2003). Cord blood can be recovered with comparative ease and without risk to either the neonatal donor or mother (Rubinstein *et al.* 1995). In comparison with adult bone marrow, umbilical cord blood possesses both a higher *in vitro* culture capacity and an increased replicative ability (Newburger and Quesenberry 1996). Isolation of mesenchymal or hematopoietic stem cells from the umbilical cord blood of horses is therefore well worth exploring, especially since the non-surgical methods required for extraction are not subject to biological product or medical practice regulations. A further advantage to using cord blood stem cells is that

cryopreservation of human cord blood mononuclear cells (white blood cells inclusive of stem cells) has been successfully developed (Rubinstein *et al.* 1995, Hunt *et al.* 2003a,b). VetCell Biosciences Ltd. is currently in the process of storing and researching equine umbilical cord blood mononuclear cells (www.vetcell.com).

We encountered several practical obstacles during trials aimed at obtaining umbilical cord blood from sheep and horses giving natural birth at full-term. Firstly, the time of labour is not accurately predictable and the animals usually gave birth at night. Secondly, there is an inability to restrain the mother sufficiently without simultaneously inducing stress related labour complications. Thirdly, it is extremely difficult to obtain clean umbilical blood samples quickly in a non-sterile environment. More often than not, by the time the collector reaches the animal the placenta has either been eaten or trampled into the ground and the blood would be coagulated. Another unforeseen complication is the latest trend of owners to let their horses give birth in paddocks instead of stables. Despite attempting both open and closed blood collection systems, it was also problematic to obtain samples of sufficient volume size. We consequently decided that caesareans would not only provide a sterile environment but also allow for the collection of larger umbilical cord blood volumes. Unfortunately, out of five caesareans conducted on sheep, no lambs were revived successfully, which precluded an autologous tendon study. Therefore we shifted our focus to stem cells present in peripheral blood, since both MSCs (Di Stefano *et al.* 2002, Quan *et al.* 2004, Roufosse *et al.* 2004) and HSCs (Gilmore *et al.* 2000, Norol *et al.* 2002) have been identified in circulating blood.

Peripheral blood progenitor cells for both HSCs and MSCs could potentially replace bone marrow transplants as result of the following advantages: (1) they can be collected without general anaesthesia in humans or analgesia in animals; (2) contrary to bone marrow, no HLA (Human Leukocyte Antigen Testing) matching is necessary, only blood type matching, and the O^+ blood group can be used with all blood types in allogeneic studies; and (3) infused cells may render a faster hematopoietic and immune reconstitution than bone marrow transplants (Bensinger *et al.* 1995, Dreger *et al.* 1995, Korbling *et al.* 1995, Ottinger *et al.* 1996). The last point may be partly explained by the relative distribution of early myeloid progenitor cells, which are higher in both peripheral and umbilical cord blood as opposed to bone marrow (Fritsch *et al.* 1996). Although less than 1 % of circulating whole blood cells are $CD34^+$ (Sutherland *et al.* 1994) and the actual percentage of circulating MSCs remains unknown, MSCs from this source have been cultured successfully and expanded to larger numbers (Di Stefano *et al.* 2002). In the case of horses we therefore used MSCs

cultured from peripheral blood for all treatment procedures. In the case of sheep, however, we were unable to form adherent MSCs from peripheral blood. In stead we utilised mononuclear cells (MNCs) obtained from the peripheral blood's buffycoat, since it is a cell source containing both MSCs and HSCs.

Stem cells derived from adipose tissue represents a third source of MSCs which is easy to obtain, inflicts minimal discomfort during harvesting and yields cell numbers that are substantial enough to obviate extensive expansion in culture (Zuk *et al.* 2001). Rangappa *et al.* (2003) used MSCs derived from adipose tissue to regenerate cardiomyocytes. Human, adipose-derived stem cells can be differentiated *in vitro* into adipogenic, chondrogenic, myogenic and osteogenic cells in the presence of lineage-specific induction factors (Zuk *et al.* 2001, Zuk *et al.* 2002). These stem cells express CD29, CD44, CD71, CD90, CD105/SH2 and CD106/SH3, which, together with SH2, are markers for MSCs (Haynesworth *et al.* 1992, Zuk *et al.* 2002). We attempted to culture MSCs from the fatty tissues of both sheep and horses. This proved to be more difficult than anticipated in the case of horses and those samples were discarded. Various culture media were tested; however, of all the samples cultured only one proliferated for a few weeks before undergoing apoptosis.

Another matter of great importance and consequence is the efficiency of transplanting allogeneic adult stem cells versus the efficiency of transplanting autologous adult stem cells. Wagner (1997) described more than 700 successful related and unrelated cord blood transplants in humans with various diseases, while Horwitz *et al.* (1999) conducted unrelated, bone marrow-derived MSC transplantation in children with osteogenesis imperfecta. In horses, bone marrow-derived stem cells obtained from an injured animal have been shown to heal and / or regenerate the tendon of that same horse (Smith *et al.* 2003). The possibility that stem cells obtained from one horse can heal and / or regenerate injured tendons of another horse would however have far-reaching implications in the equine clinical setting. Since horse and sheep monoclonal antibodies for stem cells were not commercially available at the time of this study, we were restricted to positively selecting $CD45^-$ cells for an allogeneic study, using a fluorescent activated cell sorter (BD FACS Aria).

Table 1.2 summarises findings from previous studies where stem cell injections were used to investigate their efficiency in tendon repair.

TABLE 1.2 - A summary of findings from previous studies investigating the use of stem cells in tendon repair.

LOCATION	STEM CELL TYPE	TREATMENT	INJURY	HEALING RESPONSE OF TENDON / LIGAMENT	REFERENCE
Bone marrow	Mesenchymal stem cells, cultured	Collagen gel gap implants	Achilles tendon of female rabbits (n = 53)	• Improved biomechanics • Improved structural and material properties • Larger cross-sectional area • Improved fibre alignment	Young *et al.* (1998)

Bone marrow	Mesenchymal stem cells, cultured	Collagen gel gap implants	Patellar tendon of female rabbits (n = 18)	• Improved biomechanics:
				  - maximum stress load increased by 26 %
				  - elasticity increased with 18 %
				  - strain energy density increased by 33 %
				• Increased number of tenocytes
				• More collagen fibre bundles
				• No improvement of fibre alignment
					Awad *et al.* (1999)

Bone marrow	Mononuclear cells, uncultured	Intralesional injection	Suspensory ligament of horses (n = 166)	• Increased soundness • No re-injury in 84 % (after 6 months)	Herthel (2001)
Bone marrow	Mesenchymal stem cells, cultured	Intralesional injection	Superficial digital flexor tendon of horse (n = 1)	• No lameness of affected limb • No swelling at injury site • No increased tendon thickening • Mild pain disappeared six weeks post treatment • Minimal change to tendon cross-sectional area • No change in substance of the tendon	Smith *et al.* (2003)

TABLE 1.2 (continued) - A summary of findings from previous studies investigating the use of stem cells in tendon repair.

LOCATION	STEM CELL TYPE	TREATMENT	INJURY	HEALING RESPONSE OF TENDON / LIGAMENT	REFERENCE
Adipose tissue	Mononuclear cells, uncultured	Intralesional injection	Tendon and ligament injuries and joint disease in horses (n = unknown)	• Decreased inflammation • Increased collagen fibre uniformity • Increased polarized collagen fibre crimping • Improved collagen fibre linearity • Improved tendon cell shape • Improved lesion size and grade	Internet Resources - Vet-Stem Ltd. 2002
Bone marrow	Mesenchymal stem cells, cultured	Intralesional injection	Tendon and ligament injuries in horses (n = unknown)	• Decreased inflammation • Hypo-echoic lesion becomes hyper-echoic • Improved longitudinal orientation of fascicles • Collagenase matrix exhibit a crimp pattern characteristic of ligament rather than scar tissue • Reduced scar tissue formation • 75 % of horses return to full training within a year	Internet Resources - VetCell Biosciences Ltd. 2002
Bone marrow	Mesenchymal stem cells, cultured	Intralesional injection	Tendon and ligament injuries in horses (n = unknown)	• Improved fibre alignment • Improved echogenicity • Decreased inflammation	Internet Resources - Vet Biotechnology Ltd. 2004

1.6 COMMERCIAL VENTURES PROMOTING THE THERAPEUTIC USE OF STEM CELLS IN

   TENDON REPAIR

Vet-Stem, Inc. is an American-based company specialising in adipose-derived stem cells, but their services are not available to any country outside of the United States. To date, this is the only company that promotes that they use stem cells derived from adipose tissue to treat tendon and ligament injuries in animals. The company also treats other diseases, such as degenerative joint disease and bone cysts, with adipose-derived MNCs (www.vet-stem.com).

Vet Cell Biosciences Ltd., established in 2002, supplies cultured autologous bone marrow-derived MSCs to Europe and France as a potential therapeutic agent in tendon and / or ligament injuries (www.vetcell.com). In 2004 another company, namely Vet Biotechnology Ltd., was established by the same researchers, extending an identical service to Australia and New Zealand (www.vetbiotechnology.com.au). Of the 250+ horses treated with these MSCs, 75 % returned to competitive work within a year and sustained no re-injury. Even lesions older than five months showed rapid ultrasonographic and histological improvement (www.vetbiotechnology.com.au).

Another service provided by the latter two companies is the storage of umbilical cord blood. It is not clear whether VetCell Biosciences Ltd. has already established a method for *in vitro* expansion of umbilical cord blood stem cells or whether cultured umbilical cord stem cell therapy is available. Of the approximately 110 000 foals born per annum globally, 20.9 % can make use of this service in Australia and New Zealand and 16.4 % in Europe. With no other companies offering this service to date, VetCell Biosciences Ltd. is trying to establish cryopreservation of umbilical cord blood cells for the global market.

Although no scientific articles have been published by any of these companies, various press articles and annual reports are available for downloading from their respective websites. Vet-Stem Ltd. include on their website, amongst others, an unpublished study compiled by Cornell University (authors A. Nixon and L. Dahlgren) portraying significant improvements in tendon recovery due to stem cell therapy (refer to the section on Internet Resources for a comprehensive list of articles downloaded from this website). VetCell Biosciences Ltd. and Vet Biotechnology Ltd. make bold statements of tendon recovery rates in response to stem cell applications - no substantiating studies are referenced on their websites, only downloadable magazine articles reporting the data (see Internet Resources). The contact details of the respective companies are provided in Addendum A.

1.7 PREVENTION BETTER THAN CURE?

It is clear that the treatment and rehabilitation of horses with SDF tendon injuries are problematic and alternative approaches are sought. Prevention of initial injury remains the safest option and requires horse owners to be aware of factors that may predispose their animals to potentially long-term and costly injuries. This includes reducing the exposure of horses to poor surface conditions (Riemersma *et al.* 1996a) and employing competent farriers to implement good shoeing practices (Riemersma *et al.* 1996b), for example, preventing poor dorsopalmar hoof balance or under-run heels and long toes.

Usage of a professional training regime is of particular importance in racehorses (Patterson-Kane *et al.* 1998). In the adult equine SDF tendon, exercise and age- associated microdamage, combined with a limited adaptive ability and narrow safety margins, may however mean that fatigue failure is inevitable; recent studies suggest that the adaptive response of tendons to exercise in young horses decrease as the horse reach maturity (Gillis *et al.* 1993, Birch *et al.* 1999, Kasashima *et al.* 2002). Modulation of tendon physiology prior to maturation may hold the key to injury prevention (Cherdchutham *et al.* 2001, Smith *et al.* 2002). For instance, the SDF tendons of foals display greater adaptive ability than those of adult horses and may be more responsive to appropriate training regimens, thereby producing more functionally adapted tendons. Despite its smaller cross-sectional area, the foal SDF tendon shows values similar to adult SDF tendons for maximal load and ultimate strain, suggesting ultimate strength may not be the most important factor in preventing tendon injury. There is also evidence that foals allowed free pasture and exercise, develop larger, stronger and more elastic tendons compared to foals that are confined or subjected to a training program. It remains unclear whether development of larger tendons is protective in later life (Dowling and Dart 2005). Allowing exercise may aid in the prevention of injury and may produce a more functionally adapted tendon, increase the safety margins and reduce the incidence of exercise-associated injury. Research into determining optimum exercise intensity and timing, and the role of the non-collagenous matrix in tendon physiology of the young horse, may hold the ultimate key to preventing SDF tendon injury in horses.

1.8 CONCLUSION AND CAUTIONARY REMARKS

Treatment of tendon and/or ligament injuries has improved significantly over the last decade due to both clinical and experimental research, and the findings of each study, whether *in vivo* or *in vitro*, contribute towards a more thorough understanding of tendon healing

mechanisms (Lin *et al.* 2004). The use of adult stem cells to promote tendon repair holds much promise and animal studies have shown the validity of using these cells. The main advantage of MSCs is their pluripotent ability to differentiate into a desired cell lineage (Lin *et al.* 2004). Another obvious significant advantage of MSCs is their potential of autologous transplantation, thereby preventing host rejection. There is however cumulative evidence that human MSCs could be used allogeneically, without the need for extensive immuno-suppressive drugs (Wagner 1997, Horwitz *et al.* 1999, Tabbarra *et al.* 2002, Barry *et al.* 2004). No studies prior to 2007 that have used bone marrow stem cells report the development of neoplasia, an adverse effect which may occur with other stem cell types, as has been reported for embryonic stem cells (Niesler 2004).

The limited number of experiments conducted on animals to date has provided promising preliminary data and it is reasonable to conclude that the use of adult stem cells is, at least in the short-term, safe. Although our cumulative knowledge of stem cells is vast, it is still incomplete. Both experimental and clinical studies are required to achieve the ultimate goal, namely, using stem cells to form tissues that are structurally and functionally comparable to normal, uninjured tissues. From a therapeutic point of view, it is critical to determine (1) an optimal stem cell source, (2) the optimal number of cells required for injection, (3) delivery route, (4) the timing of injection and delivery, and (5) long-term safety implications. Once these factors have been researched thoroughly, stem cell therapy may offer an alternative treatment modality that restores the normal function of damaged horse tendons, thereby increasing the extent and duration of their competitive performances.

Most horses, especially performance horses, are athletes sustaining injuries similar to their human counterparts. Therefore, once *in vitro* culture methods for MSCs have been successfully optimised, their therapeutic use could be extended to treatment of human tendon injuries. One should however be cautious in extrapolating results directly to humans, just as care must be taken when extrapolating *in vitro* results for *in vivo* settings (Lin *et al.* 2004). Nevertheless, investigations into umbilical cord blood, bone marrow, peripheral blood and fatty tissue MSCs represent a fast expanding and exciting research area at the forefront of scientific discovery. The considerable potential of stem cells already recognised in human medicine warrants further investigation into tendon and ligament injuries in both the equine and human field.

1.9 STUDY OBJECTIVES

- *Objective 1:* Isolate, culture and characterise mononuclear cells (which include mesenchymal and hematopoietic stem cells) from the peripheral blood of adult sheep and horses (Chapter 2).

- *Objective 2:* Establish an injury model that mimics natural tendon injury and describe such a site of injury (Chapter 3).

- *Objective 3:* Determine the degree of tendon healing and/or regeneration in a sheep model after injection of cultured (1) autologous and (2) allogeneic adult sheep mononuclear cells (Chapter 4), as derived from adult peripheral blood.

- *Objective 4:* Re-implant autologous MNCs obtained from adult horse peripheral blood into traumatised horse tendons to assess the degree of healing and/or regeneration over a seven week period (Chapter 5).

- *Objective 5:* Isolate, culture and characterise mononuclear cells derived from adult sheep adipose tissue (Chapter 6).

- CHAPTER 2 -

ISOLATION, CULTURE AND CHARACTERISATION OF MONONUCLEAR CELLS DERIVED FROM ADULT PERIPHERAL BLOOD OF SHEEP AND HORSES

2.1 INTRODUCTION

ADULT stem cells derived from bone marrow have shown to improve ligament repair in horses. Herthel (2001) treated suspensory ligament injuries of 100 horses with uncultured bone marrow aspirates. The overall prognosis for a return to performance was 84 % (compared to 15 % in a group of 66 horses treated conservatively), providing encouraging support for the use of adult stem cells in ligament repair. Bone marrow yields a fair number of hematopoietic stem cells (HSCs) but unfortunately low numbers of mesenchymal stem cells (MSCs) (Zuk *et al.* 2001), necessitating extensive *ex vivo* expansion of the latter to obtain clinically significant cell numbers for application. In addition, bone marrow procurement is often a painful process which requires general or spinal analgesia in humans. Using bone marrow as an adult stem cell source is therefore time consuming, expensive and often painful to the donor.

In contrast, peripheral blood is an easily accessible adult tissue that can be collected with comparative ease from the jugular vein or carotid artery. The process results in minimal discomfort and has no adverse effects to the individual. Compared with bone marrow, peripheral blood yields a greater initial number of cells, which can be expanded further *in vitro*. Mesenchymal stem cells (MSCs) and HSCs are pluripotent stem cells and are present in peripheral blood. Furthermore, both stem cell types have the ability to differentiate into various cell types, potentially including tenocytes (Zvaifler *et al.* 2000, Zhao *et al.* 2003). However, to date there is no well defined isolation or characterisation protocol for adult peripheral blood stem cells in animals. Our main objective for this subsection of the study was to isolate, culture and characterise mononuclear cells (MNCs) from the peripheral blood of adult sheep and horses. Mesenchymal stem cells, HSCs and their progenitors, along with white blood cells and macrophages, are MNCs which can be isolated from the buffycoat of blood.

Although no single specific marker has yet been identified for MSCs, these stem cells express a panel of antigens and receptors for cell adhesion molecules and are devoid of typical hematopoietic antigens (Minguell *et al.* 2001, Zuk *et al.* 2002). Specifically, peripheral blood MSCs are negative for CD34, CD45 and CD133, all of which are all HSC markers. The

acquisition and identification of MSCs is based on the capacity of these cells to adhere to plastic polymer surfaces (Minguell *et al.* 2001, Villaron *et al.* 2004). Studies of HSCs have recognised that, when samples of whole bone marrow are placed in culture dishes without any additional cytokines, the small number of cells that adhere to the plastic provides an important microenvironment for both the growth of HSCs and their differentiation into granulocytes and erythrocytes (Prockop 1997). In our study we cultured MNC populations which were identified via flow cytometry.

2.2 MATERIALS AND METHODS

All procedures and protocols involving animals in this experiment were approved by the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council (application numbers P04/06/010 and P04/06/012)[2]. All blood collection procedures were conducted by a registered veterinarian.

2.2.1 *Study groups and sample size*

Peripheral blood samples were collected from 21 of the 27 Black-headed Dorper sheep used to study tendon healing by means of MNC injections as described in Chapter 4. Four of these sheep, denoted by Group 3 in Table 2.1, did not receive any MNC treatment following injury - here they will be referred to as the Control Group. Eleven of the sheep, labelled as Group 4 and Group 5 in Table 2.1, received autologous MNC injections post-injury – in this chapter they constitute the Autologous Group. The last six sheep, identified as Group 6 in Table 2.1, received allogeneic MNC injections following injury – for the time being they will be referred to as the Allogeneic Group. Peripheral blood samples were also collected from all four of the Thoroughbred horses used to examine the effect of autologous MNC injections on tendon recovery in Chapter 5. The isolation, culturing and characterisation protocols are examined separately for sheep and horses in the following sections.

2.2.2 *Care of animals*

All our sheep and horses were housed at the Welgevallen experimental farm in Stellenbosch, South Africa. The animals were examined by a veterinarian prior to experimentation and declared healthy and clinically normal.

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[2] The University of Stellenbosch committee for animal research was only re-established in 2005, at which point we had already obtained ethical approval for our experiments from the national body, ECRA.

TABLE 2.1 - Mononuclear cells were isolated from 21 of the 27 experimental sheep referred to in this thesis. These sheep are indicated below in the non-shaded areas containing Groups 3 to 6. The remaining sheep from Groups 1 and 2 were used to study tendon healing following artificial injury and will be referred to in subsequent chapters.

			GROUP	FRONT LEG	INJURY (WEEK 0)	TREATMENT	ID	SACRIFICE
	Chapter 3		1 (n = 3)	Left	Skin incision	None	Ctrl	Week 1
				Right	Cut tendon	None	Cut	Week 1
	Chapter 3		2 (n = 3)	Left	Insert needle into tendon	None	Ctrl	Week 1
				Right	Collagenase	None	Coll	Week 1
Chapter 2	Chapter 3	Chapter 4	3 (n = 4)	Left	Saline	Saline	SS	Week 7
				Right	Collagenase	Saline	CS	Week 7
Chapter 2	Chapter 3	Chapter 4	4 (n = 5)	Left	Saline	Saline	SS	Week 7
				Right	Collagenase	Autologous MNCs	CMNC	Week 7
Chapter 2	Chapter 3	Chapter 4	5 (n = 6)	Left	Collagenase	Saline	CS	Week 7
				Right	Collagenase	Autologous MNCs	CMNC	Week 7
Chapter 2	Chapter 3	Chapter 4	6 (n = 6)	Left	Collagenase	Saline	CS	Week 7
				Right	Collagenase	Allogeneic MNC	CCD45	Week 7

The sheep used in this experiment were between eight months and four years of age and were housed indoors in stables. They were provided with sheep feed and hay two times per day and fresh water daily. The stables were swept each day and cleaned thoroughly each week. The horses, aged between two and three years, were housed outdoors in grass paddocks containing sufficient grazing and water. In addition, they were maintained on a diet of 10 % horse feed (Classic Feeds (SA) Ltd.) and hay, provided twice daily.

2.2.3 *Sheep peripheral blood*

2.2.3.1 <u>Blood sample collection</u>

Peripheral blood samples (n = 21) were collected by venupuncture of the jugular vein. At ± 10 ml / kg, this delivered an average blood sample volume of ± 200 ml per animal. The sheep were manually restrained in lateral recumbency without any use of sedatives. The neck area was prepared for aseptic blood collection by shaving the wool and cleansing the skin with a Hibitane (chlorhexidine gluconate) and 70 % ethanol solution. A 19-gauge needle was used to draw blood, led through sterile tubing to 50 ml Falcon tubes containing 0.1 ml heparin as anticoagulant. Samples were transported to the laboratory for immediate processing.

2.2.3.2 <u>Red blood cell lysis</u>

Each whole blood sample was centrifuged for 20 minutes at 3 000 g. The plasma was pipetted off and stored at 4 ºC for later inclusion (sections 2.2.3.5 and 2.2.3.6). The buffycoat was pipetted into a sterile 15 ml Falcon tube and the red blood cell pellet discarded. Mononuclear cells were isolated from the buffycoat through lysis of any remaining red blood cells. A 10x lysis solution (custom-made by Highveld Biologicals), containing ammonium chloride, potassium hydrogen carbonate and ethylenediaminetetraacetic acid (EDTA), was added to the buffycoat in a 4:1 ratio. After gently inverting the Falcon tube, it was placed in a 37 ºC water bath for 15 minutes to activate the lysing agents. Thereafter the cells were washed twice with Dulbecco's Phosphate-Buffered Saline (D-PBS; Gibco[TM] Cat. no. 14190) nutrient medium, containing 2 % Bovine Serum Albumin (BSA; Sigma® Cat. no. A7034) and 1 % Antibiotic-Antimycotic (AA; Sigma® Cat. no. A5955). The Falcon tube was centrifuged again, for five minutes at 350 g, and the supernatant discarded.

The remaining cell pellet was re-suspended in 1 ml Dulbecco's Modified Eagle's Medium (DMEM; Gibco[TM] Cat. no. 32430) culture medium, containing 1 % AA and 20 % Foetal Bovine Serum (FBS; Gibco[TM] Cat. no. 26170). Of this ± 1 ml cell suspension: (1) a 100 µl sub sample was extracted to conduct cell count and viability analyses, as described in

section 2.2.3.3, (2) a second 100 µl sub sample was extracted for flow cytometric analysis with CD45 cell surface marker, as described in section 2.2.3.4, and (3) the remaining volume was used to culture MNCs, as described in sections 2.2.3.5 and 2.2.3.6. A flow diagram of the red blood cell lysing procedure is presented in Figure 2.1.

2.2.3.3 <u>Cell count and viability</u>

To count nucleated cells, the first 100 µl sub sample of the cell suspension was diluted with D-PBS nutrient medium. The dilution factor, if used, was taken into account when comparing samples statistically. Approximately 1 – 3 µl was loaded into a haemocytometer and examined under a light microscope. The remaining 97 – 99 µl of this sub sample was used to determine cell viability with a propidium iodide (PI) dye exclusion test, in which cells were stained with PI (Sigma® Cat. no. 70335) and processed with a flow cytometer. Results were analysed digitally with CellQuest software (BD Biosciences). Viability tests with PI, using a flow cytometer, were also conducted after culturing, prior to injection of cells.

2.2.3.4 <u>Flow cytometry</u>

The second 100 µl sub sample of the cell suspension (refer to section 2.2.3.2, last paragraph) was washed with D-PBS nutrient medium and centrifuged at 350 g. The supernatant was discarded and resuspended in D-PBS nutrient medium to make up a total volume of 100 µl.

Half (50 µl) was incubated with mouse anti-ovine CD45 primary monoclonal antibody (Serotec Cat. no. MCA2220F) for 30 minutes at room temperature and in the dark. The CD45 was fluorescein isothiocyanate (FITC) conjugated at purchase. CD45 recognises antigens on lymphocytes, granulocytes, monocytes and macrophages, which allows selective removal of white blood cells from samples via flow cytometry analysis, if required. Martin *et al.* (2002) showed that feline, bone marrow-derived MSCs were negative for CD45. Following incubation, 450 µl D-PBS was added to form a total volume of 500 µl. Cell fluorescence was then evaluated by flow cytometry using a BD Biosciences FACSAria, equipped with an argon-ion laser tuned to 488 nM. Fluorescence was measured using 530 nM (FITC) band pass filters. Approximately 100 000 events were acquired and analysed by CellQuest software.

The other half of the suspension (50 µl) was incubated with matched immunoglobulin (IgG) isotypes, also for 30 minutes at room temperature, in the dark. These IgG isotypes were
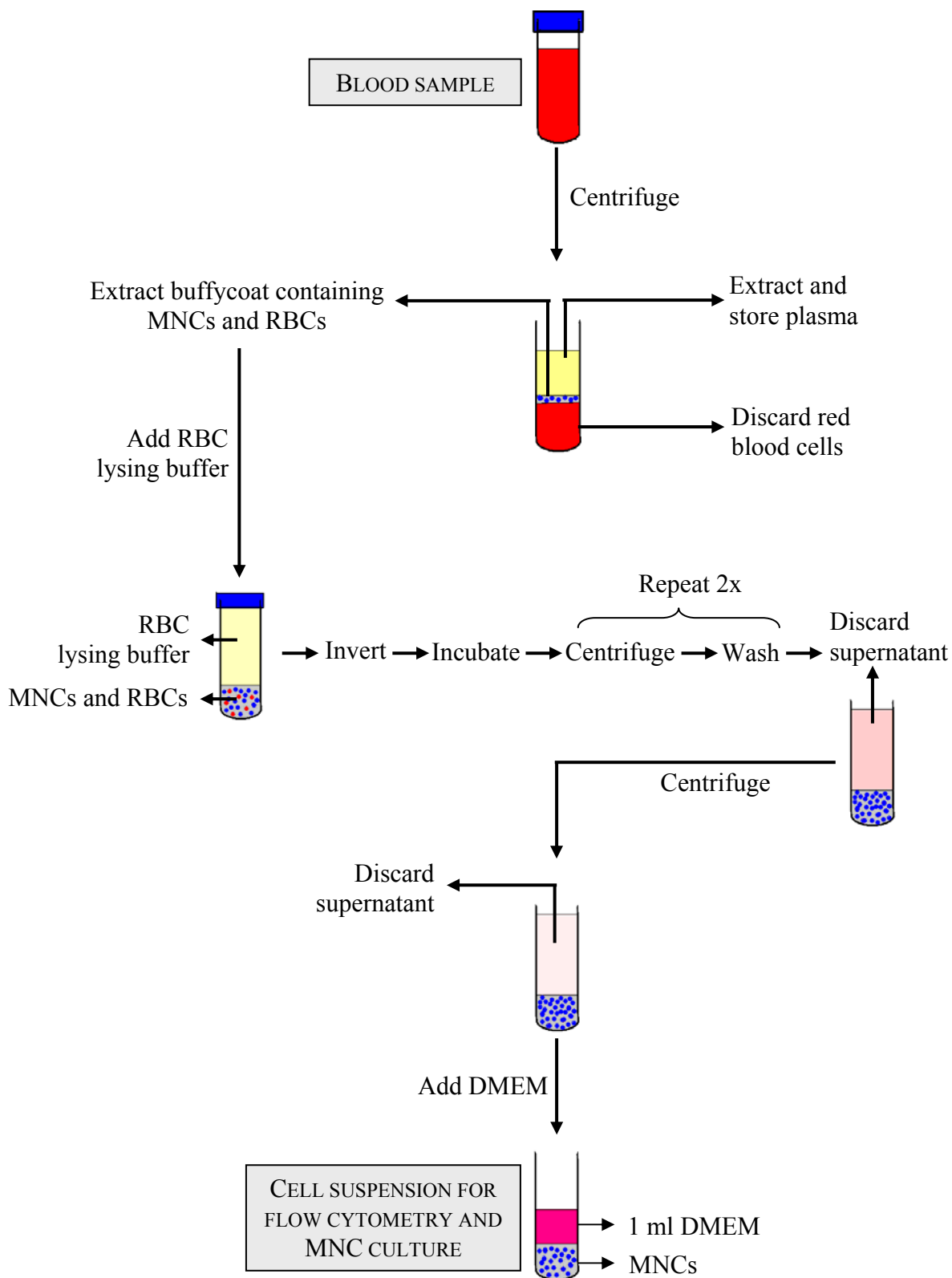
FIGURE 2.1 - Flow diagram illustrating red blood cell lysis and sample preparation for flow cytometry analysis and mononuclear cell culture (MNC = mononuclear cell; RBC = red blood cell; DMEM = Dulbecco's Modified Eagle's Medium).

similarly conjugated to FITC. The IgG-FITC conjugated samples served as a negative control to verify that no auto-fluorescence occurred during flow cytometry.

2.2.3.5 Culture of autologous mononuclear cells

The remaining sub sample of cell suspension (refer to section 2.2.3.2) was used to culture MNCs for the Autologous Group. A flow diagram of the culture protocol is presented in Figure 2.2. Using results obtained from the cell count determination (section 2.2.3.3), this suspension was diluted with DMEM culture medium to obtain a total cell count of 4.5×10^6 cells per 1 ml of diluted solution.   Accordingly, 1 ml of this solution was extracted and incubated at 37 ºC and 5 % CO_2.

After 24 hours, a culture medium change was conducted by discarding three quarters of the supernatant and replacing it with DMEM culture medium, also containing a final concentration of 10 µM BrdU (Bromo-deoxyuridine, BD Pharmingen^{TM} Cat. no. 551321) solution. Bromo-deoxyuridine is a nucleotide derivative (an analogue of the DNA precursor thymidine), which is incorporated into the growing nucleotide chain during replication or normal cell cycling. The cell marker can be stained with anti-BrdU antibodies for detection in living tissues through flow cytometry or fixed tissues through histology (refer to Chapter 4).

After two days of culture with BrdU, the cells were re-suspended in basal liquid culture medium (DMEM, 1 % AA, 10 % FBS). Viability analysis was performed at this point on a small sub sample (see section 2.3.1.1 for results). The cell suspension was finally centrifuged at 350 g for five minutes, the supernatant discarded and re-suspended in a vial with 1 ml of the blood plasma from which it originated. Vials were placed on ice for immediate transport to the animal facility for injection into our study animals (see Chapter 4).

2.2.3.6 Culture of allogeneic mononuclear cells

The remaining sub sample of the cell suspension (refer to section 2.2.3.2, last paragraph) was used to culture MNCs for the Allogeneic Group. This suspension was diluted with D-PBS nutrient medium to obtain a total volume of 10 ml. The resulting suspension was incubated for 30 minutes with the same mouse anti-ovine CD45 primary monoclonal antibody as describe in section 2.2.3.4 above, at room temperature and in the dark. The exact concentration of CD45 to be added was calculated using the results obtained from our initial cell count. The flow cytometer was set to extract a total of 3×10^6 CD45$^-$ events. Usage of CD45$^-$ MNCs implies exclusion of lymphocytes, granulocytes, monocytes and macrophages
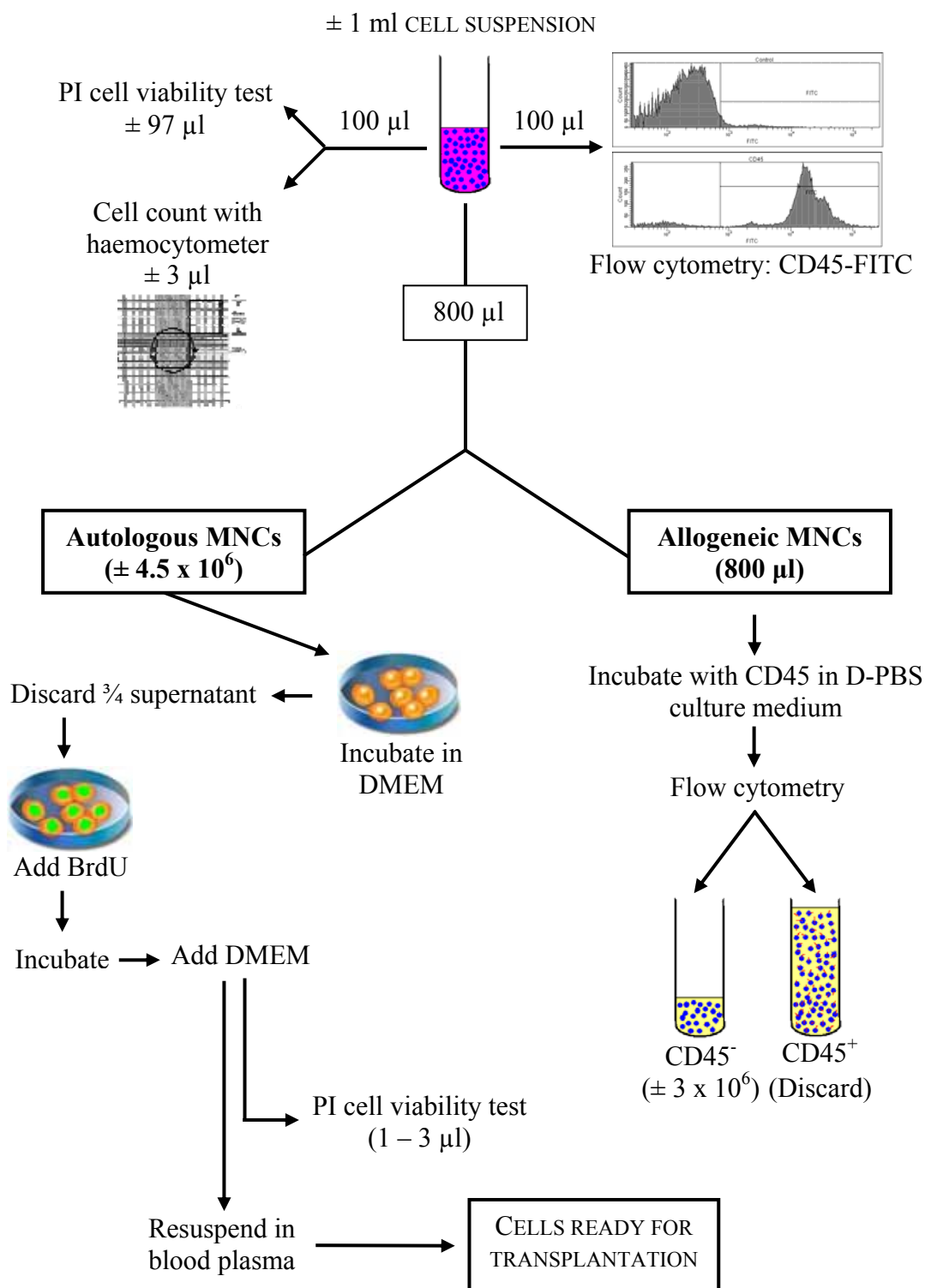
FIGURE 2.2 - Flow diagram illustrating analyses conducted on sheep blood samples in which red blood cells had been lysed. This includes cell counts, viability tests, flow cytometry and the culture of both autologous and allogeneic mononuclear cells for injection purposes (PI = propidium iodide; FITC = fluorescein isothiocyanate; MNC = mononuclear cell; BrdU = Bromo-deoxyuridine; DMEM = Dulbecco's modified Eagle's medium; D-PBS = Dulbecco's phosphate-buffered saline).

from the MNC population. This is necessary to prevent immune rejection when injecting allogeneic cells.

The 3×10^6 CD45⁻ MNCs were incubated with ± 5 ml DMEM culture medium at 37 ºC at 5 % CO_2. After 24 hours, a culture medium change was conducted by discarding three quarters of the supernatant and replacing it with DMEM culture medium also containing BrdU solution at a final concentration of 10 µM. After two days of culture in BrdU, as for the autologous MNCs, these cells were re-suspended in basal liquid culture medium (DMEM, 1 % AA, 10 % FBS). Viability analysis was once more performed on a small sub sample using PI and flow cytometry (refer to section 2.3.1.1 for results). The cell suspension was also centrifuged at 350 g for five minutes and the supernatant discarded. As opposed to the autologous MNCs, allogeneic MNCs were re-suspended in a vial with 1 ml of blood plasma from the recipient sheep. Vials were placed on ice for immediate transport to the animal facility and injection into our study animals (see Chapter 4).

2.2.4   Horse peripheral blood

2.2.4.1 Blood sample collection

Peripheral blood samples (n = 4) were collected by venupuncture of the jugular vein, at a volume of 200 ml per animal. The horses were manually restrained, without the use of sedatives. The neck area was prepared for aseptic blood collection by cleansing the skin with a Hibitane and 70 % ethanol solution. Blood was collected into sterile 500 ml blood bags containing the required amount of citrate phosphate dextrose (anticoagulant) and transported to our laboratory for immediate processing.

2.2.4.2 Red blood cell lysis

Adult horse peripheral blood samples were processed using the same red blood lysing protocol as described for sheep (section 2.2.3.2). The resulting ± 1 ml of cell suspension was, in this case, subdivided into (1) a 100 µl sub sample for a cell count, as described in section 2.2.4.3, (2) a further two 100 µl sub samples for flow cytometric analyses, as described in section 2.2.4.4, and (3) the remaining volume was used to culture MNCs, as described in section 2.2.4.5.

2.2.4.3 Cell count and viability

The first 100 µl sub sample of the cell suspension was used to determine a cell count, following the same protocol as described for sheep in section 2.2.3.3. This was also followed

up by a PI dye exclusion test for cell viability. Another PI viability test was conducted after culturing.

2.2.4.4 <u>Flow cytometry</u>

One of the further two 100 μl sub samples was washed twice with D-PBS nutrient medium, centrifuged at 350 g, the supernatant discarded and resuspended in D-PBS to make up a total volume of 100 μl. Half of this suspension (50 μl) was incubated with mouse anti-equine CD4 (Serotec, Cat. no. MCA1078) primary monoclonal antibodies for 30 minutes, at room temperature and in the dark. This antibody marks lymphocyte T-helper cells. After two washing cycles in D-PBS, the cells were incubated for a further 30 minutes in a rabbit anti-mouse IgG secondary antibody that was FITC conjugated (Serotec, Cat. no. STAR9B). Again, 450 μl D-PBS was added and the total volume of 500 μl was used for flow cytometry analysis. The other half of the suspension (50 μl) served as negative control and was only incubated with the secondary IgG antibody for 30 minutes at room temperature, in the dark.

The second of the 100 μl sub samples received exactly the same treatment, except that mouse anti-equine CD11a/18 (Serotec, Cat. no. MCA1081) was used as primary monoclonal antibody whereas the secondary antibody remained the rabbit anti-mouse, FITC conjugated IgG antibody. The CD11a/18 antibody is a combination marker for all leucocytes.

All of the above mentioned analyses were conducted both prior to culturing (fresh samples) and subsequent to the 14-day culture period.

2.2.4.5 <u>Culture of autologous mononuclear cells</u>

The remaining sub sample of cell suspension (refer to section 2.2.4.2, last paragraph) was used to culture autologous MNCs. Immediately following cell count determination (section 2.2.4.3), this suspension was diluted with DMEM culture medium to obtain a total cell count of 7.2×10^6 cells per 1 ml of diluted solution. Accordingly, 10 ml of this solution was extracted and incubated at 37 ºC and 5 % CO_2 in a humidified chamber on day one.

After 24 hours, three quarters of the culture medium was replaced with fresh DMEM culture medium. This was supplemented with the following recombinant mouse (rm) or recombinant human (rh) cytokines: rhSCF (50 ng/ml, Cat. no. S7901) + rm IL-3 (50 ng/ml, Cat. no. I4144) + rm IL-6 (50 ng/ml, Cat. no. I9646) + rm TPO (50 ng/ml, Cat. no. T4184) + rh Flt3/Flk2 (50 ng/ml, Cat. no. F3422) (Flores-Guzmán *et al.* 2002, Denning-Kendall *et al.* 2003). Stem cell factor (SCF) acts synergistically with other growth factors, including interleukin 3 (IL-3) and interleukin 6 (IL-6) to increase the number and size of colonies of

hematopoietic progenitors (Nocka *et al.* 1990, Nocka *et al.* 1990, Zsebo *et al.* 1990). Stem cell factor also plays an important role in the survival, proliferation or migration of progenitor stem cells during both development (Silvers 1979) and maturation (Manova *et al.* 1990, Orr-Urtreger *et al.* 1990). Interleukin 3 supports the formation of multilineage colonies in the early development of multipotent hematopoietic progenitor stem cells (Cerny 1974, Schrader *et al.* 1983). Interleukin 6 appears to be directly involved in the responses that occure after infection and injury. It acts on a variety of cells including fibroblasts, activated T-cells, activated monocytes or macrophages and endothelial cells, and also potentiates the proliferative effect of IL-3 on mulipotential hematopoietic progenitors (Van Snick *et al.* 1986, Gauldie *et al.* 1987, Van Snick 1990). The exact mode of action of Thrombopoietin (TPO) is difficult to determine due to the general hematopoietic and thrombopoietic actions of several cytokines, but it does appear to influence the entire thrombopoietic process as well as is necessary for complete maturation of megakaryocytes through the formation of platelets (Kaushansky 1995). The last cytokine FLK-3/FLK-2 ligand, synergises with other hematopoietic cytokines to stimulate the growth and differentiation of early hematopoietic progenitors (Small *et al.* 1994, Ray *et al.* 1996). All cytokines were purchased from Sigma®.

On day four, the supplemented DMEM culture medium was replaced a second time. During the third culture medium change, on day eight, the percentage of FBS was reduced to 10 % (original culture medium described in 2.2.3.2). On day 12, a fourth culture medium change again contained 10 % FBS. A final concentration of 10 μM BrdU was also added on day 12, in which the cells were incubated for a further 48 hours.

At the end of the 14-day culture period, all culture medium was discarded. The remaining, adherent cells were washed twice with D-PBS and trypsinised with 0.25 % Trypsin-EDTA until they became non-adherent. These cells were re-suspended in DMEM (1 % AA, 10 % FBS) and both cell count and PI cell viability analyses were conducted as described in section 2.2.4.3. After calculating the appropriate volume by using this cell count, ± 10 - 15 x 10^6 cells were collected, re-suspended in 1 ml autologous blood plasma and placed on ice for immediate transport to the animal facility (refer to Chapter 5 for application of these adherent MNCs).

Another 1 x 10^6 of the MNCs were used for the characterisation protocol described in the following section. The remainder of the MNCs were analysed by flow cytometry, following the sequence described in section 2.2.4.4.

2.2.4.6 <u>Characterisation of adherent, fibroblast-like stromal cells</u>

The 1 x 10^6 autologous horse MNCs (mentioned in section 2.2.4.5) were re-plated in four wells of a six-well plate at a density of approximately 0.1 x 10^6 cells per well. They were cultured with basal liquid DMEM culture medium (containing 1% AA, 10 % FBS) and incubated at 37 ºC with 5 % CO_2 in a humidified chamber until 60 % confluence. At this point the cells from three of the four wells were exposed to freshly prepared, myogenic differentiation medium for a further week, replacing medium every three days. The myogenic differentiation medium was prepared by supplementing DMEM with 1 % AA and 1 % horse serum (Gibco^{TM} Cat. no. 26050). The fourth well served as a control well and only continued receiving the aforementioned basal liquid DMEM culture medium. To assess myogenic differentiation, two of the three differentiated wells and the control well were stained with anti-human myogenin monoclonal antibody (Santa Cruz, Cat. no. 12732) and prepared for immunoblotting. The remaining differentiated well of cells was washed twice with D-PBS and stained with Hoechst nuclear stain (Sigma® Cat. no. H6024) to assess the morphology of the cells under a inverted ultraviolet (UV) fluorescence microscope (Nikon Eclipse E400).

For western-blotting analyses, cells were trypsinised with 0.25 % Trypsin-EDTA, washed twice in D-PBS and centrifuged at 225 g for two minutes. The supernatant was discarded and cells were lysed in 100 µl RIPA-buffer (refer to Table 2.2 for RIPA-buffer constitution), sonicated and stored at -4 ˚C. Standard western analysis procedures of cell lysates were performed. The whole cell homogenate (50 µl) of each duplicate sample was loaded onto 10 % acrylamide SDS-page gels. Mini-gels were run at 100 V for 120 minutes using a Mini-Protean 3 Gel System (Bio-Rad). Proteins were transferred from SDS-page gels onto PVDF membranes (Immuno-Blot 0.2 µm) using a Mini Trans-Blot Cell blotting apparatus (Bio-Rad). Protein transfer to chromatographic sheets was carried out by applying a current of 200 V for one hour in a gel holder cassette. Gels were stained with Coomassie-blue to determine transfer effectiveness. Antigen-antibody complexes were visualised by enhanced chemiluminescence according to the manufacturer's instructions (AE Amersham Life Sciences Inc.). Membrane surfaces were exposed to detection reagents for five minutes, placed in a cassette, covered with transparency paper and developed using hyperfilm.

2.2.5 *Statistical analyses*

Data were analysed using the SAS Enterprise Guide V3.0 software package. Student's t-tests were used to determine (1) significance of changes in cell viability prior and subsequent to the MNC culturing procedure and (2) any statistical differences within Groups.

TABLE 2.2 - Protocol for preparing RIPA-buffer (refer to the List of Abbreviations for full names of constituents).

(RIPA^{++}-BUFFER: $^{+}$PROTEIN PHOSPHATASE INHIBITORS; $^{+}$PROTEASE INHIBITORS)

Step 1	*1*x *Hanks Balanced Salt Solution (HBSS)*:	2.5 mM tris-HCl pH 7.4
		1 mM EDTA
		1 mM EGTA
		250 mM sucrose or mannitol
		50 mM NaF
		50 mM NaPPi
		1 mM DTT
Step 2	*Add protease inhibitors*:	0.1 mM PMSF
		4 µg/mℓ SBTI
		10 µg/ mℓ leupeptin
		1 mM benzamidine
Step 3	*Add detergents*:	1% NP40
		0.1% SDS
		0.5% Na deoxycholate
Step 4	*Make up*:	desired volume with dH$_2$0

Analysis of Variance General Linear Models (ANOVA-GLM), followed by Tukey-Kramer's post-hoc tests, were used to determine (1) statistical differences between Groups for both ovine and equine data sets and (2) statistical differences between data sets prior to and subsequent to culturing.

2.3 RESULTS

2.3.1 Sheep peripheral blood

2.3.1.1 Cell count and viability

In the Autologous Group, the initial cell count per given blood sample was purely used to calculate the dilution factor required to obtain 4.5×10^6 cells per 1 ml of diluted solution, for that particular sample. In the Allogeneic Group, the initial cell count per given blood sample was purely used to calculate the amount of CD45 antibody required to mark MNCs sufficiently in each sample during flow cytometry analysis.

The percentage of dead cells in freshly processed, autologous MNC blood samples constituted 1.13 ± 0.02 %. Subsequent to further culturing of MNCs in this group, viability tests indicated a slight increase in dead cells to 3.15 ± 0.10 %, although this was not significant. A similar pattern was observed for the Allogeneic Group. In this instance, the dead cell percentage increased from 1.25 ± 0.09 % in freshly processed samples to 3.97 ± 0.91 % after culturing, once again not a significant change.

2.3.1.2 Flow cytometry

The percentages of FITC$^+$ fluorescence were analysed for all uncultured sheep blood samples in the Control, Autologous and Allogeneic Groups. Each of these groups contained an IgG-FITC subset (negative control) as well as a CD45-FITC subset (test sample). Between the three groups, there were no significant differences in the percentages for CD45$^+$ fluorescence. Within each group, there was a significant increase of fluorescence in the CD45 subset when compared to the IgG negative control (Figures 2.3a-d and Figure 2.4a). The number of CD45$^+$ cells present in uncultured allogeneic MNC samples dropped significantly following post-culture cell sorting (Figure 2.4b). At this point there was no significant difference in FITC fluorescence between IgG negative controls and CD45 samples.
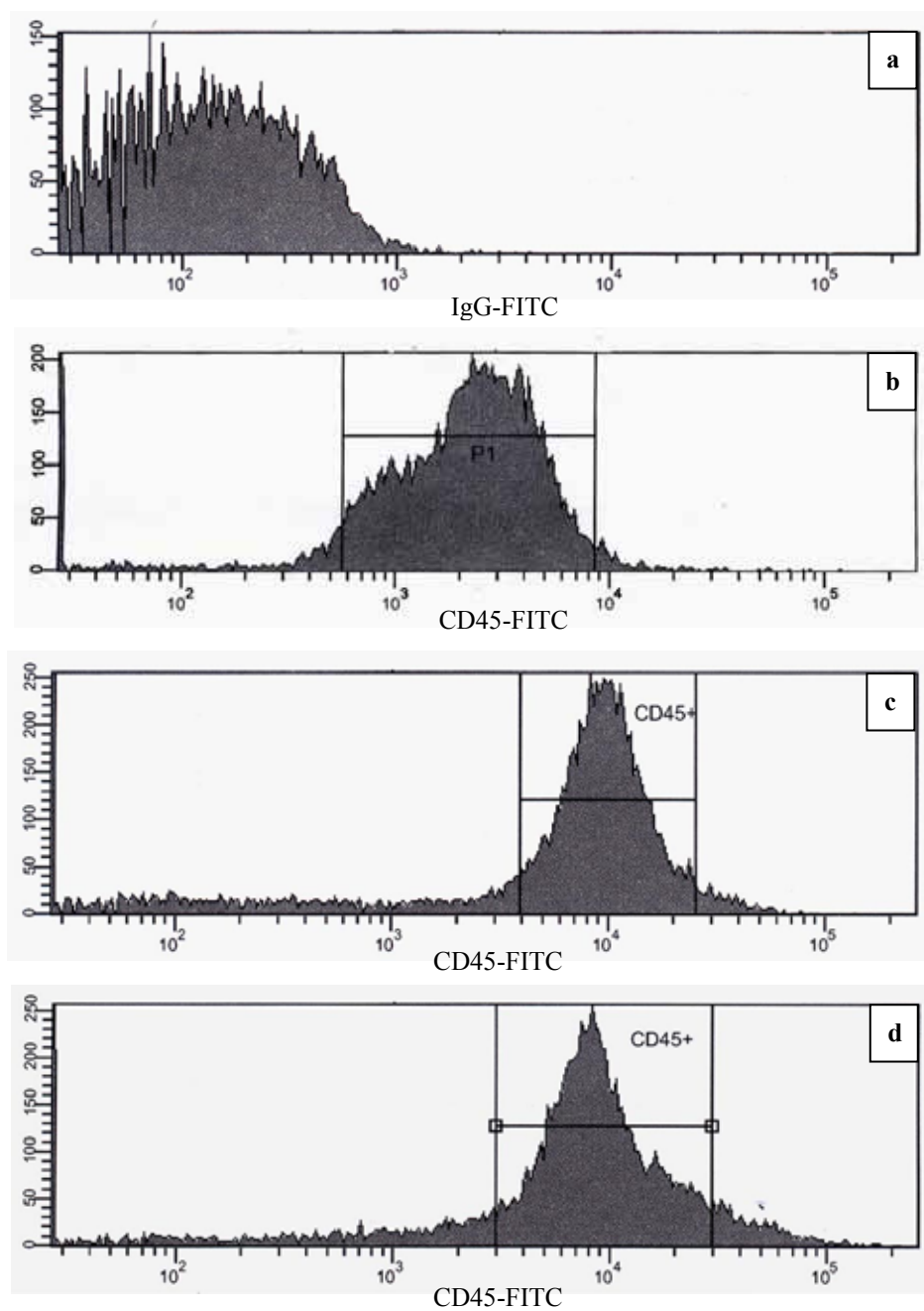
FIGURE 2.3 - Immunophenotypes of uncultured sheep mononuclear cells, which expressed CD45 when compared to the immunoglobulin (IgG) negative controls: (a) IgG negative control, (b) Control Group, (c) Autologous Group, (d) Allogeneic Group (FITC = fluorescein isothiocyanate).
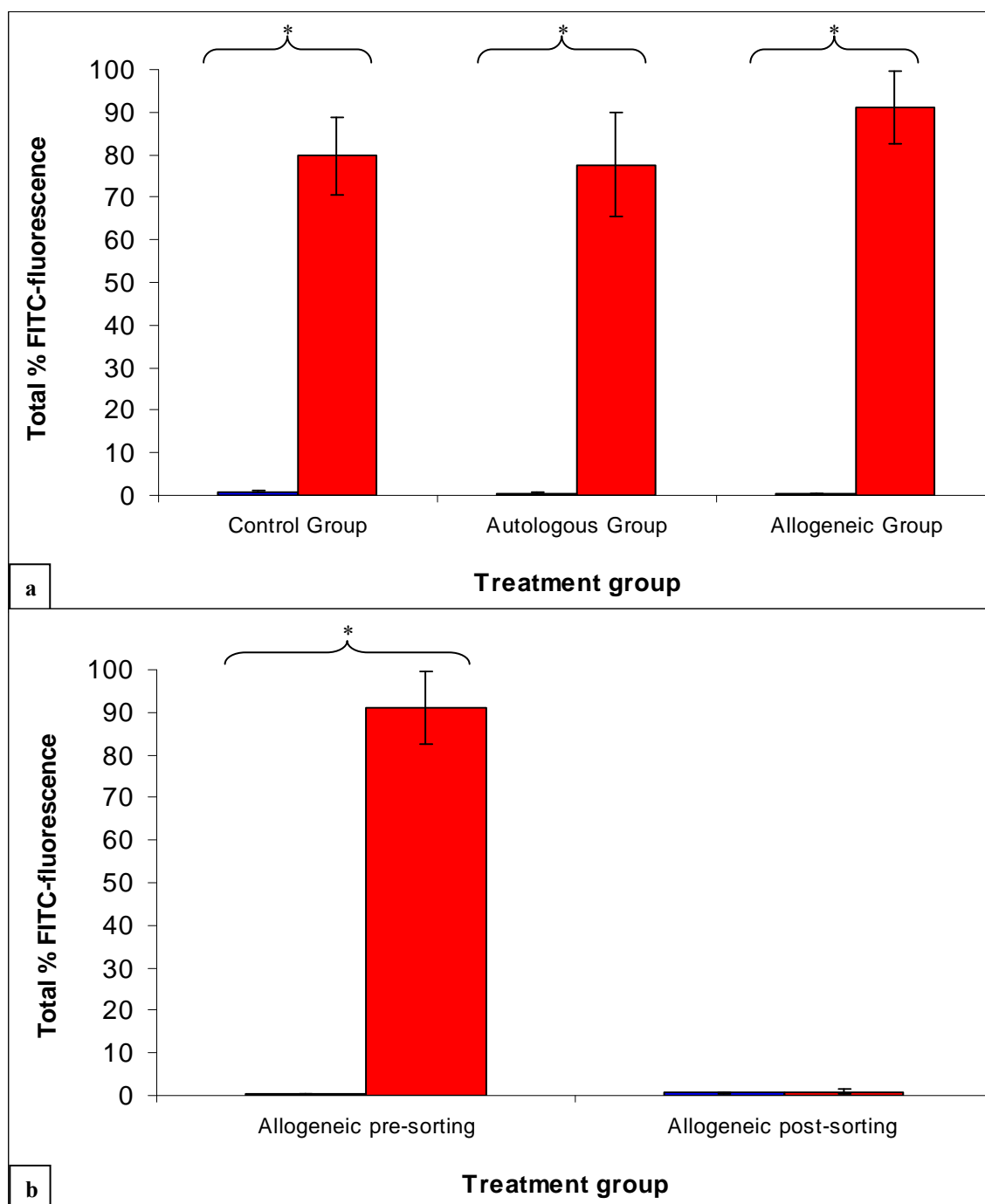
FIGURE 2.4 - Total percentages of fluorescein isothiocyanate[+] (FITC^{+}) mononuclear cells identified in the adult sheep peripheral blood samples. Graph (a) compares differences between the immunoglobulin (IgG) negative controls (blue bars) and CD45^{+} sub samples (red bars) among the Control, Autologous and Allogeneic Groups. Graph (b) compares differences between IgG negative controls (blue bars) and CD45^{+} sub samples (red bars) of the allogeneic samples prior and subsequent to flow cytometric cell sorting. Significant differences ($p < 0.05$) within groups are indicated using brackets with asterisks.

2.3.2 Horse peripheral blood

2.3.2.1 <u>Cell count and viability</u>

As was the case in the ovine Autologous Group, the initial cell count obtained from the uncultured blood samples was purely used to calculate the dilution factor required to obtain 7.2×10^6 cells per 1 ml of diluted solution for each given sample. At this point the viability of MNCs was found to be 97.51 ± 0.49 %. After the horse MNCs had been cultured for 14 days, a second cell count was conducted to determine the dilution factor required to extract $10 - 15 \times 10^6$ MNCs per 1 ml for each sample, to be used for injection in Chapter 5. A second viability test was performed on the remainder of each sample just prior to injection of the MNCs into horse tendons. It indicated that the number of dead cells had increased significantly from under 3 % to 28.79 ± 1.61 %. In other words, our horse injections each contained a live cell count of $\pm 7 - 10 \times 10^6$ live MNCs.

2.3.2.2 <u>Flow cytometry</u>

The percentages of FITC$^+$ fluorescence were analysed for horse peripheral blood in sub samples marked with the negative control IgG, those marked with CD11a/18 as well as those marked with CD4. Results for the above were obtained both prior to culturing MNCs and subsequent to the 14-day culture period (Figure 2.5).

The IgG negative controls produced virtually no FITC fluorescence, rendering their percentage values significantly less than those of sub samples marked with CD11a18 and CD4, both prior to and after culture. The number of CD11a/18$^+$ cells present decreased significantly after culturing of the samples. In contrast, the CD4$^+$ population had increased significantly at termination of the culture period.

2.3.2.3 <u>Culture of autologous mononuclear cells</u>

On the fourth day of culture, some adherent fibroblast-like or stromal-like cells were visible between lots of other cells (Figure 2.6a). The adherent cells were much larger in size than surrounding round, non-adherent cells. Larger round adherent cells were also visible. The population contained a mixture of small and large colonies of stromal cells. These adherent colonies grew rapidly until the eighth day of culture (Figure 2.6b), displaying a similar proliferation pattern to that of adult bone marrow MSCs (Smith *et al.* 2003). By the twelfth day of culture, some cells started undergoing apoptosis. This may be due to over-confluence, since the cells had not been trypsinised or passaged during the twelve day period.
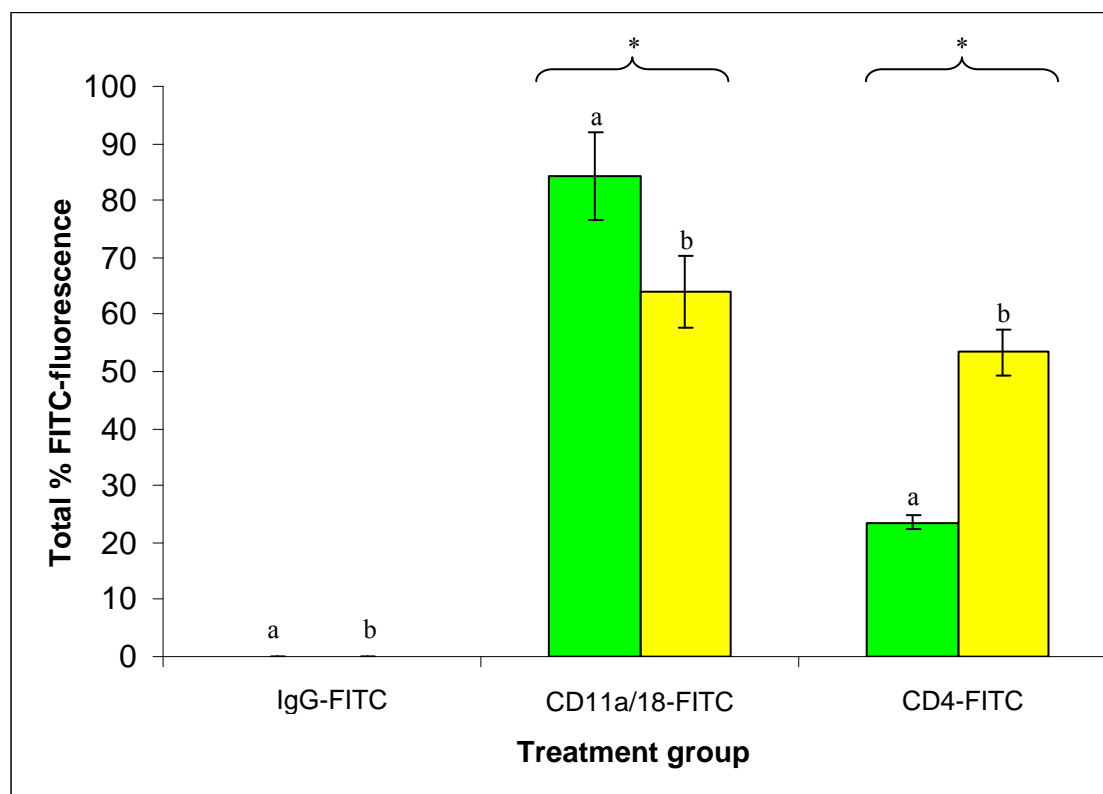
FIGURE 2.5 - Total percentage of fluorescein isothiocyanate[+] (FITC^{+}) mononuclear cells derived from the adult horse peripheral blood samples. Comparisons are made between observed fluorescence in uncultured samples (green bars) versus 14-day cultured samples (yellow bars) among treatment groups marked with the immunoglobulin (IgG) negative control, those marked with CD11a/18 primary antibody and those marked with CD4 primary antibody. Significant differences ($p < 0.05$) within groups are indicated using brackets with asterisks. Significant differences ($p < 0.05$) between the IgG negative control samples and those marked with primary antibodies are indicated for uncultured samples (a) and cultured samples (b) respectively.
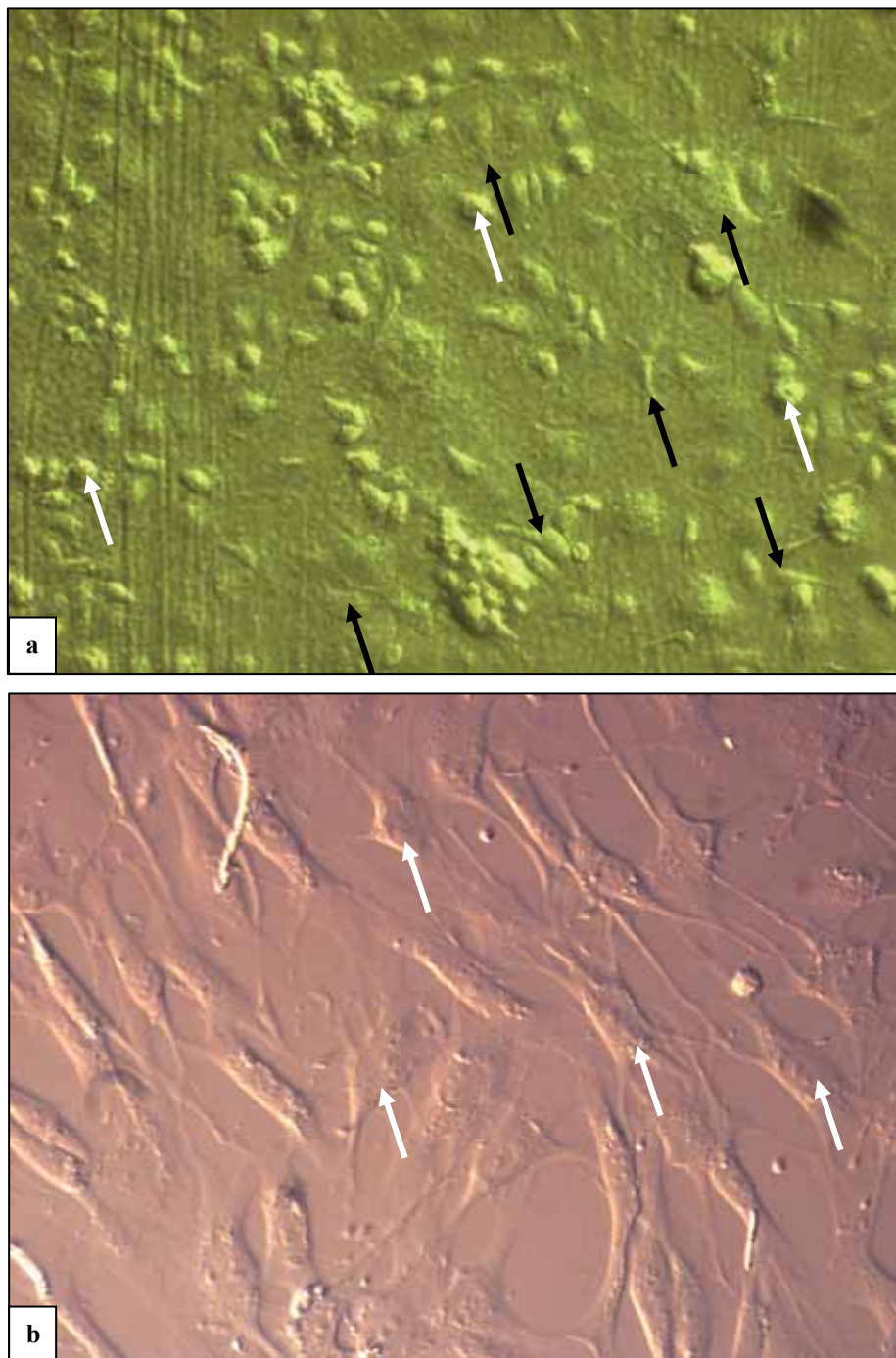
FIGURE 2.6 - Adherent, fibroblast-like cells (black arrows) and adherent round cells (white arrows) were cultured from adult horse peripheral blood after four days in basal liquid culture medium, depicted in (a) above. In figure (b) the white arrows indicate adherent stromal cells as visible on the eighth day of culture.

2.3.2.4 <u>Characterisation of adherent, fibroblast-like stromal cells</u>

Of the four samples that were analysed for myocyte differentiation, three stained positive for myogenin (Figure 2.7). Differentiated cells contained two to three nuclei which could be observed with Hoechst nuclear stain (Figure 2.8a-b). Such multi-nucleated, tube-like cells are indicative of myotubes (Ross and Reith 1985).

2.4 DISCUSSION

Our collection of sheep blood and subsequent lysing of red blood cells to isolate MNCs was conducted in a sterile environment with an efficient protocol that rendered a very low dead cell count in uncultured samples. The percentage of dead cells did not increase significantly during the culture phase, indicating the use of an appropriate culture medium for MNCs. The deaths that did occur can most likely be attributed to cells reaching the end of their natural cycles and apoptosing.

The MNC population contains both stem cells and supportive cells, with the latter producing cytokines and growth factors which may aid engraftment of cells (Prockop 1997). Peripheral blood plastic-adherent MSCs have been characterised by the positive expression of SH2 and SH3 and negative expression of CD14, CD34 and CD45 (Tondreau *et al.* 2005). However, these monoclonal antibodies were not commercially available for ovine or equine purposes. The development of ovine and equine MSC stem cell markers may be an area of focus for future research. We made use of negative selection (hematopoietic lineages) by using CD45 for ovine studies and CD11a/18 and CD4 for equine studies. These are cell surface markers for monocytes, lymphocytes, granulocytes and macrophages, which indicates the presence and amount of leucocytes in our samples for injection. Combined with *in vitro* differentiation we could thus ascertain the mesenchymal properties of cultured cells. Although this method is not ideal, it allowed us to partially characterise our MNCs used for injections.

Our study showed that FITC-conjugated IgG negative controls displayed virtually no fluorescence, whereas CD45-marked samples originating from the same sheep had significantly higher fluorescence during flow cytometry analyses. This was true for our Autologous, Allogeneic and Control Groups. This verified that no auto-fluorescence was present in the FITC batch and that it indeed marked the primary antibody, in other words, that we received no false positive results. FITC-conjugated CD45 antibodies could thus be used for positive staining of monocytes, lymphocytes, granulocytes, and macrophages in sheep,

FIGURE 2.7 - Western blot analysis of myogenin protein indicating differentiation of mononuclear cells cultured from horse peripheral blood. Plasma membrane proteins (50 μg) were separated on 10 % acrylamide SDS-page gels, probed with myogenin antibody and visualised using a chemiluminescence technique. This analysis shows representative results obtained from sample well one (1), sample well two (2) and a control well (3).
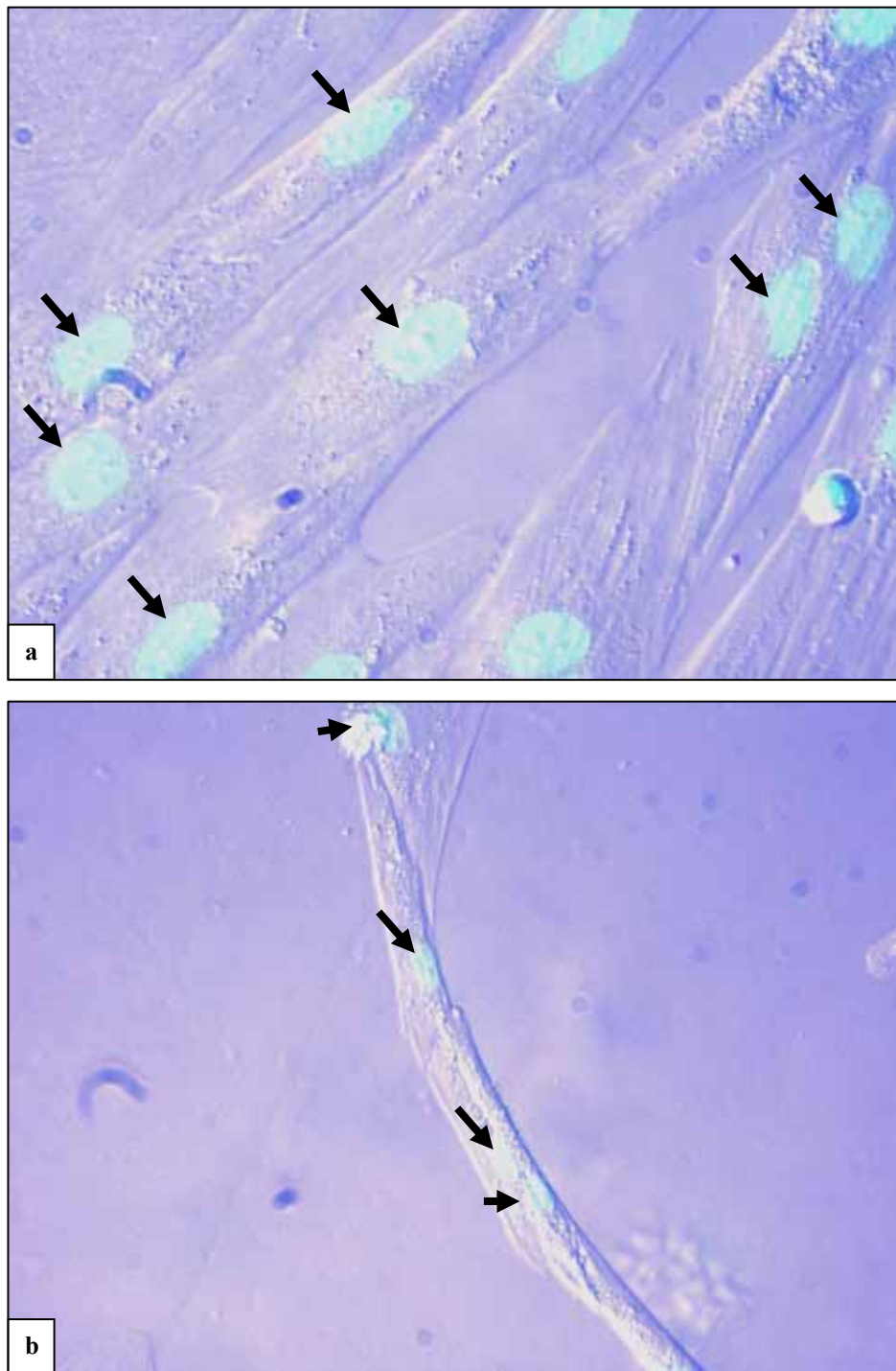
FIGURE 2.8 - Differentiation of adherent, fibroblast-like cells from adult horse peripheral blood. Adherent cells were cultured in myogenic differentiation liquid culture medium for seven days and stained with Hoechst nuclear stain. In picture (a) at 40x magnification and picture (b) at 20x magnification, the tube-like morphology of these cells can be observed. Some cells contain more than one nucleus (black arrows).

and showed that these cells make up more than 70 % of the total sheep MNC population.

In the Allogeneic Group, a significant decrease in $CD45^+$ was visible when comparing uncultured samples with FACS $CD45^-$ sorted cells. This once again points to successful FACS analyses and verifies that the majority of leucocytes were removed from our allogeneic MNC samples. This is of critical importance in minimising the risk of immune rejection when we inject MNC harvested from one sheep into the injured tendon of another individual (refer to Chapter 4 for allogeneic injections).

Furthermore, there were no significant differences among the $CD45^+$ levels observed in our Autologous, Allogeneic and Control Groups, prior to MNC culture. It indicates that our three groups possessed similar leucocyte counts in their blood which implies a similar state of health before we proceeded to injure their tendons. This allows us to make reasonable comparisons in tendon recovery rates when exposing these groups to different treatments, since such differences can not be attributed to unequal leucocyte levels inducing dissimilar immune responses.

In our culture of MNCs from horse peripheral blood, we similarly found a lack of fluorescence in all FITC-conjugated negative controls, once more verifying that flow cytometry analysis did not provide false positive results. Only adherent cells were selected for injection into injured tendons (as mentioned in section 2.3.2.1). Both CD 11a/18 and CD4 leucocytes were still present after 14 days of culture. This may either be due to differentiation of stem cells into leucocytes during culture or as a result of insufficient culture medium replacement. At this point the dead cell count had also increased to over 25 %, which could explain why there was still a high frequency of CD11a/18 at time of injection since only three quarters of the medium was extracted at culture medium changes.

Experiments have demonstrated that bone marrow (Pittenger *et al*. 1999) and foetal blood (Campagnoli 2001) include a population of mesenchymal-like stem cells with multilineage potential. We showed similar results in our study of horse peripheral blood through the differentiation of our MNC population into myotubes, which stained positive for myogenin. This suggests the presence of MSC in our horse autologous MNCs injections. The following chapter will establish the best way to mimic natural tendon injuries before we proceed to inject the MNCs harvested here into our sheep and horse study population.

- CHAPTER 3 -

ESTABLISHMENT OF A TENDON INJURY MODEL AND DESCRIPTION OF TENDON INJURY IN SHEEP

3.1 INTRODUCTION

TENDONITIS of the superficial digital flexor (SDF) tendon is a common and debilitating injury among elite equine athletes. Many studies have focused on normal and abnormal or injured tendons - a sound knowledge of these structures is essential before embarking on a study mimicking spontaneous tendon rupture (Webbon 1977 and 1978, Williams *et al.* 1984a and b, Jann *et al.* 1992).

Upon spontaneous complete or partial tendon rupture, extensive haemorrhage occurs and a haematoma may form within the tendon. The immediate result of fibre rupture is fibrin deposition and ischemia at the site of injury with concomitant congestion, tenocyte necrosis and fluid accumulation between fibres (Williams *et al.* 1984b). The injection of an enzyme collagenase type I, which dissolves tendon collagen fibres, is an attempt to mimic spontaneous tendon rupture. Many studies have reported favourable results, finding that lesions are reproducible and that collagenase injections mimic many aspects of a naturally occurring traumatic injury (Williams *et al.* 1984b, Dahlgren *et al.* 2002). Surgically induced tendon injury has also been used to test implant grafts (Strömberg and Tufvesson 1977, Awad *et al.* 1999). In smaller animals collagenase injury is seen as a very invasive form of injury.

The first objective of this experiment was therefore to determine whether, in a sheep animal model, surgically-induced injury or collagenase-induced injury best mimicked spontaneous tendon rupture at one week post-injury. The second objective was to describe the tendon injury site, both microscopically and macroscopically, at one week and seven weeks post injury.

3.2 MATERIALS AND METHODS

Use of animals was conducted in accordance with protocols approved by the Ethics Committee for Research on Animals of the Medical Research Council (application number P04/06/010). All surgical procedures and anaesthetic administration were conducted by a registered veterinarian.

3.2.1 Housing of experimental sheep

A total of 27 Black-head Dorper sheep were housed at the Welgevallen experimental farm in Stellenbosch, South Africa. The sheep, ranging between eight months and four years of age, were randomly divided into six groups. Within each group, two separate tests were administered per animal, namely, one to the left forelimb and one to the right forelimb. Table 3.1 provides a synopsis of the resulting twelve injury-treatment subsets with the identification (ID) assigned to each. Treatments CMNC and CCD45 (shaded blocks) were not used in the establishment of our injury model and will only be referred to in Chapter 4.

All sheep were weighed on the first day of the experiment and again just prior to sacrifice, providing live-mass. The sheep were housed in stables for one week prior to injury (to acclimatise) and for 14 days following injury (for observations); at this point they received sheep feed and hay twice daily, fresh water daily, and the stables were swept each day and cleaned thoroughly each week. The sheep's injured legs were provided with cold hydro therapy twice daily for three days post-injury. Thereafter they were observed twice daily for any discomfort or behavioural problems for the duration of experiments. Fourteen days after injury, following removal of the skin sutures, the sheep from Groups 3 – 6 were moved to grass paddocks where sufficient food, water and shelter were present.

3.2.2 Injury and treatment procedures

All tendons were ultrasonographically scanned prior to injury and were found to be without prior injury (sound), that is, they displayed no disruption of echogenicity (transverse and longitudinal) and no disruption of fibre alignment (linearity). Details of the ultrasound scanning procedure are provided in Chapter 4, section 4.2.3.

The time at which any given group was provided with tendon injuries was designated as Week 0. Animals in Group 1 and Group 2 were not supplied with any subsequent treatment and were sacrificed one week post-injury, referred to as Week 1. These sheep were used to establish whether a collagenase-induced or a surgically-induced injury best mimics a naturally occurring tendonitis.

Based on this data, as presented in this chapter, we decided to make use of collagenase-induced tendon injuries. Individuals in Group 3, Group 4, Group 5 and Group 6 accordingly received either collagenase-induced injury or saline control injections on Week 0. All of these sheep received a second injection, consisting of saline solution, on Week 1 and were sacrificed on Week 7. Each week post-injury the forelimbs of these sheep were

TABLE 3.1 - A summary of the injury-treatment subsets among our six groups of experimental sheep (n = 27). Subsets in shaded blocks were not used in Chapter 3. In the text any particular subset is labelled by (1) its group number and (2) its identification (ID).

			GROUP	FRONT LEG	INJURY (WEEK 0)	TREATMENT	ID	SACRIFICE
	Chapter 3		1 (n = 3)	Left	Skin incision	None	Ctrl	Week 1
				Right	Cut tendon	None	Cut	Week 1
	Chapter 3		2 (n = 3)	Left	Insert needle in tendon	None	Ctrl	Week 1
				Right	Collagenase	None	Coll	Week 1
Chapter 2	Chapter 3	Chapter 4	3 (n = 4)	Left	Saline	Saline	SS	Week 7
				Right	Collagenase	Saline	CS	Week 7
Chapter 2	Chapter 3	Chapter 4	4 (n = 5)	Left	Saline	Saline	SS	Week 7
				Right	Collagenase	Autologous MNCs	CMNC	Week 7
Chapter 2	Chapter 3	Chapter 4	5 (n = 6)	Left	Collagenase	Saline	CS	Week 7
				Right	Collagenase	Autologous MNCs	CMNC	Week 7
Chapter 2	Chapter 3	Chapter 4	6 (n = 6)	Left	Collagenase	Saline	CS	Week 7
				Right	Collagenase	Allogeneic MNC	CCD45	Week 7

manually examined to assess whether the injected SDF tendons could be palpated. Animals from Groups 3 – 6 were used to describe the site of tendon injury seven weeks post-injury.

The experimental SDF tendons of all sheep were dissected immediately *post mortem* for macroscopic and microscopic evaluations.

3.2.2.1 Group 1: surgically-induced injury

To serve as tranquiliser and provide some degree of analgesia, each sheep received an intravenous injection of Chanazine mixed with Antropine10, according to the manufacturer's instructions. The sheep were placed in dorsal recumbency and restrained. Regional nerve blocks were used for pain management and to desensitise the skin for surgery - nerve blocking was obtained distal to the accessory carpal bone by blocking the lateral and medial palmar digital nerves just below the carpometacarpal joint with 2 % lignocaine hydrochloride (approximately 2 - 5 ml per limb, depending on skin sensitivity). Forelimbs were prepared for aseptic surgery by shaving the hair over the flexor tendons in the mid-metacarpal region and cleansing the skin with a Hibitane (chlorhexidine gluconate) and 70 % ethanol solution.

After surgical draping, the right front legs (Group 1 Cut) were surgically injured by making a 4 cm skin incision on the lateral side of the leg, approximately 5 cm distal to the accessory carpal bone, using a sterile number 20 scalpel blade. The SDF tendon and deep digital flexor (DDF) tendon were separated with surgical scissors. A 2 cm longitudinal incision (palmar to dorsal), bisected with a transverse stab incision (lateral to medial), were made into the SDF tendon using a sterile number 11 scalpel blade (Figure 3.1). The left forelimbs (Group 1 Ctrl) served as controls and were handled in exactly the same manner, except that no tendon incisions were made. Six to ten sutures were applied to skin injuries using sterile surgical nylon line. Post-operatively, an intramuscular injection of Peni la Phenix (benzathine penicillin) was administered according to the manufacturer's instructions and the wound was sprayed with Necrospray (Reg. no. G2313 Act 36/1947) to prevent infections.

3.2.2.2 Group 2: collagenase-induced injury

A 26-gauge hypodermic needle was inserted into the SDF tendon core of all right forelimbs (Group 2 Coll). The exact area was located in the palmar midline, approximately midway between the carpometacarpal and metacarpophalangeal joints, or ± 6 cm as measured from the accessory carpal bone towards the proximal sesamoid bone (the mid-metacarpal region). The insertion was guided by ultrasonography. A total of 2097 units of bacterial
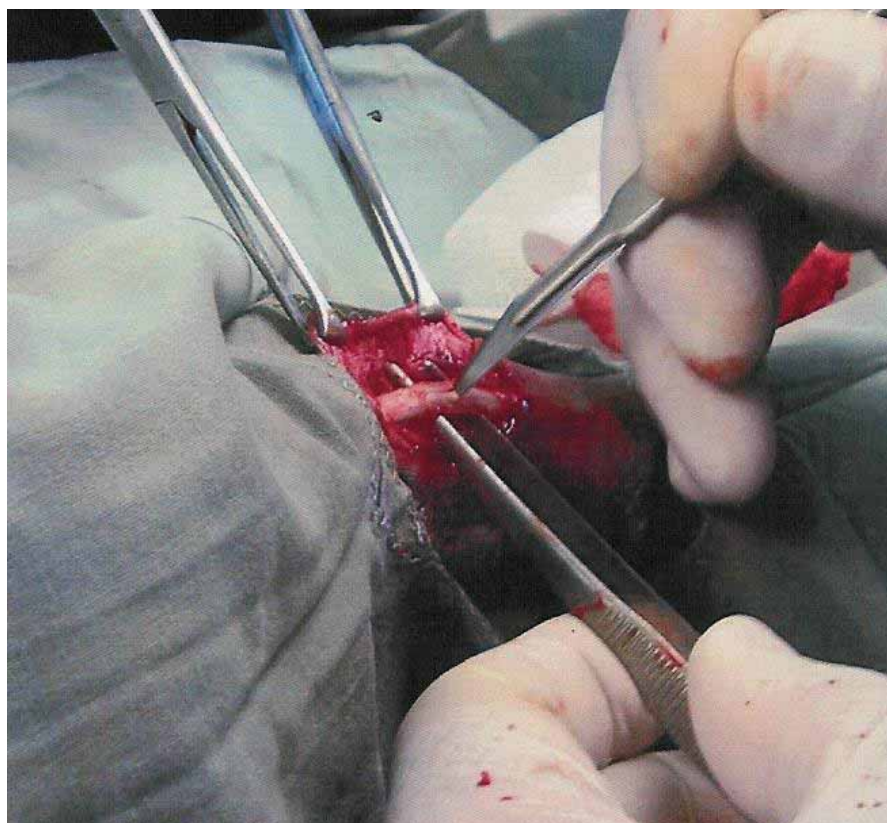
FIGURE 3.1 – Application of a transverse stab incision (lateral to medial) into the superficial digital flexor (SDF) tendon of a sheep using a sterile number 11 scalpel blade, bisecting the visible longitudinal (palmar to dorsal) incision.

collagenase type I were injected (at 300 units/mg; Gibco[TM] Cat. no. 17100). The collagenase
was dissolved in 0.9 % saline and sterile filtered through a 0.22 μm filter, injected as a
volume of 0.1 ml (dosage adapted from Dahlgren *et al.* 2005). Left forelimbs (Group 2 Ctrl)
once more served as controls. Although a hypodermic needle was similarly inserted into their
SDF tendons, no actual injection was administered.

3.2.2.3 Group 3 and Group 4 SS: saline-injected control

The left forelimbs of these sheep were prepared for aseptic surgery (refer to section
3.2.2.1). The SDF tendon was exposed and 0.1 ml of a 0.9 % saline solution was injected into
it as control, as opposed to either a surgically induced-injury or a collagenase-induced injury.
In Week 1 these tendons were treated with another 1 ml of a 0.9 % saline solution. This was
administered subcutaneously in the area of injury (approximately 10 injections of 0.1 ml
each). The sheep were not sedated in any way when receiving their treatment, but were tied
to a surgical table for immobilisation.

3.2.2.4 Group 3, Group 5 and Group 6 CS: collagenase-induced injury

Due to the difficulty of discerning between the SDF tendon and the DDF tendon
ultrasonographically in sheep, it was decided to surgically display the tendon for accurate
placement of the needle point. The sheep were prepared for aseptic surgery as described in
section 3.2.2.1. A collagenase injection (refer to section 3.2.2.2 for collagenase solution) was
administered into the mid-metacarpal regions of SDF tendons from the right forelimbs of
sheep in Group 3 and the left forelimbs of sheep in Group 5 and Group 6 (Figure 3.2). This
point in time was designated as Week 0. A 1 ml 0.9 % saline solution was injected as
treatment on Week 1, administered as 10 injections of 0.1 ml each in the tendons. The sheep
were immobilised as described previously, but not sedated in any way.

3.2.3 Macroscopic observations

On Week 1 (Group 1 and Group 2) and Week 7 (Groups 3 – 6), 5 mg Domesedan
(Detomidine – Novartis AH) was administered into the jugular vein of each sheep prior to
mechanical stunning. *Post mortem* examinations were initialised by disarticulating the
relevant limbs. The metacarpal region of injury was exposed by cutting the skin on the side
of the leg, from accessory carpal bone to proximal sesamoid bone, and pulling the skin aside.
Images of all injured tendons were taken with a Nikon digital camera in order to document

FIGURE 3.2 – Injection of collagenase into the core of the superficial flexor digital (SDF) tendon of a sheep using a 26-gauge hypodermic needle.

differences in tissue colouration. Thereafter SDF tendon specimens were carefully dissected from rest of leg and placed on ice for immediate mechanical testing.

3.2.4 Macroscopic measurements

During *post mortem* data collections, digital images were captured of the SDF tendons. Maximum tendon diameter (MTD) measurements were subsequently calculated for given points along the tendon using image analysis software (analySIS Image Processing Version 3.2). Each tendon was divided along its length into a proximal, middle and distal region (refer to Chapter 4, Figure 4.1), with the focal point of injury serving as central reference point since all tendons were injured at approximately the same anatomical locality. We collected twenty MTD measurements ± 5 mm apart - six in the distal region, seven in the middle region and seven in the proximal region - and calculated a mean MTD value per region. These measurements of diameter were used to examine tendon swelling as an indicator of inflammatory response and recovery across a seven week period.

3.2.5 Mechanical testing

Modulus of elasticity was evaluated using a servo hydraulic testing station (Instron model 4444, Apollo Scientific, Figure 3.3). Each tendon was affixed vertically between two stainless steel clamps, with the top clamp holding the proximal side of the tendon, and leaving 80 mm of tendon exposed between clamps. The clamps were aligned so that the tissue and load cell axes were co-linear. Data acquisition was achieved at a rate of 1 000 data points/sec (Smith *et al.* 2002). Load was applied with a crosshead speed of 2 400 mm/min (Dahlgren *et al.* 2002) and all tendons were loaded to failure (complete rupture).

The following variables were measured and analysed digitally: (1) maximum load at tendon failure, calculated as load (N) versus displacement (mm), (2) maximum tensile stress, calculated by dividing maximum tendon diameter (mm) with maximal load at failure, (3) ultimate tensile strain, by expressing specimen displacement (mm) at maximum load as a percentage of initial specimen length (mm), and (4) tendon stiffness (N/mm). Young's modulus of elasticity (E) was calculated from the slope of the linear region in a curve drawn of load versus displacement (Smith *et al.* 2002). This modulus provides an accurate indication of varying degrees of elasticity and stiffness in the tendons between treatment types and over time.
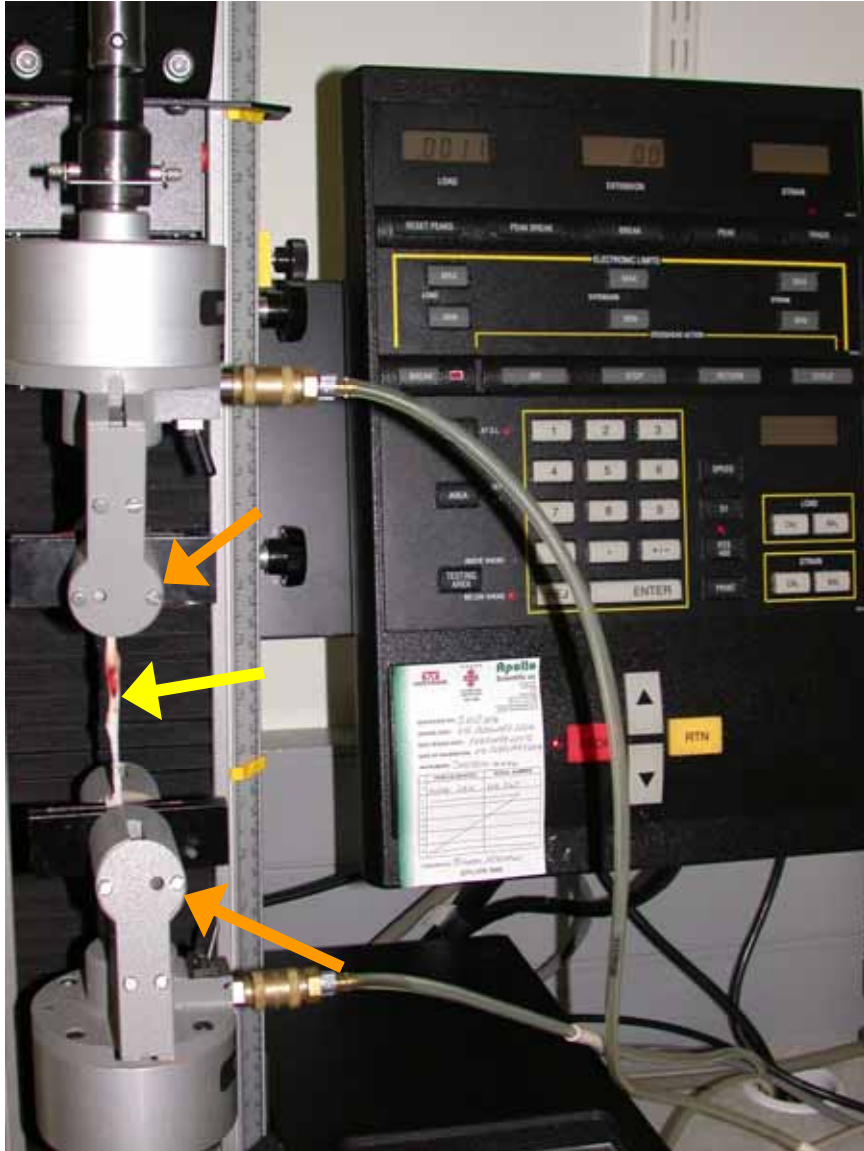
FIGURE 3.3 – Evaluation of tendon modulus of elasticity using an Instron servo hydraulic testing station. A tendon (yellow arrow) is affixed between stainless steel clamps (orange arrows) with the top clamp holding the proximal aspect of the tendon.

3.2.6 Microscopic observations and measurements

Standard histological procedures were followed to prepare cross-sectional sections out of approximately 5 mm transverse tendon cuts. Since contradictory opinions exist as to the most suitable fixing technique for tendons, we used three different tissue techniques (Humason 1967, Bancroft and Stevens 1977, Sterchi and Furell 1995). Each sheep tendon could be divided into approximately four to six 5 mm cuts per fixing technique, ranging from proximal to distal.

In the first technique, the tendon tissues were fixed in 70 % ethanol in distilled water at room temperature. Thereafter they were dehydrated in graded alcohols, cleared in toluene and embedded in paraffin wax (Paraplast plus, melting point 56 °C) (Humason 1967). Unfortunately these samples were too dehydrated and hard, preventing the wax from penetrating sufficiently and samples had to be discarded.

A second batch of tendon tissues was prepared for cryostat histology, using tissue freezing medium (Reichardt Jung OCT) as the cryoprotectant. Each 5 mm cut was dropped in cold N-pentane [$CH_3(CH_2)_3CH_3$], immediately frozen in liquid nitrogen and stored at -80 °C. These samples also proved extremely difficult to section on a cryostat and could not be used for further analysis.

As a third technique, tendon tissues were fixed in 4 % paraformaldehyde in 0.1 M sodium phosphate buffer, at pH 7.4 and a temperature of 4 °C. These tissues were also dehydrated in graded alcohols, cleared in toluene and embedded in paraffin wax. A Jung rotary microtome was used to prepare 5-10 μm cross-sections successfully, subsequently treated with Masson's Trichrome collagen stain and mounted on microscope slides.

Histological sections were examined under 20x magnification with a Nikon Eclipse E400 inverted microscope. A blue and red macro was used in the Simple PCI Analysis software package to quantify collagen present (the blue macro being a figure indicative of the amount of blue present in the viewing field) and to assess infiltration of inflammatory cells and fibroblasts (the red macro similarly indicating the amount of red present). Analysis was performed at five viewing fields within the three tendon regions (proximal, middle and distal).

3.2.7 Statistical analysis

Data were analysed using the SAS Enterprise Guide V3.0 software package. Differences in sheep mass before and after treatment were determined by paired t-tests. Independent Student's t-tests were used for all further analyses between two treatment groups. Wherever there were no statistically significant differences between the identical SS injury-

treatment subsets of Group3 and Group 4, such data were pooled for analysis. An analysis of variance (One-way ANOVA) was similarly carried out to test for significant differences between the CS injury-treatment subsets of Group 3, Group 5 and Group 6 in order to ascertain whether data could be pooled. Level of significance was accepted at $p < 0.05$. All data in graphs represent means ± standard deviation.

3.3 RESULTS

Induced injuries did not appear to have adverse effects on the general health of the sheep as was seen by the fact that they maintained their average mass during the study period. This was quantified as an average mass of 42.06 ± 7.18 kg before the onset of our experiment as opposed to 42.55 ± 7.31 kg at completion of the study, which was not statistically different. All tendons scanned ultrasonographically were found to be without any lesions and were declared within normal limits before initiating experiments. Figure 3.4 provides illustrations of the relative positions of palmar metacarpal structures visible on ultrasonographs. This is followed by Figure 3.5a-d, containing typical ultrasound scans obtained during our assessment. During the course of our study, it was observed that some sheep developed black pigmented spots on the skin areas where regular shaving occurred.

3.3.1 Establishment of injury model

3.3.1.1 External observations

Serving as controls for the surgically-induced injuries of Group 1 Cut, tendons of Group 1 Ctrl were not injured, but the overlying skin was opened to mimic access of the tendon. Considerable local inflammation and leg swelling occurred within six hours, but had subsided considerable by Week 1. The SDF tendon could not be palpated until approximately four days post-injury, probably due to the formation of haemorrhage and oedema (Figure 3.6a) as a result of the incision. There was no marked lameness of the forelimb at any point. The surgically-induced injuries of Group 1 Cut displayed external symptoms identical to those reported for their controls above (Figure 3.6b).

Group 2 Ctrl similarly acted as a control for collagenase-induced injuries – a needle was inserted into these tendons without actual application of any injections or skin surgery. Group 2 Ctrl displayed no inflammation, no tendon swelling and no lameness. In contrast, collagenase-induced injuries in Group 2 Coll produced rapid local inflammation and swelling. Partial lameness was apparent within six hours of injury. One day post-injury, the animals
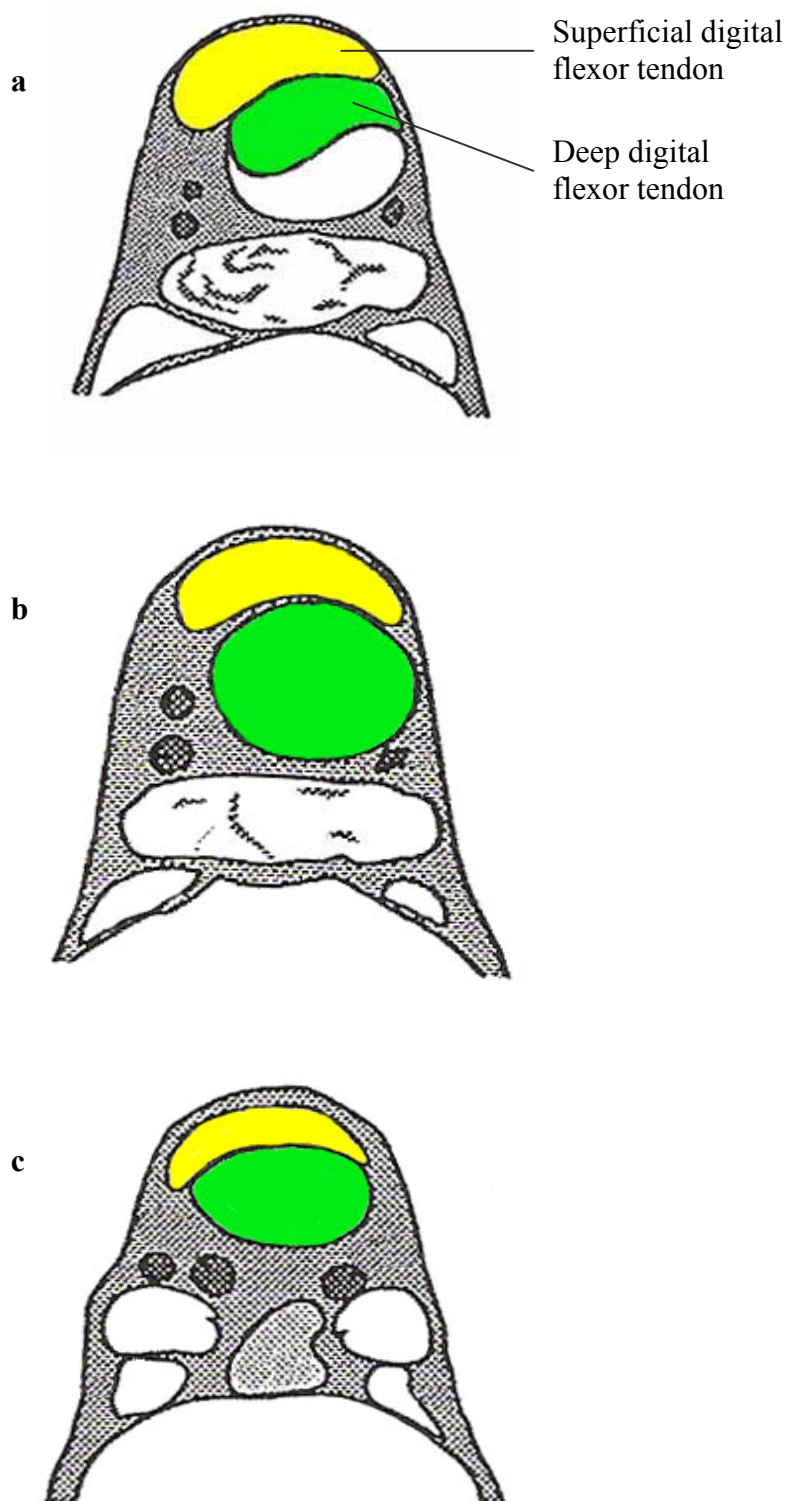
FIGURE 3.4 - Illustrations of the palmar metacarpal structures visible on transverse ultrasound scans of (a) the proximal region, (b) the middle region and (c) the distal region as defined in the text. Of significance is the superficial digital flexor tendon (yellow), with the deep digital flexor tendon (green) located adjacent to it.
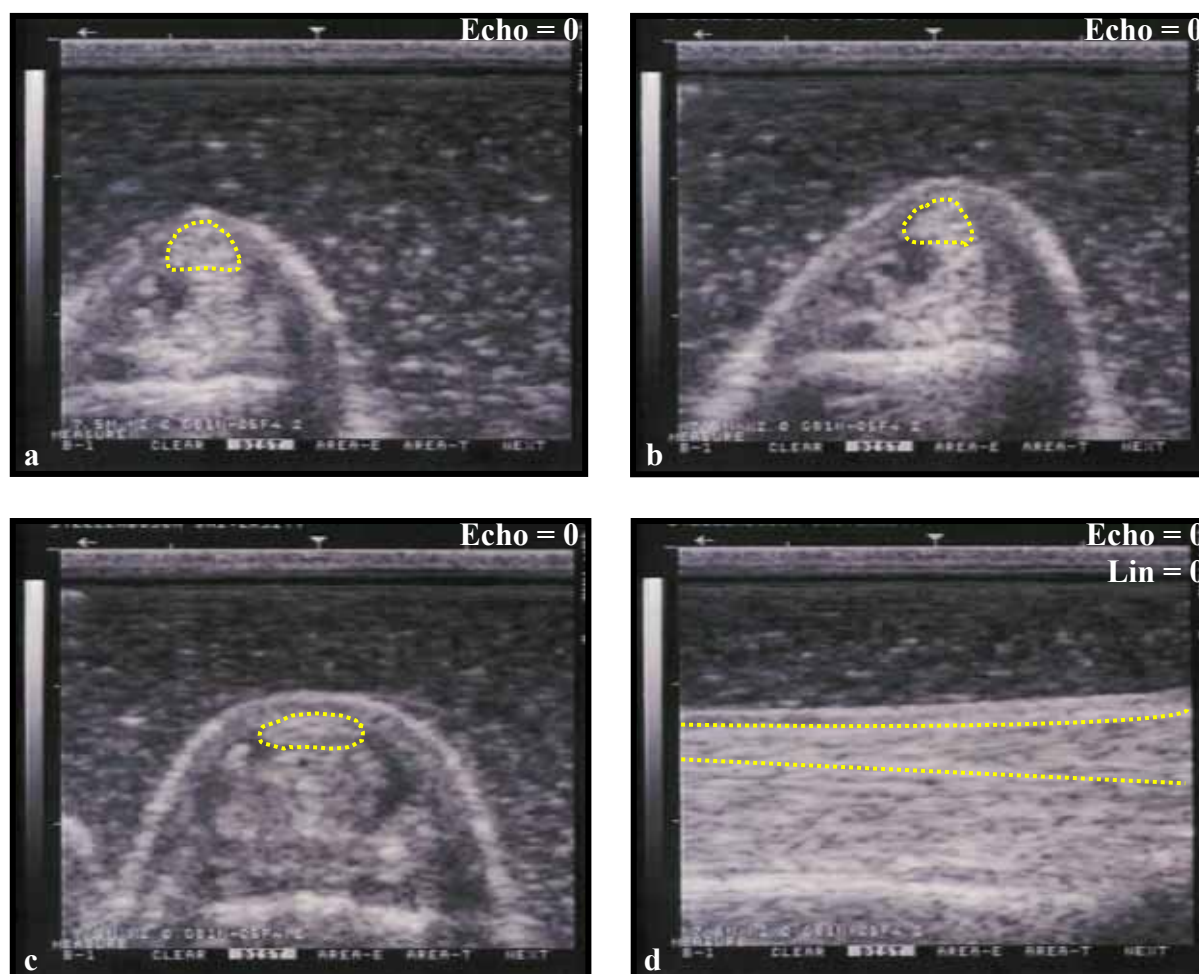
FIGURE 3.5 - Ultrasonographs of the sheep palmar metacarpal structures: (a) transverse aspect of the *proximal* region, ± 5 cm distal to the accessory carpal bone; (b) transverse aspect of the *middle* region, ± 6.5 cm distal to the accessory carpal bone; (c) transverse aspect of the *distal* region, ± 8 cm distal to the accessory carpal bone; and (d) longitudinal aspect of the middle region, ± 6.5 cm distal to the accessory carpal bone with the proximal side on the left of the image. The superficial digital flexor tendon is enclosed within yellow outlines. Echogenicity (Echo) and / or linearity (Lin) indices occurring in the upper right corner of each image are all 0, revealing normal echoic (homogenously hyperechoic) tendon tissues. Note the parallel fibre alignment in (d).
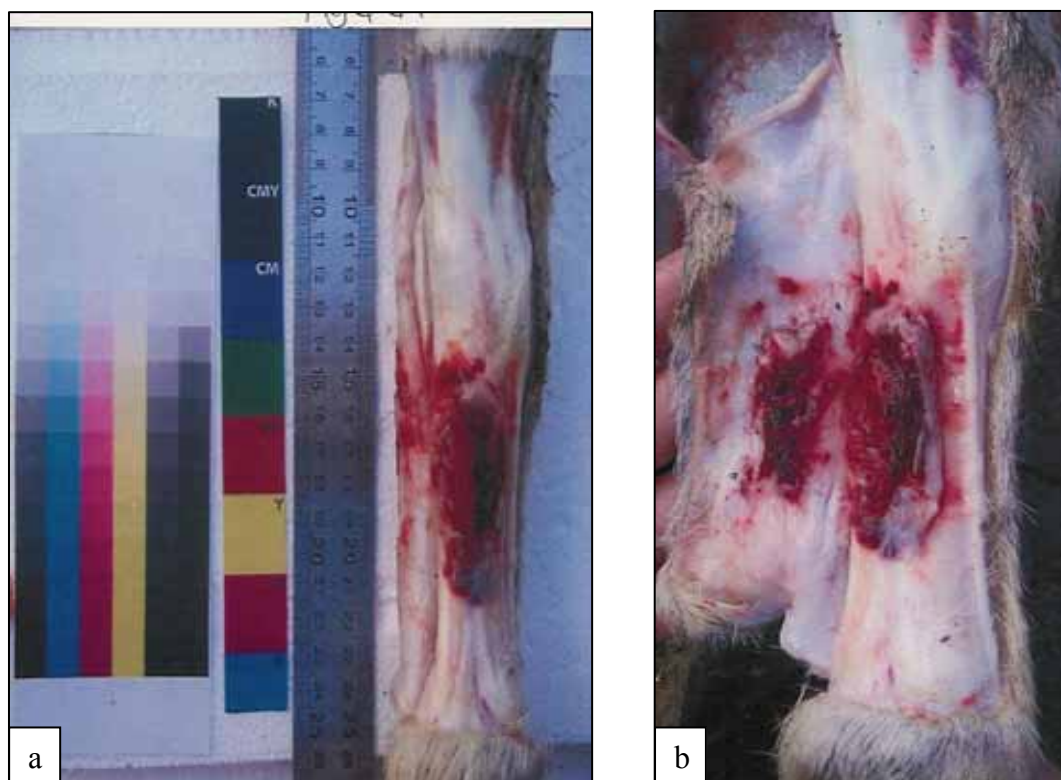
FIGURE 3.6 – Haemorrhage and oedema in subcutaneous tissues due to aseptic surgery as visible at one week post injury in (a) Group 1 Ctrl and (b) Group 1 Cut. Top of images = proximal region; left of images = lateral region.

could stand on both front legs, but walked with caution. The injured tendon had a characteristic bow appearance, similar to that of naturally occurring tendonitis in horses (McCullagh *et al.* 1979). On palpation the skin was warm and stiff under pressure and the SDF tendon could not be palpated up to Week 2.

3.3.1.2 Macroscopic observations

Control tendons from Group 1 displayed a hard, smooth exterior surface and an even white appearance (Figure 3.7a). Mock surgery and subsequent suture of the skin caused substantial subcutaneous haemorrhage and oedema, as depicted in Figure 3.6. Surgically-injured tendons from Group 1 Cut revealed disruption of the parallel alignment along the 2 cm longitudinal incision, with associated haemorrhage and oedema (Figure 3.7b). There was no apparent damage to the proximal and distal region of the tendon.

Group 2 controls also displayed a hard, smooth exterior surface and an even white appearance, as seen in Figure 3.7a. Collagenase-induced injuries of Group 2 revealed severe disruption of the tendon and peritendinous connective tissue with haemorrhage in and around the site of injection (Figure 3.7c). The haemorrhage extended mostly towards the distal region, which could be due to the way the injection was administered; the insertion point being in the middle region, but pointing in the distal direction. At site of injury the SDF tendon was almost completely lysed, embedding the disrupted fibres in a soft mass of reddish to pink coagulated blood. Peritendinous tissue at site of injury were adherent to the SDF tendon. In the proximal region, the tendon seemed to maintain a solid white appearance with little to no disruption of fibre alignment. The collagenase injection did not spread to the DDF tendon and subcutaneously the injury resembled.

3.3.1.3 Macroscopic measurements

Across all groups, the proximal region of the affected tendon displayed the lowest mean MTD, indicating that most swelling and inflammation occurred in the middle and distal regions (Figure 3.8). Within Group 1, there was an increase in mean overall MTD from the control tendons to the surgically cut tendons, but it was not statistically significant. Within Group 2, however, a similar increase in mean overall MTD from control tendons to collagenase-injured tendons was significant. Analysis of differences between treatments indicated that collagenase-injured tendons possessed a significantly higher mean overall MTD than surgically-injured tendons.
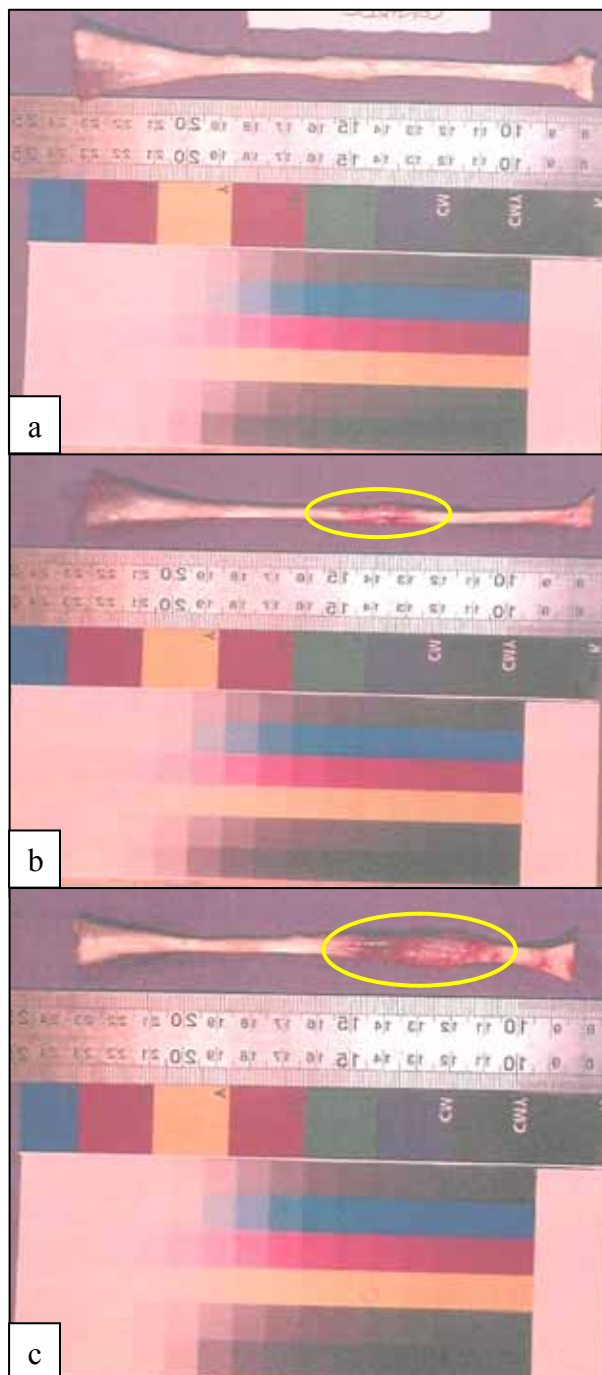
FIGURE 3.7 – Macroscopic appearance of a superficial digital flexor tendon removed from: (a) Group 1 Ctrl, displaying an even, smooth white appearance; (b) a surgically-injured specimen in Group 1 Cut, displaying haemorrhage and oedema (yellow oval); and (c) a collagenase-injured specimen in Group 2 Coll, displaying severe haemorrhage and oedema extending to distal region (yellow oval). Proximal sides of tendons are located to the left of all images.
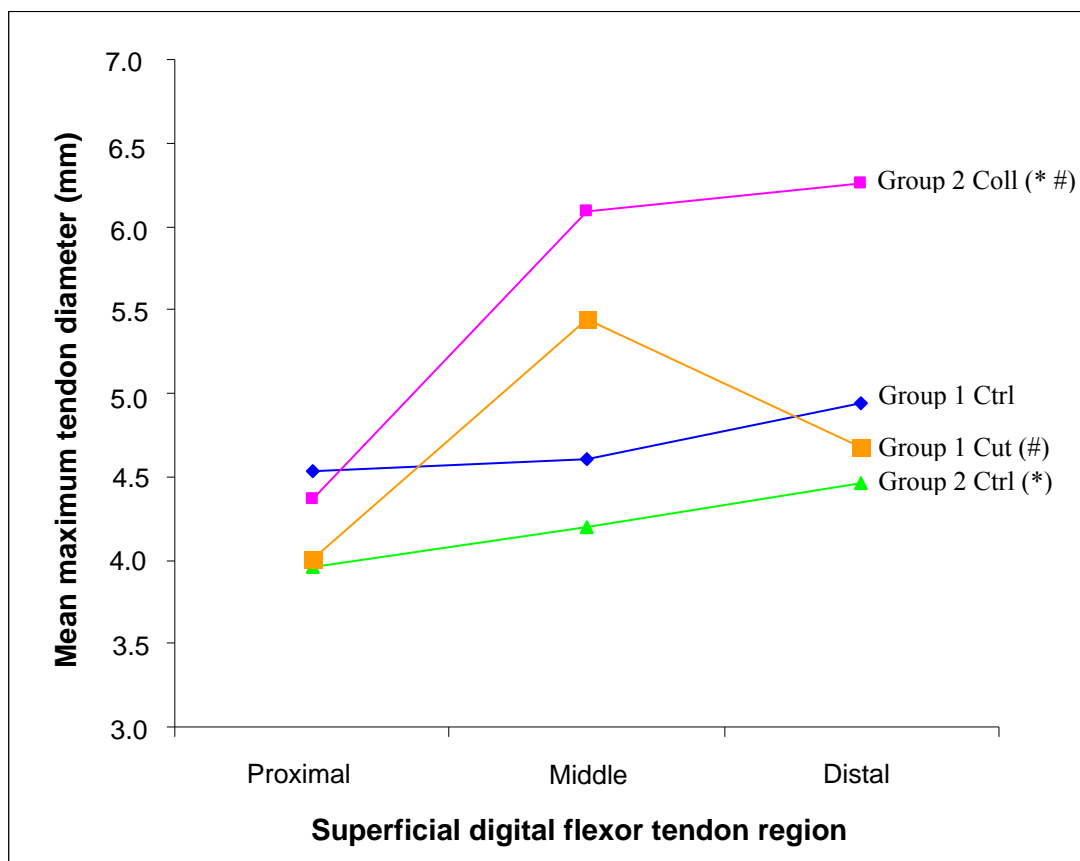
FIGURE 3.8 - Mean maximum tendon diameters (mm) of the proximal, middle and distal regions of the superficial digital flexor (SDF) tendons of Group 1 Ctrl (blue line), Group Cut (orange line), Group 2 Ctrl (green line) and Group 2 Coll (pink line). Statistically significant differences in overall means ($p < 0.05$) were recorded between Group 2 Coll and Group 2 Ctrl (*), as well as between Group 2 Coll and Group 1 Cut (#).

3.3.1.4 <u>Mechanical testing</u>

A summary of mechanical testing results for Group 1 and Group 2 is provided in Table 3.2. Elasticity decreased in both surgically-injured and collagenase-injured tendons when compared with their respective controls, although this decrease was only significant in the case of collagenase-injured tendons. The aforementioned pattern was repeated for measurements of maximum load, maximum stress resistance and stiffness. Both forms of injury resulted in tendons being able to handle less strain when compared with their respective controls, although this decrease in mechanical capacity was not significant for either form of injury.

When comparing the two forms of injury with each other, collagenase-induced injury showed significantly lower elasticity, stiffness and maximum strain resistance. Collagenase-injured tendons also exhibited a smaller load-carrying capacity and reduced stress resistance, though these differences were not significant.

At the time our sheep were sacrificed, one week post-injury, the core lesions of both surgically-injured and collagenase-injured tendons had not regained sufficient strength following injury to resist mechanical testing as in the same manner as normal uninjured tendons. This was apparent in the fact that all tendons failed at the lesion during mechanical testing.

3.3.1.5 <u>Subjective microscopic observations</u>

Group 1 and Group 2 control tendons exhibited identical histological patterns to those of normal, uninjured tendons (Figure 3.9a-b). The collagenous tissue was densely packed with a homogeneous distribution of tenocytes, which stain uniformly blue with red nuclei. Irregular, red-staining leucocytes were present and blood vessels appeared unexcited by saline-injury. The fact that no cellular abnormalities were found also corresponded with published studies (Williams *et al.* 1984b, Dahlgren *et al.* 2005).

Surgically-injured tendons (Group 1 Cut) displayed signs of inflammation, visible as haemorrhage and blood coagulation, but restricted mainly to the area of incision (Figure 3.10). Surrounding tissues still possessed a fair degree of collagenous organisation. There was no substantial increase in the number of blood vessels present.

All collagenase-injured tendons, however, revealed a highly inflamed tendon matrix, visible as a gelatinous mass of coagulated blood, fibrin and damaged extracellular matrix. The matrix was fragmented with plump fibroblasts that invaded the damaged area and appeared to be secreting a loose connective tissue matrix at random. A wide range of structural alterations

TABLE 3.2 - Variation in the modulus of elasticity (MPa), maximum load (N), maximum stress (MPa), maximum strain (%) and stiffness (N/mm) within and between Group 1 and Group 2. Data are presented as means ± SD. Statistically significant differences (*p < 0.05*) within respective columns were recorded between Group 1 Cut and Group 2 Coll (#), as well as between Group 2 Ctrl and Group 2 Coll (*).

SUBSET ID	n	ELASTICITY (MPa)	MAXIMUM LOAD (N)	MAXIMUM STRESS (MPa)	MAXIMUM STRAIN (%)	STIFFNESS (N/mm)
Ctrl 1	3	645.93 ± 51.72	442.73 ± 102.11	55.34 ± 12.76	13.19 ± 1.76	5.53 ± 1.28
Cut	3	515.05 ± 64.26[#]	351.07 ± 26.06	45.00 ± 3.95	12.82 ± 1.86[#]	4.39 ± 0.33[#]
Ctrl 2	3	668.10 ± 207.20[*]	415.57 ± 162.93	51.95 ± 7.86[*]	12.50 ± 3.29	4.75 ± 0.83[*]
Coll	3	281.97 ± 52.39[*#]	290.73 ± 56.01	36.34 ± 7.00[*]	8.67 ± 0.74[#]	2.38 ± 0.42[*#]

N: Newton (1 kg = 9.8 N)
MPa: MegaPascals = 10^6 x Pa = 10^6 x 1 M/m^2 (Force / unit area)
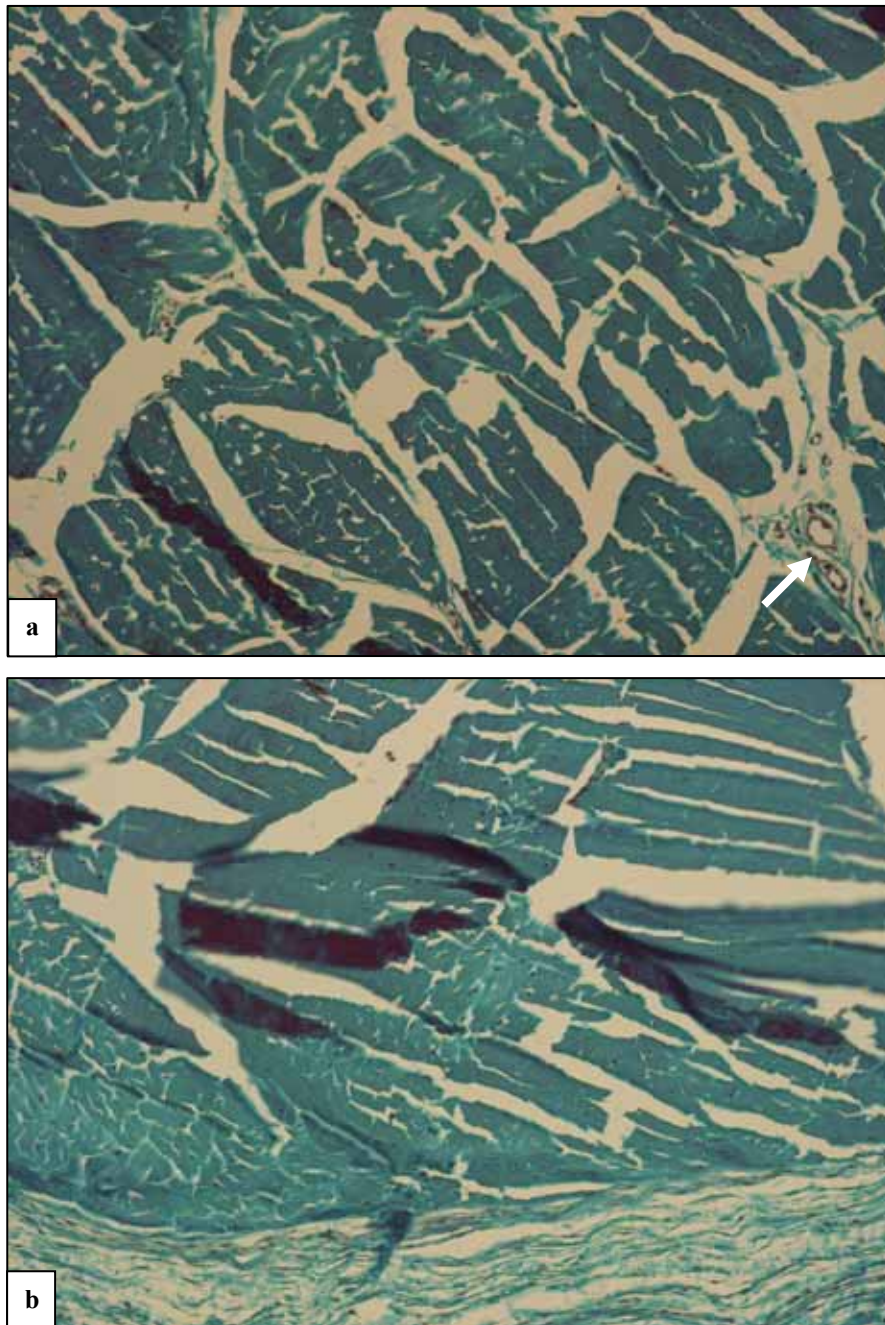
FIGURE 3.9 – Transverse histological sections of (a) a normal uninjured tendon and (b) a typical tendon from Group 1 Control; tendons from Group 2 Control and the saline-injected SS subset all displayed a similar histological appearance to that of Group 1 Control. The white arrow indicates blood vessels entering collagenous tissue. (Masson's trichrome collagen staining, 20x magnification).
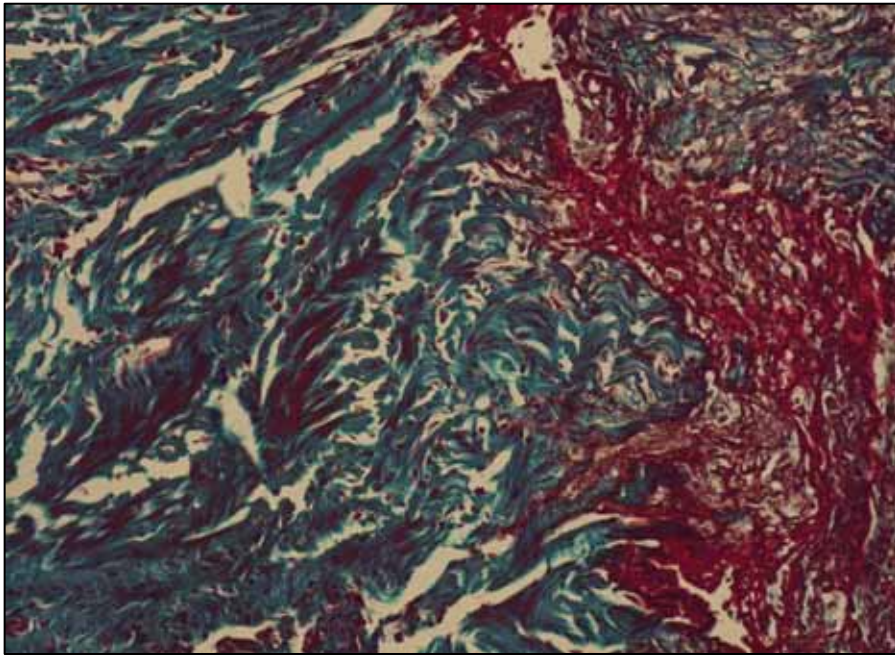
FIGURE 3.10 – Transverse section displaying haemorrhage and blood coagulation (stained red), present due to inflammation in the surgically-injured tendons of Group 1 Cut (Masson's trichrome collagen staining, 20x magnification). Blue staining areas are aligning collagen fibres.

and cellular abnormalities were evident. The leucocyte population was polymorphonuclear and numerous small blood vessels had started to form in the reparative granulation tissues (Figure 3.11a-b), in accordance with observations by Williams *et al.* (1984b).

3.3.1.6 <u>Microscopic measurements</u>

Simple PCI software was used to analyse Masson's-stained sections and to determine (1) the amount of collagen laid down in the tendons via the blue macro and (2) infiltration of inflammatory cells via the red macro. In Group 1, there were significantly lower collagen levels (Figure 3.12a-b) and a significant increase in inflammatory cells (Figure 3.13a-b) of surgically-injured tendons when compared to their controls. Within Group 2, there was similarly lower collagen levels (Figure 3.12a-b) and concomitant increase of inflammatory cells (Figure 3.13a-b) in collagenase-injured tendons as compared to their controls, in both cases significant. These changes were more pronounced in the collagenase-injured tendons as opposed to surgically-injured tendons, but not significantly so. Both forms of injury displayed the greatest impact in the middle region.

3.3.2   Description of collagenase-induced injury

3.3.2.1 <u>External observations</u>

All data for both the SS subsets and the CS subsets were respectively pooled due to a lack of statistical differences. Within six hours of tendon injury, saline-injected tendons displayed considerable local inflammation and swelling, which subsided to basal levels by Week 1. The SDF tendon could not be palpated one day post-injury, probably due to haemorrhage and oedema resulting from the skin incision. It was however possible to palpate the tendon by Week 1. There was no marked lameness of the affected limb at any point.

Collagenase-injured tendons (CS) similarly produced rapid local inflammation and swelling. At the site of injury, the SDF tendon was swollen, displaying varying degrees of enlargement. The swelling usually extended ± 2 - 3 cm proximal and distal to the injection site but never reached the carpometacarpus or metacarpophalangeal joints. The SDF tendon assumed a characteristic bow appearance, similar to that of naturally occurring tendonitis in horses (McCullagh *et al.* 1979), which lasted for the duration of the experiment. Partial lameness of the affected limb appeared within six hours of injury. The sheep could walk cautiously one day post-injury and lameness disappeared completely by Week 2.   On palpation the skin was warm and stiff under pressure and the line of the SDF tendon could not be palpated until Week 1. Over the next two to three weeks the soft tissue in the injured areas
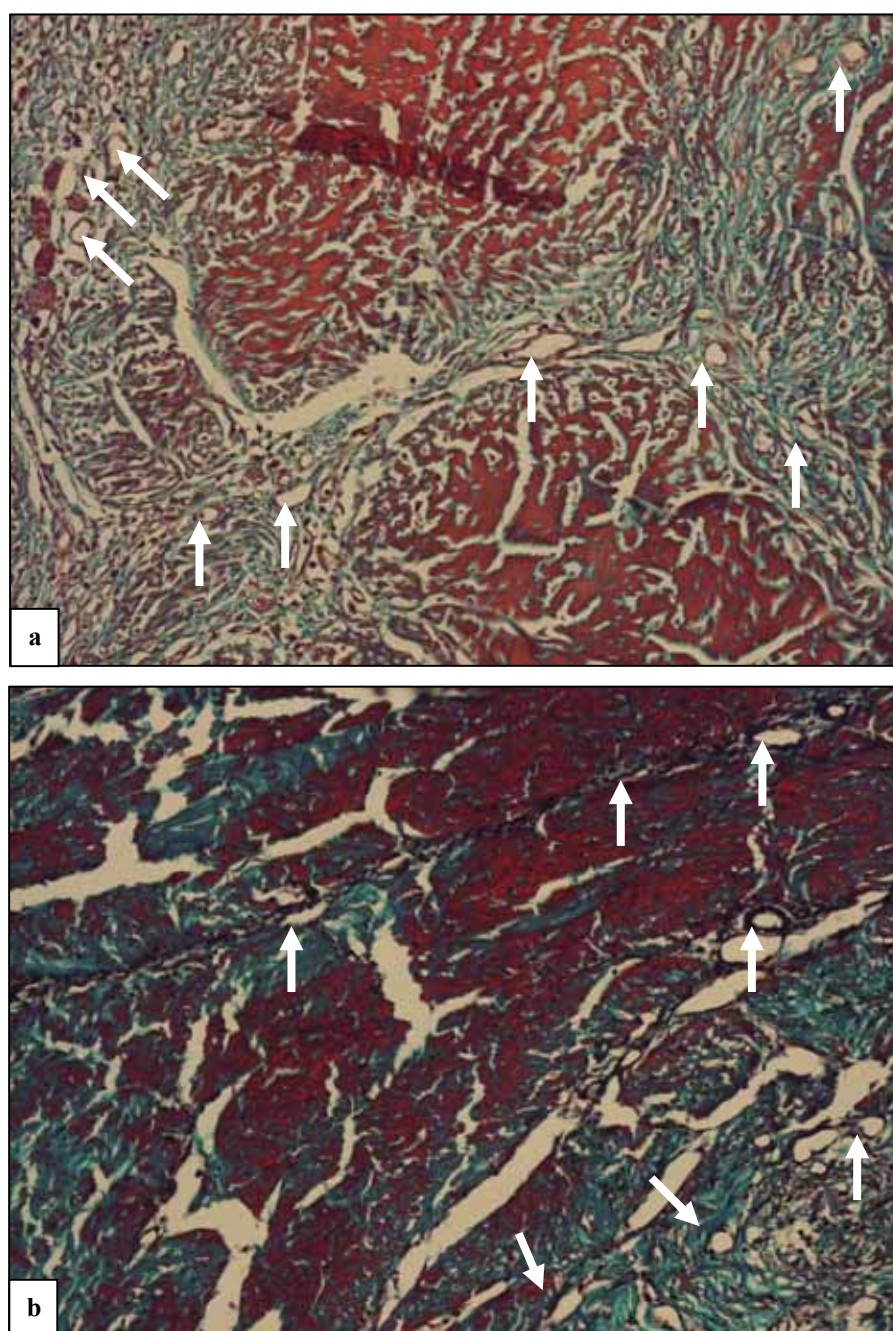
FIGURE 3.11 – Transverse section of collagenase-injured tendon tissue from Group 2 Coll. The majority of the injured area in the (a) middle region and (b) distal region has been replaced by randomly orientated infiltrating red-staining cells and by a loose matrix, containing numerous small capillaries. Note the frequent occurrence of small and densely cellular blood vessels (white arrows). The red staining areas indicate inflammation and the blue staining areas indicate disrupted collagen fibres (Masson's trichrome collagen staining, 20x magnification).
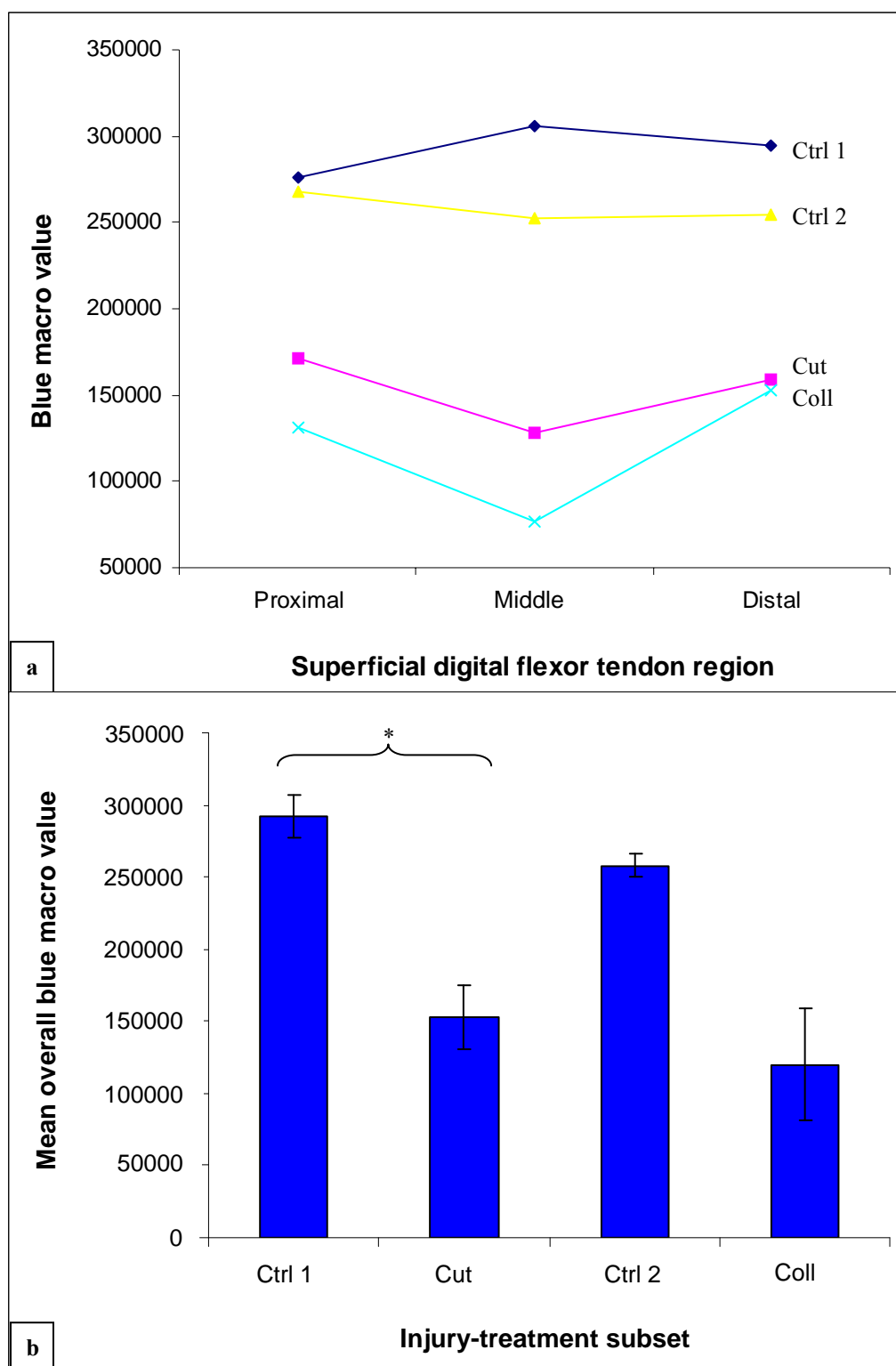
FIGURE 3.12 - Blue macro depictions (the amount of collagen present) indicating (a) distribution of collagen damage among the proximal, middle and distal regions and (b) mean overall collagen damage among surgically-injured tendons, collagenase-injured tendons and their respective controls in Groups 1 and 2 at Week 1. Significant differences (*) were accepted at $p < 0.05$.
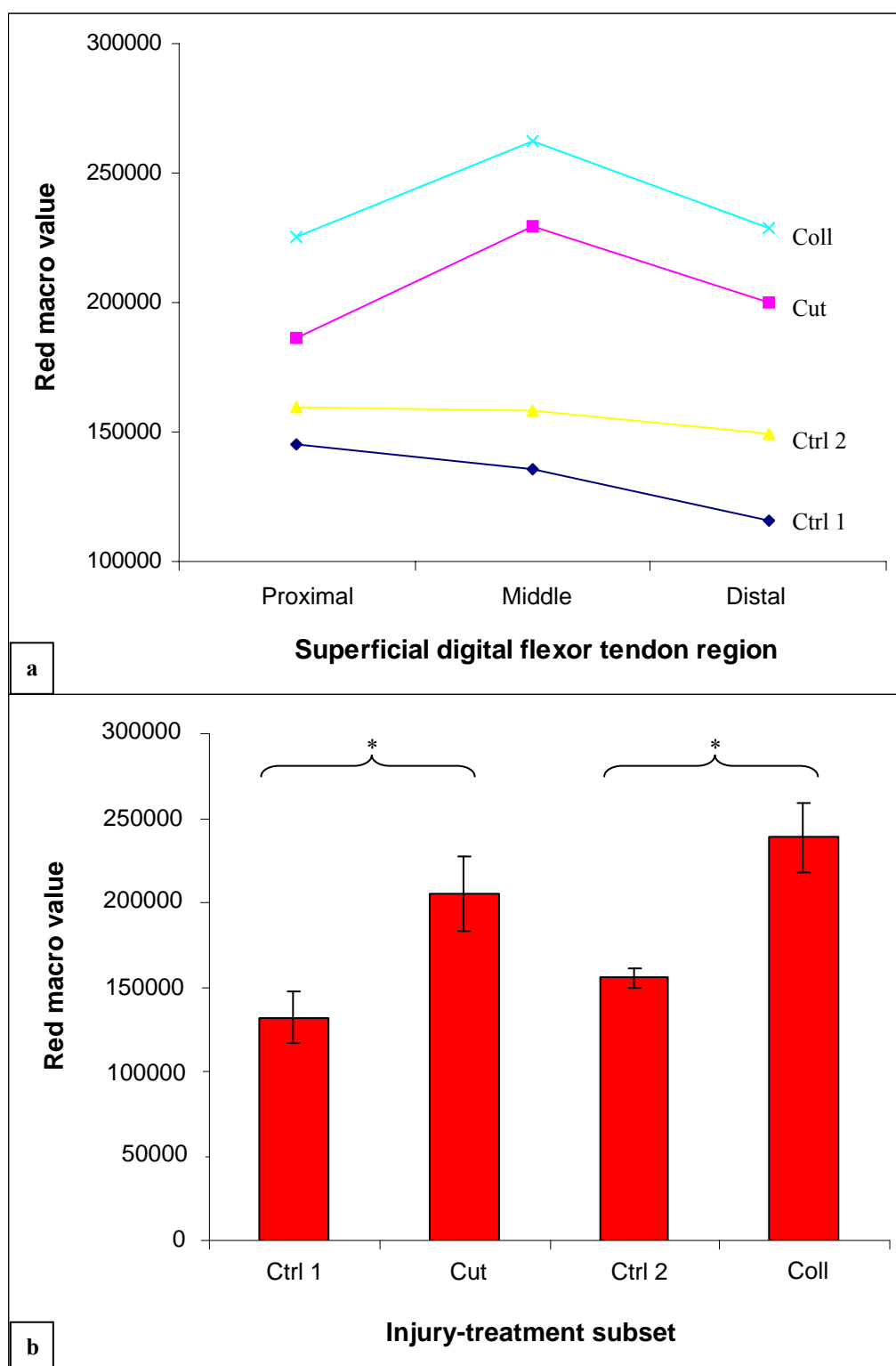
FIGURE 3.13 - Red macro depictions (the amount of inflammatory cells) of (a) inflammatory response among the proximal, middle and distal regions and (b) mean overall inflammation among surgically-injured tendons, collagenase-injured tendons and their respective controls in Groups 1 and 2 at Week 1. Significant differences (*) were taken at a probability value of $p < 0.05$.

was presumably replaced by firmer healing tissue, however, there was no obvious regression in the size of the scarred tendon up to Week 7.

3.3.2.2 Macroscopic observations

All saline-injected controls (SS) had a slight yellowish discolouration in the middle region of the tendon by Week 7. At this point skin haemorrhage had disappeared and only remnants of white to yellowish scar tissue were observed where the skin was cut (Figure 3.14a). It was evident that no extensive damage or fibre disruption occurred to the tendon itself (Figure 3.14b).

Collagenase-induced injuries of all CS subsets showed almost completely lysed and translucent SDF tendons with a white to light pinkish appearance, both distal and proximal to the injury site (Figure 3.14c). In two cases the SDF tendon had attached to the DDF tendon and, in almost all cases, to the peritendinous tissue. Fibre alignment was clearly still highly disrupted by Week 7, although the tendinous connective tissue was firm and solid. Subcutaneous skin lesions appeared the same as for the saline-injected controls (Figure 3.14a).

3.3.2.3 Macroscopic measurements

Maximum tendon diameter data for the SS subsets of Groups 3 and 4 showed no statistical difference and were pooled for further analyses. The same is true for our CS data subsets from Groups 3, 5 and 6. Seven weeks after sustaining a collagenase-induced injury, tendons still possessed a significantly higher overall mean MTD than saline-injected controls (Figure 3.15). Though control tendons displayed a steady increase in mean MTD from proximal to middle to distal region, our CS tendons showed most swelling in the middle-distal region.

3.3.2.4 Mechanical testing

Results for saline-injected control subsets (SS) were pooled due to lack of any statistical difference between Groups 3 and 4. Data for collagenase-injured tendons were pooled for the same reason. Significant differences among measured variables for the resultant SS and CS data sets describe how injury applies serious damage to the strength of a tendon. This can be expounded as collagenase-injured tendons displaying less elasticity, being capable of carrying smaller maximum loads and maximum stress, and possessing less stiffness due to collagen fibre disruption. Results are summarised in Table 3.3. Collagenase-
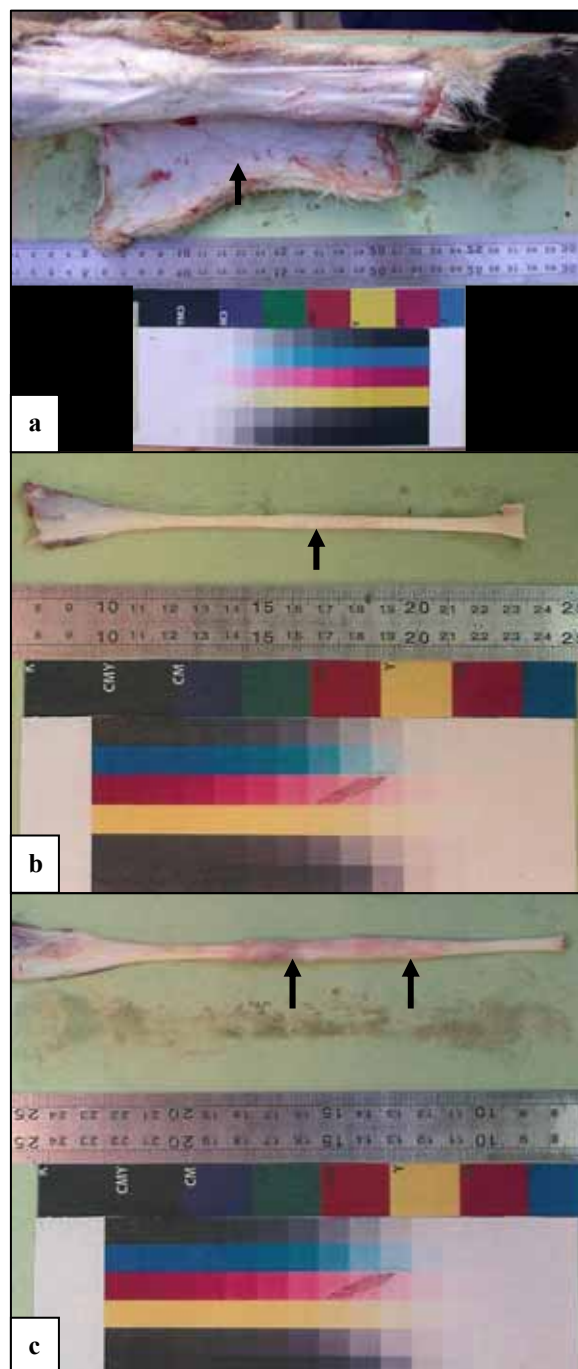
FIGURE 3.14 - Macroscopic appearance of: (a) the skin and subcutaneous area from the saline-injured group after seven weeks - the black arrow indicates a scar lesion; (b) a superficial digital flexor (SDF) tendon from one of the saline-injected controls after seven weeks - the black arrow indicates slight discolouration in the middle region; and (c) a collagenase-injured SDF tendon from one of the collagenase-injured subsets after seven weeks - black arrows indicate discolouration in the middle region. The proximal side is located on the left of all images.
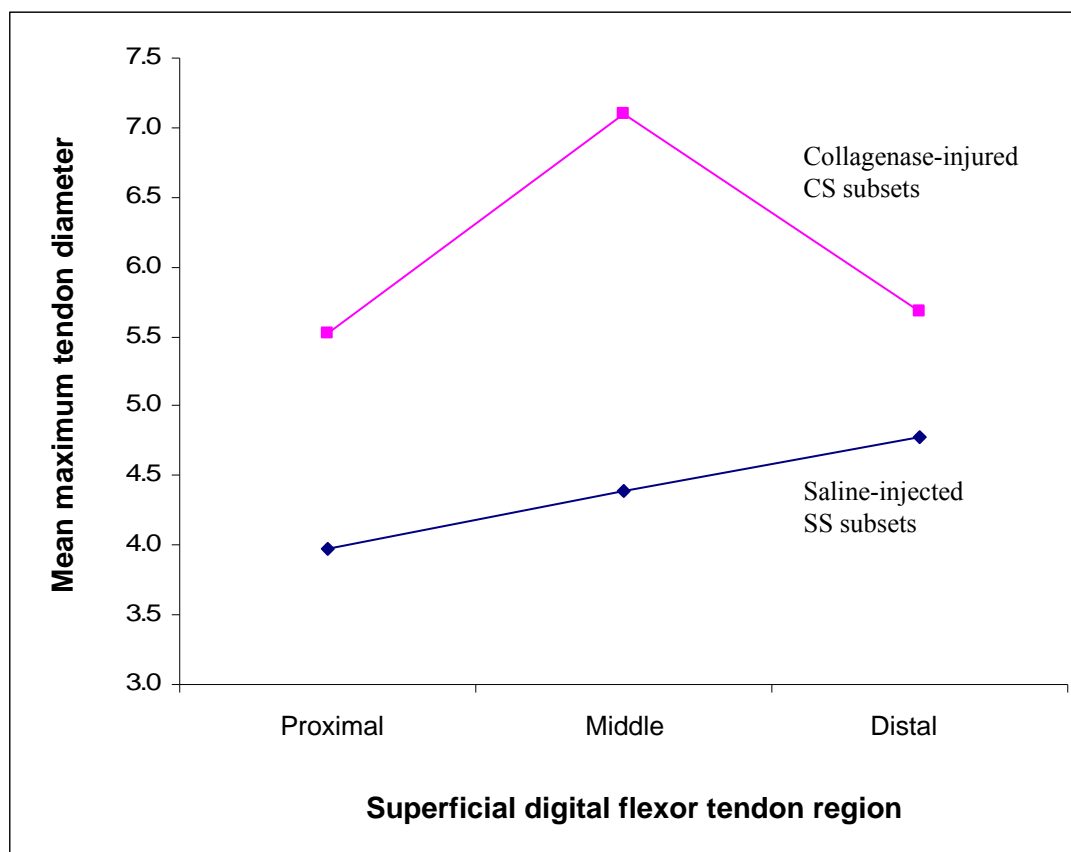
FIGURE 3.15 - Mean maximum tendon diameters (mm) of the proximal, middle and distal regions of the superficial digital flexor (SDF) tendons of saline-injected control subsets (blue line, SS) and collagenase-injured tendons (pink line, CS) of Groups 3 – 6 at seven weeks post-injury. There was a statistically significant difference in overall means between the two data sets ($p < 0.05$).

TABLE 3.3 – Pooled data for SS versus CS variation in modulus of elasticity (MPa), maximum load (N), maximum stress (MPa), maximum strain (%) and stiffness (N/mm) between saline-injected control tendons (SS) and collagenase-injured tendons (CS) of Group 3, Group 5 and Group 6 at Week 7. Data are presented as means ± SD. Statistically significant differences ($p < 0.05$) within respective columns are indicated by asterisks.

SUBSET ID	n	ELASTICITY (MPa)	MAXIMUM LOAD (N)	MAXIMUM STRESS (MPa)	MAXIMUM STRAIN (%)	STIFFNESS (N/mm)
SS	9	568.87 ± 146.08*	437.09 ± 94.40*	54.64 ± 11.80*	11.62 ± 1.98	5.46 ± 1.18*
CS	16	147.32 ± 46.97*	104.60 ± 30.16*	14.78 ± 6.34*	9.26 ± 1.72	1.31 ± 0.38*

N: Newton (1 kg = 9.8 N)
MPa: MegaPascals = 10^6 x Pa = 10^6 x 1 M/m^2 (Force / unit area)

injured tendons showed a decreased capacity to handle strain, although this did not constitute a significant difference.

By Week 7 it seemed that subsets of some CS tendons had re-gained enough strength to resist mechanical testing. Only four of the sixteen tendons tested failed at the lesion. Six tendons failed distal to the lesion and three failed proximal to the lesion. The remaining three failed at one of the two clamps.

3.3.2.5 Subjective microscopic observations

Saline-injected SS subsets exhibited the same histological patterns as normal, uninjured tendons as seen in Figure 3.11a-b. Collagenous-injured CS subsets appeared similar to collagenase-injected tendons of Group 2 (refer to section 3.3.1.5), with highly inflamed tendon matrices and cellular abnormalities. However, it did appear that the healing tissue was a bit more organised and that blood vessels were larger in CS subsets in comparison with tendons from Group 2 Coll (Figure 3.16).

3.3.2.6 Microscopic measurements

Simple PCI software was used to analyse the amount of collagen laid down in the tendons (blue macro, Figure 3.17a-b) as well as infiltration of inflammation into the tendons (red macro, Figure 3.18a-b). The SS subsets were not statistically different in their data sets for either blue macro or red macro, thus these two sets were pooled separately. The same was true for blue macro and red macro data sets in collagenase-injected CS subsets.

Collagenase-injured tendons displayed significantly more inflammation than their SS controls. At the same time, tendons injected with collagenase showed a significant reduction in collagen fibres as opposed to saline-injected controls.

3.3 DISCUSSION

Tendons subjected to surgical incisions displayed a significant (1) decrease in collagen content on the cellular level and (2) increase in inflammatory response in comparison with their controls. Surgically-injured tendons furthermore exhibited a weakening in mechanical strength for all variables measured, as well as haemorrhage, oedema and blood coagulation at the immediate site of injury – stimulating formation of numerous small blood vessels – and increased overall tendon swelling.
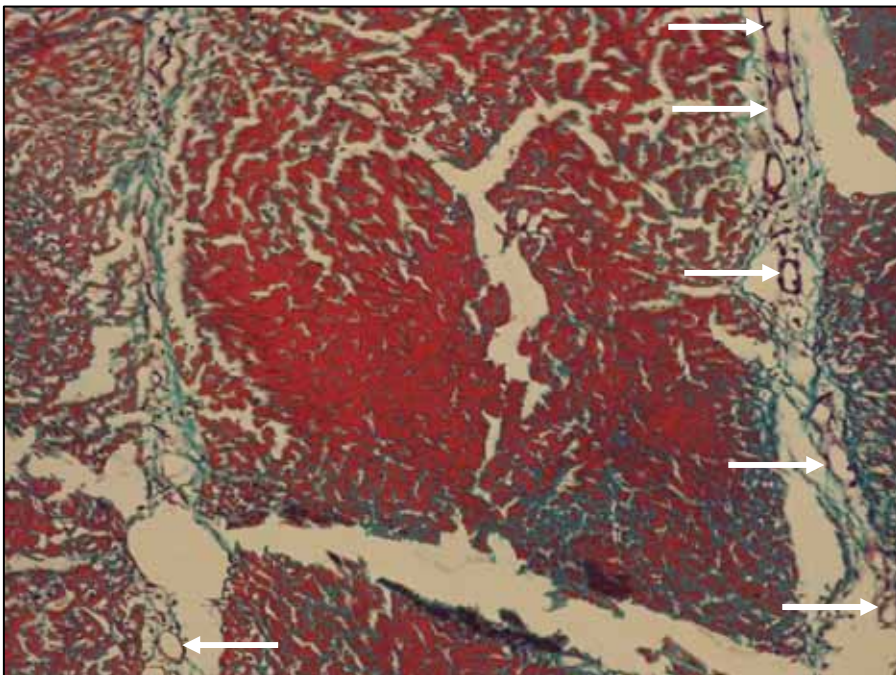
FIGURE 3.16 - Collagenase-injured tendon tissue from a CS subset sample. The tissue is highly inflamed (red areas) and granular. Blood vessels are small and numerous, as indicated by the white arrows (Masson's trichrome collagen stain, 20x magnification).
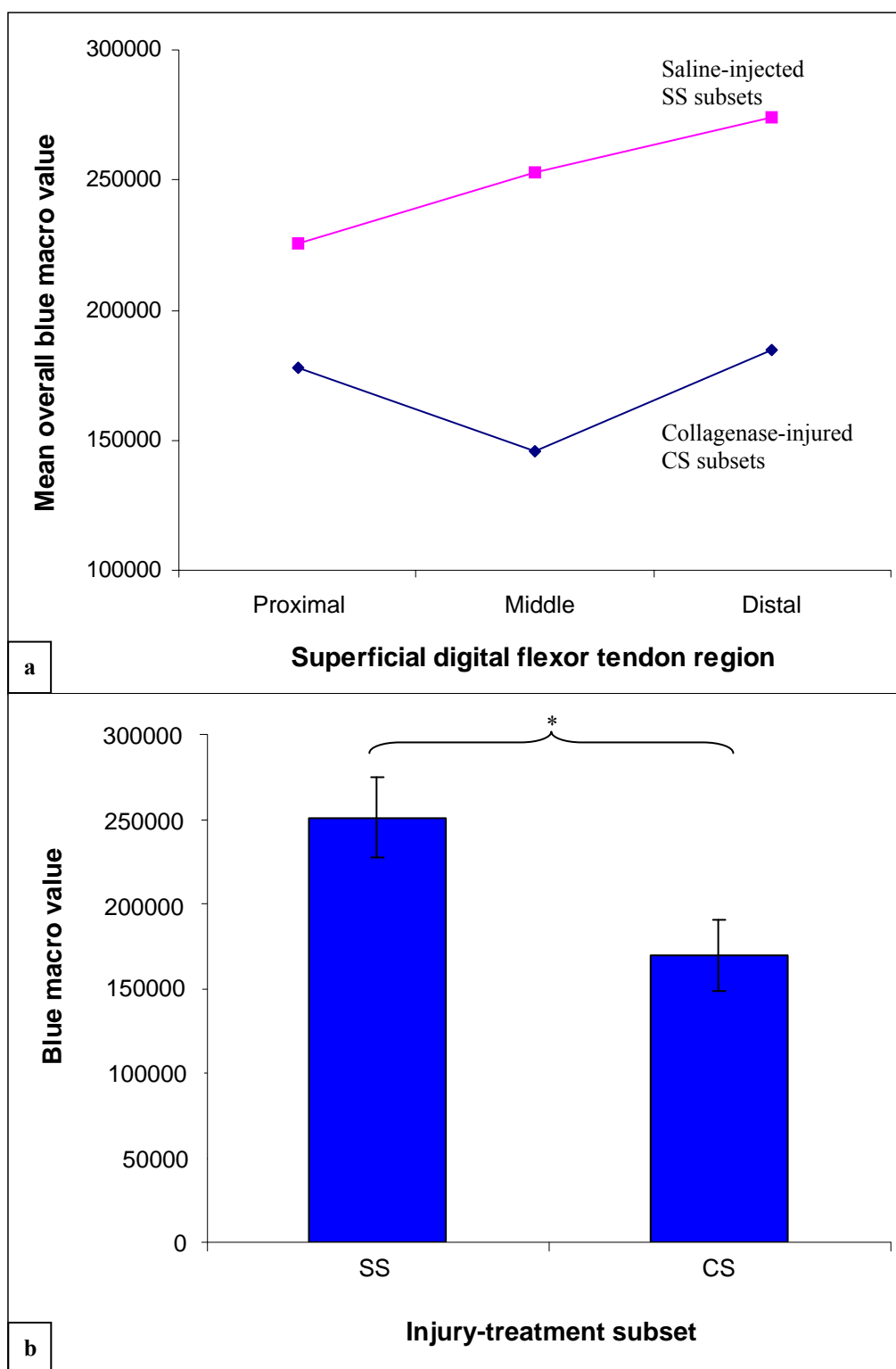
FIGURE 3.17 - Blue macro depictions (the amount of collagen present) indicating (a) distribution of collagen damage among the proximal, middle and distal regions and (b) mean overall collagen damage between collagenase-injured tendons (CS) and saline-injected controls (SS) from Groups 3 to 6 at Week 7. Statistically significant differences ($p < 0.05$) between groups are indicated by an asterisk and bracket.
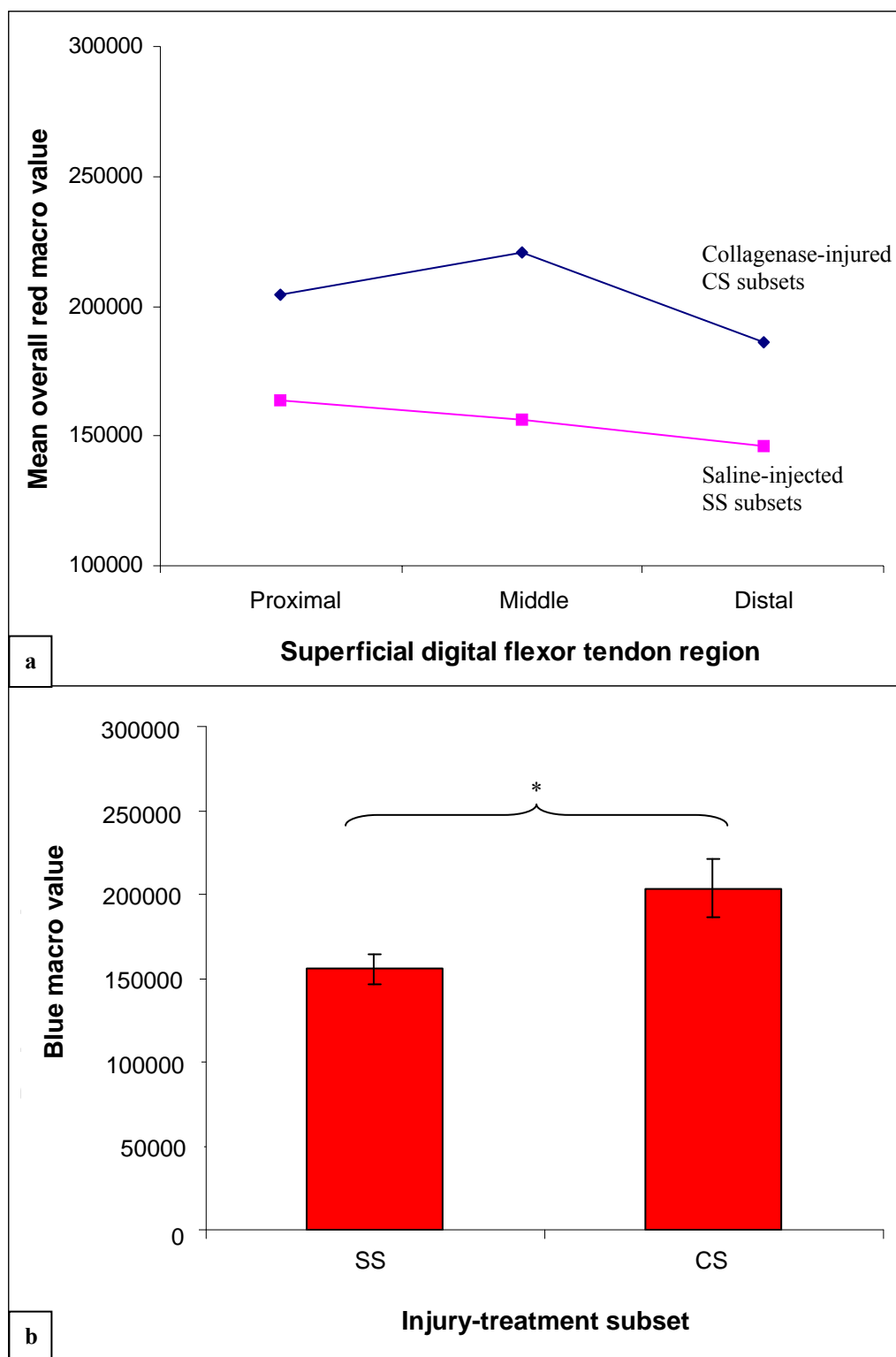
FIGURE 3.18 - Red macro depictions (the amount of inflammatory cells) of (a) inflammatory response among the proximal, middle and distal regions and (b) mean overall inflammation between collagenase-injured tendons (CS) and saline-injected controls (SS) from Groups 3 to 6 at Week 7. Statistically significant differences ($p < 0.05$) between groups are indicated by an asterisk and bracket.

Collagenase-injured tendons displayed a more acute response to injury. This impact on tendon tissues was quantified as a significant (1) increase in overall tendon swelling, (2) loss of elasticity, (3) decrease in stress resistance, (4) loss of stiffness, (5) decrease in collagen content and (6) increase in inflammatory response in comparison with controls. In addition, collagenase-injured tendons assumed the bow appearance characteristic of tendonitis, coinciding with severe tendon lysis, adherence of tendon tissues to peritendinous tissues, haemorrhage and oedema. On the cellular level, severe fibre disruption was noted along with damage to the extracellular matrix, presence of polymorphonuclear lymphocytes and blood vessel formation. Structural damage was not restricted to the middle tendon region, as was found for surgically-injured tendons, but had spread to both proximal and distal regions.

Having established that both surgically-induced and collagenase-induced injuries effected significant damage when comparing either to their respective controls, we compared the two forms of injury themselves. On all accounts collagenase-injured tendons displayed a greater degree of tendon degradation than their surgically-injured counterparts. Collagenase-induced injuries resulted in significantly higher overall tendon swelling and a significantly greater loss of elasticity, stiffness and capacity to handle strain. These results were supported by collagenase-injured tendons (1) inducing lameness within the affected limbs over a longer period, (2) rendering the tendon more difficult to palpate, (3) assuming a bow appearance, (4) experiencing extensive and overflowing haemorrhage, (5) undergoing severe lysis and (6) losing their capacity to carry loads and stress.

William *et al.* (1984b) found that the tissue damage and inflammation from a collagenase-induced injury appeared macroscopically and histologically very similar to that following spontaneous tendon rupture. He also found that the time of repair between the two groups were indistinguishable, and therefore came to the conclusion that a collagen-induced injury would provide a useful model of natural tendon injury and scarring. Our study agrees with these findings. This form of mimicked injury, used by numerous scientists working on equine models, was deemed to be the most relevant for analysing tendon repair following stem cell transplantation (presented in subsequent chapters).

We continued to describe the site of injury in collagenase-injured SDF tendons at seven weeks post-injury, comparing it with uninjured tendon tissues from saline-injected controls. Williams *et al.* (1984a and 1984b) reported that new, small blood vessels would colonise areas of haemorrhage - in which fibrin and damaged collagen fibres were still present - and invade freshly produced connective tissue in the adjacent matrix. Our results supported these findings. Williams *et al.* (1984a) also showed that collagen concentration only returned

to normal approximately six months post-injury, which could explain why we still recorded a high inflammation rate as well as low collagen levels at seven weeks post-injury. McCullagh *et al.* (1979) and Williams *et al.* (1984b) further reported that tendons remained enlarged, that fibre orientation was incomplete and that extensive adhesions persisted between scarred tendons and subcutaneous structures for up to six months post collagenase-induced injury. We noted similar observations for the seven week period.

Using the above data, we proceeded to examine whether the application of peripheral blood MNCs effected a significant improvement in tendon healing. We continued to use our sheep animal model for this purpose in Chapter 4, before conducting a pilot study on horses in Chapter 5.

– CHAPTER 4 –

APPLICATION OF ADULT SHEEP PERIPHERAL BLOOD MONONUCLEAR CELLS TO TRAUMATISED TENDONS

4.1 INTRODUCTION

TENDON and ligament injuries affect up to 46 % of performance and competitive horses in Britain alone (Williams *et al.* 2001). During *post mortem* examinations of 71 Thoroughbred forelimbs, it was found that the superficial digital flexor (SDF) tendon was torn in 48 % of the cases, as opposed to only 1 % torn deep digital flexor (DDF) tendons (Webbon 1977). When this study was expanded to include 140 forelimbs from different breeds, a similar pattern was apparent with 30 % of the limbs displaying torn SDF tendons versus only 4 % torn DDF tendons. It was concluded that injury in horses occur most commonly in the SDF tendon and in the mid-metacarpal region, where the maximum tendon diameter (MTD) of the tendon is smallest and it is not enclosed within a synovial sheath (Webbon 1977 and 1978, Marr *et al.* 1993b).

Injured tendons may take as long as 9 - 12 months to heal (Goodship 1993). Conventional treatment regimes of tendon injuries prevent early formation of collagen cross-links. This allows a controlled exercise regime to improve functionality of the scar tissue, but it does not actually regenerate tendon tissue, and scar tissue is not as functional as tendon tissue (Smith *et al.* 2003). Therefore, although 20 - 60 % of injured horses return successfully to racing, up to 80 % of those horses may sustain re-injury (Sawdon *et al.* 1996).

Effective treatment of tendon injuries requires (1) minimising inflammation, which causes further micro-injury (Brickson *et al.* 2001, Schneider *et al.* 2002, Toumi and Best 2003); (2) minimising formation of rigid and non-elastic scar tissue; and (3) restoring normal tendon structure and function (Henniger 1994). Stem cell therapy is currently receiving much attention as an alternative modality in tendon healing, with promising initial results being advocated by Bruder *et al.* (1994), Wakitani *et al.* (1994), Young *et al.* (1998), Herthel 2001 and Smith *et al.* (2003). Bone marrow aspirates are most commonly used as source of undifferentiated mesenchymal stem cells (MSCs), but bone marrow yields low numbers of MSCs and is painful and difficult to acquire (Herthel 2001, Zuk *et al.* 2001, Smith *et al.* 2003). Peripheral blood represents an alternative source of MSCs (Yasui *et al.* 2003, Zhao *et al.* 2003, Roufosse *et al.* 2004, Villaron *et al.* 2004). In animals, peripheral blood can be

collected with comparative ease through non-surgical methods. The collection poses no pain or risk to the donor and is not subject to biological product regulations.

We decided to study the application of peripheral blood mononuclear cells (MNCs), which incorporates both stem cells and leucocytes, to injured tendons. Sheep were used as animal model in this chapter to establish our methodology and to determine the efficacy thereof before initiating a pilot study on four horses (Chapter 5). Natural tendon injuries were mimicked by administering collagen injections into the SDF tendons (as had been determined in Chapter 3). Both autologous and allogeneic MNCs were used as treatment (extractions described in Chapter 2). Since the goal of efficacious treatment includes regeneration of actual tendon tissue, our main objective was to determine whether either autologous or allogeneic peripheral blood MNCs affect tendon tissue regeneration – and to what degree - within seven weeks post-injury.

4.2 MATERIALS AND METHODS

Use of animals was conducted in accordance with protocols approved by the Ethics Committee for Research on Animals of the Medical Research Council (application number P04/06/010). All surgical procedures and anaesthetic administration were conducted by a registered veterinarian.

4.2.1 Experimental sheep

In this chapter we utilised the same 21 black-head Dorper sheep from which we had isolated and cultured peripheral blood MNCs in Chapter 2, demarcated as Group 3 to Group 6 in Table 4.1. Within each group, two separate tests were administered per animal, namely, one to the left forelimb and one to the right forelimb. A test consisted of (1) administering either a control saline solution or a collagenase-induced injury to the SDF tendon on the first day of experimentation, Week 0, and (2) treating CS injured tendons on Week 1 with a control saline solution, autologous MNCs, or allogeneic CD45$^-$ MNCs. Table 4.1 provides a synopsis of these injury-treatment subsets with the ID assigned to each. All experimental sheep were sacrificed on Week 7 and their tendons dissected for macroscopic and microscopic evaluations.

The sheep were housed at the Welgevallen experimental farm in Stellenbosch, South Africa, and ranged between eight months and four years of age. They were weighed on the

TABLE 4.1 - Only 21 of the 27 experimental sheep referred to in this thesis were used for studying the application of mononuclear cells (MNCs) to injured tendons. These sheep are indicated below in the non-shaded areas containing Groups 3 to 6. The text refers to sheep according to their injury-treatment subset according to the identification (ID) labels provided below: SS, CS, CMNC and CCD45.

			GROUP	FRONT LEG	INJURY (WEEK 0)	TREATMENT	ID	SACRIFICE
	Chapter 3		1 (n = 3)	Left	Skin incision	None	Ctrl	Week 1
	Chapter 3		1 (n = 3)	Right	Cut tendon	None	Cut	Week 1
	Chapter 3		2 (n = 3)	Left	Insert needle into tendon	None	Ctrl	Week 1
	Chapter 3		2 (n = 3)	Right	Collagenase	None	Coll	Week 1
Chapter 2	Chapter 3	Chapter 4	3 (n = 4)	Left	Saline	Saline	SS	Week 7
Chapter 2	Chapter 3	Chapter 4	3 (n = 4)	Right	Collagenase	Saline	CS	Week 7
Chapter 2	Chapter 3	Chapter 4	4 (n = 5)	Left	Saline	Saline	SS	Week 7
Chapter 2	Chapter 3	Chapter 4	4 (n = 5)	Right	Collagenase	Autologous MNCs	CMNC	Week 7
Chapter 2	Chapter 3	Chapter 4	5 (n = 6)	Left	Collagenase	Saline	CS	Week 7
Chapter 2	Chapter 3	Chapter 4	5 (n = 6)	Right	Collagenase	Autologous MNCs	CMNC	Week 7
Chapter 2	Chapter 3	Chapter 4	6 (n = 6)	Left	Collagenase	Saline	CS	Week 7
Chapter 2	Chapter 3	Chapter 4	6 (n = 6)	Right	Collagenase	Allogeneic MNC	CCD45	Week 7

first day of the experiment and again just prior to sacrifice. Provisioning of food, water and shelter is described in detail in Chapter 2.

4.2.2 Injury and treatment procedures

During application of a tendon injury in Week 0, each sheep was prepared for surgery as follows. An intravenous injection of Chanazine mixed with Antropine10 was administered (according to manufacturer's instructions) in the jugular vein, to serve as tranquiliser and provide some degree of analgesia. The animal was placed in dorsal recumbency andrestrained to prevent it from kicking and injuring itself. Nerve blocking was obtained and the forelimbs were prepared for aseptic surgery as described in Chapter 3 section 3.2.2.1.

To expose the SDF tendon, a sterile number 20 scalpel blade was used to make a 4 cm skin incision on the lateral side of the forelimb, approximately 5 cm distal to the accessory carpal bone. A 26-gauge hypodermic needle was used to administer a 0.1 ml injury injection directly into the core of the SDF tendon in the palmar midline. This is located approximately midway between the carpometacarpal and metacarpophalangeal joints, or ± 6 cm as measured from the accessory carpal bone towards the proximal sesamoid bone. Following application of injury, six to ten sutures were applied to skin using sterile surgical nylon line.

Post-operatively the sheep were injected with an antibiotic as described in Chapter 3 section 3.2.2.1 and observed in their stables until they recovered from the sedatives. The sheep's injured legs received cold hydro therapy twice daily for three days post-injury. Thereafter, were observed twice daily for any discomfort or behavioural problems.

When applying treatments in Week 1, the animals were not sedated in any way, but were restrained as mentioned earlier. A 26-gauge hypodermic needle was used to administer 10 treatment injections of 0.1 ml each, applied subcutaneously in the area of injury. The following injury-treatment subsets were investigated.

4.2.2.1 <u>Saline-saline (SS)</u>

In Week 0, 0.1 ml of a 0.9 % saline solution was injected as an injury control into the SDF tendons of this subset's sheep. In Week 1, the same tendons received another 1 ml of a 0.9 % saline solution, serving as treatment control.

4.2.2.2 <u>Collagenase-saline (CS)</u>

In Week 0 a single 0.1 ml injection bacterial collagenase type 1 was administered into the centre of the SDF tendon (refer to Chapter 3 section 3.2.2.2 for collagenase solution).

Following this collagenase-induced injury, the tendons received 1 ml subcutaneous injections of a 0.9 % saline solution as treatment in Week 1.

4.2.2.3 Collagenase-autologous mononuclear cells (CMNC)

Similar to the CS injury-treatment subset, sheep in the CMNC group received an intratendinous 0.1 ml injection of collagenase in Week 0 as injury. In Week 1, however, they were treated subcutaneously with 1 ml of autologous MNCs, containing ± 4.5 x 10^6 cells. For sample preparation of the MNC injections, refer to Chapter 2 section 2.2.3.

4.2.2.4 Collagenase-allogeneic mononuclear cells (CCD45)

Sheep from this injury-treatment subset once again received a 0.1 ml injection of collagenase in Week 0 as injury. In Week 1, they were treated subcutaneously with 1 ml of allogeneic MNCs, containing ± 3.0 x 10^6 CD45$^-$ cells (excluding leucocytes which might prompt an immune response in a foreign body). For sample preparation of these CD45$^+$ MNC injections, refer to Chapter 2 section 2.2.3.

4.2.3   Ultrasonographic evaluations

Ultrasonographic echogenicity (the brightness of the evenly white / hyperechoic appearance of tendon tissues) can be used to diagnose and monitor healing in tendons (Genovese *et al.* 1987, Micklethwaite *et al.* 2001). Each experimental SDF tendon was ultrasonographically scanned eight times over a period of eight weeks. The first inspection, referred to as the pre-scan, was performed in Week 0 immediately prior to induction of tendon injury. This data was purely used to determine whether the SDF tendon was ultrasono-graphically normal as described in Chapter 3 section 3.2.2. Thereafter the tendon was scanned on a weekly basis until termination of the experiment in Week 7.

Limbs were prepared for ultrasound scanning by shaving the hair, cleaning the skin with metholated spirits (to clear it of fatty deposits) and applying an echolucent gel. Scans were conducted using a custom made stand-off pad with an Aloka 500 portable ultra-sonographic unit equipped with a 7.5 MHz linear array transducer. Examinations were undertaken in the palmar midsagittal aspect of each forelimb. The scanning area extended from ± 2 - 3 cm from the accessory carpal bone to ± 2 - 3 cm proximal to the proximal sesamoid digit. This area was divided into a proximal, middle and distal region (each ± 3 - 5 cm in length). One transverse image was recorded per region for lesion evaluations.

Two longitudinal images (overlapping in the middle region) were recorded for echogenicity and linearity evaluations (Figure 4.1).

All images were captured using a Panasonic VHS video recorder and downloaded onto DVD, from which still images were obtained using PowerDVD XP software package. Still images were assessed qualitatively by adapting the lesion echogenicity and fibre alignment classification index from Marr *et al.* (1993b) and Reef (1998, 2001). Refer to Table 4.2 for a description of our echogenicity indices and Table 4.3 for our fibre alignment indices. To ensure consistency, one investigator performed all ultrasound and computer-aided analyses. Since only the lesion itself was scored according to aforementioned indices, a mean was calculated for the area of overlap when considering the two longitudinal measurements per tendon. The three non-overlapping transverse images were considered separately according to region. Ultrasound data were normalised by expressing each index number as a proportion of the index number assigned for Week 1.

4.2.4   Macroscopic observations

At Week 7 all sheep were injected intravenously with 5 mg Domesedan (Detomidine – Novartis AH) and stunned mechanically. The forelimbs were disarticulated *post mortem* at the carpus joint. The skin was opened laterally from the accessory carpal bone to the metacarpophalangeal joint and pulled back to expose the palmar metacarpal region of injury. A Nikon digital camera was used to capture images of all injured limbs while differences in colouration were noted. Thereafter the SDF tendons were removed and placed on ice for immediate mechanical testing (refer to section 4.2.6).

4.2.5   Macroscopic measurements

The maximum tendon diameter of the proximal, middle and distal region of each SDF tendon was measured with image analysis software (analySIS Image Processing Version 3.2), following all procedures as described in Chapter 3 section 3.2.4.

4.2.6   Mechanical testing

Modulus of elasticity and mechanical properties of each SDF tendon were measured using a servo hydraulic testing station, following all procedures as described in Chapter 3 section 3.2.5.
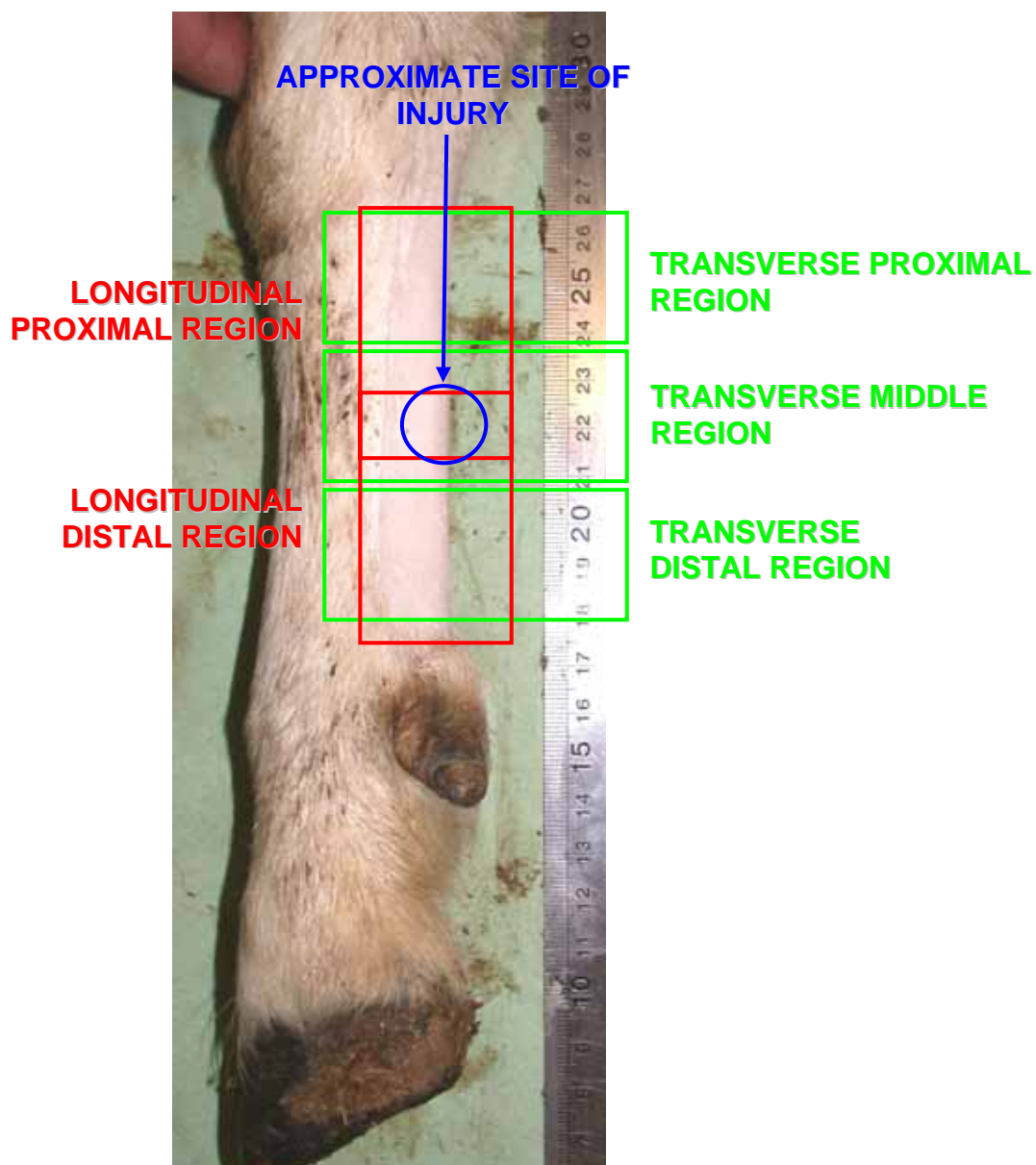
FIGURE 4.1 - Image of the left forelimb of a sheep with schematic insertions to illustrate the relative locations of the transverse and longitudinal regions that were examined ultrasonographically each week and the approximate site of injury.

TABLE 4.2 - Description of the lesion echogenicity index used for ultrasound image analyses.

INDEX	ECHOGENICITY	COLOURATION	STRUCTURAL DAMAGE
0	100 % Hyperechoic	Even white	• None
1	> 70 % Hyperechoic	Mostly white	• Minimal fibre disruption • Minimal inflammation
2	70 % Hyperechoic 30 % Hypoechoic	70 % White 30 % Grey	• Varying degrees of fibre disruption • Moderate local inflammation
3	40 % Hyperechoic 60 % Hypoechoic < 1 % Anechoic	40 % White 60 % Grey < 1 % Black	• Varying degrees of fibre disruption • Moderate local inflammation
4	Mostly Anechoic	Mostly black	• Total fibre tearing • Haematoma / oedema formation

TABLE 4.3 - Description of the parallel fibre alignment index used for ultrasound image analyses.

INDEX	DESCRIPTION	PROPORTION OF LINEARITY
0	Normal long linear echoes	76 – 100 %
1	Irregular long linear echoes	51 – 75 %
2	Numerous short, irregular linear echoes	26 – 50 %
3	No or minimal short linear echoes	0 – 25 %

4.2.7   Microscopic observations and measurements

Superficial digital flexor tendons were fixed in 4 % paraformaldehyde, dehydrated in graded alcohols, cleared in toluene and embedded in paraffin wax before preparing 5-10 μm cross-sections and staining with Masson's Trichrome collagen stain, using the equipment and following all protocols described in Chapter 3 section 3.2.6. Similarly, Simple PCI Analysis software was used in conjunction with inverted microscope to analyse a blue macro (denoting collagen content) and a red macro (indicating degree of inflammation) across three tendon regions (refer to Chapter 3 section 3.2.6 and Figure 4.1).

In addition, tendons from the CMNC and CCD45 injury-treatment subsets had been injected with MNCs containing a BrdU cell marker (refer to Chapter 2 section 2.2.3.5). Bromodeoxy-uridine was used as a MNC marker to determine whether MNCs could be detected in the lesion or whether these cells had migrated from the area of damage. Separate histological sections (two per region of each forelimb) were prepared for BrdU identification. Tissue sections were deparaffinised with xylene and graded alcohols before blocking endogenous peroxidase activity with 3 % hydrogen peroxide. Slides were incubated at 89 °C with BD^{TM} Retrievagen antigen retrieval. A second incubatory period with biotin anti-BrdU monoclonal antibody was conducted in a humidified chamber. The last incubation was performed with Streptavidin-HRP enzyme complex before adding DAB substrate for signal detection. Haematoxylin was used for counterstaining. Sections were ultimately dehydrated in alcohols, cleared in xylene and mounted on microscope slides for examination under an inverted light microscope (Nikon Eclipse E400).

4.2.8   Statistical analysis

Data were analysed using the SAS Enterprise Guide V3.0 software package. Differences in sheep mass before and after treatment were determined by paired t-tests. Independent Student's t-tests were used for all further analyses between two treatment groups. Where there were no significant differences between the two SS injury-treatment subsets, such data were pooled for analysis. The same was true for the two CMNC injury-treatment subsets. One-way Analysis of Variance (One-way ANOVA) was similarly performed to test for significant differences between the three CS injury-treatment subsets in order to ascertain whether data could be pooled. One-way ANOVA and ANOVA General Linear Models (ANOVA-GLM) were used to test for statistical differences in the means among three or more treatment groups, followed up with the Tukey *post-hoc* test for multiple comparisons.

Level of significance was accepted at *p < 0.05*. All data in graphs represent means ± standard deviation (SD).

4.3 RESULTS

4.3.1 Clinical observations

Induced injuries did not appear to have adverse effects on general health of the sheep used for these experiments, as the sheep had maintained their average mass during the study period. This was quantified as an average mass of 42.20 ± 6.86 kg before the onset of our experiment as opposed to 42.70 ± 7.30 kg at completion of the study, which is not statistically different. All tendons were ultrasonographically scanned, found to be without any lesions and were declared normal before starting experiments.

4.3.1.1 Saline-saline (SS)

Observations for the SS injury-treatment subsets are noted in detail in Chapter 3 section 3.3.2.1. To summarise, initial local inflammation and swelling quickly subsided, tendons could be palpated by Week 1 and there was no marked lameness of affected limbs.

4.3.1.2 Collagenase-saline (CS)

Observations for the CS injury-treatment subsets are noted in detail in Chapter 3 section 3.3.2.1. To summarise, rapid inflammation and severe swelling occurred and there was no regression in size of the scarred tendon up to Week 7. The forelimb took on a characteristic bow appearance and could not be palpated at Week 1. The affected forelimb displayed partial lameness until Week 2.

4.3.1.3 Collagenase-autologous mononuclear cells (CMNC)

The initial response to collagenase-induced injury was the same as observed for the CS injury-treatment subsets. By Week 1 legs from the CMNC sheep appeared the same as those of CS sheep, displaying rapid local inflammation and swelling (Figure 4.2a). There was no obvious regression in size of the scarred tendon by Week 2 (Figure 4.2b). At termination of the experiment by Week 7, however, most of the surrounding swelling in the CMNC injury-treatment subset had disappeared almost completely, leaving only minor swelling in the direct vicinity of the injury site (Figure 4.2c). By Week 3, there was no lameness of the affected limbs.
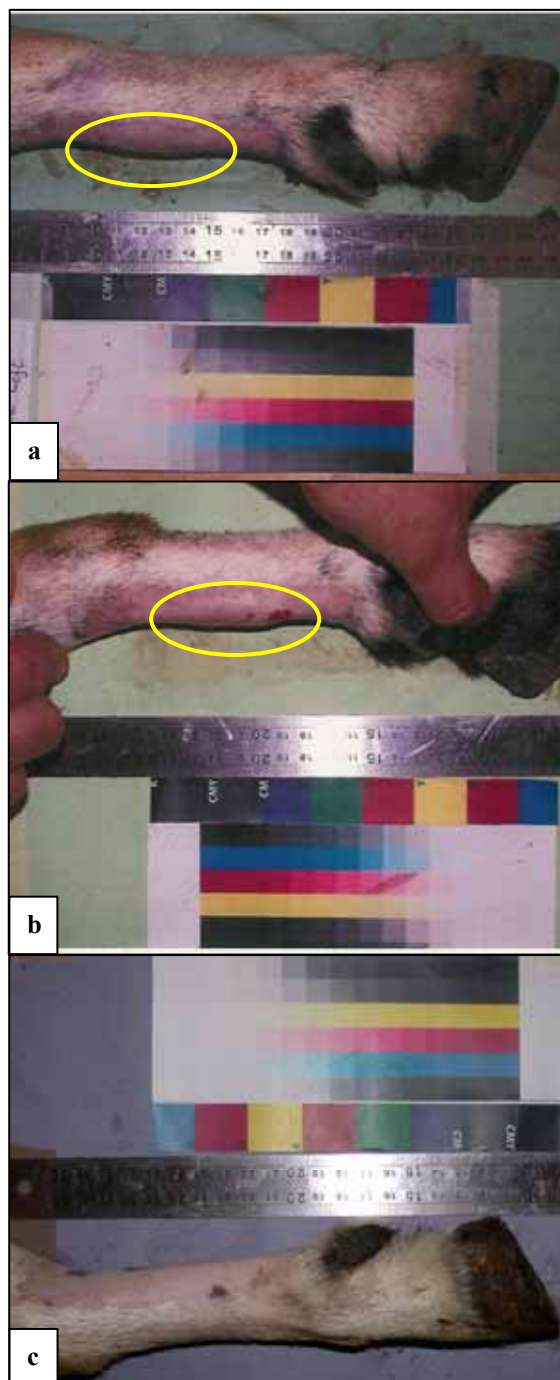
FIGURE 4.2 - Appearance of the forelimb of a sheep from the CMNC injury-treatment subset (a) at Week 1, showing extensive leg swelling (yellow oval), (b) at Week 2, with little reduction in the swelling (yellow oval), and (c) at Week 7, at which point the swelling had subsided considerably. The proximal end of each limb is located on the left side of its image.

4.3.1.4 <u>Collagenase-allogeneic mononuclear cells (CCD45)</u>

The sheep legs for this injury-treatment subset displayed the same trend as observed in the CMNC subsets. The legs remained in a bowed state up to Week 4 and by Week 7 the surrounding swelling had subsided to only the area of injury (Figure 4.3a-c). There was similarly no lameness visible by Week 3.

4.3.2 Ultrasonographic evaluations

Data could be pooled for the different SS subsets due to lack of statistical difference between them; the same was true for the CS and CMNC subsets. Ultrasonographically, healing progressed satisfactorily when the lesion site displayed a significant improvement in either echogenicity or parallel fibre alignment.

For transverse echogenicity, collagenase-injured tendons from the CS subset were significantly more hypoechoic to anechoic (grey to black ultrasound appearance) than the SS subset (more white appearance), resulting from increased oedema, haemorrhage and collagen fibre denaturation. This was the case for the proximal, middle and distal regions of SDF tendons. Both subsets that received treatment with MNCs (CMNC and CCD45) provided ultrasound scans that had an appearance intermediate to those of SS and CS subsets, but did not differ significantly from one another. The CMNC injury-treatment subset had significantly more hypoechoic to hyperechoic lesions than the collagenase-injured CS subset for mean transverse echogenicity in the proximal, middle and distal regions. At the same time all three tendon regions of the CMNC subset were significantly less hyperechoic than those of the saline-injected SS controls. The CCD45 injury-treatment subset had significantly more hypoechoic to hyperechoic lesions than the collagenase-injured subset for mean transverse echogenicity as well in the middle and distal tendon regions; this difference was, however, not significant in the proximal tendon region. Although all three CCD45 tendon regions were less hyperechoic than those of the saline-injected SS controls, differences were only significant for the proximal and middle regions.

In the longitudinal viewing fields, a similar trend emerged. Collagenase-injured tendons from the CS subset displayed a significant increase in echogenicity and parallel fibre disruption (grey to black) as opposed to tendons from the saline-injected SS controls (more white appearance). The CMNC and CCD45 subsets once again did not differ significantly from one another but displayed index scores intermediate to those of the CS and SS subsets. In comparison with collagenase-injured tendons (CS), tendons treated with MNCs were significantly more hypoechoic to hyperechoic and showed a significant increase in fibre
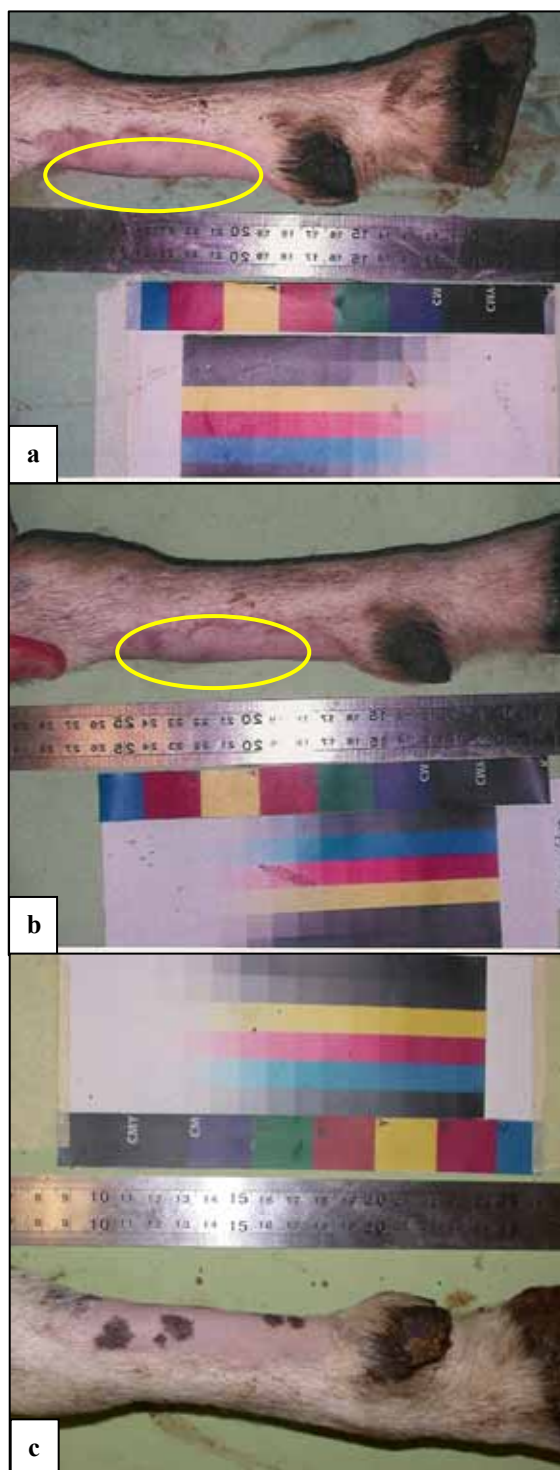
FIGURE 4.3- Appearance of the forelimb of a sheep from the CCD45 injury-treatment subset (a) at Week 1, showing extensive leg swelling, (b) at Week 2, with little reduction in the swelling, and (c) at Week 7, at which point the swelling had subsided considerably. The proximal end of each limb is located on the left side of its image.

alignment. These treated lesions were at the same time significantly less hyperechoic and displayed significantly more parallel fibre disruption compared with SS controls.

Rate of tendon recovery was analysed by plotting normalised index allocations across time for the different injury-treatment subsets and comparing their resultant gradients. Separate analyses were conducted on the resulting gradients for (1) mean transverse echogenicity as determined from the graph in Figure 4.4, (2) mean longitudinal echogenicity, as calculated from the graph in Figure 4.5, and (3) mean longitudinal fibre linearity, as assessed from the graph in Figure 4.6. Typical temporal ultrasound sequences for each injury-treatment subset are additionally provided for (1) mid-region transverse echogenicity, in Figures 4.7 to 4.10, and (2) mid-region longitudinal echogenicity, in Figures 4.11 to 4.14.

Tendons treated with MNCs (CMNC and CCD45) showed significant increases in their rate of tendon recovery over time when compared to the collagenase-injured CS subset. Despite this increased healing rate, tendons from the CMNC and CCD45 subsets were still significantly more hypoechoic to anechoic and displayed significantly more fibre disruption than saline-injected controls seven weeks post-injury.

4.3.3 Macroscopic observations

At Week 7, saline-injected tendons from the SS subset displayed a hard, smooth exterior surface with slight yellowish discolouration at the site of injury (refer to Chapter 3 Figure 3.14b). There was no subcutaneous haemorrhage or swelling, just a thin white line representing potential scar tissue remnants from the skin incision (refer to Chapter 3 Figure 3.14a). It was evident that no extensive damage occurred.

Tendons from the collagenase-injured CS subset, on the contrary, displayed an almost completely lysed and translucent SDF tendon both distal and proximal to the injury site (refer to Chapter 3 Figure 3.14c). In two cases the SDF tendon had attached to the DDF tendon, and in most cases the SDF tendon was seen to have attached to the peritendinous tissue. Fibre alignment was clearly still highly disrupted by Week 7, although the connective tissue of the lysed tendon was firm and solid with no haemorrhage. By Week 7 all appeared normal subcutaneously, haemorrhage had disappeared and only white to yellow scar tissue remained where the skin was cut, as displayed in Figure 3.14a.

In the CMNC subset the SDF tendon revealed slight yellowish discolouration with no haemorrhage, while leg swelling reduced appreciably across the study period. In some areas the tendon appeared slightly translucent but in others there was distinct yellow to pink
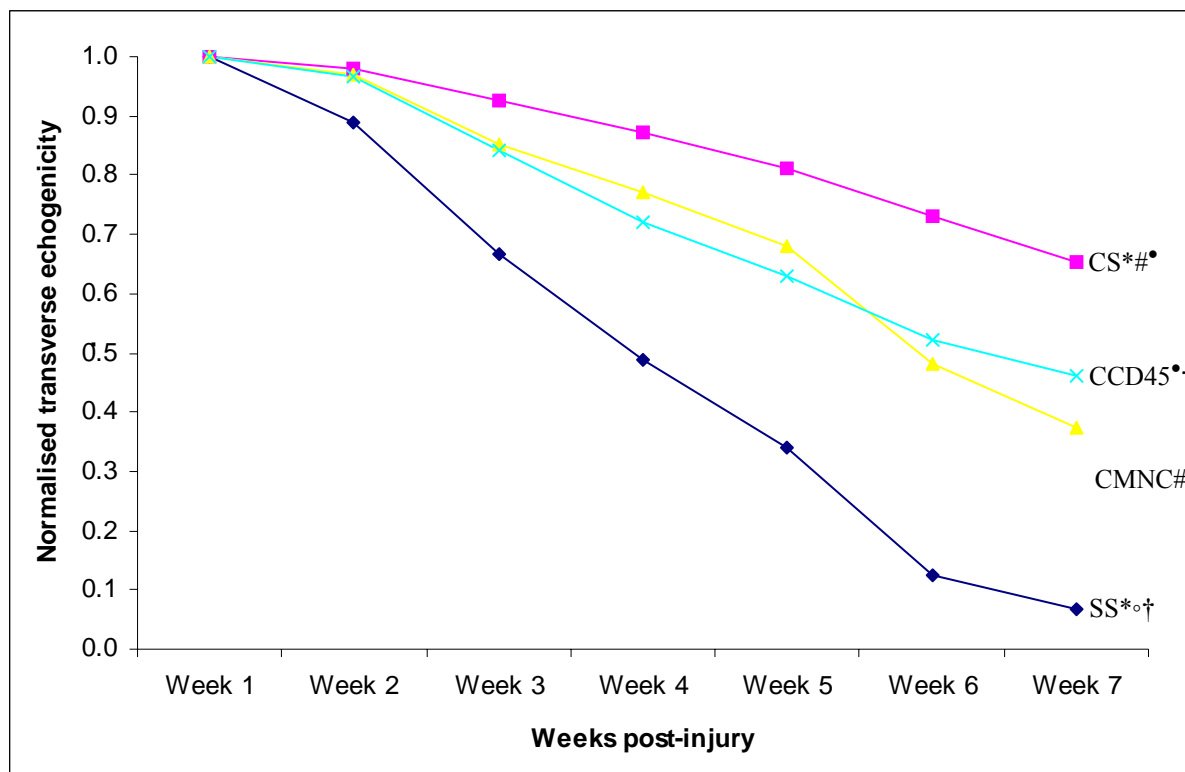
FIGURE 4.4 – Normalised echogenicity of transverse scans of the superficial digital flexor tendons, as obtained ultrasonographically over seven weeks for the different injury-treatment subsets: SS (dark blue line), CS (pink line), CMNC (yellow line) and CCD45 (turquoise). Values were normalised by dividing each index number assigned within a subset with the index score recorded for that particular subset in Week 1. Statistically significant differences ($p < 0.05$) were recorded between: (*) the collagenase-injured CS subset and saline-injected SS controls, (#) the CS and CMNC subsets, (•) the CS and CCD45 subsets, (º) the CMNC and SS subsets, and (†) the CCD45 and SS subsets.
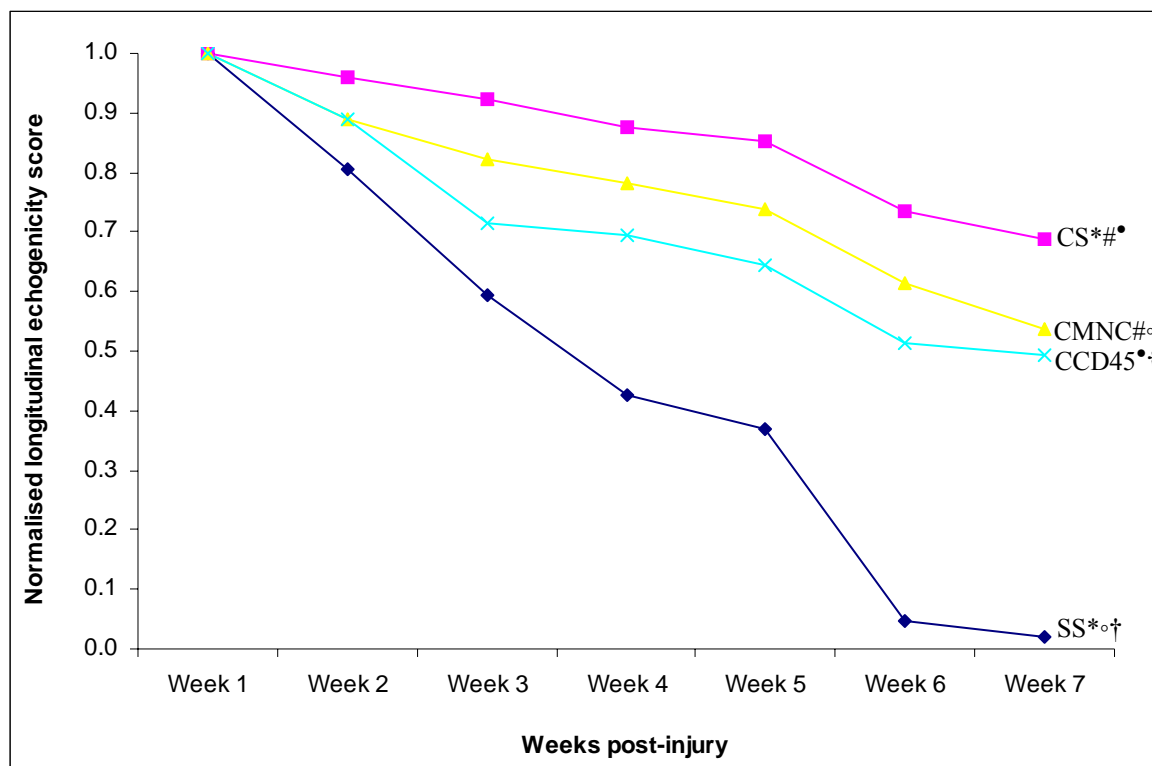
FIGURE 4.5 – Normalised echogenicity of longitudinal scans of the superficial digital flexor tendons, as obtained ultrasonographically over seven weeks for the different injury-treatment subsets: SS (dark blue line), CS (pink line), CMNC (yellow line) and CCD45 (light blue line). Values were normalised by dividing each index number assigned within a subset with the index score recorded for that particular subset in Week 1. Statistically significant differences ($p < 0.05$) were recorded between: (*) the collagenase-injured CS subset and saline-injected SS controls, (#) the CS and CMNC subsets, (•) the CS and CCD45 subsets, (º) the CMNC and SS subsets, and (†) the CCD45 and SS subsets.
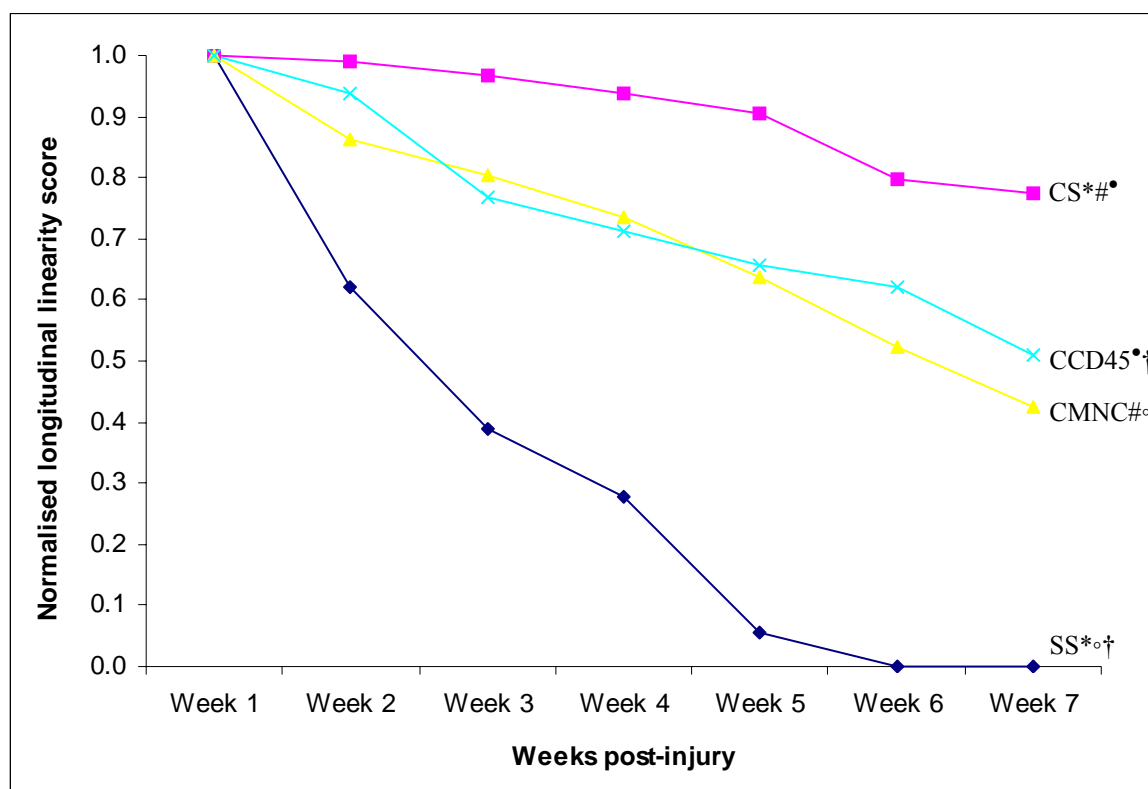
FIGURE 4.6 – Normalised linearity of longitudinal scans of the superficial digital flexor tendons, as obtained ultrasonographically over seven weeks for the different injury-treatment subsets: SS (dark blue line), CS (pink line), CMNC (yellow line) and CCD45 (light blue line). Values were normalised by dividing each index number assigned within a subset with the index score recorded for that particular subset in Week 1. Statistically significant differences ($p < 0.05$) were recorded between: (*) the collagenase-injured CS subset and saline-injected SS controls, (#) the CS and CMNC subsets, (•) the CS and CCD45 subsets, (º) the CMNC and SS subsets, and (†) the CCD45 and SS subsets.
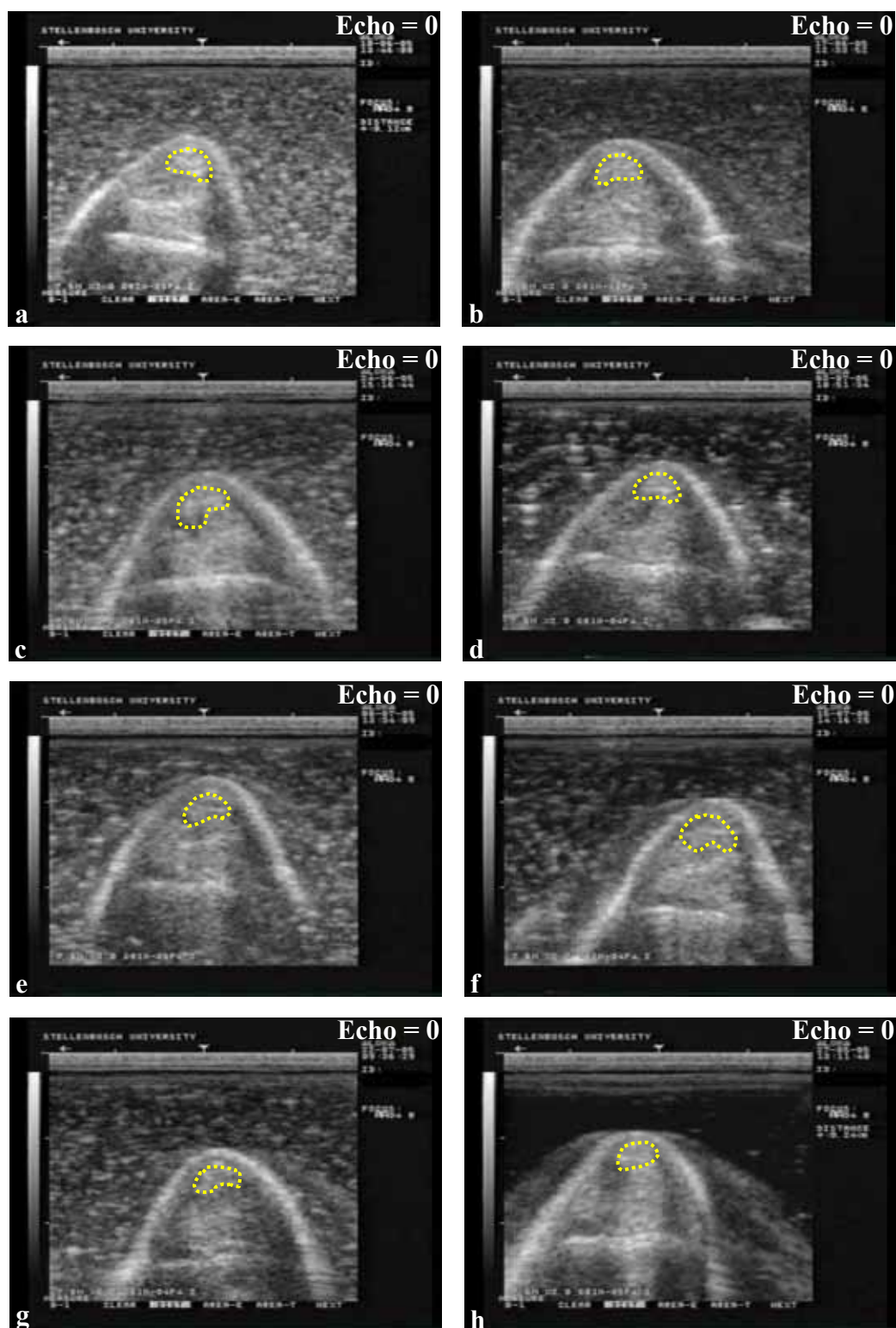
FIGURE 4.7 – Typical ultrasonographs of palmar metacarpal structures of the SS injury-
treatment subset, viewing the transverse aspect of the middle region ± 6.5 cm distal to
the accessory carpal bone, as taken (a) prior to injury and in temporal sequence from
(b) Week 1 to (h) Week 7. Echogenicity indices (Echo) appear in the top right corner
of each image. The superficial digital flexor tendon is enclosed within yellow outlines.
(Left of image = medial aspect; right of image = lateral aspect.)
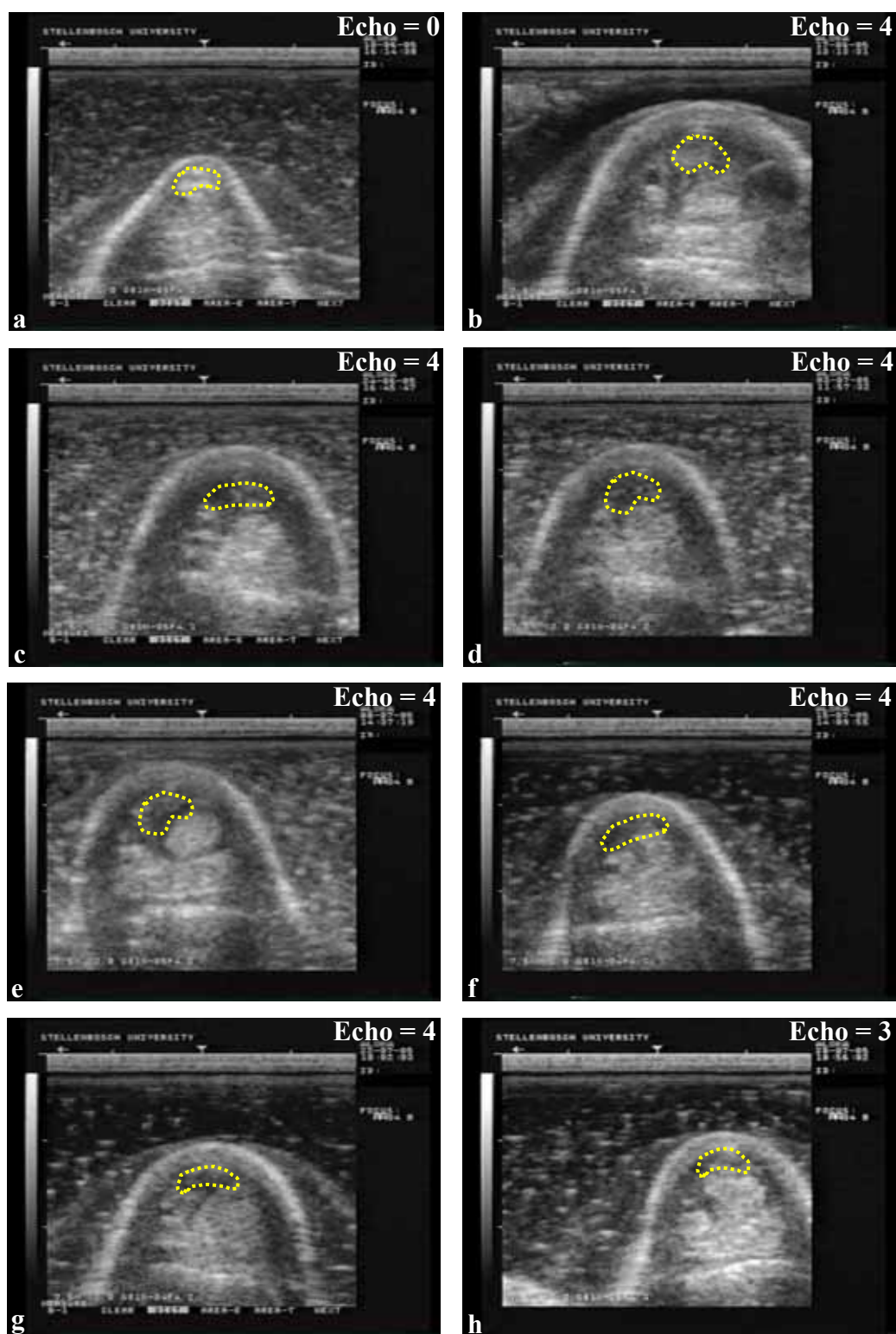
FIGURE 4.8 – Typical ultrasonographs of palmar metacarpal structures of the CS injury-
treatment subset, viewing the transverse aspect of the middle region ± 6.5 cm distal to
the accessory carpal bone, as taken (a) prior to injury and in temporal sequence from
(b) Week 1 to (h) Week 7. Echogenicity indices (Echo) appear in the top right corner
of each image. The superficial digital flexor tendon is enclosed within yellow outlines.
(Left of image = medial aspect; right of image = lateral aspect.)
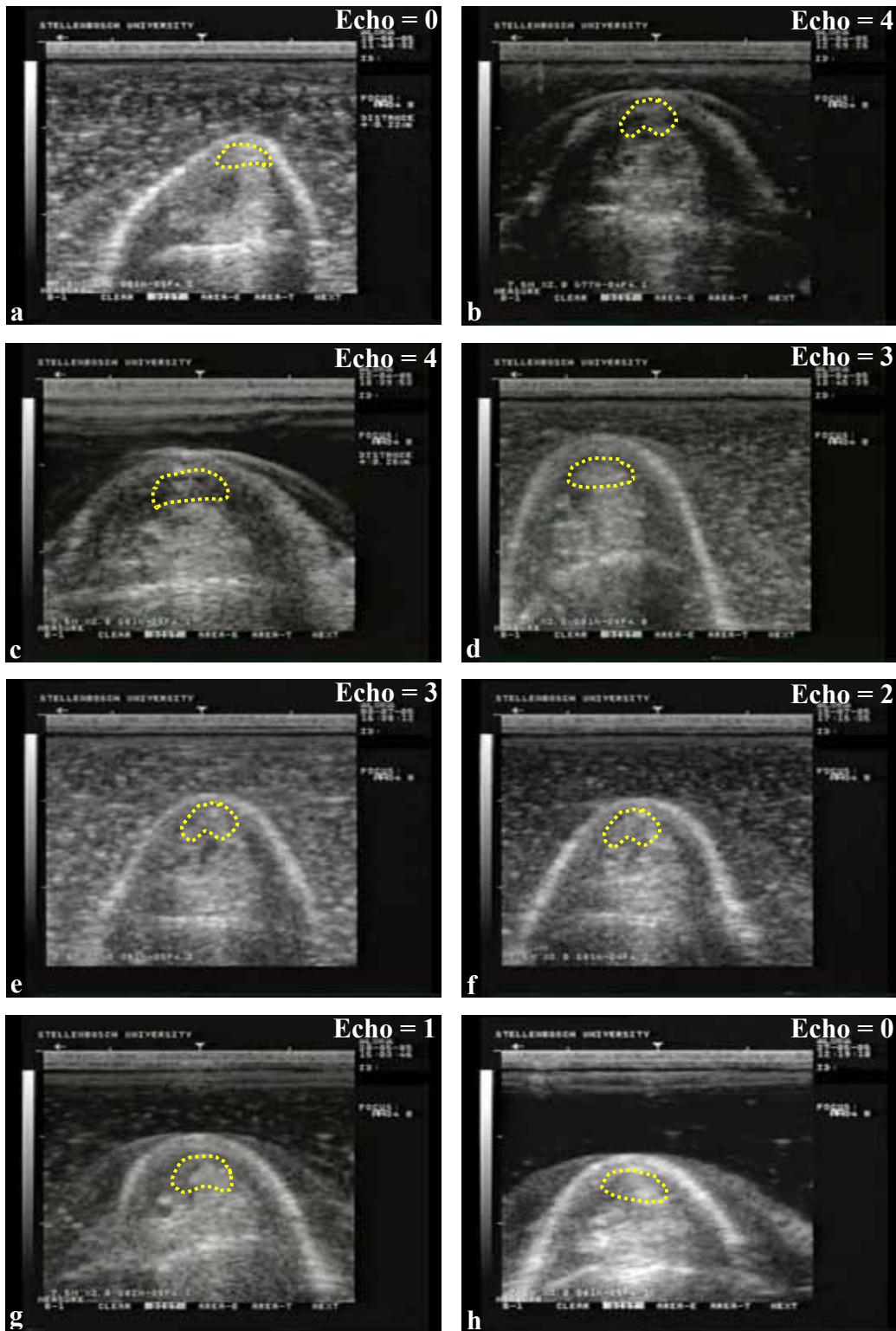
FIGURE 4.9 – Typical ultrasonographs of palmar metacarpal structures of the CMNC injury-treatment subset, viewing the transverse aspect of the middle region ± 6.5 cm distal to the accessory carpal bone, as taken (a) prior to injury and in temporal sequence from (b) Week 1 to (h) Week 7. Echogenicity indices (Echo) appear in the top right corner of each image. The superficial digital flexor tendon is enclosed within yellow outlines. (Left of image = medial aspect; right of image = lateral aspect.)
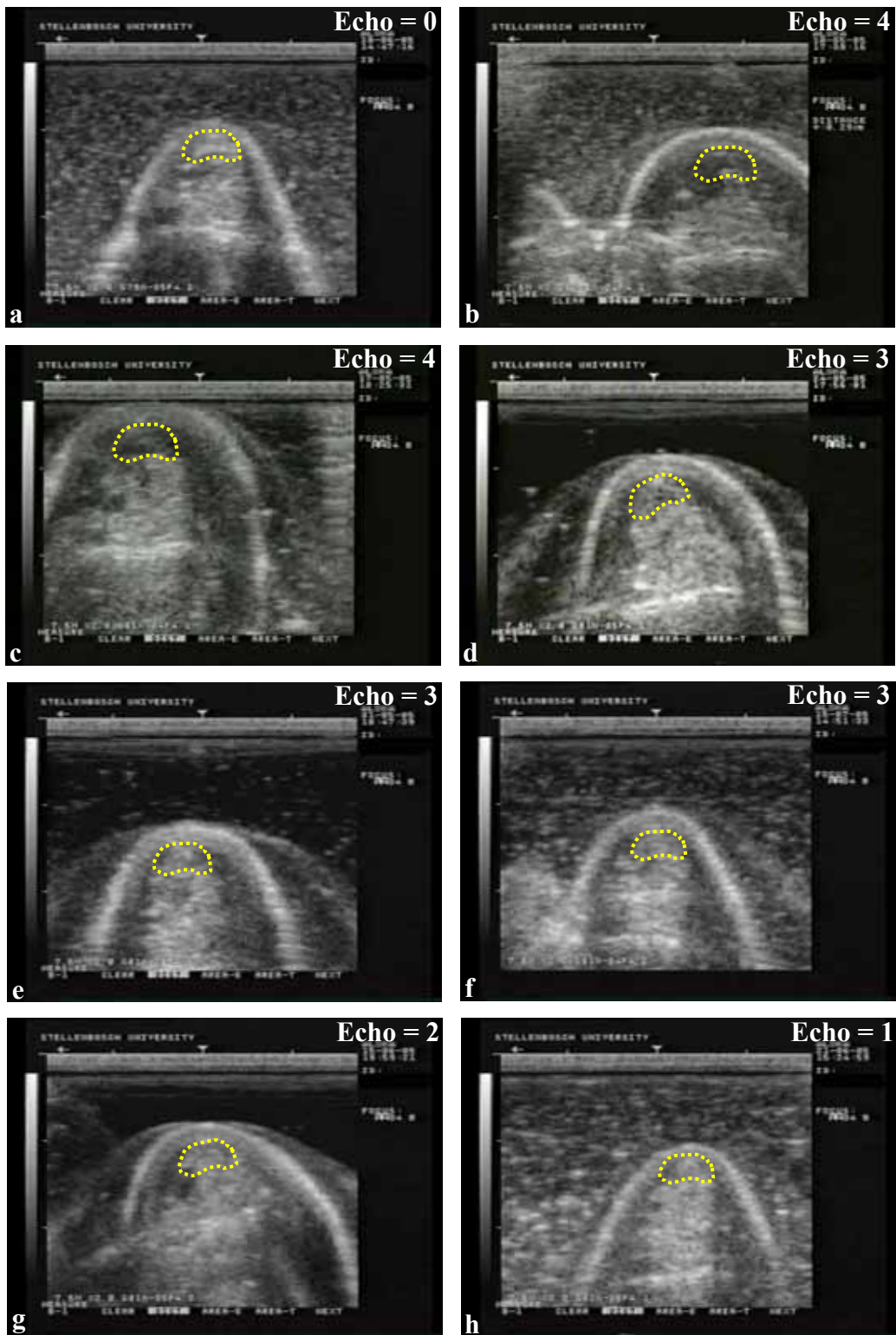
FIGURE 4.10 – Typical ultrasonographs of palmar metacarpal structures of the CCD45 injury-treatment subset, viewing the transverse aspect of the middle region ± 6.5 cm distal to the accessory carpal bone, as taken (a) prior to injury and in temporal sequence from (b) Week 1 to (h) Week 7. Echogenicity indices (Echo) appear in the top right corner of each image. The superficial digital flexor tendon is enclosed within yellow outlines. (Left of image = medial aspect; right of image = lateral aspect.)
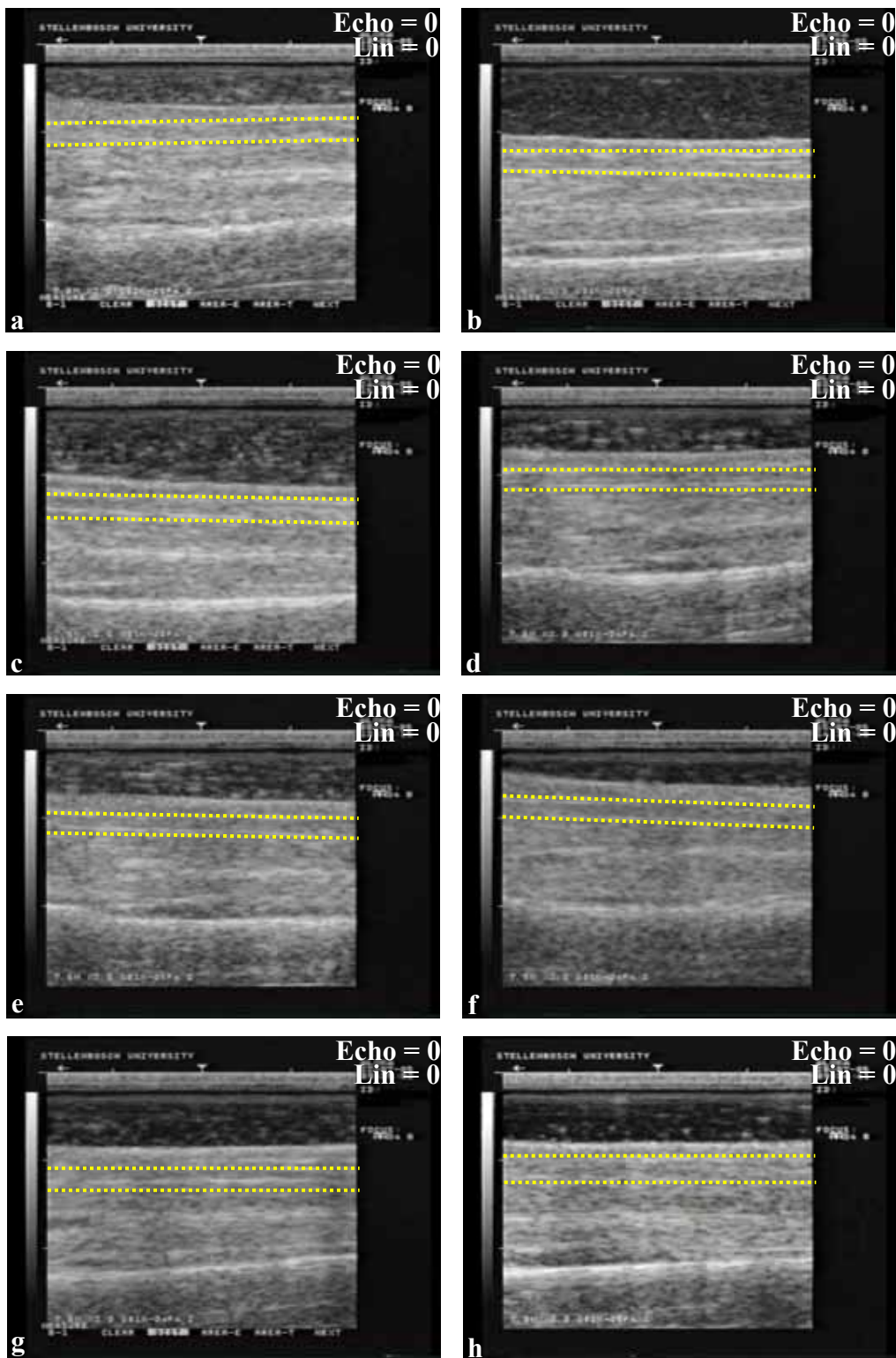
FIGURE 4.11 – Typical ultrasonographs of palmar metacarpal structures of the SS injury-treatment subset, viewing the longitudinal aspect of the area of injury as taken (a) prior to injury and in temporal sequence from (b) Week 1 to (h) Week 7. Echogenicity and linearity (Lin) indices (Echo) appear in the top right corner of each image. The superficial digital flexor tendon is enclosed between the two yellow dashed lines. (Left of image = medial aspect; right of image = lateral aspect.)
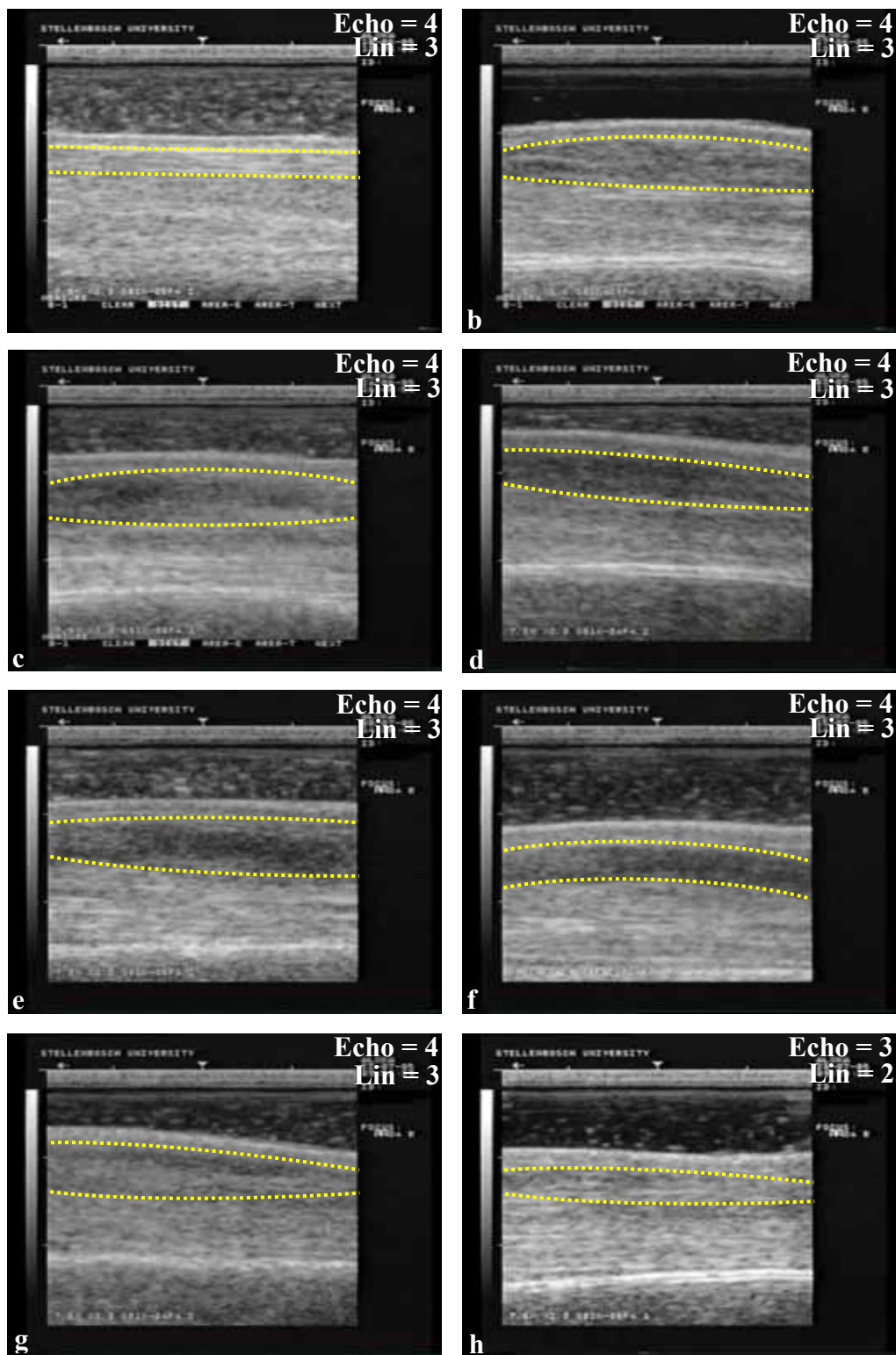
FIGURE 4.12 - Typical ultrasonographs of palmar metacarpal structures of the CS injury-treatment subset, viewing the longitudinal aspect of the area of injury as taken (a) prior to injury and in temporal sequence from (b) Week 1 to (h) Week 7. Echogenicity and linearity (Lin) indices (Echo) appear in the top right corner of each image. The superficial digital flexor tendon is enclosed between the two yellow dashed lines. (Left of image = medial aspect; right of image = lateral aspect.)
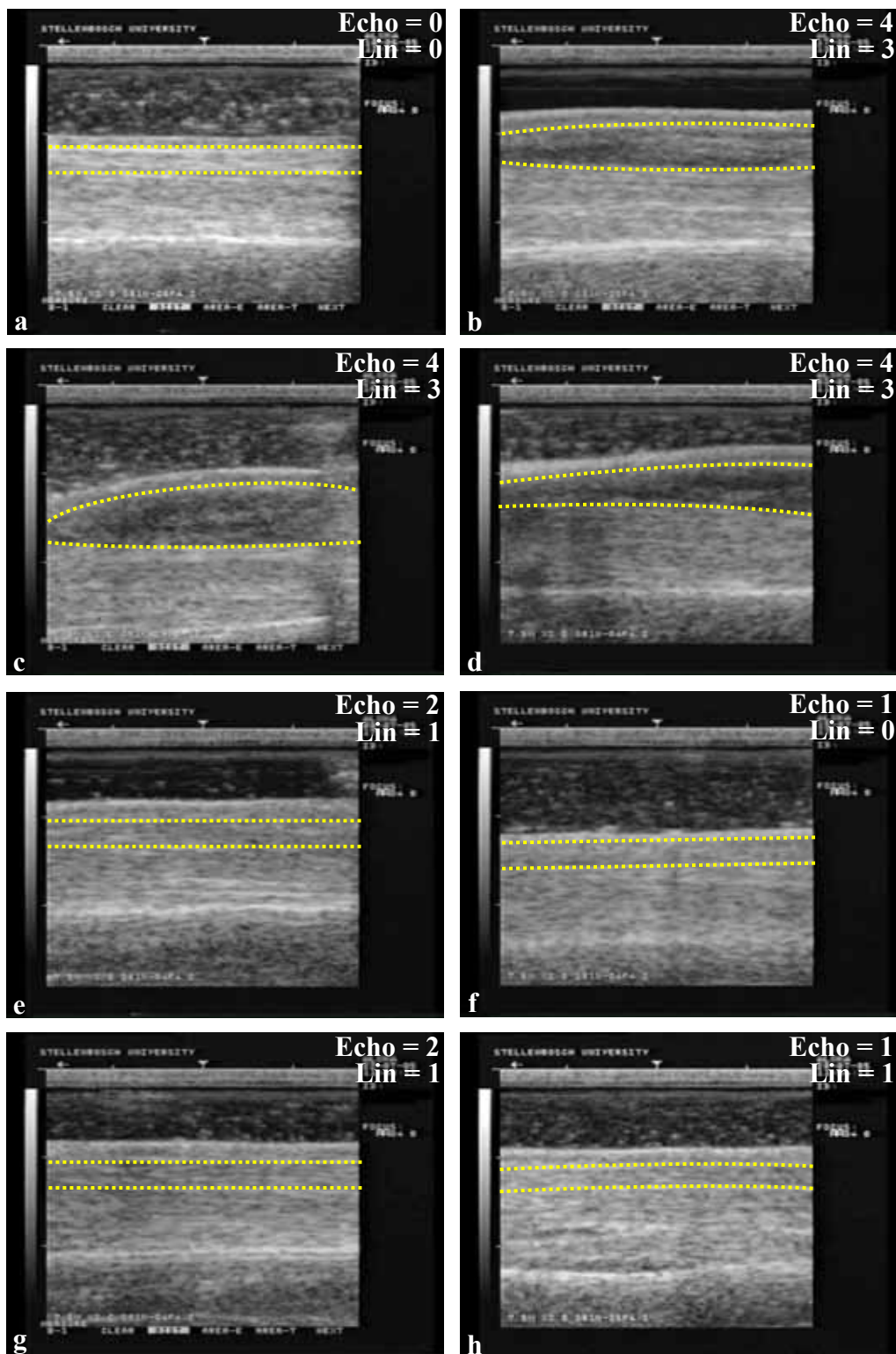
FIGURE 4.13 - Typical ultrasonographs of palmar metacarpal structures of the CMNC injury-
treatment subset, viewing the longitudinal aspect of the area of injury as taken (a) prior
to injury and in temporal sequence from (b) Week 1 to (h) Week 7. Echogenicity and
linearity (Lin) indices (Echo) appear in the top right corner of each image. The
superficial digital flexor tendon is enclosed between the two yellow dashed lines.
(Left of image = medial aspect; right of image = lateral aspect.)
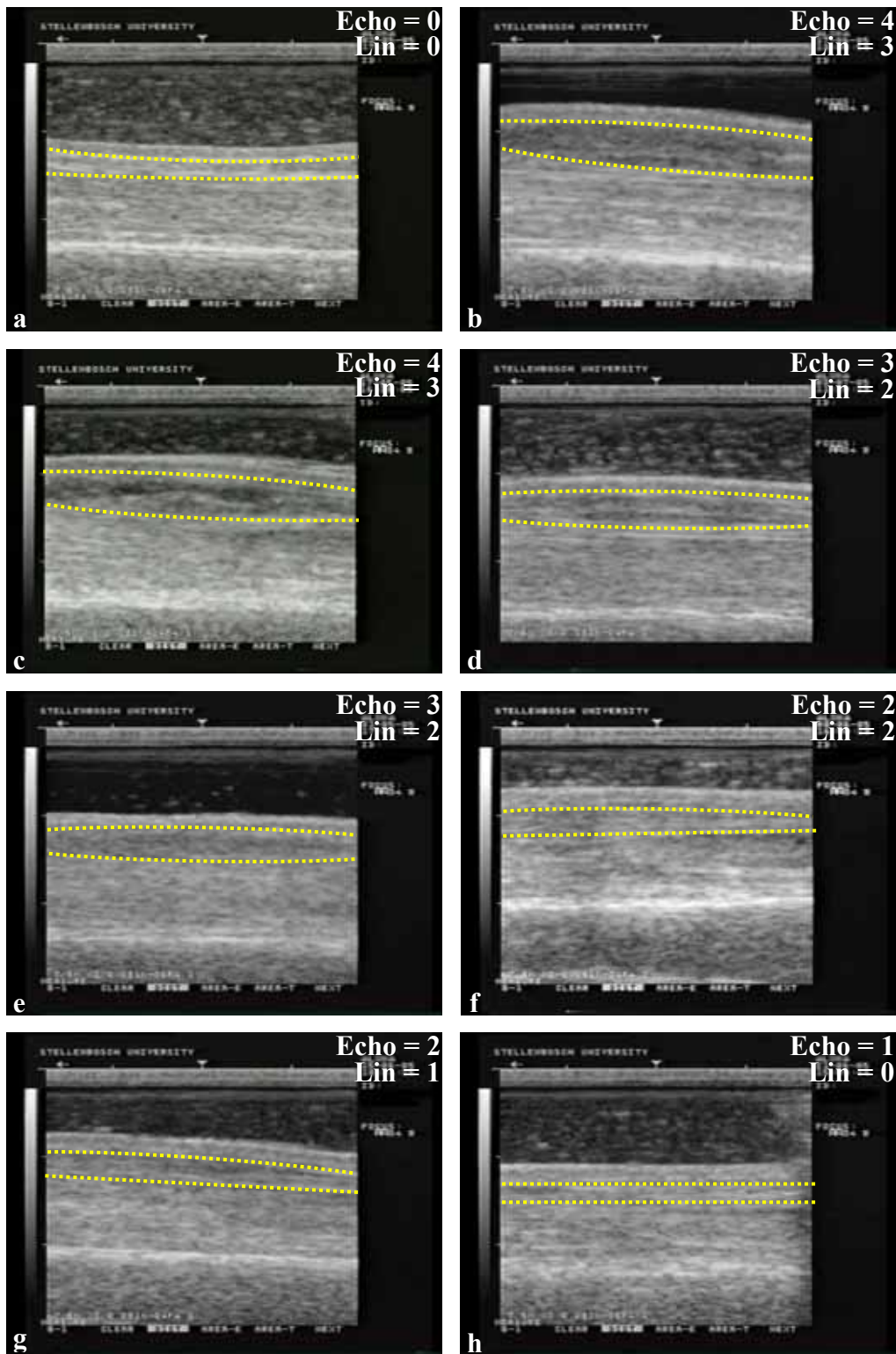
FIGURE 4.14 - Typical ultrasonographs of palmar metacarpal structures of the CCD45 injury-treatment subset, viewing the longitudinal aspect of the area of injury as taken (a) prior to injury and in temporal sequence from (b) Week 1 to (h) Week 7. Echogenicity and linearity (Lin) indices (Echo) appear in the top right corner of each image. The superficial digital flexor tendon is enclosed between the two yellow dashed lines. (Left of image = medial aspect; right of image = lateral aspect.)

discolouration (Figure 4.15a). The connective tissue of the treated tendon was firm and solid and appeared normal subcutaneously.

Superficial digital flexor tendons from the CCD45 subset were similar to those of the collagenase-injected CMNC subset. The tendon was translucent at the sight of injury with pink to yellow discolouration at the edges (Figure 4.15b). Fibre alignment was similarly disrupted, but in no cases were the SDF tendon and DDF tendon attached to each other or were the SDF tendon attached to the peritendinous tissue.

4.3.4 Macroscopic measurements

Maximum tendon diameter data for the SS subsets showed no statistical differences and were pooled for further analyses. The same was true for our CS data subsets and CMNC data subsets. Tendons from the SS control subset displayed an increase in MTD from proximal to middle to distal region. Tendons from all other subsets had received an initial collagenase-induced injury and the resulting swelling, caused by inflammation and subsequent collagen breakdown, rendered their overall mean MTD values significantly higher than that of the SS subset (Figure 4.16). This is accentuated by the fact that maximum MTD of the collagenase-injured tendons of the CS, CMNC and CCD45 subsets were observed in their middle regions, where the injection was administered and inflammation was most severe. Both CMNC and CCD45 subsets, which received subsequent treatment of autologous and allogeneic MNC injections respectively, displayed lower mean tendon MTD in comparison with the untreated CS subset, although this was not significant. Neither was there a significant difference in mean MTD between the CMNC subset and CCD45 subset.

4.3.5 Mechanical testing

All tendons from the saline-injected control subset (SS) failed at either of the two clamps on the servo hydraulic testing station (Table 4.4), not within the tendons themselves. For both the MNC and CCD45-treated subsets, the greatest percentages of failures were at the testing station clamps. Some failures occurred in either the proximal or distal region of the tendons themselves, but no tendons failed in the middle region. Collagenase-injured tendons from the CS subset were the only tendons to show failures in the middle region of the tendon itself, plus failure at all other sites.

Results of mechanical testing were pooled for saline-injected controls (SS) due to lack of any statistical difference between the subsets. Data for the CS subset were pooled for the same reason, as were those of the CMNC subsets. A similar trend was observed for all
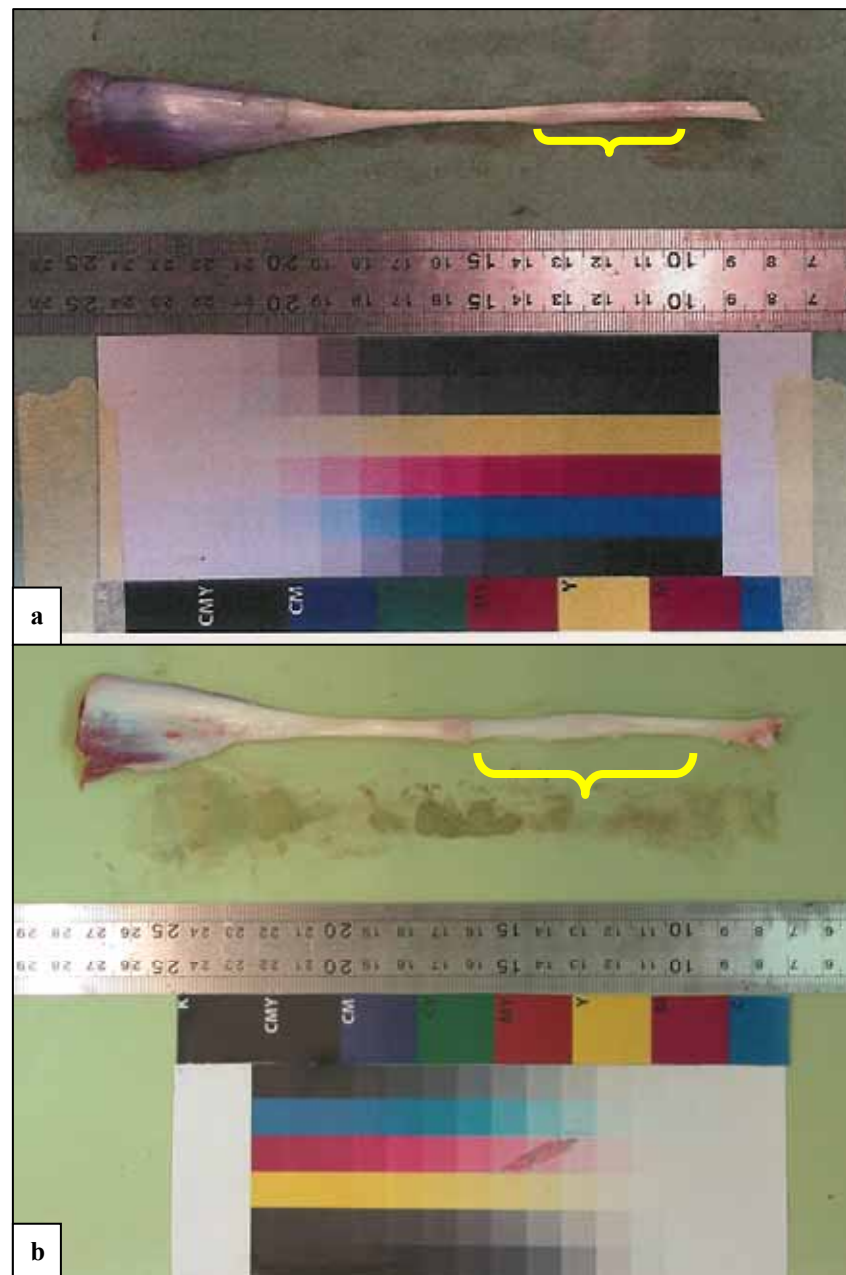
FIGURE 4.15 - Discolouration of the superficial digital flexor tendon was apparent in sheep from the (a) CMNC injury-treatment subset and (b) CCD45 injury-treatment subset. Brackets indicate the areas of discolouration. The proximal end of each tendon lies on the left of its image.
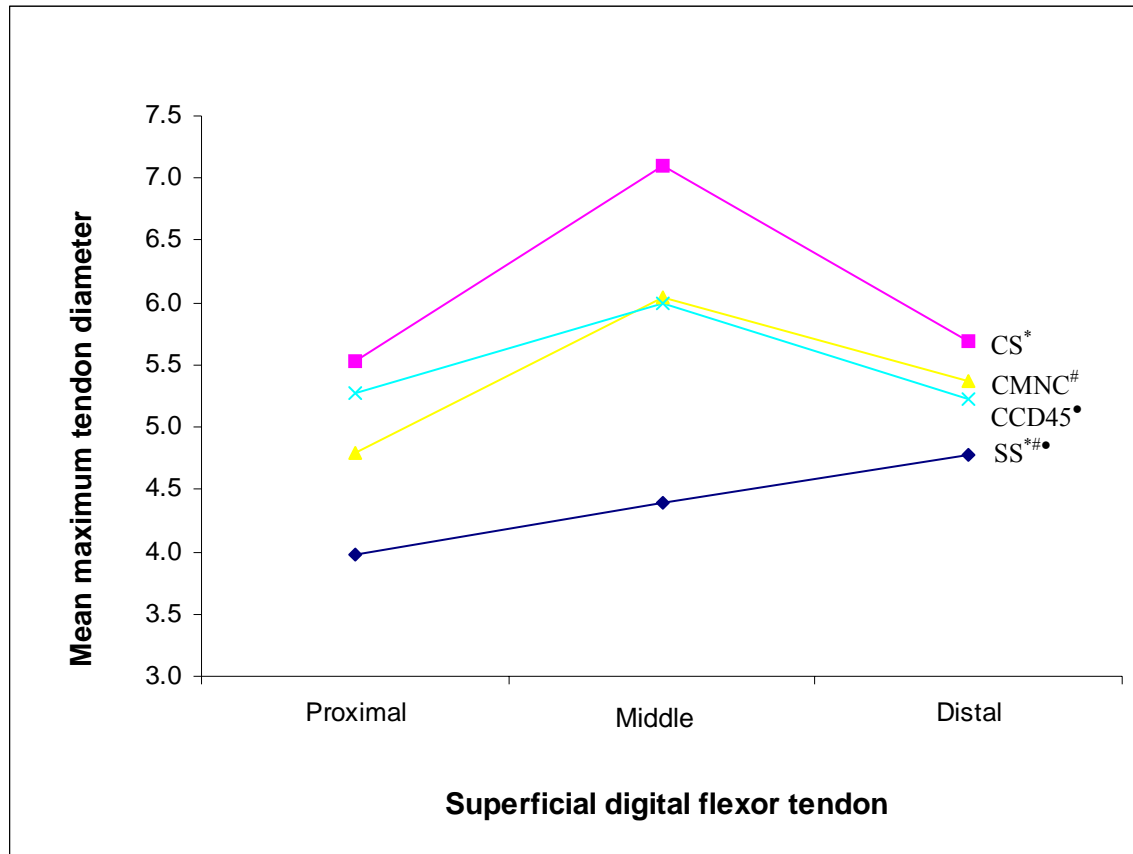
FIGURE 4.16 - Mean maximum tendon diameters (mm) of the proximal, middle and distal regions in the superficial digital flexor tendons of the SS (dark blue line), CS (pink line), CMNC (yellow line) and CCD45 (light blue line) injury-treatment subsets. Statistically significant differences in overall means ($p < 0.05$) were recorded between groups CS and SS (*), between groups CMNC and SS (#) and between groups CCD45 and SS ($\bullet$).

TABLE 4.4 - A comparison of area of failure within the superficial digital flexor (SDF) tendon on the servo hydraulic testing station between the four injury-treatment subsets. Results are given as the percentage of tendons per subset sample size that failed at either of the two testing station clamps, the proximal region of the tendon, the middle region of the tendon and the distal region of the tendon.

| SUBSET ID | n | AREA OF SUPERFICIAL DIGITAL FLEXOR TENDON FAILURE | | | |
		Testing station clamp	*Proximal region*	*Middle region*	*Distal region*
SS	4	100 %	0 %	0 %	0 %
CS	16	18.75 %	18.75 %	25 %	37.5%
CMNC	11	72.73 %	18.18 %	0 %	9.09 %
CCD45	6	66.67 %	16.67 %	0 %	16.67 %

variables, wherein the saline-injected control subset (SS) produced values associated with mechanically strong tendons, the collagenase-injured subset (CS) had values associated with loss of structural strength, and both MNC-treated subsets (CMNC and CCD45) displayed intermediate values (Table 4.5). In all instances the values between our SS and CS subsets, on opposing sides of the data range, differed significantly. Conversely, differences between the intermediary data sets for our CMNC and CCD45 samples were never significant.

Although both CMNC and CCD45 tendons were significantly less elastic than the saline-injected controls, only tendons treated with autologous MNCs (CMNC) were significantly more elastic than collagenase-injured SDF tendons of the CS subsets. The same pattern emerged when analysing maximum load and stiffness: the initial collagenase injury applied to the CMNC and CCD45 subsets significantly lessened the maximum load their tendons could bear and decreased their stiffness due to collagen breakdown; yet, although both CMNC and CCD45 tendons could carry more load and were stiffer than CS tendons, these differences were only significant for the CMNC group.

All subsets that received collagenase injuries (CS, CMNC and CCD45) were capable of enduring significantly lower maximum stress before failure than the saline-injected (SS) controls. Although both of the subsets that received treatment with MNCs (CMNC and CCD45) had a higher tolerance to stress than the collagenase-injured tendons that received only saline treatment (CS), this difference was not significant for either subset.

In terms of maximum strain, the only significant difference observed was in the decreased capacity of untreated, collagenase-injured tendons (CS) to handle strain as opposed to saline-injected controls (SS). Both groups treated with MNCs (CMNC and CCD45) displayed an intermediary ability to endure strain to the tendon, but not significantly more so than the CS subset or less than the SS subset.

4.3.6 Subjective microscopic observations

Histological sections of SS saline-injected controls (refer to Chapter 3, Figure 3.9b) appeared similar to normal, uninjured tendons (refer to Chapter 3, Figure 3.9a). The collagenous tissue is densely packed with a homogeneous distribution of tenocytes which stain uniformly blue with red nuclei. Irregular, red staining leucocytes are present and blood vessels appear normal. No cellular abnormalities were found in any of the tendons, corresponding with other studies (Williams *et al.* 1984a and b, Dahlgren *et al.* 2005).

In contrast, tissues from collagenase-injured tendons from the CS subset (refer to Chapter 3, Figure 3.17) displayed a highly inflamed, fragmented and granular red-staining

TABLE 4.5 - Variation in the modulus of elasticity (MPa), maximum load (N), maximum stress (MPa), maximum strain (%) and stiffness (N/mm) between the SS, CS, CMNC and CCD45 injury-treatment subsets. Data are presented as means ± SD.

SUBSET ID	n	ELASTICITY (MPa)	MAXIMUM LOAD (N)	MAXIMUM STRESS (MPa)	MAXIMUM STRAIN (%)	STIFFNESS (N/mm)
SS	9	568.88 ± 146.08	437.09 ± 94.43	54.64 ± 11.80	11.62 ± 1.08	5.46 ± 1.18
CS	16	147.32 ± 46.97	104.60 ± 30.16	14.78 ± 6.34	9.26 ± 1.02	1.31 ± 0.38
CMNC	11	296.11 ± 56.74	192.65 ± 17.65	24.81 ± 7.42	9.99 ± 1.34	2.41 ± 0.83
CCD45	6	246.38 ± 49.17	172.14 ± 44.49	21.53 ± 5.56	11.14 ± 1.14	2.15 ± 0.56

N: Newton (1 kg = 9.8 N)
MPa: MegaPascals = 10^6 x Pa = 10^6 x 1 M/m^2 (Force / unit area)

matrix. Plump fibroblasts invaded the damaged area, secreting a blue-staining loose connective tissue matrix. Less inflamed sections contained a high, polymorphonuclear leucocyte population with ample large blood vessels. The parallel fibre alignment was highly disrupted with red staining inflammation containing increased numbers of activated leucocytes.

Tissues from the CMNC subset did not differ appreciably from those of the CCD45 subset. Collagen fibre alignment was loosely packed and irregularly organised. In comparison with sections from the CS subset, there was less red-staining inflammation, more polymorphonuclear cells and a large number of infiltrated leucocytes.

The cell marker BrdU, which was incorporated into the DNA of MNCs during preparation of treatment injections, could not be identified on any of the slides studied (Figure 4.17 and Figure 4.18).

4.3.7 Microscopic measurements

Simple PCI software was used to analyse the amount of collagen laid down in the tendons (blue macro) as well as infiltration of inflammatory cells into the tendons (red macro). Due to lack of any statistical significance between similar subsets, blue macro data were pooled and red macro data were similarly pooled for the SS, CS and CMNC subsets.

In observations of the blue macro, it was evident that the most collagen damage occurred in the middle region (Figure 4.19a). Mean overall values for Group SS were significantly higher than Groups CS, CMNC and CCD45 (Figure 4.19b). Although the CMNC and CCD45 subsets displayed higher overall mean blue macro values than the CS subset, this did not constitute a significant difference. The red macro revealed that inflammation once again mostly occurred in the middle region (Figure 4.20a). There was no significant difference in inflammation levels between the CS and CMNC subsets, though both displayed significantly more inflammation than the saline-injected SS controls (Figure 4.20b). Tendons from the CCD45 subset showed significantly less inflammation than the CMNC subset, but otherwise did not differ from any other injury-treatment subsets.

4.4 DISCUSSION

The findings from this study support the hypothesis that autologous peripheral blood MNCs and allogeneic peripheral blood MNCs (CD45⁻), when injected subcutaneously into collagenase tendon injuries, provide material and structural benefits to healing tendons. This

CS   **CMNC**
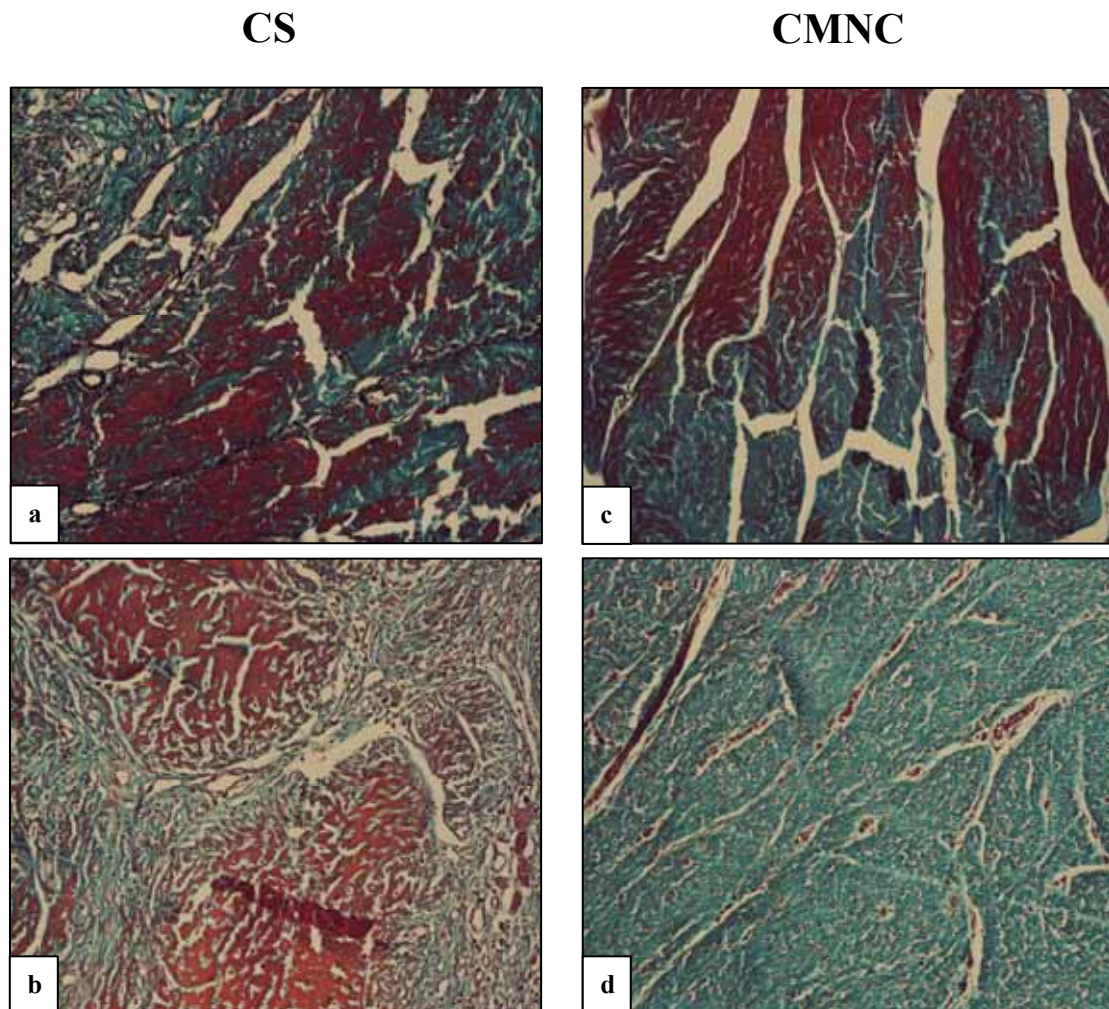
FIGURE 4.17 - A comparison between transverse histological sections of superficial digital flexor tendons from the injured CS subset (a and b, on the left) and those of mononuclear cell treated CMNC subset (c and d, on the right) at Week 7. Note the decrease in inflammatory cells, decrease in vascularisation and increase in collagen deposition from CS sections (a) to CMNC sections (c), coupled with a decrease in leucocyte infiltration from CS sections (b) to CMNC sections (d). (Masson's trichrome collagen staining, 20x magnification).

CS   **CCD45**
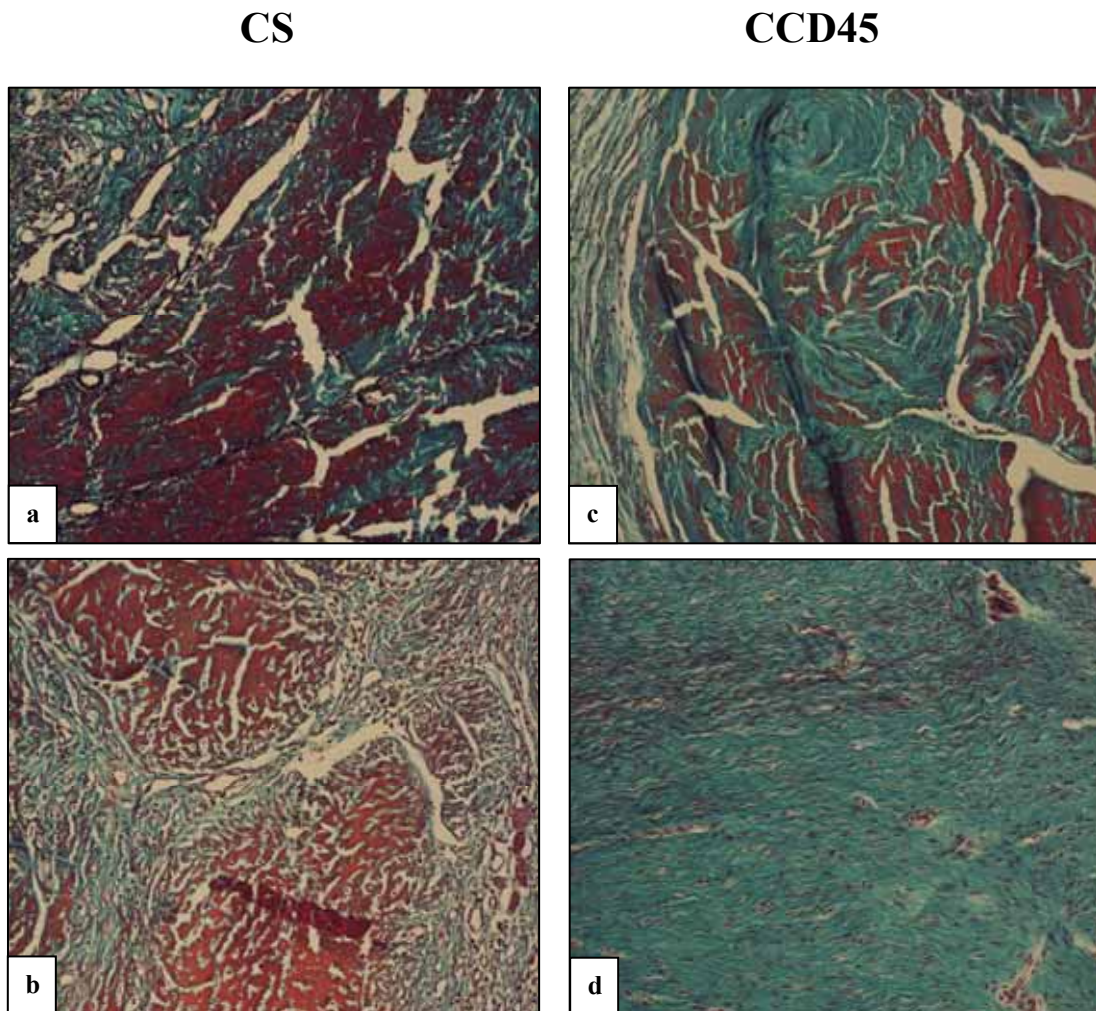
FIGURE 4.18 - A comparison between transverse histological sections of superficial digital flexor tendons from the injured CS subset (a and b, on the left) and those of mononuclear cell treated CCD45 subset (c and d, on the right) at Week 7. Note the decrease in inflammatory cells, decrease in vascularisation and increase in collagen deposition from CS sections (a) to CMNC sections (c), coupled with a decrease in leucocyte infiltration from CS sections (b) to CMNC sections (d). (Masson's trichrome collagen staining, 20x magnification).
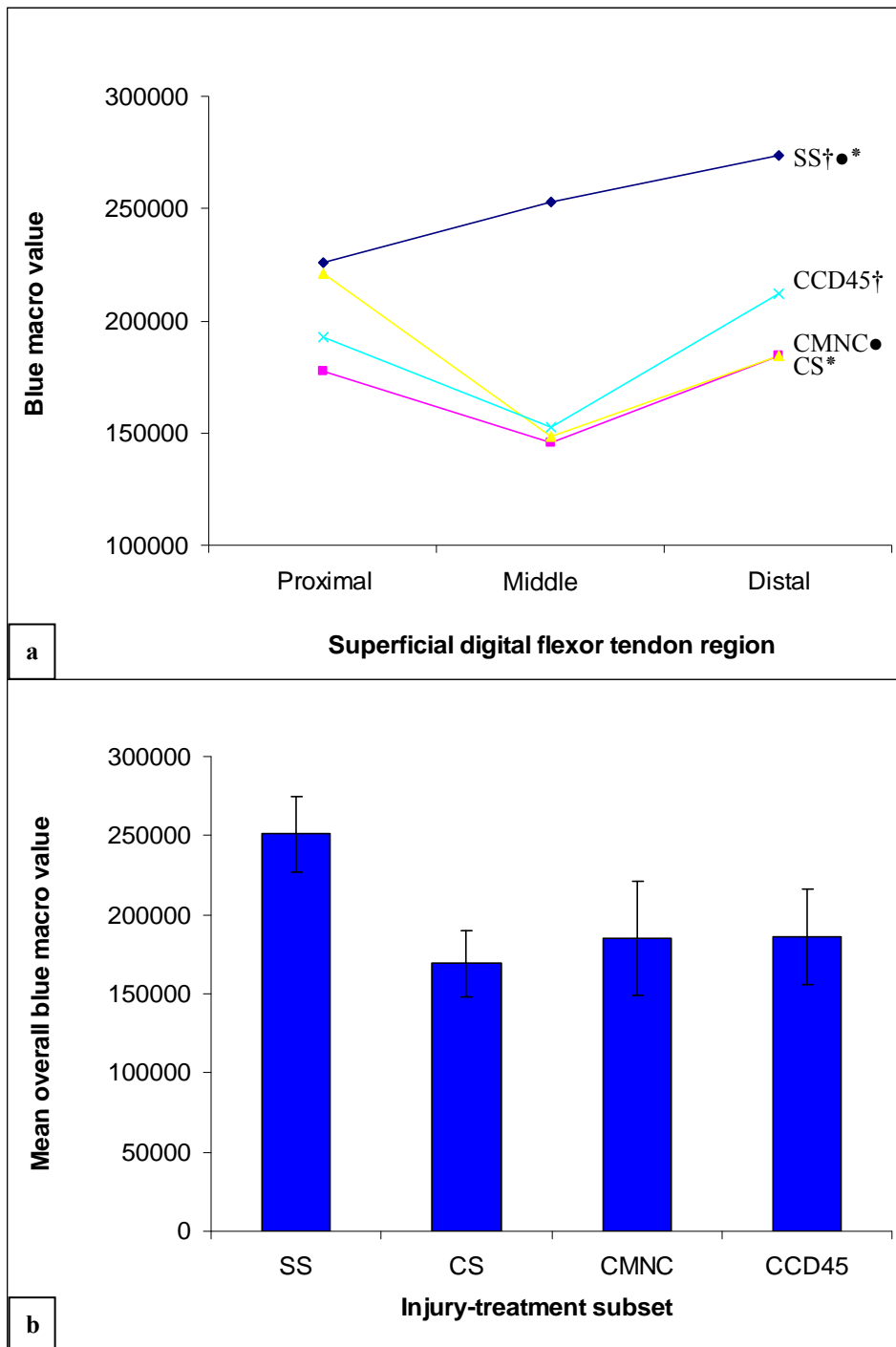
FIGURE 4.19 - Blue macro depictions (amount of collagen present) indicating (a) distribution of collagen damage among the proximal, middle and distal regions of the SS (dark blue line), CS (pink line), CMNC (yellow line) and CCD45 (light blue line) injury-treatment subsets, and (b) mean overall collagen damage among these subsets at Week 7. The mean overall blue macro value differed significantly ($p < 0.05$) between the SS subset and all the other subsets, CCD45 (†), CMNC (●) and CS (*).
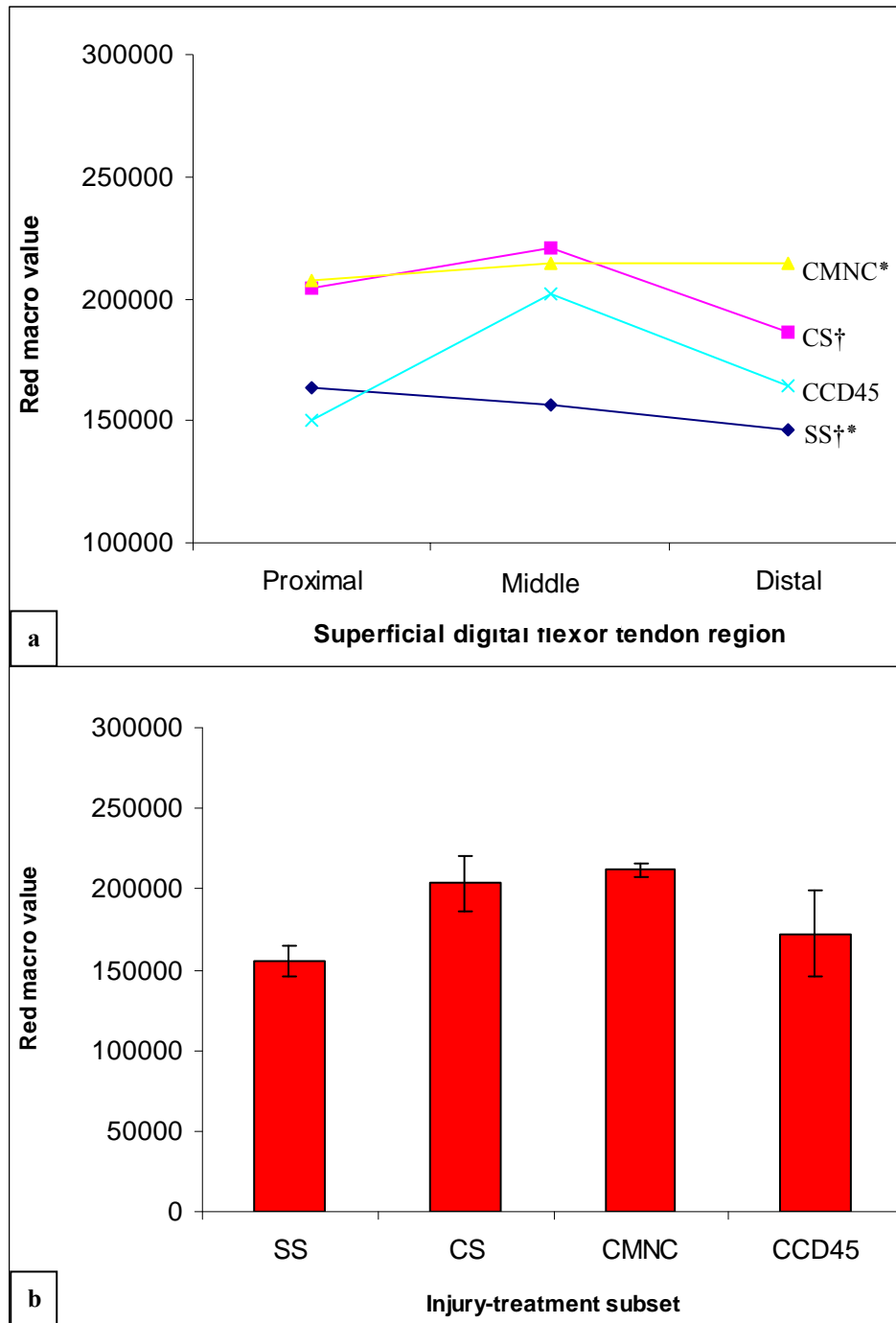
FIGURE 4.20 - Red macro depictions (amount of inflammatory cells) of (a) inflammatory response among the proximal, middle and distal regions of the SS (dark blue line), CS (pink line), CMNC (yellow line) and CCD45 (light blue line) injury-treatment subsets, and (b) mean overall inflammatory response among these subsets at Week 7. The only significant differences recorded ($p < 0.05$) were between the mean overall red macro values for SS and those of both the CS (†) and CMNC (*) subsets, respectively.

strong trend towards an increased healing rate over seven weeks may well result in more substantial long-term benefits.

The improvement in mechanical properties of SDF tendons due to MNC injections, when compared with those of collagenase-injured CS tendons that received no treatment, was observed as (1) increased elasticity, (2) an increased capacity to carry loads, (3) increased stiffness due to collagen formation, (4) an increased capacity to resist stress and (5) an increased ability to resist strain. In the case of our allogeneic CCD45 subsets, none of these differences were significant. For the autologous CMNC subset, on the other hand, these differences were significant for elasticity, stiffness and maximum load.

Ultrasonography is known to be a useful aid in the diagnosis of soft tissue injury and has been adopted by equine veterinarians for the assessment and monitoring of healing tendon injuries (Nicoll *et al.* 1992, Marr *et al.* 1993b, Micklethwaite *et al.* 2001, Pickersgill *et al.* 2001, Reef 2001). This study showed a significant improvement over a seven week period in the echogenicity and linearity of MNC-injected tendons when compared with untreated, collagenase-injured tendons. Marr *et al.* (1993b) demonstrated a consistent relationship between the ultrasonographic and histological methods used during assessment of the SDF tendon recovery of 40 horses. This was not quite the case in our study, where ultrasound analyses revealed faster recovery than what was evident from our histological assessments. Variation in ultrasound results from separate studies could be due to subjective quantification of (1) individual fibre patterns, (2) the proportion of SDF tendon injured, (3) boundary selection for echogenicity measurements using an ordinal scale and (4) echogenicity measurements using an ordinal modified scale (Pickersgill *et al.* 2001).

The histological characteristics of healing tendons described in this study are consistent with previous reports using the collagenase-induced injury model (Silver *et al.* 1983, Williams *et al.* 1984b, Spurlock *et al.* 1988, Tkach *et al.* 1993, Dahlgren *et al.* 2002). Since Masson's trichrome staining technique gives an indication of collagen content, it appears that an increase in MTD was mainly due to enhanced collagen deposition. This correlates with the hypothesis of Kobayashi *et al.* (1999) that particles on the surface of collagen fibrils may be related to an increased matrix and therefore increased MTD. Total collagen content was however only slightly higher than normal in CMNC and CCD45 subsets. Collagen produced by fibroblasts initially forms fibrils of small diameter which are randomly oriented in the damaged area (Kobayashi *et al.* 1999). Our microscopic evaluation revealed that, in the collagenase-injured area, collagen fibres were loosely packed and

irregularly organised for the CS subset and more organised for the CMNC and CCD45 subsets at seven weeks post-injury.

Normal tendons contain relatively acellular tissues in which the degradation and production of extracellular matrix molecules are held in equilibrium. With injury, this equilibrium is disrupted and upregulation of individual cell metabolism can be inadequate to repair the damage (Dahlgren *et al.* 2002). Fibroblast proliferation and increased production of extracellular matrix products are crucial (Dahlgren *et al.* 2002). Cell numbers must increase by cell division and/or migration, which was confirmed in our study.

The absence of BrdU stained cells noted may have been due to increased cell proliferation of the injected cells, thereby causing a loss of the marker. A second explanation is the migration of marked cells to other areas of the body where they are required to affect repair. Once the stem cells reach their target, there are at least four different mechanisms which may explain how they can contribute to repair:

1) Transdifferentiation or transdetermination: committed cell lineages (mature cells) are redirected to produce and assume the function of other types of cells, for example, peripheral blood stem cells may in fact start producing tenocytes.

2) De-differentiation: mature cells are triggered to revert to more basic, unspecialised forms and become multipotent or pluripotent.

3) Fusion: stem cells fuse with existing cells to create primordial cells which can access suppressed DNA in order to perform repair.

4) Trophic support: stem cells produce growth factors or cytokines that encourage existing cells to conduct repairs or produce new cells.

Thirdly, technical issues relating to antigen retrieval in the specimens may have been responsible for a failure to identify BrdU cells that were in fact present in the tissues examined. Unfortunately no positive control was conducted to ascertain the efficiency of the staining method.

In conclusion, our results support the further investigation of adult peripheral blood MNCs as a treatment for tendonitis. Subcutaneous injections of MNCs resulted in increased cell proliferation, improved sonographic appearance of lesions, increased collagen content and decreased peritendinous swelling at seven weeks post injury, which was not the case in collagenase-injured tendons left untreated. Subcutaneous injections in close proximity to regions requiring the most urgent treatment both accelerate the onset of improvements and extend the range of benefits experienced. Although this has not been verified empirically, a number of hypotheses have been put forward to explain such a potential phenomenon.

Regardless of its veracity, the potential existence of such a benefit justified a preference for subcutaneous injection, coupled with it being a relatively easier route of administration. However, for further research, administration of treatment could be injected intralesionally instead of subcutaneously (Smith *et al.* 2003). The time given for healing should be increased to at least three to six months to allow for sufficient time to obtain significant improvements.

- CHAPTER 5 -

APPLICATION OF MONONUCLEAR CELLS FROM THE PERIPHERAL BLOOD OF ADULT HORSES TO TRAUMATISED TENDONS – A PILOT STUDY

5.1 INTRODUCTION

TENDONITIS of the superficial digital flexor (SDF) tendon is a common occurrence within the horse industry. In Europe's racehorse industry 46 % of horses are annually removed from the track due to suspensory ligament or flexor tendon injuries (Williams *et al.* 2001). Most tendon and ligament injuries require 9-12 months of rest for optimal healing and up to 80 % of these horses sustain re-injury (Silver *et al.* 1983, Sawdon *et al.* 1996). There is little objective evidence that conservative tendon injury treatment has consistent and long-lasting beneficial effects (Dowling *et al.* 2000).

Promising results have been advocated by a number of researchers who utilised undifferentiated stem cells in the regeneration of tendons and ligaments in animals. These studies differed in that they used alternative stem cell sources: Bruder *et al.* (1994) provides a review article adressing mesenchymal stem cell regeneration of various animals; Wakitani *et al.* (1994) used adherent bone marrow cells from rabbits; Young *et al.* (1998) used fibroblasts from rabbits; Herthel (2001) used whole bone marrow extracts from horses; and Smith *et al.* (2003) used mesenchymal stem cells cultured from bone marrow. Therefore the aims of this experiment were (1) to re-implant autologous mononuclear cells (MNCs) into a collagenase-induced tendon injury in the horse's forelimbs and b) to analyse tendon recovery by means of macroscopic observations, ultrasonography and microscopic analysis. Extraction and culturing of autologous stem cells from horse peripheral blood, with concomitant preparation of injections, are described in detail in Chapter 2.

5.2 MATERIALS AND METHODS

All protocols involving use of live animals were approved by the Ethics Committee for Research on Animals of the Medical Research Council (application number P04/06/012). All surgical procedures and anaesthetic administration were conducted by a registered veterinarian.

5.2.1 Housing of horses

Four healthy Thoroughbred horses, between two and three years of age, were used in this pilot study. The horses were housed in grass paddocks at the Welgevallen experimental farm in Stellenbosch, South Africa, and were supplied with sufficient shelter and water. The animals were fed twice daily on a diet of 10 % horse feed from Classic Feeds (SA) Ltd. and hay.

5.2.2 Injury and treatment procedures

The horses were positioned in a crush and mildly sedated with Domesedan (Detomidine – Novartis AH), administered via slow intravenous injection in the jugular vein (dosage according to manufacturer's instructions). The area between the accessory carpal bone and proximal sesamoid bone of both forelimbs was shaved and prepared aseptically. Skin incisions were not required, since the SDF tendons could be palpated and fixed between thumb and forefinger. Once sedated, collagenase-induced lesions were created in the midmetacarpal region of the SDF tendons of both forelimbs. A 26-gauge hypodermic needle was inserted into the centre of the SDF tendon, with the needle tip directed towards the carpometaphalangeal joint (Figure 5.1). This area of injection was located in the palmar midline, approximately 12 - 15 cm as measured from the accessory carpal bone towards the proximal sesamoid bone (Cheng *et al.* 2004, Dahlgren *et al.* 2002). A dose of 2 097 units of bacterial collagenase type I was administered (at 300 units/mg; Gibco^{TM} Cat. no. 17100). The collagenase was dissolved in 0.9 % saline and sterile filtered through a 0.22 μm filter, injected as a volume of 0.3 ml (dosage adapted from Dahlgren *et al.* 2005).

The horses were treated with an intramuscular injection of Peni la Phenix (benzathine penicillin) against pain and inflammation according to the manufacturer's instructions. After being led back to the paddocks, the horses were observed until they recovered from the sedative. Thereafter they were examined daily for evidence of lameness, excessive pain, behavioural problems and discomfort. All horses' injured legs received cold hydro therapy on both forelimbs twice daily for three days post-injury. Two horses received this therapy for a further seven days to alleviate inflammation and discomfort.

Treatments were administered one week post collagenase-induced injury. Superficial digital flexor tendons on all left forelimbs, labelled HCS (collagenase-saline), served as controls: 0.9 % saline solution was injected subcutaneously in the area of injury. The SDF tendons on all right forelimbs, designated HCMNC (collagenase-autologous MNCs), received stem cell treatment: a total of 10 - 15 x 10^6 peripheral blood adherent MNCs were

FIGURE 5.1 - Injection of 0.3 ml (2 097 units) collagenase I into the superficial digital flexor (SDF) tendon centre, midway between the accessory carpal and proximal sesamoid bones.

administered subcutaneously around the area of injury (see Chapter 2, section 2.2.4, for cell culture and pre-injection preparation). In both instances the dosage was divided into ten injections of 0.1 ml each. All horses were sacrificed seven weeks post-injury and their SDF tendons were dissected for macroscopic and microscopic evaluations.

5.2.3   Ultrasonographic evaluations

As for the sheep experiments (Chapter 4, section 4.2.3), a ultrasound scan was conducted prior to induction of injury (pre-scan) to assess whether the forelimb SDF tendons were ultrasonographically normal and to rule out pre-existing tendonitis. Thereafter both forelimbs were scanned at weekly intervals for the duration of the experiment.

Limbs were prepared for ultrasound scanning as described in Chapter 4, section 4.2.3, and scans were conducted using the same Aloka 500 portable ultrasonographic unit equipped with a 7.5 MHz linear array transducer. Examinations were undertaken in the palmar midsagittal aspect of each forelimb. The scanning area extended from ± 10 cm distal of the accessory carpal bone to ± 10 cm proximal to the proximal sesamoid bone. This area was divided into a proximal, middle and distal region (each ± 3 - 5 cm in length). One transverse image was recorded per region for lesion evaluations; one longitudinal image was also recorded per region, centred in the middle of the region and with the proximal and distal scans overlapping the middle scan (Figure 5.2). The mean of the three overlapping longitudinal measurements were used in analyses, since only the lesion was scored according to our indices. For the transverse images, regions were analysed separately.

Images were processed and analysed as described in Chapter 4 section 4.2.3. Still images were assessed qualitatively by using our adapted indices for lesion echogenicity and fibre alignment (as laid out in Chapter 4, Table 4.2 and Table 4.3 respectively). Echogenicity and linearity data were normalised by expressing each index as a proportion of the index assigned for Week 1.

5.2.4   Macroscopic observations and measurements

At seven weeks post-injury, animals were injected intravenously with 15 mg Domesedan (Detomidine – Novartis AH) and stunned mechanically. The forelimbs were disarticulated *post mortem* at the carpometacarpus joint and the skin was removed at the mid-metacarpal region of injury. Macroscopic observations were conducted in the same manner as described in Chapters 3 and 4, sections 3.2.3 and 4.2.4. *Post mortem* data collection and data analyses were conducted as described in Chapters 3 and 4, sections 3.2.4 and 4.2.5.
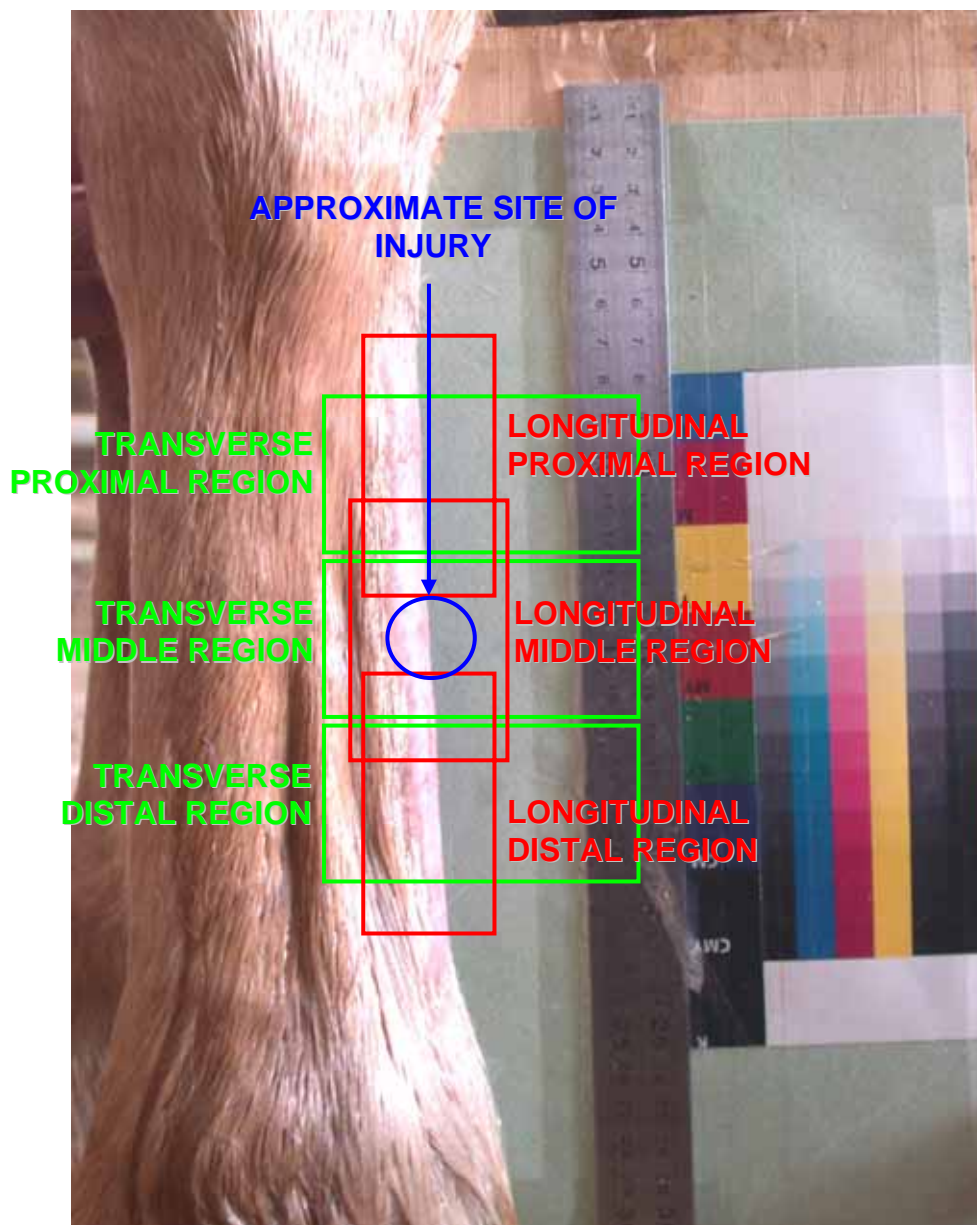
FIGURE 5.2 - Image showing the lateral side of the left forelimb of a horse with schematic insertions to illustrate the approximate site of injury and the six regions that were examined ultrasonographically each week.

5.2.5   Microscopic observations and measurements

The SDF tendons were cut into transverse slices ± 10 mm thick and fixed in 4 % paraformaldehyde in 0.1 M sodium phosphate buffer at a pH of 7.4 and 4 ºC (Humason 1967, Bancroft and Stevens 1977). The slices were processed and stained with Masson's trichrome stain, using standard histological procedures as described in Chapter 3 section 3.2.6.

A second set of histological slides (two per region of each forelimb) were prepared for BrdU identification following the protocols described in Chapters 2 and 4, sections 2.2.3.5 and 4.2.7 respectively. All histological sections were examined under 20x magnification with a Nikon Eclipse E400 inverted microscope. A blue and red macro was set up using Simple PCI Analysis software package and were used to assess the amount of blue staining, indicating collagen content, and the amount of red staining, indicating inflammatory cells and fibroblasts on histological sections. Analyses were performed at five viewing fields within each of the three tendon regions: proximal, middle and distal (see Chapter 3, section 3.2.6).

5.2.6   Statistical analysis

Independent Student's t-tests were used for all analyses between two treatment groups. One-way ANOVA and General Linear Model ANOVA tests were conducted to explore significant differences in the means among three or more treatment groups, in conjunction with the Tukey *post-hoc* test for multiple comparisons. Level of significance was accepted at $p < 0.05$. All data in graphs represent means ± standard deviation.

5.3 RESULTS

5.3.1   Clinical observations

Collagenase injections simulated severe tendonitis lesions in all injected forelimbs. Within 6 – 8 hours of injection the horses showed signs of lameness. Lameness was most apparent during the initial two days post-injury but then gradually improved until two weeks-post injury, at which point the horses were walking freely and without signs of discomfort. An inflammatory reaction caused severe swelling of the forelimbs during the first day following collagenase-induced injury (Figure 5.3a-c). The affected SDF tendons were extremely swollen, displaying a bowed appearance characteristic of tendonitis (McCullagh *et al.* 1979). Within three days the heat and inflammatory swelling had partially subsided in two of the horses and cold hydro therapy was stopped. In the other two horses, cold hydro therapy was continued for an additional seven days before the heat and inflammation subsided.   The
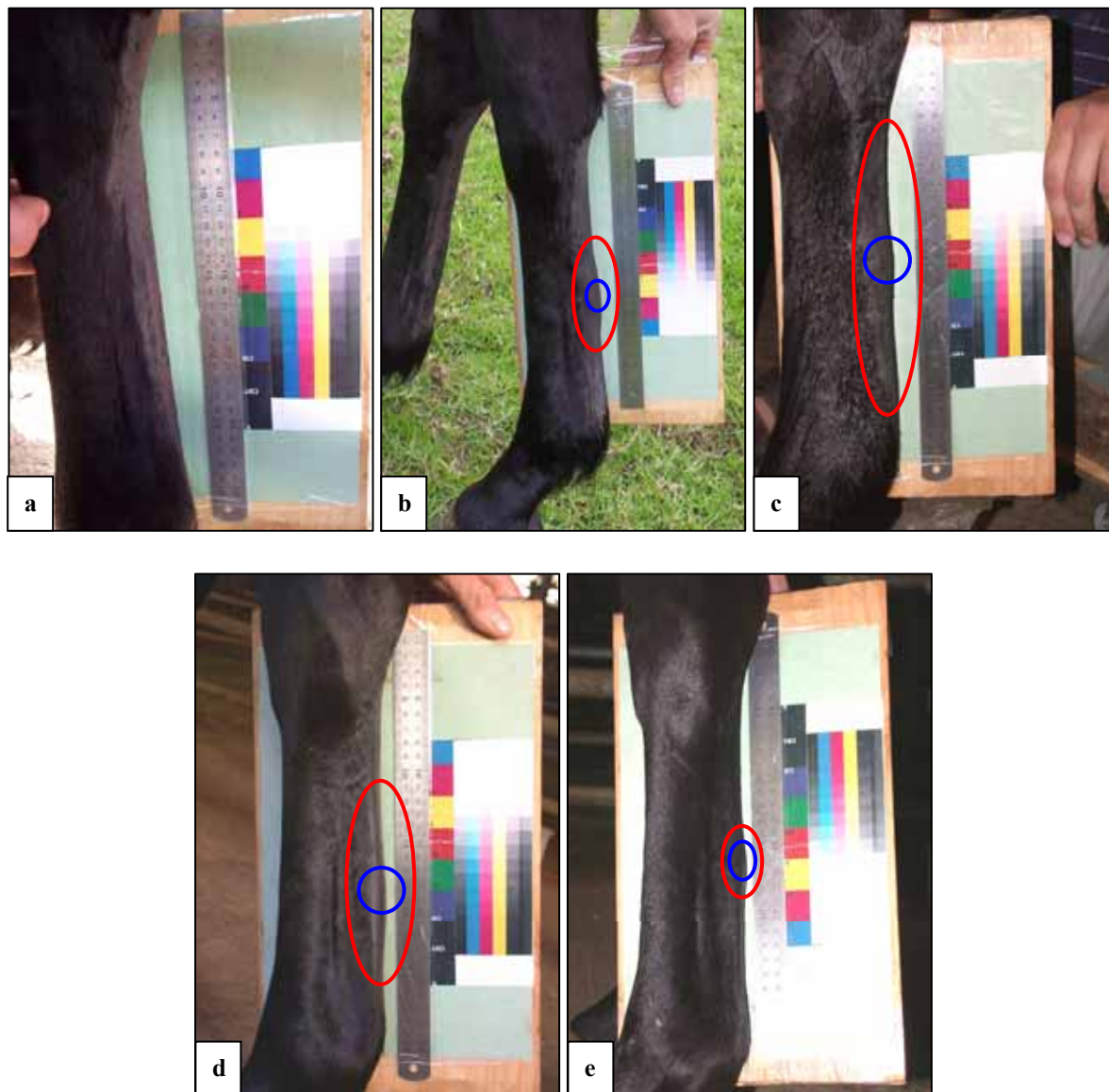
FIGURE 5.3 - Lateral images of the forelimb of a horse (a) prior to collagenase induced tendonitis, (b) six hours post-injury, (c) one day post-injury, (d) seven weeks post-injury and treated with a saline control and (e) seven weeks post-injury and treated with mononuclear cells (MNCs). Blue circles indicate the approximate original site of injury and red circles indicate the area of swelling. Dorsal aspects are located on the left hand side of each image.

injured areas on both front legs remained enlarged, bowed, soft and malleable. Over the subsequent two to three weeks the soft tissue in the injured areas was presumably replaced by firmer fibrous tissue. By Week 7 the swelling and oedema in surrounding tissues had regressed completely, leaving only minimal swelling in the vicinity area of injection (Figure 5.3d-e).

5.3.2   Ultrasonographic evaluations

Proximal region: Tendons receiving MNC treatment (HCMNC) showed significant improvement compared to saline-injected controls (HCS) on the transverse ultrasound images. There were however no significant differences between the two groups for either longitudinal echogenicity or longitudinal linearity indices. Middle region: There were no significant differences between the HCMNC and HCS tendons for either transverse echogenicity or longitudinal echogenicity. Tendons receiving MNC treatment however displayed a significant improvement in fibre linearity. Distal region: In this region tendons displayed a significant improvement in transverse echogenicity following treatment with MNCs. No significant differences were observed for either longitudinal echogenicity or longitudinal linearity.

To determine whether rate of tendon recovery differed between our controls and MNC treated tendons, our standardised indices were plotted against time and the resultant gradients were compared (Figure 5.4a-c). Neither transverse nor longitudinal echogenicity provided significant differences in recovery rates between the two groups. Treated tendons however displayed a significantly faster improvement in fibre linearity. Typical ultrasound scans obtained across the seven week period are depicted in Figure 5.5 to Figure 5.8.

5.3.3   Macroscopic observations

Observations of peritendinous tissue *post mortem* revealed some swelling at the site of injection for all tendons, as well as a yellowish discolouration (Figure 5.9a-b). The peritendinous tissue was not adherent to the SDF tendons, the SDF tendons and DDF tendons were not attached to one other, and the DDF tendons showed no superficial injury at all. In saline-injected controls, the SDF tendons themselves displayed similar yellow discolouration (Figure 5.10a) and swelling, with evidence of haemorrhage in and around the site of collagenase injection. Tendons treated with MNCs also revealed the yellow discolouration and haemorrhage, but less swelling (Figure 5.10b).
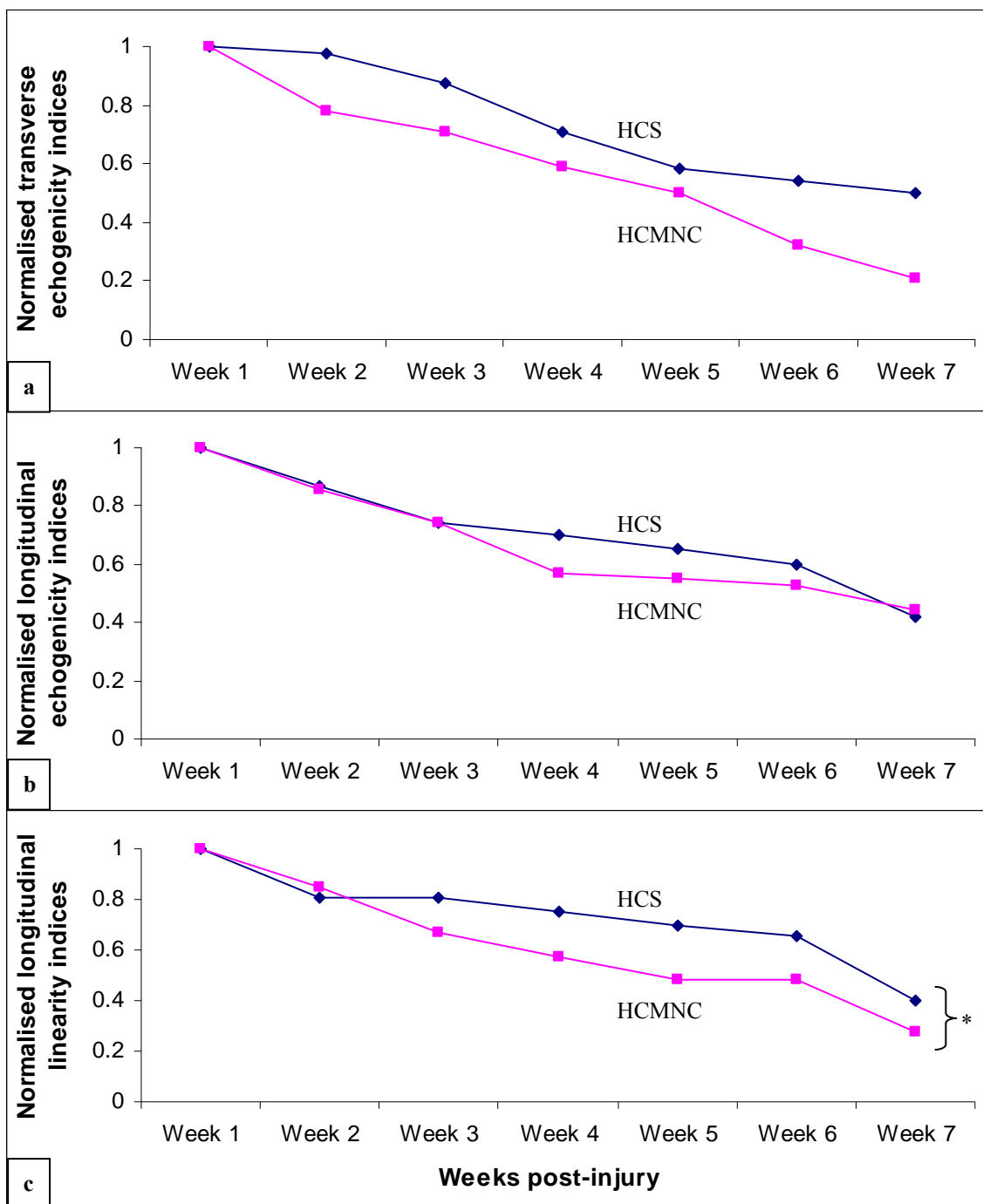
FIGURE 5.4 - Mean (a) transverse echogenicity, (b) longitudinal echogenicity and (c) longitudinal linearity of SDF tendons as obtained from ultrasound scans across seven weeks for saline-injected controls (HCS, blue line) and tendons treated with adherent mononuclear cells (HCMNC, pink line). Normalised index scores are calculated as proportions of index assessments in Week 1. ($* = p < 0.05$)
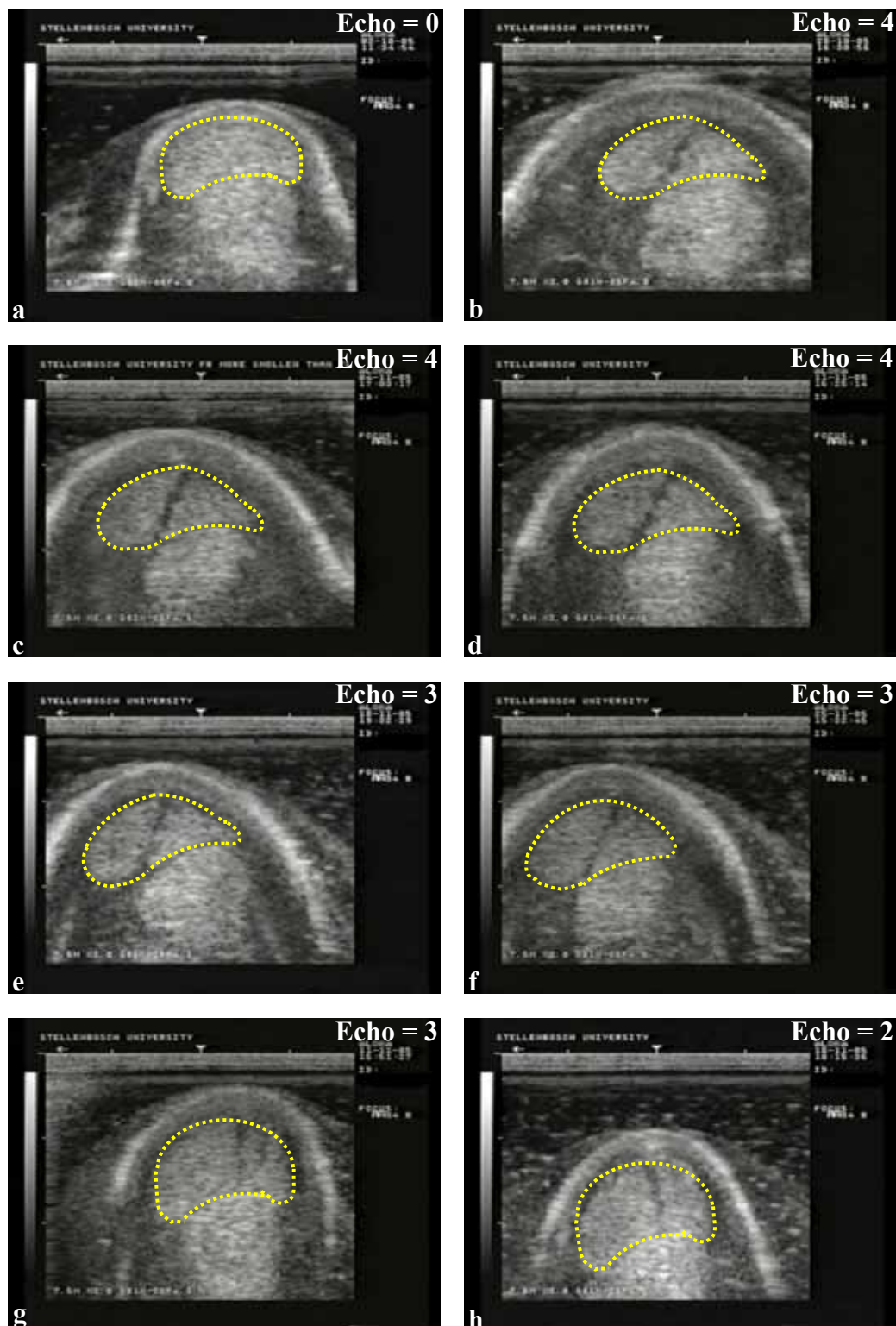
FIGURE 5.5 - Typical ultrasonographs of the palmar metacarpal structures of the CS subset's horses, viewing the transverse aspect of the middle region ± 15 cm distal to the accessory carpal bone, as taken (a) prior to injury and in temporal sequence from (b) Week 1 to (h) Week 7. Echogenicity indices (Echo) appear in the top right corner of each image. The superficial digital flexor tendon is enclosed within yellow outlines. (Left of image = medial aspect; right of image = lateral aspect.)
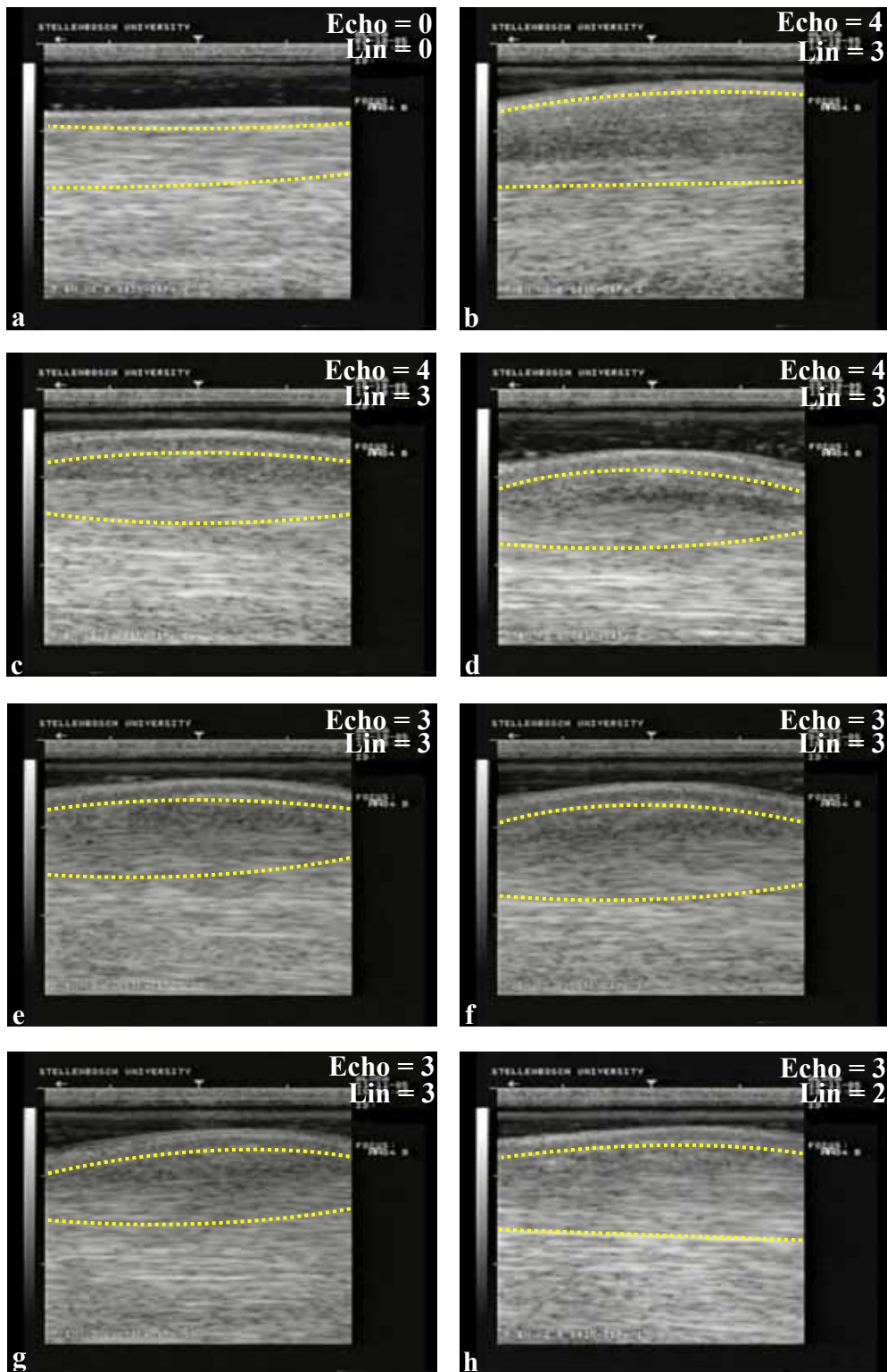
FIGURE 5.6 - Typical ultrasonographs of the palmar metacarpal structures of the CS subset's horses, viewing the longitudinal aspect in the area of tendon injury as taken (a) prior to injury and in temporal sequence from (b) Week 1 to (h) Week 7. Echogenicity (Echo) and linearity (Lin) indices appear in the top right corner of each image. The superficial digital flexor tendon is enclosed within yellow outlines. (Left of image = medial aspect; right of image = lateral aspect.)
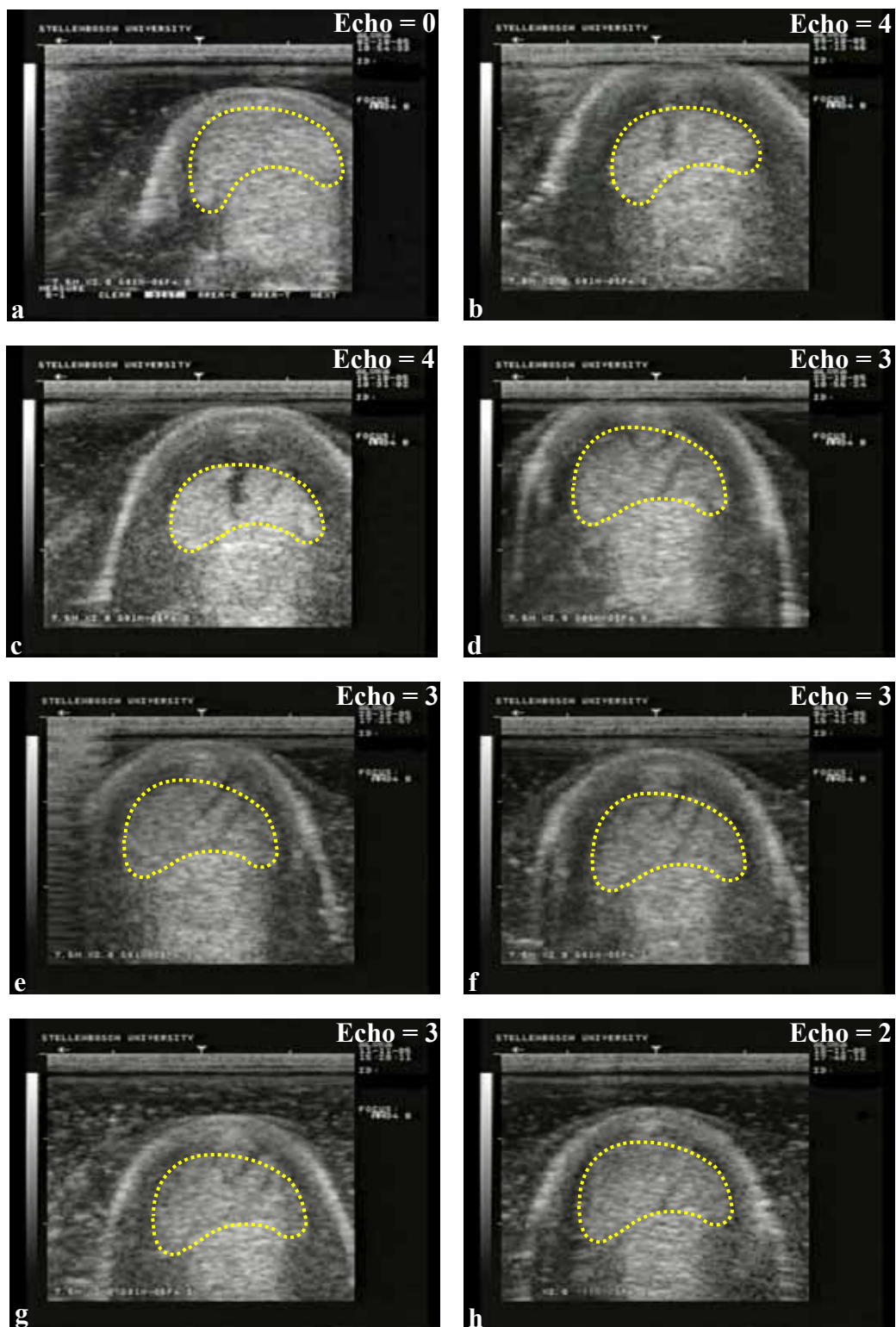
FIGURE 5.7 - Typical ultrasonographs of the palmar metacarpal structures of the CMNC subset's horses, viewing the transverse aspect of the middle region ± 15 cm distal to the accessory carpal bone, as taken (a) prior to injury and in temporal sequence from (b) Week 1 to (h) Week 7. Echogenicity indices (Echo) appear in the top right corner of each image. The superficial digital flexor tendon is enclosed within yellow outlines. (Left of image = medial aspect; right of image = lateral aspect.)
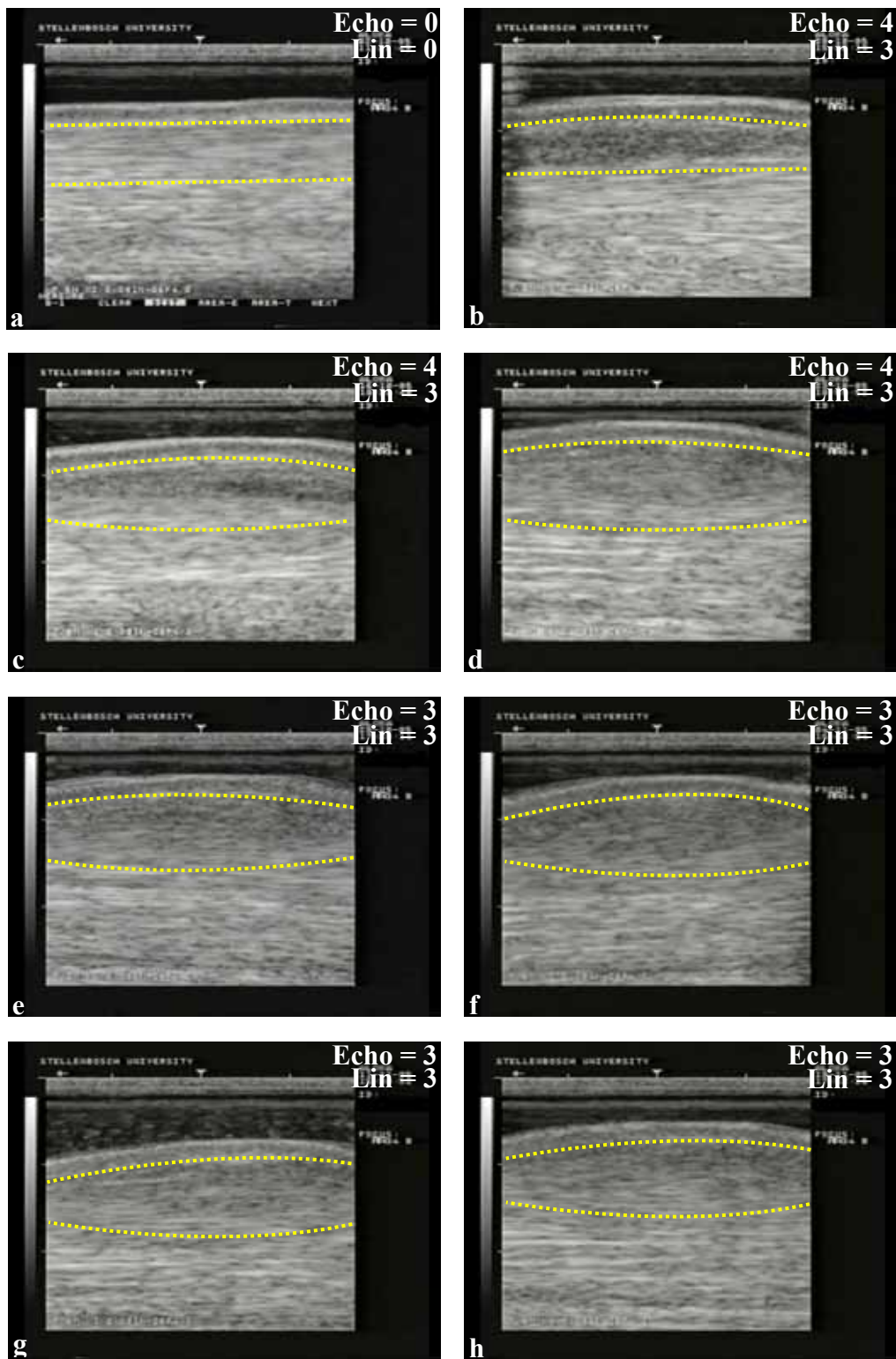
FIGURE 5.8 - Typical ultrasonographs of the palmar metacarpal structures of the CMNC subset's horses, viewing the longitudinal aspect in the area of tendon injury as taken (a) prior to injury and in temporal sequence from (b) Week 1 to (h) Week 7. Echogenicity (Echo) and linearity (Lin) indices appear in the top right corner of each image. The superficial digital flexor tendon is enclosed within yellow outlines. (Left of image = medial aspect; right of image = lateral aspect.)
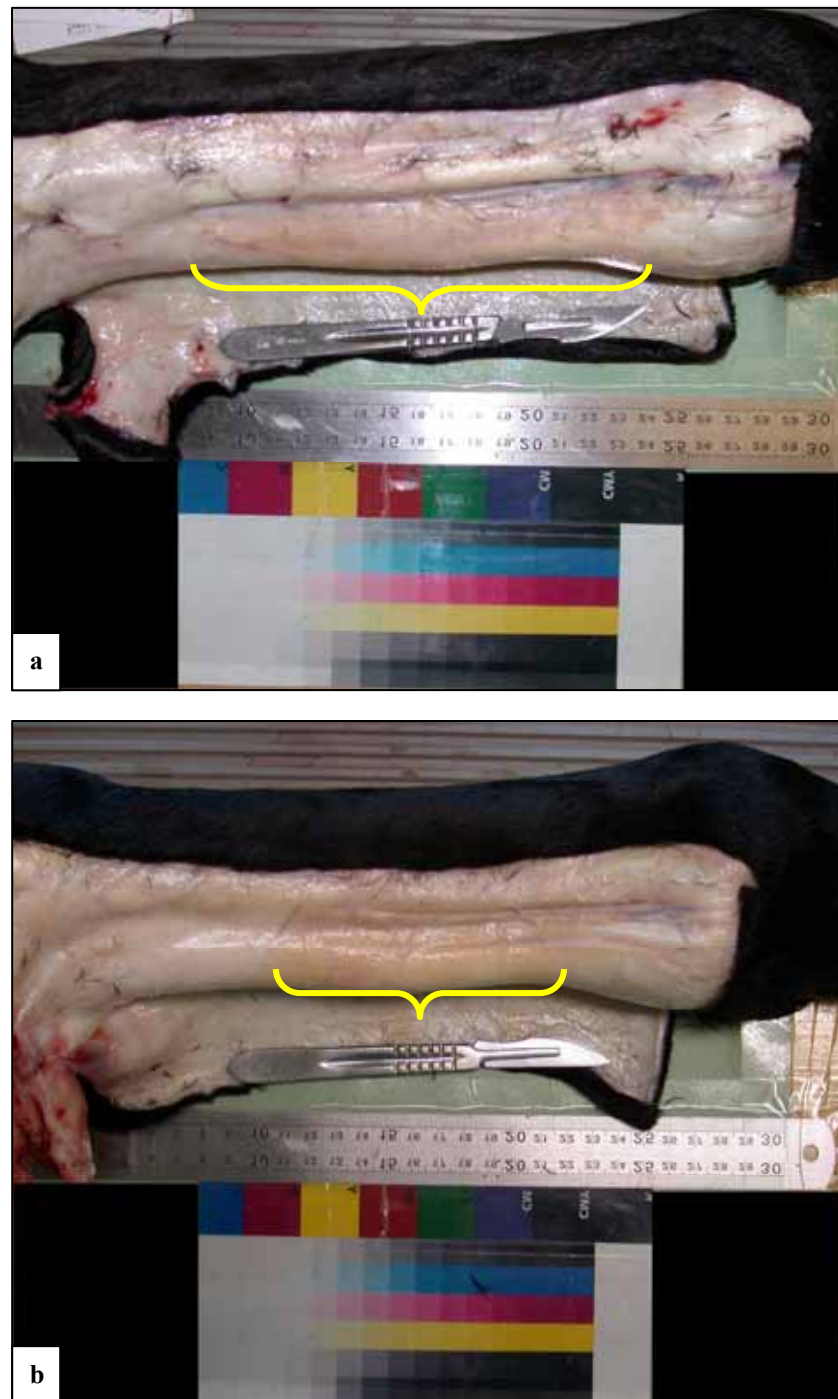
FIGURE 5.9 - Peritendinous tissue revealed swelling and yellow discolouration *post mortem* for both (a) saline-injected controls and (b) tendons treated with autologous mononuclear cells (MNCs). Yellow brackets indicate areas of discolouration. Note the decrease in swelling in the treated tendon. Proximal aspects are located on the left hand side of each image.
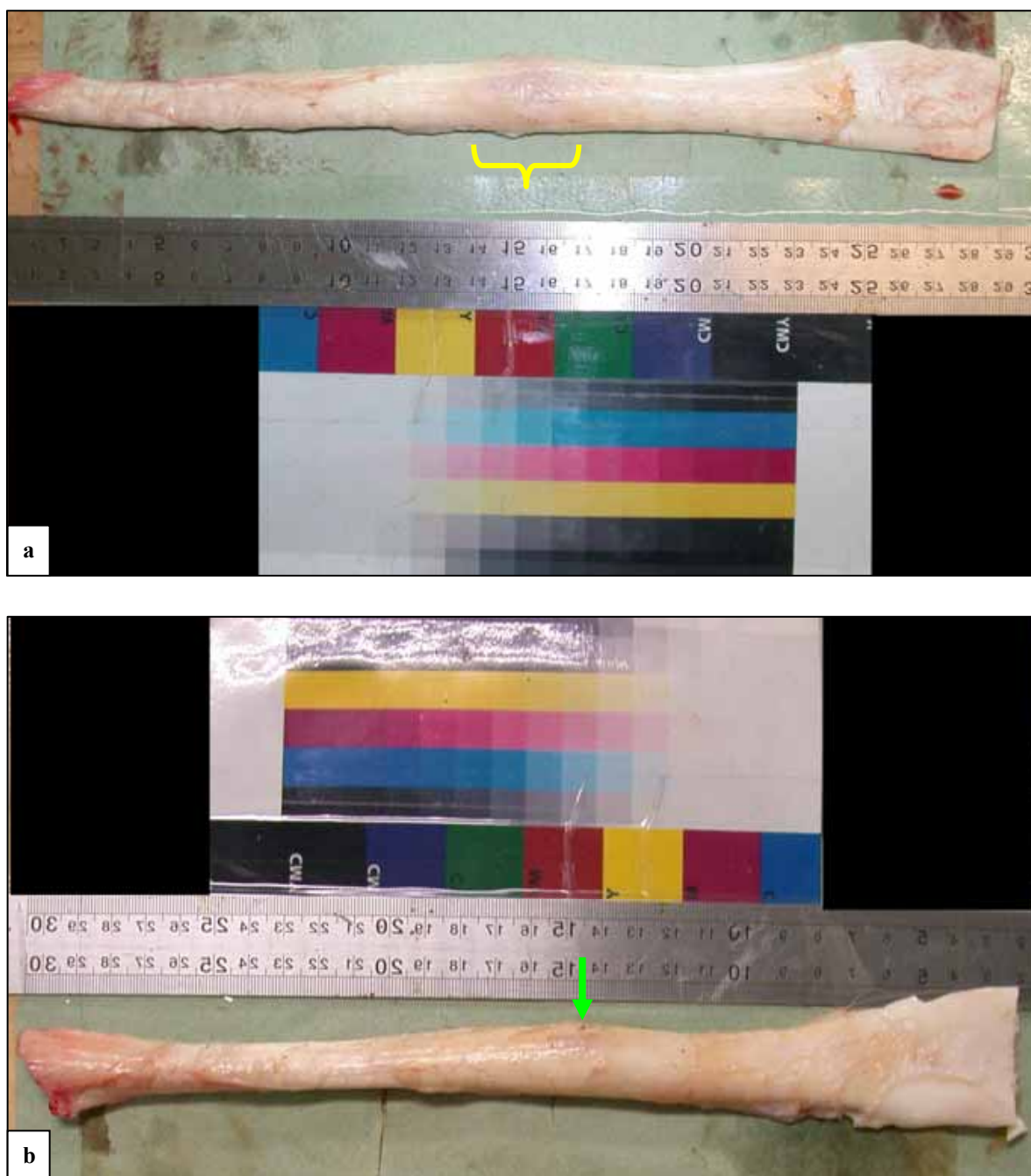
FIGURE 5.10 - The superficial digital flexor tendons of (a) saline-injected controls and (b) limbs that received treatment with mononuclear cells (MNCs) both displayed yellow discolouration and swelling at the site of injection, however more so in the saline treated group. Yellow bracket and green arrow indicate area of discolouration. Proximal aspects are located on the left hand side of each image.

5.3.4   Macroscopic measurements

The SDT tendons of both experimental groups showed an increase in maximum tendon diameter (MTD) from proximal to middle to distal region (Figure 5.11). Comparisons of mean MTDs indicated no significant differences between CS and CMNC tendons of any regions. There was no significant difference in overall mean MTD between the two groups.

5.3.5   Subjective Microscopic observations

All horse tendons showed marked increases in cellularity and neovascularisation after collagenase-induced injury. Proximal and distal regions displayed densely packed collagenous tissue with a homogenous distribution of blue tenocytes with red nuclei. The matrix was fragmented and plump fibroblasts had invaded the damaged areas, randomly secreting a loose connective tissue matrix. In the middle regions of all tendons the matrix was highly inflamed, displaying a gelatinous mass of coagulated blood, yet still retaining definite structure. Examples of histological sections from each region are provided in Figure 5.12a-c.

5.3.6   Microscopic measurements

Simple PCI software was used to analyse the amount of collagen laid down in the tendons (blue macro) as well as levels of inflammation in the tendons (red macro). Tendons injected with MNCs (HCMNC group) displayed a non-significant increase in collagen levels when compared to saline injected controls (HCS group); there were also no significant differences in overall mean red and blue macro values (Figure 5.13a). There were once again no significant differences between HCS and HCMNC tendons when comparing either the blue collagen values or red inflammation values among the middle and distal tendon regions, however this was not true for proximal region which were significant (Figure 5.13b). This may have been related to the fact that the collagenase injection was administered from proximal to distal in a downward angle.

Following microscopic analysis, we were unable to find any BrdU-positive cells in the tendon sections studied. This may be due to increased cell proliferation, and therefore rapid dilution of the BrdU within the DNA, or even migration of marked cells to other areas of repair.
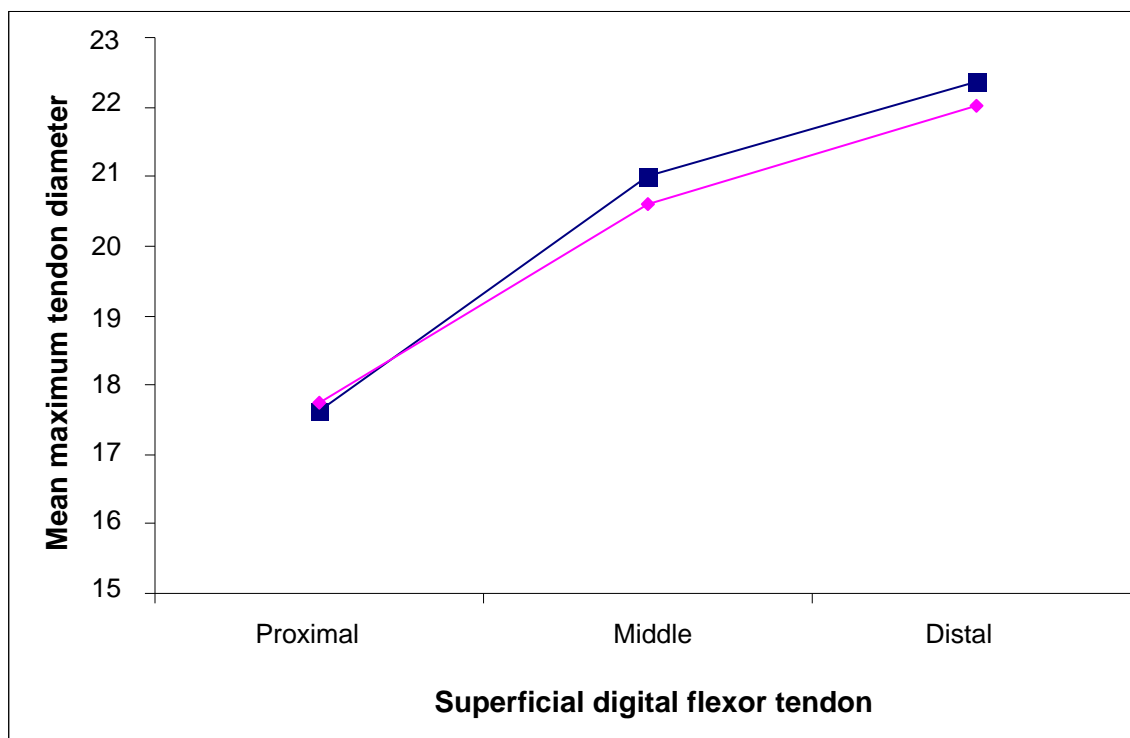
FIGURE 5.11 - Comparison of the mean maximum tendon diameters of the superficial digital flexor tendons as measured in the proximal, middle and distal regions of saline-treated controls (dark blue line) and of tendons treated with mononuclear cells (pink line).
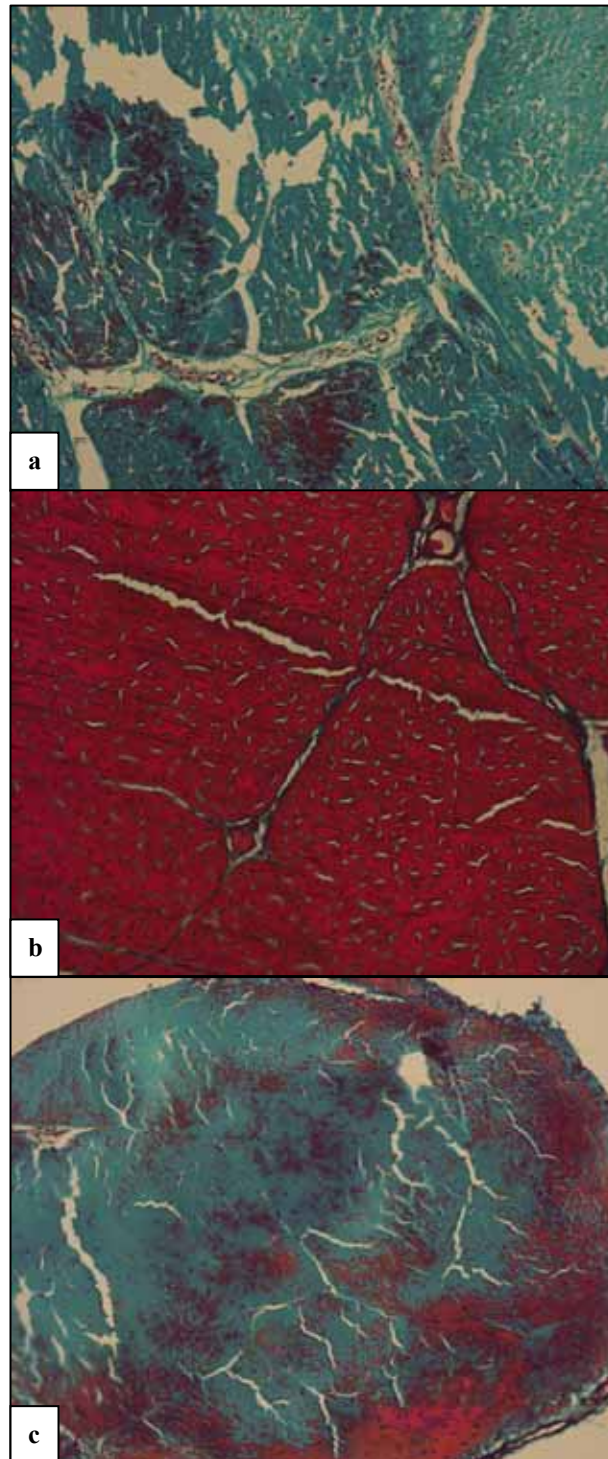
FIGURE 5.12 - Histological sections of collagenase injured horse superficial digital flexor tendons showing (a) the proximal region, with blue-staining collagen and some red-staining inflammation, (b) the middle region, with mostly red-staining inflamed tissue, and (c) the distal region, containing blue-staining collagen tissue and red-staining inflamed tissues. (Masson's trichrome collagen stain, 20x magnification).
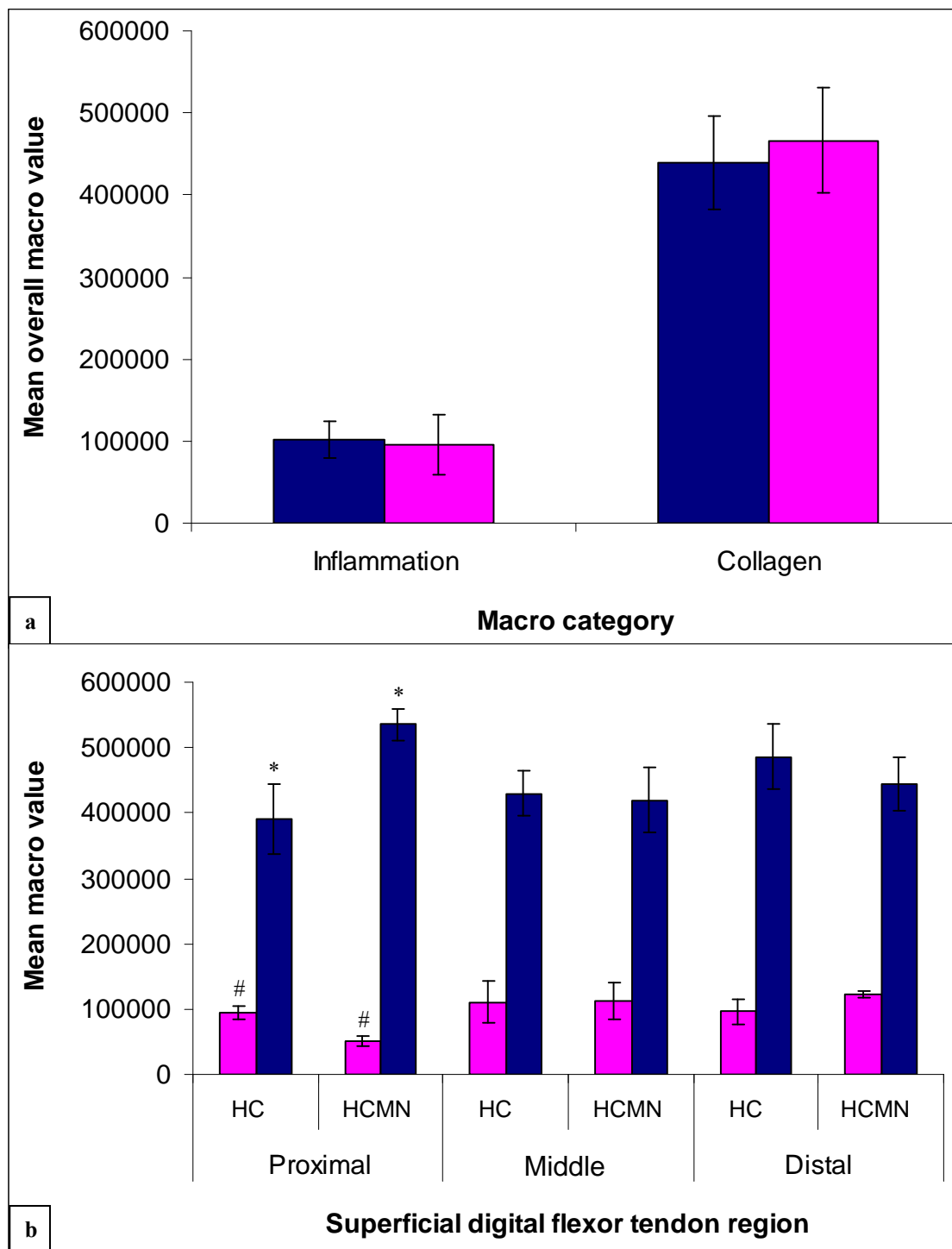
FIGURE 5.13 - Analyses of macro values displaying (a) a comparison of inflammatory response and collagen content between saline treated HCS tendons (blue bars) and mononuclear cell treated HCMNC tendons (pink bars), and (b) differences in inflammatory cell (pink bars) and collagen (blue bars) content among the three regions of HCS and HCMNC tendons. Statistically significant differences ($p < 0.05$) were found between the proximal blue macro values of the HCS and HCMNC subsets (*) as well as between the proximal red macro values for these subsets (#).

5.4 Discussion

Peripheral blood isolation and subsequent cell culture generated large numbers of autologous MNCs, containing both mesenchymal and hematopoietic stem cells, for re-implantation into damaged horse tendons. Although we could not provide conclusive evidence of improved tendon healing over a seven week period, there were sufficient changes to suggest that, given a longer time period and increased sample size, stem cells may enhance recovery rate significantly. Tendons treated with MNCs displayed significant improvements in (1) transverse echogenicity of the proximal regions, (2) transverse echogenicity of their distal regions, (3) fibre linearity of the middle regions, and (4) recovery rate of mid-region fibre linearity when compared with saline-injected controls.

Ultrasonographic analysis is generally employed by equine veterinarians to diagnose and monitor healing of tendon injuries (Nicoll *et al.* 1992, Marr *et al.* 1993b, Micklethwaite *et al.* 2001, Pickersgill *et al.* 2001, Reef 2001). In utilising this technique, we observed significant improvement in the echogenicity and linearity of both treated and untreated tendons over a seven week period. Compared to untreated tendons, those injected with adherent MNCs only displayed significantly enhanced recovery in parallel fibre alignment at the mid-regional site of injury.

Marr *et al.* (1993b) demonstrated a consistent relationship between ultrasonographic and histological assessments of SDF tendon recovery in 40 horses – this was mirrored in our study which was limited to four horses. Both ultrasound and histological examinations revealed signs of inflammation and tendon tissue disruption around the mid-regional site of injury. The histological characteristics we described for healing tendons are consistent with previous reports using collagenase-induced injury (Silver *et al.* 1983, Williams *et al.* 1984a and b, Spurlock *et al.* 1988, Tkach *et al.* 1993, Dahlgren *et al.* 2002). Since Masson's trichrome staining technique gives an indication of collagen content, an increase in echogenicity of lesions can be attributed to collagen deposition. Total collagen content was however only slightly higher in treated tendons, with inflammation persisting in both treated and untreated tendons.

Tenocyte proliferation and increased production of extracellular matrix products are crucial in tendon healing (Dahlgren *et al.* 2002). Normal tendons consist of relatively acellular tissues in which the degradation and production of extracellular matrix molecules are held in equilibrium. With injury, this equilibrium between cellular degradation and production is disrupted. Increased cell metabolism may be inadequate to repair the damage

(Dahlgren *et al.* 2002), but an increase in the overall number of cells, cell division or cellular migration could regain equilibrium by enhancing production rate of the extracellular matrix molecules. The observed increase of small blood vessels in injured areas might have been a reflection of such activity.

In conclusion, results of this study support further investigation into the utilisation of adult peripheral blood adherent MNCs for treating tendonitis in horses. Subcutaneous injection of these cells resulted in increased cell proliferation, improved sonographic linearity and rate of lesion healing, a slight increase in collagen content of affected tissues and decreased peritendinous swelling in comparison with saline treated, collagenase-injured tendons. It is recommended that future treatments be administered intralesionally instead of subcutaneously, in accordance with other research results (Smith *et al.* 2003). Recovery time should also be increased to at least three to six months before final analysis, to allow for expansion of marginal differences obtained in early healing stages.

- CHAPTER 6 -

ISOLATION AND CHARACTERISATION OF MONONUCLEAR CELLS FROM SHEEP ADHERENT ADIPOSE TISSUE

6.1 INTRODUCTION

ADULT mesenchymal stem cells (MSCs) can differentiate into cells from several cell lineages. Friedenstein originally identified adherent, spindle-shaped MSCs in bone marrow stroma during the mid-1970's (Prockop 1997) and these findings were researched further in the 1980's. To date, it has been established that MSCs isolated from bone marrow can differentiate into osteoblasts (Bruder *et al.* 1997, Jaiswal *et al.* 1997, D'Ippolito *et al.* 1999, Pittenger *et al.* 1999), chondroblasts (Caplan 1991, Berry *et al.* 1992, Pittenger *et al.* 1999), adipocytes (Berry *et al.* 1992, Pittenger *et al.* 1999), tenocytes (Young *et al.* 1998, Awad *et al.* 1999), myoblasts (Wakitani *et al.* 1994, Ferrari *et al.* 1998) and neuronal or glial cells (Kopen *et al.* 1999, Reyes and Verfaillie 1999).

Since the escalation of stem cell research in the late 1980's, MSCs have also been identified in peripheral blood (Huss *et al.* 1997, Lange *et al.* 1999, Huss *et al.* 2000, Wexler *et al.* 2003), umbilical cord blood (Hou *et al.* 2002, Romanov *et al.* 2003, Wexler *et al.* 2003), liver (Hu *et al.* 2001, Gotherström *et al.* 2003), pancreas (Hu *et al.* 2003) and adipose tissue (Zuk *et al.* 2001 and 2002, Morizono *et al.* 2003). Adipose-derived stem cells (ADSCs) can differentiate into all mesenchymal cell lineages in humans (Zuk *et al.* 2001 and 2002, Safford *et al.* 2002), mice (Cousin *et al.* 2003, Safford *et al.* 2002, Ogawa *et al.* 2004), rabbits (Rangappa *et al.* 2003) and rats (Niesler *et al.* unpublished data). These cell lineages include muscle, bone, cartilage (Zuk *et al.* 2001 and 2002, Rangappa *et al.* 2003, Ogawa *et al.* 2004), neuronal or glial cells (Zuk *et al.* 2002, Safford *et al.* 2002) as well as hematopoietic cells (Cousin *et al.* 2003). Adipose and bone marrow-derived MSCs are similar in morphology, functionality, transcriptional profile and phenotype (Lee *et al.* 2004).

The adipose tissue itself is known to contain a significant percentage of $CD34^+$ and $CD45^+$ cells (Cousin *et al.* 2003), possibly due to the blood vessels integrated in fatty tissue. Cultured ADSCs are however generally negative for CD31, CD34 and CD45, whereas they are positive for CD29, CD44, CD71, CD90, CD105/SH2, SH3 and STRO-1 (Zuk *et al.* 2002). There exists some controversy in the literature with regards to the expression of CD34, a cell marker for hematopoietic stem cells and progenitor cells, ranging from low or absent

expression (Zuk *et al.* 2002, De Ugarte *et al.* 2003, Wagner *et al.* 2005) to much higher expression (Gronthos *et al.* 2001, Festy *et al.* 2005).

Adipose-derived stem cells possess a number of clinical advantages over bone marrow-derived stem cells. These advantages include tissue accessibility and ease of isolation, as well as good cell yield and frequency (Zuk *et al.* 2001). Whereas 20 000 - 40 000 freshly isolated bone marrow-derived cells must be plated to achieve confluence within five to seven days, only 3 500 cells are required from adipose tissue digestion to reach confluence within the same period (Gronthos *et al.* 2001, De Ugarte *et al.* 2003).

The aim of this study was to confirm the existence of adherent mesenchymal-like stem cells in sheep adipose tissue and to investigate whether they are capable of mesenchymal lineage differentiation. We accordingly set out to isolate, culture, characterise and mononuclear cells (MNCs) from sheep adipose tissue.

6.2 MATERIALS AND METHODS

Collection for research purposes was approved by the Ethics Committee for Research on Animals of the Medical Research Council (application number P04/06/011) and conducted by a registered veterinarian.

6.2.1 *Adipose tissue collection*

Subcutaneous adipose tissue was harvested from six sheep, ranging between one and four years of age. The tail base was prepared for aseptic surgery by spraying shaved skin with providone-iodone and 70 % alcohol and administering local anaesthesia (5 ml of 2 % lignocaine hydrochloride). A 3 cm skin incision was made on the left side of the tail base, using a sterile number 20 scalpel blade. In each sheep, approximately 2 g of subcutaneous fat was removed aseptically and placed in a sterile 50 ml Falcon tube containing DMEM and 2 % AA agent. Samples were transported to the laboratory for immediate processing. Skin injuries were sutured with five to eight sutures using sterile surgical nylon line. An intramuscular injection of Peni la Phenix (benzathine penicillin) was administered post-operatively according to the manufacturer's instructions and the wound was sprayed with Necrospray to prevent infections.

6.2.2   Sample processing

Collagenase type II (Gibco^{TM} Cat. no. 17101) was used to isolate MNCs from the sheep adipose tissue by means of tissue digestion (refer to flow diagram in Figure 6.1). The protocols of Prins *et al.* (1997), Zuk *et al.* (2001) and Aust *et al.* (2004) were followed, all of whom reported successful isolation of multipotent MSC populations from rodent adipose tissue and human lipoaspirates.

The adipose tissue was rinsed twice with wash medium (DMEM containing 2 % AA and 2 % BSA) and finely diced. It was then suspended in an equal volume of collagenase digest solution, comprised of HBSS (Gibco^{TM} Cat. no. 14170), BSA and collagenase type II (3 mg/ml). The suspension was placed in a shaking water bath at 37 ^{o}C for one hour, receiving additional manual shakes every 20 minutes. Following digestion, each sample was centrifuged at 1 600 g for 30 minutes. The supernatant included a layer of mature adipocytes,which was aspirated off the adherent, fibroblast-like stromal cell pellet. This pellet was treated twice with wash medium before being filtered through a 70 µm syringe filter to remove cellular debris. The resulting cell pellet was re-suspended in 1 ml DMEM containing 1 % AA and 20 % FBS. Thereafter 500 µl was extracted for flow cytometry: 100 µl for each of the four cell surface markers analysed and another 100 µl as control sample. The remaining 1 500 µl of the suspension was cultured as described below.

6.2.3   Culturing of cells

The remainder of each suspension was placed in one well of a six-well culture plate and maintained for 24 hours before being washed with D-PBS to remove non-adherent cells and associated debris (Figure 6.1). Cultures were maintained at 37 ^{o}C and 5 % CO_2 in a humidified atmosphere until 60 - 100 % confluence (four to six days), implementing a culture medium change every two days. After reaching 60 % confluence, contents of each well were trypsinised (passage one, or P1), re-plated into three wells of another six-well plate and supplemented with fresh culture medium. After once again achieving 60 % confluence, the cells were washed with D-PBS before being subjected to culture in myogenic differentiation medium (see next section).

6.2.4   Characterisation of cells

6.2.4.1 <u>Myogenic differentiation</u>

For the following seven days, two of the three wells received myogenic differentiation medium while the third received culture medium, the latter well serving as control for

ADIPOSE SAMPLE

DMEM

± 2 g adipose tissue

Rinse 2x → Dice adipose tissue → Resuspend in digest solution

Adipose tissue (discard) ← Digestion: shaking water, 37 ºC, 1 hr ← Centrifuge

Digest solution (discard)

Wash cell pellet 2x and push through 70 µm filter

Add 2 ml DMEM culture medium

5 x 100 µl   1 500 µl

Flow cytometry analysis for CD1, CD31, CD34, CD45

Culture 24 hrs at 37 ºC 5 % CO$_2$

At 60-70 % confluence, trypsinise and replate

FIGURE 6.1 - Flow diagram illustrating adipose tissue processing to isolate adherent mesenchymal-like cells, together with subsequent culturing of these cells and flow cytometry analysis (DMEM = Dulbecco's Modified Eagle's Medium).

myogenic determination through use of anti-human myogenin antibody (refer to flow diagram in Figure 6.2). Myogenic differentiation medium was prepared by supplementing DMEM with 1% AA and 1% horse serum (Kubo 1991, Pontiér *et al.* 1999, Chen *et al.* 2004). To assess myogenic differentiation after the one week of culture, one treated well and one control well were prepared for immunoblotting (see Chapter 2, section 2.2.2.6, for full description of technique). The remaining differentiated well was washed twice with D-PBS and stained with Hoescht nuclear stain, following the protocol described in Chapter 2, section 2.2.4.6.

6.2.4.2 Flow cytometry

We followed the protocols described in Chapter 2, section 2.2.3.4. Cells from one of the wells cultured in culture medium were washed twice with D-PBS containing 2% BSA, trypsinised and separated into five vials for antibody staining. One vial served as control whereas the other four were incubated respectively with CD45 (Serotec Cat. no. MCA2220F), CD1 (Serotec Cat. no. MCA2212F), CD34 (Serotec Cat. no. MCA1825F) and CD31(Serotec Cat. no. MCA1097F) FITC-conjugated, primary monoclonal antibodies. All antibodies were diluted according to manufacturer's protocol. Incubation proceeded for 30 minutes at room temperature and in the dark. Thereafter the samples were washed with D-PBS, centrifuged at 350 g and re-suspended in 500 µl D-PBS. Cell fluorescence was evaluated by flow cytometry using the equipment and following all protocols described in Chapter 2 section 2.2.3.4.

Mouse anti-ovine CD45 antibody marks lymphocytes, granulocytes and macrophages, thus indicating the presence of leucocytes. Martin *et al.* (2002) demonstrated that feline MSCs were negative for CD45, while being strongly positive for CD44. Ovine specific CD44 was however not available commercially at time of experimentation. Mouse anti-ovine CD1 marks the cell surface antigen primarily found in dendritic and cortical thymocyte cells of the dermis and other tissues. Mouse anti-rat CD34 is a marker that recognises hematopoietic stem cells and endothelial cells. CD34 is expressed on ± 1 - 4 % of nucleated bone marrow cells and < 0.1 % of nucleated cells in human peripheral blood (Wognum *et al.* 2003). Although not ovine specific, we were expecting some degree of cross reactivity for mouse anti-rat CD34 due to the manufacturer proclaiming that they have had reports of cross-reactivity with sheep tissue. Mouse anti-CD31 predominantly labels PECAM-1 found on the surface of endothelial cells. This antigen is also expressed on endothelial progenitor cells, the precursors which differentiate into mature endothelial cells (Kocher *et al.* 2001).

Control well   Experimental well 1   Experimental well 2

At 60-70 % confluence, wash 2x with D-PBS   At 60-70 % confluence, wash 2x with D-PBS

Add basal liquid DMEM culture medium   Add DMEM myogenic differentiation medium

Control well   Experimental well 1   Experimental well 2

Immunoblotting analysis with anti-human myogenin antibody   Add Hoescht nuclear stain to assess myotubes nuclei

FIGURE 6.2 - Flow diagram illustrating the myogenic cell differentiation of mesenchymal-like cells cultured from adult sheep adipose tissue (D-PBS = Dulbecco's Phosphate Buffered Saline; DMEM = Dulbecco's Modified Eagle's Medium).

6.3 RESULTS

6.3.1 Culturing of cells

Within our initial cell cultures, samples from only four of the six sheep started to proliferate. These adherent cells took two to four days to start proliferating and reached 60 - 100 % confluence within another two to three days (Figure 6.3a). Wells were trypsinised for differentiation at this point, because cells either became apoptotic (Figure 6.3b) or displayed spontaneous differentiation (Figure 6.4a). Cell colonies were often seen during initial proliferation and grew in size over the time period (Figure 6.4b).

6.3.2 Characterisation of cells

For the characterisation of adherent adipose tissue cells, one well was incubated with myogenic differentiation medium for seven days and stained with Hoechst nuclear dye to identify cells with more than one nucleus. After seven days these cells became apoptotic and adherent cells were few and far between. Evidence of myogenic fusion (i.e., the creation of multinuclear, tube-like cells) were however visible (Figure 6.5a-b). Further characterisation revealed that all samples stained positive for anti-human myogenin monoclonal antibody, hence, myogenic differentiation (Figure 6.6). With regards to flow cytometry, all cultured ADSCs were found to be negative for CD45, CD1, CD31 and CD34 (Figure 6.7).

6.4 DISCUSSION

The aim of this study was to confirm the existence of mesenchymal-like stem cells in sheep adipose tissue that are capable of mesenchymal differentiation. In the present study, we indeed confirmed that ovine adipose-derived MNCs can differentiate into a myogenic lineage (lack of a protocol for tendon differentiation at time of experimentation prohibited more specific analysis). Previous studies have characterised this type of cell population in other species, revealing that ADSCs may have multi-lineage potential (Minguell *et al.* 2001, Zuk *et al.* 2001 and 2002, Rangappa *et al.* 2003). Our findings indicated that sheep adipose-derived adherent cells are capable of differentiation into a lineage besides adipose tissue, as was shown by the expression of myogenin as well as the presence of multinuclear myotubes. Furthermore, our sheep ADSCs also did not express any of the non-MSC specific surface markers CD1, CD31, CD34 and CD45.

In the mid-1970's, Friedenstein identified bone marrow adherent cell populations as heterogeneous in appearance and described the most tightly adherent cells as spindle-shaped
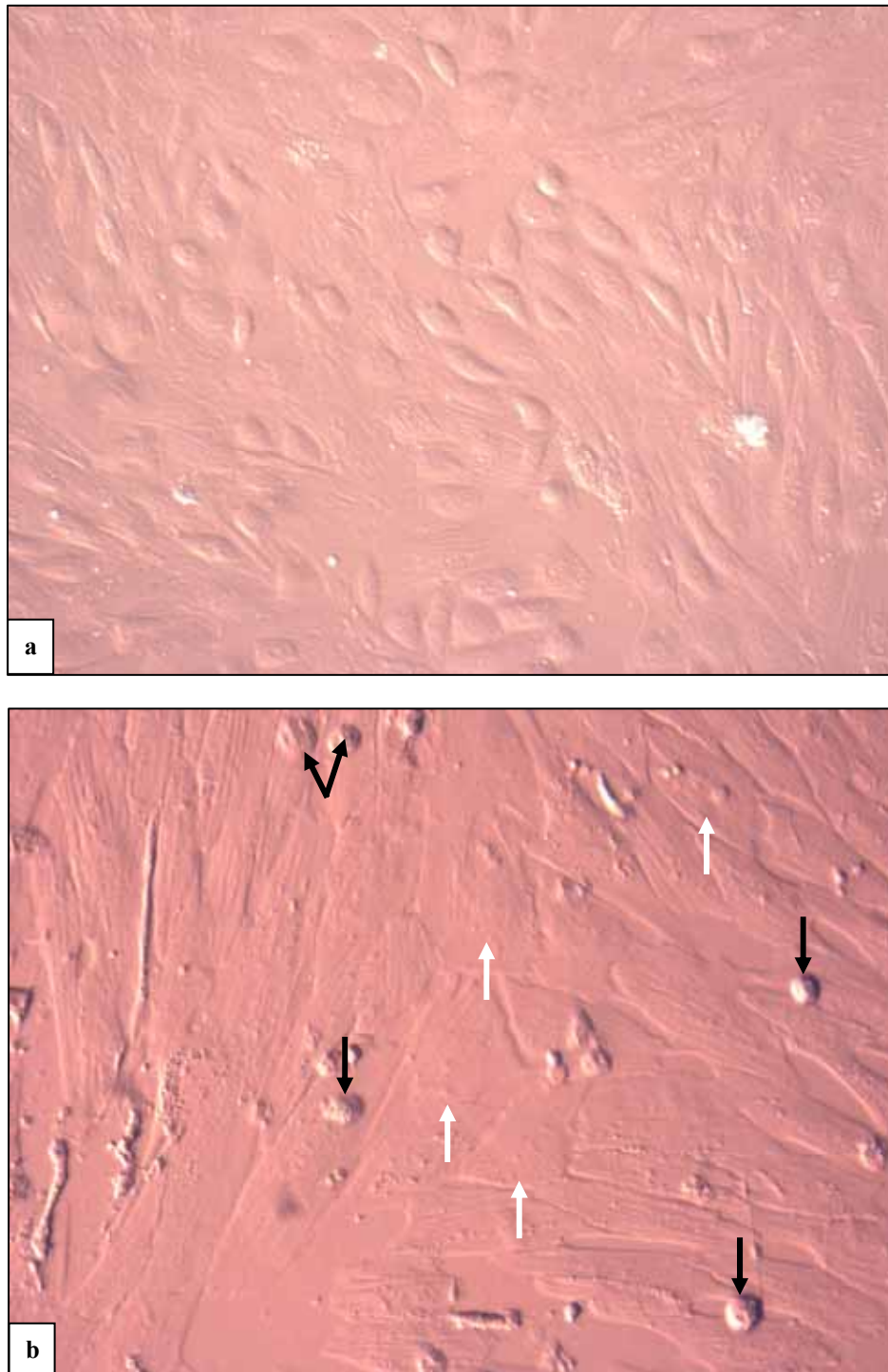
FIGURE 6.3 - (a) Spindle-shaped, fibroblast-like cells become visible in a homogenous cell population after two to four days of proliferation; adherent cells at 70 % confluence. (b) At 100 % confluence, cells become enlarged and square-like and dead cells are apparent (white arrows indicate square-like cells and black arrows indicate apoptotic cells) (20x magnification).
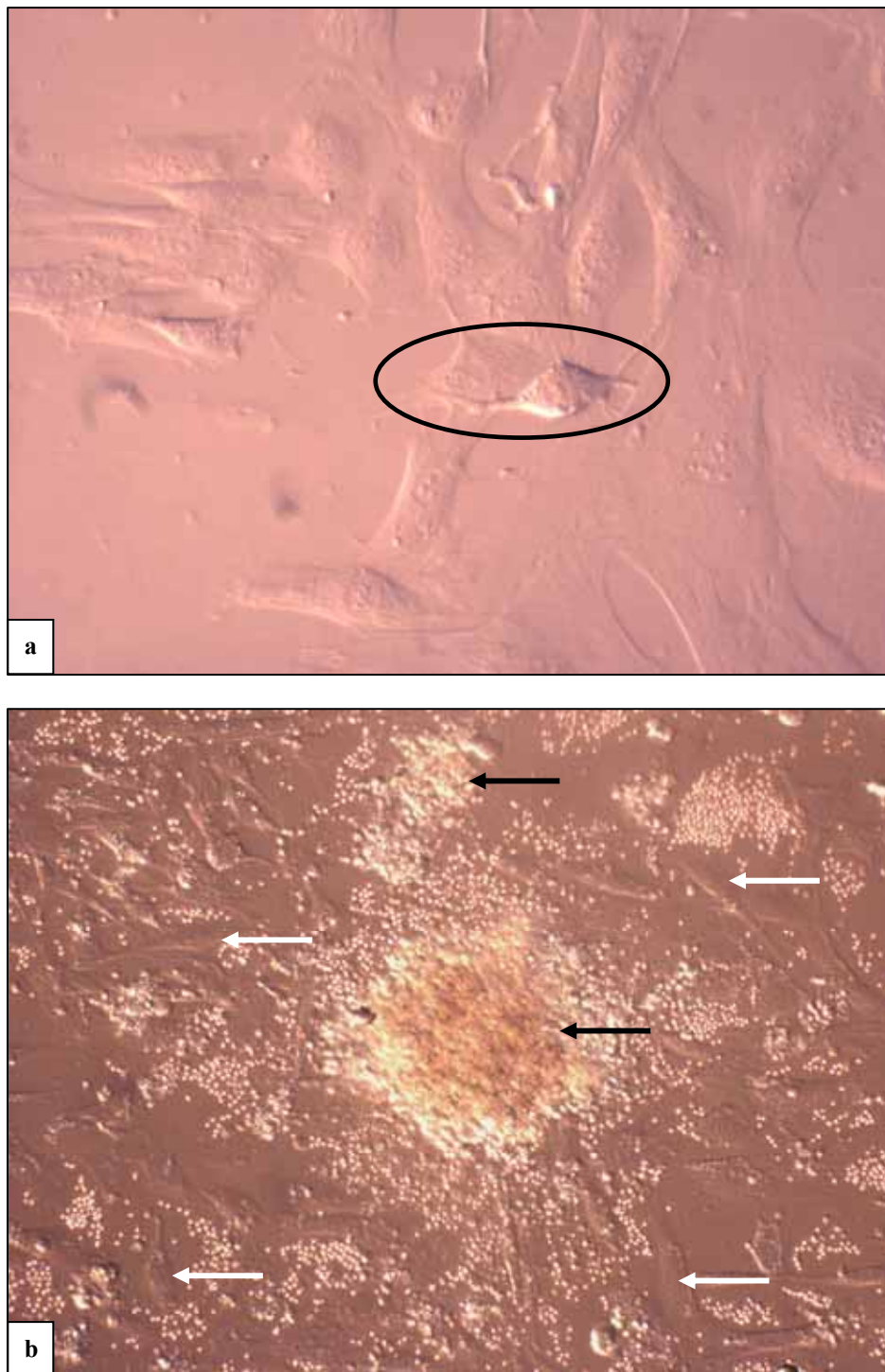
FIGURE 6.4 - (a) After 100 % confluence some cells spontaneously start to differentiate into what appear to be neuronal cells, circled above (40x magnification). (b) Cell colonies (black arrows) were often observed during initial proliferation, surrounded by adherent spindle-shaped, fibroblast-like cells (white arrows) (10x magnification).
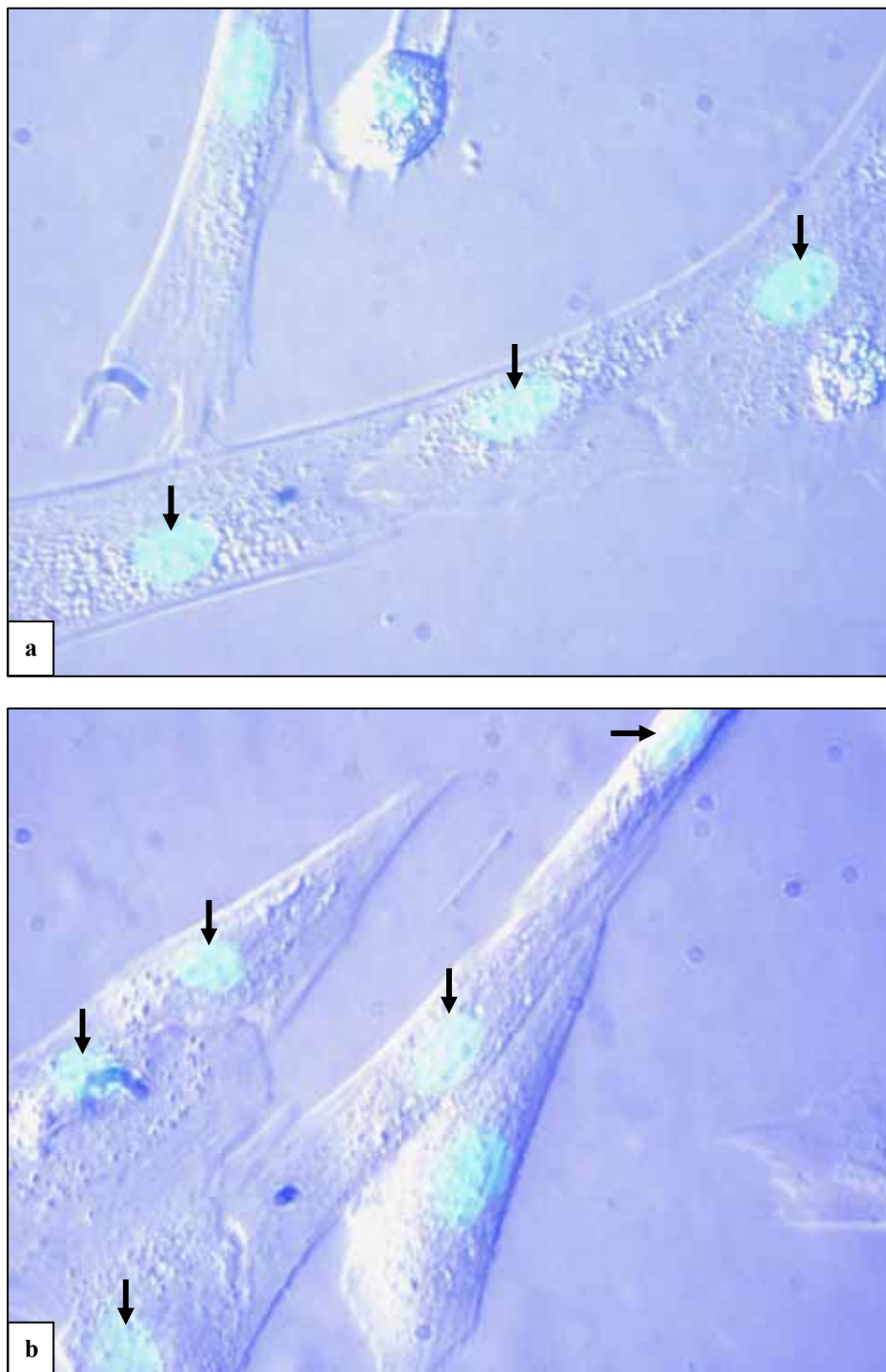
FIGURE 6.5 - After seven days of incubation in myogenic differentiation liquid culture medium, cells display a tube-like morphology and more than one nucleus (black arrows). (Hoechst nuclear stain, 40x magnification)
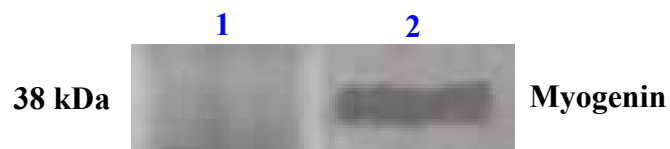
FIGURE 6.6 - Western blot analysis of myogenin protein (red arrow) in cultured, adherent mononuclear cells (MNCs) derived from sheep adipose tissue. Plasma membrane proteins (50 μg) from the indicated samples were separated on 10 % acrylamide SDS-page gels, probed with myogenin antibody and visualised using a chemiluminescence technique. This analysis shows representative results obtained from a control well (1) and a sample well (2), indicating successful myogenic differentiation in the latter.
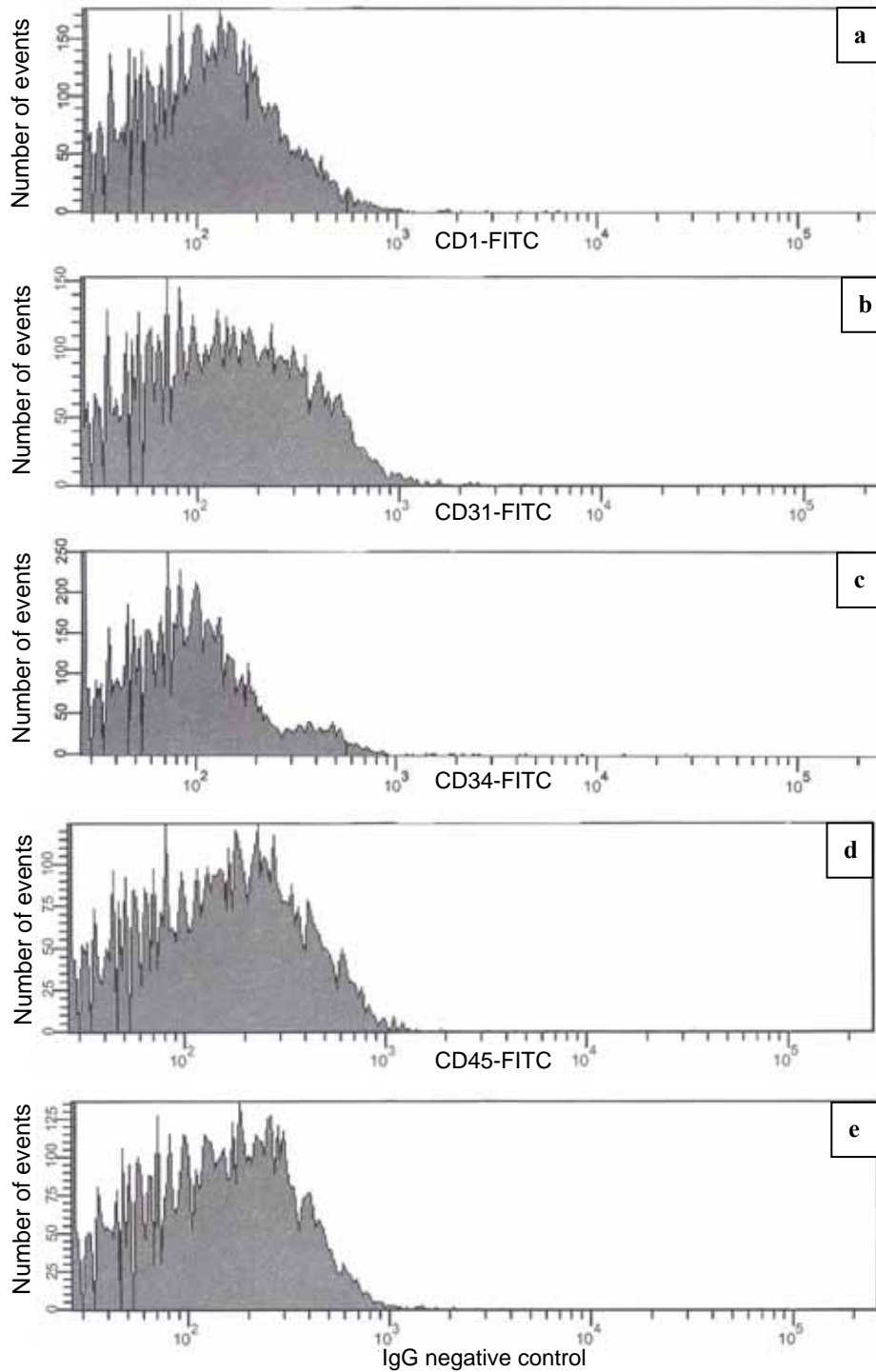
FIGURE 6.7 – Histograms obtained from flow cytometry analyses demonstrated that mononuclear cells (MNCs) derived from ovine adipose tissue test negatively for the FITC-conjugated antibodies (a) CD1, (b) CD31, (c) CD34 and (d) CD45 as opposed to (e) negative controls. Values on the x-axes denote FITC fluorescence whereas the y-axes provide number of events. Positive controls are represented in Figure 2.3, as indicated by CD45 markers showing fluorescence.

with foci containing two to four cells. The cells in these foci remained dormant for two to four days before beginning to multiply rapidly. The most striking feature of these cells was their ability to form colonies (Prockop 1997). Our results agreed with the aforementioned findings. Cells in our samples displayed reluctance to grow for two to four days, followed by rapid proliferation and the formation of cell colonies. Although most of our cells were similarly spindle-shaped (Figure 6.3a), the population appeared homogenous. If left to become 100 % confluent, cells changed to a more block-like appearance at the onset of apoptosis (Figure 6.3b) and/or started differentiating into what appeared to be neuronal cells (Figure 6.4a).

The conditions governing cell differentiation are species-dependant and are influenced by undefined variables, such as the specific horse serum used (Prockop 1997). We were unsuccessful in our attempt to differentiate cultured cells into adipocytes (assessed by Oil Red O lipid accumulation stain) and osteoblasts (assessed by silver nitrate stain), potentially due to these species-dependant factors. However, it would be very beneficial for future research to concentrate and develop differentiation protocol for peripheral blood stem cells, especially differentiation into cell types such as chondrocytes, osteocytes and tenocytes, since these cell types are directly related to tendon tissue.

In contrast, the cells differentiated into early myotubes (Figure 6.5a-b). Zuk *et al.* (2001 and 2002) reported that human adherent cells derived from adipose tissue expressed MyoD and myosin, usually expressed by differentiating myoblasts. We did not test for MyoD or myosin but instead utilised myogenin, which is expressed by differentiating myoblast precursor cells.

Further characterisation of sheep ADSCs were performed by testing for negative expression of the cell-specific surface markers CD1, CD31, CD34 and CD45. These surface markers are reported to be absent on human MSCs (Zuk *et al.* 2002); we found similar results for our ovine MNCs. The CD34 surface marker was not ovine specific suggesting the possibility that we merely failed to detect it due to a lack of cross reactivity. The same antibody was however used in a study by Young *et al.* (unpublished research paper) to characterise cells isolated from adult ovine peripheral blood. In this instance CD34 stained 32 % of a population of 100 000 recorded events, verifying that the antibody indeed cross-reacts with ovine CD34.

- CHAPTER 7 -

GENERAL DISCUSSION

RECENT evidence suggests that both mesenchymal stem cells (Di Stefano *et al.* 2002, Villaron *et al.* 2004) and hematopoietic stem cells (Gilmore *et al.* 2000, Norol *et al.* 2002) are found in circulating peripheral blood, which constitutes an ideal target for autologous and allogeneic therapeutic benefits. Yet little data exist on the characteristics of these stem cells and their microenvironments during early and adult life (Campagnoli, 2001); in the case of sheep and horses, this amounts to virtually no scientific data. The first aim of our study was to provide data regarding the isolation, culture and characterisation of adult peripheral blood mononuclear cells (MNCs) and to determine their potential as transplantable stem cells in sheep and horses. The second aim of this PhD study was however to determine whether autologous or allogeneic peripheral blood MNCs could improve tendon healing in sheep and horses. The third objective was to characterise MNCs isolated from adipose tissue in adult sheep, as an alternative source of MSCs.

7.1 ISOLATION, CULTURE AND CHARACTERISATION OF MONONUCLEAR CELLS DERIVED FROM ADULT PERIPHERAL BLOOD OF SHEEP AND HORSES

As reported in Chapter 2, peripheral blood MSCs was not successfully cultured long term from MNCs in over 20 sheep samples. In adult horses, on the contrary, peripheral blood MSCs were successfully cultured and differentiated into myotubes, a mesenchymal cell lineage (Figure 2.8a-b). These isolated, cultured horse cells displayed the same spindle-like appearance as described for bone marrow MSCs (Fortier *et al.* 1998, Smith *et al.* 2003, Smith and Webbon 2005).

Clinical data has shown that, irrespective of source, the infused cell load of both mesenchymal cells (CD29$^+$) and hematopoietic cells (CD34$^+$) cells play an important role in achieving successful transplantation - the lower the cell dose, the poorer the outcome (Humpe *et al.* 1997, Gluckman *et al.* 1998, Lim *et al.* 1999, Migliaccio *et al.* 2000, Michel *et al.* 2003). Based on data available in the literature, we used the body mass of our study animals to determine injection requirements. We accordingly determined to use ± 4.5 x 10^6 live MNCs for autologous sheep injections, ± 3.0 x 10^6 live CD45$^-$ cells for allogeneic sheep injections and ± 10-15 x 10^6 MNCs for autologous horse injections.

Results obtained from our sheep experiments are comparable to the findings of Lazarus *et al.* (1997) and later by Wexler *et al.* (2003). They were similarly unable to culture peripheral blood MNCs, even with the administration of granulocyte-monocyte colony stimulating factor (GM-CSF). During the course of our project, we conducted a pilot study on sheep (n = 5) to see whether we could increase the percentage of CD45$^+$ cells upon administration of 5 μg/kg Neupogen (which contains GM-CSF as active ingredient). Three days following injection, there was a significant increase in the percentage of CD45$^+$ cells. After five days this percentage, although still elevated, did not differ significantly from control samples and by day seven the percentage of CD45$^+$ cells had returned to normal (results not shown). Since MSCs could not even be cultured from stimulated peripheral blood (Lazarus *et al.* 1997, Campagnoli 2001, Wexler *et al.* 2003), and due to the cost of the Neupogen injections, we did not continue with this experiment. However, in a study done by Fernandez *et al.* (1997), GM-CSF-stimulated peripheral blood from cancer patients adhered to culture dishes and formed spindle-shaped, fibroblast-like MSCs. These cells were positive for the cell surface markers SH2 and SH3 and negative for CD45, CD14 and CD34. Another study by Kuznetsov *et al.* (2001) reported similar results, although they were not able to obtain MSCs from all of their samples.

Although the same protocol was used for both our sheep and horse samples, horse MNCs in contrast did adhere to plastic culture dishes and proliferated to form spindle-like MSCs. The percentage of CD11a/18 was significantly reduced in these samples; if the culture medium had been completely removed with each change (instead of only removing three quarters) there would probably have been no leucocyte trace, as reported in other research (Fernandez *et al.* 1997, Campagnoli 2001, Kuznetsov *et al.* 2001).

7.2 ESTABLISHMENT OF A TENDON INJURY MODEL AND DESCRIPTION OF TENDON INJURY IN SHEEP

Results from Chapter 3 demonstrated that a collagenase-induced injury mimicked naturally occurring tendon injury more accurately than a surgically-induced injury. The experimental sheep did not display undue adverse effects from collagenase-induced injuries, as measured by the absence of significant mass loss across the study period. Tissue damage and inflammation due to collagenase-induced injury appeared macroscopically and histologically very similar to those reported in other studies (McCullagh *et al.* 1979, Williams *et al.* 1984b), hence our decision to use this injury model for the purposes of this PhD study.

Seven weeks post collagenase-induced tendon injury, the affected area had a translucent appearance, with heavily disrupted fibre alignment and extensive adhesions between the injured tendon and surrounding subcutaneous structures. Williams *et al.* (1984b) published similar findings and also demonstrated that the tendons remained enlarged in the area of injury as late as six months post-injury. We observed a non-significant decline in inflammation following histological analysis.

7.3 APPLICATION OF ADULT SHEEP PERIPHERAL BLOOD MONONUCLEAR CELLS TO TRAUMATISED TENDONS

In Chapter 4 we demonstrated that uncultured MNCs derived from the peripheral blood of adult sheep improve tendon repair following a collagenase-induced injury; the effect was however not significant in comparison with the control group. For some of our measurements, $CD45^-$ allogeneic MNCs appeared to increase the rate of tendon healing, though significant results only emerged from our ultrasound data. Marr *et al*. (1993b) reported consistency of results between ultrasonographic and histological methods; this is in contrast to our study where ultrasound measurements reflected a faster healing rate than what was observed in histological sections. This may in part be due to differences in analytical methodology: ultrasound scans were evaluated according to subjective indices whereas histological sections were assessed by objective computer analyses. In accordance with other studies (e.g., Dahlgren *et al.* 2002), we noted a significant increase in cell numbers post-injury and with the application of treatment.

We detected no traces of cells marked with BrdU after seven weeks of therapy. According to manufacturer's notes, cells are guaranteed to retain BrdU for up 72 days post incorporation. It is possible that the cell marker intensity reduced at a faster rate than anticipated. Another potential explanation is that the transplanted MNCs were immuno-logically rejected due to the foreign protein products contained in the BrdU marker (Hildebrand *et al*. 2002). Lastly, injected cells might have undergone apoptosis, followed by reabsorption in the body and concurrent loss of BrdU at the injury site. Our results however warrant further research over a longer recovery period.

7.4   APPLICATION OF MONONUCLEAR CELLS FROM THE PERIPHERAL BLOOD OF
        ADULT HORSES TO TRAUMATISED TENDONS – A PILOT STUDY

Tendon healing is a long process and restitution of tissue structure requires more than a year for completion (Williams *et al.* 1984ab). Even under ideal conditions in young adults, the restitution of normal tendon morphology is a gradual process and complete repair with restoration of normal functionality is unlikely (Webbon 1977, Williams *et al.* 1984b). Estimates of the time required for natural tendon lesions to repair satisfactorily vary and are dependent on both the severity of the injury and the criteria used in judging recovery. McCullagh *et al.* (1979) draw on experimental and clinical veterinary experience when suggesting that healing of partial tendon rupture may take at least nine months before the structure approaches normal function; this coincides with the observations of Williams *et al.* (1984b). Tests of mechanical function by Silver *et al.* (1983) and Williams *et al.* (1984b) found that normal weight bearing characteristics were not fully restored even within a year after severe injury.

We generated promising results through our preliminary investigations of the therapeutic use of cultured peripheral blood MNCs, reported in Chapter 5. Smith *et al.* (2003) and Smith (2005) reported that cultured, bone marrow-derived MSCs only effect significant improvements two months post-treatment. In our study, there were trends towards faster structural improvement after two months and it is possible that, given a longer recovery period, significant results may have been observed. Horses treated clinically by VetCell (www.vetcel.com) entered a controlled exercise regime following stem cell injection to align and strengthen tendon deposition, as prescribed by Smith *et al.* 2005. Other studies confirmed that controlled exercise programs produce significantly better results for tendon healing when compared to conventional treatments (Gills 1997, Genovese *et al.* 1997). This could possibly explain the absence of significant results in our study. For future projects we would therefore suggest that (1) the sample size be increased, (2) separate control animals are included, (3) the recovery period should be at least six months, rendering results more comparable with similar research, and (4) that an experimental group with its relevant control is added, in which a controlled exercise program is followed.

7.5   ISOLATION AND CHARACTERISATION OF MONONUCLEAR CELLS FROM SHEEP

ADHERENT ADIPOSE TISSUE

Selective proliferation and characterisation of adipose-derived MNCs in sheep generated promising results, as reported in Chapter 6. The adherence of spindle-shaped, fibroblast-like cells, original lag phase of proliferation and sudden explosion of expansion after two to four days of culture have been documented by others (Prockop 1997, Zuk *et al*. 2001 and 2002, Rangappa *et al*. 2003). Zuk *et al*. 2002 also similarly reported the differentiation capacity of such cells. We furthermore established that our cells did not express the CD1, CD31, CD34 and CD45 cell surface markers for hematopoietic stem cells.

7.6   FUTURE RESEARCH

When injury occurs in the tendon or ligament, the body follows a certain pattern to restore functionality to the tendon, this includes (as mentioned in Chapter 1): (1) haemorrhage and oedema, (2) inflammation, (3) proliferation/ fibroplasias, and (4) remodelling / maturation. During the proliferation stage there is an influx of leucocytes and fibroblasts to the area of damage to digest and remove damaged or dead tissue and to stabilise the tendon to ensure that further restoration can take place while the tendon is semi-functional, therefore slight locomotion is possible. During this stage Type III collagen is laid down, and is later replaced with Type I collagen (which is present in normal uninjured mature tendons) to ensure strength and elasticity, however because the linearity is disrupted, it is hypothesised that the collagen is not laid down in the exact same manner as it was originally designed due to the removal of coagulated blood and dead tissue while new collagen is being formed. This would then provide evidence that interstitial collagens are not laid down in the same manner due to injury, which results in extensive fibrosis or scar formation. The extensive fibrosis of both clinically and experimentally injured tendons and their slow remodelling contribute to the difficulty of devising an effective treatment for equine tendonitis (Williams *et al*. 1984b).

In recent years there has been much excitement about the potential of MSCs to improve this repair process. Herthel (2001), Smith *et al*. (2003) and Smith and Webbon (2005) have demonstrated that uncultured or cultured bone marrow can produce clinical improvements, with over 85 % of horses returning to competition without re-injury. Vet-Stem recorded a high success rate with the injection of autologous adipose-derived MNCs (www.vet-stem.com). In our preliminary study on both sheep and horses, we observed improvements in just six weeks post-treatment following injection of cultured peripheral

blood MNCs, as well as CD45^{-} cultured peripheral blood MNCs. Had we allowed more time for the initial inflammatory debridement phase to subside and a suitable granulation bed to form, the injected cells may have found better support. This could have produced more significant outcomes as well as the detection of BrdU-positive cells in the tendon tissue itself. All clinical cases recorded thus far have allowed at least one month to pass post-injury before transplanting stem cells; it is however critical to inject stem cells prior to fibrosis, which occurs around two months post-injury (Smith and Webbon 2005). However, it is still not clear whether the stem cells that are injected for regeneration of tendon tissue are directly responsible for collagen deposition after injury or whether the stem cells transdifferentiate, de-differentiate, fuse with existing cells to form collagen and tenocytes, or whether the cells only provide trophic support. Trophic support might stimulate the body to produce collagen and form tenocytes or tendon instead of forming the initial fibroblasts which is more likely to follow the normal sequence of injury and form fibrotic scar tissue.

Bone marrow-derived stem cells have predominated studies in the field of MSC differentiation capacity. Transplantation of bone marrow-derived MSCs are known to be feasible for ligaments and tendons (Awad 1999, Young *et al.* 1998, Smith *et al.* 2003, Smith and Webbon 2005). In tendons, preliminary studies indicate an early beneficial effect of collagen gel seeded with autologous MSCs (Awad 1999, Young *et al.* 1998). Bone marrow-derived from rhesus monkeys has been differentiated into tenocytes by adding bone morphogenetic protein to the cell culture (Wang *et al.* 2005). However, as mentioned earlier, the factors used for stem cell differentiation can be species specific. Research on the potential of MSCs to differentiate into tenocytes, plus the growth factors and mediators required to promote such differentiation, have been limited (Smith and Webbon 2005). Tendon-derived stem cells therefore represent exciting prospects.
In a preliminary study conducted in our laboratory, we were able to culture and expand adherent cells derived from adult equine tendon tissue (Figure 8.1a). The majority of these cells were spindle-shaped, yet the population appeared heterogenous (Figure 8.1b-c). The adherent cells were cultured for up to four weeks with one weekly medium change, which could explain the large amount of floating dead cells, and no passaging, which could explain the heterogenous population. Other cells from the population appeared flat and square (Figure 8.1b) or semilunar (Figure 8.1c).

It is widely believed that the differential expression of growth factors and cytokines in foetal tissue during the early gestational period, in comparison with expression during the late gestational period or adulthood, plays an important role in distinctive healing responses
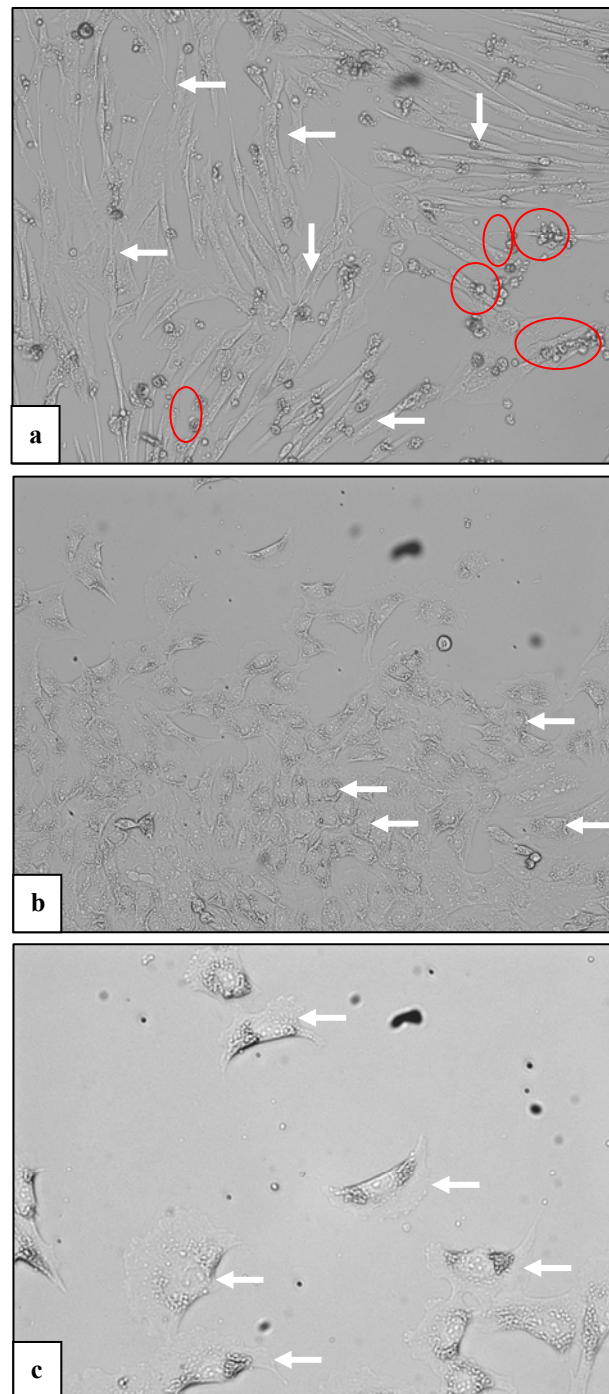
FIGURE 8.1 - Photographs of cultured adherent mononuclear cells (MNCs) from adult horse tendons. The heterogenous cell population contains (a) spindle-shaped cells (white arrows), similar to MSCs cultured from bone marrow and adipose tissue, (b) flat, square-like adherent cells (white arrows) and (c) cells with a semilunar shape (white arrows). Four weeks of culture without passaging shows indications of confluence and differentiation (b and c) while other cells became apoptotic (red circles in a). (Magnification is 10x for a and b 40x for c).

(Burrington 1971, Adzick and Longaker 1992). It is also believed that the regenerative process of tendon tissue is governed by inherent tissue properties and not the environment (Beredjiklian 2003). Preliminary research on scar healing in tendons of foetal sheep revealed that foetal tendon tissue experiences regenerative healing without scarring (Flanagan *et al.* 1999; Beredjiklian 2003). This implies that therapeutic use of allogeneic tendon MSCs derived from foals is a field well worth exploring.

It is furthermore well recognized that motion prevents fibrous tissue formation (Gelberman and Manske 1985). Controlled exercise programs have become the norm in treating tendon injuries, whether as a post-treatment measure or as part of a conventional resting regime (Gills 1997).

Due to their pluripotent nature, mesenchymal stem cells secrete growth factors and produce extracellular matrix components critical for tendon repair. In this way they provide an attractive alternative for improving tendon healing (Beredjiklian 2003). In addition, allogeneic and autologous cells have already been successfully transplanted (Bellincampi *et al*. 1998, Young *et al.* 1998, Awad *et al.* 2000) so these cells are in theory not expected to elicit an immune response, if transplantation is conducted intraspecifically.

It is clear from our data that peripheral blood mononuclear cells do have a more positive effect on tendon healing than their conservative conventional treatment regimes, which warrants further investigation into the mechanisms of stem cell tendon regeneration. Therefore, in conclusion, our data suggests that stem cells derived from peripheral blood, adipose tissue and tendons are important candidate cell types which may improve tendon repair in horses. Sufficient time must be allowed following injury and prior to stem cell treatment (at least one month) and a controlled exercise program should be followed post-treatment. A large enough sample size is required and at least six months of recovery before repair is analysed macroscopically and histologically. Ultrasonography can be carried out on a continuous basis due to its non-invasive nature.

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- ADDENDUM A -

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