Antioxidant (Oxiprovin™) supplementation and muscle recovery from contusion injury – an *in vivo* study

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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.

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ABSTRACT

Human studies on the response of muscle to contusion injury are limited, probably due to the large variability in injury severity and the non-specificity of clinical symptoms reported. To circumvent this problem, several experimental animal models have been designed to study muscle damage and regeneration after contusion injuries. However, the majority of techniques currently used to induce contusion injury are very invasive and therefore not optimal. Furthermore, published studies regarding clinical treatment of such injuries are limited. The main aims of this study were therefore: a) to establish and characterise an *in vivo* model of non-invasive contusion injury, and b) to assess the effect of pre-injury chronic administration of the antioxidant supplement Oxiprovin™ - a natural grape seed extract (GSE) - on skeletal muscle recovery after experimentally-induced injury.

Two groups of male Wistar rats were subjected to 14 days of oral administration of isovolaemic placebo (sterile isotonic saline) or GSE (20 mg/kg/day) prior to induced contusion. Contusion injury was induced with the mass-drop technique, and recovery parameters assessed for up to 14 days post-injury. Placebo-treated rats on average exhibited a 56 % higher creatine kinase (CK) activity when compared to the GSE-treated rats when area under the curve (AUC) was calculated for 14 days post-injury ($p < 0.001$). In the placebo group, plasma oxygen radical absorbance capacity (ORAC) was unchanged over time, but muscle ORAC was significantly increased by day 7 post-injury ($p < 0.001$). In the GSE group, a significant decrease in both plasma ($p < 0.01$) and muscle ORAC ($p < 0.001$) was evident 4 hr after injury, followed by a significant increase by day 3 ($p < 0.05$ and $p < 0.001$ respectively). CD34$^+$ satellite cell (SC) numbers

(quiescent and activated) peaked earlier in GSE-treated rats when compared to placebo-treated rats (4 hours *vs.* day 7 post-injury). Total satellite cell number (CD56$^+$) also peaked earlier in GSE-treated rats than in placebo-treated rats (4 hours *vs.* 3 days post-injury), while M-cadherin$^+$ SC numbers (quiescent, activated or proliferating) in both treatment groups were significantly increased 4 hours post-injury ($p < 0.001$), but more so in the placebo group. In GSE-treated rats when compared to placebo-treated rats, newly generated muscle fibres (displaying central nuclei and MHC$_f^+$) both appeared (day 3 *vs.* day 7 post-injury) and peaked in number (day 3 *vs.* day 7 post-injury; increase from baseline $p < 0.001$ for both) earlier.

The results of this study demonstrate that we have successfully established an *in vivo* model for non-invasive contusion injury in rats. Furthermore, we have shown that Oxiprovin™: a) increased the ability to scavenge reactive species generated after injury and b) resulted in the activation of satellite cells and formation of newly generated muscle fibres at an earlier time point, thus accelerating the recovery of skeletal muscle after a standardised contusion injury.

OPSOMMING

Eksperimente aangaande die reaksie van spier op kneusings in mense is beperk, waarskynlik as gevolg van die groot verskeidenheid simptome wat mag voorkom en die verskille in die ernstigheid van beserings. Ten einde hierdie problem te oorbrug, is verskeie eksperimentele diermodelle opgestel om kneusings en die herstel van spier daarna te ondersoek. Die tegnieke wat grootendeels vandag gebruik word om kneusings te veroorsaak, maak inbraak op die spier deur die spier te ontbloot voor besering, en is dus nie ideaal nie. Daar is ook nie baie bewyse aangaande die mees geskikte manier om so 'n besering klinies te behandel nie. Die doel van hierdie studie was dus om: a) 'n *in vivo* model van kneusings op te stel en te omskryf, en b) die effek van chroniese toediening van die antioksidant Oxiprovin™ - 'n natuurlike druifsaad ekstrak (DSE) – op die herstel van skeletspier na 'n kneusing te ondersoek.

Twee groepe manlike Wistar rotte is onderwerp aan mondelikse toediening van isovolemiese plasebo (steriele isotoniese soutoplossing) of DSE (20 mg/kg/dag) vir 'n tydperk van 14 dae voor kneusing. Kneusing is geïnduseer met die "mass-drop" tegniek, en parameters van herstel is ondersoek tot en met 14 dae na besering. Plasebo-behandelde rotte het gemiddeld 56 % hoër kreatien kinase (KK) aktiwiteit in vergelyking met DSE-behandelde rotte ($p < 0.001$), toe die oppervlak onder die kurwe (OOK) tot en met 14 dae na besering bereken is. Geen verskil oor tyd is in die plasebo groep opgemerk toe plasma suurstof radikaal absorpsie kapasiteit (SRAK) bepaal is nie, maar 'n betekenisvolle toename in spier SRAK teen dag 7 ($p < 0.001$) is waargeneem. 'n Betekenisvolle afname in beide plasma ($p < 0.01$) en spier ($p < 0.001$) SRAK van die DSE is teen 4 hr waargeneem, gevolg deur 'n betekenisvolle toename teen dag 3 na

besering ($p < 0.05$ en $p < 0.001$ onderskeidelik). Die aantal $CD34^+$ satelliet selle (SS – rustend en geaktiveerd) het beduidend vroeër in die DSE groep gestyg in vergelyking met die plasebo groep (4 uur *vs.* 7 dae na besering). Die totale aantal SS ($CD56^+$) het ook vroeër in die DSE-behandelde rotte as die plasebo-behandelde rotte gestyg (4 uur *vs.* 3 dae na besering), terwyl die aantal M-cadherin$^+$ SS (rustend, geaktiveerd of prolifererend) betenisvol gestyg het in beide groepe teen 4 uur ($p < 0.001$) na besering, maar hoër in die plasebo groep was. Die aantal nuutgevormde spiervesels (met sentraal geleë nukleï en MHC_f^+) het beide vroeër verskyn en gepiek in die DSE-behandelde rotte in vergelyking met die plasebo-behandelde rotte (dag 3 *vs.* dag 7 na besering).

Die resultate van hierdie studie dui aan dat ons instaat was om 'n *in vivo* model van nie-indringende kneusing in rotte op te stel. Verder, het ons ook bewys dat Oxiprovin™ toediening die vermoë verleen het om: a) reaktiewe spesies wat na beserings gevorm word, meer doeltreffend te verwyder en b) satelliet selle vroeër te aktiveer en die vorming van nuwe skeletspiervesels te vervroeg, om sodoende die herstel van skeletspier na 'n gestandardiseerde kneusing vinniger te bewerkstellig.

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And lastly, but most importantly, the Lord, Jesus Christ.

LIST OF ABBREVIATIONS

AAPH	2,2'Azobis-(2-methylpropinalmidine)-dihydrochloride
ANOVA	analysis of variance
ATP	adenosin triphosphate
AUC	area under the curve
BrdU	Bromodeoxyuridine (5-bromo-2-deoxyuridine)
BSA	bovine serum albumin
C	control
Ca^{2+}	calcium
Cat	catalase
C-GSE	chronic grape seed extract group
CK	creatine kinase
C-P	chronic placebo group
CSA	cross sectional area
ddH$_2$O	distilled water
DOMS	delayed onset muscle soreness
FGF	fibroblast growth factor
FITC	fluorescein streptavidin
GPX	glutathione peroxidase
GSE	grape seed extract
GSH	glutathione
GSPE	grape seed proanthocyanidin
H&E	haematoxylin and eosin
HCl	hydrogen chloride
HGF	hepatocyte growth factor

HPLC	high performance liquid chromotography
HSC	haematopoietic stem cells
H_2O_2	hydrogen peroxide
IFN-γ	interferon gamma
IGF	insulin-like growth factor
I-GSE	grape seed extract injury group
IL1,2,6,8	interleukins
I-P	placebo injury group
K_2HPO_4	dipotassium hydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
LIF	leukaemia inhibitory factor
LTB_4	leukotrienes
mATPase	myosin adenosine triphosphatase
M-cad	M-cadherin
$MgSO_4$	magnesium sulphate
MHC	myosin heavy chain
MHC_f	foetal myosin heavy chain
MI	mild injury
MIF	migration inhibition factor
MPCs	myogenic precursor cells
MRFs	myogenic regulatory factors
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
NCAM	neural cell adhesion molecule
NO	nitric oxide
NOS	nitric oxide synthase
NSAIDs	nonsteroidal anti-inflammatory drugs

O2·⁻	superoxide
OH·	hydroxyl radical
OPC	oligomeric proanthocyanidin
ORAC	oxygen radical absorbance capacity
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PGE$_2$, PGF$_2$α	pro-inflammatory prostaglandins
RICE	rest, ice, compression and elevation treatment
ROS	reactive oxygen species
SC	satellite cells
SD	standard deviation
SEM	standard error of the mean
SI	severe injury
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TGFβ	transforming growth factor beta
TNFα/β	tumour necrosis factor alpha or beta
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TxA$_2$	thromboxane A$_2$
VEGF	vascular endothelial growth factor
WBC	white blood cell
w/v	weight per volume

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

According to published studies in the United states of America [1-3], contusion and strain injuries account for approximately 90% of all sports-related injuries and contusion caused by a blunt, non-penetrating object is the most frequent type of injury [4] reported in athletes – contributing up to 60% of all reported injuries. Muscle groups in the arms, hands, legs, feet and buttocks are most commonly affected [5], and apart from the expected mechanical damage to muscle cells themselves, at a microstructural level, skeletal muscle contusion injury involves capillary rupture and infiltrative bleeding, oedema, and inflammation. These changes may lead to haematoma (blood clot) formation and can cause compartment syndrome in areas where volumes are limited by fascial planes [6]. This phenomenon is characterised by severe pain. In addition, oxygen and nutrients are prevented from reaching nerve and muscle cells [5, 6] due to the disrupted blood supply, thus exacerbating the extent of injury and the ability to resolve the injury. The magnitude of the inflammatory response and the time it takes for muscle to heal largely depends on the severity of the injury and the degree of vascularisation of the tissue [7].

Interactions between the immune system and skeletal muscle may play a significant role in modulating the course of both the muscle injury and the repair process after a contusion injury. As a result of muscle injury and capillary rupture, mast cells present within the damaged area release histamine, which causes an increase in blood flow to the site of injury, thereby allowing more blood-borne inflammatory cells

to gain direct access to the site of injury [8]. In the early acute phase following an injury to skeletal muscle, neutrophils are the most abundant immune cells at the injury site, but within the first day neutrophil numbers start to decline and the number of macrophages increases [9].   However, neutrophils remain functionally active and their numbers elevated from baseline at the site of injury for approximately 5 days, after which their activity gradually returns to pre-injury levels. Although the function of neutrophils in response to muscle injury is well described (see later), the specific roles for macrophages *in vivo* are poorly understood [10, 11]. In addition, although it is generally accepted that cytokines (secreted by neutrophils and macrophages) control the events following an injury, the exact role players and their respective contributions remain unclear, since e.g. damaged skeletal muscle may itself also produce cytokines and the roles of particular cytokines are difficult to unravel.

Satellite cells, a population of mononucleated progenitor cells specific to skeletal muscle, also play an important role after injury, in replacing or repairing the damaged muscle fibres. Satellite cells are located on the surface of myofibres but beneath the basement membrane [12-14]. When the muscle is injured, satellite cells become activated and start to proliferate, differentiate and fuse either with other myoblasts, or with damaged muscle fibres [15, 16]. In mature skeletal muscle, satellite cells are normally quiescent, but are activated not only in response to muscle damage, but growth and hypertrophy as well [14]. The most reliable way to identify satellite cells is on the basis of their position using various forms of microscopy with or without the use of antibodies raised against proteins expressed by the satellite cells themselves, or against adjacent structural proteins. Therefore much interest has been placed on

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antibodies to identify specific proteins in quiescent and activated satellite cells *in vivo* [17, 18].

While the clinical aspects of a contusion injury have been well documented in the sports-related clinical literature, symptoms of contusion injuries are often non-specific and include soreness, pain with active and passive motion, and a limited range of motion [19]. Despite occurrence of these debilitating symptoms, the large variability in the severity of injury and the multiple underlying processes complicate research efforts, so that an universally accepted treatment modality is still elusive [6, 20]. One way of treating skeletal muscle contusion injury could be through the diet. Diets rich in fruits and vegetables supply antioxidants [21], which can then convey protection against reactive oxygen species (ROS) generated in both the skeletal muscle and other cell types after sustaining a contusion injury [22]. However, before attempting to find a remedy for this problem, a better understanding of the mechanisms and role players involved in the healing of muscle after injury is required, as well as a reproducible model of contusion injury in which all these factors can be fully researched.

1.2 Skeletal muscle

1.2.1 Introduction

Skeletal muscle, a complex structure consisting of muscle cells, organized networks of nerves and blood vessels, and an extracellular connective-tissue matrix, represents the largest tissue mass in the body, constituting approximately 40 to 45% of total body weight in males (healthy young male adults,weight: 60-80 kg; age: 20-

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35 yr) [23]. This framework is necessary to produce joint movement and locomotion, for postural behaviour as well as for breathing [24]. However, skeletal muscle is susceptible to injury, which results in the production of free radicals by neutrophils and macrophages [25], and if not repaired properly, a loss of muscle mass, locomotive deficiency and in the worst cases lethality may occur [24]. One possible reason for the body not repairing itself completely, might be inadequate intake of essential vitamins and minerals [26]. However when the body has an adequate intake of supplements, the body repairs itself after injury, through a process known as skeletal muscle regeneration. This process is finely regulated by various cellular responses, and supports skeletal muscle following injury to prevent any of the above mentioned detrimental outcomes [27].

1.2.2 Basic structure and function

Muscle cells are in many ways similar to any other bodily cell, but because skeletal muscle cell function is highly specialised to produce force and movement [28], the cellular components responsible for maintaining this function must also be highly specialised [29].

A cross section of muscle at various areas through the basic structures of the muscle indicates the three types of connective tissue which surrounds the muscle (see figure 1.1). Each muscle is surrounded by a connective tissue sheath, the epimysium, which is surrounded by fascia, separating the muscles from one another. Portions of the epimysium project inward to divide the muscle into compartments, each compartment containing a bundle of muscle fibres called a fasciculus.   The fasciculus is surrounded by a layer of connective tissue called the perimysium

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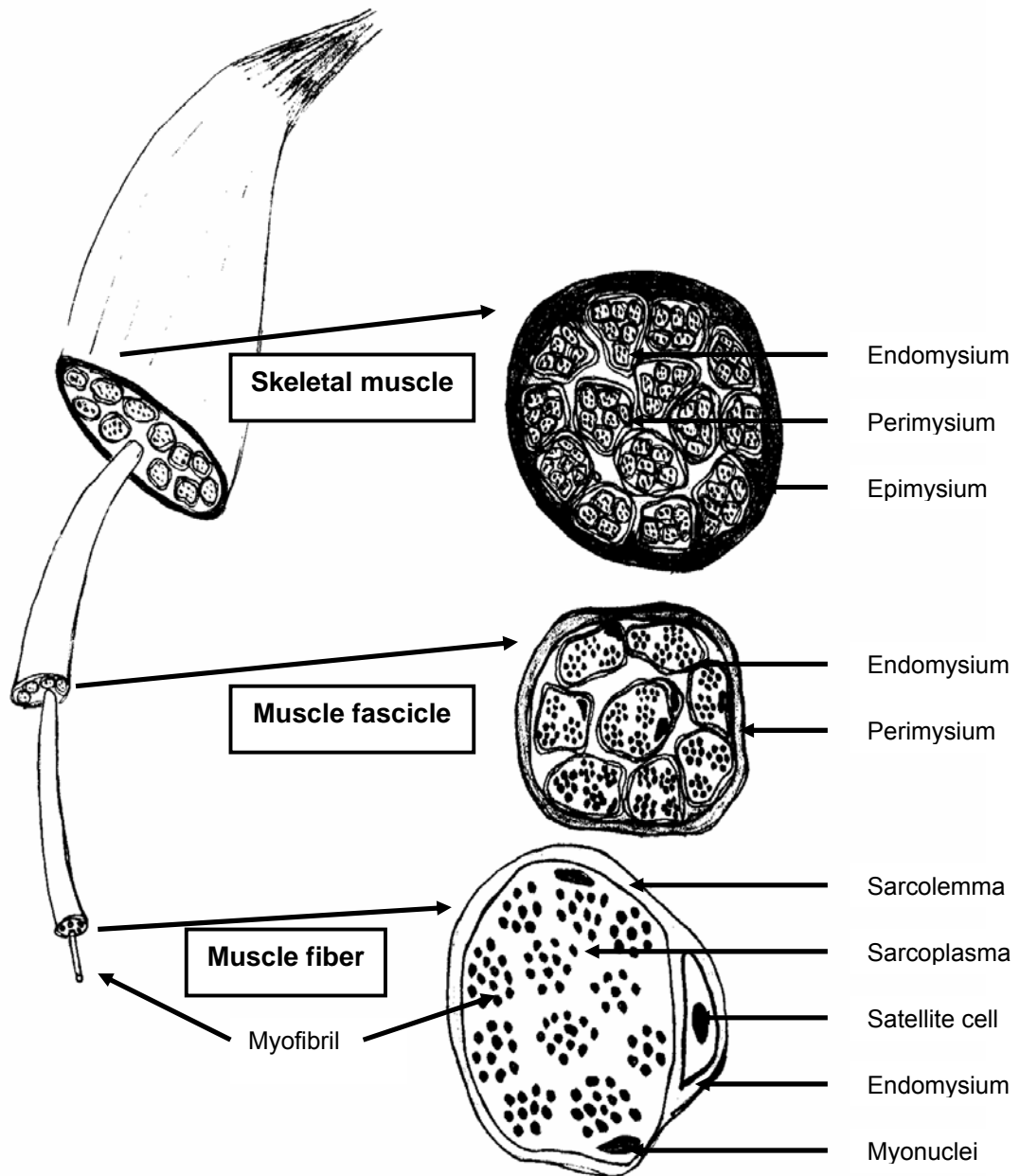
Figure 1.1: Schematic representation of skeletal muscle showing the gross components. Modified from Martini (1998) [29].

containing muscle fibres, each of which is a single cell surrounded by the endomysium. Muscle fibres (groups of muscle cells, also knows as myofibres) have an elongated, cylindrical shape, and are multinucleated.   The nuclei of these myofibres (myonuclei) are located just under the plasma membrane, with the central part of the muscle fibre free of nuclei [29] (Figure 1.1). Scattered satellite cells lie between the endomysium and the muscle fibres and function to repair damaged muscle tissue (discussed in detail later) [14].

1.2.3 Skeletal muscle fibre types

The existence of different fibre types in skeletal muscle is readily apparent and has long been recognized by the development of highly sensitive enzyme assays. One of these assays make use of adenosine triphosphate (ATP) utilizing enzymes that operate during contraction and relaxation [30, 31]. The muscle fibres are extremely adaptable, and although the fibre type distribution is genetically determined at birth, an appropriate training programme will have a major effect on the metabolic potential of the muscle, irrespective of the fibre types present [32].

Myosin is the largest and hence the most abundant contractile molecule in mammalian skeletal muscles. This polymorphic molecule exists in various different isoforms. These isoforms can be separated based on their electrophoretic mobility or, based on histochemical staining using the myosin adenosine triphosphatase (mATPase) assay into various myosin heavy chain (MHC) isoforms. In rodents, the MHC I is expressed in type I fibres (slow twitch-fatigue resistant), MHC IIa in type IIA (fast twitch-fatigue resistant), MHC IIb in type IIB (fast twitch-fatiguable) and MHC IIx or d in type IIX fibres (fast twitch-fatiguable) [33]. Adult human skeletal muscle on

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the other hand appears to express each of these muscle isoforms, with the exception of the fast type IIb MHC isoform. Although Weiss *et al.* (1999) recently cloned the human fast type IIb gene, it is expressed in selected muscle groups [34]. The biochemical characteristics of the three major fibre types, type I, IIA and IIB, are summarised in Table 1.1.

Table 1.1: Biochemical characteristics of human muscle fibre types.
The relative metabolic characteristics of type I, type IIA and IIB fibres are indicated. Adapted from Seiler (1996) [35].

Characteristic	Type I	Type IIA	Type IIB
Nomenclature	Slow Red Fatigue resistant Oxidative	Fast White Fatigue resistant Oxidative/glycolytic	Fast White Fatiguable Glycolytic
Capillary density	High	Intermediate	Low
Mitochondrial density	High	High	Low
Oxidative capacity	High	High	Low
Glycolytic capacity	Low	High	High
Activity used for	Aerobic (long term)	Aerobic (short term)	Anaerobic (short term)
Force production	Low	High	Very high
Major storage fuel	Triglycerides	Glycogen	Glycogen

Depending on the kind of contractile activity the different skeletal muscles are required to perform, the fibres are able to respond to altered demand by undergoing a series of structural and biochemical adaptations, resulting in switching from one

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fibre type to another [36]. Conversion from type IIB to IIA, and IIA to IIB has been observed in humans in response to various stimuli. Type IIB conversion to IIA has been observed in response to different exercise regimes [37-40], whereas conversion from type IIA to IIB took place after detraining or denervation [41, 42]. Conversion from type I to IIX has also been shown to occur in human muscle after denervation [41], but this switch in fibre type took approximately 20 months, indicating that conversion between fast-twitch and slow-twitch fibres might involve more extreme adaptations. The mechanism for conversion from type II to I fibres still remains elusive, due in part to the inconsistency in results [43-48] and in part because the genes for the MHC I protein are situated on a different chromosome, while those for MHC IIa and b are close together on a single chromosome so that control of switching is a single process [49]. It has also been shown that hybrid fibres (containing more than one MHC isoform) exist in human, rat and dog muscle and are formed in response to exercise [50-52]. Putman *et al.* (2004) proposed that hybrid fibres might reflect fibres that are in the process of expressing either the one or the other MHC isoform whilst the previously expressed protein is still present [50]. On the other hand, Stephenson (2001) and Wu *et al.* (2000) are of the notion that the occurrence of hybrid fibres is an adaptation involving expression of both isoforms in order to provide the muscle with a wider variety of functional properties [53, 54].

Type I fibres are small diameter blood cells with a red colour that contain relatively slow acting myosin ATPases and hence contract slowly. The red colour is due to the presence of myoglobin, an intracellular respiratory pigment, capable of binding oxygen and only releasing it at very low partial pressure (as found in the proximity of the mitochondria) [55, 56]. Type I fibres have numerous mitochondria, mostly

located close to the periphery of the fibre, near the blood capillaries which provide a rich supply of oxygen and nutrients [57]. These fibres possess a high capacity for oxidative metabolism, are extremely fatigue resistant and specialised for the performance of repeated contractions over prolonged periods [55, 56].

Type IIB fibres are much paler than type I, because they contain little myoglobin. They possess more rapidly acting myosin ATPases and so their contraction (and relaxation) time is relatively fast. They have fewer mitochondria, a poorer capillary supply [57], but greater glycogen and phosphocreatine stores compared to the type I fibres. High activities of glycogenolytic and glycolytic enzymes cause type IIB fibres to have a high capacity for rapid (but relatively short-lived) ATP production in the absence of oxygen (anaerobic capacity). As a result lactate can accumulate quickly in these fibres. They also fatigue rapidly. Hence, these fibres are best suited for delivering more rapid, powerful contractions for brief periods. The metabolic characteristics of type IIA fibres lie between the extreme properties of the other two fibre types. They contain fast-acting myosin ATPases like the type IIB fibres, but have an oxidative capacity more akin to that of the type I fibres [55, 56].

Different myofibre subtypes are also detected during embryonic life [58], and patterning of fibre types within major muscle groups is established postnatally depending on the functional requirements of the muscle [59]. Most noticeably during the first postnatal week, the myosin heavy chain transition is complex and as many as five different isoforms are expressed concurrently in a particular muscle [60]. In fast-contracting rat muscle, neonatal myosin replaces the embryonic isoform and is the predominant fibre type by days 7-11 after birth, followed by the replacement of

the neonatal myosin by the adult fast isoforms [61]. Development of slow muscle fibres can occur by several pathways, but similarly involves myosin isoform transitions [62]. Studies on mouse [63], rat [64], and human [65] show that during the foetal stages of development, most fibres express the embryonic and neonatal myosin isoform, while a small percentage of fibres express the slow type 1 isoform. However, it was concluded in a study by McKoy *et al.* (1998) that this expression pattern of different myosin heavy chain isoforms is not related to muscle-specific activity [66]. As the animal matures, individual muscles become adapted to perform highly specialised functions by predominantly expressing one or two myosin isoforms. In early postnatal development the speed of contraction of slow muscle changes less than the fast. During the first 20 days of life, both fast and slow muscles of rats increase their speed of contraction significantly, and by day 30, each muscle fibre has stabilized at the adult value [60].

Staining muscle sections for mATPases histologically, clearly indicates the difference in Type I and Type II fibres according to their myofibrillar ATPase activity, since ATPases of different fibre types display differential pH sensitivity [30, 56].

1.3 Skeletal muscle injury and repair

1.3.1 Introduction

Tissues in the body are not isolated: they combine to form organs with diverse functions. Any injury to the body affects several different tissue types simultaneously, and these tissues must respond in a coordinated manner to preserve homeostasis [27]. The basal lamina, which surrounds muscle fibres plays

an important role during recovery from injury. In injuries where the basal lamina remains intact (e.g. contusion injury), recovery is relatively complete. Conversely, in cases where the basal lamina is destroyed (e.g. injury causing ischemia) [67], fibre regeneration first requires the laying down of a new scaffold by newly generated fibres with the help of satellite cells, in the early stages of recovery [28]. In both cases, recovery will take place, but the latter injury is more severe and recovery might take longer. After an injury, the healing of skeletal muscle can take several weeks and although injured muscles can initiate regeneration promptly, the healing process is often inefficient and hindered by the formation of scar tissue, which may contribute to the tendency for muscle injury to occur again [27]. The response of skeletal muscle following an injury will be discussed in more detail in the next section.

1.3.2 The muscle's response to injury

There are many ways in which muscle fibres can be damaged. External causes include contusion and laceration injuries to the body, or extremities of heat or cold, whereas internal causes include muscle tears and tendon ruptures following sudden forceful contractions [14]. The response of skeletal muscle to injury follows a fairly consistent pattern, irrespective of its cause. The response of muscle to contusion injury will be the focus of this thesis.

Usually, the underlying bone is not broken during a contusion injury, but when an injury results in the breaking of bone, the healing tissue (the bone) is identical to the tissue that existed there before. This process is termed regeneration. However, the healing of injured muscle may include the formation of a scar, which is termed repair

alongside the generation of new contractile tissue. Therefore, regeneration is not only used to refer to the healing of bone, but also to the healing of muscle after injury. Although these two processes are different from one another, the terms regeneration and repair are often used interchangeably by researchers [19].

Although the overall injury response process in skeletal muscle, is most correctly termed regeneration, it can be divided into three distinct phases (for review see Jarvinen *et al.* 2005) [19]. The first is actually a *destruction phase*, which is characterised by the disruption of the vasculature and muscle ultrastructure, followed by the formation of a haematoma, necrosis of the damaged muscle fibres, and a pro-inflammatory immune response. This is followed by the *repair phase*, which consists of numerous overlapping processes, including phagocytosis of the damaged muscle tissue, activation of satellite cells, production of a connective-tissue scar and capillary revascularisation. During the third and final phase, the *remodelling phase*, formation of new or regenerated muscle fibres reaches completion along with reorganisation of the scar tissue. These three phases are usually closely associated or overlapping as graphically illustrated in Figure 1.2, making temporal resolution difficult.

For the purpose of this thesis, further discussion of the events following an injury will focus on three main topics with particular reference to reactive species production: the first part will focus on the early events following a contusion injury (pro-inflammatory response included), the second part will focus on the resolution of inflammation and concomitant resolution of muscle damage, and the last part on the necessity of satellite cells in regeneration.

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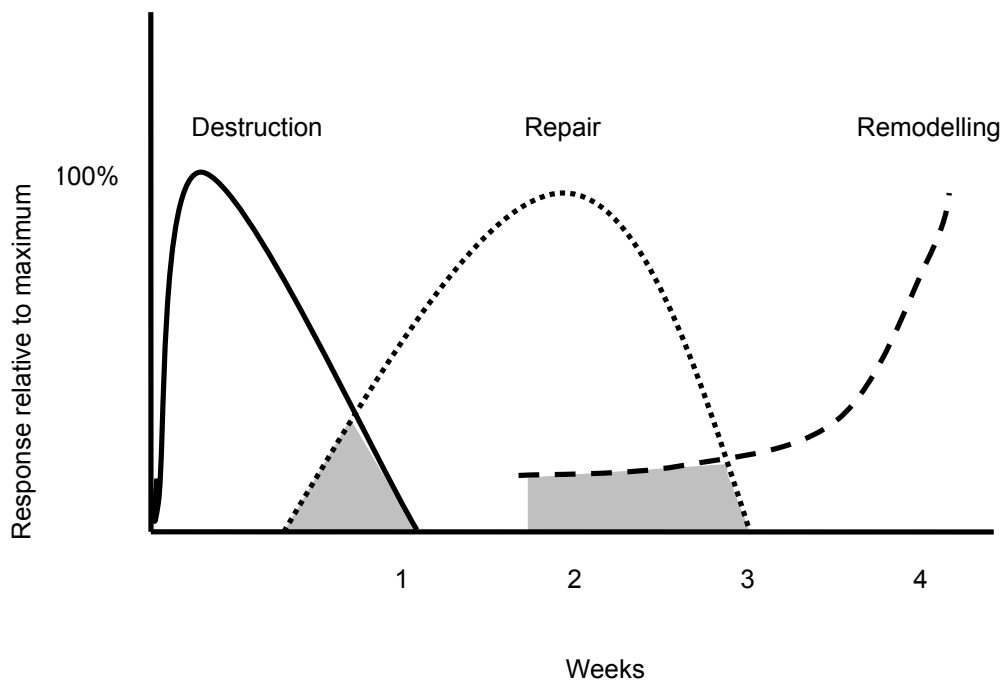
Figure 1.2: Time course of regeneration of injured muscle.

1.3.3 Inflammation, leukocyte infiltration and secondary damage: the early response

Following a mild contusion injury, the vasculature in the skeletal muscle is usually bruised but not disrupted, although the muscle fibres themselves rupture at or adjacent to the impact area [19]. Therefore, the arterioles within the injured area can dilate, in response to histamine release from mast cells as well as from activated platelets (both residing in the tissues near blood vessels), which in turn will increase the blood flow to the site of injury [68]. A second effect of this localised histamine release is an increase in capillary permeability at the site of injury, which causes the endothelial cells that line the blood vessels to contract, so that they round up and pull

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away from one another [69]. When the cells retract from one another, gaps are formed, known as capillary endothelial pores, that permit fluid and plasma molecules to flow freely from the bloodstream out into the tissues [69]. As a result, an increase in the numbers of phagocytic leukocytes and plasma proteins, both crucial to the inflammatory response [68], are seen in and around the damaged tissue [70]. This process does not damage the endothelial cells, and after roughly 1 hr, they spread back out and re-establish connections with their neighbours [69].

With severe injury, much of the vasculature is also extensively disrupted, exposing the collagen in the subendothelial layers of blood vessels, which occurs after the muscle fibres and sarcoplasm have ruptured and torn. Following this, a process of "damage control" is initiated immediately. Platelets adhere to the exposed collagen, become activated as a result and start to release pro-inflammatory mediators such as 5-hydroxy tryptamine (serotonin), histamine and thromboxane A_2 (TxA_2). Following formation of a platelet plug and the control of haemorrhage, blood-borne immune cells begin to migrate into the area of tissue damage [19]. A chronological illustration of white blood cell (WBC) involvement in the response to skeletal muscle injury is presented in Figure 1.3.

Immediately following the injury, neutrophils are the predominant cell infiltrate. For mild and moderate damage, they enter the injured area by way of rolling (via selectins), adhesion (via integrins) and migration through areas of intact capillary endothelium and across layers of basement membrane and sarcolemma by means of pro-inflammatory mediators (released by the activated platelets), as well as

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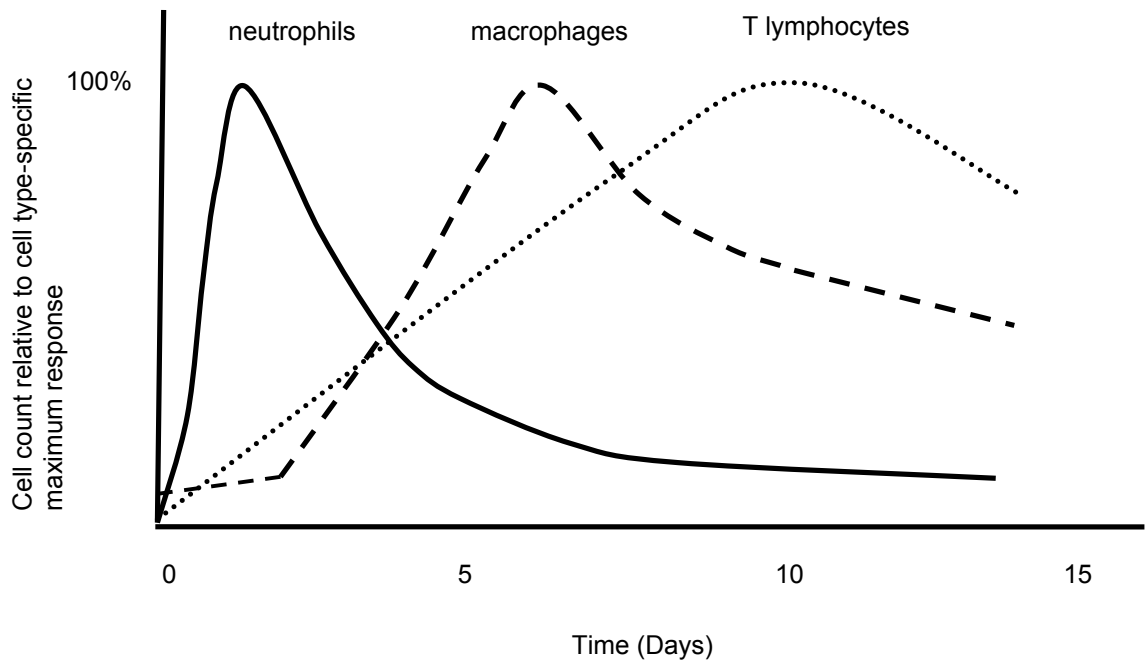
Figure 1.3: Illustration of chronological involvement of peripheral immune cells following a contusion injury. Cell counts are represented relative to the maximum response seen. The duration of increased cell numbers in circulation may vary depending on the extent of tissue trauma.

complement component C5 [71]. Although the literature does not clearly compare possible differences due to injury extent, it is likely that in severely injured muscle, neutrophils spill into the injured area from disrupted capillaries and adhere to exposed integrins in fragments of disrupted sarcolemma. Neutrophil infiltration can be detected in the damaged muscle within the first hour following injury. Neutrophil cell numbers peak in approximately 24-48 hr and can remain elevated for up to 5 days [11]. The dominance of neutrophils during the early phase after injury – relative

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to other immune cells – is partly due to the transfer into the blood of large numbers of preformed neutrophils (most abundant WBC in the circulation) from the bone marrow, and also an increase in production of new neutrophils in the bone marrow, stimulated by the release of chemical mediators from the inflamed region [11, 70], including complement components (C5), pro-inflammatory prostaglandins (PGE$_2$ and PGF$_{2\alpha}$) and leukotrienes (LTB$_4$) [72], as well as factors released by activated platelets (TxA$_2$, serotonin and histamine) [73].

Neutrophils contribute to the post-injury events in 2 ways: firstly, the invading neutrophils have a phagocytic function [74], clearing the wound of blood-derived fibrin [19] and necrotic debris and secondly they initiate the inflammatory process via the release of pro-inflammatory cytokines such as IL-6 and TNF-α [11, 75, 76].

During the early phase of inflammation, pro-inflammatory cytokines are secreted by not only neutrophils, but also activated macrophages which also migrated from blood into the damaged tissue [76]. This acute inflammatory response develops rapidly and only returns to baseline approximately 10-14 days later when the injured area becomes cleared of all damaged tissue [75, 76]. Table 1.2 summarises the different cytokines produced by the major cell types present in muscle and their involvement in muscle injury and repair processes. These data, and the numerous studies published on the topic, illustrate the complexity of the inflammatory process which involves several interrelated steps. Although IL-6 and TNFα are considered most frequently, other cytokines such as IL-1, -2 and IL-8 [10, 11] are also involved, as

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Table 1.2: Involvement of various cell types in inflammation and muscle injury.

Cell type	Cytokines/ Growth factors secreted			Injury-related activity	Reference
Neutrophils	IL-1 TGF-β	IL-6 TNF-α	IL-8	Source of pro-inflammory cytokines First leukocyte to infiltrate injury site Phagocytosis of necrotic myofibres and cellular debris	[76] [76] [11]
Monocytes/ Macrophages	FGF-2 IL-6 LIF	IGF-1 TGF-β	IL-1 TNF-α	Source of growth factors, cytokines & reactive species Sending survival factors to regenerative cells Promote muscle injury or proliferation *in vitro & in vivo*	[11] [19] [76]
Fibroblasts	IL-1	IL-6	IL-8	Produce chemotactic signals for circulating inflammatory cells Help formation of connective tissue scar	[19]
T lymphocytes	IL-1 TNF-α IFN-γ	IL-2 TGF-β TNF-β	IL-6 MIF	Involved in immediate hypersensitivity via IL-1 & 6 Involved in delayed sensitivity reactions via IFN-γ, TNF-β & IL-2	[10]
B lymphocytes	IL-1 TNF-β	IL-2	IL-6	Involved in antibody formation	[78]
NK cells	IL-1	TNF-β	IFN-γ	Causes an increase in lymphocyte concentration	[79]
Eosinophils	IL-6			Capable of generating reactive oxygen species	[80]
Platelets	TGF-β			Secretion of adherence factors (P-selectin) to help neutrophils gain access to site of injury	[81] [10]
Injured skeletal muscle cells	IL-1 FGF LIF	IL-6 IGF	TGF-β HGF	Release growth factors and cytokines which helps to activate regeneration	[19] [77]
Keratinocytes	IL-1	TNF-α		Act as "signal transducers", which converts exogenous stimuli into production of cytokines, adhesion molecules, and chemotactic factors	[82]

well as related cytokines such as leukaemia inhibitory factor (LIF) [11, 77], migration inhibitory factor (MIF) [10] and interferon γ (IFN-γ) [10].

Neutrophils are generally accepted to play the main role in the early inflammatory response to contusion injury, but the involvement of macrophages cannot be excluded. Phagocytosis is a vital process in the recovery from muscle injury, but it can damage the injured muscle even further, and may result in secondary damage to the healthy surrounding tissue by the generation of reactive species. The processes by which this secondary damage occurs are somewhat controversial, and the extent to which neutrophils and/or other cell types contribute is not completely understood. The role of neutrophils and macrophages in reactive species production will be discussed below, but first a brief overview on free radicals and reactive species is needed.

1.3.3.1 Free radicals and reactive species

A free radical is any atom (e.g. oxygen, nitrogen) with at least one unpaired electron in the outermost shell, and is capable of independent existence [83]. When a free radical reacts with a molecule that is a non-radical, the molecule becomes a new radical, and this can result in a radical chain reaction as further reactions with non-radicals take place [84]. As most biological molecules are non-radicals, the generation of reactive radicals *in vivo* will usually set off a chain of radical reactions. Although most of the biologically important free radicals and reactive species are derived from or are associated with molecular oxygen, they are not limited to oxygen species. Therefore, for the purposes of this thesis, reactive species will be used as

the collective term for all free radicals and other reactive species, including reactive oxygen species and reactive nitrogen species.

Reactive species are naturally formed within the body by various physiological processes to maintain homeostasis [85]. Reactive species can also be generated from exposure to certain chemicals, environmental pollutants, sunlight, radiation, burns, cigarette smoke, drugs, alcohol, viruses, bacteria, parasites, dietary fats, and more [86-88]. Interestingly, superoxide dismutase, an antioxidant in the body may result in free radical generation in a process known as the Fenton reaction [83]. Superoxide ($O_2^{\bullet-}$) is produced by the addition of a single electron to oxygen (Equation 1). As a result of a spontaneous dismutation reaction, which is catalysed by superoxide dismutase, superoxide will form hydrogen peroxide (H_2O_2) (Equation 2). Although hydrogen peroxide is less reactive than other oxygen-derived reactive species, it is a biologically important oxidant due to its ability to diffuse considerable distances from its site of production but also because it can react with reduced metal ions in the Haber-Weiss reaction (referred to as the Fenton reaction) when it is iron catalyzed (Equation 3) forming the highly reactive and damaging hydroxyl radical ($OH^{\bullet}$). In healthy humans, extra cellular fluids have essentially no transition metal ions that can catalyse free radical reactions. However, extracellular unbound iron may be increased in some cases, such as in iron-overload diseases or where iron intake is very high (as can occur through supplementation) and this free iron is then available to drive the Fenton reactions.

$$O_2 + e^- \rightarrow O_2^{\bullet-} \qquad\qquad\qquad\qquad\qquad\qquad \text{Equation 1}$$
$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad\qquad\qquad\qquad\qquad \text{Equation 2}$$
$$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^- + Fe^{3+} \qquad\qquad\qquad \text{Equation 3}$$

Thus the incomplete reduction of oxygen may result in the formation of superoxide radical, hydrogen peroxide, and hydroxyl radical. However, within the cell, antioxidants can also protect against oxidative damage at different levels including preventing radical formation, intercepting formed radicals, repairing damage caused by radicals, eliminating damaged molecules and preventing mutations from occurring [26]. The role of antioxidants will be discussed in detail later.

Reactive species have been implicated in the pathogenesis of a wide spectrum of diseases as well as in the aging process [89]. In addition to these primary sources of reactive species, they can also be formed in the haematoma after sustaining an injury, exacerbating the muscle necrosis by extending the zone of injury to include neighbouring healthy myofibres [19]. These secondary sources of reactive species, may amplify the body's general inflammatory response and promote further cell injury [89]. A number of other secondary sources of reactive species within muscle are likely to be important after the onset of damage (e.g. contusion injury) initiated by other mechanisms. This secondary generation of reactive species may be important in spreading and worsening the damaging processes, or may merely be part of the body's adaptive responses to ensure that efficient preparation of the damaged tissue allows regeneration to occur [90]. Other sources of reactive species, apart from the reactive species generated by free iron in the Fenton reaction, include: reactive specie generation by phagocytic white blood cells, mainly neutrophils and macrophages following injury.

1.3.3.2 Reactive species generation by phagocytic white cells and macrophages

It is clear that substantial injury to muscle fibres is followed by the invasion of the area by macrophages and other phagocytic cells from the blood and interstitium [9]. These infiltrating cells appear to be essential to prepare the tissue to allow for fast, effective regeneration to occur. As part of the phagocytic process, they release substantial amounts of reactive species to aid in the degeneration of necrotic areas, but can also contribute to damage of surrounding healthy tissue [91]. This increase in reactive species generation is independent of the type of tissue injury and will occur in all tissue damaged *in vivo*. Thus, direct trauma to muscle during exercise or by means of contusion can cause damage that will eventually lead to a secondary increase in intramuscular reactive species generation from phagocytic cells [90, 92].

An *in vivo* study by Nguygen and Tidball (2003) shows that neutrophils lyse muscle cells through superoxide-dependent mechanisms [93], which is consistent with other *in vitro* findings [94-96]. However, no significant lysis through superoxide-dependent mechanisms was detected when macrophages were also present at macrophage/neutrophil ratios that usually occur in muscle injury and inflammation. Furthermore, the addition of low concentrations of neutrophils (lower than the usual concentrations required after muscle injury) to macrophages in muscle co-cultures resulted in the activation of more macrophages, thereby promoting the overexpression of nitric oxide [93]. Also, the presence of muscle cells increased nitric oxide production by macrophages even further, suggesting that there might be a feedback mechanism promoting the production of nitric oxide by macrophages [93]. However, it should be mentioned that the ratio of neutrophils to macrophages

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used in the study by Nguuyen and Tidball (2003), is not the same as it would be *in vivo*.

Macrophages also increase muscle membrane (sarcolemma) lysis *in vivo* in the mdx mouse model of muscular dystrophy. In the presence of macrophages, an increased susceptibility of the cell membrane to mechanical damage during muscle contraction, which leads to muscle inflammation and membrane lysis, were apparent [97]. In another study, production of macrophages was inhibited by injecting mdx mice with an antibody (anti-F4/80), depleting the muscle of macrophages and therefore reducing the amount of nitric oxide produced. This led to an 80 % reduction in sarcolemma lysis *in vivo* [98]. These findings show that not only do neutrophils play a major role in promoting secondary muscle damage, but macrophages also contribute in promoting muscle damage through the increased production of reactive species (nitric oxide - NO) apparent after injury. Although not mentioned in the vague studies above, it is also possible that other membranes beside the sarcolemma could be involved e.g. the mitochondrial membrane and nuclear envelope. However, no evidence has been provided to include these membranes mentioned above.

1.3.4 Resolution of skeletal muscle damage: the late response

Initiation of injury resolution may be defined as the point in time following the injury when the number of neutrophils in the region of damage begins to decrease [19]. A concomitant rise in the number of macrophages is seen with this decline in neutrophil number. The recruitment of circulating macrophages to the site of injury is secondary to chemotactic factors released by both platelets and neutrophils [9, 19].

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The main function of macrophages is thought to be the removal of damaged muscle fibres from the tissue by means of phagocytosis [99, 100]. Approximately nine different macrophage subtypes exist [76], but these subtypes will not be discussed further, as this is not the main focus of this thesis. Although the exact contribution of the different macrophage subtypes to the healing process is unclear, a number of studies reported a beneficial role for these macrophages in regenerating muscle fibres, suggesting that these macrophages may be involved not only in the pro-inflammatory process but maybe also serve as a major source of growth factors and cytokines that promote healing [101]. One specific aspect of macrophages' beneficial role has previously been discussed in section 1.3.3.2 where it was reported that when the numbers of neutrophils decrease and macrophages increase (as happens during the late phase), the production of NO by macrophages is increased, with no significant muscle cell lysis occurring [93].

Not only do macrophages and neutrophils play an important role in the healing of injured skeletal muscle, but satellite cells also contribute significantly to skeletal muscle regeneration. Indeed these satellite cells may be the mechanism mediating the positive effects of the growth factors. The different cells supplying growth factors can be seen in Table 1.2 and their effect on satellite cells will be explained in the next section.

1.3.5 Involvement of satellite cells during skeletal muscle regeneration

Adult mammalian skeletal muscle is a stable tissue with little turnover of nuclei; only approximately 1-2 % of myonuclei (nuclei of muscle fibres) are replaced each week [102]. Nonetheless, mammalian skeletal muscle has the ability to regenerate injured

myofibres after extensive muscular damage through the involvement of satellite cells.

As mentioned earlier, satellite cells are a population of mononucleated progenitor cells specific to skeletal muscle and reside under the basal lamina and one of the layers of the sarcolemma in mature and growing muscle (see figure 1.1 for detail). In contrast to myonuclei, satellite cells are quiescent under normal circumstances. In response to muscular trauma, enhanced recruitment and activation of satellite cells is evident [12-14]. Upon muscle injury, reactive species (refer to section 1.3.3) are formed when satellite cells exit their normal quiescent state, become activated and start proliferating to form adult myoblasts, also known as myogenic precursor cells (MPCs) [16]. The resulting myoblasts, after several rounds of proliferation, differentiate and can either fuse with other myoblasts to form new myofibres or with damaged fibres in order to repair them [15]. It should be noted that satellite cell activation is not restricted to the damaged area – many satellite cells within a fibre get activated, even when only one end of the myofibre is injured, compared to when the whole fibre is damaged [103, 104]. However, recruitment of satellite cells from adjacent muscle fibres is very rare and thought to only take place if the connective tissue between the muscle fibres is also damaged [103, 104]. Following satellite cell activation, the proliferation and differentiation cycle is characterised by expression of the myogenic regulatory factors (MRFs). These MRFs consist of MyoD, Myf5, myogenin and MRF4. MyoD and Myf5 are also expressed during muscle development, and are involved in the determination of the myogenic lineage. They are also expressed early during satellite cell activation and proliferation. Myogenin

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and MRF4 are expressed later as myoblasts progress through differentiation and are thought to act specifically as differentiation factors [105].

Skeletal muscle regeneration is a highly orchestrated process, which is regulated through mechanisms involving cell-cell and cell-matrix interactions as well as extracellularly secreted factors [24]. Mechanisms that are controlled or altered by growth factors (Table 1.2) include satellite cell activation, migration to the injury site, proliferation of satellite cell-derived progeny MPCs and the differentiation of these MPCs into myotubes and myofibres. *In vitro* studies making use of monolayer cell lines in culture have identified several factors responsible for the activation of satellite cells [106-108]. These include fibroblast growth factor (FGF) [106-108], hepatocyte growth factor (HGF) [109], insulin-like growth factor (IGF) [110, 111], leukaemia inhibitory factor (LIF) [112, 113], vascular endothelial growth factor (VEGF) [114] and interleukins, including IL-6 and IL-1 [112, 113], and nitric oxide (NO) [115]. All these role players are involved in maintaining a balance between proliferation (growth) and differentiation of satellite cells to restore normal muscle architecture. The next section will focus on the interaction between NO and satellite cells.

1.3.5.1 Satellite cells regulate reactive species production

Muscle-derived nitric oxide appears to be a particularly important regulator of muscle inflammation and muscle damage by invading inflammatory cells. Due to the fact that satellite cells are positioned so close to fibres and often stay attached to the external lamina as the sarcolemma buckles after injury [116], they are ideally positioned to be the "first responders" to an injury-induced release of nitric oxide from

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nitric oxide synthase (NOS).   Anderson (2000) demonstrated that satellite cell activation that occurs immediately upon muscle injury, is mediated by nitric oxide released from circulating macrophages and is ultimately required for muscle hypertrophy [117].   However, some evidence suggests that the effect of nitric oxide is concentration and time dependent, evident only at the onset of differentiation, and directed on the fusion process itself [118].   Also, recent investigations have provided new insights into specific mechanisms through which both satellite cells and muscle fibres can regulate reactive species production by inflammatory cells, contributing to either muscle injury or growth.   Satellite cells themselves, after activation and prior to proliferation, also release factors which attract more macrophages and monocytes to the injured area, these cells being responsible for the production of more nitric oxide [119].   The build-up of huge quantities of nitric oxide could result in nitric oxide-dependent cytotoxicity, as described earlier.

1.3.5.2 Satellite cell identification

According to the literature, adult muscle satellite cell nuclei represent about 3-6 % of all muscle nuclei.   From animal experiments, it has been reported that their percentage differs in different types of muscles and muscle fibre types and may also differ between animal species [120].   Satellite cells are therefore associated with all muscle fibre types, albeit with unequal distribution.   Higher numbers of satellite cells are found adjacent to slow-twitch muscle fibres in comparison to fast-twitch muscle fibres [120].   Increased density of satellite cells have also been identified at the motor neuron junctions and adjacent to capillaries, suggesting that some factors originating from these structures may play a role in either homing satellite cells to specific locations or in regulating the satellite cell pool by other means [24].

Therefore, although satellite cells are so widely distributed, the most reliable way to identify satellite cells is on the basis of their position, situated between the sarcolemma and basal lamina of skeletal muscle, using fluorescence or electron microscopy. Although different markers have been introduced for satellite cell identification, such as M-cadherin (M-cad) [121], NCAM (CD56) [122], CD34 [123], Pax7 [124], c-met [15], myoD [125] and myogenin [126], direct comparison of identification by these different markers has not been done. Therefore it is difficult to judge which marker stains all satellite cells. The satellite cell markers used for the purpose of this study will be discussed below.

M-cadherin

One of the most widely used markers for satellite cells appears to be the cell surface protein M-cadherin (M-cad) which is located at the interface of the satellite cell and the underlying myofibre [12, 13]. M-cad, a particular member of the cadherin family, has been identified in skeletal muscle cell lines *in situ,* in prenatal and postnatal skeletal muscle, and in developing and regenerating muscle [121, 127-129]. The cadherins are members of a multigene family of transmembrane calcium-dependent intercellular adhesion molecules which influence morphological processes such as tissue development and maintenance, and in particular the establishment of intercellular junctions [128, 130].

Functional assays performed in cell culture have implied a role for M-cad during fusion of mononucleated myoblasts into multinucleated myotubes [130], a process which involves extensive changes in components of the cytoskeletal network. In line with this are observations showing that M-cad expression was upregulated during

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myotube formation during development and although expression declined after completion of this process, it was still present in quiescent satellite cells [123, 131-133]. But not only is M-cad expressed in quiescent satellite cells and just prior to fusion, M-cad[+] labeling is also seen in activated satellite cells, and according to Cooper *et al.* (1999) it is a reliable marker staining all satellite cells [125]. It has also been shown that in activated satellite cells in regenerating muscle (in adult Sprague-Dawley rats and BALB C and CBA inbred mice), M-cad expression is markedly induced, suggesting a functional role in the repair process rather than simply a marker carried over from development [121, 133]. However, although it has been postulated that M-cad may be essential for the fusion of myobasts into multinucleated myofibres [130, 134], Hollnagel *et al.* (2002), using M-cad-knockout mice, found that M-cad is not required for normal skeletal muscle development [135]. Interestingly, the satellite cells in these mutant mice proliferated and formed myotubes in culture and also contained increased amounts of N-cad, suggesting that this molecule may substitute for the lack of M-cad.

It has been proposed that satellite cells contain a subpopulation of cells with stem-like characteristics that serve to replenish the satellite cell compartment, and this is the subject of a review by Zammit and Beauchamp (2001), although there is no evidence to confirm this [18]. Kuschel *et al.* (1999), used a satellite cell culture model within normal and denervated muscle [136], in order to characterise the proliferative and differentiative potential of satellite cells. In this study it was demonstrated that in normal muscle, a second satellite cell phenotype exists in addition to the proliferating-differentiating compartment, which is M-cad[+]/myogenin[-] and which is therefore non-differentiating. The reliability of M-cad as a molecular

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marker of satellite cells has however also been questioned [15]. Cornelison *et al.* (1997) found that M-cad mRNA was only present in a small subset of satellite cells at early times after myofibre explant, and not in all quiescent satellite cells. After activation however, M-cad was expressed by all activated satellite cells. Therefore care should be taken when interpreting results using M-cad as a molecular marker staining all satellite cells.

Subsequently, Wernig *et al.* (2004) also investigated the proportion of M-cad$^+$ satellite cells present in normal mouse muscle. Of importance is that these researchers observed that approximately 94-100 % of all quiescent satellite cells are M-cad$^+$ [137], indicating that M-cad is a reliable marker of quiescent satellite cells. However, they also concluded that although M-cad is present in most quiescent precursor cells, the small fraction of the satellite cell pool which is M-cad$^-$ cannot be excluded, as was previously claimed by Beauchamp *et al.* (2000).

CD34

Structurally, CD34 is a highly O-glycosylated, transmembrane sialomucin, expressed by haematopoietic stem cells (HSC) and progenitors [138] and by small-vessel endothelium [139]. The expression of CD34 on quiescent adult skeletal muscle satellite cells extends the role of CD34 as a marker in the field of progenitor cell biology. CD34 expression has also been associated with activation and progress towards self-renewal or differentiation [140]. Recently the status of CD34 as a marker of quiescence has come into question, because of the identification of no or very low levels of CD34 in HSCs [141]. In quiescence, CD34 is expressed in a truncated form, but following activation there is expression of the full length CD34

isoform. Taken together, it seems therefore likely that these two isoforms of CD34 could have distinct roles in the maintenance and activation of quiescent lineage-primed progenitors during adult tissue renewal and regeneration. Co-expression of CD34 with M-cad is restricted to the myogenic lineage, suggesting that in adult skeletal muscle, CD34 does not mark stem cells but is expressed by precursors that are committed to a specific fate and have become arrested and held in reserve for subsequent activation [123].

However, although most myogenic cells are CD34^{+}, it is considered not to be a useful marker for satellite cells on tissue sections, because CD34 is also present on many cells of the vasculature and on HSC [18]. Nevertheless, CD34 is still widely used as a marker for quiescent satellite cells, but making use of morphological features at the same time which may be considered subjective. Therefore care should be taken when interpreting results using CD34.

CD56/NCAM

The CD56 antibody recognizes the neural cell adhesion molecule (NCAM), expressed during developmental myogenesis and in adult muscle satellite cells [142]. It also stains nerves, neuromuscular junctions and the cytoplasm of an occasional myofibre [122, 143]. According to various researchers, satellite cells are closely attached to the plasma membrane of the adjacent muscle fibre and express the adhesion molecules NCAM and M-cad in the quiescent state [116, 121, 129]. CD56 is also considered to be one of the most useful markers for satellite cells as it binds to both quiescent and activated satellite cells without binding to myonuclei [144].

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Due to the dynamic nature of the satellite cells, various immunohistochemical markers, as mentioned above for CD34, require additional information on the specific location of the marked cell. M-cad is also one such marker, which by some scientists is believed to be reliable, at least in murine muscle (no mention of reliability in rodent muscle).

Summary

Although our knowledge on the roles of various immune and satellite cells after injury is growing, several confounding factors contribute to our lack of understanding of the exact roles of these cells in damaged tissue after a contusion injury or injury of any kind. These include inter-individual variations in the severity of injuries that occur in humans, as well as the invasive nature of the methods of inflicting injury in some models. In the next section, animal models of muscle injury (invasive and non-invasive) will be described, with particular emphasis on the variability between the different models and the suitability of each for inflammation-related research.

1.4 Contusion injury models

1.4.1 Introduction

Skeletal muscle contusion injury is a proven method of inducing mechanical injury in skeletal muscle [145-147]. However, despite being reliable and standardised, several factors could lead to variation in injury severity from researcher to researcher. Technical considerations relevant to this model, which may influence the physiological results obtained, are discussed in the next few paragraphs.

1.4.2 Invasiveness of injury model

The model most commonly described in the literature makes use of a single impact trauma to the muscle, either with or without prior surgical exposure of a selected muscle group [145-150].   For example, the mass-drop injury model originally described by Stratton *et al.* (1984) [151] involves dropping a solid weight with a flat impact surface (varying in diameter and mass) from various heights onto the specific muscle (See Figure 1.4 for a representative illustration).
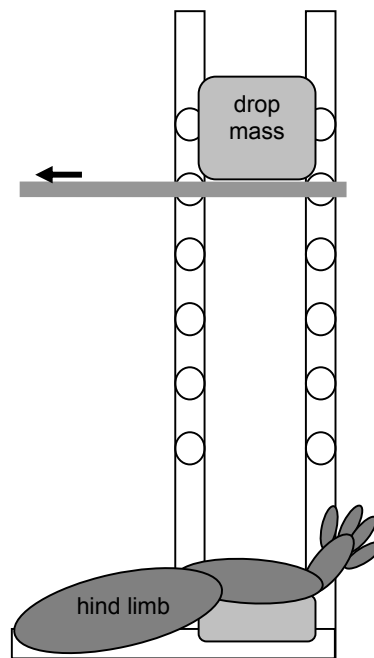
Figure 1.4: Representative illustration of a non-invasive, standardised 'mass-drop injury jig'.

Both localised and systemic inflammation have been studied using the invasive version of this model [145]. Although changes in skeletal muscle expression of different pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) were reported, the lack of sham operated groups resulted in failure to account for cytokines released as a result of the surgical procedure itself [145, 148]. Therefore, ideally, one should choose a non-invasive model of injury, 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.

A variation of this model is to place a heavier weight on the muscle, but with no impact force (i.e. no impact damage) and leave it in place for a given period of time, usually two or more hours [152, 153]. Apart from this model resulting in more severe damage due to longer-term occlusion of blood flow, it more closely simulates muscle injury incurred in accidents where the subject is trapped, and is therefore not ideally suited for studying sports-related injuries [154].

Forceps crush injury is another invasive model of contusion injury. Prior to the injury, the muscle is surgically exposed (as with invasive mass-drop), placed between the jaws of forceps and then bruising is achieved by pinching the forceps manually or by dropping a weight onto the forceps. Apart from the invasiveness of this method, manual contusion injury is difficult to deliver reproducibly. Despite these complications, this model has been used, with success, to study skeletal muscle regeneration following a contusion injury [149, 150, 155].

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1.4.3 Characteristics of drop-mass weight

Both murine and rat models have been used to study contusion injury. Given the difference in body size between mice and rats, it is not unexpected that the mass of drop-weights and hence the impact force used to induce injury also differed. The specific protocols previously used in rats to induce contusion injury with the mass-drop technique were either relatively heavy weights from a relatively small height (640 and 700 g from 27 and 25 cm respectively) [151, 156-158], or a smaller weight released from a greater height (171 g from 102 cm) [1, 159-161]. Injuries to mice have also typically been induced using one of these strategies [145, 148, 162].

The impact surface influences the impact area size and severity of the skeletal muscle injury. When a weight with a flat impact surface is used, an injury of uniform severity is achieved, but if a weight with a spherical impact surface is used, the injury induced is more severe in the middle of the impact area compared to the periphery. The infiltration of immune cells into the site of injury was reported to be more concentrated to the middle of the injury when a spherical impactor tip was used than with a flat surface [6, 159, 162]. The diameter of the impact surface of weights previously reported in the literature also varies, which will also influence the impact area. If a mass with a larger impact surface hits the muscle belly, the injured area would be wider than a mass with a smaller impact surface. Therefore both characteristics, the shape and diameter of the impact surface, will influence the pattern of infiltration of the immune cells, and thus also impact on secondary damage.

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1.4.4 Selection of muscle group

Due to the different metabolic phenotypes seen in different muscle fibre types, the degree of vascularisation differs between fibre types. Type I fibres have a high demand for oxygen and show a high degree of vascularisation, which provides a rich source of oxygen and nutrients to the fibres. In contrast, type IIB fibres are oxygen independent, with a reduced capillary supply when compared to the type I fibres [163]. Although whole muscles most frequently contain a mixture of these two and an intermediate fibre type, the proportions in which they are found differ substantially between different muscles and can also differ between different species or individuals within a specific species [164]. To date, the majority of research on skeletal muscle crush injury has made use of the *gastrocnemius* muscle [1, 6, 156-158, 160-162, 165, 166], or the *tibialis anterior* muscle [148, 150, 153, 155]. Both of these muscles possess type II fibres, but only the *gastrocnemius* muscle is a mixed muscle, also containing type I fibres [167]. In addition, the *gastrocnemius* muscle is relatively large, which makes it possible to injure the muscle without bone involvement. The belly-shaped/fusiform nature of *gastrocnemius* muscle is similar to that of the biceps muscle, which frequently suffers contusion injuries in many contact sports, particularly the game of rugby. Therefore, in our opinion, the *gastrocnemius* muscle is the better candidate to use when studying sports-related injuries.

With regard to the immune and cytokine system, it has been shown that muscle fibres secrete cytokines in a fibre-type specific manner. For example, in the *triceps, vastus* and *soleus* muscles of humans at rest, TNF-α and IL-18 were expressed in type II fibres only, while IL-6 expression was more prominent in type I than in type II [168].

1.4.5 Contractile status of the muscle

The contractile status of the muscle at the time of injury has been shown to influence the muscle's susceptibility to injury [159]. In this study, muscle was shown to be less susceptible to injury when in the maximally contracted state. However, the extent of muscle relaxation was also a factor. Muscles were only fully relaxed after general anaesthesia – highlighting the importance of similar protocols for animal treatment during such experiments.

In sports, contusion injuries occur both during practices and in competition. Thus, all contusion injuries will be accompanied by recent prior exercise and, if not severe, also by subsequent exercise. Treatments to augment the normal repair and regenerative processes are important to a wide variety of patients ranging from elite athletes to the elderly, who want to return to their previous levels of function as quickly as possible.

1.5 Current and experimental therapeutics of skeletal muscle injuries

1.5.1 Introduction

Blunt-force muscle trauma is frequently seen in contact sports in which opposing players cause a muscle contusion by direct impact on the muscle belly. This 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 [169]. Traditionally, treatments have focused on conservative techniques such as rest, ice, compression, and elevation (RICE), heat, or immobilisation. Other treatment options include continuous passive motion machines and anti-

inflammatory or other drugs [2]. Some of the different treatments mentioned above will be discussed in detail below.

1.5.2 Cryotherapy

The application of ice (cryotherapy) facilitates recovery from contusion injuries as determined by decreased nerve conduction, reduced muscle spasms, and limited oedema [170], resulting in a decrease in the severity of local cellular damage by restricting the haemorrhage and reducing the metabolic demands of the injured tissue after injury [171]. However, the exact mechanism associated with the therapeutic efficacy of this treatment is still unclear. In contrast to the use of cryotherapy, some researchers believe that application of heat to the site of injury, accompanied by careful passive and active stretching, has a better effect on healing [172-174]. According to their reports, warm muscles have improved elasticity and could stretch further before failure, therefore reducing the chance of re-injury when stimulated. But, nonetheless, warm muscles were more deformed and absorbed less energy than cold muscles. Furthermore, all the measurements used in the studies above were objective and focussed on the functional aspects of the damaged muscle, irrespective of the extent of damage and healing of the injured muscle.

1.5.3 Nonsteroidal anti-inflammatory drugs

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is frequently supported because animal studies have shown a marked inflammatory response to an experimental contusion injury [175]. However, some controversy about the safety and efficacy of NSAID treatment in the acute and subacute phase after injury does

exist. On the one hand, NSAIDs may decrease the inflammatory response and thereby the pain and swelling [176, 177], but on the other hand, NSAIDs treatment has been reported to delay muscle regeneration [178]. Since the inflammatory response is a necessary phase during soft tissue healing, inhibition of this response could result in poor healing [6]. Another potential concern is that NSAIDs have no direct effect on satellite cell activation and proliferation [166], processes which are required for the regenerative response after loss of muscle fibres. Although certain concerns exist regarding the safety of these drugs, a recent review concluded that NSAIDs have a role in the short-term treatment of delayed onset muscle soreness (DOMS) in humans [179]. NSAIDs have also been shown not to affect long-term muscle repair, if administered at low doses after contusion injury, since the inflammatory response (important in muscle regeneration) is most probably not completely suppressed at low doses [150].

1.5.4 Steroids

Corticosteroids, despite promoting catabolism of muscle and inhibiting the healing process, have been used to treat muscle contusion injuries and are usually injected directly into the site of injury. However, corticosteroids were only shown to alleviate pain and thereby to expedite a player's return to active status. With no added beneficial effect on the regenerative capacity of the muscle, this may therefore not be at all beneficial to the athlete [180-183]. Anabolic steroids have also been contrasted with corticosteroids with regard to their effects on muscle tissue, with widely conflicting reports on their advantages and disadvantages. Although not considered to have anti-inflammatory properties, anabolic steroids have been shown to promote muscle growth and regeneration in some circumstances [184-186] and

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are now being considered as a possible treatment option following severe contusion injuries [6]. Beiner *et al.* (1999) found that nandrolone decanoate, a testosterone derivative, was able to increase the number of muscle progenitor cells in injured muscle, rapidly healing and restoring force generation capacity. The effect was also associated with an increase in inflammatory cells. This therefore suggests that the initial inflammatory process is a crucial part of the entire healing response [6].

1.5.5 Therapeutic ultrasound

Therapeutic ultrasound is one of the most frequently used treatment modalities for a variety of both athletic and nonathletic injuries, and although it has been used for decades as treatment, there is no good evidence which suggests its effectiveness for treating pain, muscoskeletal injuries or soft tissue injuries [187, 188]. Despite this, ultrasound has been extensively used for treatment of sports-related contusion injuries to skeletal muscle. According to Wilkin *et al.* (2004), for ultrasound to be an effective treatment, it should speed up the regenerative process, primarily by increasing cell permeability, thereby permitting an earlier entry of macrophage and other WBC into the damaged area, which will further speed up the inflammatory process. This should then stimulate the release of growth factors that will initiate the regenerative process [161]. The results of their experiments, however, suggested that following contusion injury, pulsed therapeutic ultrasound treatment had no influence on measured markers of skeletal muscle regeneration – changes in muscle mass, protein concentration, cross-sectional area (CSA), and myonuclear number, thereby suggesting that ultrasound is actually not that effective. However, the markers of regeneration mentioned above, are not considered to be the best markers for regeneration. Further research is therefore needed to include more

markers of regeneration, mainly focussing on satellite cells and immune cells known to be involved in the healing of skeletal muscle following injury.

1.5.6 Gene therapy

Gene therapy is another technique that could be used to deliver growth factors to injured skeletal muscle. The common means of delivery are direct injection, transmission via a viral vector, and transmission via a nonviral vector. One potential advantage associated with using human recombinant growth factors in the treatment of muscle injuries is the ease and safety of the injection procedure into rodent models. However, the direct injection of recombinant proteins (growth factors) is limited by the high concentration of the factor typically required to produce a substantial effect. The vascular system's rapid clearance of the molecules and the molecules' relatively short biological half-lives are the main reasons that large concentrations of growth factor are required [189, 190]. Both viral vectors (adenovirus, retrovirus, herpes simplex virus, and adeno-associated virus) and nonviral vectors (plasmid DNA and liposomes) have been used to deliver genes to injured skeletal muscle. Each of these vector systems has advantages and disadvantages, but the ability of the adenovirus to efficiently transduce regenerating myofibres has prompted researchers to focus on adenoviral vectors as promising gene delivery vehicles [191, 192].

1.5.7 Nutritional supplements

Nutritional supplements have become increasingly popular in the treatment of many conditions. Of particular interest is the belief that pre-supplementation before exercise may induce a preventive effect if an injury is sustained during exercise, by

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targeting reactive species production (particularly ROS), which is excessively produced after sustaining an injury. The following section will focus on antioxidants as a possible treatment regime for contusion injuries.

1.5.8 Antioxidants

Antioxidants are important protectors of health because they provide electrons that neutralize reactive species [26]. Antioxidants are naturally occurring substances which function to protect against the harmful effects of pro-oxidants by inhibiting their oxidation. Antioxidant compounds must however be present in biological systems in sufficient concentrations to prevent accumulation of pro-oxidant molecules, a state known as oxidative stress [193].

In general, the mammalian cell has adequate antioxidant reserves to cope with ROS production under normal physiological conditions as well as following an insult. A variety of proteins and enzymes in the body function as antioxidants. Such substances include catalase (Cat), reduced glutathione (GSH), selenium-dependent glutathione peroxidase (GPX), copper and zinc-dependent superoxide dismutase (SOD), uric acid and the transition metal-binding proteins, such as transferrin and ceruloplasmin [193, 194]. The levels of these protein-derived antioxidants are determined by their rate of synthesis, and therefore cannot be manipulated easily. In addition to these endogenous antioxidants, one can obtain oxygen scavengers from dietary sources. The common dietary antioxidants are tocopherols (vitamin E), ascorbic acid (vitamin C), carotenoids, selenium and flavonoids [193, 194]. Since these dietary antioxidants are exogenous in nature, their levels can be manipulated by supplements and dietary modifications [193].

Skeletal muscles are rarely thought of as a primary target of oxidative stress. In fact, they can withstand various stressors. However, during a sustained bout of exercise for example, there is increased mitochondrial respiration, allowing for greater radical species production. In addition to these findings, in response to exercise-related muscle damage, neutrophils and macrophages migrate to the site of injury, infiltrate injured muscle tissue, express and release certain cytokines (Table 1.2), and produce additional reactive species [195, 196]. Excess generation of reactive species may overwhelm natural cellular antioxidant defences such as muscle membrane vitamin E, and further contribute to muscle damage [197, 198].

Some authors have claimed that antioxidants including vitamin C, vitamin E, carotenoids and flavonoids can act as pro-oxidants and can stimulate oxidative damage *in vivo* and *in vitro*, especially ascorbate, alleged in several studies to increase oxidative DNA damage (reviewed in Duarte and Lunec (2005)) [199]. These antioxidants are able to reduce transition metals to generate transition metal ions that can stimulate reactive species generation. However, overall, the data suggesting that antioxidants may be harmful by acting as pro-oxidants are unclear and inconclusive. In the next section attention will be focused on an antioxidant potentially more powerful than some of the most widely known antioxidants.

1.5.7.1 Oxiprovin™

Oxiprovin™ grape seed extract (GSE) is an antioxidant manufactured from grape seeds in a water and ethanol extraction process [200]. Oxiprovin™ is one of several GSE products available, eg. Procydin, but is the only GSE supplement made from South African raw products, whereas all other products used imported components.

GSE contains an active component of oligomeric proanthocyanidins (OPCs - approximately 100 %). These proanthocyanidins are a class of polyphenolic compounds found throughout nature in plant species and may be present as individual monomers or, in some cases as oligomeric units, as is the case with Oxiprovin™ [201, 202]. Typically, OPCs are found in the outer shells of seeds and barks, where they protect the plants against reactive species (free radical) damage. They are one of the most potent antioxidants found in nature [201-203]. These OPCs are part of the flavonoid family - natural plant components that strengthen and protect living tissue [201-203]. This flavonoid family includes anthocyanidins, monomeric flavanols - epicatechin and catechin - which when bound together form oligomeric proanthocyanidins, flavanones, flavones and flavonols (see Table 1.3 for dietary sources of flavonoids) [204].

Table 1.3: Dietary sources of flavonoids

Flavonoids	Dietary sources	References
Flavanone	Peel of citrus fruits, citrus fruits	[222-224]
Flavonol	Endive, leek, broccoli, radish, grapefruit, black tea, onion, lettuce, cranberry, apple skin, berries, olive, red wine, grapes	[215, 216, 224-226]
Flavone	Fruit skin, celery, parsley	[215, 224, 226]
Anthocyanidins	Red grapes, red wine, cherry, raspberry, strawberry, coloured fruit and peels	[213, 227]

Flavanols
 Monomeric flavanols
  Catechin
  Epicatechin
Proanthocyanidins
	Wheat, corn, rice, tomatoes, spinach, cabbage, asparagus, white grapes, white wine, olives, olive oil, coffee, apples, pears, cherries, plums, peaches, apricots, blackberries, blueberries, cranberries, anis, green and black teas, red wine, chocolate, beans, grape seeds and skins	[213, 228, 229]

Diets rich in fruit and vegetables, thus high in the antioxidants vitamin C, β-carotene, carotenoids and plant phenolics including flavonoids, have been shown to be beneficial in various situations [205]. The biological, medicinal and pharmacological properties of the flavonoids have been extensively reviewed [21]. Flavonoids and other plant phenolics are reported, in addition to their free radical scavenging activity [206-210], to have multiple biological activities including vasodilatory [211-213], anticarcinogenic [214-216], anti-inflammatory [217], antibacterial [218], immune-stimulating [219], anti-allergic [217], and antiviral effects [218]. Flavonoids have also been reported to exert inhibitory effects on phospholipase A2, cyclooxygenase, and lipoxygenase, glutathione reductase, and xanthine oxidase [21, 206, 214, 220, 221].

In contrast to the beneficial effects, some flavonoids have also been found *in vitro* to be mutagenic [230, 231]. These harmful effects were suspected to result from the pro-oxidant rather than antioxidant action of these flavonoids. The biological and pharmacological effects of a flavonoid compound may depend upon its behavior as either an antioxidant or a pro-oxidant. Using the ORAC assay, it was demonstrated that the same flavonoids could behave as both antioxidants and pro-oxidants, depending on concentration and free radical source [221]. Flavonoids acted as antioxidants against free radicals but demonstrated pro-oxidant activity when a transition metal was available. The antioxidant activities and the copper-initiated pro-oxidant activities of these flavonoids depended on the number of free OH substitutions on its structure, the more OH substitutions, the stronger the pro-oxidant activity [21]. But, although it seems that flavonoids, as well as other antioxidants can behave as pro-oxidants, the copper-initiated pro-oxidant actions of flavonoids and

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other antioxidants including ascorbic acid and α-tocopherol may not be important *in vivo*, where copper ion will be largely sequestered, except perhaps in certain metal overload diseases. However, the pro-oxidant effect of flavonoids *in vivo* is still unclear.

The free radical scavenging abilities of flavonoids have been well documented and command the most attention relative to other antioxidants [206, 207]. However, research on the bioavailability of flavonoids suggests that they are poorly absorbed [232]. Studies regarding proanthocyanidins and their bioavailability have also only recently been investigated (reviewed in Rasmussen *et al.* (2005)) [233]. According to these studies, only the monomeric and dimeric proanthocyanidins are able to be absorbed in their intact form and only to a very limited extent, whereas the polymers are non-bioavailable and transfer unaltered through the gastro-intestinal system. A few studies indicate that some procyanidin dimers are able to be absorbed intact in rats and humans, whereas others have not been able to detect any intact dimers. Nevertheless, *in vivo* studies on mice have shown 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) [206]. Oxidative tissue damage in this study was assessed by the following assays: the chemiluminescence assay as an index of ROS production, the cytochrome C assay as an index of superoxide anion production, and the thiobarbituric acid reactive substances (TBARS) assay as an index of lipid peroxidation. Recently, grape seed proanthocyanidins (GSPE) were also found to possess cardioprotective abilities by functioning as an *in vivo* antioxidant in rats and

by virtue of their ability to directly scavenge reactive oxygen species including hydroxyl and peroxyl radicals [213].

For a proanthocyanidin to be defined as an antioxidant it must satisfy two basic conditions: (1) when present in low concentrations relative to the substrate to be oxidized it should be able to delay, retard, or prevent auto-oxidation or free radical-mediated oxidative injury; and (2) the resulting product formed after scavenging must be stable (through intramolecular hydrogen bonding) on further oxidation [234]. Proanthocyanidins have also attracted increased attention in the fields of nutrition, health, and medicine largely due to their potent antioxidant capacity and possible beneficial implications on human health. But how does one measure the antioxidant capacity of a particular sample?

1.5.7.2 Measuring of antioxidant capacity

Several methods have been developed to assess the total antioxidant capacity of human serum or plasma because of the difficulty in measuring each antioxidant component separately and the interactions among different antioxidant components present in the serum or plasma [235].

The oxygen radical absorption capacity (ORAC) assay is a method for measuring the total antioxidant activity of a biological sample, including serum, agricultural products, food products, food ingredients, pharmaceutical products and animal tissues [236]. The assay measures the effectiveness of various natural antioxidants present in the tested sample, in preventing the loss in the fluorescence intensity of the fluorescent marker protein, fluorescein, during peroxy radical induced free radical

damage [237]. Assay results are quantitated by allowing the reaction to read until the fluorescence decay plateaus (e.g. for 35 minutes, i.e. 37 reads, approximately 1 read per minute) and then integrating the area under the kinetic curve relative to a blank reaction containing no added antioxidants. This method is based on the method of Huang *et al.* (2002), where the area under the curve is proportional to the concentration of all the antioxidants present in the sample. Each reaction is calibrated using known standards of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water soluble vitamin E analog. The results of the assay are reported on the basis of 1 ORAC unit = 1 micro-M Trolox [238].

The ORAC assay provides a way of evaluating the potential antioxidant activity of various compounds and biological samples. This method, although only water-soluble antioxidants are assessed, is superior to other methods in determining the antioxidant properties of a sample. Firstly, the ORAC assay is very economical, the reagents needed to perform the ORAC assay are relatively cheap and only a small volume of the reagents and a small volume of samples are needed. Secondly, the ORAC assay is quick and simple to use, as well as reliable (personal communication: Dr. Theo Nell, Department of Physiological Sciences, Stellenbosch University). However, although other more reliable methods exist for measuring the antioxidant status of a particular component in the sample, i.e. high performance liquid chromatography (HPLC), these methods are more difficult to use, much more expensive and time consuming.

1.6 Summary

When damage to skeletal muscle occurs (e.g. contusion injury sustained after sporting accident), it causes the rupturing of muscle fibres at or adjacent to the impact area, and depending on the injury severity, the underlying vasculature may also be disrupted. Neutrophils first infiltrate the damaged area, followed by macrophages, both of which generate free radicals, causing the area of necrosis to extend to affect uninjured tissue as well. Satellite cells become activated by cytokines, growth factors and by the reactive species (generated by macrophages), whereafter they start to proliferate, fuse and form myoblasts. These myoblasts proliferate, differentiate, and fuse with the damaged fibres to repair them.

Currently there are many ways in which to accelerate the healing process after injury, mostly focussing on enhancing regeneration and minimizing fibrosis so as to reduce the amount of scar tissue formed within the muscle. The ideal treatment for muscle contusions remains elusive, due to the fact that injuries of different severities can occur and due to the many different role players in the response to injury. A single treatment is unlikely to prove optimal for all cases of contusion injury. Rather, complementary groups of treatments should be considered. The use of antioxidants, to scavenge reactive species (expression of free radicals over and above the quantity needed for the repair process) in particular Oxiprovin™ might prove a reliable method of treating contusion injuries, by reducing additional secondary damage.

1.7 Aims

Given the positive results reported in the literature about flavonoids and their various beneficial effects on the body, we hypothesise that Oxiprovin™ (part of the flavonoid family) supplementation will improve the speed of muscle recovery after a contusion injury.

Therefore, the main aims of this study are:

(a) to induce a standardised skeletal muscle contusion injury in rats using a non-invasive technique,

(b) to determine the effects of long term (both prior to and after injury) oral GSE supplementation on total body antioxidant status, by assessment of snap-frozen muscle samples and plasma samples using the ORAC assay,

(e) to determine the effects of long term (both prior to and after injury) oral GSE supplementation on muscle physiology through immunohistochemical staining of satellite cells with CD34, CD56 and M-cad, and immunohistochemical staining of regenerating muscle fibres with foetal myosin heavy chain (MHC$_f$).

CHAPTER 2: MATERIALS AND METHODS

2.1 Study design

2.1.1 Experimental animals

All experimental protocols were approved by the Animal Ethics Committee of Sub-Committee B of Stellenbosch University (project # 2006 Smith01). Male Wistar rats were used for this study. Animals were bred and housed in the Small Animal Unit in the Department Physiological Sciences at Stellenbosch University. Animals were divided and housed in groups of four for the duration of the study, starting at the age of three weeks once weaned. All animals had access to food (standard rat chow – 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 6:30). Ambient temperature was controlled at 21 ºC, and rooms ventilated at 10 changes/hour.

A couple of preventative measures were taken to minimise possible influences by confounding factors. Firstly, rats were handled and weighed (twice daily) to ensure that at the start of the supplementation and injury protocols, rats were accustomed to the specific researcher. Secondly, rats were orally gavaged with tap water (weight in µl) 2 weeks prior to the onset of the injury protocol, to habituate them to this procedure prior to supplementation with the grape seed extract (GSE). All rats were allowed to reach maturity (± 7 weeks; mean weight of 286.6 ± 23.5 g), before subjection to any intervention.

2.1.2 Experimental groups

Preceding the onset of the supplementation protocol and contusion injury, experimental rats were randomly divided into 4 groups:

A) Non-injured rats receiving 2 weeks of placebo (C-P, n=8)

B) Non-injured rats receiving 2 weeks of GSE supplementation (C-GSE, n=8)

C) Rats injured after 2 weeks of placebo supplementation, with continued placebo supplementation post-injury (I-P; n=32, i.e. 8 rats per time point, time points were 4 hr, 3 d, 7 d and 14 d post-injury)

D) Rats injured after 2 weeks of GSE supplementation, with continued GSE supplementation post-injury (I-GSE; n=32, i.e. 8 rats per time point, time points were 4 hr, 3 d, 7 d and 14 d post-injury)

Refer to section 2.1.3 for details on the preparation of the supplement and placebo.

2.1.3 Intervention protocols

The study included 2 intervention protocols, namely the supplementation and the injury. Briefly, the 2-week supplementation intervention was followed by the mass-drop contusion injury, after which blood and muscle samples were collected at different time points during recovery from injury for subsequent analysis. Supplementation with GSE or placebo was continued throughout the protocol up to sacrifice.   The study design is outlined in Figure 2.1.

2.1.3.1 Supplementation

Preparation: The 20 mg/kg/day GSE (Oxiprovin™) was prepared early in the morning, before supplementation. Briefly, 20 mg of GSE was dissolved in 1 ml of 0.9 % saline. 0.9 % saline was used as vehicle for the placebo rats.

Administration: Rats were orally gavaged with either 20 mg/kg/day GSE or 0.9 % saline for the duration of the experimental procedure. Control (C) rats were gavaged for 2 weeks without injury and contusion injury groups received GSE or placebo for 2 weeks prior to injury as well as after injury (Figure 2.1).

2.1.3.2 Injury

Apparatus: Contusion of the hind limb was produced by an apparatus (Figure 2.2), designed and manufactured especially for our study in collaboration with the Central Engineering Services, Stellenbosch University.   In short, 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 the 200 g weight from the desired height to the area of the muscle identified for injury. A peg through a hole in the plastic tube kept the mass in place at the desired height above the muscle, so that its removal resulted in a standardised mass dropping speed.

Procedure: All injury groups were gavaged for 2 weeks, after which they were anaesthetised (75 mg/kg ketamine, 0.5 mg/kg medetomidine and 0.9 % saline; 1

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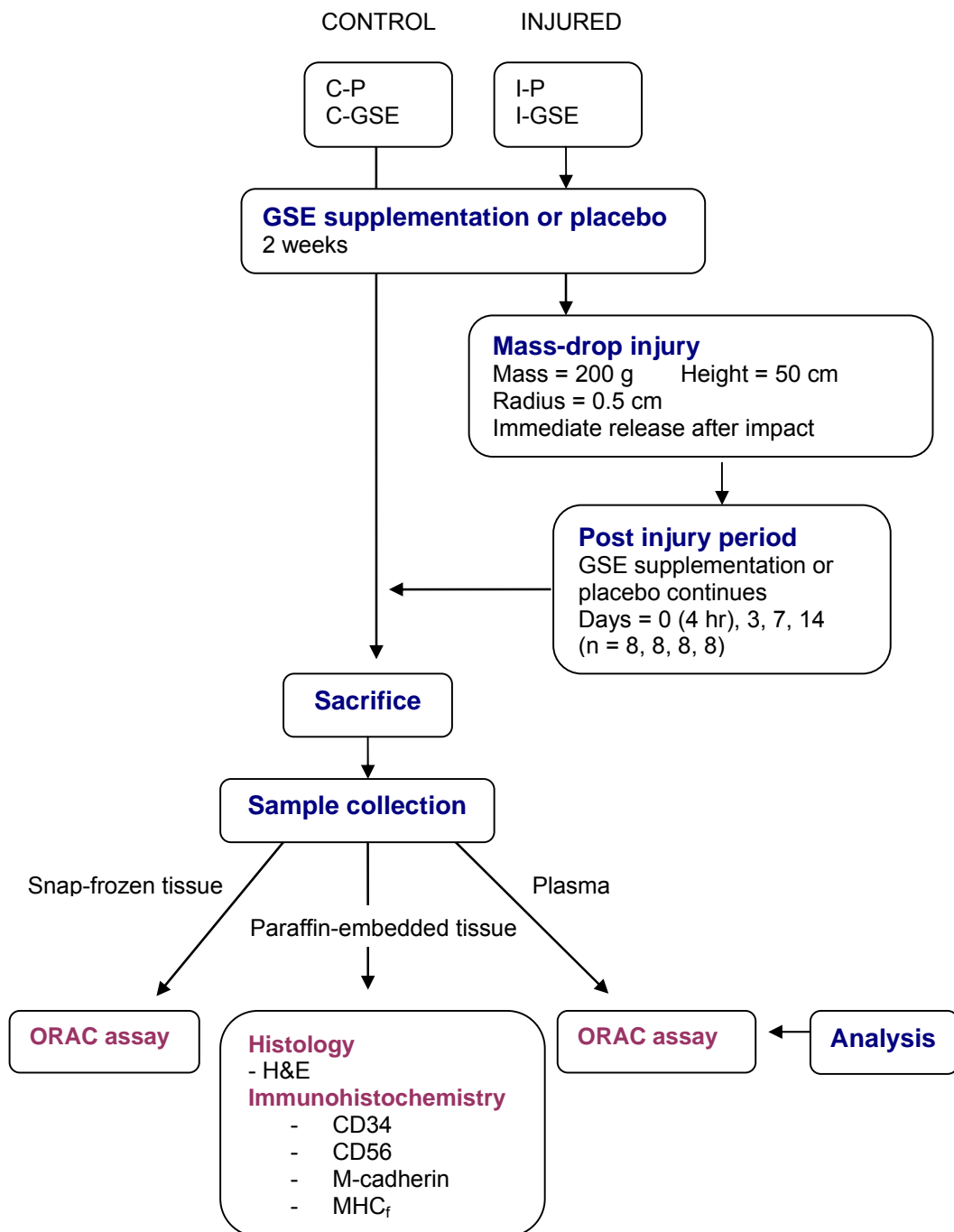
Figure 2.1: Study design.

Figure 2.2: The muscle contusion injury jig.

ml/kg body weight i.p.). The right hind limb of the animal was placed on the platform, and moderately extended away from the animal, to prevent injuring the underlying bone. Injury was produced by dropping a weight with a mass of 200 g (through the plastic tube) from a height of 50 cm onto the medial surface of the right *gastrocnemius* muscle. The weight dropped had a flat, circular impact surface, with a diameter of 1 cm, therefore an impact area of 0.785 cm^2 that directly contacted the skin. Directly post-injury, rats were allowed to recover under infrared lights until fully conscious.

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2.2 Sacrifice and sample collection

2.2.1 Sacrifice

Entrance of rats into the intervention protocols was staggered, so that no more than 9 rats were sacrificed per day. All sacrifice procedures took place between 08:00 and 10:00 in the morning. With exception of the 4 hr injury time point, all rats were sacrificed 24 hr after the last intervention procedure. All experimental animals received an overdose of pentobarbitone sodium (200 mg/kg i.p.). On day 0, control rats were sacrificed (i.e. after 14 days of supplementation or placebo). Eight rats from each of the 2 injury groups (I-P and I-GSE), were sacrificed at each of the following time points after subjection to hindlimb muscle contusion injury (4 hr, on day 3, 7 and 14 after injury). After sacrifice, the right injured *gastrocnemius* muscle was excised (see section 2.2.2.2) and whole blood samples obtained (see section 2.2.2.1).

2.2.2 Sample collection

2.2.2.1 Blood collection

Following euthanasia by pentobarbitone overdose, the heart was exposed by opening the chest cavity. 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 minutes at 4 °C. Plasma obtained was aliquoted into 1.5 ml reaction vials and stored at -80 °C until subsequent analysis. Batches of plasma were used to analyse CK activity as an indirect indicator of

muscle damage (see section 2.3.1) [239]. For ORAC analysis, which was used to determine the oxygen radical absorbance capacity, plasma samples were kept frozen for no longer than 1 to 1½ weeks after collection before analysis.

2.2.2.2 Muscle collection

After the procedures for cardiac puncture, but within 5 minutes of euthanase administration, the *gastrocnemius* muscle of the right hind leg was exposed by cutting and removing the skin and connective tissue surrounding the muscle. The medial central section of the *gastrocnemius* muscle (Figure 2.3) was harvested by cutting away the unwanted muscle after which this section was divided into two, so that one part was processed for immunohistochemistry (see section 2.3.3), and the other snap-frozen for biochemical analysis.
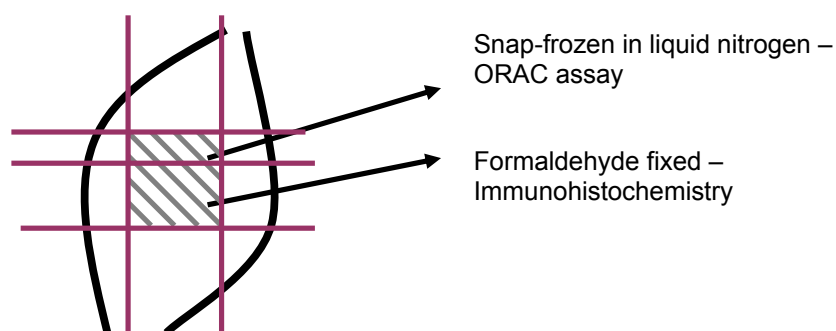
Figure 2.3: The *gastrocnemius* muscle complex with grid superimposed to indicate cutting during the harvesting process. The central medial (hatched) section was used for analysis.

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2.3 Sample analysis

Four different analytical procedures were used: a) creatine kinase (CK) activity assays performed on plasma, b) ORAC assays performed on both plasma and snap-frozen muscle tissue, c) histological staining and visualisation of muscle sections and d) immunohistochemical analysis of muscle sections.

2.3.1 CK activity

Total CK, as an indicator of 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 of serum CK activity is 15 – 195 u/L (at 37 ^{o}C).

2.3.2 Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed on plasma using the method of Huang *et al.* (2002) no later than 10 days after sample collection, because the antioxidants present in the sample decrease with time. The ORAC assay is based on the measurement of free radical-induced quenching of a fluorescent probe. Through the change in the fluorescence intensity of the probe in solution over time, an index of the extent of free radical quenching capacity of the probe in the sample solution can be determined. In the presence of antioxidants, free 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 [170–174]. After antioxidant supplementation, a higher ORAC compared to the controls is expected. The ORAC assay procedure is identical for assessment of plasma/serum or tissue homogenates. See appendix A for summary of reagents and laboratory method.

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2.3.2.1 Standard preparation

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used as standard. To generate the standard curve, the following Trolox standards were prepared in 75 mM phosphate buffer, pH 7.4: 50, 25, 12.5 and 6.25 μM.

2.3.2.2 Solution and sample preparation

A fresh 153 mM 2,2'-Azobis-(2-methylpropionamidine)-dihydrochloride (AAPH) working solution was made, by completely dissolving 0.414 g AAPH in 10 ml phosphate buffer (75 mM, pH 7.4) and then kept on ice. Fluorescein stock solution (5×10^{-3} nM) was made in 75 mM phosphate buffer (pH 7.4) and was kept at 4 ºC in dark conditions. The fluorescein stock solution, if stored appropriately, can last for several months [238]. A 8.16×10^{-5} mM fluorescein working solution was made daily by further diluting the stock solution in 75 mM phosphate buffer (pH 7.4). Plasma samples were defrosted and diluted 75x (personal communication: Dr. Dee Blackhurst, Cape Town University) in 75 mM phosphate buffer, pH 7.4.

Crude tissue extracts were prepared by adding approximately 10 mg of the snap-frozen muscle tissue to 75 mM phosphate buffer (pH 7.4) (1:75 w/v). The tissue samples were sonicated on ice for approximately 1 minute continuously with a tissue sonicator (Virsonic 300; The Virtis Company, Inc; Gardiner, N.Y; setting 3), whereafter, the homogenates were centrifuged for 30 min at 8853 g (12000 RPM) at 4 °C with a multispeed refrigerated centrifuge (model: PK121R centrifuge, brand: ALC international SRL, Milano, Italy) and the supernatant immediately used for the ORAC assay. The values obtained after performing the ORAC assay on the muscle were standardised for protein by determination of total protein concentration of the

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muscle supernatants used, by means of the Bradford assay [240] (described in Appendix B). Results for ORAC were expressed as µmol Trolox equivalents/µg protein.

2.3.2.3 Analytical method

The figure in Appendix A illustrates the 96-well plate used for the ORAC assay. The quantity of blank, standard or sample per well was 25 µl added in triplicate to their respective wells, as well as 150 µl of the Fluorescein working solution. The plate was then covered and allowed to equilibrate for 10 minutes at 37ºC in the fluorometer, whereafter 25 µl of the AAPH solution was added for the reaction to start. The fluorescence intensity of the solutions in all wells was read 36 times, at one minute intervals according to the method of Huang *et al.* (2002).

2.3.2.4 Calculation of the ORAC values

The net area under the curve (AUC) of all standards and samples were calculated by plotting time (in minutes) against relative fluorescence intensity (Figure 2.4). The standard curve is obtained by plotting the four different Trolox standard concentrations against the average net AUC of these standards (Figure 2.5). The final ORAC values are calculated using the regression equation between Trolox concentration and the net AUC and are expressed as µmol Trolox equivalents per liter for liquid samples (plasma) and µmol Trolox equivalents per µg protein for solid samples (homogenised muscle).

The AUC was calculated using Microsoft Excel XP as

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

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.

$$\text{Net AUC} = [(AUC_{\text{sample or Trolox}} - AUC_{\text{blank}}) \qquad\qquad (2)$$
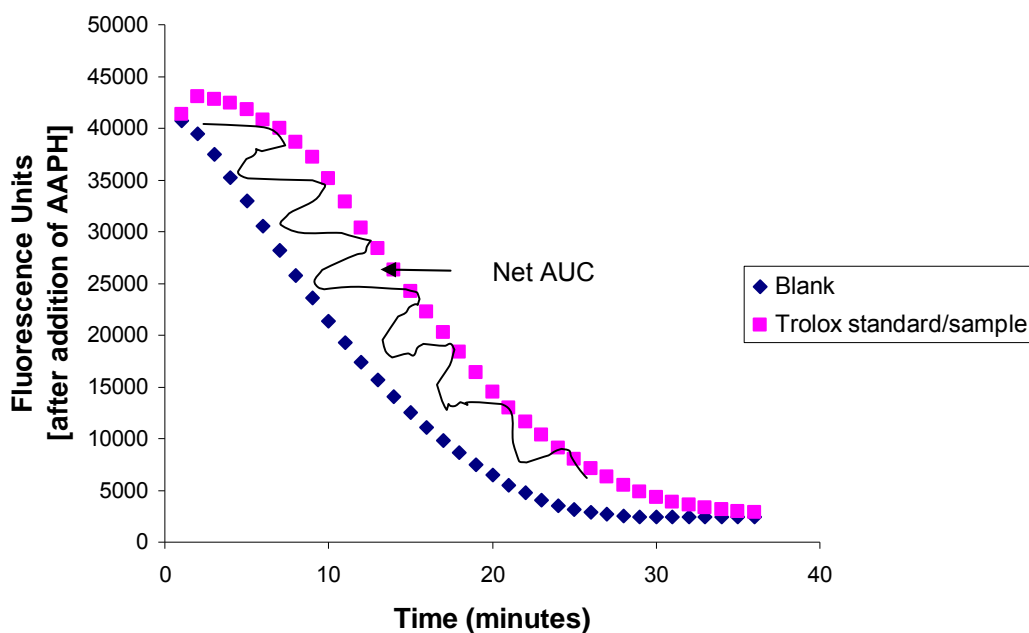
Figure 2.4: An illustration of the calculation of the net AUC, calculated as difference in fluorescence of the Blank and the Trolox standards or samples.

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The standard curve is obtained by plotting Trolox concentrations against the average net AUC of the Trolox standards. An example is illustrated in Figure 2.5. Final ORAC values are calculated using the regression equation between Trolox concentration and the net AUC and are expressed as µmol Trolox equivalents per liter. It should be noted that the dilution factor (in this study it was 75x) 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 B).
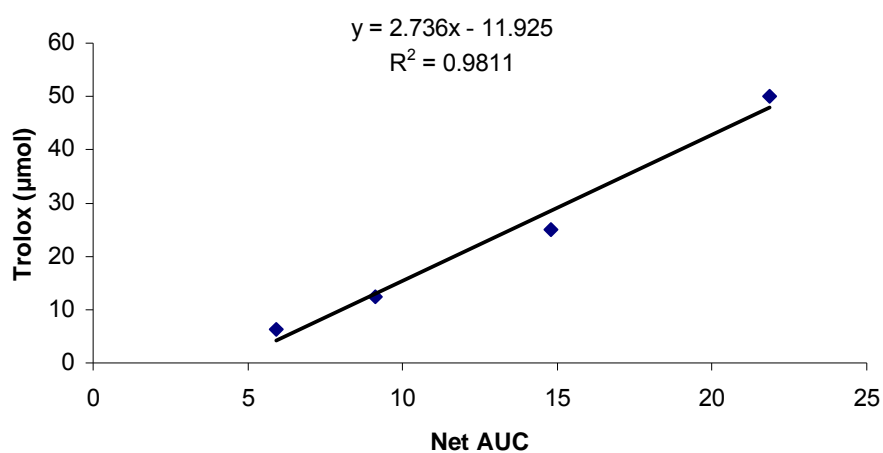
Figure 2.5:   Example of the linear relationship between net area under the fluorescence decay curve and the Trolox concentrations.

2.3.3 Histology

One representative muscle sample from each of the 4 experimental groups was preserved in 10 % formaldehyde-saline for 72 hours. The samples were then cut to size, placed into embedding cassettes, processed and impregnated with paraffin wax (Histosec, Merck) (see Appendix C 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 cross-sections were cut using a rotary microtome (Reichert Jung, Heidelberg, Austria) and stained with haematoxylin and eosin (H&E – see Appendix D for detailed method) to visualise subjectively the extent of immune cell infiltration into the injured area.

2.3.3.1 H&E staining

The tissue used for H&E staining is embedded in paraffin wax, and, in order for water soluble dyes to penetrate the tissue sections, the slides are deparaffinized and rehydrated (Appendix D) [241]. Haematoxylin stains the basophilic structures blue-purple and alcohol-based eosin stains the eosinophilic structures bright pink. The basophilic structures usually contain nucleic acids, such as ribosomes and cell nuclei, and cytoplasmic regions rich in RNA. The eosinophilic structures are intra- or extracellular proteins (mostly cytoplasm and red blood cells).

2.3.4 Immunohistochemistry

2.3.4.1 Reagents

The antibodies used for immunohistochemistry, are summarised in Table 2.1.

Table 2.1: Antibodies used to identify vascular endothelium (CD31), satellite cells (CD34, CD56, M-cadherin), and regenerating skeletal muscle fibres (MHC_f).

Antibodies	[Stock] (µg/ml)	Dilution	Catalog # and supplier
Primary antibodies:			
Monoclonal mouse anti-human CD31 (JC70A)		1/100	M0823, Dako
Monoclonal mouse anti-human CD34 (ICO115)	100	1/100	sc-7324, Santa Cruz
Polyclonal rabbit anti-human NCAM (H-94)	1.7×10^5	1/100	sc-8305, Santa Cruz
Mouse anti-human foetal myosin heavy chain (MHC_f)		1/25	F1.652-s, Developmental Studies Hybridoma Bank
Polyclonal rabbit anti-human M-cadherin (H-71)	200	1/100	sc-10734, Santa Cruz
Biotinylated secondary antibodies:			
Donkey anti-rabbit	1.3×10^3	1/1200	711-065-152, Jackson ImmunoResearch
Donkey anti-mouse	1.3×10^3	1/1000	715-065-150, Jackson ImmunoResearch
Tertiary antibodies:			
Fluorescein Streptavidin (FITC)		1/500	SA-5001, Vector Laboratories
Texas Red Streptavidin		1/500	SA-5006, Vector Laboratories

2.3.4.2 Procedure

Slides, each containing three serial sections per slide for each sample were labelled using each of the following markers: CD31 (staining the vascular endothelium – used only in the pilot study discussed in section 2.6), CD34 (satellite cells), CD56 (satellite cells), M-cadherin (satellite cells) and foetal myosin heavy chain (MHC_f – regenerating skeletal muscle fibres). All dilutions were made with phosphate-buffered saline (PBS). Sections stained for CD34 were co-stained with CD56 and M-

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cadherin, whilst MHC$_f$ and CD31, were used as individual markers. Slides were first taken through a conventional rehydration sequence followed by 0.1 % trypsin at 37 °C for 20 minutes prior to immunostaining (See Appendix E for detail). The immunostaining procedure for paraffin sections is summarised in Appendix F. Briefly, slides were dried, and sections were encircled with a wax pen. Non-specific binding sites were blocked by incubating the sections with 5 % donkey serum at room temperature for 30 minutes.   The serum was then drained off and primary antibody added to the slides and left for 4 hours at room temperature. Slides were washed with 0.1 M PBS (1 ℓ of 1 M phosphate buffer, 90 g NaCl, 9 ℓ ddH$_2$O; pH 7.4) prior to addition of biotinylated secondary antibody (1/1200 anti-rabbit, 1/1000 anti-mouse), raised in donkey. After 40 minutes of incubation, slides were washed with PBS and then the tertiary antibody (1/500) was added and left for 30 minutes at room temperature followed by the addition of the nuclear stain, Hoechst (1/200), for 10 minutes.   To co-stain, either M-cadherin (1/100) or CD56 (1/100) were added to different CD34-stained slides and incubated overnight at 4 °C. The following morning slides were washed as previously described before incubation with a biotinylated secondary antibody raised in donkey (1/1200) for 40 minutes. Slides were washed 3 times in PBS before adding Texas Red-labelled antibody (1/500) for 30 minutes at room temperature. The nuclear marker was added as previously described before washing with PBS and mounting the slides with fluorescent mounting medium (Dako, Diagnostech). During the whole staining protocol, slides were kept in the dark in a humidified environment. PBS controls were used to test the specificity of the antibodies.

2.3.4.3 Image analysis

All imaging data were obtained by analysing two serial sections (5 µm apart) from each muscle sample, at each time point for each antibody. Six fields of view per section were imaged using a microscope (Nikon ECLIPSE E400; 400x objective used), equipped with a colour digital camera (Nikon DXM1200).   Photos were used to count positively stained satellite cells as well as the total number of muscle fibres and regenerating muscle fibres per field of view using a computer programme (*Simple PCI* version 4.0, Compix Inc., Imaging Systems, USA). If necessary, photos were enlarged and the color of the stain enhanced after importation into *Simple PCI* to assist with identification. The images displayed are only partial images of those taken at 400x. All photos of injured muscle were taken at borderline zones, next to the severely injured areas. Satellite cell data were expressed as the number of satellite cells per myofibre, whereas MHC_f data were expressed as the percentage of regenerated myofibres per total myofibre count in the viewed area.

CD34: CD34 expression in muscle sections was quantified as the number of $CD34^+$ satellite cells per myofibre number. Due to the fact that other cells, including fibroblasts and hematopoeitic cells, also express CD34, only $CD34^+$ cells in contact with the sarcolemma and causing an indentation in the myofibre, were recorded as $CD34^+$ satellite cells (Figure 2.6, yellow arrows). When more than one nucleus was present in the satellite cell pocket, each one of these satellite cells was counted individually. The nuclei of the $CD34^+$ cells which did not make an indentation in a myofibre in most cases appeared round and/or were situated clearly outside the myofibrillar space. Alternatively, some cells made an indentation, but CD34 staining

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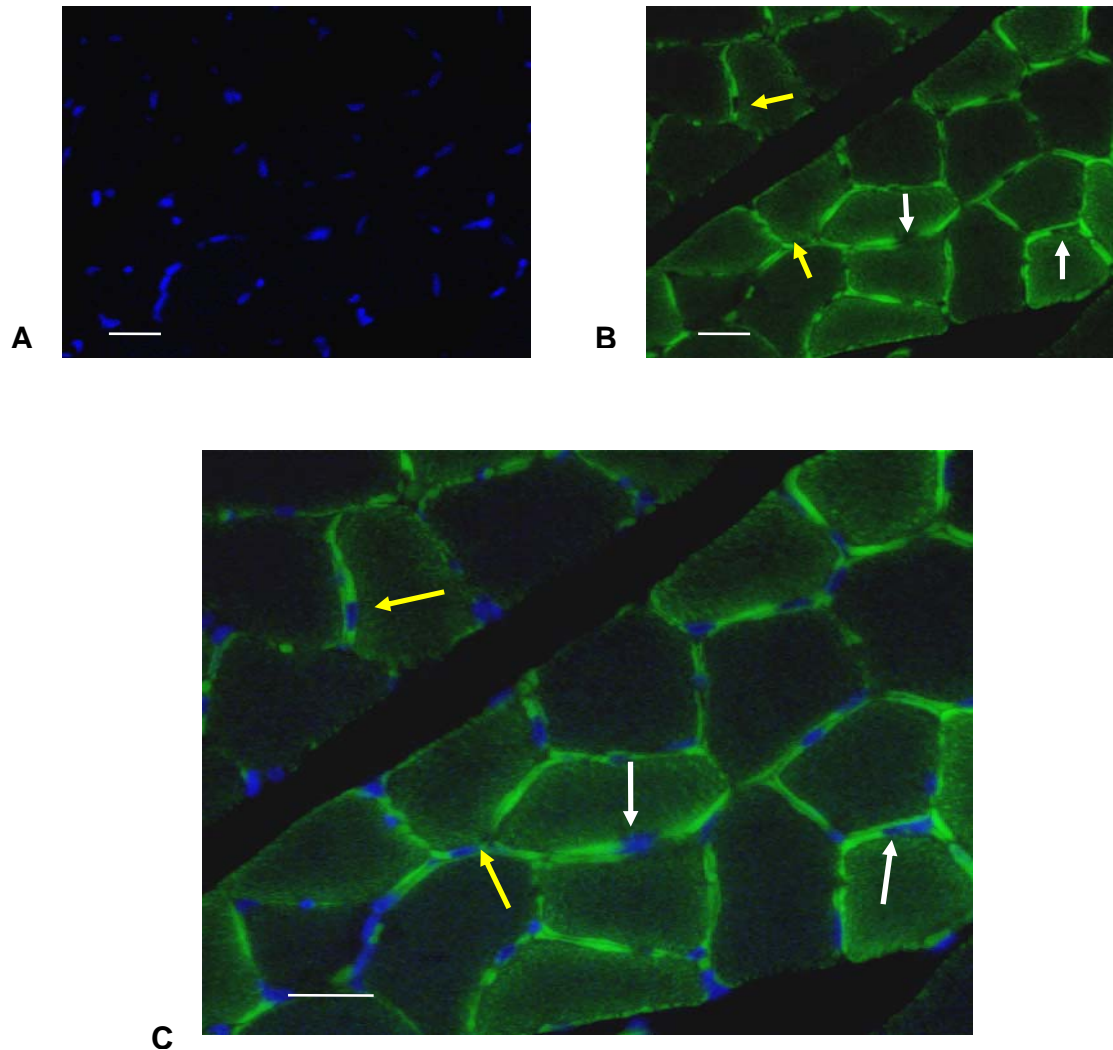
Figure 2.6: CD34 staining in a biopsy of the uninjured *gastrocnemius* muscle (original magnification x 400). Figure 2.6A represent a PBS control. Figures 2.6B and C indicate CD34 expression visualised by FITC (green) alone, or FITC and Hoechst (blue – stains all nuclei) respectively. The yellow arrows indicate CD34^{+} satellite cells whereas the white arrows demonstrate CD34^{+} cells not identified as satellite cells (see text). Scale bar represents 10 µm.

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was not intense enough, or the nucleus was absent. These cells, although CD34$^+$, were not counted (Figure 2.6, white arrows).

CD56: CD56 was used as a marker to investigate the number of activated satellite cells [144]. The identification of satellite cells with this specific marker, was done making use of the same criteria discussed in the previous section using CD34 as a satellite cell marker.

M-cadherin: M-cadherin does not stain all satellite cells, but it does stain most quiescent, activating and proliferating satellite cells [121, 123, 125]. Counting of satellite cells stained positive for M-cadherin was done using the same criteria as counting satellite cells stained for CD34.

Foetal myosin heavy chain (MHC$_f$): Using the MHC$_f$ antibody, one can distinguish between adult muscle fibres and newly formed or regenerating muscle fibres [242]. The number of foetal or regenerating muscle fibres that are formed after sustaining a contusion injury are expressed as a percentage of the total number of muscle fibres viewed (all muscle fibres = adult + foetal). Identifying fibres positive for this particular antibody can be problematic, as it labels not only the foetal muscle fibres, but some mature fibres as well. To be counted as MHC$_f^+$, the staining inside the muscle fibre had to comply with the following criteria: i) At least 3 or more sides of the muscle fibre had to be either bright green (if FITC labelled secondary was used) or bright red (if Texas red labelled secondary was used), ii) Central nuclei must be present inside the fibre, iii) The positively stained fibre also had to have a somewhat hazy shade compared to the other fibres. Figure 2.7 illustrates labelling with MHC$_f$.
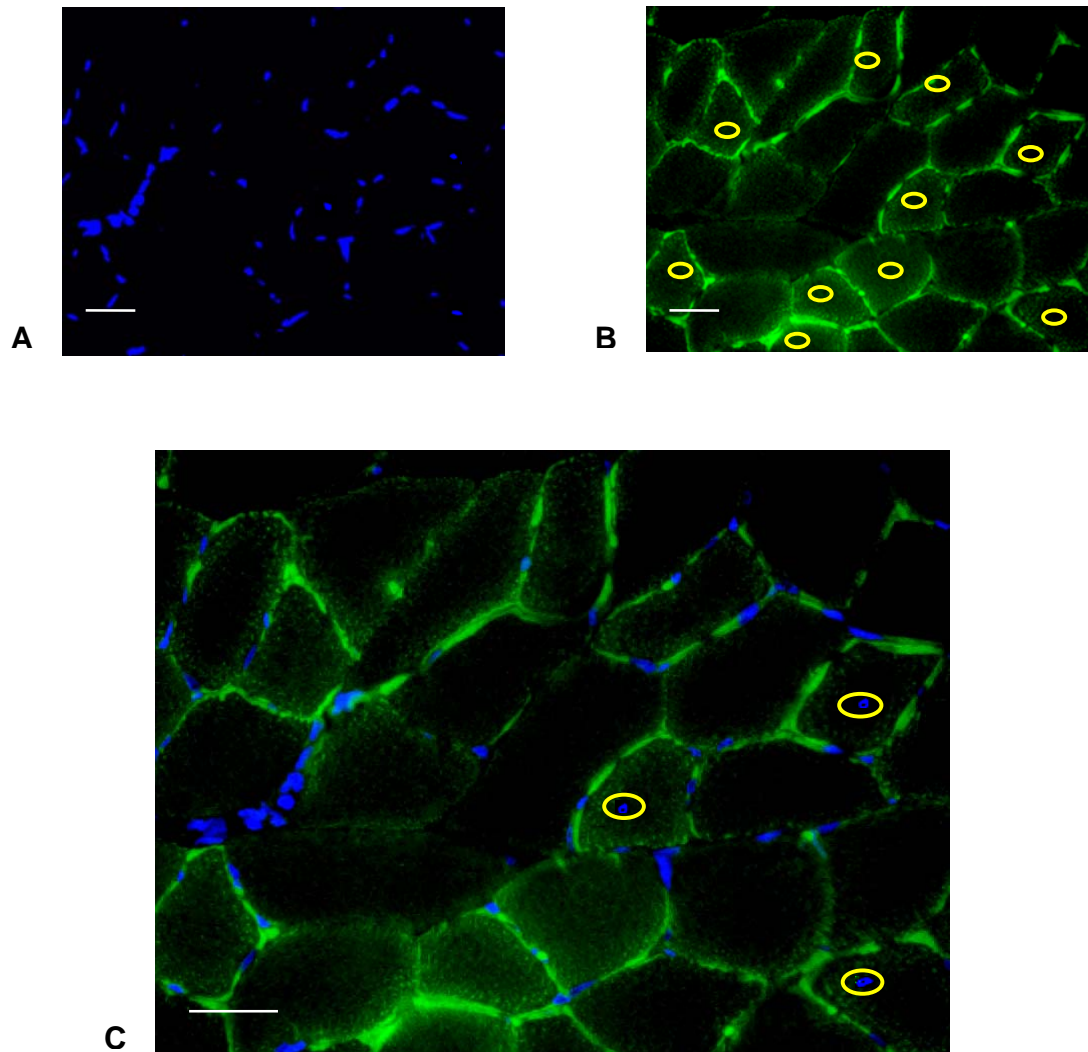
Figure 2.7: Foetal myosin heavy chain (MHC$_f$) staining of newly formed muscle fibres or regenerating muscle fibres. Panel A is a representative PBS control. FITC (green) is visualized in Panel B, and FITC and Hoechst (blue – stains all nuclei) in Panel C (original magnification x 400). Images taken of borderzone areas from muscle sections from a GSE supplemented rat, 3 d after injury. The yellow circles in image B represent the fibres positive for MHC$_f$, however, only the muscle fibres in image C are truly regarded as being MHC$_f^+$, due to the presence of central nuclei. Scale bar represents 10 μm.

2.4 Statistical analysis

A regression analysis was performed to compare growth curves in the different experimental groups. Differences between time points for other results were analysed using factorial analysis of variance (ANOVA) with Bonferonni *post hoc* tests and an unpaired, two-tailed student t-test for comparison of ORAC data between the control groups. All statistical analyses were done using the computer software Statistica version 7 (StatSoft Software), with the exception of CK AUC, which was determined using NCSS 2000. The accepted level of significance was $p < 0.05$. All results are reported as means ± standard deviation (SD), unless otherwise specified.

2.5 Additional experimental issues

Due to the variability in methods reported in the literature to achieve a contusion injury, it was necessary to optimise the experimental procedure. In order to achieve this, two pilot studies were carried out. These are discussed below in section 2.6.

2.6.1 Pilot 1: Injury optimisation in terms of drop distance

2.6.1.1 Introduction

The drop distance needed to be optimised in order to determine which height would give a moderately severe contusion injury, but not as severe as the injuries obtained with e.g. compartment syndrome or cryoinjury, to investigate muscle recovery. Muscle recovery includes two possible ways in which the fibres can heal. Firstly, there is recovery of injured fibres, i.e. regeneration of already existing fibres, and secondly there is formation of completely new fibres. Recovery from a contusion

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injury in our model, is likely to include a combination of both newly generated muscle fibres, as well as regenerated muscle fibres, however, because we investigated cross-sections (example in Figure 2.8), we cannot distinguish between the two types of fibre repair mentioned.

2.6.1.2 Methods

Following anaesthesia (75 mg/kg ketamine, 0.5 mg/kg medetomidine and 0.9 % saline; 1 ml/kg body weight i.p.), 13 male Wistar rats (367.5 ± 17.7 g) received either a severe injury (SI – n=6), mild injury (MI – n=3) or sham injury (C – n=4) to their right *gastrocnemius* muscle. Drop distances were 50 cm for SI and 25 cm for MI, the weight used had a mass of 200 g in both groups. Rats were sacrificed 3 days after the injury intervention by overdose of pentobarbitone sodium (200 mg/kg i.p.), whereafter the medial central part of the *gastrocnemius* muscle complexes were harvested, and fixed in 10% formaldehyde, processed, and wax blocks prepared as described earlier. Muscle sections (5 μm) were stained with H&E and examined using light microscopy for signs of immune cell infiltration and tissue disruption. In order to also investigate the extent of vascular disruption, sections were stained with CD31, the vascular endothelial marker.

2.6.1.3 Results

No quantitative data were obtained for the images captured. H&E stains are presented in row 1 of Figure 2.8, whilst row 2 contains phase contrast images showing the outlines of the muscle fibres, whilst the images in row 3 indicate fluorescent labelling using CD31 (see appendix F and section 2.3.3 for immunohistochemistry). The columns represent the 3 different groups, with the
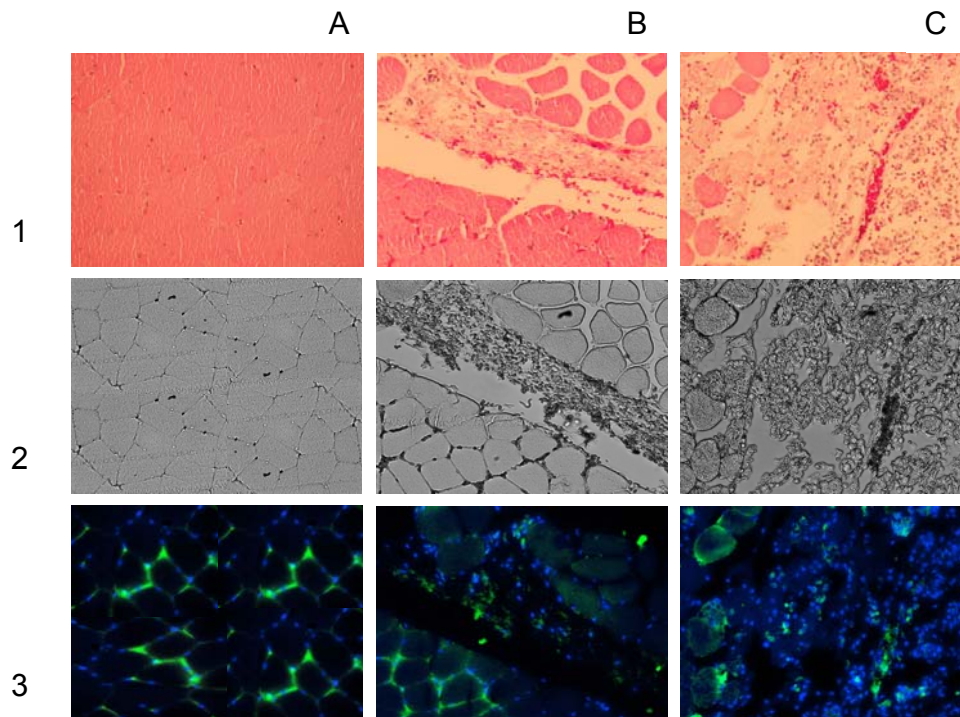
Figure 2.8: The *gastrocnemius* muscle 3 days post injury. The columns indicate the following: **(A)** uninjured transverse section; **(B)** injured transverse section (weight of 200 g from a distance of 25 cm) and **(C)** injured transverse section (weight of 200 g from a distance of 50 cm). The rows indicate the following: **(1)** H&E stain; **(2)** phase contrast indicating the outline of the muscle fibres; and **(3)** fluorescent staining with CD31 (capillary endothelial marker – green) and Hoechst (nuclear stain – blue). Original magnification (x 400).

control (C) group in column A, the mild injury (MI) group in column B and severely injured (SI) group in column C. Image A1, a section of the uninjured *gastrocnemius* muscle, illustrates normal skeletal muscle fibre ultrastructure. When compared to images A1 and B1, it can be seen that a more profound immune cell infiltration

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accompanied the total disruption of the muscle fibre ultrastructure in image C1. In image B1 and B2, only mild disruption of skeletal muscle fibre ultrastructure is visible, as well as a small amount of immune cell infiltration.   In image C3, the large degree of vascular disruption is apparent. As a result of these findings, the height of 50 cm was chosen as the optimal height for the purpose of this contusion injury experiment.

2.6.2 Pilot 2: Grape seed extract (GSE) dose determination

2.6.2.1 Introduction

The recommended method of supplementation with grape seed extract (GSE) is by daily oral intake of two tablets, each containing 70 mg proanthocyanidin. Assuming a standard human body mass of 70 kg, this translates to a dose of 2 mg/kg/day. Taking into account the higher metabolic rate of rats relative to humans, it was possible that the 2 mg/kg/day dose of GSE would not be sufficient to allow sustained detection of GSE in rats. Therefore, we investigated supplementation with various concentrations of the extract.

2.6.2.2 Methods

Rats were divided into four groups of 3 rats each. Rats received either the prescribed dose for humans of 2 mg/kg/day GSE (n = 3), or higher doses of 10 mg/kg/day GSE (n = 3) or 20 mg/kg/day GSE (n = 3), or placebo (0.9 % saline, n = 3) administered by oral gavage once daily. Supplementation with GSE or placebo was started once animals reached maturity (i.e. at about 7 weeks of age) and was administered for 14 days. Rats were sacrificed 24 hr after last gavage and whole

blood collected by cardiac puncture into heparinized tubes for determination of the oxygen radical absorbance capacity (ORAC assay – see section 2.4.1).

2.6.2.3 Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) with Bonferonni *post hoc* test. The accepted level of significance was $p < 0.05$. All results are reported as means ± standard error of the mean (SEM), unless otherwise specified. Statistical analysis for this particular pilot study was done with the aid of the computer software Prism version 2.01 (GraphPad Software Inc, San Diego, California, USA).

2.6.2.4 Results

Figure 2.9 illustrates the different dosages of GSE and placebo (supplement) on the x-axis plotted against the ORAC value (µg Trolox equiv/ℓ) on the y-axis. The free radical scavenging capacity of the plasma was higher when GSE was administered to the rats at a dose of 20 mg/kg/day compared to the free radical scavenging effects of the 10 mg/kg/day and 2 mg/kg/day doses. There were significant differences between the 20 mg/kg/day GSE groups and all the other groups receiving either a lower dose of GSE or 0.9 % saline (see Figure 2.9). There was also a significant difference between the 10 mg/kg/day and the 0.9 % saline ($p < 0.01$). However, there was no statistically significant difference between the 2 mg/kg/day GSE and the saline group. Furthermore, as a result of the data obtained and due to the fact that the metabolic rate of rats is 7-10 times quicker than that of humans, the 20 mg/kg/day dose was chosen.

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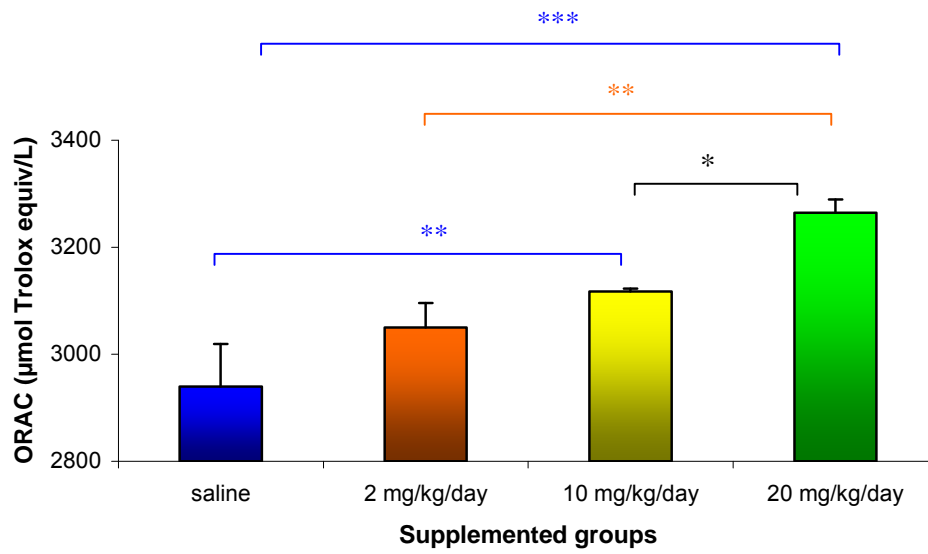
Figure 2.9: ORAC results of the second pilot study, determining the response to 3 concentrations of GSE. Data are indicated as mean ± SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

CHAPTER 3: RESULTS

Results of all figures are given as mean ± SD, unless otherwise indicated. Data presented graphically are also present in Tables in Appendix G.

3.1 Body mass

The daily averages for body mass of both the injury and control GSE and placebo groups are illustrated in Figure 3.1.
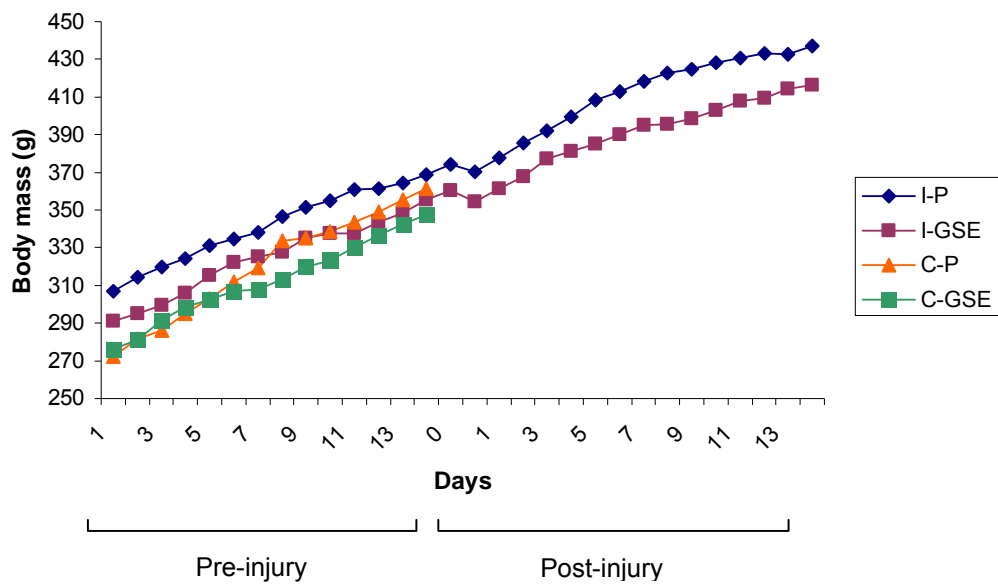
Figure 3.1: Daily body mass, with n = 8 rats per time point per group.

When regression analysis was performed on the same data for time points prior to injury (Figure 3.2), the C-P group displayed a steeper slope compared to the other groups.   However, since this group was neither injured or GSE treated, this difference, which may indicate a slightly higher metabolic rate in this group, should not influence other results obtained.   It should also be noted that directly following the contusion injury (Figure 3.1), there was a decrease in body mass in both the placebo and GSE groups, but this initial body mass loss was regained by day 1 after injury, whereafter rats continued to gain body mass until sacrifice.
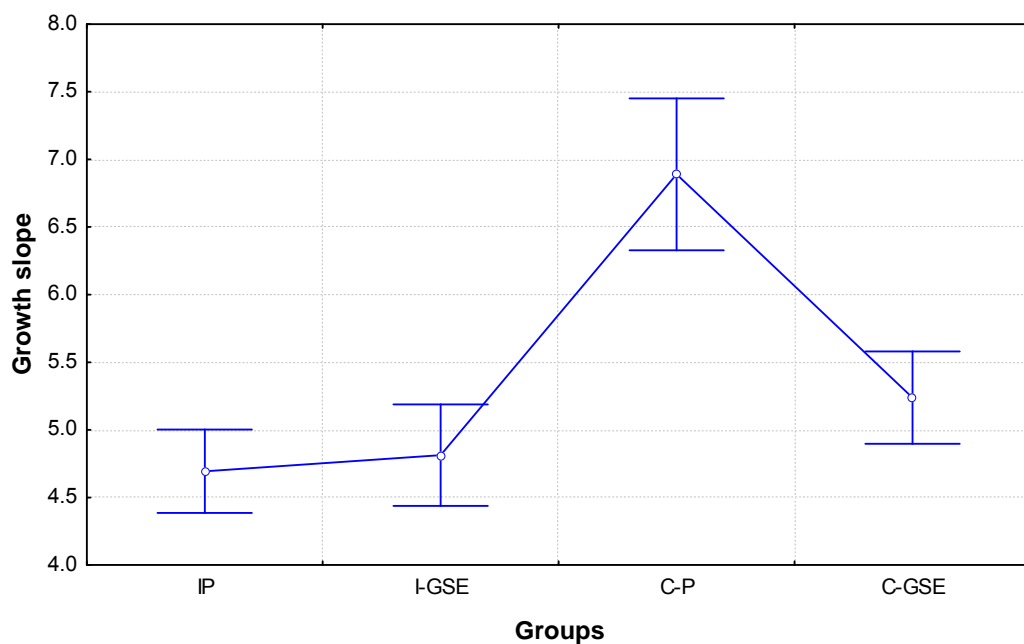
Figure 3.2: Differences in growth over time. The dots represent the slope and the bars represent 95% confidence intervals of all groups.   Statistics: Multiple regression analysis, n = 8 rats per time point per group.

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Table 3.1 indicates the average body mass of all four groups at two specific time points prior to sacrifice/injury. A main effect of time was apparent for all treatment groups before sacrifice/injury ($p < 0.001$). Over the 7 day period during which all groups were subjected to daily gavage, mean increases in body mass were similar for C-GSE, I-P and I-GSE and ranged from 36.2g (I-GSE) to 40.3 (I-P), whereas only C-P increased significantly ($p < 0.01$).

Table 3.1: Body mass (g) prior to injury.

Intervention time	C-P	C-GSE	I-P	I-GSE
Gavaging (2 wk pre-injury)	272.3 ± 20.0**	276.0 ± 30.3	306.8 ± 19.8	291.3 ± 7.5
Gavaging (1 wk pre-injury)	333.8 ± 20.9	313.5 ± 22.2	346.5 ± 22.1	327.5 ± 3.9

Data presented as mean ± SD. Abbreviations: Control Placebo (C-P), Control GSE (C-GSE), Injury Placebo (I-P), Injury GSE (I-GSE). Statistics: Factorial analysis of variance (ANOVA) with Bonferonni *post hoc* test. ** $p < 0.01$ for difference in the C-P group between 2 wk and 1 wk to injury. n = 8 rats per time point per group.

Table 3.2 indicates the average body mass of both injury groups at different time points following contusion injury. After injury, a main effect of time and treatment ($p < 0.001$ for both) were visible between the two groups. However, the main effect of treatment was the result of a consistent difference in mean body mass of the randomly divided groups at the start of the experimental procedures (Figure 3.1) that remained fairly consistent throughout ranging between 14 and 23 g. Seven days after injury, there was a significant difference in body mass in the placebo group compared to body mass before injury ($p < 0.05$). In the GSE group, a significant difference in body mass was only apparent 14 days after injury compared to before injury ($p < 0.001$).

Table 3.2: Body mass (g) after injury.

Intervention times	I-P	I-GSE
Before injury	374.5 ± 18.9	360.5 ± 15.7
4 hr after injury	370.5 ± 20.2	354.3 ± 10.0
3 days after injury	392.0 ± 16.1	377.0 ± 6.8
7 days after injury	418.5 ± 18.1	395.0 ± 2.6
14 days after injury	437.3 ± 17.2	416.5 ± 13.4

Data presented as mean ± SD. Abbreviations: Injury Placebo (I-P), Injury GSE (I-GSE). Statistics: Factorial analysis of variance (ANOVA) with Bonferonni *post hoc* test. n = 8 rats per time point per group.

3.2 Creatine kinase activity

Main effects ANOVA indicated significant differences in plasma CK levels over time ($p < 0.05$), but there were no differences between treatment groups at any time point. Figure 3.3, suggests a transient increase in CK levels 4 hr post-injury in both the treatment groups. However, when comparing 4 hr post-injury time points to all other time points, *post hoc* analysis only indicated significant differences in the GSE group. In contrast to the higher initial CK level of the GSE group, when AUC of plasma CK was calculated, the placebo group displayed a 56 % greater AUC over time than the GSE group (Figure 3.4).

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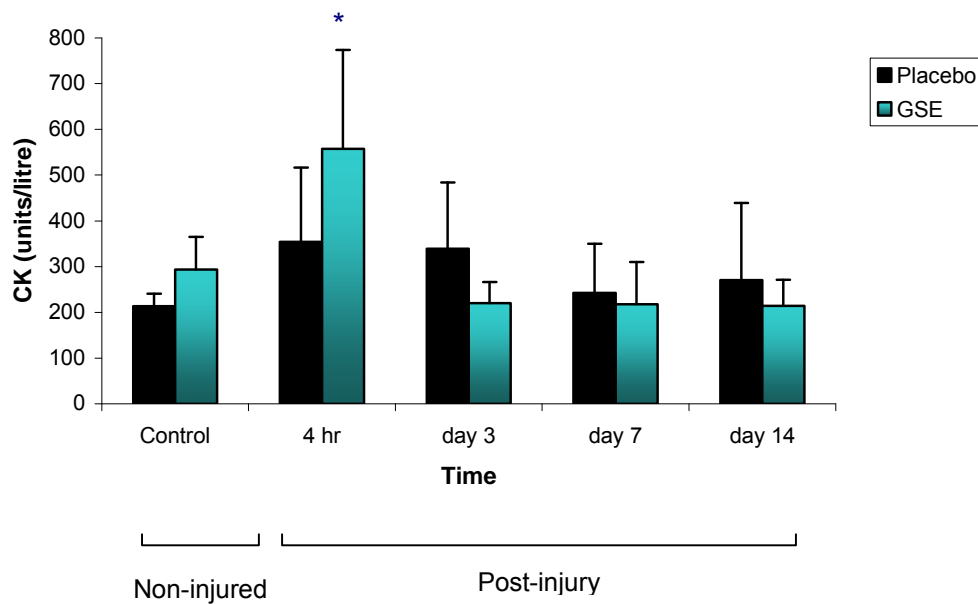
Figure 3.3: Total plasma creatine kinase (CK) activity (mean $\pm$ SD) of the control non-injured groups and the injury groups at several time points after the contusion injury. Statistical analysis: Factorial analysis of variance (ANOVA) with Bonferroni *post hoc* test (* $p < 0.05$ for the 4 hr time point compared to all other time points of the GSE group, except control). Main effect of time, $p < 0.05$. n = 4 rats per time point per group.

3.3 Haematoxylin and eosin staining

Figure 3.5 illustrates histological staining with haematoxylin and eosin. Using this staining technique, qualitative changes in the muscle can be visualised over time. In the control groups (A and F), no muscle fibre ultrastructure damage is apparent. Soon after injury (4 hr, B and G), the disruption of the muscle fibre ultrastructure was accompanied by vascular disruption and immune cell infiltration in both the treatment groups. A qualitative assessment of day 3, resulted in an estimation of more
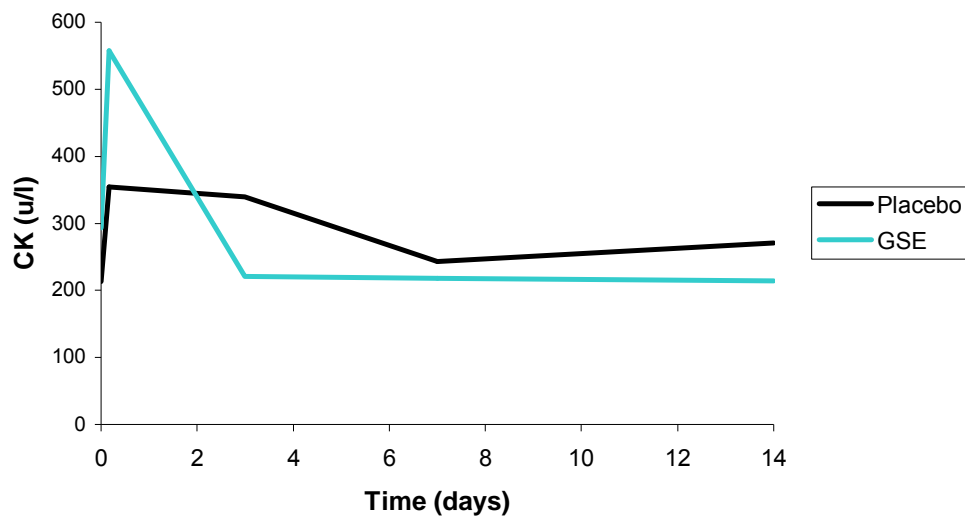
Figure 3.4: AUC of plasma CK calculated for all time points in the placebo group and the GSE group. n = 4 rats per time point per group. AUC was calculated using NCSS 2000.

immune cells and less vascular damage at this time (C and H). It would appear that at this time point, in the GSE group (H) the immune cell infiltrated area was surrounded by newly formed/regenerating muscle fibres. In the placebo group on the other hand, newly formed/regenerating muscle fibres were only apparent 7 days post-injury (D). By day 14, the GSE group's muscle ultrastructure was normal (J), whereas a few immune cells were still present in the muscle fibres of the placebo group (E), and all fibres were not yet well connected.
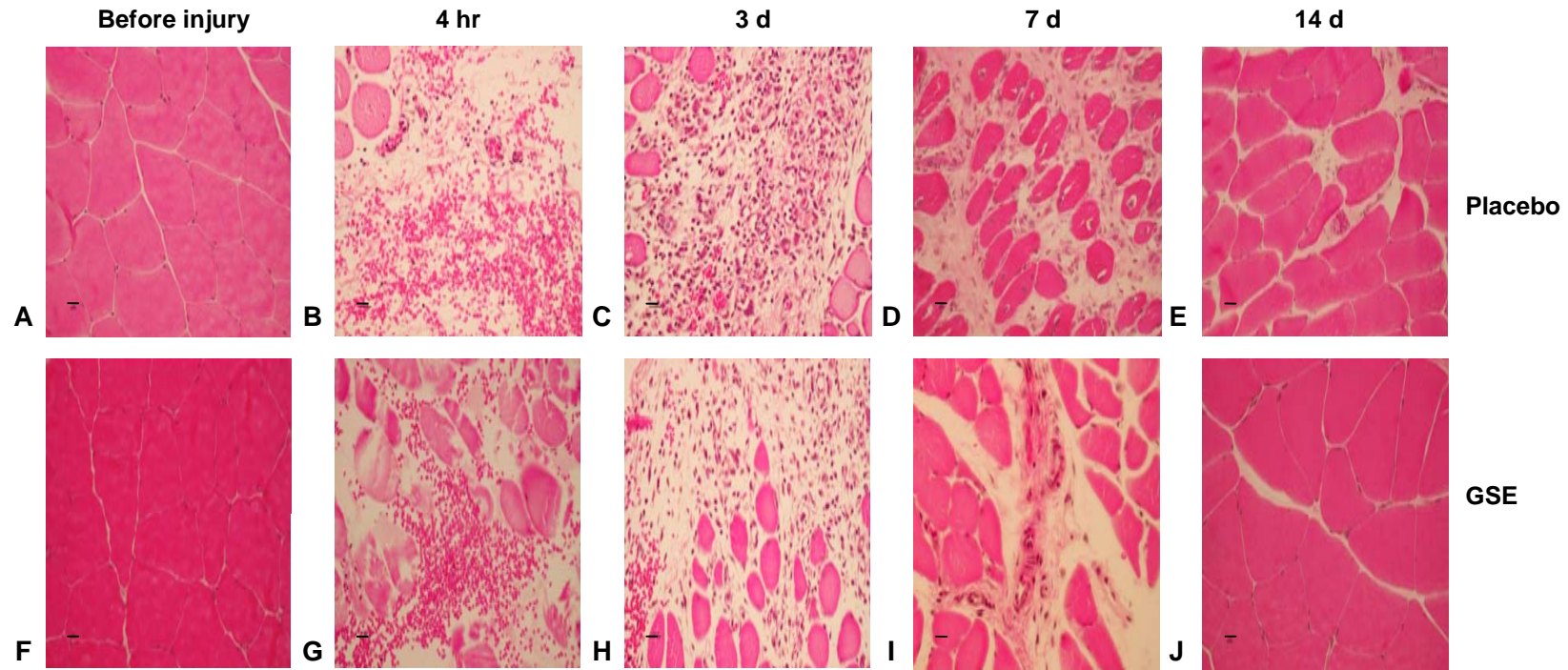
Figure 3.5: H& E staining illustrating the clearing of inflammation after injury. Photos A – E represent samples taken from placebo-treated animals at 14 d after gavaging began: before injury (A), 4 hr (B), 3 d (C), 7 d (D) and 14 d (E) post-injury. Photos F-J represent the same time points in the GSE group. Scale bar represents 10 μm. Photo B and G represent muscle fibre destruction and vascular disruption. Immune cells infiltrated the injured area (photo C and H). New muscle fibres are formed by day 3 in the GSE group (H), whilst new muscle fibres are only formed by day 7 in the placebo group (D). The GSE group (J) displayed normal muscle fibre ultrastucture 14 days after injury, whilst a small amount of immune cells were still present in the placebo group (E).

3.4 The oxygen radical absorbance capacity (ORAC)

Figure 3.6 illustrates the oxygen radical scavenging capacity of the control groups in the plasma and muscle. Significant differences were noted in both the plasma (Panel A) and muscle (Panel B) between the placebo and GSE groups ($p < 0.001$). Figure 3.7 illustrates the free radical scavenging capacity of plasma from chronically supplemented rats sacrificed at various time points after injury, whereas Figure 3.8 illustrates the muscle's scavenging capacity.
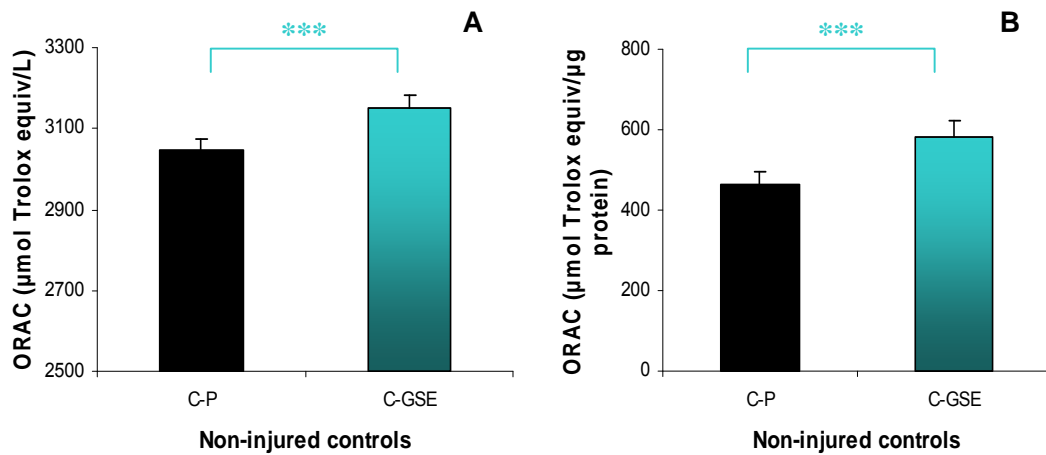
Figure 3.6: Plasma (A) and muscle (B) oxygen radical absorbance capacity of the control groups (Figure detail: *** $p < 0.001$). Statistics: Unpaired, two-tailed student t-test. n = 8 rats per group.

Statistical analysis of the results of the plasma ORAC assay indicated a main effect of time ($p < 0.05$), but no main effect of treatment. *Post hoc* analysis indicated no significant differences in the ORAC of the placebo group between any time points. However, 4 hr following the contusion injury, a significant decrease was seen in

ORAC in the GSE group compared to its non-injured control. By day 3 after injury, ORAC increased significantly compared to early after injury, revealing a capacity equivalent to control values ($p < 0.05$). Between the two treatment groups, the only significant difference was seen on day 3, when the GSE group had a higher ORAC value than the placebo group.
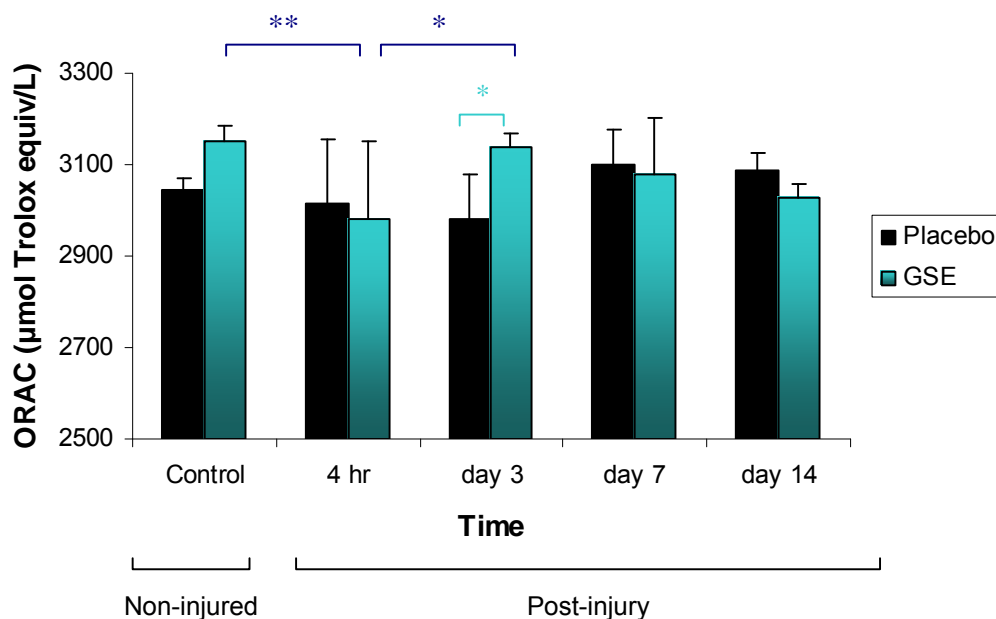
Figure 3.7: Plasma oxygen radical absorbance capacity over time (Symbols: * $p < 0.05$, ** $p < 0.01$). Statistics: Factorial analysis of variance and Bonferonni *post-hoc* test. n = 8 rats per time point per group.

In the muscle samples (Figure 3.8), statistical analysis of the ORAC assay results indicated a main effect of both time and treatment ($p < 0.001$). A significant increase in ORAC was seen by day 7 post-injury in the placebo group compared to placebo

control. The ORAC of the GSE group on the other hand had a similar pattern to that seen in the plasma.   Between the treatment groups, significant differences were noted by day 3, and day 14 post-injury where values similar to control values were reached. ORAC values by day 7 were also similar to control.
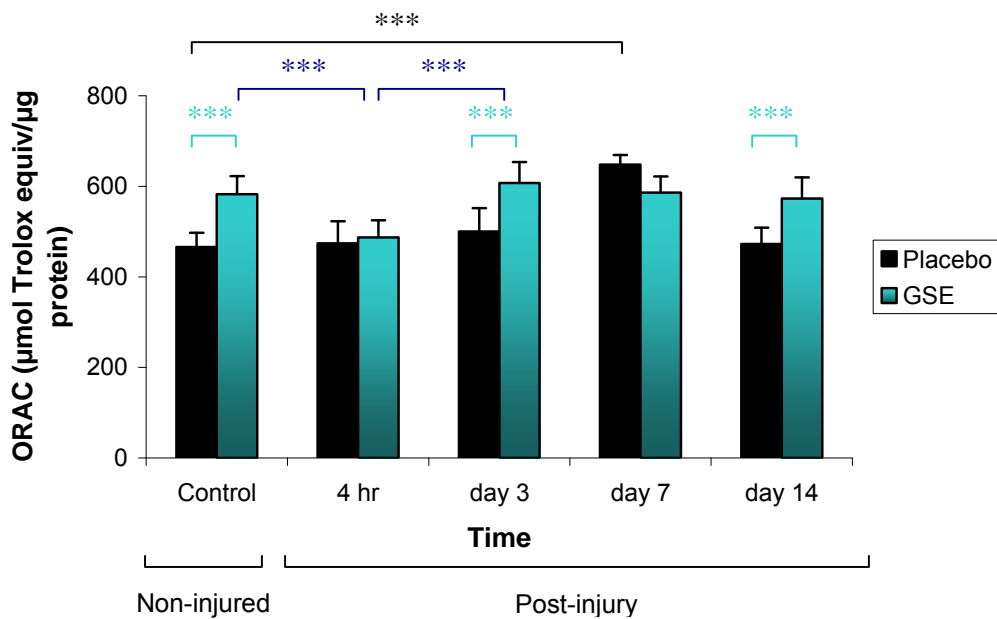
Figure 3.9: ORAC assay results from analysis performed on snap-frozen muscle sections of the *gastrocnemius* muscle (Figure detail: *** $p < 0.001$).   Statistics: Factorial analysis of variance and Bonferonni *post-hoc* test. n = 8 rats per time point per group.

3.5 Satellite cell response to injury

An overview of this section's results indicates that satellite cell (SC) responses to injury were significant (main time effect), the presence of GSE affected the response

(main treatment effect) and interaction between time and treatment were seen at various time points (p < 0.001 for all marker antibodies used). Detail for each marker follow.

3.5.1 CD34

The number of CD34$^+$ SC per myofibre at different time points and for different groups is presented in Figure 3.9. A main effect of time was seen (p < 0.001). Four hours after injury, there was a significant 2.69-fold increase in the number of CD34$^+$ SC per myofibre in the placebo injured (I-P) group compared to placebo control (C-P). A further increase in CD34$^+$ SC numbers in the placebo group was seen on day
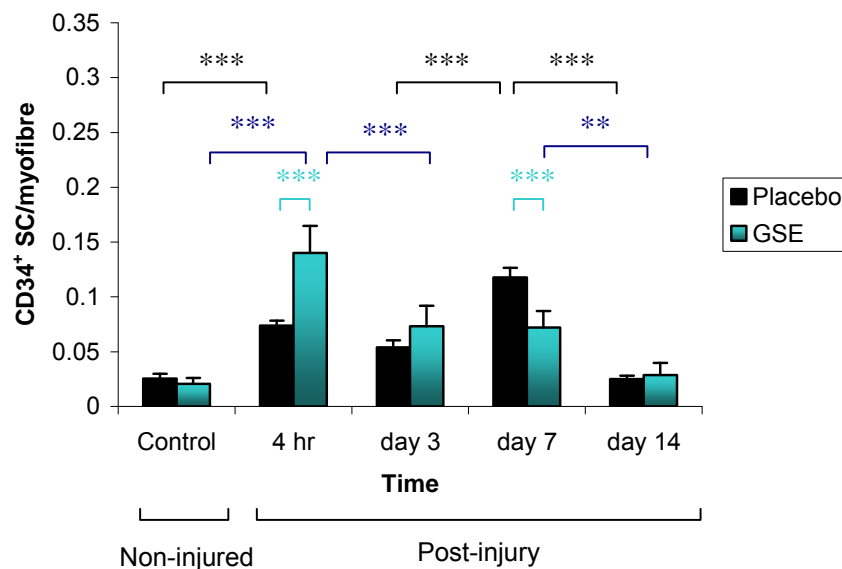
Figure 3.9: CD34$^+$ satellite cell (SC) count normalised for the myofibre number (SC/myofibre) (mean ± SD). Statistical analysis: Factorial analysis of variance (ANOVA), with Bonferonni *post-hoc* test demonstrating significance between the two specific data points (** p < 0.01, *** p < 0.001). n = 4 rats per time point per group.

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7 following injury at which time the increase was 4.62-fold higher than placebo control. In the GSE group, CD34$^+$ SC numbers also showed a significant increase at 4 hr which in this case was already 5.38-fold, but by day 3, CD34$^+$ SC in the GSE group had already significantly decreased to 3.51-fold above GSE control. By day 14, the CD34$^+$ SC number returned to levels similar to control levels in both of the two treatment groups. GSE treatment also had a main effect on CD34$^+$ SC number ($p < 0.001$), which was most evident at two specific time points. Although there were no differences between the two groups pre-injury, 4 hr after injury the GSE group had a significantly higher number of CD34$^+$ SC than the placebo group, whereas on day 7, this pattern was reversed with the placebo group having more CD34$^+$ SC at this later time point. Representative images illustrating increased CD34 expression in the GSE and placebo groups are presented in Figure 3.10. Control images are also included for comparison.

3.5.2 CD56

There was a main effect of time and treatment for CD56 expression ($p < 0.001$). Figure 3.11 indicates the differences between groups over time for the number of CD56$^+$ SC. There was a significant 4.19-fold increase in the number of CD56$^+$ SC in the placebo group by day 3 after injury compared to the placebo control ($p < 0.001$). On day 7 the number of CD56$^+$ SC was similar to that on day 3 for the placebo group, but by day 14, levels correlating to control levels were reached. At this time point, the CD56 levels differed once again, significantly from those seen on day 7 ($p < 0.001$). CD56 expression in the GSE group on the other hand showed a highly significant increase of 9.33-fold at the 4 hr after injury time point compared to its control ($p < 0.001$). From 4 hr onwards, the GSE group had a gradual decrease in
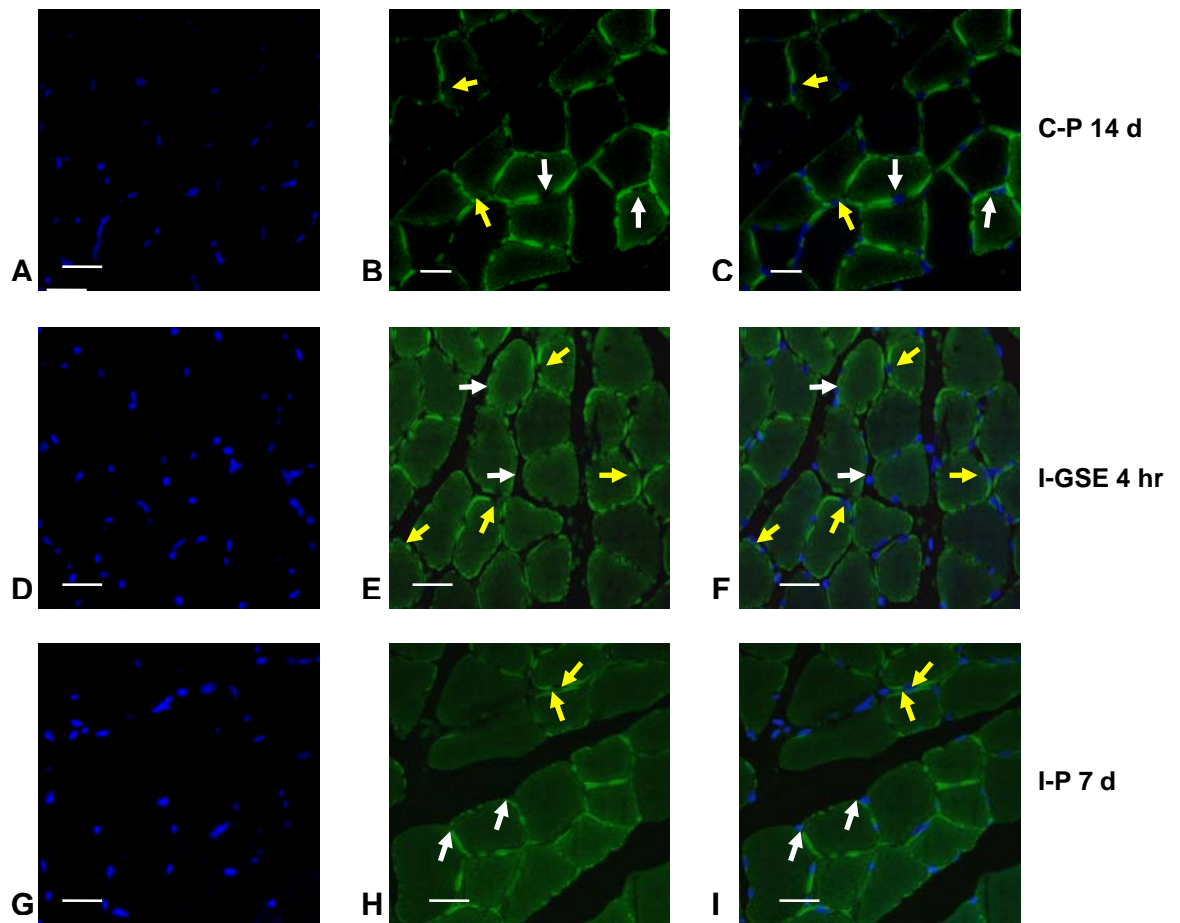
Figure 3.10: CD34 expression (green, FITC) in muscle samples taken at (B) 14 d after placebo treatment in the control group, (E) 4 hr post-injury in the injured leg of the GSE group and (H) 7 d post-injury for the placebo group. The photos on the right hand side (C, F, I) correspond to the time points of the photos in the middle, representing merged images of CD34 expression and that of Hoechst (blue) that stains all nuclei (original magnification x 40). PBS controls are presented in photos A, D and G. CD34^{+} satellite cell examples are indicated by yellow arrows, whereas the white arrows indicate non-satellite cells. Scale bar represents 10 µm. Border zone images were analysed for injured muscle.
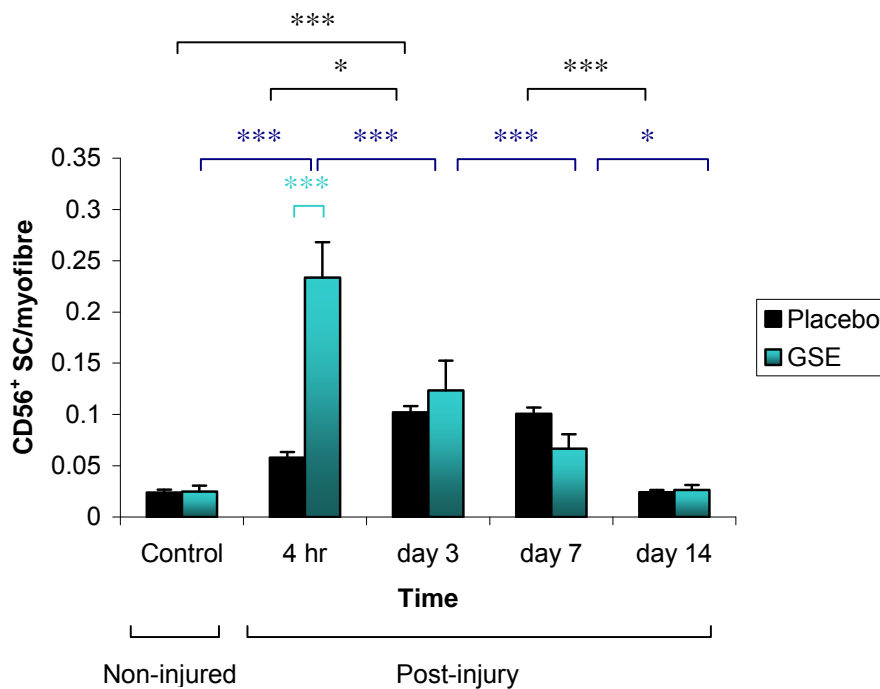
Figure 3.11: CD56^{+} satellite cells expressed per myofibre number (CD56^{+} SC/myofibre number) (means ± SD) at baseline (non-injured) and at 4 hr, day 3, 7 and 14 post-injury. Statistical analysis: Factorial analysis of variance and Bonferonni post-hoc test (* $p<0.05$, *** $p<0.001$). n = 4 rats per time point per group.

the number of CD56^{+} SC until reaching levels similar to control levels on day 14. Treatment also had an effect, with a highly significant difference between the two groups at one time point, 4 hr after injury. Representative images illustrate three time points to indicate the increases in the number of CD56^{+} SC (F and I *vs* C) (Figure 3.12).
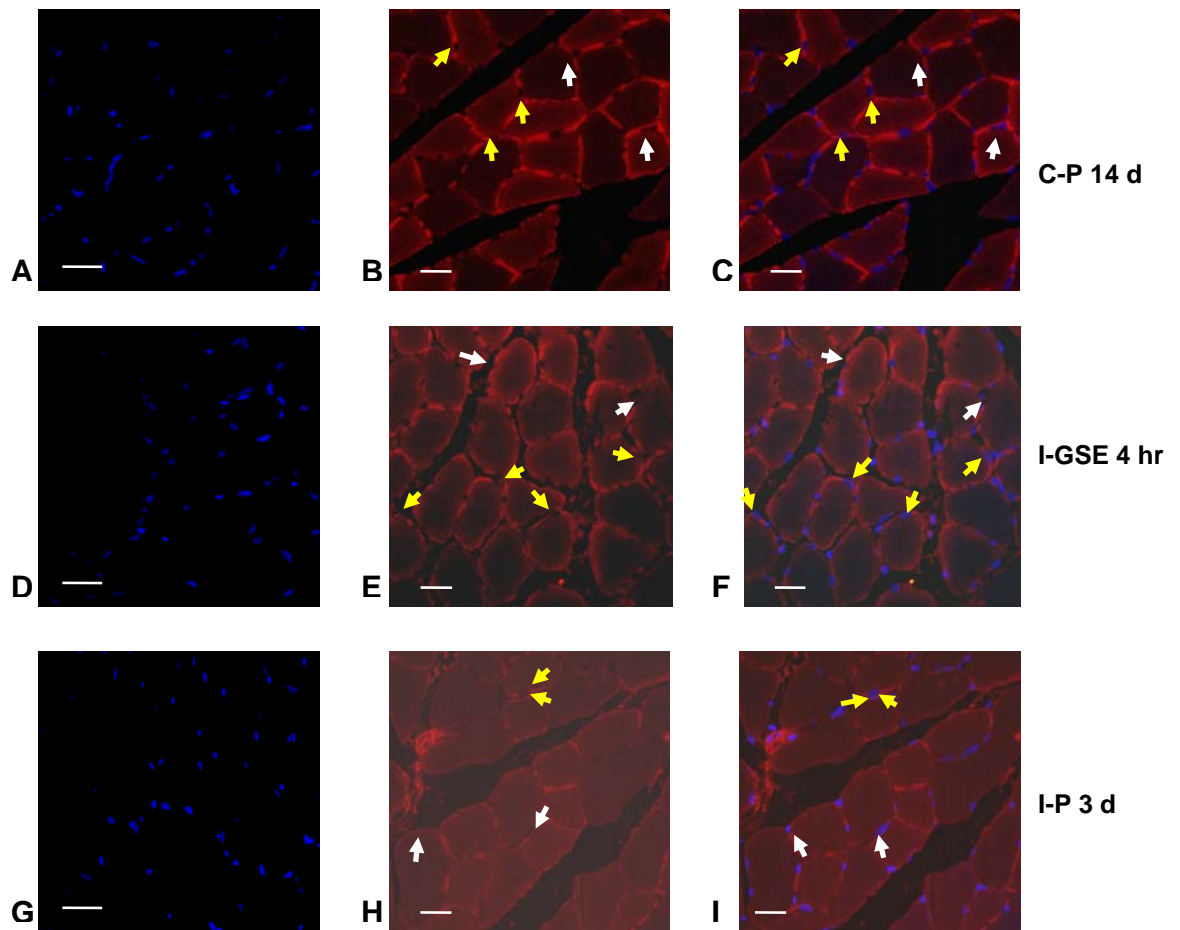
Figure 3.12: CD56 expression (green, FITC) in muscle sections taken at (B) 14 days after placebo treatment in the control group, (E) 4 hr post-injury in the injured leg of the GSE group and (H) 3 d post-injury of the placebo group. The photos on the right hand side (C, F, I) correspond to the time points of the photos in the middle, representing merged images of CD56 expression and that of Hoechst (blue) that stains nuclei (original magnification x 40). PBS controls are presented in photos A, D and G. CD56^{+} satellite cells are indicated by the yellow arrows. White arrows indicate non-satellite cells. Scale bar represents 10 μm. Borderzone images of injury were captured and analysed.

3.5.3 M-cadherin

M-cadherin[+] SC numbers, expressed per myofibre are presented in Figure 3.13. There was a main effect of time and treatment (p < 0.001). Four hr after the contusion injury, both the GSE and placebo group had significantly higher M-cadherin expression, compared to their respective controls (p < 0.001 for both), whereafter the number of M-cadherin[+] SC gradually decreased, until day 14.
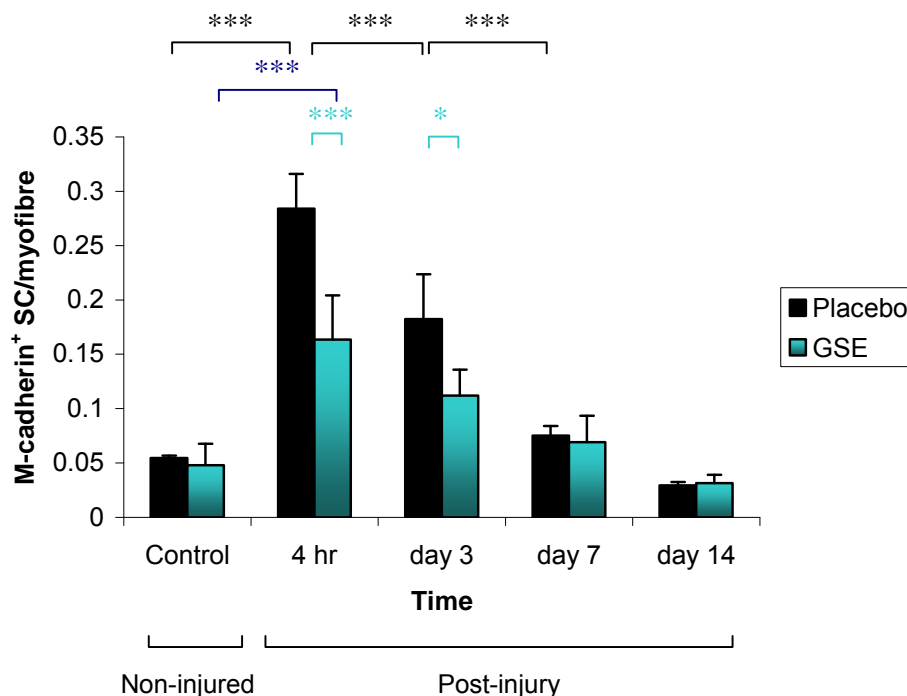
Figure 3.13: M-cadherin^{+} satellite cells expressed per myofibre number (M-cadherin[+] SC/myofibre number) (means ± SD) at control levels (non-injured) and at 4 hr, day 3, 7 and 14 post-injury. Statistical analysis: Factorial analysis of variance with Bonferonni *post hoc* test (* p<0.05, *** p<0.001). n = 4 rats per time point per group.
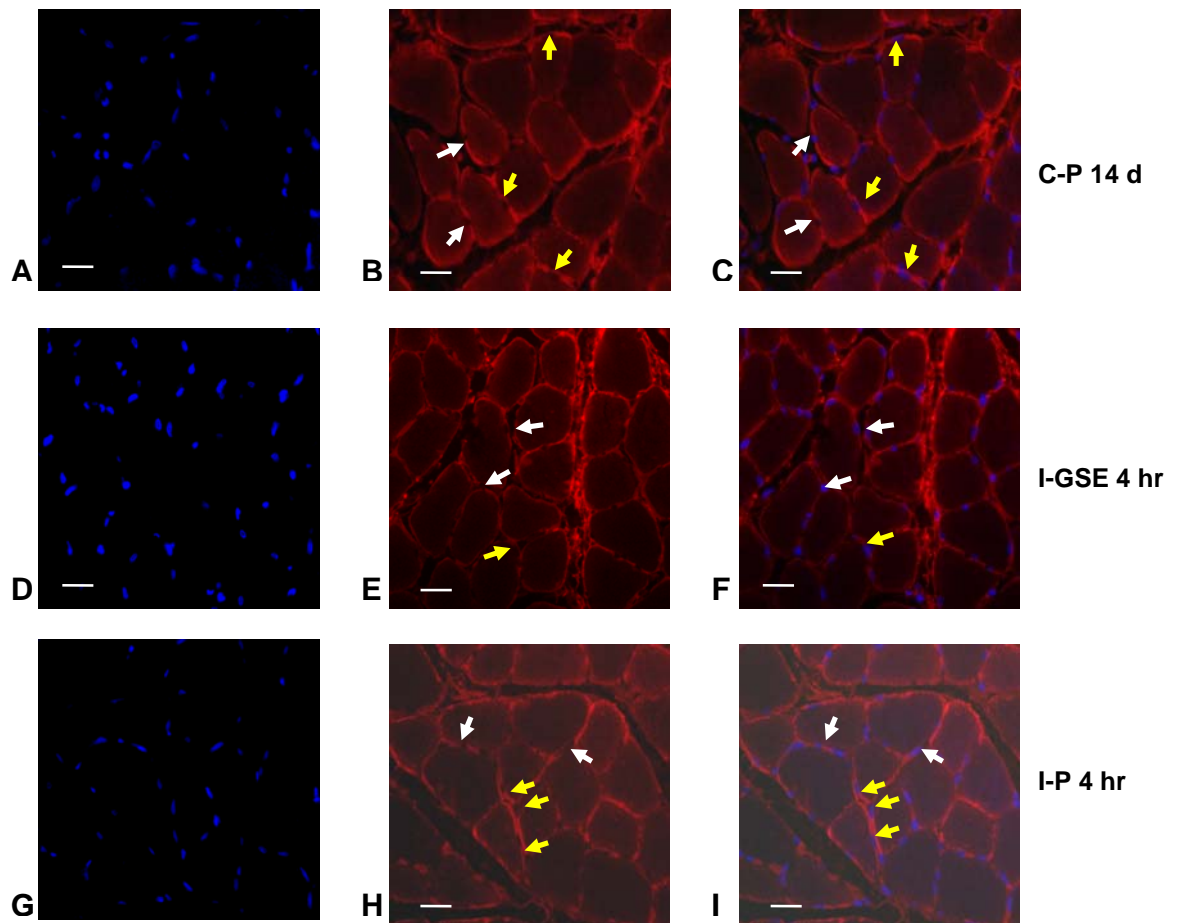
Figure 3.14: M-cadherin⁺ satellite cells visualised in the right *gastrocnemius* muscle by Texas red (B) 14 days after placebo treatment in the control group, (E) 4 hr post-injury in the GSE group and (H) 4 hr post-injury in the placebo group. Figures C, F and I represent merged images of the Figures in B, E and H, stained with both Texas red and Hoechst (original magnification x 40). PBS controls are presented in photos A, D and G. CD56⁺ satellite cells are indicated by the yellow arrows. White arrows indicate non-satellite cells, whilst yellow arrows indicated satellite cells positive for M-cadherin.   Scale bar represents 10 µm. Borderzone images were captured and analysed for the injured muscle.

Significant differences in M-cadherin expression were noted at 4 hr ($p < 0.001$) and on day 3 ($p < 0.05$) post-injury between the two treatment groups. For this particular marker, the placebo group had higher values at both those time points. Representative images illustrating M-cadherin expression 4 hr after injury of both the GSE and placebo groups are presented in Figure 3.14.

3.6 Foetal myosin heavy chain (MHC$_f$)

Figure 3.15 indicated the percentage of MHC$_f^+$ myofibres, with central nuclei. A time effect was seen in both the GSE- and placebo-treated groups ($p < 0.001$).
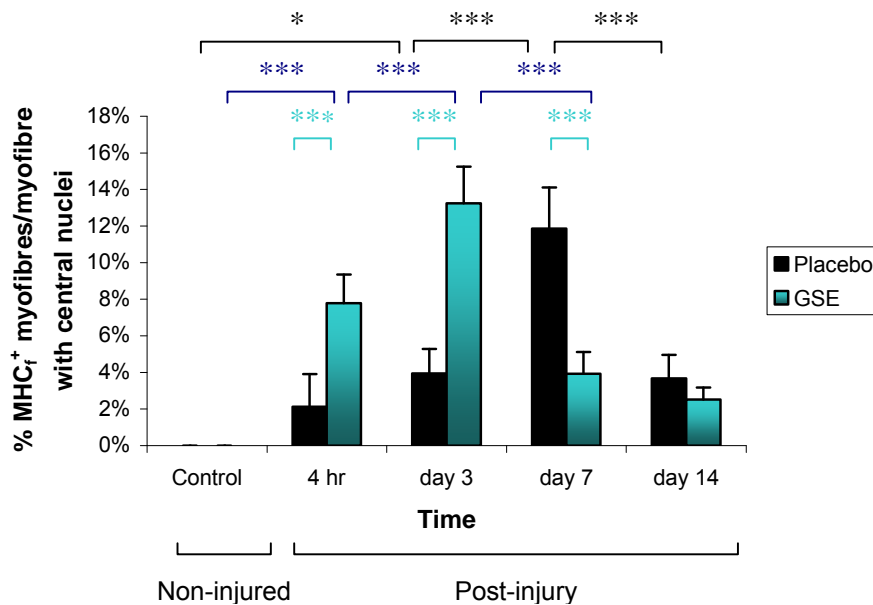
Figure 3.15:   Foetal myosin heavy chain (MHC$_f$) positive myofibres expressed relative to myofibre number (% MHC$_f^+$ myofibres) at control levels and at 4 hr, day 3, 7 and 14 post-injury (* $p<0.05$, *** $p<0.001$).   Statistics: Factorial analysis of variance with Bonferonni *post hoc* test. n = 4 rats per time point per group.
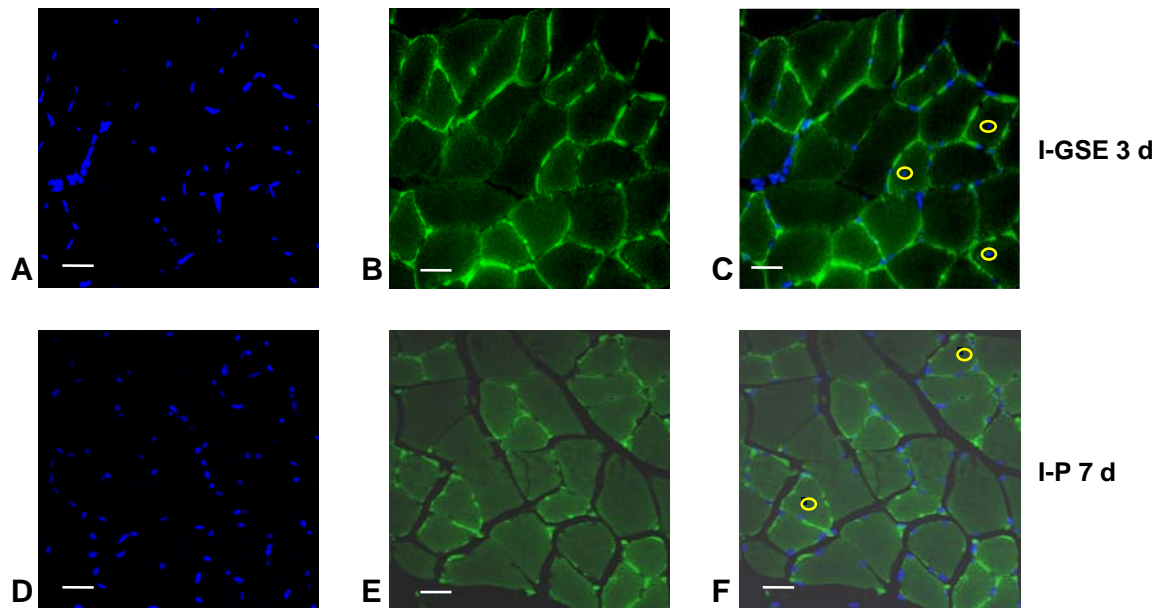
Figure 3.16: Foetal myosin heavy chain expression is illustrated in Panels B,C, E and F. Panels B and C represent a muscle section of a GSE supplemented rat 3 days post-injury, whereas Panels E and F represent a muscle section of a placebo supplemented rat, 7 days post-injury. FITC (green) was used to visualise the antibody and Hoechst (blue) was used as a nuclear marker. Panels A and D represent PBS controls. The yellow circles in Panels A and C represent the fibres positive for MHC_f, however, only the muscle fibres in Panels B and D are truly regarded as being MHC_f^+, due to the presence of central nuclei. Scale bar represents 10 µm. Borderzone images of the injured muscle were captured and analysed.

Following the contusion injury, the placebo group had a significant increase in the number of MHC_f^+ myofibres by day 3 after injury ($p < 0.05$), while reaching peak values by day 7. However, by day 14, the MHC_f content of the placebo group had significantly decreased compared to day 7 ($p < 0.001$). A significant increase in

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MHC$_f^+$ myofibres in the GSE group were already apparent 4 hr after injury compared to its respective control ($p < 0.001$). A further increase in the percentage of MHC$_f^+$ myofibres was seen by day 3, after which the MHC$_f$ content of the GSE group decreased. There was also a main effect of treatment, at 4 hr and day 3 after injury (higher in GSE *vs* placebo), and by day 7 (lower in GSE *vs* placebo). Representative images illustrating the increased number of MHC$_f^+$ fibres found by day 3 in the GSE group and day 7 in the placebo group are presented in Figure 3.16.

CHAPTER 4: DISCUSSION

To our knowledge, this is the first study to investigate the effect of supplementation with a natural plant product possessing antioxidant properties on skeletal muscle following a contusion injury. Specifically, this is the first study investigating Oxiprovin™ administration daily to rats over a 2 to 4 week period to determine ORAC status and skeletal muscle SC activity following a contusion injury.

4.1 Body mass

Rats in all four experimental groups gained approximately the same amount of weight every day, irrespective of treatment. However, rats belonging to the C-P group had a steeper growth slope when regression analysis was performed. This steeper slope of the C-P group can be ascribed to the initial slightly lower body mass of the rats (not significantly different), which could influence the metabolic rate of the animals in that particular group. Also, rats belonging to the injury groups lost weight directly after injury (i.e. 4 hr after injury), possibly due to the anaesthetics used and the amount of time it took for them to wake up after the procedure. This weight loss was recovered 24 hr post-injury and no further differences were seen between the two treatment groups.

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4.2 Creatine kinase

As mentioned in Chapter 1, skeletal muscle contusion injury causes damage to the underlying muscle and plasma CK activity is often used as an indicator of the presence of muscle damage in humans [243-245] and animals [152, 246, 247]. However, CK activity does not correlate proportionately to the extent of muscle damage, but rather that an association between elevated plasma CK activity and damage exist [152, 243-247].

In a study by Akimau *et al.* (2005), the hind limb of rats was compressed for 6 hr with a weight of 3 kg [152]. Results indicated normal CK activity in animals receiving sham injury (placebo group) as 146 ± 100 U/L. The results presented for the control groups in this thesis were just as widely spread and variable as the results obtained by Akimau *et al.* (2005). The CK activity in the control placebo group was 214 ± 27 U/L, whereas that in the control GSE group was 294 ± 71 U/L (see Figure 3.4). In their study, the CK activity results in the placebo group stayed the same irrespective of injury. However, after the hind limb was allowed to be reperfused for 3 hr following the compression in their study, the serum CK activity increased significantly (2641 ± 1683 U/L *vs* control values, $p < 0.05$).

In contrast, in the current study, 4 hr after injury, CK activity only tended to increase (non-significantly), after which the CK activity gradually decreased again. In the placebo group, the 4 hr and 3 d CK activities were still very similar, indicating a slow clearance of CK out of the muscle into the blood. Moreover, even though the GSE group displayed significant differences over time with a greater CK peak 4 hr after injury, the placebo group had a 56 % larger CK activity AUC. This can be explained

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by significantly lower CK activities in the GSE group, seen already by day 3 after injury compared to 4 hr (p < 0.05), indicating on the one hand that the GSE supplement was associated with a quicker clearance of CK into the blood (4 hr), but also less sustained loss of CK by day 3 from the muscle due to less membrane/ECM damage.

Two possible mechanistic explanations for the higher CK activity seen at 4 hr in the GSE group, as well as two possible mechanistic explanations for the decrease by day 3 after injury in the same group are presented below:

i) GSE supplementation may have resulted in a more rapid increase in the inflammatory response. This in turn would result in more immune cells infiltrating the damaged tissue, which could lead to secondary damage. This explanation is possible, as it is known that GSE supplementation results in improved blood circulation [248].

ii) Alternatively, improved circulation may simply have effectively cleared the leaking creatine kinase enzymes from the injured muscle bed to the systemic circulation. Proanthocyanidin, the active component of GSE, binds to collagen, strengthening the vasculature, especially capillaries [249]. Therefore, GSE may have reduced the damage done to the vasculature.

iii) It is possible that a similar mechanism acting on muscle extracellular matrix resulted in lower CK release by day 3.

iv) Furthermore, the decrease seen by day 3 may also be due to an improved and more rapid phagocytic process, as a result of better supply of neutrophils and monocytes to the site of injury. A study by Loike *et al.* (1984) demonstrated that increased phagocytosis is positive correlated with a decrease in CK activity, supporting this abovementioned assumption [250].

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A limitation of the current study with regard to the response of CK activity to injury is that its activity was not measured at more time points, allowing a better understanding of the time course of events following an injury. Furthermore, muscle damage assessed in this way by means of CK activity is an indirect way of measuring skeletal muscle damage, because it measures circulating CK activity in the plasma/serum and not the actual amount of damage done to the muscle itself.

4.3 Histology

Previous studies on skeletal muscle contusion injury have focused on ultrastructural changes in the muscle during the recovery from a contusion injury [6, 156, 157]. Histological staining with haematoxylin and eosin was also used in the current study, as well as in the studies mentioned above, to illustrate the extent of damage to the muscle following a contusion injury. Similar time points were used in the current study to allow comparisons of muscle fibre ultrastructure to the other studies mentioned.

Fisher *et al.* (1990) investigated the ultrastructure of skeletal muscle 6 hr after injury, as well as at various other time points after injury [156]. Since this 6 hr time point was the earliest time point available in the literature, the results obtained will be compared to our earliest time point of 4 hr. At 6 hr in the injured muscle, a variable pattern of tearing and disruption of normal muscle fibre ultrastructure and small blood vessels was apparent. Due to this local disruption of capillaries and small blood vessels, cellular constituents of the blood including erythrocytes and

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neutrophils escaped into the intercellular space [156]. This qualitative observation was also evident in our study at 4 hr after injury independent of treatment.

At 3 days after injury, the degree of vascular damage evident was reduced (also seen in the studies by Beiner *et al.* (1999) [6] and Kami *et al.* (1993) [157]), possibly as a result of either the large number of immune cells that had infiltrated the injured area resolving the damage or a rapid time course of vascular healing. In the study by Kami *et al.* (1993), 2 days after injury, fibronectin (an extracellular matrix component responsible for modulating the activity of VEGF and angiogenic factor [251]) was observed filling the widened endomysium, indicating that the vasculature had started to heal [157].

By day 7, the muscle at the site of trauma appeared to have regenerated to a significant extent compared to 4 hr and day 3 after injury. At this time point of 7 days, muscle fibres displayed multiple subsarcolemmal and central nuclei, indicating that the muscle fibres were in the process of regenerating themselves. Central nucleation has long been recognised as a crucial feature of the regenerating process [252]. These results are also comparable to the results obtained by Beiner *et al.* (day 7), Fisher *et al.* (day 6) and Kami *et al.* (day 8) [6, 156, 157]. Fourteen days after injury, our GSE and placebo groups had relatively normal muscle ultrastructure, similar to results obtained by Beiner *et al.* (1999) [6] and Fisher *et al.* (1990) [156]. However, in the placebo group, a few immune cells were still visible, indicating that the muscle had not healed completely compared to the GSE group. Also, a qualitative assessment indicates that the intercellular space was still enlarged only in the placebo group. Future studies should investigate whether fibronectin and VEGF

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responses in the post-injury phase are modulated by GSE supplementation (or other antioxidant treatments) and whether this particular supplement may be involved in accelerating the vascular healing process after injury.

Focussing on the muscle cells themselves, the qualitative results/data obtained for the GSE groups were similar to that of the placebo group at 4 hr after injury. However, by day 3, in the GSE group, more new muscle fibres were evident which could be compared to day 7 of the placebo group, suggesting that the GSE supplement accelerated the recovery process after injury by allowing earlier skeletal muscle fibre formation and therefore a quicker return of normal skeletal muscle fibre ultrastructure.

Of the three studies mentioned above, only Beiner *et al.* (1999) investigated the effect of an intervention on the outcome of the muscle ultrastructure following a contusion injury [6]. In that study, the effect of anabolic steroids and corticosteroids on the healing of skeletal muscle after a contusion injury was investigated. The results indicated that in an animal model corticosteroids may be beneficial in the short term, but they cause irreversible damage to healing muscle in the long term, including disordered fiber structure and a marked diminution in force-generating capacity. Anabolic steroids may aid in the healing of muscle contusion injury to speed up the recovery of force-generating capacity, however, the use of anabolic steroids did not seem to accelerate new fibre formation as was apparent after Oxiprovin™ supplementation. Future studies using the GSE supplement should also investigate the time couse of recovery of force-generating capacity of the muscle following a contusion injury.

4.4 ORAC assay

The ORAC assay measures the total amount of antioxidants present in an aqueous system, therefore we do not know what the exact contribution of the lipophilic antioxidants is. However, because the GSE supplement we used in our study was dissolved in 0.9% saline, an aqueous solution, the ORAC assay was considered to be the best available method to determine the oxygen radical scavenging capacity.

Mazza *et al.* (2002) investigated the consumption of proanthocyanidins by humans and its effect on the serum antioxidant status was assessed by the ORAC assay [253].   In this particular study, it was found that there was a 1.16 fold increase in ORAC in the proanthocyanidin group already apparent 4 hr after consumption compared to the placebo group. However, this increase was not statistically significant, although a tendency towards significance was reported ($p < 0.06$). In the current study, there was a significant difference in plasma ORAC between the placebo and the GSE group, 2 weeks after treatment ($p < 0.05$). It can therefore be concluded that although 4 hr after proanthocyanidin consumption, a difference is apparent between the two treatment groups as seen in the study by Mazza *et al.* (2002), a longer timeframe is needed for the proanthocyandins to reach a peak value and to remain stably elevated. In the current study, ORAC was also measured in the muscle. Just as in the plasma, the ORAC results indicated a significant difference between the placebo and GSE control groups (1.25 fold increase). This significantly higher ORAC value of the GSE group therefore indicates that the antioxidants present in the GSE (proanthocyanidins – a compound of the flavonoid family), provided a better free radical scavenging ability, than is normally present in the placebo group.

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Few statistically significant differences in plasma ORAC were apparent over time in response to injury. Although no statistically significant differences were seen between any time points in the placebo group's plasma ORAC, the GSE group had a significant decrease in ORAC 4 hr after injury. This is possibly due to the generation of reactive species by circulating macrophages and neutrophils, and antioxidants themselves (as described in the literature), which neutralised a significant amount of the available antioxidant capacity. Although reactive species lead to secondary damage [91, 92, 254], no literature is available on the possible connection between GSE and secondary damage. The decrease in ORAC seen at 4 hr is in accordance with the increase seen in CK activity at 4 hr in the GSE group. Together these data indicated an enhanced early response to injury. Three days after injury, the ORAC of the GSE group was similar to the GSE pre-injury control values. At this time point, there was a significant difference between the two treatment groups, indicating on the one hand that more radical species were still being generated in the placebo group, and on the other hand the GSE group had regained its radical quenching ability.

In contrast to the lack of significant change in plasma ORAC, muscle ORAC was significantly increased by day 7 after injury in the placebo group. There are several possible explanations for this result:

i) More cytosolic proteins may leak into the muscle after injury including antioxidant and reactive species neutralizing proteins [254].

ii) Reactive species generating compounds may not be present to the same extent in the newly generated muscle fibres, hence the inherent quenching ability is not

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required as much and the net effect is elevated muscle ORAC. Possible candidates include amongst other things iron or iron-containing proteins like myoglobin [254].

iii) Thirdly, although no studies are available on this particular aspect, I speculate that the composition of the regenerating proteins present in the muscle could also have changed, offering better reactive species buffering/antioxidant quenching capacity.

From day 7 to day 14 after injury in the placebo group, there was a significant decrease in muscle ORAC, probably as a result of increased protein expression and protein turnover, as seen in a study by Gierer *et al.* (2003) regarding myosin heavy chain expression in denervated muscle [255].   In addition to the proteins present in the muscle, SC might also play an essential role, because it is known that reactive species are generated upon SC activation, proliferation and fusion [16], indicating a possible role for SC in ORAC decrease in inflamed regenerating muscle. Therefore, SC by day 7 in the placebo group could comprise a greater proportion of the cytoplasmic to nuclear content of the muscle after injury indicating that SC might be busy proliferating and fusing. This explanation is most probably the main reason, as seen in our SC data (see figure 3.11), where a decreased number of CD56^{+} SC were observed by day 7 and 14 in the placebo group. In turn, this could mean that more processes might occur in these nuclei, which could produce reactive species.

The GSE group had a significant decrease in muscle ORAC 4 hr following injury compared to the control GSE group. Four hours after injury, the disrupted vasculature may have caused a rapid loss of antioxidant delivery to the site of injury or the immune and satellite cells generated more ROS which needed to be

quenched. In addition, one could postulate that the change in ORAC is the result of inflammation and could therefore be the influence of neutrophils and macrophages. As it is known that neutrophils (activated very early after injury – as early as 4 hr) and macrophages are responsible for reactive species generation as part of the inflammatory process [89-92], it might possibly explain the events following a contusion injury. As part of the phagocytic process, neutrophils produce substantial amounts of ROS [254, 256], which play an essential role in the regeneration of tissue, by activating more neutrophils to remove damaged proteins [254]. Although Child *et al*. (1999) reported that inflammation did not alter the antioxidant status after multiple bouts of eccentric exercise over 12 days [257], a possible influence of inflammation on ORAC (total antioxidant capacity – TAC used in their study) may be an early response (as early as 4 hr as seen in our study), when CK was elevated after eccentric exercise. However, TAC was not investigated at earlier time points prior to 4 days, therefore the possibility that their biopsy sampling missed the peak in neutrophil-mediated oxidative stress cannot be discounted. Furthermore, although this conclusion regarding antioxidant status and inflammation was drawn, the study design also included the multiple bouts of exercise to which muscle could have adapted the endogenous antioxidant capacity, therefore masking the effect of an inflammation on ORAC. In the current study however, only a single contusion injury was applied to the muscle, therefore, inflammation may have caused the changes in ORAC seen over time.

The muscle's cellular integrity recovered rapidly by day 3 as indicated by H&E. At this time point, the GSE group had a significantly higher ORAC than the placebo group, indicating that the chronic antioxidant supply in the GSE group was once

again delivered to the muscle. Alternatively, adaptation of antioxidant enzymes in rat skeletal muscle may have occurred in response to the chronic GSE supplementation by further increasing the number of enzymes present. Similar findings were seen in an exercise study, where it was found that chronic vitamin E supplementation may influence the adaptation of glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities in rat skeletal muscle [258]. By day 14, the ORAC in both treatment groups returned to control values, indicating that the muscle had recovered and that the differences seen in ORAC here were due to supplementation only.

4.4 Satellite cells

Various articles report on the expression of different SC markers during recovery from exercise (e.g. strength training, eccentric exercise or stimulation) [122, 144, 259], and these studies have been performed in humans, animals and cell culture models.   However, limited data are available on SC marker expression after sustaining a contusion injury, and the change in expression during the recovery process [260]. Moreover, research has focused on ultrastructural events following injury and no data has been presented on Oxiprovin™ or other proanthocyanidin products and possible effects on SC expression following a contusion injury. Controversies also exist in the literature on specific SC markers and when exactly they are expressed [15, 123, 125, 140, 141, 144]. For the purpose of this study, the following conclusions were drawn regarding the SC markers used and their expression pattern: CD34 can be used as a marker of quiescence and early activation (taking into account position), CD56 can be used as a marker of SC in

either quiescence, activation or proliferation phases and similarly M-cadherin can be a marker of either quiescence, activation or proliferation.

4.4.1 CD34

We showed time and treatment effects on CD34 expression *in vivo* at almost all time points. In our study, no significant difference in CD34 expression was evident between the two pre-injury control groups, but a biphasic response to injury was apparent in both treatment groups. Four hr after injury, the placebo group displayed a significant increase in the number of CD34^{+} SC, which may be due to the activation and migration of SC from another area into the injured area still carrying "the quiescent marker" (CD34). Another explanation might be that newly activated SC still expressed the "quiescent marker", even though they may also express an activation marker. This is quite possible, as CD34 labels both quiescent and early activated SC [123, 140]. Migration and activation, according to Hirata *et al.* (2003), occur simultaneously [261], therefore both of the abovementioned explanations may be true.

In the placebo group, 7 days after injury, there was an even more significant increase in CD34 expression than that seen at 4 hr and day 3 after injury. During this time SC would be proliferating but may also be returning to quiescence after fusion has taken place, although the return of a SC to quiescence hasn't been as intensely studied as SC activation. In a study by Allen *et al.* (2005), SC from 9-month-old adult rats (quiescent *in vivo)* were isolated and cultured [262]. These quiescent SC displayed a lag phase prior to division and under normal growth conditions, divided for the first time between 42 and 60 hr. However, in the presence

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of HGF, SC proliferated sooner. Therefore it is logical that the increased expression of CD34 by SC by day 3 indicates proliferation of SC. Fourteen days after injury however, the CD34 levels in the placebo group had returned to control values indicating that the SC had fused.

The GSE group had a similar CD34 pattern to the placebo group, although the increase in the number of CD34^{+} SC occurred earlier in the GSE group (already at 4 hr following injury) compared to the placebo group. Since at this early time point proliferation cannot be an explanation, this indicated that the GSE group had a better migration of immune cells into the injured area, as a result of improved blood flow to the site of injury [248], sufficient to secrete the cytokines required to attract the SC for regeneration. Another explanation for this earlier increase in CD34 expressing cells may also be that migration is present at this time, but not fusion. A possible mechanism is a better suppression of cell surface or chemoattractive proteins (chemokines) in the GSE group. A study by Roebuck *et al.* (1999) illustrated that antioxidants inhibited the TNF-α mediated stimulation of chemokine expression [263], indicating that the suggestion made here is quite valid. By day 14, the GSE group had similar CD34^{+} SC numbers than that of controls.

In contrast to CD34, CD56 has been used more frequently to assess SC in human and mice muscle biopsies, mainly making use of the *vastus lateralis* and *gastrocnemius* muscle [143, 144, 259, 260]. However, CD56 stains both quiescent and activated SC and no distinction between the quiescent and activated SC pool has been attempted in any of these studies. A possible way in which to distinguish between these two SC pools will in the end be to use a combination of markers of

quiescence, in particular Pax7 [124] and CD34 [123], or activation, particularly M-cad and CD56 [122], or Pax3 [17] and CD56 [122]. Pax7 is probably the most useful current marker for identifying quiescent satellite cells [124], whereas Pax3 is also now accepted as a good marker for activation and proliferation [17].

4.4.2 CD56

In the current study, similar results were obtained when staining muscle sections with CD56 compared with CD34, possibly indicating co-expression of CD34 and CD56 during the quiescent and early activation stages of SC during recovery. However, more SC expressed CD56 than CD34 in the GSE group 4 hr after injury and by day 3 after injury. Resolution also followed a relatively similar pattern to that seen for CD34. CD56 peaked in the placebo group 3 days after injury and stayed elevated until day 7. This increase in CD56 may indicate that SC are starting to proliferate, however, co-staining with other markers of proliferation, bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) and proliferating cell nuclear antigen (PCNA) are needed to try and verify whether this is true [17, 24]. These markers are generally regarded as true markers of proliferation, as opposed to other markers which labels quiescent, activated and proliferating SC [17, 24].

Nitric oxide, expressed by activated macrophages [93], has been suggested as one of the activators of SC [115] and may be involved in inducing CD56 expression. However, CD56 has also been shown to be diffusely distributed in denervated human skeletal muscle, whereas there was no expression of the sarcolemma-associated nitric oxide synthase (NOS I) [264]. As the muscle started to heal and an increase in the number of re-innervated muscle fibres was apparent, the intensity of

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CD56 expression decreased progressively, whereas that of NOS I increased. Thus, changes in CD56 expression after injury, as seen here, may indicate a change in neural stimulation or release of neural factors during the injury. For future studies, it might be useful to determine the exact time course of re-innervation, as well as to investigate the interaction between CD56 and other neural factors known to be expressed during re-innervation.

Fourteen days after injury, there was a significant decrease in CD56 expression in the placebo group, again similar to the data obtained for CD34. In an *in vitro* study it was shown that CD56 expression enhances fusion of myoblasts to form myotubes [265], thus suggesting that CD56 has a role in myoblast fusion. Therefore, the decrease in CD56 expression seen 14 days after injury could indicate that the SC have fused, as the number of SC were similar to the placebo control.

CD56 also followed a similar pattern to CD34 in the GSE group. Four hr after injury, there was an increase in CD56 expression, after which the number of CD56^{+} SC gradually decreased. During this time point, a significant difference was seen between the two supplementation groups, providing evidence that SC are activated earlier after injury in the presence of Oxiprovin™. By day 14, all SC levels were similar to control values, indicating that the repair phase had reached completion.

4.4.3 M-cadherin

Pre-injury, no difference in M-cadherin (M-cad) expression was seen in the control groups. However in comparison to CD34 and 56, almost double the number of SC were identified using this marker. A possible explanation for the higher number of

SC seen when labelling muscle sections with M-cad could be due to the normal calcium-potassium balance or the release of calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR) [266]. During contraction, calcium enters the muscle cytoplasm, while potassium leaves the cells, therefore there will always be calcium present inside and outside the muscle cells, even at rest, thus M-cad will always be expressed. Although it is known that M-cadherin is a calcium-dependent adhesion molecule [128, 130], it is not known how the interaction between M-cadherin and Ca^{2+} causes M-cadherin to be expressed on the outside of the muscle beneath the basement membrane.

Four hr after injury, there was a significant increase in the number of M-cad^{+} SC in the placebo group to levels similar to the CD56^{+} SC in the GSE group. Thereafter the number of M-cad^{+} SC gradually decreased from day 3 until day 14. In a study by Ozawa *et al.* (1990), removal of Ca^{2+} ions, usually required for cadherin-mediated adhesiveness, mimicked the inhibitory effect of the M-cad peptide 040/M [267], and a decreased M-cad expression should be expected.   Conversely, immediately following a contusion injury (within 15 min), the calcium-potassium balance inside the muscle cell is disrupted and the SR membranes leak more Ca^{2+} ions, causing the build-up of Ca^{2+} inside the muscle cell. The rate at which Ca^{2+} accumulates inside the cell, is far greater than the rate at which the potassium inside the cell is able to difuse to the outside, causing a negative shift in the electrical potential [268]. Therefore, due to the fact that M-cad is a calcium dependent adhesion molecule [128, 130], an increase in Ca^{2+} would therefore cause an increase in M-cad adhesion to the muscle membrane, as seen with an increase in M-cad expression at 4 hr after

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injury in the placebo group. Therefore, it is plausible that the increase in M-cad at 4 hr after injury is the result of increased Ca^{2+} influx to the site of injury.

Furthermore, the gradual decrease in M-cad expression seen by day 3 in the current study can also be ascribed to the change in the Ca^{2+} balance in the muscle, as the recovery of the Ca^{2+} balance ($[Ca^{2+}]$ returning to normal) in the muscle would therefore cause a decrease in the number of M-cad$^+$ SC. In the study by Leybaert and De Ley (1994) already mentioned [268], the interstitial ionic changes were completely reversible within 90 min following injury, however their study made use of a cat model with a less severe injury when compared to the rat model of contusion injury used in the current study. Therefore it is possible that in the current study, the time it took for the calcium balance to return to normal, took relatively longer. It should also be noted that no other time points between 4 hr and day 3 were observed, in which the M-cad expression could also have shown a decrease prior to the decrease seen by day 3.

Similarly, the GSE group also displayed an increase in M-cad expression directly after injury, followed by a gradual decrease. However, the number of M-cad$^+$ SC in the placebo group was significantly higher than that of the GSE group. Due to the fact that proanthocyanidin comprise a group of polyphenolic bioflavonoids ubiquitously found in fruit and vegetables [201-203], it is likely that proanthocyanidins may interact with intracellular Ca^{2+} as well as with other components to elicit its protective effect. A possible way in which proanthocyanidins can work, may include the interaction with Ca^{2+} ions, leading to a reduction in the ionized Ca^{2+} content inside the cell. By this mechanism, proanthocyanidins may increase the binding

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affinity of a substrate to improve the transfer of antioxidant enzymes to the injured muscle, thereby providing further protection against injury-induced calcium overload. However, this particular aspect of proanthocyanidins mechanism of action involving Ca^{2+} still merits research in order to clarify discussion. A possible way of investigating the effect of proanthocyanidins on the Ca^{2+} balance would be to tag the Ca^{2+} ions with a fluorescent marker to visualise the changes in calcium balance immunohistochemically.

4.5 Foetal myosin heavy chain (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 [242]. Therefore, it was assumed that MHC_f^+ staining in this study represents regenerating muscle fibres formed after sustaining the contusion injury. However, due to the fact that the particular antibody used was a mouse-anti human antibody, it may not have been specific only to the MHC$_f$ isoform and regenerating myofibres, but may have labelled another MHC isofom in other fibres without central nuclei as well. Although no articles are available on possible sequence homology between MHC$_f$ and other MHC isoforms, it is not certain which isoform is also stained positive with this particular marker. However, for future studies, this problem can be overcome by co-staining with other adult MHC markers making use of immunohistochemistry, mATPase staining techniques can also be used, as well as Western blotting to possibly identify the other isoform. This will then enable us to use this marker in other rat-based studies to determine the MHC content of a particular muscle sample.

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In light of the above, in order to calculate the actual number of regenerating myofibres, counting of positively stained MHC_f myofibres with central nuclei was needed. To our knowledge, this is the first time that the immunohistochemical expression of MHC_f has been used in conjunction with a contusion injury. However, this particular antibody was also used in a cryoinjury experiment to rat hearts, but no mention of central nuclei was made. The data of the MHC_f expression found in the current study supports the findings of $CD56^+$ staining since the biggest differences were seen between the 2 groups in the early time points (4 hr and 3 d). As it is known that CD56 is a marker of activation of satellite cells and possibly fusion into myotubes, it was expected that an increase in CD56 should be followed closely by an increase in MHC_f. In future, markers of fusion could possible be of use, however no markers of fusion for use in immunohistochemical staining or any in culture techniques exist.

Both control groups showed no centrally nucleated fibres with MHC_f expression. The placebo group displayed an early increase in the number of MHC_f^+ myofibres by day 3 after injury (compared to control), but reached a peak by day 7. Similar qualitative histological findings of regenerating myofibres by day 6-8 were found in placebo controls in our study (section 3.3), as well as other studies [6, 156, 157]. However, in these studies immunohistochemistry was not used to quantitate the data obtained, but rather histological staining with haematoxylin and eosin was used. By day 14 in the placebo group, the number of MHC_f^+ myofibres decreased again. During this time point, it was noticed that some of the myofibres displayed central nuclei, without MHC_f^+ staining, thereby possibly indicating that these fibres had already matured, but that the nuclei had not migrated to the sub-sarcolemma region yet. A possible

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way to investigate the migration of nuclei, may be to inject BrdU to label the proliferating pool of SC in order to identify nuclei and possibly their migration to the sarcolemma. However, no such studies have been attempted.

The GSE group displayed a similar rise and then fall in MHC_f^+ staining compared to the placebo group. However, the number of MHC_f^+ myofibres showed an earlier significant increase at 4 hr, which increased even further and peaked on day 3, whereafter, MHC_f expression decreased. Again here, these data illustrated GSE's effect in accelerating the recovery process after injury, and was also validated by histological staining. In the GSE group, the presence of myofibres with central nuclei (negative for MHC_f), were also observed. However, in this particular group this phenomenon was seen by day 7 already.

4.6 Summary and conclusion

A contusion injury is the leading cause of discomfort in athletes participating in high contact sports, such as rugby and soccer today, and although several therapeutic strategies have been developed to treat the symptoms of such an injury, few of them have been successful. Through the use of an *in vivo* model of skeletal muscle contusion injury, we were able to mimic a contusion injury in rats. This enabled us to successfully determine the severity of injury, firstly by investigating its effect on skeletal muscle fibre ultrastructure and the inflammatory response (haematoxylin and eosin) and secondly by investigating qualitatively its effect on the vascular system (CD31). Although both treatment groups showed a similar pattern of damage to the skeletal muscle fibre ultrastructure and the vasculature, followed by a

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rapid immune cell infiltrate in the injured area, the GSE-supplemented group showed a much better and quicker recovery time following the contusion injury. This is the first study, which has shown quicker recovery from such an injury through the use of antioxidant supplements (Oxiprovin™) in a rat model. ORAC results indicated that groups supplemented with GSE had a better free radical scavenging effect, and in conjunction with the lower plasma CK activities found in the GSE group, it was concluded that GSE supplementation could protect the skeletal muscle against secondary damage.

Labelling muscle sections with different SC markers enabled us a better understanding of the involvement of SC in the recovery process following a contusion injury. CD34 and CD56 showed quite similar expression patterns and both the two treatment groups had similar resolution patterns following injury except that the GSE group had an earlier elevation of SC compared to the placebo group. In light of the more specific results obtained in the current study, it can be concluded that CD34 labeled quiescent as well as early activated SC and that CD34$^+$ SC migrated to the area of damage. Furthermore, interpreting CD34 data was complicated by the fact that CD34 was not specific to all activated and quiescent SC, but only to a proportion of SC. M-cadherin (calcium dependent), according to the literature is a similar marker to CD56 [121, 128], labelling activated, quiescent and proliferating SC, surprisingly showed completely different results than those for CD56. In this study, the number of M-cadherin$^+$ SC in the placebo group were higher at all time points following injury, indicating that GSE supplementation may have exerted its protective function by influencing calcium homeostasis during the recovery process. The data therefore suggested that CD34 is a marker of a subset

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of quiescent as well as activated SC, and that CD56 labels quiescent, activating and proliferating SC.   However, co-staining with other markers of quiescence and activation is needed. Furthermore, care should be taken when interpreting data, as even a slight disruption in calcium homeostasis could influence the relationship between results obtained with M-cadherin and CD56.

We can also conclude that supplementation with Oxiprovin™ (GSE) did in fact accelerate the healing process. Activation of SC occurred quicker in the GSE-supplemented group compared to the placebo group, as seen with CD34 and CD56 staining. Furthermore, muscle fibres showed regenerating capacity much quicker in the GSE group compared to the placebo group, as seen through histological staining with H&E, as well as labeling with MHC_f.

Although in the current study, Oxiprovin™ was successfully proven to be beneficial in treating contusion injuries, this study had a few limitations. Firstly, the control uninjured groups were sacrificed 14 days earlier than the injured groups, allowing no direct time comparisons between groups. This omission was a planned one, to reduce the number of animals required for the study, to reduce the cost of the study, as well as due to the limited capacity of the small animal house.   Secondly, additional earlier time points prior to 7 days post-injury may potentially elucidate the exact timecourse of events following a contusion injury. The time points chosen were done after consultation of the literature, and were also limited for the same reasons stated above.

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Since this is the first study to show an effect of Oxiprovin™ supplementation at tissue level in animals prior to and after sustaining a contusion injury, we cannot rule out the importance of investigating other parameters such as immune cells, which may also play an important role in further elucidating the results obtained. Furthermore, we cannot rule out the possibility that acute Oxiprovin™ supplementation in the post-injury period only, could also be beneficial. The possibility of not only using Oxiprovin™ as a preventative measure, but also as a complementary remedial treatment, is worth investigating further. In addition, a live cell culture model of injury, might also be useful in determining the effect of Oxiprovin™ as well as the exact time course of events following such an injury *in vitro* and therefore relating it to the *in vivo* conditions seen in the current study.

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Appendix A: ORAC assay

Reagents

Table presenting the reagents used for the ORAC assay

Reagent	[Stock]	[Working]	Supplier
AAPH*		153 mM 0.414 g in 10 ml Phosphate buffer pH 7.4	Sigma-Aldrich
Fluorescein	5×10^{-3} mM	8.16×10^{-5} mM Made up in phosphate buffer, pH 7.4	Sigma-Aldrich
Potassium Phosphate buffer		75 mM (pH 7.4) 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
Trolox**	0.02 M	6.25 µM; 12.5 µM; 25 µM; 50 µM in phosphate buffer, pH 7.4	Sigma-Aldrich

* 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.

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

3. Add 25 µl TROLOX standards or plasma in triplicate to respective wells.

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

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

5. Add 25 µl AAPH rapidly to each well.

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

Appendix B: Bradford assay

Reagents

1. 200 µg/ml BSA (bovine serum albumin) stock solution – Sigma-Aldrich

2. Bradford reagent – Sigma-Aldrich

Method

1. Generating 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)

2. Vortex and leave on ice for 5 min.

3. Zero the spectrophotometer with the BLANK and read the absorptions of the standards at 595 nm.

4. Prepare the samples – 5 µl of sample, 95 µl distilled water and 900 µl Bradford reagent. Vortex and read the absorption at 595 nm (*Note: samples stable for up to 1 hr after preparation*).

5. Plot the standard curve and calculate the protein concentration in µg.

Appendix C: Automatic tissue processing.

Reagents

1. Alcohol (70 %, 90 %, 95 %, 100 %)

2. Xylene – Sigma-Aldrich

3. Paraffin wax – Merck, Histosec melting point 56 °C

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

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Appendix D: 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

   <u>Stock solution:</u>

   10 g Eosin dissolved in 1 ℓ distilled water

   <u>Working solution:</u>

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

   Prepare fresh daily.

   <u>For staining:</u>

   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

   <u>To prepare:</u> 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.

   <u>For staining:</u> 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 E: Conventional deparaffinization and dehydration sequence of paraffin embedded tissue prior to immunohistochemistry.

Reagents

1. Alcohol (50 %, 80 %, 95 %, 100 %)

2. Xylene – Sigma-Aldrich

3. 0.1 % Trypsin – Highveld

   0.1 g trypsin in 100 ml PBS

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 °C) 0.1 % trypsin for 20 min

Appendix F: Immunohistochemistry staining procedure (2 markers)

Reagents

1. PBS, pH 7.4

     1 ℓ of 1 M phosphate buffer, 90 g NaCl, 9 ℓ ddH$_2$O

2. Donkey serum – Jackson Immunoresearch Inc.

3. For antibodies used, see Chapter 2, section 2.3.4 for reagents

Method

1. Wash slides in PBS after trypsin (step 7, Appendix E).

2. Encircle samples with a wax pen.

3. Block for 20 min in 5% serum at room temperature (RT). (*Note: use the same serum in which the secondary antibody is raised*)

4.   Shake off serum and incubate sections for 4 hr at RT with the 1st primary antibody. (*Note: Do not wash after serum blocking step*)

5. Wash slides with PBS and add the secondary antibody (1/1000 for anti-mouse) to the sections. Incubate for 40 min at RT.

6. Wash slides and incubate slides with 1/500 FITC for 30 min at RT.

7. Wash slides with PBS and add the 2nd primary antibody overnight at 4°C.

8. Add the secondary antibody (1/1200 for anti-rabbit) for 40 min after washing the sections thoroughly with PBS.

9. Wash sections and add 1/500 Texas Red tertiary antibody for 30 min at RT.

10. Add Hoechst (1/200) for 15 min to the Texas red.

11. Wash slides well and mount with DAKO fluorescent mounting medium.

(Note: if only use 1 antibody, apply steps 1-6 and then 10 and 11)

Appendix G: Results of figures in Chapter 3 indicated as mean ± SD

Figure 3.2: Difference in growth over time

Group	Slope	Upper limit	Lower limit
I-P	4.69	4.38	5.00
I-GSE	4.81	4.44	5.19
C-P	6.89	6.33	7.45
C-GSE	5.24	4.90	5.58

Figure 3.3: Total plasma creatine kinase (CK) activity expressed as units/liter (u/ℓ)

Time point	Placebo	GSE
Control (non-injured)	214 ± 27.1	294 ± 71.3
4 hr (post injury)	354 ± 162	557 ± 216
Day 3 (post injury)	339 ± 145	221 ± 46.1
Day 7 (post injury)	243 ± 108	218 ± 92.4
Day 14 (post injury)	271 ± 168	214 ± 57.0

Figure 3.7: Plasma oxygen radical absorbance capacity expressed as µmol Trolox equiv/ℓ

Time point	Placebo	GSE
Control (non-injured)	3045 ± 26.4	3152 ± 31.3
4 hr (post injury)	3016 ± 137	2980 ± 172
Day 3 (post injury)	2981 ± 98.4	3140 ± 28.6
Day 7 (post injury)	3100 ± 77.1	3078 ± 124
Day 14 (post injury)	3089 ± 38.0	3029 ± 30.0

Figure 3.8: Muscle oxygen radical absorbance capacity expressed as µmol Trolox equiv/µg protein

Time point	Placebo	GSE
Control (non-injured)	466 ± 32.0	583 ± 39.6
4 hr (post injury)	474 ± 48.9	487 ± 37.6
Day 3 (post injury)	500 ± 52.0	607 ± 46.2
Day 7 (post injury)	648 ± 21.1	586 ± 35.5
Day 14 (post injury)	473 ± 35.7	573 ± 46.4

Figure 3.9: The number of CD34$^+$ satellite cells/myofibre

Time point	Placebo	GSE
Control (non-injured)	0.026 ± 0.004	0.026 ± 0.005
4 hr (post injury)	0.074 ± 0.004	0.140 ± 0.025
Day 3 (post injury)	0.054 ± 0.007	0.073 ± 0.019
Day 7 (post injury)	0.121 ± 0.009	0.072 ± 0.015
Day 14 (post injury)	0.025 ± 0.003	0.029 ± 0.011

Figure 3.11: The number of CD56$^+$ satellite cells/myofibre

Time point	Placebo	GSE
Control (non-injured)	0.024 ± 0.003	0.025 ± 0.006
4 hr (post injury)	0.060 ± 0.005	0.233 ± 0.035
Day 3 (post injury)	0.102 ± 0.006	0.123 ± 0.029
Day 7 (post injury)	0.101 ± 0.006	0.067 ± 0.014
Day 14 (post injury)	0.025 ± 0.002	0.026 ± 0.005

Figure 3.13: The number of M-cadherin$^+$ satellite cells/myofibre

Time point	Placebo	GSE
Control (non-injured)	0.055 ± 0.002	0.048 ± 0.020
4 hr (post injury)	0.285 ± 0.026	0.164 ± 0.040
Day 3 (post injury)	0.018 ± 0.041	0.112 ± 0.024
Day 7 (post injury)	0.076 ± 0.008	0.069 ± 0.024
Day 14 (post injury)	0.029 ± 0.003	0.031 ± 0.008

Figure 3.15: Percentage of MHC_f^+ myofibres

Time point	Placebo	GSE
Control (non-injured)	0.00 ± 0.00	0.00 ± 0.00
4 hr (post injury)	2.12 ± 1.34	7.78 ± 1.56
Day 3 (post injury)	3.95 ± 1.79	13.2 ± 2.00
Day 7 (post injury)	11.9 ± 2.25	3.93 ± 1.18
Day 14 (post injury)	3.68 ± 1.28	2.52 ± 0.66