IMMUNE AND SATELLITE CELLS: IMPORTANT ROLE PLAYERS IN MUSCLE RECOVERY AFTER INJURY

Maria Jacoba Kruger

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Promoter: Dr. Carine Smith

Co-promoter: Prof. Kathryn H Myburgh

Faculty of Science

Department of Physiological Sciences

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

Signature: ..…….....

Date: ……...…….

DEDICATED TO:

All my loved ones, my friends and family.

For the inspiration you've been to me.

ABSTRACT

Muscle injuries are associated with changes in skeletal muscle as well as the immune system. All studies investigating possible treatment modalities have found both positive and negative effects on muscle recovery. Since no universally accepted treatment modality exists, this thesis aims to determine whether a plant-derived antioxidant, proanthocyanidolic oligomer (PCO), might prove beneficial as treatment for sports injuries in order for athletes to return to the sports field quicker. The difference in recovery of muscle following both chronic (supplementation started 14 days prior to injury and continued thereafter) and acute supplementation (supplementation started two hours after injury) were also investigated.

Both chronic and acute PCO supplementation in a rat hindlimb contusion injury model resulted in earlier muscle recovery, verified by an earlier satellite cell response compared to the placebo group. This effect was most prominent already at the four hour time point following injury, compared to day seven and three after chronic and acute placebo treatment respectively. PCO supplementation also resulted in quicker foetal myosin heavy chain (MHC_f) expression compared to placebo treatment. Chronic supplementation specifically resulted in a blunted circulatory pro-inflammatory cytokine response, whilst allowing for a significant increase in IL-10, an anti-inflammatory cytokine, on day three (in the PCO group only). At tissue level, the response of the muscle pro-inflammatory cytokines, TNF-α and IL-6, coincided with the satellite cell response. Macrophage infiltration into the injured muscle also followed a similar pattern to that seen for the pro-inflammatory cytokines. Macrophages invaded the injured area quicker when supplemented with PCO chronically, however, macrophage infiltration could not explain the cytokine response seen with acute supplementation. Both chronic and acute supplementation with PCO was responsible for a severely blunted neutrophil response, a novel finding of this particular antioxidant.

The main findings of the *in vivo* rodent study were that PCO was able to blunt the neutrophil response, whilst allowing for earlier macrophage infiltration. To establish possible mechanisms by which PCO might exert these beneficial effects, further analysis included

determining macrophage phenotypes and neutrophil numbers in circulation. An *in vitro* neutrophil migration assay was also employed to further elucidate PCO's ability to blunt neutrophil infiltration into the injured area. For this study, conditioned plasma were harvested from experimental animals and added together with neutrophils from control rats and granulocyte colony stimulating factor (G-CSF) to the insert of the migration chamber. A chemotactic factor, *N*-formyl methionine-leucine-phenylalanine (fMLP), was added to the bottom well and neutrophils were allowed to migrate for two hours. Results from this study indicated that neutrophil migration was attenuated *in vitro* in the presence of conditioned plasma from PCO supplemented rats only.

The studies in this thesis on the effect of PCO on parameters of muscle and the immune system led to the following main conclusions: a) PCO supplementation resulted in earlier muscle recovery as a result of earlier satellite cell activation and MHC_f synthesis; b) PCO favours an anti-inflammatory cytokine reaction, whilst blunting the pro-inflammatory cytokine response; and c) PCO blunted the neutrophil response whilst facilitating earlier macrophage infiltration into the injured area. The specific mechanism of action of PCO to blunt the neutrophil response specifically, possibly includes the ability to suppress adhesion molecule expression on the neutrophils themselves. However, this warrants further investigation.

OPSOMMING

Spier beserings word geassosiëer met veranderinge in skeletspier sowel as die immuunstelsel. Meeste studies wat moontlike behandelingsopsies ondersoek, het beide positiewe en negatiewe spierherstel gerapporteer. Omrede daar geen universele behandelingsmoontlikheid bestaan nie, is die doel van hierdie tesis om die effek van ‘n plantgebaseerde anti-oksidant, pro-antosianiedoliese oligomeer (PSO), as ‘n voordelige behandelingstrategie vir sportbeserings te toets. Die verskil in spierherstel na beide kroniese (supplementering wat 14 dae voor besering begin is, en volgehou is daarna) en akute supplementering (supplementering het twee uur na besering begin), is ook ondersoek.

Beide kroniese en akute PSO supplementering, in ‘n rot agterbeen-kneusbeseringmodel, het gelei tot vroeë spierherstel. Die bevindinge is geverifiëer deur ‘n vroeë satelietselrespons in vergelyking met die plasebo groep. Hierdie effek was reeds opvallend vier uur na besering, in vergelyking met die dag sewe en dag drie tydpunt tydens kroniese en acute plasebo behandeling onderskeidelik. In vergelyking met die kontrole groep, het PSO supplementering ook gelei to vininger uitdrukking van miosienswaarketting (MHC_f). Kroniese supplementering het spesifiek gelei to ‘n onderdrukte sirkulatoriese pro-inflammatoriese sitokien response, terwyl ‘n betekenisvolle toename in IL-10 op dag drie (in die PSO groep alleenlik) waargeneem is.

Op weefselvlak, het die pro-inflammatoriese sitokiene, IL-6 en TNF-α, dieselfde patron gevolg as die van satelietselle. Makrofaaginfiltrasie binne die beseerde spier het ook ‘n soorgelyke patroon gevolg. Makrofage het die beseerde area vinniger geïnfiltreer in die kronies PSO-gesupplementeerde groep, maar kon nie die sitokienrespons, wat waargeneem is met akute supplementasie, verklaar nie. Beide kroniese en akute PSO supplementering was verantwoordelik vir ‘n onderdrukte neutrofiel respons, wat ‘n nuwe bevinding is vir hierdie spesifieke anti-oksidant.

Die hoof bevindinge in die *in vivo* rotstudies, is dat PSO instaat is om die neutrofielrespons te onderdruk, en sodoende vroeë makrofaaginfiltrasie teweeg te bring. Om meganismes waarby PSO hierdie voordelige effekte veroorsaak te ondersoek, is verdere analises gedoen om makrofaagfenotipe en neutrofielgetalle in die sirkulasie te bepaal. 'n *In vitro* neutrofielmigrasie studie is ook aangewend om PSO se vermoë om neutrofielinfiltrasie in die beseerde area te onderdruk, te ondersoek. Neutrofiele van kontrole rotte, tesame met gekondisioneerde plasma van eksperimentele diere en granulosiet-kolonie stimulerende faktor (G-KSF), is toegelaat om vir twee ure in die teenwoordigheid van 'n chemotaktiese faktor, *N*-formiel metionien-leusien-fenielalanien (fMLP) te migreer. Resultate van hierdie studie het aangetoon dat neutrofielmigrasie, *in vitro*, alleenlik onderdruk word in die teenwoordigheid van gekondisioneerde plasma van PSO-gesupplementeerde rotte.

Die studies in hierdie tesis oor die effek van PSO op parameters van spier en die immuunsisteem, het tot die volgende hoofgevolgtrekkings gelei: a) PSO supplementering het vroeë spierherstel, as gevolge van vroeë satelietselaktivering en MHC*f* sintese, teweeg gebring; b) PSO verkies 'n anti-inflammatoriese sitokien reaksie, terwyl dit die pro-inflammatoriese sitokienrespons onderdruk; en c) PSO onderdruk die neutrofielrespons, terwyl vroeë makrofaaginfiltrasie in die beseerde area gefasiliteer word. Die spesifieke meganisme van aksie van PSO, om die neutrofielrespons te onderdruk, kan moontlik die vermoë van neutrofiele om adhesie molekule uit te druk, insluit. Hierdie aanname moet egter verder ondersoek word.

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LIST OF PUBLICATIONS AND CONGRESS CONTRIBUTIONS

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Peer-reviewed Paper

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INTERNATIONAL

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Posters

Kruger MJ, Smith C, Smith RM, Myburgh KH. Antioxidant supplementation enhances muscle recovery from contusion injury in rats. The America Physiology Society Intersociety Meeting: The integrative biology of exercise-V, Hilton Head, South Carolina, 2008

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LIST OF ABBREVIATIONS

AA	- arachidonic acid
ANOVA	- analysis of variance
APC	- antigen presenting cell
Asc	- ascorbic acid
bFGF	- basic fibroblast growth factor
BrdU	- bromodeoxyuridine
C5	- complement components
CAM	- cell adhesion molecule
cAMP	- cyclic adenosine monophosphatase
CBA	- Cytometric Bead Array
CCR	- chemokine receptor
CD	- cluster of differentiation
CINC	- cytokine-induced neutrophil chemoattractant
CK	- creatine kinase
COX	- cyclooxygenase
C-PCO	- control proanthocyanidolic oligomer group
C-PLA	- control placebo group
CRP	- C-reactive protein
CTRL	- control
Cu	- copper
DNA	- deoxyribonucleic acid
DFM	- diferuloylmethane
DMSO	- dimethyl sulphoxide

DMTU - dimethylthiourea

DOMS - delayed onset muscle soreness

e^- - electron

EGCG - (–)epigallocatechin gallate

ELISA - enzyme-linked immunosorbent assay

ERK - extracellular signal-regulated kinase

E-selectin - endothelial selectin

FGF - fibroblast growth factor

fMLP - *N*-formyl methionine-leucine-phenylalanine

formylmethionyl-leucyl-phenylalanine

G-CSF - granulocyte colony stimulating factor

GM-CSF - granulocyte macrophage colony stimulating factor

GSE - grape seed extract

GSH - glutathione

GSSG - glutathione, oxidised form

GSTα1 - glutathione-S-transferase α1

H^+ - hydrogen ion

HBOT - hyperbaric oxygen therapy

H&E - haematoxylin and eosin

HGF - hepatocyte growth factor

H_20_2 - hydrogen peroxide

HO^- - hydroxyl radicals

HSC - haematopoietic stem cells

HSVEC - human saphenous vein endothelial cells

HUVEC - human umbilical vein endothelial cells

ICAM - intercellular adhesion molecule

IFN - interferon

IGF - insulin-like growth factor

IL - interleukin

i-NOS - inducible nitric oxide synthase

i.p. - intraperitoneal

I-PCO - injury proanthocyanidolic oligomer group

I-PLA - injury placebo group

JAK - janus activating kinase

JNK - c-Jun NH2-terminal protein kinase

KO - knock out

LFA - lymphocyte function associated antigen

LIF - leukemia inhibitory factor

$LOO^{\bullet}$ - peroxyl radical

L-selectin - leukocyte selectin

LTB_4 - leukotriene B_4

L-NAME - Nω-nitro-L-arginine methyl ester

LPS - lipopolysaccharide

MAdCAM - mucosal addressin cell adhesion molecule

MAPK - mitogen activating protein kinase

M-cad - M-cadherin

MCP - monocyte chemotactic protein

M-CSF - macrophage colony stimulating factor

MEE - middle ear effusion

MGF - mechano growth factor

MHC_f - foetal myosin heavy chain

MIP - macrophage inflammatory protein

MMP - matrix metalloproteinase

Mn - manganese

MNF - myocyte nuclear factor

MP - methylprednisolone

mpc - myogenic precursor cells

MPO - myeloperoxidase

MRFs - myogenic regulatory factors

m-RNA - messenger ribonucleic acid

MSC - mesenchymal stem cells

NAC - N-acetyl-L-cysteine

N-cad - neural cadherin

NCAM - neural cell adhesion molecule

NFκB - nuclear factor-kappa B

NGF - nerve growth factor

NK - natural killer cell

nNOS - neuronal nitric oxide synthase

NO - nitric oxide

NOS - nitric oxide synthase

NOx - nitrite/nitrate

NSAIDs - non-steroidal anti inflammatory drugs

NSAIAODs - non-steroidal anti-inflammatory and antioxidant drugs

$1O_2$ - singlet oxygen

0_2^- - superoxide anions

OPCs - oligomeric proanthocyanidins

ORAC - oxygen radical absorbance capacity

OTC - 2-oxothiazolidine-4-carboxylate

PAF - platelet activating factor

PBMC - peripheral blood mononuclear cell

PBS - phosphate-buffered saline

PCNA - proliferating cell nuclear antigen

PCO - proanthocyanidolic oligomers

PDGF - platelet derived growth factor

PDTC - pyrrolidine dithiocarbamate

PECAM - platelet-endothelial cell adhesion molecule

PG(E_2)/(F_2) - prostaglandin E_2 or prostaglandin F_2

PKC - protein kinase C

PI3-K - phosphatidylinositol-3-kinase

PI-PCO - post-injury proanthocyanidolic oligomer group

PI-PLA - post-injury placebo group

PLA - placebo

P-selectin - platelet selectin

Reparixin - R(-)-2-(4-isobuthylphenyl)propionyl methanesulfonamidel

RICE - rest, ice, compression and elevation

RNA - ribonucleic acid

RNS - reactive nitrogen species

ROS - reactive oxygen species

rpm - repetitions per minute

SCs - satellite cells

SD - standard deviation

SEM - standard error of the mean

SOD - superoxide dismutase

STAT - signal transducer and activator transcription protein

SUSPI - Stellenbosch University Sport Performance Institute

TA - tibialis anterior

TBARS - thiobarbituric acid-reactive substances

TGF - transforming growth factor

T_H0 - naive helper T cells

T_H1 - helper T lymphocyte type I

T_H2 - helper T lymphocyte type II

TNF-α - tumour necrosis factor alpha

$ToC^{\bullet}$ - tocopherol radicals

TXA_2 - Thromboxane A_2

$UQ_{10}H_2$ - reduced form of Ubiquinone Q10

VCAM - vascular cell adhesion molecule

VEGF - vascular endothelial growth factor

VLA-4 - very-late antigen 4

WBC - white blood cell

Zn - zinc

CHAPTER 1

Background

1.1 General introduction

In sports, contusion injuries - defined as an impact by a blunt non-penetrating object - occur both during practice and in competition (Kibler 1993). Muscle groups most commonly affected by these types of injuries, which result in mechanical damage to muscle fibres, capillary rupturing, infiltrative bleeding, oedema and inflammation, are those in the arms, legs, hands, feet and buttocks (Kearns *et al.,* 2004). The trauma can produce significant disability because of inflammation-related pain and impaired muscle function, often resulting in a loss of flexibility and strength, placing the player at increased risk of re-injury (Gates and Huard 2005) if returning to the sports field before full muscle recovery.

Satellite cells (SCs), precursor/stem cells specific to skeletal muscle, are located on the surface of muscle fibres (myofibres), beneath the basement membrane (Bischoff 1997; Grounds 1999; Grounds *et al.,* 2002; McComas 1996). SCs function to replace or repair damaged muscle fibres. Upon muscle damage, SCs become activated and start to proliferate, differentiate and fuse either with other myoblasts, or with damaged muscle fibres in order to repair muscle fibre ultrastructure (Cornelison and Wold 1997; Scime and Rudnicki 2006). It is also well known that the entire process of muscle regeneration is regulated by the myogenic regulatory factors (MRFs – discussed in section 1.2.3) (Florini *et al.,* 1991a; Grounds 1999).

The interaction between the immune system and skeletal muscle is regarded as an important regulatory process, modulating the events during repair and remodelling. Upon muscle injury, muscle fibres and capillaries located around the muscle fibres rupture at or adjacent to the impact area, the latter resulting in exposed collagen. Platelets adhere to the exposed

collagen, become activated as a result and release pro-inflammatory mediators such as 5-hydroxy tryptamine (serotonin), histamine and thromboxane A_2 (TxA_2). Mast cells in the tissue also release histamine, which increases the blood flow to the injury site, allowing blood-borne inflammatory cells to gain direct access to the injury area (Pedersen *et al.,* 1998; Jarvinen *et al.,* 2005). Neutrophils are the most abundant immune cells at the injury site during the first couple of hours and days after injury, whilst macrophages (M1 followed by M2) appear a couple of days later (Li *et al.,* 2001). For a chronological illustration of immune cell involvement after injury, see Figure 1.1. As part of the phagocytic process, these immune cells release substantial amounts of reactive species to aid in the degeneration of necrotic areas, but this process can involve surrounding healthy tissue, thereby contributing to secondary damage (Fialkow *et al.,* 2007). In addition, both neutrophils and macrophages also differentially secrete cytokines to attract more immune cells to the injured area for fast and effective regeneration (see Table 1.3 on pg 27).

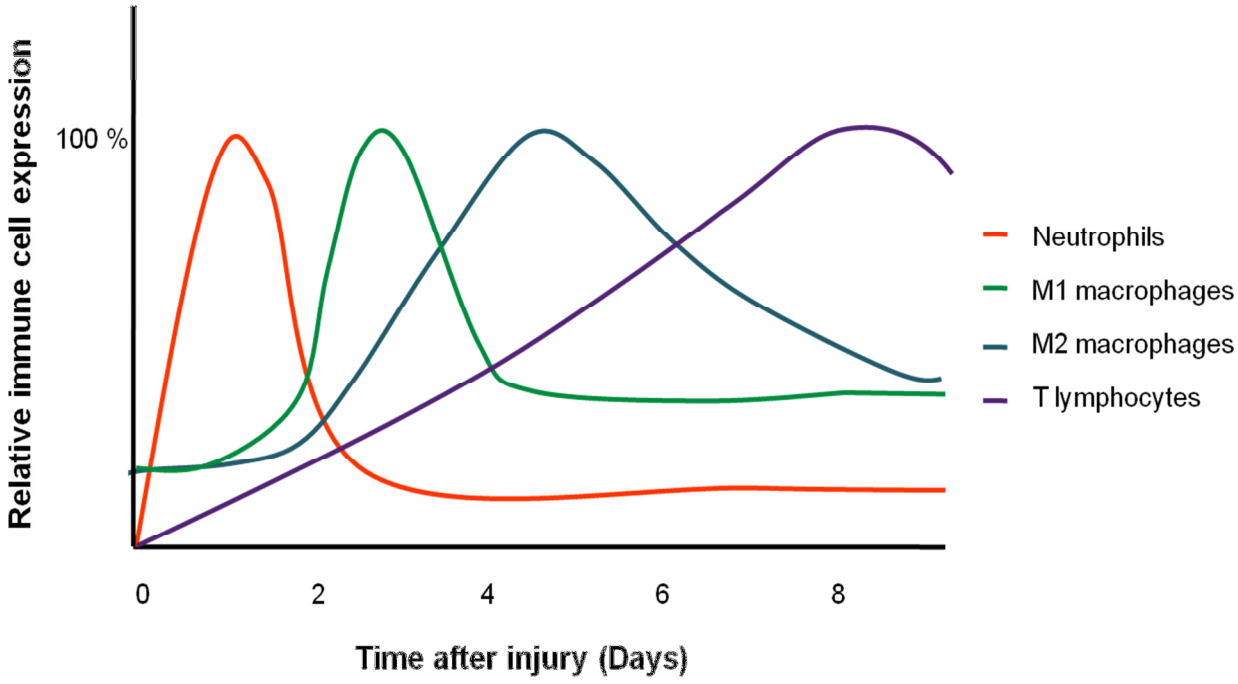


Figure 1.1: An illustrative time course of inflammatory immune cells after injury. A 100 % cell presence indicates a specific point in time where the specific immune cell had a peak response after injury. The duration of this inflammatory response depends on the type and severity of the injury. Reproduced from Smith *et al.* (2008).

To date, interactions between the immune system and skeletal muscle after trauma have not been studied as extensively as the histological assessment of muscle inflammation, following acute muscle injury or diagnosis of inflammatory myopathies (Authier *et al.,* 1997; Liprandi *et al.,* 1999; Confalonieri *et al.,* 2000). Nevertheless, the scientific literature suggests that immune cells may play a greater role in the regulation of muscle development, repair and regeneration than only the removal of debris. The purpose of this thesis is therefore to further elucidate the relationship between these two systems (specifically inflammatory immune cells and satellite cells) during the response to a contusion injury, focussing on the various role players involved in the inflammatory process.

In this chapter, I will give a basic overview of satellite cell and immune cell physiology and how they interact with one another. I will also provide an overview of the available literature on the reaction of these cells to a muscle contusion injury. In Chapter 2, I will discuss the different treatment regimens for muscle injury in general, and then focus on grape seed-derived proanthocyanidin (PCO, Oxiprovin™), a natural supplement with potential to aid muscle recovery. Chapter 3-5 will focus on experiments investigating both mechanism of action and *in vivo* efficacy of treating muscle contusion injury with proanthocyanidin using two different treatment regimens. A synthesis of results and conclusions are presented in Chapter 6.

1.2 Satellite cells

Skeletal muscle regeneration after muscle injury involves the degeneration of damaged muscle fibres, removal of debris and the formation of new myofibres through myogenesis. As mentioned in the introduction, activation of satellite cells usually results in the formation of reactive species (Scime and Rudnicki 2006), which will be discussed in more detail below (refer to section 1.2.4). The involvement of these satellite cells in muscle regeneration is also characterised by the expression of different myogenic regulatory factors (MRFs) (see section 1.2.3).

In order to understand the literature on satellite cell function in muscle recovery, it is necessary to first consider different labelling systems used to identify and/or quantify these cells in their different stages of activation, proliferation and differentiation.

1.2.1 Choosing the appropriate satellite cell marker(s)

Satellite cells can be characterized by their expression of conserved molecules such as M-cadherin (M-cad) (Irintchev *et al.,* 1994), CD34 (Beauchamp *et al.,* 2000), CD56 (Kadi and Thornell 2000), Msx1, cMet (Cornelison and Wold 1997), MNF or Foxk1 (Garry *et al.,* 2000) and Pax-7 (Cornelison and Wold 1997; Beauchamp *et al.,* 2000; Cornelison *et al.,* 2000; Seale and Rudnicki 2000), as well as by their typical morphological appearance. However, although all of these markers can be used to identify satellite cells, SCs do not represent a unique cell type but a rather heterogeneous population of muscle precursor cells in various stages of activation, proliferation and differentiation (see Table 1.1 on p 5). Furthermore, although some satellite cell markers, such as Pax-7, CD56, or CD34 are expressed at various stages during injury, the expression pattern differs, with expression being high during quiescence, followed by decreasing expression during activation and proliferation. Also, not all the molecular markers are unique to SCs, e.g. CD56 is also present on a number of other cell types. Although a comprehensive list of SC markers are presented in Table 1.1, only SC markers used in the experimental sections of this study will be discussed in detail below.

CD34 has been widely used as a marker of quiescent satellite cells (Beauchamp *et al.,* 2000). However, it became apparent that CD34 is not only expressed by satellite cells in their quiescent state, but also by haematopoietic stem/progenitor cells (Krause *et al.,* 1996), as well as by small-vessel endothelial tissue (Baumhueter *et al.,* 1994). Although researchers have commonly used CD34 as a marker of quiescence, CD34 expression has also been present during activation and progression towards self-renewal or differentiation (Sato *et al.,* 1999b). Therefore, CD34 may not be such a good singular marker for distinguishing only one population of satellite cells.

Table 1.1: Expression patterns of satellite cell markers in adult skeletal muscle after injury. Compiled from Charge and Rudnicki (2004), Hawke and Garry (2001) and Zammit *et al.* (2006).

Molecular Marker	Quiescent SCs	Activated SCs	Proliferating SCs	Myoblasts	Adult myofibres
MNF	High	Low	Low		
Pax-7	High	Low	Low		
c-Met	High	Low	Low		
CD56/NCAM	High	Low	Low		
CD34	High	Low	Low		
VCAM-1	High	Low	Low		
M-cadherin	Low	High	Low		
myf5		High	Low		
Pax-3		Low	High, Low	Low	
BrdU (uptake)			High, Low		
PCNA			High, Low		
[3H]thymidine (uptake)			High, Low		
MyoD		Low	High	Low	Low
Desmin		Low	Low	High	High
Myogenin			Low	Low	High
MRF4				Low	High

High indicates that the level of the expression of the various markers is at its highest, whereas low indicates where the expression is at its lowest. High, followed by low as is the case with Pax-3, BrdU, PCNA and [3H]thymidine, indicates that expression is high early on in proliferation, and low later in the proliferation cycle. Abbreviations: MNF, myocyte nuclear factor; NCAM, neural cell adhesion molecule; VCAM, vascular cell adhesion molecule; BrdU, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; MRF, myogenic regulatory factor.

CD56/NCAM recognizes the neural cell adhesion molecule (NCAM), which is expressed during developmental myogenesis and in adult muscle satellite cells (Schubert *et al.,* 1989). However, CD56 is also expressed in nerves, natural killer (NK) cells, neuromuscular junctions and even occasionally in the cytoplasm of myofibres (Kaufmann *et al.,* 1999; Kadi

and Thornell 2000). According to various researchers, satellite cells are closely attached to the plasma membrane of the adjacent muscle fibre and express CD56 in the quiescent state (Bornemann and Schmalbruch 1994; Irintchev *et al.,* 1994; Schultz and McCormick 1994), as well as in the activated state (Malm *et al.,* 2000), indicating once again the non-specificity of singular markers. Due to this non-specificity of the satellite cell markers, analysis to distinguish satellite cells from other cells, often requires not only the satellite cell marker but also co-immunostaining to identify the basal lamina, with for example an antibody that recognises laminin.

Pax-7 has been described as a member of the paired homeobox gene family, which is expressed during somite development (Jostes *et al.,* 1991). In a study on Pax-7 knockout (KO) mice, it was revealed that Pax-7 is an essential transcription factor for satellite cell formation (Seale *et al.,* 2000). In this study, wild-type or Pax-$7^{-/-}$ KO mice exhibited severe muscle deficiencies at birth and premature mortality, and were completely devoid of satellite cells. More recently, Pax-7 was suggested to be a better marker due to its specificity in adult muscle for satellite cells (SCs) and the availability of effective antibodies against Pax-7 (Zammit *et al.,* 2006; Day *et al.,* 2007). Pax-7 is expressed in the nucleus of the majority of, if not all, satellite cells [quiescent, active, and proliferating SCs, but not myonuclei] (Seale and Rudnicki 2000; Collins *et al.,* 2005; Relaix *et al.,* 2005; Shefer *et al.,* 2006). In a study by Halevy *et al.* (2004), Pax-7^{+} cells were present in the intact muscle underneath the myofiber basement membrane, and usually associated with the SC nucleus. Pax-7 expression also declined as the cells moved into the myogenin-expression state, while the expression of MyoD was still sustained. Since some cells within the Pax-7^{+} cell population were positive for both Pax-7 and other markers, while some were only positive for Pax-7, it was thought that the latter population of cells might represent “reserve” myogenic progenitors (Halevy *et al.,* 2004). It was also postulated that Pax-7 is a major regulator of SC renewal. This function of Pax-7 was suggested because a proportion of activated satellite cells that remained undifferentiated, still retained their Pax-7 expression and was therefore thought to reconstitute the satellite cell pool (Olguin and Olwin 2004; Oustanina *et al.,* 2004; Zammit *et*

al., 2004). Pax-7 expression may be influenced by age and muscle fibre type, and is also species specific (Hawke and Garry 2001), indicating that if injury was sustained in a slow-twitch fibre type-specific muscle, Pax-7 expression would differ compared to the expression in a fast-twitch muscle. After reviewing the literature on satellite cell markers, Pax-7 is considered the most suitable marker and should be used in combination with a basal lamina component such as laminin to enable more accuracy.

1.2.2 Satellite cell response after injury

Skeletal muscle regeneration is regulated through mechanisms involving cell-cell and cell-matrix interactions as well as extracellularly secreted factors (Charge and Rudnicki 2004). In mature muscle, satellite cells are quiescent but can re-enter the cell cycle in response to a variety of stimuli, which promote their activation and proliferation to form myoblasts, which differentiate and fuse to each other or to damaged myofibers (Hawke and Garry 2001). Satellite cell activation may be restricted to the damaged area. However, if the connective tissue between muscle fibres is also damaged, satellite cells may also be recruited from adjacent fibres (Schultz *et al.,* 1985; Schultz *et al.,* 1986).

Perhaps the most extensive and reproducible model for severe muscle injury is the delivery of cardiotoxin (purified from the venom of the *Naja nigricollis* snake) into the hindlimb skeletal muscle of the mouse (Doroshow *et al.,* 1985; Nicolas *et al.,* 1996; Garry *et al.,* 2000). The intramuscular injection of 100 µl of 10 µM cardiotoxin into the *gastrocnemius* muscle results in 80–90% muscle degeneration. After cardiotoxin-induced injury, satellite cells become activated within 6 hr of injury (Garry *et al.,* 2000). In response to locally released growth factors from injured myotubes and macrophages, the satellite cells proliferate extensively within 2–3 days of injury (Garry *et al.,* 1997; Garry *et al.,* 2000). Approximately 5 days after injury, the satellite cells withdraw from the cell cycle and either self-renew or form differentiated myotubes containing a central nucleus (Garry *et al.,* 1997; Garry *et al.,* 2000).

In this cardiotoxin-induced injury model, the architecture of the injured muscle is largely restored within 10 days after injection.

In a study by Oustanina *et al.* (2004) on freeze-crush or cardiotoxin-induced skeletal muscle damage, both the number and the staining intensity of Pax-7-lacZ (allele for β-galactosidase activity) -positive cells increased, reflecting an expansion of satellite cell-derived myoblasts in the injured muscle and an upregulation of Pax-7 gene expression in proliferating myoblasts. Satellite cells on single fibres (*in vitro*) become activated by an extract of crushed muscle (Bischoff 1986). The relevant factors in the extract could be numerous and required more specific studies to be determined. Mice deficient for fibroblast growth factor (FGF)-6 (i.e., knockout mice at the FGF-6 locus) have impaired satellite cell proliferation and a subsequent defect in muscle regeneration in response to a crush injury. However, it is not only external factors that play crucial roles, but also the balance between factors endogenous to the satellite cells themselves, as these are not all similarly activated and regulated by the injury.

Kuang *et al.* (2006) established that Pax-7 is a prerequisite for muscle regeneration. With the use of Pax-7/LacZ and Pax-7-null/LacZ mice, it became apparent that in the absence of Pax-7, the presence of resident stem cells in the muscle was not sufficient to compensate for a loss of satellite cell function, severely affecting regenerating muscle (Kuang *et al.,* 2006). In addition, several recent studies have reported that newly activated satellite cells and proliferating myoblasts co-express Pax-7 and MyoD *in vitro* (Halevy *et al.,* 2004; Olguin and Olwin 2004; Zammit *et al.,* 2004), but that overexpression of Pax-7 down-regulates MyoD in response to injury, and promotes cell-cycle withdrawal from the proliferating state prior to commitment to differentiation, therefore playing a critical role in the maintenance of the satellite cell pool (Olguin and Olwin 2004; Zammit *et al.,* 2004). The extent to which SCs respond to damage-induced myogenesis, relies on the expression of Pax-7 and the myogenic regulatory factors (MRFs) (Zammit *et al.,* 2006), which will be the focus of the next section.

1.2.3 Myogenic regulatory factors

The regeneration of muscle following injury, occurs *via* a series of SC stages (activation, proliferation and differentiation of SCs) which are subjected to positive and negative regulatory influences, facilitated by the myogenic regulatory factors (MRFs), MyoD, Myf5, myogenin and MRF4 (Yablonka-Reuveni *et al.,* 1999a). MRFs are only expressed in the nucleus when satellite cells are activated, and contain a conserved basic DNA binding domain (Weintraub *et al.,* 1991). This is in contrast to adhesion molecules which are found on either the satellite cell niche membrane or expressed as nuclear adhesion molecule.

Different studies have identified specific time of first appearance/expression and function of the myogenic basic helix-loop-helix transcription factors, the first of which are Myf5 and MyoD. MyoD and Myf5 are expressed early during satellite cell activation and proliferation, during muscle development, and are involved in the determination of the myogenic lineage (Braun *et al.,* 1992; Rudnicki *et al.,* 1992; Rudnicki *et al.,* 1993; Weintraub 1993). Myf5 and Pax-7 are expressed in quiescent satellite cells (Beauchamp *et al.,* 2000), while MyoD is expressed as the cells become activated, proliferating and subsequently differentiate (Yablonka-Reuveni and Rivera 1994) (Figure 1.2). The expression of myogenin and MRF4 are upregulated in myoblasts beginning their terminal differentiation program, which is followed by permanent exit from the cell cycle (Yablonka-Reuveni *et al.,* 1999a; Yablonka-Reuveni *et al.,* 1999b).

These MRFs also activate the transcription of muscle-specific proteins through binding to a DNA consensus sequence known as the E-box which is present in the promoter of numerous muscle genes - such as acetylcholine receptor α subunit (Piette *et al.,* 1990), creatine kinase (CK) (Lassar *et al.,* 1989) and sarcomeric myosin. In a small number of activated satellite cells, Myf5/MyoD expression is down-regulated, resulting in them returning to a quiescent state to maintain a more or less constant pool of satellite cells (Zammit *et al.,* 2006).

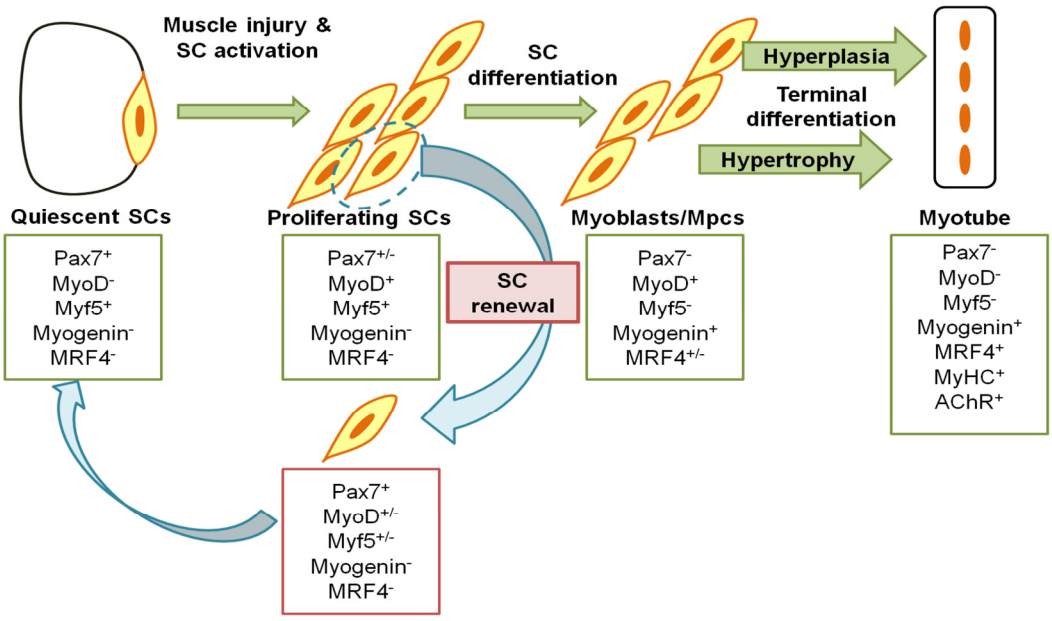


Figure 1.2: The role of MRFs in muscle regeneration. In response to injury, normally quiescent satellite cells, re-enter the cell cycle, and start their proliferation and differentiation cycle in order to either fuse to produce new myofibers (hyperplasia) or fuse to damaged myofibers (hypertrophy) to regenerate muscle fibres. The myogenic regulatory factors (MRFs) play a key role in driving the regeneration process. Abbreviations: AChR (acetylcholine receptor α); MyHC (myosin heavy chain). Adapted from Zammit *et al.* (2004).

1.2.4 Muscle-derived reactive species and secondary damage

Even in the absence of injury or pathology, reactive oxygen species (ROS) are continuously being produced by skeletal muscle to stimulate local adaptive responses, as well as to mediate some degenerative processes (McArdle *et al.,* 2001; Murrant and Reid 2001). Oxidative stress is quantified in more than one way: (a) using indirect markers based on end-point products of the reactions of ROS and reactive nitrogen species (RNS) with lipids, proteins, or DNA (Davies *et al.,* 1982; Jackson *et al.,* 1985), or (b) by evaluating the activities of the main antioxidant enzymes, which include manganese superoxide dismutase (SOD), copper, zinc SOD, catalase, and glutathione peroxidase (Murrant and Reid 2001). Skeletal muscle tissue and particularly the role of ROS within muscle have been examined using both *in vivo* (O'Neill *et al.,* 1996) and *in vitro* (Reid *et al.,* 1992) studies. In the next few

paragraphs, I will focus on studies elucidating sources of ROS as well as their effect in muscle tissue within the context of injury.

Nitric oxide (NO) appears to be one of the most important regulators of muscle inflammation and subsequent muscle damage by invading inflammatory cells and is considered to be one of the most common reactive species produced in muscle as a result of injury. However, it should be noted that it is not clear whether NO production has a positive or negative effect after injury, since various studies have been done which justify both outcomes.

By focussing on the positive effects first, an *in vitro* co-culture study has indicated that muscle-derived NO reduces neutrophil-mediated lysis of damaged muscle cells, resulting in decreased superoxide production (Nguyen and Tidball 2003). A possible explanation for this positive effect is that NO scavenged the superoxide radicals released by neutrophils in order to prevent its conversion into a more cytotoxic oxidant (Rubanyi *et al.,* 1991). Another possibility is that NO inhibited the activity of NADPH oxidase, so that superoxide production was reduced (Clancy *et al.,* 1992). Satellite cells are one of the "first responders" to an injury-induced release of nitric oxide *via* nitric oxide synthase (NOS) (Schultz and McCormick 1994), demonstrating that satellite cell activation (*in vivo* and *in vitro*) is mediated, at least in part, by NO (Anderson 2000). NO is known to be released by the vascular endothelium, smooth muscle cells, myocardium, platelets, monocytes and macrophages after an injury (Moncada 1997). Inhibition of NO, through the injection of a NOS inhibitor (Nω-nitro-L-arginine methyl ester, L-NAME), results in the suppression of satellite cell activation (Anderson 2000). NO is also able to inhibit the transcriptional activation of the intercellular adhesion molecule (ICAM), necessary for the adhesion of neutrophils to the vascular endothelium (Berendji-Grun *et al.,* 2001), thereby limiting the number of neutrophils gaining access to the injured area *via* blood (Gauthier *et al.,* 1994; Niu *et al.,* 1994; Akimitsu *et al.,* 1995; Adams *et al.,* 1997; Aljada *et al.,* 2000). Therefore, since it is known that NO inhibits the infiltration of neutrophils into the injured area, an injury in which the vasculature remains intact (e.g. mild contusion injury), will result in a decrease in neutrophils infiltrating the injured

area compared to an injury in which the vasculature is disrupted (e.g. severe contusion injury) – which will result in free flow of neutrophils into the injured area and thus exacerbation of injury. These studies therefore indicate that NO plays a role in muscle regeneration through the activation of SCs and though the suppression of neutrophil infiltration into the injured area.

Moving on to the negative aspects of NO: *in vitro* and *in vivo* studies indicate that specifically M1 macrophages can promote muscle damage by the production of cytotoxic levels of NO generated by inducible nitric oxide synthase (iNOS) (Nguyen and Tidball 2003; Villalta *et al.,* 2009). This is most probably due to the fact that satellite cells, after activation and prior to proliferation, release factors which attract more macrophages and monocytes to the injured area and these cells produce more NO than needed, which could result in NO-dependent cytotoxicity (Chazaud *et al.,* 2003). These studies illustrating negative effects of NO indicate that the amount of NO expressed plays a very important role in facilitating either muscle regeneration or further muscle damage.

Thus from these studies it is clear that NO, released by both neutrophils and M1 macrophages, influences satellite cells, which suggests that an increase in NO should be expected during the early stages of muscle injury (Tidball and Villalta 2010), i.e. during day 1 and 2 after injury (see Figure 1.1). In a study on spinal cord injury, an increased number of iNOS positive cells in the perivascular space were evident on day 1 and day 2 after injury (Satake *et al.,* 2000), corroborating the abovementioned statement of an increase in NO in the early stages after injury.

Although one of the best known reactive species studied is NO, it was demonstrated in a study by O'Neill (1996) that contracting skeletal muscle is also able to generate hydroxyl radicals, detectable in muscle microvasculature. The cellular origin of these radical species was at first not defined due to the fact that muscle tissue *in vivo* contains numerous other cell types, which includes endothelial cells, fibroblasts, and lymphocytes, that may potentially contribute to the generation of free radical species. However, an *in vivo* study on contracting

myotubes, demonstrated that skeletal muscle cells, rather than other cells in the tissue, are able to release superoxide when stimulated by means of an acute bout of unaccustomed exercise (McArdle *et al.,* 2001).

After muscle injury, myocytes and other cells release a number of cytokines, such as interleukin (IL)-1β, IL-6, IL-8, and tumour necrosis factor (TNF)-α, that cause neutrophils to become activated (Best *et al.,* 1999; Brickson *et al.,* 2001). These cytokines all stimulate pathways that contribute to activation of the enzyme NADPH oxidase, which generates a "respiratory burst" and the subsequent release of reactive oxygen species, such as hydrogen peroxide (H_20_2), superoxide anions (0_2^-), and hydroxyl radicals (HO^-) (Abramson and Weissmann 1989; Kerr *et al.,* 1996; Ali *et al.,* 1997). Although phagocytosis and the neutrophil respiratory burst are important mechanisms in the early phase inflammatory response to muscle injury, they can damage the injured muscle even further, and may result in damage to the healthy tissue bordering the injured area.

Therefore, the immune system is a critical component and is involved in both the injury and repair process.

1.3 The inflammatory immune system

The immune system, which involves immune organs and systems (bone marrow, spleen and lymphatic system), the immune cells (leukocytes/white blood cells – WBCs) and cytokines, plays a very important role during all types of inflammation. For the inflammatory response to be beneficial it must first allow for a quick and efficient inflammatory stimulus, which then subsides to allow the tissue to return to its pre-inflamed state.

Clinically, the inflammatory reaction is characterised by various symptoms, such as fever, redness, swelling and pain. These symptoms are mainly caused by downstream effects of histamine, which include vasodilation, increased vascular permeability, oedema and appearance of acute phase proteins. Within seconds after tissue injury, the permeability of

the surrounding capillaries is altered, leaking fluid, plasma proteins and immune cells into the surrounding tissue (Deal *et al.,* 2002; Sherwood 2004b). In case of substantial tissue damage, this initial reaction is quickly followed by an infiltration of white blood cells, predominantly neutrophils, from blood vessels into the tissue (Jarvinen *et al.,* 2005). This pro-inflammatory response can reach major proportions within a few hours of injury, most probably due to the increased transfer of neutrophils from the bone marrow and spleen into the blood, as a result of chemical mediators released from the inflamed tissue (Sherwood 2004b; Tidball 2005). During the next few days, neutrophil numbers decrease, while the affected tissue is infiltrated by mononuclear cells, primarily macrophages (Li *et al.,* 2001). For the purpose of this thesis, in the next few sections, specific attention will be paid to the inflammatory WBCs, specifically neutrophils and macrophages, and their respective roles after contusion injury. Both neutrophils and macrophages are phagocytic – however, their roles in inflammation are quite different, as discussed below.

1.3.1 Neutrophils

Neutrophils are blood-borne leukocytes which originate in the bone marrow, and migrate into the tissue upon injury or trauma. Almost immediately following an injury (as soon as 1 hr), neutrophils form the predominant immune cell infiltrate. Neutrophils migrate into the injured area through areas of intact capillary endothelium and across layers of basement membrane and sarcolemma (Menger and Vollmar 1996). This occurs by way of rolling along (*via* selectins), adhesion to (*via* integrins) and then migration into the injured tissue. The triggers for neutrophils to enter the injured area are usually chemical mediators from the injured area itself (Sherwood *et al.,* 2004; Tidball and Wehling-Henricks 2005) - including complement components (C5), pro-inflammatory prostaglandins (PGE_2 and $PGF_{2\alpha}$) and leukotrienes (LTB_4) (Shen *et al.,* 2006), as well as factors released by activated platelets (TxA_2, serotonin and histamine) (Marder *et al.,* 1985).

Neutrophils have 2 main functions after injury. Firstly, the infiltrating neutrophils have a phagocytic function (Tiidus 1998), clearing the wound of blood-derived fibrin (Jarvinen *et al.,*

2005) and necrotic material. Secondly, they strengthen the inflammatory process *via* the release of pro-inflammatory cytokines such as IL-6 and TNF-α (Rosenberg and Gallin 1993; Cannon and St Pierre 1998; Tidball and Wehling-Henricks 2005) (For more information regarding the inflammatory cytokines, see section 1.5.2). However, both these functions may have undesired side-effects. Firstly, an *in vivo* study by Nguygen and Tidball (2003) reported that neutrophils lyse muscle cells through superoxide-dependent mechanisms, which is consistent with earlier *in vitro* findings (Simchowitz and Spilberg 1979; Markey *et al.,* 1990; Srinivasan *et al.,* 1997). In addition, myeloperoxidase (MPO), an enzyme present in neutrophils and macrophages, can generate hypochlorous acid, a highly reactive oxidising agent. Secondly, pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α), which result in the activation of more neutrophils, also stimulate pathways that contribute to activation of the enzyme NADPH oxidase (located on activated neutrophils), which generates a neutrophil respiratory burst and the subsequent release of reactive oxygen species. Specifically, superoxide anions can quickly be converted into hydrogen peroxide (Best *et al.,* 1999; Brickson *et al.,* 2001) [See Figure 1.4 for more detail]. Although it has been observed in studies of single stretch that peak damage occurs at the same time as maximal neutrophil infiltration of the injured tissue (Brickson *et al.,* 2001; Schneider *et al.,* 2002), the exact time course of the appearance of inflammatory cells and secondary muscle damage may vary depending on the extend and type of muscle damage. Identification and inhibition of the molecule(s) responsible for promoting neutrophil migration into and within skeletal muscle may lead to treatment options for alleviating neutrophil-mediated muscle injury. Furthermore, inhibiting or limiting neutrophil infiltration into the injured area may also play an important role in restoring normal muscle fibre ultrastructure sooner. Methylprednisolone (MP – a corticosteroid) decreased the expression levels of adhesion molecules and integrins on the leukocyte surface, therefore resulting in less neutrophils infiltrating the injured muscle area (Bartholdi and Schwab 1995; Droogan *et al.,* 1998; Xu *et al.,* 1998; Rabchevsky *et al.,* 2002). Another study, using R(-)-2-(4-isobuthylphenyl)propionyl methanesulfonamidel (reparixin, for 7 days), a small-molecule inhibitor of human CXCR1/R2 and rat CXCR2 receptor activation, was also very effective in preventing neutrophil infiltration into injured spinal cord, and

therefore significant improvement in hind limb recovery of function (Bertini *et al.,* 2004; Cugini *et al.,* 2005; Garau *et al.,* 2005). These studies indicate that by limiting the neutrophil response, muscle regeneration may be accelerated.

Various cytokines and chemokines play a significant role as chemoattractants for neutrophils. Skeletal muscle cells can produce and release a variety of molecules, including cytokines and CXC and CC chemokines (Nagaraju *et al.,* 1998; De Rossi *et al.,* 2000; Reyes-Reyna and Krolick 2000; Alvarez *et al.,* 2002) in order to promote neutrophil chemotaxis (Olson and Ley 2002; Gouwy *et al.,* 2005). Most of these studies have focussed on IL-8, since it has been shown that low IL-8 concentrations (10-50 ng/ml) activate the two IL-8 receptors (CXCR1 and CXCR2), either by inducing neutrophil accumulation at inflammatory sites or by increasing the number of circulating neutrophils, and therefore resulting in neutrophil chemotaxis (Leonard *et al.,* 1991; Kurdowska *et al.,* 1994; Ben-Baruch *et al.,* 1995; Ben-Baruch *et al.,* 1997a; Ben-Baruch *et al.,* 1997b; Ludwig *et al.,* 1997; Wolf *et al.,* 1998; Zaslaver *et al.,* 2001). High doses on the other hand (1000 ng/ml) prevented specific neutrophil migration into inflamed tissue, indicating a dose dependent shift from activation to attenuation, which suggests that the interplay between different cellular factors are regulated by dose (Leonard *et al.,* 1991; Ben-Baruch *et al.,* 1997a; Ben-Baruch *et al.,* 1997b; Ludwig *et al.,* 1997).

Another potential candidate is IL-1, which has been shown to be involved in the regulation of neutrophil adhesion and migration *in vivo* and *in vitro* in several species (Bevilacqaa *et al.,* 1985; Sayer *et al.,* 1988; Moser *et al.,* 1989). In one of the *in vivo* studies, direct injection of 0.005-5 ng/ml of either IL-1α or β resulted in increased neutrophil chemotaxis into the peritoneal cavity (Sayer *et al.,* 1988). However, the role of IL-1 in stimulating neutrophils to migrate across cellular barriers is a bit more complex in *in vitro* conditions. When neutrophils were stimulated with IL-1, it did not result in neutrophil chemotaxis, suggesting that IL-1's role in neutrophil chemotaxis could be more indirect than direct and possibly involve the activation of other cytokines or chemokines, such as TNF-α, to allow for neutrophil

chemotaxis. Similar results were also evident when injecting granulocyte macrophage colony stimulating factor (GM-CSF), IL-6, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and TNF-α into skeletal muscle alone or after mechanical loading of skeletal muscle (Cassatella 1999; Wolach *et al.,* 2000).

It is clear that not only one chemokine is responsible for neutrophil chemotaxis. Furthermore, various adhesion molecules are also involved in regulating neutrophil migration, e.g. ICAM and vascular adhesion molecule (VCAM).

The pro-migratory/chemotactic responses of neutrophils and macrophages, and endothelial cells, are mediated by integrins, cellular adhesion and the rearrangement of the actin cytoskeleton. The selectin family of adhesion molecules plays a very important role in the attachment or tethering of flowing neutrophils to the vessel wall through adhesions that permit neutrophils to roll along the blood vessel wall (Lawrence and Springer 1991; Mayadas *et al.,* 1993). Three members form part of the selectin family, namely L (leukocyte) -selectin, P (platelet) -selectin, and E (endothelial) -selectin (Bevilacqua and Nelson 1993). L-selectin is the only selectin expressed on all leukocytes, except for a subpopulation of memory lymphocytes (reviewed in Springer 1995). The immunoglobulin superfamily acts as integrin ligands and include the ICAM-1 (CD54), ICAM-2 (CD102), VCAM1 (CD106), platelet-endothelial cell adhesion molecule-1 (PECAM-1 or CD31), and the mucosal addressin (MAdCAM-1) (Carlos and Harlan 1990). Among these, ICAM-l and ICAM-2 are the major endothelial receptors for the neutrophil β2 integrins. Activation of neutrophils by chemotactic factors upon injury, and the interaction between selectins, the immunoglobulin superfamily members and integrins, results in transmigration of neutrophils into the injured area from the vasculature (Jones *et al.,* 1995; Springer 1995).

In summary, once acute inflammation occurs, circulating peripheral neutrophils accumulate at the inflammatory site, a phenomenon established by the production of various chemoattractants, activation of neutrophils and especially the expression of ICAM on the cell

surface. This, in combination with chemotactic factors will therefore result in more immune cells gaining access to the injured area.

1.3.2 Monocytes and macrophages

Monocytes arise from haematopoietic stem cells in the bone marrow (Sherwood 2004a). Under steady-state conditions, low-level recruitment of monocytes to tissues *via* circulation, followed by their transition into macrophages, accounts for resident macrophage populations (McLennan 1993). Following a local insult, production and recruitment of monocytes to the injured area are greatly increased (Figure 1.3, p 19). The recruited macrophage populations may also undergo further cytokine-mediated migration to the site of injury, resulting in even greater macrophage recruitment to the injured site. Their cell numbers peak roughly on day 5 after injury, although significant numbers are already present on day 1 or 2, when neutrophil numbers start to decline (Tidball and Wehling-Henricks 2005).

Unlike neutrophils, macrophages can be divided into subpopulations depending on their localization (ED typing) or manner of activation (M typing). An update on the literature regarding time course of macrophages and their subpopulations, as well as their specific roles during various types of injuries, was the focus of a recent review by Tidball and Villalta (2010). M1 macrophages (ED1) are normally recruited by increased expression of interferon (IFN)-γ, TNF-α and GM-CSF as a result of muscle damage. Due to the fact that M1 macrophages are the first subpopulation of macrophages to invade the injured area, they are thought to be important in removing tissue debris *via* phagocytosis (McLennan 1996). However, these macrophages also promote inflammation through the release of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, IL-12 and IL-23, as well as the production of other effector molecules such as reactive oxygen and nitrogen intermediates and thereby might exacerbate the injury process in a manner similar to that of neutrophils (Goerdt *et al.,* 1999; Mills *et al.,* 2000; Mosser 2003). M1 macrophages promote muscle damage *in vitro* and *in vivo* in the presence of neutrophils at relatively low levels. In an *in*

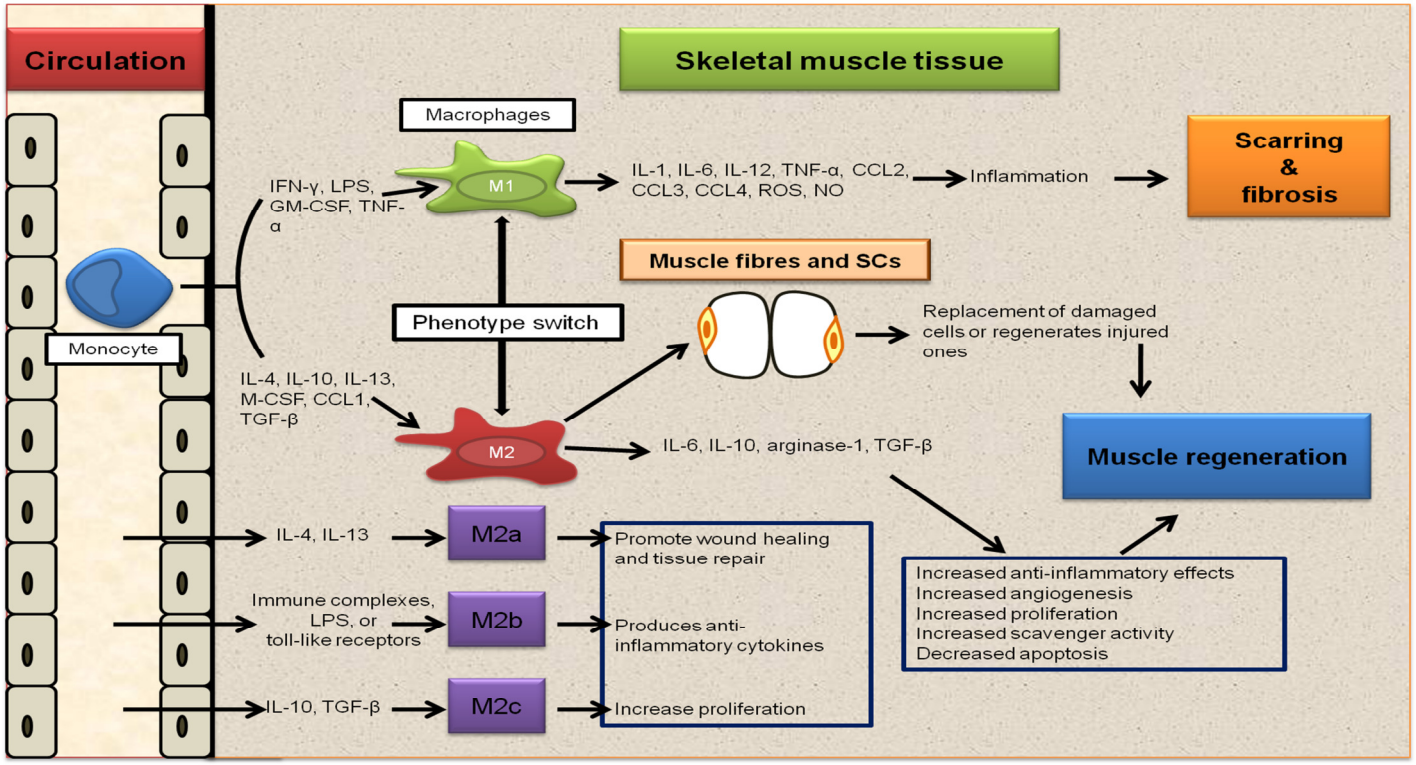


Figure 1.3: The role of the different macrophage subpopulations in muscle recovery from injury. Upon injury, monocytes are recruited to the injured area, and are activated as a result of various pro- or anti-inflammatory cues. These macrophages can then either worsen the inflammatory process (M1 macrophages - early response), or result in muscle regeneration (M2 - later response), either by the factors it releases, or by direct interaction with injured muscle fibres and satellite cells. Furthermore, depending on the time of the response, M1 macrophages can switch to a M2 phenotype, although the mechanism that regulates this transition is still unclear. Abbreviations: IFN, interferon; LPS, lipopolysaccharide; GM-CSF, granulocyte macrophage colony stimulating factor; TNF, tumour necrosis factor; M-CSF, macrophage colony stimulating factor; TGF, transforming growth factor; IL, interleukin; ROS, reactive oxygen species; NO, nitric oxide; SCs, satellite cells. Adapted from the following authors: (Mantovani *et al.,* 2004; Ricardo *et al.,* 2008; Tidball and Villalta 2010).

vitro study by Nguyen and Tidball (2003), muscle membrane damage (lysis) of myotubes through superoxide-dependent mechanisms was also detected when macrophages (> 90 % ED1 and the rest ED2) were present at macrophage/neutrophil ratios (10:1). This is the ratio that usually occurs in muscle injury and inflammation at the stage of injury when muscle membrane lysis occurs (roughly on day 2-4) (Tidball *et al.,* 1999). In this study, the neutrophils and M1 macrophages were added in relatively low numbers (500 cells/mm^2 and 5000 cells/mm^2 respectively) to muscle co-cultures, compared to the normal levels (25 000 cells/mm^2) and this resulted in the activation of a greater proportion of the macrophages, thereby promoting their overproduction of NO and thus greater myoblast cytotoxicity. Since it is also known that muscle cells can produce NO, it is quite possible that the high levels of NO detected in the study by Nguyen and Tidball (2003) may be as a result of myoblasts themselves. SCs within injured muscle can release NO and other free radicals upon activation (Schultz and McCormick 1994; Anderson 2000; Chazaud *et al.,* 2003; Pisconti *et al.,* 2006), and may therefore contribute towards increased muscle membrane damage or lysis. However, the increase in NO in Nguyen and Tidball's study (2003) was as a result of iNOS (specific to immune cells) in macrophages and not nNOS (specific to skeletal muscle) in muscle, indicating that muscle cells might not have contributed to the NO-dependent cytotoxicity in this specific situation.

After M1 macrophages have reached their peak concentration in injured and regenerating muscle, they are either replaced by or switched to a population of M2 (alternatively activated) macrophages that can attenuate the inflammatory response and promote tissue repair. M2 macrophages in general, are activated by a variety of T helper 2 (T_H2) cytokines, namely IL-4, IL-10 and IL-13 (Gordon 2003). ED2 macrophages differ from total M2 macrophages in the sense that the ED2 macrophage population is comparable with M2 macrophages with an immunosuppressive profile, in other words the M2c macrophages (Mantovani *et al.,* 2004). In a cell culture study by Massimino *et al*. (1997), it became evident that M2 macrophages played a role in activating satellite cells and myoblast proliferation, thereby contributing to skeletal muscle regeneration and tissue repair. From the reviewed literature on

macrophages, it is evident that M1 macrophages plays a predominant role in removing debris but also contributing to further muscle injury, whereas M2 macrophages aid muscle recovery.

It is also possible that M1 macrophages can change to M2 macrophages when stimulated with different factors (see Figure 1.3). This will not only result in a shift from one phenotype to the next, but will also result in a shift in cytokines produced thereafter. Although it has been proposed that macrophages can switch phenotypes during the course of muscle regeneration, little is known regarding the specific time after injury, although the trigger mechanisms that regulate this transition in macrophage phenotype are better understood. Quantifying the different macrophage subpopulations over time, after an injury, should provide a more complete time line of when the switch from a M1 to a M2 phenotype occurs in that particular injury model. Inconsistencies also exist regarding time courses of inflammation in various different models of injury. For example, ED1 macrophages are evident at 24 and 48 hr after downhill running (Lapointe *et al.,* 2002; Tsivitse *et al.,* 2003), while ED2 macrophages accumulate in injured muscle 2-3 days post-injury (Tidball *et al.,* 1999; Frenette and Tidball 2000; Frenette *et al.,* 2002). In one specific study, a downhill-running study (humans), no elevations in ED2 macrophages were evident 72 hours afterwards (Tsivitse *et al.,* 2003), whereas, lengthening contractions of the dorsiflexor muscles of the ankles (rats) resulted in elevated ED2 macrophages 48 hr afterwards (Lapointe *et al.,* 2002).

Therefore, care should be taken when comparing immune cell infiltration into the injured area after different exercise or different types of injury regimes, as the timing of appearance of the different macrophage populations will vary considerably.

1.4 Effect of WBCs on muscle after injury

During acute muscle injury, the regeneration process of muscle activates a sequence of interactions between muscle and inflammatory cells (see Figure 1.4, pg 23). From the literature it is known that an acute injury can result in the release of chemoattractant molecules that initially attract neutrophils or M1 macrophages into the muscle (Tidball and Villalta 2010) for the inflammatory response. However, this process also differs depending on the amount of time that has elapsed since injury. Human myogenic precursor cells (mpcs, SCs), *in vitro*, were found to selectively and specifically attract monocytes through an endothelial layer in a dose-dependent manner (Chazaud *et al.,* 2003). This property of mpcs to attract monocytes varied according to their differentiation stage: individual chemotactic activity of satellite cells was highest shortly after their release from quiescence and then declined progressively to reach levels similar to that of other cell types at a time corresponding to the late or terminal differentiation SC stage (Chazaud *et al.,* 2003).

However, not only is there communication from muscle cells to WBCs, but a close reciprocal relationship also exist between the WBCs and the muscle. Upon muscle injury, neutrophils release several cytokines (TNF-α, IFN-γ, IL-1β and IL-12) and growth factors (transforming growth factor (TGF-β)) (Cassatella 1999), which are known to inhibit either myoblast proliferation and/or differentiation *in vitro* (Garrett *et al.,* 1992; Hawke and Garry 2001; Langen *et al.,* 2001). Some of the interactions may be more direct: neutrophils have been shown to be involved in satellite cell activation and proliferation *in vitro via* the interaction of the neutrophil α4-integrin receptor with VCAM-1, which is expressed by both myoblast and myotubes (Seale and Rudnicki 2000). Furthermore, neutrophils could also facilitate the resolution of the injury by releasing cytokines, (HGF for SC activation and migration and IL-6 for maintaining SC proliferative state) (Austin and Burgess 1991; Allen *et al.,* 1995; Cassatella 1999; Miller *et al.,* 2000); and/or by promoting the accumulation of macrophages (Merly *et al.,* 1999; Cantini *et al.,* 2002).

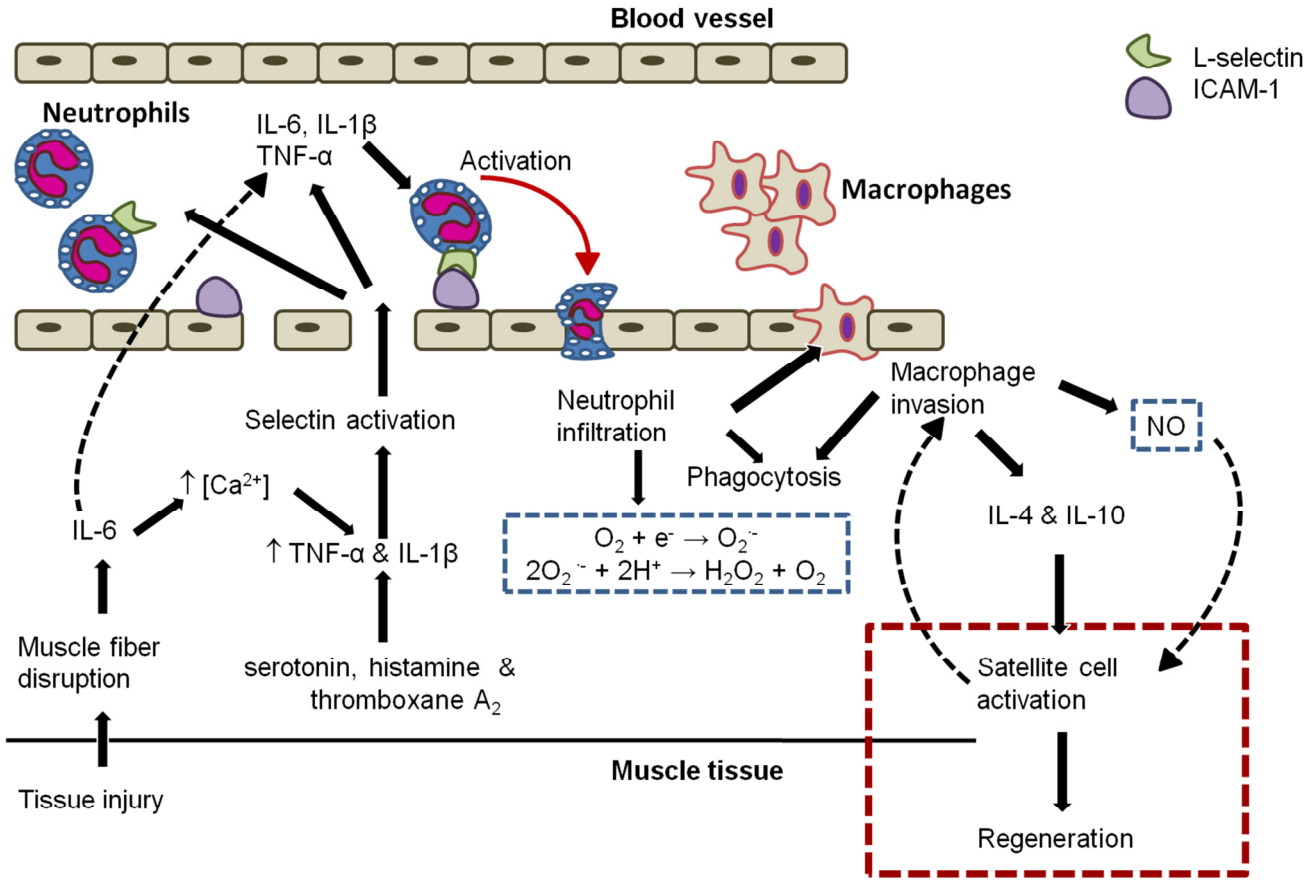


Figure 1.4: The interaction between muscle and immune cells drive the regeneration process. At the onset of an injury, the disruption of muscle fibres, together with products released by activated mast cells and platelets result in the production of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α). These cytokines result in selectin activation and extravasation of immune cells out of the blood into the injured area. Neutrophils result in ROS production, whereas macrophages produce anti-inflammatory cytokines (IL-4 and IL-10) which, together with NO, drives the satellite cell activation process in order to restore normal muscle fibre ultrastructure. O_2: oxygen, e^-: electron, $O_2^{\cdot-}$: superoxide radical, H^+: hydrogen ion, H_2O_2: hydrogen peroxide, NO: nitric oxide.

Macrophages on the other hand play their own vital role in muscle regeneration, since myogenesis is impaired in the absence of macrophage infiltration (Lescaudron *et al.,* 1999). Macrophages, particularly ED2 macrophages were able to increase the number of $MyoD^+$ cells and thus cause myoblast proliferation *in vitro* (Massimino *et al.,* 1997; Giurisato *et al.,* 1998; Merly *et al.,* 1999), indicating that they may contribute to satellite cell activation and proliferation *in vivo* as well. This is probably likely, since it is known that M2 macrophages release cytokines that can promote satellite cell activation and proliferation (Tidball and Villalta 2010). ED1 macrophages on the other hand have been thought to be involved in negatively regulating satellite cells *in vivo* (Kuschel *et al.,* 2000) and can promote muscle damage through the production of cytotoxic levels of NO (Nguyen and Tidball 2003; Villalta *et al.,* 2009).

It is therefore apparent that immune cells do not only have a phagocytic and inflammatory function, but are also able to influence satellite cell activation and muscle regeneration after injury. It is also evident that an inter-relationship exists between muscle cells, macrophages and neutrophils, in that myoblasts recruit macrophages and neutrophils to sites of muscle injury *via* secreting chemotactic factors *vs.* immune cells themselves secreting cytokines to activate more satellite cells.

1.5 Other role players in restoring normal muscle function

In addition to their roles in attracting immune cells to the injured site, cytokines along with growth factors also play very important roles in controlling SC activation, migration of satellite cells to the injury site, proliferation of SC-derived myogenic precursor cells (mpcs) and fusion into myotubes and myofibres. The role of growth factors and cytokines involved in restoring normal muscle function will be discussed in detail in the next few paragraphs. Due to the emphasis in this thesis on the immune system, the effect of growth factors will not be discussed in depth.

1.5.1 Growth factors

Growth factors are proteins that bind to receptors on the cell surface, with the primary result of activating cellular proliferation and/or differentiation. Post-injury, these factors are especially important for maintaining a balance between proliferation and differentiation of SCs to restore normal muscle architecture. Many growth factors are quite versatile, stimulating cellular division in numerous different cell types, while others are specific to a particular cell type (see Table 1.2).

Table 1.2: Growth factors that play a role during muscle contusion injury and recovery.

Growth factor	Source cell	Function	References
TGF-α	Transformed cells	May be important for normal wound healing	(Peters *et al.,* 2005)
TGF-β	Neutrophils Monocytes/macrophages T lymphocytes (TH1) Natural killer cells Platelets Injured skeletal muscle	Promotes wound healing Promotes neutrophil recruitment Inhibits pro-inflammatory cytokine synthesis Increases satellite cell proliferation	(Peters *et al.,* 2005) For review, see (Hawke and Garry 2001) (Haugk *et al.,* 1995)
FGF-2	Monocytes/macrophages Injured skeletal muscle	Promotes proliferation of many cells Attenuates satellite cell differentiation to myotubes Important role in muscle regeneration	(Sheehan and Allen 1999) For review, see (Hawke and Garry 2001) (Floss *et al.,* 1997)
IGF-1	Monocytes/macrophages Injured skeletal muscle	Promotes proliferation of many cell types, including satellite cells Regulates skeletal muscle regeneration Increases satellite cell differentiation	(Chakravarthy *et al.,* 2000) (Florini *et al.,* 1991b)
HGF	Injured skeletal muscle	Role as chemotactic factor Activates satellite cells and promotes their proliferation Increases mpc migration into injured tissue	(Bischoff 1997) (Allen *et al.,* 1995) (Florini *et al.,* 1991a; Christov *et al.,* 2007)
VEGF	Activated SCs Regenerating myotubes	Induces vasodilation Enhances vascular permeability Stimulates proliferation, migration, and survival of endothelial cells Modulates skeletal muscle function	(Germani *et al.,* 2003) (Becker *et al.,* 2006; Ochoa *et al.,* 2007)

Abbreviations: TGF, transforming growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor.

1.5.2 Cytokines

Cytokines are small proteins that carry signals locally between cells as well as in the circulation, and thus have an effect on other cells. Cytokines are also produced by immune cells and various other non-immune cells of the body (see Table 1.3, pg 27). Many are known as interleukins, since they are not only secreted by leukocytes, but also able to affect the cellular responses of other leukocytes. Cytokines play very important roles in restoring normal muscle structure, and specifically IL-6, IL-1β and TNF-α have been shown to be involved in regeneration [for review see Smith *et al*. (2008)]. Only the main cytokines important in muscle regeneration, and thus relevant to this study, will be addressed in the next few paragraphs.

Tumour necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine that is produced by macrophages and monocytes, but also other body cells. TNF-α is known to have pleiotropic effects – it mediates inflammatory and apoptotic responses, and modulates growth and differentiation of many cell types. In a review regarding TNF-α, it was reported that this particular cytokine plays a predominant role in skeletal muscle, contributing to both degeneration and regeneration (Li and Reid 2001). In a study by Wilcox *et al.* (1994), direct administration of TNF-α (60 μg/kg) to experimental animals (dogs) inhibited contractile function, possibly through platelet activating factor (PAF)-dependent synthesis of NO (Alloatti *et al.,* 2000). In a study by Li *et al.* (1998), when exposing skeletal muscle myocytes to TNF-α (physiological doses, ranging from 1 ng/ml – 6 ng/ml), it resulted in a nuclear factor kappa B (NFκB)-dependent loss of protein, a process known to mediate muscle atrophy (Li *et al.,* 1998). It also plays a predominant role in promoting early differentiation of C2C12 murine myoblasts in an autocrine fashion (Li and Schwartz 2001). In contrast to the positive results found with TNF-α, other studies by this particular group, established that prolonged incubation with repeated higher doses of TNF-α (10 ng/ml or higher) killed cultured myocytes (Li *et al.,* 1998; Li and Reid 2000). Other studies have also reported a negative effect of

Table 1.3: Cytokines with pro- and anti-inflammatory activities.

Cytokines	Cell sources	Major activity	References
IL-1β	Neutrophils Macrophages Fibroblasts B and T cells NK cells Injured skeletal muscle Keratinocytes	Stimulates selectin expression Plays a role as chemotactic agent Facilitates increased expression of G-CSF and GM-CSF Inhibits satellite cell differentiation, thus increases proliferation Upregulates IL-6 and MCP-1 expression Promotes muscle inflammation	(Cannon and St Pierre 1998) (Zsebom *et al.,* 1988) (Allen and Boxhorn 1989) (Gallucci *et al.,* 1998; Pedersen *et al.,* 1998; Li *et al.,* 2001) (Guo *et al.,* 2004) (Barr *et al.,* 2004)
IL-4	B and T cells (T_H2) Mast cells Stromal cells Myotubes	Promotes T_H2 lymphocyte development Inhibits LPS-induced proinflammatory cytokine synthesis B cell proliferation Controls recruitment of further myoblasts	(O'Garra and Murphy 1994) (Brown and Hural 1997) (Grabstein *et al.,* 1986) (Horsley *et al.,* 2003)
IL-6	Neutrophils Macrophages B and T cells Eosinophils Fibroblasts Injured skeletal muscle	Inhibits TNF-α, MIP-2, GM-CSF and IFNγ secretion Stimulates acute phase response Upregulates IL-1β secretion at rest only Stimulates proliferation of various cell types Promotes degradation of necrotic tissue Induces apoptosis of macrophages following injury Stimulates secretion of glucocorticoids directly	(Caiozzo *et al.,* 1996; Cai *et al.,* 2000) (Heinrich *et al.,* 1990) (Panzer *et al.,* 1993) (Massimino *et al.,* 1997) (Cantini and Carraro 1996) (Cantini and Carraro 1996) (Steensberg *et al.,* 2003)
IL-8	Neutrophils Fiboblasts	Promotes macrophage chemotaxis Decreases neutrophil infiltration	(Li *et al.,* 2001) (Gimbrone Jr *et al.,* 1989)
IL-10	Macrophages T cells (T_H2) B cells	Inhibition of monocytes/ macrophages and neutrophil cytokine production Inhibits T_H1-type lymphocyte responses	(De Waal Malefyt *et al.,* 1991; Fiorentino *et al.,* 1991 ; Giannoudis *et al.,* 2000) (Moore *et al.,* 2001)
TNF-α	Macrophages	Upregulates IL-1β and IL-6 secretion Increase selectin expression Indirectly related to muscle atrophy *via* NFκB stimulation Promotes muscle inflammation Stimulates satellite cell proliferation, not differentiation Induces production and attraction of macrophages to injury site	(Panzer *et al.,* 1993 ; Gallucci *et al.,* 1998) (Cannon and St Pierre 1998) (Li *et al.,* 1998) (Barr *et al.,* 2004) (Foulstone *et al.,* 2004; Langen *et al.,* 2004; Stewart *et al.,* 2004; Langen *et al.,* 2006) (Tidball and Wehling-Henricks 2005)
IFN-γ	T cells NK cells	Facilitate neutrophil infiltration Stimulates myoblast proliferation	(Gao and Issekutz 1996) (Olsson *et al.,* 1994)

Abbr: IL, interleukin; TNF, tumour necrosis factor; IFN, interferon; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; MCP, monocyte chemotactic protein; LPS, lipopolysaccharide; MIP, macrophage inflammatory protein; NFκB, nuclear factor kappa beta.

TNF-α on muscle gene expression when treating myoblasts with repeated doses of 25 ng/ml (Miller *et al.,* 1988), which is several times higher than the serum levels found in humans with catabolic diseases (Nakashima *et al.,* 1995). From the above, it is therefore clear that doses between 1-6 ng/ml TNF-α results in positive effects, whereas supraphysiological doses may result in cytotoxicity and negative outcomes.

Interleukin-6 (IL-6) is a pleiotropic cytokine mainly secreted by skeletal muscle fibres, fibroblasts, endothelial cells, keratinocytes and peripheral blood mononuclear cells (PBMCs), specifically known as T_H2 cells and macrophages (Biffl *et al.,* 1996). Like many other cytokines, IL-6 has both pro-inflammatory as well as (indirect) anti-inflammatory properties. Most often IL-6 is referred to as a pro-inflammatory cytokine rather than an anti-inflammatory cytokine, as its main immune-related function is to increase lymphocyte proliferation and differentiation and to activate the release of other pro-inflammatory cytokines and acute phase proteins (Heinrich *et al.,* 1990). However, many of its actions also have an indirect anti-inflammatory outcome, *via* stimulating cortisol release, inducing hepatic synthesis of antioxidants and protease inhibitors (Steensberg *et al.,* 2003). IL-6 is also a myokine, released from muscle, in response to muscle contraction, including exercise, and precedes the appearance of other cytokines in the circulation (Febbraio and Pedersen 2005; Petersen and Pedersen 2005).

Interleukin-10 (IL-10), otherwise known as human cytokine synthesis inhibitory factor (CSIF) is a key regulator of immune responses. This anti-inflammatory cytokine is produced primarily by monocytes and to a lesser extent by lymphocytes, T_H2, B cells and mast cells (Asadullah *et al.,* 2003). IL-10 has pleiotropic effects in immunoregulation and inflammation, by downregulating the expression of Type 1 helper (T_H1) cytokines and myosin heavy chain (MHC) class II antigens (Moore *et al.,* 2001). *In vitro* studies showed that IL-10 can directly inhibit cytokine production by T_H1 and T_H2 cells by acting on the antigen-presenting cells (APC). Further investigations have demonstrated that the immunosuppressive effects of IL-10 are more often at the level of the APC and not directly at the level of the T cell (Moore *et*

al., 2001). In context of this thesis, the specific IL-10 function of inhibiting the synthesis of pro-inflammatory cytokines, such as IFN-y, IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-8, IL-12, TNF-α and GM-CSF made by cells such as macrophages and the T_H1 cells (De Waal Malefyt *et al.,* 1991; Fiorentino *et al.,* 1991; Giannoudis *et al.,* 2000; Hackstein *et al.,* 2003) is most important. In recent studies, it was also demonstrated that IL-10 is also able to activate and attract M2 macrophages to the injured area, aiding muscle regeneration by facilitating the synthesis of more anti-inflammatory cytokines from these macrophages (De Waal Malefyt *et al.,* 1991; Fiorentino *et al.,* 1991 ; Giannoudis *et al.,* 2000).

Interleukin-4 (IL-4) is produced by activated T-lymphocytes, mast cells, eosinophils and basophils and myotubes. It is a highly pleiotropic cytokine that plays a major role in T cell development, where it induces differentiation of naive helper T cells (T_H0 cells) to T_H2 cells (Brown and Hural 1997). T_H2 cell secretion of IL-4 and IL-10, leads to the suppression of T_H1 responses by down-regulating the production of macrophage-derived IL-12 and inhibiting the differentiation of T_H1-type cells (Levings and Schrader 1999). IL-4 has a marked inhibitory effect on the expression and release of pro-inflammatory cytokines. It is able to suppress monocyte-derived cytokines, including IL-1, TNF-α, IL-6, IL-8 and MIP-1α when stimulated with LPS, TNF-α or IL-1 respectively (Standiford *et al.,* 1990). It has also been shown to suppress macrophage cytotoxic activity, parasite killing and macrophage-derived NO production. IL-4 has a direct effect on satellite cells by recruiting more myoblasts to the injured area upon trauma (Horsley *et al.,* 2003). In addition to its inhibitory effect on the production of inflammatory cytokines, it stimulates the synthesis of the cytokine inhibitor IL-1Rα.

From the abovementioned literature regarding the different cytokines, it is evident that these cytokines play very important roles in muscle regeneration. These cytokines will be discussed in context of muscle injury in section 1.7.3.

1.6 Skeletal muscle contusion injury

1.6.1 Overview

There are many ways in which muscle fibres can be damaged. External causes include contusion and laceration injuries to the body, or extremes of heat or cold, whereas internal causes include muscle tears and tendon ruptures following sudden forceful contractions (McComas 1996). For the purpose of this thesis, only contusion injuries will be discussed in detail.

Clinical contusion injuries are caused by a blunt non-penetrating impact to the muscle belly. The process of soft tissue wound healing is complex and progresses through 3 distinct, yet overlapping phases, namely: destruction, repair and remodelling, which can take between 14-21 days for full recovery. For a detailed description of the 3 phases, see Jarvinen *et al.* (2005). When bone or tendon is involved during these types of injuries, it complicates the recovery process even further. **Bone injury** occurs when an injury, including a contusion injury, occurs too close to the bone. Bone healing involves physiological and cellular processes to regenerate broken bones (Einhorn 1998; Vortkamp *et al.,* 1998). New bone matrix mineralization can take several months before healed bone has maximum strength (Klug *et al.,* 1986). These physiological and cellular processes involved include localized tissue hypoxia, fracture hematoma formation, and subsequent inflammation and thus migration of immune cells into the fracture site, as well as fracture callus remodelling with the help of bone-forming cells (osteoblasts) and bone-resorptive cells (osteoclasts) (Brighton *et al.,* 1991; Bolander 1992). Production or release of specific growth factors, cytokines, and local hormones at the fracture site aid the recovery process (Brighton *et al.,* 1991; Bolander 1992). If a contusion or any type of soft tissue injury occurs, with subsequent bone involvement, it results in a greater immune response. Previous studies have demonstrated that 24 hr after trauma–hemorrhage (Fx-TH), proliferation of splenic T lymphocytes occurred, whereas IL-2, and IFN-γ expression were depressed in 2–3 month old male mice compared to their respective sham-injured group (Kahlke *et al.,* 2000). Studies by the same group

found a significant decrease in splenocyte proliferation and a significant increase in bone marrow cell proliferation in these mice following Fx-TH injury compared to their respective sham groups (Kang *et al.,* 2004). In addition, plasma IL-6 and IL-10 levels were significantly higher 2 hr following Fx-TH compared to control groups, as well as compared to contusion injury only.

With **tendon injuries**, the healing tendons may take up to 1 year to reach maximal strength and might need reconstructive surgeries (Mishra *et al.,* 2009). Tendon injury also results in a different immune response compared to contusion injury. A study in a rabbit model of Achilles paratendonitis and tendinosis, using radiolabelled microspheres, indicated that blood flow is increased in the damaged tendons (Backman *et al.,* 1990). Increased levels of TGF-β2 have been reported in pathologically inflamed human Achilles tendons and in rabbit flexor tendons after injury. TGF-β2 results in scar formation and fibrosis (Ernst *et al.,* 1996). The response to cytokines may be site-specific, and insulin-like growth factor-1 (IGF-1) induces a higher rate of collagen synthesis in rabbit flexor tendons than it does in rabbit Achilles tendons (Abrahamsson and Lohmander 1996). The use of cytokines and growth factors to enhance tendon healing remains largely experimental and has been restricted to *in vitro* studies and animal models (for a review on the available literature, see Sharma and Maffulli, 2005). In human studies, the application of cyclic strain increases production of prostaglandin E_2 (PGE_2) in human patellar tenocytes, and also increases IL-6 secretion and IL-1β gene expression in human flexor tenocytes (Tsuzaki *et al.,* 2003a; Tsuzaki *et al.,* 2003b). IL-1β is released on mechanical stretching of rabbit Achilles tendons resulting in increased production of matrix metalloproteinase (MMP)-3 (Archambault *et al.,* 2002). In all of these studies, tendon injury resulted in higher cytokine levels when compared to muscle-only contusion injury.

Therefore, it is clear that when designing a study simulating a contusion injury, it is imperative that bone or tendon is not involved. If contusion injuries occur in close proximity to bone or tendon, it will take longer for the injury to heal and other complications such as re-

injury may occur if an athlete were to return to the sports field too soon. Thus, ideally a study investigating bone or tendon injuries should look at later time points – months up to 1 year post-injury, while contusion injuries should usually not take longer than 21 days to heal, and should therefore be monitored over a more acute post-injury period.

1.6.2 Models used to study mechanical injuries

Currently, the only way to investigate the effect of contusion injury in humans, is to use athletes who have been injured accidentally as a result of participation in their sport. A complication with such studies is that the subjects investigated will have various degrees of injury severity, and as such may be subjected to very different treatment modalities. Also, no pre-injury control can be used, so, the effect of injury is not completely clear. Another potential model to investigate muscle damage in human subjects makes use of eccentric exercise. In this type of exercise, subjects have to perform various sets of repeated muscle contractions, which may result in morphological changes (Takekura *et al.,* 2001) and delayed onset muscle soreness (DOMS) (Yu *et al.,* 2002), and is not ideal for investigating contusion injuries. Therefore, animal models may provide more information on muscle regeneration from impact trauma.

Skeletal muscle contusion injury is a proven method of inducing mechanical injury in skeletal muscle of rodents (Bunn *et al.,* 2004; Darmani *et al.,* 2004; Gierer *et al.,* 2005). The most common models used are those producing a single impact trauma to a particular muscle group (**mass-drop injury** model), with or without prior surgical exposure of the muscle (Rushton *et al.,* 1997; Bunn *et al.,* 2004; Darmani *et al.,* 2004; Gierer *et al.,* 2005; Squarzoni *et al.,* 2005; Vignaud *et al.,* 2005). The non-invasive model was first described by Stratton *et al.* in 1984, and involved the dropping of a solid weight with a flat impact surface, varying in diameter and mass, from various heights onto the specific muscle studied. In a recent study by our group (MSc thesis, Kruger 2007), a moderately severe, non-invasive mass-drop injury model, similar to the model first described by Stratton, was employed. A standardised injury to the *gastrocnemius* muscle of rats was delivered by dropping a mass of 200 g from a height

of 50 cm through a plastic tube fastened perpendicularly and directly above the muscle impact zone. This model produced reproducible contusions that were moderately severe, and took about 14 days for full recovery in terms of inflammation.

Variations of the mass-drop model have been described in rodents and mice. For example, **forceps crush injury** is one of the ways used to study a contusion injury, where contusion injury can either be delivered invasively or non-invasively. Prior to the injury, the muscle is surgically exposed (invasive), or kept intact (non-invasive), placed between the jaws of forceps and then bruised by either pinching the forceps manually or by dropping a weight onto the forceps. Apart from the invasiveness of this method (if exposed surgically), manual injury is difficult to deliver in a standardised manner. Despite these complications, this model has been used to study skeletal muscle regeneration following a contusion injury (Collins and Grounds 2001; Squarzoni *et al.,* 2005; Vignaud *et al.,* 2005).

A different model entails placing a much heavier weight on the muscle (i.e. no impact force) for 2 or more hours, a model used when studying compartment syndrome (Rubinstein *et al.,* 1998; Akimau *et al.,* 2005). Due to its extreme nature, and the lack of perfusion followed by hyper-perfusion, this is not a relevant model for studying impact contusion injury.

The current classification of muscle injuries identifies mild, moderate, and severe injuries based on the clinical impairment they bring about. Mild strain/contusion represents a tear of only a few muscle fibres with minor swelling and discomfort accompanied by no or only minimal loss of strength and restriction of movement. In moderate strain/contusion injuries, a greater extent of muscle damage with a clear loss in function (ability to contract) is present, whereas a tear extending across the entire cross section of the muscle, resulting in a virtually complete loss of muscle function, is termed severe strain/contusion (Jarvinen *et al.,* 2005). Therefore, according to these standards, severe contusion injuries should be thought of as clinical, i.e. operations are often needed to repair the damage, whilst mild and moderate contusion injuries are more physiological.

1.7 Response to contusion injury

1.7.1 Satellite cell response

Acute skeletal muscle injuries result in enhanced recruitment and activation of satellite cells (Grounds 1999; Grounds *et al.,* 2002; McComas 1996). Satellite cell activation is usually restricted to the damaged area, however, if the connective tissue between muscle fibres are also damaged, satellite cells may also be recruited from adjacent fibres (Schultz *et al.,* 1985; Schultz *et al.,* 1986). In general, contusion injured rat skeletal muscle rapidly regenerates, forming myotubes within 3-7 days after injury, with full recovery around day 14 post-injury (MSc thesis, Kruger 2007).

In a study on contusion injury by Thorsson *et al.* (1998), the number of proliferating satellite cells within regenerating muscles was counted in order to provide an assessment of the extent of early muscle regeneration. Satellite cells labelled with bromodeoxyuridine (BrDU – proliferating SC marker) were abundant in the early stages of skeletal muscle regeneration (day 3), both between surviving basal laminae and between muscle fibres in the regenerating muscle stumps, diminishing later on (Thorsson *et al.,* 1998). In the basic histological studies, all muscle injuries regenerated normally. The first matured myofibers with desmin-positive cross-striation were detected at Day 9, still with some centrally located nuclei. Similar results were also found in a subsequent study by Rantanen *et al.* (1999). In a rodent study by our group (MSc thesis, Kruger 2007), it was demonstrated that in response to experimental *gastrocnemius* muscle contusion injury, SC markers CD34 and CD56 were readily expressed 4 hr after injury, with a peak at 7 days after injury. The peak on day 7 indicates either that SCs were proliferating, or that SCs had stopped fusing and were returning to quiescence. It also correlated with the formation of new regenerated muscle fibres, suggesting that the time course for the skeletal muscle regeneration may take up to 7-14 days.

From these studies, it is apparent that the skeletal muscle regeneration process after contusion injuries has not been studied as comprehensively as other models in terms of the

role of satellite cells. Furthermore, questions still remain, especially regarding the effect of the inflammatory immune system, as well as the specific role players involved in the repair process. The next section will focus on the inflammatory immune system and its role in myogenesis, and will specifically focus on the limited research available on the effect of immune cells on the regenerating process specifically related to contusion injury.

1.7.2 Immune cell response

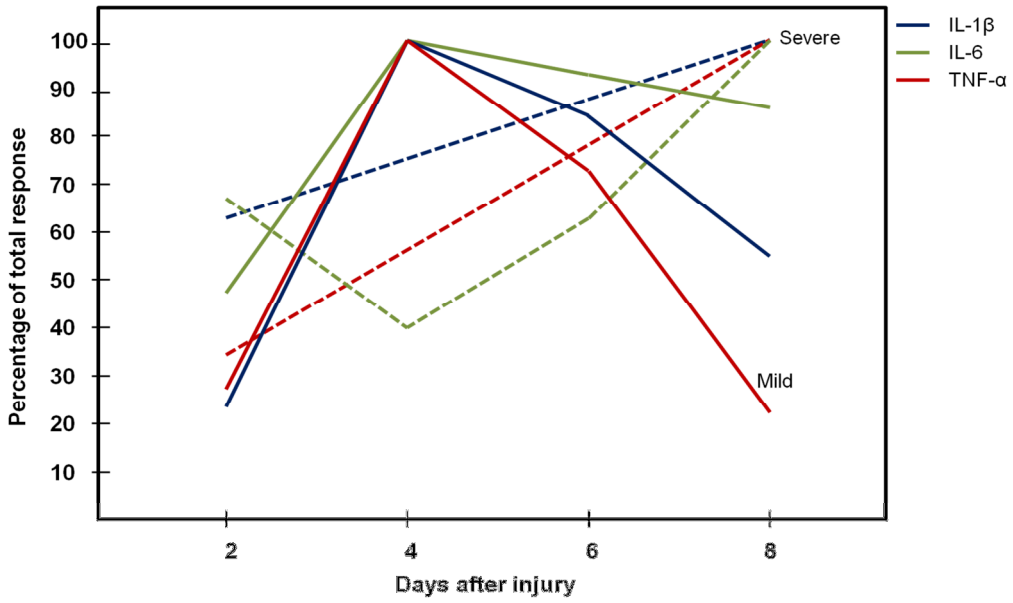
Time courses of the immune cell response after contusion injury are quite similar compared to the response to other types of acute injuries, with the exception of the appearance of M2 macrophages. In a contusion injury study by Thorsson *et al.* (1998), a small number of neutrophils were still present at day 3 after injury, but virtually none were detected on day 6. In contrast, large numbers of monocytes were still present at Day 6, only disappearing by Day 11.

1.7.3 Cytokine response

Limited studies have been done to investigate the effect of a contusion injury on the cytokine response. To date, only three research groups actually measured cytokine protein concentrations or gene expression levels in this type of injury (Kami and Senba 2002; Bunn *et al.,* 2004; McBrier *et al.,* 2007). Figure 1.5A represents the muscle cytokine response after contusion injury of different severities, whereas Figure 1.5B illustrates the plasma cytokine response after exercise.

In one particular study, (Figure 1.5A) only localized inflammation was studied extensively in an invasive model of contusion injury (Bunn *et al.,* 2004). In this study, in a model of mild contusion injury (100 g weight dropped from 13 cm – solid lines), similar expression patterns were evident for all three pro-inflammatory cytokines measured (IL-1β, IL-6 and TNF-α), with peak expression occurring on day 4. Moderate contusion injury (200 g weight dropped from 13 cm – dashed lines) on the other hand resulted in a much later peak (day 8). However,

A: Animal model – Intramuscular



B: Human subjects - Circulation

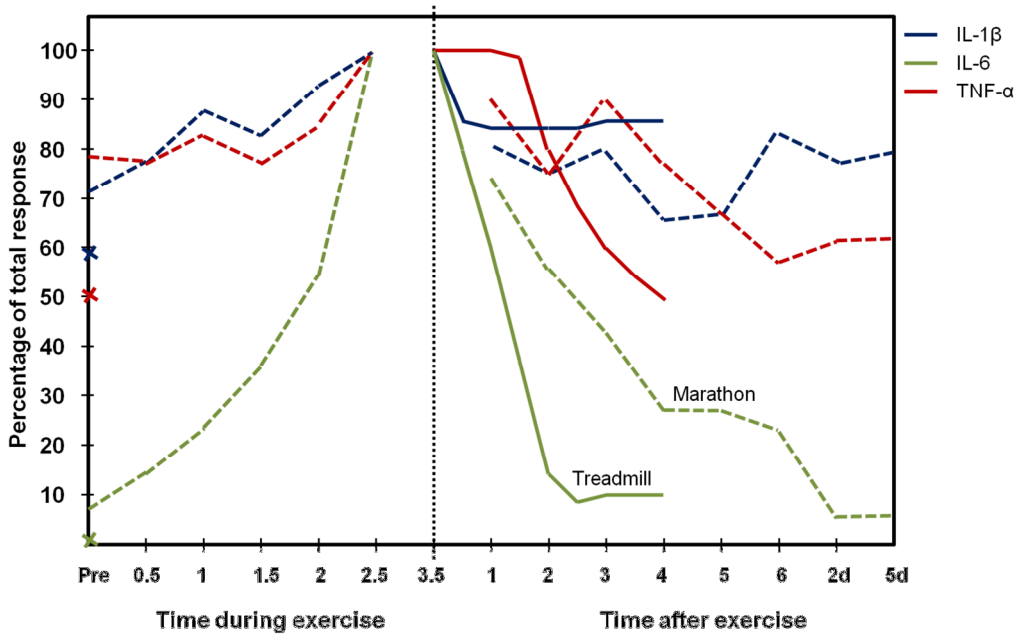


Figure 1.5: Time course pattern of the cytokines, IL-1β, IL-6 and TNF-α in animal and human injury. (**A**) represents the cytokine pattern in the muscle after contusion injury of different severities, whereas (**B**) represents the circulatory cytokine pattern after exercise. Contusion injury severities include: Mild (100g from a height of 13 cm, indicated by solid lines), moderate (200 g from a height of 13 cm, indicated by dashed lines). Exercise includes 2.5 hr of treadmill running (TR, indicated by solid lines) or an average of 3 hr 27 min marathon running (MR, indicated by dashed lines). Adapted from Bunn *et al.*, 2004 (muscle cytokines); Ostrowski *et al.*, 1998 and 1999 (circulatory cytokines).

since no data are available for day 0 (day of injury, before injury) or even a couple of hours after injury, it is not clear whether these cytokines actually peaked earlier than day 2. Also, no statistical analysis was performed to determine whether mild and moderate injury had a significant effect on cytokine levels over time. This information would have been more valuable, since it is clear from the figure that there were big differences over time. This study also lacked a proper control for the surgery prior to injury and therefore the cytokine levels reported were not corrected for cytokines released as a result of the injury caused by surgery. It is imperative that in studies involving damage to the skin, proper controls are required due to the fact that particularly TNF-α and IL-6 are known to increase a couple of hours after surgery (Nossuli *et al.,* 2000). In a study by Farnebo *et al.* (2009), the contralateral leg was used as control when rats were exposed to severe contusion injury (1250 g weight dropped from 50 cm respectively). Unfortunately, the researchers only investigated time points up to 3 hr after injury, and might have missed critical information regarding cytokine expression at later time points (not indicated in Figure 1.5). From the abovementioned studies on muscle cytokines, it is clearly evident that these studies lack proper controls as well as the necessary statistics to investigate data over time. Further research is needed to determine the effect and timing of cytokine peaks after injury. What is clear though from the study by Bunn *et al.* (2004), is that the curve shifts to the right, with cytokine expression occurring later, the more severe the injury is. A possible explanation for this phenomenon might be that during mild injury, cytokine expression occurred earlier, since there is not a lot of vascular disruption and fibre tearing. Thus, cytokines are not that necessary to recruit neutrophils and macrophages to the injured area at later time points to mop up the debris. Furthermore, the intact muscle could have been the main source for cytokine expression during mild injury. However, after moderate injury, other cell sources, including the injured muscle itself, could be responsible for cytokines expression. Furthermore, since it is known that neutrophils are responsible for secondary damage roughly on day 3 after injury, it is possible that cytokine expression only peaked at later time points in the moderate injury group, as their expression is necessary for the recruitment of immune cells to the injured muscle. However, it is important to note that no definite

conclusion can be made regarding the cytokine patterns from these studies, since no control data, or cytokine data at earlier time points were measured.

Plasma cytokines, on the other hand, displayed different expression patterns. No data are available on plasma cytokines after contusion injury. Figure 1.5B represent plasma cytokine expression after different forms of exercise. Adult men participated in either 2.5 hr of treadmill running (Ostrowski *et al.,* 1998) or roughly 3 hr and 27 min of strenuous marathon running (Ostrowski *et al.,* 1999). It is evident from the graph that irrespective of the type of exercise, all three cytokines peaked at the end of the exercise protocol, followed by a rapid decrease in IL-6 and a more gradual decrease in IL-1β and TNF-α, all of which returned to baseline levels roughly 4 hr after exercise.

Apart from assessment of actual cytokine levels, information can also be gained by considering assessment of related receptors or downstream signalling intermediates. For example, the signal transducer and activator of transcription 3 protein (STAT3) was shown to be exclusively induced in the activated satellite cells, proliferating myoblasts and surviving myofibres in the regenerating muscle, but not in quiescent SCs, differentiating myoblasts or myotubes (Kami and Senba 2002). However, IL-6 receptor α (IL-6Rα), the ligand binding site in a functional IL-6 receptor complex, was exclusively upregulated in interstitial mononuclear cells but not in myofibres or mpcs during muscle regeneration (Kami *et al.,* 2000). This suggests that the IL-6 response is not directly induced in myofibres and mpcs after contusion injury, but that IL-6 merely acts on interstitial cells. It might also suggest that there are sufficient receptors, or its function is not induced *via* the cell surface receptor. Investigations into the IL-6R system provided evidence that IL-6 does not act only on IL-6R, but can also bind to a silencing (s)IL-6R that express gp130 (receptor subunit shared by several IL-6 cytokine receptors). The authors therefore suggested that IL-6 in myofibres might be binding to the (s)IL-6R after contusion injury instead of binding to IL-6R itself (Kishimoto *et al.,* 1994). There is also some evidence showing beneficial effects of leukemia inhibitory factor (LIF) on muscle regeneration (Barnard *et al.,* 1994; Kurek *et al.,* 1996; Kurek

et al., 1997). All of these studies indicate a possible role for IL-6 and IL-6 related family members in regenerating muscle.

1.8 Summary

This summary will highlight some of the specific difficulties encountered in this field and conclusions made from the literature discussed above.

i) Various proteins exist in order to identify satellite cells at different stages after muscle injury, however, not one single protein exists that can identify SCs at one specific stage. To date, due to the fact that Pax-7 is the major protein expressed only by satellite cells, it is considered to be the best suitable marker for identifying satellite cells, compared to other markers.

ii) Reactive oxygen species are produced at many sites and in many cells after muscle injury. Although SC are also able to generate reactive species after muscle injury and may contribute to secondary damage, these cells are the major role players in the muscle regeneration process responsible for muscle recovery. SCs are not solely responsible for further damaging muscle tissue, but macrophages and neutrophils are also involved in this particular process.

iii) Neutrophils are the first immune cells to infiltrate the injured area, followed by macrophages, both of which have two main functions, which are phagocytosis (clearing the wound debris) and production of pro-inflammatory cytokines and reactive species which will exacerbate the initial injury even further.

iv) Many other factors also contribute to muscle injury and repair and involve the myogenic regulatory factors, growth factors, cytokines and chemotactic factors. It has also become apparent that not only one chemokine is at play when attracting macrophages or neutrophils to the injured area, but rather that a combination of chemokines might be responsible. Therefore, it is very important to determine which chemotactic cytokines are responsible for attracting immune cells to the injured area, after contusion injury.

v) The interaction between muscle cells and immune cells play a very important role in modulating the events following an injury.

vi) A non-invasive model of contusion injury is the best suitable model to investigate all of these issues, as this type of model excludes the possibility of infections, as well as immune system activation (which could change the local and systemic oxygen radical absorbance capacity) as a result of tissue damage (e.g. muscle, skin etc.) due to the surgical procedure itself.

vii) The specific timing of events after contusion injury could potentially be modulated by supplements or medication, but not necessarily at similar times or by similar interventions. If known, it will allow health care professionals to administer certain treatments (aimed at either enhancing or suppressing certain processes) at specific time points which might promote quicker healing of muscle tissue after injury. The different treatment regimes will be discussed in the next chapter.

CHAPTER 2

Literature review: Treatments for muscle injury

2.1 General introduction

Muscle repair after injury involves a complex interaction between local muscle cells and immune cells, with interplay of other regulatory factors. If these factors function optimally, muscle function and muscle fibre ultrastructure will be restored to its original form, if not, regeneration is delayed and scarring will occur. Upon skeletal muscle damage, muscle fibres rupture at or adjacent to the impact area, and depending on the severity of the injury, the underlying vasculature may also be disrupted. Neutrophils are the first immune cell type to infiltrate the damaged area, followed by pro-inflammatory types of macrophages, both of which generate free radicals, causing the area of necrosis to extend to affect surrounding uninjured tissue (secondary damage). Satellite cells become activated, through the expression and release of many growth factors, cytokines, as well as reactive species (e.g. nitric oxide – NO, generated by macrophages). These satellite cells then start to proliferate, differentiate, and fuse with damaged muscle fibres to repair them, with concomitant capillary revascularisation and the possible production of a connective tissue scar.

Skeletal muscle injuries are a frequent occurrence in sporting events, and can produce significant disability because of pain and impaired muscle function often resulting in a loss of flexibility, and strength, placing the player at an increased risk of re-injury (Gates and Huard 2005). Therefore, treatments are now focused on augmenting the normal repair and regenerative processes in order to allow athletes to return to their previous levels of function as quickly as possible. Anti-inflammatory medications (including corticosteroids), extensive growth enhancing treatments, and exercise protocols have been studied extensively (Beiner and Jokl 2001; Järvinen *et al.,* 2007). Other approaches include the experimental use of

stem cells (including skeletal muscle-derived stem cells) or biological growth factors to repair and/or regenerate skeletal muscle (Bach *et al.,* 2006). While these treatment options offer considerable promise for the treatment of muscle damage, side effects are also evident and realistically it will take many years before emerging techniques such as stem cell treatment are perfected and become mainstream clinical treatments. Even then, this treatment will probably be reserved for the most extreme cases. Therefore, the "ideal" treatment for the majority of injuries still remains to be elucidated, mainly because the large variability in the severity of injury and the multiple underlying processes complicate research efforts aimed at finding the best treatment (Beiner *et al.,* 1999; Beiner and Jokl 2001). In this chapter, I will review the existing literature on traditional clinical treatment modalities for muscle injury. I will also introduce more recently identified potential therapeutic agents and finally focus on those most closely related to the treatment that will be used in the experimental chapters of this thesis.

2.2 Traditional treatments

Commonly known treatments for muscle injury are largely aimed at inhibiting the inflammatory response to alleviate the clinical signs of inflammation – swelling and pain – and to limit fibrosis. These treatments include both steroidal and non-steroidal anti-inflammatory drugs, as well as alternative treatments such as cryotherapy, hyberbaric oxygen therapy and pulsed ultrasound. More recently, administration of growth promoting agents and light/moderate exercise have received more attention.

2.2.1 Anti-inflammatory treatments

2.2.1.1 Steroids

Corticosteroids are often injected directly into the site of injury. However, depending on the manner of administration (injection *vs.* oral ingestion), the timing of, as well as the optimum

dose of a specific corticosteroid, will determine whether the therapy is effective for muscle regeneration or not.

In an animal study by Mitchell *et al.* (1991), low doses of glucocorticoids (1 and 10 µg/kg dexamethasone per day in the contralateral *gastrocnemius* muscle, starting on the day prior to injury, and on day 1 up to day 9 post-injury), did not significantly affect muscle regeneration, although glucocorticoids is known to have anti-inflammatory actions. In this study, although a reduction in the proportion of necrotic muscle tissue was evident, there was no noticeable new muscle fibre formation. In another animal study, oral administration of corticosteroids after a contusion injury resulted in delayed resolution of the haematoma, retarded muscle regeneration and decreased tensile properties (Jarvinen *et al.,* 1992). In contrast, in the animal study by Beiner *et al.* (1999), corticosteroids (acute dose of 25 mg/kg methylprednisolone acetate – long acting anti-inflammatory steroid), resulted in an early, positive, muscle sparing effect, with improved muscle function on day 2 following the injury, indicating a positive effect of corticosteroids. However, by day 7 in the post-injury period, a marked disruption of the healing process with poor muscle function was evident, indicative of an incomplete healing process. In all these studies, induced muscle tension and basic structural histology were used to assess muscle function and muscle regeneration respectively. No additional quantitative measurements were made to assess markers of muscle regeneration or mechanisms involved.

In human studies, although corticosteroids are able to alleviate pain (Blair *et al.,* 1996), the overall incidence of positive effects, as well as side effects after local corticosteroid injection is variable. Corticosteroid injection into injured tendon has been shown to result in tendon rupture (Unverfirth and Olix 1973; Gottlieb and Riskin 1980). Several clinical studies have also shown that high doses of corticosteroids in a short period of time can result in acute corticosteroid myopathy (van Marle and Woods 1980; Faragher *et al.,* 1996; Hanson *et al.* 1997). This process involves general weakening of both the distal and proximal muscle groups with degeneration of type I and type II muscle fibres. Muscle fibre necrosis also

occurs, resulting in a marked elevation of myoglobin (Hanson *et al.* 1997). However, corticosteroids do not only affect the muscle, but can also influence the immune system, by decreasing the percentage of T helper cells (CD4) (Cupps *et al.,* 1984). Thus, although corticosteroids have been beneficial in alleviating pain and have early muscle sparing effects, it seems to inhibit the healing process later on, with no added beneficial effect on the regenerative capacity of the muscle, indicating that this treatment may not be beneficial to the athlete, and may even have undesired effects. Animal studies that support this, include, the use of a higher dosage of dexamethasone than mentioned above (1 mg/kg daily for 12-16 days subcutaneously – starting the same day as venom injection), which severely decreased muscle regeneration after injury (Noirez *et al.,* 1999). Even a dosage as low as 100 µg/kg daily continued for 9 days, resulted in more necrotic tissue than that of controls indicating that doses higher than 100 µg/kg might not be beneficial (Mitchell *et al.,* 1991).

On the other hand, the injection of the testosterone derivative, nandrolone decanoate (20 mg/kg, once only), was shown to result in more rapid healing and restoration of force generating capacity after contusion injury in rats (Beiner *et al.,* 1999). Also shown in this study, is the ability to increase the number of muscle progenitor cells in the injured area. These effects were all associated with an initial increase in inflammatory cells, suggesting that the initial inflammatory response is part of the crucial process of healing of muscle. Although anabolic steroids were beneficial for quicker muscle regeneration in this study, the continued use of anabolic steroids may increase the initial inflammatory reaction even further which would exacerbate tissue injury. Due to the fact that anabolic steroids play a role in enhancing muscle mass, without any added effect on the tendons, athletes might be at a higher risk for tendon rupture as reviewed by Shahidi (2001).

It is also important to note that the dosage of steroids needed to have a similar effect will differ depending on the manner of administration. A clear example is that administering NSAIDs or any type of drug by means of an oral route is considered to be the most convenient and safest way (Birckelbaw Kopacek 2007), but, a bigger dose is needed to

obtain the same effect as that obtained when administering drugs by means of injection. This is due to the absorption rate of the specific drug, suggesting a slower and lower level of distribution to the muscle, which might not be sufficient for quicker healing.

2.2.1.2 Non-steroidal anti-inflammatory drugs (NSAIDs)

In conjunction with temporary immobilization, elevation and local cryotherapy, the administration of non-steroidal anti-inflammatory drugs has become a therapeutic standard in the acute routine treatment of soft-tissue injuries, particularly after an athletic injury (Cohn *et al.,* 1989; Buckwalter 1995). These drugs are still recommended and prescribed for their analgesic purposes rather than for enhanced healing, as the evidence to support the latter remains limited. The ability of non-steroidal anti-inflammatory drugs to reduce inflammation and pain after injury is mainly based on the inhibition of prostaglandin synthesis by cyclooxygenase (COX), which has a direct effect on muscle cells (Brooks and Day 1991b). COX is present in at least two isoforms, namely COX-1 and COX-2. COX-1 is a predominantly constitutive form and is involved in cellular homeostasis with maintenance of tissue physiology, whereas COX-2 is a rapidly inducible isoform upregulated by reactive oxygen species, inflammatory cytokines and mitogens (Dubois *et al.,* 1998).

Although NSAIDs are commonly used for muscle injuries (personal communication: Dr Pierre Viviers, Stellenbosch University Sports Performance Institute, SUSPI), studies regarding its ability to blunt the body's inflammatory response to injury in order to facilitate faster recovery, are not in agreement, with only some reporting positive effects, while many others have shown quite the opposite. Although certain concerns exist regarding the safety of these drugs and their involvement in causing myocardial infarction and other cardiovascular and gastrointestinal defects (Hippisley-Cox and Coupland 2005; Scheiman and Fendrick 2005), a relatively recent review concluded that certain NSAIDs have a role in both long-term or short-term treatment of delayed onset muscle soreness (DOMS) after injuries in humans (Connolly *et al.,* 2003). This is possibly due to the fact that some NSAIDs, especially diclofenac, can

act as a dual-action NSAID, inhibiting both the COX and lipooxygenase pathways of arachidonic acid (AA) metabolism, thus providing a potentially greater anti-inflammatory effect (Brooks and Day 1991a).

Different types of injuries will result in different magnitudes of the inflammatory response that will either be alleviated with a particular NSAID dosage or not be affected at all. Also, the type of NSAID used, the specific dosage, the duration of the treatment, as well as the onset of first dosage might all play a predominant role in NSAIDs having a positive or negative outcome on muscle regeneration (Smith *et al.,* 2008). In a study by Vignaud *et al.* (2005), it was proposed that long-term non-steroidal anti-inflammatory and antioxidant drugs (NSAIAODs) could markedly reduce the speed of subsequent muscle recovery after severe injury. Two models of injury were used in this study: (1) the *tibialis anterior* muscle was injected with 20 µl of 0.9 % saline containing a myotoxic agent (2 µg/kg per muscle of snake venom from *Notechis scutatus scutatus*), or (2) it was mildly crushed twice for 5 seconds with forceps placed all the way from the distal tendon to the proximal extremity. Different NSAIAODs [diclofenac, diferuloylmethane (DFM), dimethylthiourea (DMTU), dimethyl sulphoxide (DMSO), indomethacin and pyrrolidine dithiocarbamate (PDTC)], with either known anti-oxidant or only anti-inflammatory action, or both, were administered. Drug injections began on the day of injury and lasted for up to 2 weeks. All the NSAIAODs used (at low doses) resulted in a decreased accumulation of inflammatory cells in the damaged muscle, as well as a limited production of free radicals/oxidants, prostaglandins, cytokines and chemokines in the first few days after injury (Vignaud *et al.,* 2005). None of the drugs had detrimental effects on long-term (42 days) muscle functional recovery (force production), except when using NSAIAODs at supraphysiological doses (> 2 mg/kg). However, it is not completely clear whether the inhibitory effects of NSAIAODs are due to the anti-oxidant or anti-inflammatory properties of these drugs, since some of the drugs displayed both anti-inflammatory and anti-oxidant properties. However, it is worth mentioning that no animals died with DMSO supplementation, indicating that this antioxidant might have protected the animals. Although the results report that low doses of NSAIDs might be beneficial in

reducing the amount of free radicals and inflammation, other studies indicate that high doses have detrimental effects.

In an earlier study, decreased neutrophil and macrophage infiltration in the recovery phase (day 2 post-injury) and slower resolution of inflammation was reported in response to 5 days of NSAID treatment (Jarvinen *et al.,* 1992). In a similar study, NSAID treatment resulted in the total inhibition of the inflammatory phase, decreasing the capacity for regeneration and thereby delaying muscle regeneration (Mishra *et al.,* 1995). Since the inflammatory response is a necessary phase during soft tissue healing, total inhibition of this response could result in poor healing (Beiner *et al.,* 1999). However, in a study by Thorsson *et al.* (1998), neither early nor late NSAID supplementation had any effect on muscle regeneration, as seen by similar SC activation and proliferation cycles in both NSAID and control groups. Late and early treated groups both received daily doses of 10 mg/kg naproxen intramuscularly. The early treated group started naproxen 6 hr after injury and were sacrificed on day 1, 3, 6 and 9, whereas the late group's treatment was delayed until day 3 and sacrifice took place on day 5, 8 and 11. The control animals received no injections, however, these animals cannot be considered to be ideal controls, since they did not receive placebo injections. Studies mentioned in this section thus far have focussed on the effect of relatively long NSAID treatment (5 or more days) for muscle recovery, or starting 2 days after the initial insult and have shown either negative or no effects at all.

In clinical practice, NSAIDs are often not prescribed for 24 to 48 hr after injury (Rahusen *et al.,* 2004), which may be too late, due to the fact that neutrophils which infiltrate the injured area already 24 hr after injury, may already have resulted in secondary damage – exacerbating the injury further. Other studies have investigated different routes of administration and different timing. In a study by Gierer *et al.* (2005), relatively low doses of NSAIDs (10 mg/kg parecoxib sodium) were infused intravenously, immediately before or 2 hr after contusion injury. In this specific study, NSAID infusion both prior to and after contusion injury, resulted in a marked decrease in the inflammatory response and almost complete

restoration of microcirculation to normal by 18 hr after trauma, indicating that despite conflicting results, NSAIDs might be a good treatment for contusion injuries (Gierer *et al.,* 2005). Administration of rofecoxib (5 mg/kg) prior to contusion injury (24 hr prior to), did not appear to dramatically influence muscle recovery (Rahusen *et al.,* 2004). This is surprising, since it would have been expected that administration of NSAIDs should have had some effect in blunting the inflammatory response as seen in the study by Gierer *et al.* (2005), rather than no effect. A possible reason for no effect might be that NSAID administration 24 hr prior to injury might not have been long enough to have a beneficial effect. However, this cannot be considered the main reason, since NSAID administration in the previous study resulted in beneficial effects (Gierer *et al.,* 2005). The most obvious reason may therefore have been the relatively small dosage, which might have been too low to have had a positive effect, indicating that that research on the timing and dosage of NSAID administration may still be of some importance. Even early post-injury rofecoxib, administered as soon as the animals recovered from the injury induction protocol (not mentioned in the study by Gierer *et al*., 2005), did not appear to affect the outcome of the recovery protocol. Although earlier administration of NSAIDs cannot be ruled out as a possibility, more research using such acute, shorter-term protocols are required, for example only for 2 or 3 days.

Traditional treatments include the use of steroids and anti-inflammatory drugs, and also alternative treatments. These treatments include the use of ice (cryotherapy), therapeutic ultrasound, hyperbaric oxygen, growth promoting agents and exercise, all of which will be discussed in more detail in the next section.

2.2.2 Alternative therapies

2.2.2.1 RICE

Rest, ice, compression and elevation (RICE) has been used extensively for the treatment of injured skeletal muscle as well as soft tissue injury, in order to minimize bleeding. These treatments are commonly used in combination, making it difficult to determine the value of

one of the components alone. However, some studies have investigated only one or a combination of two of these treatments in order to allow us to determine their function more clearly.

a) Rest

It has been shown that early but not immediate mobilization – walking around – after contusions helps restore muscle function to previous levels earlier than usual (Jackson *et al.,* 1974). Mobilising the injured limb immediately after injury is not advised, since it results in the formation of scar tissue (Jarvinen 1975), whereas mobilisation starting 2-3 days after injury resulted in better healing and restoring of muscle strength (Jarvinen and Lehto 1993). This therefore suggests that the muscle needs to be immobilised (rested) for a couple of days after injury.

b) Rest and elevation

Rest in combination with elevation immediately after injury prevented the injured area from growing in size, limited the size of the haematoma and the size of the subsequent connective tissue scar (Jarvinen and Lehto 1993), further supporting that rest and elevation is needed prior to mobilisation of the injured limb.

c) Ice and compression

The combination of both ice application and compression can limit bleeding, reduce pain as well as result in a faster return to the sports field (Levy and Marmar 1993). Although various studies have tried to compare the use of ice in combination with compression, as reviewed by Bleakly *et al.* (2004), it was difficult to compare the efficacy of each treatment modality alone, since the compression bandages varied with each study. Most of the studies found no difference between the groups, in terms of function, pain or swelling. Although two studies reported a difference in favour of ice and compression, inadequate data were provided and these studies were of low quality (see studies reviewed in Beakly *et al.* (2004).

d) Ice/cryotherapy

The application of ice alone was shown to limit the area of the haematoma between the ruptured muscle stumps, resulting in less inflammation and to accelerate early regeneration (Hurme *et al.,* 1993; Deal *et al.,* 2002), indicating a possible positive outcome after injury with the use of this particular technique. Cryotherapy is often prescribed for 10 to 20 min, 2 to 4 times per day for the first 2 to 3 days (Kellet 1986). Temperatures around 25 ℃ are considered optimal for cold vasoconstriction. Treatment with ice also seems to provide short-term analgesic effects (Olson and Stravino 1972; McMaster and Liddle 1980; Meeusen and Lievens 1986; Grana 1993; Ernst and Fialka 1994). However, the exact mechanisms, as well as the specific time of administration of ice are still debated. When applying ice immediately post-injury, it reduces metabolism and bleeding, thereby minimizing secondary hypoxic injury and the degree of tissue damage (Knight 1989; Knight *et al.,* 2000). In contrast, when applied for rehabilitative purposes, it is used primarily to relieve pain, in order to return to the sports field earlier than usual (Knight 1989; Knight *et al.,* 2000). Currently, many clinicians do not fully understand the pathophysiological rationale at each stage and may not be using ice to its full advantage (Knight *et al.,* 2000).

In studies by Curl *et al.* (1997) and Smith *et al.* (1994), cryotherapy resulted in an acute vasoconstrictive effect on capillaries and a significant decrease in tissue perfusion following contusion of striated muscle. Decreased oedema after ice therapy was also apparent, but vasoconstriction and decreased tissue perfusion is unlikely to be the sole cause, since inflammatory actions could also have contributed and could have mopped up some of the debris (Smith *et al.,* 1994; Curl *et al.,* 1997). A more recent study by Deal *et al.* (2002) illustrated that cryotherapy also decreased neutrophil-endothelial interactions, indicating that ice resulted in decreased microvascular permeability at a time-point at which neutrophil-endothelial interaction would usually take place maximally.

In various rat studies, it has been shown that decreased temperature decreases histamine release and is responsible for the disruption of mast cells (Chakravarty 1960; Hogberg and

Uvnas 1960). In later studies, it was found that histamine release was maximal when mast cells were incubated with an ionophore A23187 (possibly resulting in a similar response to that seen during inflammation) at a temperature ranging from 33-39 ℃ (Johansen 1978). Below and above this temperature range, histamine release was significantly reduced. Other studies using histamine release stimulants have shown similar results regarding temperature sensitivity (Kazuhiko *et al.,* 2000; Deal *et al.,* 2002). These studies suggest that ice application would decrease histamine release and therefore decrease the number of leukocytes infiltrating the injured area.

However, ice treatment can also be detrimental. The lowering of tissue temperature decreases cellular metabolism and thereby diminishes oxygen and nutrient needs, but decreasing the temperature of muscle below 25 ℃ for a lengthy period can dilate blood vessels, resulting in increased hemorrhage and inflammatory response (Kellet 1986; Meeusen and Lievens 1986). As a result of the detrimental effects of ice, as seen above, Hurme *et al.* (1993) applied ice to contusion injured muscle, immediately after trauma for 5 min and repeated 4 times at 12 min intervals. The intramuscular temperature was also measured in this study and it was found that this protocol kept the muscle at ± 3 ℃ of the recommended temperature of around 25 ℃. The same protocol was used 24 hr after trauma. In this particular study it was evident that the extravasation of plasma and erythrocytes in and around the injured area was more limited in the cryotherapy treated group. But, the muscle fiber regeneration process was slower in the cold treatment group, as indicated by a later appearance of desmin-positive myoblasts. This was most probably due to a delayed inflammatory reaction, since phagocytosis of necrotic debris appears to be an important aspect for satellite cell activation (Hurme and Kalimo 1992). However, the differences of regeneration between the control cryotherapy and injured cryotherapy groups were no longer visible towards the end of the observation period (day 7), so that cryotherapy did not alter the final outcome of skeletal muscle regeneration.

From the studies mentioned above, it is evident that early application of ice (immediately after injury) for a prolonged period of time can have detrimental effects. However, limiting the duration of ice treatment, can decrease oedema, as well as reduce excessive leukocyte infiltration into the injured area. Ice should therefore be applied as early as possible, but for a short period of time. Also, although the use of the separate components in the RICE technique have beneficial effects on muscle healing, the combination of these treatments might be more beneficial if administered properly and at an adequate time after injury.

2.2.2.2 Therapeutic ultrasound

Therapeutic ultrasound is widely recommended by clinicians (physiotherapists and other practitioners), as this technique promotes healing with the use of high-frequency sound waves (Roebroeck *et al.,* 1998; Speed 2001; Warden and McMeeken 2002). Deep heat is produced (3-4 ℃ higher than tissue temperature) (Draper *et al.,* 1995; Draper and Richard 1995) when applied directly to the injured area, while its high-frequency vibrational energy generates friction on the interfaces of tissues, leading to a "micromassage" effect (Johns 2002). In a review on ultrasound, it was concluded that the deep-heating effect had no influence on tissue, therefore a pulsed form of ultrasound application was suggested to minimize the deep tissue heating and thus allow greater amounts of energy to be transmitted for the nonthermal effects (Holmes and Rudland 1991).

In a study by Rantanen *et al*. (1999), rat muscle was treated with ultrasound starting either 3 days or 6 hr after injury (6 min daily) for two consecutive days, followed by one day rest, until animals were sacrificed. Results indicated that ultrasound can enhance both satellite cell-derived myogenic precursor cell (mpc) and fibroblast proliferation on day 3 after injury, but that myotube production was unaffected. Therefore, although ultrasound resulted in enhanced satellite cell proliferation, it did not translate into increased myotube formation. Furthermore, enhanced proliferation of fibroblasts could result in more scar tissue forming in the regenerating muscle.

In a later study, ultrasound was also applied to contused muscle, starting 6 hr after injury, for 6 min over a period of 7 days (Wilkin *et al.,* 2004). In this particular study, ultrasound did not have any beneficial effect on skeletal muscle regeneration. However, the markers used to assess muscle regeneration in this study included muscle mass, fibre cross sectional area, total protein content and the number of myonuclei per fibre, and might not have been sensitive enough to reflect changes. Better markers include assessment of the number of fibres with central nuclei, or the number of activated satellite cells (see section 1.2). In a study by Markert *et al.* (2005), ultrasound treatment was initiated 24 hr after injury (once daily for 5 min), starting on day 1 after contusion injury until sacrifice (day 3). In this study, ultrasound was also used either with or without exercise (Markert *et al.,* 2005). Exercise alone, ultrasound alone, or a combination of the two had no effect on any markers of regeneration measured in this particular study, 96 hr after injury. However, 3 days is not enough time to see a regenerating effect. Furthermore, this group assessed fibre cross sectional area, mononuclear number, percentage of contractile protein, and *gastrocnemius* muscle mass as markers of regeneration, none of which can be considered actual markers of regeneration.

In a more recent study, ultrasound (6 min duration, once only) was applied to injured animals (after blunt injury) at the following time points: 2, 12, 24, 48, 72, 96, and 120 hrs after muscle trauma (Silveira *et al.,* 2010). This treatment was used in combination with dimethylsulfoxide (DMSO, 15 mg/kg), a highly permeable, non-enzymatic, sulphur-containing antioxidant and primarily hydroxyl radical (OH^-) scavenger. In this particular study, markers of muscle damage included creatine kinase (CK) and acid phosphatase, whereas markers of oxidative damage included superoxide anion, lipid peroxidation, and protein carbonyls production, while antioxidant enzymes, such as superoxide dismutase and catalase were analysed in serum samples. The results of this study demonstrated that ultrasound (only 1 application) was only effective in reducing the presence of muscle damage and oxidative stress after injury, when used in combination with DMSO treatment. Since DMSO alone had similar

effects, it is believed that it is the antioxidant rather than therapeutic ultrasound that was beneficial.

From the literature it is therefore apparent that ultrasound may not be an effective therapeutic treatment after contusion injury. However, due to the short time frame studied after injury, as well as the various markers studied, which are not considered to be useful markers of regeneration, it is not certain whether this therapy might have been successful if better markers of regeneration were studied. Further studies looking at better markers are therefore necessary to determine the efficiency of this particular therapy.

2.2.2.3 Hyperbaric oxygen

Hypoxic wounds often have a reduced oxygen supply (hypoxia), which impairs leukocyte activities and wound healing, and it has been thought that improving the oxygenation of the area surrounding the wound, would improve wound healing (Wang *et al.,* 2003). Therefore, the benefit of hyperbaric oxygen therapy (HBOT) for wound healing is based on the idea that raising tissue oxygen levels will enhance wound healing ability (Grim *et al.,* 1990; Jonsson *et al.,* 1991; Kurz *et al.,* 1996; Hopf *et al.,* 1997). HBOT has been used for the treatment of deafness and acute tinnitus (Aslan *et al.,* 2002), hypoxic wounds and diabetic ulcers (Wang *et al.,* 2003), bone infections and osteonecrosis (Migliorati *et al.,* 2005), radiation side effects; which includes mucositis (inflammation and ulceration of mucous membranes), trismus (spasm of the jaw muscles), dysphagia (painful and difficult swallowing) and xerostomia (dry mouth) (Bui *et al.,* 2004), diving injuries (decompression illness) (Camporesi and Bosco 2009), and burn injury (Zamboni *et al.,* 1993).

Possible explanations for the effects of HBOT might be attributed to its potential ability to inhibit the release of oxygen free radicals, which may result in decreased ability to recruit damaging immune cells to the injured area. Furthermore, it is also possible that HBOT may result in the production of free radical scavengers that can modulate muscle healing (Niinikoski *et al.,* 1970). HBOT has also been shown to play a significant role in promoting

revascularization while stimulating fibroblast activity and collagen production (Niinikoski *et al.,* 1970). However, relatively few studies have investigated the effects of this non-drug therapy on muscle repair. In a study by Best *et al.* (1998), HBOT was applied to stretch-injured muscle during the early stages of muscle recovery. In this particular study, HBOT improved muscle recovery as assessed by ankle isometric torque, and by qualitative investigations of muscle ultrastructure after haematoxylin and eosin staining, which indicated that HBOT resulted in fewer immune cells and also less muscle fibre damage (Best *et al.,* 1998). In a recent review by Bennet *et al.* (2006) regarding different forms of exercise-induced injury, specifically eccentric exercise, it has been shown that HBOT plays a significant role in reducing oedema, enhancing oxygen delivery and inactivating white cell adhesion by inhibiting neutrophil β2 integrin function (Nylander *et al.,* 1985; Thom *et al.,* 1994; Staples *et al.,* 1995; Hills 1999).

Although it has been proven that hyperbaric oxygen therapy might be beneficial for wound healing, a great deal of uncertainty still exists concerning risks and side effects (Esterhai *et al.,* 1987), especially because exposure to pressures exceeding the normal 100 kPa at sea level and the application of dramatically raised oxygen partial pressures of about 1500–2000 mmHg might cause complications, for example affecting the middle ear, nasal sinuses or lungs, temporary worsening of short-sightedness, claustrophobia and oxygen poisoning (Plafki *et al.,* 2000). Although reports of serious adverse events are rare, the side-effects that do arise should still be taken into consideration when deciding on the best treatment option. In contrast to the beneficial effects seen with HBOT in other models, studies in muscle by Nelson *et al.* (1994) (crush injury) and Thorsson *et al*. (1997) (contusion injury), showed no significant beneficial effects, most probably due to the fact that muscle tissue is still readily supplied with oxygen during muscle contusion injury and that the injury was not severe enough for HBOT to be able to have a beneficial effect.

From the studies mentioned thus far, it is apparent that HBOT may not be beneficial as treatment for muscle injuries. However, positive effects in muscle regeneration have not been established.

2.2.2.4 Stem cell therapy

The use of muscle stem cells for therapeutic purposes holds much promise in situations where chronic disease affects skeletal muscle, such as muscular dystrophy (Gussoni *et al.,* 1997; Gussoni *et al.,* 1999). In one particular study, human haematopoietic stem cells (HSC) were infused into the muscle of *mdx* mice, a mouse model for muscular dystrophy (Gussoni *et al.,* 1999). Four to six weeks later, muscle fibers from the hind limb were able to express human dystrophin, and the synthesis of mechano growth factor (MGF), a protein that controls repair of normal muscle, was also restored, indicating that this particular stem cell population is most effective in disease states. However, only a small population of myofibres expressed dystrophin, and even though the number of fibers expressing dystrophin increased later on in time, it suggests that in order for stem cell therapy to be successful for treatment of disease states, multiple local, intramuscular injections might be needed.

Since it is known that most tissue injuries can lead to scar formation, different studies have turned to using adult-derived mesenchymal stem cells (MSCs) to treat animals with injuries or defects affecting bone, ligaments, cartilage and/or tendons, as a way in which to rather regenerate tissue through the anti-fibrotic properties they possess (Bruder *et al.,* 1998; Young *et al.,* 1998; Awad *et al.,* 1999). In a study by Pulavendran *et al.* (2010), they postulated that MSC treatment prevented fibrosis by protecting the neutrophils from undergoing apoptosis, as lower myeloperoxidase (MPO – index of neutrophil activity) and interleukin (IL)-6 expression were detected. This was further supported in another study (Raffaghello *et al.,* 2008). These stem cells were collected from either bone marrow or adipose tissue and then infused back into the patients (autologous), in human studies; or

from a genetically different donor of the same species (animals), since the immune system usually mounts a response to transplanted cells (Kraus and Kirker-Head 2006).

Studies have shown that stem cells obtained from different tissues and infused into spinal cord-injured animals or animals suffering from stroke have a great ability to migrate to sites of tissue damage and stimulate repair either by differentiating into tissue-specific cells, or by enhancing the repair by endogenous cells (Hofstetter *et al.,* 2002; Li *et al.,* 2002). A study by De Bari *et al.* (2003) found similar results when MSCs from human synovial membranes were infused into the *tibialis anterior* muscle of nude mice after cardiotoxin injury. In this study, it was found that approximately 10 % of the infused cells were present in the muscle after day 1, whereas almost twice as much were present between 2 weeks and 1 month after injury, indicating that MSCs are beneficial in repairing muscle cells. The authors were also able to show that these cells differentiated into muscle cells, which indicates that stem cells might be potentially beneficial, but only as a longer-term treatment option.

However, there is one particularly important aspect to take into account when applying stem cell therapy. Whether the stem cells are to heal bone or any other type of tissue, the microenvironment into which a group of introduced stem cells is placed, is very important for effective healing (Singec *et al.,* 2007). Often platelet-rich plasma is used in conjunction with bone-marrow derived stem cells as a matrix which supplies growth factors and the scaffold needed to induce tissue regeneration (Yamada *et al.,* 2004). Alternatively, adipose tissue also contains other cell types that are rich in growth factors to support tissue regeneration (Nakagami *et al.,* 2006; Varma *et al.,* 2007). This transfer medium is very important and has been shown to aid muscle healing in three ways. Firstly, it helps the formation of new blood cells from endothelial progenitor cells, secondly, it prevents apoptosis of cells at the damaged site, and thirdly, it reduces inflammation (Singec *et al.,* 2007).

From this, it is evident that different stem cell treatments exist which have therapeutic potential in various types of muscle injury. However, more research is needed to investigate

stem cells' behaviour and their underlying mechanisms of action before these experimental treatments can become a reality (Singec *et al.,* 2007) for the treatment of chronic disease.

2.2.2.5 Growth promoting agents

The rationale behind therapy employing growth promoting agents is that it may hasten muscle regeneration by increasing the size and number of existing and newly regenerating muscle fibres and thereby improving muscle function (Kasemkijwattana *et al.,* 1998b). Muscle growth promoting agents include, but are not limited to, growth hormone, β2-adrenoceptor agonists, testosterone-derived or testosterone-like hormones (anabolic steroids, discussed in 2.1.1.1) and growth factors such as insulin-like growth factor-I (IGF-1).

Growth hormone: Growth hormone has various beneficial effects after severe burn injury. These include the ability to: (a) decrease whole body catabolism (Byrne *et al.,* 1993), (b) improve muscle protein synthesis (Gore *et al.,* 1991), (c) accelerate wound healing (Herndon *et al.,* 1990; Gilpin *et al.,* 1994) (d) attenuate prolonged hyperactivity of the hepatic acute-phase response (Jarrar *et al.,* 1997; Jeschke *et al.,* 2000) and (e) promote growth. Although growth hormone is more commonly used for treatment of prolonged protein breakdown after burn injury (Rutan and Herndon 1990; Hart *et al.,* 2000), no literature is available on the effect of growth hormone treatment on muscle following a contusion injury. Given all the beneficial effects of growth hormone treatment, it might potentially aid muscle regeneration, since it has been shown to promote wound healing and improve muscle protein synthesis. Longer term administration (0.05 mg/kg daily for 12 months) after burn injury has also displayed improvement of metabolic disturbances (Hart *et al.,* 2001), whilst dosages as high as 0.2 mg/kg have been shown to further increase wound healing abilities, although increasing the frequency of hyperglycemia (Herndon *et al.,* 1990; Gilpin *et al.,* 1994).

β2-adrenoceptor agonists: Skeletal muscle has a significant amount of the β_2 subtype adrenoceptors (Kim *et al.,* 1991; Sillence and Matthews 1994; Sillence *et al.,* 1995). However, these adrenoceptors also exist in the heart and care should be taken when

systemically administering adrenoceptor agonists, because they could potentially be harmful to the heart, although having positive effects on skeletal muscle. In a clinical trial, β_2 adrenoceptors for the treatment of different types of dystrophies resulted in heart palpitations, although showing beneficial effects on cardiac muscle strength (Kissel *et al.,* 2001). In two specific studies, it has been demonstrated that synthetic β2-adrenoceptors agonists promote skeletal muscle hypertrophy *via* activation of the cyclic adenosine monophosphatase (cAMP)-dependent mechanisms that increase protein synthesis and inhibit protein degradation pathways (Lynch *et al.,* 2007; Lynch and Ryall 2008). Recently, it has been shown that the phosphatidylinositol-3-kinase (PI3-K)-Akt signalling pathway is also involved (Kline *et al.,* 2007). In other studies it was found that administration of the β2-adrenoceptor agonists, feneterol (1.4 mg/kg per day, i.p.) (Beitzel *et al.,* 2004) or clenbuterol (2 mg/kg per day, by means of oral gavage) (Briscout *et al.,* 2004), were able to promote regeneration of injured skeletal muscles after myotoxin injury, indicating a positive role for these agonists.

Growth factors: During muscle regeneration, growth factors and cytokines released by the injured muscle are believed to activate satellite cells (for review, see Smith *et al*., 2008). Preliminary data suggest that growth factors play additional roles, such as promoting neutrophil recruitment (Peters *et al.,* 2005), increasing satellite cell proliferation (Allen *et al.,* 1995), and differentiation (Haugk *et al.,* 1995) during muscle regeneration. A whole host of growth factors have been shown to affect the behaviour of skeletal muscle cells – these are expressed predominantly in skeletal muscle tissue itself, but also in various other tissue types, and include fibroblast growth factor (FGF), transforming growth factor (TGF), hepatocyte growth factor (HGF), IGF, platelet derived growth factor (PDGF), nerve growth factor (NGF).

Three of these growth factors have shown the most promise, namely basic FGF (bFGF), NGF and IGF-1 (Kasemkijwattana *et al.,* 1998a; Kasemkijwattana *et al.,* 1998b; Kasemkijwattana *et al.,* 1999; Menetrey *et al.,* 2000). In a study by Kasemkijwattana *et al.*

(1998), the *gastrocnemius* muscles of mice were subjected to experimental injury, and the growth factors bFGF, NGF, and IGF-1, were injected separately into the injured area. Although the direct injection of bFGF and NGF conveyed some beneficial effects on muscle healing after injury, IGF-1 mediated the greatest improvement in muscle healing across all muscle injury types tested (laceration, contusion and strain). These growth factors were able to enhance muscle regeneration by increasing the number and diameter of regenerating myofibres, and thus indirectly also tetanic force of the muscles, but were not able to prevent the formation of scar tissue that ultimately impairs the healing process. No study has been published focussing on the speed of muscle recovery following injection of growth factors after a injury.

Likewise, supplementation with growth factors like LIF, HGF, and bFGF by bolus injections or pumps leads to increase of muscle fibre density and muscle fibre size after injury (Lefaucheur and Sebille 1995b). More direct evidence for the important roles of bFGF and IGF-1 have also been reported, as separate injections of neutralizing antibodies against these two growth factors, inhibited muscle regeneration (Lefaucheur and Sebille 1995a).

Other treatment modalities in this class are focused on limiting fibrosis as a means to facilitate quicker and more effective muscle functional recovery. TGF-β has been shown in various studies not only to regulate proliferation, but also differentiation of skeletal muscle myoblasts (Allen and Boxhorn 1989; Greene and Allen 1991; Zentella and Massague 1992; Cook *et al.,* 1993; Zhang *et al.,* 2000). In skeletal muscle, during muscle injury, TGF-β is released as a result of the inflammatory reaction, and triggers fibrosis *via* the deposition of the extracellular matrix and the expansion of fibrous connective tissue (Confalonieri *et al.,* 1997). Three different isoforms exist, namely TGF-β1, - β2, and - β3, all of which have been shown to be key modulators in the wound healing process (Shah *et al.,* 1995; Stewart *et al.,* 2003; Sinha *et al.,* 2004).

Many *in vivo* studies used knock-out mice in order to investigate the importance of TGF-β in wound healing and scar formation after skin laceration. In TGF-β1 knock-out mice, wound

healing followed a relatively normal pattern for the first 10 days, followed by an increased inflammatory response, a decrease in the abilities of wounds to close, re-epithelialisation, granulation tissue formation, collagen deposition and vasculogenesis (Brown *et al.,* 1995). TGF-β2 knock-out mice on the other hand, die during, shortly before or shortly after birth, due to developmental defects of the heart (Sanford *et al.,* 1997).

In contrast to TGF-β1, TGF-β3 knock-out mice have revealed an important role for limiting scars during wound repair (Ferguson and O'Kane 2004). In other important studies, the neutralisation of TGF-β1 and -β2 resulted in improved wound repair by modulating the fibrotic response, whereas the addition of TGF-β1 elicited a similar response (Shah *et al.,* 1995). Furthermore, both the structure and function of lacerated muscle reached near complete recovery when injected with suramin (2.5 mg injected daily for 7 or 14 days), a direct antagonist of TGF-β1, whilst the amount of scar tissue was reduced when compared to controls (Chan *et al.,* 2003). Therefore, the presence of all three TGF-β isoforms is of particular importance in wound healing after injury. Despite distinct isoform-specific effects of TGF- β1, -β2 and –β3, a comparison of the effect of these three isoforms on myogenesis *in vitro* has not been carried out. Only one study thus far has been able to demonstrate that TGF-β was able to increase proliferation and decrease differentiation of C2C12 myoblasts in an isoform-independent manner. Results from this cell culture study also indicated variable effects regarding TGF-β treatment, depending on the muscle cell type and duration of TGF-β administration (Schabort *et al.,* 2009).

Therefore, the TGF-β isoforms are especially important in promoting tissue regeneration. However, since the isoforms induce different effects on wound repair, fibrosis and scarring, it is probably very important that all 3 isoforms are present in sufficient quantities to allow for optimal healing (Sporn and Roberts 1993), although TGF-β2 might not be as important.

However, although growth factors are beneficial, various studies have also indicated that the overexpression of some growth factors can have negative effects. High levels of IGF-1 are associated with the development of different types of cancer (Chan *et al.,* 1998; Hankinson *et*

al., 1998; Ma *et al.,* 1999; Yu *et al.,* 1999), and animal experiments have indicated that IGF-1 overexpression can result in tumour development in certain tissues (Rogler *et al.,* 1994; Bates *et al.,* 1995). IGF-2 has also been shown to result in spontaneous lung tumours (Moorehead *et al.,* 2003). FGF-2 is overexpressed in tissues other than skeletal muscle, including neuronal tissues (Gonzalez *et al.,* 1990) and the heart where it may affect growth and survival of cardiomyocytes and growth of the coronary blood vessels during hypertrophy and ischemia (Kardami and Fandrich 1989; Yanagisawamiwa *et al.,* 1992). These studies suggest that an even more increased growth hormone expression – by direct injection – may have detrimental outcomes on other tissues, although having a beneficial effect on skeletal muscle tissue after injury. Care should therefore be taken when using growth factor injections. However, since growth factors can enhance exercise performance, the use of growth factor injections has been banned in the sports arena.

2.2.2.6 Exercise

Light exercise (walking) is commonly prescribed by clinicians as treatment for musculoskeletal injuries, mainly to reduce DOMS. Only one research study focused on the effects of exercise on markers of skeletal muscle regeneration following muscle injury using human subjects, which suggested that exercise is efficient in resolving injured skeletal muscle (Gregory *et al.,* 1995). There is also evidence to support the results from animal studies, investigating both *in vitro* and *in vivo* approaches. In these studies, using a variety of species, it has been demonstrated that exercise assists in promoting normal growth and repair of mammalian skeletal muscle after exercise damage (Vandenburgh 1982; Darr and Schultz 1987; Esser and White 1995; Wanek and Snow 2000). Furthermore, there is also evidence that exercise promotes myonuclear accumulation in injured muscle (Smith *et al.,* 2001), although factors such as species, age, and training status also play a role.

In a review by Buckwater (1995), it was reported that the effects of activity on muscle healing depend to some extent on the onset of activity following injury. If injured muscle is mobilized immediately following injury, it may increase scar formation and interfere with normal muscle

regeneration (Jarvinen and Lehto 1993). Fibroblasts play an important role after injury, by invading the injured gap and producing an extracellular matrix in order to restore the connective tissue framework (Lehto *et al.,* 1985; Hurme *et al.,* 1991b), enabling the use of the injured limb before the completion of the repair process (Kalimo *et al.,* 1997). In extensive muscle trauma, which includes strains, contusions and lacerations, proliferation of fibroblasts can quickly lead to an excessive formation of dense scar tissue, which impedes muscle regeneration and results in incomplete recovery (Carlson and Faulkner 1983; Nikolaou *et al.,* 1987; Garrett 1990; Hurme *et al.,* 1991a; Bornemann and Schmalbruch 1992; Kasemkijwattana *et al.,* 1998a; Kasemkijwattana *et al.,* 1998b), an effect which is exacerbated during early mobilisation, due to the fact that early mobilisation results in increased blood flow (up to five-fold) and an increased influx of damaging neutrophils (results in secondary damage) immediately upon the release of muscle contraction (Robergs *et al.,* 1997).

Furthermore, if injured muscles are immobilized for a prolonged period of time (5-7 days), it results in muscle atrophy and poor organization of the regenerating myofibers, due to the fact that blood flow is reduced (McDonald *et al.,* 1992) to the injured area for too long to ensure optimal restoration. In contrast, if injured muscles are mobilized following a short period of immobilization (hours), it results in a rapid disappearance of haematoma and inflammatory cells, followed by a more rapid and organized myofiber regeneration, with a rapid increase in tensile strength and stiffness (Lehto *et al.,* 1985; Jarvinen and Lehto 1993), suggesting that a short period of rest, followed by controlled muscle use will provide optimal healing (Lehto *et al.,* 1985; Jarvinen and Lehto 1993). An increase in blood flow could therefore be a mechanism for the enhanced recovery of muscle force when light exercise is performed during recovery, but only when following a short period of immobilisation. Increased blood flow has also been established as an important factor in reducing pain, improving the healing of damaged muscle, reducing swelling and improving circulation (Mohr *et al.,* 1987), as well as improving the efficiency of muscle contraction (Clemente *et al.,* 1991).

Therefore, exercise may be an effective therapy after injury, but timing is an important factor in determining whether this therapy is successful.

2.2.3 Summary

The cellular and molecular mechanisms of normal, unassisted muscle regeneration after injury have been described extensively in the literature. Recovery is often not complete or delayed if the injury is not properly treated, regarding dosage or timing of treatment, or if the athlete returns to the sports field earlier than usual, resulting in re-injury and extensive tissue scarring. Thus, it is crucial to consider other potentially therapeutic strategies to optimise the regeneration process, to hasten restoration of muscle, and shorten the time athletes are away from the sports field.

From the literature presented in this section, it is clear that therapies have both positive and negative outcomes. However, it is important to consider the fact that great skill and sometimes individualisation is required for application of these treatments, in order to ensure optimal results. Since some of the treatments that might aid muscle regeneration such as growth factor injections are banned, it is imperative to continue the search for the optimal treatment, which can minimise secondary damage, minimise fibrosis and modulate inflammation, and can be easily administered in order to quicken the recovery process.

One of the possible exacerbating factors in contusion injuries, is the generation of reactive oxygen species (ROS) during the early recovery process, which could result in oxidative stress and subsequent secondary damage. The next section will review the literature on antioxidants and the possibility of enhancing the antioxidant status of the muscle in order to counteract the effect of ROS formation, thereby assisting recovery.

2.3 Antioxidants

The role of oxidative stress in increasing the magnitude of damage after primary injury was reviewed in Chapter 1. Given the major role that oxidants play in cellular damage, it is not surprising that myocytes and other cells contain several endogenous anti-oxidant defence mechanisms to prevent oxidative injury. Two major classes of endogenous protective mechanisms work together to reduce the harmful effects of oxidants in the cell: enzymatic defence (e.g. superoxide dismutase - SOD, glutathione peroxidase and catalase); and 2) nonenzymatic antioxidants (e.g. glutathione - GSH, ascorbic acid, α-tocopherol, β-carotene, uibiquinol-10, albumin, ceruloplasmin (copper part of it) and ferritin) (Yu 1994; Rice-Evans *et al.,* 1995; Basu 1999) – see Table 2.1. Although mammalian cells seem to have adequate antioxidant reserves to cope with ROS production under normal physiological conditions (see Figure 2.1), these antioxidant reserves may be inadequate to cope when additional ROS is produced. Therefore, supplementing the diet with dietary anti-oxidants or vitamins might prove beneficial to scavenge the extra ROS generated.

Several studies have been conducted on the properties and effectiveness of dietary anti-oxidants [which include vitamin C (ascorbic acid), vitamin E (tocopherols, tocotrienols), and vitamin A (retinol) or its precursor, β-carotene] to scavenge free radicals and therefore to decrease oxidative stress. Their importance was highlighted in a study by Basu (1999), in which deficiencies in some of these anti-oxidants were associated with oxidative stress. Similarly, another study linked acute dietary supplementation to a decrease in lipid peroxidation (Brown *et al.,* 1994). In the following few paragraphs, the anti-oxidants used commonly by athletes, will be discussed in more detail.

Vitamin C is arguably the anti-oxidant most widely used. As a result of exercise (even in the absence of clinical injury, but presence of oxidative damage), there is a reduction in plasma concentrations of vitamin C a day after exercise (Gleeson *et al*. 1987), wich coincides with the point in time when neutrophils are present in greatest numbers in injured muscle.

Table 2.1: Essential nutrients with antioxidant functions are able to scavenge multiple free radicals and interact with other endogenous antioxidants. Adapted from Machlin *et al.* (1987).

Nutrients	Free radicals to scavenge	Reaction
Vitamin E (α-tocoperol)	Peroxyl radical ($LOO^{\bullet}$) Hydroxyl radicals ($OH^{\bullet}$) Superoxide ($O_2^{\bullet-}$) Singlet oxygen ($^1O_2$)	$LOO^{\bullet} + TocH \rightarrow LOOH + Toc^{\bullet}$ $OH^{\bullet} + TocH \rightarrow H_2O + Toc^{\bullet}$ $2O_2^{\bullet-} + TocH + H^+ \rightarrow Toc + H_2O_2 + O_2$ $^1O_2 + TocH \rightarrow Toc\text{-}OOH$
Vitamin C (ascorbic acid)	Superoxide ($O_2^{\bullet-}$) Hydroxyl radicals ($OH^{\bullet}$) Singlet oxygen ($^1O_2$)	$O_2^{\bullet-} + AscH + H^+ \rightarrow Asc + H_2O_2 + O_2$ $OH^{\bullet} + AscH \rightarrow H_2O + Asc^{\bullet}$ $^1O_2 + AscH \rightarrow Asc\text{-}OOH$
Vitamin A (β-carotene)	Singlet oxygen ($^1O_2$)	$^1O_2 + CarH \rightarrow Car\text{-}OOH$
Catalase Iron present	Hydrogen peroxide (H_2O_2)	$H_2O_2 \rightarrow O_2 + 2H_2O$
Glutathione peroxidase Selenium present	Hydrogen peroxide (H_2O_2)	$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$ GSH=glutathione, reduced form GSSG=glutathione, oxidised form
Superoxide dismutase Zinc and copper present	Superoxide ($O_2^{\bullet-}$)	$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$
Ubiquinone-10 Reduced form ($UQ_{10}H_2$)	Tocopherol radicals ($ToC^{\bullet}$) Peroxyl radicals ($LOO^{\bullet}$)	$ToC^{\bullet} + UQ_{10}H_2 \rightarrow TocH + UQ_{10}H^{\bullet}$ $LOO^{\bullet} + UQ_{10}H_2 \rightarrow LOOH + UQ_{10}H^{\bullet}$

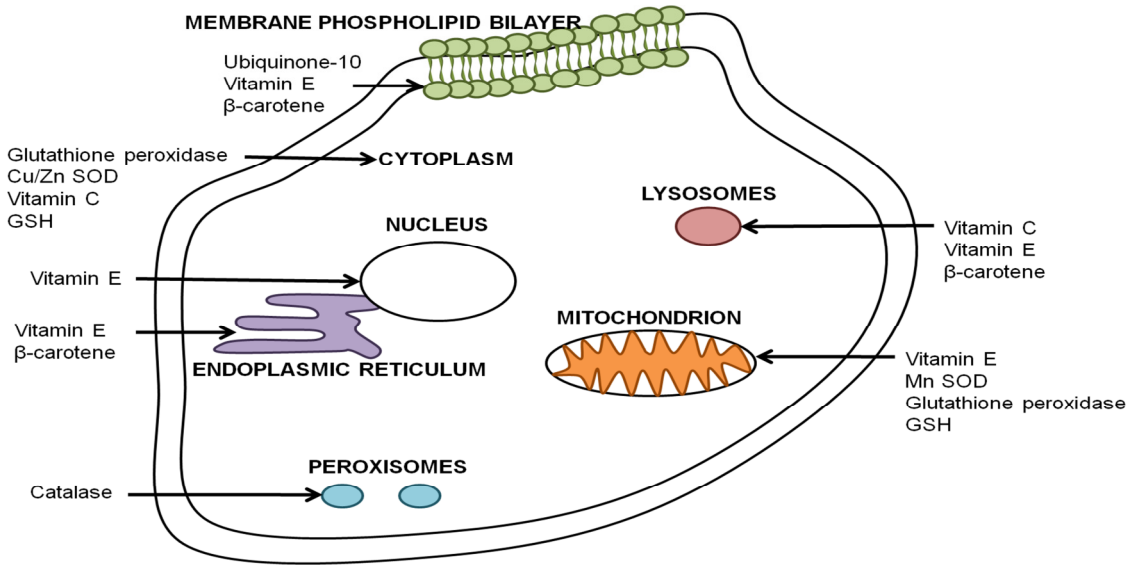


Figure 2.1: Antioxidant defence targets and locations within the cell. Adapted from Machlin *et al.* (1987). Abbreviations: Cu, copper; GSH, glutathione; Mn, manganese; SOD, superoxide dismutase; Zn, zinc.

Vitamin C has been implicated in the neutralisation of phagocyte-derived oxidants, indicating that this antioxidant is of particular importance in injuries associated with a significant inflammatory component (Anderson and Lukey, 1987).

Turning attention to administration or supplementing protocols, investigations where vitamin C supplementation was continued after eccentric exercise, have shown evidence of improved clinical recovery demonstrated by speed of recovery of muscle contraction and a decrease in muscle soreness (Kaminski and Boal 1992; Jakeman and Maxwell 1993). In the one study, 1 g of vitamin C was taken 3 times daily, starting 3 days prior to exercise and up to 7 days thereafter (Kaminski and Boal 1992), whereas in the other study 400 mg was taken for 21 days prior to and 7 days afterward (Jakeman and Maxwell 1993). Vitamin C supplementation also attenuated cortisol release after exercise in humans, and resulted in significantly lower levels of acute phase reactants such as C-reactive protein (CRP) and CK (Peters *et al.,* 2001b), as well as significantly reduced concentrations of anti-inflammatory cytokines such as IL-1 receptor antagonist and IL-10 (Nieman *et al.,* 2000; Peters *et al.,* 2001a). This may be explained by the fact that high doses of vitamin C may act as a "pro-oxidant", rather than an anti-oxidant by reducing transition metal ions, which in turn drives the Fenton reaction, potentially resulting in oxidative stress through the production of superoxide, hydrogen peroxide, and hydroxyl radicals (Halliwell 1996), as follows:

$$O_2 + e^- \rightarrow O_2^{\bullet -} \qquad \text{Equation 1}$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{Equation 2}$$

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + Fe^{3+} \qquad \text{Equation 3}$$

In contrast, acute supplementation (300 mg, starting 2 hr prior to exercise), had no effect on various measures of recovery, despite increasing plasma concentrations of vitamin C (Thompson *et al.,* 2001a). In a follow-on study by the same group (Thompson *et al.,* 2003), vitamin C supplementation for 14 days prior to exercise, resulted in a pronounced elevation in plasma IL-6, followed by a significant decline 2 hr after exercise – lower than controls (Thompson *et al.,* 2001b). However, if supplementation started 2 hr after injury (2x daily for 3

days), no significant effect on plasma CK, myoglobin, malondialdehyde and IL-6 was observed (Thompson *et al.,* 2003). These studies suggest that short-term as well as post-exercise supplementation is not sufficient to allow for a beneficial effect on recovery.

A combination of vitamin C and E (500 mg vit C and 400 mg vit E), administered daily for 2 weeks prior to exercise and continued for up to 1 week after exercise, did not alter exercise-induced muscle damage as measured by CK levels (Petersen *et al.,* 2001). There was no significant difference in plasma IL-1ra and IL-6ra between the supplemented and placebo groups after injury. Plasma levels increased irrespective of supplementation, although additional vitamin supplementation resulted in even further increases. These results suggest that endogenous antioxidants may be sufficient enough to quench free radicals in periods of greater oxidative stress, without the need for extra supplementation. This suggestion is quite valid, since in one particular study it was shown that elite athletes with a higher training load had more elevated baseline antioxidant levels compared to sedentary subjects (Child *et al.,* 1999), indicating that the natural increase in plasma antioxidant vitamin concentrations during and after exercise could possibly abolish the effect of additional vitamin supplementation.

Vitamin E can serve as a potent peroxyl radical scavenger, and recent research has shown an increase in its utilization following intense exercise (Mastaloudis *et al.,* 2001). This suggests that acute supplementation with vitamin E might be needed to maintain the antioxidant stores in situations of increased ROS production. Even though it has been postulated that adequate dietary amounts of vitamins should be consumed to protect against free radicals, it has been thought that the liver and kidney function as vitamin reservoirs (Blomhoff *et al.,* 1990; Warren and Reed 1991), and that some of the vitamins may act in synergy with one another to spare excess vitamin utilization. For example, vitamin C has the ability to regenerate the reduced form of vitamin E (Bendich *et al.,* 1984; Bendich *et al.,* 1986), thereby either decreasing the rate of vitamin E consumption or replenishing the reduced form of vitamin E (Niki 1991). In muscle fibers, vitamin E may also have the added

ability to stabilize membranes, by anchoring to membrane polyunsaturated fatty acids through its side-chain (Phoenix *et al.,* 1991), but the literature is not in agreement. Meydani *et al.* (1993), reported that oral vitamin E supplementation (physiological dose for 48 days) after downhill-running resulted in a muscle protective effect 5 days after injury, based on decreased (compared to control) plasma creatine kinase (CK) levels, while a similar study (5 days of intravenous injections of vitamin E (200 µl of a 70 % (w/v) α-tocopherol in ethanol)) reported no effect on markers of muscle damage (CK, serum pyruvate kinase) after plyometric contractions (Van der Meulen *et al.,* 1997). These two studies suggest that a longer intake of vitamin E is necessary to reduce muscle damage.

For vitamin A, chronic supplementation seems the best option, as chronic vitamin A supplementation (1000 IU – daily for 13 weeks) resulted in suppression of lipid peroxidation 24 hr after injury, although baseline oxygen radical absorbance capacity (ORAC) was not improved (Sacheck *et al.,* 2003). From these studies, it is apparent that vitamin supplementation may have positive effects on some aspects of the muscle response to damage. However, these vitamin supplementation studies (exercise-related) investigated only pro-inflammatory markers or indicators of damage, such as plasma CK, rather than markers of recovery, such as satellite cell number and number of regenerating muscle fibres. Other important processes to consider would be satellite cell activity, formation of regenerated muscle fibres and the magnitude and time course of the anti-inflammatory response. Thus, since the possibility exists that vitamin supplementation may have positive effects, more studies are needed, which include some of the abovementioned processes, in order to confirm a possible positive effect especially after injury.

Plant-derived compounds, or phytochemicals, also have demonstrated anti-oxidant capacities. These include carotenoids, bioflavonoids, flavonoids, and others as reviewed in various articles (Rice-Evans *et al.,* 1995; Rice-Evans *et al.,* 1996; Basu 1999). In a study by Chao *et al.* (1999), US Marine Corps personnel participated in a 14-day field-training event during the winter season. The oxidative stress in this model was associated with intense

physical exertion, ultraviolet light exposure, and fluctuating temperatures. Subjects received a daily dose of either α-tocopherol, ascorbic acid or β-carotene, or a combination of all three. The combination of these antioxidant vitamins was most effective in limiting increases in oxidative stress from baseline. It is possible that a synergistic effect could be achieved when combining phytochemicals with vitamin antioxidants or other compounds. In an *in vitro* study by Stahl *et al.* (1998), combinations of carotenoids (13 different combinations) were also more effective in reducing oxidative stress and protecting multilamellar liposomes than individual carotenoids, a result ascribed to their different physiochemical properties. The effects of single carotenoids and α-tocopherol on lipid peroxidation were measured by levels of thiobarbituric acid-reactive substances (TBARS) and indicated that the most efficient carotenoid was lycopene, followed by α-tocopherol, α-carotene, β-cryotoxanthin, β-carotene, zeaxanthin and lutein (Stahl *et al.,* 1998). Unfortunately, no placebo control was used in this study, the researchers used the total combination of carotenoids as "control". Thus, in light of these results, supplementing with a combination of vitamins, minerals, and phytochemicals known individually to have direct or indirect antioxidant characteristics, may therefore expand the body's natural defence system against oxidative stress.

Another potential beneficial effect of β-carotene, is its ability to protect against lipid peroxidation induced by methyl mercuric chloride infusion in mouse models (Andersen and Andersen 1993) and to reduce indices of lipid peroxidation in serum (Mobarhan *et al.,* 1990; Gottleib *et al.,* 1993). An important consideration when investigating vitamin studies in the literature, is that many studies do not intervene with a potential stressor of the antioxidant system, thus resulting in controversial results.

To date, only 5 studies have shown that polyphenols are beneficial for muscle repair (Kato *et al.,* 2000; Buetler *et al.,* 2002; Dorchies *et al.,* 2006; Hofmann *et al.,* 2006; Nakazato *et al.,* 2010). Not only were they able to decrease muscle necrosis and elevate twitch tension in *mdx* mice (Buetler *et al.,* 2002; Dorchies *et al.,* 2006), but they also resulted in suppression of oxidative stress and earlier recovery due to a significantly lower force deficit in the rat

skeletal muscle (Kato *et al.,* 2000) after subjection to treadmill-induced exercise. Furthermore, in a study by Nakazato *et al.* (2010), it was shown that apple polyphenols significantly decreased the levels of serum TBARS, the major indicator of oxidative damage according to the researchers, compared to the control group after lengthening contraction exercise. Analysis of the mRNA expression of antioxidative proteins such as glutathione-S-transferase α1 (GSTα1) was significantly higher in the polyphenol group, suggesting that these mice possessed a higher antioxidative ability (Nakazato *et al.,* 2010). This finding was also confirmed in an earlier study (Hofmann *et al.,* 2006).

Amongst the polyphenols that have attracted research attention recently are flavonols, catechins and quercetin. Results have suggested that certain flavonols have the ability to prevent collagen breakdown by inhibiting collagenase (Sin and Kim 2005). Similarly, catechins, in particular (–)epigallocatechin gallate (EGCG) have been shown to inhibit membrane type I matrix metalloproteinase (MMP), which hydrolyzes type I collagen (Yamakawa *et al.,* 2004). Therefore, since the inhibition of collagen breakdown by some flavonoids is a property relating positively to cases of hindered skeletal muscle healing, compounds capable of reducing collagen breakdown could modulate the deposition of the extracellular matrix, thus favouring the repair process with very little fibrosis.

In an *in vitro* study it was demonstrated that quercetin (30 μM) was able to inhibit the expression of pro-inflammatory cytokines in human mast cell line by blocking the activation of p38 mitogen activating protein kinase (MAPK) and nuclear factor kappa beta (NFκB) (Min *et al.,* 2007). However, a high dose of quercetin (1,000 mg/day) ingestion over a period of 24 days in cyclists did not alter muscle NFκB content, exercise-induced increases in muscle cyclooxygenase (COX)-2 mRNA or muscle mRNA expression for IL-6, IL-8, IL-1β, and TNF-α (Nieman *et al.,* 2007). It did attenuate increases in postexercise blood leukocyte IL-8 and IL-10 mRNA and tended to lower plasma levels of IL-8 and TNF-α, whilst reducing blood leukocyte IL-10 mRNA expression after the first day of exercise, without any measurable effect on plasma IL-10 levels. Although these data might indicate that quercetin is beneficial

by blocking NFκB activation in some cell types and thus attenuating pro-inflammatory actions, RNA levels are not conclusive evidence of clinical effects.

Although no direct evidence exists to show efficacy of any of these substances to improve or hasten recovery, with the exception of polyphenols, all reviewed studies suggest that vitamins A and E and other dietary flavonoid antioxidants are beneficial in scavenging free radicals, so as to reduce oxidative stress. However, these products are not without risks, as high doses of a single vitamin or high doses as a result of multiple vitamins at a time – particularly after physical exercise - can be toxic (Ristow *et al.,* 2009). Therefore, other products should be investigated for efficacy, but with lower associated risk to the user.

Numerous plant derivatives claim to have the ability to promote wound healing, such as *Angelica sinensis* (Ye *et al.,* 2001), *Eucommia ulmoides* (leaf extract) (Li *et al.,* 2000), *Arnica*, *Astragalus*, *Calendula*, *Hyssop*, *Myrrh* and *Paud'arco* (Williamson and Wyandt 1997). Proanthocyanidins are a group of biologically active polyphenolic bioflavonoids, which are synthesized by many plants, vegetables and fruit, especially grape seeds. The particular importance of these compounds in muscle physiology will be discussed in detail in the next section.

2.4 Grape seed extract

Flavonoids and other plant phenolics are reported to have multiple biological activities, in addition to their free radical scavenging (anti-oxidant) activity (Kandaswami and Middleton 1994; Chen *et al.,* 1996; Bagchi *et al.,* 1997; Bagchi *et al.,* 1998; Orozco *et al.,* 2003). Flavonoids, which include grape seed proanthocyanidolic oligomers (PCO) were reported to possess cardioprotective abilities by directly scavenging reactive oxygen species such as hydroxyl and peroxyl radicals (Sato *et al.,* 1999a). Other protective abilities include vasodilatory (Duarte *et al.,* 1993a; Duarte *et al.,* 1993b; Sato *et al.,* 1999a), anticarcinogenic (Hertog *et al.,* 1992; Hertog *et al.,* 1993; Birt *et al.,* 2001), anti-inflammatory (Middleton and

Kandaswami 1992; Sud'ina *et al.,* 1993; Salah *et al.,* 1995; Rice-Evans *et al.,* 1996; Cao *et al.,* 1997; Bagchi *et al.,* 1998; Birt *et al.,* 2001), antibacterial (Surico *et al.,* 1987), anti-allergic (Sud'ina *et al.,* 1993), and antiviral effects (Surico *et al.,* 1987). Although most of these effects of flavonoids have been studied in the context of cardiovascular diseases, conclusions regarding some of these effects could also be applicable to injured skeletal muscle. The most important effects to take note of include their free radical scavenging/ antioxidant properties, vasodilatory and anti-inflammatory effects. These effects involve the inhibition of the enzymes in the arachidonic acid pathway, such as phospholipase A2, lipoxygenase and cyclooxygenase, glutathione reductase, and the xanthine oxidase pathway (Middleton and Kandaswami 1992; Sud'ina *et al.,* 1993; Salah *et al.,* 1995; Rice-Evans *et al.,* 1996; Cao *et al.,* 1997; Bagchi *et al.,* 1998; Birt *et al.,* 2001), all of which are activated as a result of oxidative stress and injury and may function to hinder adequate muscle healing.

When muscle is damaged, free radicals are formed by various cell types, which include satellite cells (upon activation) and immune cells, and can result in secondary damage if damage spreads to include the healthy surrounding tissue (Brickson *et al.,* 2001; Schneider *et al.,* 2002). Therefore, in order to prevent secondary damage, it is necessary to have an increased ability to scavenge free radicals (flavonoids are oxidised by radicals, resulting in a more stable, less reactive radical – see equation 1) (Halliwell 1995), as well as a decreased production of free radicals in order to restore normal muscle structure and function and thus to improve muscle recovery. Selected flavonoids can directly scavenge superoxides, whereas other flavonoids can scavenge the highly reactive oxygen-derived radical called peroxynitrite.

$$\text{Flavonoid(OH)} + R^{\cdot} \rightarrow \text{flavonoid}(O^{\cdot}) + RH \qquad \text{Equation 4}$$

($R^{\cdot}$ is a free radical and $O^{\cdot}$ is an oxygen free radical)

In response to injury, increased immobilization and adhesion of leukocytes to the endothelial wall results in the production of free radicals upon neutrophil degranulation. This release of cytotoxic oxidants and inflammatory mediators is also responsible for further activation of the

complement system, which has been associated with an increased inflammatory reaction, further exacerbating the initial injury (Bury and Pirnay 1995; Belcastro *et al.,* 1996). This occurs during episodes of ischemia and inflammation, and may result in injury to other tissues as well. Therefore, in order to prevent the degranulation and adhesion of leukocytes to the endothelial wall, one either has to mobilise the leukocytes by means of chemotactic factors, or prevent the degranulation of the neutrophils, thereby inhibiting the formation of free radicals. However, blood-borne immune cells, including neutrophils and macrophages must gain direct access to the site of damage, as a result of vasodilation and a combined effect of adhesion molecules and chemotactic factors, to aid muscle recovery (Deal *et al.,* 2002; Sherwood 2004a). Anti-inflammatory cytokines play an additional role in allowing immune cells to enter the injured area. These cytokines are particularly important for attraction of M2 macrophages - macrophages important for muscle regeneration (Mantovani *et al.,* 2004).

Considering the fact that reactive oxygen species are such a problem after injury and the beneficial effects of flavonoids in scavenging these free radicals, as well as their role in suppressing the various enzymes and key role players in the arachidonic acid and xanthine oxidase pathway (Hilário *et al.,* 2006), flavonoids might play a crucial role in possibly limiting the time required for muscle regeneration. Since the focus of this thesis is proanthocyanidins, the next few paragraphs will focus on the available literature regarding proanthocyanidin and their possible beneficial effects.

An *in vivo* study in mice showed grape seed proanthocyanidin extract (100 mg/kg) to be a better free radical scavenger and inhibitor of oxidative tissue damage than vitamin C (100 mg/kg), E succinate (100 mg/kg), and β-carotene (50 mg/kg) (Bagchi *et al.,* 1998). Proanthocyanidin monomers are rapidly absorbed into the blood, where they bind to heavy metal ions, resulting in the scavenging of free radicals (Gonthier *et al.,* 2003; Sano *et al.,* 2003). Oligomeric proanthocyanidin are not as readily absorbed and may therefore not be regarded as the main flavonoid responsible for beneficial effects (Rios *et al.,* 2003).

However, when comparing studies done in rodents *vs.* human subjects, most of the studies in the past did not take the metabolic rate, or manner of administration, into account. Administering a similar dosage of flavonoids to humans or rats will have a different effect, since rats have an approximately 7-10 times higher metabolic rate, therefore one might only see a positive effect in rats and not humans. Also direct gavaging (directly into the stomach) compared to taking a tablet will also result in a different dosage reaching the stomach, with gavaging allowing the full concentration to reach the stomach *vs.* a lower concentration with a tablet. Over the past few decades, numerous *in vivo* studies (mainly cardiovascular, inflammation and cancer research) have also demonstrated that flavonoids, including proanthocyanidins are safe and produce no clinical side effects, except perhaps quercitin (Dunnick and Hailey 1992; Zhu *et al.,* 2001).

Specific functions of proanthocyanidins include the ability to strengthen and protect living tissue, through inhibiting the activity of the proteolytic enzymes collagenase and elastase, which are directly involved in the turnover of the main structural components of connective tissue, collagen and elastin (Tixier *et al.,* 1984; Hyun *et al.,* 2003; An *et al.,* 2005; Balu *et al.,* 2005). Proanthocyanidins have been shown to mediate several anti-inflammatory mechanisms, in particular those involved in the development of cardiovascular disease. Studies have indicated that these mechanisms are of particular importance in aiding the inflammatory process after atherosclerosis to allow monocytes to adhere to the endothelial wall and entering the "area of damage". In one particular *in vitro* study, grape seed proanthocyanidins were responsible for down-regulating the TNF-α-induced expression of vascular adhesion molecule (VCAM)-1 on human endothelial cells, therefore playing a pivotal role in the inflammatory process as a result of its effect on migration (Sen and Bagchi 2001). In contrast to the study focussing on expression of VCAM, other studies have taken into account the metabolism of the cell, and showed positive results: moderate red wine consumption for 28 days abolished the adhesion of monocytes (isolated from healthy males) to endothelial cells (Badia *et al.,* 2004; Estruch *et al.,* 2004). Markers influenced in these studies, included VCAM, intercellular adhesion molecule (ICAM), CRP and IL-1α, all of which

are usually elevated in vascular disease associated with inflammation. The vascular endothelium is important for synthesizing and releasing nitric oxide (NO), which in turn promotes vasorelaxation and reduces platelet aggregation, and limits the influx of acute phase proteins into the injured area (Palmer *et al.,* 1988). Various studies have indicated that proanthocyanidins may have favourable effects on these vascular endothelial functions (Duffy and Vita 2003; Dell'Agli *et al.,* 2004). Furthermore, grape juice administration specifically was able to improve endothelium-dependent vasodilation in coronary artery disease patients with impaired endothelial function (Stein *et al.,* 1999). Although most of these studies investigating the positive effects of proanthocyanidins have looked at cardiovascular diseases, the general beneficial effect of proanthocyanidins on inflammation might also be true for skeletal muscle injury models.

In order for a natural product to be considered an effective therapy for skeletal muscle injury, it must display the following properties. Firstly, for effective healing, a natural plant-derived product should have an anti-inflammatory property – the ability to attenuate pro-inflammatory cytokine release, whilst increasing anti-inflammatory cytokine release. Inflammatory cytokines play a predominant role in aiding muscle regeneration by facilitating immune cell recruitment to the injured area. Pro- and anti-inflammatory cytokines are responsible for attracting macrophages, whilst only pro-inflammatory cytokines are involved in neutrophil and macrophage recruitment. Therefore, a second property of a potential product is the ability to recruit more macrophages, whilst decreasing the number of neutrophils to the injured area. Thirdly, the natural product should have a potent free radical scavenging ability, eliminating or reducing the amount of ROS, therefore resulting in quicker and faster healing. Fourthly, satellite cells should be activated earlier to facilitate faster recovery. Lastly, if the natural product can strengthen plasma membranes, it will limit the amount of damage, and therefore healing will occur sooner. Oxiprovin™ grape seed extract (GSE) is believed to be one such product (Oxiprovin™, Brenn-O-Kem, Wolseley, South Africa). It is a natural South African plant antioxidant product extracted from grape seeds in a water and ethanol extraction process (Brenn-O-Kem 2006). Oxiprovin™ contains 45 % proanthocyanidins and less than 5

% monomers, the remainder of which is made up of of long chain sugars and glycosides attached to the oligomers.

With particular relevance to muscle injury, the effect of oral grape-seed derived proanthocyanidin (Oxiprovin™) supplementation on muscle recovery after contusion injury were investigated by our group (MSc thesis, Kruger 2007). In this study, Oxiprovin™ supplementation resulted in faster skeletal muscle fiber regeneration, as assessed by magnitude and speed of satellite cell activation, and foetal myosin heavy chain (MHC_f) expression in regenerating muscle fibres. These data suggest that chronic Oxiprovin™ supplementation may facilitate quicker recovery of muscle following muscle injuries of mild to moderate severity. The exact mechanism(s) by which Oxiprovin™ achieves its effect of increasing muscle recovery is not clear. Furthermore, the minimum duration of supplementation for beneficial effects has not been determined.

2.5 Summary

The ideal treatment for skeletal muscle injuries is still lacking, mainly due to the extent and severity of injuries reported in the literature as well as the multiple underlying processes which complicate the research.

In spite of numerous research with experimental and clinical studies, the effect of different treatment regimes on muscle recovery have been disappointing. RICE has been shown to be beneficial, but timing is important, since the various components it consists of will allow for better recovery of muscle if applied at the correct time. Cryotherapy (the application of ice), one of the key components of RICE, for example, should be applied as soon as possible, however, it only alleviates pain. Therapeutic ultrasound on its own does not seem to be beneficial for muscle regeneration, however, if used in combination with antioxidant drugs it has been shown to be beneficial. Light exercise or early mobilization of the injured leg after a short period of immobilization has proven to be beneficial for rapid regeneration.

Although some of the studies have shown positive effects, the negative effects cannot be ignored. Steroids have only been shown to alleviate pain, with no beneficial effect on muscle regeneration. NSAIDs, although showing some promise in limiting inflammation when administered at low doses, higher doses have been shown to be detrimental. Furthermore, most studies started NSAID administration 2-5 days after injury, points in time when inflammation is already prominent, and neutrophils might already have contributed to secondary damage as a result of their respiratory burst. Further studies looking at earlier time points might therefore be beneficial. Most studies investigating regeneration have also looked at markers not considered to be good markers of muscle damage and recovery. More specific markers should therefore be investigated in order to determine if these drugs and treatment modalities are beneficial. In this regard, one excellent study has investigated growth factor injection into the injured area, specifically IGF-1. Beneficial effects were measured directly, e.g. increased number of regenerating myofibres compared to their respective control (Kasemkijwattana *et al.,* 1998a; Kasemkijwattana *et al.,* 1998b).

With all the studies mentioned and considered in this chapter, few studies have investigated speed of muscle recovery, as well as the potential of the treatment to reduce reactive species, which are generated upon muscle injury. Thus ideally if one can limit reactive species formation, one can potentially allow for quicker muscle recovery. Treatment with vitamin supplements was hypothesized to be beneficial after muscle injury. However, although vitamin supplements have been shown to be able to reduce oxidative tissue damage, the only marker of damage and recovery investigated was plasma CK, and therefore not conclusive enough evidence for quicker muscle recovery. Polyphenol treatment, on the other hand, has been shown to be beneficial for healing of pathology, especially with regard to the *mdx* model. The evidence for speeding up the recovery process after acute injury is still lacking.

A previous study by our group has found that chronic Oxiprovin™ supplementation is beneficial in speeding up the recovery of rat muscles after a contusion injury (MSc thesis,

Kruger 2007). However, it is not known how Oxiprovin™ exerts its beneficial effect. In order to investigate Oxiprovin™'s mechanism of action (Chapter 3), the local and systemic cytokine profiles, as well as the infiltration of immune cells (neutrophils and macrophages) into the injured muscle were assessed. Since most athletes cannot predict the outcome of a game, the issue of acute supplementation were also addressed (Chapter 4). In this thesis, Oxiprovin™'s ability to: (a) scavenge free radicals, (b) speed up the recovery from a contusion injury, (c) blunt the inflammatory cytokine status and (d) drive the repair phase of muscle recovery in favour of early immune cell infiltration after sustaining a contusion injury were also investigated.

Different macrophage phenotypes are involved in the inflammation and repair phase following an injury. Macrophages can also switch from being "damaging" (M1) to "repairing" (M2). Thus, investigating the presence of the different macrophage phenotypes will provide us a better understanding of when and how Oxiprovin™ might exert its beneficial effect (Chapter 5). Also, since cytokines and chemotactic factors play a major role in attracting neutrophils and macrophages to the injured area, an *in vitro* migration assay will further promote our understanding of the particular mechanisms of action of Oxiprovin™ to enable or prevent neutrophil migration. In this last study, neutrophils are allowed to migrate, in the presence of various plasma cytokines at different time points after injury, towards a specific chemotactic factor (Chapter 5). This will enable the determination of the specific timing of neutrophil migration from circulation.

CHAPTER 3

The effect of chronic proanthocyanidolic oligomer supplementation on the inflammatory response to contusion injury in rat hindlimb

3.1 Introduction

A contusion injury, an injury caused by a blunt non-penetrating object, result in the rupturing of muscle fibres at or adjacent to the injured area (Jarvinen *et al.,* 2005). The inflammatory process is a very important event following an injury, contributing to both secondary damage and recovery. As mentioned before, neutrophils are the first immune cell to infiltrate the injured area, playing a predominant role, from approximately 1 hr after injury, in clearing the injured area of any debris (Tiidus 1998), whilst macrophage infiltration of the tissue is initiated roughly 24-48 hr after injury, playing a role in both phagocytosis and muscle repair (Duffield 2003). Inflammation is regarded as a complex process, involving a whole host of events which are regulated by various role players, involving both pro- and anti-inflammatory cytokines and various other mediators - for a recent review on cytokines and growth factors in this context, see Smith *et al.* (2008).

Although it is known that immune cells, as well as injured muscle cells themselves, release cytokines (Feldmann and Maini 2003), it is not known which cell is responsible for injury-related cytokine release. Nevertheless, cytokines associated with injury include the pro-inflammatory cytokines interleukin (IL)-6, IL-1β and tumour necrosis factor (TNF)-α, which coincide with the early phase after injury, in other words destruction, and the anti-inflammatory cytokines IL-4 and IL-10, which coincide with muscle recovery (Mantovani *et al.,* 2007). Pro-inflammatory cytokine expression also correlates with the activation of the oxidative-stress sensitive nuclear transcription factor NFκB (Blanchard *et al.,* 2000). These pro-inflammatory cytokines are produced within the injured muscle, resulting in the release of more cytokines into circulation. It also results in the activation of white blood cells, as well as

inducing satellite cell (SC) proliferation. For example, macrophages can stimulate pro-inflammatory cytokine production (IL-1β and TNF-α), which recruits more monocytes and aids SC activation, ultimately resulting in muscle regeneration (Rosenberg and Gallin 1993; Cannon and St Pierre 1998; Tidball 2005).

There is normally a fine balance between the interaction of different immune cells and the cytokines and growth factors in response to an injury. This interaction will determine: 1) whether the immune response is of short-duration or a longer-term adaptation, 2) the speed of the inflammatory response and 3) whether a shift in favour of one particular response (pro *vs.* anti-inflammatory) will result in another response being compromised.

The effect of contusion injury on immune cells and cytokines has not been studied extensively and data presented are not consistent. The differences in the severity of injury (i.e. with or without bone or tendon involvement) and the models used to produce experimental contusion injury (invasive *vs.* not), result in variations in the inflammatory response reported (e.g. long *vs.* short duration, or no *vs.* extensive inflammatory responses) (Raj *et al.,* 1998; Tidball *et al.,* 1999; Kami *et al.,* 2000; St. Pierre Schneider *et al.,* 2002; Bunn *et al.,* 2004; Farnebo *et al.,* 2009). Little is known regarding the involvement of the inflammatory immune cells and the specific cytokines in recovery from a contusion injury, thus more work is required to elucidate their specific contributions to skeletal muscle injury and recovery.

While the phagocytic and inflammatory responses are considered critical in the recovery from muscle injury, oxidants released from neutrophils, macrophages and SCs can all contribute to promote further muscle damage, also termed oxidative stress. Oxidative stress occurs when production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) overwhelms the endogenous antioxidant system of enzymes (i.e. superoxide dismutase, glutathione peroxidase) and substrates (i.e., vitamin E, reduced glutathione), and is considered to be a key contributor to inflammation (McArdle and Jackson 2000). It has been suggested that the dynamic balance between inflammatory cell- and muscle-derived free

radicals may play important roles in modulating the course of muscle injury and repair after modified muscle loading (Tidball 2002). ROS can be important in the initiation of exercise-induced muscle damage and in the initiation and propagation of the subsequent acute muscle inflammatory response (Tiidus 1998). Furthermore, in this specific study, it was demonstrated that oxygen radicals generated *via* the neutrophil respiratory burst are vital in clearing away the damaged muscle tissue, but that an excessive oxygen radical response may result in the propagation of further tissue damage (Tiidus 1998). More specifically, Finkel and Holbrook (2000) proposed that oxidative stress is secondary to the recruitment of inflammatory cells which contain the potent NADPH oxidase system (present in neutrophils, macrophages, vascular cells and cell membranes), which produces large amounts of superoxide (O_2^-). O_2^- reacts rapidly with nitric oxide (NO) to form peroxynitrite, overproduction of which is also a causative factor of tissue-damage, particularly in the case of chronic lung inflammation (Kooy *et al.,* 1995), but also in brain or vessel injury (Kontos and Wei 1986). Macrophages produce large amounts of NO that attract SCs to the injured area, and consequently result in SC proliferation and maturation, as well as recruitment of more macrophages (Anderson 2000; Chazaud *et al.,* 2003), forming a positive feedback loop. NO production is time and concentration dependent and that if more macrophages are present at a given time, this will result in the recruitment and activation of more SCs (Anderson 2000). Therefore, it is evident that if one can limit oxidative stress, one can also limit the magnitude of inflammation and associated damage in the injured area, and potentially speed up the recovery process. However, it is also possible that limiting oxidative stress too much will lead to insufficient phagocytosis and/or too little activation of repair.

Oxiprovin™ is a commercially available extract prepared from grape seeds, and contains the active ingredient proanthocyanidolic oligomers (PCO), which form part of the flavonoid family, and have anti-inflammatory properties (Gonzalez-Gallego *et al.,* 2007). Previous results in rats have indicated that PCO supplementation is positively associated with muscle recovery (MSc thesis, Kruger 2007). In this study, chronic PCO supplementation (2 weeks prior to and after contusion injury) resulted in quicker SC activation (CD34 and CD56

expression were elevated 4 hr after injury in the PCO group, compared to day 3 and day 7 in the PLA group) and earlier skeletal muscle regeneration (foetal myosin heavy chain – MHC_f was elevated by day 3 in the PCO group compared to day 7 in the PLA group, Figure 3.1), indicating that PCO supplementation had a positive effect on muscle recovery (MSc thesis, Kruger 2007). Oxygen radical absorbance capacity (ORAC) results also indicated that groups supplemented with PCO had a better free radical scavenging effect, and in conjunction with lower plasma CK activities found in the PCO group, it was concluded that PCO supplementation could protect the skeletal muscle against secondary damage (MSc thesis, Kruger 2007).

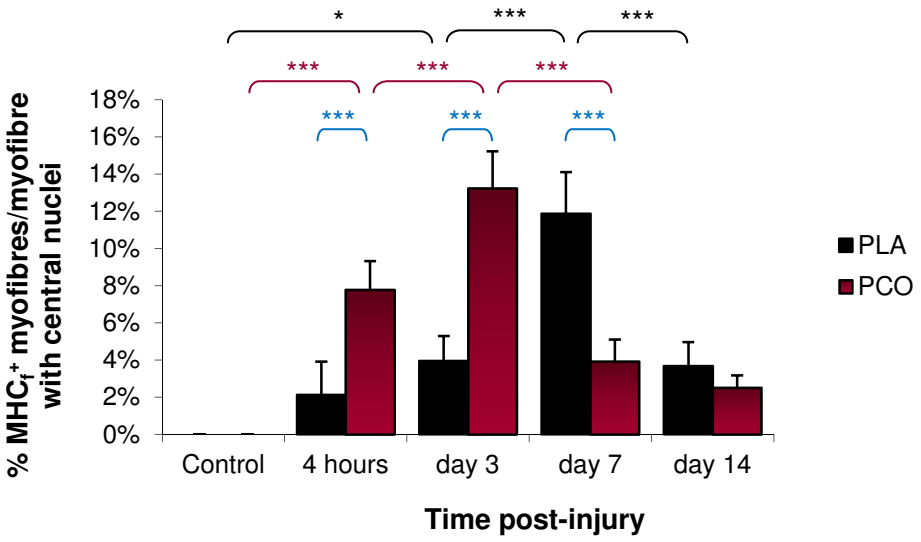


Figure 3.1: Foetal myosin heavy chain (MHC_f) positive myofibres with central nuclei are expressed as percentage of the total myofibre number (% MHC_f^+ myofibres) (means ± SD) at control levels (non-injured) and at 4 hr, day 3, 7 and 14 post-injury (* $p<0.05$, *** $p<0.001$). Statistics: Factorial analysis of variance with Bonferonni *post hoc* test. n = 4 rats per time point per group (MSc thesis, Kruger 2007).

Considering the above data and other literature, it was hypothesised that PCO in Oxiprovin™ would alter the inflammatory response, most probably in favour of an anti-inflammatory response. To assess this hypothesis, the magnitude and duration of changes in pro- and anti-inflammatory cytokine levels in circulation and tissue, as well as the magnitude and time

course of neutrophil and macrophage infiltration into the injured muscle tissue following chronic PCO supplementation were assessed. In order to verify SC results seen in previous studies, recent and more specific marker for SCs were used.

3.2 Methods

3.2.1 Animals and interventions

3.2.1.1 Experimental animals

Adult, male Wistar rats (bred and housed in the small laboratory animal facility at the Department of Physiological Sciences, Stellenbosch University) weighing approximately 280 g were used for this study. All animals had access to food (standard rat chow pellets, supplied by the Medical Research Council animal unit, Parow) and tap water *ad libitum* and were exposed to a 12 hr light/dark cycle (lights on at 6h30). Ambient temperature was controlled at 21 ºC, and rooms ventilated at a rate of 10 changes/hour. Prior to the initiation of intervention protocols, rats were accustomed to the researcher by daily handling and oral gavage (tap water only) for a period of 2 weeks. All experimental protocols were approved by the Animal Research Ethics Committee of Sub-Committee B of Stellenbosch University (reference # 2006 Smith01).

Experimental rats were divided into 4 groups, a control group and an injury group (see Figure 3.2). The control group rats were divided into a control placebo group receiving 2 weeks of 0.9 % saline treatment (C-PLA, n=8) and a control proanthocyanidolic oligomer (PCO) group, receiving 2 weeks of Oxiprovin™ supplementation (C-PCO, n=8). Rats belonging to the injury group were also subdivided into two groups, an injury-placebo group, injured after 2 weeks of placebo supplementation, with continued placebo supplementation post-injury (I-PLA; n=48, i.e. 8 rats per time point, time points were 4 hr, 1 d, 3 d, 5 d, 7 d and 14 d post-injury) and an injury PCO group, injured after 2 weeks of Oxiprovin™ supplementation, with

continued Oxiprovin™ supplementation post-injury (I-PCO; n=48, i.e. 8 rats per time point, time points were 4 hr, 1 d, 3 d, 5 d, 7 d and 14 d post-injury).

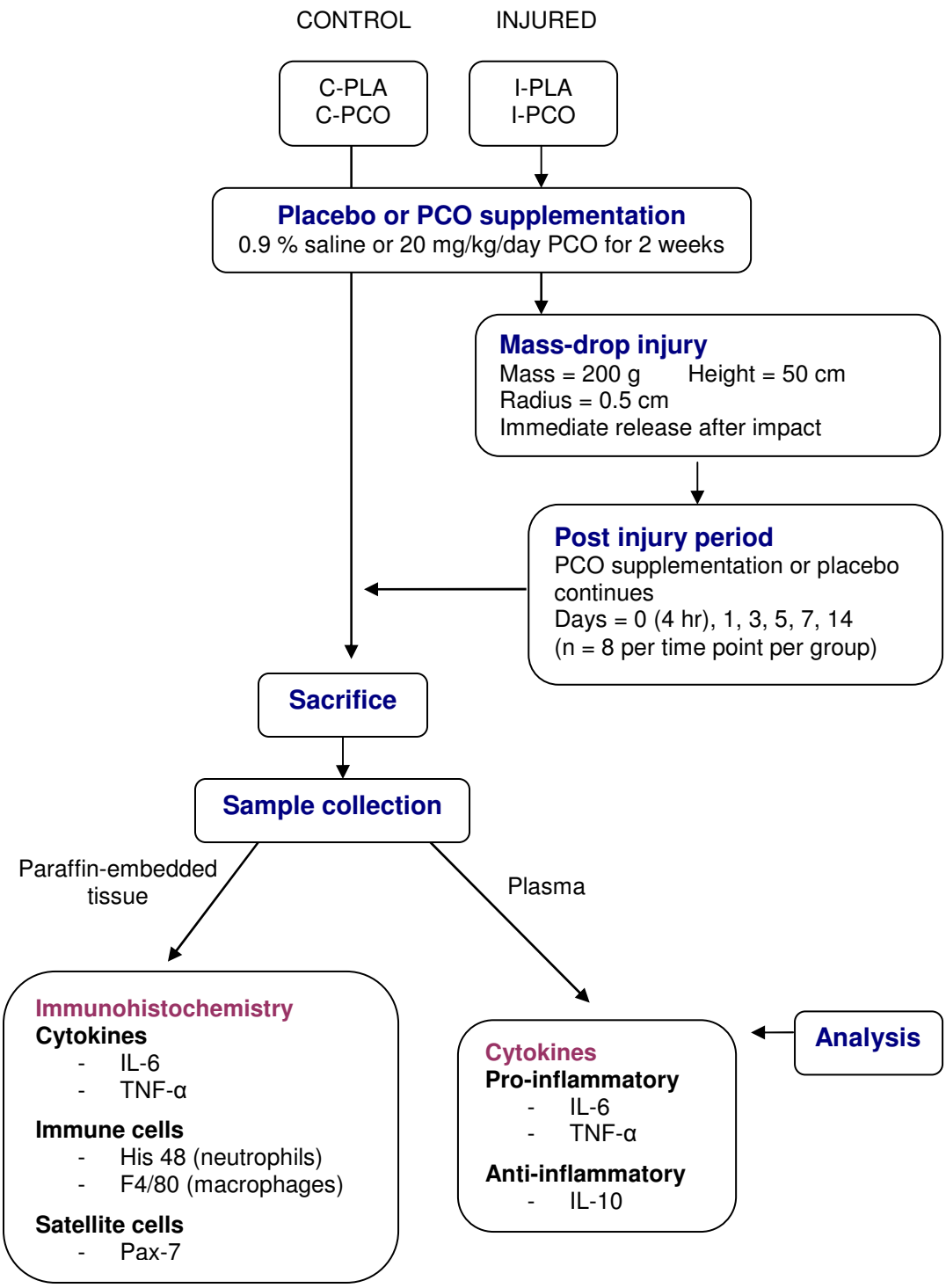


Figure 3.2: Experimental design.

3.2.1.2 PCO administration

Oxiprovin™ is a natural South African plant antioxidant product extracted from grape seeds in a water and ethanol extraction process (Brenn-O-Kem 2006), which contains 45 % proanthocyanidins, less than 5 % monomers and the remainder made up of long chain sugars and glycosides attached to the oligomers. Rats belonging to the PCO group, both injured and controls, were administered a daily dose of 20 mg/kg/day Oxiprovin™ (dissolved in 0.9 % sterile saline) by means of oral gavage (1 ml/kg body mass). Rats in the PLA group received a daily dose of 0.9 % saline (1 ml/kg body mass) for 14 days, also by means of oral gavage. Additionally, rats were also treated with Oxiprovin™ or saline for up to 14 days after injury.

3.2.1.3 Induction of muscle injury

Contusion of the hind limb was produced by a mass-drop jig (Figure 3.3), similar to the model first described by Stratton *et al.* (1984) and later used by Kami *et al.* (1993, 1995). Briefly, the lower part of the apparatus comprised a large round metal platform, with a small cylindrical platform in the centre, on which the hind limb of the animal rested just prior to and during injury. A plastic tube fastened perpendicularly to and directly above the smaller platform directed the passage of a 200 g flat, circular bottomed weight from a height of 50 cm onto the medial surface of the right *gastrocnemius* muscle. A peg through a hole in the plastic tube kept the weight in place at the desired height above the muscle, so that its removal resulted in a standardised mass dropping speed. Rats were anaesthetised with 75 mg/kg ketamine, 0.5 mg/kg medetomidine and 0.9 % saline (1 ml/kg body mass), administered intraperitoneally (i.p.), whereafter the right hind limb was moderately extended away from the hip joint, and the muscle was injured. After this procedure, the rats were unconscious for up to 2 hr, after which they still remained slow in motion for a further 2 hr. However, there was no limping and no uneven gait.

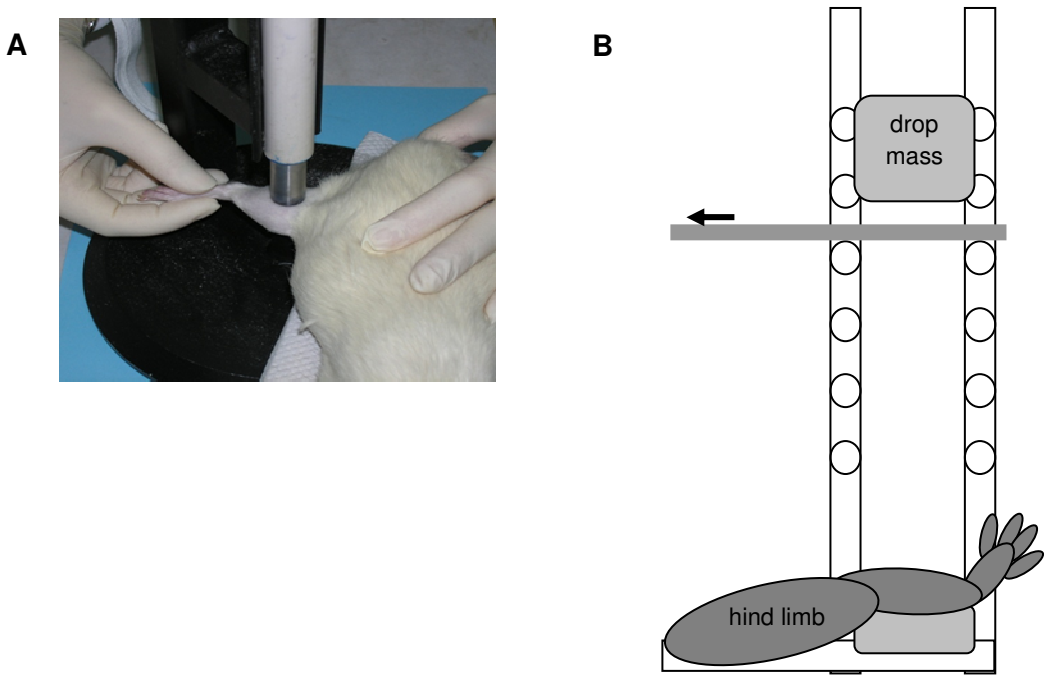


Figure 3.3: Muscle contusion injury jig. **A** represents a real-time photo of our muscle contusion injury jig, whereas **B**, represents a schematic illustration of the non-invasive, standardised 'mass-drop injury jig'.

3.2.2 Sacrifice and sample collection

3.2.2.1 Sacrifice

All sacrifice procedures took place between 08:00 and 10:00 in the morning, with the exception of the 4 hr time point. In this group, rats were sacrificed at 12:00, 2 hr after injury and 2 hr after the last supplementation treatment.

During the sacrifice procedures, all animals received an overdose of pentobarbitone sodium (200 mg/kg i.p.), and eight rats from each of the 2 control groups (C-PLA and C-PCO) or injury groups (I-PLA and I-PCO) were sacrificed at each of the following time points after subjection to hindlimb muscle contusion injury (4 hr, on day 1, 3, 5, 7 and 14 after injury).

3.2.2.2 Sample collection

Blood collection: Following euthanasia by pentobarbitone overdose, the chest cavity was opened and the heart exposed. Whole blood samples were obtained by means of right ventricular cardiac puncture. Blood was collected using a 20 gauge, 1½ inch needle into a 5 ml syringe and immediately transferred to heparinised tubes (Vacutainer, Beckton Dickinson). Blood samples were immediately mixed and placed on ice prior to centrifugation (within 2 hr) at 3000 g for 10 min at 4 °C. Plasma obtained was aliquoted into 1.5 ml reaction vials and stored at -80 °C until subsequent analysis.

Muscle collection: After the procedures for cardiac puncture, the *gastrocnemius* muscle of the right (injured) hind leg was exposed by cutting and removing the skin and connective tissue surrounding the muscle. The damaged/recovering medial central section of the *gastrocnemius* muscle was harvested and the section randomly divided so that one part was processed for immunohistochemistry, and the other snap-frozen for biochemical analysis.

3.2.3 Sample analysis

Two different analytical procedures were used: a) immunohistochemistry on muscle, and b) flow cytometry for plasma pro- and anti-inflammatory cytokines.

3.2.3.1 Immunohistochemistry

For immunohistochemistry, muscles were fixed in 4 % formaldehyde (or 10 % formal saline) for 7 days. The samples were then cut to size, placed into embedding cassettes, processed and impregnated with paraffin wax (Histosec, Merck – see Appendix A for details of tissue processing) using an automated tissue processor (TISSUE TEK II, model 4640B, Lab-Tek division, Miles Laboratories Inc, Naperville, IL). Impregnated tissue samples were embedded in paraffin wax.

Five µm thick cross sections were cut using a rotary microtome (Leica Microsystems CM1850, Nussloch, Germany). Sections were adhered to poly-L-lysine (Sigma Aldrich) coated slides (3 per slide), deparaffinised, digested in 0.1 % Trypsin (Highveld) to open up the binding sites for antigens and then blocked with 5 % serum to limit non-specific binding. Donkey serum were used for all staining procedures, with the exception of Pax-7 double labelling with laminin, where goat serum was used. Antibodies for pro-inflammatory cytokine labelling included anti-TNF-α and anti-IL-6, whereas His48 (plus nuclear presence) was used to label neutrophils and F4/80 to label macrophages. Phosphate buffered saline (PBS) controls were used to determine the specificity of the primary antibodies and all primary antibodies were optimised prior to immunohistochemistry. All antibodies used were suitable for recognising specific antigens on rat satellite cells, neutrophils and macrophages. For detailed staining procedure, see Appendix B.

Image acquisition: All imaging data were obtained by analysing two serial sections from each muscle sample, at each time point for each antibody. Six or more fields of view per section were imaged using a fluorescence microscope (Nikon ECLIPSE E400; 40x objective used; actual enlargement thus 400x), equipped with a colour digital camera (Nikon DXM1200).

Image analysis: All pictures were overlayed using a computer software program, *Simple PCI* (version 4.0, Compix Inc., Imaging Systems, USA). A standard way of identifying immune cells, is to enlarge photos and to enhance the color of the stain after importation into *Simple PCI*. *Simple PCI* was used to acquire and overlay images, whereas *Image J* was used in addition to *Simple PCI* for quantify cytokine levels. Note that the images presented here are only partial images of those taken at 400x. All cytokine and immune cell staining were assessed in sections including border zones (areas right next to the severely injured areas, where muscle fibres are intact) and injured areas. The threshold for all images were set to the same level throughout the experiment to enable comparisons of cytokine data over time and between groups. Cytokine data were expressed as the relative fluorescence area containing either IL-6 or TNF-α per field of view in either the injured area or border zone

area. Immune cell data were counted manually and expressed as the number of positively labeled immune cells per field of view (350 μm^2) in both the injured and border zone areas. To ensure accurate counting of neutrophils, multilobular nuclei had to be present. Satellite cell data were assessed in border zone areas and expressed as the number of satellite cells per myofibre. In order for a cell to be classified a true satellite cell, it had to comply to a few criteria: (a) Pax-7^+ cells had to be in contact with the sarcolemma, (b) the satellite cell had to form an indentation into the muscle fibre, and (c) an elongated nucleus had to be present. If one of these criteria were not present, the cell could not be classified a true SC.

3.2.3.2 Flow cytometry

Plasma from controls and animals subjected to contusion injury sacrificed at different time points (4 hr, 3 d, 7 d and 14 d) post-injury were analysed for IL-4, IL-6, IL-10, and TNF-α with a Cytometric Bead Array (CBA) Rat Flex Set kit from BD Biosciences according to the manufacturer's instructions using a FACSArray Bioanalyser flow cytometer (see Appendix C for catalogue numbers of antibodies and kits used as well as for protocol specificity). The CBA assay kit consisted of four types of beads (IL-4, IL-6, IL-10, and TNF-α), uniform in size, each of which measures the presence of cytokines in the sample. Each bead population were given an alpha-numeric position indicating its position relative to other beads in the BD CBA Flex Set system, therefore, beads with different positions can be combined in assays to create a multiplex assay, such as in this study. The four different capture beads in this study were pooled, diluted 50x and added to each well (50 µl/well). Standards from each flex set were diluted with 4.0 ml of Assay Diluent (Top standard) and allowed to equilibrate for 15 min before making the following serial dilutions: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256. Fifty µl of each of the diluted standards, including a blank (assay diluent only), were added to the first 10 wells, whereas the rest of the wells were filled with different samples and allowed to incubate for 1 hr. Four different capture antibodies, each of which were covalently coupled to each type of bead, were diluted 50x and added to each well (50 µl/well). Each of these capture antibodies have different intensities of red-emitting fluorescence dye (PE) enabling

detection of cytokines. After incubation for 2 hr, the fluid was aspirated and wash buffer was added to each assay well (150 µl/well). The fluorescence measured was proportional to the cytokine concentration in the sample and was quantified from a calibration curve. The detection limits for the cytokines used in this kit are: (a) 1.4 pg/ml for IL-6, (b) 27.7 pg/ml for TNF-α, (c) 3.4 pg/ml for IL-4 and (d) 19.4 pg/ml for IL-10.

3.2.4 Statistics

Values presented are means ± standard errors of the mean (SEM), unless otherwise specified. Differences between time points and treatment groups were analysed using factorial analysis of variance (ANOVA). When significant differences were found, Bonferroni or Fischer's *post hoc* tests were done to identify specific differences. All statistical analyses were done using Statistica version 9 (StatSoft Software). The accepted level of significance was $p < 0.05$.

3.3 Results

3.3.1 Satellite cell (Pax-7)

Repeated measures analysis of variance indicated a significant effect of both time and treatment separately, and treatment and time ($p < 0.001$ for all 3). After injury, similar to previous results (MSc thesis, Kruger 2007), an increase in SC number in the PLA group was seen at a much later time point (evident on day 7), compared to Pax-7 expression in the PCO group (4 hr after injury). Pax-7 expression in both supplementation groups also returned to control levels by day 14. Figures 3.4A-D visually display representative sections at points in time where the PLA and PCO groups were significantly different from one another, whilst Figure 3.4E illustrates the number of Pax-7^+ SCs per myofibre at different time points and for different groups. The Pax-7^+ nuclei can be described as elongated

compared to myonuclei and are situated in the satellite cell pocket. A clear example of a Pax-7$^+$ cell is indicated by the top yellow arrow in Figure 3.4C.

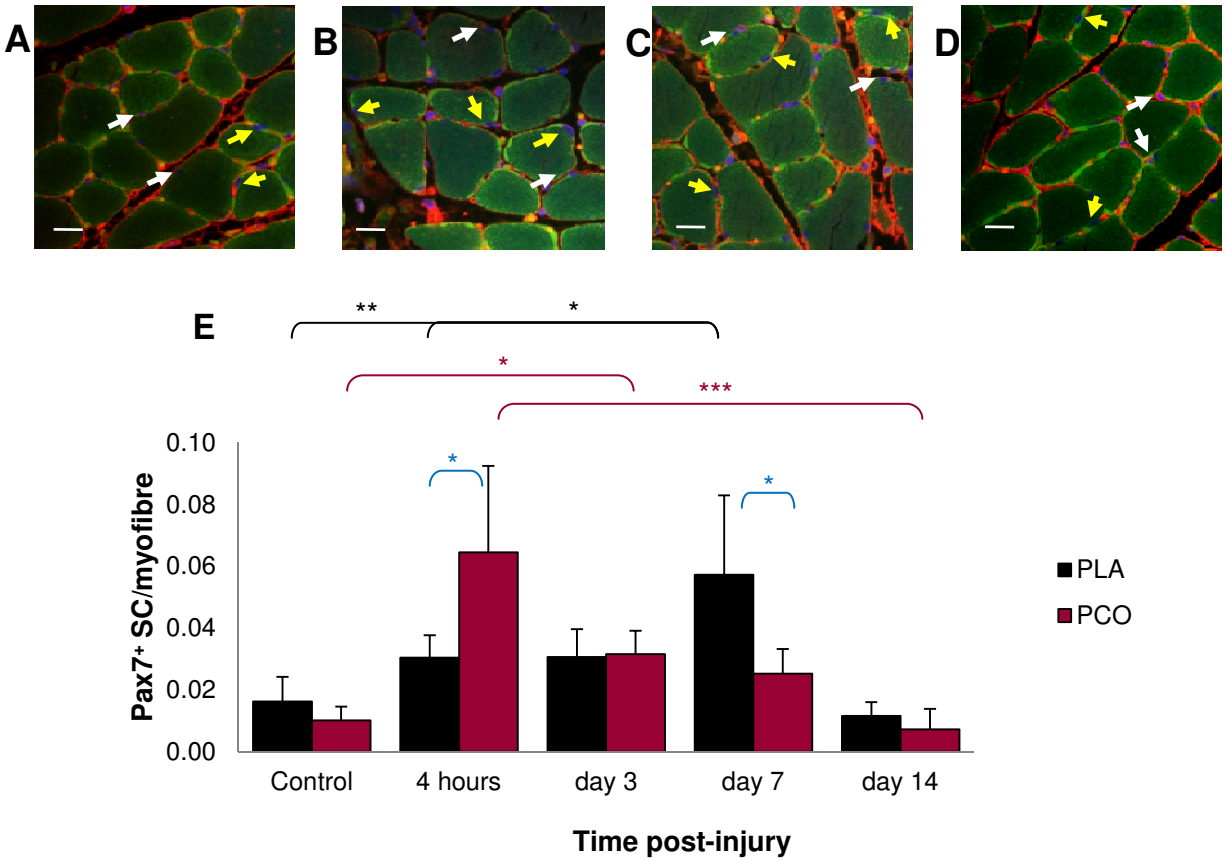


Figure 3.4: Pax7 staining in the PLA and PCO group at 4 hr (**A**&**B**) and day 7 (**C**&**D**) respectively. FITC (green) was used to visualise Pax7, Texas Red (red) was used to visualise laminin and Hoechst (blue) was used as a nuclear marker. Yellow arrows indicate true satellite cells, whereas white arrows indicate non-satellite cells. Scale bar represents 10 µm. Pax-7$^+$ satellite cells expressed per myofibre number (Pax-7$^+$ SC/myofibre number) (means ± SD; **E**). Statistical analysis: Factorial analysis of variance with Bonferroni *post hoc* test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). n = 4 rats per time point per group.

3.3.2 Plasma pro- and anti-inflammatory cytokine expression

ANOVA indicated a main effect of time for both pro-inflammatory cytokines assessed (TNF-α and IL-6; $p < 0.05$ for both), but this seemed to only be significant in the PLA group. PCO cytokine levels remained unchanged throughout the protocol. *Post hoc* tests indicated a

significant peak in both TNF-α and IL-6 levels in PLA group on day 7 ($p < 0.05$ *vs.* control, $p < 0.01$ *vs.* 4 hr) (Figure 3.5A&B).

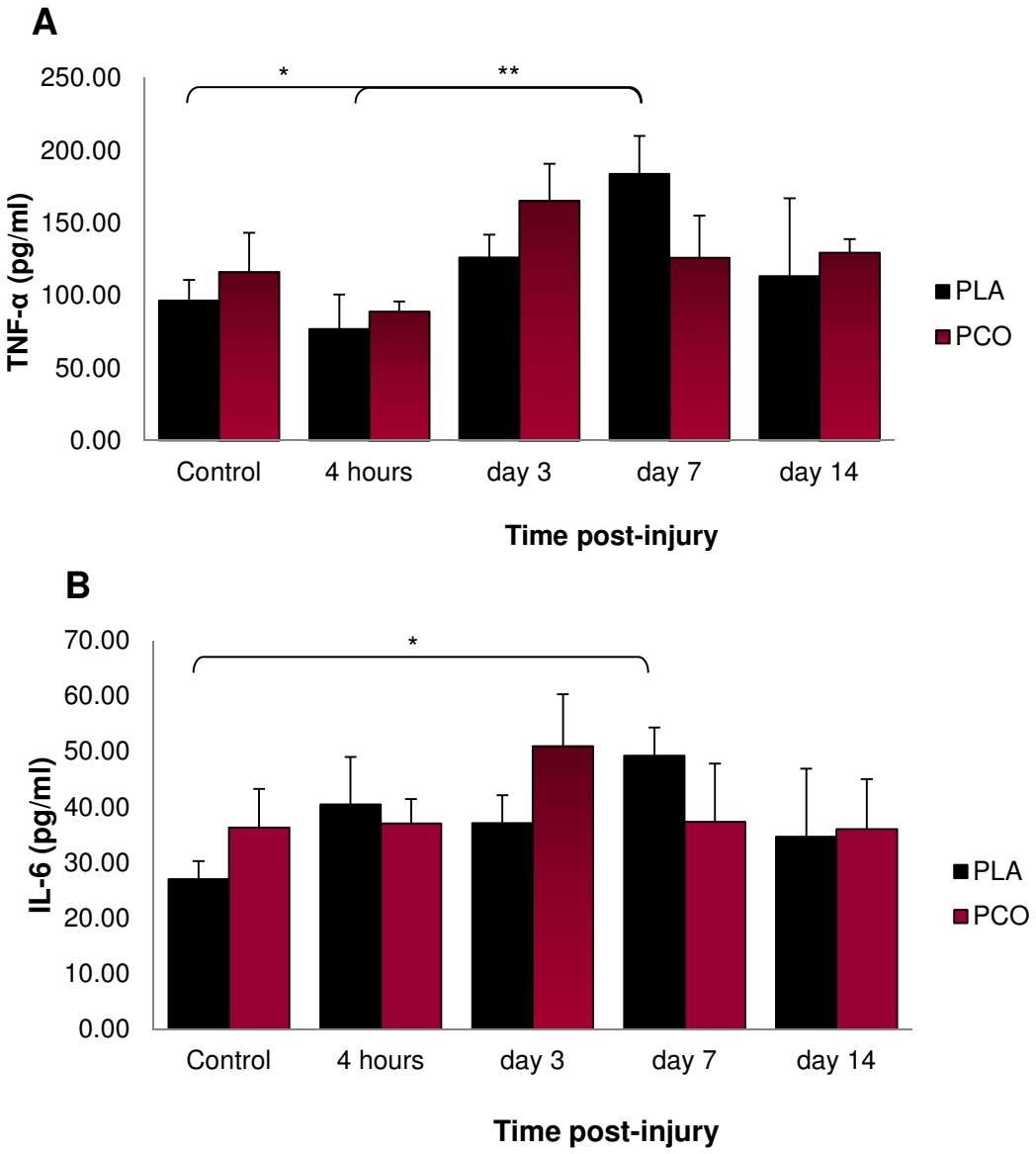


Figure 3.5: Pro-inflammatory cytokine response to contusion injury, assessed by plasma levels of TNF-α (**A**) and IL-6 (**B**). Statistical analysis: Factorial analysis of variance (ANOVA) with Bonferroni *post hoc* test (* $p < 0.05$ and ** $p < 0.01$). n = 8 rats per time point per group.

A main effect of time was observed for the plasma anti-inflammatory cytokine, IL-10 ($p < 0.01$), but again there was no ANOVA main effect of treatment. Although levels of anti-inflammatory cytokines in the PLA group were similar at all time points assessed, there was

a statistically significant increase in IL-10 (Figure 3.6) in the PCO group on day 3, at which point IL-10 were significantly higher than PLA ($p < 0.01$). IL-4 values were all below the detection limit of the assay kit used and thus excluded.

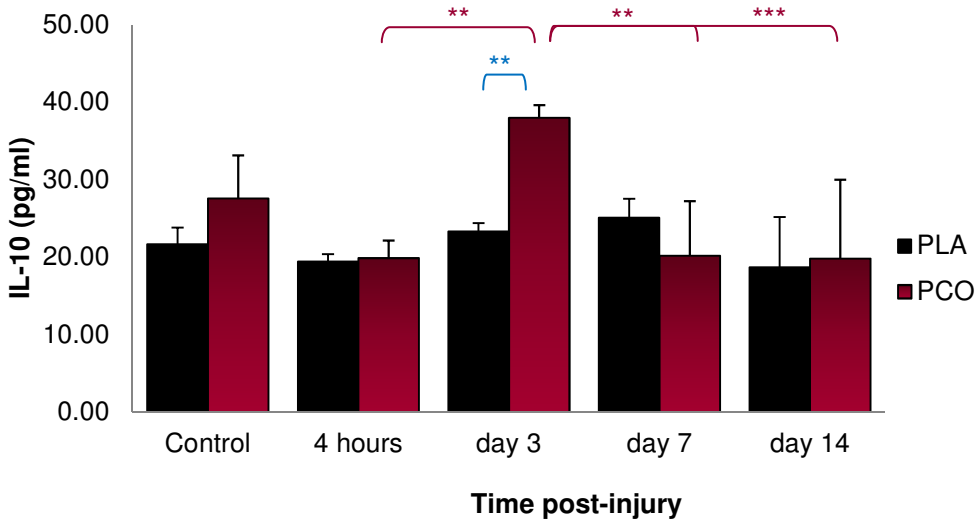


Figure 3.6: Plasma anti-inflammatory cytokine, IL-10 response to contusion injury. Statistical analysis: Factorial analysis of variance (ANOVA) with Bonferroni *post hoc* test (** $p < 0.01$, and *** $p < 0.001$). n = 8 rats per time point per group.

3.3.3 Muscle pro-inflammatory cytokine response

Statistical analysis of the pro-inflammatory cytokine, TNF-α in the injured area and border zone indicated a main effect of treatment ($p < 0.05$ for the injured area and $p < 0.001$ for the border zone), as well as a main effect of time ($p < 0.001$ for both). In the injured area, both treatment groups displayed an early elevation in TNF-α, 4 hr after injury, followed by a significant increase on day 3 and a subsequent decline thereafter (day 5) (Figure 3.7A). On day 3, the time point at which both groups had peak TNF-α levels, the PCO group's TNF-α was significantly lower. In the border zone, TNF-α peaked in both groups on day 1, followed by a significant decrease (day 3), only seen in the PCO group over time (Figure 3.7B). The TNF-α levels in the PLA group on the other hand remained elevated throughout the rest of the protocol. Representative images of day 3 in the injured area and day 1 in the border

zone area, which were significantly different between the two groups are displayed in Figures 3.8A-D.

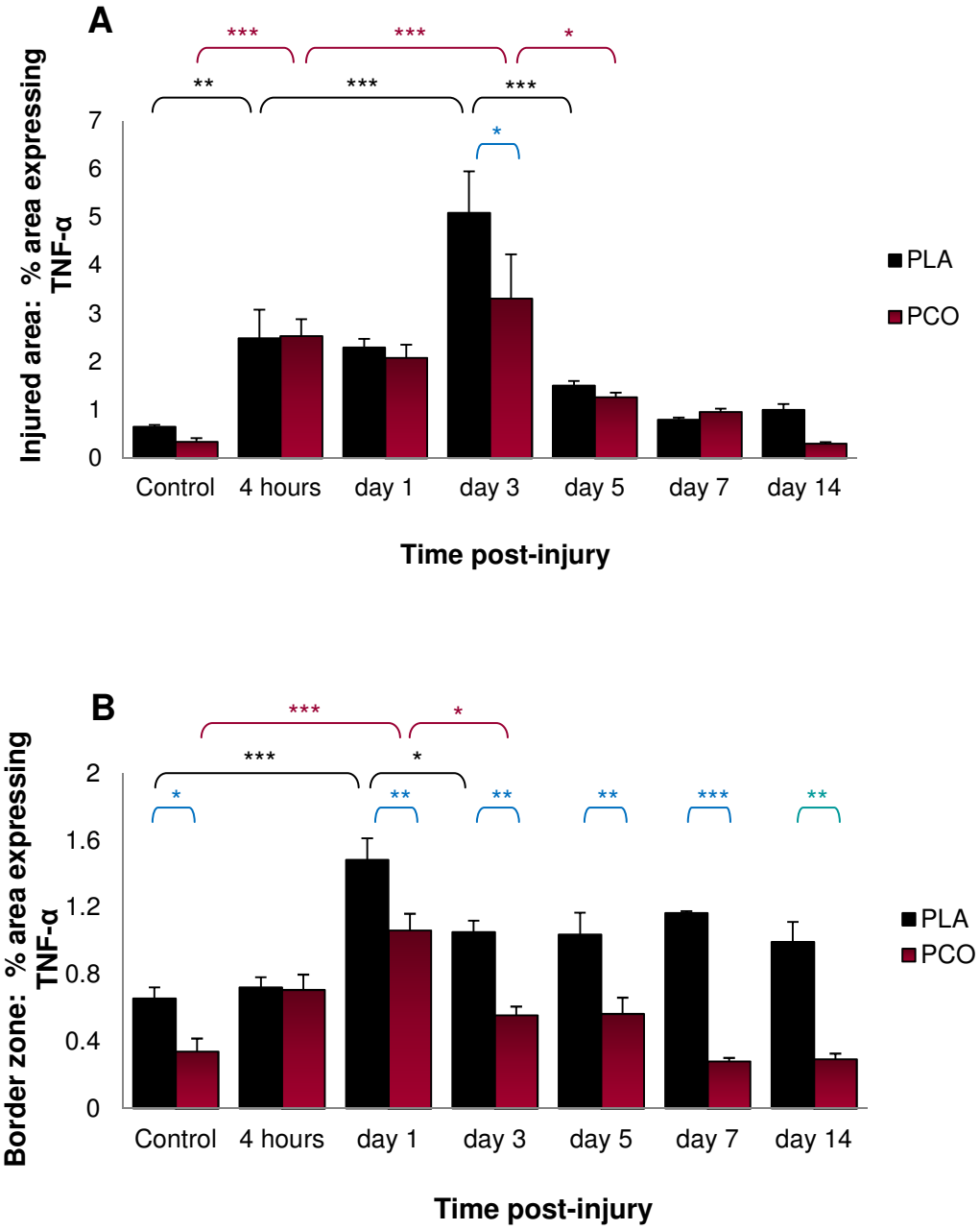


Figure 3.7: TNF-α (mean ± SEM) expressed as relative percentage fluorescence in the injured area (**A**) and border zone areas (**B**). Statistical analysis: Factorial analysis of variance (ANOVA), with Fisher's *post-hoc* test demonstrating significance between the two specific data points (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). n = 4 rats per time point per group.

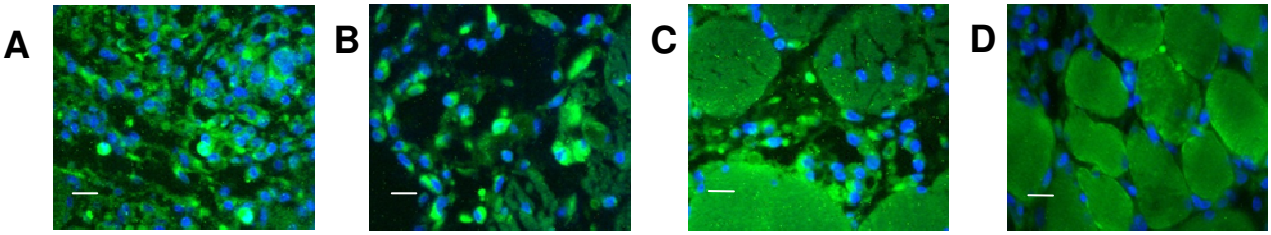


Figure 3.8: TNF-α expression (green, FITC) in muscle samples taken at 3 d post-injury in the injured area of the PLA group (**A**), and the PCO group (**B**), and at 1 d post-injury in the border zone of the PLA group(**C**), and the PCO group (**D**). Hoechst was used to visualise nuclei (blue). All images displayed are enlargements of original pictures taken at 400x magnification. Scale bar represents 10 µm.

IL-6 also displayed a significant effect of time in both the injured area and border zone area ($p < 0.001$ for both), however, no effect of treatment was evident. Similar to the results seen with TNF-α, IL-6 was significantly increased 4 hr after injury and continued to rise further untill day 3 in both groups in the injured area (Figure 3.9A). However, IL-6 decreased earlier in the PCO group (day 5) compared to the PLA group (day 7). In the border zone, although similar elevations were seen in IL-6 compared to that observed for TNF-α, IL-6 did not remain elevated after peaking on day 1 (Figure 3.9B). Both treatment groups had a significant decrease in TNF-α on day 3 after injury, however, the PCO group had an even further decrease in IL-6 on the day 3 time point compared to the PLA group at that specific time point. IL-6, in both the injured and border zone areas on day 3 are displayed in Figures 3.10A-D.

3.3.4 White blood cell response

A main effect of treatment ($p < 0.001$), time ($p < 0.001$) and treatment and time ($p < 0.001$) was evident for neutrophils in the injured area, whereas, a main effect of time ($p < 0.001$) and treatment and time ($p < 0.05$) was evident for neutrophils in the border zone.

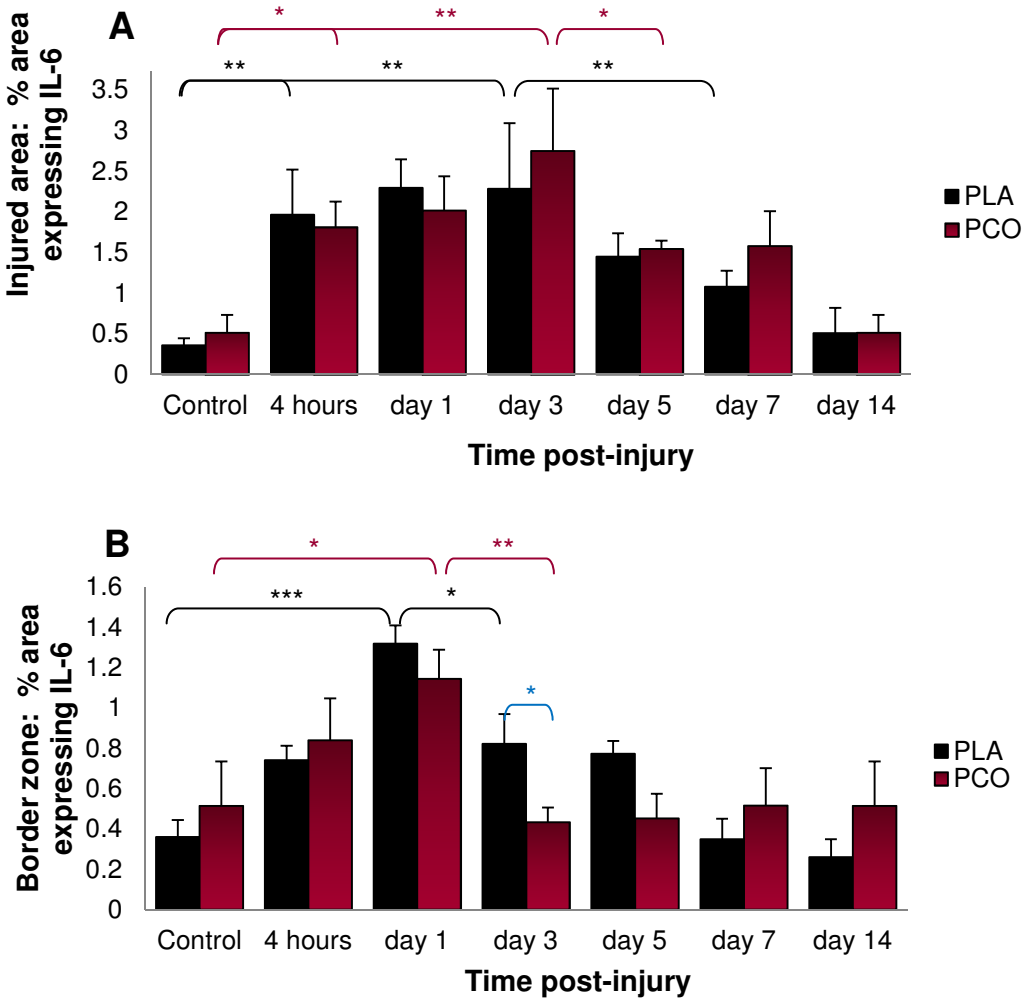


Figure 3.9: IL-6 (mean ± SEM) are expressed as relative percentage fluorescence in the injured area (**A**) and border zone areas (**B**). Statistical analysis: Factorial analysis of variance (ANOVA), with Fisher's *post-hoc* test demonstrating significance between the two specific data points (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). n = 4 rats per time point per group.

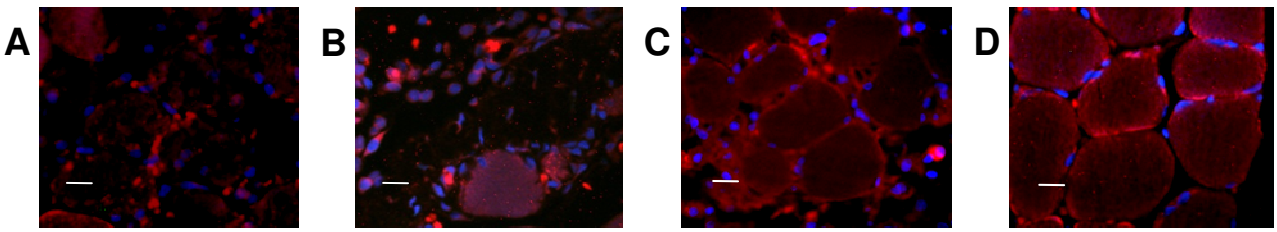


Figure 3.10: IL-6 expression (red, Texas Red) in muscle samples taken at 3 d post-injury in the injured area of the PLA group (**A**), and the PCO group (**B**), and at 3 d post-injury in the border zone of the PLA group (**C**), and the PCO group (**D**). Hoechst was used to visualise nuclei. All images displayed are enlargements of original pictures taken at 400x magnification. Scale bar represents 10 µm.

The neutrophil response in the PLA and PCO group was similar in both the injured area and border zone (Figures 3.11A and B respectively). No neutrophils were present in the control samples (i.e. no injury). At day 1 after injury, a neutrophil peak was evident in the PLA group (evident in both the injured area and border zone; $p < 0.001$), followed by a gradual decrease. No elevation in neutrophils in the PCO group was evident. The images displayed in Figures 3.12 A-H and I-P are images respresenting neutrophil expression at the 4 hr and day 1 time point of the injured and border zone areas respectively.

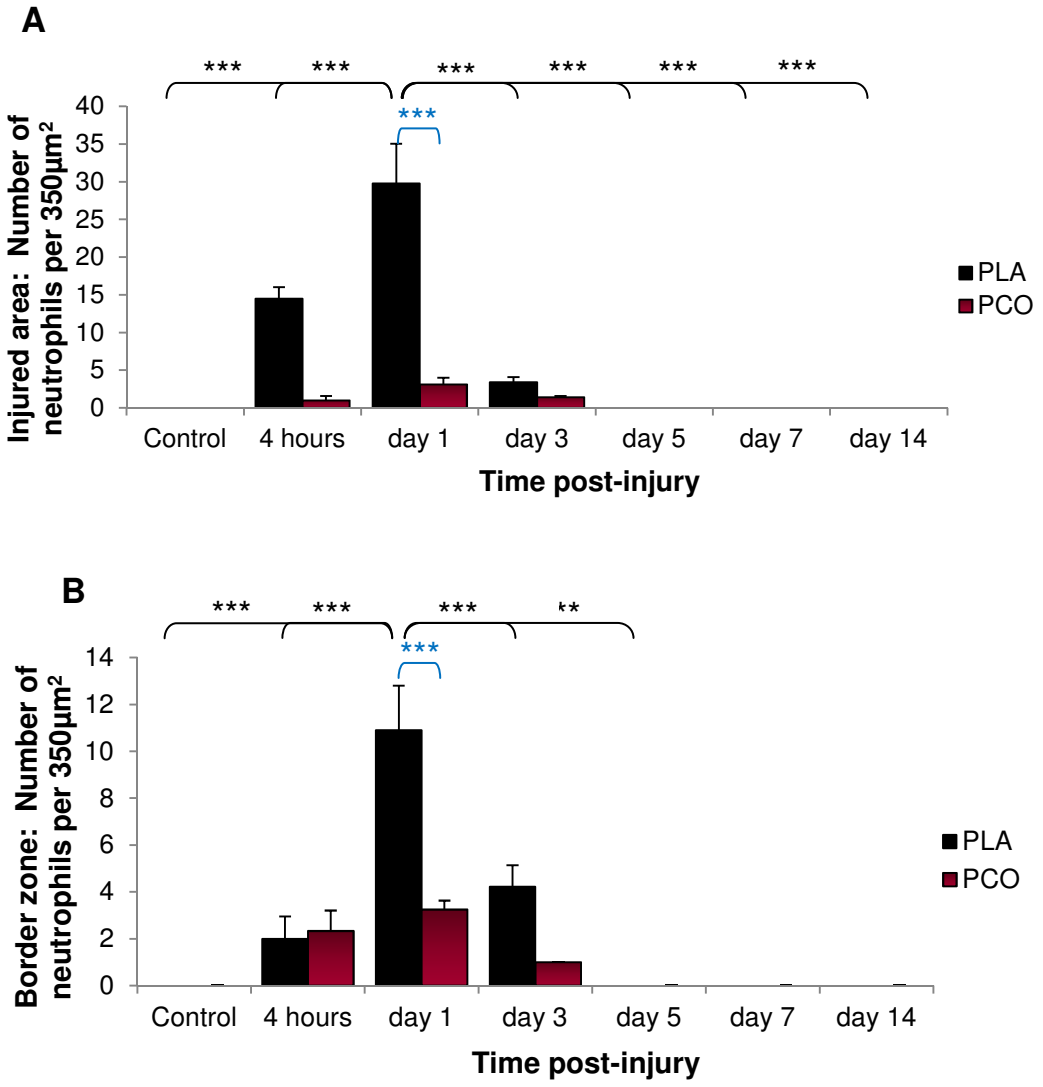


Figure 3.11: Neutrophil infiltration in the injured area (**A**) and border zone area (**B**) of muscle are expressed as the number of neutrophils per field of view (means ± SEM) at baseline (control) and at 4 hr, day 1, 3, 5, 7 and 14 post-injury. Statistical analysis: Factorial analysis of variance and Fisher's *post-hoc* test (** $p < 0.01$; *** $p<0.001$). n = 4 rats per time point per group.

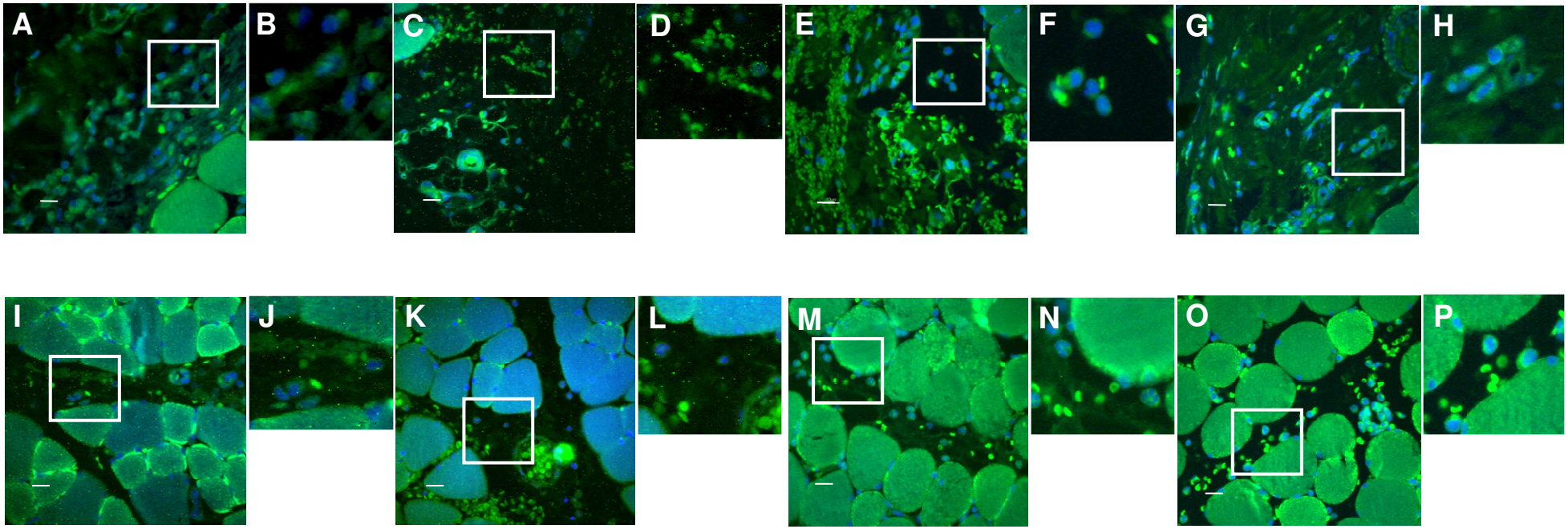


Figure 3.12: Neutrophil (His48) expression (green, FITC) and infiltration into the injured area (**A-H**) and border zone (**I-P**) of muscle, 4 hr post-injury (**A, B** and **I, J** indicates the PLA group; **C, D** and **K, L** indicates the PCO group) and on day 1 post-injury (**E, F** and **M, N** indicates the PLA group; **G, H** and **O, P** indicates the PCO group) respectively. Images **B**, **D**, **F**, **H**, **J**, **L**, **N** and **P** are enlargements of the area surrounded by the white block of the figures on the left respectively. Hoechst was used as nuclear dye. Scale bar represents 10 µm, with the original magnification of 400x used for the image acquisition.

Analysis of macrophage infiltration into the injured and border zone areas indicated a main effect of treatment, time and treatment and time ($p < 0.001$ for all 3). Significant macrophage infiltration is visible on days 3 and 5 in the PLA group, with resolution of inflammation shortly thereafter. PCO supplementation on the other hand resulted in an earlier macrophage response in both the border zone (Figure 3.13B) and injured areas (Figure 3.13A). Significant differences between the 2 treatment groups were evident on day 1 and day 5 in the injured area, as well as in the border zone area. Figure 3.14 indicates the time points, day 1 and 5, at which the 2 treatment groups differed significantly from one another in the border zone and injured area respectively.

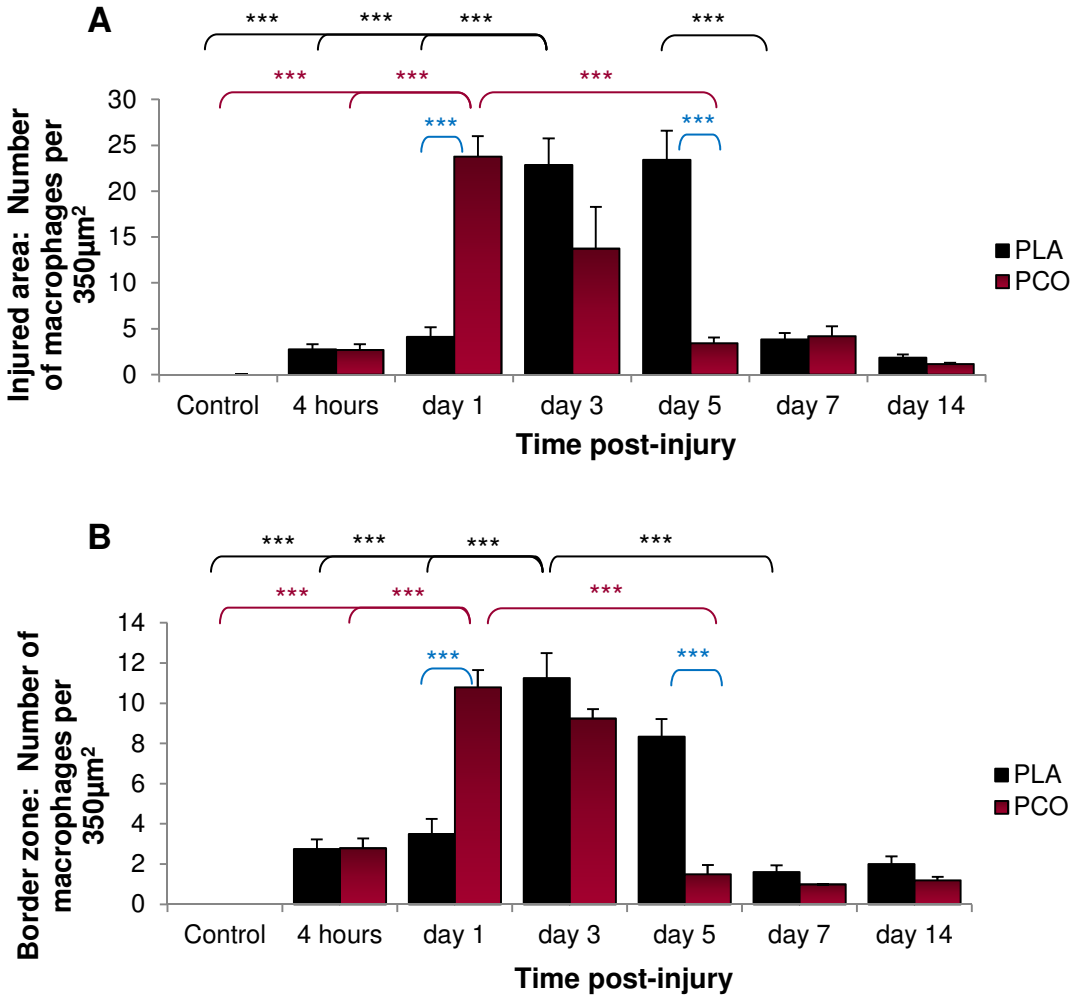


Figure 3.13: Macrophage infiltration in the injured area (**A**) and border zone area (**B**) of muscle are expressed as the number of macrophages per field of view (means ± SEM) at baseline (control) and at various time points after injury. Statistical analysis: Factorial analysis of variance and Fisher's *post-hoc* test (*** $p<0.001$). n = 4 rats per time point per group.

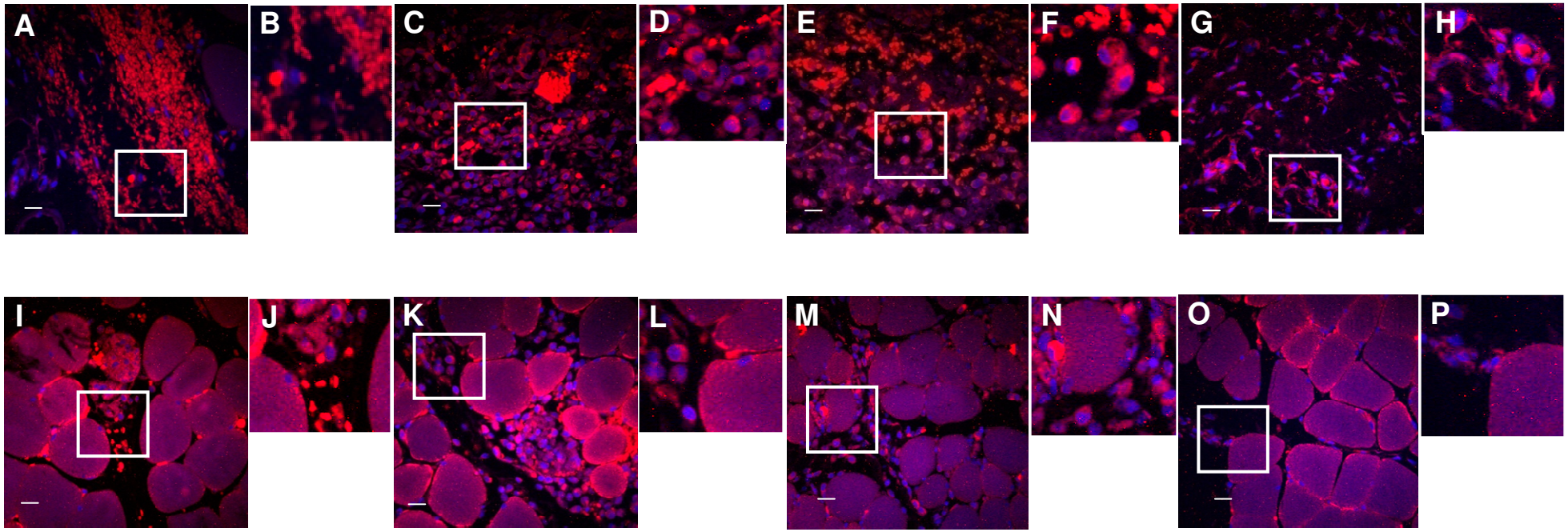


Figure 3.13: Macrophage (F4/80) expression (red, Texas Red) and infiltration into the injured area (**A-H**) and border zone (**I-P**) of muscle, on day 1 post-injury (**A, B** and **I, J** indicates the PLA group; **C, D** and **K, L** indicates the PCO group) and on day 5 post-injury (**E, F** and **M, N** indicates the PLA group; **G, H** and **O, P** indicates the PCO group) respectively. Images **B**, **D**, **F**, **H**, **J**, **L**, **N** and **P** are enlargements of the area surrounded by the white block of the figures on the left respectively. Hoechst was used as nuclear dye. Scale bar represents 10 µm, with the original magnification of 400x used for the image acquisition.

3.4 Discussion

The main findings of this study were that chronic PCO supplementation (Oxiprovin™, 14 days prior to and after injury) resulted in: (a) a much earlier increase in Pax-7 expression on satellite cells, (b) a significant circulatory anti-inflammatory response (IL-10) on day 3, (c) attenuated pro-inflammatory responses (IL-6 and TNF-α) both in circulation and locally (muscle and extracellular fluid) in the border zone, and also TNF-α in the injured area (including injured muscle and extracellular fluid). There were some differences between IL-6 and TNF-α responses which will be highlighted. An important novel result was the extremely small number of neutrophils that infiltrated the injured and border zone areas of muscles, whilst macrophage numbers were elevated significantly earlier and decreased relatively quicker than PLA, suggesting earlier resolution of inflammation.

3.4.1 Pax-7

Post-injury in placebo: Previously, it was demonstrated that a contusion injury resulted in satellite cell ($CD34^+$ and $CD56^+$) activation on day 3 followed by significantly elevated numbers on day 7 and a return to control levels on day 14 (MSc thesis, Kruger 2007). In the current study, Pax-7 displayed a similar expression pattern compared to CD56, possibly indicating co-expression of these satellite cell markers during different stages of SC activation and proliferation following contusion injury. The only difference observed was in the absolute count of satellite cells expressing the markers. Fewer satellite cells expressed Pax-7 compared to SC counts obtained with other SC markers (CD34 and CD56) as seen in the previous study (MSc thesis, Kruger 2007). However, it should be mentioned that CD56, as well as other SC markers, are not exclusive markers of SCs. Therefore, using a SC marker in conjunction with a basal lamina marker is more accurate when counting SCs. Pax-7 in the current study was used in conjunction with laminin, a basal lamina marker, to ensure that only SCs (situated beneath the basal lamina) were counted.

In the PLA group, a slight increase in Pax-7 is evident at 4 hr and on day 3 after injury, although only reaching statistical significance by day 7 after injury. During this time SC are activated and start to proliferate. NO is synthesized by activated macrophages (Nguyen and Tidball 2003), and has been suggested as one of the activators of SCs (Ryten *et al.,* 2002; Pisconti *et al.,* 2006). This raises the possibility that macrophage-derived NO may be involved in a bigger increase in SC Pax-7 expression on day 7 after injury in the PLA group, but this is unlikely since no elevation in the number of macrophages infiltrating the injured area was observed at that time (Figure 3.13). However, there are other sources of NO. Inducible nitric oxide synthase (iNOS) is expressed in a wide variety of cell types, including myocytes (Riede *et al.,* 1998) and myeloid cells (Tripathi *et al.,* 2007), and is responsible for NO production. Its activity is regulated at the transcriptional level by cytokines and by exposure of the cells to inflammatory stimuli (Nathan 1992). iNOS expression is also mediated by the activation of the transcription factor NFκB (Brady *et al.,* 1997). It has been shown in a study by Fuchs *et al.* (2001), that TNF-α can activate the NFκB pathway, therefore increasing iNOS expression and the possibility for increased NO synthesis. Also, in a study by Merly *et al*. (1999), it was suggested that IL-6, TNF-α and probably other inflammatory cytokines are associated with enhanced SC proliferation. It is therefore possible that an increase in TNF-α and thus a secondary increase in NO might be possible candidates in causing elevated SC numbers through the NFκB pathway. This latter explanation is very likely, given the peak in both IL-6 and TNF-α on day 7 in the PLA group (Figure 3.5), which coincided with the Pax-7 peak. Since Pax-7 is a good marker labelling quiescent, activated and proliferating SCs, and is subsequently absent from differentiated myonuclei (Halevy *et al.,* 2004; Olguin and Olwin 2004; Zammit *et al.,* 2004), it was expected that MHC_f (expressed during differentiation) expression would be increased following an increase in Pax-7 expression. In a previous study, MHC_f (see Figure 3.1) and Pax-7 expression were both significantly elevated by day 7 (MSc thesis, Kruger 2007), indicating that Pax-7 is also expressed during differentiation of myoblasts, which is puzzling, since Pax-7 expression should be downregulated just prior to differentiation. However, since no analysis was done at an early time point between day 3 and day 7, such as day 5, it is not

known whether Pax-7 expression could have been even more elevated compared to day 7 after injury. Fourteen days after injury however, the Pax-7 levels in the PLA group had returned to control values indicating that the SCs had fused, and this coincided with a decrease in MHC_f expression to levels higher than that of control levels.

Post-injury in PCO: Increases in Pax-7 expression was evident earlier in the PCO group (already at 4 hr following injury), compared to the PLA group. This could possibly indicate that PCO supplementation indirectly played a role in secreting cytokines, which could have mobilized more immune cells, specifically macrophages to migrate to the injured area, as a result of improved blood flow to the site of injury (Shi *et al.,* 2003). These immune cells could then potentially secrete other pro-inflammatory cytokines to attract more SCs to the injured area. However, it should be noted that the muscle pro-inflammatory cytokines in the PCO supplemented group decreased faster and thus had a shorter duration compared to the cytokines in the PLA group, suggesting that the pro-inflammatory cytokines in the PCO group were more potent for a relatively short period of time. Macrophage numbers were elevated by day 1 in the PCO group (Figure 3.13) in the current study, whereas local release of pro-inflammatory cytokines, TNF-α and IL-6, was also significantly elevated on day 1 in the border zone, indicating that the abovementioned statement is a valid suggestion made here. However, peak Pax-7 expression preceded the cytokine peak, arguing against the abovementioned statement. Since Pax-7 expression on day 1 after injury were not investigated, it is possible that Pax-7 expression could have been further elevated at this time point, so that the peak may in fact have occurred in line with the above interpretation. Hepatocyte growth factors (HGFs) are also able to activate SCs (Allen *et al.,* 1995) and could have contributed to the increase in SC expression seen early on (4 hr) in the current study. It has been shown that IL-6 expression stimulates proliferation of various cell types (Massimino *et al.,* 1997), therefore IL-6 expression in this study in the injured area (Figure 3.9) could also potentially account for the increase seen in Pax-7 expression in the PCO group. Another possibility might be that SCs may also be migrating into the injured area from healthy

surrounding tissue, although this is less likely, since the epimysium surrounding intact muscles is a barrier that prevents SC migration (Schultz *et al.,* 1985; Schultz *et al.,* 1986).

By day 3, a significant decrease in Pax-7 expression was evident in the PCO goup. In a study by various researchers (see Table 1.3, pg 27) it was found that IL-10 resulted in the inhibition of macrophage and neutrophil pro-inflammatory cytokine production (De Waal Malefyt *et al.,* 1991; Fiorentino *et al.,* 1991; Giannoudis *et al.,* 2000), and thus may result in a decreased ability of attracting SCs to the injured area. IL-10 in the current study was significantly elevated by day 3 in the PCO group, compared to a blunted pro-inflammatory cytokine response, indicating that PCO administration may have blunted or decreased pro-inflammatory cytokine production at that specific time point (seen in this study), which could explain the reduction in Pax-7 expression seen at this time point. By day 14, the PCO group had similar Pax-7 levels to that of controls indicating that SCs have fused and regeneration is almost complete.

3.4.2 Cytokine release in circulation

3.4.2.1 Pro-inflammatory cytokines

From the literature it is known that skeletal muscle and various immune cells continuously produce cytokines at low levels (IL-6: ± 2 pg/ml and TNF-α: ±3.5 pg/ml) in order to maintain homeostasis and to regulate cell function (Lu *et al.,* 2004). These cytokines bind at the surface of the target cells and mainly alter cell function. Changes in skeletal muscle fibre ultrastructure, as reported after eccentric exercise or injury (Malm *et al.,* 2000), result in marked increases in the release of pro-inflammatory cytokines, of which IL-1β and TNF-α are mainly the focus in research reports. The increase in circulatory IL-6 post-eccentric exercise-induced damage or contusion injury has long been assumed to originate from the inflammatory cells infiltrating damaged skeletal muscle (Hellsten *et al.,* 1997; Pedersen *et al.,* 2001). However, it is also known that contracting muscle can produce and secrete relatively large amounts of IL-6 (Steensberg *et al.,* 2000). Therefore, although it is quite feasible that

inflammatory cells infiltrating the injured area prior to day 7 might be responsible for the increase in IL-6, one cannot rule out the possibility that muscle contraction during normal activity could also have contributed. However, this interpretation does not explain the elevation seen in PLA plasma IL-6 on day 7, since the changes in everyday activity are too small to cause differences of this magnitude. Also, rats in both the injured groups were handled and injured in exactly the same way, with no elevation in IL-6 in the PCO group. A more likely explanation is that these cytokines are leukocyte-derived.

Post injury in placebo: In the current study the circulatory pro-inflammatory cytokines TNF-α and IL-6 were significantly elevated on day 7 after injury in the PLA group, compared to control values. However, research regarding the systemic inflammatory cytokine response is conflicting in nature mainly due to the difference in the type of injury, as well as the severity of injury when the same model is used. In a recent study, the bilateral femoral vessels in the lower limb of rats were ligated with microvascular clamps for 3 hr and then reperfused for 3 hr in order to study ischemia/reperfusion injury (Shih *et al.,* 2010). In this particular study, circulatory IL-6 (probably mostly muscle-derived) increased almost 5-fold after injury compared to sham, whereas no significant difference in TNF-α was evident. A possible explanation for only an increase in IL-6 and not TNF-α is that IL-6 down-regulates TNF-α levels (Caiozzo *et al.,* 1996; Cai *et al.,* 2000). Ischemia/reperfusion injury is very different from contusion injuries, as it prevents the normal circulation of cytokines from the blood into the muscle, which is possibly why circulatory IL-6 in their study is more elevated, and also why circulatory IL-6 in the current study was approximately 10 times lower compared to their study. However, due to the fact that muscle also produces IL-6 and TNF-α (see Table 1.3, Chapter 1), and the researchers did not investigate the contribution of pro-inflammatory cytokines from the muscle, it is not known whether muscle-derived cytokines could have leaked into circulation. Also, since the researchers only investigated plasma cytokine levels on the day of injury (6 hr afterwards), it is likely that they missed the peak in TNF-α, since it is known that IL-6 is the earliest cytokine expressed after contraction-type injuries a couple of hr after injury (Febbraio and Pedersen 2005; Petersen and Pedersen 2005). Although this is

a likely conclusion, other studies have shown elevated TNF-α levels early (2-6 hr) after injury (Warren *et al.,* 2002; Vassilakopoulos *et al.,* 2003). TNF-α expression in sham rats was also relatively high, and even in the presence of injury, no significantly higher level of TNF-α was evident, suggesting that the elevated TNF-α levels (irrespective of injury), might be due to the invasive procedure prior to injury and not the injury itself. On the other hand, in a study by Schmidtz *et al.* (2010), blunt trauma in mice resulted in no significant differences in TNF-α or IL-6 over time (3 hr, 24 hr, 36 hr, 48 hr and 72 hr). The researchers argue that their model of blunt trauma might not have been severe enough to result in changes in circulating cytokines. However, it could also be argued that their model of injury was severe enough or even more severe than the current model - 20 g from a height of 120 cm in mice. It has also been suggested that the TNF-α response coincides with inflammatory cell infiltration, predominantly macrophages (Ralph *et al.,* 1983), which occurs only at later time points than those assessed in the study by Schmidtz *et al.* (2010). Therefore, if later time points were assessed, they might have seen significant differences in TNF-α. In the current study, macrophage infiltration in the PLA group preceded circulatory TNF-α release (see Figure 3.5), suggesting that local TNF-α production rather than circulatory cytokine production might be involved in attracting more macrophages to the injured area. Furthermore, it should also be noted that immune cells also produce inflammatory cytokines (see Table 1.3), allowing for the possibility that relatively high macrophage counts on day 3 and 5 in the muscle may have been responsible for the systemic increase in cytokines on day 7. Already mentioned in the Pax-7 section, it has been suggested that TNF-α expression is associated with enhanced SC activation and proliferation (Merly *et al.,* 1999), which is a likely possibility, since SC activation and circulatory TNF-α release in this study coincided (day 7 in both).

Post injury in PCO: In order to test the effectiveness of chronic PCO supplementation in blunting the inflammatory response, rats were supplemented prior to and after contusion injury. The few studies addressing the role of antioxidants in strenuous exercise-induced cytokine production in humans were not able to show significant effects, although a tendency for a blunted IL-6 response was reported after vitamin C supplementation (Nieman *et al.,*

2000; Petersen *et al.,* 2001). Treatment with a combination of vitamins C and E (29 days), followed by 3 hr of dynamic two-legged knee extensor exercise and 3 hr of recovery, inhibited IL-6 release into circulation and mRNA expression in contracting muscle (Fischer *et al.,* 2004). Similarly, in the current study, 2 weeks of PCO supplementation also resulted in significantly decreased IL-6 levels both in circulation and in muscle. Pre-treatment with vitamin D3 for 5 days prior to bilateral femoral artery occlusion for 3 hr, followed by 3 hr reperfusion, also resulted in a marked decrease in IL-6 after injury, with no effect on TNF-α. This is not surprising, since it is known that IL-6, rather than TNF-α, is considered to be an early phase indicator of inflammation (Shih *et al.,* 2010). However, it should be noted that in the presence or absence of vitamin D3, with or without injury, TNF-α was already elevated, indicating that vitamin D3 had no effect on TNF-α levels. In a study by Vassilakopoulos *et al.* (2003), a combination of antioxidants (vitamin A, C, E, *N*-acetylcysteine (NAC) for 60 days) after 45 min of cycling, resulted in plasma IL-1β to be undetectable, and the TNF-α and the IL-6 responses to be blunted compared to PLA, suggesting that a combination of antioxidant vitamins might be more promising. The antioxidant supplementation blunted but did not completely abolish the cytokine response to exercise, and this effect is most likely as a result of the combined effect of the antioxidants used in their study (Vassilakopoulos *et al.,* 2003). However, increased cytokine clearance cannot be ruled out since a complex relationship between cytokine production and clearance is known to determine the concentration of cytokines, and the effect of oxidative stress on cytokine clearance is largely unknown. In another study, Yamashita *et al.* (1999), an acute bout of treadmill exercise increased the TNF-α and IL-1β content of the myocardium, whereas in the presence of the antioxidant *N*-2-mercaptoproprionyl glycine, no increase in either cytokine was observed, because of abolishment of ROS production. Similar to the abovementioned studies, the current study also provided evidence that the antioxidant Oxiprovin™ played a significant role in blunting the pro-inflammatory cytokine response in the circulation.

Possible mechanisms whereby antioxidants might exert beneficial effects have been investigated to some extent. For example, resveratrol, an antioxidant found in grapes, was

able to suppress rat carrageenan-induced paw oedema (Jang *et al.,* 1997) by the selective inhibition of cyclooxygenase (COX)-1, *via* impairment of prostaglandin (PG) synthesis. Furthermore, in an *in vitro* study by Subbaramaiah *et al*. (1998) using human mammary and oral epithelial cells, it was reported that resveratrol was also able to inhibit protein kinase C (PKC) translocation and the enhancement of COX-2 promoter activity (mediated by PKC) through extracellular signal-regulated kinase-1 (ERK-1), and c-Jun (Lee and Lin 1997). Also, when resveratrol was administered to murine peritioneal macrophages (stimulated by lipopolysaccharides (LPS) or phorbol myristate acetate (PMA), it suppressed the production of O_2^- and hydrogen peroxide (H_2O_2), impairing arachidonic acid (AA) mobilization, COX-2 overexpression, and subsequently PGE2 release, indicating that antioxidants found in grapes might work through the AA and mitogen activating protein kinase (MAPK) pathways specifically (Martinez *et al.* 2000). Similar macrophage stimulation studies (*in vitro*) were also done with vitamin E (Hempel *et al.,* 1994; Rao *et al.,* 1994; Tetsuka *et al.,* 1996; Davi *et al.,* 1997) and showed similar results. In future studies, one should investigate whether the PCO, Oxiprovin™, is able to inhibit or suppress certain upstream targets involved in the AA, MAPK or NFκB pathway in order to determine whether PCO also has the same effect as those seen in the studies mentioned above.

Inflammatory cytokines (specifically IL-6 and IL-1β), injury, and oxidative stress can activate NFκB (Schreck *et al.,* 1992) *via* the phosphorylation and release of the inhibitory subunit (IκB) from NFκB in the cytoplasm, rendering NFκB inactive. This pathway is one of the most important ones in the inflammatory process, and is usually activated when oxidant production (ROS) overwhelms the antioxidant system, resulting in oxidative stress. A potential mechanism through which oxidant production can increase with inflammation and thus contribute to skeletal muscle atrophy or damage and weakness is iNOS, possibly activated by NFκB (Kondo 2000). Due to the fact that PCO supplementation in the current study resulted in a reduction in TNF-α and IL-6 in muscle, it is quite possible that this supplement's mechanism of action is by blunting NFκB or some of the upstream targets involved in this specific pathway. In a study by Nakao *et al.* (2000), it was demonstrated that the free radical

scavenger pyrrolidine dithiocarbamate (PDTC) inhibited NFκB activation, after inhibiting tyrosine kinase phoshorylation in the Janus activating protein kinase/signal transducer and activator transcription protein (JAK/STAT) pathway in human gingival fibroblasts, and subsequently reduced PGE2 production. This suggests that other antioxidants, such as PCO in the current study, might also result in a blunted inflammatory cytokine response, which subsequently blunts/inhibits NFκB activation and thus COX-2 activation and PGE2 production.

In a study by Aronson *et al.* (1998), it was demonstrated that injury to skeletal muscle of human subjects, either by needle biopsy or surgical open muscle biopsy, resulted in the activation of multiple components in the MAPK signalling cascade (Raf-1/MEK-1/ERK/RSK) and increased the activity of both c-Jun NH_2-terminal protein kinase (JNK) and p38, possibly as a result of an increased TNF-α expression. However, the MAPK pathway and its downstream targets have not been studied as extensively as the NFκB and AA pathway after an injury. Thus, since its known that TNF-α and IL-6 can result in MAPK activation (Baud and Karin 2001), it's quite possible that PCO also inhibits the MAPK pathways as a result of TNF-α and IL-6 inhibition. Therefore, applying standard western blotting techniques one can test whether PCO supplementation prior to and following injury, influenced the various components of the MAPK pathway.

3.4.2.2 Anti-inflammatory cytokines

Post-injury in placebo: Most studies on the effects of mechanical trauma on circulating cytokine profiles do not include anti-inflammatory cytokines in the assessment panel. Only two studies investigated anti-inflammatory cytokines. In the study by Schmitz *et al.* (2010), although the anti-inflammatory cytokine IL-10 was measured, no data were displayed as it was below the detection limit (17.5 pg/ml) for both uninjured and injured mice. This might indicate that the injury in their study was not severe enough, however, their injury was more severe than the injury used in the current study. Another possibility might be that the assay kits used to analyse IL-10 were not sensitive enough, however, the assay kit in the current

study was less sensitive. Their study is not considered an optimal study for comparison, since the increased TNF-α, seen even in the sham group (discussed in the previous section), indicates that their model is pro-inflammatory in nature and could have accounted for undetectable IL-10. In another study in mice, elevated serum levels of IL-10 were found after fracture-associated soft tissue injury (Kobbe *et al.,* 2008). However, in their study, both upper legs were injured, the bone was fractured and the mass-drop protocol more severe. The severity of injury could have played a role in the expression pattern of anti-inflammatory cytokines. In the current study, the injury was much less severe than that of Kobbe *et al.* (2008). It is not surprising that in this particular study, the anti-inflammatory cytokine IL-10 in the PLA group was unchanged over time. Another possibility might be that IL-10 levels in the PLA group never increase, because it is secreted and works locally, and therefore never clears into the circulation. However, this suggestion is unlikely, given the increased IL-10 seen in the PCO group.

Post-injury in PCO: In the PCO group, an elevation in circulatory IL-10 level was evident on day 3 after injury, indicating that PCO has a primary or secondary anti-inflammatory property and aids the recovery process by resulting in increased anti-inflammatory actions. It is known that M2 macrophages are primarily responsible for IL-10 expression (Asadullah *et al.,* 2003). Therefore, it is likely that PCO elevated M2 macrophage levels in the injured area and border zone earlier when compared to PLA, accounting for increased circulatory IL-10 expression on day 3. However, the different subpopulations and their contribution towards muscle recovery were not investigated in this particular study.

The mechanism of IL-10 action is widely accepted to be inhibition of the expression of pro-inflammatory cytokine genes, as well as inhibition of the production of granulocyte macrophage colony stimulating factor (GM-CSF), granulocyte colonly stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF) and platelet activating factor (PAF) produced by activated monocytes/macrophages (De Waal Malefyt *et al.,* 1991; Fiorentino *et al.,* 1991; D'Andrea *et al.,* 1993; de Waal Malefyt *et al.,* 1993; Gruber *et al.,* 1994). These inhibitory effects of IL-10 are thought to be crucial and may reduce the responses of the pro-

inflammatory cytokines, as seen in the current study. One of the best characterized IL-10 signalling pathways is the JAK/STAT system (Shimodo and al 1996; Takeda *et al.,* 1996). IL-10 induces tyrosine phosphorylation and activation of the transcription factors STAT3 and STAT1 (Finbloom and Winestock 1995; Lai *et al.,* 1996; Weber-Nordt *et al.,* 1996; Wehinger *et al.,* 1996). STAT3 is recruited directly to the IL-10/IL-10R complex *via* either of the two tyrosine residues in the IL-10R1 cytoplasmic domain that become phosphorylated in response to IL-10 (Weber-Nordt *et al.,* 1996) and is required for IL-10 signalling (Ho *et al.,* 1995; O'Farrell *et al.,* 1998; Riley *et al.,* 1999). It has also been demonstrated that IL-10 is able to inhibit macrophage activation, as well as NFκB activation in response to stimuli *in vitro* (Wang *et al.,* 1995; Clarke *et al.,* 1998; Ehrlich *et al.,* 1998; Schottelius *et al.,* 1999), the latter in two different ways: either by inhibiting activation of IκB kinase (Yin *et al.,* 1998), or by inhibiting NFkB DNA binding activity (Schottelius *et al.,* 1999), although, the latter mechanism is not properly understood. It should be mentioned that the majority of studies investigating the role of IL-10 have been done *in vitro* using monocytes/macrophages, T cells, and B cells, which would exclude the possibility of involvement of other signalling pathways, so results may not be directly applicable to an *in vivo* situation. Since satellite cells are also involved in this particular *in vivo* model, it is not clear which pathway might be activated, or whether the same pathways are involved when PCO is administered. Although SCs themselves do not secrete IL-10, the pathways mentioned above are also involved here. From this section, it is evident that PCO can also be considered to be an effective anti-inflammatory supplement, since it is able to increase anti-inflammatory cytokines, whilst blunting pro-inflammatory cytokines.

3.4.3 Cytokine levels in muscle

In the current model of contusion injury, IL-6 and TNF-α had similar patterns in the injured area in the PLA group, but different in the border zone. Only one previous study has investigated muscle pro-inflammatory cytokine levels after a invasive contusion injury (Bunn

et al., 2004). In Bunn *et al.*'s study, mild contusion injury (100g from a height of 13 cm) resulted in an increase in pro-inflammatory cytokines on day 4, whilst moderate injury (200 g from a height of 13 cm) resulted in an even later peak – on day 8 (Bunn *et al.,* 2004). TNF-α and IL-6 in the PLA group in the current study (200 g from a height of 50 cm) peaked on day 3 (injured area) and day 1 (border zone) respectively, although being elevated already 4 hr after injury in the injured area. However, although the current results deviates from that of Bunn *et al.* (2004), there are many differences between the 2 studies that may account for inconsistencies. Firstly, the effect of cytokine release as a result of invasiveness of the intervention (removing skin from muscle and incisions into the muscle) was not quantified in the earlier study, while the current study was non-invasive. Secondly, the study by Bunn *et al.* (2004) was done in mice, and this study used rats. From Bunn's study, it is possible that with bigger injuries, inflammatory cytokine release is sustained longer. It has also been suggested that if the centre of the injured area lies too far from an intact blood supply, it will result in a delayed release of cytokines from the blood into tissue and thus delayed progression of inflammation (Bunn *et al.,* 2004). This may be true in the current study, since the increase/changes in cytokine expression in the border zone occurred earlier compared to the injured area.

In a freeze-injury study by Warren *et al.* (2002), the time courses were similar for IL-6 and TNF-α mRNA expression in the *tibialis anterior* (TA) muscle post-injury. In their study, an increase in TNF-α mRNA expression occurred within 5 hr following injury and peaked at 24 hr after injury, followed by a gradual decline from days 3-7 and a return to control levels by day 13. mRNA levels were measured to assess the cytokine response in the study by Warren *et al.* (2002), while protein levels were measured in the current study. Results indicate that for IL-6 release in the border zone, the protein levels correspond to mRNA levels observed in Warren *et al.*'s study. On day 1, IL-6 levels in the PLA group were at their highest, at a point in time when neutrophils are the predominant immune cell infiltrate in the injured area, suggesting that neutrophils in both the injured area and border zone were the source of the elevated IL-6 levels in the muscle. In a study by Kami and Senba (1998), it

was found that IL-6 expression in injured muscle does not increase SC proliferation. However, although in the PLA group, an increase in Pax-7 SCs was only observed on day 7 – a point in time when IL-6 expression was already decreasing (in both the injured area and border zone) – the possibility that IL-6 is responsible for SC activation cannot be ruled out, since increases in Pax-7 expression and elevations in muscle IL-6 levels in the PCO group coincided.

TNF-α (in the border zone) of PLA displayed a somewhat similar pattern, also peaking on day 1, but in this case, TNF-α levels were still significantly elevated up to day 14. A likely reason for this sustained elevated TNF-α level is the possibility that TNF-α could have circulated in from blood, given the increased TNF-α in the plasma on day 7 after injury. Another possibility could be the production of cytokines by immune cells at different time points after injury. Collins and Grounds (2001) observed the presence of increased TNF-α in the TA muscle following crush or autograph (stretch injury until muscle failure), localized to inflammatory cells as well as atrophic muscle fibres. During the early time points after injury in the current study, a high number of immune cells were evident in the injured area, therefore it is not surprising that cytokine expression is at its highest on day 1 and day 3 after injury, points in time when neutrophils and macrophages respectively infiltrated the injured area maximally.

In the injured area, TNF-α and IL-6 displayed similar inflammation resolution patterns, and even though both IL-6 and TNF-α were expressed early in the PLA groups (4 hr), only TNF-α expression was further increased by day 3. Determining the effect of PCO supplementation on muscle cytokines after injury indicated that PCO was also effective in blunting the inflammatory response in the muscle, similar to that seen for plasma cytokines.

To determine the mechanisms responsible for the increases in IL-6 and TNF-α production in skeletal muscle following exposure to ROS-quenching agents (PCO), it is necessary to investigate signalling pathways and downstream targets involved. The three main pathways involved after injury, as already discussed in the section on circulatory cytokines, are the

NFκB, AA and the MAPK pathways. However, since a variety of other cells could have contributed to the increases in inflammatory cytokines, such as SCs, macrophages, neutrophils, etc., different inflammatory pathways might be activated in different cell types in response to different treatment regimes.

Of interest, in the current study, when assessing muscle IL-6 and TNF-α expression by means of immunohistochemistry (qualitatively), these cytokines seemed to be localized predominantly within inflammatory cells at the early time points (1 day and 3 days post-injury), similar to the observations made by Warren *et al.* (2002). However, on days 5 and 7, inflammatory cytokine levels were decreased in white blood cells, while in the regenerating myofibers, slight cytoplasmic staining and staining around the borders appeared.

3.4.4 Immune cell infiltration into the injured area

Immune cell infiltration have been studied extensively in various models of injury, which include stretch (St. Pierre Schneider *et al.,* 2002), hindlimb-suspension (Tidball *et al.,* 1999; Frenette and Tidball 2000; Frenette *et al.,* 2002), exercise (Fielding *et al.,* 1993), downhill running (Tsivitse *et al.,* 2003), Achilles tendon injury (Marsolais *et al.,* 2001) and cardiotoxin-injury (Sun *et al.,* 2009). However, very little is known regarding the effect and specific time frame of immune cell infiltration after a contusion injury. The only study regarding immune cell infiltration after contusion injury was performed by Thorsson *et al.* (1998), who assessed whether neutrophils were present after injury, but no actual cell counts were performed. Neutrophil numbers peak predominantly on day 1 after injury, followed by resolution of inflammation roughly on day 2-5. However, time points at which neutrophils start to infiltrate the injured area and resolution of inflammation differ when different injury models are used. For example, after excessive and invasive non-physiological stretch injury in rabbits, neutrophils were already evident 4 hr after injury and undetectable 48-72 hr later (St. Pierre Schneider *et al.,* 2002). In contrast, after exercise (in men) and Achilles tendon injury (in rats), neutrophils were still elevated for up to 5 days after injury (Fielding *et al.,* 1993;

Marsolais *et al.,* 2001). In the current study, 4 hr after contusion injury, a large number of neutrophils were evident in the injured area of the PLA group, and also to a lesser extend in the border zone, adjacent to the injured area. On day 1 after injury, a 30-fold increase is evident in the injured area, whereas the neutrophils in the border zone increased only 10-fold, a point in time corresponding to maximal neutrophil numbers similar to all studies mentioned. In most studies mentioned, the results only mentioned the most predominant increase in neutrophils on day 1, without actually quantifying it. The big difference in neutrophil numbers between the border zone and injured area in the current study can predominantly be attributed to the fact that injury was more severe in the injured area compared to the border zone. A minor contribution might be attributed to vascular disruption which is evident in the injured area, allowing free flow of red blood cells and immune cells into the injured area, compared to less or no vascular disruption in the border zone. Also, similar to the previous studies mentioned, in the current study in the PLA group, increased neutrophils were still evident on day 3 after injury, but significantly lower, indicating resolution of inflammation, with no detectable neutrophils on day 5.

On the other hand, in this study, macrophages started to infiltrate the injured area roughly 4 hr to day 1 after injury, peaking on day 3, a point in time when the number of neutrophils started to decrease. At day 5 after injury, macrophage numbers were still significantly elevated and only started to decrease on day 7 onwards. In contrast to this study's findings, almost no detectable macrophage levels were evident in the study by St Pierre Schneider *et al.* (2002) on stretch injury, assessing time points 4 hr, 24 hr, 48 hr and 72 hr post-injury. However, the antibody used to identify macrophages in that study does not bind to any peripheral blood cells, including monocytes and granulocytes, and is therefore not ideal for identification of all macrophages in skeletal muscle after injury (St. Pierre Schneider *et al.,* 2002). Also, with stretch injury, the basal lamina is usually not disrupted and might not have the need for macrophages to infiltrate the injured area, since damage was not as excessive as with other injury types. In agreement with this study's data, in the contusion injury study by Thorsson *et al.* (1998), macrophages were still numerous at Day 6, but they too had

disappeared by Day 11. However, again this phenomenon was only qualitatively assessed. In the current study, the co-localisation of both local pro-inflammatory cytokines (IL-6 and TNF-α in the injured area) and macrophages were evident on day 3 after injury, indicating macrophages as predominant cytokine source.

With PCO treatment, almost no neutrophils were detected in the border zone or injured area, indicating that at least one mechanism of PCO is the blunting of neutrophil infiltration into the injured area. It is known that PCO binds to collagen, thereby strengthening the vasculature, especially capillaries (Teixeira 2002). It is possible that in the current study, there is a decrease in hypoxia as a result of a strengthened vasculature and therefore a decrease in damage. Also, a lower number of neutrophils infiltrated the injured area, since damage of the vasculature results in a free flow of immune cells into the injured area, which although excessive, is short of duration. This is corroborated in previous experiments, where haematoxylin and eosin (H&E) stains indicated less vascular disruption in the PCO group (MSc thesis, Kruger 2007). It is also possible that PCO supplementation resulted in better suppression of cell surface or chemoattractive proteins (chemokines) for neutrophil migration to the injured area. In support of this, Sato *et al.* (1996) illustrated that the antioxidants NAC or 2-oxothiazolidine-4-carboxylate (OTC) inhibited the TNF-α-mediated stimulation of chemokine expression, critical for neutrophil migration (IL-8 and monocyte chemoattractant protein (MCP)-1). This therefore suggests that PCO's effect of limiting the number of neutrophils infiltrating the injured and border zone area on day 1 might have occurred *via* its blunting of plasma pro-inflammatory cytokines IL-6 and TNF-α. Alternatively, there may not be an inhibition of the neutrophil response, and PCO could increase the activity of individual neutrophils, thereby allowing fewer neutrophils to infiltrate the injured area, ultimately having a greater effect. This latter hypothesis was also tested and will be discussed in Chapter 5, where the aim was to identify why fewer neutrophils infiltrate the injured area after PCO supplementation.

Macrophages infiltrated the different muscle areas earlier in PCO, indicating that Oxiprovin™ accelerated the macrophage response (day 1 *vs* day 3 and 5 in the PLA group). The

macrophage response in the PCO group also coincided with the cytokine patterns. Two distinct subpopulations of macrophages are known to invade injured muscle tissue at different time points after injury (Chazaud *et al.,* 2009; Tidball and Villalta 2010). The early invading macrophages, M1, have been shown to reach maximal numbers in damaged muscle at about 24 hr after the onset of injury and then rapidly decline (Chazaud *et al.,* 2009; Tidball and Villalta 2010). These inflammatory macrophages secrete pro-inflammatory cytokines, such as TNF-α and IL-1β, and are responsible for the phagocytosis of necrotic tissue (Chazaud *et al.,* 2009; Tidball and Villalta 2010). The second population of macrophages, M2, possibly derived from inflammatory macrophages by phenotype switch, reach their peak at 2 to 4 days after injury (Chazaud *et al.,* 2009; Tidball and Villalta 2010). These macrophages secrete anti-inflammatory cytokines, such as IL-10, that contribute to the termination of inflammation and thus muscle regeneration (Chazaud *et al.,* 2009; Tidball and Villalta 2010). In the current study, the appearance of macrophages in the border zone and injured area in the PCO group coincided with the increases in pro-inflammatory cytokines in the border zone. Also, on day 3 after injury, the macrophage population in the injured area and border zone were still high, coinciding with increased circulatory anti-inflammatory cytokine, IL-10. Although the different subtypes of macrophages were not characterized, current macrophage infiltration and cytokine time courses suggest that chronic PCO supplementation can result in an earlier macrophage phenotype switch in favour of an M2 response following injury.

3.5 Conclusion and directions for future research

The results obtained in this study indicate that (a) the earlier satellite cell activation response, and (b) the changed inflammatory response in favour of (c) a blunted neutrophil response and (d) an anti-inflammatory reaction are the result of chronic PCO supplementation. This study also highlights the importance of illuminating the precise mechanism of action of anti-oxidants such as PCO in Oxiprovin™. From the literature it is known that IL-6 and TNF-α are important activators of three main inflammatory pathways, namely NFκB, AA or MAPK.

Since PCO significantly blunts the pro-inflammatory response, it therefore suggests that PCO might function by blunting or completely inhibiting one or all of these signalling pathways. Assessment of the anti-inflammatory cytokines (IL-4 and IL-10) in the muscle, as done in the plasma, could have provided a better indication of what anti-inflammatory processes occur within the muscle. Also, the two macrophage subtypes, M1 and M2, were not determined which could have provided for a better interpretation of the data (one of the focus points in Chapter 5). Considering the positive effects of chronic PCO supplementation, it is also of particular interest to note whether acute supplementation might have similar effects, which is the focus of the next chapter.

CHAPTER 4

Effect of acute proanthocyanidolic oligomer (PCO) supplementation on muscle recovery and the inflammatory response to contusion injury in rats

4.1 Introduction

Various therapeutic interventions exist for treating muscle after injury. Currently, all of these treatments are directed to the restoration of full skeletal muscle function, augmenting normal repair and regeneration processes and limiting inflammation and muscle fibrosis to reduce scar formation. The basic biological processes which occur during the healing of muscle are similar, irrespective of the underlying cause of the injury (Hurme *et al.,* 1991a; Kalimo *et al.,* 1997). However, differences may occur, since the severity of injury plays a predominant role in determining the magnitude and possibly onset and/or duration of these phases (Bunn *et al.,* 2004). This suggests that timing of first treatment, will play an important role in determining the best treatment options for muscle following injuries of different severities.

For example, actively using the injured muscle after a short but adequate period of rest (hr), results in faster disappearance of haematoma and inflammatory cells, followed by a more rapid and organized myofiber regeneration (Lehto *et al.,* 1985; Jarvinen and Lehto 1993). However, premature mobilisation of the injured leg can have detrimental outcomes and may result in increased scar formation and increased potential for re-injury of the muscle (Jarvinen and Lehto 1993).

Other non-invasive strategies are often used in combination. Rest in combination with elevation may decrease the accumulation of blood in the injured limb, thus reducing swelling and pain (Zhang *et al.,* 2001). The combination of ice and compression limits bleeding, reduces pain and facilitates healing and thus faster return to the sports field, while

compression alone does not seem to have any significant effect (Levy and Marmar 1993). Ice is usually applied 2 to 4 times per day, starting on the day of the injury (Olson and Stravino 1972; McMaster and Liddle 1980; Kellet 1986; Meeusen and Lievens 1986; Grana 1993; Ernst and Fialka 1994). What is also important is that understanding the basic principles of muscle healing and the physiological or biochemical effect of the treatment, will allow for more informed choices.

Ice applied immediately post-injury reduces metabolism and bleeding, which decreases neutrophil infiltration and minimizes secondary hypoxic injury (Knight 1989; Knight *et al.,* 2000; Deal *et al.,* 2002). However, if one lowers the temperature of the muscle below 25 ℃, it can dilate blood vessels, which increases hemorrhage and exacerbates the inflammatory response (Kellet 1986; Meeusen and Lievens 1986).

Hyperbaric oxygen therapy (HBOT) has been shown, in *in vivo* and *in vitro* studies, to be an effective treatment for different forms of exercise-related damage, reducing oedema, enhancing oxygen delivery and inactivating white cell adhesion (Nylander *et al.,* 1985; Thom *et al.,* 1994; Staples *et al.,* 1995; Hills 1999). However, it does not seem to be effective after muscle crush or contusion injury (Nelson *et al.,* 1994; Thorsson *et al.,* 1997), possibly due to the fact that blood flow is too severely disrupted, thus limiting oxygen delivery to the injured tissue early after injury. Stem cell therapy has also been shown to be potentially effective after various types of muscle injury (Singec *et al.,* 2007), but more research is needed to determine these beneficial effects.

Although short-term corticosteroid usage is known to benefit muscle regeneration (Beiner *et al.,* 1999), long-term use may increase the initial inflammatory reaction even further and may inhibit the healing process later on, resulting in exacerbation of tissue injury. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used after sports-related injuries, and are prescribed by clinicians quite often for their analgesic purposes rather than for enhanced healing, as the evidence to support the latter remains limited (Ong *et al.,* 2007; Xian and Zhou 2009). The most common clinical approach is to start treatment two days after injury,

when swelling caused by inflammation results in pain, and to continue treatment for up to 7 days (personal communication: Dr Pierre Viviers, Stellenbosch University Sport Performance Institute, SUSPI). Although this regime results in a decreased inflammatory response (decreased neutrophil and macrophage infiltration) and alleviation of pain and swelling, it may also delay muscle regeneration (Jarvinen *et al.,* 1992; Mishra *et al.,* 1995). Research using small animals and *in vitro* models, have shown that short-term NSAID treatment during the early repair phase (1-3 days) may result in the reduction of inflammatory symptoms (swelling and pain) (Tidball and Wehling-Henricks 2007), which suggests that if treated earlier (as soon as possible after injury) but for a much shorter period (discontinued after two days), it could potentially limit the side-effects of the treatment (e.g. bleeding complications – personal communication: Dr Pierre Viviers, SUSPI).

Therapeutic ultrasound is another treatment option, usually started a couple of hr after injury, and done for a couple of minutes each day (Rantanen *et al.,* 1999; Wilkin *et al.,* 2004). Ultrasound on its own normally does not have any beneficial effect on skeletal muscle regeneration (Wilkin *et al.,* 2004; Silveira *et al.,* 2010). However, if administered in combination with antioxidants (dimethylsulfoxide – DMSO), it effectively reduces the presence of muscle damage (creatine kinase – CK and acid phosphatase), oxidative stress (superoxide anion, lipid peroxidation and protein carbonyls production) and possibly secondary damage after injury (Silveira *et al.,* 2010). Most ultrasound studies however assessed sub-optimal markers for determining muscle regeneration, so that the effectiveness of this therapy is largely based on clinical observations of parameters such as decreased pain, which is not necessarily indicative of recovery.

Upon muscle injury, cells are continuously attacked by reactive oxygen species (ROS), which arise as natural byproducts of metabolic processes – this occurs no matter what type of injury. Normally, most of these ROS produced are neutralized by natural antioxidant defences such as glutathione peroxidase and catalase (Rice-Evans *et al.,* 1995; Basu 1999). However, in situations where ROS production is increased, e.g. after injury or any type of "hyper" inflammatory response, these natural antioxidants are often not enough to neutralise

the free radicals generated (Bowles *et al.,* 1991). Despite the increase in clinical use of antioxidants and the interest in nutrition for treatment of muscle injuries, only a limited number of studies have focussed on antioxidant treatment of muscle injury in otherwise healthy individuals.

The most comprehensively researched antioxidants in exercising subjects are the vitamins C and E. Chronic supplementation with vitamin C, has improved muscle recovery (speed of recovery of muscle contraction and a decrease in muscle soreness), and decreased plasma interleukin (IL)-6, 2 hr after injury (Kaminski and Boal 1992; Jakeman and Maxwell 1993; Thompson *et al.,* 2001b), whereas acute treatment had no effect on various measures of antioxidant status and damage (plasma CK, myoglobin, malondialdehyde and IL-6) (Thompson *et al.,* 2003), despite increasing plasma concentrations of vitamin C (Thompson *et al.,* 2001a). Acute oral vitamin E supplementation yielded variable results: protection was reported after downhill-running (Meydani et al., 1993), but no effect after plyometric contractions (Van der Meulen *et al.,* 1997). From these studies, vitamins may have positive effects when consumed as a chronic supplement, but short-term supplementation may not be sufficient enough to allow for a beneficial effect on recovery.

Most often antioxidants such as vitamin C and E are taken chronically as a means of boosting the immune system. However, since most recreational and especially competitive athletes take a variety of supplements chronically, they may be at risk for toxicity, since megadoses of vitamins and other antioxidants can be toxic (Ristow *et al.,* 2009). Therefore, the ideal antioxidant treatment for muscle injury would be one that can be administered acutely post-injury rather than by preventative supplementation.

Recently, polyphenols have been shown to benefit muscle repair (Kato *et al.,* 2000; Buetler *et al.,* 2002; Dorchies *et al.,* 2006; Hofmann *et al.,* 2006; Nakazato *et al.,* 2010) *via* suppression of oxidative stress (Kato *et al.,* 2000). The efficacy of chronic PCO supplementation was discussed in the previous chapter. The aim of the following study was to determine whether acute post-injury PCO supplementation might prove as beneficial for

muscle recovery as chronic PCO treatment. We hypothesized that acute PCO supplementation will have similar effects on muscle recovery after a contusion injury, but that its effects on the acute period after injury (neutrophil phase and satellite cell activation) may not be optimal, due to the time required for its absorption into blood and tissue. The main objectives of this study were to determine (a) changes in satellite cell (SC) activation and (b) foetal myosin heavy chain (MHC_f) expression as an indication of muscle regeneration, and (c) the effects on the inflammatory immune cells and (d) cytokines.

4.2 Methods

4.2.1 Study design

4.2.1.1 Experimental animals

Adult, male Wistar rats, weighing approximately 280 g were used for this study. All animals were handled exactly the same and were subjected to exactly the same conditions inside the animal house as the animals in the previous study (see chapter 3).

Preceding the contusion injury protocol, experimental rats were randomly divided into three groups: a placebo control group receiving 2 weeks of 0.9 % saline treatment without injury (C-PLA, n=8), a placebo post-injury group, injured and then treated with placebo for up to 2 weeks after injury (PI-PLA; n=38, i.e. 6-8 rats per time point, time points were 4 hr, 1 d, 3 d, 5 d, 7 d and 14 d post-injury) and a proanthocyanidin post-injury group, injured and then supplemented with Oxiprovin™ for up to 2 weeks post-injury (PI-PCO; n=38, i.e. 6-8 rats per time point, time points were 4 hr, 1 d, 3 d, 5 d, 7 d and 14 d post-injury). See Figure 4.1 for more detail.

4.2.1.2 PCO administration

Rats were orally gavaged with tap water for two weeks prior to injury, followed (post-injury) by a daily dose of 20 mg/kg/day PCO (dissolved in 0.9 % saline) or 0.9 % saline (placebo) for

up to 2 weeks after injury. The first post-injury dose was administered 2 hr after injury, when the rats where fully conscious. Control animals received 2 weeks of placebo treatment, without any injury intervention.

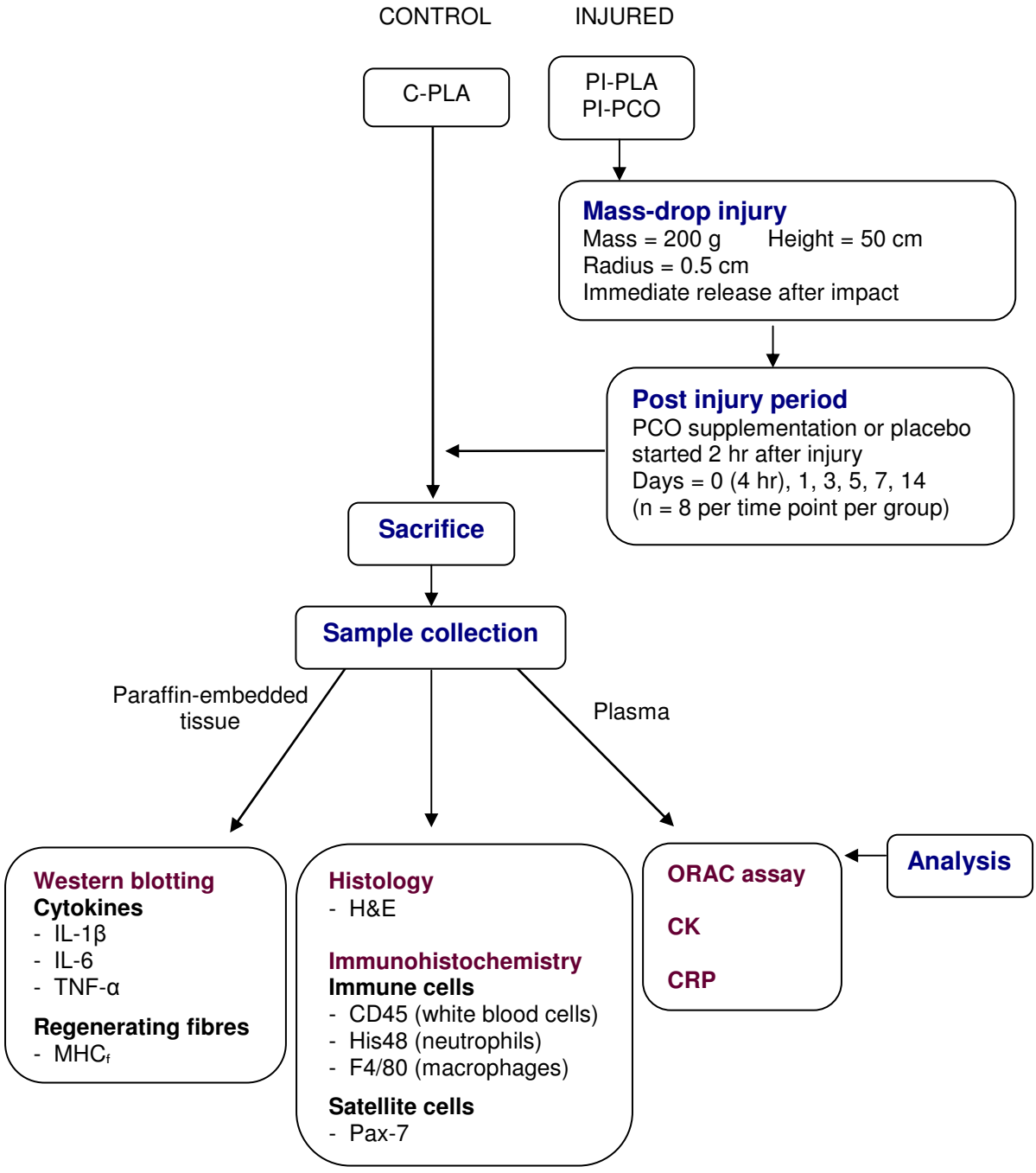


Figure 4.1: Experimental design.

4.2.1.3 Induction of muscle injury

Contusion of the hind limb was produced by a mass-drop injury jig. This method was fully described in Chapter 3, section 3.2.1.3.

4.2.2 Sacrifice and sample collection

All sacrifice and sampling procedures were exactly the same as those in the previous chapter. For a detailed description of sacrifice and sample collection, see the previous chapter, section 3.2.2.

4.2.3 Sample analysis

The analytical profile included the following parameters and procedures: a) plasma CK concentration, b) plasma C-reactive protein (CRP) level, c) plasma and muscle oxygen radical absorbance capacity (ORAC), d) protein concentration, e) immunohistochemical staining and visualisation of muscle sections for SCs measuring Pax-7, f) qualitative histological analysis of muscle recovery and g) western blotting for quantification of cytokines (IL-1β, IL-6 and tumour necrosis factor (TNF)-α) and MHC_f (indication of regenerating muscle fibres).

4.2.3.1 CK concentration

Total CK, an indirect marker for the presence of muscle damage, was determined by *PathCare* pathology laboratory (Vergelegen Medi Clinic, Somerset West, South Africa) using an automated enzymatic method. The normal range for rat plasma CK concentration is 15 – 195 international units per liter (IU/L) at 37 ^{o}C.

4.2.3.2 CRP level

Total CRP, an indirect marker for the presence of inflammation, was determined by means of a 96-well high sensitivity ELISA assay (eBioscience, 88-7501-28). CRP standards and plasma samples were prepared according to the manufacturer's instructions. Standard or sample (100 µl) were added to each CRP-antibody-pre-coated well in duplicate and allowed to incubate for 2 hr. After washing, 100 µl of diluted HRP-conjugated anti-rat CRP were added to each well and allowed to incubate for 1 hr. The plate was washed again and 100 µl of TMB substrate solution was added to each well for 5-10 min. The reaction was stopped by adding 100 µl of stop solution (H_2SO_4) and the absorbance was read on an ELISA plate reader equipped with a 450 nm filter, corrected to 570 nm. A standard curve was used to determine the amount of rat CRP present in the samples.

4.2.3.3 ORAC assay

The ORAC assay was performed on plasma no later than 10 days after collection and is based on the measurement of oxygen radical-induced quenching of a fluorescent probe (Huang *et al.,* 2002). Through the change in the fluorescence intensity of the probe in solution over time, an index of the extent of oxygen radical quenching capacity of the probe in the sample solution can be determined. In the presence of antioxidants, oxygen radical-induced quenching of the probe is inhibited, and the extent of delay in quenching reflects the antioxidant capacity of the sample and is called the ORAC (Meeusen and Lievens 1986; Safran *et al.,* 1989; Noonan *et al.,* 1993; Olson and Ley 2002). After antioxidant supplementation, a higher ORAC compared to the controls is expected. See Appendix D for a summary of reagents and the laboratory method.

4.2.3.4 Determination of protein concentrations

To estimate muscle total protein concentrations, either for making up of samples for western blotting or to include as measurement in the ORAC assay, the Bradford method was used (Appendix E).

4.2.3.5 Histopathology and immunohistochemistry

All histopathological and immunohistochemical evaluations were conducted on at least four rats per time point per treatment group.

Haematoxylin and eosin (H&E) stained sections were used to qualitatively assess the recovery process. For H&E staining, 5 µm muscle cross-sections were briefly deparaffinised and then stained with H&E (Appendix F). Haematoxylin stains the basophilic structures blue-purple and alcohol-based eosin stains the eosinophilic structures bright pink. The basophilic structures include nucleic acids, such as in ribosomes and cell nuclei, and cytoplasmic regions rich in RNA. The eosinophilic structures are intra- or extracellular proteins (mostly cytoplasm and red blood cells).

Using immunohistochemistry techniques, 5 µm cross-sections were labelled with: (a) Pax-7 for labelling SCs, (b) His48 for labelling neutrophils and (c) F4/80 for labelling macrophages. PBS controls were also used in this part of the study and the antibodies used were specific for rat samples. For appropriate dilutions and suppliers of antibodies used, see Table 1 in Appendix B.

Image analysis: All imaging data were handled and analysed exactly as previously described in Chapter 3.

4.2.3.6 Western blotting analysis for cytokine and MHC_f levels

Protein levels were determined by standard Western blotting techniques. Briefly, 40 µg muscle homogenate of each sample was prepared with sample and RIPA buffer. Samples

were then analysed by either 8 or 12 % gel electrophoresis and were immunoblotted for pro-inflammatory cytokines, IL-6 and TNF-α, as well as for MHC_f. β-actin was used to determine equal loading. For a full-detailed description of all the reagents used, as well as the method, see Appendix G.

4.2.4 Statistics

Results are presented as means ± standard error of the mean (SEM), unless otherwise specified. A regression analysis was performed to compare growth curves in the different experimental groups. Differences between time points for other parameters within and between groups were analysed using factorial analysis of variance (ANOVA). When significant interactions were found, Bonferroni *post hoc* tests were performed. However, for immune and cytokine data, a Fisher's *post hoc* test was used. All statistical analyses were done using the computer software Statistica version 9 (StatSoft Software). The accepted level of significance was $p < 0.05$.

4.3 Results

4.3.1 Body weight

Rats in the different groups were matched for body mass at the start of the protocol. Contusion injury in the two injury groups resulted in a slight decrease in body mass 4 hr after the injury (Figure 4.2), which might be attributed to the fact that rats were asleep for up to 2 hr after anaesthesia and probably dehydrated. However, approximately 1 day after the injury, rats had recovered this loss.

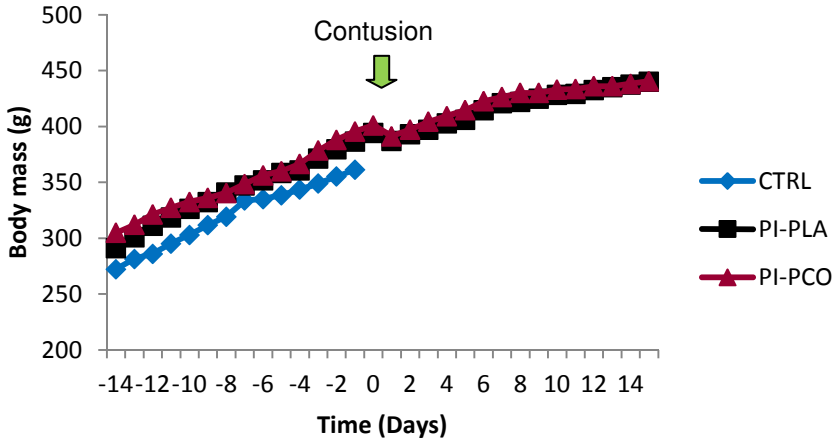


Figure 4.2: Comparison of mean body mass in control PLA rats and in rats supplemented with either PLA or PCO, after subjection to contusion injury. Days -14 to 0 represent 2 weeks prior to injury, whereas time point 0 indicates the day of injury and the start of the supplementation procedure (2 hours after injury). n = 8 rats per time point per group.

4.3.2 H&E

Figure 4.3 illustrates histological staining with H&E. Using this staining technique, qualitative changes in the muscle can be visualised over time. In the muscle of the control group (A), no ultrastructural damage is apparent. Soon after injury (4 hr, B and H), the disruption of the muscle fibre ultrastructure and vascular disruption are apparent in both treatment groups. Immune cell infiltration was seen at 4 hr (H) in the PCO group and on day 1 (C) in the PLA group. Immune cells stayed visible in the injured area up to day 7 (F) in the PLA group, compared to day 5 in the PCO group (K). Newly formed/ regenerating muscle fibres were only apparent at day 14 (G) in the PLA group, while peaking already at day 7 in the PCO group. By day 14, the PCO and PLA group did not display normal muscle ultrastructure, indicating that regeneration is not complete, however, the PCO group's ultrastructure were almost complete.

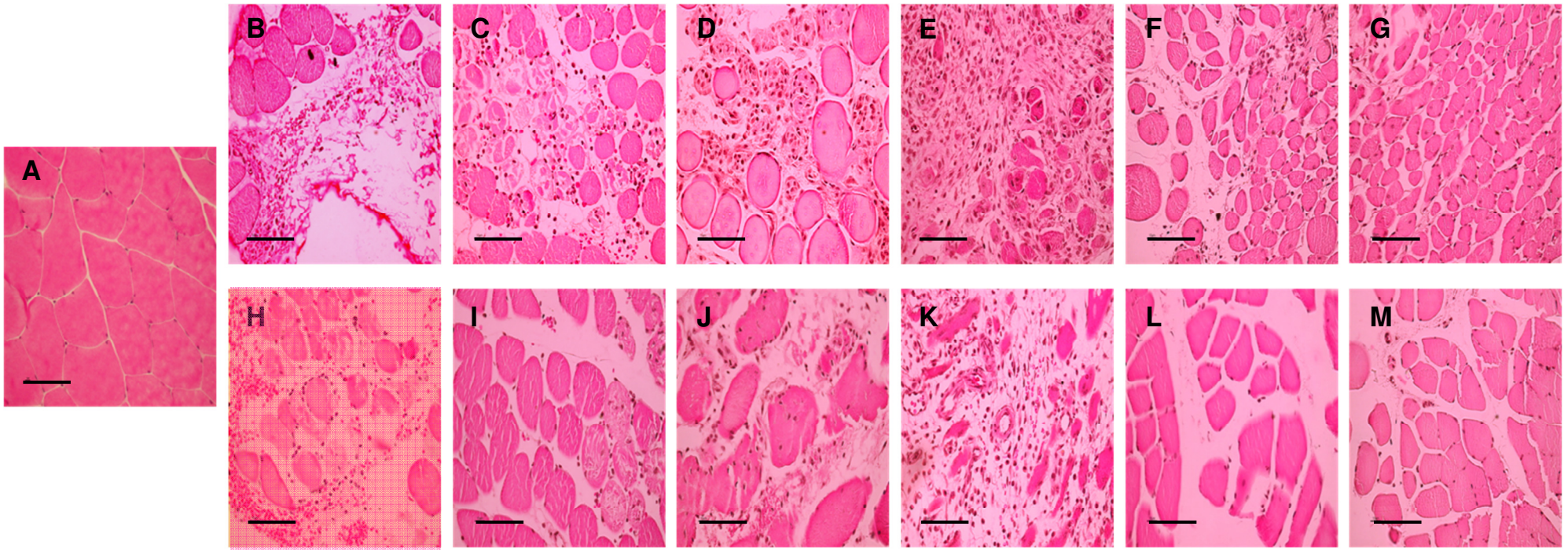


Figure 4.3: H& E staining illustrating the clearing of inflammation after injury. Photo A represent control samples with no injury. Photos B–G represent samples taken from PLA-injury animals at (B) 4 hr, (C) 1 d, (D) 3 d, (E) 5 d, (F) 7 d and (G) 14 d post-injury. Photos H-M represent similar time points in the PCO group. Scale bar represents 40 µm. Photo B and H represent muscle fibre destruction and vascular disruption. Immune cells infiltrated the injured area (photo C and I) and remain visible for up to day 7 in the PLA group and day 5 in the PCO group. Neither the PLA, nor the PCO group displayed normal muscle fibre ultrastucture 14 days after injury, indicating that muscle fibre regeneration is not complete.

4.3.3 Plasma CK level

There was a trend towards a time effect (p = 0.068) for CK, with a slight increase in plasma CK in both the PLA and PCO group 4 hr after injury (Figure 4.4). No treatment effect was evident.

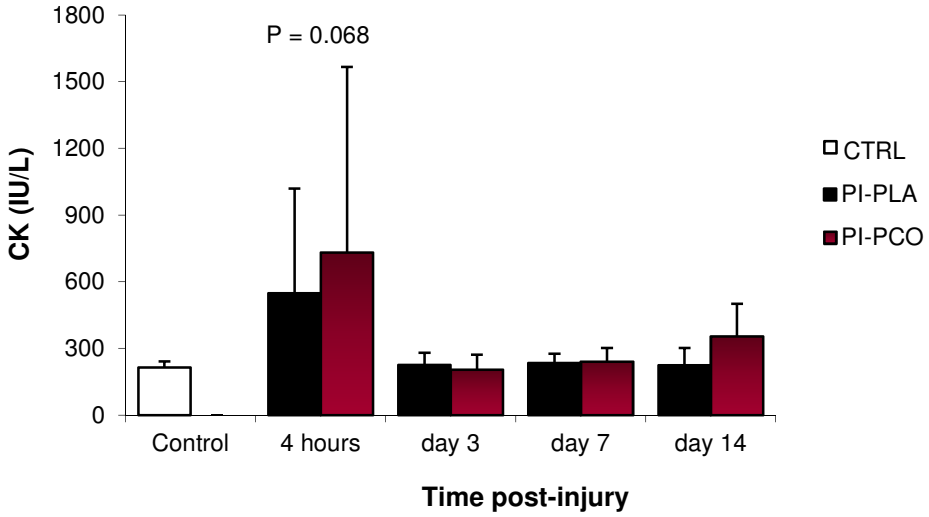


Figure 4.4: Total plasma creatine kinase (CK) activity (mean ± SD) of the acute supplemented control and injury groups at several time points after the contusion injury. Statistical analysis: Factorial analysis of variance (ANOVA) with Bonferroni *post hoc* test, n = 4 rats per time point per group.

4.3.4 Plasma and muscle ORAC assay

Statistical analysis of the results of plasma ORAC indicated a main effect of time ($p < 0.001$), and a main effect of treatment-time ($p < 0.05$) (Figure 4.5A). In the PLA group, a significant but transient decrease in plasma ORAC was evident on day 3 compared to day 1 after injury. This was followed by a significant increase by day 5, whereas ORAC values in the PLA group on day 14 were similar to that of controls. No significant changes were seen in the PCO group, however, on day 14, the PCO group had a significantly higher plasma ORAC compared to the PLA group.

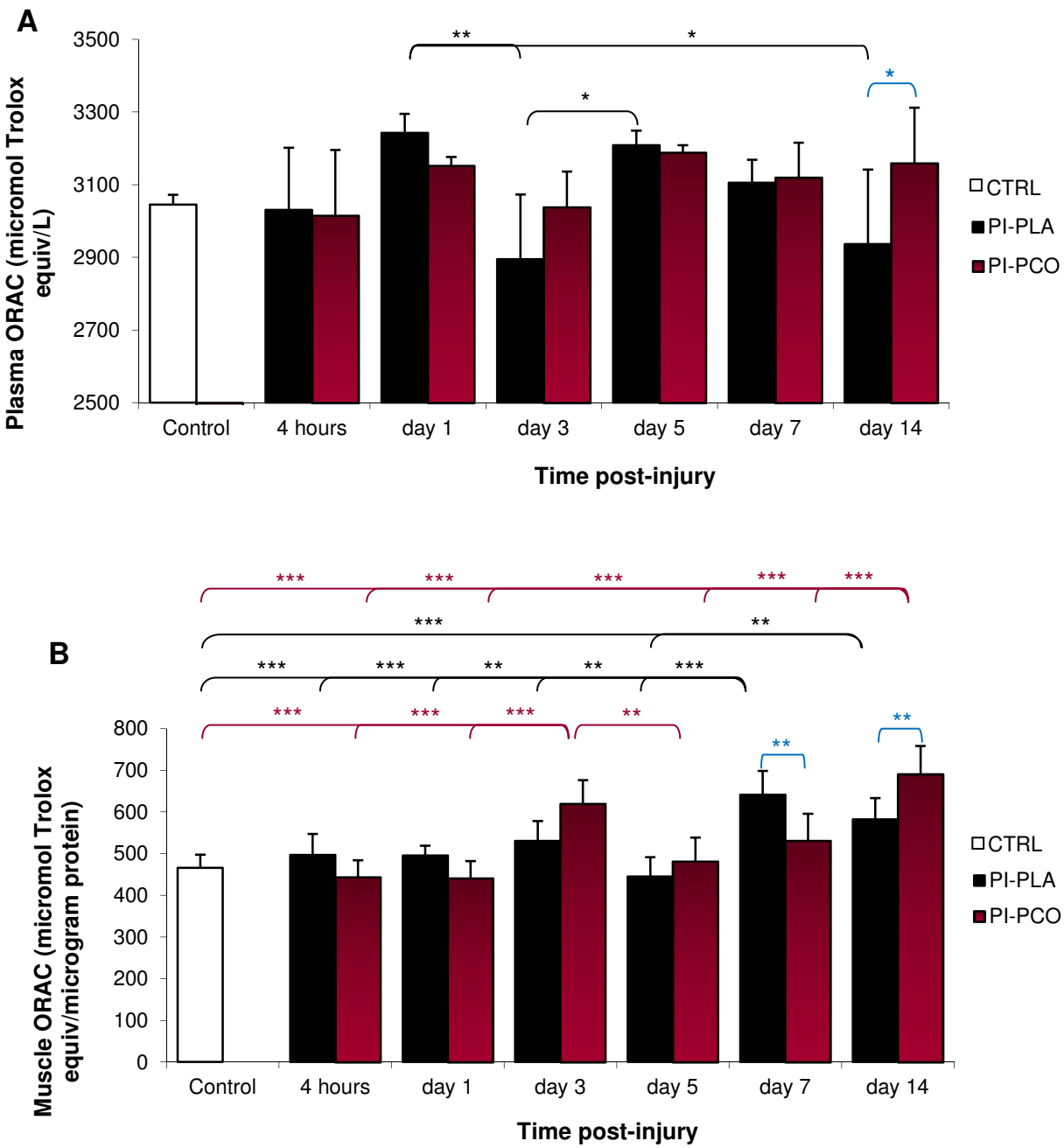


Figure 4.5: The effect of muscle contusion injury with or without acute PCO supplementation on plasma (**A**) and muscle (**B**) oxygen radical absorbance capacity (ORAC) over time. (Figure details: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Statistics: Factorial analysis of variance and Bonferroni *post-hoc* test. n = 8 rats per time point per group.

In the muscle samples, statistical analysis also indicated a main effect of both time and a treatment-time effect ($p < 0.001$ for both) (Figure 4.5B). Muscle ORAC were significantly elevated by day 7 in the PLA group, whilst the PCO group displayed a more variable ORAC

status. ORAC in this group increased significantly earlier (day 3), with a secondary peak on day 14 after injury, compared to control. Significant differences between groups were also evident on day 7 – the PLA group had a higher ORAC – and day 14 post-injury – the PCO group had a higher ORAC.

4.3.5 Plasma CRP level

No effect of time or treatment or treatment-time on plasma CRP was evident (Figure 4.6).

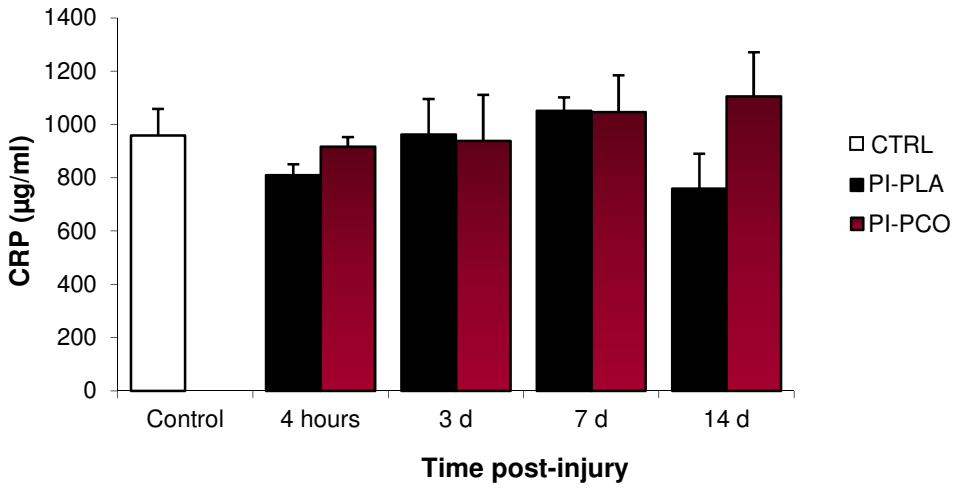


Figure 4.6: Plasma C-reactive protein (CRP) activity (mean ± SD) of the control non-injured groups and the injury groups at several time points after contusion injury and acute supplementation. Statistical analysis: Factorial analysis of variance (ANOVA) with Bonferroni *post hoc* test indicated no effect of treatment or time. n = 8 rats per time point per group.

4.3.6 Immune cell infiltration

4.3.6.1 Neutrophils

Repeated measures ANOVA indicated a similar and significant effect of treatment ($p < 0.01$), time, and treatment-time ($p < 0.001$ for latter 2) in both the border zone and injured area. No neutrophils were present in control pre-injured samples, whereas neutrophils peaked on day

1 in the PLA group, a point in time when the PCO group had a significantly lower number of neutrophils (Figure 4.7). This significant early increase was followed by a quick decrease on day 3 after injury. Almost no neutrophils were detected in the PCO group. Neutrophil

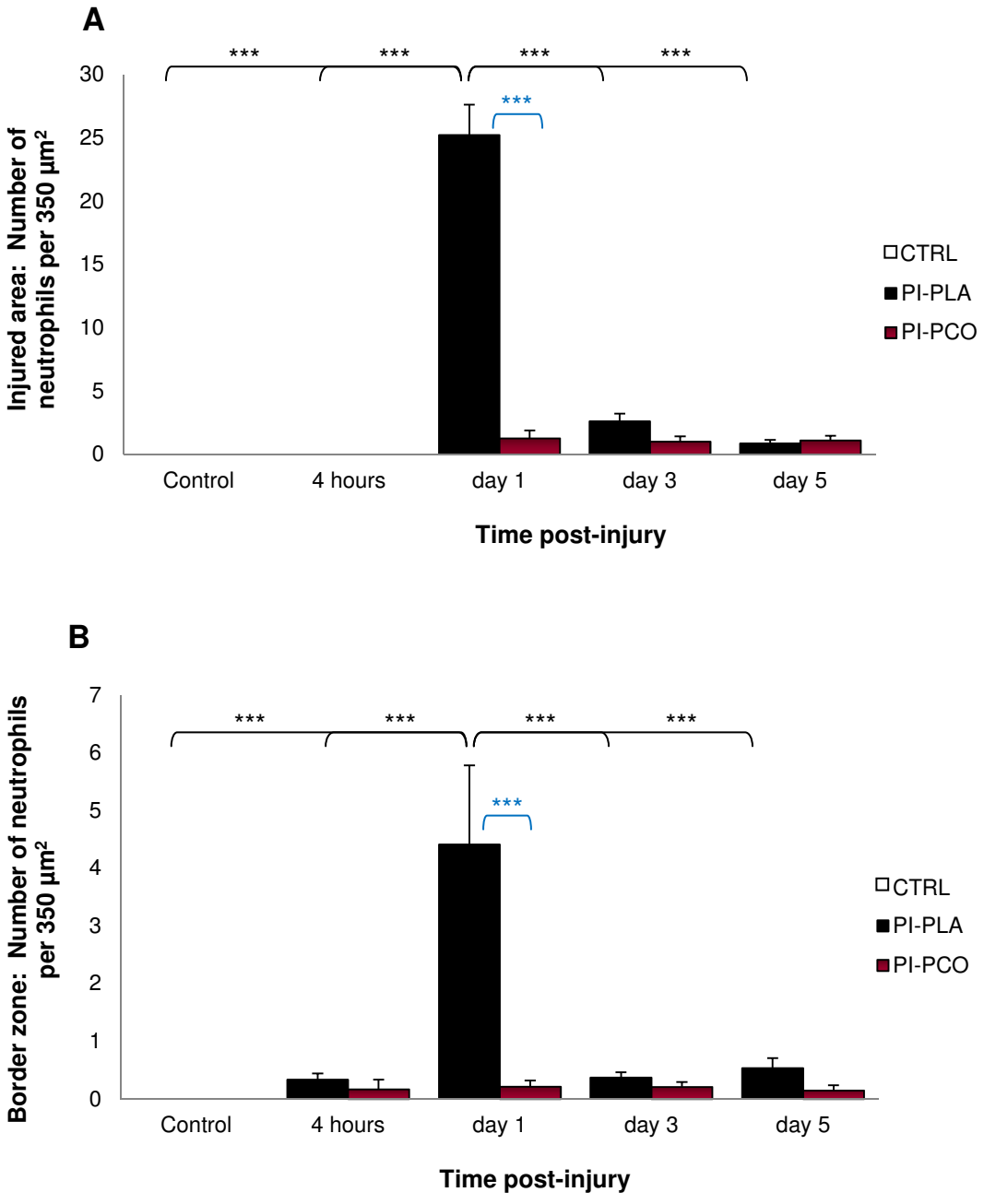


Figure 4.7: Neutrophil infiltration into the injured area (**A**) and border zone (**B**) after contusion injury and subsequent supplementation. Statistics: Factorial ANOVA with Fisher's post-hoc test; n = 4 per time point per group. Significance (*** $p < 0.001$).

numbers were generally higher in the injured area than in the border zone. Neutrophil infiltration on day 1 after injury is represented by Figure 4.8 – note that more neutrophils are apparent in the PLA group.

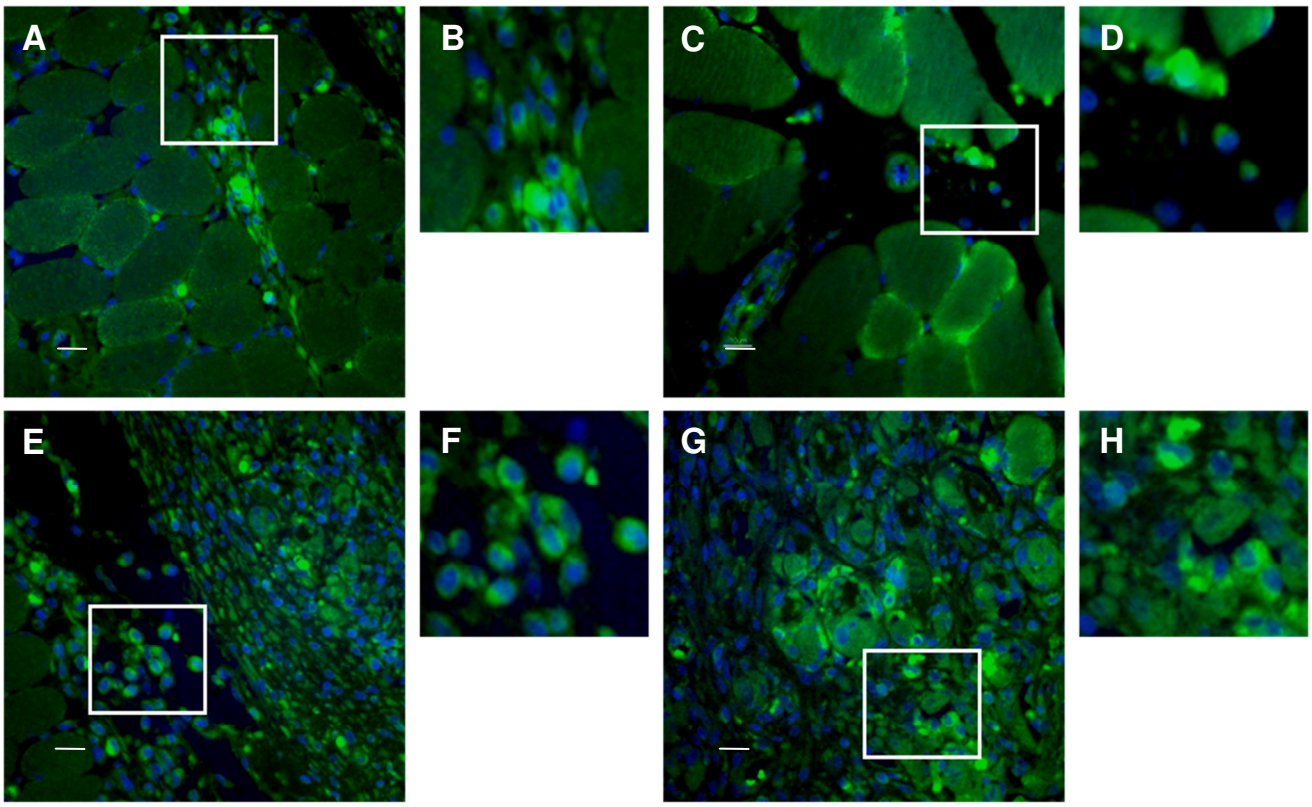


Figure 4.8: Neutrophil (His48) expression (green, FITC) and infiltration into the border zone (**A-D**) and injured area (**E-H**) of muscle, on day 1 post-injury (**A, B** and **E,F** indicates the PLA group; **C, D** and **G, H** indicates the PCO group). Images **B**, **D**, **F**, and **H** are enlargements of the area surrounded by the white block of the figures on the left respectively. Hoechst was used as nuclear dye. Scale bar represents 10 µm, with the original magnification of 400x used for the image acquisition.

4.3.7.2 Total white blood cells (WBC) and macrophages

White blood cells and macrophages showed similar results and it was therefore decided to discuss these results together. A main effect of time and treatment-time ($p < 0.001$ for both) were evident in the injured area for both WBCs and macrophages (Figures 4.9 A&C). There was a trend towards a main effect of treatment alone ($p = 0.065$). In the PLA group, only WBCs infiltrated the injured area on day 1 after injury, whereas macrophages were only detected by day 3 after injury. However, both macrophages and WBCs reached peak values by day 5 after injury. In the PCO group on the other hand, macrophages and WBCs had already infiltrated the injured area 4 hr after injury, followed by a significant elevation by day 5. Other time points where macrophage and WBC levels significantly differed between the groups were the 4 hr (both macrophages and WBCs) and day 1 time point (only for WBCs).

The factorial analysis of variance indicated a main effect of treatment ($p < 0.001$), time ($p < 0.05$ for WBCs and $p < 0.01$ for macrophages) and a treatment-time ($p < 0.05$ for WBCs and $p < 0.01$ for macrophages) effect in the border zone area (Figures 4.9 B&D). Both WBCs and macrophages in the PLA group were significantly elevated already on day 3 after injury, and even more so on day 5 after injury. No elevation in WBCs or macrophages in the PCO group were evident at any point in time. Treatment groups differed from one another in terms of WBCs and macrophages numbers, with the PLA group having significantly more WBCs and macrophages on day 1 (only for WBCs), 3, and 5 compared to the PCO group. Figure 4.10 (A-H) indicates WBC infiltration in the injured area, whilst Figure 4.10 (I-P) indicates WBC infiltration in the border zone, on day 1 and 5 respectively, where significant differences between groups were evident. Figures 4.11 (A-H) and (I-P) is representative of macrophage infiltration in the injured area and border zone respectively on days 3 and 5.

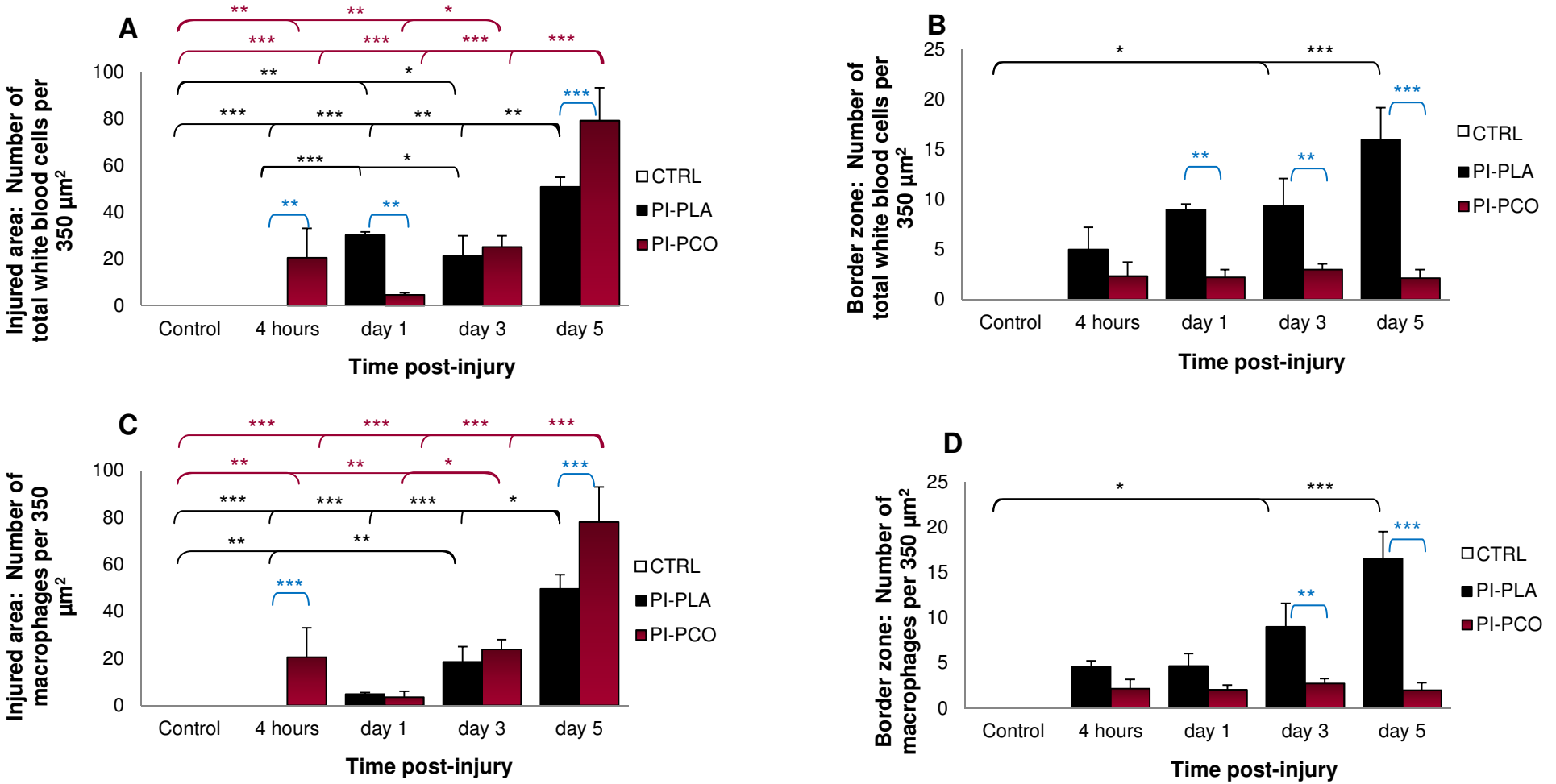


Figure 4.9: The effect of contusion injury and post-injury PCO treatment on WBC and macrophage infiltration into the injured area (**A&C**) and border zone (**B & D**) of acutely supplemented rats respectively. Statistics: Factorial ANOVA with Fischer's post-hoc test; n = 4 per time point per group. Significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

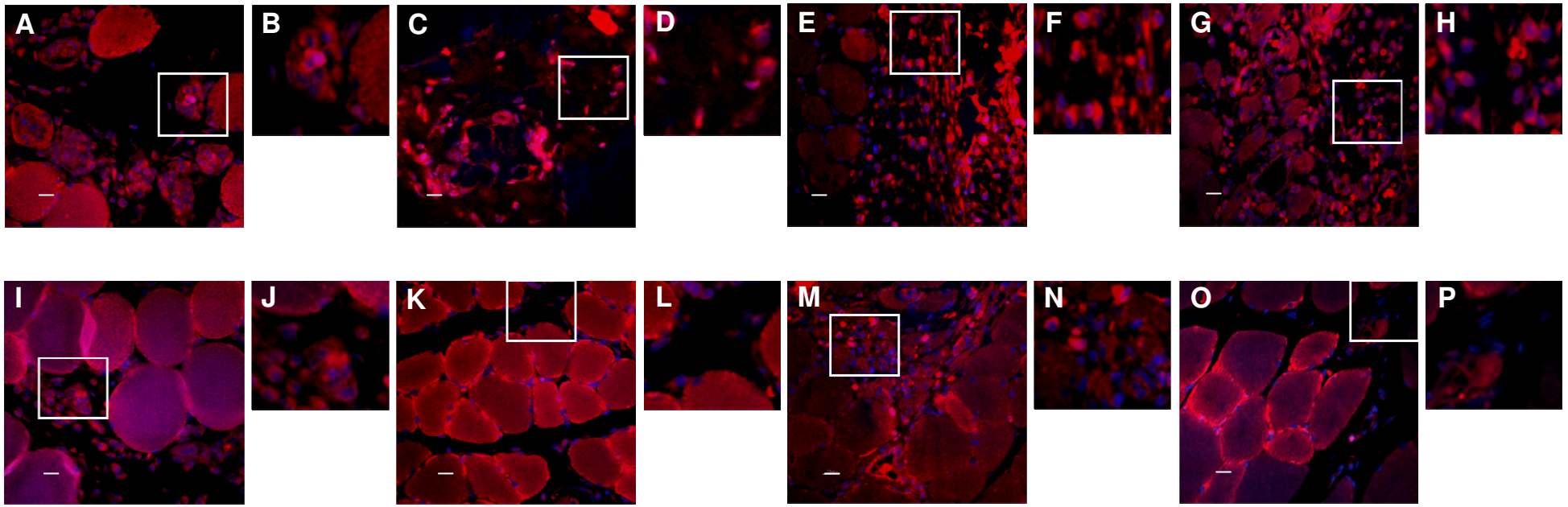


Figure 4.10: White blood cell (CD45) expression (red, Texas Red) and infiltration into the injured area (**A-H**) and border zone (**I-P**) of muscle, on day 1 post-injury (**A, B** and **I, J** indicates the PLA group; **C, D** and **K, L** indicates the PCO group) and on day 5 post-injury (**E, F** and **M, N** indicates the PLA group; **G, H** and **O, P** indicates the PCO group) respectively. Images **B**, **D**, **F**, and **H** are enlargements of the area surrounded by the white block of the figures on the left respectively. Hoechst was used as nuclear dye. Scale bar represents 10 µm, with the original magnification of 400x used for the image acquisition.

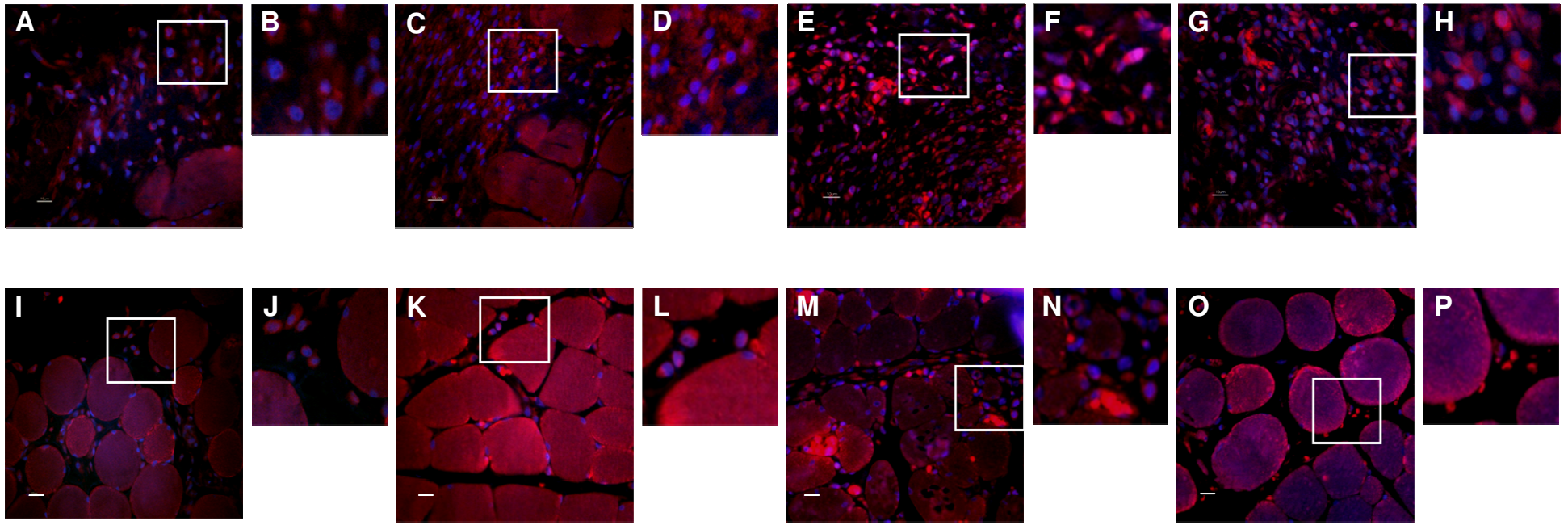


Figure 4.11: Macrophage (F4/80) expression (red, Texas Red) and infiltration into the injured area (**A-H**) and border zone (**I-P**) of muscle, on day 3 post-injury (**A, B** and **I, J** indicates the PLA group; **C, D** and **K, L** indicates the PCO group) and on day 5 post-injury (**E, F** and **M, N** indicates the PLA group; **G, H** and **O, P** indicates the PCO group) respectively. Images **B**, **D**, **F**, and **H** are enlargements of the area surrounded by the white block of the figures on the left respectively. Hoechst was used as nuclear dye. Scale bar represents 10 µm, with the original magnification of 400x used for the image acquisition.

4.3.7 Inflammatory cytokines

4.3.7.1 IL-1β content

No main effect of time or treatment on IL-1β content was evident (Figure 4.12). However, there was a main treatment-time effect ($p < 0.05$), with the PCO group displaying a higher IL-1β content on day 1 compared to the PLA group.

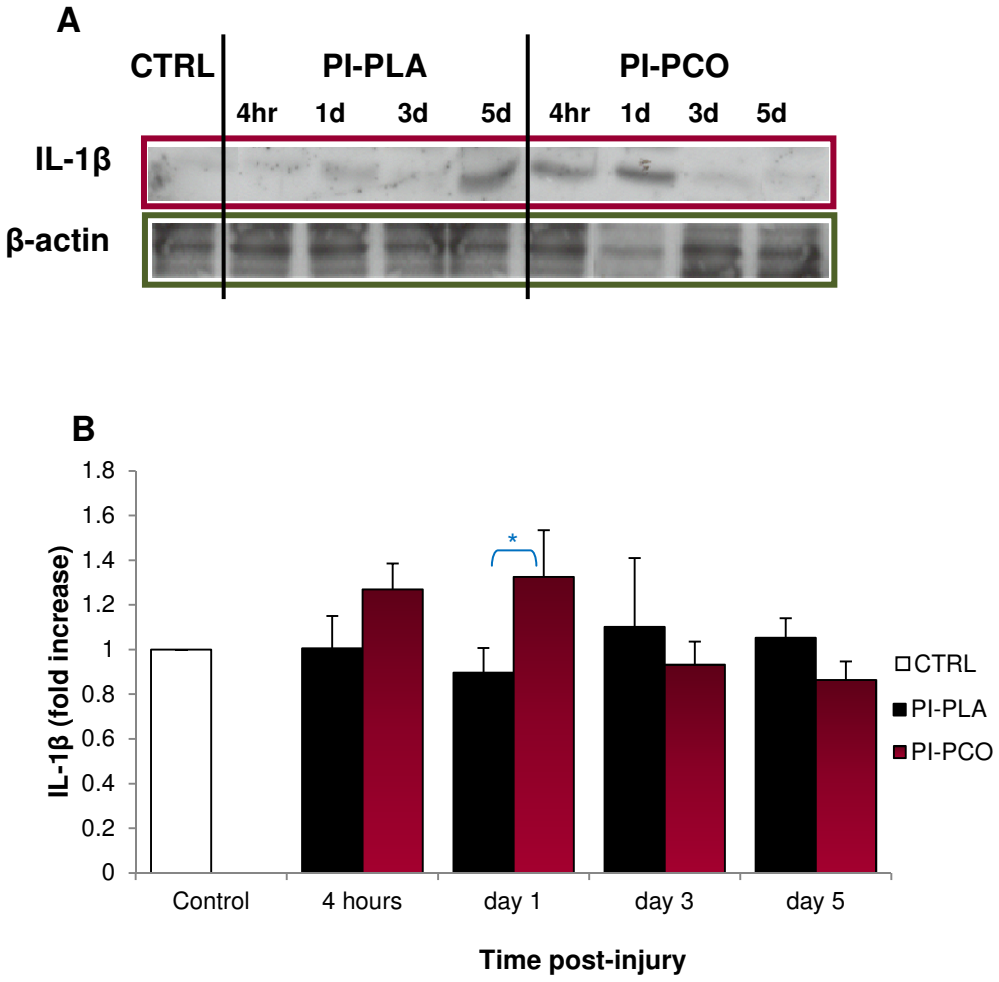


Figure 4.12: IL-1β expression in skeletal muscle following a contusion injury. (**A**) represents a western blot probed for IL-1β and β-actin and (**B**) represents a bar graph illustrating the date observed in A. Values are expressed relative to the control values (mean ± SEM). Statistics: Factorial analysis of variance with Fischer's *post-hoc* test, n = 3 per time point per group (* $p < 0.05$).

4.3.7.2 IL-6 content

A main effect of treatment and time ($p < 0.01$ for both) were evident when analysing IL-6 content. Although IL-6 content in the PLA group was similar at all time points assessed, a gradual, but significant decrease in IL-6 content was visible in the PCO group over time (Figure 4.13). Significant differences were also evident between the two groups, with the PCO group having less IL-6 at both time points (day 3 and 5).

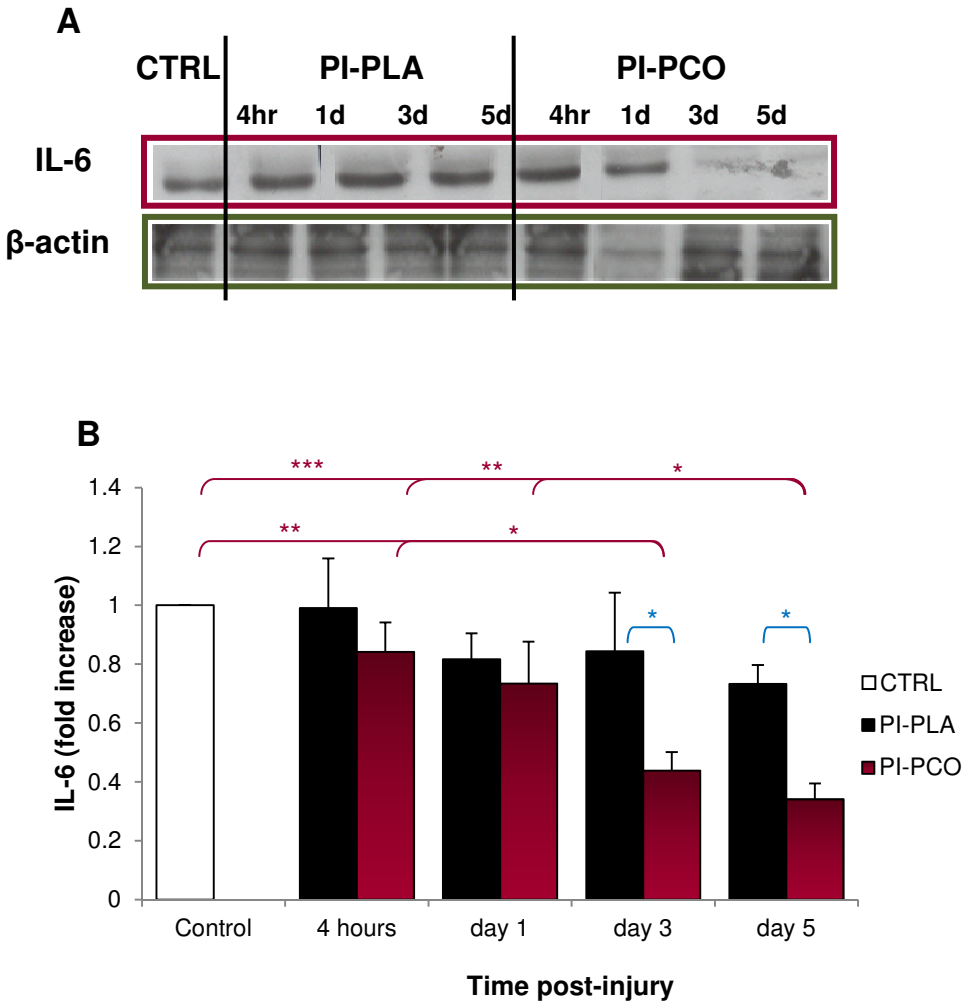


Figure 4.13: IL-6 content in skeletal muscle following a contusion injury. (**A**) represents a western blot probed for IL-6 and β-actin and (**B**) represents a bar graph illustrating the date observed in A. Values are expressed relative to the control values (mean ± SEM). Statistics: Factorial analysis of variance with Fischer's *post-hoc* test, n = 3 per time point per group (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.7.3 TNF-α content

No main effects were evident when TNF-α was compared between groups or over time. However, there was a trend towards a main effect of time ($p = 0.07$). No significant differences were evident in the PLA group over time (Figure 4.14). However, the PCO group also displayed a gradual decrease in TNF-α (from 4 hr to day 5), similar to that observed with IL-6.

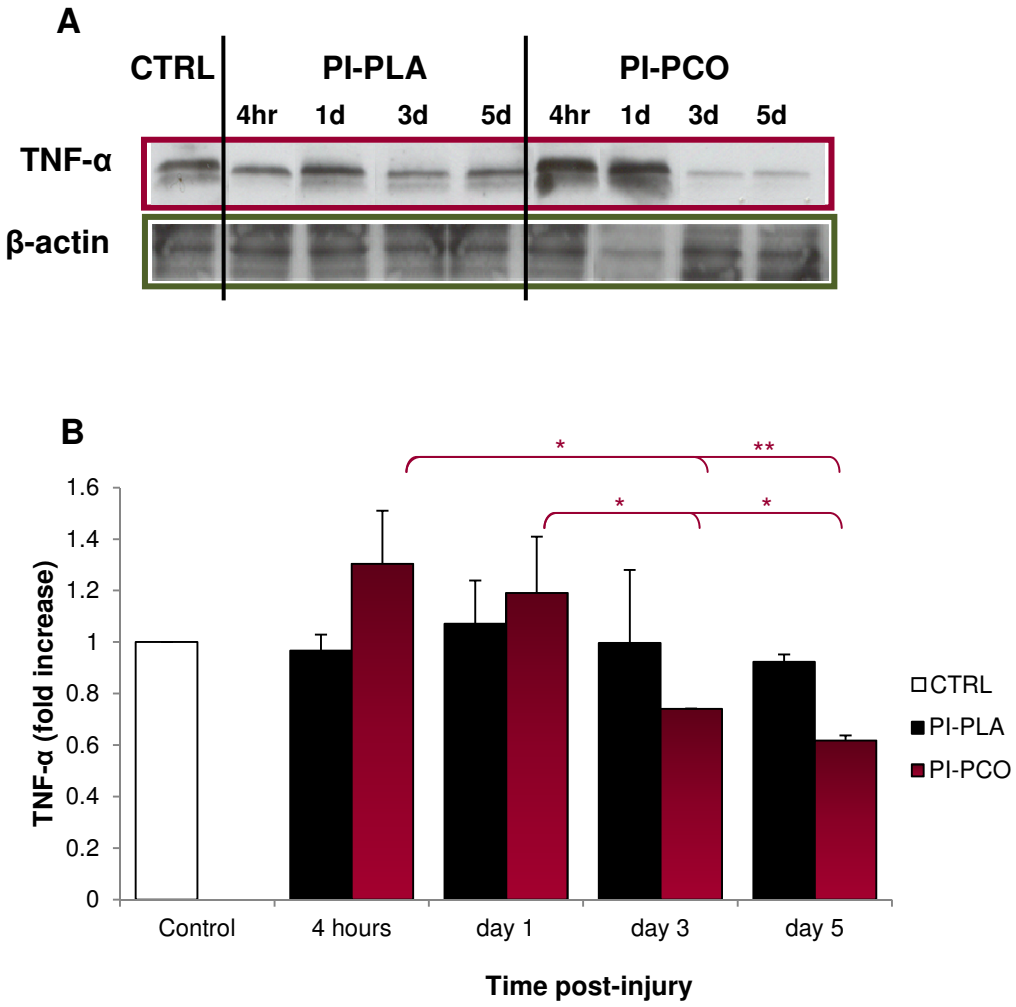


Figure 4.14: TNF-α expression in skeletal muscle after contusion injury. (**A**) represents a western blot probed for TNF-α and β-actin and (**B**) represents a bar graph illustrating the date observed in A. Values are expressed relative to the control levels (mean ± SEM). Statistics: Factorial analysis of variance with Fischer's *post-hoc* test, n = 3 per time point per group (* $p < 0.05$; ** $p < 0.01$).

4.3.8 Pax-7$^+$ cell count

Repeated measures analysis of variance indicated a significant effect of time, as well as a treatment-time interaction ($p < 0.001$ for both). Similar to the results obtained with chronic supplementation, an increase in SCs/myofibre in the PLA group was evident at a later time point than that observed for the PCO group (day 3 compared to 4 hr). SC expression in the PCO group also returned to values seen in control groups earlier than that observed for the PLA group (Figure 4.15). Significant differences between the two treatment groups were also evident at 4 hr (PCO group had more SCs, thus activation and/or increased migration) and on day 3 (PLA group had more SC, indicating activation).

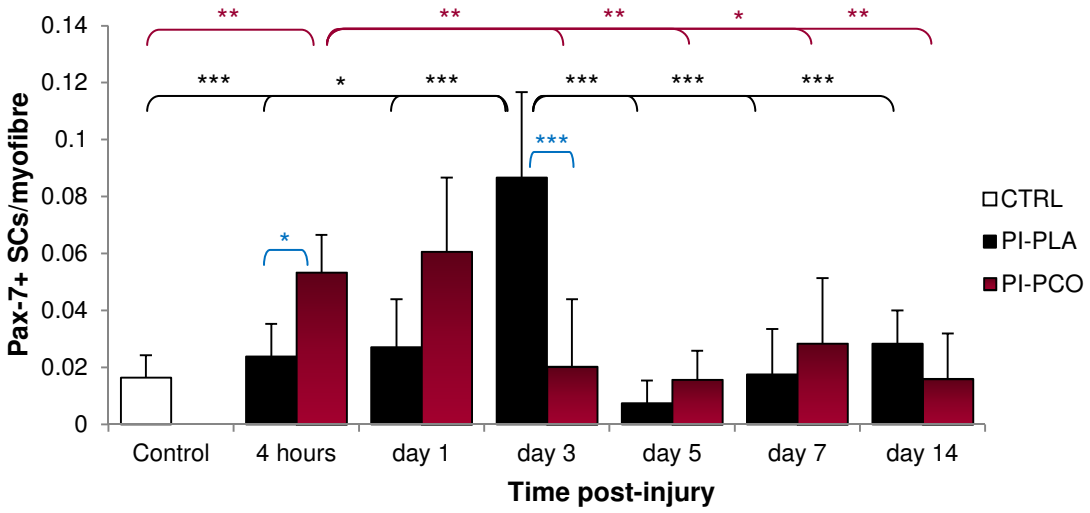


Figure 4.15: Pax-7$^+$ satellite cell (SC) count normalised for the myofibre number (SC/myofibre) (mean ± SD). Statistical analysis: Factorial analysis of variance (ANOVA), with Bonferonni *post-hoc* test demonstrating significance between the two specific data points (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). n = 4 rats per time point per group.

Due to the large standard deviations at various time points (Figure 4.15), in part due to varying degrees of fibre damage and swelling (more swelling and thus fewer intact myofibres evident in the PLA group on day 3 – figure 4.17C), the number of Pax-7$^+$ SC per area (field of view - 350 µm^2) were also taken into account. Similar to the previous results, a main effect of treatment, time and treatment-time interaction were evident ($p < 0.05$ for all). In Figure 4.16,

it is apparent that the number of Pax-7^{+} SCs in the PLA group is unchanged at all time points following injury. The PCO group on the other hand showed a significant increase in Pax-7 4 hr after injury, with normalisation by day 3 after injury. *Post hoc* tests indicated that PCO Pax-7 was significantly higher than PLA 4 hr after injury. Figure 4.17 illustrates Pax-7 expression at 4 hr and on day 3 after injury in representative sections. Take note that at both the 4 hr and day 3 time point, the PLA group had relatively fewer myofibres per field of view, as these fibres were more swollen compared to the fibres in the PCO group.

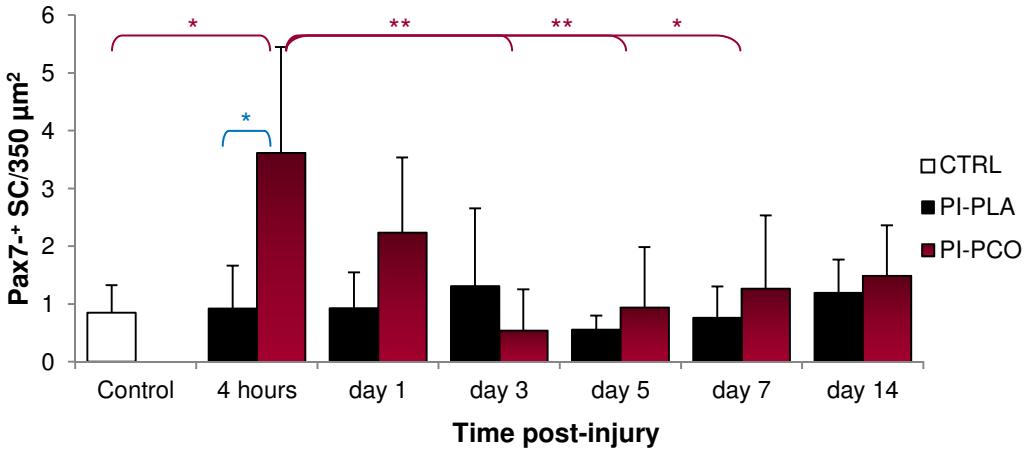


Figure 4.16: Pax-7^{+} satellite cell (SC numbers per field of view (mean ± SD). Statistical analysis: Factorial analysis of variance (ANOVA), with Bonferonni *post-hoc* test demonstrating significance between the two specific data points (*$p < 0.05$, ** $p < 0.01$). n = 4 rats per time point per group.

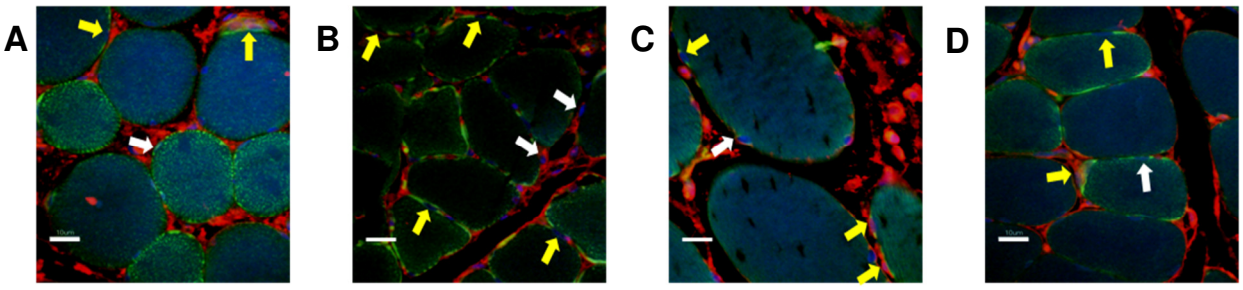


Figure 4.17: Pax-7 staining in the PLA and PCO group at 4 hr (**A**&**B**) and day 3 (**C**&**D**) respectively. FITC (green) was used to visualise Pax-7, Texas Red (red) was used to visualise laminin and Hoechst (blue) was used as a nuclear marker. The yellow arrows indicate true satellite cells, whereas the white arrows indicate non-satellite cells. Scale bar represents 10 µm.

4.3.9 Foetal myosin heavy chain (MHC_f) expression

ANOVA indicated a main effect of time ($p < 0.05$) for MHC_f content. After injury, the PLA group displayed no difference, whereas MHC_f expression in the PCO group was significantly elevated on day 5 after injury (Figure 4.18B). Figure 4.18A indicates a representative western blot probed for MHC_f and β-actin respectively.

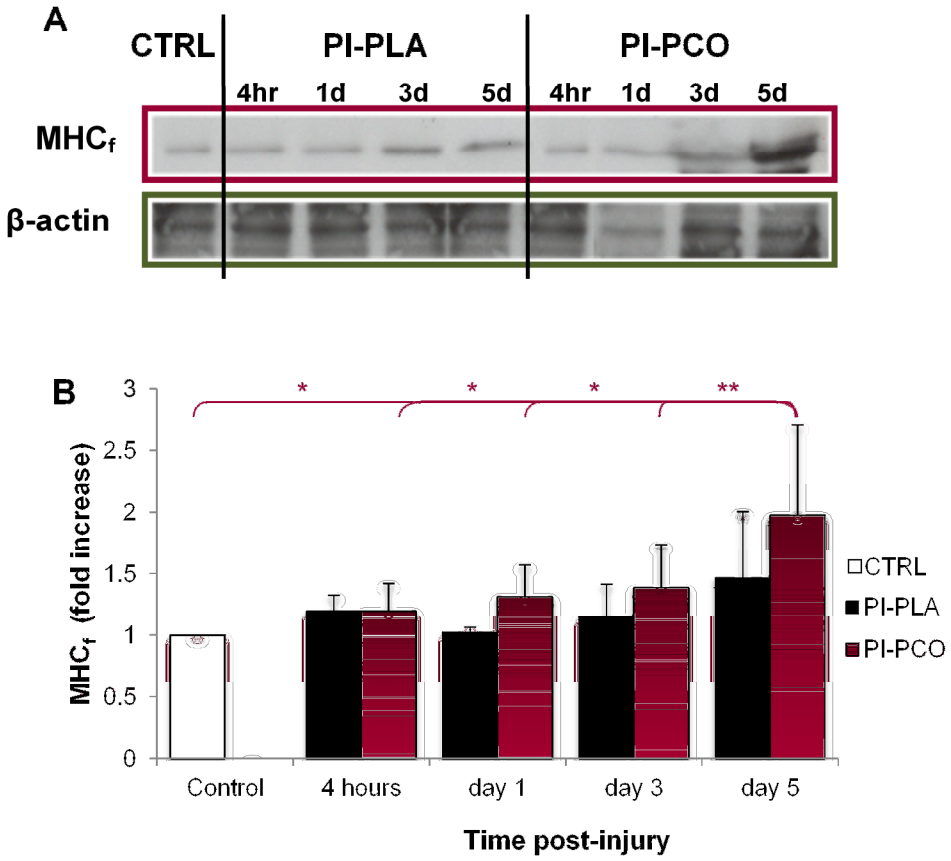


Figure 4.18: MHC_f expression in skeletal muscle after contusion injury. (**A**) represents a western blot probed with MHC_f and β-actin, and (**B**) represents a bar graph illustrating the data seen in A. Values are expressed relative to the control values (mean ± SEM). Statistics: Factorial analysis of variance with Fischer's *post-hoc* test, n = 3 per time point per group (* $p < 0.05$, ** $p < 0.01$).

4.4 Discussion

The main findings of this study were that acute PCO supplementation blunted the pro-inflammatory response and possibly promoted an earlier anti-inflammatory response. The evidence for the former was three-fold: faster decline in TNF-α and IL-6 levels and a blunted neutrophil response in both the border zone and injured area. The evidence for the latter was an earlier macrophage infiltration in the injured area. Other main findings were a decreased number of macrophages infiltrating the border zone which appeared to limit secondary damage in this area, and an earlier increase in SC count.

However, before considering results of individual parameters, it is necessary to consider the relative severity of primary damage in PCO *vs.* PLA, since differences between groups over time will impact on the interpretation of data.

4.4.1 Creatine kinase (CK) activity

CK is still used as gold standard for measuring damage (Neubauer *et al.,* 2008). In this study, CK did not change significantly over time in either PLA or PCO, except for a similar tendency for increased CK 4 hr after injury in both groups, which did not reach significance due to the increased inter-individual variability in response. However, since day 1 and day 5 time points were not investigated, the peak in CK could have been missed, which probably would have been evident on day 1 or 2. Since the responses in the two groups were similar (peaking non-significantly at 4 hr compared to all other time points), one can assume that the technique indeed produced a similar, standard injury across groups, so that observed differences between groups may be ascribed to treatment only. On a qualitative level. The histology sections at 4 hr post-injury also reveal a similar degradation pattern.

4.4.2 Antioxidant capacity

The ORAC assay measures the total amount of antioxidants present in an aqueous system, and since the PCO supplement was dissolved in 0.9 % saline, an aqueous solution, the ORAC assay was used. A decreased ORAC is indicative of a decreased ability of the total antioxidants present in plasma or muscle to scavenge free radicals, whereas a higher ORAC correlates with an increased ability to scavenge free radicals (Cao *et al.,* 1998; Mazza *et al.,* 2002). However, in a dynamic system of free radical overload, such as contusion injury, the ORAC can also reflect how many free radicals were in the area that have used the quenching ability already, so that the ORAC measured is interpreted as the remaining ability. Because of this, the interpretation of results should be cautious.

The finding in the PLA group of a significant decrease in plasma ORAC from day 1 to day 3 is possibly due to the generation of reactive species (Abramson and Weissmann 1989; Kerr *et al.,* 1996; Ali *et al.,* 1997) by predominantly circulating neutrophils (Li *et al.,* 2004), and possibly also macrophages (Knight 1999; Jackson 2000; Fialkow *et al.,* 2007). However, the conditions in the interstitial space of the injured area also influences the conditions in the plasma. This is most likely since in this study this decrease in plasma ORAC followed after an increased neutrophil invasion into the muscle, therefore corresponding with neutrophil oxidative burst in muscle or interstitial space rather than circulation. Further support for this interpretation is the decreased IL-1β content on day 1 in the PLA group compared to PCO, which suggests that the activation of NADPH oxidase and xanthine oxidase (involved in respiratory burst) is inhibited or suppressed (Abramson and Weissmann 1989; Kerr *et al.,* 1996; Ali *et al.,* 1997), whilst an increase in muscle IL-6 in the PLA group on day 3 after injury (significantly higher than PCO) suggests the stimulation of the pathways mentioned above, and thus respiratory burst. Although the respiratory burst activity of blood neutrophils was not measured, by specifically investigating MPO (expressed on activated neutrophils) activity, NADPH oxidase and xanthine oxidase, the possibility that circulating neutrophils could have contributed to the decrease in plasma ORAC on day 3 cannot be excluded.

Macrophages were also significantly elevated in the injured area on day 3 compared to control levels and might also result in the release of free radicals. However, it is not known whether the pro- or anti-inflammatory subtypes are responsible, making it difficult to draw a clear conclusion. Another potential contributor to the decreased ORAC is the satellite cell population, which are known to generate reactive species upon activation, proliferation and fusion (Scime and Rudnicki 2006). In the PLA group, Pax-7^+ SC were significantly increased on day 3. From day 3 to 5, ORAC increased significantly in the PLA group, possibly as a result of the decline in pro-inflammatory signal, as well as a decrease in neutrophils and potentially M1 macrophages. On day 5, in accordance with the literature, macrophage infiltration was at its peak. It is possible that on approximately day 5 after injury, a switch from M1 to M2 macrophages occurred, with M2 macrophages playing a predominant role in muscle regeneration and thus the decrease of free radical production (Massimino *et al.,* 1997). The fact that muscle IL-6 level was still significantly elevated in the PLA group on day 5 argues against this and thus it is more difficult to explain, given its many sources even within muscle. It may indicate a continued inflammatory signal in the early stages of a macrophage phenotype switch. On day 14, the ORAC of the PLA group was similar to that of controls.

In contrast to PLA, PCO administration prevented significant changes in plasma ORAC over time, and on day 14 significantly higher ORAC compared to PLA group was observed. This suggests that more free radicals were still being generated in the PLA group at this specific time point, or that the PCO group had a better ability to respond to free radical production, possibly as a result of an increased antioxidant enzyme activity (Gulgun *et al.,* 2010). This result corresponds to a study in humans, which reported improved ORAC after post-exercise supplementation with vitamin C (12.5 mg/kg body weight) and N-acetyl-cysteine (NAC – 10 mg/kg body weight) (Childs *et al.,* 2001).

Most studies on the effect of antioxidants investigate ORAC in plasma only, without any indication of what is actually happening in the muscle. This is one of the few studies that measured ORAC in the muscle.

Muscle ORAC results differed from plasma results, since a whole host of cells, including injured muscle fibres can all contribute to an increase or decrease in antioxidant status and thus overall ORAC status. In muscle, no difference in the ORAC in the PLA group is evident until day 7 after injury, at which time the PLA group had a significantly higher ORAC compared to control levels, which was sustained at day 14 (similar to the results seen with chronic supplementation). This suggests that the PLA group's antioxidant status has probably recovered only on day 7, with replenishment of the endogenous antioxidant pool, leaking into the interfiber muscle space (Jackson 2000). This is possible, since muscles are starting to regenerate in the PLA group as seen with H&E staining, although not to the same extent as with PCO supplementation.

In the current study, PCO, a water-soluble antioxidant was used and rats were only supplemented starting 2 hr after injury, daily. Muscle ORAC in the PCO group increased significantly on day 3 (an earlier response was probably prevented by the fact that the first supplement was only administered 2 hr after injury). In a study by Kayali *et al.* (2005), it was shown that β-Glucan, an antioxidant, worked as a scavenger and had suppressive effects on lipid peroxidation after spinal cord contusion injury. Furthermore, in a review by Teixeira *et al.* (2002), it was concluded that PCO has the ability to limit oxidative stress *via* several mechanisms, including decreased tissue degradation by inhibition of proteolytic enzymes, limiting the production of oxygen free radicals and limiting hypoxia by improving local tissue circulation. In the current study, a low number of macrophages and neutrophils were present on day 3 in both the injured area and border zone, supporting the idea of a limited free radical production by these cells. On day 5, ORAC in the PCO group was decreased again compared to day 3, possibly as a result of satellite cells fusing, and therefore increased MHC_f expression (evident in the current study on day 5), which suggests regeneration and higher metabolism. By day 14 after injury, average ORAC was at its peak, and even more increased than both control and PLA (day 14), indicating that PCO was being restored in response to the daily supplementation, rather than actively participating in any further quenching requirement. This is supported by histology (H&E – Figure 4.3), where the PCO

group displayed better structural recovery by day 14 after injury compared to the PLA group. Chronic PCO supplementation on the other hand (MSc thesis, Kruger 2007) resulted in complete muscle regeneration on day 14 after injury, suggesting that chronic rather than acute supplementation is more beneficial for a quicker recovery.

4.4.3 Satellite cells

The process of SC activation in muscle regeneration has been described in detail in many recent studies and has been used as a basic measurement of initiation of muscle regeneration (Grounds 1991; Hurme *et al.,* 1991a). Although the activation of SCs have been studied extensively in models of injury and disease, the time course of first appearance as well as their persistence differs depending on the type of injury or severity. In a study by Kuang *et al.* (2006), it was established that Pax-7 is very important in the muscle recovery process, and that if Pax-7 is absent (knocked out), it can severely inhibit muscle regeneration.

In the current study, the number of Pax-7^+ SCs per myofibre in the PLA group was relatively high on day 3 after injury compared to the control group. This suggests that satellite cells were activated on day 3 in response to injury and might have proliferated significantly thereafter (Garry *et al.,* 1997; Garry *et al.,* 2000). IL-6 (produced by activated macrophages and injured muscle fibres themselves) is one of the cytokines which has been shown to stimulate SC activation and proliferation (Merly *et al.,* 1999). In the current study, macrophages were elevated in the border zone on day 3, which may have stimulated SCs to proliferate, increasing Pax-7^+ SC numbers. However, since IL-6 was not significantly elevated on day 3 compared to day 1 in the PLA group, it could not explain the increase in Pax-7. Other potential role players in SC activation that were not assessed, included neutrophil or macrophage-derived NO and growth factors (released by injured fibres and macrophages) including hepatocyte growth factor (HGF), fibroblast growth factor (FGF) and insulin-like growth factor (IGF) (Ryten *et al.,* 2002; Pisconti *et al.,* 2006). From day 5,

however, fewer Pax-7$^+$ SCs are present in the PLA group, and numbers stayed low until day 14 after injury, suggesting that SCs are returning to quiescence or differentiating. This is a likely conclusion, since after cardiotoxin-injury in mice, activated SC numbers remained elevated up to day 5 (as seen with immunohistochemistry), whereafter they started to differentiate (Garry *et al.,* 1997; Garry *et al.,* 2000). This early decrease (day 5) in Pax-7$^+$ SCs in the PLA group is quite surprising, since chronic PLA supplementation resulted in Pax-7$^+$ SCs still being elevated on day 7, suggesting that chronic supplementation resulted in increased proliferation and thus more stem cells involved in improving the quality of recovery. Prompted by large standard deviations for this parameter in most groups, raw data were re-assessed. This revealed that the muscle fibres in the PLA group were rather swollen, resulting in few intact muscle fibres per field of view (see Figure 4.17), which may have influenced results. On the other hand, SCs are usually associated with fibres, so it remains problematic how to quantify SCs when fibre size changes so much. When plotting Pax7$^+$ SC per field of view in Figure 4.16, no significant increase in Pax-7$^+$ SCs/field of view were evident in the PLA group over time, suggesting that SC response to contusion injury – where sever fibre disruption is clearly evident – is difficult to quantify.

In contrast, Pax-7$^+$ SC number in the PCO group was significantly elevated 4 hr after injury and on day 1, suggesting significant recruitment of SCs to the injured area or possibly an increase in Pax-7 expression by existing SCs. A study by Dumont *et al.* (2008), showed that depletion of neutrophils may spare muscle from excessive membrane damage, suggesting that PCO's mechanism of action might be through limiting neutrophil infiltration, followed by reduced sarcolemma damage, thereby allowing SC to migrate to the injured area despite excessive basal lamina damage. However, this is unlikely, since PCO here was first administered at 2 hr after injury and by 4 hr, there were no neutrophils evident in the injured area. Further, these early increases did not correspond to any changes in leukocyte infiltration or cytokine levels assessed, therefore, other cells, or even the damaged muscle itself, must have been responsible for signalling the increases seen in SC activation or migration, or both. From day 3, Pax-7 numbers were similar to that of control, indicating that

these SCs are undergoing terminal differentiation to form new muscle fibres or fusing with injured fibres, whereas a subgroup returns to quiescence. MHC_f was also significantly higher on day 5 in the PCO group compared to the PLA group, making this a likely conclusion (when plotting Pax-7^+ SCs per field of view, similar results were evident in the PCO group over time than for Pax-7^+ SCs per muscle fibre). These findings of earlier SC activation and return to quiescence in the PCO group over time is similar to the findings seen when rats were supplemented chronically with PCO.

4.4.4 MHC_f

The embryonic or foetal myosin heavy chain isoform is expressed predominantly in developing skeletal muscles, but can also be detected in the adult muscle in regenerating fibres, where central nuclei are apparent (d'Albis *et al.,* 1988). When immunohistochemically staining muscle sections for MHC_f, it not only labels regenerating muscle fibres with central nuclei, but also some muscle fibres without central nuclei. Therefore in order to distinguish and accurately count regenerating fibres, one should take central nuclei into account. Since the specific time course of appearance and disappearance of MHC_f after injury and chronic supplementation is known (MSC thesis, Kruger 2007), the next study was only aimed at determining whether a similar MHC_f time course is evident with acute supplementation.

Post-injury in placebo: No significant difference was seen in MHC_f content in the PLA group over time (4 hr to day 5), suggesting that no significant muscle differentiation had taken place yet. This finding of no change in MHC_f was also similar to no change in Pax-7^+ SCs per muscle area (Figure 4.16) corroborating the abovementioned statement that regeneration has not occurred yet significantly. By day 14 it is more clearly evident (see Figure 4.3 G). In a previous study using the same contusion injury model, MHC_f expression was only evident on day 7 in the PLA group (MSc thesis, Kruger 2007). Similar qualitative histological findings of regenerating myofibres by day 6-8 were also found in PLA controls in the current study and other studies (Fisher *et al.,* 1990; Kami *et al.,* 1993; Beiner *et al.,* 1999). Since only the

acute post-injury time points were assessed, it is possible that a peak could have been detected if later time points were included in the analysis. Also, since tissue used for western blotting analysis in this study included a mixture of both injured and border zone areas, it is possible that effects in the injured area could have been different from effects of the regenerating fibres in the border zone area, which most probably have not regenerated yet.

Post-injury in PCO: In the PCO group on the other hand, MHC_f was elevated on day 5 after injury (compared to PLA), suggesting that acute PCO resulted in earlier regeneration of muscle fibres. From previous studies regarding chronic PCO supplementation (MSc thesis, Kruger 2007), it was evident that MHC_f was present already on day 3 after injury, indicating that acute supplementation had a response that occurred slightly later in the recovery process.

4.4.5 Immune cells

In earlier studies, inflammatory cell infiltration in the injured muscle (after stretch-injury) was assessed using general H&E staining (Best *et al.,* 1999) and the biochemical assay, muscle myeloperoxidase (MPO) activity (Brickson *et al.,* 2001). These studies suggested that neutrophils infiltrate the injured area 24 hr post-injury and that this is associated with indicators of fibre tearing and oxidative stress (Best *et al.,* 1999; Brickson *et al.,* 2001). Results from the studies in this thesis regarding neutrophils are in agreement with these studies. Neutrophil numbers in the PLA group after contusion injury were significantly elevated on day 1 in both the injured area and border zone, followed by a significant decrease from day 3 onward, similar to the results obtained with chronic PLA supplementation. However, the peak increase in the injured area was 25-fold and in the border zone 4-fold, compared to a 30-fold increase and 10-fold of the injured area and border zone respectively of chronically supplemented rats. One possibility is that acute supplementation reduced neutrophil infiltration. The decrease in neutrophil numbers by day

3 could indicate that neutrophils have undergone respiratory burst and that free radicals were already released into the injured area (Best *et al.,* 1999; Brickson *et al.,* 2001). Plasma ORAC was significantly decreased in the PLA group on day 3, indicative of a decreased ability of the antioxidants present in a sample to scavenge free radicals. This would suggest that significant scavenging ability had been utilised to quench free radicals prior to this time point, corroborating the abovementioned statement. Similar to data presented in Chapter 3 regarding chronic PCO supplementation, acute PCO was also responsible for blunting the neutrophil response, even if taken acutely. A potential mechanism whereby PCO blunts the neutrophil response may be reduction of the adhesion molecule expression on circulating immune cells. In one particularly relevant study, a grape seed-derived PCO was indeed able to reduce the disease-associated increase in circulating soluble adhesion molecules, intercellular adhesion molecule (ICAM) and vascular adhesion molecule (VCAM), endothelial (E)-selectin and platelet (P)-selectin, thereby reducing the number of neutrophils migrating to muscle (Kalin *et al.,* 2002).

However, neutrophils may not be solely responsible for inducing oxidative stress in injured skeletal muscle. Other non-immune cells are also responsible for increases in MPO activity and not all neutrophils express MPO (Derby-Dupont *et al.,* 1999). These neutrophils are normal and even have a normal or increased metabolic burst by means of other mechanisms, such as the enzyme NADPH oxidase (Best *et al.,* 1999; Brickson *et al.,* 2001). Macrophages should also be considered as sources of increased oxidative stress (Ysebaert *et al.,* 2000; Sugiyama *et al.,* 2001). This is a likely possibility, since macrophages were detected already 48 hr after a single stretch injury (Almekinders and Gilbert 1986; Nikolaou *et al.,* 1987), a point in time when MPO activity was significantly increased (Brickson *et al.,* 2001). However, other studies suggested that activated adult macrophages lack MPO (Yamada and Kurahashi 1984; Koeffler *et al.,* 1985; Tobler *et al.,* 1988; Ulvestad *et al.,* 1994), and also have other ways of undergoing respiratory burst. Thus, although MPO is high when macrophages are at their peak number, as in the study by Brickson *et al.* (2001), it does not necessarily imply that MPO is synthesized by macrophages. Rather, MPO is more

associated with the time of neutrophil respiratory burst, which occurs after day 1, and it is possible that it remains elevated for a few days. This interpretation would require a sampling time at 2 days as well.

Macrophages in the PLA group significantly increased on day 3 after injury in both the border zone and injured area. In the border zone however, by day 5 after injury, there was an even more significant increase in the PLA group. On the other hand, a low number of macrophages were present in the PCO group in the border zone. Differences between treatment groups were evident on day 3 and day 5, with the PCO group exhibiting a lower number of macrophages in the border zone. This could indicate that there is less secondary damage in the border zone in the PCO group compared to the PLA group. In the injured area however, there are more macrophages in the PCO group compared to the PLA group at the 4 hr and day 5 time points. Two different types of macrophages exist, M1 macrophages are involved in further promoting muscle damage, and infiltrate the injured area early after injury, whereas M2 macrophages promote muscle repair and infiltrate the injured area somewhat later (Frenette and Tidball 2000; Frenette *et al.,* 2002; Tsivitse *et al.,* 2003). Therefore it is possible that 4 hr after injury, M1 macrophages were present to enhance the pro-inflammatory phase, whereas on day 5, M2 macrophages might be present to enhance resolution of inflammation. A more detailed analysis of macrophages is required to elucidate this.

4.4.6 Pro-inflammatory cytokines

Post-injury in placebo: According to Ostrowski *et al.* (1999), and Xing *et al.* (1998), the magnitude and duration of an inflammatory response may be restricted, due to the balance that exists between the pro-inflammatory cytokines, such as IL-6, and their inhibitors (including, but not exclusively the anti-inflammatory cytokines). IL-1β, IL-6 and TNF-α are released in response to injury, as a result of the rupturing of the muscle fibres and the release of histamine from mast cells and platelets exposed after injury (Rosenberg and Gallin

1993; Cannon and St Pierre 1998; Tidball and Wehling-Henricks 2005). One might therefore expect increased levels of all three cytokines after a contusion injury. Even though this injury is similar to the injury produced in the experiment described in Chapter 3, different cytokine patterns were observed in the PLA group in the previous study *vs.* this one. With chronic PLA treatment, TNF-α and IL-6 peaked on day 3 and day 1 in the injured area and border zone respectively. However, no changes in IL-6 and TNF-α levels were evident with acute PLA treatment. Similarly, IL-1β also showed no change over time compared to the control group. In this particular study, western blotting was used to determine the levels of cytokines, whereas in the previous chapter, immunohistochemistry was used to identify cytokines in both the injured area and border zone area. The rationale behind using western blotting was to determine in a more time-efficient manner whether the cytokine profile will be affected to the same extent following acute supplementation. A confounding factor in using western blotting is that it is not known whether the muscle sample used contains only injured area or possibly some intact or border zone areas. Therefore, no response does not necessarily indicate no cytokine response at all – it might only indicate that the combination of either the injured or border zone areas might have masked the real effect. The possibility of such technical masking was less apparent in the PCO group.

Post-injury in PCO: IL-1β was significantly higher than PLA in the PCO group on day 1 after injury, however, both treatment groups were not significantly different from control. This might indicate an earlier or more potent inflammatory response in the PCO group. Neutrophil numbers in the PCO group were low throughout. On day 1, macrophage number was also not elevated and could therefore not have contributed to the increase in IL-1β on day 1. However, 4 hr after injury, macrophage numbers were significantly higher in the injured area and might have contributed to an increase in IL-1β on day 1 if this effect is delayed. Fibroblasts express IL-1β and are also present early in the injured area (Jarvinen *et al.,* 2005). Fibroblasts rather than macrophages and neutrophils may therefore be responsible for the increase on IL-1β on day 1. Regardless of the origin of the increased IL-1β, it is known that IL-1β facilitates increased expression of macrophage colony stimulating factor

(M-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) by endothelial cells, which both play a predominant role in attracting more neutrophils and/or macrophages to the injured area (Zsebom *et al.,* 1988; Allen and Boxhorn 1989). We did observe increased macrophage number on day 3 and a more significant increase on day 5 in the injured area, although not the border zone.

The other inflammatory cytokines (IL-6 and TNF-α) measured, significantly decreased earlier (day 3) over time compared to the PLA group, in which cytokine concentrations remained constant, indicating an active anti-inflammatory response in PCO. Further studies are needed to determine if this effect of PCO supplementation is directly/indirectly blunting the inflammatory process.

4.5 Conclusion

The results obtained in this study indicate that, similar to chronic preventative supplementation, acute post-injury PCO supplementation also influenced muscle fibre regeneration, as seen through MHC_f, which was elevated on day 5. It is also apparent that acute PCO supplementation resulted in a better ability to maintain oxygen radical scavenging capacity after injury compared to PLA. Contusion injury resulted in an inflammatory response, but PCO supplementation altered the inflammatory response, through limiting neutrophil infiltration into the injured area and allowing an earlier macrophage response. A decrease in pro-inflammatory cytokines within 3 days supports this anti-inflammatory action.

4.6 Limitations

In this study, all cytokine and immune cell data only represent time points up to day 5. Since previous results in the chronic supplementation study showed all differences in responses prior to day 5 after injury, it was not considered necessary to investigate time points further than day 5. In retrospect, it would potentially have been better to be able to determine

whether resolution of inflammation did actually occur earlier in PCO, by addition of one or two later time points. A major limitation was not typing macrophages in order to better make sense of results obtained. Since it is known that the ORAC only measures the water-soluble portion, another potential limitation is that the lipophilic component of plasma were not assessed in addition to the water-soluble portion. However, a study by Wu *et al.* (2004) found that the antioxidant capacity in the lipophilic content after proanthocyanidin supplementation (in berries and fruits) is negligible. Since the manner in which satellite cell numbers were expressed changed the outcome of the results in the PLA group, determining satellite cell numbers in the interstitium between fibres, in addition to the numbers at the fiber surface, could also illuminate whether satellite cell migration or proliferation were primarily affected in the treatment groups over time.

CHAPTER 5

Effect of chronic oligomeric proanthocyanidin supplementation on *in vivo* macrophage subpopulation distribution and *in vitro* neutrophil migration following contusion injury

5.1 Introduction

Results from Chapter 3 and 4 indicated that both chronic and acute PCO supplementation were able to hasten the regeneration response. Chronic PCO supplementation resulted in earlier and increased satellite cell activation or migration or both, and earlier synthesis of foetal myosin heavy chain (MHC_f), indicative of muscle regeneration. Furthermore, the same treatment protocol was also able to blunt the pro-inflammatory cytokine response, both in circulation and in the injured muscle. Two of the most significant and consistent findings at muscle level, was that both acute and chronic PCO not only blunted the neutrophil response substantially, but also resulted in a shift to the left in time course for macrophage infiltration and resolution.

Various plant-derived antioxidants have been shown to exert anti-inflammatory effects. Vitamin E and C are considered two of the most important dietary antioxidants. Previous studies have shown that short-term supplementation with high doses of vitamin E (600-1200 I.U./day) resulted in decreased production of pro-inflammatory cytokines, tumour necrosis factor (TNF)-α (Mol *et al.,* 1997; Devaraj and Jialal 2000b; van Tits *et al.,* 2000), interleukin (IL)-1β (Devaraj *et al.,* 1996; Devaraj and Jialal 2000b; van Tits *et al.,* 2000) and IL-6 (Devaraj and Jialal 2000a) by blood monocytes *in vitro*. However, long-term supplementation (3 years) with a combination of α-tocopherol (vitamin E; 91 mg or 136 I.U./day) and vitamin C (250 mg) had no detectable anti-inflammatory effect in healthy men (Bruunsgaard *et al.,* 2003), most probably due to the low concentration of vitamin E compared to that used in the other studies mentioned above. Resveratrol is one of the most

researched grape-seed derived antioxidants. In a recent review, all its anti-inflammatory mechanisms were summarized in detail, in terms of model used, mechanism and effective concentration (de la Lastra and Villegas 2005). The anti-inflammatory mechanisms of resveratrol include (a) its ability to inhibit the production of pro-inflammatory mediators, IL-8 and IL-6 (Donnelly *et al.,* 2004), (b) alter eicosanoid synthesis, and (c) inhibit the action of activated immune cells, primarily neutrophils and macrophages. More specifically resveratrol supplementation resulted in the inhibition of neutrophil adhesion to stimulated human umbilical vein endothelial cells (HUVEC) by directly inhibiting the expression of the adhesion molecules, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Ferrero *et al.,* 1998; Bertelli *et al.,* 2001). The effect of resveratrol on eicosanoid production was demonstrated in a rat model of carrageenan-induced paw oedema (Jang *et al.,* 1997). In this study, resveratrol inhibited the synthesis of prostaglandin (PG) *via* the direct inhibition of the cyclyooxygenase (COX) activity of COX-1, but not COX-2. In contrast, another study investigating resveratrol activity in human mammary epithelial cells suggested quite the opposite, that PG expression *via* COX-2 specifically was affected (Subbaramaiah *et al.,* 1998), which clearly demonstrated that depending on the model of inflammation, and the cell type involved, the arachidonic acid pathway will be influenced differently either through COX-1 or COX-2. Collectively, these studies indicate that plant-derived antioxidants have anti-inflammatory properties, some of them similar to that seen in our previous studies.

Since it has been shown that other plant-derived antioxidants were able to exhibit anti-inflammatory properties, similar to those found in previous studies in this thesis (Chapter 3 and 4), the ability of PCO to inhibit/blunt neutrophil recruitment to injured tissue may be due to PCO's effect on (a) reducing the number of circulating neutrophils, (b) limiting the number of activated neutrophils, or (c) interfering with mechanisms for neutrophil migration into the injured tissue. The latter include the inhibition of neutrophil chemotaxis, possibly due to (a) the decrease in pro-inflammatory cytokine expression (seen in this thesis in Chapter 3), (b) alterations in expression of other chemotactic factors, or (c) reduced expression of adhesion molecules on neutrophils.

Neutrophil adhesion to and migration through the vascular endothelium is mediated by membrane adhesion molecules (β2 integrins) expressed on neutrophils, which include leukocyte function antigen-1 (LFA-1/CD11a), Mac-1 (CD11b/CD18), and very-late antigen 4 (VLA-4) (Smith *et al.,* 1989; Furie *et al.,* 1991) and their respective vascular endothelial ligands, endothelial (E)-selectin, platelet (P)-selectin, ICAM-1, VCAM-1, and connective tissue components such as fibronectin (Albelda *et al.,* 1994; Carlos and Harlan 1994). Neutrophil migration into the injured area is further mediated by chemical mediators released from the injured area itself (Sherwood *et al.,* 2004; Tidball and Wehling-Henricks 2005), which include pro-inflammatory prostaglandins (PGE_2 and $PGF_{2\alpha}$) and leukotrienes (LTB_4) (Shen *et al.,* 2006), as well as factors released by activated platelets (thromboxane (Tx)A_2, serotonin and histamine) (Marder *et al.,* 1985). Chemoattractants (cytokines and CXC and CC chemokines) released from the injured area as well as from immune cells also play a very important role (Nagaraju *et al.,* 1998; De Rossi *et al.,* 2000; Reyes-Reyna and Krolick 2000; Alvarez *et al.,* 2002). In *in vitro* studies by Cassatella (1999) and Wolach *et al.* (2000), it has been demonstrated that neutrophil migration was either directly or indirectly influenced by granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1, IL-6, IL-8, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and TNF-α after mechanical loading of skeletal muscle, due to their effect on the inflammatory response. Granulocyte colony-stimulating factor (G-CSF) also enhances neutrophil motility and chemotaxis, thereby increasing the ability of neutrophils to migrate across the vascular endothelium (Dale *et al.,* 1995; Nelson and Bagby 1996). Pro-inflammatory cytokines, including IL-1β, TNF-α, and IFN-γ, have the ability to increase ICAM-1 expression on different cell types (Dustin *et al.,* 1986; Pober *et al.,* 1986a; Pober *et al.,* 1986b), and TNF-α specifically, have been shown to be responsible for upregulating the expression of some of the β2 integrins (CD11b/CD18) (von Andrian *et al.,* 1992; Condliffe *et al.,* 1996; Yoshida *et al.,* 1997). Controversy exists regarding the expression of the different adhesion molecules in different types of tissue injury, since CD11b/CD18 or expression of ICAM-1 may or may not be responsible for neutrophil migration out of circulation (Doerschuk *et al.,* 1990; Bullard *et al.,* 1995). Since the previous studies in this thesis were able to indicate that PCO was

able to blunt the neutrophil response at tissue level, the first step would be to prove an effect on migration *in vitro*, followed by a second step, which would be to determine the exact molecule(s) involved in neutrophil migration.

Turning attention to macrophages, those found in injured tissue have been linked to both inflammation and repair (Leibovich and Ross 1975; Tsunawaki *et al.,* 1988; Diez-Roux and Lang 1997; Kodelja and al 1997; Zhang-Hoover *et al.,* 2000; Danenberg and al 2002). Following neutrophil invasion, macrophages of a M1 phenotype (classically activated, ED1, phagocytic) enter tissue, reaching significantly elevated concentrations at about 24 hours post-injury and continuing to increase in numbers until about 2 days post-injury, whereafter their numbers begin to decline rapidly (St. Pierre and Tidball 1994; Tidball *et al.,* 1999; Frenette and Tidball 2000; Selva-O'Callaghan *et al.,* 2006; Contreras-Shannon *et al.,* 2007). Although the specific timing of the M1 phenotype seems to remain similar irrespective of injury type, limited studies are available on the onset of M2 (alternatively activated) macrophage infiltration. Only two models of injury thus far have reported on the presence of these different macrophage phenotypes, which include downhill running (Tsivitse *et al.,* 2003) and hindlimb unloading (Tidball *et al.,* 1999; Frenette and Tidball 2000; Frenette *et al.,* 2002). In these models it was shown that ED2 macrophages infiltrated the injured area on day 2 or 3. Further proof of macrophages of a M2 phenotype were evident in a study by Tidball and Wehling-Henricks, (2007), where it was shown that depleting injured muscle of macrophages on day 2-4 during hindlimb reloading, resulted in an inability to regenerate, suggesting that macrophages at this time point are required for muscle regeneration.

Considering the positive effects of PCO (at tissue level) to hasten the macrophage response, secondary to a blunted neutrophil response, we hypothesized that chronic PCO supplementation will result in an altered monocyte phenotype expression during resolution of inflammation. PCO may also be directly involved in limiting the ability of neutrophils to migrate to the injured area at circulatory level. In order to investigate these hypotheses, the number of macrophages (M1 – $CD68^+$ and M2c – $CD163^+$) and neutrophils in circulation

were quantified. The possible mechanisms whereby PCO blunted the neutrophil response were also investigated, by determining changes in *in vitro* neutrophil migration, in the presence of conditioned plasma collected at different time points from PLA and PCO-supplemented rats subjected to contusion injury.

5.2 Method

5.2.1 Animals and interventions

5.2.1.1 Experimental animals

Fifty five male Wistar rats were used in this study. All rats were housed in groups of 5 in standard rat cages and fed rat chow and tap water *ad libitum*. All conditions inside the animal house were kept exactly the same as those mentioned in the two previous chapters (Chapter 3 and 4). Fifty animals were used for *in vivo* compartment of the study, while 5 extra control animals (non-injured and non-supplemented) were used for preparation of primary neutrophil cultures used in the *in vitro* study.

Preceding the onset of the supplementation protocol and contusion injury, experimental rats were randomly divided into 4 groups (see Figure 5.1). These groups consisted of a control placebo (C-PLA; n=5), a control proanthocyanidin (C-PCO; n=5), a chronic injury placebo (I-PLA; n=20, i.e. 5 rats per time point which include 12 hr, 1 d, 3 d, and 5 d post-injury) and a chronic injury proanthocyanidin (I-PCO; n=20, i.e. 5 rats per time point which include 12 hr, 1 d, 3 d, and 5 d post-injury) group. All groups were treated with either placebo or proanthocyanidin similar to that of the rats used for the study in chapter 3, with the exception that the protocol here was terminated at day 5 after injury and not day 14 as in chapter 3, since this time point was deemed long enough after injury to inform on inflammatory cell behaviour.

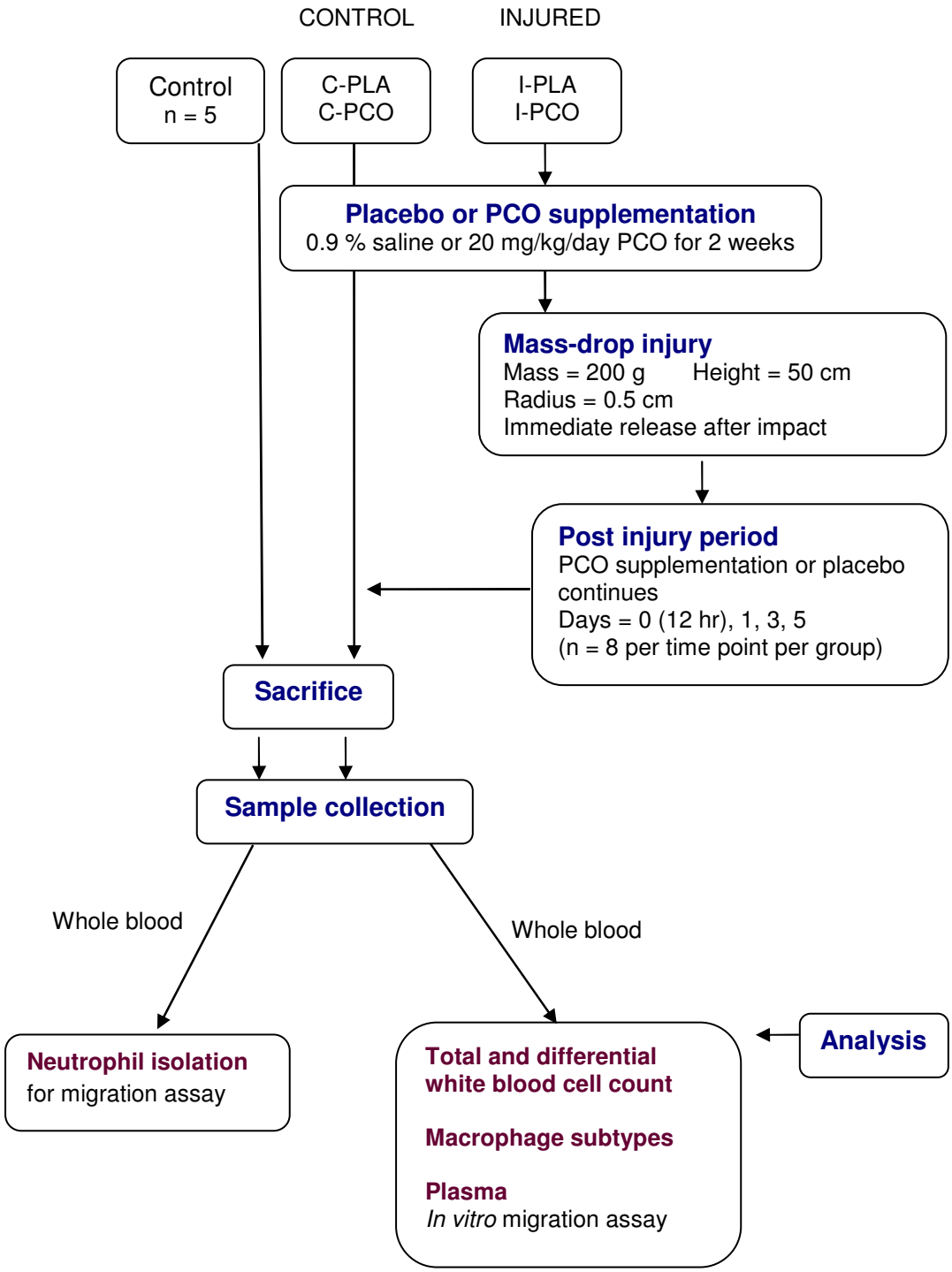


Figure 5.1: Experimental design

5.2.1.2 PCO administration

Rats, with the exception of the animals used for neutrophil isolation, were orally gavaged with either 20 mg/kg/day Oxiprovin™ (dissolved in 0.9 % saline) or 0.9 % saline (placebo) for the

duration of the experimental procedure. Control (C) rats were gavaged for 2 weeks without injury and contusion injury groups received Oxiprovin™ or placebo for 2 weeks prior to injury, as well as up to 5 days after injury.

5.2.1.3 Induction of muscle injury

Rats were anaesthetised with isofluorane (inhaled anaesthetic, Isofor, Safe Line Pharmaceutricals, South Africa) 5 minutes prior to injury (as described in chapter 3) by means of a vaporiser (Ohmeda Isotec 3 unit) and kept under general anaesthesia while injury was experimentally achieved [an anaesthetic environment was induced in rats with 4 % (v/v) isoflurane in medical oxygen (Affrox, South Africa), and anaesthetic conditions were maintained with 2 % (v/v)]. In this specific study, animals regained consciousness within 1 minute of removal of the anaesthesia. All rats were injured at 8:00 in the morning, with the exception of the 12 hr group, where rats were injured at 20:00 to allow all sacrifice time points to coincide.

5.2.2 Sacrifice and sample collection

Sacrifice procedures and sample collection in this study is similar to that already described in Chapter 3, section 3.2.1, with the exception that muscle samples were not obtained. Sacrifice took place at 12 hr, day 1, 3, and 5. Another exception was that plasma samples were aliquoted into sterile 1.5 ml eppendorff tubes and stored at -80 °C until needed for the migration assay. After plasma collection, the whole blood was reconstituted with sterile PBS.

5.2.3 Sample analysis

5.2.3.1 Total and differential white blood cell and subpopulation counts

Heparinised whole blood samples were analysed for total and differential white blood cell count using a CellDyne 3700 CS Haematology Analyzer (Abbott Diagnostics, Fullerton, CA).

Whole blood was used to determine total and differential white blood cell counts, as well as selected macrophage subpopulation at various time points after injury, whereas plasma samples were used to study neutrophil migration. Whole blood samples from the extra 5 rats were used to isolate neutrophils, which were also used in the migration study.

For flow cytometry (BD FACSAria™ Cell Sorter, BD Biosciences, San Jose, CA), monoclonal antibodies were used to identify neutrophils (FITC-conjugated anti-His48), total macrophages (Texas Red-conjugated anti-F4/80) and the two different macrophage subpopulations (FITC-conjugated anti-CD68 for M1 and APC-conjugated anti-CD163 for M2c) in samples of PCO and PLA-treated rats at different time points after injury.

Briefly, this entailed incubation of whole blood with CD32, an Fc block antibody, to block the Fc receptors on particularly monocytes and macrophage surfaces. This will limit background staining, since some antibodies may bind to specific cells *via* antibody Fc portions. Thereafter, the whole blood was incubated with the respective antibodies and red blood cells lysed before flow cytometry (see Appendix H for detailed method). A PBS control (whole blood incubated with PBS instead of antibodies) was also used to optimise the method. The neutrophil population was set as gate and M1 and M2c macrophages were calculated per 5000 neutrophils. Since 500 µl of whole blood was used for flow cytometry purposes for neutrophil or macrophage runs, and the total number of neutrophils were also quantified, the volume used to count 5000 neutrophils could be calculated. Macrophage counts were multiplied with this volume and expressed as the number of cells per µl.

5.2.3.2 Cell migration

Cell migration of neutrophils from control rats was measured using 24-well plates (BD Biosciences) with 3 µm pore size polycarbonate inserts (Falcon, BD Biosciences) (see Appendix I). Briefly, RPMI 1640 media with the chemotactic factor, *N*-formylmethionyl-leucylphenylalanine (fMLP, $1x10^{-7}$ M) were added to the bottom wells, whereas RPMI media containing 200 000 cells/well with 100 ng/ml G-CSF (stimulate neutrophils) and 20 %

conditioned plasma from either control (C-PLA or C-PCO) or supplemented rats (I-PLA or I-PCO) at different time points after injury were added to the inserts (see Figure 5.2). The inserts and bottom wells were then allowed to incubate for 15 minutes at 37 ℃ separately prior to migration, after which the inserts were placed into their respective wells using sterile tweezers and allowed to migrate for 2 hr at 37 ℃. Neutrophils still left in suspension in the top chamber (A) and migrated but non-adherent neutrophils (D) were collected into eppendorff tubes (Figure 5.2), transferred to TruCOUNT tubes (BD Biosciences) and analysed using a Flow Cytometer (see Appendix I for specifics). Cells attached to the insert filter (top – B; bottom – C) and to the bottom of the culture well (E) were stained with anti-His48 (the `neutrophil marker) and Hoechst (nuclear marker) and visualised with a fluorescent microscope (see section 5.2.4.1).

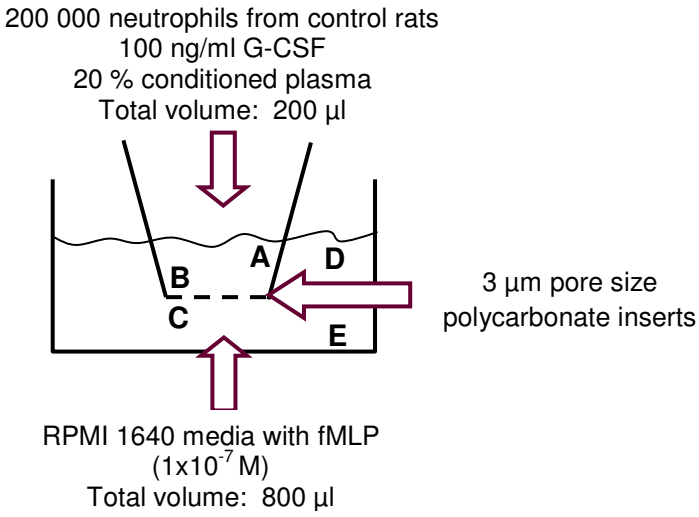


Figure 5.2: An illustration of the neutrophil migration study setup. Neutrophils, together with G-CSF and conditioned plasma were added to the top of the insert and allowed to migrate towarts fMLP in the bottom of the well. (**A**) and (**D**) represents non-adherent neutrophils still in suspension, (**B**) and (**C**) represent neutrophils adherent to the top en bottom of the insert filter respectively and (**E**) neutrophils adherent to the bottom of the culture well.

In order to identify and utilize the best possible method to isolate neutrophils prior to migration, as well as to identify and quantify non-migrating and migrating neutrophils following the migration assay, several methods were tested. The next section will focus only on the methods used.

Neutrophil isolation: Neutrophils were isolated, making use of a two-step process involving Ficoll-Histopaque-1077 (Sigma) and BD Cell lysis solution (BD), which yielded almost 95 % pure neutrophils. See Appendix I for a full detailed description of the method.

Non-migrated neutrophils in suspension: BD TruCOUNT tubes may be used for determining absolute counts of leukocytes in blood. For the purposes of this study, neutrophils (in suspension) were added directly to the TruCOUNT tubes. After the pellet was allowed to dissolve, a known number of fluorescent beads was released and used to obtain the absolute number of neutrophils (cells/µl) in the sample, compared to 1000 bead events. This method allowed for an accurate estimation of neutrophils, relative to manual counting of cells using a haemocytometer or on a slide after haematoxylin and eosin staining or staining with Rapidiff.

Migrated neutrophils: After cell migration, the media was aspirated and the insert and bottom well fixed in acetone:methanol (50:50) and airdried. Inserts and wells were then stained with His48 (1/1000) and Hoechst (1/200). This staining method resulted in clear staining of neutrophils on top of the insert and those busy migrating through the pores, as well as neutrophils adherent to the bottom wells. This method also resulted in preserving neutrophils during staining, compared to methods where cells were fixed after staining using 2 % paraformaldehyde.

5.2.4 Data collection

5.2.4.1 Immunofluorescent image collection with the Olympus Cell^{-R} system

Neutrophils adherent to the top and bottom of the insert filter, as well as to the bottom of the well, were visualised on an Olympus Cell^{-R} system attached to an IX-81 inverted fluorescence microscope equipped with a F-view-II cooled CCD camera (Soft Imaging Systems). Using a Xenon-Arc burner (Olympus Biosystems GMBH) as light source, images were excited with the 360 nm or 472 nm excitation filter for DAPI and FITC respectively. Emission was collected using a UBG triple-bandpass emission filter cube (chroma). The cells on the bottom surface of the culture well (migrated and adherent) were evenly distributed in all samples and thus only one 20x image was taken. Using this image, an area of 39.6 µm^2 of the total area of 132.67 mm^2 (insert) and 606.5 mm^2 (well) was analysed for number of neutrophils. Due to the conical shape of the insert, there was a greater density of cells in the peripheral regions and a lower, even distribution of cells towards the centre of the insert. Images were thus taken in the central region, and the left top quarter of the image analysed. Since cells were also adherent to the bottom of the insert, the microscope focus was repositioned and cells viewed and counted in the same relative regions on the bottom surface of the insert filter. Hoechst staining for nuclei was used to assist counting cells, since visibility was a bit impaired when His48 was also present.

5.2.5 Statistical analysis

Data were analysed using a Factorial ANOVA with Fisher *post hoc* tests to assess differences between subgroups. All data are presented as means ± SEM, with $p < 0.05$ regarded as statistically significant. All statistical analyses were done using the computer software Statistica version 9 (StatSoft Software).

5.3 Results

5.3.1 Total and differential white blood cell counts in peripheral blood

5.3.1.1 Neutrophils

For neutrophils, a main effect of time ($p < 0.05$) was observed in both PLA and PCO groups for circulating neutrophil numbers (Figure 5.3). Over time, both groups showed an early increase 12 hours after injury, with an early reduction on day 1 after injury in the PLA group which was not apparent in the PCO group ($p < 0.05$) which remained high. Both groups were at normal levels on day 3.

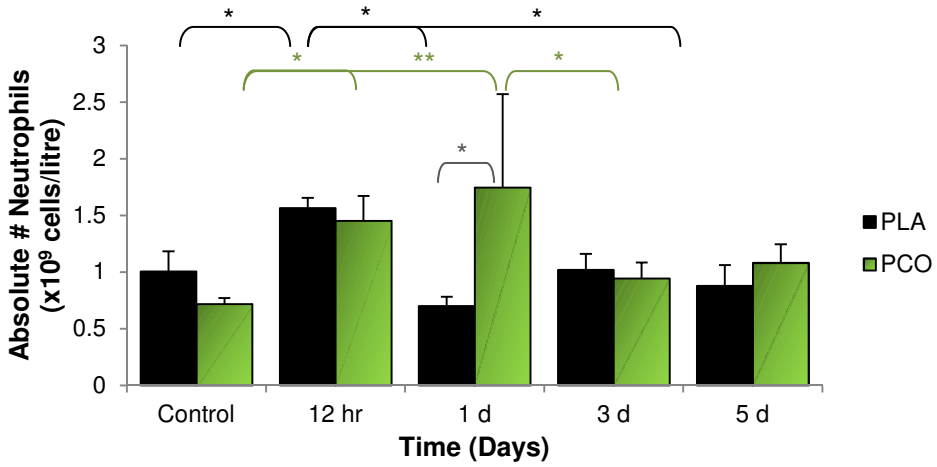


Figure 5.3: Number of neutrophils present in whole blood in control PLA and PCO rats and PLA and PCO rats with continued supplementation after contusion injury. Statistical analysis: Factorial analysis of variance with Fischer *post hoc* test (* $p<0.05$, ** $p<0.01$). n = 5 rats per time point per group. Error bars indicate SEM.

5.3.1.2 Macrophage subtypes in circulation

The scatter plot in Figure 5.4A illustrates the typical distribution of neutrophils (in the P3 zone) compared to all other white blood cells in the P1 zone. Figure 5.4B represent the typical scatter plot of macrophages co-stained with anti-CD163 (FITC-labelled M2c macrophages) and anti-CD68 (APC-labelled M1 macrophages). Cells in Q1 are positive for

CD68, whilst cells in Q4 are positive for CD163. Cells in Q3 are negative for both CD68 or CD163.

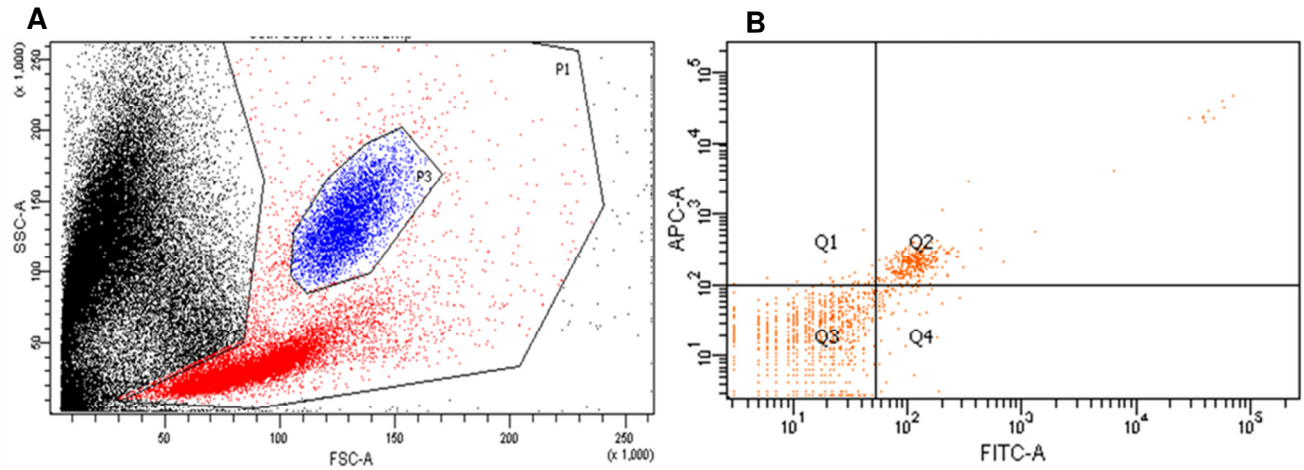


Figure 5.4: Representative image of a scatter plot of all white blood cells (**A**) and the macrophage subtypes (**B**). Neutrophil identity (P3) were confirmed with FITC-anti-His48, whereafter macrophage numbers were normalised to neutrophil counts based on forward and side scatter characteristics (A). Q1 indicate all macrophages of an M1 phenotype, whereas Q2 indicate all macrophages of an M2c phenotype.

A main effect of time ($p < 0.01$) and a treatment-time effect ($p < 0.01$) was evident for M1 macrophages. The PLA group showed a significant transient increase in M1 macrophages, peaking on day 5 after injury (Figure 5.5), whilst M1 macrophages in the PCO group peaked significantly earlier (day 3). Differences between groups occurred on day 3, with the PCO group having more M1 macrophages, and day 5, with the PLA group having more M1 macrophages.

Pre-injury the PLA and PCO groups differed significantly, due to the chronic nature of the PCO supplementation. Main effects ANOVA indicated an effect of time ($p < 0.01$) and a time-treatment effect ($p < 0.01$) for M2c macrophages. In response to injury, the PLA group displayed a significant early decrease in M2c macrophage number 12 hours after injury compared to its respective control, followed by a significant increase on day 3 (*vs.* 1d - Figure 5.6). M2c macrophage numbers in the PCO group were unchanged over time, untill a significant increase was evident on day 5 after injury (different from all other PCO time

points), a point in time when the PCO group had significantly more M2c macrophages compared to the PLA group. Overall, the M2c macrophages were lower and had a lower response than the M1 macrophages.

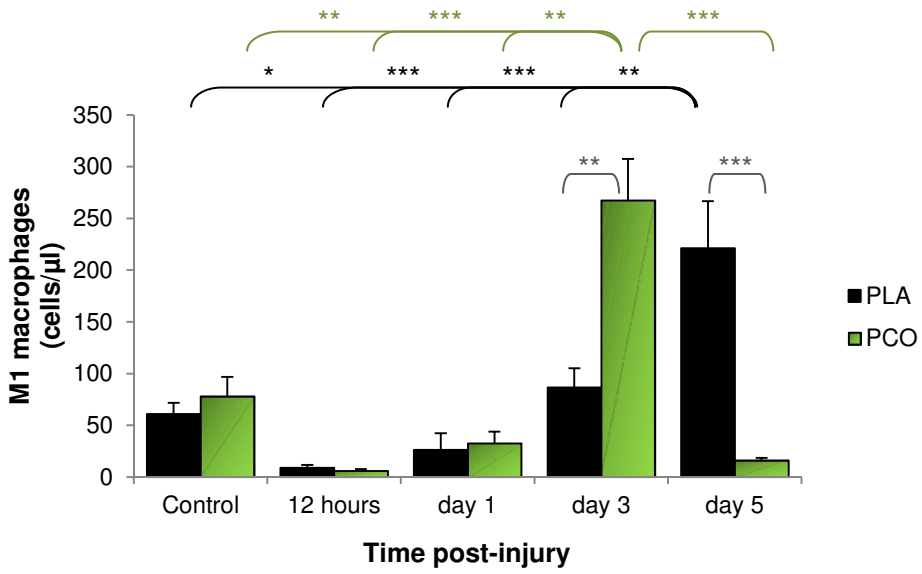


Figure 5.5: Number of M1 macrophages present in 1 µl of whole blood samples of control and injury rats expressed per number of neutrophils. Statistical analysis: Factorial analysis of variance with Fischer *post hoc* test (* p<0.05, ** p<0.01, *** p < 0.001). n = 5 rats per time point per group. Error bars indicate SEM.

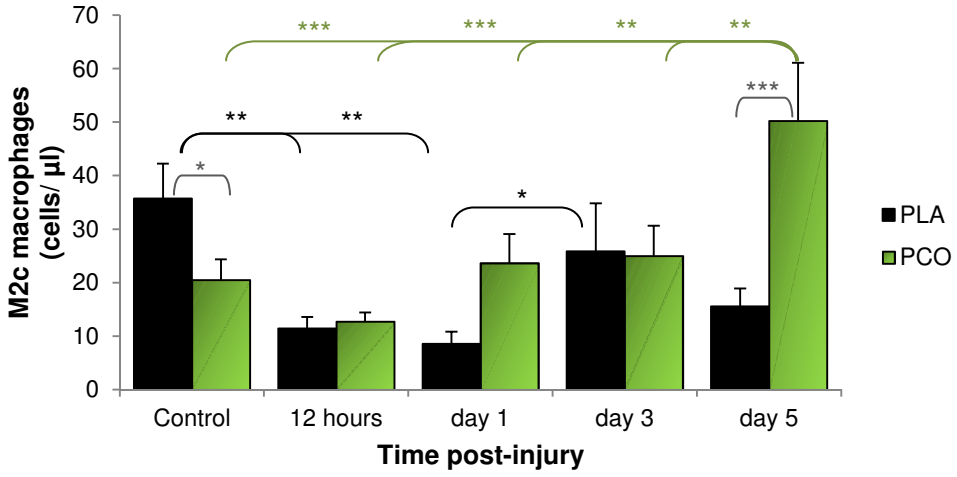


Figure 5.6: Number of M2c macrophages present in blood samples of control and injury rats expressed per 5000 neutrophils. Statistical analysis: Factorial analysis of variance with Fischer *post hoc* test (* p<0.05, ** p<0.01, *** p < 0.001). n = 5 rats per time point per group. Error bars indicate SEM.

5.3.2 Neutrophil migration *in vitro*

5.3.2.1 Fractions probably not indicative of migratory ability

No effect of time or treatment or a treatment-time effect was evident when neutrophils in solution in the top (A) and bottom wells (D) were analysed. Neutrophils from these fractions probably do not represent significant phases of neutrophil migration, or that the timing of migration (2 hr) may have been too short for cells to adhere. Table 5.1 represent the total number of cells that stayed in suspension and did not contribute to neutrophil migration.

Table 5.1: Total number of non-migrated neutrophils in solution on top of the insert filter, as well as non-adherent neutrophils in the bottom well 2 hr after initiation of the migration assay.

	Top of the insert filter		**Bottom well**	
Days post-injury	**PLA**	**PCO**	**PLA**	**PCO**
Day 0	39535 ± 1861	36637 ± 3032	26207 ± 1048	28116 ± 1711
12 hours	33611 ± 3439	39922 ± 2416	26233 ± 911	24703 ± 1256
Day 1	37789 ± 1688	42916 ± 2390	26809 ± 1127	25838 ± 3271
Day 3	38211 ± 1766	36104 ± 2045	26655 ± 1467	29251 ± 936
Day 5	42364 ± 1883	41134 ± 3030	25373 ± 1326	27738 ± 1597

Assessing the number of adherent neutrophils at the bottom of the well, factorial ANOVA indicated no main effects. Although not statistically significant, 25 % fewer neutrophils adhered to the bottom of the culture well as a result of plasma from PCO-supplemented rats on day 1 after injury compared to the PLA group on that specific time point (Figure 5.7).

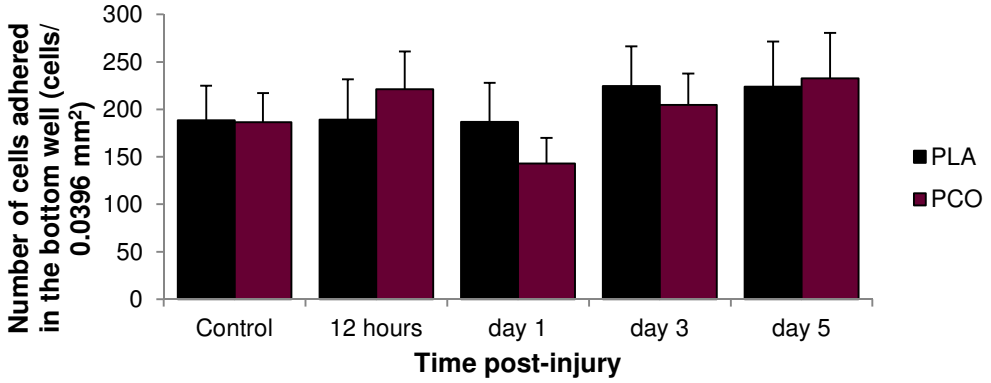


Figure 5.7: Number of migrated neutrophils adherent to bottom well after 2 hours of migration stimulated by fMLP (bottom well) and in the presence of plasma from either PLA- or PCO-supplemented rats (in the insert) harvested at various time points after injury. Statistical analysis: Factorial analysis of variance (ANOVA) with Fischer *post hoc* test indicated no statistical significance. n = 5 rats per time point per group. Error bars indicate SEM.

5.3.2.2 Migrating neutrophils

Cells migrated through and adherent to the bottom of the insert filter: Repeated measures ANOVA indicated a main effect of treatment ($p < 0.05$), time ($p < 0.001$) and a treatment-time effect ($p < 0.05$). Major responses were seen in the PLA group on day 1 after injury, a point in time where the PLA group had significantly more neutrophils in this position compared to all other PLA time points and compared to the PCO group on that day (Figure 5.8).

Cells adherent to the top of the insert filter: Both a main effect of treatment ($p < 0.01$) and a treatment-time effect ($p < 0.05$) were evident. The major difference was seen on day 1: significantly fewer neutrophils in the PLA group were present in comparison to all other time points in this group, and also when compared to day 1 PCO (Figure 5.9). Figure 5.10 illustrates representative images of a field of view of cells adhering to the top of the insert filter (A) and the bottom of the insert filter (B).

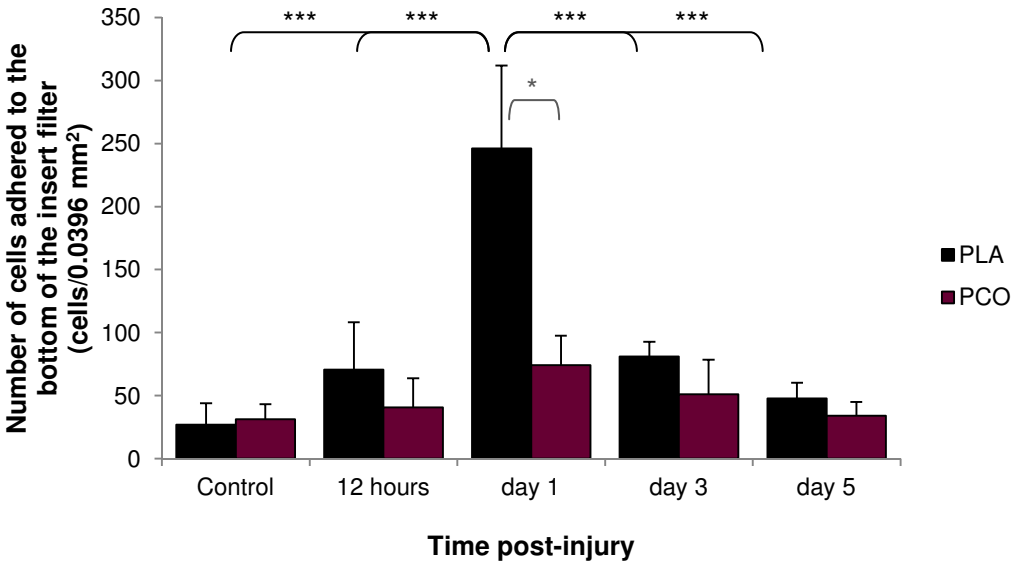


Figure 5.8: Number of migrated neutrophils adherent to bottom of the insert after 2 hours of migration stimulated by fMLP (bottom well) and in the presence of plasma from either PLA- or PCO-supplemented rats (in the insert) harvested at various time points after injury. Statistical analysis: Factorial analysis of variance with Fischer *post hoc* test (* $p<0.05$, *** $p < 0.001$). n = 5 rats per time point per group. Error bars indicate SEM.

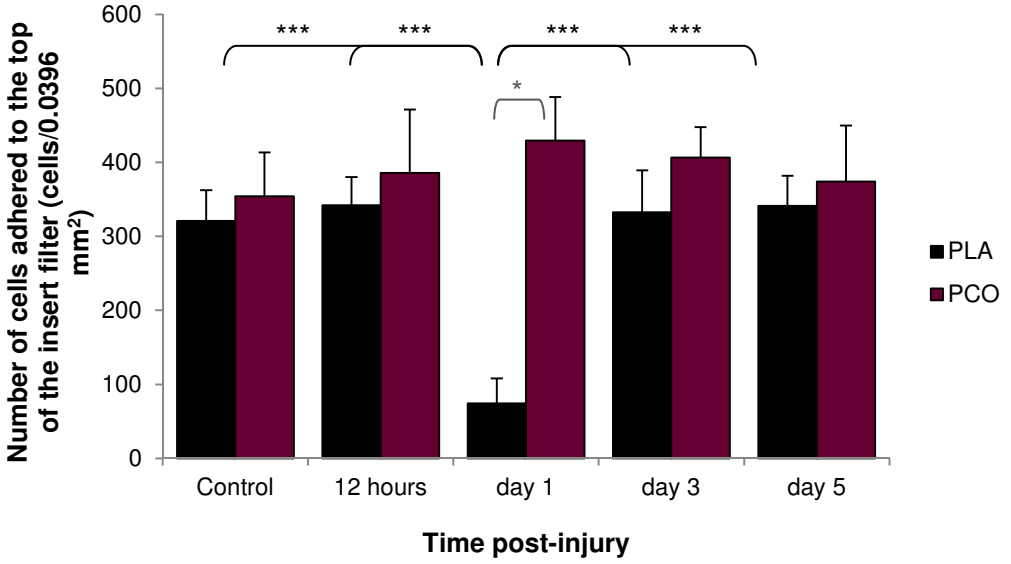


Figure 5.9: Number of non-migrated neutrophils adherent to top of the insert after 2 hours of migration stimulated by fMLP (bottom well) and in the presence of plasma from either PLA- or PCO-supplemented rats (in the insert) harvested at various time points after injury. Statistical analysis: Factorial analysis of variance with Fischer *post hoc* test (* $p<0.05$, *** $p < 0.001$). n = 5 rats per time point per group. Error bars indicate SEM.

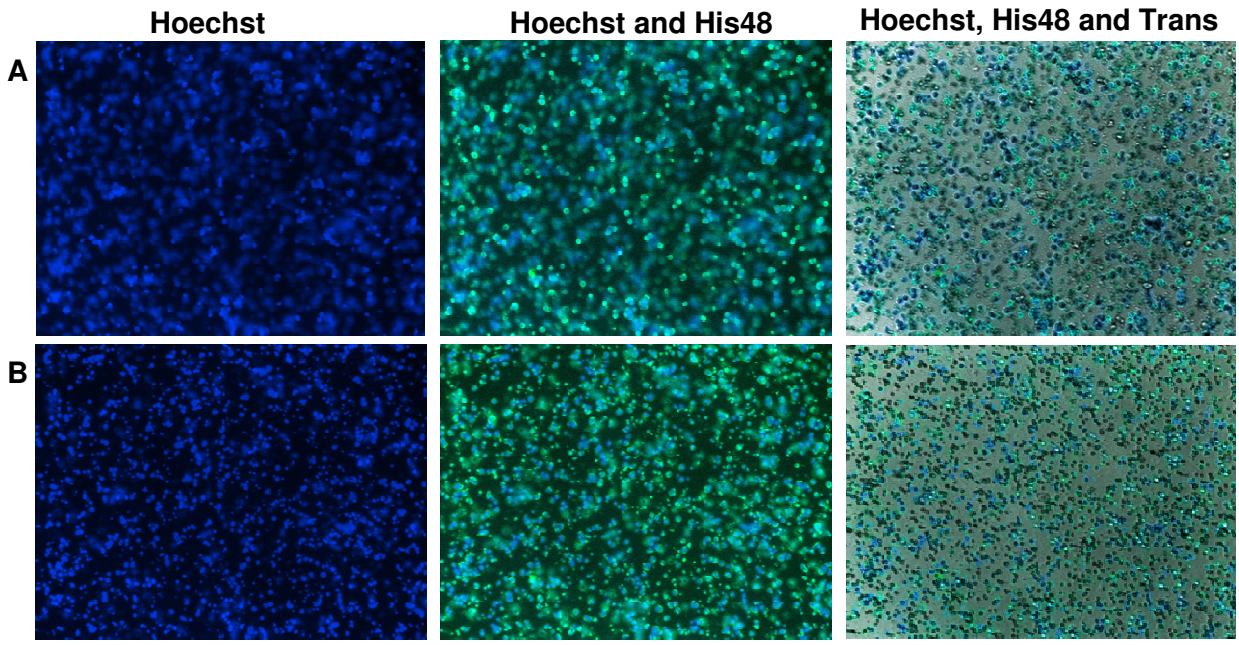


Figure 5.10: Representative image of cells adherent to the top of the insert filter (**A**) and the bottom of the insert filter (**B**). Note that the cells adherent to the top of the insert filter were relatively few, compared to those adherent to the bottom of the insert filter (representative image of the PLA group on day 1). The trans image was used to visualise the filter pores for easy identification. Neutrophils were labelled with His48 (FITC, green) and the nuclei with Hoechst (blue).

5.4 Discussion

We report 3 main findings for this study. Firstly, circulatory neutrophils were significantly reduced on day 1 after injury in the PLA group whilst they were significantly elevated at this time point following chronic PCO supplementation. Secondly, chronic PCO supplementation resulted in a significantly early increase and then decrease in M1 macrophage numbers (day 3 and day 5 respectively) in circulation, with a concomitant rise in M2 macrophages on day 5, at which time the PLA group had a similar elevation in M1 subtype than was seen 2 days earlier in PCO. Thirdly, neutrophil migration *in vitro* was blunted as a result of conditioned plasma from PCO supplemented rats compared to PLA supplemented rats on the day 1 time point specifically.

5.4.1 Neutrophil count and migration study

White blood cells (leukocytes) are continuously being circulated from the blood into various immune compartments, a function essential for the maintenance of an effective immune defence system (Sprent and Tough 1994). The absolute numbers and relative proportions of these leukocytes in the blood provide important information regarding the distribution of leukocytes in the body and possibly also the activation status of the immune system.

Numerous studies have investigated the effect of exercise on circulating blood leukocytes absolute number, percentage distribution and *in vitro* function (MacIntyre *et al.,* 1996; Smith *et al.,* 1998; MacIntyre *et al.,* 2000; Malm *et al.,* 2000; Quindry *et al.,* 2003). These studies are regarded as important in order to understand the function of the immune system. Collectively, these findings also highlight whether there is an inflammatory process and what the magnitude of the response is. The recent history or current use of muscle, and possibly injury-specific interactions between muscle and inflammatory cells will influence these parameters.

Inflammation begins at the onset of exercise or damage, when the circulating level of neutrophils increases significantly, at a point in time that differs depending on the type of injury (MacIntyre *et al.,* 1996; Smith *et al.,* 1998; MacIntyre *et al.,* 2000; Quindry *et al.,* 2003). In two different exercise studies, it was evident that neutrophils appear in the blood at different time points depending on the type of exercise, but their disappearance from the blood coincided (Malm *et al.,* 2000; Starkie *et al.,* 2001). Neutrophils in the current study in the PLA group were significantly increased 12 hr after contusion injury and decreased on day 1, a time point similar to that seen by Malm and Starkie's groups (Malm *et al.,* 2000; Starkie *et al.,* 2001). This decrease in circulatory neutrophil numbers could explain the increase observed in neutrophils infiltrating the injured and border zone areas on day 1 after injury in the PLA group (see Chapter 3). What this indicates is that in order for damaged muscle to repair itself, neutrophils need to migrate into the injured area from circulation, a statement supported by the literature (Robertson *et al.,* 1992). However, many studies only

investigated neutrophils in circulation or in the injured or inflamed tissue, without assessing both compartments. It is therefore a strength of this study that neutrophil infiltration into the injured tissue was also assessed using the exact same protocol.

In the PCO group, although neutrophil numbers were also elevated 12 hr after injury, it remained elevated on day 1 and only decreased 3 days after injury. However, no significant elevations in neutrophil number were evident in the PCO group in the muscle on day 3 or any other time point (see Chapter 3). This might suggest that although neutrophil numbers decrease in circulation, neutrophils are not able to migrate into the injured area.

It is well known that in order for neutrophils to enter the tissue from circulation (migration/diapedesis), it requires the communication between a whole range of cells and tissue itself, which include myocytes, together with mast cells from local connective tissue (Fielding *et al.,* 1993). If the muscle fibre is injured, e.g. as a result of an active stretch, or contusion injury, the myocyte interacts with the endothelial wall of the adjacent blood vessel, releasing a series of signalling molecules or cytokines, ultimately resulting in the infiltration of a cascade of events and neutrophil chemotaxis (Fielding *et al.,* 1993). It is therefore possible that PCO alters the ability of neutrophils to migrate to the injured tissue.

The neutrophil chemotaxis assay enabled the determination of whether PCO or PLA supplementation could influence the magnitude of neutrophil migration. In this study, a transwell assay system was used to test neutrophil migration towards the chemotactic agent, fMLP under standard conditions. Neutrophil migration has been previously studied *in vitro* through 3 µm pores in a transwell system (Smith *et al.,* 1993), but an endothelial cell monolayer was necessary to separate the chamber containing the cells from the chamber containing the chemoattractant. Since the goal was to determine the effect of conditioned plasma from PCO supplemented rats on neutrophil migration, it was decided to use uncoated 3 µm pore membranes without an endothelial layer.

After migration, equal numbers of neutrophils were evident in suspension in the bottom well, irrespective of the addition of plasma from PCO or PLA supplemented rats and irrespective of time post-injury, therefore suggesting that gravity might have played a role. Although it may indicate that plasma from PCO supplemented rats had no effect/benefit on neutrophil migration, 25 % more neutrophils were evident at the bottom of the migration chamber when incubated with conditioned plasma from PLA supplemented rats a day after injury compared to all time points in the PCO group. Similarly, and significantly so, more neutrophils were also seen at the bottom of the insert at that specific time point in the PLA group compared to the PCO group, verifying the results of blunted neutrophil infiltration after PCO supplementation seen in the injured muscle (Chapter 3).

As highlighted in the introduction, a number of possible mechanisms could explain this finding. It is known that pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) are released as a result of injury, and that these cytokines can indirectly cause chemotaxis of granulocytes and monocytes, these leukocytes in turn producing further cytokine expression and the expression of other pro-inflammatory mediators (Utsunomiya *et al.,* 1996). TNF-α specifically has been shown to be responsible for the release of chemotactic factors such as cytokine-induced neutrophil chemoattractant (CINC-1, equivalent to rat IL-8), an important mediator promoting neutrophil migration and also further exacerbating inflammation (Clozel *et al.,* 1993). CINC-1 can increase the expression of LFA-1 integrin on rat neutrophils (Frevert *et al.,* 1995) and because expression of leukocyte adhesion molecules such as E-selectin is dependent on CINC (Harris *et al.,* 1996), the likely outcome is an increase in neutrophil chemotaxis. In Chapter 3, in the PLA group, both pro-inflammatory cytokines, TNF-α and IL-6 were elevated locally, on day 1 and 3 after injury in the border zone and injured area respectively. In the PCO group on the other hand, a blunted pro-inflammatory cytokine response was evident in both circulation and in the muscle. Although these results suggest that the pro-inflammatory cytokines might be directly responsible for upregulation of integrin expression on neutrophils and adhesion molecule expression on endothelial cells, cytokine

release from injured muscle is an effect of injury and may not be a direct cause of a blunted neutrophil response, although it might contribute to the effect seen.

In a study by Garbacki *et al.* (2004), proanthocyandin from *Ribes nigrum* leaves was harvested and supplemented after carrageenin injection into the plantar region of rat paws. Supplementation resulted in the inhibition of TNF-α and CINC-1 levels in pleural exudates of the paw membrane indicating that proanthocyanidin might either be directly or indirectly responsible for reducing neutrophil migration (Garbacki *et al.,* 2004). Proanthocyanidin has also been shown to reduce the expression of soluble adhesion molecules, ICAM-1, VCAM-1 and E-selectin in the plasma of systemic sclerosis patients (Kalfin *et al.,* 2002), suggesting that PCO might rather have a direct effect on adhesion molecule expression instead.

A second possibility is a decreased expression of chemotactic factors as a result of conditioned plasma from PCO-supplemented rats. However, since an equally low number of neutrophils migrating through the insert filter at all time points in the PCO group was observed, as well as all time points in the PLA group, except day 1, the explanation for the inhibition of chemotaxis cannot be as a result of a general effect on chemotactic factor expression.

Another possibility to take into account is the potential contribution of G-CSF, which was added to all wells. In a study by Yong (1996), it was demonstrated that G-CSF increases the adhesion of neutrophils to both resting and TNF-α-stimulated microvascular endothelial cells. Data have also shown that G-CSF upregulates expression of neutrophil adhesion molecules in mature neutrophils *in vitro* (Carulli 1997), as well as increases neutrophil migration (*in vitro*) across a chemotactic gradient in samples from healthy controls and patients with liver cirrhosis (Fiuza *et al.,* 2002). The researchers suggested that this effect of G-CSF is most probably due to the direct interaction of G-CSF with neutrophils, as G-CSF failed to enhance the secretion of pro-inflammatory cytokines such as TNF-α and GM-CSF or to upregulate the expression of adhesion molecules on endothelial cells (Yong 1996; Carulli 1997; Fiuza *et al.,* 2002). Although the studies mentioned above suggest that G-CSF can increase the

adhesion and migration of neutrophils, the *in vivo* conditions are more complex to explain. In one specific study, intravenous administration of G-CSF resulted in a decrease in the absolute neutrophil count, suggesting that the neutrophils might be bound to the endothelium instead, and not migrating (de Haas *et al.,* 1994). In the current study, G-CSF treatment alone, without plasma from PCO- or PLA- supplemented rats, had no effect on neutrophil migration, indicating that G-CSF only stimulated neutrophils.

Thirdly, it is known that neutrophils need to be activated prior to adherence to the endothelium and migration. PCO might have exerted its effect in blunting migration by altering neutrophil activation. However, since treatment of neutrophils with G-CSF in combination with plasma from PCO supplemented rats decreased the number of neutrophils migrating, but increased the number of neutrophils in the top of the insert, it indicates that neutrophils were activated, and therefore they might have been bound too tightly to the insert filter itself. Thus, the possible explanation for PCO's ability to blunt neutrophil migration at the day 1 time point specifically is its ability to alter adhesion molecules on neutrophils and possibly endothelium (although not measured here) instead for neutrophil adhesion. Another possibility is that PCO might alter the expression of the adhesion molecule, platelet-endothelial cell adhesion molecule (PECAM)-1, necessary for neutrophil transmigration. In an *in vitro* study, it was demonstrated that antibodies against PECAM-1 could significantly block neutrophil migration through an endothelial monolayer, without influencing neutrophil adhesion (Muller *et al.,* 1993). This effect has also been confirmed *in vivo* (Vaporciyan et al., 1993). One specific study investigated the effect of resveratrol on PECAM-1 expression (Robich *et al.,* 2010). In this particular study, resveratrol was able to reduce the expression of PECAM-1 in blood vessels, which suggests that it can potentially limit subsequent neutrophil migration. This might also be true for PCO in the current study. The mechanism by which PECAM may regulate migration is largely unknown, but it has been thought that it involves regulating the expression of the other cellular adhesion molecules (CAMs) on neutrophils themselves (Muller *et al.,* 1993).

Studies have shown that the CAMs play vital roles in neutrophil migration. More specifically, the interaction between one of the β2 integrins [lymphocyte function associated antigen-1 (LFA-1, CD11a/18), L-selectin/CD62L or CD11b/Mac-1] and the ligand of immunoglobulin intercellular adhesion molecule-1 (ICAM-1) are involved in the process of neutrophil migration to an inflammatory lesion (Fiuza *et al.,* 2002). It has also been shown that after neutrophil migration, the expression of surface molecules such as CD29 (β1-integrin) and CD18 (β2-integrin) are significantly increased (Kubes *et al.,* 1995; Roussel and Gingras 1997; Poon *et al.,* 1999), whereas L-selectin is shed (Lewinsohn *et al.,* 1987; Jutila *et al.,* 1989).

Antioxidants such as the glutathione (GSH) precursor and radical scavenger N-acetyl-l-cysteine (NAC) and the antioxidant pyrrolidine dithocarbamate have been shown to inhibit adhesion molecule expression (ICAM-1 and VCAM-1 *in vitro*) induced by cytokines or oxidants in different cell types (Marui *et al.,* 1993; Weber *et al.,* 1994; Kawai *et al.,* 1995; Aoki *et al.,* 1996). Other *in vitro* studies also found that coincubation of resveratrol (another grape-derived polyphenol) with different endothelial cell lines (HUVEC, human saphenous vein endothelial cells – HSVEC or EA.hu926 cells) and lipopolysaccharide (LPS) or TNF-α respectively, resulted in a significant decrease in ICAM-1 and VCAM-1 expression on the endothelium (Ferrero *et al.,* 1998). This therefore suggests that resveratrol may exert its beneficial effect by modulating endothelial activation and adhesion rather than neutrophil activation (Weber *et al.,* 1995; May *et al.,* 1996; Ferrero *et al.,* 1998). In studies by Judge *et al.* (2008) and Novelli *et al.* (1997), it was shown that vitamin E has the ability to attenuate neutrophil infiltration following prolonged ischaemia-reperfusion in skeletal muscle, possibly due to a decreased expression of VCAM (Zapolska-Downar *et al.,* 2000), ICAM and E-selectin (Formigli *et al.,* 1997; Wu *et al.,* 1999). However, not only do antioxidants modulate the expression of adhesion molecules on endothelial cells, they also influence the expression of the β2 integrins on neutrophils. In a study regarding the grape seed derivative, resveratrol, it was shown that resveratrol has the ability to decrease the expression of Mac-1 (Rotondo *et al.,* 1998). These studies therefore suggests that antioxidant supplements might

modulate neutrophil migration, either by decreasing adhesion molecule expression on endothelial cell, or decrease β2 integrin expression on neutrophils, or both.

Currently, the specific mediators responsible for the PCO's ability to blunt neutrophil migratory behaviour are unknown. Expanded efforts making use of cell culture models have the potential to clarify these, and could include the incubation of neutrophils either with or without an endothelial cell layer with PCO for a period of time, followed by staining for soluble receptors or adhesion molecules on neutrophils and endothelial cells specifically. From the literature it is clear that PECAM-1, ICAM-1 and VCAM-1 are worth investigating, as well as the Mac-1 integrin on neutrophils.

5.4.2 Macrophage subtypes

Another goal of the present study was to identify the different subpopulations of monocytes in normal rat peripheral blood and in blood harvested at different time points after injury with respect to phenotype. The criteria used to distinguish monocytes were their expression of different receptors present on the membrane of monocytes. Different subpopulations of human monocytes can be distinguished according to their expression of CD64 or CD14 and CD16. In mice different markers are used, which include CX_3CR1, CCR2, and L-selectin (CD62L). It has been shown in different studies that low expression of CX3CR1 and high levels of CCR2 and CD62L are indicative of an M1 phenotype and are recruited to sites of inflammation, whereas monocytes expressing high levels of CX3CR1 are predominantly recruited to non-inflamed sites (Geissmann and Littman 2003). This therefore suggests that M1 monocytes are the first macrophage to infiltrate the injured area and are involved with inflammation, whereas M2 macrophages are only evident when it can be observed that inflammation is decreasing. Another study also identified two very distinct populations of F4/80-positive mononuclear cells in peripheral blood of $CX_3CR1^{GFP/+}$ in mice, of which only one population expressed CD62L (Palframan *et al.,* 2001), similar to the results seen by

Geissmann and Littman (2003). Together these studies all concur, even though different methods were used.

Results from a study by Sunderkotter *et al.* (2004) illustrated that distinct subpopulations of monocytes can be identified in peripheral blood of normal mice by differential expression of various surface markers, in particular, Ly-6C. Monocytes in circulation are immature and predominantly express high levels of Ly-6C (ED1 in rats). However, monocytes are able to mature in circulation under steady state conditions, a process which is reflected by down-regulation of Ly-6C expression (Sunderkotter *et al.,* 2004). During inflammation, it has been shown that only Ly-6C$^{med/high}$ monocytes (still immature) are recruited to the affected sites to become inflammatory macrophages. During inflammation, monocytes also enter the bloodstream from bone marrow as immature Ly-6C^{high} monocytes, and while some of these monocytes migrate to the injured area, the rest gradually down-regulate their Ly-6C expression (M2 phenotype) and mature in blood approximately 2 days later (de Bruijn *et al.,* 1994), and are unable to migrate into the injured area or only to a lesser extent. What this section therefore suggests is that although the M2 macrophage subtypes are found in circulation, only M1 macrophage can play a predominant role and can enter the injured area.

In the current study, CD68 was used to determine M1 monocytes, whereas CD163 was used to determine M2c monocytes. It has been suggested that under normal conditions, human and rat macrophages stain brightly for CD163 and play a role in the resolution of inflammation as they are found in high numbers in inflamed tissue (Zwadlo *et al.,* 1987), whereas CD68 plays a role in phagocytosis and has been shown to release pro-inflammatory mediators (Zwadlo-Klarwasser *et al.,* 1995). In the studies mentioned above, it was shown that the anti-inflammatory cytokine, IL-10, but not IL-4 or IL-13, has the ability to increase the expression of CD163 on cultured monocytes. Together these studies suggest that the M1 monocytes (CD68^{+}) may be able to convert themselves to M2 in the presence of IL-10. In one specific study, circulating monocytes were labelled with both CD163 and CD14 (M2 phenotype), and it was found that CD163 is expressed on nearly all circulating M2c

monocytes (Sulahian *et al.,* 2000). However, although it has been found that CD163 is expressed on circulating M2c monocytes, the presence of this M2 phenotype in circulation has not been studied as extensively as the existence of the M1 phenotype.

The current study indicated that relatively few M1 monocytes/macrophages were evident in the PLA group on day 3 after injury, compared to that in the PCO group. On day 5 on the other hand, M1 monocytes/macrophages were significantly elevated in the PLA group, but reduced again in the PCO group. In contrast, more monocytes/macrophages in the PCO group (day 5) expressed the chosen marker of the M2c phenotype (CD163), indicating that PCO supplementation resulted in, not necessarily a monocyte phenotype switch from M1 to M2c, but mobilisation of the majority of the M1 monocytes into the injured tissue, whilst a number of the M1 monocytes might mature into M2c macrophages instead in the blood. Since the M1 monocytes on day 5 were significantly lower than that observed for the PLA group, it could also indicate that PCO might exert its beneficial effect on not only blunting neutrophil migration, but also inhibiting M1 monocytes early on.

In the previous study in Chapter 3, in the muscle tissue, macrophages were significantly elevated in the PCO group on day 1 and 3 respectively and only declined on day 5, suggesting that the presence of macrophages at this time point in tissue might come from circulation and were of the M1 phenotype. Similarly, macrophages in the PLA group were elevated on day 3 but also on day 5, and only decreased in numbers on day 7, suggesting the presence of M1 macrophages lasted for several days longer. However, considering all the abovementioned literature, it is still unclear whether macrophages with an M2 phenotype are able to infiltrate the injured area from circulation. Rather, it is thought that M1 monocytes infiltrate the injured area from circulation and potentially switch to M2c. Taking into account the results from the current study and previous chapters, since high levels of IL-10 were detected in circulation in the PCO group on day 3, at the same time as the high M1 macrophages, it is possible that PCO mediated IL-10 release in some way, and therefore resulted in an increase in M2c macrophages in the circulation which was evident on day 5

after injury (Figure 5.6). Although the M2c macrophages increased significantly in PLA between days 1 and 3, this was mainly because they were significantly decreased from control at 12 hr and day 1, and returned to normal levels on day 3. At no time did the M2c macrophages in the PLA group exhibit an increase relative to the control.

5.5 Conclusions and limitations

In this study, it was determined that PCO in Oxiprovin™ do indeed have an anti-inflammatory action. Results from this chapter suggested that chronic PCO supplementation results in a macrophage switch from a M1 (pro-inflammatory) to a M2c (anti-inflammatory) phenotype on day 5 after injury, a point in time when M1 macrophages were expressed at a high number in the PLA group. Data on the effects of PCO on the relative proportions of M1 and M2 macrophages within the muscle would be valuable for addressing the relative importance of diapedesis and phenotype switching in the changes observed in circulating macrophages and should be included for future studies. Conditioned plasma from PCO-supplemented rats also resulted in decreased chemotaxis of control neutrophils towards the chemoattractant fMLP. Because neutrophils have been demonstrated to injure skeletal muscle *in vitro* and *in vivo*, this specific mechanism of action of PCO provides a potential avenue for alleviating neutrophil-mediated secondary muscle injury. Oxiprovin™ may inhibit the early process of neutrophil activation (possibly through inactivating adhesion molecules), and it speeds up the mobilisation of M1 macrophages, thus allowing an earlier inflammatory macrophage infiltration and phenotype switch.

CHAPTER 6

Synthesis

Both skeletal muscle and immune cells are severely affected by contusion injuries. Although our group have previously shown beneficial effects of a plant-derived polyphenol antioxidant (proanthocyanidolic oligomers (PCO) in Oxiprovin™) on muscle recovery, these benefits have not been comprehensively investigated. Therefore, it was the aim of this thesis to determine its effect and possible mechanisms of action.

Initial qualitative comparison of two different treatment regimes revealed that chronic PCO supplementation resulted in earlier ultrastuctural recovery than acute post-injury treatment, as seen with the H&E sections of the 14 day time point (Figure 6.1). Muscle from chronically supplemented rats illustrated full muscle regeneration, while muscle from acutely supplemented rats still displayed some fiber disorganisation – the result of either residual oedema or possibly altered adhesion molecule profile – although both supplemented groups displayed better ultrastructural recovery when compared to placebo groups.

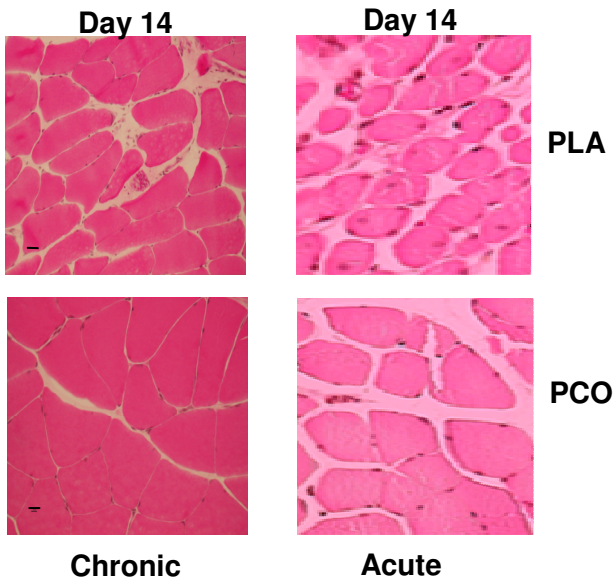


Figure 6.1: Histological comparison of muscle recovery after chronic *vs.* acute PCO supplementation.

The recovery process involves multiple processes and multiple contributing cells and factors. Therefore in order to explain the qualitative results, it is necessary to consider more than one potential role player. A particular strength of this thesis is that a variety of contributors were investigated in more than one compartment.

In the tissue compartment, satellite cells are arguably the most important role player in muscle regeneration. For a treatment to be effective in resulting in quicker muscle regeneration, satellite cell activation needs to occur much quicker. With chronic supplementation, the supplement was already available at tissue level at the time of injury, which was not the case with acute supplementation, where supplementation only started two hours after injury. Therefore, the difference in recovery can be ascribed at least in part, to earlier PCO-induced satellite cell activation. This was evident in the Pax-7 data, which indicated earlier satellite cell activation in the supplementation group.

However, satellite cells were clearly not the only cell type affected by supplementation. The inflammatory process was significantly altered. Chronic and acute PCO supplementation resulted in similarly suppressed neutrophil profiles, indicating that at least one possible mechanism of action of PCO is limiting neutrophil-associated secondary damage (potential mechanisms investigated will be discussed later). However, the macrophage data is less clear cut. Both chronic and acute supplementation had a similar effect of macrophage infiltration after injury. Chronic PCO supplementation resulted in a shift to the left of time course for macrophage infiltration compared to acute supplementation, suggesting that if PCO is taken chronically, it will result in a quicker inflammatory response. Interestingly though is the suppression of macrophage infiltration in the border zone after acute supplementation, which provides further support for PCO's proposed mechanism of limiting secondary damage.

The macrophage response in the chronic PCO group also coincided with tissue cytokine and satellite cell responses. This could indicate that PCO, at least as a chronic supplement, was able to alter the time course of appearance of macrophages, due to its direct effect on

satellite cells and possibly cytokine production. Since the macrophage response in the acute supplementation protocol did not coincide with either the tissue cytokine profile or satellite cell response (these two latter responses coincided with one another), it suggests that supplementation started 2 hr after injury might not provide adequate timing to affect the early responses after injury. Perhaps starting supplementation at the time of injury might have provided a clearer picture or early immune cell infiltration and cytokine responses, closer to that seen with chronic supplementation. However, in this study, this option was not available, since oral gavage is not possible with anaesthetised animals.

Cytokines are not only produced locally (at tissue level) as a result of injury, but also appear in circulation later on. Interestingly, PCO supplementation resulted not only in a blunted pro-inflammatory cytokine response in the injured muscle, but also a blunted circulatory pro-inflammatory cytokine response, and an increased circulatory anti-inflammatory cytokine response. Since the response of the muscle pro-inflammatory cytokines, TNF-α and IL-6, coincided with the satellite cell response, after both acute and chronic supplementation, the direct effect of PCO on satellite cell activation could have accounted for this effect. Although it is also known that macrophages has the ability to influence the pro-inflammatory cytokine response, the later appearance of macrophages in the injured area after acute supplementation cannot explain the cytokine response. This suggests that although satellite cells and macrophages both produce cytokines, they do not necessarily have to be dependent on one another and only one cell type may be influenced by PCO supplementation.

The finding of significantly higher IL-1β content on day 1 after injury in the acute PCO group, could indicate an earlier or more potent inflammatory response as a result of PCO supplementation. However, since macrophage and neutrophil numbers (known to be responsible for IL-1β release) at that specific time point in the PCO group were relatively low, they could not have contributed to an elevation in IL-1β. PCO could have stimulated fibroblasts instead to express IL-1β.

Turning attention to circulatory cytokines, no data are available on the effect of contusion injury on circulatory inflammatory cytokines, indicating that the findings in this study of an altered inflammatory profile in circulation are quite novel. PCO supplementation resulted in a blunted circulatory pro-inflammatory response, which could be explained by PCO's ability to blunt neutrophil infiltration whilst facilitating an earlier increase in macrophage numbers. Also, since IL-6 and TNF-α are produced locally as well, these cytokines might have exerted their effect locally, rather than "spilling" over in circulation. The timing of appearance of cytokines in the blood was also delayed compared to their appearance in tissue in the current model, supporting this option. Since an increase in tissue cytokines after chronic PCO supplementation were observed on day 1 and 3 respectively, it might suggest that time points between day 1 and 3, e.g. day 2 or 36 hr, might have been better suited for detection of a possible increase in pro-inflammatory cytokines. Whilst blunting the pro-inflammatory cytokine response, PCO was also responsible for a significant increase in circulatory anti-inflammatory cytokines on day 3 after injury, suggesting an anti-inflammatory action of macrophages. M2 macrophages are known to be responsible for the release of anti-inflammatory cytokines, one of which includes IL-10, suggesting that the elevations in total macrophages seen in the PCO group in the injured muscle on day 3 might potentially contain predominantly M2 macrophages.

To enable a better understanding of the mechanism of chronic PCO in blunting the inflammatory response, whilst allowing for an earlier macrophage response, the presence of the M1 (classically activated) and M2c (alternatively activated) monocyte/ macrophage subtypes were determined in circulation. We were able to show that PCO supplementation resulted in an earlier disappearance of M1 macrophages in circulation, whilst allowing the expression of the M2 phenotype. Various studies in the literature have shown that only the M1 phenotype migrates to the injured area and then switch into a M2 phenotype, whereas others have not been able to detect the M2 phenotype. Thus, although our results might potentially indicate that PCO supplementation resulted in a switch in macrophage subtypes

already in circulation, it does not necessarily imply that M2 macrophages were able to infiltrate the injured area.

From the results regarding contusion injury, it is evident that a decreased neutrophil response in circulation coincided with increased neutrophil migration into the injured tissue, which suggests that neutrophils migrated to the injured tissue from blood. The neutrophil response as a result of PCO supplementation did not follow the same pattern. Since PCO supplementation resulted in a blunted neutrophil response at tissue level, the finding of decreased neutrophil numbers in circulation was somewhat surprising. These results therefore implied that although neutrophil numbers decrease in circulation, PCO might function by binding neutrophils too tightly to the vascular endothelium itself, thereby suppressing its ability to migrate to the injured area. Results from the migration study indicated that conditioned plasma from PCO-supplemented rats was able to blunt neutrophil migration towards a chemotactic factor at all time points after injury. A couple of mechanisms whereby PCO might exert its beneficial effect on blunting neutrophil migration have been proposed and include: (a) its potential to influence local cytokine release; (b) its involvement in chemotactic factor release, (c) its ability to possibly alter the activation status of neutrophils, or (d) a direct effect on adhesion molecule expression either on the endothelium or on neutrophils themselves. Although all of these mechanisms seem probable, results from the migration study, as well as support from other studies on PCO and antioxidants, suggested that the mechanism of action of PCO most probably involves modulating neutrophil migration, by decreasing both adhesion molecule expression on endothelial cell as well as β2 integrin expression on neutrophils, thereby limiting diapedesis of neutrophils. However, although its ability to modulate neutrophil adhesion might be the likely option, the combined effect of PCO in blunting the circulatory inflammatory cytokine response might also be involved in limiting neutrophil chemotaxis.

6.3 Conclusions and recommendations for future studies

This study indicated that PCO does not only have an antioxidant effect, but also anti-inflammatory properties. Since it is known that cytokines are important activators or suppressors of various inflammatory pathways, including the nuclear factor kappa B (NFκB), arachidonic acid or mitogen activating protein kinase (MAPK) pathways, and PCO blunts the pro-inflammatory cytokines whilst increasing anti-inflammatory responses, it is worth investigating which of the particular upstream and downstream effectors is primarily influenced. Applying western blotting techniques could enable one to test which of the various components of the inflammatory pathways are influenced by both acute or chronic PCO supplementation. Also, since we did not determine the different macrophage subpopulations at tissue level, immunohistochemical staining of muscle samples at different time points after injury will provide valuable insight into a possible phenotype switch after injury as a result of PCO supplementation. In addition, a cell-culture based study can be implemented in which either tissue or blood macrophages can be stimulated with various inflammatory cytokines, followed by administration of PCO, which will also allow for the determination of a possible phenotype switch and the effect of PCO.

Our results regarding blunting of neutrophil migration at all time points after injury with this particular PCO supplement are novel. Further studies are needed to elucidate the exact mechanism(s) of action of PCO resulting in this effect. Possible adhesion molecules worth investigating include CD11b (Mac-1) integrin on neutrophils, although the CD11a/CD18 integrin might also potentially be involved. ICAM-1 and VCAM-1 expression on endothelial cells should also be measured. Though our migration study excludes the expression of these latter adhesion molecules (we did not add an endothelial cell layer to the culture study), the contribution of the endothelial cell adhesion molecules cannot be excluded. Finally, although this study provided valuable insight into the various role players involved in the recovery process after an injury, more studies are needed in order to answer some of the questions that arose during the course of this study.

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Appendix A: Automated tissue processing

Reagents

1. Alcohol (70 %, 90 %, 95 %, 100 %)
2. Xylene – Sigma-Aldrich
3. Paraffin wax – Merck, Histosec melting point 56 ℃

Method

Processing time:

A) Dehydration

1) 70 % alcohol – 1.5 hr
2) 70 % alcohol – 1.5 hr
3) 90 % alcohol – 1.5 hr
4) 95 % alcohol – 1.5 hr
5) 95 % alcohol – 1.5 hr
6) 100 % alcohol – 1.5 hr
7) 100 % alcohol – 1.5 hr
8) 100 % alcohol – 2.0 hr

B) Clearing

9) Xylene – 1.5 hr
10) Xylene – 2.0 hr

C) Impregnation

11) Paraffin wax – 2.0 hr
12) Paraffin wax – 2.0 hr

Thus Total processing time = 20 hr

Appendix B: Conventional staining procedure for paraffin-embedded tissue

Reagents

1. Alcohol (50 %, 80 %, 95 %, 100 %)
2. Xylene – Sigma-Aldrich
3. 0.1 % Trypsin – Highveld

   0.1 g trypsin in 100 ml PBS
4. PBS, pH 7.4

   1 ℓ of 1 M phosphate buffer, 90 g NaCl, 9 ℓ ddH_2O
5. For antibodies and serum used, see Table 1 (catalogue numbers and supplier provided)

Method

1. Incubate sections in Xylene: 2 changes, 5 min each.
2. 100 % absolute alcohol: 2 changes, 3 min each.
3. 95 % alcohol: 2 changes, 3 min each.
4. 80 % alcohol: 3 min.
5. 50 % alcohol: 3 min.
6. Rinse in distilled water: 2 changes, 3 min each.
7. Place slides in prewarmed (37 ℃) 0.1 % trypsin for 20 min.
8. Wash slides in PBS after trypsin (step 7, Appendix B).
9. Encircle samples with a wax pen.
10. Block for 20 min in 5% serum at room temperature (RT). (*Note: Use the same serum in which the secondary antibody is raised.*)
11. Shake off serum and incubate sections for 4 hr at RT with the 1st primary antibody. (*Note: Do not wash after serum blocking step.*)
12. Wash slides with PBS and add the secondary antibody (1/250) to the sections. Incubate for 40 min at RT. *(Note: From here on, all steps should be performed in the dark.)*
13. Wash slides with PBS and add the 2nd primary antibody overnight at 4℃.

14. Add the 2nd secondary antibody (1/250) for 40 min after washing the sections thoroughly with PBS.
15. Wash sections and add Hoechst (1/200) for 15 min.
16. Wash slides well and mount with DAKO fluorescent mounting medium.

Notes: If only use 1 antibody, apply steps 1-12 and then 15 and 16

All dilutions and wash steps were done using phosphate buffered saline (PBS)

If anti-human primary antibodies were used, I made sure that it also cross-reacted with rat tissue

Table 1: Antibodies used to identify satellite cells (Pax-7), pro-inflammatory cytokines (IL-6, TNF-α), neutrophils (His48), macrophages (F4/80), and basal lamina (Laminin).

Antibodies	**[Stock]**	**Dilution**	**Catalogue number and supplier**
Primary antibodies:			
Monoclonal mouse Pax-7	Not stated	1/200	Pax-7, Developmental Studies Hybridoma Bank
Goat polyclonal IL-6 (M-19)	200 µg/ml	1/100	sc-1265, Santa Cruz
Goat polyclonal TNF-α (L-19)	200 µg/ml	1/100	sc-1351, Santa Cruz
Mouse monoclonal anti-rat His48	0.5 mg/ml	1/200	554905, BD Biosciences
Goat polyclonal anti-mouse F4/80 (A-19)	200 µg/ml	1/200	sc-26642, Santa Cruz
Rabbit polyclonal anti-human Laminin	Not stated	1/200	Z 0097, Dako Diagnostec
Fluorescent labelled secondary antibodies:			
Texas red donkey anti-goat	100 µg/ml	1/250	sc-2783, Santa Cruz
FITC donkey anti-rabbit	100 µg/ml	1/250	sc-2090, Santa Cruz
Alexa Fluor 594 goat anti rabbit	2 mg/ml	1/250	A11012, Invitrogen
Alexa Fluor 488 goat anti-mouse	2 mg/ml	1/250	A11029, Invitrogen

Appendix C: Flow cytometry - measuring cytokines with a CBA kit

Reagents

1. Rat soluble protein master buffer kit – BD558267
2. Rat IL-10 – BD558306
3. Rat IL-4 – BD558307
4. Rat IL-6 – BD558308
5. Rat TNF-α – BD558309

Sample preparations

1. Perform instrument setup procedure.
2. Dilute serum or plasma samples 1:4 with Sample Diluent and mix thoroughly before transferring the samples to the assay wells containing the Capture Beads.
3. Reconstitute Standards mix and prepare serial dilutions using the Assay Diluent.

   3.1 Pool one lyophilized standard vial from each BD CBA Rat Soluble Flex Set to be tested into one tube (Recommended 15 ml Conical Tube, BD Falcon Cat.No.352097) and label it Top Standard.

   3.2 Reconstitute the standards with 4.0 ml of Assay Diluent and allow to equilibrate for 15 min before making serial dilutions. Do not vortex or mix standards vigorously, use only a pipette.

   3.3 Label 10 x 75 mm tubes (BD Falcon Cat.No.352008) and arrange them in the following order, according to dilution: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256.

   3.4 Pipette 500 µl of Assay Diluent in each of the labelled tubes.

   3.5 Perform a serial dilution by transferring 500 µl from the Top Standard to the 1:2 dilution tube and mix thoroughly. Continue making serial dilutions by transferring 500 µl from one tube into the next one and continue up to the tube labelled 1:256 (see table 2). Remember to mix by pipette only. Prepare one tube containing Assay Diluent to serve as the 0 pg/ml negative control.

3.6 The first ten wells in the experiment should be used for the standards. Standards should be run in order from least concentrated (0 pg/ml) to most concentrated (Top Standard).

Table 2: BD CBA Rat Flex Set Standard concentrations after dilution

BD CBA Rat Soluble Protein Flex Set	Top Standard	1:2 Dilution Tube	1:4 Dilution Tube	1:8 Dilution Tube	1:16 Dilution Tube	1:32 Dilution Tube	1:64 Dilution Tube	1:128 Dilution Tube	1:256 Dilution Tube
Protein (pg/ml)	10000	5000	2500	1250	625	312.5	156	80	40

4. Prepare the diluted BD CBA Rat Soluble Protein Flex Set Capture Beads using Capture Bead Diluent. The Capture Beads provided in each BD CBA Rat Soluble Flex Set are at a 50x concentration and must be diluted before adding to a given assay well.

4.1 Vortex each Capture Bead stock vial for at least 15 seconds to resuspend beads thoroughly.

4.2 Determine the total volume of diluted beads needed for the experiment. Each well requires 50 µl of the diluted beads. The total volume of diluted beads can be calculated by multiplying the number of wells by 50 µl.

eg, 35 tests × 50 µl = 1750 µl total volume of diluted beads

4.3 Determine the volume needed for each Capture Bead. Beads are supplied so that 1.0 µl = 1 test. Therefore the required volume (µl) of beads is equal to the number of tests.

eg, 35 tests requires 35 µl of each Capture Bead included in the assay

4.4 Determine the volume of Capture Bead Diluent needed to dilute the beads. The volume of Capture Bead Diluent can be calculated by subtracting the volume for each bead tested from the total volume of diluted beads needed to perform the assay.

eg, 1750 µl Tot vol dil beads - 35 µl for each bead = vol Capture Bead Diluent

eg, if testing one analyte: 1750 µl – (35 µl x 1) = 1715 µl diluent

eg, if testing 5 analytes: 1750 µl – (35 µl x 5) = 1575 µl diluent

4.5 Pipette the Capture Beads and Capture Bead Diluent into a tube labelled Mixed Capture Beads.

5. Dilute BD CBA Rat Soluble Protein Flex Set PE Detection Reagents. The PE Detection Reagent is a 50x stock (1 µL/test) and should be mixed with other Flex Set PE Detection Reagent (i.e. of different cytokine beads) and diluted to their optimal volume per test (50 µl/test) before adding to a given assay well. The calculations are the same as in the previous section.

   5.1 Pipette the Detection Reagents and Detection Reagent Diluent into a tube labeled Mixed PE Detection Reagents. (*Note: Protect the PE Detection Reagents from exposure to direct light because they can become photobleached and will lose fluorescent intensity and store at 4℃ until ready to use.)*

Method

1. Wet filter plate with 100 µl of Wash Buffer and aspirate using a vacuum manifold.
2. Transfer 50 µl of capture beads to each assay tube or well.
3. Add Standard Dilutions and serum/plasma samples to the appropriate wells (50 µl/well). Incubate for 1 hr at room temperature and protect from light (tinfoil). *(Note: Everything should be done in duplicate.)*
4. Add mixed PE Detection Reagent to each assay well (50 µl/well). Incubate for 2 hrs at room temperature, protected from light.
5. Aspirate fluid.
6. Add Wash Buffer to each assay well (150 µl/well) and analyze samples.

Appendix D: ORAC assay

Reagents

Reagents used for the ORAC assay are tabulated below:

Reagent	[Stock]	[Working]	Supplier
AAPH*		153 mM 0.414 g in 10 ml Phosphate buffer, pH 7.4	Sigma-Aldrich 44,091-4
Fluorescein	$5x10^{-3}$ mM (4 ℃) Light-sensitive	$8.16x10^{-5}$ mM (4 ℃) Made up in phosphate buffer, pH 7.4	Sigma-Aldrich F6377-1006
Potassium Phosphate buffer		75 mM (pH 7.4) (4 ℃) 6.5 g K_2HPO_4 in 500 ml dist. H_2O 5.1 g KH_2PO_4 in 500 ml dist. H_2O	Sigma-Aldrich 60353 (Fluka) 60218 (Fluka)
Trolox**	0.02 M (-20 ℃)	6.25 µM; 12.5 µM; 25 µM; 50 µM in phosphate buffer, pH 7.4	Sigma-Aldrich 56510 (Fluka)

* 2,2'-Azobis-(2-methylpropionamidine)-dihydrochloride

** 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

Method

1. Defrost plasma samples at room temp and dilute them 75x with phosphate buffer (personal communication: Dr. Dee Blackhurst, Cape Town University).

2. Take approximately 10 mg of snap-frozen muscle tissue and add to phosphate buffer (1:75 w/v). Continuously sonicate tissue samples on ice for approximately 1 minute using a tissue sonicator (Virsonic 300; The Virtis Company, Inc; Gardiner, N.Y; setting 3) and then centrifuge for 10 minutes at 8853xg (12 000 rpm) and 4 ℃ in a multispeed refrigerated centrifuge (PK121R, ACL International SRL, Milano, Italy).

3. Add 150 µl fluorescein working solution to each well of a black plate.

4. Add 25 µl TROLOX standards, plasma or tissue extract (supernatant) in triplicate to respective wells.

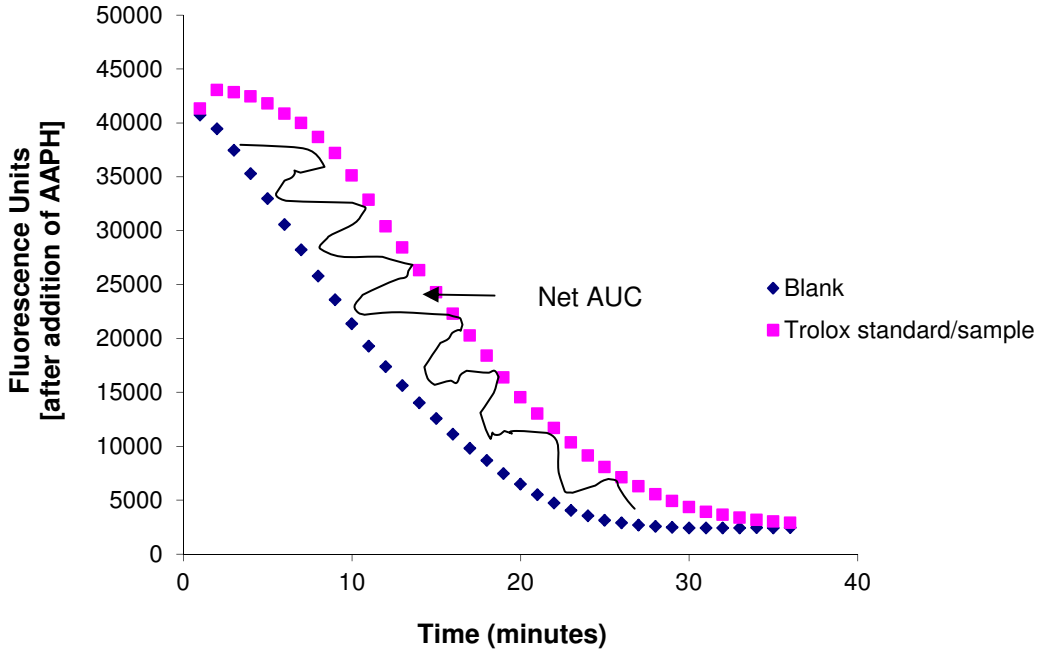
	BUFFER	TROLOX	PLASMA/TISSUE EXTRACT	AAPH	FLUORESCEIN
BLANK	25 µl	-----	-----		150 µl
STD	-----	25 µl	-----		150 µl
SAMPLE	-----	-----	25 µl		150 µl

5. Cover plate with lid and incubate in preheated reader (37°C) for 10 min with 3 min shaking.

6. Rapidly add 25 µl AAPH to all wells.

7. Read for 35 min with fluorescence measured every minute. (Excitation wavelength = 485 ± 20 nm; emission wavelength = 530 ± 25 nm).

8. The net area under the curve (AUC) of all standards and samples are calculated from a graph of time (in minutes) against relative fluorescence intensity, as illustrated below:



The AUC was calculated using Microsoft Excel XP as

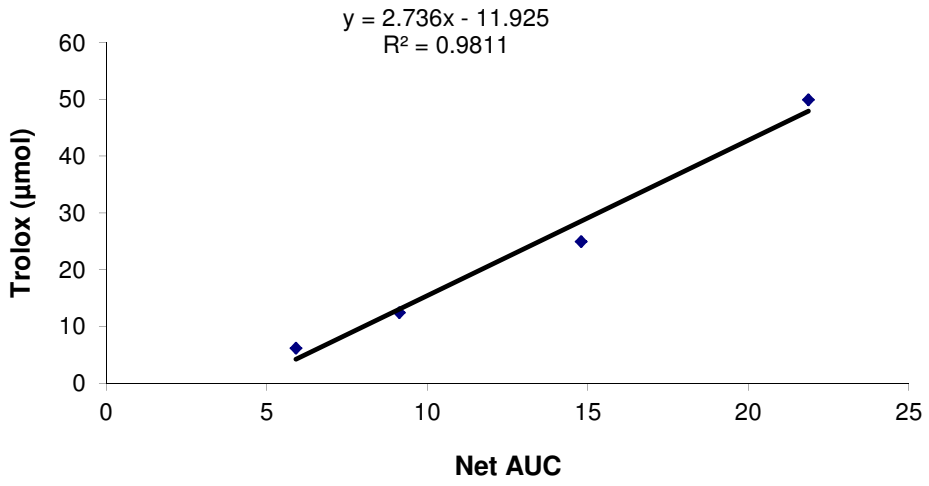
$$0.5 + f_1/f_0 + \ldots f_i/f_0 + \ldots + f_{34}/f_0 + f_{35}/f_0 \qquad (1)$$

where f_0 = initial fluorescence reading at 0 minutes and f_i = fluorescence reading at time i.

The net AUC is obtained for all the samples, by subtracting the AUC of the blank from that of a sample or a Trolox standard.

$$\text{Net AUC} = [(AUC_{\text{sample or Trolox}} - AUC_{\text{blank}}) \qquad (2)$$

9. The standard curve is obtained by plotting the Trolox standard concentrations against the average net AUC of these standards:



10. The final ORAC values are calculated using the regression equation between Trolox concentration and the net AUC. Results are expressed as µmol Trolox equivalents per liter for liquid samples (plasma) and µmol Trolox equivalents per µg protein for solid samples (e.g. homogenised muscle).

Note: The dilution factor (75x in this study) needs to be included for final ORAC value calculation. For the muscle samples, the ORAC values were obtained in the same way as that for the plasma samples, but in addition, the ORAC value was divided by the protein concentration determined using the Bradford assay (Appendix E).

Appendix E: Bradford assay

Reagents

1. 1 mg/ml bovine serum albumin (BSA) stock solution – Sigma-Aldrich
   Diluted to 200 µg/ml
2. Bradford reagent – Sigma-Aldrich, B6916

Method

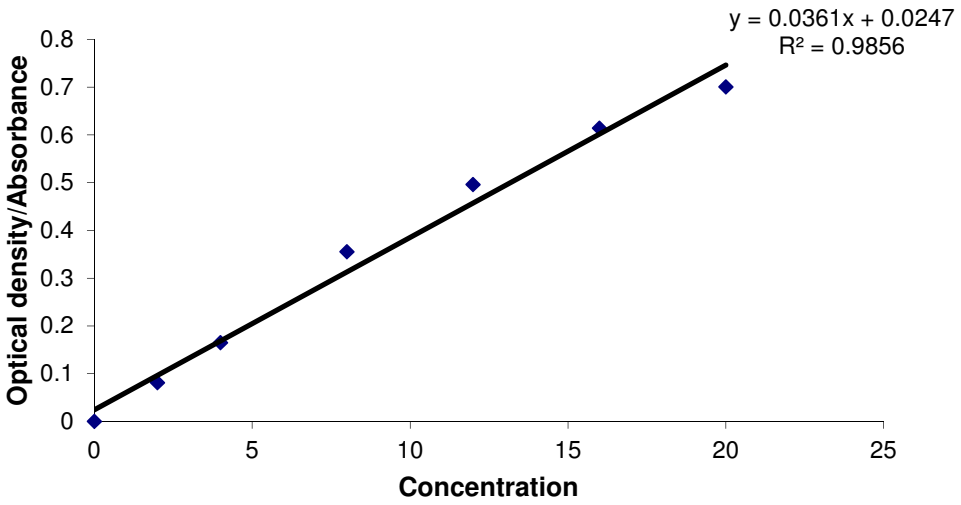
1. Prepare phosphate buffer (ORAC) or RIPA (Western blotting, see Appendix G).
2. Add all of the reagents mentioned in the table below to their respective tubes and generate the BSA standard curve:

[ ] µg/ml	BSA stock	Distilled water	Bradford reagent
20	100 µl	0 µl	900 µl
16	80 µl	20 µl	900 µl
12	60 µl	40 µl	900 µl
8	40 µl	60 µl	900 µl
4	20 µl	80 µl	900 µl
2	10 µl	90 µl	900 µl
0	0 µl	100 µl	900 µl (BLANK)

3. Vortex and leave on ice for 5 min.
4. Zero the spectrophotometer with the BLANK and read the absorptions of the standards at 595 nm.
5. Add a small piece of snap-frozen tissue samples to either Phosphate buffer (ORAC assay) or RIPA buffer (Western blotting) and sonicate on ice for approximately 1 minute with a tissue sonicator (Virsonic 300; The Virtis Company, Inc; Gardiner, N.Y; setting 3)
6. Centrifuge the homogenates for 10 min at 8853xg (12000 rpm) at 4 ℃ with a multispeed refrigerated centrifuge (model: PK121R centrifuge, brand: ALC international SRL, Milano, Italy).
7. Prepare the tissue samples – 5 µl of muscle sample, 95 µl distilled water and 900 µl Bradford reagent. Vortex and read the absorption at 595 nm (UV-visible

spectrophotometer, Cary 50) using the computer software Simple Read (version 2, WinUV, Cary 50). *(Note: samples stable for up to 1 hr after preparation.)*

8. Plot the standard curve and calculate the protein concentration in µg:



Appendix F: H&E staining protocol

Reagents

1. 10 % Acid alcohol

   10 ml 1 % HCl dissolved in 1 ℓ 70 % alcohol

2. Alcohol (70 %, 95 %, 100 %)

3. Eosin

   Stock solution:

   10 g Eosin dissolved in 1 ℓ distilled water

   Working solution:

   10 ml Eosin stock solution dissolved in 90 ml distilled water.

   Prepare fresh daily.

   For staining:

   Add 2 – 3 drops glacial acetic acid per 100 ml before use.

4. Haematoxylin

   5 g Harris haematoxylin

   100 g Ammonium Alum

   50 ml 100 % alcohol

   1 ℓ distilled water

   2.5 g Mercuric oxide

   To prepare: Dissolve haematoxylin in alcohol.

   Add Ammonium Alum to distilled water and heat to boiling point.

   Immediately add mercuric Oxide and shake until solution has purple-black colour.

   Cool rapidly in fridge.

For staining: Filter before use.

Add 4 ml glacial acetic acid per 100 ml of haematoxylin.

5. Scott's tap water

   3.5 g $NaHCO_3$

   20 g $MgSO_4$

   10 ml 37 % Formalin

   1 ℓ tap water

   To prepare: Dissolve $NaHCO_3$ in tap water first.

   Add $MgSO_4$ and formalin.

6. Xylene

Method

1. Xylene (10 min)
2. 100 % alcohol (10 dips)
3. 100 % alcohol (10 dips)
4. 95 % alcohol (10 dips)
5. 95 % alcohol (10 dips)
6. 70 % alcohol (10 dips)
7. Rinse in distilled water
8. Haematoxylin (3 min)
9. Rinse in distilled water
10. Rinse in acid alcohol
11. Rinse in distilled water
12. Blue in Scott's tap water

13. Rinse distilled water
14. 2 min in Eosin
15. Rinse in distilled water
16. 70 % alcohol (10 dips)
17. 95 % alcohol (10 dips)
18. 95 % alcohol (10 dips)
19. 100 % alcohol (10 dips)
20. 100 % alcohol (10 dips)
21. Xylene (10 dips)
22. Xylene (10 dips)
23. Mount with coverslip

Appendix G: Western blotting

Reagents (Gels and buffers)

1. **RIPA buffer**

100mM Tris-HCl, 300mM Nacl (stock)

To 350 ml distilled water (dH_2O), add 7.9 g Tris, 9 g NaCl and pH to 7.4 using HCl.

Make up to 500 ml with dH_2O and keep at room temperature or in the fridge until needed.

On the day of extraction:

Take 25 ml of RIPA stock and add 5 ml of a 10 % NP-40 stock solution, 1.25 ml of 10 % Na-deoxycholate stock solution (an ionic detergent to extract protein, protect from light), 1 Roche complete protease inhibitor tablet, 1 Roche Phostop tablet and 0.25 ml of a 200 mM Phenylmethylsulfonyl flouoride (PMSF) stock solution (made up in isopropanol, stored at room temperature).

Make up to 50 ml with dH_2O and use immediately.

2. **3x Sample buffer** (*pre-made, bench*)

For a 0.5 M Tris solution, add 3.03 g to 50 ml distilled water and adjust the pH to 6.6.

Take 33.3 ml and add 8.8 g SDS, 20 g Glycerol and a tiny bit Bromophenol blue.

Fill up to 75 ml with dH2O.

For making up samples, take 850 µl of the 3x sample buffer and add 150 µl β-mercaptoethanol.

SDS = denature proteins, constant anionic charge-to-mass ration

Glycerol = give sample a higher density than buffer to “sink” to the bottom of the well

Mercaptoethanol = reduce disulfide bonds present in protein sample

3. **Resolving gel** (2 gels)	**12 %**	**10 %**
Millipore H_2O	3.35 ml	3.850 ml
1.5 M Tris-HCl, pH 8.8 *(store in fridge)* (68.1 g Tris base dissolved in 1 liter dH_2O)	2.5 ml	2.5 ml
10 % SDS stock *(recipe below)* (10 g APS dissolved in 100 ml dH_2O)	100 µl	100 µl
Acrylamide (*carcinogenic*)	3 ml	2.5 ml
10 % APS (*fresh every day*) (0.1 g APS dissolved in 1 ml dH_2O)	50 µl	50 µl
Temed (*add at very end in fume hood*)	20 µl	5 µl

4. **Stacking gel** (2 gels)	**4 %**	**5 %**
Millipore H_2O	3.05 ml	1.875 ml
0.5 M Tris-HCl, pH 6.8 *(store in fridge*) (6.06 g Tris base dissolved in 1 liter dH_2O)	1.25 ml	0.75 ml
10 % SDS-stock	50 µl	30 µl
Acrylamide	500 µl	375 µl
10 % APS (0.1 g/ml)	50 µl	20 µl
Temed (*add at very end in fume hood*)	10 µl	10 µl

5. **10X running buffer** (STOCK – store in fridge)

Tris base 60.6 g

Glycine 288 g

10 % SDS 20 g

Dissolve in 2 liter distilled water.

6. **10 x TBS** (STOCK – store in fridge)

   48.4 g Tris

   160 g NaCl

   Dissolve in 500 ml distilled water, set pH to 7.6 with concentrated HCl and then make up to 2 liters.

7. **Coomassie**

   Stain: 40 % methanol; 10 % acetic acid; 50 % water; 0.1 (w/v) Coomassie Brilliant Blue R250 (27816, Sigma-Adrich)

   De-stain: 40 % methanol; 10 % acetic acid; 50 % water

Method

Setting up and casting the gels:

1. Clean glass plates with 70 % ethanol and assemble the plates into the casting frame and casting stand. (*Note: The grey gaskets must be present underneath the glass plates to allow proper sealing of the system.)*
2. Place a comb between the gel plates and mark 1 cm below the bottom of the comb and then remove the comb.
3. Pour freshly prepared resolving gel up to the marked level using a pasteur pipet. (*NB: The size of the gel will depend on the size of the protein. If the protein of interest is smaller than 40 kDa, use a 12 % gel; if the protein is bigger than 40 kDa, but smaller than 100 kDa, use a 10 % gel; and if it is bigger than 100 kDa, use a 8 % or 6 % gel.)*
4. Pour few ml isobutanol on top of the gel to straighten it out. Let gel polymerise for 30 min to 1 hr. *(Note: Watch progress by observing spare gel solution left in your tube.)*
5. Pour off the top isobutanol layer by tilting the apparatus and wash a couple of times with distilled water. Use filter paper strips or paper towels to dry the space. *(Note: Be careful at this stage not to touch the gel, as it will cause the gel to stick to the paper strips)*
6. Pour in stacking gel till it overflows. Immediately put in the comb and let gel polymerise for 30 min.

7. After gels have set, remove the comb prior to disassembling the glass plates from the casting apparatus.

Electrophoresis:

8. Assemble glass plates in the gel chamber with the thin plate facing inward, and pour 200-300 ml 1x running buffer into the middle of the gel chamber. *(Note: Put paper towels underneath to see if buffer leaks out. If it leaks, reclamp plates, otherwise the level of the buffer will be too low and the electrical circuit will be broken, therefore no electrophoresis will occur.)*

9. Boil samples for 5 min at 95 ℃ and spin them down quickly with a benchtop centrifuge. *(Note: Do not boil the protein marker (ladder) – just take it out of the freezer and allow to reach room temperature.)*

10. Load 5 µl of the protein marker (27-2110, PeqGOLD protein marker IV, Peqlab) into the first well of each gel, followed by the samples. *(Note: Do not let one sample overflow into the next well.)*

11. Place the gel chamber into the electrophoresis mini tank and pour the rest of the running buffer into the tank, making sure to fill up the gel chamber properly. *(Note: Be careful not to pour too close to the gel wells, because the samples might spill out of the wells and thus protein concentration will not be equal.)*

12. Connect the electrical leads to a power source and run the gels in two individual sessions. The first run set at 100 V, 400 mA for 10 minutes will allow the proteins to reach the stacking gel, and the second set to run at 200 V, 400 mA for 50 minutes. *(Note: The time of the second run will depend on the size of the protein that you're looking for.)*

13. Just before the run is over, soak 2 pieces (per gel) of thick blotting paper into transfer buffer. Soak PVDF membranes in methanol for 15 sec and then in transfer buffer.

14. When the run is complete, remove the spacers and separate the glass plates from one another. Cut off the stacking gel with the glass plate and put in transfer buffer.

15. Put one blotting paper, the gel, the membrane and then the other blotting paper on the semi-dry apparatus, Press out all the bubbles by using a wet test tube.

16. Transfer the proteins to the membrane by using the following settings for 1-2 hours: 0.5 A, and 15 V.

17. Make up the blocking solution by adding 10 g milk powder to 200 ml of a 1x TBS-Tween solution (1 ml Tween-20 per 100 ml of 1x TBS). *(Note: Use 50 ml blocking buffer per membrane.)*

18. After transfer, remove the membrane and place in methanol for couple of seconds, followed by 10 min airdry.

19. Add the membranes to the blocking solution for 2 hr in plastic tub.

20. Place the gel in a plastic container with lid containing enough Coomassie stain to cover the gel. Put lid on and microwave on high for 1 min, and then stain while shaking for 2 min to detect proteins. Pour out the stain, add a large amount of de-stain and de-stain on rocker at room temperature for a few minutes. If no proteins are detected on the gel, then all proteins have transferred to the membranes.

21. Make up primary antibody in TBS-T in 50 ml falcon tube according to recommended dilution on antibody data sheet just prior to taking the membranes off the milk. *(Note: The usual recommendation is 1:1000, thus 5 µl antibody in 5 ml TBS-T.)* Antibodies used are tabulated below *(Note: Store made up primary antibodies in the fridge.)*:

Antibodies	**[Stock]**	**Dilution**	**Catalogue number and supplier**
Primary antibodies:			
Mouse monoclonal IL-1β	200 µg/ml	1/1000	sc-7884, Santa Cruz
Goat polyclonal IL-6 (M-19)	200 µg/ml	1/1000	sc-1265, Santa Cruz
Goat polyclonal TNF-α (L-19)	200 µg/ml	1/1000	sc-1351, Santa Cruz
Rabbit polyclonal β-actin		1/1000	sc-81178, Santa Cruz
Human anti-mouse MHC_f		1/1000	F1.652, Developmental Studies Hybridoma Bank (DSHB)
Horseradish peroxidase conjugated secondary antibodies:			
Donkey anti-rabbit		1/4000	sc-2313, Santa Cruz
Donkey anti-mouse		1/4000	sc-2318, Santa Cruz
Donkey anti-goat		1/4000	sc-2768, Santa Cruz

22. After blocking is complete, rinse the membranes in TBS-T and place the membrane inside the falcon tube containing the primary antibody.

23. Allow to rotate overnight at 4 ℃ in the cold room.

Developing:

24. Take out the membrane from the falcon tube and wash the membranes (3x 5 min each) with TBS-T.

25. Make up the horseradish peroxidase-conjugated secondary antibody in TBS-T (Amersham Life Science; 1/4000) in a 50 ml falcon tube.

26. Incubate the membranes for 1 hr at room temperature in the secondary antibody.

27. After incubation with the secondary antibody, warm up the ECL kit (Amersham Life Science Inc., Arlignton Heights, IL, USA) containing solution A and solution B to room temperature.

28. Wash membrane 3x 5 min each with TBS-T.

29. Whilst washing, prepare dark room cassettes by cleaning them with alcohol first and allowing to air dry. Now cut a transparency in half and stick to cassette.

30. Make up the ECL (500 µl of solution A + 500 µl of solution B per membrane) in 15 ml falcon tube. *(Note: Protect from light.)*

31. Pour 1 ml of ECL on top of each membrane in the specific area where the protein of interest is located and leave on for 1 min.

32. Blot excess ECL, place between transparencies and close the cassette.

33. In the dark room, cut a piece of hyperfilm (RPN-2103K, Amersham BioSciences), place on top of the transparencies, and allow to expose for 5 min or longer depending on the clarity of the bands.

34. Develop and fix the film (Fixing and Developing Solutions from Axim, 9x23013 and 9x23018, respectively) and analyse with the program, UNSCANIT (Silk Scientific Corporation (SilkScience), USA, version 5.1)

Appendix H: Labelling immune cells in whole blood for flow cytometry

Reagents

1. Fc Block/CD32 (550271, BD)
2. BD FACS™ Lysing Buffer (349202, BD)
3. FITC-conjugated mouse anti-rat granulocyte marker (anti-His48, BD Pharmingen, 554907)
4. PE-conjugated mouse anti-rat macrophage subset marker (anti-F4/80, BD Pharmingen, 554901)
5. Alexa Fluoro mouse anti-rat CD68 (anti-M1, Serotec, MCA647)
6. FITC-conjugated mouse anti-rat CD163 (anti-M2c, Serotec, MCA341)

Method

1. Label two 15 ml falcon tubes (c and d) per sample (four tubes when setting up the method).
    a. Unstained (only needed for optimisation).
    b. Stained with neutrophil marker, His48 (determine neutrophil population – used as gate to enable counting of other immune cells).
    c. Stained with total macrophage marker, F4/80.
    d. Stained with M1 macrophage marker, CD68 and M2c macrophage marker, CD163.
2. Add 500 µl of whole blood to the respective tubes.
3. Block by incubating the cell suspension with Fc block (CD32) for 10 min at 4℃ (≤ 1 µg/million cells in 100 µl). Do not rinse. Proceed with staining. (*Note: It is very important to block Fc receptors for certain cell types, including, but not limited to, mouse and rat blood, mouse spleen, mouse bone marrow, etc.)*

    Thus, for 1 million cells, add 2 µl Fc block/100 µl RPMI medium or add to whole blood as is.
4. Add primary antibodies (≤ 1 µg/million cells) to the respective tubes and incubate for 20-30 min at 4 ℃ in dark on ice.

Primary antibody	Concentration	For staining
Anti-F4/80	200 µg/ml	5 µl
Anti-His48	500 µg/ml	2 µl
Anti-CD68	500 µg/ml	2 µl
Anti-CD163	500 µg/ml	2 µl

5. For each 500 µl of blood, add 5 ml of BD Lysing solution (1:10 in distilled water) to lyse the red blood cells. Blood needs to be lysed for 5 min at room temperature. (*Note: If cells are lysed beyond 5 min, then the white blood cells might also be lysed. Also, if the lysing solution is cold, the cells will not lyse correctly.)*
6. Centrifuge for 5 min at 2000 rpm.
7. To wash off excess antibody following staining, add 1.5-2 ml of PBS to each tube. Centrifuge in tabletop microfuge for 5 min at 2000 rpm. Repeat 1-2x.
8. Resuspend in 2 ml PBS.
9. Acquire results.

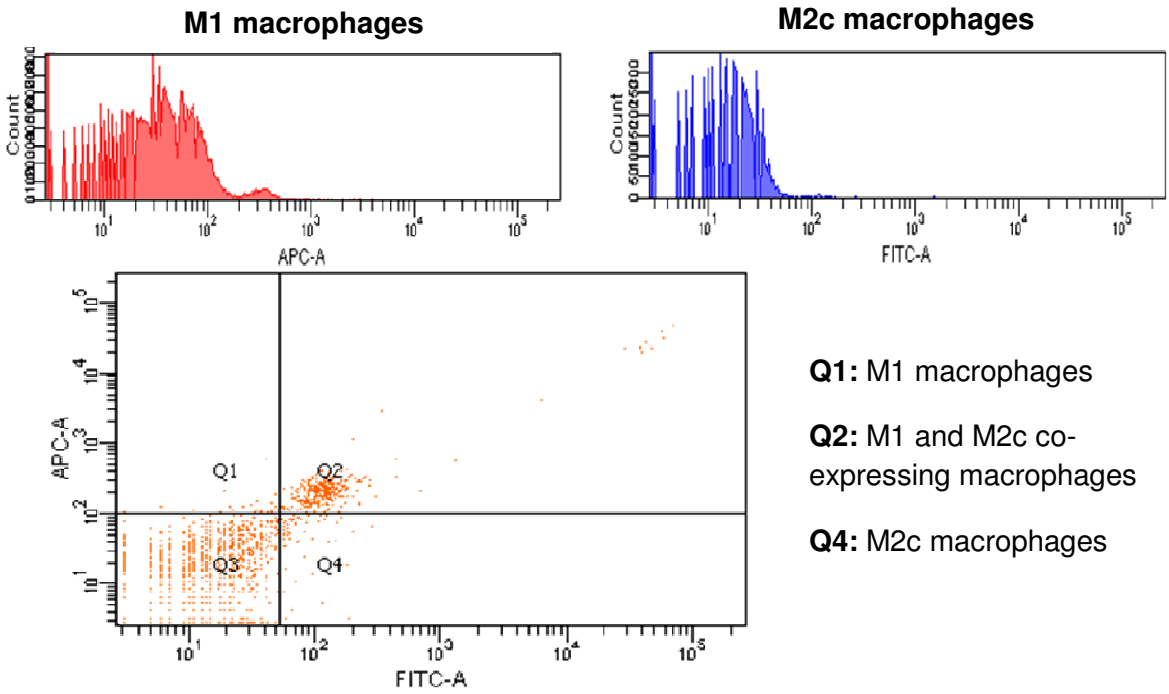
Notes on determining macrophage subpopulation distribution:

A pilot study was done to optimise the method for macrophage identification. Firstly, a scatter plot was generated without using a cellular marker antibody according to forward scatter (x-axis) and side scatter (y-axis) to determine different populations of white blood cells according to size and granularity. A gate is set around the neutrophil population (the easiest to identify in non-human samples), and this population is then used as reference cell population. Secondly, a similar scatter plot was generated, but in the presence of a fluorescence-labelled antibody directed against His48, a neutrophil marker. This neutrophil population identified by the fluorescent marker was identical to the population indicated by the arrow, and it was decided that a neutrophil marker is not necessary in this particular study.

a) Using scatter characteristics or fluorescently labelled antibodies

Macrophages within whole blood were either labelled with a PE-conjugated total macrophage marker (anti-F4/80) or with markers specifically identifying the APC-labelled M1 (anti-CD68) and FITC-labelled M2c (anti-CD163) population of macrophages. M1 and M2c macrophages were counted relative to 5000 neutrophils. Since the starting blood volume (used for staining) and the total number of neutrophils were known, the volume used to count 5000 neutrophils could be calculated. This volume was used and cells were expressed as the number of M1 and M2c macrophages per 1 µl of blood.

A representative image of the cell scatter for each of these markers are indicated in the images below.



Flow settings for the FACSAria:

All flow cytometric analysis were performed on a FACSAria Cell sorter flow cytometer (Becton Dickinson Biosciences, San Jose, CA) equipped with a 488 nm Coherent Sapphire solid state laser (13-20 mW), 633 nm JDS Uniphase HeNe air-cooled laser (10-20 mW) and 407 nm Point Source Violet solid state laser (10-25 mW). For each sample, population information from a minimum of 5000 events (gated around the neutrophil population to identify M1 and M2c macrophages) or 1000 events (gated around the beads in the TruCOUNT tubes to identify migrated and non-migrated neutrophils – see Appendix I) was acquired using a 70 µM nozzle. Instrument setup and calibration were performed prior to each data acquisition. FACSDiva Version 6.1 was used to display data acquired from each population.

Appendix I: Migration study

Reagents

1. Ficoll-Histopaque (10771, Sigma)
2. BD FACS™ Lysing Buffer (349202, BD)
3. FITC-conjugated mouse anti-rat granulocyte marker (anti-His48, BD Pharmingen, 554907)
4. fMLP (F3506, Sigma)
5. G-CSF (G8160, Sigma)
6. RPMI 1640 media (GIBCO, 21875)
7. Plasma from control and injured rats, PLA and PCO supplemented
8. Cell culture inserts for 24-well plates (3 µm pores, BD, 353492)
9. Cell culture plates (24-well)

Method

1. Pipette 3 ml of Ficoll-Histopaque into a 15 ml Falcon tube.
2. Layer 4 ml of whole blood slowly on top of the Ficoll by holding the tube at a 30° angle.
3. Spin the tube for 25 min at 4 °C at a speed of 1500 rpm using a low brake.
4. Discard the supernatant and add Lysis buffer to the red blood cell containing solution according to manufacturer's instructions. *(Note: Add 10 parts of 1x Lysis buffer and 1 part red blood cell solution, thus 10 ml 1x Lysis buffer and 1 ml red blood cell solution.)*
5. Allow to stand for 5 min at room temperature.
6. Spin down at 1500 rpm for 5 min at 4 °C.
7. Discard supernatant and resuspend pellet in 2 ml RPMI medium.
8. Perform a cell count using a haemocytometer.
9. Resuspend 200 000 neutrophils per tube in pre-warmed RPMI media and add to separate sterile eppendorff tubes to a total volume of ± 156.8 µl.

10. To this, add 3.2 µl G-CSF of a 5 µg/ml stock solution (final G-CSF concentration of 100 ng/ml) and 40 µl plasma from rats supplemented with either placebo or PCO from control and rats at different time points after injury to respective eppendorff tubes containing neutrophils.
11. Add the abovementioned cocktail to the insert, and 800 µl media containing fMLP at a concentration of $1x10^{-7}$ M to the bottom well of the migration chamber and allow to incubate separately for 15 min prior to the actual migration assay. (*Note: Do not add the inserts to the wells right away, but allow to incubate separately in order for the G-CSF to stimulate the neutrophils prior to migration.)*
12. Following the pre-incubation period, place the inserts over the media containing fMLP and allow migration to take place for 2 hr. (*Note: Carefully place the inserts over the wells containing medium, as bubbles trapped underneath the insert will prevent migration in that specific area to take place.)*
13. After migration, take off the media containing cells from the insert and the bottom well respectively and place into separate eppendorff tubes.
14. Label 2 TruCOUNT tubes per sample either as “top” or “bottom”.
15. Add 400 µl of cell suspension (from either the inserts and the culture wells) to the respective TruCOUNT tube and allow the pellet to dissolve.
16. Vortex the tubes and read using a flow cytometer.
17. Neutrophils are expressed relative to 1000 beads, whereafter, taking total beads and volume into account, the results were expressed as the total number of cells per “top” and “bottom” of the insert. *(Note: This method also resulted in an accurate counting of neutrophils and was decided to be the best possible method.)*
18. Add ice cold acetone:methanol (50:50 v/v) to all inserts and wells and incubate for 10 min at 4 ℃.
19. Aspirate acetone:methanol off and allow samples to airdry for 20 min.
20. Add 1 ml hoechst (1/200) to all inserts and wells for 15 min at room temperature.
21. Remove hoechst and wash twice with PBS.
22. Incubate plates in the fridge overnight, covered with PBS.

23. Aspirate PBS off and add FITC-conjugated His48 (1/1000) to all respective inserts and wells for 1 hr at room temperature.
24. Wash inserts and wells twice with PBS and visualise using a fluorescence microscope.
25. Express cells as the number of migrated/non-migrated neutrophils per area.

Appendix J: Methods tested but not used for migration study

For neutrophil isolation: Dextran sedimentation method

Assay principle: This method is based on firstly separating neutrophils from other white blood cells and red blood cells by means of sedimentation, and then lysing any further red blood cells that might contaminate the separation procedure.

Analytical procedure:

Reagents

1. **ACD:**

To 250ml ddH_2O, add:

7.36 g Citric Acid

14.71 g Sodium Citrate

9.91 g Dextrose

Store at 4℃

2. **6% Dextran:**

To 250ml ddH_2O, add:

15.00 g Dextran (at least 100 000 MW)

2.25 g NaCl

Store at 4℃

3. **0.6M KCl:**

To 250ml ddH_2O add:

11.18 g KCl

Store at 4℃

4. Ficoll-Histopaque 1077 (Sigma-Aldrich, 10771)

Method

1. Pipette 4 ml of ACD into a 50 ml falcon tube and pipette 20 ml of whole blood down the side of the tube. Gently invert the tube several times to mix (or 1 ml for each 5 ml).

2. Pipette 12 ml (50% of blood volume in 1) of 6 % Dextran / 0.9 % NaCl solution into the ACD/blood mixture and invert 18-20 times to ensure adequate mixing. Pipette the mixture into 4, 15 ml tubes (10 ml/tube). Let the four tubes stand at RT for 45 min – 1 hr, or until separation is complete.

3. After separation, pipette the yellowish supernatant into a 50 ml tube. Spin at 1150 rpm for 12 min at 4℃ using a low brake.

4. Discard the supernatant and resuspend in 12 ml of ice-cold dH_2O to break the pellet. After 20 sec, add 4 ml of 0.6 M KCl and mix several times. Dilute the solution to 50 ml with PBS and spin at 1300 rpm for 6 min at 4℃ using a high brake.

5. Repeat Step 4, 1-2 times, until no RBC's remain.

6. Discard supernatant and resuspend the pellet in 2.5 ml of PBS.

7. Layer the cell suspension over 3 ml of Ficoll-Histopaque in a 15 ml tube. Spin at 1500 rpm for 30 min at 4℃ using a low brake.

8. When the cells have finished spinning suck off the supernatant, using a pasteur pipette.

9. Resuspend the pellet in 2 ml RPMI at 4℃. Determine the cell concentration using a haemocytometer, or other device.

Results: Although the neutrophils isolated with this method were relatively pure, with limited or no red blood cells, it resulted in a relatively low yield. This method is also very long (2-3 hrs) and involves multiple steps, making it difficult to determine whether some of the neutrophils might have become activated as a result.

Quantifying migrated vs non-migrated neutrophils

<u>In solution</u>

A) Haemocytometer counting

Method:

1. Two eppendorff tubes per sample were labelled either as “top” or “bottom”.

2. Cells in solution from the top or bottom of the inserts were added to their respective 1.5 ml eppendorff tube.
1. Cells were resuspended and 20 µl from each tube were added to a haemocytometer and cells counted.

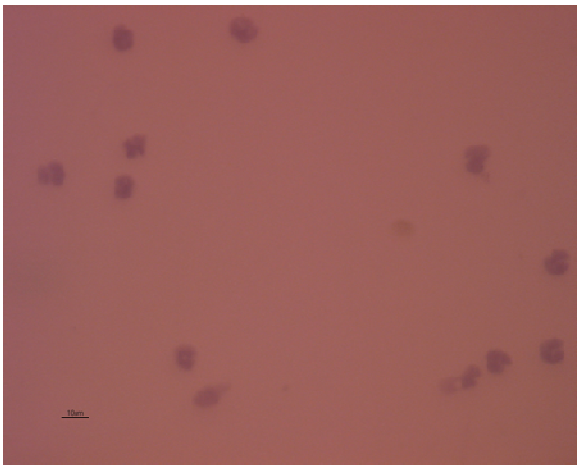
Results: Since some of the bottom wells after migration, only had a limited number of neutrophils, it resulted in a relatively low number of neutrophils to count and thus potentially resulting in an inaccurate cell count. This method was therefore not considered to be a good method to count cells after migration.

B) Grid counting on a slide

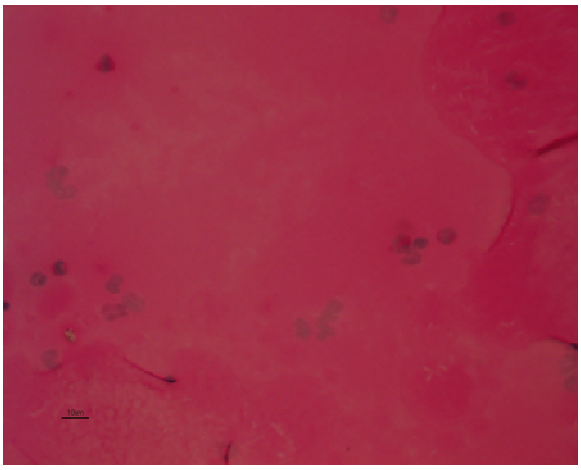
Method:

1. Two tubes per sample were labelled either as “top” or “bottom”.
2. Cells in solution from the top or bottom of the inserts were added to the eppendorff tubes.
3. Cells were spun down at 3000 rpm for 5 min.
4. The supernatant were aspirated off and the cells resuspended in 30 µl of PBS.
5. Cells were pipetted on a slide and a smear was mad.
6. Cells were then stained with either Rapidiff, which consists of a fixing solution, Stain A and stain B, or Haematoxylin and Eosin (H&E) (steps 6-20, Appendix F).
7. Slides were then left to dry and viewed with a light microscope.

A) Rapidiff staining



B) Haematoxylin and Eosin staining



Results: Cells stained with Rapidiff, as well as with H&E appear very light with intense background staining with H&E. Since the whole slide mostly contains cells, and possibly not equally distributed, it is not easy to randomly select different fields of

view to acquire images. It is also not known whether some of the cells washed away with H&E staining, since cells are not properly adhered to the slide prior to staining. This method was not considered to be a good method for counting cells. A indicates a picture of neutrophils stained with Rapidiff, whilst B is an H&E stained image.

Adherent

A) *Scraping cells from bottom of the insert after staining and fixing to slides*

Method:

1. After cells in solution were placed into eppendorff tubes, ice cold acetone:methanol were added to all inserts and wells and left for 10 min at 4 ℃.
2. Acetone:methanol were aspirated off and left to airdry for 20 min.
3. Hoechst (1/200) were added to all inserts and wells for 15 min at room temperature.
4. Hoechst were aspirated off and inserts and wells washed twice with PBS.
5. Plates were placed in the fridge overnight.
6. PBS were aspirated off and FITC-conjugated His48 (1/1000) were added to all respective inserts and wells for 1 hour at room temperature.
7. Inserts and wells were washed twice with PBS.
8. Cells from the bottom of the inserts were scraped to remove all cells that have possibly migrated and the inserts were adhered to slides with fluorescent mounting medium.

Results: After scraping, all pores were stained blue (i.e. cells most probably pushed back into pores) and no clear identification of migrated *vs.* non-migrated neutrophils could be made. Scraping of cells were therefore not considered to be the best possible method of identifying migrated *vs.* non-migrated neutrophils.

B) *Paraformaldehyde fixing after staining of intact insert*

Method:

1. After cells in solution were placed into eppendorff tubes, inserts and wells were stained with FITC-conjugated His48 (1/1000) for 1 hr at room temperature.
2. Inserts and wells were washed twice with PBS and Hoechst (1/200) added for 15 min.
3. After washing inserts and wells with PBS again, 2 % paraformaldehyde were added to the cells and left overnight in the fridge.

4. Inserts and wells were washed twice and visualised.

Results: Due to the fact that cells were only fixed after staining with paraformaldehyde, it resulted in a lower number of intact neutrophils. This method was therefore not considered to be the best possible option for counting adhered neutrophils.